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Biochemical responses of postlarval Penaeus vannamei to infection by the virus Baculovirus penaei.

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BIOCHEMICAL RESPONSES OF POSTLARVAL PENAEUS VANNAMEI TO INFECTION BY THE VIRUS BACULOVIRUS PENAEI

by

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Title Biochemical responses of postlarval Penaeus vannamei to infection by the virus Baculovirus penaei

In a series of five studies, the effect of the virus Baculovirus penaei, commonly known as BP, on larval and postlarval Pacific white shrimp (Penaeus vannamei) was investigated. In the first study, time required for development of patent BP infections and persistence of those infections was determined. Pre-patent infections were detected in larval shrimp at 12 hours post-inoculation (p.i). Patent infections, characterized by the presence of viral polyhedra, developed in some shrimp 18-24 hours p.i., increased in prevalence to 100% 3 to 17 days p.i. and were not detectable in most shrimp after 30 days p.i. In the second study, the effects of BP on the survival and growth of postlarval shrimp were investigated. An age-dependent pattern of disease was observed in which shrimp initially infected as larvae and young postlarvae experienced higher mortality and reduced growth, compared to older postlarvae and juveniles exposed to the virus. Immediately after a patent infection was established, postlarvae experienced high mortality in response to nutritional stress. In the third study, the biochemical response to starvation in postlarval shrimp was determined to provide a basis of comparison for nutritional and BP-induced stress. Biochemical indices, especially dry weight, RNA:DNA, protein:DNA, spermidine:

DNA, spermine:DNA, and two unidentified amines expressed as a ratio to spermine, appear to be useful indicators of severe nutritional stress. In the fourth study, the relationship between BP and energy reserves in larval and postlarval shrimp was investigated. In some cases, patent BP infections were associated with a significant post-infection reduction of triacylgycerol (TAG). Experimental reduction of TAG content immediately prior to viral inoculation delayed the development of a patent infection. High pre-inoculation TAG levels were associated with increased susceptibility to BP infections. In the fifth study, the effect of BP on biochemical indices of growth was investigated. The biochemical response to BP was different from the response to nutritional stress observed earlier. The rapid and significant increases in putrescine levels of inoculated shrimp observed in this study are useful indicators of BP-induced subacute stress.

Abstract Approved by: Committee Chairman Stephen J. Watts

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BIOCHEMICAL RESPONSES TO BACULOVIRUS PENAEI INFECTIONS IN LARVAL AND POSTLARVAL PACIFIC WHITE SHRIMP, PENAEUS VANNAMEI: NUCLEIC ACIDS AND POLYAMINES

INTRODUCTION

Over the last decade, the high demand for penaeid shrimp has stimulated the emergence of a substantial and rapidly expanding worldwide aquaculture industry. Although the vast majority of cultured shrimp are currently produced in South America and South East Asia, production of farm raised shrimp in the United States has increased from 2.3 million pounds in 1988 to 5.1 million pounds in 1993 (Dill et al., 1994). Because of high production cost in the United States, future expansion of the domestic shrimp farming industry will depend in large part on the successful development of high-intensity culture methods. The major impediment to maintaining current production levels and the further development of intensive culture methods, both in the United States and abroad, is the detrimental influence of viral related diseases.

There are currently at least 15 viruses known to infect cultured and wild penaeid shrimp, and most are associated with disease in at least one or more species; but "despite the economic importance of these viruses to the world's aquaculture industry, relatively little is known about them" (Lightner et al., 1994). To develop and implement effective disease control procedures in aquaculture, the viral-host interactions of each of these pathogens must be characterized, and the full extent of the resulting disease determined. Methods to assess both acute and subacute

pathogenicity of viruses are therefore needed. The development of such methods will also be of value in determining the effects of viruses in wild populations of penaeid shrimps.

Baculovirus penaei, commonly known as BP and designated as PvSNPV by the International Committee on Taxonomy of Viruses (Francki et al., 1991), was the first virus reported from penaeid shrimp. BP was originally described in wild pink shrimp, Penaeus duorarum, from the northern Gulf of Mexico (see Couch, 1974a,b), and the morphology and cytopathic effects of the virus have been extensively studied in that species (Couch, 1989,1991). Couch et al. (1975) reported that the prevalence of BP in wild populations of pink shrimp from different locations in northwest Florida ranged from 0 to 50%. BP has now been reported from at least 13 additional penaeid species and can cause serious disease in most of those (Lightner et al., 1994; Overstreet, 1994). LeBlanc et al. (1991) investigated the relative susceptibility of wild caught brown shrimp, P. aztecus and several other crustaceans to BP infection. Overstreet (1994) reported that the natural prevalence of the virus in wild populations of brown shrimp from the northern Gulf of Mexico may seasonally exceed 30%.

The pathogenic effects of BP on cultured penaeids have been best documented and studied in the Pacific white shrimp, Penaeus vannamei, the primary species used in commercial farming operations in the Americas and Hawaii. BP infections in P. vannamei can cause serious epizootics with high mortality of larval and young postlarval shrimp (Lightner, 1988). In aquaculture operations, BP causes economic losses from mass mortalities, primarily in the hatchery phase of production. Overstreet et al. (1988) developed a procedure for experimentally infecting larval and

postlarval P. vannamei with BP. The age-dependent susceptibility of P. vannamei to BP infections (LeBlanc and Overstreet, 1990) and procedures for deactivation of the virus have also been reported (LeBlanc and Overstreet, 1991a,b). In all previously published studies, the pathogenic effects of BP have been assessed in terms of prevalence and intensity of infection and viral-induced mortality. Because subacute effects of the virus are more difficult to determine, particularly in wild populations of shrimp, the full extent of BP-induced disease in penaeid shrimp is not completely known.

Because of the practical application of baculoviruses as pesticides, there is a substantial volume of literature on the biological and molecular properties of these viruses in insects (see Granados and Federici, 1986; Adams and McClintock, 1991). Many aspects of baculovirus infections, including the sequence of events and time course of viral replication, viral-host interactions, and mechanisms of disease, have been extensively investigated in insects. Previous studies on BP in shrimp have shown both similarities (Couch, 1989; Bruce et al. 1994) and differences (Summers, 1977; Overstreet, 1988) with baculovirus in insects. The extensive characterization of baculoviruses in insects may provide valuable insights into understanding possible mechanisms of transmission and BP-induced pathology in shrimp.

The primary purpose of this investigation was to determine the extent and nature of acute and subacute responses of Penaeus vannamei to experimental BP infections. When appropriate, the effects of BP in P. vannamei were compared to baculovirus infections in insects. The dissertation consists of five studies; each study has been prepared as a manuscript, which has either been accepted or submitted for

publication in an appropriate international peer-reviewed journal. In the first study, traditional and recently developed molecular detection methods for BP were used to document the time course for the establishment and retention of infections. In the second study, subacute effects of the virus were characterized by reductions in growth and decreased resistance to nutritional stress of infected shrimp. Because BP infects and can cause substantial tissue destruction in the hepatopancreas (Couch 1981), the primary organ responsible for nutrient absorption, synthesis of digestive enzymes, and storage of lipid reserves in crustaceans (Gibson and Baker, 1979), it is hypothesized that BP-induced subacute effects may be similar to nutritional stress. Therefore, during the third study a biochemical characterization of nutritional stress in postlarval shrimp was conducted to provide a basis for comparison with BP-induced stress. In the fourth study, the relationship between BP and host energy reserves was investigated. Finally, the effects of BP infections on biochemical growth indices were determined during the fifth study. The information obtained during this investigation should provide a better understanding of viral-host interactions and pathogenicity of BP in penaeid shrimp.

ESTABLISHMENT AND PERSISTENCE OF BACULOVIRUS PENAEI INFECTIONS IN CULTURED PACIFIC WHITE SHRIMP, PENAEUS VANNAMEI

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ABSTRACT

The time course for establishment of a baculovirus (BP) infection and the persistence of infections in larvae and postlarvae of the Pacific white shrimp, Penaeus vannamei, were investigated. In two preliminary studies, postlarvae were inoculated with BP and the prevalence of infections was monitored over 138-day and 73-day periods. Viral polyhedra characteristic of patent infections first appeared 24 hr after inoculation, were present in all shrimp examined 14 - 17 days post-inoculation (p.i.), and then decreased in prevalence during the remainder of each study. BP infections could not be detected by *in situ* hybridization in shrimp collected during the final day of each study. In a third and more comprehensive study, PCR-based diagnostic procedures were used to detect BP in experimentally infected shrimp over a 120-day period. In that study, BP was first detected in viral exposed mysis stage shrimp 12 hr p.i., was present in all shrimp examined by 72 hr p.i., and decreased in prevalence during the remainder of the study. BP infections could not be detected by PCR-based diagnostics in shrimp collected on the final day of the study. Only 1 of 10 previously infected shrimp collected at 31 days p.i. and diagnosed for BP by bioassay was infected. Results of this study indicate similarities between Penaeus vannamei and some species of insects in the time required for establishment and loss of a patent baculovirus infection.

INTRODUCTION

Baculovirus penaei, commonly known as BP and designated as PvSNPV (Francki *et al.*, 1991), is a baculovirus that was originally described from pink shrimp, Penaeus duorarum, by Couch (1974a,b). BP is now known from at least 14

species of penaeid shrimp (Lightner et al., 1994) and occurs in both wild and cultured populations. Epizootics of BP can result in high mortality or reduced growth in larvae and early postlarvae of cultured shrimp (LeBlanc and Overstreet, 1990; Stuck and Overstreet, 1994). Although susceptible to infection, the effects of BP on survival and growth of late postlarval and juvenile P. vannamei appears to be negligible.

Patent BP infections are characterized by the presence of viral polyhedra or tetrahedral occlusion bodies in epithelial cells of the hepatopancreas (HP). Polyhedra are easily observed by examination of wet mount squashes of the HP following procedures described by Overstreet *et al.* (1988). Histological and DNA *in situ* hybridization methods have been used to detect BP infections prior to the formation of polyhedra (Bruce *et al.*, 1994). Recently, diagnostic methods employing polymerase chain reaction (PCR) have been developed for the detection of BP infections (Wang *et al.*, submitted; Poulos *et al.*, 1995). Because of the availability of molecular detection methods, detailed investigations of the life history of BP in both wild and cultured populations of penaeid shrimp are now possible.

The time sequence for baculovirus replication and the establishment of persistent infections has been extensively studied in insects (see Burand *et al.*, 1986; Granados and Williams, 1986; Adams and McClintock, 1991). Similar information on baculovirus life history in penaeid shrimps is sparse. Couch (1989, 1991) proposed a replication cycle for BP in shrimp that was parallel in many aspects to that observed in insects. Bruce *et al.* (1994) observed that replication of BP in experimentally infected Penaeus vannamei may be similar in some respects to insect

baculoviruses. The objectives of the present study were to (1) investigate the time course of BP infections in P. vannamei, (2) determine if BP establishes persistent infections in P. vannamei, and (3) document similarities and differences in the development and persistence of baculovirus infections among insects and shrimp. This investigation provides basic information about BP life history in P. vannamei that should prove useful in prevention and management of viral epizootics in aquaculture operations.

MATERIALS AND METHODS

In two preliminary studies, high health shrimp (see Wyban, 1992) were obtained as nauplii from Harligen Shrimp Farm, Los Fresnos, Texas (study 1), and Waddell Mariculture Center, Bluffton, South Carolina (study 2), and reared to 9-day-old postlarvae following the procedures described by Stuck and Overstreet (1994). In both preliminary studies, postlarvae, stocked at density of approximately 10 per liter, were inoculated with BP by introducing a single dose of homogenized infective stock (10 mg per liter) directly into a 150-liter culture. A second culture, which served as the negative control, was administered an identical amount of homogenized BP-free shrimp tissue. The BP infective stock used in these studies was originally collected in Ecuador (see Overstreet *et al.*, 1988). During the first preliminary study, samples (N = 15-20 shrimp) were periodically collected and examined for the presence of viral polyhedra over a 138-day period. The wet mount squash procedure described by Overstreet et al. (1988) was used in this and all subsequent studies to detect polyhedra. In the second preliminary study, samples (N = 10-15 shrimp) were periodically collected and examined for polyhedra over a 73-day period. At the

termination of both preliminary studies, samples (N = 4 shrimp heads) were collected from BP-inoculated and control groups and fixed in Davidson's solution for *in situ* hybridization. These shrimp were later diagnosed for the presence of BP by personnel in D. Lightner's laboratory in the Department of Veterinary Science, University of Arizona, Tucson, Arizona, using probes and procedures described by Bruce *et al.* (1993).

A third and more comprehensive study was then undertaken to further investigate the development and persistence of BP infections. High health nauplii were obtained from The Oceanic Institute, Waimanalo, Hawaii, and reared to mysis stage following procedures described by Stuck and Overstreet (1994). A 150-liter culture of mysis I-II, stocked at density of approximately 50 per liter, was inoculated with BP following procedures identical to those described in the preliminary studies. A corresponding negative control was also maintained. Samples (N = 10-17 shrimp) collected from both control and BP-inoculated treatments at 0, 12, 15, 18, 24, and 72 hr and 7, 31 and 120 days post-inoculation (p.i.) were examined for the presence of viral polyhedra. During those same sampling periods, and at 4 and 8 hr p.i., additional samples (N = 20-40 shrimp) were collected, frozen in liquid nitrogen, and stored at -70°C for later use in PCR-based and bioassay diagnostic procedures.

The sample extraction and PCR-based diagnostic procedures, source, and sequence of the DNA primers used in this study are described in detail by Wang *et al.* (submitted). Template DNA was extracted from whole mysis stage shrimp sampled at 0-72 hr p.i. and from the head only for shrimp sampled on Day 7 p.i. Individual shrimp were homogenized in 75 μl of digestion buffer (DB). For the

larger Day 31 and Day 120 p.i. shrimp, either the head (if TL < 15mm) or HP only (if TL > 15mm) from individual shrimp was homogenized in 400 μl DB. Regardless of the homogenizing volume, a 50-μl aliquot of each homogenate was used to isolate template DNA. The samples were extracted with phenol and chloroform, and the ethanol precipitated DNA dissolved in 30 μl of resuspension buffer (RB). Primers BPA and BPB and AmpliTaq thermal-stable DNA polymerase (Perkin Elmer, Norwalk, CT) were used in the PCR reactions. Each time PCR was performed, positive control reactions containing DNA isolated from shrimp known to be infected with BP were used to verify that the reagents were functional. Negative control reactions containing DNA isolated from high health shrimp were also used each time to verify that the reagents were not contaminated with BP DNA.

A combination of fresh squash, PCR-based and bioassay diagnostic procedures was used to determine if shrimp established detectable persistent infections by Day 31 p.i. Ten individual Day 31 p.i. shrimp, a positive control consisting of 10 mg of 72 hr p.i. infected mysis stage shrimp, and a negative control consisting of 10 mg of 0 hr uninfected mysis stage shrimp were used in the bioassay. The heads (if TL < 15mm) or the HP only (if TL > 15 mm) of individual Day 31 p.i. shrimp and the positive and negative controls were homogenized with 10 μl dH_2O. A pre-bioassay diagnosis of each sample was conducted using a 1-μl aliquot homogenized in 75 μl of DB for PCR, and a 1-μl aliquot was examined for viral polyhedra. The remainder of each sample was used as an inoculum for a 7-day bioassay. The bioassay was conducted in 1-liter Immhoff cones generally following procedures outlined by Overstreet *et al.* (1988). Each of 12 cones was stocked with

100 mysis I-II stage high health Penaeus vannamei obtained from the maturation facility at Gulf Coast Research Laboratory. Inoculums from the 10 individual Day 31 p.i. shrimp, and the positive and negative controls were then added to the separate cones. After 7 days, a sample of 20 shrimp from each cone was examined for viral polyhedra. Eight individuals from each cone were also examined for BP using PCR-based diagnostic procedures described previously for Day 7 p.i. shrimp.

RESULTS

In the two preliminary studies, patent infections diagnosed by presence of viral polyhedra first appeared in a few individuals at 24 hr p.i. In the first of those studies, the prevalence of infection was 100% (N = 15-20) at 17 days p.i., declined to 10% by 43 days p.i., and was undetectable at 138 days p.i. In the second study, the prevalence of infection was 100% by 14 days p.i. (N = 10-15), decreased to 6% by 41 days p.i., and was undetectable at 73 days p.i. BP was not detectable by *in situ* hybridization from shrimp (N = 4) collected during the last sampling period from BP-inoculated or control groups from either preliminary study. Mortality in both groups of infected postlarvae and the corresponding uninfected controls was inconsequential.

The time course of BP replication and the persistence of patent infections were investigated in greater detail during the third experimental infection in which wet mount squash and PCR-based diagnostics were used to detect the presence of the virus (Table 1). BP infections were first detected by PCR among viral exposed larvae 12 hr p.i. and increased in prevalence to 100% by 72 hr p.i. Viral polyhedra were first observed at 24 hr p.i. and were present in all shrimp examined at 72 hr and

Table 1
Prevalence of Baculovirus penaei in Experimentally Infected and Uninfected "Control" Larvae of Penaeus vannamei Over a 120-Day Period

	DIAGNOSIS			
	Squash		PCR	
Time p.i.	N	%	N	%
0 hr	0/10	0	0/8	0
4 hr	NC	-	0/8	0
8 hr	NC	-	0/16	0
12 hr	0/15[a]	0	1/15	7
15 hr	0/15[a]	0	3/12	25
18 hr	0/15[a]	0	10/17	59
24 hr	6/17[a]	35	7/8	88
72 hr	15/15[a]	100	8/8[b]	100
Day 7	16/16[a]	100	8/8	100
Day 31	1/10[a]	10	5/31	16
Day120	0/10[a]	0	0/6	0

Note: Infections were detected using wet mount squash and PCR-based diagnostics. The time post-inoculation (p.i.), number of infected individuals/total number examined (N), and percent infected (%) are listed for each sampling period and diagnostic method. NC = data not collected.

[a] Ten individuals from uninfected control group were negative.

[b] Eight individuals from uninfected control group were negative.

7 days p.i. By day 31 p.i., only 10% of the shrimp examined had viral polyhedra, and 12% had BP infections detectable by PCR. BP was not detected from inoculated shrimp at 120 days p.i. using either squash or PCR-based diagnostic methods. Although dead shrimp were observed in the viral exposed group 4-7 days p.i., substantial mortality after that time was not evident. BP infections were not detectable among uninfected control larvae and mortality was inconsequential.

Ten of the Day 31 p.i. shrimp from the third experiment were diagnosed for BP infections by bioassay (Table 2). The PCR-based and squash prebioassay diagnosis showed that only one of those shrimp and the positive control was infected. Inoculums prepared from the single BP-infected Day 31 shrimp and the positive control resulted in heavy infections during the 7-day bioassay. Inoculums prepared from the other Day 31 p.i. shrimp used in the bioassay and the negative control did not cause infections that could be detected either by squash or PCR-based diagnostic methods. Survival during the 7-day bioassay was $\geq$ 90% in the nine cultures inoculated with Day 31 p.i. shrimp that did not have detectable infections and the negative control, 86% in the culture inoculated with the single Day 31 p.i. shrimp that had an infection, and 72% for the positive control.

DISCUSSION

The time sequence of BP replication implied from results of our study is similar to that reported from previous studies of shrimp and insects. Using PCR-based diagnostics, the earliest we were able to detect BP from homogenates of inoculated Penaeus vannamei larvae was 12 hr p.i. Patent infections, characterized by the presence of viral polyhedra, were first evident at 24 hr p.i. and reached 100%

Table 2
Detection of Baculovirus penaei Infections in Penaeus vannamei
31 Days Postinoculation (p.i.) as Determined by Bioassay

DIAGNOSIS

	Pre-bioassay		Post-bioassay		
Sample	Squash	PCR	Squash	PCR	% Survival
D31-1	-	-	0/20	0/8	98
D31-2	+	+	20/20	8/8	86
D31-3	-	-	0/20	0/8	91
D31-4	-	-	0/20	0/8	96
D31-5	-	-	0/20	0/8	100
D31-6	-	-	0/20	0/8	100
D31-7	-	-	0/20	0/8	90
D31-8	-	-	0/20	0/8	99
D31-9	-	-	0/20	0/8	92
D31-10	-	-	0/20	0/8	98
+ control	+	+	20/20	8/8	72
- control	-	-	0/20	0/8	99

Note: Homogenates prepared from ten individual day 31 p.i. shrimp and positive and negative control groups were first examined for BP infections (pre-bioassay) using wet mount squash and PCR-based diagnostic methods, and then used as inoculum in a 7-day bioassay. The % survival and number of infected shrimp/number examined by squash and PCR diagnostic methods was determined at the termination (post-bioassay) of the study.

prevalence at 72 hr p.i. Bruce *et al.* (1994) used an *in situ* hybridization technique to determine the time sequence of BP replication in larval P. vannamei. In that study,

viral infections were first detected at 12 hr p.i. and increased in prevalence to 100% by 48 hr p.i. The minimum time required for the *in vivo* development of viral polyhedra (18-24 hr) reported in this and previous studies on BP (Overstreet *et al.*, 1988; Bruce *et al.* 1994; Stuck and Overstreet, 1994; Stuck *et al.*, submitted) is almost identical to that reported for many species of insects (see Granados and Williams, 1986; Adams and McClintock, 1991).

Kelly *et al.* (1978) used an enzyme linked immunosorbent assay to study replication of an insect baculovirus in Heliothis armigera larvae. They reported that virus antigens could first be detected in extracts of whole larvae at 12 hr p.i. and reached maximum titers by 72 hr p.i. The 12 hr p.i. detection of BP infections in Penaeus vannamei observed in the present study and by Bruce *et al.* (1994) generally corresponds with the time required for development of virogenic stroma and viral progeny reported from insects (see Granados and Williams, 1986; Adams and McClintock, 1991). It appears that the early stages of a BP infection, such as the entry of virions into epithelial cells of the HP and uncoating at the nuclear pores as proposed by Couch (1989), are not detectable by currently available molecular diagnostic methods.

Stuck and Overstreet (1994) reported a decrease in the prevalence of BP over time among experimentally infected Penaeus vannamei diagnosed by wet mount squash. In the present study, the prevalence of shrimp with detectable BP infections decreased substantially between Day 7 p.i. and Day 31 p.i. and by 120 days p.i. was not detected in any of the shrimp examined by wet mount squash or PCR-based diagnostic methods. Mortality during that period of time was inconsequential and

could not possibly account for the apparent loss of infected individuals. Results of the preliminary investigations using *in situ* hybridization diagnostic methods also indicated a loss of infection. However, we have already demonstrated that molecular diagnostic methods used in this study were not capable of detecting extremely low levels of BP, such as occurs during the initial stages of an infection. Therefore, a group of Day 31 p.i. shrimp were also diagnosed for the presence of BP by bioassay. Only 1 of the 10 shrimp used in the 7-day bioassay was infective. It is unlikely that the loss of infectivity of those shrimp can be attributed to deactivation of the virus during storage because the positive control used in the bioassay, which was stored for a longer period of time than the Day 31 p.i. shrimp, was very infective. Jarvis and Garcia (1994) demonstrated that frozen baculovirus stocks retain their infectivity during long-term storage, as long as they are not exposed to light. Another possible explanation for the apparent loss of infectivity may be due to an extended prepatent period (longer than 7 days) required for nonpatently infected shrimp to cause infections detectable by bioassay. However, homogenates prepared from nonpatently infected P. vannamei caused patent infections in a bioassay system identical to that used here within 3-6 days p.i. (Stuck *et al.*, 1994).

The apparent *in vivo* loss of BP from previously infected individuals observed in this and previous studies using Penaeus vannamei (LeBlanc and Overstreet, 1990; Stuck and Overstreet, 1994) is similar to that reported for baculovirus infected Fall Webworm larvae, Hyphantria cunea (Yamaguchi, 1979). However, the establishment of persistent baculovirus infections in many species of insects is well documented (see Burand *et al.*, 1986). Such persistent infections can be caused by defective

interfering viral particles that continue to replicate but do not result in the formation of viral occlusion bodies. It is possible that defective viral particles, which may not be detectable by the diagnostic methods used in this study, also develop in BP infected shrimp.

Crawford and Sheehan (1983) reported that persistent baculovirus infections in an insect cell line occurred in three stages. The first is characterized by high levels of infection (cells containing polyhedra) and death, the second is characterized by decreasing levels of infection, and the third is where less than 1% of the cells become persistently infected. They reported that persistently infected cells could then be "cured" by dilution to give virus-free clones. The "cured" cells also developed resistance to reinfection by the same or other baculoviruses. The *in vivo* development of BP infections in Penaeus vannamei appears to go through similar stages. Shrimp that survive the initial infection rapidly replace virally damaged cells with new growth (Stuck and Overstreet, 1994), which dilutes the original infection with BP resistant cells. Yamaguchi (1979) reported midgut epithelial cells regenerated in response to a baculovirus infection in insect larvae were immune to subsequent reinfection. This may be a mechanism by which individuals in a culture of experimentally infected shrimp can be "cured" and not continually reinfected by the remaining infected shrimp in the culture. Immunity to reinfection may also be related to the development of resistance to baculoviruses infections with increasing host age as observed in shrimp (Sano *et al.*, 1985; LeBlanc and Overstreet, 1990; Stuck and Overstreet, 1994) and insects (see Briese, 1986).

In insects, persistent baculovirus infections can be induced into productive infections by a variety of stressors, such as changing environmental conditions, diet, chemical exposure, and ingestion of a heterologous virus (see Burand *et al.*, 1986). These inapparent but inducible baculovirus infections, also referred to as latent infections (Podgwaite and Mazzone, 1986), have been extensively studied in insects. Factors controlling the maintenance and induction of latent infections in insects are not well understood and even less is known about latency of baculoviruses in crustacean hosts. Couch (1974b) reported that chemically induced stress increased the prevalence of patent BP infections in pink shrimp, Penaeus duorarum, with "probable" latent infections. In a series of experiments using wild caught shrimp, Couch (1976) attempted to increase the prevalence of BP by exposure to several chemicals. Although the prevalence of detectable BP infections was higher in some chemically exposed groups compared to nonexposed control shrimp, no consistent pattern of increase in viral prevalence was observed. The initial or base prevalence of infection was determined in two of the seven reported exposures. In one of those, the prevalence of infection changed little over a 10-day exposure period. In the second, the prevalence of BP infection decreased over a 25-day period from an initial level of 36% to 0% in both exposed and control groups. Although the observed reduction in prevalence of infected individuals might be attributable to selective mortality of infected shrimp (Couch, 1976), those results are consistent with the observations from this study in which the prevalence of detectable infections is substantially reduced over a 3- to 4-week period. Couch and Courtney (1977) later reported a significant increase in patent BP infections in pink shrimp exposed to

Aroclor® 1254 for a 35-day period relative to an unexposed control. Additional investigations are needed to conclusively determine if persistent BP infections are established in different species of shrimp and the role of persistence, if any, in maintenance and transmission of BP in both feral and cultured populations.

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EFFECT OF BACULOVIRUS PENAEI ON GROWTH AND SURVIVAL OF EXPERIMENTALLY INFECTED POSTLARVAE OF THE PACIFIC WHITE SHRIMP, PENAEUS VANNAMEI

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ABSTRACT

In a series of experiments conducted to investigate age and size-dependent effects of the baculovirus BP on postlarvae of the Pacific white shrimp, Penaeus vannamei, six groups of specific pathogen-free shrimp of different ages (mysis 2-3 through PL 25) were exposed to the virus and cultured for 15 to 21 days. All BP-exposed groups of early postlarvae (PL 9 or younger) became heavily infected within 2-5 days of initial exposure to the virus, and some of those groups experienced high mortalities compared to the noninfected controls. Postlarvae that survived the infection had highly variable and significantly reduced growth, as determined by dry weight, compared to controls. Exposure of older postlarvae to BP produced a high prevalence of infection, but with little effect on either survival or growth. One group of shrimp exposed to BP at PL9 was cultured for 49 days. Postlarvae that survived the infection were significantly smaller than the noninfected controls for the first 4 weeks following exposure to the virus; however, the effect of BP on long-term growth of infected postlarvae appeared minimal. To determine the effect of BP on nutritionally stressed shrimp, groups of noninfected and previously infected postlarvae (PL13-14) of similar size were deprived of food for 10 days. Less than 2% of the infected postlarvae survived the 10-day starvation period compared to 52% survival of the noninfected postlarvae.

INTRODUCTION

The virus commonly known as BP, originally named Baculovirus penaei by Couch (1974) and designated as PvSNPV (Francki *et al.*, 1991), occurs in both wild and cultured penaeid shrimp throughout the Americas, including Hawaii. BP has been

reported from 14 species of Penaeus (see Lightner and Redman, 1991; Lightner, University of Arizona, pers. commun.) and is known to cause serious epizootics with high mortality of both larval and postlarval stages in several species, including P. vannamei (see Overstreet *et al.*, 1988; LeBlanc and Overstreet, 1990). In aquaculture operations, the virus causes economic losses from mass mortality in the hatchery phase as well as legal restrictions on transport of infected postlarvae for use in stocking grow-out ponds.

Mortality of penaeids from baculoviruses is not restricted to BP. The nonoccluded virus that causes baculoviral midgut gland necrosis (BMN) causes mortality of Penaeus japonicus experimentally infected as mysis or Day 2 postlarvae (PL 2), but not in that shrimp when infected as PL 9 (Sano *et al.*, 1985). The widespread monodon baculovirus (MBV) infects all stages of P. monodon and causes mortalities of that species in juveniles and senescent adults (Lightner, 1988).

BP infects the epithelium of the hepatopancreatic tubules (HP) and anterior midgut; it produces polyhedra, or tetrahedral occlusion bodies, in the nuclei of infected cells. In the later stages of infection, the hypertrophied nucleus packed with viral polyhedra ruptures from the infected cells and the polyhedra pass through the intestine with feces (Couch, 1991, and Fig. 5 therein). The amount of tissue destruction associated with the release of polyhedra depends at least on the age when infected, size of infected shrimp, and severity of infection. Consequently, the loss of significant amounts of hepatopancreatic epithelium at critical points in postlarval development results in a variety of adverse affects on the host (Couch, 1981).

The susceptibility of Penaeus vannamei to BP infection and the effects of the infection on host survival are known to be age-dependent. Infections occurring during early larval development are often acute with associated mortalities approaching 100% within 3 to 4 days after exposure to the virus (Overstreet *et al.*, 1988). LeBlanc and Overstreet (1990) found that the virus has little effect on survival of postlarvae older than 63 days, whereas younger postlarvae occasionally experience high mortalities. Lightner (1983) reported that BP infections among postlarvae and juveniles are subacute or chronic and may result in reduced feeding and growth rates of the hosts. Specific information about the effects of BP on growth and survival of postlarval and juvenile P. vannamei is limited. The chronic or subacute effects of the virus have not been adequately documented, and the effects of BP on nutritionally stressed shrimp are unknown. The purpose of this study is to provide such information on both the acute and subacute effects of BP on postlarval and juvenile P. vannamei, the stages that are typically stocked into nursery or grow-out ponds. That information is essential to a complete understanding of how BP acts as a disease agent in penaeid shrimp; consequently, it should be useful in management of aquaculture operations.

MATERIALS AND METHODS

Age and Size-Dependent Effects of BP on Growth and Survival

A series of six experiments were condusted in which groups of specific pathogen-free (SPF) Penaeus vannamei (see Wyban, 1992) of different ages (mysis 2-3 through PL 25) were experimentally infected with BP. The source of shrimp, age at infection, stocking densities, date and duration of experiment after initial viral exposure, temperature and salinity of cultures, and presence of Infectious Hypodermal

and Hematopoietic Necrosis Virus (IHHNV) in BP-exposed shrimp are listed in Table 1. Spawns of shrimp originating as SPF stocks from BP-free facilities in Hawaii were routinely monitored for BP before supplying other facilities from which we obtained shrimp. A subsample of 20 shrimp from each of the 6 experimental groups was initially examined for the presesce of BP polyhedra in the HP following the diagnostic procedures for fresh shrimp described by Overstreet *et al.* (1988). Lack of BP in any of the control groups confirms the BP-free status of our experimental stocks. Shrimp used in most of these experiments were checked for the presence of IHHNV using a gene probe developed by researchers at the University of Arizona. Because this probe was not available during the earlier phases of this study, data on the IHHNV status for all experimental groups are incomplete.

The shrimp used in each experiment were originally obtained as nauplii from one of three sources: (1) The Oceanic Institute, Honolulu, Hawaii, (2) Harligen Shrimp Farm, Los Fresnos, Texas, and (3) Waddell Mariculture Center, Bluffton, South Carolina. Nauplii were placed in 200-liter rectangular glass aquaria containing 150 liters of 30-ppt salt water produced from hw-Marinemix (Hawaiian Marine Imports, Houston, Texas) and deionized water. Larvae were reared to the desired age at 27±1°C on a diet consisting of the diatom Chaetoceros neogracilis during protozoeal stages 1-3 and brine shrimp during protozoeal stage 3-postlarvae.

With the exception of the experiment with PL 14-16's, all exposures were conducted in two 200-liter glass aquaria containing 150 liters of salt water. Shrimp in one of those aquaria were exposed to BP by introducing 8 mg per liter of homogenized BP-infected postlarvae directly into the culture. The second aquarium,

Table 1

Culture Parameters for BP-Exposed and Nonexposed "Control" Treatments for Six Experimental Groups of Penaeus vannamei

Stage at infection and source of shrimp	Date and duration of experiment (days postinfection)	IHHNV status	Initial stocking density (shrimp per liter)	Salinity (ppt)	Temperature (°C)
MYSIS 2-3 (SC)	7-92 (15)	Negative	20.0	30±1	28±1
PL 8-9 (H)	7-92 (21)	Negative	8.0	25±1	27±1
PL 8-9 (SC)	7-91 (21)	N/A	8.0	25±1	27±1
PL 8-9 (T)	3-93 (21[a])	Negative	8.0	25±1	27±1
PL 14-16 (H)	4-91 (21)	N/A	5.0	20±1	27±1
PL 23-25 (T)	4-93 (21)	Negative	2.5	20 - 15[b]	27±1

Note. Source of larvae: SC = South Carolina; H = Hawaii; T = Texas. N/A = data not available.

