

## NOTE

## FIRST REPORT OF VIRAL PATHOGENS WSSV AND IHHNV IN ARGENTINE CRUSTACEANS

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## ABSTRACT

The pathogenic penaeid shrimp viruses white spot syndrome virus (WSSV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) are reported for the first time from Argentina. They both occurred in wild crustaceans in Bahia Blanca Estuary, with WSSV detected by polymerase chain reaction (PCR) or real time quantitative PCR (qPCR) methods as infecting samples as high as 56% of the penaeid *Artemesia longinaris* Bate, 1888, 67% of the grapsoid *Cyrtograpsus angulatus* Dana, 1851, and 40% of the introduced palaemonid *Palaemon macrodactylus* Rathbun, 1902. The highest detected copy number was 39,600 copies/ $\mu$ g DNA. IHHNV was tested for using PCR in only *A. longinaris*, in which it infected 30% of the sample. The presence or absence of cuticular white spots, diagnostic features of a chronic WSSV infection, was not a reliable indicator of WSSV detected by PCR or qPCR methods. The qPCR method was more sensitive than three PCR methods for detecting WSSV, presumably because of low copy numbers ( $< 10^3$  copies/ $\mu$ g DNA) in several hosts. The low numbers may be related to temperature, season, IHHNV co-infection, or perhaps host. Introductions of the viruses could have originated from contamination by infected living animals, frozen products, or from alongshore currents from Brazil bringing the agents from as far away as 1700 or perhaps 5500 km.

Highly pathogenic penaeid shrimp viruses have caused severe losses to commercial shrimps in the northern countries of South America and are a threat to Argentina. One of two such viruses is referred to as white spot syndrome virus (WSSV), originating from Southeast Asia where it emerged in the western hemisphere in the mid 1990s. It had a severe impact on commercial penaeid aquaculture in 1999, first in Central America and then in Ecuador (e.g., Lightner, 2004). Infections spread south, finally to Brazil by late 2004 (available at [http://www.oie.int/eng/info/hedbo/AIS\\_58.htm](http://www.oie.int/eng/info/hedbo/AIS_58.htm) on 7 August 2009, previously on [ftp://ftp.oie.int/infos\\_san\\_archives/eng/2005/en\\_050121v18n03.pdf](ftp://ftp.oie.int/infos_san_archives/eng/2005/en_050121v18n03.pdf); Cavalli et al., 2008; Marques, 2008). This large double-stranded DNA virus in the nimavirid genus *Whispovirus* produces mortalities in several penaeid shrimp species as exemplified by the loss of about 10 billion US dollars attributed to WSSV infections in penaeid aquaculture (e.g., Lightner, 2004; Lo, 2006). Once established, the infections had an impact on wild crustaceans (e.g., Sánchez-Martínez et al., 2007). Another virus is infectious hypodermal and hematopoietic necrosis virus (IHHNV), which appeared to have reached the Americas from the Philippines in the early 1980s (Tang et al., 2003b). It is abundant in shrimp farms in northeastern Brazil (Braz et al., 2009) and documented earlier from cultured Pacific white shrimp, *Litopenaeus vannamei* (Boone, 1931), in southern Brazil (Moser, 2005). The virus can be fatal to 90% or more of some stocks of *Litopenaeus stylirostris* (Stimpson, 1874) and produce a runtting syndrome in the giant tiger prawn, *Penaeus monodon* (Fabricius, 1798), and *L. vannamei*, reducing the latter's market value by 10%–50% (Lightner and Redman, 1998). This small, non-enveloped, icosahedral, single-stranded linear DNA virus with virions averaging 22–23 nm in diameter is

capable of vertical transmission through spawning females (Motte et al., 2003). It belongs to the family Parvoviridae in the genus *Brevidensovirus* as *Penaes stylirostris densovirus* (PstDNA) in the subfamily Densoirinae (Shike et al., 2000; Tattersall et al., 2005). Here we document *Artemesia longinaris* Bate, 1888 as a new wild host for WSSV and IHNV, supported by histopathological sections and molecular polymerase chain reaction (PCR) and real time quantitative PCR (qPCR) methods. We also report two other crustacean hosts for WSSV, establishing the extension of geographic ranges of the crustacean viruses into Argentina, and comparing grossly visible white spots with confirmed WSSV cases.

