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Detection and tissue tropism of white spot syndrome baculovirus (WSBV) in captured brooders of *Penaeus monodon* with a special emphasis on reproductive organs

Chu-Fang Lo[1], Ching-Hui Ho[1], Chau-Huei Chen[1], Kuan-Fu Liu[2], Ya-Lin Chiu[1], Pei-Yan Yeh[1], Shao-En Peng[1], Hui-Chen Hsu[1], Hwei-Chung Liu[1], Chen-Fang Chang[2], Mao-Sen Su[2], Chung-Hsiung Wang[3], Guang-Hsiung Kou[1,*]

[1]Department of Zoology, National Taiwan University, Taipei, Taiwan, ROC
[2]Tung Kang Marine Laboratory, Taiwan Fisheries Research Institute, Tung Kang, Ping Tung, Taiwan, ROC
[3]Department of Plant Pathology and Entomology, National Taiwan University, Taipei, Taiwan, ROC

ABSTRACT: In cultured shrimp, white spot syndrome 'baculovirus' (WSBV) infection is characterized by a wide range of target tissues, rapid disease onset and high mortality. During the viremic phase of infection, the virus is present in many organs. However, the situation in the natural environment remains unclear. To identify the pattern of the tissue tropism of WSBV infection in adult *Penaeus monodon* (black tiger shrimp) of wild origin, we conducted a combined study using currently available nucleic acid diagnostic tools and conventional histological observations using light (LM) and transmission electron (TEM) microscopy to examine the sites for virus multiplication. Sixteen parts excised from shrimp specimens were examined: pleopods, gills, stomach, abdominal muscle, hemolymph, midgut, heart, pereiopods, lymphoid organs, integument, nervous tissue, hepatopancreas, testes, ovaries, spermatophores, and eye stalks. All these tissues/organs were found to support WSBV replication. For the first time, *in situ* hybridization and TEM showed evidence of WSBV in reproductive organs of black tiger shrimp. In testes, WSBV-positive cells were located in the connective tissue layer surrounding the seminiferous tubules and no germ cells were found to be infected. In the spermatophore, only muscle and connective tissue cells were WSBV positive. In the ovary, follicle cells, oogonia, oocytes and connective tissue cells were WSBV positive. However, the fact that we were unable to find any infected mature eggs suggested that infected egg cells were killed by the virus before maturation.

KEY WORDS: WSBV · Captured brooders · Tissue tropism · PCR · *In situ* hybridization · Electron microscopy · Reproductive organs · *Penaeus monodon*

INTRODUCTION

White spot syndrome (WSS) is a disease of cultured shrimp caused by white spot syndrome 'baculovirus' (WSBV), an enveloped non-occluded rod-shaped virus. The disease is characterized by the display of white spots in the exoskeleton (Chou et al. 1995, Wang et al. 1995, Lightner 1996). WSBV has been recognized since 1993, and it has become a significant pathogen of cultured shrimp in Asia.

WSBV was previously classified by our team as the genus *Non-Occluded* [sic] *Baculovirus* (*NOB*) of the subfamily *Nudibaculovirinae* of family *Baculoviridae* in accordance with the fifth report of the International Committee on the Taxonomy of Viruses (ICTV) (Francki et al. 1991). In the sixth report of the ICTV however, the subfamily *Nudibaculovirinae* and the genus *NOB* are not listed. The insect viruses Oryctes rhinoceros virus (OrV) and Heliothis zea virus 1 (HzV-1)

*Addressee for correspondence.
E-mail: ghkou@ccms.ntu.edu.tw

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which were previously classified as non-occluded members of the family *Baculoviridae* are now placed in the list of unassigned invertebrate viruses (Murphy et al. 1995). We have done a sequence analysis of WSBV genomic DNA (about 15% of the whole genome). The comparison of sequence data has shown WSBV to be unique, that is, it has no homology with any viral sequences in Genbank or other databases (authors' unpubl. data). For the moment, we still do not have sufficient viral sequence or replication cycle data to conclusively establish WSBV's taxonomic position. However, we are confident that WSBV is at least a new virus if not a new virus group. Notwithstanding the uncertainty in the taxonomic position of non-occluded baculoviruses, in a recently published handbook of pathology and diagnostic procedures for diseases of penaeid shrimp edited by the shrimp pathologist Dr Lightner in 1996, this virus is called white spot syndrome baculovirus (WSBV). Consequently, in the interests of consistency, we continue to use the name WSBV until we have sufficient data to do otherwise (Lo et al. 1996b). However, we take the precaution of putting 'baculovirus' in quotation marks.

WSBV has been shown to target various tissues originating from both the mesoderm and the ectoderm, as evidenced by histopathological observations and *in situ* hybridization (for example, see Wongteerasupaya et al. 1995, 1996, Chang et al. 1996). However, all of the published studies were conducted with cultured and/or experimentally infected shrimp, and this may not reflect the pattern of WSBV infection in wild shrimp populations. Lo et al. (1996a, b) have shown that WSBV could be detected in wild shrimp populations, with infected specimens showing WSBV-positive signals by either 1-step or 2-step WSBV diagnostic polymerase chain reaction (PCR). However, most of these WSBV diagnostic PCR-positive shrimp specimens were evidently in latent or pre-patent stages of WSBV infection, because no obvious WSS was observed (Lo et al. 1996b). Thus, the pathogenicity and tissue tropism of WSBV in wild shrimp populations remained unclear. Furthermore, brooders of cultured shrimp species such as *Penaeus monodon* (black tiger shrimp) are usually not available from production ponds since they are normally marketed long before sexual maturity (Chen 1990). Thus, to date, there are no data on the susceptibility of reproductive organs to infection by WSBV. Tissue tropism, replication rate and/or spread of a virus generally play key roles in determining the outcome of infection of the host (Zurbriggen et al. 1991, Ren & Racaniello 1992, Suresh & Sharma 1996). Thus, we conducted combined observations using light (LM) and transmission electron (TEM) microscopy to examine tissues that might support the multiplication of WSBV in adult, naturally infected, wild black tiger shrimp *P. monodon*.

MATERIALS AND METHODS

Prevalence of WSBV in captured brooders of black tiger shrimp *Penaeus monodon* detected by PCR. From July 1995 to February 1996, brooders of black tiger shrimp were captured from their natural environment in the coastal waters around southern Taiwan and then immediately transported to the Tung Kang Marine Laboratory of the Taiwan Fisheries Research Institute in Ping Tung Prefecture in southern Taiwan. Upon arrival, a pereiopod from each shrimp was immediately subjected to 2-step WSBV diagnostic PCR (Lo et al. 1996a, b). To minimize struggling during pereiopod removal, each brooder was wrapped in a wet towel so that only the appendage to be removed remained exposed. The pereipod was then excised at the carpopodite segment with a red-hot pair of forceps in a manner similar to that for eye stalk ablation usually done in hatcheries. Specimens (T1 to T20) were dissected for the tissue tropism study, while the captured brooders (S1 to S56) were maintained for spawning in reproduction facilities (500 l tanks kept in the dark and containing 450 l aerated, sand-filtered, 33‰ seawater which had previously been sterilized with active carbon for 24 h and ozone for 1 h, at 30 ± 1°C, pH 8.3) at Tung Kang Marine Laboratory. The brooders were routinely fed frozen captured marine crabs of various species. The offspring of some brooders were tested for the presence of WSBV by PCR. Whenever any brooder died, another perieopod was removed and also subjected to WSBV diagnostic PCR for comparative studies.

WSBV tissue tropism in captured brooders. When the results of 2-step WSBV PCR became available 7 h after their arrival at Tung Kang Marine Laboratory, 20 brooders (T1 to T20) were selected for WSBV tissue tropism analysis. These comprised 10 specimens which were 2-step negative, 4 specimens positive only after re-amplification and 6 specimens positive in the first step of amplification. From these specimens, the heart, gills, stomach, midgut, hepatopancreas, abdominal muscle, integument covering the gill chamber underneath the carapace, ventral nerve cord, lymphoid organ, hemolymph, testes or ovaries, spermatophore, pleopods, pereiopods and eye stalks were collected. In order to minimize post-mortem changes, the operations were performed on ice and finished within 10 min for each shrimp specimen. WSBV tissue tropism analysis was carried out by using the methods described below.

WSBV diagnostic PCR: Hemolymph (100 µl) and tissue samples (100 mg) removed from each specimen were placed in separate microfuge tubes, frozen rapidly in liquid nitrogen and then stored at −70°C until used for PCR template DNA preparation. DNA from solid tissue samples was isolated following the

method described by Lo et al. (1996a), with some modification. Briefly, 1.2 ml digestion buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8, 25 mM EDTA, pH 8, 0.5% N-lauryl sarcosine, 0.5 mg ml^{-1} proteinase K) was added to the tissue in each microfuge tube and the tissue was then crushed with a disposable stick. After 1 h incubation at 65°C, the NaCl concentration of the DNA solution was adjusted with 5 M NaCl to 0.7 M. The solution was then treated with 1% N-cetyl N,N,N-trimethylammonium bromide (CTAB) for 10 min at 65°C followed by successive extractions with an equal volume of chloroform/isoamyl alcohol once, an equal volume of phenol 2 to 3 times, and a double volume of chloroform/isoamyl alcohol once. The DNA was recovered by ethanol precipitation, dried and resuspended in 0.1 × TE buffer (1 × TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 7.6) at 65°C for 30 min, and then stored at 4°C until used for WSBV diagnostic PCR. Each sample of hemolymph was digested with 500 µl of the digestion buffer before undergoing the same incubation and DNA extraction procedures as performed for solid tissues. For each sample, the quality of the extracted DNA was checked by PCR with a decapod 18S rRNA gene specific primer pair before it was subjected to WSBV diagnostic PCR (Lo et al. 1996a). WSBV diagnostic PCR was performed as described previously (Lo et al. 1996b).

Histological observation by LM: Tissues were treated in Davidson's fixative for 48 h. The specimens were then processed routinely for tissue sections and stained with hematoxylin and eosin (H&E) (Humason 1979). Microphotographs were taken under an Olympus Research Microscope Model AHBT3.

WSBV detection in histological sections by in situ hybridization: For histological observation, 5 µm sections from the same tissue blocks were placed onto 2% silane (r-methacryloxypropyl tri-methoxysilane) pretreated slides for *in situ* hybridization by a modified version of the method described previously (Chang et al. 1996). Briefly, deparaffinized sections were permeabilized by exposure to 0.02 N HCl. After washing in phosphate-buffered saline (PBS), the slides were treated for 2 to 30 min (depending on the type of tissues) with 100 µg ml^{-1} proteinase K in PBS at 37°C. Hybridization with a digoxigenin (DIG)-labeled probe and coloration were as described previously. After coloration, the sections on the slide were mounted with a drop of 1 × TE buffer and a coverglass, the circumference of which was sealed with fingernail polish. For comparison, some tissue sections were counterstained with 1% aqueous neutral red for 10 s and then rinsed with water, and finally mounted with a drop of water and a coverglass sealed with fingernail polish. Microphotographs were taken under an Olympus Research Microscope Model AHBT3.

Samples for *in situ* hybridization included WSBV PCR-positive and -negative tissues (see Table 5) and tissues from diseased cultured shrimp with WSS as positive controls. A reaction control with no nucleic acid probe was also included in order to demonstrate the non-specific reaction of the cuticle during *in situ* hybridization.

