Immunomodulatory effects of hyperthermia on resisting WSSV infection in *Procambarus clarkii*

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Abstract

White spot disease remains a constant threat to aquaculture worldwide. Hyperthermia has been shown to reduce mortality in white spot syndrome virus (WSSV)-infected shrimps, but the mechanism still remains unclear. In this study, we sought to identify host immune factors that contribute to inhibition of WSSV infection during hyperthermia. In WSSV-infected red swamp crayfish *Procambarus clarkii* (Girard) cultured at 24 ± 1 °C, transcriptional levels of the heat shock protein 70 (Hsp70) gene showed a modest, 2.2-fold increase in haemocytes following 48 h post-infection (hpi). In contrast, in WSSV-infected crayfish cultured at 32 ± 1 °C, Hsp70 gene expression showed a rapid, 19.5-fold induction by 4 hpi. This suggests that Hsp70 plays a positive regulatory role in resistance to WSSV infection during hyperthermia. Furthermore, total haemocyte counts (THC) and phenoloxidase (PO) activity were both significantly increased in WSSV-infected crayfish cultured at 32 ± 1 °C by 48 hpi. Both may be critical for crayfish survival in the late stages of WSSV infection. Collectively, the up-regulation of host protein Hsp70 expression and increase in THC and PO activity suggest that hyperthermia has immunomodulatory effect that enhanced the resistance of *P. clarkii* to WSSV infection.

Keywords: Hsp70, hyperthermia, immune response, *Procambarus clarkii*, white spot syndrome virus.

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Introduction

White spot syndrome virus (WSSV) is one of the most serious viral pathogens threatening shrimp aquaculture worldwide. WSSV outbreaks cause up to 100% cumulative mortality within 3–10 days after the onset and have devastating effects on the shrimp culture industry (Chou *et al.* 1995; Wang *et al.* 1998; Lightner 2011). As a new member of the genus *Whispovirus* of the family *Nimaviridae* (Vlak 2004), WSSV has a wide host range, which includes shrimp, crayfish, crabs, lobsters and copepods (Lo *et al.* 1996). Hence, it is also regarded as a major threat to marine environments.

Temperature is one of the most important environmental factors in shrimp. It can influence the metabolism, oxygen consumption, growth rate, moult cycle and survival rate directly. Outbreaks of WSSV occur less frequently during warm seasons, and there is evidence suggesting that hyperthermia protects shrimps from WSSV infection (Withyachumnarnkul *et al.* 2003). Nowadays, warm water for shrimp cultivation has generally been accepted as beneficial and is already applied in some commercial shrimp farms. However, the exact mechanisms of hyperthermia inhibiting WSSV infection are still unknown. Previous studies proposed that hyperthermia could interfere with the WSSV lifecycle inhibiting WSSV replication (Du *et al.* 2006) or reducing the viral load (Granja *et al.* 2006). In the host, increases in cellular apoptosis may contribute to the suppression of WSSV replication (Wu *et al.* 2012). Hyperthermia has also been shown to inhibit vesicular stomatitis virus (Demarco & Santoro 1993), Mayaro virus (Virgilio, Motta & da Gloria 1997) and rhinovirus (Conti *et al.* 1999) replication in mammalian cells. An abrupt short-term or long-term heat shock has shown to increase Hsp70

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expression with regard to microbial tolerance in *Penaeus monodon* (de la Vega *et al.* 2006) and *Artemia* (Sung *et al.* 2007). Induction of heat shock protein 70 (Hsp70) has been suggested as a key step in the inhibition of viral replication (Demarco & Santoro 1993; Virgilio *et al.* 1997; Conti *et al.* 1999). In this study, we attempted to identify other host immune factors especially Hsp70 that play a role in mediating resistance to WSSV as well under hyperthermic conditions. Using red swamp crayfish, *Procambarus clarkii* (Girard), as an infection model, we first examined the survival rate and viral load of WSSV-infected crayfish after hyperthermic treatment. Then, we measured changes of expression of Hsp70, total haemocyte counts (THC) and phenoloxidase (PO) activity.

Materials and methods

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Procambarus clarkii (15–20 g) were purchased from Hangzhou (Eastern China) and maintained in 40 L tanks (100 × 50 × 55 cm) containing sand-filtered, ozone-treated, flow-through fresh water. Crayfish were fed with commercial feed pellets at 5% of body weight per day. The WSSV strain used originated from wild *Penaeus chinensis* (Osbeck) collected from the East China Sea in 2001. The virus inoculum was semipurified from gill tissues of experimentally infected *P. clarkii* by differential centrifugation (Du *et al.* 2007). Prior to WSSV challenge, crayfish were confirmed to be free of WSSV by PCR analysis (Du *et al.* 2006) and acclimated to the desired temperatures (24 ± 1 and 32 ± 1 °C) for 1 week. For challenge tests, two groups of 24 crayfish were injected intramuscularly with 0.1 mL of WSSV inoculum (1×10^7 copies mL^{-1}). At various times (0, 4, 8, 16, 24, 48, 72 and 96 h) after infection, three crayfish were removed at random from each group and subjected to analysis. To monitor WSSV survival, two additional groups of crayfish were challenged with WSSV, while control crayfish were injected with 0.1 mL of 330 mM NaCl. The number of dead crayfish was recorded, and freshly dead crayfish were tested for WSSV by PCR (Du *et al.* 2006).