[a]At day 21, control and BP-exposed cultures were restocked at a density of 4.0 postlarvae per liter and maintained for a total of 49 days postinfection.

[b]Salinity was gradually reduced during the course of the experiment.

which served as the source of the negative control group of shrimp, was administered an identical amount of homogenized BP-free shrimp tissue. An identical second dose of BP-infected or uninfected tissue was introduced 24 hr after the initial dose. The strain of BP used in this study was originally collected in Ecuador (see Overstreet *et al.*, 1988) from wild broodstock and pond-reared juveniles of Penaeus vannamei and then passed through numerous lots of hatchery-spawned larval P. vannamei experimentally infected at the Gulf Coast Research Laboratory. Approximately 36 hr after the initial introduction of viral material, 50 to 100 BP-exposed and corresponding negative control shrimp were removed from the 200-liter cultures, counted, and placed in separate 19-liter glass aquaria containing 12 liters of water from the original culture. These aquaria were maintained under conditions identical to the primary cultures and were used to estimate survival in the original cultures at the termination of the experiment. The experiment with the PL 14-16 group was conducted in a series of six 38-liter glass aquaria, each containing 20 liters of salt water. Three replicate aquaria were administered BP-infected tissue, and three others served as negative controls. The method and quantity of tissues administered to both the exposed and control aquaria were identical to those used for the 200-liter cultures. Survival was determined at the end of the experiment by counting the number of shrimp remaining in each of the six aquaria. All cultures were fed newly hatched brine shrimp and Zeigler pellets (Zeigler Bros., Gardners, PA) *ad libitum*. Temperature and salinity of all cultures were routinely monitored, and excess food and feces were siphoned from the cultures daily. Water was never exchanged; it

passed through biological sponge filters and received constant aeration. The culture tanks were monitored daily for mortality, and dead shrimp were removed.

Samples of 15-20 individuals were assessed for BP-infection from the 200-liter BP-exposed and control cultures several times during and at the termination of each experiment. Prevalence of infection among the PL 14-16 group was determined only at the termination of the experiment. Individual shrimp were examined for the presence of viral polyhedra following the diagnostic procedures for fresh shrimp described by Overstreet *et al.* (1988). At the termination of each experiment, each individual from a sample of 25-30 shrimp from both the exposed and control cultures was measured (total length, TL) and weighed wet. Those individual shrimp were then dried at 60°C for 48 hr and reweighed to obtain their dry weight.

Long-Term Effects of BP on Growth

A portion of one of the PL 8-9 groups of postlarvae, the one obtained from Texas, was maintained in culture for 49 days after initial exposure (postinfection, p.i.) to BP. Samples each consisting of a minimum of 30 individuals were collected from both the exposed and control cultures on Days 3, 7, 14, 21, 28, 35 and 49 p.i. Total length, as well as wet and dry weights, was determined for individual shrimp in each sample. Prevalence of infection in a sample of 20 shrimp from both the BP-exposed and control tanks was monitored daily for the first 3 days after introduction of the virus and then twice a week until termination of the experiment. Because of high mortality during the first week following introduction of the virus in the BP-exposed culture, stocking densities in both the exposed and control groups were readjusted to 4.0 shrimp per liter at Day 21 p.i. Routine removal of shrimp for

weight determinations and BP diagnosis resulted in an average reduction of the stocking densities from 4.0 to 0.5 shrimp per liter by Day 49 p.i. Salinity of the water for both the exposed and control cultures was also gradually reduced by the addition of deionized water from 25 ppt to 15 ppt between Days 14 and 35 p.i.

Effect of BP on Survival of Nutritionally Stressed Postlarvae

Upon termination of the short-term growth study using mysis 2-3 from South Carolina, we determined the prevalence of infection in an isolated group of postlarvae (PL13-14) within a standardized size range of 8-10 mm TL (estimated to be 0.3 to 0.5 mg dry weight per postlarvae) from both the control and infected cultures. Based upon examination of the entire HP from each of 20 individuals from each group, we found the negative control group to be free of BP polyhedra while the infected group exhibited a 90% prevalence of infection. Subsequently, postlarvae from control and infected groups were placed into plastic trays with 18 compartments, one postlarva per compartment. Three trays were stocked with the previously infected postlarvae and three trays with noninfected control postlarvae. The trays were placed in a constant temperature incubator and maintained for 10 days at 27°C and 25.0 $\pm$ 0.5 ppt under constant light. During this 10-day period, postlarvae in both the control and infected groups were not fed. Trays were checked daily for the presence of dead shrimp, at which time 50% of the water in each compartment was exchanged.

Analysis of Data

Size of negative control and BP-exposed shrimp during growth studies was quantified as the mean and standard deviation of the dry weights obtained from subsamples of each treatment group. Mean and standard deviation were also

determined for survival data collected during the starvation experiment. Size differences between control and BP-exposed treatments in growth experiments and differences in survival during the starvation experiments were tested for significance using the students *t* test. Differences in survival between control and BP-exposed treatments in growth experiments were tested for significance using the Chi-squared (X^2) statistic.

RESULTS

Age and Size-Dependent Effects of BP on Growth and Survival

Significant differences were observed in the short-term growth of some of the six groups of postlarval shrimp used in this phase of the study (Table 2). Significantly ($\alpha = .05$) lower final mean weights were observed for BP-exposed in comparison to control treatments when infections were initiated at either mysis 2-3 or PL 8-9. For the group of shrimp infected at PL 14-16, two replicates had slightly higher final mean weights compared to controls, but the mean weight in a third replicate was lower than the means for all control replicates. The group of shrimp exposed to BP at PL 23-25 had slightly higher final weights compared to the control treatment. The differences, however, in growth observed between BP-exposed and control treatments in neither the PL 14-16 nor PL 23-25 experimental groups were significant. Highly variable growth in postlarvae, as exemplified by the greater standard deviations relative to the mean of infected versus control treatment groups, was especially evident in all three groups of shrimp from different sources infected with BP at PL 8-9.

Table 2

Size/Age-Dependent Effects of BP on the Growth and Survival of Postlarval (PL) Penaeus vannamei

Stage at infection and source of shrimp	Preinfection dry weight (average mg per PL)	Treatment[c]	Final dry weight (Average mg per PL)	Maximum prevalence of BP (%)	Final prevalence of BP (%)	Survival %
Mysis 2-3 (SC)	0.06±.01	I[a]	0.33±.08	100	70	71[b]
		U	0.79±.54	0	0	98
PL 8-9 (H)	0.20±.09	I[a]	1.40±1.74	100	78	89
		U	3.32±2.37	0	0	95
PL 8-9 (SC)	0.18±.05	I[a]	2.75±3.04	95	80	95
		U	4.67±2.79	0	0	98
PL 8-9 (T)	0.20±.06	I[a]	2.61±3.03	100	75	66[b]
		U	5.80±3.52	0	0	100

Note. Mean weights and standard deviations (SD) were determined from a sample of 25 or more individuals, and the source of shrimp was SC = South Carolina; H = Hawaii; T = Texas.

[a]Denotes significant difference between paired treatments by students t-test at $\alpha = .05$.

[b]Denotes significant difference between paired treatments by Chi-squared (X^2) statistic at $\alpha = .05$.

[c]Treatment: I = Infected; U = Uninfected. N/A = Data not available.

Table 2 (Continued)
Size/Age-Dependent Effects of BP on the Growth and Survival of Postlarval (PL) Penaeus vannamei

Stage at infection and source of shrimp	Preinfection dry weight (average mg per PL)	Treatment[c]	Final dry weight (Average mg per PL)	Maximum prevalence of BP (%)	Final prevalence of BP (%)	Survival %
PL 14-16 (H)	0.64±.23	I-1	22.96±10.25	N/A	28	96
		I-2	22.12±11.71	N/A	36	97
		I-3	16.43±6.65	N/A	32	94
		U-1	20.56±8.56	N/A	0	98
		U-2	19.76±6.50	N/A	0	94
		U-3	20.04±6.75	N/A	0	95
PL 23-25 (T)	2.51±1.46	I	31.25±23.54	80	49	94
		U	28.90±17.36	0	0	100

Note. Mean weights and standard deviations (SD) were determined from a sample of 25 or more individuals, and the source of shrimp was SC = South Carolina; H = Hawaii; T = Texas.

[a]Denotes significant difference between paired treatments by students t-test at $\alpha = .05$.

[b]Denotes significant difference between paired treatments by Chi-squared (X^2) statistic at $\alpha = .05$.

[c]Treatment: I = Infected; U = Uninfected. N/A = Data not available.

Progression of the viral infection, determined by the presence of viral polyhedra, was monitored during each experiment, with the exception of that using the PL 14-16 group. In those experiments polyhedra appeared 18 to 24 hr after initial introduction of viral material, reached a maximum prevalence of infection 3 to 7 days p.i., and then gradually decreased in prevalence through the remainder of the culture period.

BP was also associated with lower survival in infected shrimp than in the controls in several experiments. Significantly lower survival ($\alpha = .05$) compared to controls was observed in the group infected at mysis 2-3 and one of the groups infected at PL 8-9. Massive mortality in those two groups was evident by the accumulation of dead shrimp 4 to 7 days p.i.; only an occasional dead shrimp was observed after that period.

Long-term Effects of BP on Growth

Shrimp in the BP-exposed treatment group were noticeably smaller than those in the control group by Day 3 p.i. (Fig. 1). The difference became significant ($\alpha = .05$) by Day 7 and remained so through Day 28 of the study. Standard deviation of the mean weight for the infected samples during that period was higher than the corresponding controls, reflecting the highly variable growth of infected individuals. An analysis of size class distribution during the course of the experiment (Fig. 2) demonstrates that the BP-exposed treatment group became negatively skewed compared to the control by Day 7 p.i. because of the proportionally higher number of small individuals. By Day 35 and 49 p.i. differences in size between BP-exposed and control shrimp were no longer significant.

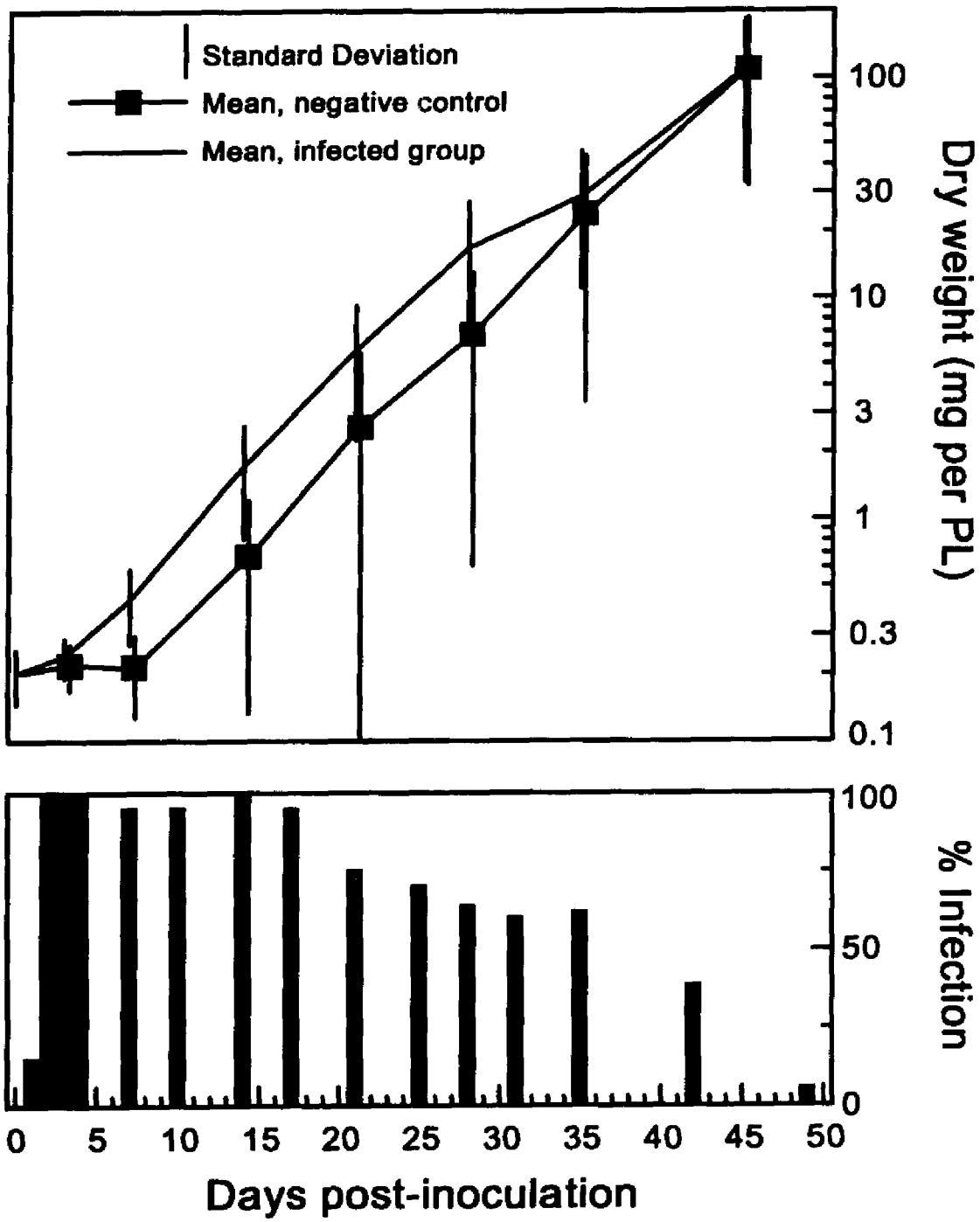

Figure 1. Effect of BP on growth rate of postlarval Penaeus vannamei over a 49-day culture period. The Y-axis for dry weight is presented as a logarithmic scale.

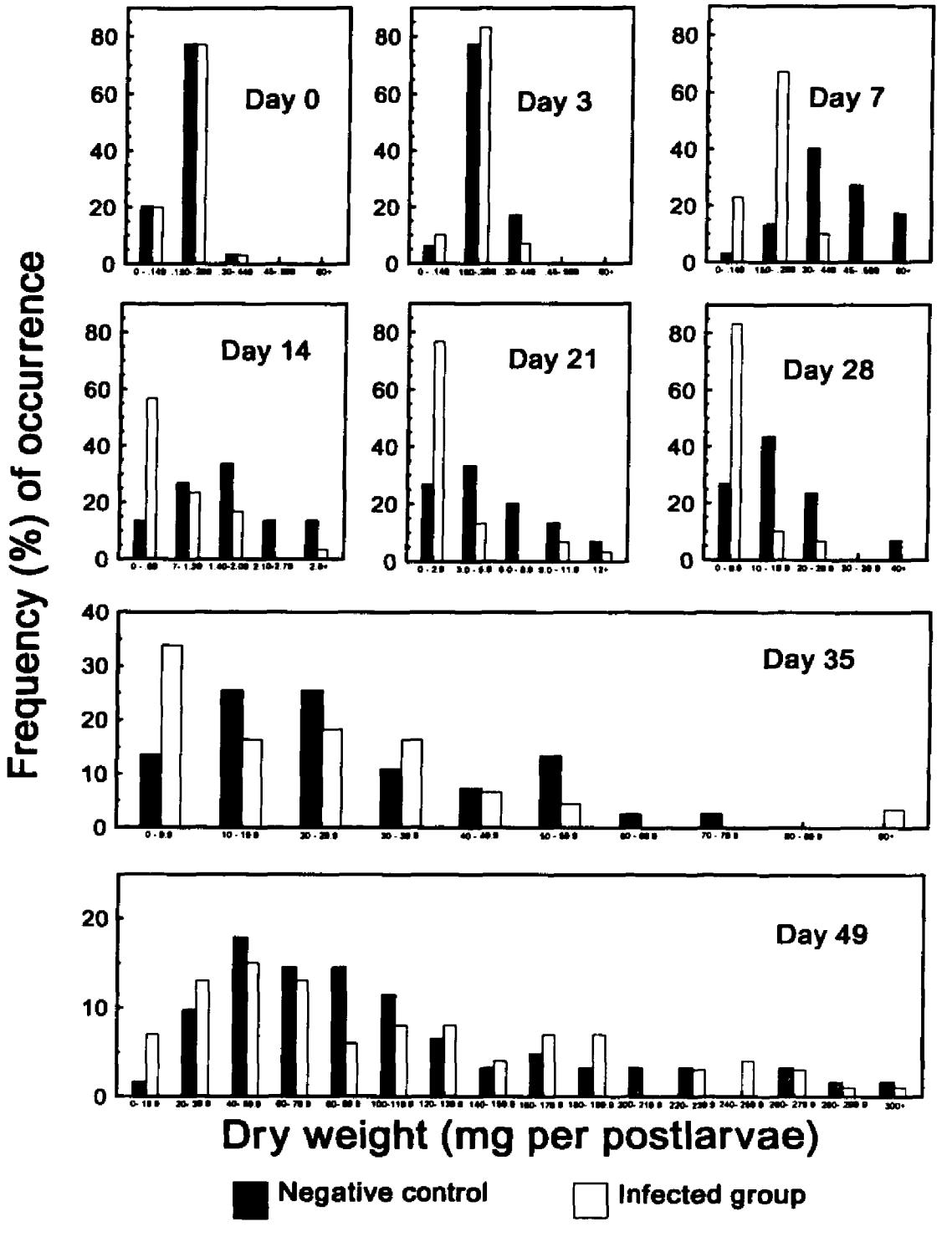

Figure 2. Weight-frequency distribution of BP-exposed and nonexposed (control) postlarvae at 0 (pre-infection), 3, 7, 14, 21, 28, 35, and 49 days postexposure. Samples taken between Day 0 and Day 28 consisted of 30 BP-exposed and 30 nonexposed shrimp, Day 35 consisted of 92 BP-exposed and 83 nonexposed shrimp, and Day 49 consisted of 98 BP-exposed and 82 nonexposed shrimp.

Viral polyhedra first appeared 24 hr after initial introduction of the virus and reached 100% prevalence of infection by 48 hr p.i. By Day 21, prevalence of shrimp with polyhedra fell to 75% and steadily decreased to 6% by Day 49. At Day 35 p.i. prevalence of infection by size class of shrimp was determined (Fig. 3), with the highest value (85%) in the smallest weight group. A substantially smaller size in the unmeasured HP of infected individuals compared to that in uninfected individuals of the same size was common in most examined individuals between Days 7 and 28 p.i.

Effect of BP on Survival of Nutritionally Stressed Postlarvae

The survival of starvated BP-infected compared with starved uninfected postlarvae over a 10-day period was significantly lower ($\alpha = 0.5$) by the second day of starvation and remained significantly lower through the duration of that period (Fig. 4). From Days 3 through 6 of starvation, the mean survival of the negative control group remained relatively constant (92-94%), while the survival of the infected group fell rapidly from 81 to 36%. By Day 10 of starvation, only 1 postlarva survived of the original 54 stocked, compared to 28 of the 54 stocked as the control group.

DISCUSSION

Results of this study corroborate the findings of previous studies that the pathogenic effects of BP are somewhat dependent on the age of Penaeus vannamei when the shrimp are initially infected, and those results provide additional details on this relationship. Overstreet *et al.* (1988) reported that the prevalence of infection and mortality of shrimp experimentally infected with BP at the protozoeal or mysis stages approached 100%, whereas older shrimp were more difficult to infect. LeBlanc and

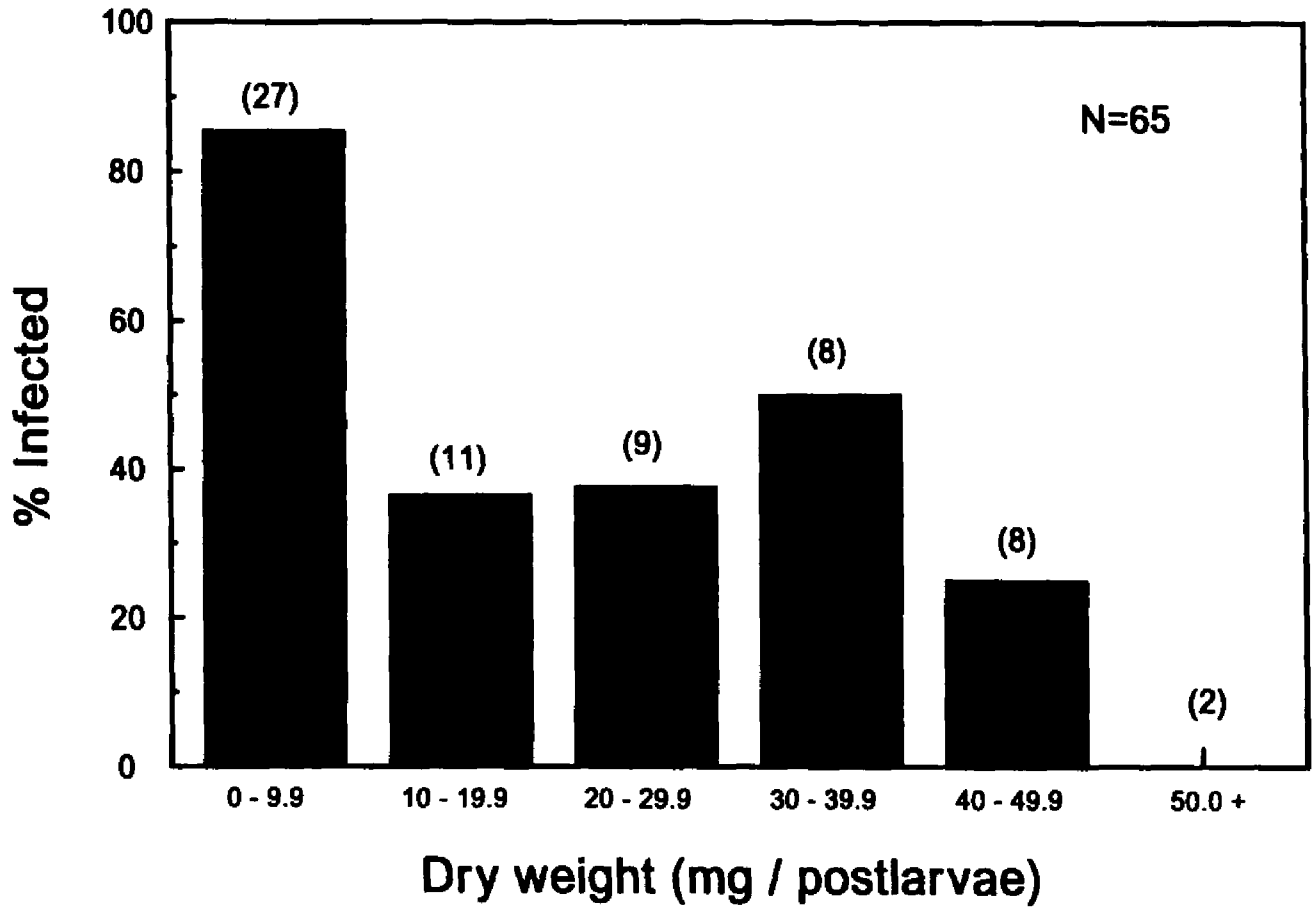

Figure 3. Prevalence of BP-infected larvae by weight class 35 days post-exposure. N = total number of shrimp examined. The number in parentheses represents the actual number of shrimp in each weight class.

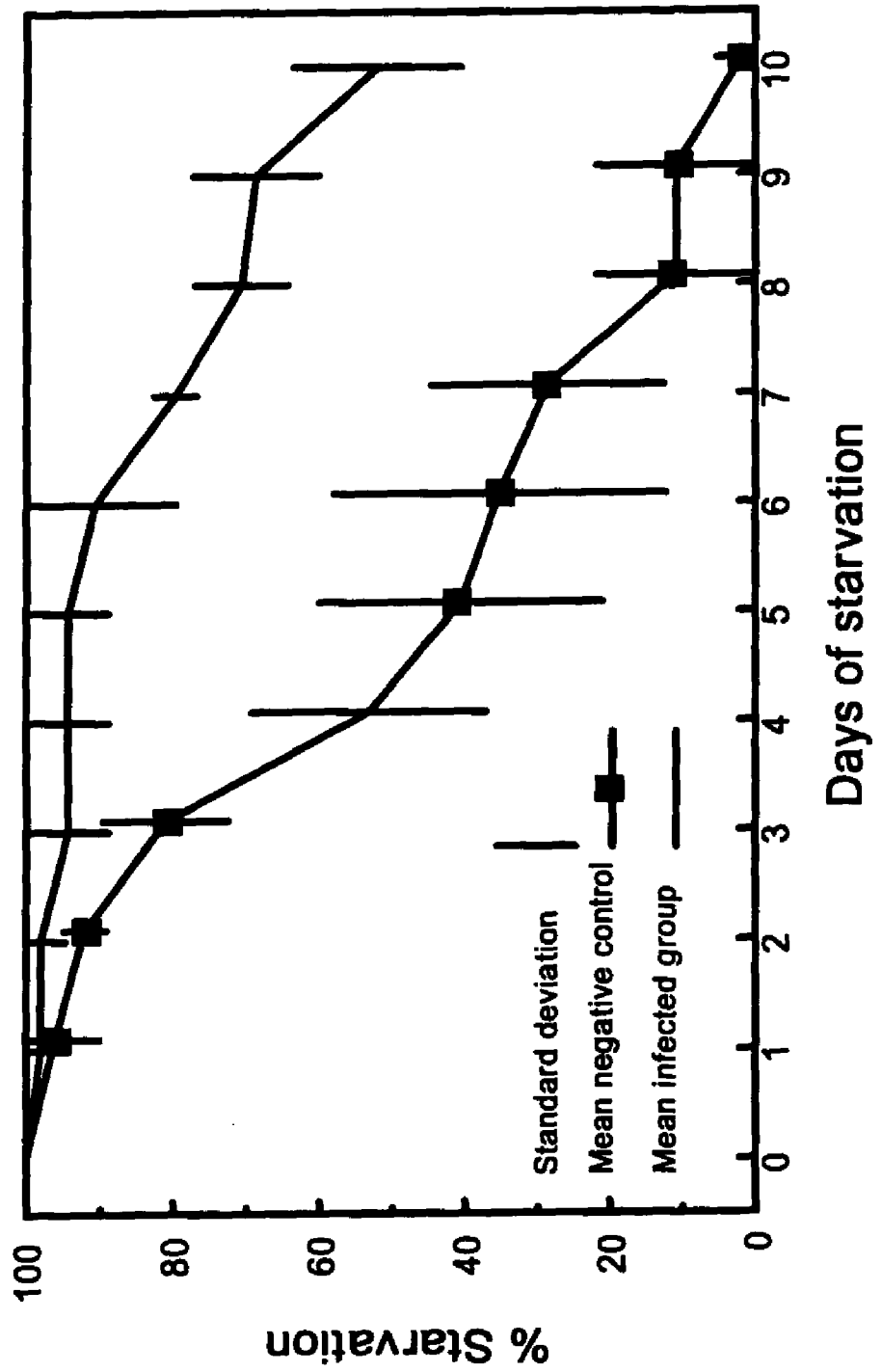

Figure 4. Survival of BP-infected and noninfected postlarvae of Penaeus vannamei during a 10-day starvation period.

Overstreet (1990) experimentally infected seven groups of P. vannamei at ages ranging from 3 to 454 days after reaching postlarvae. Three-day-old postlarvae (PL 3) became 100% infected within 9 days after exposure to the virus, and those shrimp experienced 100% mortality by 14 days p.i. Older postlarvae (PL 39) exposed to BP developed a 30% prevalence of infection and did not experience the extensive mortality observed in the younger group. Based on those data and our preliminary observations, we designed experiments to investigate infections during the first few months of postlarval development. Since previously published studies were restricted to the pathogenic effects of BP in terms of prevalence of infection, extent of polyhedra in the individual HP, and occurrence of significant mortality in cultured populations, we stressed the effects on shrimp growth immediately following BP infection, long-term growth of infected postlarvae, and depletion of energy reserves.

Pathogenicity of BP in Penaeus vannamei appears to undergo a change from the host-age range of mysis 2-3 to PL 23-25. This trend is not readily evident from data on prevalence of infection or mortality alone, but it is best demonstrated by data on growth. By combining our observations with those of previous studies (Overstreet *et al.*, 1988; LeBlanc and Overstreet, 1990), we noticed a distinct age-dependent pattern of disease. The effect of BP on larvae and early postlarvae is often acute, and it frequently, but not always, concludes in mortality. Should postlarvae become infected with BP at approximately PL 8-9, the age at which they are commonly stocked into nursery or grow-out ponds, the response by the shrimp to the virus is primarily subacute, resulting in slow and highly variable growth immediately following infection. Exposure of older postlarvae to BP may produce a high

prevalence of infection, associated with little recognizable effect on either survival or growth rates. Juveniles and subadults are less susceptible to infection, and they may become completely resistant to infection. This pattern of pathogenicity is by no means invariable, and factors, such as culture conditions, virulence of virus, and host nutritional condition at the time of infection, may have considerable influence on the pattern of disease. For example, LeBlanc and Overstreet (1990) reported 100% mortality of a 63-day-old group of postlarvae 16 days after exposure to BP, although the maximum prevalence of infection recorded was only 42% at 14 days.

The overall detrimental effect of BP on long-term growth of postlarvae infected at PL 8-9 appears to be minimal. Infected postlarvae showed little or no growth for a short period of time immediately following exposure to the virus, and moderate mortality (estimated to be about 25%) occurred from 5 to 7 days following exposure to the virus. Of particular interest is the substantial difference in weight frequency of BP-exposed and control treatment groups by Day 7. The selective absence of large individuals among the BP-exposed group suggests that the virus is initially most pathogenic to faster growing individuals. We have made similar unpublished observations for protozoeae of Penaeus vannamei exposed to BP. After 7 days p.i., growth observed in the infected culture group closely paralleled and eventually equalled that of the uninfected culture group; still, growth within the infected group remained highly variable, as indicated by the standard deviations. Some variability, as indicated by the PL8-9 tests, may relate to source of larvae, date when tested, and possibly other factors.

The significant reduction in prevalence of viral infection that we observed from 21 to 49 days p.i. corroborates similar findings reported by LeBlanc and Overstreet (1990). The reduction in prevalence of infection with time in our study generally corresponded with the appearance by Day 35 of a few large, rapidly growing individuals in the BP-exposed cultures. Weight-frequency analysis of the prevalence of infection in the exposed group at day 35 showed that the largest individuals had the lowest prevalence of infection. Those data support our unpublished observations that, once BP-infected shrimp have lost or significantly reduced the extent of their infection, they have the potential for accelerated growth.

Based on our results we suggest that BP can substantially reduce energy reserves in postlarval Penaeus vannamei and that reduction may contribute to the high mortality of young infected individuals in response to nutritional stress. The HP, the primary organ infected by BP, is the major site of lipid storage in decapods (Gibson and Barker, 1979). O'Leary and Matthews (1989) reported that the highest level of triacylglycerides, the class of lipids utilized primarily as energy reserves, in P. monodon occurs in the HP. Vogt (1992) observed that in advanced stages of a different baculovirus infection (MBV), P. monodon exhibited reduced lipid reserves. He speculated that lipids in the HP were being utilized to supply energy for viral replication. We have also observed a similar reduction in the number and size of lipid droplets in the HP cells of BP-infected larvae and postlarvae of P. vannamei immediately following exposure to the virus. The association between lipid reserves and viral replication may be related to our observation that, among larvae and early postlarvae, the larger or more rapidly growing individuals, those that are likely to

have substantial energy reserves, develop a patent infection faster and are initially more susceptible to the harmful effects of the virus than are slower growing individuals. However, once the infection is established and lipid reserves are depleted, small shrimp are most susceptible to the harmful effects of the virus, especially under conditions of nutritional stress.

Reduction in the size of the HP in infected postlarvae compared to that of uninfected individuals from the same size group probably resulted from the destruction of infected HP cells as nuclei containing polyhedra are released into the lumen of the midgut. We have made similar observations for protozoea and mysis stages infected with BP. The loss of hepatopancreatic tissue further reduces the capacity of the HP to store or utilize energy reserves and probably contributes to the high mortality observed among nutritionally stressed postlarvae.

The age-dependent pathogenic response to BP observed in this and previous studies may also be related to the ontogeny of the HP in penaeid shrimps. Lovett and Felder (1989) reported the period from PL 1 - PL 10 as a "critical" time during which high rates of mortality are often observed in cultured penaeid shrimps. During this period, the entire digestive system is undergoing extensive morphogenesis, and the ratio of HP to body weight is extremely low. In cultured Penaeus setiferus, there is no significant change in the volume of the HP from mysis 2 through PL 4, and the rate of increase in the HP volume does not equal that of the body until about PL 10, when tubule ramification of the HP becomes significant. We noticed a similar pattern of HP development in P. vannamei (see Overstreet *et al.*, 1988). The loss of a significant portion of the HP resulting from viral infection during this critical period

of development may account for much of the acute pathogenicity observed among larval and early postlarval shrimp. The relationship between development of the HP in larval shrimp and acute effects of BP was first proposed by Couch (1981). Infections occurring after this period may be less acute because of the accelerated growth of the HP and that organs ability to quickly replace virally damaged cells. Age-dependent growth data from this study generally supports this theory.