Ultrastructural observations by TEM: 1-step WSBV PCR-positive abdominal muscles and reproductive organs were pre-fixed in 2.5% glutaraldehyde in 0.1 M cold phosphate buffer solution (pH 7.2) for 3 h at 4°C and then post-fixed in 1% osmium tetraoxide in the same buffer for 2 h at 4°C. The fixed samples were then dehydrated in an alcohol gradient series and embedded in Spurr epon. Ultrathin sections were cut on a Reichert OMU3 ultramicrotome and stained with uranyl acetate and lead citrate. The transmission electron micrographs were made with a Hitachi H-600 electron microscope at 100 kV.

Detection of WSBV in captured *Portunus sanguinolentus* crab fed to shrimp brooders. To ascertain whether or not WSBV existed in the captured crabs *Portunus sanguinolentus* that were served as food to the *Penaeus monodon* brooders, dactylopods of the 5th pereiopod of randomly chosen specimens were subjected to WSBV PCR, histopathological observation, *in situ* hybridization analysis, and TEM. (A report of our investigation of the various other species of marine crabs that were also used to feed the *P. monodon* brooders will appear elsewhere.)

RESULTS

Prevalence of WSBV in captured brooders

From July 1995 to February 1996, 40 male and 48 female brooders were captured from the wild. Among the males, 40% were positive in the first step of amplification, 27.5% were positive only in the second step of amplification and 32.5% were 2-step PCR negative. Among the females, 12.5% were positive in the first step of amplification, 62.5% were positive only in the second step of amplification and 25% were 2-step PCR negative (Table 1). The prevalence of WSBV detected in female brooders varied with season; the prevalence in summer and autumn (July to November) was higher than in winter (December to February) (Table 2). Although some female brooders did not spawn during the observation period (14 d), others spawned a few hours or a few days after arrival at Tung Kang Marine Laboratory (Table 3). Of those that did spawn successfully, none had been 1-step WSBV PCR positive; conversely, brooders that were 1-step WSBV PCR positive invariably died 1 to 4 d after being captured and before

Table 1. Detection of white spot syndrome baculovirus (WSBV) in captured brooders of *Penaeus monodon* using 2-step WSBV diagnostic PCR with DNA templates prepared from brooder pereiopods

WSBV diagnostic PCR	Prevalence of WSBV in brooders detected by PCR			
	Female		Male	
	No.	%	No.	%
Positive in the 1st step	6	12.5	16	40
Positive only in the 2nd step	30	62.5	11	27.5
Negative in both steps	12	25	13	32.5
Total	48	100	40	100

spawning. Of these brooders that spawned, all subsequently died, were 1-step PCR positive after death (Table 4) and had very tiny white spots visible on the carapace (Fig. 1). Interestingly, some of the brooders that were 2-step WSBV PCR positive, produced offspring that were 2-step WSBV PCR negative, while the offspring of some uninfected brooders were found to be 2-step WSBV PCR positive (Table 3).

WSBV tissue tropism analysis in captured *Penaeus monodon* brooders with WSBV diagnostic PCR

Of the 20 selected brooders shown in Table 5, 10 had pereiopods that were negative in 2-step WSBV PCR, 4 were positive only after re-amplification, and 6 were positive in the first step of amplification. For tissue tropism analysis, all the selected tissues of shrimp with 1-step WSBV-positive pereiopods were subjected to 1-step PCR, while those from the other 14 were subjected to 2-step PCR. The brooders were then divided into 3 groups. Group I comprised those whose tissues were all 2-step WSBV PCR negative. Group II comprised those which had at least some tissues positive after re-amplification. Group III comprised those whose tissues tested mostly 1-step PCR positive (Table 5).

Table 2. Seasonal change in the prevalence of WSBV in captured brooders *Penaeus monodon* detected by 2-step WSBV diagnostic PCR with DNA templates prepared from brooder pereiopods. Shrimp collected from July 1995 to February 1996

WSBV diagnostic PCR	Prevalence of WSBV in female brooders					
	Jul		Aug–Nov		Dec–Feb	
	No.	%	No.	%	No.	%
Positive in the 1st step	2	7.4	4	44.4	0	0
Positive only in the 2nd step	16	59.2	5	55.6	3	25
Negative in both steps	9	33.3	0	0	9	75
Total	27	100	9	100	12	100

Table 3. Detection of WSBV in *Penaeus monodon* brooders and their offspring by 2-step WSBV diagnostic PCR. S1, S2, etc.: no. of brooders maintained in tanks for spawning; nd: not determined; where no values for egg, nauplius or zoea are given, the brooder died before spawning or did not spawn during the 14 d observation period

	Brooder		Egg		Nauplius		Zoea	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd
S1	–	+						
S2	–	+						
S3	–	+	–	–	–	–	–	–
S4	–	–	–	–	–	–	–	+
S5	–	+	–	–	–	–	–	–
S6	–	–						
S14	–	+	–	–	–	–	–	3/6[a]
S15	–	–						
S16	–	+						
S17	–	+						
S18	+	+						
S19	–	–	–	–	–	–	–	1/5[a]
S20	–	+	–	+	–	+	–	–
S21	–	–	–	–	–	–	–	–
S22	–	+	–	–	–	–	–	–
S23	–	+	–	–	–	–	–	–
S24	–	–	–	–	–	nd	–	nd
S25	–	–	–	–	–	–	–	+
S26	–	–	–	–	–	+	–	nd
S27	–	–	–	–	–	–	–	–
S28	–	+	–	–	–	–	–	–
S29	–	–	–	–	–	–	–	nd
S30	–	+	–	+	–	1/2[a]	–	–
S51	+	+						
S52	+	+						
S53	–	+						
S54	+	+						
S55	–	+						
S56	–	+						

[a]Values represent the number of tanks with 2-step WSBV-positive offspring per number of tanks in which the offspring were stocked

Upon arrival, all the shrimp listed in Table 5 were regarded as either healthy or at worst only prepatently or latently infected; they looked healthy and had no obvious white spots in their exoskeleton. In fact, the prevalence of WSBV detected by PCR was moderately high in female brooders (6/11 = 55%) and very high in male brooders (9/9 = 100%). WSBV DNA-positive results were found in 15 checked parts (Table 5). Emphasis is made here that in Group II, the prevalence of WSBV DNA in the lymphoid organ (4/9; 44%) and integument (3/9; 33%) was surprisingly low, indicating that they were not main targets for WSBV in latently or very lightly infected shrimp. In Group III, prevalence of WSBV in the nervous tissue and hepatopancreas was also low (as

Table 4. Detection of WSBV by 2-step WSBV diagnostic PCR in 10 female brooders of *Penaeus monodon* before and after spawning. WSS: white spot syndrome on carapace of the shrimp. Dates given as day/month in 1995; nr: not recorded

Brooder	Capture	Spawning	Death	WSBV infection					
				1 d post-capture			After death		
				WSS	PCR		WSS	PCR	
					1st	2nd		1st	2nd
S3	18 Jul	27 Jul	12 Aug	–	–	+	+	+	+
S5	20 Jul	27 Jul	nr	–	–	+	+	+	+
S14	24 Jul	24 Jul	29 Jul	–	–	+	+	+	+
S20	26 Jul	26 Jul	27 Jul	–	–	+	+	+	+
S21	26 Jul	27 Jul	8 Aug	–	–	–	+	+	+
S26	27 Jul	28 Jul	2 Aug	–	–	–	+	+	+
S27	28 Jul	29 Jul, 3 Aug	12 Aug	–	–	–	+	+	+
S28	27 Jul	28 Jul	8 Aug	–	–	+	+	+	+
S29	27 Jul	28 Jul	1 Aug	–	–	–	+	+	+
S30	27 Jul	28 Jul	3 Aug	–	–	+	+	+	+

revealed by 1-step PCR) while it was high (100% 1-step PCR positive) in the integument and other parts. This indicated that WSBV replicated and spread rapidly among these latter tissues. Very tiny white spots were also observed in the carapace removed from the carcass of each Group III brooder. Examples of WSBV-DNA detected in various organs of 1 female and 1 male shrimp specimen by WSBV diagnostic PCR analysis are shown in Fig. 2.

No eyestalk sample (with compound eye attached) from 20 initial shrimp specimens gave PCR amplification products, even with shrimp-specific DNA primers (data not shown). Consequently, 4 more specimens (T21–T24) were used to check the suitability of the compound eye for PCR. From these specimens, all pereiopods and eye stalks (with compound eye removed) gave positive amplification products with the shrimp specific primers (Fig. 3A). When the compound eye was still attached, however, eyestalks consistently gave negative results. In completed PCR reaction mixtures (10 µl) containing compound eye DNA, neither PCR products nor the primer dimmers usually seen as a faint band in PCR negative reactions were observed (Fig. 3A & B).

Histopathology by LM

Typical WSBV histopathology was observed in tissues of 1-step WSBV diagnostic PCR-positive specimens (T16 and T18) (Fig. 4). Almost all the tested tissues of these 2 shrimp were WSBV positive by 1-step amplification (Table 5). The pathological changes in shrimp T18 were more severe than those in shrimp T16, but the cytopathic effects in both shrimp were very similar. Infected nuclei in the cuticular epidermis of the pleopods, gills, stomach, integument, pereiopods and eye stalks were dramatically hypertrophied (Fig. 4B). Their diameters were 2 to 3 times greater than those of normal nuclei. They stained homogeneously with H&E and were mostly basophilic, although acidophilic nuclei were occasionally found. In the abdominal muscle, heart, lymphoid organ, midgut, nervous tissue, hepatopancreas, testes, ovaries, and spermatophores, infected nuclei were not so obviously hypertrophied and remained close to normal size (for example, see Fig. 4), so they were not readily distinguishable. However, they stained homogeneously with H&E similarly to clearly hypertrophied nuclei.

