Evidence for cell apoptosis suppressing white spot syndrome virus replication in *Procambarus clarkii* at high temperature

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ABSTRACT: In shrimp, higher water temperatures (~32°C) can suppress the ability of white spot syndrome virus (WSSV) to replicate and cause mortality, but the mechanisms remain unclear. To investigate whether cell apoptosis might be involved, a Tdt-mediated dUTP nick-end label (TUNEL) method was used to assess levels of chromosomal DNA fragmentation in hepatopancreas and gill cells of *Procambarus clarkii* crayfish infected with WSSV and maintained at either 32 ± 1°C or 24 ± 1°C. Based on relative cell numbers with yellow-green colored TUNEL-positive nuclei, the apoptotic index was elevated in WSSV-infected crayfish maintained at 32°C. In gill tissue sections examined by transmission electron microscope, cells with nuclei displaying apoptotic bodies or marginated, condensed and fragmented chromatin without concurrent cell cytoplasm damage were also more prevalent. Flow cytometry sorting of annexin-stained cells showed apoptosis to be most prevalent in granular haemocytes, and assays for caspase-3 activity showed it to be most elevated in hepatopancreas tissue. Despite these indicators of cell apoptosis but consistent with WSSV replication being restricted at elevated temperatures, no increases in transcription of the viral anti-apoptosis genes *ORF390* and *ORF222* were detected by RT-PCR in shrimp maintained at 32°C, possibly due to the elevated levels of cellular apoptosis.

KEY WORDS: WSSV · Crayfish · TUNEL

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INTRODUCTION

Since it first emerged in the early 1990s, white spot disease (WSD) has spread to severely impact aquaculture of penaeid shrimp and other crustaceans in most regions of the world (Lightner 1996). WSD is caused by white spot syndrome virus (WSSV), an enveloped rod-shaped virus containing a large (~300 kb) double-stranded DNA (dsDNA) genome classified in the genus *Whispovirus* of the family *Nimaviridae* (Vlak 2004), and can result in complete losses of shrimp in farm ponds within 3 to 10 d of gross signs becoming evident (Wang et al. 1998). However, WSD outbreaks tend to occur less frequently during warm seasons (Withyachumnarn-

kul et al. 2003). Bioassays at various water temperatures have indicated that while 24 to 28°C is ideal for WSSV to replicate and induce mortality (Vidal et al. 2001, Du et al. 2006, You et al. 2010), its replication and thus ability to cause mortality in either shrimp (Vidal et al. 2001) or crayfish (Du et al. 2006, Gao et al. 2011) is suppressed at temperatures above ~32°C. Shrimp cultivation during summer months is now accepted widely as a practical means of reducing WSD impact. It has been suggested that this protective effect is not due exclusively to temperature-mediated inhibition of viral replication (Vidal et al. 2001, Jiravanichpaisal et al. 2004); however, the mechanisms involved have yet to be elucidated.

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Apoptosis is a distinctive mechanism of programmed cell death that can be triggered in response to cell invasion by viruses (O'Brien 1998), thus limiting virus shedding and spread (Barber 2001), and is the main adaptive anti-viral response mounted by terrestrial invertebrates (Koyama et al. 2000). Cell apoptosis has been suggested as a possible mechanism by which *Litopenaeus vannamei* shrimp survive WSSV infection when maintained at 32°C (Granja et al. 2003). Thus, to investigate this possibility further, approaches to detecting and quantifying cell apoptosis levels including the Tdt-mediated dUTP nick-end label (TUNEL) assay, a caspase-3 activity assay, flow cytometry of annexin-stained haemocytes as well as transmission electron microscopy (TEM) were undertaken on crayfish *Procambarus clarkii* acclimated to moderate (24°C) or high (32°C) water temperatures before challenge with WSSV.

MATERIALS AND METHODS

Virus

The WSSV stain used originated from wild *Penaeus chinensis* collected from the East China Sea in 2001. Using a homogenate of gill tissue from experimentally infected *Procambarus clarkii*, a semi-purified virus inoculum was prepared using the differential centrifugation method described previously (Du et al. 2007) and stored at –80°C.

Crayfish

Adult *Procambarus clarkii* (15–20 g, 8–10 cm long) were purchased from Hangzhou, China, and maintained in 40 l tanks (100 × 50 × 55 cm) containing sand-filtered, ozone-treated, flow-through freshwater. Crayfish were fed with commercial feed pellets at 5 % of body weight per day. When being acclimated at 24 ± 1°C for a week, a small pleopod piece was excised from each crayfish to confirm absence of WSSV by PCR as described previously (Du et al. 2006).