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BIOCHEMICAL RESPONSES DURING STARVATION AND SUBSEQUENT RECOVERY IN POSTLARVAL PACIFIC WHITE SHRIMP, PENAEUS VANNAMEI

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ABSTRACT

Postlarval shrimp, Penaeus vannamei Boone 1931, were held individually in cages and exposed to two feeding regimes. One group was starved for 12 d and then fed during the following 12 d. A second group was fed throughout the 24-d study. Four individuals were sampled from each of the two groups on days 0, 1, 2, 4, 8, 12, 13, 14, 16, 20, and 24. Molting and growth among the starved-fed postlarvae stopped after 2 d of starvation, while fed postlarvae increased significantly in size during the 24-d study. Among the starved-fed postlarvae, water content increased rapidly in response to starvation. DNA and sterol concentrations increased significantly during starvation due to selective catabolism of cellular components. After 12 d, RNA concentration was not significantly different between the starved-fed and fed postlarvae, but became significantly higher in the starved-fed postlarvae 48 h after feeding resumed. Triacylglycerol reserves were severely depleted during the first day of starvation, while protein concentrations began to decrease after the second day of starvation. Protein, RNA, and the polyamines, spermidine and spermine, when expressed as a ratio to DNA, decreased in response to starvation. Concentrations of all measured parameters in the starved-fed postlarvae returned to levels similar to the fed group 8-12 d after feeding resumed. Results of this study suggest that triacylglycerol provides energy during short periods of starvation, while protein is utilized during prolonged starvation. The ratios of RNA:DNA, protein:DNA, spermidine:DNA, spermine:DNA, % water content, and two unidentified amine compounds are all useful indicators of prolonged nutritional stress in postlarval P. vannamei.

INTRODUCTION

Crustaceans occasionally experience nutritional stress, particularly during the planktonic phases of larval and early postlarval development (Anger and Dawirs 1981). The ability to withstand and recover from periods of nutritional stress is an important adaptation for survival of any organism that must sporadically endure limited food supply. Starvation during early development has been recognized as an important factor influencing successful recruitment of decapods (Dawirs 1987). While several studies have investigated the effects of starvation on reptant megalopae or postlarvae (Anger and Dawirs 1981; Anger 1984; Sasaki et al. 1986), relatively little information is available on the effects of starvation among natant postlarvae. In wild populations, postlarval shrimp in transition from planktonic to benthic habitats may experience nutritional stress, similar to that suggested for lobsters and crabs (Sasaki et al. 1986; Anger et al. 1989), while searching for a suitable habitat and adopting new feeding behavior. In aquaculture operations, postlarvae used as seed shrimp may experience nutritional stress during handling, shipping, and stocking into grow-out ponds.

Biochemical responses to nutritional stress induced by prolonged starvation have been examined in a number of crustacean groups including crabs (Heath and Barnes 1970; Wallace 1973; Wang and Stickle 1986), lobsters (Dall 1974, 1975; Sasaki 1984; Juinio et al. 1992;), isopods (Steeves 1963; Alikan 1972), crayfish (Speck and Urich 1969; Hazlett et al. 1975), and several planktonic species (e.g. Hassett and Landry 1988, 1990; Virtue et al. 1993). The effects of starvation on the utilization of energy reserves have been examined for sub-adult and adult Penaeus

duorarum (Schafer 1968), P. japonicus (Cuzon et al. 1980), and P. esculentus (Barclay et al. 1983; Chandumpai et al. 1991). Moss (1994a) investigated the effects of starvation on nucleic acid content in juvenile P. vannamei. There is no information on the utilization of energy reserves during nutritional stress in postlarval penaeids, or the relationship between energy utilization and biochemical growth indicators, such as nucleic acids.

A variety of biochemical parameters have been used to assess the effects of starvation or nutritional stress in decapod crustaceans. Dall (1975) reported a reduction in dry weight and corresponding increase in tissue water content as a response to starvation in the lobster, Panulirus longipes. A reduction in RNA:DNA ratios in response to starvation was observed in postlarvae of the lobster, Homarus americanus (Juinio et al. 1992), the crab, Callinectes sapidus, (Wang and Stickle 1986), and juvenile Penaeus vannamei (Moss 1994a). In P. esculentus, the effects of starvation were characterized by significant reductions in total lipid and protein (Barclay et al. 1983). Rapid utilization of triacylglycerol (TAG) reserves in response to starvation has been reported in sub-adult P. esculentus (Chandumpai et al. 1991) and larval H. americanus (Sasaki 1984). TAG content has been used to assess growth and nutritional condition of snow crab, Chionoecetes opilio, zoeae (Lovrich and Ouellet 1994) and predict survival of shrimp, Pandalus borealis, larvae (Ouellet et al. 1992). Fraser (1989) proposed the use of a TAG:sterol ratio as an indicator of nutritional stress in crustaceans. Numerous studies (Jungreis 1968; Schafer 1968; Heath and Barnes 1970; Vogt et al. 1985), have reported that carbohydrates are a minor energy reserve in crustaceans and contribute little or no energy during

starvation. Other biochemical parameters that have potential value in assessing nutritional stress are the polyamines and related amine compounds. The polyamines putrescine, spermidine and spermine are organic cations associated with nucleic acid and protein synthesis (Heby 1981) and have been used as biochemical markers of normal and pathological cell growth in vertebrates (Pegg 1988). Relatively few studies have investigated the relationship between polyamines and growth or condition of aquatic organisms. The effects of toxic chemicals, temperature induced stress, and diet on polyamine metabolism have been investigated in fish (Corti et al. 1988; Davalli et al. 1990). Watts et al. (1992) and Stuck et al. (1994) proposed the use of polyamine ratios to assess the condition and growth potential of postlarval shrimp.

In this study we monitored changes in % water content, RNA, DNA, total protein, triacylglycerol, sterol, polyamine, and related amine content during the course of starvation and subsequent refeeding of individual postlarval shrimp. Our primary objective was to identify biochemical changes that occur during prolonged starvation and subsequent recovery in postlarval Penaeus vannamei. We also wanted to investigate the inter-relationship of selected biochemical parameters in response to changing nutritional conditions and identify potentially useful biochemical indicators of nutritional stress.

MATERIALS AND METHODS

Culture experiment

Specific pathogen free (SPF) nauplii of Penaeus vannamei (see Wyban et al. 1992) were obtained from the Oceanic Institute, Honolulu, Hawaii, and reared in a 200-liter glass aquaria containing 30 ppt artificial seawater produced from hw-

Marinemix (Hawaiian Marine Imports, Houston, Texas) and deionized water. Nauplii, stocked at an initial density of 100 l^{-1}, were reared to postlarvae at 27 $\pm$ 1C° on diets consisting of the diatom Chaetoceros gracilis during protozoeal stages 1-3, brine shrimp during protozoeal stage 3-early (10d) postlarvae, and brine shrimp and No. 1 Zeigler postlarval shrimp pellets (Zeigler Bros., Gardners, PA) for older postlarvae. All diets were provided ad libitum. When shrimp reached the postlarval stage, biological sponge filters were placed in the culture tanks and stocking densities were reduced to 20 postlarvae $liter^{-1}$. Postlarvae were reared together for 30 d until they reached an average size of approximately 150 mg wet weight (based on the average weight of a sub-sample of 10 individuals). During the first 14 d of that period, salinity was gradually reduced ($<$ 1.0 ppt d^{-1}) to 20 ppt to maintain optimal growth conditions (Ogle et al. 1992).

Postlarvae ranging in size from 100 to 200 mg were placed in 10-cm diameter cages constructed from glass petri dishes and 1-mm nitex netting. Two groups, each consisting of 48 individually caged postlarvae, were placed into separate 200-liter aquaria containing 20 ppt salt water at 27 $\pm$ 1C°. Both groups were initially maintained on Zeigler pellets for 2 d, after which all of the excess food and feces were siphoned from the cages of one group. That group, referred to as "starved-fed" postlarvae, was maintained without food until the shrimp appeared very lethargic (12 d) and then fed pellets for the next 12 d. The second group, referred to as "fed" postlarvae, were provided pellets ad libitum throughout the 24-d study. Both groups were checked daily for mortalities and molting, and any excess food and feces were siphoned from the cages immediately before the next feeding. On days 0, 1, 2, 4, 8,

12, 13, 14, 16, 20 and 24, 4 postlarvae that had not molted within the past 24 h were sampled at random. Each sample was rinsed with deionized water and placed on a piece of Whatman No. 1 filter paper for about 10-15 sec to remove external moisture. Individual postlarvae were weighed to the nearest mg, placed in 2 ml cryo-vials, and frozen in liquid nitrogen. Frozen postlarvae were stored at -70C°, for later biochemical analysis.

Biochemical analysis

In this study only the cephalothorax (head) of the postlarvae was used for biochemical analysis. Preliminary investigations and previous studies suggested that the hepatopancreas (HP), located in the head, is the organ most responsive to short-term nutrient deprivation and that many of the energy reserves are stored there. For example, the concentration of TAG in the HP of a 0.5 g Penaeus vannamei was 471.0 $\mu g \cdot mg^{-1}$ dry weight (dw) compared to 0.80 $\mu g \cdot mg^{-1}$ dw in tail muscle. RNA concentrations and RNA:DNA ratios were also substantially higher in HP (92.72 $\mu g \cdot mg^{-1}$ dw and 36.5:1, respectively) compared to tail muscle (36.6 $\mu g \cdot mg^{-1}$ dw and 2.11:1, respectively). Chandumpai et al. (1991) reported that TAG in P. esculentus is present in substantial amounts only in the HP and is rapidly exhausted after short periods of starvation. Watts et al. (1992) reported that polyamine levels in postlarval P. vannamei were generally much higher in the head than in the tail and concluded that biochemical changes in the head reflect growth and development more than total body. Therefore, to optimize the detection of an early starvation response in this study, only the heads were used for biochemical analysis.

Each head was weighed to the nearest 0.1 mg and homogenized in 20 volumes, or a minimum of 1200 μl of cold distilled water using a small hand-held electric tissue grinder. Each sample was then briefly sonicated using a VirTis model 50 sonicator (10 - 0.5 sec pulses) and partitioned for the different biochemical analyses.

Two 50-μl aliquots of each head were dried in pre-weighed aluminum micro weigh pans at 80 C° for 24 h. After cooling to room temperature in a desiccator, each pan was re-weighed to the nearest 0.1 ug using a Cahn Electrobalance. The total dry weight of the head was calculated using the equation: TDWT = DWT (V_1/V_2) where TDWT = total dry weight, DWT = dry weight of the sample (from 50-μl aliquots), V_1 = total volume of the homogenate, V_2 = volume used to determine dry weight. Percent water content of the head was calculated as (1-TDWT/wet weight of head)·100.

A 25-μl aliquot was used for protein determination. Protein concentration was determined using a Bio-Rad protein assay kit, which is based on the Bradford method (Bradford 1976), and bovine serum albumin as the standard.

Two 200-μl aliquots of each sample were used for nucleic acid determinations, one for DNA and the other for RNA. Each aliquot was prepared as described by Wang et al. (1993). DNA and RNA concentrations were determined using the diphenylamine procedure (Burton 1956) and Schmidt-Thannhauser procedure (Munro and Fleck 1966), respectively, as described by Wang et al. (1993).

A 400-μl aliquot of each sample was used for lipid extraction following the procedures of Bligh and Dyer (1959). An internal standard, palmitic acid propyl ester, was added to the extracted lipids, and the entire sample was concentrated by blowing

gently with a stream of N_2 gas. Neutral lipids were separated by thin layer chromatography on Type S-III chromarods using the solvent system dichloroethane:chloroform:acetic acid (926:31:1). TAG and sterol were quantified using an Iatroscan TH-10 Mark IV. The concentrations of TAG, sterol, RNA, DNA, and protein were calculated and expressed as $ug \cdot mg^{-1}$ dry weight of the head.

A 200-μl aliquot of each sample homogenate was used for polyamine determinations. Polyamines were solubilized by the addition of 8 μl of 11.5 N perchloric acid (final concentration 0.45 N) and incubated on ice for a minimum of 20 min. Samples were then centrifuged at 13,000 RFC at 4 C°, after which the supernatant containing the polyamines was recovered. The samples were then derivitized with dansyl chloride and analyzed for polyamine content by high performance liquid chromatography following procedures described by Watts et al. (1994). The concentrations of putrescine, spermidine, and spermine were calculated and expressed as $nmole \cdot g^{-1}$ dry weight of the head.

Statistical analysis

The experimental design used in this study may represent pseudoreplication as described by Hurlbert (1984). However, based on historical comparisons of penaeid shrimp culture in our laboratory, tank effects are substantially less than the extreme treatment (fed vs starved) effects examined here. Consequently, differences in biochemical parameters between starved-fed and fed postlarvae were tested for significance using t-tests and Bonferroni 95% t-critical values. Comparisons were made at each sampling time. Correlation values (r) between paired parameters in

either the fed or starved-fed postlarvae were determined by simple linear regression. Analysis of variance was used to test for significance of the regressions.

RESULTS

Growth and survival

Over the 24-d study, the starved-fed postlarvae exhibited little or no growth compared to fed postlarvae (Fig. 1). Between the second and final day of the study, including the 12-d period after feeding resumed, molting was not observed among starved-fed postlarvae. After 12 d of starvation, postlarvae appeared lethargic, but immediately consumed food pellets when they were offered. Fed postlarvae continued to grow throughout the study and weighed significantly more ($p \leq 0.05$) than starved-fed postlarvae by day 16. Mortalities were first observed on the 11th day among the starved-fed postlarvae. Three starved-fed postlarvae and two fed postlarvae died during the study.

Water content

The mean % water content determined from heads of the starved-fed postlarvae increased steadily from 78.7% to 87.6% after 12 d of starvation and reached a maximum of 88.3% on day 13 (Fig. 2). Two days after feeding resumed, the % water content began decreasing and reached pre-starvation levels by the final day of the study. Between days 4 and 16, the % water content of individual starved-fed postlarvae ranged from 83.5% to 91.8%. During that same time period, the % water content of individual fed postlarvae ranged from 75.1% to 82.8%. Significant differences ($p \leq 0.05$) in % water content between fed and starved-fed postlarvae were observed on days 8, 13, 14, and 16.

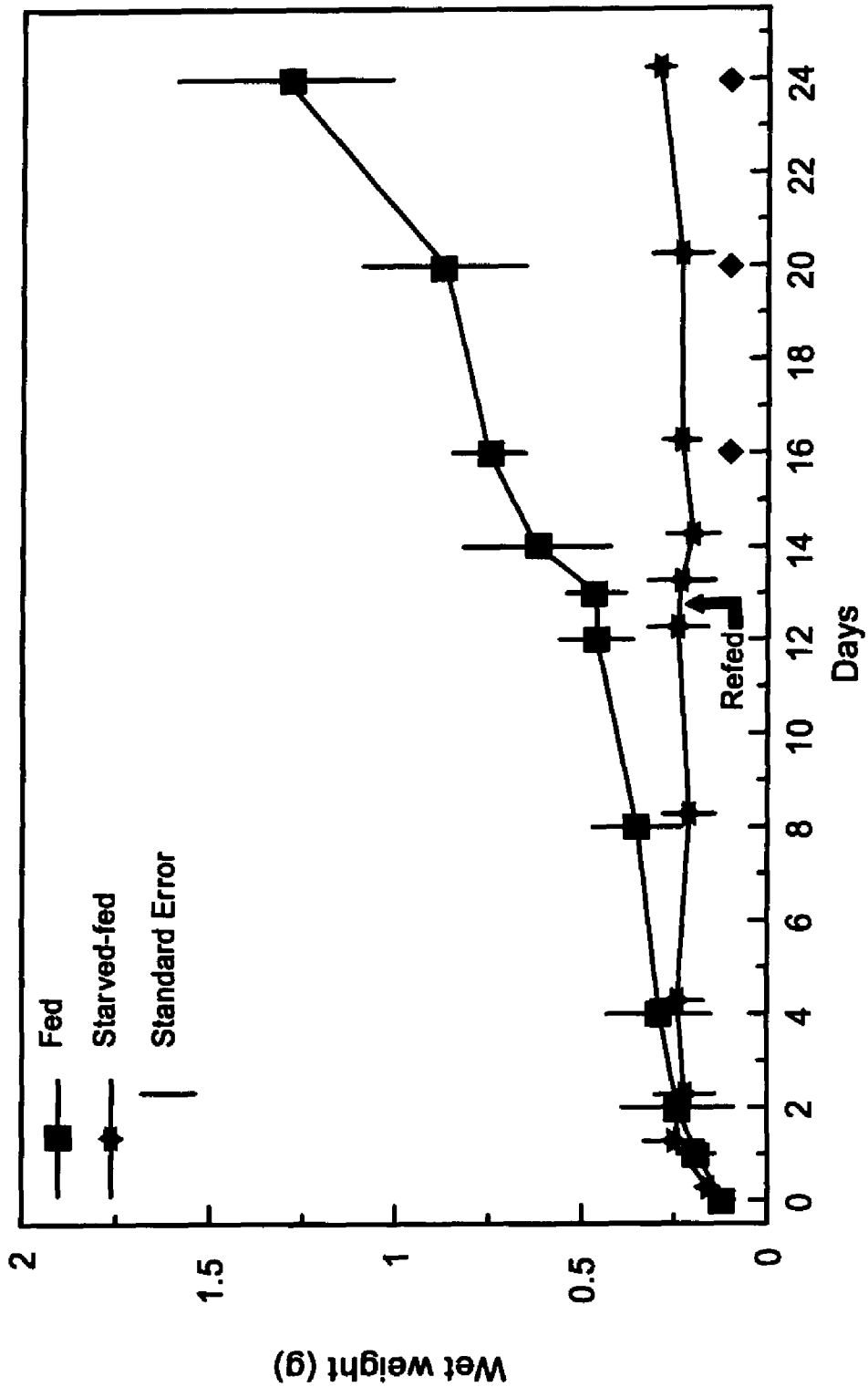

Fig. 1 Penaeus vannamei. Total body wet weight of fed and starved-fed postlarvae over 24 d. Values are expressed as means ±SE (n = 4). ◆ - denotes significant differences ($p \leq 0.5$) between fed and starved-fed postlarvae.

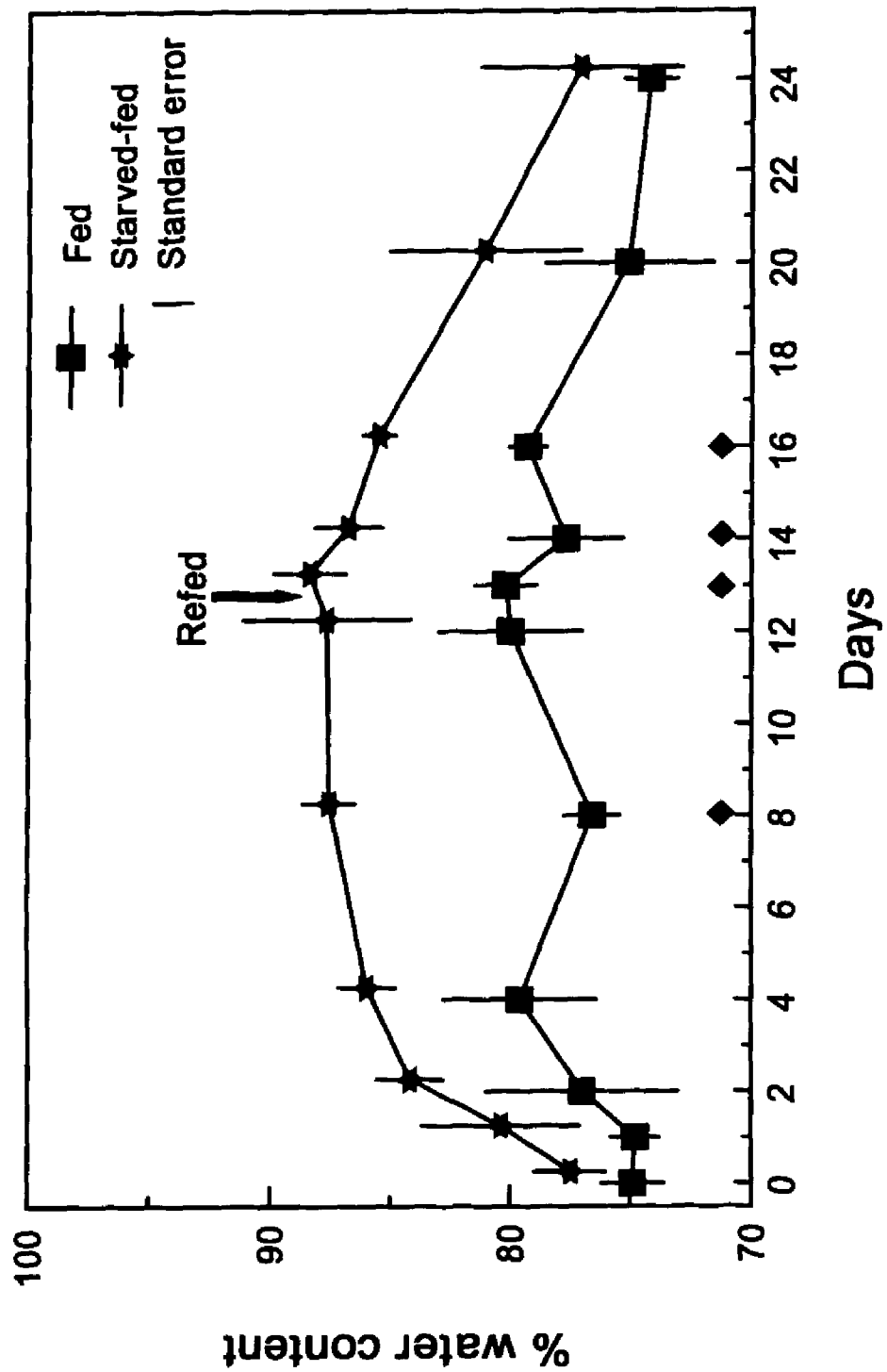

Fig. 2 Penaeus yannamei. Percent water content determined from heads of fed and starved-fed postlarvae over 24 d. Values and symbols as in Fig. 1.

Nucleic acids and protein

After the second day of the study, DNA concentrations (ug·mg dry weight^{-1}) of starved-fed postlarvae began increasing relative to the fed group and continued to increase through day 13 (Fig. 3A). The DNA concentrations of starved-fed postlarvae were significantly higher ($p \leq 0.05$) than fed postlarvae on sampling days 4 through 16 and day 24 of the study. After feeding of starved-fed postlarvae resumed, the DNA concentration decreased to pre-starvation levels by day 20. The concentration of DNA among fed postlarvae decreased through the course of the study. As postlarvae in the fed group grew, total DNA (heads only) increased from a mean of 122.1 ug on day 0 to 403.7 ug by day 24 (Fig. 3B). In contrast, after the second day of starvation and continuing through the duration of the study, the mean total DNA content of starved-fed postlarvae did not change significantly. Significant differences ($p \leq 0.05$) in total DNA content between starved-fed and fed postlarvae were observed on days 16 and 24.

RNA concentrations among starved-fed postlarvae generally decreased during the first 12 d of the study, but after 8 d were slightly higher than those of fed postlarvae (Fig. 3C). RNA concentrations were significantly higher ($p \leq 0.05$) in starved-fed compared to fed postlarvae 2 and 4 d after feeding resumed, and then declined rapidly to levels similar to the fed group by the end of the study. RNA concentrations among fed postlarvae declined steadily throughout the 24-d study.

Protein concentration of fed and starved-fed postlarvae fluctuated similarly during the first 2 d of the study (Fig. 3D), after which the concentration decreased rapidly in the starved-fed postlarvae. Protein concentration was significantly lower (p

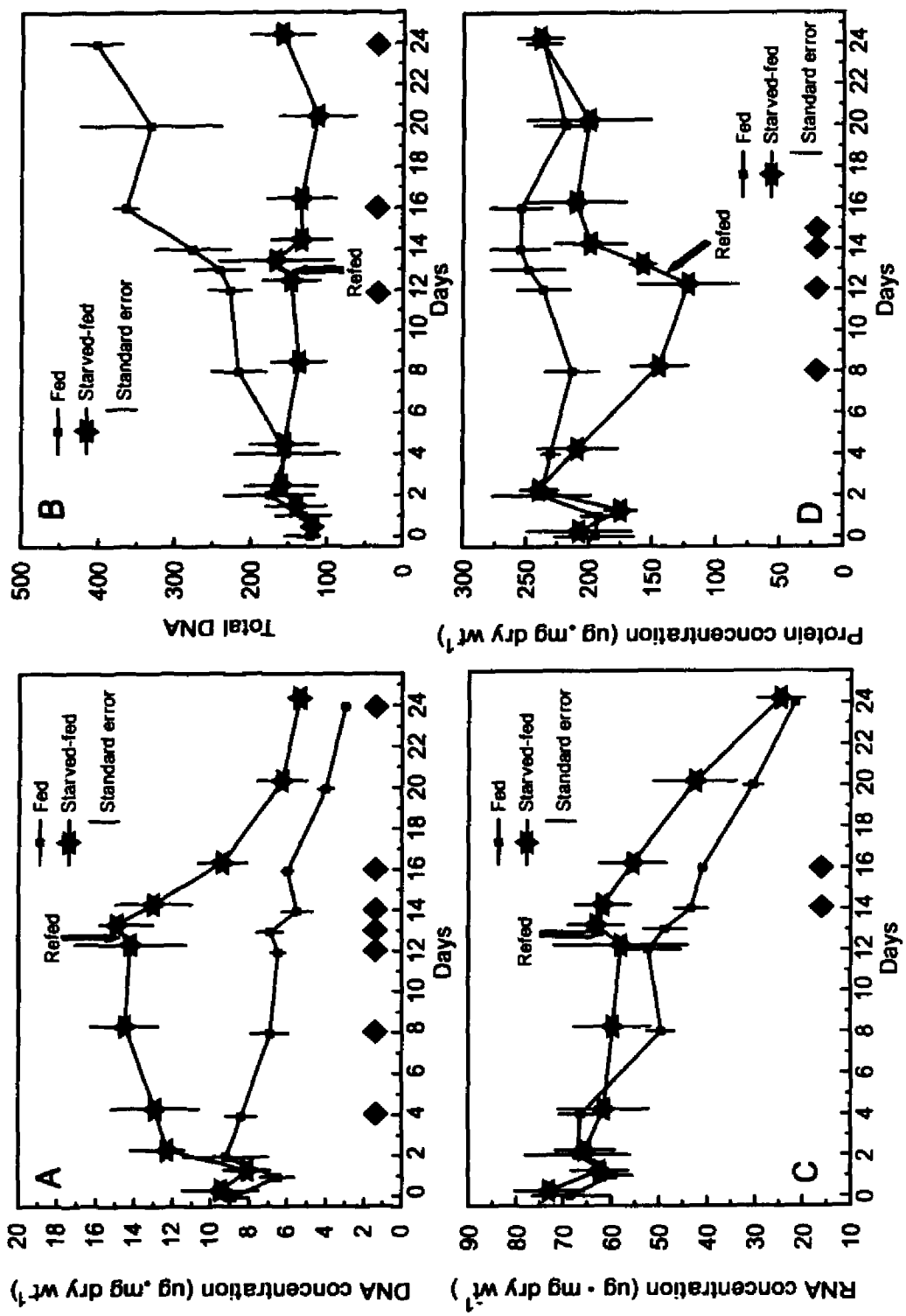

Fig. 3 Penaeus vannamei. Nucleic acid and protein levels determined from heads of fed and starved-fed postlarvae over 24 d. (A) DNA concentrations; (B) Total DNA contents; (C) RNA concentrations; (D) protein concentrations. Values and symbols as in Fig. 1.

≤ 0.05) in the starved-fed compared to fed postlarvae after 8 d of starvation and continued to be significantly lower through day 14 of the study. Protein concentration of starved-fed postlarvae increased rapidly once feeding resumed and reached a level similar to fed postlarvae by the end of the study.

We determined the correlation of RNA, DNA, and protein with % water content (Table 1). Biochemical parameters of fed and starved-fed postlarvae were tested separately for possible correlations. Percent water content was positively correlated with DNA concentration ($r = 0.74$; $p \leq 0.001$) and negatively correlated with RNA concentration ($r = -0.61$; $p \leq .001$) and protein concentration ($r = -0.36$; $p \leq 0.05$) in starved-fed postlarvae. However, % water content and protein concentration were positively correlated ($r = 0.51$; $p \leq .01$) in fed postlarvae. A positive correlation ($r = 0.66$; $p \leq 0.001$) was observed between RNA and DNA concentrations from fed, but not starved-fed, postlarvae. RNA and protein concentrations exhibited a weak negative correlation ($r = -0.39$; $p \leq .01$) among fed, but not starved-fed, postlarvae.

RNA:DNA ratios of starved-fed postlarvae rapidly declined between the first and second day of starvation and continued to gradually decrease through day 12 (Fig. 4A). Once feeding resumed, RNA:DNA ratios of starved-fed postlarvae immediately began to increase, reaching levels similar to fed postlarvae by day 16. Differences in RNA:DNA ratios obtained from the two groups were significant ($p \leq 0.05$) between days 2 and 13 of the study. The RNA:DNA ratio (Table 2) was significantly correlated with % water content among starved-fed ($r = -0.43$; $p \leq 0.01$), but not fed, postlarvae.

Table 1 Penaeus vannamei. Pairwise correlation matrix of concentrations of biochemical parameters determined from individual fed and starved-fed postlarvae. Correlation values (r) and level of significance are listed for each correlation. Abbreviations: Put - putrescine, Spd - spermidine, Sp - spermine. Level of significance: * ($p \leq .05$); ** ($p \leq .01$); *** ($p \leq .001$)

FED POSTLARVAE

	DNA	RNA	Protein	TAG	Sterol	Put	Spd	Sp	% Water
DNA	-								
RNA	0.66***	-							
Protein	-0.03	-0.039*	-						
TAG	-0.03	-0.11	-0.07	-					
Sterol	0.33*	0.24	0.36	-0.15	-				
Put	-0.01	0.04	-0.12	-0.01	0.26	-			
Spd	0.48**	0.35**	-0.08	-0.18	-0.20	-0.29	-		
Sp	0.48**	0.37*	0.15	-0.05	0.05	-0.32	0.65***	-	
% Water	0.28	-0.18	0.51***	-0.21	0.51**	0.17	-0.11	0.18	-

Table 1 (Continued) Penaeus vannamei. Pairwise correlation matrix of concentrations of biochemical parameters determined from individual fed and starved-fed postlarvae. Correlation values (r) and level of significance are listed for each correlation. Abbreviations: Put - putrescine, Spd - spermidine, Sp - spermine. Level of significance: * ($p \leq .05$); ** ($p \leq .01$); *** ($p \leq .001$)

STARVED-FED POSTLARVAE

	DNA	RNA	Protein	TAG	Sterol	Put	Spd	Sp	% Water
DNA	-								
RNA	-0.33*	-							
Protein	-0.30	0.13	-						
TAG	-0.04	-0.06	0.22	-					
Sterol	0.78***	-0.53***	-0.15	0.05	-				
Put	0.03	-0.25	-0.14	0.17	0.23	-			
Spd	-0.29	0.61***	0.30	-0.12	-0.33*	0.0 9	-		
Sp	-0.28	0.41**	0.27	0.02	-0.22	0.15	0.66***	-	
% Water	0.74***	-0.61***	0.36*	0.05	0.84***	0.40***	-0.37*	-0.17	-

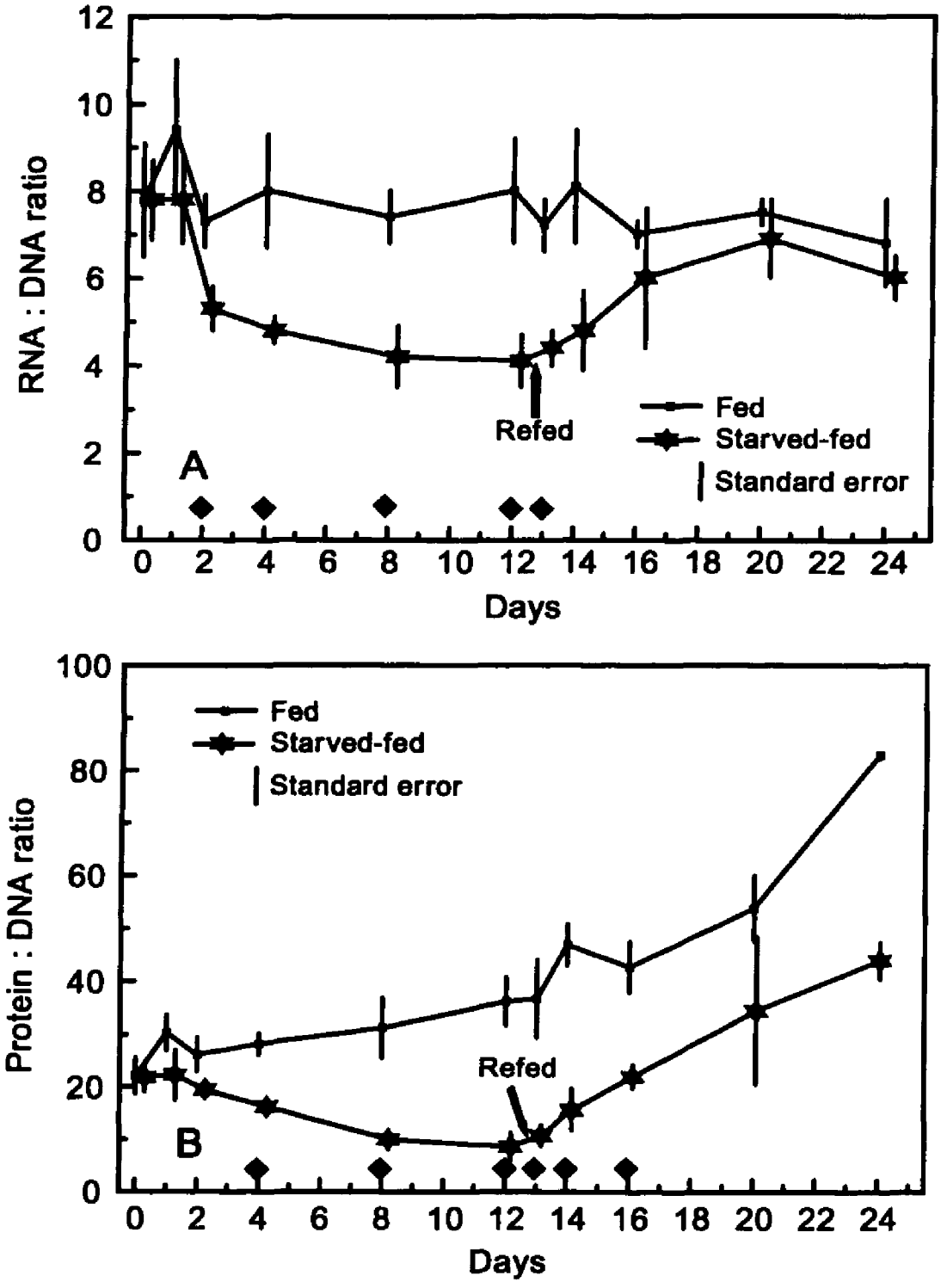

Fig. 4 Penaeus vannamei. (A) RNA:DNA ratios, and (B) protein:DNA ratios determined from heads of fed and starved-fed postlarvae over 24 d. Values and symbols as in Fig. 1.