## METHODS

The commercially important penaeid shrimp *A. longinaris* was collected from Bahía Blanca Estuary, Argentina (Fig. 1; 38°46' S, 62°20' W). Samples of about 40 individuals of both *A. longinaris* and the solenoceric penaeoidean *Pleoticus muelleri* (Bate, 1888) (Argentine red shrimp) were purchased fresh at the Ingeniero White Port each year from 2003 to 2008, usually in February–March. These shrimps were captured by relatively large commercial vessels trawling channels of the estuary. Individual shrimp were kept on ice and examined grossly for signs of WSSV, and two or three representatives of each yearly sample were immediately fixed and sectioned. Also in February and March of 2008, a total of 20 live specimens of *A. longinaris* were collected by cast net from a pier at General Daniel Cerri, an oligohaline region of the Estuary. An additional 20 live specimens were obtained from commercial vessels. These live shrimp were transported to the laboratory in plastic bags with aerated sea water from the source. After viral infections were confirmed, additional specimens from General Daniel Cerri were collected and the gills immediately fixed in 95% molecular grade ethanol for molecular analyses. In February 2009, 102 specimens of *A. longinaris* were cast-netted from a fishing pier, and the gills were removed from 18 individuals. In April 2009, the gills were fixed from six specimens of the intertidal mud crab *Cyrtograpsus angulatus* Dana, 1851 (Grapsoidae: Varunidae) that were caught in crab-traps and from five of the introduced (Spivak et al., 2006) oriental shrimp, *Palaemon macrodactylus* Rathbun, 1902, (Caridea: Palaemonidae) that were cast-netted. The remainder of those animals was fixed in Davidson's fixative (alcohol, formalin, and acetic acid) for histological evaluation. Once living crustaceans were brought into the laboratory, they were either fixed for analysis immediately or kept alive for 1–14 d in aerated static aquaria until dissected. For histological examination of these and the field-fixed samples, representatives were either fixed in 10% phosphate buffered formalin or injected and saturated in Davidson's fixative for 24–48 hrs and then transferred to 50% ethyl alcohol for storage until processed, using the methods of Bell and Lightner (1988). For molecular studies, samples of skeletal muscle, hepatopancreas, appendages, and primarily gill tissue were fixed in 95% molecular grade ethanol and sent to the Gulf Coast Research Laboratory (GCRL) in Ocean Springs, Mississippi.

DNA was extracted from gills, skeletal muscle, hepatopancreas, and cephalothorax appendages using the DNeasy (Qiagen) kit methodology. PCR-amplification was conducted on the extracted DNA using illustra™ PuReTaq Ready-To-Go PCR beads (GE Healthcare) containing a concentration of 200  $\mu$ M dNTP's in 10 mM Tris-HCl pH 9.0, 50 mM KCl and 1.5 mM MgCl<sub>2</sub> in a 25- $\mu$ l reaction volume and specific primer pairs for detection of WSSV, WS500F: CCT TCC ATC TCC ACC ACA CTT and WS500R: CTG TTC CTT GGC AGA GCA TTC that yields a 490 bp amplicon; WS800F: CTC ACC TGC GCT TCT CAC CTC ATG CTT and WS800R: GCC GGC CAG TTC GTT CAC GAT AAC CTT that yields a 783 bp amplicon (Nunan et al., 1998, D. Lightner, University of Arizona, pers. comm.). Both one-step and nested reactions were carried out using these primers with the WS500F/R as the internal primer set. A nested two-step PCR using the World Animal Health Organization (or Office



Figure 1. Map of South America showing areas where infected shrimp occur in shrimp farms and surrounding water in the states of Santa Catarina and Ceara, Brazil, and in the natural waters of Bahía Blanca Estuary, Argentina, where wild samples were collected.

International des Epizootics, OIE) WSSV protocol with primers developed by Lo et al. (1996) was also performed on 23 of the *A. longinaris* specimens.

The IHNV-PCR assay was conducted on the same 23 specimens of *A. longinaris* indicated above using IHNV309F/R primers and methodology developed by Tang et al. (2007). All amplified PCR products were run by gel electrophoresis on a 1.2% agarose gel and stained with ethidium bromide to determine whether bands were present at the expected bp amplicon corresponding with known positive controls from WSSV-positive shrimp or IHNV plasmids run in conjunction with each PCR reaction.

A real-time, quantitative PCR (qPCR) analysis was performed on the same extracted DNA to detect and confirm the WSSV infection and determine copy number. The qPCR reaction was run in duplicate on an iCycler (Bio-Rad) using iQ™ Supermix with a dual-labeled 5'FAM and 3'TAMRA probe: AGC CAT GAA GAA TGC CGT CTA TCA CAC A with forward and reverse primers WSSV1011F: (TGGTCCCCTCCTCATCTCAG) and WSSV1079R: (GCTGCCTTGCCGGAAATTA) in a 25- $\mu$ l reaction volume (Durand and Lightner, 2002). We used the WS800/WS500 primers for PCR and the qPCR method indicated above on all of the samples. All listed positive qPCR values comprised those detected within the range of our developed standard curve between the lowest standard copy number ( $2.8 \times 10^1$ ) and the highest ( $2.78 \times 10^8$ ).

## RESULTS

Specimens of the crustaceans *A. longinaris*, *P. muelleri*, *C. angulatus*, and others except *P. macrodactylus* collected from the Bahía Blanca Estuary from 2003 to 2007