D m C

White spot syndrome virus was detected by quantitative real-time PCR (qPCR). DNA was extracted from the gills of three crayfish at each time point using the Viral DNA Extracted Kit (Sangon) according to the manufacturer's instructions. qPCR amplification and quantification of WSSV DNA in comparison with a 69 bp plasmid DNA internal standard was performed using WSSV69F/R primers (Table 1) (Durand & Lightner 2002). Plasmid DNA was purified and quantified to calculate DNA copy number. Standard curves were constructed using qPCRs undertaken using 10-fold serial dilutions ranging from 10^8 to 10 plasmid DNA copies. qPCR utilized the Step One plus Real-time PCR System (ABI Applied Biosystem) and Bio-Easy SYBR Green Kits (Bioer). The number of WSSV DNA copies in any sample was determined from standard curves.

H m

Gills 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 (60–90 nm) of silver-grey interference were collected on copper grids, stained with uranyl acetate/lead citrate and viewed using a Hitachi H-300 transmission electron microscope (TEM).

A H 70

Total RNA extraction was performed using TRIzol Reagent (Invitrogen), and first strand cDNA was synthesized according to the RevertAid TM H Minus First Strand cDNA Synthesis Kit (Fermentas). The abundance of Hsp70 transcripts was measured by real-time quantitative reverse transcription PCR. The reactions were carried out in a Step One plus Real-time PCR System (ABI Applied Biosystem)

T 1 Primers used for real-time PCR

Primer	Sequence (5′–3′)	Amplicon size (bp)
WSSV334 F	CTTTCACTCTTTCGGTCGTG	334
WSSV334 R	TTCTGCCCCACAGTCACTTC	
WSSV69 F	TGGTCCCGTCCTCATCTCAG	69
WSSV69 R	GCTGCCTTGCCGGA AATTA	
Hsp70 F	TGGGTTGATTGATTTGTTGAGTT	151
Hsp70 R	CCAGTGGAGAAGGCTTTGAG	
18sRNA F	TGGTGCATGGCCGTTCTTA	100
18sRNA R	AATTGCTGGAGATCCGTCGAC	

WSSV, white spot syndrome virus.

using Maxima™ SYBR Green qPCR Master Mix (Sangon) with primer pairs for Hsp70 (Table 1). Host 18sRNA was used as an internal control. The expression of Hsp70 genes was calculated as fold expression relative to 18sRNA according to the $2^{-\Delta\Delta C_T}$ methods (Livak & Schmittgen 2001). For Western blot analysis, tissues of gill, hepatopancreas, muscle and eyestalk of collected crayfish ($n = 3$) were ground, respectively, in liquid nitrogen and homogenized with PBS containing a proteinase inhibitor cocktail (Merck). The total protein was quantified using Bradford reagents (Bio-Rad), and equal amounts of protein were loaded on SDS-PAGE gels enabling direct comparison between different samples in Western blot. Rabbit polyclonal anti-Hsp70 (Cayman) and peroxidase-conjugated goat anti-rabbit polyclonal antibodies (Sigma) were used for immunoblotting.

m mm

The haemolymph was withdrawn from the ventral sinus of each sampled crayfish with a 1.5-mL sterile syringe fitted with 25-gauge needle and divided into two aliquots. An aliquot was thoroughly mixed with a 0.1-mL anticoagulant solution (26 mM citrate, 30 mM sodium citrate, 0.45 M sodium chloride, 10 mM EDTA, 0.1 M glucose, pH 7.6) and used for THC. THC was counted manually by hemocytometer under phase contrast microscope (Moullac *et al.* 1997). Another aliquot was centrifuged for 5 min at 4000 g at 4 °C to obtain plasma for PO analysis. PO activity was spectrophotometrically analysed by recording the formation of dopachrome (Hernandez-Lopez, Gollas-Galvan & Vargas-Albores 1996). The L-3,4-dihydroxyphenylalanine (L-DOPA; Sigma) and trypsin (Sigma) were served as a substrate and an elicitor, respectively.