Table 2 Penaeus vannamei. Pairwise correlation matrix of biochemical ratios determined from individual fed and starved-fed postlarvae. Correlation values (r) and level of significance are listed for each correlation. Abbreviations: Pro - protein, Str - sterol, Put - putrescine, Spd - spermidine, Sp - spermine, 3.4 - unknown amine with retention time 3.4 min, 7.5 - unknown amine with retention time 7.5. Level of significance: * ($p \leq .05$); ** ($p \leq .01$); *** ($p \leq .001$)

FED POSTLARVAE

	RNA:DNA	Pro:DNA	TAG:Str	Put:DNA	Spd:DNA	Sp:DNA	3.4:Sp	7.5:Sp	% Water
RNA:DNA	-								
Pro:DNA	0.06	-							
TAG:Str	0.11	0.15	-						
Put:DNA	0.06	0.65***	-0.08	-					
Spd:DNA	0.11	0.36*	0.02	0.19	-				
Sp:DNA	0.26	0.85***	0.22	0.51**	0.53***	-			
3.4:Sp	0.31	0.39**	0.20	0.36*	0.15	0.31	-		
7.5:Sp	-0.26	-0.19	-0.19	-0.11	0.30	-0.38*	0.05	-	
% Water	-0.24	-0.11	-0.35*	-0.10	-0.32	-0.21	-0.16	-0.16	-

Table 2 (Continued) Penaeus vannamei. Pairwise correlation matrix of biochemical ratios determinedfrom individual fed and starved-fed postlarvae. Correlation values (r) and level of significance are listed for each correlation. Abbreviations: Pro - protein, Str - sterol, Put - putrescine, Spd - spermidine, Sp - spermine, 3.4 - unknown amine with retention time 3.4 min, 7.5 - unknown amine with retention time 7.5. Level of significance: * ($p \leq .05$); ** ($p \leq .01$); *** ($p \leq .001$).

STARVED-FED POSTLARVAE

	RNA:DNA	Pro:DNA	TAG:Str	Put:DNA	Spd:DNA	Sp:DNA	3.4:Sp	7.5:Sp	% Water
RNA:DNA	-								
Pro:DNA	0.43**	-							
TAG:Str	0.04	0.26	-						
Put:DNA	0.38*	0.43**	0.08	-					
Spd:DNA	0.58***	0.75***	0.27	0.51**	-				
Sp:DNA	0.57***	0.81***	0.21	0.57***	0.84***	-			
3.4:Sp	0.16	0.53***	0.43**	0.36*	0.37*	0.34*	-		
7.5:Sp	-0.34*	-0.57***	-0.45**	-0.31	-0.54***	-0.69***	-0.52***	-	
% Water	-0.43**	-0.65***	-0.21	-0.09	-0.70***	-0.61***	-0.50**	0.57***	-

The protein:DNA ratio of starved-fed postlarvae began to gradually decrease after the first day of starvation and continued to decrease through day 12 (Fig. 4B). Once feeding resumed, the protein:DNA ratio of starved-fed postlarvae increased, but remained lower than that of fed postlarvae for the duration of the study. Differences in protein:DNA ratios between fed and starved-fed postlarvae were significant ($p \leq 0.05$) on sampling days 4 through 16 and day 24 of the study. Protein:DNA ratios of fed postlarvae steadily increased during the study period. The protein:DNA ratio (Table 2) was significantly correlated with % water content ($r = -0.65$; $p < 0.001$) and the RNA:DNA ratio ($r = 0.43$; $p \leq .01$) among starved-fed but not fed postlarvae.

Neutral lipids

After 1 d of starvation, TAG concentrations were severely depleted and after 8 d were undetectable in the starved-fed postlarvae (Fig. 5A). Within 24 h of the resumption of feeding, TAG concentrations returned to pre-starvation levels and decreased later to a concentration similar to that of fed postlarvae by the end of the experiment. Throughout the study, TAG concentrations obtained from fed postlarvae were extremely variable both within and between sampling periods. As a result of the variability, differences in TAG concentrations between the fed and starved-fed postlarvae were not significant. Mean TAG concentrations were, however, always higher in fed compared to starved-fed postlarvae during the starvation phase of the study.

In contrast to TAG, the concentration of sterol in starved-fed postlarvae increased rapidly between the first and second day of starvation, and then continued to

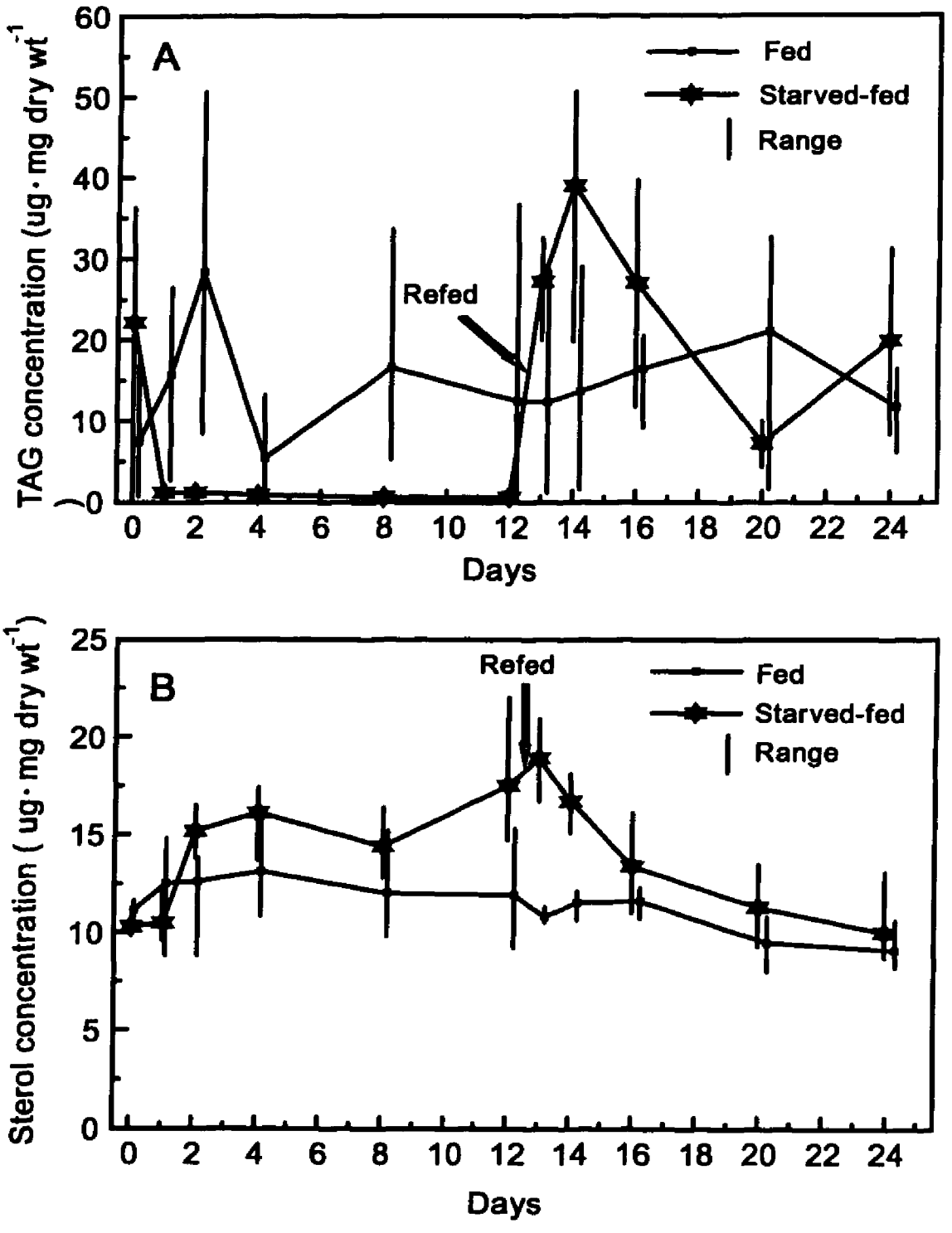

Fig. 5 Penaeus vannamei. Concentrations of neutral lipids determined from heads of fed and starved-fed postlarvae over 24 d. (A) triacylglycerol concentrations; (B) sterol concentrations. Values and symbols as in Fig. 1.

gradually increase through day 12 (Fig. 5B). One day after feeding resumed, sterol concentrations in starved-fed postlarvae began to decrease and reached levels similar to fed postlarvae by the end of the study. Significant differences ($p \leq 0.05$) in sterol concentrations between fed and starved-fed postlarvae were observed only during days 13 and 14. Among the fed postlarvae, sterol concentrations remained relatively constant through the duration of the study.

TAG and sterol concentrations from individual postlarvae were tested for possible correlations with other measured biochemical parameters (Table 1). There were no significant correlations between TAG and % water content, RNA, DNA, protein or sterol concentrations among either fed or starved-fed postlarvae. A strong positive correlation ($r = 0.84$; $p \leq 0.001$) was obtained between % water content and sterol concentration from starved-fed postlarvae, and a similar but weaker correlation ($r = 0.51$, ≤ 0.01) was observed among fed postlarvae. Sterol and DNA concentrations were positively correlated in the starved-fed postlarvae ($r = 0.78$, $p \leq 0.001$) and, to a lesser extent, among the fed postlarvae ($r = 0.33$; $p \leq 0.05$). Sterol and RNA concentrations were negatively correlated ($r = -0.53$; $p \leq 0.001$) in the starved-fed but not fed postlarvae. A weak positive correlation ($r = 0.36$; $p \leq 0.05$) was obtained between sterol and protein among fed but not starved-fed postlarvae.

Polyamines and related amines

The concentration of the polyamines, putrescine (Put), spermidine (Spd), and spermine (Sp), of both starved-fed and fed postlarvae was highly variable within sampling periods. Throughout the study, differences in polyamine concentrations between starved-fed and fed postlarvae were not significant. However, a rapid

increase in putrescine concentrations among starved postlarvae was observed immediately after feeding resumed. Polyamine ratios, including Put:Sp, Put:Spd, and Spd:Sp, were also not significantly affected by starvation.

Polyamine concentrations from individual postlarvae were tested for possible correlations with other biochemical parameters including % water content, DNA RNA, protein, TAG, and sterol (Table 1). There was a significant correlation ($r = 0.40$; $p \leq .01$) between % water and putrescine concentrations in starved-fed but not fed postlarvae, otherwise, significant correlations were not observed between putrescine and all other measured biochemical parameters. Positive correlations between spermidine and RNA concentrations were observed in both the fed ($r = 0.35$; $p \leq 0.05$) and starved-fed ($r = 0.61$; $p \leq 0.001$) groups of postlarvae. Spermidine and DNA concentrations were positively correlated in fed ($r = 0.48$; $p \leq 0.01$), but not starved-fed, postlarvae. A positive correlation ($r = 0.48$; $p \leq 0.01$) between spermine and DNA concentration was observed in the fed but not starved-fed postlarvae and between spermine and RNA in both fed ($r = 0.37$; $p \leq 0.05$) and starved-fed ($r = 0.41$; $p \leq 0.01$) postlarvae. Spermidine and spermine concentrations were also positively correlated in both starved-fed ($r = 0.66$; $p < 0.001$) and fed ($r = 0.65$; $p < 0.001$) postlarvae.

The effect of starvation on polyamine levels was evident when polyamine concentrations were expressed as a ratio to DNA concentrations (n moles polyamines:mg DNA^{-1}). After 2 d of starvation, Put:DNA ratios (Fig. 6A) were consistently lower in the starved-fed compared to fed postlarvae and immediately increased when feeding resumed; differences were significant ($p \leq 0.05$) on day 4.

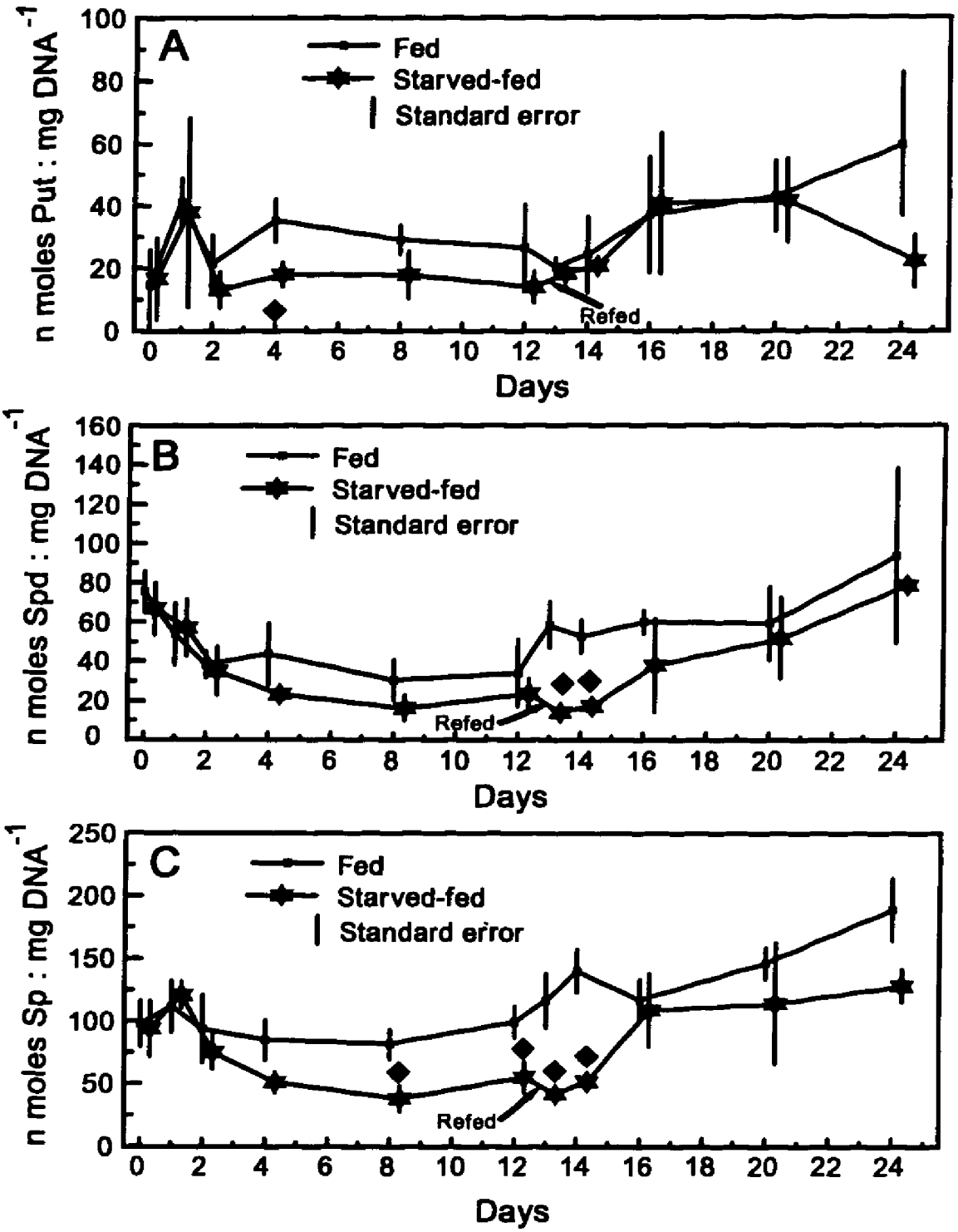

Fig. 6 Penaeus vannamei. Polyamine:DNA ratios determined from heads of fed and starved-fed postlarvae over 24 d. (A) putrescine:DNA ratio; (B) spermidine:DNA ratio; (C) spermine:DNA ratio. Values and symbols as in Fig. 1.

Spd:DNA ratios (Fig. 6B) were consistently lower in starved-fed compared to fed postlarvae between day 4 and day 24; differences were significant ($p \leq 0.05$) on days 13 and 14. Beginning with the second day of starvation and continuing through to the end of the study, Sp:DNA ratios (Fig. 6C) were lower in starved-fed compared to fed postlarvae; differences were significant ($p \leq 0.05$) between days 8 and 14. Put:DNA was positively correlated with protein:DNA ($r = 0.65$; $p \leq .001$) and Sp:DNA ($r = 0.51$; $p \leq .01$) among fed postlarvae and protein:DNA ($r = 0.43$; $p \leq .01$), Spd:DNA ($r = 0.51$; $p \leq .01$) and Sp:DNA ($r = 0.57$; $p \leq .001$) among starved-fed postlarvae (Table 2). Spd:DNA was also significantly correlated with protein:DNA ($r = 0.36$; $p \leq .05$) and Sp:DNA ($r = 0.53$; $p \leq .001$) among fed postlarvae and RNA:DNA ($r = 0.58$; $p \leq .001$), protein:DNA ($r = 0.75$; $p < .001$) and % water ($r = -0.70$; $p \leq .001$) among starved-fed postlarvae. Sp:DNA was significantly correlated with protein:DNA ($r = 0.85$; $p \leq .001$) among fed postlarvae and RNA:DNA ($r = 0.57$; $p \leq .001$), protein:DNA ($r = 0.81$; $p \leq .001$) and % water ($r = -0.61$; $p \leq .001$) among starved-fed postlarvae.

Two amines that appeared on the same HPLC chromatogram as the polyamines exhibited a definite response to starvation. The identities of those compounds were not known at the time of the assay, therefore, standards needed for their quantification were not available. For the purpose of the present analysis, these unknown amines were identified by their retention time in minutes (Fig. 7) and levels expressed as a ratio of the peak area of the unknown to spermine.

Between the first and second day of the study, the ratio of amine 3.4:Sp (Fig. 8A) from starved-fed postlarvae abruptly decreased and then continued to

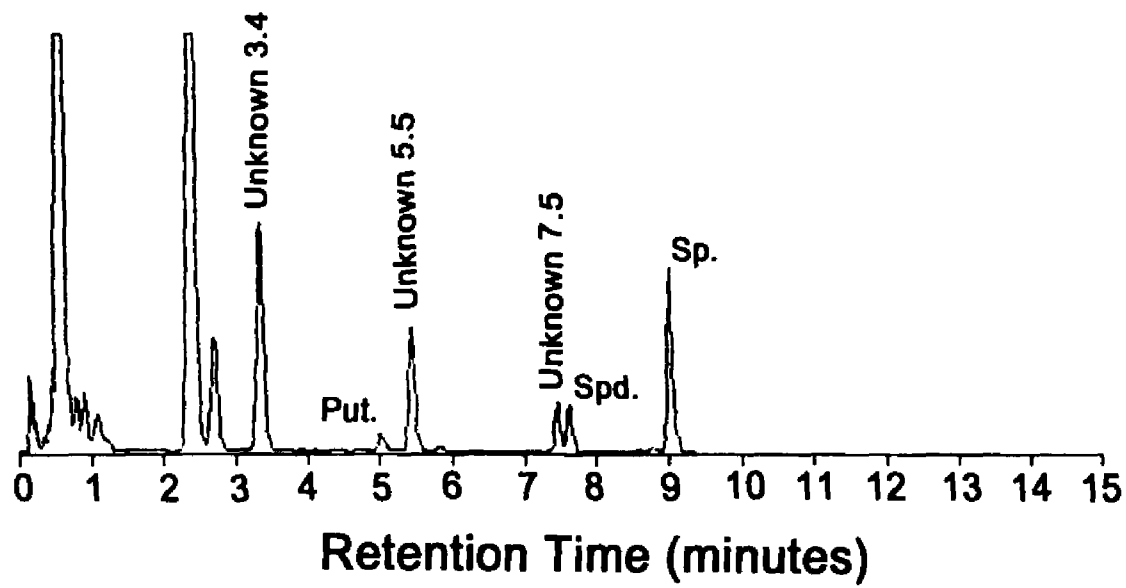

Fig. 7 HPLC chromatogram showing the relative positions of unknown amine compounds and their retention times compared to the polyamines putrescine, spermidine, and spermine.

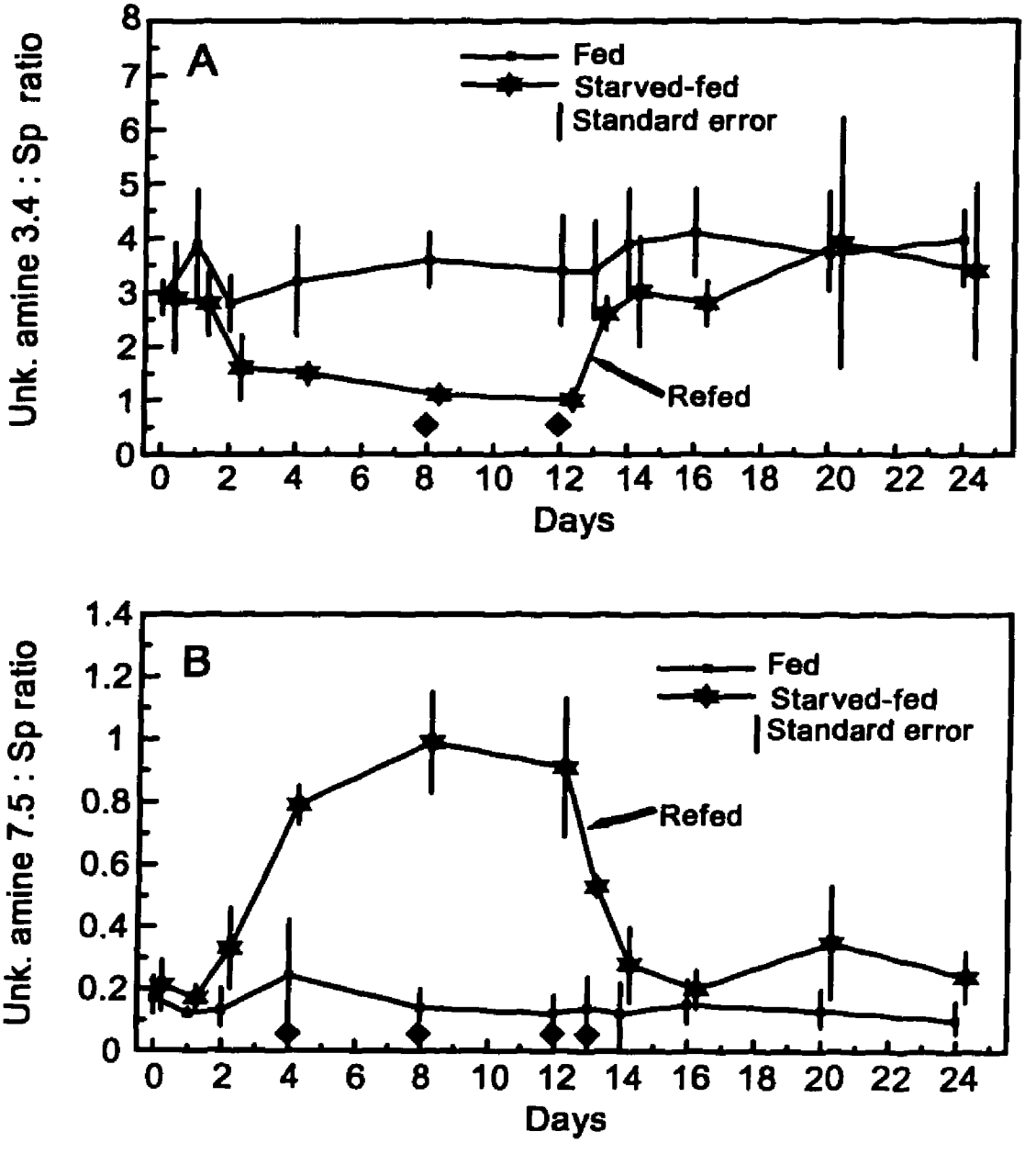

Fig. 8 Penaeus vannamei. Concentrations of unknown amines relative to spermine determined from heads of fed and starved-fed postlarvae over 24 d. (A) unknown amine 3.4:spermine ratio; (B) unknown amine 7.5:spermine ratio. Values and symbols as in Fig. 1.

gradually decrease compared to fed postlarvae during the remainder of the starvation phase of the study. Once feeding resumed, the ratio of amine 3.4:Sp quickly increased and returned to levels similar to the fed group by the end of the study. Differences in the amine 3.4:Sp ratio between fed and starved-fed postlarvae were significant ($p \leq 0.05$) on days 8 and 12 of the study.

After 1 d of starvation, the ratio of amine 7.5:Sp (Fig. 8B) began increasing in starved-fed compared to fed postlarvae and remained high though day 12. Once feeding resumed, the amine 7.5:Sp ratio rapidly declined to levels similar to fed postlarvae by day 16. By comparison, the amine 7.5:Sp ratio of fed postlarvae showed little change during the course of the study. Differences in the amine 7.5:Sp ratio obtained from starved-fed compared to fed postlarvae were significant ($p \leq 0.05$) on sampling days 4 through 13. Among starved-fed postlarvae, unknown amines 3.4:Sp and 7.5:Sp were significantly correlated with most of the other ratios and each other (Table 2). Amine 3.4:Sp was negatively correlated with % water ($r = -0.50$; $p \leq .01$) and positively correlated with TAG:sterol ($r = 0.43$; $p \leq .01$), while amine 7.5:Sp was positively correlated with % water ($r = 0.57$; $p \leq .001$) and negatively correlated with TAG:sterol ($r = -0.45$; $p \leq .01$).

DISCUSSION

Growth and survival

Crustaceans are generally able to withstand and recover from prolonged periods of starvation. Adult crayfish (Speck and Urich 1969) and lobsters (Dall 1974) have been maintained in the laboratory without food for 28 and 41 d, respectively. The adult shore crab, Carcinus maenas, can survive up to 3 mo of starvation with

only 50% mortality (Wallace 1973), and juvenile blue crabs, Callinectes sapidus, have a high capacity for recovery from prolonged starvation (Wang and Stickle 1986). Small planktonic species, such as the copepod, Calanus hyperboreus, have been maintained without food for up to 80 d (Conover 1964). In contrast, larval and postlarval crustaceans are far less tolerant to prolonged starvation and may reach a "point of no return" after only a few days of food deprivation (Anger et al. 1985)

Although we could not determine actual mortality of postlarvae in our study due to the periodic sampling for biochemical analysis, mortality resulting from starvation appeared to be minimal. Stuck and Overstreet (1994) reported that 13- to 14-d-old postlarvae of Penaeus vannamei, which were substantially smaller than the postlarvae used in our study, experienced 42% mortality after 10 d of starvation. Vogt et al. (1985) reported that 50-d-old postlarval P. monodon were able to withstand an absence of food for a maximum of 15 d with mortalities first appearing after 5 d. Postlarvae refed after 13 d of starvation were able to survive but required a 1- to 7-d period of recovery to re-establish the structure of the hepatopancreas (HP). In our study, a recovery period of more than 12 d was required before molting resumed. Juinio et al. (1992) reported that molting in postlarval lobsters, Homarus americanus, was arrested in response to starvation, but survival through 12 d of starvation was high. We observed a similar inhibition of molting in response to starvation in this study.

Water content

One of the most pronounced responses to starvation we observed was a significant increase in water content and corresponding decrease in % dry weight.

Similar responses to starvation have been reported for lobsters (Dall 1974; Sasaki 1984) and other penaeid shrimp (Cuzon et al. 1980; Barclay et al. 1983). During starvation, energy is derived solely from endogenous resources and tissues are lost due to catabolic activities. Should starvation also arrest molting activity, as was observed in our study, the volume of a crustacean becomes fixed by its exoskeleton. To maintain the necessary body volume and internal turgidity during starvation, the lost tissue mass must be replaced by water (Dall 1974; Wilcox and Jeffries 1976).

We observed a 42% decrease in tissue dry weight among postlarvae starved for 12 d. Regnault (1981) reported a 25% drop in total dry weight of the adult caridean shrimp, Crangon crangon, after 14 d of starvation. Wilcox and Jeffries (1976) found that the degree of tissue hydration in the caridean shrimp, Crangon septemspinosa, varied with the nutritional quality of the diet. They concluded that hydration can be used as a short-term, sensitive indicator of nutritional stress in crustaceans. The rapid changes in tissue water content in response to starvation and refeeding observed in our study support that conclusion.

Nucleic acids and proteins

During starvation in other crustaceans, protein and RNA levels decline, while DNA is generally conserved (Barclay et al. 1983; Wang and Stickle 1986; Juinio et al. 1992; Moss 1994a). A similar pattern was observed in our study. Between the second and twelfth day of starvation, protein concentration in the head was reduced by 48%. Since molting did not occur after the second day of starvation, the loss of protein must be attributed to catabolism. A 50% loss of total body proteins after 30 d of starvation was observed in adult caridean shrimp (Regnault 1981). Protein was the

major source of energy used during prolonged starvation in sub-adult penaeid shrimp, Penaeus esculentus (Barclay et al. 1983), and postlarval lobsters (Juinio et al. 1992). The utilization of protein as an energy source during starvation was also reported by Schafer (1968) for P. duorarum and Cuzon et al. (1980) for P. japonicus. In addition, we observed a 30% reduction in the protein:DNA ratio, a measure of cell biomass, after 12 d of starvation for P. vannamei. Juinio et al. (1992) observed a similar response to starvation in postlarval lobsters and suggested that the ratio can serve as an indicator of protein catabolism in crustaceans.

The significant increase in DNA concentrations and, to a lesser extent, RNA concentrations that we observed among starved postlarvae of Penaeus vannamei probably resulted from the selective catabolism of cellular components other than nucleic acids for energy. While storage materials, such as proteins and lipids, were depleted and replaced by water, nucleic acids, especially DNA, were conserved. That resulted in an increase in the concentration of nucleic acids relative to the remaining tissue solids. We also observed a sudden increase in RNA concentration immediately after feeding of starved postlarvae resumed. That increase was likely associated with the rapid increase in protein synthetic activity. Wang and Stickle (1986) reported that starvation resulted in a gradual reduction in RNA and, to a lesser extent, DNA in blue crabs. Moss (1994a) observed a rapid and significant reduction in RNA, but not DNA, in juvenile P. vannamei in response to starvation. Since both of those studies expressed nucleic acid concentrations in terms of wet weight, the effects of tissue hydration and differential catabolism of tissue solids were minimized. Juinio et al. (1992) reported that RNA content of postlarval lobsters, expressed as mg·individual^{-1},

declined in response to starvation, while DNA content remained relatively stable. Wang and Stickle (1986) did observe a rapid increase in RNA concentration, similar to that observed in our study, shortly after feeding of starved crabs resumed.

In this and previous studies (Wang and Stickle 1986; Juinio et al. 1992; Moss 1994a) that have examined the effect of starvation on nucleic acids in decapod crustaceans, the ratio of RNA:DNA has proven to be a reliable and sensitive indicator of recent food deprivation. In all those studies, significant decreases in RNA:DNA ratios were observed within 1 to 3 d of the initiation of starvation. The decline in RNA:DNA ratio reported in previous studies was due primarily to decreases in RNA with respect to DNA, while, in our study, the decline in RNA:DNA was due primarily to a relative increase in DNA concentration. The rapid recovery of the RNA:DNA ratio we observed after the resumption of feeding shows that the ratio is closely correlated with the nutritional condition of the shrimp. In studies with fish, RNA:DNA ratios have proven valuable in assessing nutritional condition (Clemmesen 1987, 1994) and anthropogenic stress (Barron and Adelman 1984; Wang et al. 1993), and in predicting growth (Buckley 1984; Malloy and Targett 1994). A similar use of RNA:DNA ratio for crustaceans may be complicated by cyclic changes in the ratio associated with the molting cycle (Anger and Hirche 1990). However, Moss (1994a) reported that the effects of molt stage on nucleic acid content of abdominal muscle of shrimp were not significant. The influence of molting on RNA:DNA ratios can also be minimized by either limiting the analysis to a specific molt stage or using a sample consisting of a large number of individuals in different molt stages. Moss (1994b) found that the RNA:DNA ratio accounted for about 80% of the variation in growth

rate of juvenile P. vannamei reared on different algal diets. While RNA:DNA ratio may be useful as a condition index for P. vannamei, its value in predicting growth in other crustaceans has been questioned (Dagg and Littlepage 1972; Ota and Landry 1984; Anger and Hirche 1990).