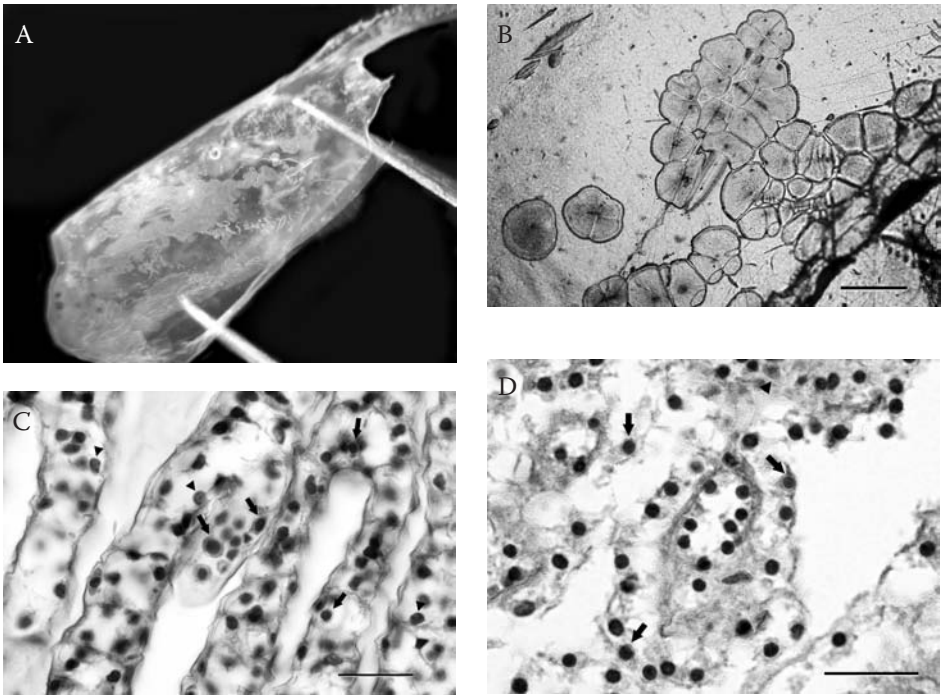


Figure 2. White spot syndrome virus (WSSV) in *Artemesia longinaris*: (A) typical “white spot” of WSSV in cuticle of cephalothorax, (B) close-up of “white spot” of WSSV in cuticle of sixth abdominal segment, scale bar = 2 mm, (C) histological section of individual without concurrent IHNV infection, showing secondary branching gill filaments with infected nuclei (arrows) and normal (arrowheads) nuclei, hematoxylin and eosin stain, scale bar = 25  $\mu$ m, (D) section of antennal gland of same individual as in Figure 2C, with infected (arrows) nuclei, hematoxylin and eosin stain, scale bar = 25  $\mu$ m.

exhibited no gross lesion characteristic of the diagnostic “white spots” of WSSV. In February/March 2008, the sample of 40 live *A. longinaris* included 24 (60.0%) that demonstrated white spots (Figs. 2A,B), and, in February 2009, 102 live shrimp consisted of 71 (69.6%) with the white spots. These spots were abnormal deposits of calcium salts in the cuticle, when examined under a stereomicroscope. *Palaemon macrodactylus*, which was examined from Mar del Plata Port in 2003 and 2004, exhibited some individuals with white spots, but the condition could not be confirmed as WSSV using a Shrimple® kit. In all of the four histologically sectioned representatives of *A. longinaris*, the gills (Fig. 2C), cuticular epithelium, and antennal gland (Fig. 2D) exhibited hypertrophied nuclei with scarce nuclei having centrally located eosinophilic inclusions, some separated from the nuclear margined chromatin by an artifactual halo. Figures 2C and 2D show sections of the same individual that did not have a concurrent IHNV infection. In 66 counted nuclei, 13 exhibited relatively small to large inclusions, with seven of those also expressing the halo. This halo pattern resembled what Lightner (1996) regarded an early phase WSSV inclusion body. Of those shrimp maintained in aquaria for 2 wks prior to critical examination and processing, none died and all demonstrated a similar normal swimming and feeding behavior. They were fed a commercial tropical fish diet of Shulet®.

White spots that commonly occurred in *A. longinaris* from Bahia Blanca in February 2009 as well as in 2008 could also clearly be seen in the dorsal carapace and

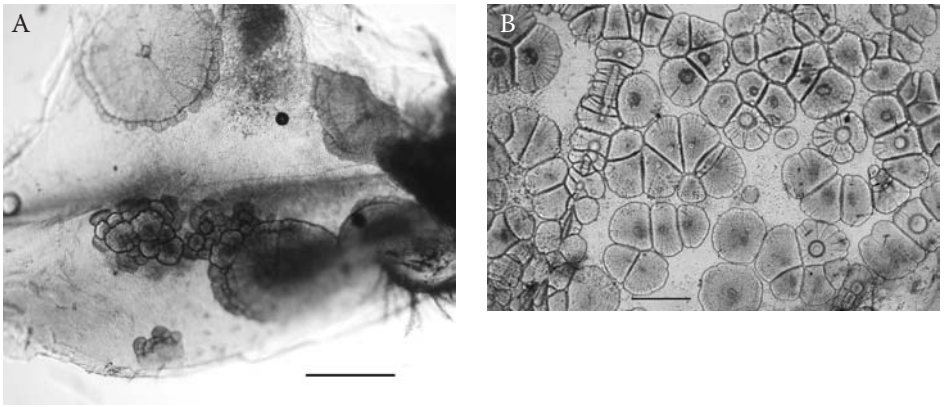


Figure 3. “White spots” in unfixed cuticle of carapace of non-penaeids, scale bars = 500  $\mu\text{m}$ . (A) varunid grapsoid, *Cyrtograpsus angulatus*, (B) palaemonid, *Palaemon macrodactylus*.

internal cuticle of the branchial chamber of three of six specimens of *C. angulatus* in April 2009 (Fig. 3A) and one of two and one of three specimens of *P. macrodactylus* in March 2008 and April 2009 (Fig. 3B), respectively (Table 1). These spots, 1–3 mm across, occurred in the internal surface of the cuticle overlying both the cephalothorax and abdomen.