Confirmation of WSBV by *in situ* hybridization

Figs. 5 to 8 give examples of *in situ* hybridization results with the DIG-labelled WSBV-specific probe used to confirm that the histopathology observed was caused by WSBV. Nonspecific probe reactions with uninfected shrimp occurred only in the cuticle (for example, see Fig. 5A–I). By *in situ* hybridization, infected and uninfected cells in various tested tissues were easily distinguished. Infected cells with clearly hypertrophied nuclei and degenerated cells with non-

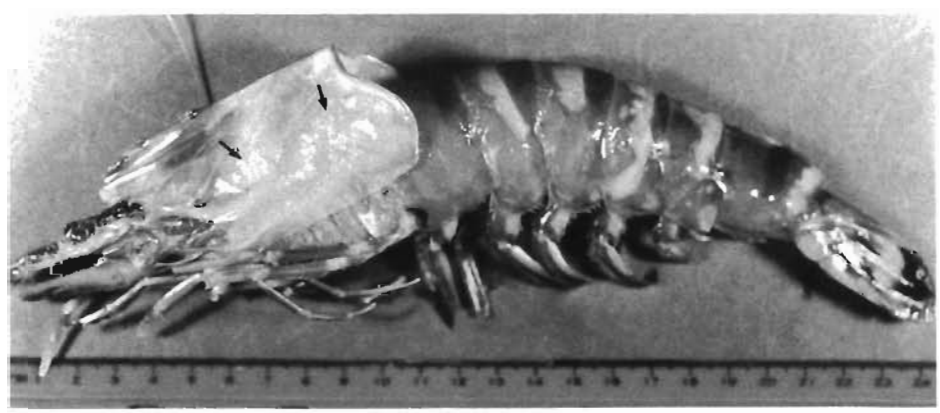


Fig. 1. *Penaeus monodon*. A brooder with very tiny white spots (arrows) in the exoskeleton of carapace after death

Table 5. WSBV tissue tropism as revealed by WSBV diagnostic PCR with 1-step and 2-step amplification protocols. T1, T2, etc: no. of shrimp dissected immediately after being captured for tissue tropism analysis; nd: not determined. For information regarding group divisions see 'Results'

Group no	Shrimp no.	Body weight (g)	Sex	Pleopod	Gill	Hemolymph	Stomach	Abdominal muscle	Midgut	Heart	Pereiopod	Lymphoid organ	Nervous tissue	Integument	Hepatopancreas	Ovary	Spermatophore	Testis	WSBV PCR
I	T1	84	F	–	–	–	–	–	–	–	–	–	–	–	–	–			2-step
	T2	115	F	–	–	–	–	–	–	–	–	–	–	–	–	–			2-step
	T3	122	F	–	–	–	–	–	–	–	–	–	–	–	–	–			2-step
	T4	126	F	–	–	–	–	–	–	–	–	–	–	–	–	–			2-step
	T5	112	F	–	–	–	–	–	–	–	–	–	–	–	–	–			2-step
II	T6	73	M	+	+	–	–	–	–	–	–	–	–	–	–		–	–	2-step
	T7	128	F	–	–	nd	–	+	+	–	+	+	–	–	–	–			2-step
	T8	59	M	+	+	nd	+	+	+	+	–	–	–	–	–		–	–	2-step
	T9	63	M	+	+	–	+	–	–	+	+	–	–	–	–		+	+	2-step
	T10	59	M	+	+	+	–	+	+	–	+	–	+	–	–		+	–	2-step
	T11	128	F	+	–	+	+	+	–	–	+	–	+	–	+	+			2-step
	T12	90	F	+	+	+	+	–	+	+	–	+	+	+	–	+			2-step
	T13	63	M	+	+	+	+	+	+	+	–	+	–	+	–		+	+	2-step
	T14	78	M	+	+	+	+	+	–	–	–	+	+	+	+		+	+	2-step
Prevalence (%)[a]				89	78	71	67	67	56	44	44	44	44	33	22	67	67	50	
III	T15	101	F	+	+	nd	+	+	+	+	+	+	–	+	–	+			1-step
	T16	134	F	+	+	+	+	+	+	+	+	+	–	+	–	+			1-step
	T17	183	F	+	+	+	+	+	+	+	+	+	+	+	–	+			1-step
	T18	48	M	+	+	+	+	+	+	+	+	+	+	+	+		+	+	1-step
	T19	79	M	+	+	+	+	+	+	+	+	+	+	+	+		+	+	1-step
	T20	75	M	+	+	+	+	+	+	+	+	+	+	+	+		+	+	1-step
Prevalence (%)[a]				100	100	100	100	100	100	100	100	100	67	100	50	100	100	100	

[a]Prevalence (%) among WSBV positive specimens within that group

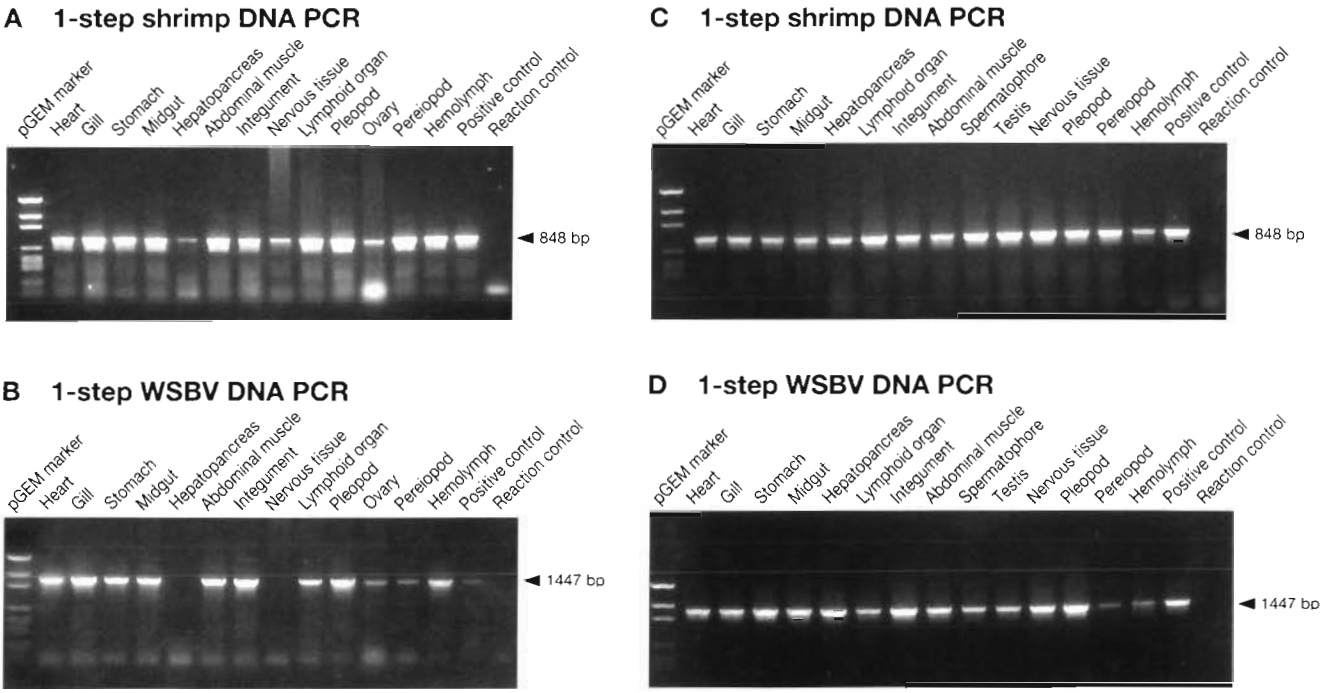


Fig. 2. Detection of WSBV DNA in various parts of captured female (A, B) and male (C, D) brooders of *Penaeus monodon* by PCR. (A, C) 1-step shrimp DNA amplification; (B, D) 1-step WSBV DNA amplification. PCR products are indicated by arrowheads

Table 6. Tissue distribution of WSBV in brooders of *Penaeus monodon* as revealed by *in situ* hybridization

Tissue	Cell type	WSBV
Heart	Myocardial	+
	Epicardial	–
	Hemocytes	+
Gill	Epithelial	+
	Vessel endothelial	–
Stomach	Epithelial	+
	Spongy connective tissue	+
	Muscle	+
Midgut	Epithelial	–
	Muscle	–
	Connective tissue	+
Hepatopancreas	Hepatopancreatocytes	–
	Connective tissue sheath	+
	Myoepithelial	+
Lymphoid organ	Stromal matrix	+
	Hemocytes	+
	Endothelial lumen	–
Integument	Epithelial	+
	Connective tissue	+
Abdominal muscle	Muscle	+
Nerve cord	Glial	+
	Nerve	–
	Connective tissue	+
Spermatophore	Sperm	–
	Muscle	+
	Epithelial	–
	Connective tissue	+
Testes	Germ	–
	Connective tissue	+
Ovary	Oogonia	+
	Chromatin nucleolus oocytes	+
	Perinucleolus oocytes	–
	Yolk stage oocytes	–
	Connective tissue	+
Pleopod	Epithelial	+
	Muscle	+
	Connective tissue	+
	Glial	+
Pereiopod	Epithelial	+
	Muscle	+
	Connective tissue	+
	Glial	+
Eye stalk	Epithelial	+
	Glial	+
	Dioptic portion	–
	Nerve	–

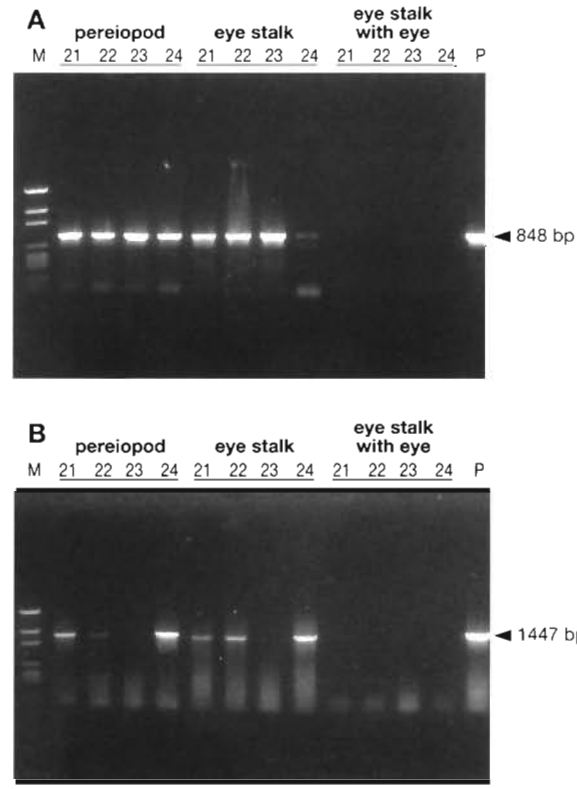


Fig. 3. Assessment of the suitability of DNA templates prepared from the compound eye for PCR. PCR templates comprise DNA prepared from pereiopods, eye stalks with compound eyes removed and eye stalks with compound eyes intact. The numbers at the top of each lane refer to shrimp specimens T21 to T24. (A) 1-step shrimp DNA amplification; (B) 1-step WSBV DNA amplification. PCR products are indicated by arrowheads. M: pGEM marker; P: positive control

hypertrophied nuclei gave positive signals (Fig. 5G). Although the positive *in situ* hybridization signal was primarily nuclear, a positive signal was also evident in the cytoplasm. A nonspecific reaction of the cuticle was demonstrated in control reactions which occurred in the absence of the nucleic acid probe (Fig. 5I). *In situ* hybridization confirmed that WSBV replication occurred in the following tissues and organs: pleopods, gills, stomach, muscle, hemolymph, midgut, heart, pereiopods, lymphoid organ, integument, nervous tissue, hepatopancreas, testes, ovaries, spermatophores, compound eyes and eye stalks (Table 6). Nonetheless, the number of positive cells in each tissue/organ was relatively low when compared to the number usually seen in cultured and/or experimentally infected shrimp.