Neutral lipids

Lipid serves as an important endogenous energy reserve in marine invertebrates, and numerous studies have reported a reduction in total lipids in response to starvation. Among crustaceans, neutral lipids are preferentially catabolized during starvation (Heath and Barnes 1970; Bourdier and Amblard 1989), while polar lipids are conserved due to their role as structural components of cell membranes. Of the different types of neutral lipids, triacylglycerol (TAG) is primarily used for energy storage in animals (Lehninger 1975). In larval lobsters, TAG is accumulated during periods when intake of exogenous energy sources exceeds immediate demand and is consumed during periods of nutritional stress (Sasaki 1984). Although sterol, another neutral lipid, serves as a precursor for steroid hormones, it is quantitatively most important as a structural component of cell membranes and represents a consistent portion of the wet weight of an animal (Nes 1974). Sterol content in larval lobsters appears to be unaffected by nutritional conditions and remains essentially unchanged during prolonged starvation (Sasaki 1984). Fraser (1989) has proposed the use of a TAG:sterol ratio as a condition index for fish and bivalve and crustacean larvae.

A reduction in total lipid as a response to starvation has been reported from adult and sub-adult Penaeus duorarum, P. japonicus, and P. esculentus (Schafer 1968; Cuzon et al. 1980; Barclay et al. 1983). Vogt et al. (1985) noted a reduction in lipid

droplets in the HP of postlarval P. monodon between the first and third day of starvation. However, those studies did not examine the effects of starvation on specific classes of lipids. In the present study we found that TAG reserves were almost totally depleted during the first day of starvation and were undetectable after 8 d. Chandumpai et al. (1991) reported that digestive gland (HP) triacylgycerol in sub-adult P. esculentus was severely depleted after 4 d of starvation and almost completely absent after 8 d. Sasaki (1984) reported a rapid depletion of TAG in starved lobster larvae. Bourdier and Amblard (1989) reported a 93% to 96% decline in TAG levels from a copepod after 7 d of starvation and restoration of TAG content 20 d after feeding resumed. We observed a rapid reestablishment of TAG reserves within 24 h after feeding of starved postlarvae resumed. The pattern of change in TAG and protein content in postlarval P. vannamei suggests that TAG is used primarily during the initial phases of nutritional stress and that energy for prolonged starvation is derived from other sources, such as protein.

In our study, the concentration of sterol in postlarval Penaeus vannamei increased in response to starvation, but returned to near pre-starvation levels after feeding resumed. This increase was probably related to selective catabolism of cellular components during starvation as discussed above. Since sterol does not appear to be utilized as an energy source in postlarval P. vannamei, the TAG:sterol ratio can potentially be used as an index of nutritional condition. In both fed and starved-fed postlarvae, changes in the TAG:sterol followed a pattern almost identical to TAG concentrations. Since molting was arrested after the second day of starvation, we believe that changes in TAG:sterol ratio of starved-fed shrimp reflected their

nutritional conditions. However, among fed shrimp, the ratio was highly variable both within and between sampling periods, probably due in part to molting activities. Chandumpai et al. (1991) reported that TAG accumulated during pre-molt in P. esculentus. While a high TAG:sterol ratio may indicate good nutritional status, a low ratio does not necessarily indicate nutritional stress unless the effects of molting have been minimized by using samples either collected during a specific molt stage, or consisting of a large number of individuals. The rapid and almost total depletion of TAG and the TAG:sterol ratio we observed after only 1 d of starvation may preclude the use of this ratio as an indicator of prolonged or severe nutritional stress in postlarval P. vannamei.

Polyamines and related amines

Although polyamines have been studied in some crustaceans (Watts et al. 1992, 1994, 1995; Stuck et al. 1994; Lovett and Watts 1995), the relationship between nutritional stress and polyamine content is poorly understood. In our study, the effects of starvation on polyamine concentrations were masked by differential catabolism as discussed above for both DNA and sterol. To compensate for those effects, we also expressed polyamine concentration as a ratio to DNA, a cellular component that is not catabolized during starvation. The levels of putrescine, spermidine, and spermine, when expressed as a ratio to DNA, generally reflected the nutritional status of the postlarvae. In mammalian studies, slow growing or quiescent cells have a lower polyamine content than rapidly growing cells (Tabor and Tabor 1984).

Several amine compounds identified by their retention times and relative concentrations of these compounds expressed as a ratio to spermine (spermine

concentration has been closely associated with DNA content; Tabor and Tabor 1984), may be useful indicators of nutritional stress. The relative concentrations of amines 3.4 and 7.5 remained stable in fed individuals, but changed rapidly in response to starvation and subsequent refeeding. Although the identity of these compounds is unknown, their relative retention times on the column and other separation parameters suggest that they are small compounds containing at least one primary amine, possibly amino acids. The inverse relation in the response observed between these two compounds at the onset of starvation and later at refeeding warrants further investigation.

Interrelationships of biochemical parameters

The positive correlation between DNA concentrations and % water content and sterol and % water content in starved-fed shrimp resulted from selective catabolism of cellular components other than sterol and DNA during starvation and replacement with water. The positive correlation between DNA and sterol concentrations in both fed and starved-fed groups of shrimp and their resistance to catabolism during prolonged starvation indicates that both are stable indicators of relative cell number. As indicators of nutritional stress, protein, RNA, TAG, or polyamine content should be expressed as a ratio to either DNA or sterol. This would minimize the problems associated with differential catabolism as was observed in this and previous studies on crustaceans.

The negative correlation obtained between % water content and protein concentrations of starved-fed shrimp suggests that proteins lost during starvation are replaced by water. The positive correlation between % water content and protein

concentrations of fed postlarvae suggests that growth during postlarval development is at least partly due to cell hypertrophy. Regnault and Luquet (1974) observed that cell hypertrophy in Crangon vulgaris occurred mainly when the shrimp increased from 40 to 300 mg, a similar size increase as the fed group of postlarvae in our study.

The polyamines, spermidine, and spermine are believed to directly interact with RNA and DNA, neutralizing the negative charges on phosphate groups and stabilizing their structure (Tabor and Tabor 1984). That interaction may account for the positive relationship we observed between DNA and spermidine and spermine in this study. Spermidine and spermine have also been shown to be involved with RNA and protein synthesis, although the exact mechanism of the interaction is not completely understood (Heby 1981). We observed positive correlations between RNA and both spermine and spermidine in fed and starved-fed postlarvae.

The lack of a significant correlation between TAG:sterol and most other parameters is due primarily to the rapid and total catabolism of TAG reserves in postlarval Penaeus vannamei compared to other labile cellular components. Changes in the unknown amines 3.4:Sp and 7.5:Sp among starved-fed postlarvae were significantly correlated with TAG:sterol because of their similar rapid and absolute responses to changing nutritional conditions. The TAG:sterol ratio may be a useful indicator of mild or short-term nutritional stress. The RNA:DNA, protein:DNA, spermidine:DNA, spermine:DNA, amine 3.4:Sp, and amine 7.5:Sp ratios of starved-fed shrimp were all significantly correlated with % water content. Those ratios, and the % water content, all appear to be useful indicators of severe nutritional stress in

postlarval shrimp. The information obtained from this study should be useful in developing a biochemical quality or condition index for postlarval shrimp.

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RELATIONSHIP BETWEEN BACULOVIRUS PENAEI AND ENERGY RESERVES IN LARVAL AND POSTLARVAL PACIFIC WHITE SHRIMP, PENAEUS VANNAMEI

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ABSTRACT: The relationship between energy reserves of the penaeid shrimp Penaeus vannamei and Baculovirus penaei, or BP, were investigated in a series of experiments using mysis stage or early postlarval shrimp. Pre-exposure and post-exposure levels of protein and triacylgycerol (TAG) were determined. The effect of pre-exposure protein and TAG levels on susceptibility to BP infections was also investigated by starving a group of shrimp immediately prior to BP exposure. There was no consistent relationship between either pre-exposure or post-exposure protein levels and the percent of shrimp developing patent BP infections. There, however, was a significant positive correlation between TAG levels immediately prior to viral exposure and prevalence of infection 72 h later. Experimental reduction of TAG reserves prior to BP exposure delayed the development of a patent infection. In some, but not all experiments, there was a significant reduction in TAG levels of infected compared with uninfected shrimp 72 h post-exposure. The effect of patent BP infections on host TAG levels are subordinate to fluctuations in TAG content associated with the ontogeny of the hepatopancreas. Results of this study support histological observations that shrimp lipid levels can be altered by baculovirus infections. Furthermore, high levels of energy reserves in the form of TAG are associated with increased susceptibility to BP infection in larval and postlarval shrimp.

INTRODUCTION

Baculovirus penaei (Couch), commonly known as BP and designated by the International Committee on Taxonomy of Viruses (Francki et al. 1991) as PvSNPV, is one of approximately 18 viruses reported from penaeid shrimp (Overstreet 1994).

Penaeus vannamei, the primary species of shrimp used for aquaculture in the Western Hemisphere, is susceptible to BP. In fact, BP can cause serious epizootics with high mortality of larval and, to a lesser extent, postlarval P. vannamei (see Overstreet et al. 1988). Typically, larval and young postlarval P. vannamei develop patent infections 18-24 h following initial exposure to BP (Stuck and Overstreet 1994). Substantial mortalities or reductions in growth begin to occur 4 to 7 days post-exposure. That pattern of infectivity and pathogenicity is by no means invariable, and exceptions have been reported for experimental studies (LeBlanc & Overstreet 1990, Overstreet 1994) and occur in commercial hatcheries (personal observations, KCS). Although variability in the apparent susceptibility of P. vannamei to BP may be related in part to the method used to detect the virus (Bruce et al. 1994), a variety of other factors undoubtedly are more significant. Overstreet (1994) has identified viral, host, and environmental factors that may influence BP infections. Among those factors is host nutritional condition.

The nutritional condition of an animal can be assessed biochemically in terms of available energy reserves. The principle energy storage materials in penaeid shrimp are lipids and protein (Schafer 1968, Barclay et al. 1983). Carbohydrates are considered to be a minor energy reserve in most decapod crustaceans (Barclay et al. 1983), including penaeid shrimp (Schafer 1968). Triacylgycerol (TAG) is the primary class of lipid used for energy storage in animals (Lehninger 1975). In penaeid shrimp, TAG reserves are rapidly depleted in response to nutritional stress (Chandumpai et al. 1991). However, during prolonged periods of starvation, protein serves as the major energy source in penaeid shrimp (Barclay et al. 1983). Stuck et al. (submitted)

reported that TAG reserves are significantly reduced in postlarval P. vannamei during the first 24 h of starvation and are rapidly re-established once feeding resumes. TAG content has been used extensively as an indicator of nutritional condition in larval crustaceans (Fraser 1989, Ouellet et al. 1992, Lovrich & Ouellet 1994). Any type of stress that causes a significant reduction in feeding activities or absorption of nutrients will result in a rapid depletion of TAG reserves in crustacean larvae.

In this study we investigated the relationship between host energy reserves and susceptibility to BP infections. Our specific objectives were (1) to determine the effect that host nutritional condition immediately prior to BP exposure has on infectivity of the virus and (2) to determine the impact that patent BP infections have on energy reserves in larval and postlarval P. vannamei. We measured pre-infection and post-infection TAG and protein levels during a series of experimental BP exposures, some of which were originally designed to assess other aspects of the relationship between BP infections and nutrition. The information obtained during this investigation contributes to a better understanding of how host factors can influence BP infections.

MATERIALS AND METHODS

BP infectivity experiments. The first phase of the study consisted of a series of eight experiments in which groups of high health P. vannamei (see Wyban et al. 1992) were experimentally infected with BP. Non-exposed controls were maintained during each experiment. The shrimp used in the experiments were obtained as either nauplii or postlarvae from one of several sources: (1) The Oceanic Institute, Waimanalo, Hawaii, (2) Amorient Shrimp Farm, Kahuku, Hawaii, (3) Harlingen

Shrimp Farm, Los Fresnos, Texas, and (4) Waddell Mariculture Center, Bluffton, South Carolina. Regardless of the source, all shrimp used in these experiments originated from shrimp spawned from specific pathogen free (SPF) broodstock produced from Kona Population 1 (see Wyban 1992). The source of shrimp, stage of development at which exposure to BP was initiated, and the approximate date and duration of each experiment are listed in Table 1. Shrimp used in experiments 1, 3, 4, and 5 were obtained as nauplii and reared to the desired age at 27 ± 1°C. The diatom Chaetoceros neogracile was fed to protozoeal stages 1-3 and brine shrimp nauplii to protozoeal stage 3 through postlarvae. Shrimp used in experiments 2, 6, 7, and 8 were obtained as postlarvae and maintained at 27 ± 1°C on a diet of brine shrimp nauplii. Throughout each experiment, food was provided ad libitum. The C. neogracile used in all experiments was initially obtained from Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, Maine (clone CCMP 1318), and reared on Fritz f/2 Algae Food® (Fritz Chemical Company, Dallas, Texas). Brine shrimp used in all experiments were obtained from Aquarium Products, Glen Burnie, Maryland (lot number 756). Water used in all the experiments, including the brine shrimp and diatom cultures, was produced from hw-Marinemix® (Hawaiian Marine Imports, Houston, Texas) and deionized water. Disodium ethylenediaminetetraacetate (EDTA-Na_2) was added to the salt water at a concentration of 10 ppm. Salinities were adjusted to 30 ppt for larval stages and 25 ppt for postlarvae.

Immediately before exposure to BP, a sub-sample of 15-20 shrimp from each experimental group was examined for the presence of BP polyhedra in the hepatopancreas (HP) following the diagnostic procedures for fresh shrimp described

Table 1. Penaeus vannamei. Source of shrimp, approximate date experiment was conducted, stage of development at which shrimp were first exposed to BP, duration for each experiment, and biochemical analyses conducted (X). Source of shrimp: SC-South Carolina, Waddell; HO-Hawaii, Oceanic Institute; HA-Hawaii, Amorient; TH-Texas, Harlingen. Stage: M=mysis; PL=postlarvae. *Postlarvae were starved for 48 h immediately prior to viral exposure.

Experiment	Source	Date	Stage	Duration (days)	Protein	TAG
1	SC	7-92	M-I	15	X	X
2	HO	7-92	PL-9	21	X	X
3	TH	3-93	PL-9	3		X
4	SC	5-93	M-II	3		X
5	HO	10-93	PL-16	3		X
6	HA	1-94	PL-9	3		X
7	HO	2-94	PL-9	25	X	X
8	HO	11-94	PL-9	3		X
9	HO	10-93	PL-18	8*	X	X

by Overstreet et al. (1988). The same diagnostic procedure was used to determine the prevalence of BP infections in all subsequent experiments. Lack of BP polyhedra in any of the pre-exposure stocks or in the unexposed controls confirmed the BP-free status of our experimental stocks. Experiments 1, 2, 3, 4, and 7 were conducted in two 200-L glass aquaria containing 150 L of salt water. Experiments 5, 6, and 8 were conducted in two 19-L aquaria containing 15 L of salt water. Stocking densities were approximately 80-100·L^{-1} for mysis shrimp and 8-10·L^{-1} for postlarvae. During each experiment, shrimp in one aquarium were exposed to BP and shrimp in the second aquarium from the pair served as the negative control. The strain of BP and the procedures for viral administration have been described previously by Stuck and Overstreet (1994).

In all experiments, three replicate samples consisting of approximately 30 mg (wet weight) of larvae or postlarvae were collected for TAG analysis immediately prior to BP-exposure. Protein samples were collected only during experiments 1, 2, and 7. If sufficient numbers of larvae or postlarvae remained, a second set of samples was collected for TAG and protein analysis 72 h after initial viral exposure. Samples for TAG and protein analysis were immediately frozen in liquid nitrogen and stored at -70°C. Beginning at 12 - 18 h post-exposure and continuing through the duration of each experiment, the prevalence of infection was determined at periodic intervals by examining a sub-sample of the BP-exposed and corresponding unexposed cultures. During three experiments (see Table 1), the effects of BP on energy reserves were monitored over extended periods of time. Through the duration of those experiments, replicate samples (n = 3-4) were periodically collected for protein and TAG analysis,

and the prevalence of infection was determined from sub-samples ($n \geq 10$) of the BP-exposed and unexposed cultures. Data collected during experiments 1-8 were used to determine the relationship between pre-exposure energy reserves and susceptibility to BP infection. Data from experiments 1-4 and 7 were used to determine the impact that patent BP infections have on post-exposure energy reserves.

In the second phase of the study (experiment 9), we continued our investigation of the effect that pre-exposure host energy reserves have on susceptibility to infection by starving a group of postlarvae prior to BP-exposure. The resulting pattern of infectivity was compared to a continually fed control group. Shrimp used in this study were obtained as nauplii from The Oceanic Institute and reared in a 95-L aquarium to the postlarval stage (PL16) following culture procedures described previously. Approximately 250 postlarvae from that common initial culture were placed into two 19-L aquaria; one culture was maintained for 48 h without providing food, and shrimp in the other were continually fed. Replicate samples ($n = 3$) were collected for TAG and protein analysis from the common initial culture immediately prior to stocking the two 19-L aquaria and 48 h later from both the fed and starved groups of postlarvae. Fed and starved postlarvae were then exposed to BP at which time feeding of starved postlarvae resumed. Beginning at 18 h post-exposure and continuing for 192 h, we periodically monitored the prevalence of infection from sub-samples ($n = 15$) of the starved-fed and fed cultures.

Protein and triacylglycerol determinations. Samples collected for protein and TAG analysis were homogenized in 20 volumes of cold distilled water using a small hand-held electric tissue grinder and then briefly sonicated. Two 50-μl aliquots of

each sample were placed in pre-weighed, aluminum, micro weigh pans and dried at 80°C for 24 h. After cooling to room temperature, each pan was reweighed to the nearest 0.1μg using a Cahn Electrobalance. The weights obtained were used to estimate the total dry weight of the aliquots taken for TAG and protein determinations.

A 25-μl aliquot was used to determine the soluble protein content of each sample. Protein concentration was determined using a Bio-Rad protein assay kit based on the Bradford method (Bradford 1976). Bovine serum albumin was used as the standard.

Lipids extracted from 400-μl aliquots were used to determine the TAG content of each sample (Bligh & Dyer 1959). An internal standard, palmitic acid propyl ester was added to the extracted lipids, and the samples were then evaporated to dryness by blowing a gentle stream of N_2 gas over the samples. Neutral lipids were separated by thin layer chromatography on Type S-III chromarods using the solvent system dichloroethane:chloroform: acetic acid (926:31:1). The TAG content of each sample was quantified using an Iatroscan TH-10 Mark IV (see Ranny 1987). Tripalmitin was used as the standard. The concentrations of TAG and protein were calculated and expressed as $\mu g \cdot mg^{-1}$ dry weight.

Statistical analysis. Mean and standard error for TAG and protein concentrations were determined for each set of replicate samples collected at each sampling time. Differences in TAG and protein levels between BP-exposed and unexposed control shrimp were tested for significance using the Student *t* test. Bonferroni corrected 95% *t*-critical values were used when multiple comparisons were made. Analysis of

differences in protein and TAG levels of infected and uninfected shrimp was limited to comparisons within individual experiments. Correlations between prevalence of infection and both protein and TAG concentrations were determined by simple linear regression. Analysis of variance was used to test for significance of the regressions.

RESULTS

Establishment of patent BP infections. In each of the eight experiments conducted during the first phase of the study, viral polyhedra characteristic of a patent BP infection first appeared in the HP 18-24 h post-exposure. However, progression of the infection after the initial appearance of polyhedra was variable between different experiments (Table 2). During experiments 3, 4, 5, 7,and 8, 86%-100% of the shrimp exposed to BP were infected within 72 h. In contrast, 35% or less of the BP-exposed shrimp in experiments 1, 2, and 6 developed patent infections by 72 h. In experiments 1 and 2, the prevalence of infection increased to 100% by 9-10 d after initial viral exposure. Substantial mortalities among BP-infected shrimp were evident only during experiments 1 and 3, and occurred 4 - 7 days after initial viral exposure.

Effects of BP infection on shrimp energy reserves: In the first phase of the study, TAG levels of BP infected and uninfected shrimp 72 h post-exposure were determined during experiments 1-4 and 7 (Table 2). In experiments 3 and 4, the mean TAG levels of infected shrimp were significantly reduced ($p \leq .01$) compared to the uninfected controls. Mean TAG levels were slightly, but not significantly, reduced in infected compared with uninfected shrimp during experiments 1 and 2. During experiment 7, the mean TAG levels of infected and uninfected shrimp were both low and nearly identical. There was no significant correlation between mean 72-h

Table 2. Penaeus vannamei. Triacylglycerol (TAG) levels in larval and postlarval shrimp immediately prior to BP inoculation and 72 h post-exposure during phase 1 of the study. TAG levels are expressed as mean ug·mg^{-1} dry weight ± standard error (n = 3-4). N = number of individuals examined for BP infections 72 h post-infection (% infected). NA = data not available.
* Significant difference ($p \leq .01$) in 72 hour post-exposure TAG levels between uninfected and infected shrimp.

Experiment	TAG (pre-exposure)	TAG (72 hr post-exposure) Uninfected	Infected	N	% Infected
1	1.8 ± 0.3	5.1 ± 0.8	4.8 ± 0.6	16	31
2	1.2 ± 0.1	2.5 ± 0.2	1.8 ± 0.3	14	14
3	3.7 ± 0.6	21.8 ± 1.3	4.6 ± 1.4*	20	100
4	8.4 ± 0.6	25.1 ± 3.5	14.4 ± 1.7*	21	86
5	12.0 ± 5.1	NA	NA	14	93
6	1.3 ± 0.2	NA	NA	29	35
7	6.7 ± 1.4	1.2 ± 0.2	1.3 ± 0.5	15	100
8	3.5 ± 0.3	NA	NA	15	100

post-exposure TAG levels of either infected or uninfected shrimp and prevalence of infection in the BP-exposed treatments due primarily to the low TAG levels observed during experiment 7.

During experiments 1, 2, and 7, protein and TAG levels were monitored for extended periods (15-25 days) following initial exposure to BP. In all three experiments, TAG content of infected and uninfected shrimp followed similar patterns of change (Fig. 1). In experiments 1 and 7, TAG levels at the termination of the experiment were substantially, but not significantly, higher in BP-exposed shrimp compared with unexposed shrimp, whereas in experiment 2 they were substantially lower. During all three experiments, differences in TAG levels observed between infected and uninfected shrimp were not significant based on Bonferroni 95% *t*-critical values. Among BP-exposed shrimp, there was no significant correlation between TAG levels and prevalence of infection. During experiments 1 and 2, protein levels (Fig. 2) were variable between sampling periods in both BP-exposed and unexposed shrimp. Protein levels among infected shrimp at the end of experiment 1 were significantly lower ($p \leq .01$) than uninfected shrimp, but were significantly higher ($p \leq .01$) in infected compared to uninfected shrimp at the end of experiment 2. In experiment 7, protein levels of BP-exposed and unexposed shrimp remained relatively stable and were nearly identical throughout the course of the experiment. In all three experiments, protein levels were not significantly correlated with either prevalence of infection or TAG levels.

Effect of pre-exposure energy reserves on infectivity of BP: In all eight experiments conducted during the first phase of the study, TAG levels were

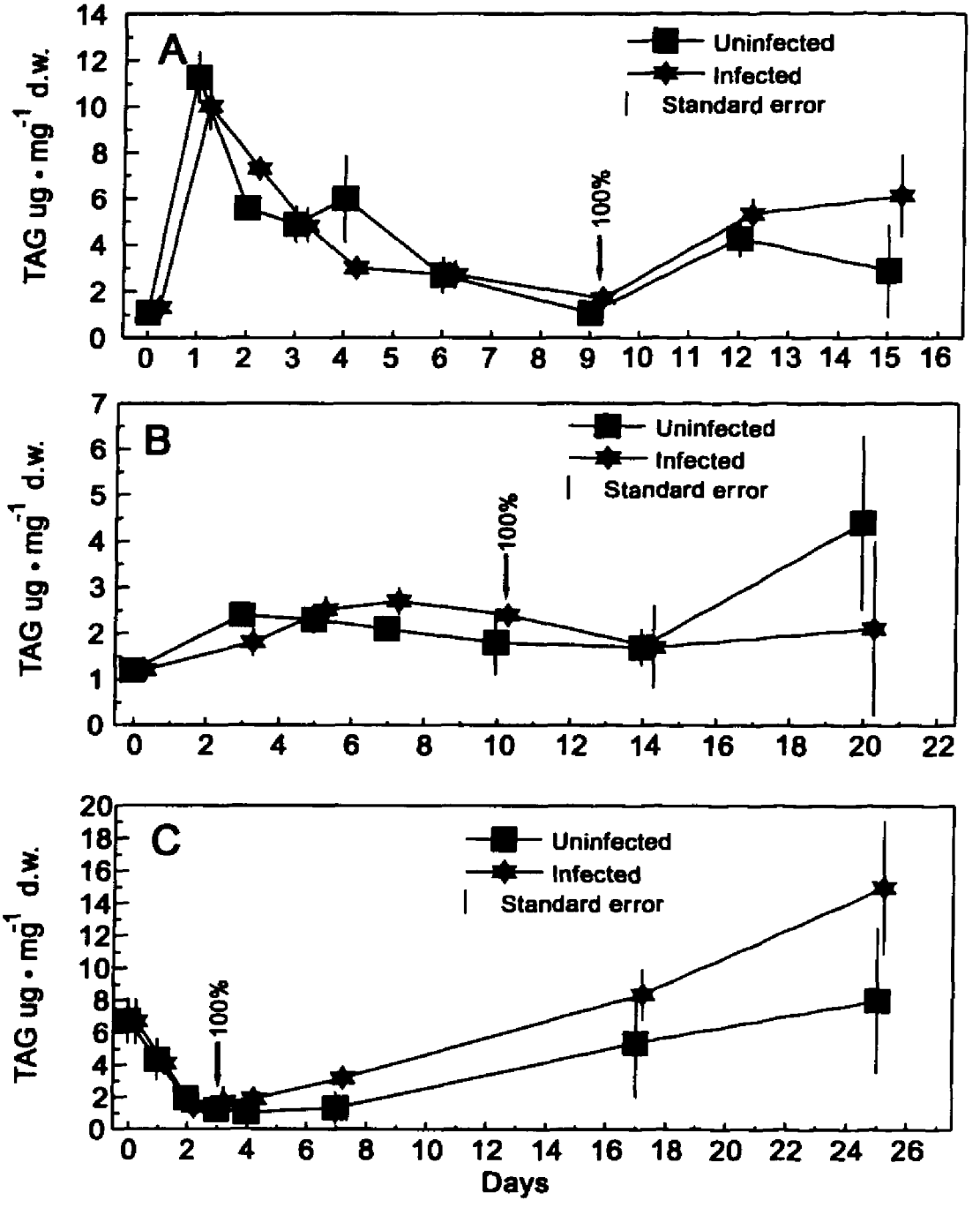

Fig. 1. Penaeus vannamei. Triacylglycerol levels of BP-infected and uninfected shrimp following initial exposure to BP. (A)- experiment 1, initial exposure to BP at mysis-I stage; (B)-experiment 2, initial exposure to BP at PL-9 stage; (C)-experiment 7, initial exposure to BP at PL-9 stage. Values are expressed as means ± standard error (n = 3-4). The sampling period at which 100% prevalence of infection among BP-exposed shrimp was obtained is indicated.

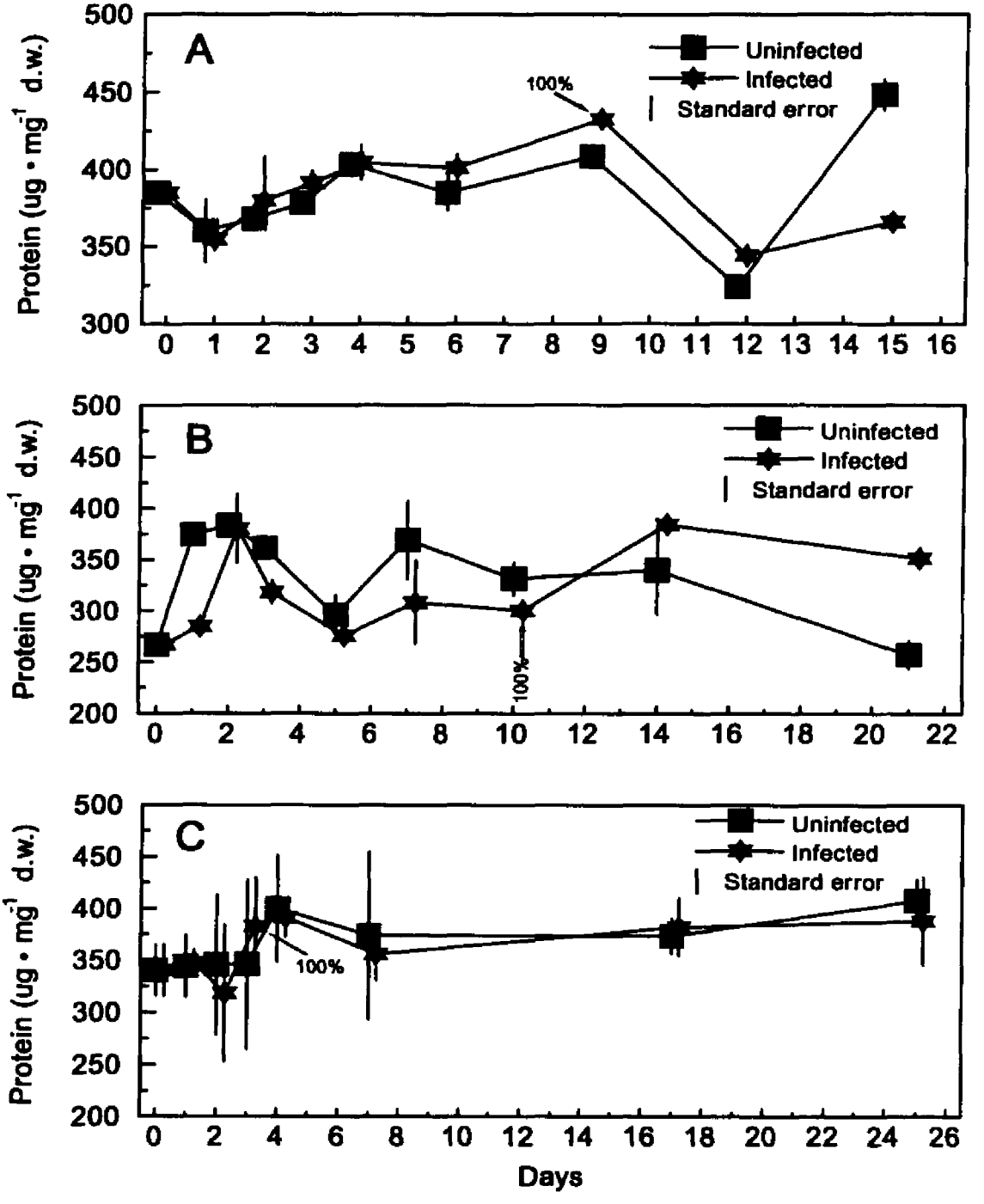

Fig. 2. Penaeus vannamei. Protein levels of BP-infected and uninfected shrimp following initial exposure to BP. (A)- experiment 1, initial exposure to BP at mysis-I stage; (B)-experiment 2, initial exposure to BP at PL-9 stage; (C)- experiment 7, initial exposure to BP at PL-9 stage. Values are expressed as means ± standard error (n = 3-4). The sampling period at which 100% prevalence of infection among BP-exposed shrimp was obtained is indicated.

determined immediately prior to BP-exposure (Table 2). There was a weak but significant positive correlation ($r^2 = 0.427$, $p \leq 0.05$) between pre-exposure TAG levels and prevalence of infection 72 h post-exposure (Fig. 3). When mean pre-exposure TAG levels were ≤ 1.8 $\mu g \cdot mg^{-1}$ the 72 h prevalence was $\leq 35\%$. When mean pre-exposure TAG levels were ≥ 3.5 $\mu g \cdot mg^{-1}$, the 72-h prevalence of infection was $\geq 86\%$. Pre-exposure protein levels, determined during experiments 1, 2, and 7, were not significantly correlated with 72-h prevalence of infection.

In the second phase of the study (experiment 9), we experimentally reduced energy reserves by starving shrimp immediately prior to BP exposure. TAG levels were significantly reduced ($p \leq 0.01$) in postlarvae that were starved for 48 h compared to either the initial common stock of postlarvae or the continually fed controls (Fig. 4). Protein levels were not significantly influenced by starvation. Among the fed controls, viral polyhedra were observed at 18 h post-exposure, and the maximum prevalence of infection (93%) occurred at 72 h (Fig. 5). Viral polyhedra were first observed at 30 h post-exposure in the previously starved group of postlarvae; however, the prevalence of infection then increased rapidly to levels similar to fed postlarvae.