Experimental design

Crayfish were acclimated for 3 d at either 24 ± 1°C or 32 ± 1°C. At each temperature, 2 groups of 30 *Procambarus clarkii* were infected intra-muscularly with 0.1 ml WSSV inoculum per crayfish. A control group of crayfish injected with 0.1 ml 330 mM NaCl was also

maintained at 32 ± 1°C. At various times (0, 24, 48, 60, 72, and 96 h) post-infection (hpi), 5 crayfish were sacrificed at random from each group for analysis.

PCR and real-time quantitative PCR

DNA was extracted from gill tissue using the Viral DNA Extracted Kit (Sangon) according to the manufacturer's instructions. DNA pooled from 3 crayfish at each time point was amplified by PCR using the WSSV 334F/R primers as described previously (Du et al. 2006) and PCR products were analyzed in 2 % agarose gels.

Real-time quantitative PCR (qPCR) based on the 69F/R primers described previously (Durand & Lightner 2002) was used to amplify and quantify WSSV DNA relative to a 69 bp plasmid DNA internal standard. Plasmid DNA was purified and quantified to calculate DNA copy number. Standard curves were constructed from qPCR data on 10-fold serial dilutions containing 10⁸ to 10 plasmid DNA copies. The qPCR used the Step One plus Real-time PCR System (ABI Applied Biosystems) and Bio-Easy SYBR Green Kits (Bioer). WSSV DNA copies in any sample were determined from the standard curve.

TUNEL assay

Gill and hepatopancreas tissues preserved in Davidson's fixative were prepared into paraffin wax and sectioned using standard methods (Lightner 1996). Apoptotic cells were detected using an *In Situ* Cell Death Detection TUNEL Assay Kit (Roche) as described in the manufacturer's protocol. Tissue sections were counterstained using a Cell Apoptosis Propidium Iodide (PI) Detection Kit (Keygen) and observed immediately by fluorescence microscopy using a green filter to detect DNA fragmentation (TUNEL-positive cells) and a red filter to highlight the cell nucleus. An apoptotic index (AI) was calculated as the percentage of TUNEL-positive nuclei per 500 cells across an ocular grid at 400× magnification. Positive controls comprised tissues from uninfected crayfish digested with DNase I to fragment nuclear DNA before processing. Negative controls comprised normal tissues treated identically but without addition of TdT enzyme.

Transmission electron microscopy

Gill tissues of WSSV-infected crayfish were fixed in 2.5 % glutaraldehyde at 4°C for 24 h, post-fixed

in 1% osmium tetroxide, dehydrated through graded ethanol concentrations and embedded in Aradite-502 resin. Ultrathin sections (60 to 90 nm) of silver-gray interference were collected on copper grids, stained with uranyl acetate/lead citrate and viewed using a Hitachi H-300 electron microscope.

Flow cytometry

Apoptotic cells in haemolymph were labeled using the fluorescein EGFP-Annexin V Apoptosis Detection Kit (Keygen) following the manufacture's protocol and quantified using a standard fluorescence-activated cell analyzer (FACScalibur, Becton Dickinson). EGFP-Annexin V labels phosphatidylserine relocated from inner to outer cell membranes at the early apoptotic stage, and PI was used to stain cellular DNA. Data were expressed as 2-dimensional dot plots and contour plots. For each haemolymph sample, 5000 haemocytes were acquired for analysis and data presented as a cell cytogram of low-angle forward-scattered (FSC) and right-angle side-scattered (SSC) laser light in linear mode (Cellquest Software). The granular cells indicative of WSSV infection stained with both EGFP-Annexin V and PI, and were analyzed as histograms at green fluorescence (FL1) and red fluorescence (FL2) heights in linear mode (Cellquest Software).

Caspase-3 activity assay

Caspase-3 activities in crayfish hepatopancreas tissues were measured using a colorimetric Caspase-3 Assay Kit (Keygen) as described in the manufacturer's protocols. Briefly, tissues were disrupted in Lysis buffer, the protein concentration adjusted to $2 \mu\text{g } \mu\text{l}^{-1}$ and 3 replicate 50 μl amounts were added to a equal volume of reaction buffer before determining optical density (OD) as absorbance with a wavelength of 405 nm ($A_{405\text{nm}}$) using a Multiskan Spectrum spectrophotometer (Molecular Devices). Caspase-3 activity was expressed as $(\text{OD}_{\text{infected}} - \text{OD}_{\text{blank}}) / (\text{OD}_{\text{control}} - \text{OD}_{\text{blank}})$.