For genetic confirmation of the histological indication of WSSV, the amplified PCR product produced an amplicon at 490 bp, designating WSSV in the gill tissue of two *A. longinaris* from the initial collection. DNA extracted from hepatopancreas of the same individuals produced no amplicon at 490 bp, although the skeletal muscle of one of them produced a faint band at 490 bp using nested WS800/500 primers; skeletal muscle samples of both were positive for WSSV by qPCR. The viral copy number/ $\mu\text{g}$  of total DNA for the two gill specimens determined by qPCR was  $4.60 \times 10^3$  and  $1.47 \times 10^3$ .

We observed that two of 25 samples of *A. longinaris* produced positive reactions for WSSV with the one-step PCR procedure using the WS800F/R primers, but 11 of those 25 were positive with the nested WS800/WS500 PCR. This finding contrasted with six of 23 positive shrimp using the OIE nested protocol, including two false positives (Table 1), if the qPCR is taken as the correct result for the sample, i.e., the “gold standard.” The qPCR yielded 14 out of 25 shrimp (56%) possessing WSSV, with a copy number/ $\mu\text{g}$  of total DNA ranging from  $7.3 \times 10^1$  to  $3.96 \times 10^4$ . The presence of white spots in the cuticle did not directly relate to whether or not the specimen tested PCR-positive for WSSV (Table 1).

The six samples of *C. angulatus* yielded four with a band in the range of 490 bp when the nested WS800/WS500 PCR was performed, and the qPCR was positive for the same four specimens. The five specimens of *P. macrodactylus* included only one positive for WSSV with the nested WS800/WS500 PCR, but qPCR revealed two. Results of the qPCR are listed in Table 1, providing relatively low WSSV copy numbers per  $\mu\text{g}$  of extracted DNA given.

To determine whether infections and gross signs of WSSV in *A. longinaris* might be influenced by a co-occurring IHNV infection, we assessed for and detected IHNV in seven of 23 (30.4%) shrimp by PCR (Table 1). Infections were confirmed by histological results (Fig. 4). Five shrimp were shown by PCR to have a dual infec-



Table 1. WSSV from *Artemesia longinaris*, *Cyrtograpsus angulatus*, and *Palaemon macrodactylus* and IHNV from *A. longinaris* in Bahía Blanca, Argentina, in 2008 and 2009. + is positive; - is negative. The asterisk\* denotes a false positive. The abbreviation < Std denotes that the qPCR value was below the lowest standard (28 copies).

Sample #	White spots in cuticle	Date	Species	Tissue	WS800	Nestd WS800/WS500	WSSV Lo nested	qPCR WSSV copy / $\mu$ g DNA	IHNV
DNA-08-649	+	Feb/Mar-08	<i>Artemesia longinaris</i>	gills	-	+	+	4,600	-
DNA-08-651	+			muscle	-	+	+	73	-
DNA-08-650	+			hepato	-	-	-	< Std	-
DNA-08-652	+	Feb/Mar-08	<i>Artemesia longinaris</i>	gills	-	+	+	1,470	-
DNA-08-654	+			muscle	-	-	-	248	-
DNA-08-653	+			hepato	-	-	-	< Std	-
DNA-09-735	+	Feb-09	<i>Artemesia longinaris</i>	gills	+	+	+	226	+
DNA-09-734	+	Feb-09	<i>Artemesia longinaris</i>	gills	+	+	+	296	-
DNA-09-723	+	Feb-09	<i>Artemesia longinaris</i>	gills	-	-	+	< Std	-
DNA-09-715	+	Feb/Mar-08	<i>Artemesia longinaris</i>	gills	-	+	-	39,600	+
DNA-09-716	+	Feb/Mar-08	<i>Artemesia longinaris</i>	gills	-	-	-	< Std	+
DNA-09-737	+	Feb-09	<i>Artemesia longinaris</i>	gills	-	-	-	330	+
DNA-09-721	+	Feb-09	<i>Artemesia longinaris</i>	gills	-	-	-	< Std	-
DNA-09-722	+	Feb-09	<i>Artemesia longinaris</i>	gills	-	-	-	< Std	-
DNA-09-724	+	Feb-09	<i>Artemesia longinaris</i>	gills	-	+	-	1,180	-
DNA-09-736	+	Feb-09	<i>Artemesia longinaris</i>	gills	-	-	-	< Std	-
DNA-09-738	+	Feb-09	<i>Artemesia longinaris</i>	gills	-	+	-	355	-
DNA-09-718	+	Feb/Mar-08	<i>Artemesia longinaris</i>	gills	-	-	-	< Std	-
DNA-09-725	-	Feb-09	<i>Artemesia longinaris</i>	gills	-	+	+	4,920	-
DNA-09-731	-	Feb-09	<i>Artemesia longinaris</i>	gills	-	-	+	2,440	-
DNA-09-732	-	Feb-09	<i>Artemesia longinaris</i>	gills	-	+	+	< Std	-
DNA-09-719	-	Feb/Mar-08	<i>Artemesia longinaris</i>	gills	-	-	-	636	+
DNA-09-727	-	Feb-09	<i>Artemesia longinaris</i>	gills	-	-	-	< Std	+
DNA-09-730	-	Feb-09	<i>Artemesia longinaris</i>	gills	-	+	-	186	+
DNA-09-720	-	Feb-09	<i>Artemesia longinaris</i>	gills	-	-	-	< Std	-
DNA-09-726	-	Feb-09	<i>Artemesia longinaris</i>	gills	-	+	-	2,440	-