DISCUSSION

There appears to be a relationship between TAG content of penaeid shrimp and baculovirus infections in this and previous studies. Stuck and Overstreet (1994) reported a reduction in the number and size of lipid droplets in the hepatopancreas (HP) of larval and postlarval Penaeus vannamei infected with BP. Vogt (1992) observed a proliferation of smooth ER into concentric membrane whorls indicative of

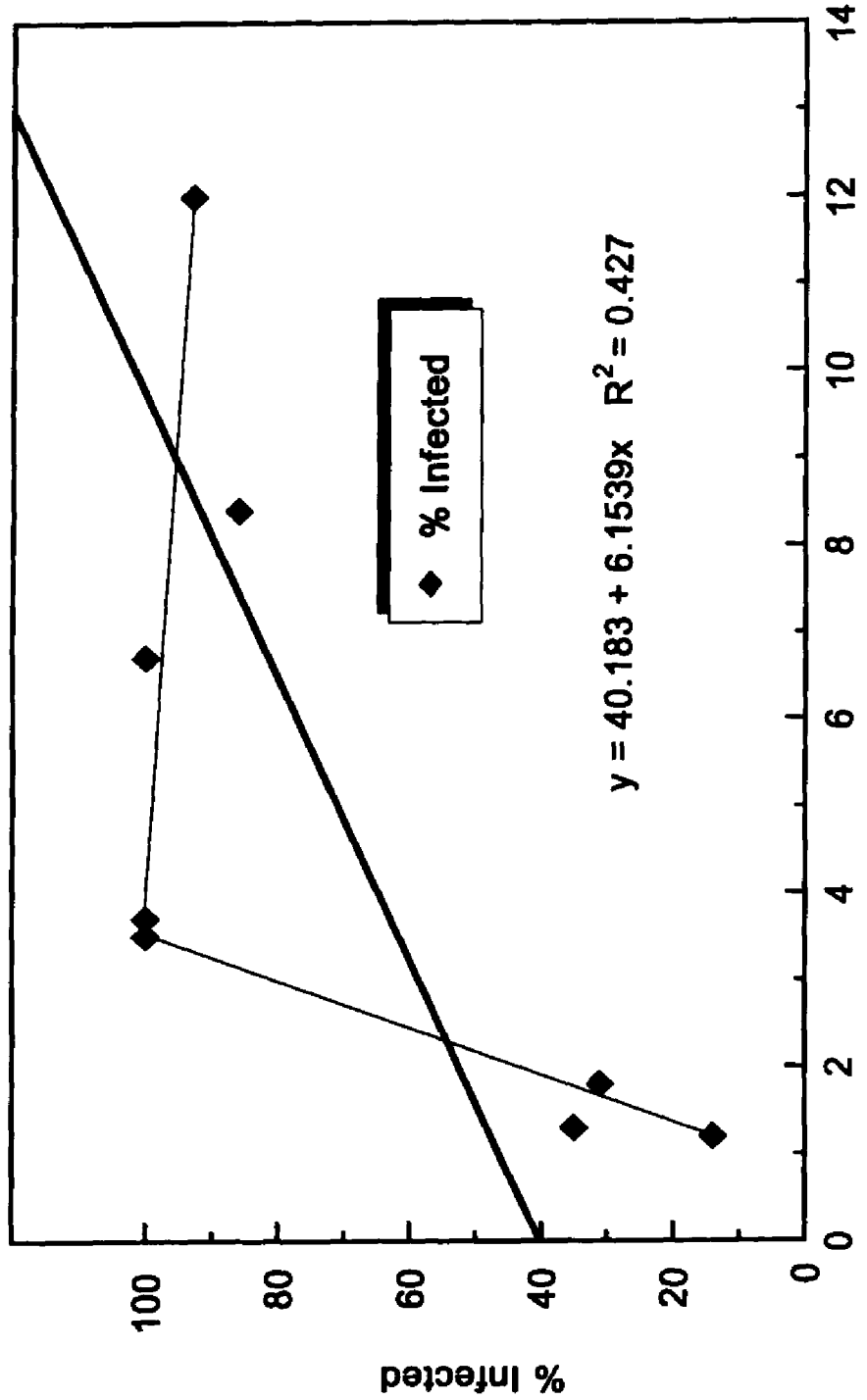

Fig. 3. Penaeus vannamei. Relationship between pre-exposure triacylglycerol (TAG) levels and 72 h post-exposure prevalence of BP infection. The relationship is described by a simple linear regression model (heavy line) and a hyperbolic line fitted to the data points.

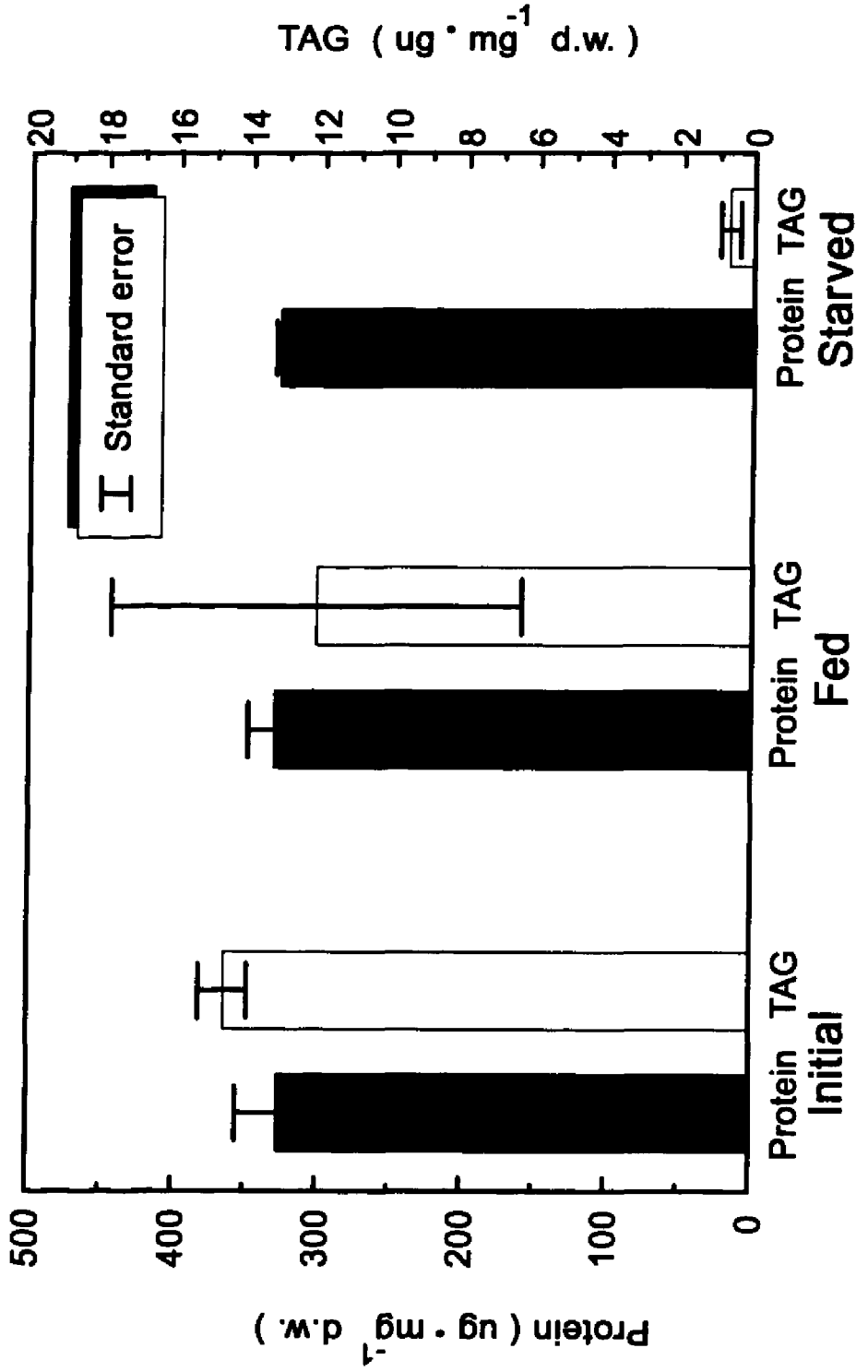

Fig. 4. Penaeus vannamei. Protein and triacylglycerol (TAG) levels of fed and starved postlarvae prior to BP exposure (experiment 9). Protein and TAG concentrations were determined from the initial common stock, and 48 h later from the continually fed and starved groups of postlarvae. Values are expressed as means ± standard error (n = 3).

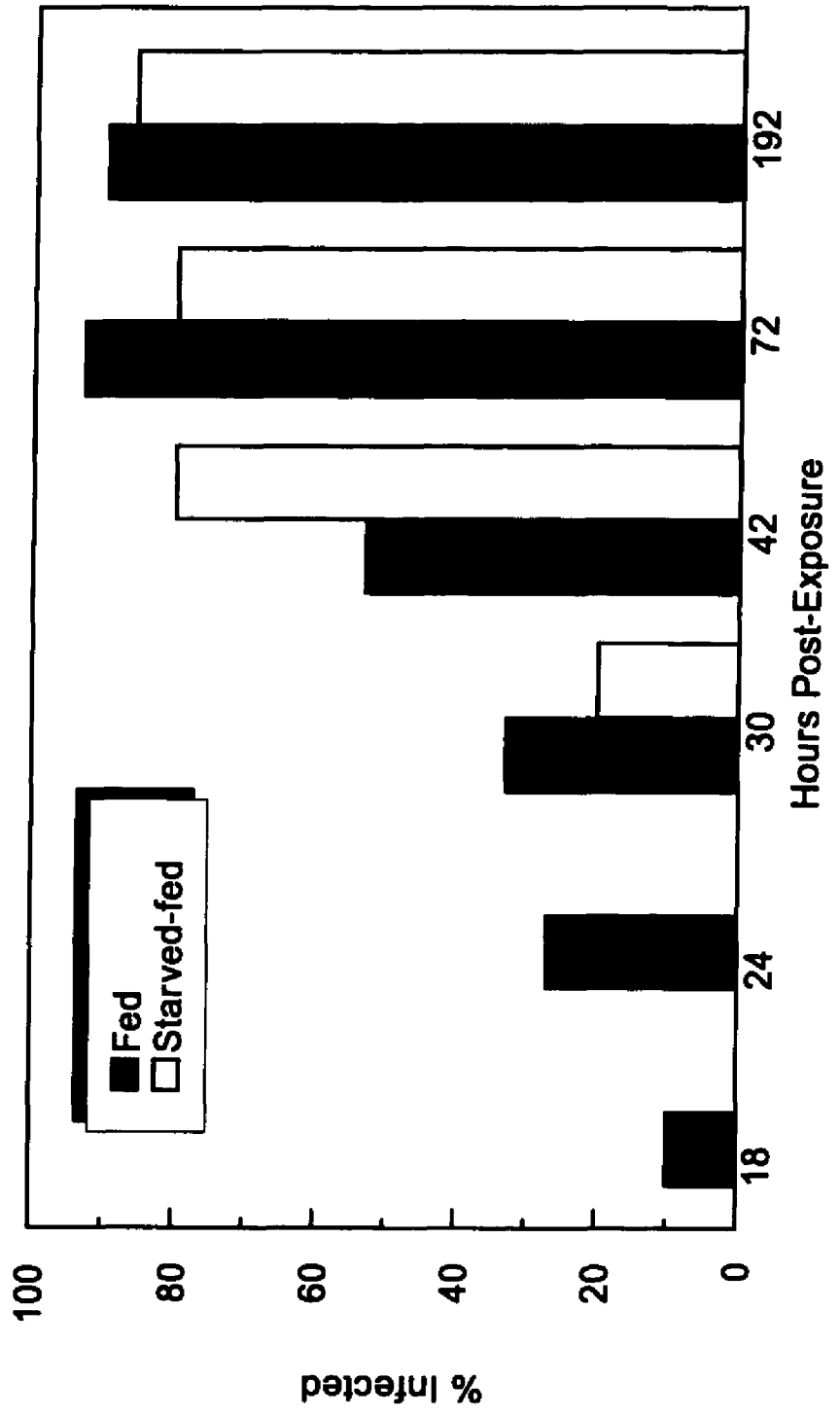

Fig. 5. Penaeus vannamei. Prevalence of BP infection in postlarvae starved 48 h prior to BP exposure compared to continually fed postlarvae (experiment 9). Values are expressed as the % of postlarvae with viral polyhedra of the total examined (n = 15) from each post-exposure sampling period. Feeding of starved postlarvae resumed after initial exposure to BP.

lipid catabolism in HP cells of P. monodon infected with the baculovirus MBV. He reported that HP cells in the advanced stages of infection lacked lipid reserves. Couch (1989) observed the formation of a membranous labyrinth (ML) from ER vesicles in BP infected P. duorarum during the early stages of viral infection. He observed that the ML is closely associated with viral replication and one of its functional roles may be related to energy demand. TAG is the primary class of lipid used for energy storage in penaeid shrimp (Chandumpai et al. 1991) and constitutes approximately 18% of the total wet weight of the HP in juvenile P. vannamei (Stuck, unpublished data). Probably, the reduction of lipids in response to baculovirus infection observed in previous studies is primarily attributable to TAG. In our study, we did not observe any significant or consistent relationship between protein levels and BP infections.

Measurements of mortality due to viral exposure were not made in all eight experiments conducted during the first phase of this study; however, data on the survival of BP-infected and uninfected control shrimp from experiments 1, 2, and 3 have been presented previously (Stuck & Overstreet 1994). Although significant viral related mortality was observed during experiments 1 and 3, that mortality was not evident until 4 to 7 days post-exposure. In other experiments conducted during this study, substantial mortalities in either control or infected treatments were not evident during the first 72 h following initial viral exposure. It is therefore unlikely that the relationship between BP and TAG levels observed in this study is the result of differential survival of infected compared to uninfected shrimp.

Investigating the relationship between BP and TAG content is complicated by a variety of non-viral factors that can also influence TAG content. When interpreting

results of this study, we must consider the possible effects of molting activities, stage of development, nutrition, and inherent capacity of a brood of shrimp to store TAG.

Chandumpai et al. (1991) observed a relative increase in TAG content in the HP of sub-adult Penaeus esculentus during early premolt, but otherwise did not find a definite trend in TAG levels during the molting cycle. Samples collected for TAG analysis during our study consisted of approximately 10 (older postlarvae) to several hundred (mysis stage) shrimp. Since molting did not appear to be completely synchronous in our experimental cultures, the samples consisted of shrimp in a variety of molt stages, thus minimizing the possible effects of molting on TAG content.

The accumulation of TAG reserves in larval and postlarval Penaeus vannamei are significantly influenced by the stage of development. Since almost all the TAG in penaeid shrimp is found in the HP (Chandumpai et al., 1991), changes in the concentration of TAG relative to the total weight of the shrimp should parallel the ontogeny of that organ. In P. setiferus, there is no significant change in the volume of the HP from mysis 2 through PL 4, and the rate of increase in the HP volume does not equal that of the body until about PL 10 (Lovett & Felder, 1989). Thus, during that period of development the relative concentration of TAG should decrease. After PL 10, the HP begins to rapidly increase in size, and there should be a corresponding increase in the relative concentration of TAG. The pattern of change in TAG levels of both BP-exposed and unexposed shrimp observed during experiments 1, 2, and 7 (see Fig. 3) generally followed the ontogeny of the HP. The post-exposure effects of BP on TAG levels appear to be subordinate to those resulting from normal developmental patterns. Patent BP infections were associated with a 72-h post-exposure reduction of

TAG levels in shrimp from some, but not all, of the experiments conducted. Shastri-Bhalla & Consigli (1994) reported a 29% reduction in TAG levels among insect larvae during the development of a patent baculovirus infection. Vogt (1992) suggested that the reduction of lipid reserves during the later phases of an MPV infection was due to energy requirements for viral replication. It is also possible that the reduction in energy reserves associated with patent baculovirus infections observed in this and previous studies resulted from reduced feeding activities of infected individuals. We also observed substantially higher TAG levels in infected compared with uninfected shrimp at the end of experiments 1 and 7. Those results support the observations by Stuck & Overstreet (1994) that after shrimp have recovered from the initial deleterious effects of BP, infected shrimp often experience accelerated growth.

Following initial viral exposure, all cultures were fed ad libitum with brine shrimp from the same lot, therefore providing a similar nutritional diet. Differences in observed TAG levels between experiments attributable to nutrition should be minimal. However, during experiments 1-4, when shrimp were transferred from a common stock to separate experimental aquaria, feeding levels were reduced for an 8-h to 12-h period immediately prior to BP exposure. That reduction in feeding may account in part for the relatively low pre-exposure TAG levels compared with the 72-h levels observed during those experiments. Despite those possible reductions in TAG, there was still a weak but significant correlation between pre-exposure TAG levels and 72-h prevalence of infection. However, because there is an upper limit (100%) for prevalence of infection, the relationship between pre-exposure TAG levels and 72 h post-exposure prevalence of infection (Fig. 3) was not linear over the entire

range of TAG levels obtained during the study. There appears to be a "threshold" concentration for TAG, beyond which larval and early postlarval shrimp are highly susceptible to BP infection.

In experiment 1 (Fig. 1A), pre-exposure TAG level was relatively low, but increased 10-fold approximately 24 h after ad libitum feeding levels resumed. Seventy-two h later, the prevalence of infection suddenly increased from 31% (72 h post-exposure) to 80% (96 h post-exposure). We observed a similar response in experiment 9 conducted during the second phase of the study in which pre-exposure TAG levels were intentionally reduced by starving postlarvae for 48 h prior to BP exposure. In that experiment, there was a 12-h delay in the development of a patent infection among postlarvae with reduced TAG levels compared to that in the fed controls. In both of those experiments, the low pre-exposure TAG levels appear to have lengthened the prepatent period of the virus. Starvation and nutritional deficiencies also have been shown to delay or suppress development of baculoviruses in some insects (see Benz 1987).

There appears to be an inherent difference in the ability of various broods of shrimp reared under similar ad libitum feeding conditions, to store TAG. For example, the maximum level of TAG recorded from experiment 1, in which mysis stage shrimp were used, was 11.4 $ug \cdot mg^{-1}$ dry weight. In comparison, TAG levels from mysis stage shrimp used in experiment 4 were as high as 25.1 $ug \cdot mg^{-1}$ dry weight. Postlarvae used in experiment 3 had TAG levels as high as 21.8 $ug \cdot mg^{-1}$ dry weight compared to 12.0 $ug \cdot mg^{-1}$ dry weight or less in other groups of postlarvae. Shrimp that have the capacity for storing large reserves of TAG, either immediately

before or after exposure to BP, appear to be most susceptible to infection. That relationship does not necessarily serve as evidence for a direct interaction between baculovirus replication and lipid reserves as suggested by Vogt (1992), although such a relationship seems plausible. TAG levels may simply reflect the inherent metabolic activity of a group of shrimp. High TAG levels may be associated with fast growing shrimp in which the rapidly dividing tissues provide an excellent multiplication ground for the virus. Stuck & Overstreet (1994) reported that BP was most pathogenic to fast growing shrimp, which are likely to have greater energy reserves than slow growing shrimp. The results of our study support that observation. Differences in susceptibility of various high health stocks to BP infection reported by Overstreet (1994) may be related to inherent differences in growth rates and ability to accumulate TAG reserves. In summary, a variety of factors may have an effect on host susceptibility of BP infections. Of those, TAG levels appear to have a significant influence.

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BIOCHEMICAL RESPONSES TO BACULOVIRUS PENAEI INFECTIONS IN LARVAL AND POSTLARVAL PACIFIC WHITE SHRIMP, PENAEUS VANNAMEI: NUCLEIC ACIDS AND POLYAMINES

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ABSTARCT

The effect of baculovirus (BP) infections on nucleic acid and polyamine levels and ratios of larval and postlarval Pacific White Shrimp, Penaeus vannamei, was investigated. During several experiments, the prevalence of infection was periodically monitored over 15 - 21-day periods from both BP-inoculated and noninoculated controls. Samples for biochemical analysis were collected from the two groups immediately prior to viral exposure and at various times postinoculation (p.i.). Nucleic acid and polyamine content were determined from pooled samples of larvae and postlarvae, while individual postlarvae were analyzed for polyamines only. In all experiments, patent BP infections developed in a few inoculated shrimp by Day 1 p.i. and subsequently increased in prevalence to 100%. RNA:DNA ratios of BP-inoculated larvae were significantly lower than controls by Day 3 p.i., compared to Day 21 p.i. for postlarvae. In experiments with both larvae and postlarvae, postinoculation putrescine levels, expressed either as a concentration or as a ratio to other polyamines, were higher in BP-inoculated compared to control shrimp during most sampling periods. Changes in putrescine levels and ratios appear to be a direct response to BP infections and are not solely attributable to the viral induced differences in size of infected and noninfected shrimp. Because of the sensitivity of the detection method and specificity of the response, polyamines appear to be useful biochemical indicators of BP-induced stress in penaeid shrimp.

INTRODUCTION

Baculovirus penaei (Couch, 1974 a,b), commonly known as BP and designated as PvSNPV (Francki *et al.*, 1991), is a virus that infects both wild and cultured

populations of penaeid shrimp throughout the Western Hemisphere and Hawaii (Lightner *et al.*, 1994). Among cultured shrimp, BP infections are often associated with high mortalities and significantly reduced growth of larval and young postlarval shrimp (Stuck and Overstreet, 1994). Although the natural prevalence of BP in wild pink shrimp, Penaeus duorarum (Couch *et al.*, 1975), and brown shrimp, P. aztecus (Overstreet, 1994), from the northern Gulf of Mexico may seasonally exceed 30%, there is currently no direct evidence that the virus causes substantial mortalities in either of these species. Because it is difficult to instantaneously identify subacute responses, the effects of BP on the growth of penaeid shrimp in wild populations are unknown.

Patent BP infections are characterized by the presence of viral polyhedra or tetrahedral occlusion bodies in the nucleus of epithelial cells of the hepatopancreas (HP) and anterior midgut. Polyhedra, which are easily observed in fresh squash preparations of the HP by light microscopy (Overstreet *et al.*, 1988), usually contain a variable number of rod-shaped viral nucleocapsids occluded in a proteinaceous crystalline matrix. In the later stages of an infection, nuclei filled with polyhedra and free virions rupture from infected cells into the lumen of the midgut and pass through the feces (Couch, 1991, and Fig. 5 therein). In larval and young postlarval shrimp, a substantial portion of the HP may be destroyed when infected cells are lysed (Couch, 1981). Although loss of HP tissue may account for much of the pathogenicity of the virus, the actual mechanism by which BP causes tissue destruction is not well understood.

In Penaeus vannamei, the principle species of shrimp used in commercial culture in the Americas and Hawaii, BP is known to cause serious epizootics primarily during the hatchery phase of production (Overstreet *et al.*, 1988). The pathogenicity of BP in cultured P. vannamei can be acute (causing death) or subacute depending on the age and size of the shrimp when first infected (Stuck and Overstreet, 1994). Determining the acute effects of BP in cultured shrimp can be easily accomplished by assessing mortality. Determining the magnitude and duration of subacute effects of the virus on cultured shrimp is far more difficult and highly problematic in wild populations of shrimp. Because BP infections are frequently associated with reduced growth, biochemical indices that reflect the level of biosynthetic activity may be useful in assessing subacute effects of the virus.

Several biochemical parameters have been proposed for assessing the condition and predicting growth of crustacean larvae, postlarvae, and juveniles. Ouellet *et al.* (1992) predicted survival of shrimp larvae, Pandalus borealis, based on lipid condition. Moss (1994 a,b) used nucleic acid concentrations and ratios to assess growth of juvenile Penaeus vannamei cultured under different nutritional conditions. He reported that RNA:DNA ratios have the potential to estimate instantaneous growth in wild populations of shrimp. Stuck *et al.* (in press) reported that polyamines and some related amine compounds are useful indicators of nutritional stress in P. vannamei and can potentially be used to assess the condition of larval and postlarval shrimp. The primary objective of the study was to identify a suitable biochemical indicator of BP-induced stress in larval and postlarval penaeid shrimp. In this study, nucleic acid concentrations and ratios, and polyamine concentrations and ratios were

examined during experimental BP infections to determine their usefulness in characterizing host-virus interactions. These data may provide insights to better understand the mechanism(s) by which BP causes disease in penaeid shrimp.

MATERIALS AND METHODS

General Culture Conditions

In this study, several groups of high health Penaeus vannamei (see Wyban *et al.*, 1992) were experimentally inoculated with BP. The shrimp used in this study were obtained as either nauplii or postlarvae from one of three sources: (1) Waddell Mariculture Center, Bluffton, South Carolina, (2) The Oceanic Institute, Waimanalo, Hawaii, or (3) Harlingen Shrimp Farms, Los Fresnos, Texas. Regardless of the source, all shrimp used in these experiments originated from shrimp spawned from specific pathogen free (SPF) broodstock produced from Kona Population 1 (see Wyban, 1992). Immediately prior to initiation of each experiment, a subsample ($N \geq 10$) of larvae or postlarvae was examined for the presence of BP polyhedra in the HP following the diagnostic procedures for fresh shrimp described by Overstreet *et al.* (1988). The same diagnostic procedure was used to determine the prevalence of BP infections in all subsequent experiments conducted during this study. Lack of viral polyhedra in the preinoculated stocks and all of the noninoculated controls confirms the BP-free status of the shrimp used to stock the experiments. At the end of each experiment, shrimp were also checked for the presence of infectious hypodermal and hematopoietic necrosis virus (IHHNV) using a gene probe (Lightner *et al.*, 1992) developed by researchers at the University of Arizona. In all experiments, both BP-inoculated and control shrimp were free of IHHNV.

Salt water used in all experiments, including diatom and brine shrimp cultures, was produced from hw-Marinemix® (Hawaiian Marine Imports, Houston, Texas) and deionized water. Disodium ethylenediaminetetraacetate (EDTA-Na_2) was added to the salt water at a concentration of 10 ppm. Salinities were adjusted to 30 ± 1 ppt for larval stages and 25 ± 1 ppt for postlarvae. Cultures were maintained at 27 ± 2°C. The diatom, Chaetoceros neogracile, was fed *ad libitum* to protozoeal stages 1-3 and brine shrimp nauplii ad libitum to protozoeal stage 3 through postlarvae.

Exposure of Mysis Larvae to BP

Nauplii were obtained from Waddell Mariculture Center and reared to the mysis 1 larval stage. Larvae were then placed into two 200-liter glass aquaria at a stocking density of approximately 20 per liter. Shrimp in one aquarium were inoculated with homogenized BP-infected tissue. The strain of BP and the procedures for viral administration used during all experiments in this study have been previously described by Stuck and Overstreet (1994). Shrimp in the second aquarium, which served as the control, were given an identical amount of BP-free tissue. At 0 (pre-inoculation), 1, 2, 3, 4, 6, 9, 12 and 15 days postinoculation (p.i.), subsamples (N = 6-8), each containing 50 to 250 shrimp (ca. 60 mg total weight; "pooled" samples), were collected for biochemical analysis from both the BP-inoculated and control aquaria. During each sampling period the prevalence of infection was also determined from a subsample (N ≥ 10) of shrimp from the BP-inoculated and control aquaria. Samples collected for biochemical analysis were placed in cryo-vials, immediately frozen in liquid nitrogen, and stored at -70°C.

Exposure of Postlarvae to BP

Four-day-old postlarvae (PL4) were obtained from The Oceanic Institute and reared to PL9. Postlarvae were placed into two 200-liter aquaria at a stocking density of approximately $8 \cdot l^{-1}$. Postlarvae were inoculated with BP-infected and BP-free tissues as described previously. At O (pre-inoculation), 1, 2, 3, 5, 7, 10, 14, and 21 days p.i., subsamples (N = 6-8), each containing 10 to 25 shrimp (ca. 80 mg total weight; "pooled" samples), were collected for biochemical analysis from both the BP-inoculated and control aquaria and frozen as described earlier. During each of those sampling periods, and at 17 days p.i., the prevalence of infection was also determined from a subsample ($N \geq 10$) of shrimp from the BP-inoculated and control aquaria.

In a subsequent experiment designed to assess the effects of BP on individual postlarvae, nauplii were obtained from Harlingen Shrimp Farm, reared to PL9, and then placed into two 200-liter aquaria at a stocking density of approximately 8 per liter. Postlarvae were inoculated with BP-infected and BP-free tissues as described previously. At 0 (pre-inoculation), 3, 7, 14, and 21 days p.i., individual shrimp (N = 10-25) were collected for biochemical analysis from both the BP-inoculated and control cultures, briefly blotted on a piece of absorbent filter paper, weighed to the nearest microgram using a Cahn Electrobalance, and frozen as described earlier. At 0 (pre-inoculation), 1, 2, 3, 7, 10, 14, 17, and 21 days p.i., the prevalence of infection was determined from a subsample ($N \geq 10$) of shrimp from the BP-inoculated and control aquaria.

Biochemical Analysis

Subsamples of pooled shrimp were analyzed for nucleic acid content. Each sample was homogenized in 10 volumes of cold TEN buffer (0.05 M Tris-HCL pH 8.0, 0.02 M EDTA, 0.1 M NaCl) using a small hand-held electric tissue grinder. Two 50-μl aliquots of each sample were dried in preweighed aluminum micro weigh pans at 80°C for 24 hr to determine dry weight. After cooling to room temperature in a desiccator, each pan was reweighed to the nearest 0.1 μg using a Cahn Electrobalance.

Two 200-μl aliquots of each sample were used for nucleic acid determinations, one for DNA and the other for RNA. Each aliquot was prepared as described by Wang *et al.* (1993). DNA and RNA concentrations were determined using the diphenylamine procedure (Burton, 1956) and Schmidt-Thannhauser procedure (Munro and Fleck, 1966), respectively, as described by Wang *et al.* (1993). RNA and DNA concentrations were calculated as $\mu g \cdot mg^{-1}$ dry weight of tissue and also expressed as a RNA:DNA ratio. Preliminary attempts to use this same procedure for analysis of small postlarvae (0.3 - 5.0 mg wet weight) did not produce consistent results due to detection limits of the assay. Therefore, nucleic acid analysis was not performed on individual postlarvae.

For polyamine analysis, pooled subsamples were each homogenized in 20 volumes of cold dH_2O using a small hand-held electric tissue grinder and then briefly sonicated with a VirTis model 50 sonicator (10-0.5 sec pulses). Dry weight of each sample was determined from two 50-μl aliquots as described previously. Individual

postlarvae were homogenized in 20 volumes, or a minimum of 400 μl of dH_2O and sonicated. A single 100-μl aliquot was used for dry weight analysis.

A 200-μl aliquot of each homogenate of pooled or individual postlarvae was used for polyamine analysis. Polyamines were solubilized by the addition of 8 μl of 11.5 N perchloric acid (final concentration 0.45 N) and incubated on ice for a minimum of 20 min. Samples were then centrifuged at 13,000 RFC at 4°C after which the supernatant containing the polyamines was recovered. The samples were then derivatized with dansyl chloride and analyzed for polyamine content by high performance liquid chromatography (HPLC) following procedures described by Watts *et al.* (1994). The concentrations of putrescine, spermidine, and spermine were calculated as nmole per gram dry weight and also expressed as ratios to each other.

Statistical Analysis

Biochemical and weight measurements determined from replicated subsamples were calculated and expressed as means $\pm$ standard error. Differences in those measurements between BP-inoculated and control shrimp at each sampling period were tested for significance using *t* test and Bonferroni 95% *t*-critical values. Estimated growth-rates ($mg \cdot day^{-1}$) of individual postlarvae were calculated at each sampling period using the equation: $(W_s - W_i)/ t$, where W_s = wet weight of shrimp (mg) on the sampling day, W_i = estimated initial wet weight of shrimp (mean pre-inoculation weight), and t = days post-inoculation sample was collected. Correlation values (r) between paired estimated growth-rate and polyamine ratios for individual postlarvae were determined by simple linear regression. Analysis of variance was used to test for significance of the regressions. Analysis of covarience (ANCOVA)

was used to determine if polyamine ratios (arcsin transformed) from individual postlarvae differed significantly among treatments (BP-inoculated vs. control) when the effect of weight was statistically eliminated.

RESULTS

Exposure of Mysis Larvae to BP

Patent infections were observed in 18% of larvae inoculated with BP at Day 1 p.i., increased to 100% by Day 9 p.i. and decreased slightly by the end of the experiment (Fig. 1A). Between Days 3 and 12 p.i., the concentration of DNA in pooled samples was significantly higher ($P \leq .05$) in BP-inoculated compared to control larvae (Fig. 2A). RNA concentrations were significantly higher ($P \leq .05$) in BP-inoculated compared to control larvae 3 - 4 days p.i., but were significantly lower ($P \leq .05$) by the end of the experiment (Fig. 2B). Beginning at Day 3 p.i. and continuing through the end of the experiment, the RNA:DNA ratio in BP-inoculated larvae was consistently lower than in control larvae (Fig. 2C).

Putrescine (Put) concentrations among pooled BP-inoculated larvae were higher than control larvae during most sampling periods (Fig. 3A). Although spermidine (Spd) concentrations were slightly higher in inoculated compared to control larvae during most of the experiment, differences were not significant (Fig. 3B). Spermine (Sp) concentrations in BP-inoculated and control larvae were generally similar throughout the experiment (Fig. 3C). By Day 9 p.i. and continuing to the end of the experiment, the ratios of Put:Spd (Fig. 4A) and Put:Sp (Fig. 4B) were consistently higher in BP-inoculated compared to control larvae.