Semi-quantitative RT-PCR

Total RNA was extracted from gills using TRIzol reagent (Invitrogen), purified by RNAClean RNA Kit (Aidlab) and reverse transcribed using the First-

Strand cDNA Synthesis kit (Fermentas). cDNA pooled from 3 crayfish at each time point (0, 24, 48, 72 and 96 hpi) was amplified by PCR using primers targeted to 2 anti-apoptosis genes: the *ORF390* gene (F, 5'-TAA GCG GGA AAG AGT AAA-3'; R, 5'-TAG GGT GAT GAG GGT C-3') and *ORF222* gene (F, 5'-CTA GCC AAC ACC TAT CCG-3'; R, 5'-TGA GTT CAA GGG CAG ATT-3') (Wang et al. 2004, Fang et al. 2006). As a control for qualification, host 18S ribosomal RNA was also amplified using PCR primers (F, 5'-GAC CAG AGC GAA AGC ATT-3'; R, 5'-TCC ACC AAC TAA GAA CGG-3').

Statistical analysis

All measurements were made in duplicate. The data were analyzed using 1-way ANOVA in SPSS 16.0. Differences were considered as significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Prior to injecting *Procambarus clarkii* crayfish with WSSV, the absence of pre-existing infection was confirmed by PCR analysis. At the first sampling point after challenge (24 hpi), WSSV was detected by both PCR and qPCR using DNA isolated from gill tissues of crayfish held in both 24°C (ambient) and 32°C (high) temperature water (Fig. 1A). In crayfish maintained at 24°C, WSSV DNA copy numbers quantified by qPCR increased ~1000-fold (2.3×10^4 to 2.2×10^7) between 24 and 96 hpi (Fig. 1B). In crayfish maintained at 32°C, however, WSSV DNA copy numbers reduced from 1.0×10^4 to 1.4×10^3 between 24 and 60 hpi, after which they remained stable (Fig. 1B). Non-progression of WSSV infection in crayfish maintained at 32°C is consistent with previous reports of elevated temperatures hindering replication (Du et al. 2006, Granja et al. 2006, Rahman et al. 2006, Gao et al. 2011).

To examine for DNA fragmentation and morphological changes indicative of cell apoptosis, crayfish tissues were examined using a TUNEL assay and TEM, respectively. In gill and hepatopancreas tissues, no TUNEL-positive cells were detected in uninfected *Procambarus clarkii* except when tissue was digested with DNase I to purposely fragment DNA as a control (Fig. 2A). In tissues of WSSV-infected crayfish sampled at 48 and maintained at 24°C, there were scattered apoptotic cells, but among those maintained at 32°C, yellow-green colored TUNEL-

Fig. 1. White spot syndrome virus (WSSV) detection in *Procambarus clarkii* at 24°C and 32°C. (A) One-step PCR amplification of WSSV DNA from gills of crayfish maintained at either 24°C or 32°C and sampled before and after 24 h acclimation at 32°C or sampled immediately before WSSV infection or between 24 and 96 h post-infection (hpi) with WSSV. M = DNA marker. (B) WSSV DNA quantification by real-time quantitative PCR. Data are mean + SD of $\log_{10}$ real-time PCR units (rtPCRU) from 2 replicate tests of DNA pooled from 3 crayfish. *Statistically significant differences ($p < 0.05$) between WSSV replication rates at the 2 incubation temperatures

positive nuclei were ubiquitous (Fig. 2A), consistent with increased rates of apoptosis. From 48 to 96 hpi, AI values (% TUNEL-positive nuclei per 500 cells) were elevated significantly ($p < 0.05$) across both gill and hepatopancreas tissues of WSSV-infected crayfish maintained at 32°C compared to 24°C (Fig. 2B). In addition, at both 32°C and 24°C, the AI peaked earlier and at higher levels in gills at 48 hpi (27.7% and 19.2%) than in hepatopancreas at 60 hpi (15.2% and 10.4%) before declining to similar levels at 72 hpi at both temperatures (Fig. 2B). This more pronounced increase and then degradation of apoptotic cells in *P. clarkii* maintained at 32°C provides clues as to how WSSV replication and cell-cell spread is precluded at elevated temperatures.