Table 1. Continued.

Sample #	White spots in cuticle	Date	Species	Tissue	WS800	Nested WS800/WS500	WSSV Lo nested	qPCR WSSV copy / µg DNA	IHHNV
DNA-09-728	-	Feb-09	<i>Artemesia longinaris</i>	gills	-	-	-	< Std	-
DNA-09-733	-	Feb-09	<i>Artemesia longinaris</i>	gills	-	-	-	3,820	-
DNA-09-717	-	Feb/Mar-08	<i>Artemesia longinaris</i>	gills	-	-	-	< Std	-
DNA-09-748	+	Apr-09	<i>Cyrtograpsus angulatus</i>	gills	+	+	-	2,320	-
DNA-09-744	+	Apr-09	<i>Cyrtograpsus angulatus</i>	gills	-	+	-	44	-
DNA-09-749	+	Apr-09	<i>Cyrtograpsus angulatus</i>	gills	-	+	-	2,380	-
DNA-09-747	-	Apr-09	<i>Cyrtograpsus angulatus</i>	gills	-	-	-	< Std	-
DNA-09-745	-	Apr-09	<i>Cyrtograpsus angulatus</i>	gills	-	+	-	95	-
DNA-09-746	-	Apr-09	<i>Cyrtograpsus angulatus</i>	gills	-	-	-	< Std	-
DNA-09-739	+	Mar-08	<i>Palaemon macrodactylus</i>	gill/ appen	-	-	-	< Std	-
DNA-09-742	+	Apr-09	<i>Palaemon macrodactylus</i>	gill/ appen	-	-	-	< Std	-
DNA-09-741	-	Apr-09	<i>Palaemon macrodactylus</i>	gills	-	+	-	205	-
DNA-09-743	-	Apr-09	<i>Palaemon macrodactylus</i>	gills	-	-	-	648	-
DNA-09-740	-	Mar-08	<i>Palaemon macrodactylus</i>	gills	-	-	-	< Std	-

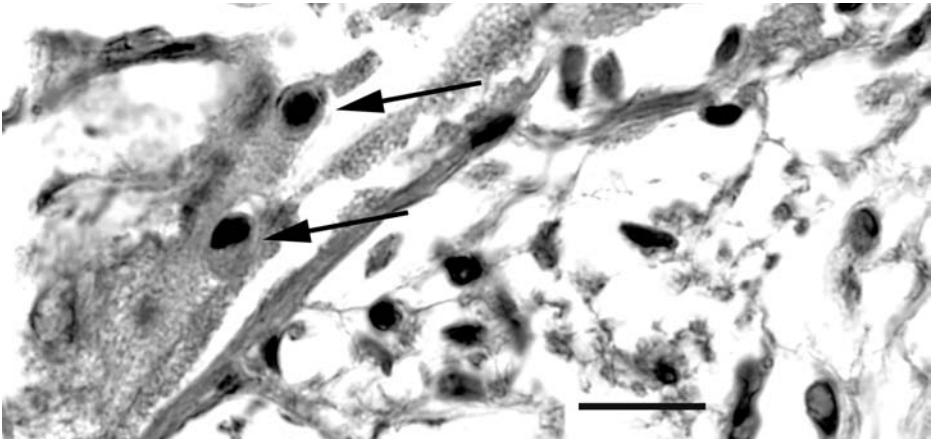


Figure 4. Infectious hypodermal and hematopoietic necrosis virus in connective tissue surrounding ventral nerve cord of *Artemesia longinaris*, hematoxylin and eosin stain, scale bar = 12.5  $\mu\text{m}$ .

tion with WSSV, and all but one (DNA-09-715) had a low copy number. Also, two of the seven shrimp positive for IHHNV did not test positive for WSSV (Table 1).