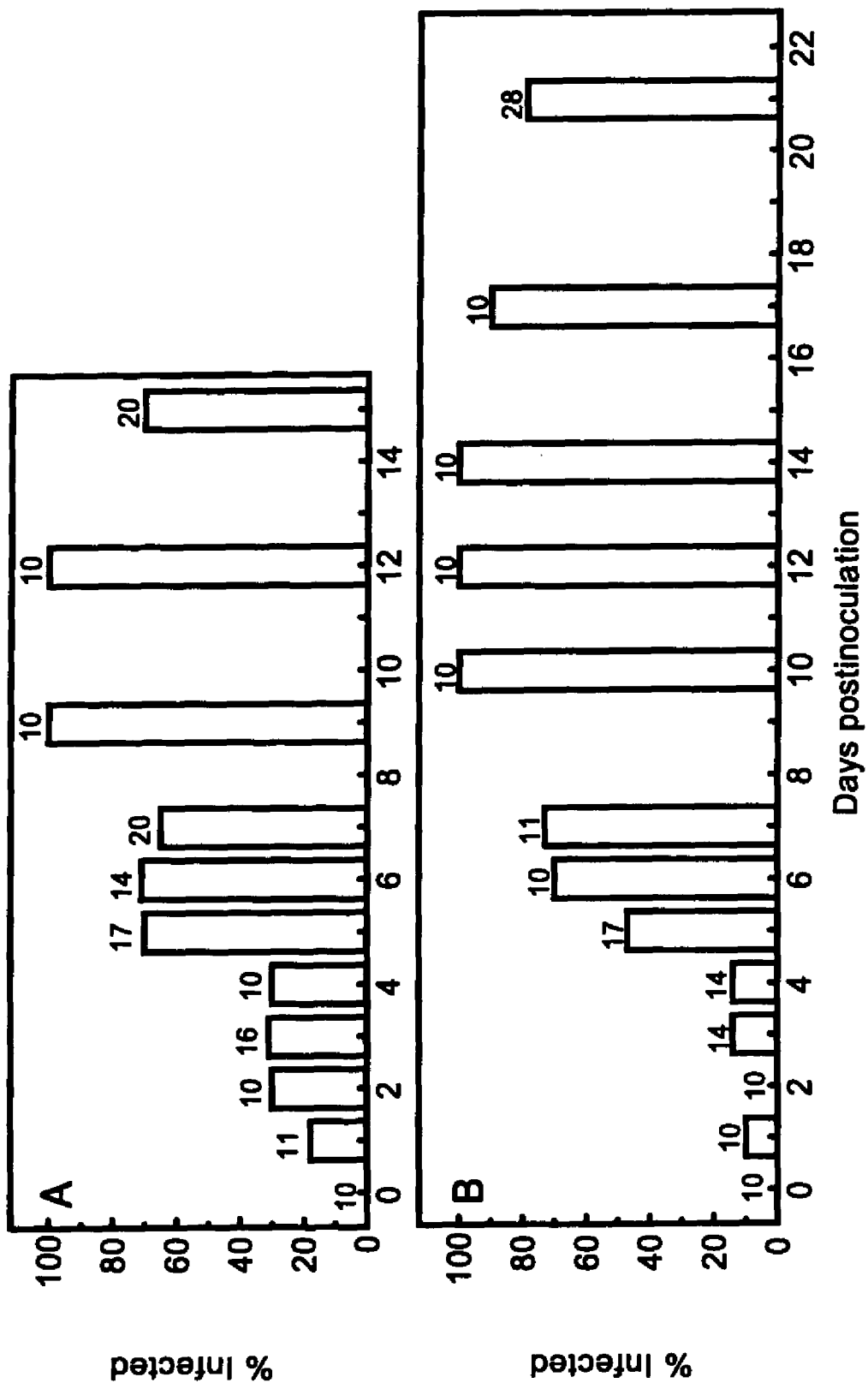

Fig. 1. Prevalence of infection in pooled samples of BP-inoculated Penaeus vannamei. (A) inoculation of mysis I larval stage; (B) inoculation of Day 9 postlarval stage. Number of shrimp examined during each sampling period is indicated above histogram bars.

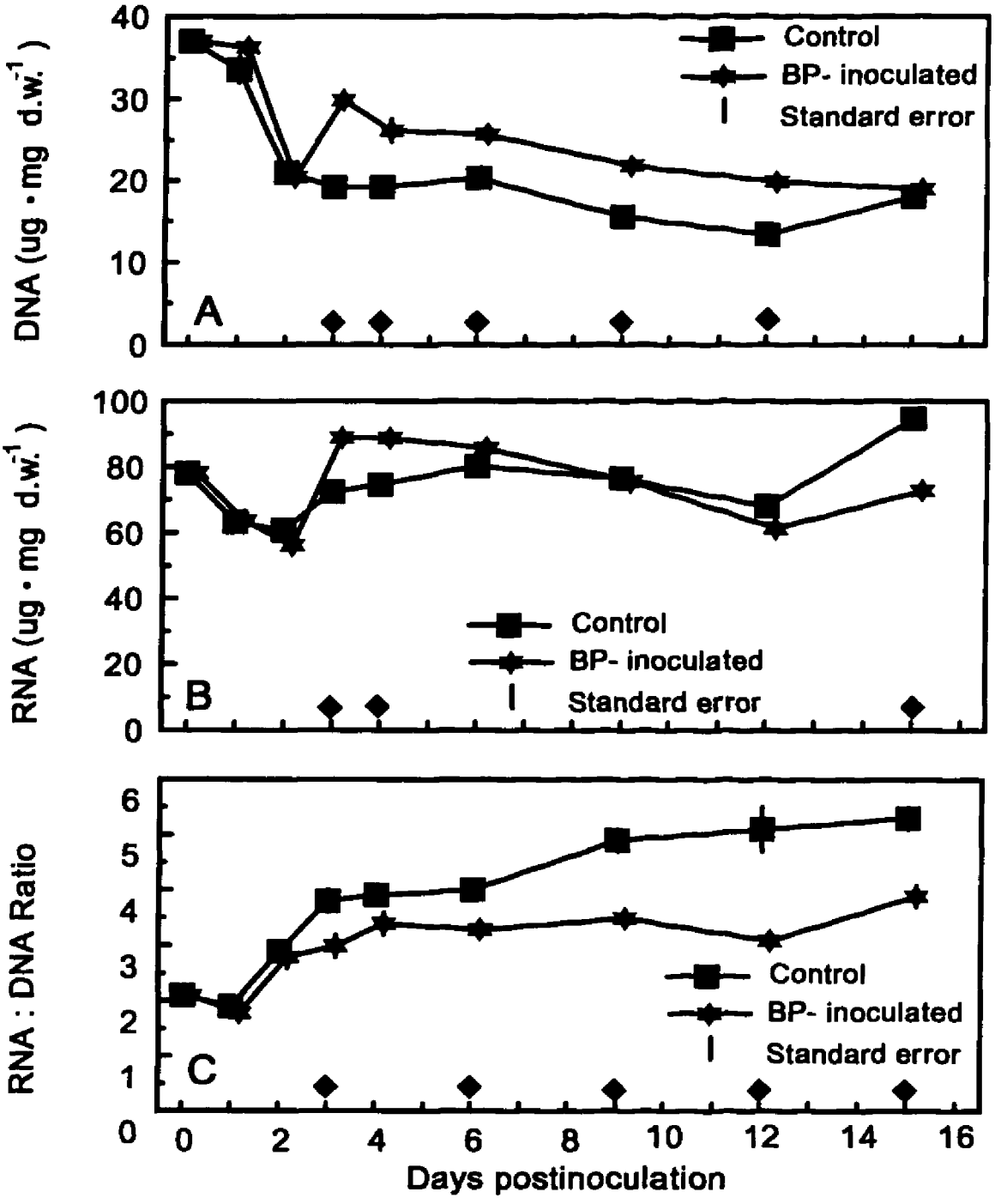

Fig. 2. Nucleic acid levels in pooled samples of BP-inoculated and control larval Penaeus vannamei. (A) DNA concentrations; (B) RNA concentrations; (C) RNA:DNA ratios. Values are expressed as means ± standard errors (N = 3). ◆ - denotes significant differences ($P \leq .05$) between BP-inoculated and control shrimp.

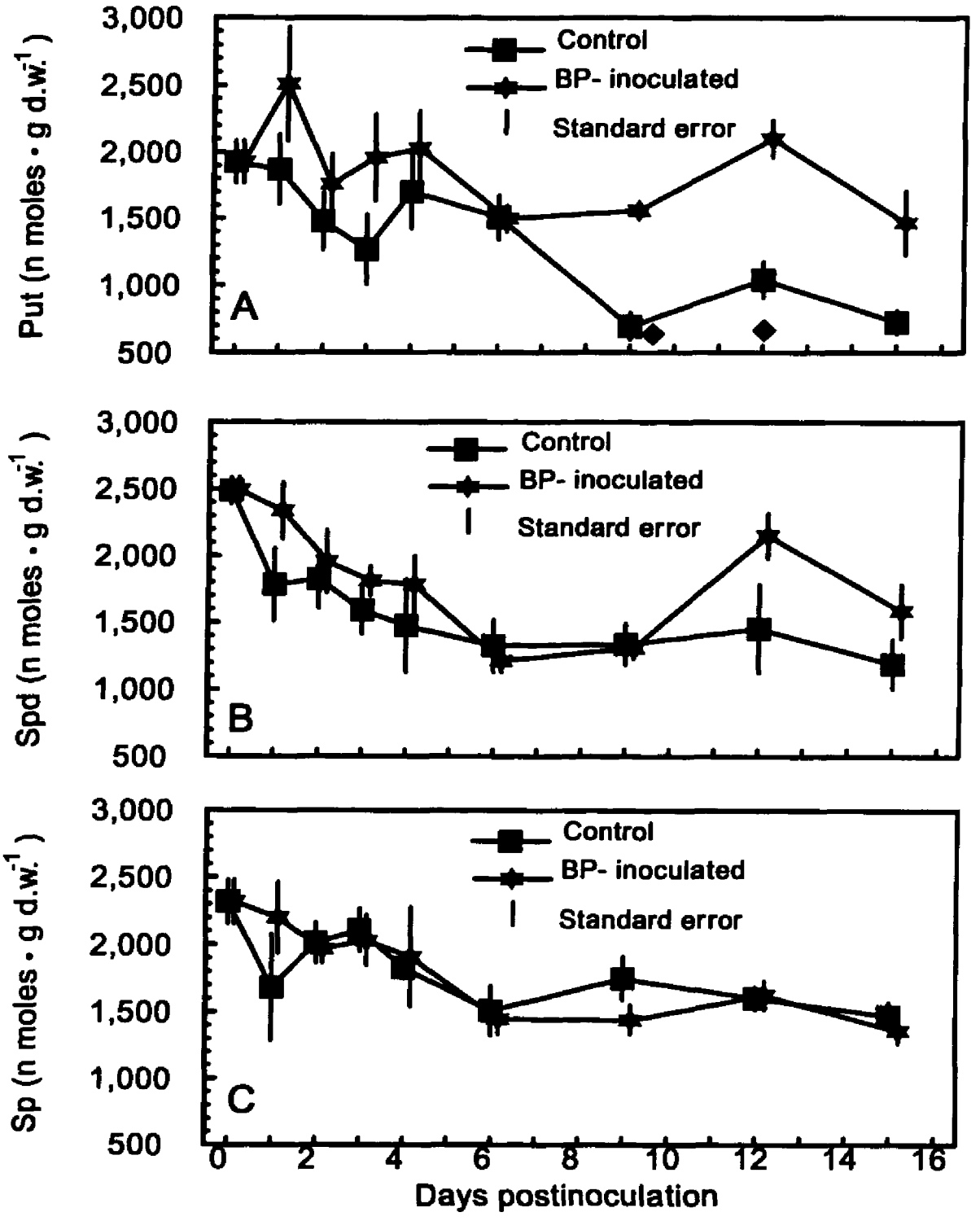

Fig. 3. Concentration of polyamines in pooled samples of BP-inoculated and control larval of Penaeus vannamei. (A) putrescine concentrations; (B) spermidine concentrations; (C) spermine concentrations. Values and symbols as in Figure 2.

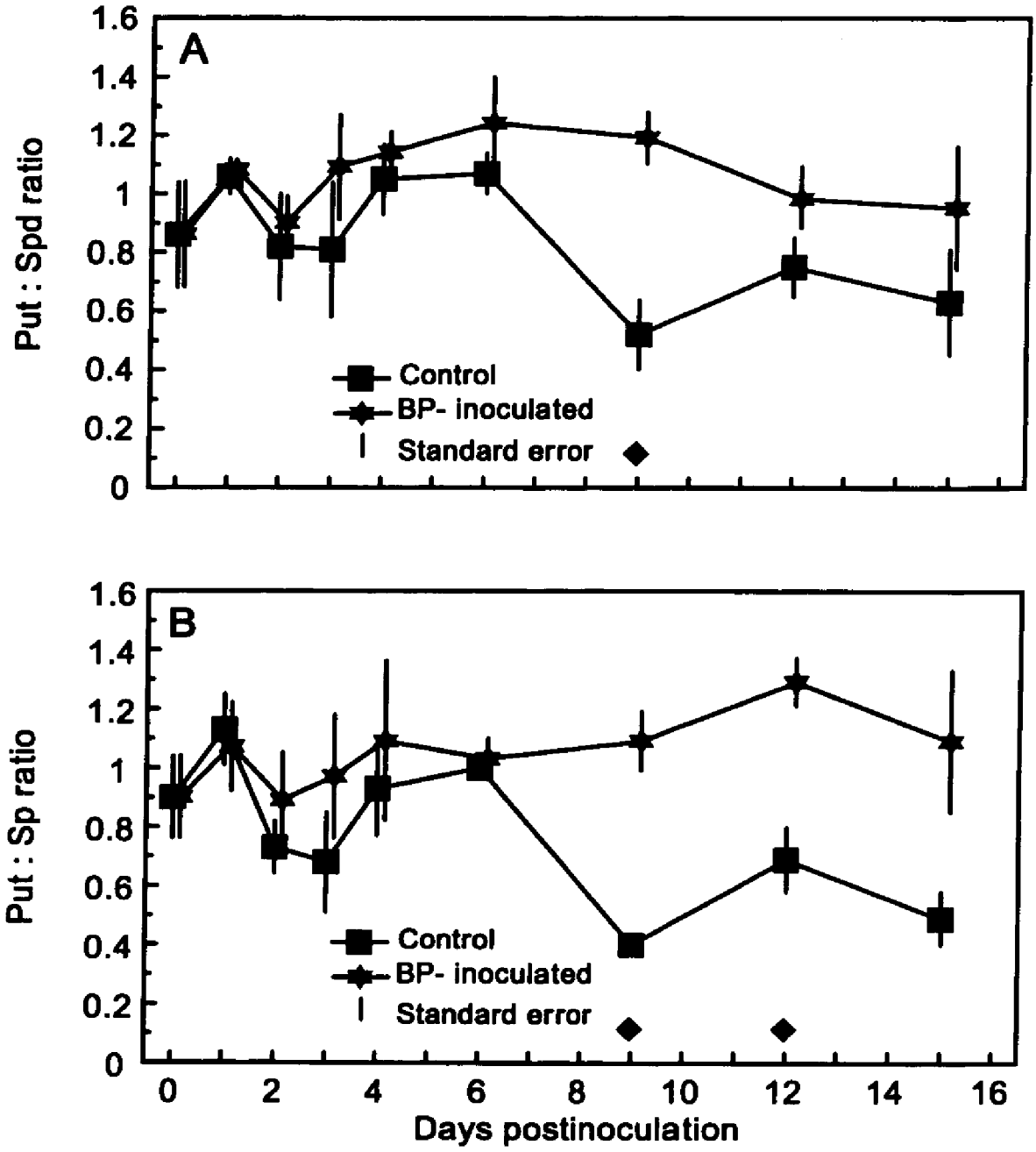

Fig. 4. Polyamine ratios in pooled samples of BP-inoculated and control larval Penaeus vannamei. (A) ratio of putrescine:spermidine; (B) ratio of putrescine: spermine. Values and symbols as in Figure 2.

Exposure of Postlarvae to BP

During the experiment in which pooled samples were collected, patent infections were observed in 10% of postlarvae inoculated with BP at Day 1 p.i., increased to 100% by Day 10 p.i. and decreased slightly by the end of the experiment (Fig. 1B). Although DNA concentrations in pooled samples of BP-inoculated compared to control postlarvae were significantly lower ($P \leq .05$) on Day 7 p.i. and significantly higher ($P \leq .05$) on Day 21 p.i., there was a gradual decline in DNA concentrations over the course of the experiment in both groups (Fig. 5A). Although RNA concentrations of BP-inoculated and control postlarvae generally followed a similar pattern of change during the course of the experiment, the concentrations were significantly lower in BP-inoculated postlarvae on Days 7 and 21 p.i. (Fig. 5B). While RNA:DNA ratios (Fig. 5C) of BP-inoculated postlarvae were significantly lower ($P \leq .05$) than in control postlarvae on Day 21 p.i., the ratios were similar during the first 14 days of the experiment.

Put concentrations were higher in pooled samples of BP-inoculated compared to control postlarvae during most sampling periods (Fig. 6A). Spd concentrations (Fig. 6B) and Sp concentrations (Fig. 6C) of BP-inoculated postlarvae at Day 1 p.i. were significantly higher ($P \leq .05$) than in control postlarvae; otherwise, Spd and Sp concentrations of BP-inoculated and control postlarvae were similar during the remainder of the experiment. Beginning at 7 - 10 days p.i., the Put:Spd ratio (Fig. 7A) and the Put:Sp ratio were consistently higher in subsamples of BP-inoculated compared to control postlarvae.

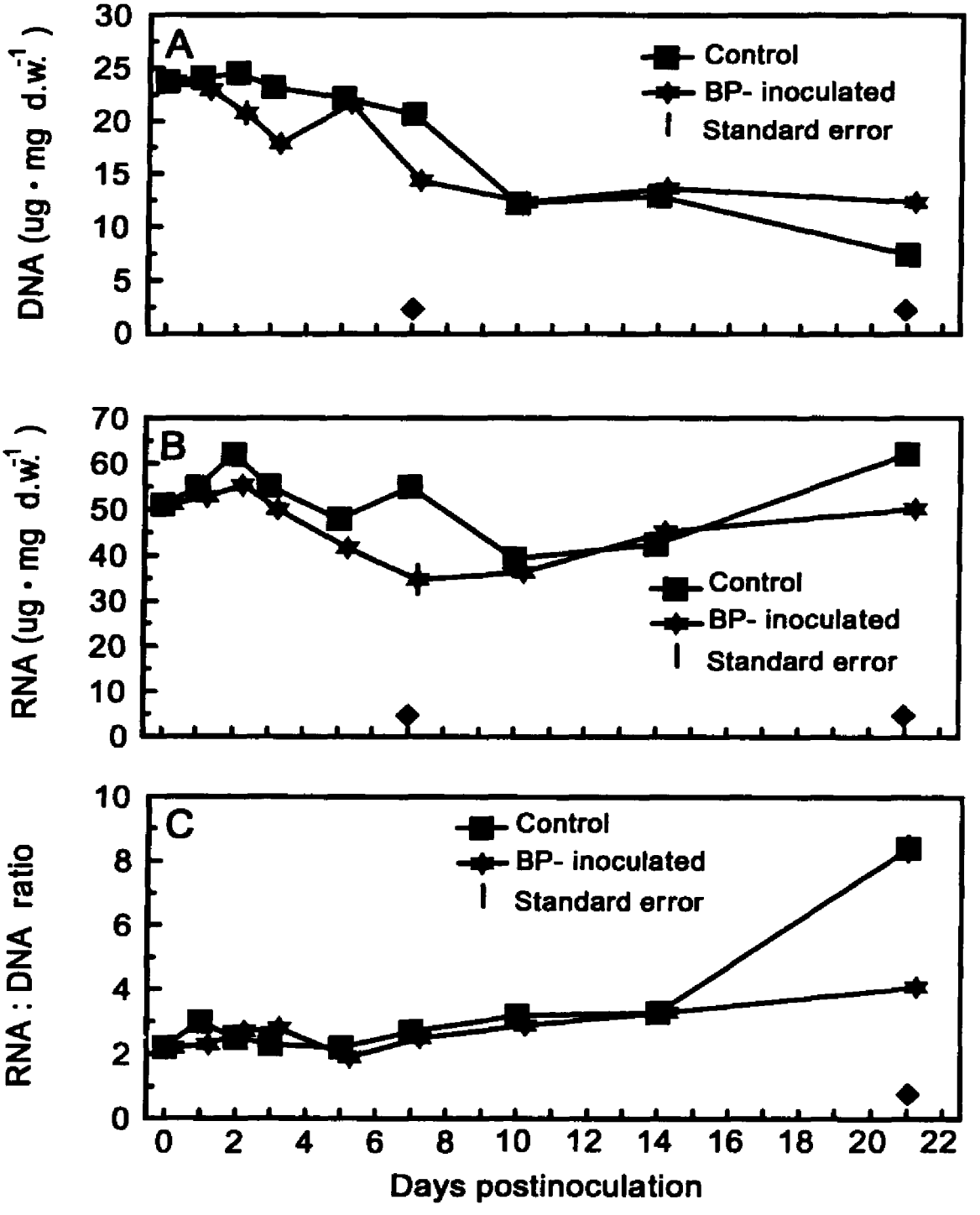

Fig. 5. Nucleic acid levels in pooled samples of BP-inoculated and control postlarval Penaeus vannamei. (A) DNA concentrations; (B) RNA concentrations; (C) RNA:DNA ratios. Values and symbols as in Figure 2.

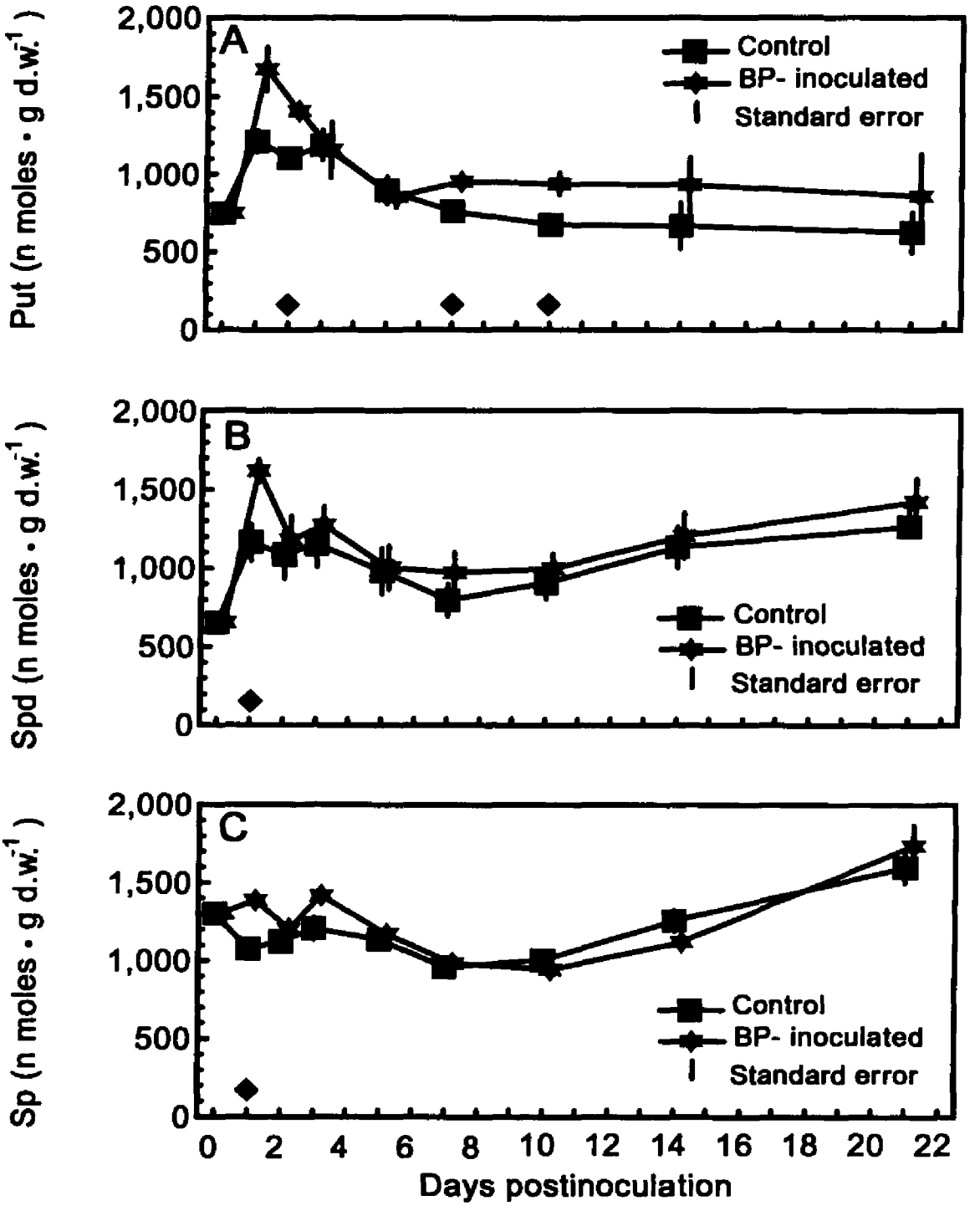

Fig. 6. Concentration of polyamines in pooled samples of BP-inoculated and control postlarval Penaeus vannamei. (A) putrescine concentrations; (B) spermidine concentrations; (C) spermine concentrations. Values and symbols as in Figure 2.

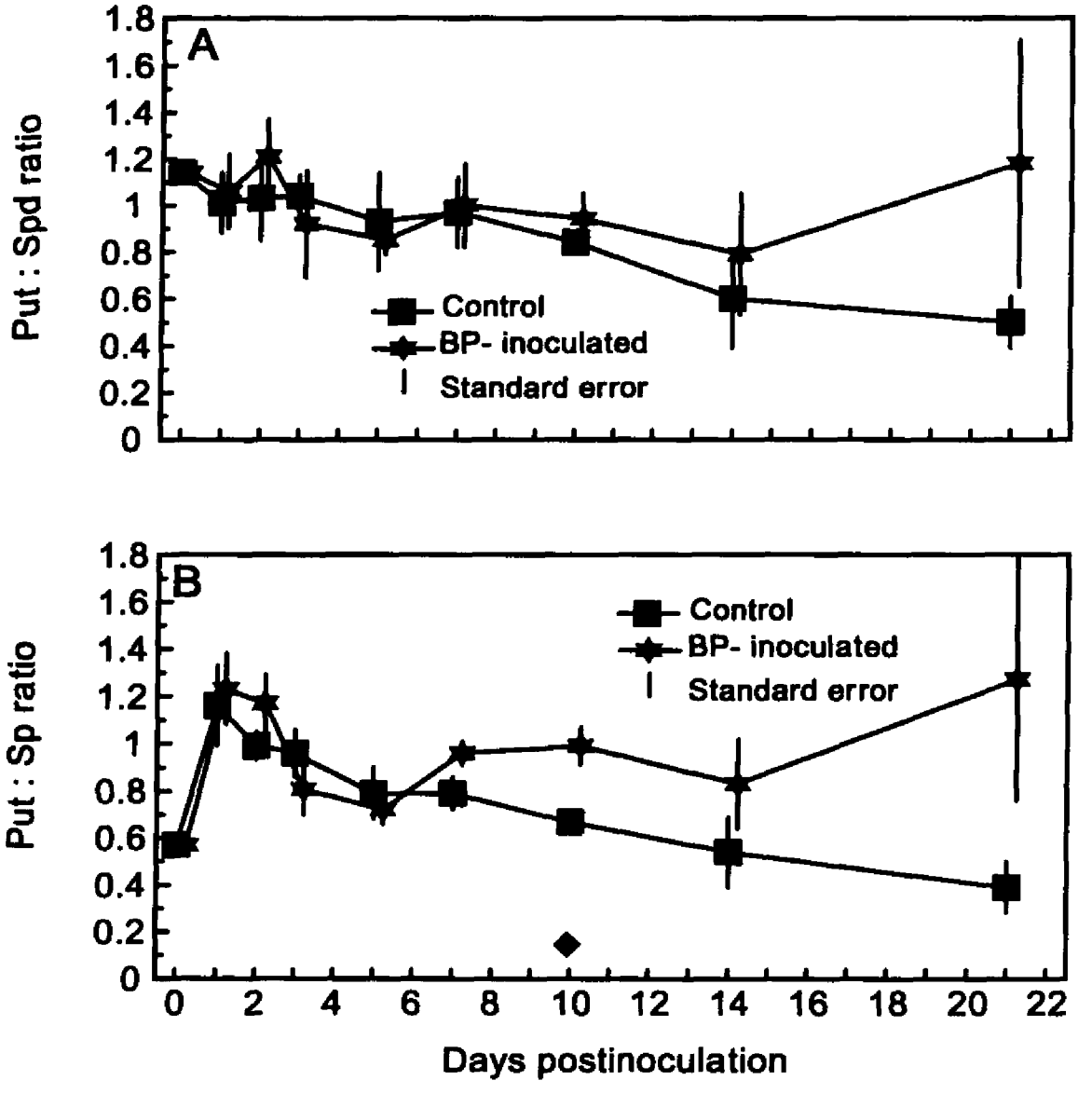

Fig. 7. Polyamine ratios in pooled samples of BP-inoculated and control postlarval Penaeus vannamei. (A) ratio of putrescine:spermidine; (B) ratio of putrescine:spermine. Values and symbols as in Figure 2.

Growth of individual postlarvae appears to have been substantially influenced by BP infection (Fig. 8). BP-inoculated postlarvae were significantly smaller ($P \leq .05$) than control postlarvae at 7 and 14 days p.i. Little growth was observed among BP-inoculated postlarvae during the first 7 days p.i. Patent infections were first detected at Day 1 p.i., increased in prevalence to 100% by Day 2 p.i., and remained high until the end of the experiment at which time a slight reduction in prevalence was observed.

The concentration of Put in individual BP-inoculated postlarvae rapidly increased between 3 and 7 days p.i. and was significantly higher ($P \leq .05$) than in control postlarvae at 7 and 14 days p.i. (Fig. 9A) . The concentration of Put in BP-inoculated postlarvae returned to levels similar to control postlarvae by the end of the study. The concentration of Spd (Fig. 9B) was substantially, but not significantly, higher in individual BP-inoculated compared to control postlarvae at Day 7 p.i., but otherwise was similar during the course of the experiment. Sp concentrations of individual BP-inoculated and control postlarvae followed similar patterns of change during the course of the experiment and were not significantly different. The ratio of Put:Spd in individual BP-inoculated postlarvae rapidly increased during the first 3 days of the experiment and was significantly higher ($P \leq .05$) than in control postlarvae on Days 3, 7, and 14 p.i (Fig. 10A) . A similar response to BP exposure was observed in the Put:Sp ratio (Fig. 10B).

During most but not all sampling periods, the ratios of Put:Spd and Put:Sp, were negatively correlated with estimated growth rates (mg per day) of individual shrimp (Table 1). While most of these correlations were weak, the strongest

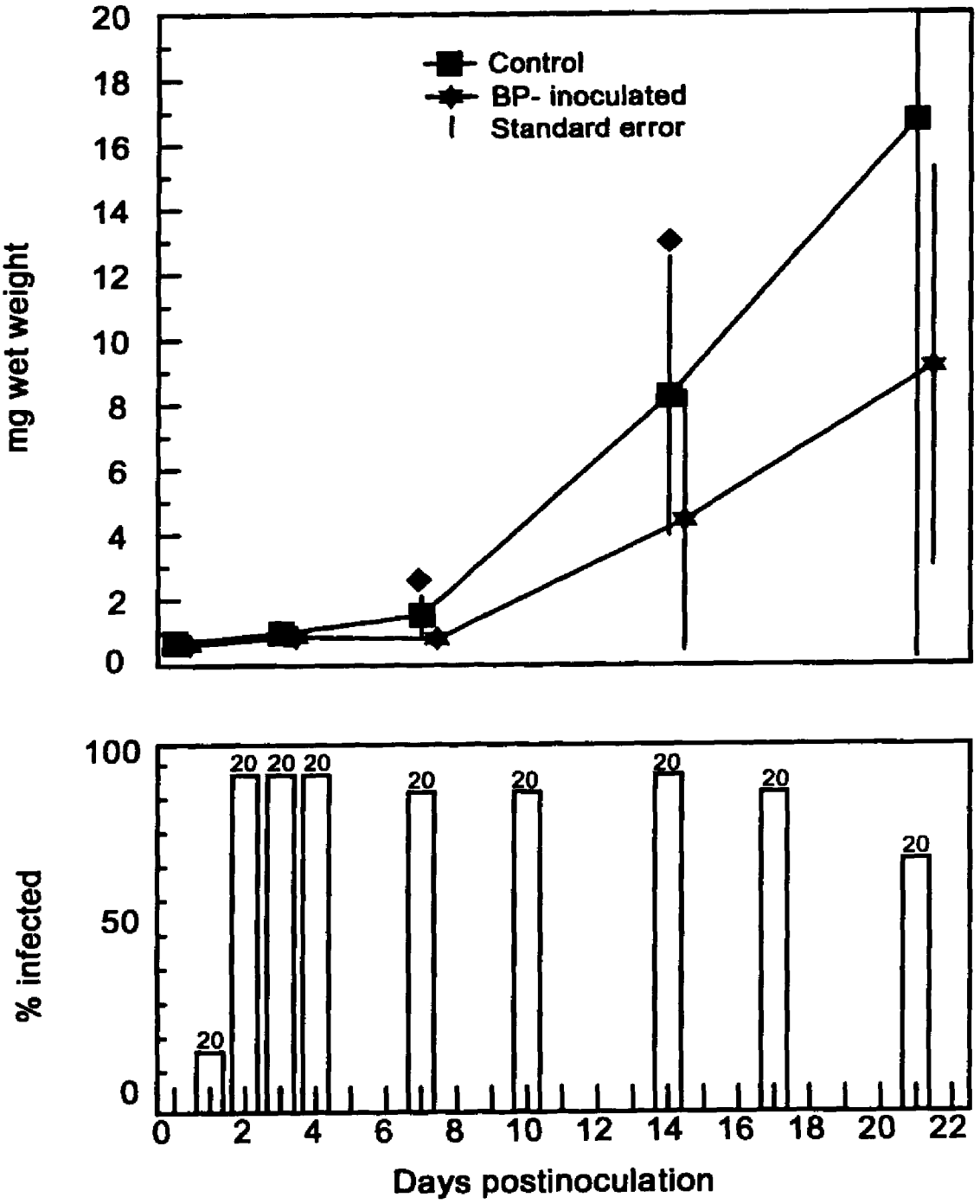

Fig. 8. Growth of individual BP-inoculated and control postlarval Penaeus vannamei and prevalence of infection in the BP-inoculated group. Number of shrimp examined for infection during each sampling period is indicated above histogram bars. Values and symbols as in Figure 2.