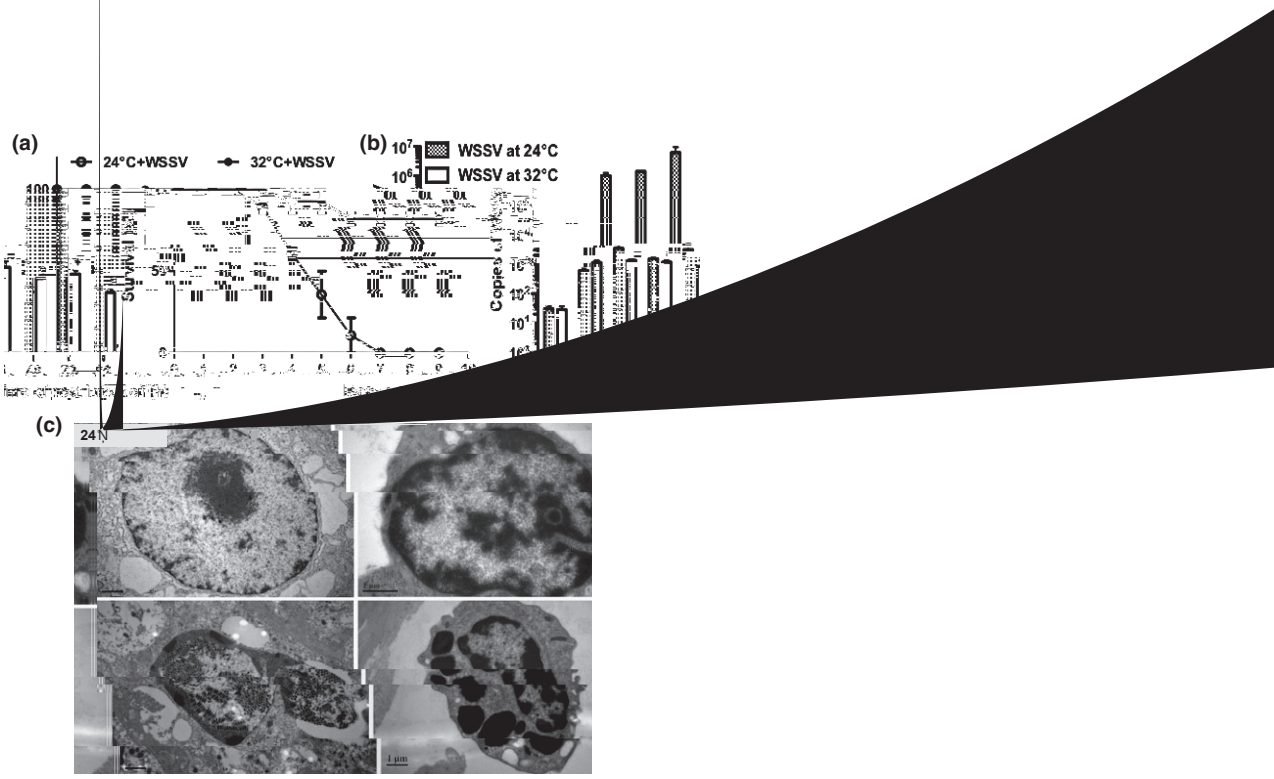
All measurements were taken in triplicate. The data were analysed using one-way ANOVA in SPSS software (IBM). Differences were considered as significant at $P \leq 0.05$.

Results

m

At 24 ± 1 °C, crayfish showed no survival at 7 days post-infection (dpi) of WSSV. However, under hyperthermic conditions, where crayfish were maintained at 32 ± 1 °C, the mean survival at 7 dpi was 80% (Fig. 1a). Under both conditions, 100% randomly sampled dead crayfish were WSSV-positive by PCR test (data not shown). This confirmed that hyperthermia increases survival of crayfish exposed to WSSV.

In crayfish maintained at 24 ± 1 °C, WSSV DNA copy numbers increased ~10^4 fold (6.1×10^2 to 6.2×10^6) between 4 and 96 h post-infection (hpi). In crayfish maintained at 32 ± 1 °C, a similar initial increase in WSSV was detected at 4 hpi (1.1×10^3). However, tgt513.fica(a)9(n)15.in(a)21.7(ai)17l7(,)-3



hepatopancreas and gills (38.4-, 5.2- and 3.1-fold of induction relative to control at 24 ± 1 °C, respectively) by 48 hpi, while it remained relatively similar in haemocyte (Fig. 3a). However, at 24 ± 1 °C, Hsp70 transcripts of WSSV-challenged crayfish were induced in gill and hepatopancreas with 3.6- and 2.2-fold increase relative to unchallenged crayfish, respectively (Fig. 3b). Furthermore, Hsp70 transcript of WSSV-infected crayfish maintained at 32 ± 1 °C was significant induced in most tissues, except in eyestalk. The transcript was the most induced in muscle with a 75.4-fold increase relative to infected crayfish maintained at 24 ± 1 °C (Fig. 3c). Western blot analysis confirmed that Hsp70 protein was induced in gill and hepatopancreas after WSSV infection and increased more significantly in muscle after hyperthermic treatment (Fig. 3d).