#### DISCUSSION

Infections of WSSV had not been confirmed from Argentina until we analyzed *A. longinaris* with gross signs collected in March and April of 2008, even though WSSV may have been present for a few years previously. The prevalence of “white spots” in those shrimp in 2008 was 60%, but the prevalence of WSSV confirmed by PCR was only 43%, including some shrimp not exhibiting white spots. The infections that we encountered, initially based on the presence of white spots, can be considered chronic infections because of the low copy number. Acute infections typically involve rapidly replicating virus with a high copy number that produces death in penaeids within a few days, and those infected penaeids rarely exhibit the white spots. Detection of acute infections and dead shrimp may be hampered because weak or moribund shrimp are vulnerable prey for several predators. Possible reasons for this discrepancy between the presence of white spots and detectable virus are discussed below. *Artemesia longinaris* also demonstrated a prevalence of 30% infected with IHHNV. The records of these two viruses constitute the first for them or for any crustacean virus in Argentina.

Problems involving the use of traditional PCR for the detection of viruses, especially when a concentration was low, making the sensitivity of qPCR greater than conventional PCR, are accepted and well recognized (e.g., Dagher et al., 2004; Kubista et al., 2006; Logan et al., 2009). We used the OIE (2006) recommended protocol for a nested (two-step) PCR based on the method by Lo et al. (1996), which resulted in six of 23 specimens of *A. longinaris* testing positive, with two of the six apparently being false positives (Table 1). Based on various studies at GCRL and on analyses by Lightner and others (e.g., Claydon et al., 2004; Sritunyalucksana et al., 2006), many users have determined that a series of modified methods are more sensitive and reliable than those suggested by OIE (2006). The nested 800WS/WS500 methodology indicated nine out of 23 being positive, and the qPCR detected at least 12 out of the same 23 as being positive. The WSSV copy number/ $\mu\text{g}$  of total DNA determined by



qPCR for all 25 shrimp ranged from  $7.3 \times 10^1$  to  $3.96 \times 10^4$ , with most of the values being low but easily detectable. Durand and Lightner (2002) reported no dose less than  $2.0 \times 10^4$  to  $9.0 \times 10^{10}$  copy number/ $\mu\text{g}$  of total DNA in moribund penaeids involving three penaeid species. Only one specimen (DNA-09-715, from February/March 2008) had a concentration within this range ( $3.96 \times 10^4$ ). The presence of WSSV in *A. longinaris* was confirmed by PCR, qPCR, and histology, but the actual prevalence of infection, based on the low copy numbers and presence of white spots in some PCR-negative shrimp, was probably higher than reported during preceding months of collection. Nevertheless, copy number in most cases was so low that prior PCR methods to detect infections were not available.

One of the reasons for the low prevalence and low copy number was the relatively low temperatures during most of the year in Bahia Blanca. Moreover, the relatively low temperatures experienced by *A. longinaris* probably controlled some harmful effects of at least WSSV. For example, WSSV injected by Du et al. (2008) into the red swamp crayfish, *Procambarus clarkii* (Girard, 1852), and held at  $24 \pm 1^\circ\text{C}$ ,  $18 \pm 1^\circ\text{C}$ , and  $10 \pm 1^\circ\text{C}$  killed all of the first two groups by 9 and 22 d, respectively. Those at  $10^\circ\text{C}$  did not die by day 24. However, crayfish maintained at  $10^\circ\text{C}$  with a viral load too low to be detected by single-step PCR (Du et al., 2008) did not die until transferred to  $24^\circ\text{C}$ . The authors also demonstrated a reduced copy number per mg of gill tissue at the low temperature. When another freshwater species, the signal crayfish, *Pacifastacus leniusculus* (Dana, 1852), was fed or injected WSSV and maintained at  $4 \pm 2^\circ\text{C}$  or  $12 \pm 2^\circ\text{C}$ , none died, but all died at  $22 \pm 2^\circ\text{C}$  unless transferred to temperatures below  $16^\circ\text{C}$  (Jiravanichpaisal et al., 2004). Hemopoietic tissue proliferated at high temperatures, possibly supporting WSSV replication and explaining mortality. The penaeid kuruma shrimp, *Marsupenaeus japonicus* (Bate, 1888), when infected, reached only 60% cumulative mortality at day 19 when maintained at  $15^\circ\text{C}$  compared with 100% mortality at day 6 when maintained at  $23^\circ\text{C}$  (Guan et al., 2003). The total hemocytic count in those at  $15^\circ\text{C}$  tended to be less than that in uninfected controls at  $15^\circ\text{C}$  but significantly more than that in those shrimp maintained at  $23^\circ\text{C}$ ,  $28^\circ\text{C}$ , and  $33^\circ\text{C}$ . When *M. japonicus* was injected with a low dose of WSSV, it developed resistance within 3 or 4 wks and would survive a challenge for a month at  $24^\circ\text{C}$  as would a naïve shrimp given plasma from a resistant shrimp containing the neutralizing factor(s) (Wu et al., 2002). The range of mean surface temperature near Lighthouse Recalada in Bahia Blanca was reported as  $18.8\text{--}20.9^\circ\text{C}$  with a maximum of  $23.3^\circ\text{C}$  from the austral summer, December through March, but declined to  $6.9\text{--}8.8^\circ\text{C}$  with a minimum of  $4.0^\circ\text{C}$  during June to August (austral winter) (available from <http://www.ceado.gov.ar/Ef/bahiabla.asp>). Similar values were reported by Gayoso (1998). Consequently, the potential could be relatively high for morbidity and mortality in infected *A. longinaris*, the only common penaeid in the Bahia Blanca Estuary, during periods of high temperature, thereby posing a health risk to the local stock. When the temperature was low, development of infections was inhibited, resulting in eosinophilic material in the nuclei, and copy number probably decreased. The reason for mortality probably reflects a high copy number related to relatively high temperature. WSSV in the gazami crab, *Portunus trituberculatus* (Miers, 1876), off Korea in the Yellow Sea had a prevalence of WSSV of 97%, 95%, and 93% in April, June, and August, respectively, but 34% in late December when the winter temperature dropped to  $10/12^\circ\text{C}$  and when the copy number also decreased significantly (Meng et al., 2009).