In situ hybridization analysis of pereiopods, gills and the stomach revealed that WSBV positive cells were most prevalent in the epidermis (Fig. 6A, B). WSBV-positive cells were also readily observed in abdominal muscle tissues in connective tissue cells and sometimes in muscle cells. In the midgut, some connective tissue cells were WSBV positive but no epithelial cells were positive (Fig. 6C, D). In the heart, some myocardial cells were WSBV positive, but the epicardial cells were WSBV negative (Fig. 6E, F). In the lymphoid organ, a moderately heavy reaction product was seen in the nucleus and cytoplasm of infected cells. These comprised mostly hemocytes, stromal matrix cells and

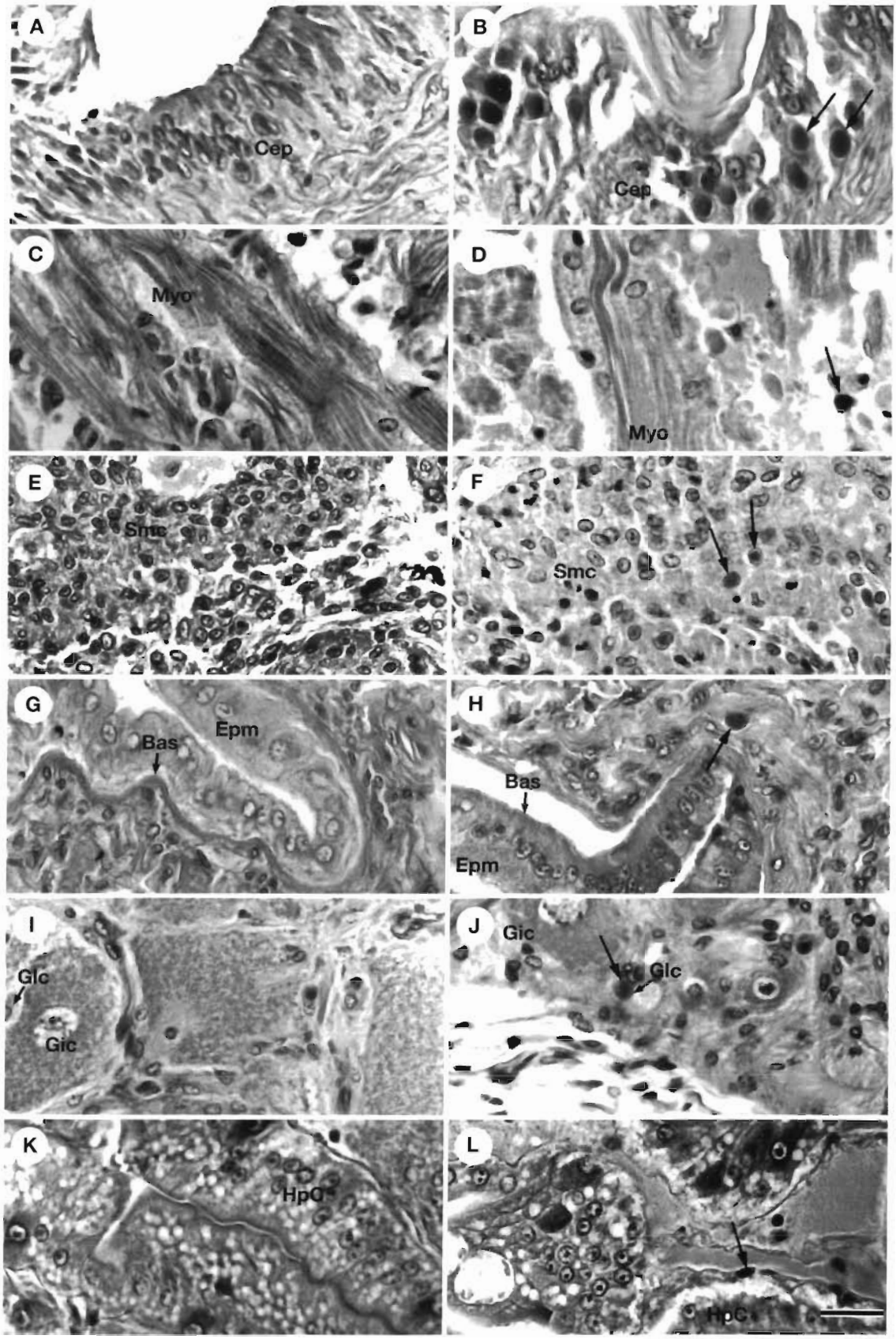


Fig. 4. Photomicrographs (LM) of cuticular epithelia of captured *Penaeus monodon*. (A, B) Eye stalk; (C, D) heart; (E, F) lymphoid organ; (G, H) midgut; (I, J) ventral nerve cord; and (K, L) hepatopancreas. (A, C, E, G, I, K) WSBV PCR-negative specimens. (B, D, F, H, J, L) WSBV PCR-positive specimens showing degenerated nuclei in the infected cells (arrows). Bas: basement membrane; Cep: cuticular epithelial cells; Epm: epithelium, midgut; Gic: giant cell; Glc: glial cell; HpC: hepatopancreatocytes; Myo: myocardium; Smc: stromal matrix cells. H&E stain, scale bar = 20 µm

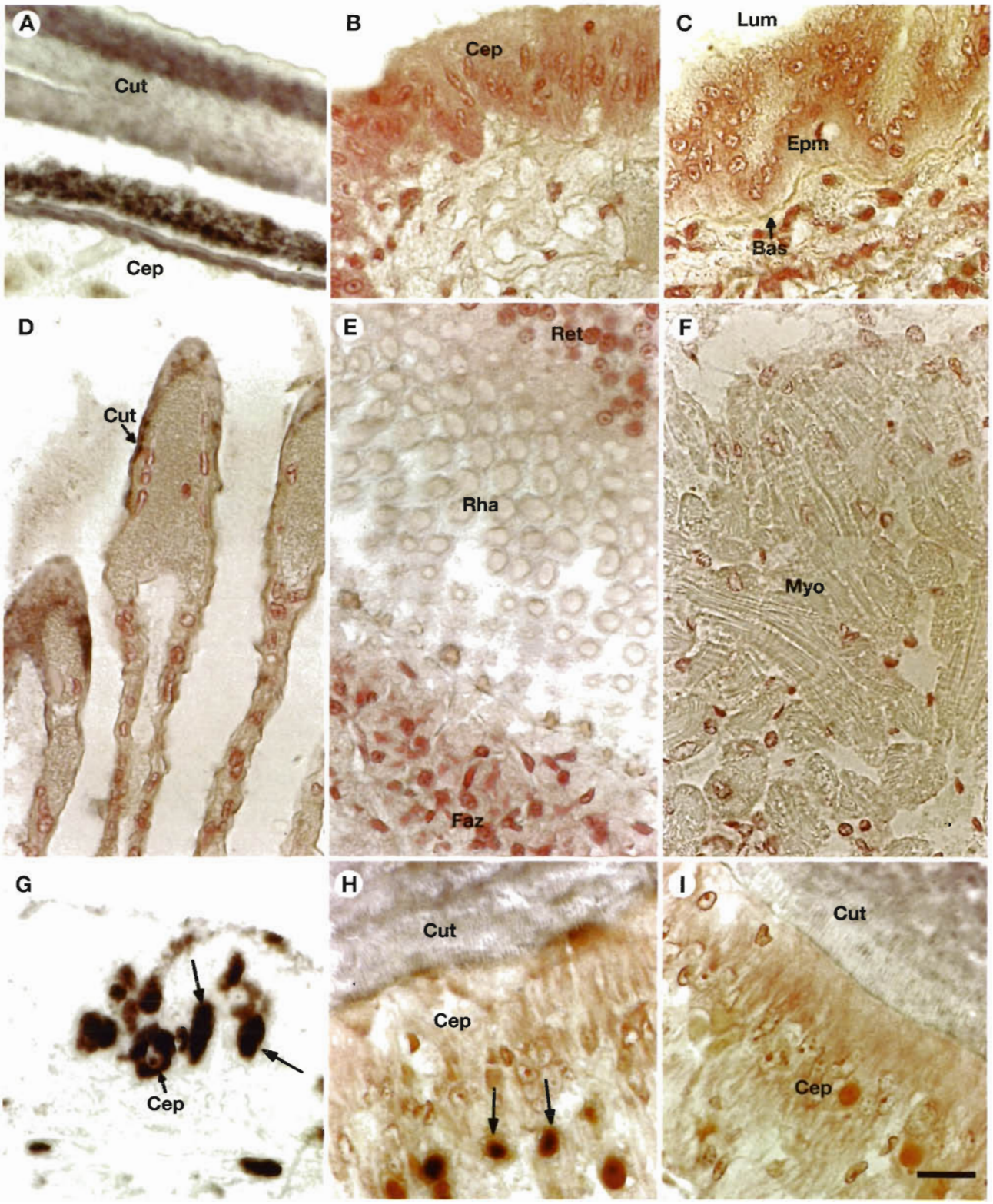
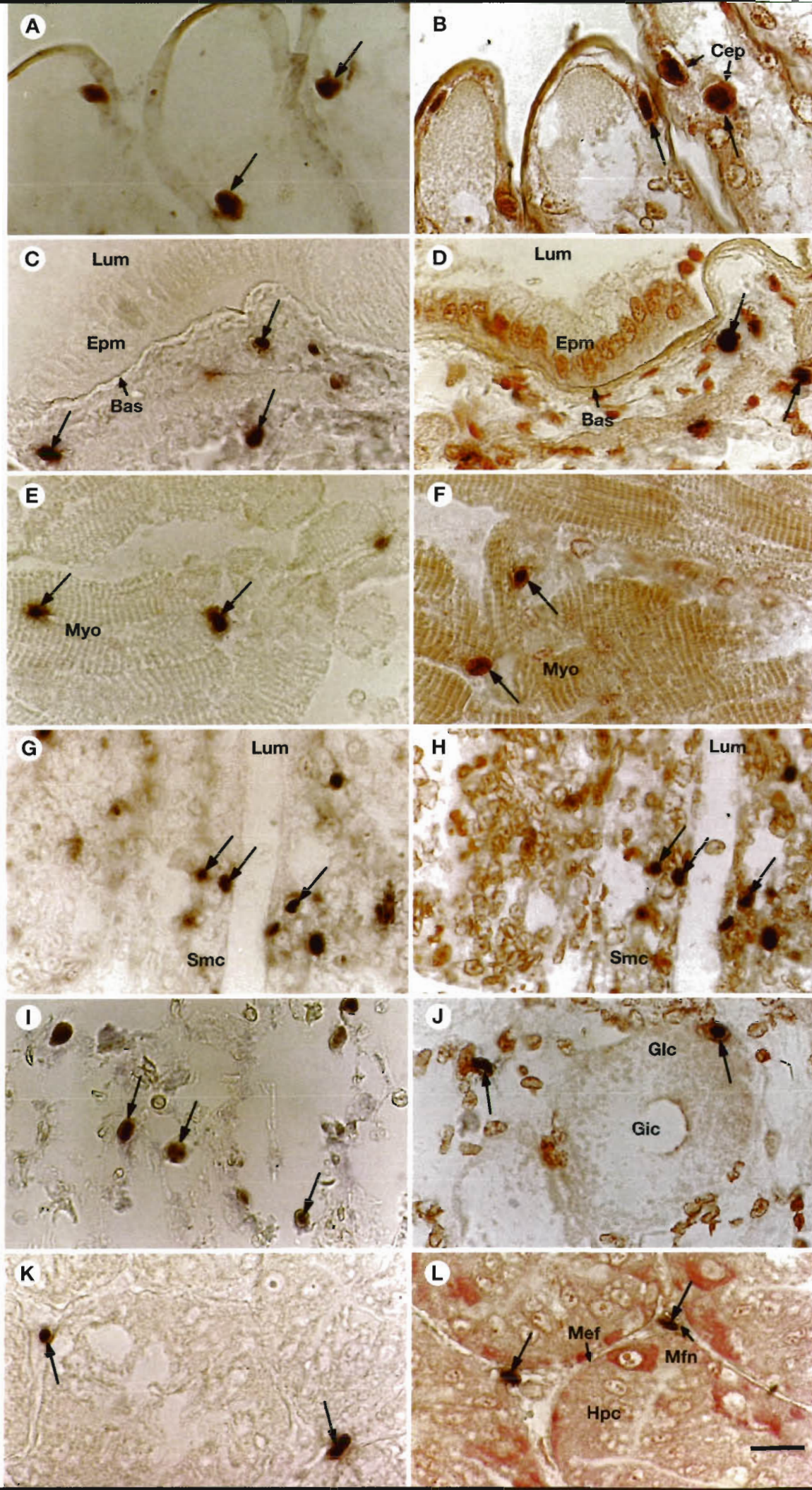