mm **m**

The THC for WSSV-infected crayfish maintained at 32 ± 1 °C was increased from 5.6×10^6 to 1.5×10^7 cells mL^{-1}, while it was decreased from 5.3×10^6 to 1.8×10^6 cells mL^{-1} at 24 ± 1 °C (Fig. 4a). It was significantly induced at 72 and 96 hpi with 3.9- and 8.6-fold increase, respectively (Fig. 4a). The PO activity in WSSV-infected crayfish maintained at 32 ± 1 °C was higher than that of crayfish maintained at 24 ± 1 °C at 48 and 72 hpi (1.5- and 1.6-fold, respectively) (Fig. 4b).

Discussion

Upon exposure to altered temperatures for prolonged periods, many animals adapt physiologically and biochemically. This is termed thermal acclimatization (Horowitz 2010). In some circumstances, hyperthermia is considered as a sign of acute inflammatory response triggered by the body as a part of host defence mechanism. Present challenge study confirmed that hyperthermia could increase WSSV-infected crayfish survival rate, inhibit WSSV replication and reduce viral load (Fig. 1).

Heat shock proteins (HSPs) are key induced indicators of hyperthermia and comprise a group of highly conserved proteins that have general protective functions against pathogens in all living organisms. In addition to serving essential functions as molecular chaperones, HSPs also participate in immunological processes in mammals (Srivastava 2002). In aquatic animals, Hsp70 has been shown to play an important role in relation to the host response to environmental pollutants, food toxins and bacterial or viral infections (Roberts *et al.* 2010; Loc *et al.* 2013). In the present study, hyperthermia or WSSV infection could

both induce the level of Hsp70 transcripts, but the former was much more significant and prompt. The Hsp70 was easily induced in hyperthermia and had a high expression level (Fig. 2). Interestingly, after WSSV infection at 24 ± 1 °C,

expression even after WSSV infection, which suggested that high level of Hsp70 might be related to fighting against WSSV infection. A previous study also showed that Hsp70 knockdown shrimps became severely infected at 32 °C (Lin *et al.* 2011). Therefore, the up-regulation of Hsp70 by hyperthermia strongly suggests that it may play a role in mounting an immune response to WSSV infection. Hsp70 serves as a danger signal to activate macrophages and has the ability to induce several cytokines and the costimulators of the adaptive immune response in mammals (Moseley 2000; Srivastava 2002; Pockley 2003), but further studies will be needed to figure out the exact role of Hsp70 in invertebrate animals.

Generally, invertebrates are believed to depend entirely on non-specific innate immunity, mediated primarily by haemocytes. The THC of crustaceans decreases significantly after infection with pathogenic bacteria (Hauton, Williams & Hawkins 1997), fungi (Goarant & Boglio 2000) and WSSV (Van de Braak *et al.* 2002; Wang & Zhang 2008; Fu *et al.* 2010). Decreases in THC in WSSV pathology are most likely due to the infection of a large proportion of haemocytes, especially semigranular cells (Jiravanichpaisal *et al.* 2006). This results in a reduction of haemocytes in circulation. In present study, the THC for WSSV-infected crayfish maintained at 32 ± 1 °C was observed to increase after 72 hpi (Fig. 4a). This increase may be due to hyperthermia protecting crayfish from cell burst during WSSV infection. Retaining haemocytes in circulation may enhance crayfish immunity during periods of stress.

In addition, hyperthermia was found to stimulate the prophenoloxidase (proPO) cascade system of shrimp, which is important for pathogen melanization by the innate immune system (Pan *et al.* 2008). After release from granulosa cells into the plasma, proPO can be converted by serine proteases into active PO, which then catalyses phenolic compounds into melanin. The melanin and its intermediate products can insolate and kill invading pathogens (Sritunyalucksana 1999). Therefore, the strength of PO activity may correlate with the resistance of crayfish to viral infection. In the present study, after hyperthermic treatment, the PO activity of infected crayfish was significantly higher at 48 and 72 hpi than that in non-hyperthermic treated infected crayfish

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(30901116 and 31272455) and Natural Science Foundation of Zhejiang Province of China (Y3080212). We wish to express our great thanks to Dr. Silvia Piccinotti, Harvard Medical School, for the English editing and valuable suggestions.

Publication History

Received: 3 March 2014
Revision received: 17 April 2014
Accepted: 23 April 2014

This paper was edited and accepted under the Editorship of Professor Ron Roberts.

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