Another reason for low copy numbers of WSSV may have involved induced resistance to WSSV resulting from pre-infection with IHNV. Tang et al. (2003a) showed that by infecting *L. stylirostris*, a highly susceptible penaeid species to fatal infections by IHNV, with IHNV that they demonstrated reduced mortality; when a high copy number of IHNV was present in a survivor, the corresponding copy number of WSSV was low. When *L. vannamei*, a species in which some IHNV-infected individuals became runted but none died, was experimentally pre-infected with IHNV at 28 °C, resistance to WSSV developed (Bonnichon et al., 2006). Mortality was delayed significantly, and WSSV decreased the copy number of IHNV. When WSSV infected the same species in culture, co-infection with IHNV made the shrimp's condition worse, suppressing a variety of its immunological defense mechanisms (Yeh et al., 2009). Responses by crustaceans to IHNV differ among species (Chayaburakul et al., 2005), and we noticed Cowdry Type A inclusions but no apparent runts. In our samples, seven of 23 specimens of *A. longinaris* were PCR-positive for IHNV. Of those, five had co-infections with WSSV and all but one had low copy numbers plus two had no detectable WSSV infection, suggesting that most shrimp could have been pre-infected with IHNV and were expressing increased resistance to replication of WSSV. The data were not significant to detect such a relationship using Fischer's exact test, but those data represented wild shrimp and the dose and origin of infection by neither virus was known. Perhaps some individual penaeids infected with WSSV only, during periods of high temperature, remained in the warm water and succumbed to infections. Moreover, other factors such as co-infection with species of *Vibrio* (e.g., Phuoc et al., 2009) and acute salinity stress (Joseph and Philip, 2007) can increase susceptibility to WSSV.

How and when WSSV and IHNV entered into Argentina and the Bahia Blanca Estuary are important questions. One possibility concerns entrance with introduced infected species on one or more occasions. For example, the palaemonid *P. macrodactylus* was recently introduced into Argentina (Spivak et al., 2006). Specimens were examined for WSSV previously from Mar del Plata Port by Martorelli and Fernandez (2005). Of 30 specimens collected bimonthly in 2003 and 2004, five exhibited white spots. Those shrimp neither exhibited histological signs of disease nor a positive reaction for the monoclonal-based commercial immunochromatographic detection assay for WSSV (Shrimple®) or IHNV. They were not then tested by PCR methods and cannot now be tested. However, we detected it in two of five recent samples in a low copy number detectable by qPCR but only one of those was determined to be positive using a two-step PCR method (WS500). Perhaps copy number diminished during periods of low temperature, and infections actually occurred in 2003 or earlier.

Ballast or bilge water from commercial cargo or fishing vessels could include WSSV-infected *P. macrodactylus* or highly susceptible larvae or adults of some other potential vectors for WSSV. The virus could also be introduced into Argentina as infected frozen seafood products, the implicated route by which WSSV was transported to the Americas (e.g., Nunan et al., 1998). Frozen penaeid products are not common in Argentina; however, SRM observed a small amount of *Farfantepenaeus paulensis* (Pérez-Farfante, 1967) originating from Brazil in a Chinese market in Buenos Aires in 2007.

Viral infections could also enter Argentina in live animals to be tested for aquaculture potential. For example, potential hosts such as *F. paulensis* imported from Brazil

were tested for culture in ponds in Samborombon Bay, Buenos Aires (Mallo and Galarza, 2002). Crustacean aquaculture in Argentina is practically nonexistent. Two freshwater decapods have been introduced for aquaculture, the palaemonid *Macrobrachium rosenbergii* (de Man, 1879) (giant river prawn) in the 1990s and more recently the parastacid *Cherax quadricarinatus* (von Martens, 1868) (Australian red claw crayfish), and both are potential vectors. The presence of white spots in 2003 and the difficulty in detecting low copy numbers of WSSV or any virus suggest that exotic aquatic viral infections in crustaceans could have been in Argentina for a few years.