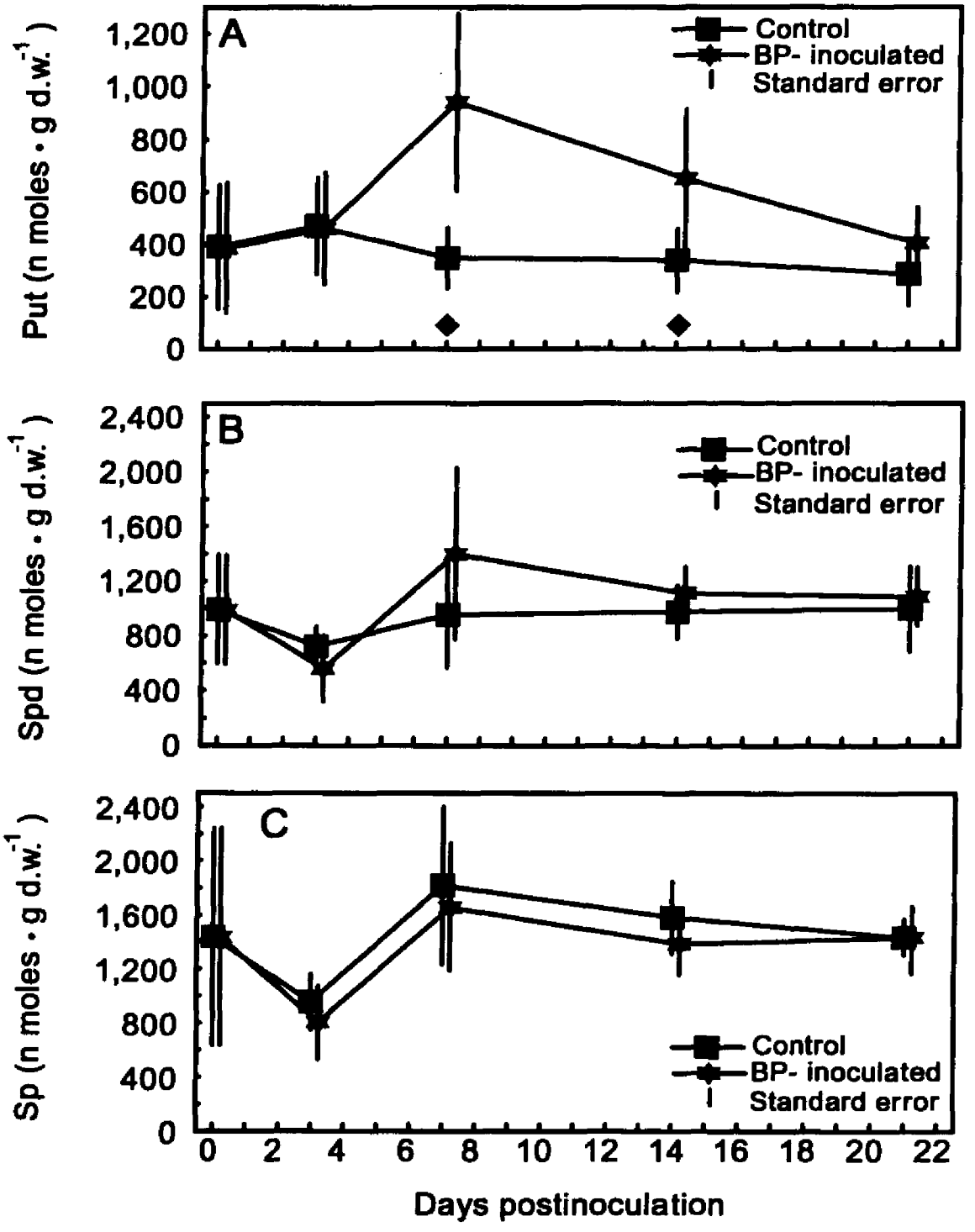

Fig. 9. Concentration of polyamines in individual BP-inoculated and control postlarval Penaeus vannamei. (A) putrescine concentrations; (B) spermidine concentrations; (C) spermine concentrations. Values and symbols as in Figure 2.

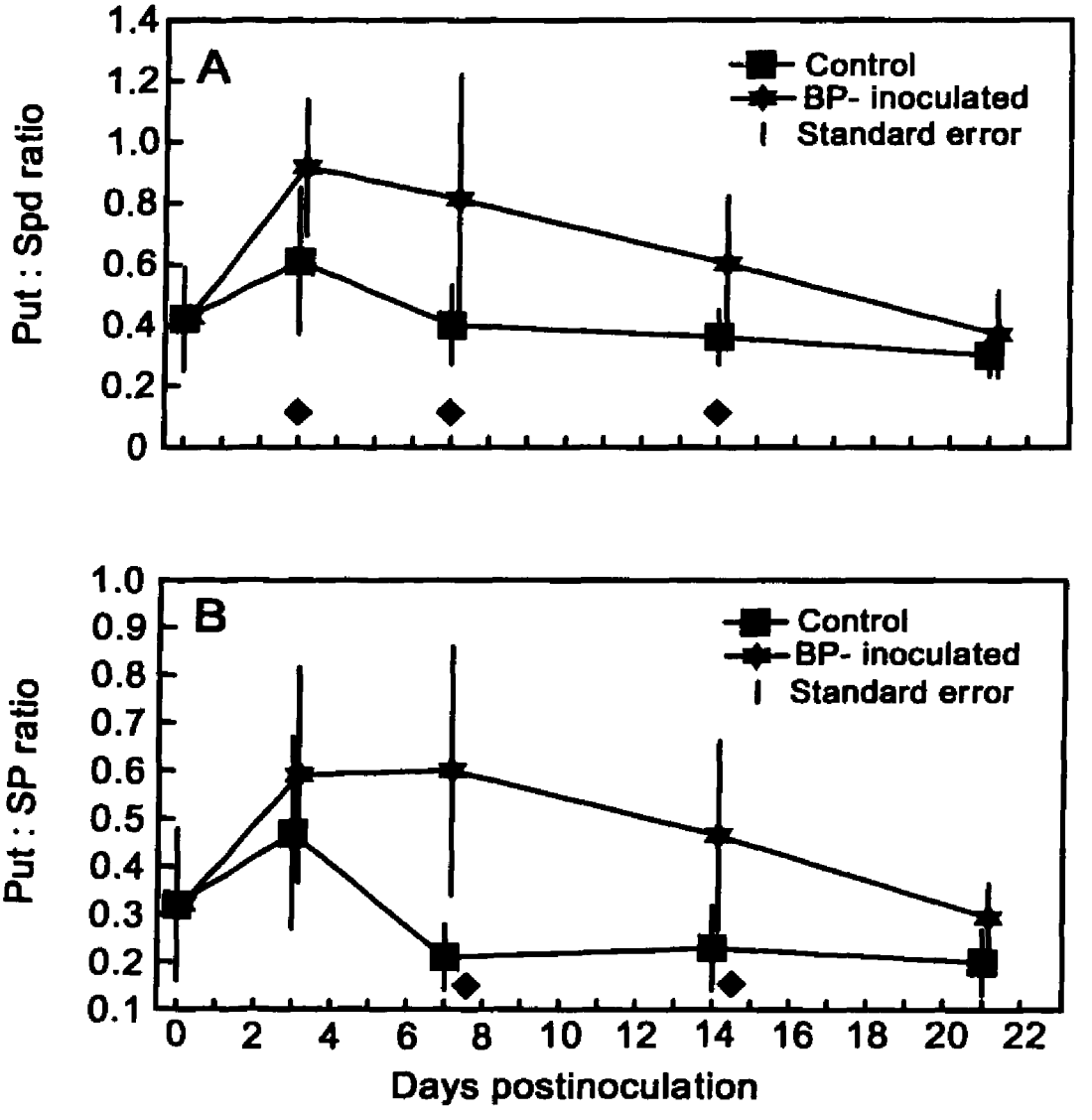

Fig. 10. Polyamine ratios in individual BP-inoculated and control postlarval Penaeus vannamei. (A) ratio of putrescine:spermidine; (B) ratio of putrescine:spermine. Values and symbols as in Figure 2.

Table 1
Correlations coefficients (r) for paired estimated growth-rates (mg per day) and polyamine ratios from individual postlarvae collected at 3, 7, 14, and 21 days postinoculation to BP

Day 3 post-inoculation			
Ratio	Control (22)	BP (22)	Combined (44)
Put:Spd	0.163	0.131	-0.002
Put:Sp	-0.016	-0.012	-0.090
Day 7 post-inoculation			
Ratio	Control (20)	BP (23)	Combined (43)
Put:Spd	-0.175	-0.062	-0.376[a]
Put:Sp	-0.049	-0.092	-0.428[b]
Day 14 post-inoculation			
Ratio	Control (22)	BP (22)	Combined (44)
Put:Spd	0.065	-0.715[c]	-0.553[c]
Put:Sp	-0.027	-0.589[b]	-0.531[c]
Day 21 post-inoculation			
Ratio	Control (10)	BP (10)	Combined (20)
Put:Spd	-0.319	-0.017	-0.254
Put:Sp	-0.333	-0.076	-0.336

Note. Correlations were run on shrimp from BP-inoculated and control groups separately and combined. The number of measurements in each group is indicated in parentheses ( ). Abbreviations: Put = putrescine, Spd = Spermidine, Sp = spermine, BP = BP-inoculated postlarvae. Control = noninoculated postlarvae.

[a]correlation significant at $P \leq .05$,
[b]correlation significant at $P \leq .01$,
[c]correlation significant at $P \leq .001$.

correlations occurred among postlarvae sampled on Day 7 p.i., when results from the BP-inoculated and control groups were combined, and on Day 14 p.i. from the BP-inoculated group of shrimp, when analyzed separately and in combination with the

control postlarvae. The strongest correlations were observed between Day 14 p.i. estimated growth-rates of BP-inoculated postlarvae and Put:Spd and Put:Sp ratios. Analysis of covarience (ANCOVA) indicated that the Put:Spd ratio of BP-inoculated and control shrimp was significantly affected by both BP-inoculation and weight of shrimp on Day 14 p.i. and when results from all sampling periods were combined (Table 2). However, on days 3, 7, and 21 p.i., the Put:Spd ratio was affected by viral inoculation to a much greater extent than weight. A similar influence of BP inoculation and weight on the Put:Sp ratio was also observed.

DISCUSSION

Previous studies (Sano *et al.*, 1985; Overstreet *et al.*,1988; LeBlanc and Overstreet, 1990; Stuck and Overstreet., 1994) have shown that the pathogenicity of baculovirus infections in penaeid shrimp, including Penaeus vannamei, is influenced by host age. Experimental BP infections in larval and early postlarval (<PL10) P. vannamei can in some, but not all, cases cause substantial mortality typically between 4 - 7 days p.i. Mortality of BP-infected shrimp after 7 days p.i. is usually low; however, surviving shrimp typically experience reduced growth (Stuck and Overstreet, 1994). Most mysis stage larvae and postlarvae of P. vannamei experimentally infected with BP appear to lose their infection within 30-40 days p.i. (Stuck and Wang, submitted). Therefore, in cultured P. vannamei, the pathogenic effects of BP, both acute and subacute, on larvae and young postlarvae typically occur between 4 and 40 days p.i. Results of the growth-rate and biochemical analysis conducted during the present study support that observation. Experimental BP infections in older postlarvae (>PL14) of P. vannamei usually have little effect on

Table 2
The influence of BP-inoculation and weight on polyamine ratios

	N	BP	WEIGHT
Put : Spd ratio			
Day 3 p.i.	44	0.010	0.696
Day 7 p.i.	43	0.007	0.541
Day 14 p.i.	44	0.007	0.001
Day 21 p.i.	20	0.136	0.516
Combined	151	<0.001	<0.001
Put : Sp ratio			
Day 3 p.i.	44	0.124	0.536
Day 7 p.i.	43	<0.001	0.970
Day 14 p.i.	44	0.001	0.029
Day 21 p.i.	20	0.086	0.450
Combined	151	<0.001	<0.001

Note. Probabilities for the dependent variable (arcsin transformed ratios of Put:Spd or Put:Sp) were determined by analysis of covariance (ANCOVA) using viral inoculation (control vs. BP-inoculated) as the independent factor, and weight as the covariate. Probabilities were calculated seperately on days 3, 7, 14, and 21 days post-inoculation (p.i.) and for all days combined. Abbreviations: Put = putrescine, Spd = Spermidine, Sp = spermine, BP = influence of virus on polyamine ratios, WEIGHT = influence of weight on polyamine ratios.

either survival or growth, while juveniles and adults may become completely resistant to infection (LeBlanc and Overstreet, 1990; Stuck and Overstreet, 1994).

Acute effects of baculovirus infections in cultured penaeid shrimp are typically assessed in terms of mortality (Sano *et al.*, 1985; Overstreet *et al.*, 1988); however, subacute effects of the virus are more difficult to determine. Stuck and Overstreet (1994) assessed subacute effects of BP in cultured Penaeus vannamei by comparing growth of infected postlarvae to uninfected controls. While this may be feasible under experimental conditions, such an approach would be impractical to use with wild populations of shrimp. Because BP infections can affect nutrient uptake and growth, or may stimulate a specific host response, biochemical parameters that reflect such changes may be useful in assessing subacute effects of the virus.

Triacylglycerol (TAG) content has been used to predict survival of shrimp larvae (Ouellet *et al.*, 1992) and assess growth of crab larvae (Lovrich and Ouellet, 1994). Preinoculation TAG levels appear to have a significant influence on the development of patent BP infections (Stuck *et al.*, submitted). However, postinoculation reductions in TAG content in response to BP in larval and postlarval Penaeus vannamei are inconsistent and subordinate to ontogenic changes. Therefore, the utility of TAG levels for assessing acute and subacute effects of the virus appears limited.

Because the RNA:DNA ratio serves as an index of cellular protein synthesis, it has been widely used to assess growth (Buckley, 1984; Malloy and Targett, 1994), nutritional condition (Clemmesen, 1987, 1994), and sublethal anthropogenic stress (Barron and Adelman, 1984, Wang *et al.*, 1993) in fish. A similar potential use of the RNA:DNA ratio in penaeid shrimp has been demonstrated (Moss, 1994a,b; Stuck *et al.*, in press). The RNA:DNA ratio of BP-infected larvae was substantially lower than

controls by Day 3 p.i. and continued to be lower through the duration of the experiment, while the RNA:DNA ratio of BP-inoculated postlarvae was not significantly lower than controls until Day 21 p.i. These results generally support the growth and mortality data from both these experiments as reported by Stuck and Overstreet (1994). Results of nucleic acid determinations in this study also corroborate the findings of previous studies that BP is generally more pathogenic to larvae than postlarvae. The sudden increase in DNA concentrations among BP-inoculated larvae observed on Day 3 p.i. may be an indication of rapid depletion of energy reserves, similar to that reported by Stuck *et al.* (in press) in response to severe nutritional stress. The low RNA:DNA ratios observed among BP-inoculated larvae and postlarvae compared to controls during the later phases of both experiments were due to a reduction in RNA relative to DNA, and are similar in response to chronic nutritional (Wang and Stickle, 1986; Moss, 1994a,b) and anthropogenic stress (Wang and Stickle, 1988) reported in other crustaceans.

The polyamines, putrescine, spermidine, and spermine are organic cations associated with nucleic acid and protein synthesis (Heby 1981); however, their specific functions in biosynthetic pathways and other cellular processes are not completely understood. Polyamines have been used as biochemical markers of normal and pathological growth in vertebrates (Pegg, 1988). In comparison to nucleic acids, relatively few studies have investigated the relationship between polyamine levels and growth or condition of aquatic animals. Corti *et al.* (1988) and Davalli *et al.* (1990) investigated the effects of toxic chemicals, temperature induced stress, and diet on polyamines in fish. Watts *et al.* (1992) proposed the use of polyamines to assess

growth in IHHNV infected and noninfected Penaeus vannamei, and Stuck *et al.* (in press) characterized the effects of starvation on polyamine content of the same species.

Polyamines were analyzed from pooled samples of larvae and postlarvae and, subsequently, from individual postlarvae to determine the response to BP infection. In all these experiments, Put levels of BP-inoculated shrimp were substantially to significantly higher than in control shrimp during most sampling periods, Spd levels were slightly higher during some sampling periods, and Sp levels were least affected by the virus. Because all three polyamines are quantified simultaneously using HPLC (Watts *et al.*, 1994), relative changes in polyamine levels can also be reliably expressed as ratios to each other, thus eliminating the time consuming effort and possible errors associated with dry weight analysis. In experiments with both larvae and postlarvae, the Put:Spd and Put:Sp ratios generally reflected the relative increase in Put in response to BP infections. The greatest differences in the Put:Sp ratio of BP-inoculated compared to control shrimp occurred on or after Day 7 p.i. This corresponds with the time period during which shrimp that survived the initial infection would be experiencing subacute effects of the virus.

An increase in Put levels in response to anthropogenic stress or injury is a common response in plants (Flores, 1991; Scoccianti *et al.*, 1995) and mammals (Corti *et al.*, 1985; Gilad *et al.*, 1993). In fact, accumulation of Put is one of the very first metabolic events detectable in many biological system under stress (Corti *et al.*, 1987). Whether the increase in Put is the result of a block in utilization, changes in the rate of polyamine degradation, or stimulation of biosynthesis is unknown (Soccianti *et al.*, 1995). However, examination of possible causes for the elevated Put

levels in infected shrimp may provide insights into the mechanism of pathology and recovery from BP infections. One possible factor affecting the rate of degradation of polyamines is associated with the phenomenon of programmed cell death or "apoptosis." Apoptosis, an active process of self-destruction, is initiated in response to baculovirus infection in insect cells (Clem *et al.*, 1991). Recent studies (Gramzinski *et al.*, 1990; Parchment, 1993) have shown a link between polyamine catabolism and programmed cell death, whereby oxidation of Spd and Sp to Put produces hydrogen peroxide, which acts as a mediator of apoptosis. The increase in Put we observed in BP-infected shrimp may be related to that process. Stuck and Overstreet (1994) reported a substantial reduction in the size of the HP in response to the establishment of patent BP infections in larval and young postlarval Penaeus vannamei. In surviving shrimp, BP-infected cells appear to be destroyed and quickly replaced by new noninfected cells (Overstreet, 1994).

Changes in polyamine levels and ratios observed in our study do not appear to be a generalized response to stress as does nucleic acid levels and ratios. In postlarval Penaeus vannamei, Put concentrations and Put:Sp ratios were not affected by prolonged starvation, and Put expressed as a ratio to DNA decreased relative to fed shrimp (Stuck *et al.*, in press). In our study of individual postlarvae, the strongest correlations between estimated daily growth rates and both the Put:Spd and Put:Sp ratios occurred within BP-inoculated postlarvae at Day 14 p.i. This corresponds to the time period in which subacute effects of the virus on surviving shrimp should be substantial. Correlations between polyamine ratios and estimated daily growth rate of noninfected control postlarvae were weak and did not exhibit a consistant relationship.

Using ANCOVA, it was determined that both weight of postlarvae and the effects of BP-inoculation may have had a significant influence on polyamine ratios. However, the influence of weight on polyamine ratios was significant only on Day 14 p.i. This apparent affect of size on polyamine ratios is probably the result of the reduced growth of surviving, but severely impacted BP-inoculated postlarvae, and was most evident by Day 14 p.i. By Day 21 p.i., some of the surviving infected postlarvae appear to have recovered and began experiencing accelerated growth, thus reducing the apparent effect of weight on polyamine ratios. The changes in polyamines and polyamine ratios observed in our study appear to be a specific response to the effects of BP infection and not simply a function of size differences of BP-inoculated and control postlarvae.

Put concentrations and the Put:Spd and Put:Sp ratios were consistently higher when determinations were made from pooled compared to individual postlarvae, whereas Spd and Sp concentrations in the two groups of postlarvae were similar. Because these two groups of postlarvae were from different sources, it was not possible to determine if the differences were due to a possible masking effect of a few large individuals in the pooled samples, or to actual inherent differences in polyamine content of the two populations. Mysis stage larvae also had substantially higher polyamine concentrations than postlarvae, suggesting there may also be an ontogenic influence on polyamine content. Additional research is needed to document the ontogenic and possible inherent differences in polyamine content of penaeid shrimp.

Results of our study suggest that Put, as well as the Put:Sp and Put:Spd ratios, may be useful biochemical indicators of subacute stress in cultured BP-infected

penaeid shrimp. The use of polyamines to asses viral induced stress has several advantages over nucleic acids: (1) HPLC determination of polyamines is very sensitive, allowing analysis of small (~0.3mg) individual postlarvae; (2) multiple polyamine ratios can be determined from a single analytical procedure; and (3) changes in polyamine ratios may be a specific response to BP infection. Additional research is needed to investigate the relationship between polyamine levels and BP infections in cultured and wild populations of penaeid shrimp.

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SUMMARY

This dissertation comprises a compendium of five interrelated publishable investigations on the virus Baculovirus penaei (BP) and its effect on the Pacific white shrimp, Penaeus vannamei. Several important aspects of the infection, the agent, and host were determined that have an influence on the production of this commercially important penaeid shrimp in aquaculture operations. Information was also obtained on viral-host interactions that should be valuable in assessing the influence of the virus on wild populations of penaeid shrimp.

The time course for development of BP infections in Penaeus vannamei observed in this study was similar to that reported in several previous studies and similar to that in many species of insects. Using both traditional and molecular diagnostic methods, this study first detected pre-patent infections in BP-exposed larvae at 12 hr postinoculation (p.i.) and patent infections by 18-24 hr p.i. The prevalence of infection increased to 100% by 3 to 17 days p.i. and was undetectable in most shrimp after 30-45 days p.i. The apparent loss of BP infections and reduced susceptibility of shrimp to reinfection suggest a possible induction of immunity in previously infected shrimp. Similar responses have been observed in some species of insects infected with baculoviruses.

The acute and subacute effects of BP on shrimp of different ages were determined by assessing survival and growth. An age-dependent pattern of

pathogenicity was observed in which BP infected larvae and young postlarvae commonly experienced high mortality and significantly reduced growth of surviving shrimp. Whereas older postlarvae inoculated with BP sometimes experienced a temporary reduction in growth, viral related mortality was rarely observed. These findings corroborate those of previous studies showing that the pathogenic effects of BP are somewhat dependent on the age and size of the shrimp when initially infected. The virus also appears to be most pathogenic to rapidly growing shrimp. Subacute effects of the virus can also be assessed by determining the susceptibility of infected shrimp to mortality induced by prolonged starvation.

Several biochemical indices, including water content, nucleic acids, and protein and polyamine ratios can be used to assess the effects of prolonged and severe nutritional stress in and subsequent recovery of postlarval penaeid shrimp. Because of the rapid and almost total depletion of triacylglycerol (TAG) in response to starvation, the TAG concentrations and TAG:sterol ratio is of limited value in characterizing chronic nutritional stress. Two unidentified amines, expressed as a ratio to the polyamine spermine, however, showed a significant response to changing nutritional conditions.

In some cases, energy reserves in the form of TAG are significantly reduced during the establishment of patent BP infections; however, such reductions are subordinate to fluctuations in TAG content associated with the ontogeny of the hepatopancreas. High pre-inoculation TAG levels were associated with rapid development of intense infections. Experimental reduction of TAG levels prior to inoculation delayed viral replication. Similar observations have been made in some

insects infected with other baculoviruses. The apparent relationship between TAG and baculovirus replication in shrimp may not be due to the direct utilization of lipid reserves for viral reproduction as suggested in a previously published study, but rather the association between growth rates and susceptibility to infection determined in this study.

The RNA:DNA ratio in BP-infected shrimp was significantly reduced compared to that in uninfected shrimp but not until patent infections were well established. The observed changes in nucleic acid levels and ratios appear to be a generalized response to stress associated with reduced growth and high mortality of infected shrimp. Inoculation and subsequent infection of larval and postlarval shrimp were associated with an increase in levels of the polyamines putrescine and, to a lesser extent, spermidine relative to those levels in uninfected shrimp. Changes in polyamine levels and the two unidentified amines reported earlier as a response to nutritional stress were not observed as a response to BP infections. Therefore, the pathogenic effects of BP are biochemically distinct from nutritional stress. Changes in putrescine levels associated with BP infections may be a specific response to viral infection. If so, this response may prove to be a valuable tool for instantaneously identifying individual shrimp experiencing subacute stress in both cultured and wild penaeid shrimp stocks infected with BP.

In addition to providing new knowledge on aspects of the biology of BP in penaeid shrimp, viral-host interactions, the extent and nature of pathology, and methods for determining subacute effects of the virus, this study has generated numerous questions not part of the original objectives. To address these questions,

additional studies are planned or are needed. The availability of molecular detection methods and biochemical procedures for assessing viral pathogenicity creates new opportunities for additional research. Investigations of the mechanisms and extent of viral mediated immunity in penaeid shrimp and the relationship between polyamine levels and pathogenicity of the virus have been initiated. Future investigations on BP should attempt to determine the mechanism(s) of transmission and assess the ecological significance and influence of the virus in wild populations of penaeid shrimp. Additional research is also planed to determine the utility of biochemical indices, such as protein and nucleic acid ratios, dry weight, and neutral lipids, to predict quality of postlarval shrimp and to determine the identity and nature of the biochemical response to nutritional stress observed in unidentified amines 3.4:Spermine, 5.5:Spermine and 7.5:Spermine.

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APPENDIX A

DETECTION OF BACULOVIRUS PENAEI DNA AMPLIFIED BY PCR

For each sample diagnosed for the presence of viral DNA as reported in Chapter 1, multiple 25-μl reactions were established using 1, 5, and 10 μl of host-viral template DNA, 1 μl each of the primer pair (5 μM stock solution), and reagents provided in a AmpliTaq thermal-stable DNA polymerase kit (Perkin Elmer, Norwalk, CT). A Perkin-Elmer model 2400 thermal cycler was used for the amplification and was programmed as follows: one cycle of 95°C for 3 minutes; 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 60 seconds; one cycle of 72°C for 5 minutes. Five microliters of 10x loading buffer was added to the sample and 10 μl of the mixture was loaded on a 2% agarose gel submerged in 1x TBE buffer. The gel was run at 79 volts for 1.5 hours and then stained for 20 minutes in a 0.5-μg/ml solution of ethidium bromide. DNA bands were visualized by placing the gel on a UV-transilluminator and the gel was photographed using Polaroid 667 B/W film. The PCR amplified viral DNA produced a single distinct band of 560 base pairs in infected shrimp (Fig. 1). DNA from uninfected shrimp did not produce a similar band.

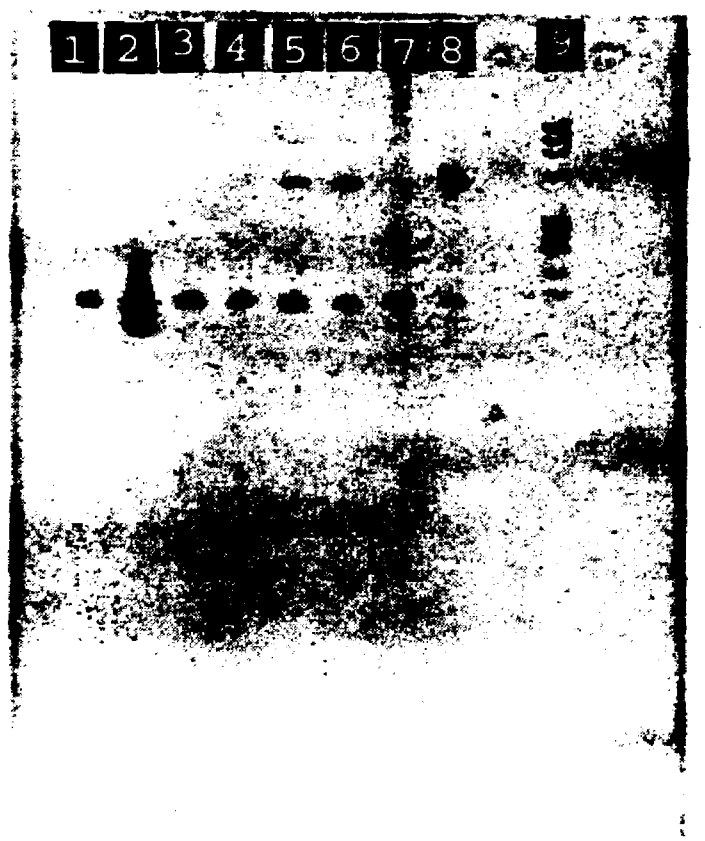

Fig. 1. Visualization of Baculovirus penaei DNA amplified by PCR. Lanes 1 - 4 from uninfected postlarval Penaeus vannamei; lanes 5, 6 from infected postlarvae; lane 7, - control; lane 8, + control. Lane 9 contains size markers (ϕX174 HaeIII Digest) expressed in base pairs (bp) from top to bottom: 1,353bp, 1,078bp, 872bp, 603bp, 310bp, 281bp, 271bp, 234bp, 194bp, 118bp, 72bp.

APPENDIX B

OCCURRENCE OF UNKNOWN AMINE 5.5 IN RESPONSE TO PROLONGED STARVATION IN POSTLARVAL PENAEUS VANNAMEI

During the study of biochemical responses to starvation and subsequent recovery in postlarval Penaeus vannamei (Chapter 3), an unidentified amine with a retention time of 5.5 minutes appeared on the HPLC chromatograms obtained during that study (see Fig. 7, page 85) and its levels were also expressed as a ratio with Sp. The levels of amine 5.5:Sp in two of four postlarvae sampled after 12 days of starvation were substantially higher than in postlarvae from the fed group (Fig. 1). By day 13, the levels of 5.5:Sp dramatically increased and then subsequently decreased to levels similar to the fed group by the end of the study. On day 13 of the study, most postlarvae in the starved-fed group were still lethargic and some appeared to be near death. Because high levels of amine 5.5:Sp first appeared in starved postlarvae, it is unlikely that the dramatic increase in that amine observed between day 12 and day 13 is a response to the resumption of feeding, but is rather a delayed response to chronic starvation. The retention time of amine 5.5 is similar to that of the polyamine cadaverine (S. Watts, personal communication). Further investigations are needed to positively identify amine 5.5 and to determine its relationship to postlarval condition.

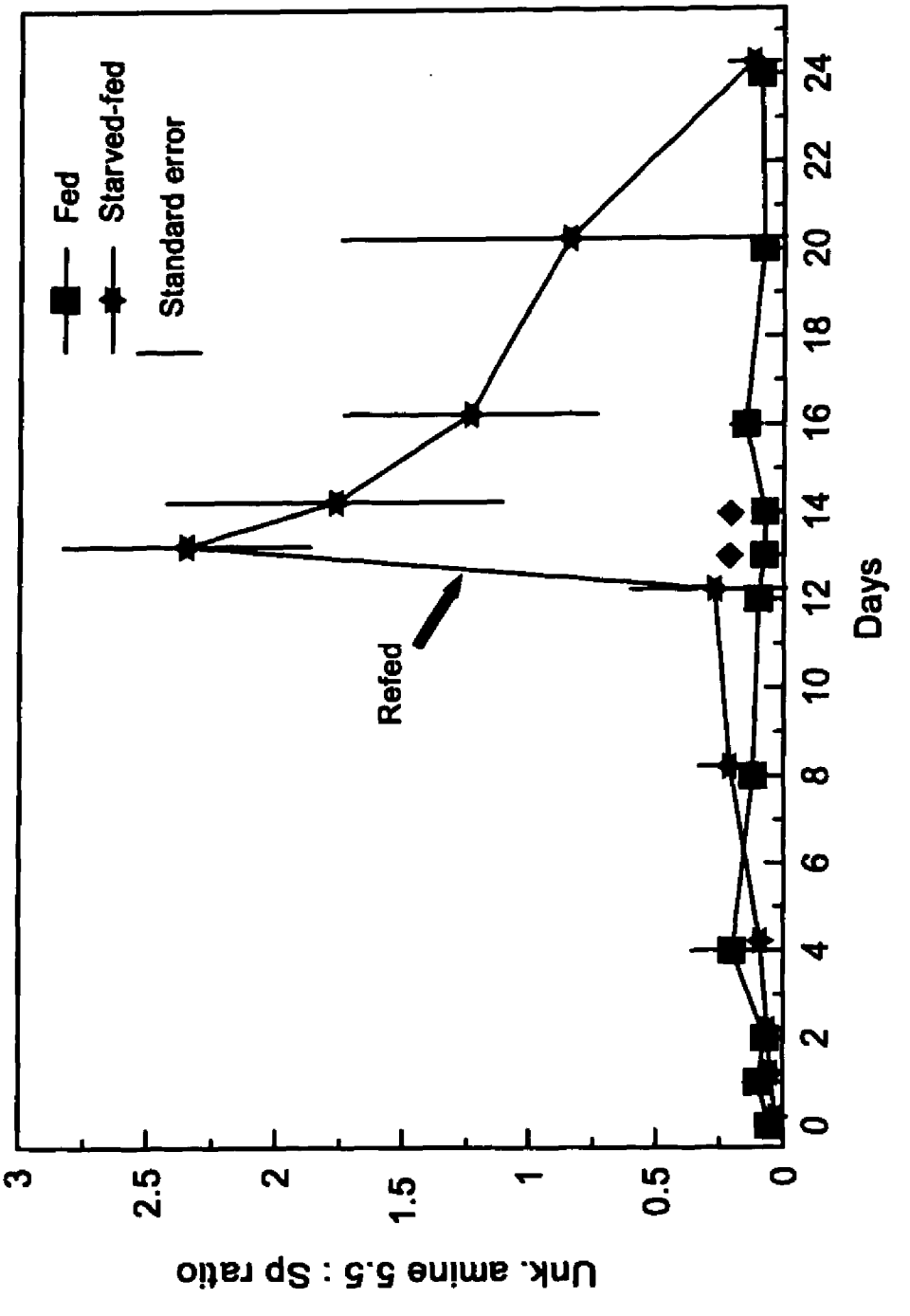

Fig. 1. Concentration of unknown amine 5.5 relative to spermine determined from heads of fed and starved-fed postlarvae of Penaeus vannamei over 24 days. Values are expressed as means ± SE (n = 4). ◆ - denotes significant differences ($p \leq 0.05$) between fed and starved-fed postlarvae.

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