Fig. 5. Detection of WSBV DNA in tissues of captured *Penaeus monodon* by *in situ* hybridization. Negative controls of the (A) integument; (B) stomach; (C) midgut; (D) gill; (E) compound eye, and (F) heart, all showing no signals except in the cuticle. (G) WSBV PCR-positive stomach showing the strong positive hybridization signals (consisting of blue-purple precipitates) in infected cells (arrows) in a section without neutral red as a counter-stain; (H) WSBV PCR-positive integument showing the strong positive hybridization signals (consisting of dark brown precipitates) of infected cells (arrows) distinguishable from the uninfected cells when seen in a section counter-stained with neutral red; (I) WSBV PCR-positive integument showing the false-positive signals of the cuticle (consisting of blue-purple precipitates) in a section treated with a hybridization without probe. (A, G) No neutral red counterstaining; (B, C, D, E, F, H, I) with neutral red as counter-stain. Bas: basement membrane; Cep: cuticular epithelial cells; Cut: cuticle; Epm epithelium, midgut; Faz: fasciculated zone; Lum: lumen; Myo: myocardium; Ret: retinular cell, nuclei; Rha: rhabdoms and retinular cell bodies. Scale bar = 20 µm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cells in the fibrous connective tissue layer. The flattened layer of endothelial cells immediately surrounding tubular lumens was consistently WSBV negative (Fig. 6G, H). In nervous tissue, nerve cells were WSBV negative while glial cells surrounding giant cells and connective tissue cells surrounding the ventral nerve cord were WSBV positive (Fig. 6I, J). In the hepatopancreas, a few WSBV-positive cells were located in the connective tissue sheath and the network of myoepithelial cells. Hepatopancreatocytes were never found to be WSBV positive (Fig. 6K, L).

In reproductive organs, it was very difficult to distinguish infected cells from uninfected cells by H&E histological staining. However, *in situ* hybridization could be used to identify WSBV target cells (Figs. 7 & 8). In testes, WSBV-positive cells were located in the connective tissue layer surrounding seminiferous tubules; no sperm cells were found to be WSBV positive (Fig. 7A–F). In the spermatophore, WSBV-positive cells were found in the thin, peripheral sheath of circular muscle (Fig. 7G). In the ovary, most of the WSBV positive cells were follicle cells and oogonia (Fig. 8D, F, J, L). A few developing oocytes gave strong positive signals for WSBV DNA in the nucleus (Fig. 8E, H, K). In one positive specimen (T16), the diameter of the nucleus of the largest oocyte was 40 µm, but positive signals were never found in oocytes with a nuclear diameter greater than 28 µm.

Confirmation of WSBV by TEM

By TEM observation, viral particles were observed in the nucleus and cytoplasm of the connective tissue layer surrounding the seminiferous tubules of the testis (Fig. 9A, B). In the ovary, viral particles were found in the nucleus and cytoplasm of infected follicle cells. In contrast, they were often seen in the nucleus of infected oogonia, but only seldom in the cytoplasm (Fig. 10A–F).

Detection of WSBV in crabs used as feed

A surprisingly high number of the captured crab *Portunus sanguinolentus* were WSBV PCR positive. Of 48 crab specimens tested, 28 were positive by 1-step WSBV PCR. The presence of WSBV in captured crabs was confirmed by histopathology, *in situ* hybridization and TEM. Typical WSBV histopathology was observed in 3 tested organs, the heart, stomach, and midgut (Fig. 11). By *in situ* hybridization analysis, infected cells gave strong WSBV DNA-positive signals (Fig. 11). Using TEM, many rod-shaped non-occluded virus particles were readily seen (Fig. 12). The morphology of the virus found in *P. sanguinolentus* was indistinguishable from that previously reported for WSBV in cultured shrimp (Wang et al. 1995) and in captured *P. monodon* (Figs. 9 & 10).

DISCUSSION

The present study showed a high frequency of WSBV by PCR in captured *Penaeus monodon* brooders. WSBV prevalence was also reported to be quite high in other wild decapod populations collected in nearby areas (Lo et al. 1996b). Furthermore, a pilot study of decapods captured from coastal waters around southern Taiwan in 1996 suggests that WSBV prevalence has not diminished since 1995 (data not included in this manuscript). It remains to be determined whether the prevalence of WSBV in decapods captured from Taiwan coastal waters will be higher than in decapods from the other Asian coastal waters. When studies are done elsewhere and comparisons made, it should be kept in mind that we used sensitive PCR diagnostic procedures which included tissue and template preparations, a 2-step amplification protocol, and a variety of positive and negative PCR controls to ensure optimal assay conditions.

Although there is general agreement that electron microscopy is important in diagnostic applications for viral diseases, the length of time required to carry out routine electron microscopy is one of the factors limiting its practical usefulness (Lightner et al. 1992). In addition, much time is needed to search for scarce virus-containing cells in lightly infected specimens or asymptomatic carriers. This limits the practicable sample size for TEM observation and makes definitive conclusions difficult. However, WSBV diagnostic PCR makes it very easy to identify even lightly infected animals and virus target sites in them. These diagnoses can then be readily confirmed by *in situ* hybridization or TEM, thus increasing the speed and efficiency of screening investigations.

Fig. 6. Detection of WSBV DNA in WSBV PCR-positive tissues of captured *Penaeus monodon* by *in situ* hybridization. (A, B) Gill; (C, D) midgut; (E, F) heart; (G, H) lymphoid organ; (I, J) ventral nerve cord; (K, L) hepatopancreas showing cells with positive signals (arrows). (A, C, E, G, I, K) Without counter-stain; (B, D, F, H, J, L) counter-stained with neutral red. Bas: basement membrane; Cep: cuticular epithelial cells; Epm: epithelium, midgut; Gic: giant cell; Glc: glial cell; Hpc: hepatopancreatocytes; Lum: lumen; Mef: myoepithelial fibers; Mfn: myoepithelial fiber nucleus; Myo: myocardium; Smc: stromal matrix cells. Scale bar = 20 µm

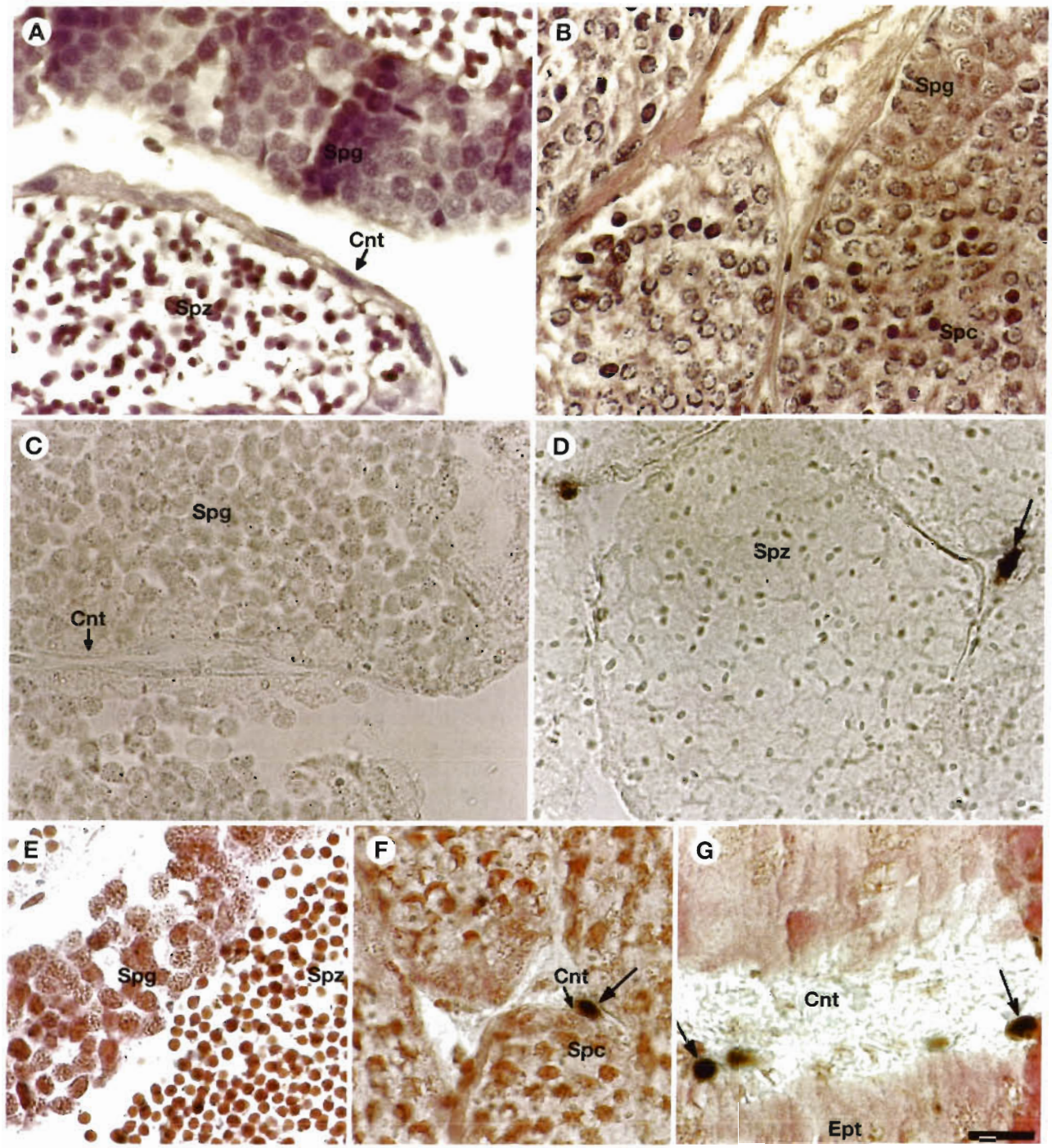
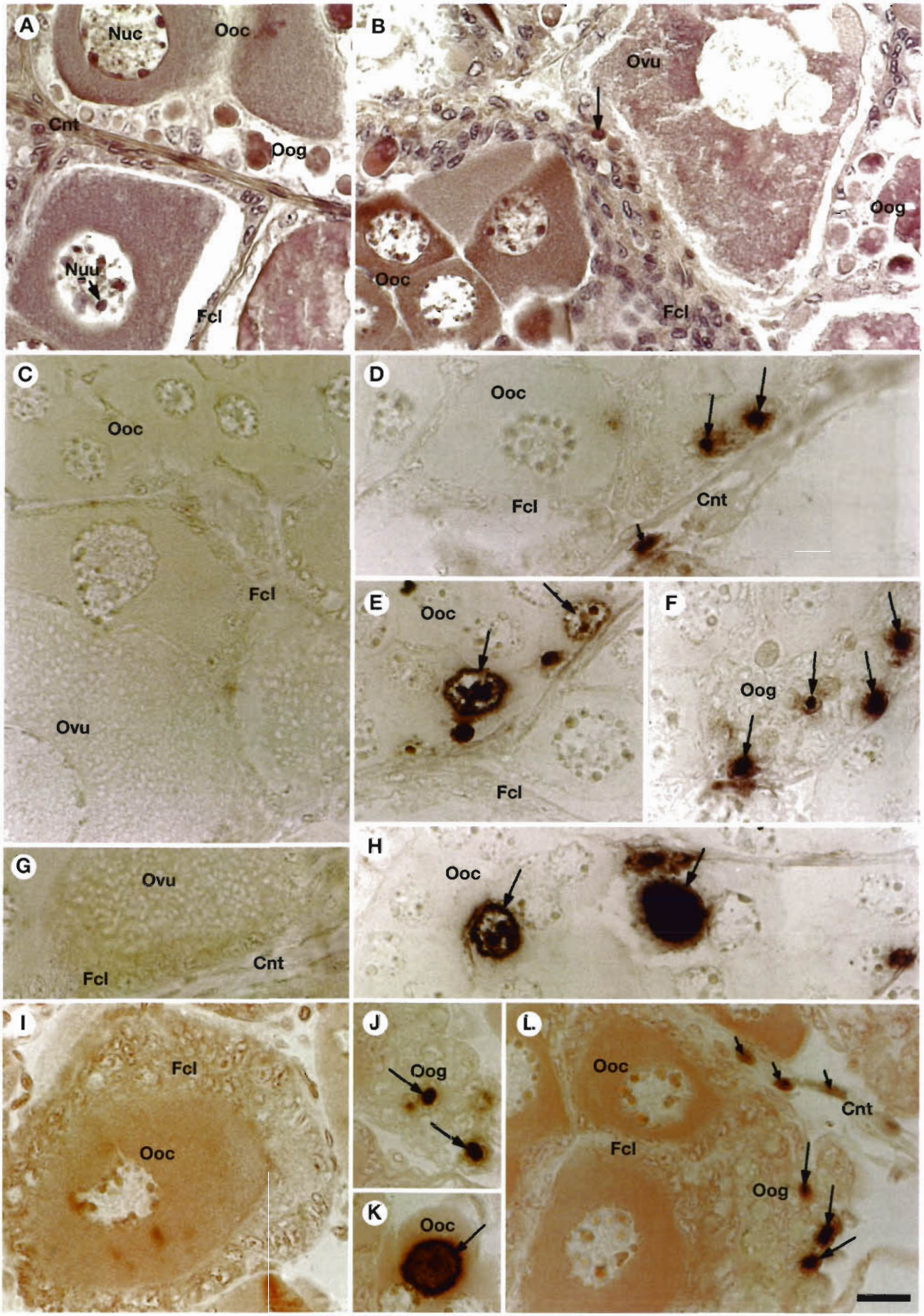