In *Procambarus clarkii* maintained at 24°C and sampled prior to WSSV infection, TEM showed gill cells to contain normal nuclei with heterochromatin in peripheral patches and as a central mass (Fig. 3A). At 72 hpi with WSSV, however, gill cell nuclei displayed dense ring-like zones containing marginated chromatin (Fig. 3B,C,D). Enveloped WSSV particles

were first observed at 48 hpi (Fig. 3C), and at 96 hpi, some enlarged nuclei displayed dense marginated chromatin around a central zone containing rod-like viral particles as well as ruptured nuclear membranes (Fig. 3E). In contrast to the crayfish maintained at 24°C, the nuclei of gill cells of those acclimated at 32°C for 3 d prior to WSSV challenge showed a broad zone of dense and marginated chromatin (Fig. 4A), suggesting that exposure to this temperature alone promoted apoptosis. In crayfish sampled over time following WSSV challenge, nuclear chromatin was observed to condense and fragment, with disruption of presumed nucleolar structures (Fig. 4B,C,D). Apoptotic bodies containing nuclear material were also frequently observed in intercellular spaces (Fig. 4E). In addition, no WSSV particles were ever observed in cells undergoing apoptotic changes (Fig. 4), confirming the inability of WSSV to replicate in such cells.

While the TUNEL assay and TEM reliably identify individual apoptotic cells, they provide only qualitative data on cell apoptosis levels. A flow cytometry (FCM) method based on reduced DNA staining by PI and cell shrinkage was thus used to quantify cells undergoing apoptosis (Sahul Hameed et al. 2006). Of the 3 haemocyte types in crayfish, only semi-granular and granular cells can be infected by WSSV (Wang et al. 2002), and granular cells play an important role in the innate immune system of shrimp (Söderhäll & Cerenius 1998). FCM analysis of cell morphology and cell granularity using FSC and SSC light scattering, respectively, was used to distinguish viable cells not binding to either PI or EGFP-Annexin-V, necrotic cells binding both EGFP-Annexin-V and PI, apoptotic cells not binding EGFP-Annexin-V but binding PI, as well as naked nuclei to estimate apoptosis levels in granular cells (Fig. 5Ai). Using these methods, apoptotic cells could be separated and quantified in linear histograms comparing green fluorescence (FL1) and red fluorescence (FL2) heights (Fig. 5Aii). In WSSV-infected crayfish, the FCM data confirmed TUNEL and TEM data, indicating that apoptosis of granular haemocytes was more prevalent in crayfish maintained at 32°C than at 24°C (Fig. 5B) and increased with time post-challenge (Fig. 5B), which supports evidence on WSSV infection triggering cell apoptosis in penaeid shrimp (Sahul Hameed et al. 2006).

As cleavage of many key proteins by caspase-3 is critical for cell apoptosis (Gerald 1997), caspase-3 activity was also investigated in hepatopancreas cells. In *Procambarus clarkii* acclimated at 32°C compared to 24°C, activity was significantly higher ($p \leq$

Fig. 2. (A) Fluorescence microscopy of contiguous sections of Tdt-mediated dUTP nick-end label (TUNEL)-stained hepatopancreas and gill tissue of WSSV-infected *Procambarus clarkii* maintained at either 24°C or 32°C. Positive (control): DNase I-treated tissues showing nuclei displaying yellow-green fluorescence typical of TUNEL staining (scale bars = 100 µm); negative (control): untreated tissues showing only the red fluorescent counterstain (scale bars = 100 µm); +WSSV: WSSV-infected crayfish, sampled at 48 h post-infection (hpi) (scale bars = 50 µm). (B) Apoptosis index (% apoptotic cells, mean ± SEM), calculated from numbers of TUNEL-positive cells detected in hepatopancreas and gill tissue from WSSV-infected crayfish maintained at either 24°C or 32°C

0.01) both pre-challenge and at all times post-infection with WSSV. Activity levels at both temperatures peaked between 60 and 72 hpi before returning to more normal levels at 96 hpi (Fig. 6).

RT-PCR tracking of mRNA expression levels of the anti-apoptosis genes *ORF390* and *ORF222* showed both to increase, although more so in crayfish maintained at 24°C than at 32°C (Fig. 7). In addition, while

expression levels continued to increase (*ORF390*) or to stabilize (*ORF222*) from 48 hpi onwards in crayfish maintained at 24°C, decreases in both were evident at 96 hpi in crayfish maintained at 32°C. These data provided no new information on whether elevated temperature itself and/or elevated cell apoptosis as a result of crayfish being maintained at 32°C restricted WSSV replication.