Virus associated with penaeid aquaculture in Brazil presents several opportunities for introductions. Infections are now known from cultured penaeids as well as from animals in nearby habitats. For example, 40% of 5.6 million cultured individuals of *L. vannamei* died about 7 February 2005 at Aracati in the northeastern state of Ceara (4°33'41.76"S, 37°46'15.0"W) ([http://www.oie.int/eng/info/heβδο/AIS\\_58.htm](http://www.oie.int/eng/info/heβδο/AIS_58.htm)), which is located approximately 5500 km by water and 4550 km by land from Bahia Blanca Estuary. Shortly before that in November 2004, a loss reaching 90% of the same shrimp species occurred in large farms of Imarui Lagoon in Laguna, State of Santa Catarina, Southern Brazil (Cavalli et al., 2008). WSSV was not mentioned as detected by PCR in cultured shrimp from the University of Santa Catarina in a 2003 report (Toschi Maciel and Freire Marques, 2003), so the infections have apparently spread rapidly in Brazil. The latter location (38°46'S, 62°20'W) is about 1700 km from the Bahia Blanca Estuary. Three penaeids in natural waters of the Laguna Estuary near the farms, *F. paulensis* (São Paulo shrimp), *Farfantepenaeus brasiliensis* (Latreille, 1817) (redspotted shrimp), and *Litopenaeus schmitti* (Burkenroad, 1936) (southern white shrimp), collected in 2006 exhibited about a 3% prevalence of PCR-confirmed WSSV (Cavalli et al., 2008). The range of *A. longinaris*, Rio de Janeiro, Brazil, to North Patagonia in Chubut Province, Argentina, (Farfante and Kensley, 1997) overlaps that area now enzootic for WSSV, but no record exists for WSSV in that shrimp. Moreover, wild non-penaeid crustaceans such as some palaemonids, portunids, grapsoids, and even non-decapods can serve as vectors (e.g., Sánchez-Martínez et al., 2007). In December 2005, the marsh crab *Chasmagnathus granulata* Dana, 1851 (Grapsidae) in ponds from Santa Catarina had a 97% prevalence and in the nearby environment had a 23% prevalence of WSSV (Marques, 2008). The crab is an important species in Bahia Blanca. To date, no documented case of WSSV in any host has been reported from neighboring Paraguay, Uruguay, or Chile. IHNV in Brazil has been reported from *L. vannamei* in shrimp farms both in the State of Santa Catarina where adults had a prevalence of 54.8% of 124 shrimp by PCR and 65.1% of 129 shrimp by dot blot (ShrimProbe®-IHNV, DiagXotics, Inc.) (Moser, 2005) and in northeastern Brazil where it occurred in 9%–81% of *L. vannamei* in 26 ponds on seven farms (Braz et al., 2009).

The viruses could reach the Bahia Blanca Estuary in penaeid hosts, reservoir hosts, or mechanical vectors carried by natural currents. The Brazil Current transports 18–28 °C water southward along the coast of Brazil from 9°S to approximately 38°S until it forms an easterly confluence with the Malvinas (Falkland) Current flowing northward. During the austral summer and autumn, the latitude of the confluence is further south, and shifting wind systems allow for coastal interactions (Peterson and Stramma, 1991). Anomalous warm water extensions could bring with them infected animals, including plankton and parasitic copepods (Zhang et al., 2008; Esparza-

Leal et al., 2009; Overstreet et al., 2009). Also during spring-summer, onshore winds in the Rio de la Plata Estuary result in surface fresh water being pushed southwards along the Argentine coast (Guerrero et al., 1997). Not only is there a seasonal and annual fluctuation in currents, there is a fluctuation in prevalence of WSSV in cultured shrimp and wild animals in southern and northern Brazil (Cavalli et al., 2008; Marques, 2008; Braz et al., 2009).

The status of "white spots" on some animals remains uncertain. Based on multiple positive tests for WSSV in several individuals, the deposits in specimens of the penaeid *A. longinaris* not testing PCR-positive for WSSV likely represent a surviving shrimp from a prior WSSV infection in which the viral copy number was presently quite low or zero. Unless there are two different agents from the palaemonid *P. macrodactylus*, the cause of the deposition of the white spots in five of 30 specimens exhibiting spots in northern Argentina in 2003 and 2004 represented WSSV. That period was earlier than the date on which WSSV was first reported to kill shrimp in farm ponds in Brazil. The Shrimple kit when used at GCRL typically produced a positive result in some animals which could not be confirmed as WSSV with PCR; it rarely produced a false negative result. The white spots in the grapsoid *C. angulatus* appeared similar to those in the two other hosts, and all those that exhibited spots produced positive PCR as did the two negative crabs. On the other hand, bacterial white spot syndrome (BWSS) is similar in appearance and has been reported from *P. monodon* in culture (Wang et al., 2000). Four bacteria were isolated from lesions in this non-fatal BWSS disease, but the probiotic *Bacillus subtilis* was dominant. Some Argentine cases exhibiting white spots may have had bacterial associates, but we did not note evidence of such bacterial involvement in representative histological preparations. In a study using WSSV-infected *P. monodon*, Leu et al. (2007) showed that the infection strongly up-regulated gene expressions of various cuticular proteins, and they suggested that WSSV affects calcium ion balance and other basic metabolic processes. Without experimental studies to determine whether WSSV replication occurs in and can harm *C. angulatus* or *P. macrodactylus*, we do not know if they are good hosts or serve as reservoirs or vectors for infections in penaeid shrimp. Based on the lack of critical inspections of crustaceans for viruses in Argentina and the rapidity with which penaeid viruses spread, we expect native and other exotic crustacean viruses to be reported soon.

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