Fig. 7. Detection of WSBV in testes and spermatophores of captured *Penaeus monodon* by *in situ* hybridization. (A, C, E) WSBV 2-step PCR-negative testis with C and E showing no positive hybridization signals. (B, D, F, G) WSBV PCR-positive testis (B, D, F) and spermatophore (G) showing strong positive hybridization signals (arrows) in connective tissue layers. (A, B) H&E stain; (C, D) *in situ* hybridization without counter-stain; (E, F, G) *in situ* hybridization counter-stained with neutral red. Cnt: connective tissue; Ept: epithelium; Spc: spermatocyte; Spg: spermatogonia; Spz: spermatozoa. Scale bar = 20 µm

Fig. 8. Detection of WSBV in ovaries of captured *Penaeus monodon* by *in situ* hybridization. (A, C, G, I) WSBV 2-step PCR-negative ovary. (B, D, E, F, H, J, K, L) WSBV 1-step PCR-positive ovary. (D, L) Small arrows: connective tissue cells; large arrows: follicle cells. (F, J) Arrows: oogonia, (E, H, K) arrows: oocytes. (A, B) H&E stain; (C, D, E, F, G, H) *in situ* hybridization without counter-stain; (I, J, K, L) *in situ* hybridization counter-stained with neutral red. Cnt: connective tissue; Fcl: follicle cell; Nuc: nucleus; Nuu: nucleolus; Ooc: oocyte; Oog: oogonium; Ovu: ovum. Scale bar = 20 µm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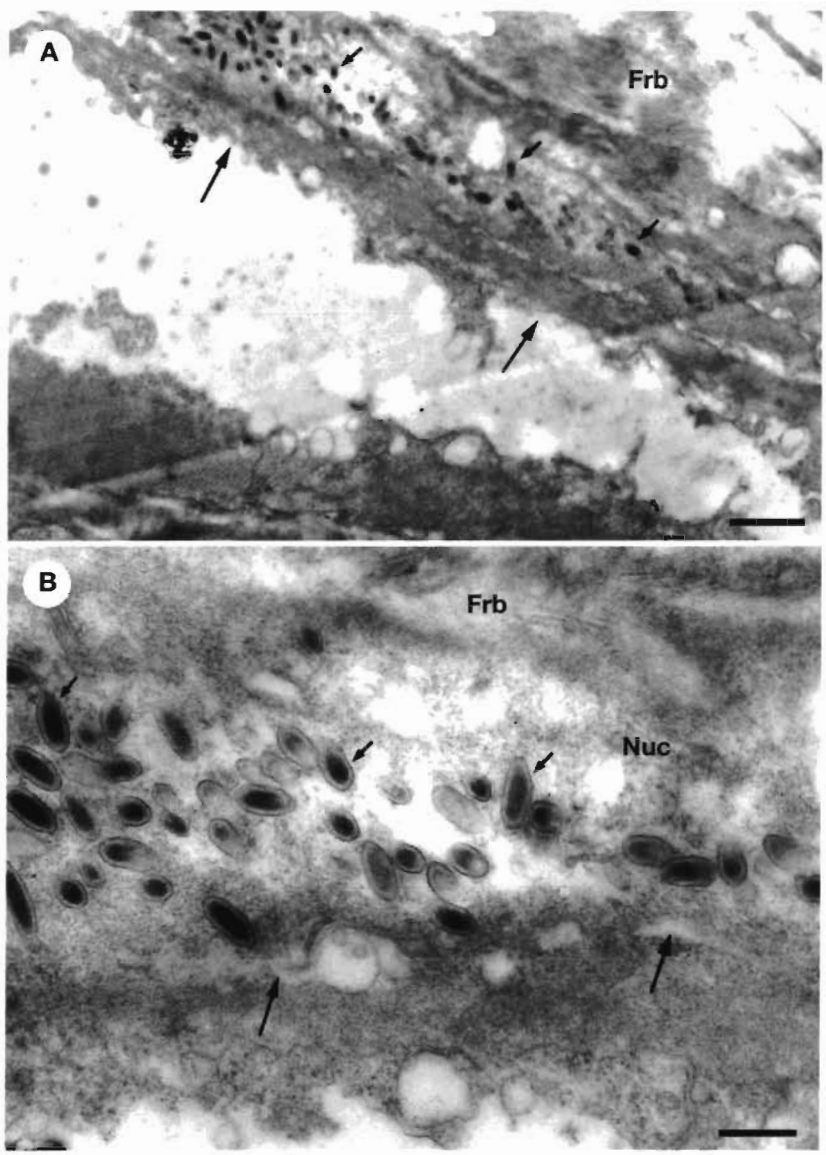
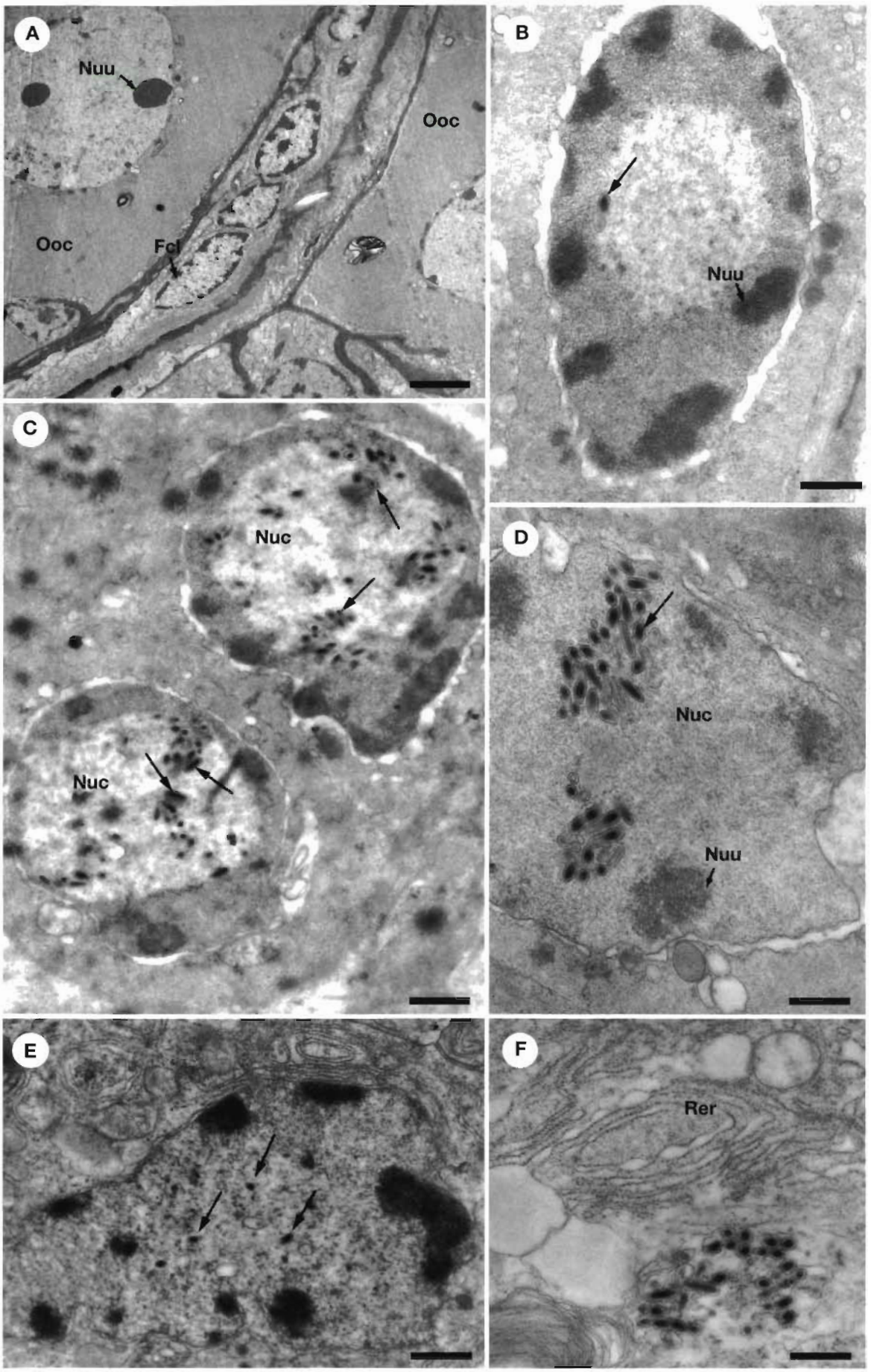


Fig. 9. Detection of WSBV in the testis of captured *Penaeus monodon* by transmission electron microscopy. (A, B): TEM micrographs of thin-sectioned testis showing viral particles (small arrows) and disintegrated nuclear envelope (large arrows) in fibroblasts surrounding the seminiferous tubules. Frb: fibroblast; Nuc: nucleus. Scale bars = 1 µm in (A), and 300 nm in (B)

For *Penaeus monodon* female brooders, WSBV was detected more frequently from July to November than from December to February (Table 2). A larger sample would be required to prove whether the indicated difference was statistically significant. However, in Taiwan coastal waters, the spawning season of *P. monodon* lasts from March to November and the brooders usually spawn repeatedly (Chen 1990). Our present study showed that, at least in aquaria, the stress inherent in spawning may trigger an outbreak of WSS in latently WSBV infected brooders and cause death after spawning. Consequently, a lower prevalence of captured *P. monodon* brooders in the winter might be due to the death of infected brooders after spawning. Although this hypothesis cannot be easily verified, we always use larvae from winter-captured brooders for experimental infection trials because these larvae are quite frequently WSBV free.