Fig. 3. Transmission electron micrographs of gills of WSSV-infected *Procambarus clarkii* maintained at 24°C. (A) 0 h post-infection (hpi): normal nuclei (n); (B) 24 hpi: nucleus showing dense ring-like zone (rz) containing margined chromatin; (C) 48 hpi: enlarged nucleus showing a dense rz and a small number of viral envelopes and particles (vp); (D) 72 hpi: enlarged nucleus with thin rz and many vp; (E) 96 hpi: damaged nucleus containing high density of vp; thin rz containing little margined chromatin with vp appearing to be released from the nucleus. Scale bars = 1 μ m

Fig. 4. Transmission electron micrographs of gills of WSSV-infected *Procambarus clarkii* maintained at 32°C. (A) 0 h post-infection (hpi): slightly enlarged nucleus with chromatin margination; (B) 24 hpi: enlarged nucleus showing a dense ring-like zone (rz) or margined chromatin; (C) 48 hpi: enlarged nucleus showing a dense rz or margined chromatin without viral particles (vp) in the central region; (D) 72 hpi: thin rz containing little margined chromatin but no vp observed in the nucleus; (E) 96 hpi: apoptotic bodies (a) containing nuclear fragments in intercellular spaces in gill cells and increased cytoplasmic vacuolization around nucleus. Scale bars = 1 μ m

Fig. 5. Flow cytometry analysis of apoptotic granular haemocytes in WSSV-infected *Procambarus clarkii* at 24°C and 32°C. (Ai) Dot plot analysis of haemocytes at 32°C stained with EGFP-Annexin-V and propidium iodide (PI) comparing forward scatter (FSC), which measures relative cell size, with side scatter (SSC), which measures cell granularity. For apoptosis analysis, granular cells were gated. (Aii) Dot plot analysis at 32°C showing viable cells not binding to either PI or EGFP-Annexin-V (lower left quadrant), necrotic cells binding both EGFP-Annexin-V and PI (upper right quadrant) and apoptotic cells not binding EGFP-Annexin-V but binding PI (lower right quadrant) with green fluorescence (FL1) or red fluorescence (FL2). (B) Percentage apoptotic granular cells (mean $\pm$ SEM) determined by flow cytometry analysis of haemocytes collected from *Procambarus clarkii* following WSSV infection (+WSSV). *Statistically significant differences ($p < 0.05$) among results from control (uninfected) and the 2 incubation temperatures

Fig. 6. Caspase-3 activity (mean $\pm$ SEM) in hepatopancreas tissue of *Procambarus clarkii* maintained at 24°C or 32°C sampled following WSSV infection, expressed as a measure of optical density (OD) at 440 nm, and calculated as $(OD_{\text{experiment}} - OD_{\text{blank}}) / (OD_{\text{control}} - OD_{\text{blank}})$. *Statistically significant differences ($p < 0.01$) among results from control (uninfected) and the 2 incubation temperatures

In WSSV-infected penaeid shrimp, progressive increases in apoptotic cells associated with disease severity has been implicated as a factor contributing to organ failure, morbidity and ultimately to death (Sahtout et al. 2001, Khanobdee et al. 2002). In the

Fig. 7. RT-PCR amplification of *ORF390* and *ORF222* mRNA in gills of *Procambarus clarkii* maintained at 24°C and 32°C and sampled following WSSV infection. As a control, 18S rRNA was amplified by RT-PCR from the same RNA samples

Procambarus clarkii examined here, acclimation to 32°C water for 3 d resulted in increased levels of cellular apoptosis compared to crayfish left in ambient temperature (24°C) water, and WSSV challenge increased apoptosis rates more in the crayfish maintained at 32°C. As WSSV infection loads only increased briefly to peak at 24 hpi in crayfish maintained at 32°C, priming for elevated cellular apoptosis might be important in restricting WSSV replication and intracellular spread. This supposition is supported by findings in insect cells infected by *Autographa californica* nuclear polyhedrosis virus (AcMNPV), where apoptosis induced by virus mutants lacking a functional p35 gene dramatically

decreased yields of progeny virus (Clem & Miller 1993). Based on this observation, it was suggested that cell suicide was the likely mechanism preventing virus from multiplying and thus infecting naïve cells. The premature demise of a virus-infected cell, curtailing the ability of a virus to multiply and spread, is generally accepted as a pivotal antiviral defense mechanism (Flint et al. 2000).

In vertebrates, physiological stress caused by exposure to elevated temperatures alone is sufficient to induce cellular apoptosis (Ashish et al. 2009, Wang et al. 2009) or to suppress viral replication (Conti et al. 1999). Data reported here in *Procambarus clarkii* crayfish are consistent with these observations and shed additional light on the possible reasons why WSSV replication does not progress to cause acute disease and mortality at 32°C (Du et al. 2006). However, while elevated cellular apoptosis correlates well with virus suppression to low levels easily tolerated by the crayfish, as WSSV-infected crayfish maintained at 24°C also displayed evidence of apoptosis, priming, apoptosis levels or other as yet to be discovered virus-specific processes also seem likely to be involved.

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