Fig. 10. Detection of WSBV in the ovary of captured *Penaeus monodon* by transmission electron microscopy. Transmission electron micrographs of (A) thin-sectioned ovary showing follicle cells and oocytes in the ovary; (B, C, D) nuclei of oogonia; (E) nucleus of a follicle cell (large arrows show viral particles); (F) cytoplasm of a follicle cell. Ooc: oocyte; Fcl: follicle cell; Nuc: nucleus of oogonia; Nuu: nucleolus of oocytes; Rer: rough endoplasmic reticulum. Scale bars = 5.0 µm in (A), 867 nm in (B, D), 500 nm in (C, F), and 850 nm in (E)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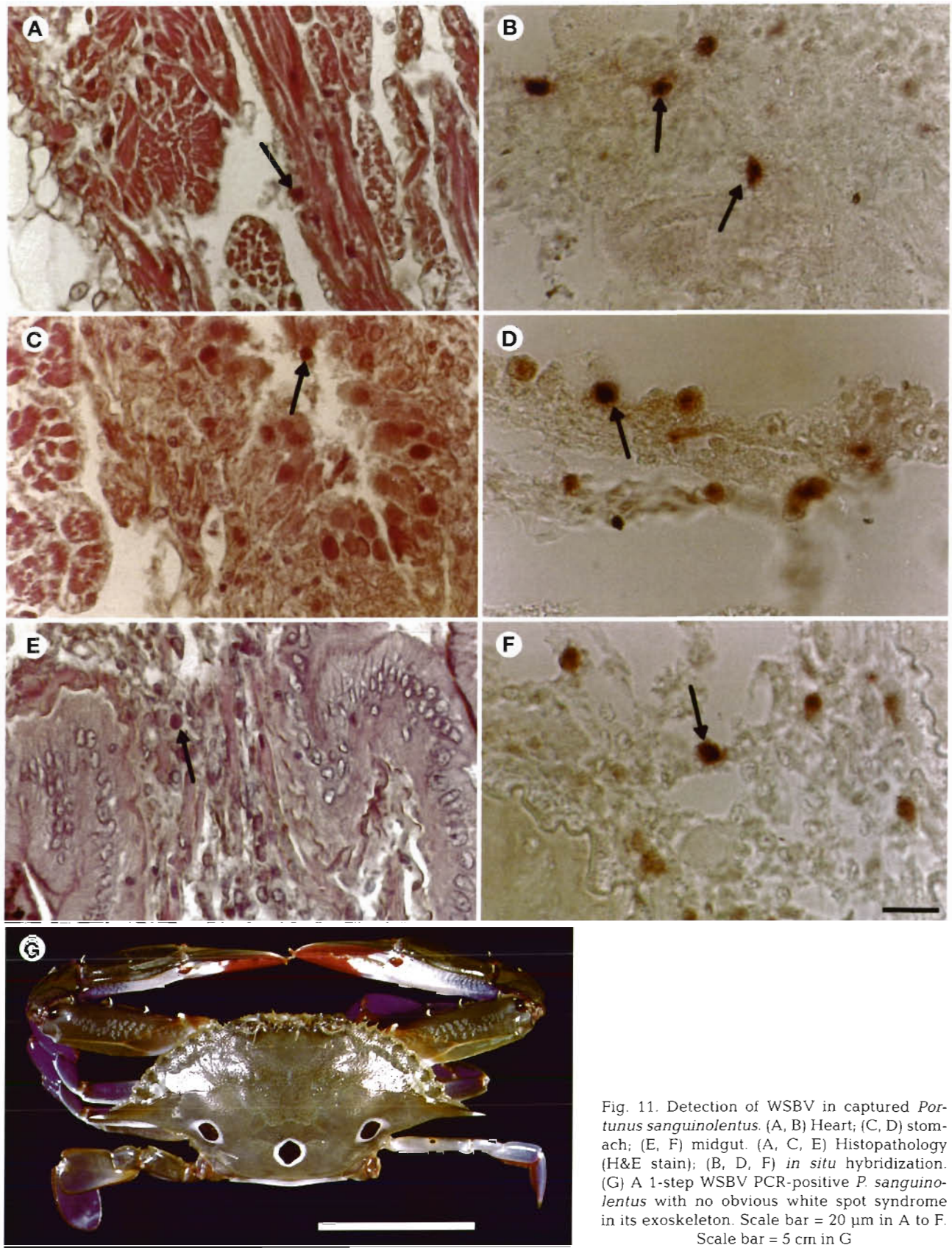

Fig. 11. Detection of WSBV in captured *Portunus sanguinolentus*. (A, B) Heart; (C, D) stomach; (E, F) midgut. (A, C, E) Histopathology (H&E stain); (B, D, F) *in situ* hybridization. (G) A 1-step WSBV PCR-positive *P. sanguinolentus* with no obvious white spot syndrome in its exoskeleton. Scale bar = 20 µm in A to F. Scale bar = 5 cm in G

None of the brooders that did spawn successfully were WSBV positive by 1-step PCR upon capture (Table 3). This result suggests that only lightly infected (and of course healthy) brooders can spawn successfully. Before the WSBV outbreak, a female brooder in a hatchery was expected to spawn repeatedly from 2 to 4 times (sometimes up to 20 times or more) once every 2 to 4 d before death (Chen 1990). However, according to personal communication with some hatchery operators in Taiwan, most female brooders have not managed to spawn repeatedly since the outbreak of WSS in 1992. This was corroborated in our study where brooders never spawned more than twice before death (usually only once) and died shortly after spawning (Table 4). This indicates that WSBV is highly pathogenic to brooders during the spawning season.

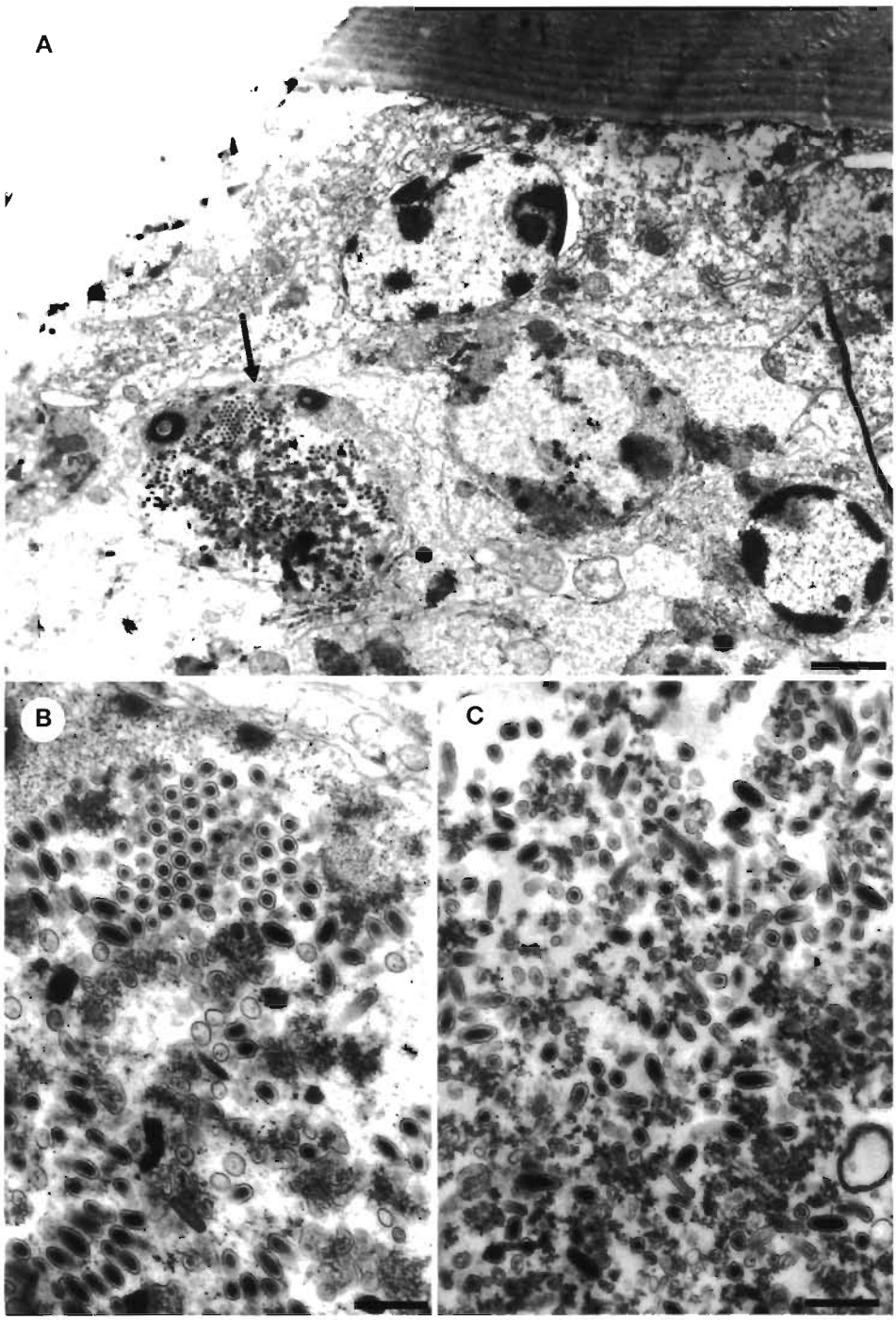

Fig. 12. Detection of WSBV in a captured crab (*Portunus sanguinolentus*) by transmission electron microscopy. (A) Infected cells (arrow) underneath the cuticle; (B, C) high magnification of virus particles. Scale bars = 2 µm in (A) and 500 nm in (B, C)

In the case of brooders collected in July or August, we found that almost all (including those that were initially 2-step WSBV PCR negative) became 1-step PCR positive after spawning and subsequent death (Table 4). Gross signs of WSS were often exhibited after death (Table 4), although the white spots were very tiny (Fig. 1). Since the brooders were held separately during the observation period, horizontal transmission was unlikely to have been the main cause of infection. We initially considered spawning stress to be the critical factor that triggered viral multiplication in the brooders. Now, however, we suspect that some of the captured crabs used to feed the brooders may have caused WSBV infection. PCR, *in situ* hybridization (Fig. 11) and TEM (Fig. 12) established that the *P. sanguinolentus* crabs used as feed were heavily infected with WSBV although they did not exhibit gross signs such as white spots. Because of this finding, farmers have been warned against using captured crabs to feed brooder shrimp. In Thailand, marine crabs were also proven to be hosts of systemic ectodermal and mesodermal baculovirus (SEMBV) (Wongteerasupaya et al. 1995), a shrimp virus to which WSBV is very closely related. Once informed, local farmers there stopped using crabs to feed brooders. Some farmers have also set up facilities to block the entrance of crabs to shrimp culture ponds and have thus effectively helped to contain the disease (Dr Timothy William Flegel pers. comm.). Before we have effective diagnostic tools for all known decapod viruses and before decapods can be certified, the use of untreated cultured or captured decapods—or indeed other possible viral reservoir hosts—should be avoided.

Critical for the reliable amplification of extracted DNA by PCR are the selection of suitable tissues for DNA extraction and the method of extracting DNA. Our evaluation of nucleic acid templates prepared from 16 shrimp parts by PCR amplification of a shrimp 18S rRNA gene fragment showed that all of them were suitable except eye stalk with the compound eyes still attached, since these produced consistently negative results. We further showed that the failure of amplification was due to the compound eye which may have contained PCR inhibitor(s). It is quite common to encounter PCR inhibitors in DNA extracted from both fixed and fresh tissues of a variety of organisms (An & Fleming 1991, Doyle & O'Leary 1992). Although no inhibitor in the compound eye has yet been identified, knowledge of this inhibition is critical for routine PCR screening of brooders for WSBV. In Taiwan, it is common to induce egg maturation in brooders through eye-stalk ablation (Chen 1990), and one might use the ablated eyestalk as a convenient source for PCR templates provided that the compound eye is removed before use.

The tissue tropism study suggested that hemolymph and pereiopods from brooders might also be good sources for PCR template preparation. The detected prevalence of WSBV in pereiopods, however, was not high in Group II brooders, with some pereiopods being 2-step WSBV PCR negative, while many other tissues were 2-step WSBV PCR positive (Table 5). Unfortunately, the other potential tissue sources are unsuitable for other reasons. Even though the pleopod, for example, which had the highest rate of WSBV detection (Table 5), would probably reduce the number of 'false negatives', its excision would be much more damaging to the shrimp (authors' pers. obs.). This is an important consideration among hatchery operators. Indeed, if the operator is not well-practiced in the withdrawal of hemolymph from live specimens, then we would definitely recommend use of either the eye stalk or pereiopod instead.

The method of DNA extraction is also critical for reliable PCR amplification. Our previously published data on WSBV diagnostic PCR (Lo et al. 1996a, b) were based solely on results obtained from deproteinized template DNA. However, we have carefully assessed the suitability of 3 DNA extraction methods for PCR template preparation. These will be briefly described and discussed in this paragraph. The preparation of deproteinized DNA with proteinase K and CTAB treatments followed by phenol-chloroform extraction and ethanol precipitation is time consuming, but it is apparently most reliable in yielding amplifible DNA templates from shrimp or crab tissues (Lo et al. 1996a, b). The deproteinized DNA template is very stable and can be stored at 4°C until use. This method is the method of choice if it is essential to test a whole sample (i.e. if there is only a small amount of tissue available or if the specimens are lightly infected). Although high-quality DNA is usually required for PCR, nucleic acid extracted by rapid proteinase K digestion or by simply boiling can also yield amplifible template. In the rapid proteinase K digestion method, a piece (ca 0.04 g) of the shrimp tissue was incubated with 500 µl of detergent buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 0.1 mg ml^{-1} gelatin, 0.45% NP-40, 0.45% Tween 20) and proteinase K (final concentration of 0.2 mg ml^{-1}) at 65°C for 1 h. Next, the reaction mixture was boiled for 10 min to inactivate the proteinase K. Immediately afterwards, 50 µl of the supernatant was used in a 100 µl PCR reaction mixture. This method is a modification of the protocol proposed by Malitschek & Schartl (1991). In the simple boiling method, a piece of shrimp tissue (0.04 g) was boiled with 500 µl distilled water for 20 min to extract the DNA template (An & Fleming 1991). Again 50 µl of supernatant was immediately used for a 100 µl PCR. Provided that failure to amplify some samples is not a major problem, both of

these methods can be used for detection of WSBV in diseased shrimp collected from farms when abundant, patently infected specimens are available and it is not necessary to keep them alive. Usually, gills and appendages are good materials for PCR template preparation using rapid DNA extraction methods. Nucleic acid extracted from shrimp by rapid extraction methods is not stable and should be used for PCR immediately after preparation. Results are not always reproducible. This most likely reflects the rapid disintegration of the DNA templates in crude shrimp nucleic acid extracts. Considering the likelihood of false negatives from crude nucleic acid extracts, it is important to use deproteinized nucleic acids to reconfirm any negative amplification results.

In cultured and/or experimentally infected shrimp in the patent stage, WSBV infection is characterized by serious infection in a wide range of target tissues (Chou et al. 1995, Wang et al. 1995, Wongteerasupaya et al. 1995, Chang et al. 1996). In the naturally WSBV-infected wild *P. monodon* specimens here which were 1-step WSBV PCR positive, *in situ* hybridization showed a similar wide range of target tissues (Figs. 5 to 8), although the number of positive cells was relatively limited when compared to cultured and/or experimentally infected shrimp. In 2-step WSBV PCR positive specimens (Table 5, Group II), the prevalence of WSBV DNA in the lymphoid organ (4/9; 44%) and integument (3/9; 33%) was relatively low; these are not main targets for WSBV replication in latent infections although they become so in patent specimens (Chang et al. 1996). Thus, the Group III shrimp specimens in Table 5 did not have any obvious white spots in their exoskeleton and looked healthy only because they had very recently made the transition from Group II to Group III.

In the ovary, we found by *in situ* hybridization that most of the WSBV-positive cells were follicle cells and oogonia. A few developing oocytes gave strong positive signals for WSBV DNA. In our WSBV-positive specimens, the diameter of the nucleus of the largest oocyte was 40 mm, but the WSBV DNA positive signals were only found in oocytes with a nucleus diameter less than 28 µm. This suggested that the infected oocytes were unable to develop further or that they died before maturation. If so, it would be unlikely for WSBV to be transmitted from brooders to their offspring through infected ova. Nonetheless, the virus may be released during spawning and thus may infect the developing larvae. This may explain the detection of WSBV in larvae (Table 3). Although this possibility has not been formally investigated, we think it is proper to recommend here that eggs or nauplii be rinsed in order to minimize the chances of water-borne infection.

Although the removal of an eye-stalk induces egg maturation in female brooders, it also reduces the level of the molt-inhibiting hormone and often results in molting and the consequent dropping of spermatophores. Shrimp need to mate immediately after molting to recharge the seminal receptacle with a spermatophore (Chen 1990). We found that the prevalence of WSBV in male brooders was very high (Table 1), and that spermatophores and testes gave WSBV-positive results by PCR (Table 5) and by *in situ* hybridization analysis (Fig. 7). Thus, to minimize the spread of WSBV, only male brooders which have tested negative for WSBV and other known viruses should be used for mating.

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LITERATURE CITED

An SF, Fleming KA (1991) Removal of inhibitor(s) of polymerase chain reaction from formalin fixed, paraffin wax embedded tissues. J Clin Pathol 44:924–927

Chang PS, Lo CF, Wang YC, Kou GH (1996) Identification of white spot syndrome associated baculovirus (WSBV) target organs in the shrimp *Penaeus monodon* by *in situ* hybridization. Dis Aquat Org 27:131–139

Chen LC (1990) Aquaculture in Taiwan. The Alden Press, Oxford

Chou HY, Huang CY, Wang CH, Chiang HC, Lo CF (1995) Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. Dis Aquat Org 23:165–173

Doyle CT, O'Leary JJ (1992) The search for the universal fixative or 'magic juice'. J Pathol 166:331–332

Francki RIB, Fauquet CM, Knudson DL, Brown F (1991) Classification and nomenclature of viruses. Arch Virol Suppl 2:1–450

Humason GL (1979) Animal tissue techniques, 4th edn. WH Freeman, San Francisco

Lightner DV (ed) (1996) A handbook of pathology and diagnostic procedures for diseases of penaeid shrimp. World Aquaculture Soc, Baton Rouge, LA, Section 3.11

Lightner DV, Poulos BT, Bruce L, Redman RM, Mari J, Bonami JR (1992) New developments in penaeid virology: Application of biotechnology in research and disease diagnosis for shrimp viruses of concern in the Americas. In: Fulks W, Main KL (eds) Diseases of cultured penaeid shrimp in Asia and the United States. The Oceanic Institute, Honolulu, HI, p 233–256

Lo CF, Leu JH, Ho CH, Chen CH, Peng SE, Chen YT, Chou CM, Yeh PY, Huang CJ, Chou HY, Wang CH, Kou GH (1996a) Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction. Dis Aquat Org 25:133–141

Lo CF, Ho CH, Peng SE, Chen CH, Hsu HC, Chiu YL, Chang CF, Liu KF, Su MS, Wang CH, Kou GH (1996b) White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods. Dis Aquat Org 27:215–225

Malitschek B, Schartl M (1991) Rapid identification of recombinant baculoviruses using PCR. Biotechniques 11: 177–178

Murphy FA, Fauquet CM, Bishop DHL, Ghabrial SA, Jarvis AW, Martelli GP, Summers MD (1995) Classification and nomenclature of viruses. Arch Virol Suppl 10:1–586

Ren R, Racaniello VR (1992) Human poliovirus receptor gene expression and poliovirus tissue tropism in transgenic mice. J Virol 66:296–304

Suresh M, Sharma JM (1996) Pathogenesis of type II avian adenovirus infection in turkeys: *in vivo* immune cell tropism and tissue disribution of the virus. J Virol 70: 30–36

Wang CH, Lo CF, Leu JH, Chou CM, Yeh PY, Chou HY, Tung MC, Chang CF, Su MS, Kou GH (1995) Purification and genomic analysis of baculovirus associated with white spot syndrome (WSBV) of *Penaeus monodon*. Dis Aquat Org 23:239–242

Wongteerasupaya C, Vickers JE, Sriurairatana S, Nash GL, Akarajamorn A, Boonsaeng V, Panyim S, Tassanakajon A, Withyachumnarnkul B, Flegel TW (1995) A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodon*. Dis Aquat Org 21:69–77

Wongteerasupaya C, Wongwisansri, Boonsaeng V, Panyim S, Pratanpipat P, Nash GL, Withyachumnarnkul B, Flegel TW (1996) DNA fragment of *Penaeus monodon* baculovirus PmNOBII gives positive *in situ* hybridization with white-spot viral infections in six penaeid shrimp species. Aquaculture 143:23–32

Zurbriggen A, Yamada M, Thomas C, Fujinami RS (1991) Restricted virus replication in the spinal cords of nude mice infected with a Theiler's virus variant. J Virol 65: 1023–1030

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