Gene expression kinetics of the yellow head virus in experimentally infected *Litopenaeus vannamei*

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Abstract

The yellow head virus (YHV) has been reported to be one of most pathogenic viruses for cultivated shrimp; however, serious problems have only been reported in farms in south and southeastern Asian. Recently, a YHV strain was detected in *Litopenaeus vannamei* cultivated in Mexican farms that lacked virus-associated mortalities or epizooties, and the animals were apparently healthy. The identity of the virus was confirmed by sequencing replicative and structural protein-encoding regions and comparing with homologous virus sequences. Phylogenetic relationships and genetic distances were also determined and, although some differences were observed, an influence on virulence was uncertain. In addition, the expression levels of several transcripts (3CL^{PRO}, POL, GP64 and GP116) were evaluated by quantitative real-time polymerase chain reaction during an experimental infection. Although the transcript showed varying kinetics, viral genes were expressed in infected *L. vannamei*, demonstrating the replicative capability of this YHV strain.

Keywords: *Nidovirales*, *Roniviridae*, YHV, glycoproteins, 3CL^{PRO}, polymerase, expression kinetics

Introduction

Shrimp aquaculture has undergone a rapid expansion in recent decades. However, bacterial and viral infections represent a major constraint affecting the industry due to significant economic losses. The yellow head virus (YHV) and the white spot syndrome virus have been reported to be the most dangerous

viruses for cultivated shrimp (Durand, Tang & Lightner 2000), and besides virulence, the epizooties are typically associated with poor management and/or environmental conditions. Yellow head virus epizooties cause up to 100% mortality 3–5 days after virus introduction (Wongteerasupaya, Sriurairatana, Vicker, Akrajamorn, Boonsaeng, Panyim, Tassanakajon, Withyachumnarnkul & Flegel 1995), and an effective treatment is not available. In addition to its high virulence and infectiveness, YHV is a very risky virus (Boonyaratpalin, Supamattaya, Kasornchandra, Direkbusaracom, Aekpanithanpong & Chantanachookin 1993), as it is also able to naturally infect other shrimp populations, which can then act as natural reservoirs (Lu, Tapay, Brock & Loh 1994; Ma, Overstreet & Jovonovich 2009).

Based on its morphology, YHV was originally classified as a *Baculovirus* (Boonyaratpalin *et al.* 1993). However, by genomic taxonomy, YHV is classified as an *Okavirus*, a member of the *Roniviridae* family that characteristically has a (+)ssRNA genome (Cowley, Dimmock, Wongteerasupaya, Boonsaeng, Panyim & Walker 1999; Cowley, Dimmock, Spann & Walker 2000; Sittidilokratna, Hodgson, Cowley, Jitrapakdee, Boonsaeng, Panyim & Walker 2002; González, Gomez-Puertas, Cavanagh, Gorbalenya & Enjuanes 2003; Gorbalenya, Enjuanes, Ziebuhr & Snijder 2006; Pasternak, Spaan & Snijder 2006). The insertion of YHV into *Nidovirales* was supported by similarities in both its genomic organization and its replication mechanism (de Vries, Horzinek, Rottier & de Groot 1997; Pasternak *et al.* 2006; Sittidilokratna, Dangtip, Cowley & Walker 2008). In *Nidovirales*, including YHV, the key biochemical machinery for virus replication is typically encoded by a large discontinuous open reading

frame (ORF), referred to as ORF1a and ORF1b, which overlap in a small area containing a – 1 ribosomal frameshift (Cowley *et al.* 2000; Sittidilokratna *et al.* 2002; Gorbalenya *et al.* 2006). Other genomic regions include ORF2, which encodes the nucleocapsid and envelope proteins (Cowley, Dimmock & Walker 2002; Jitrapakdee, Unajak, Sittidilokratna, Hodgson, Cowley, Walker, Panyim & Boonsaeng 2003; Cowley, Cadogan, Spann, Sittidilokratna & Walker 2004; Gorbalenya *et al.* 2006; Sittidilokratna, Phetchampai, Boonsaeng & Walker 2006); ORF3, which encodes the precursors for the envelope glycoproteins GP116 and GP64 (Cowley *et al.* 2002; Jitrapakdee *et al.* 2003); and ORF4, which is interrupted by multiple stop codons (Nadala Jr, Tapay & Loh 1997; Sittidilokratna *et al.* 2008).

Even though considerable progress has been made in the characterization of the YHV genome (Sittidilokratna *et al.* 2008), information on viral gene expression during infection is limited. Using specific monoclonal antibodies, expression of the envelope (GP64 and GP116) and nucleocapsid (N) proteins was monitored in five species of prawns (*Macrobrachium rosenbergii*, *Macrobrachium lanchesteri*, *Macrobrachium sintangense*, *Palaemon styliferus* and *Penaeus serriifer*) that had been experimentally infected (Longyant, Sithigorngul, Chaivisuthangkura, Rukpratanporn, Sithigorngul & Menasveta 2005). *Penaeus serriifer*, *P. styliferus* and *M. sintangense* exhibited mild to moderate YHV infection symptoms 3 days postinjection, and the infection severity was significantly reduced after 10 days. Conversely, *M. rosenbergii* and *M. lanchesteri* did not show symptoms, even though viral proteins were detected in the first hour post-infection, suggesting a natural resistance to YHV infection and/or the ability to efficiently eliminate the virus. A similar response was observed in *Penaeus (Metapenaeus) affinis* and *Pseudocarea brevicornis*, suggesting that some prawns are able to gradually eliminate the virus by an unknown mechanism (Longyant, Sattaman, Chaivisuthangkura, Rukpratanporn, Sithigorngul & Sithigorngul 2006). Interestingly, experimentally infected *Litopenaeus vannamei* and *Litopenaeus stylirostris* show symptoms and mortality profiles similar to those of naturally infected cultivated *Penaeus monodon* (Lu, Tapay, Loh, Brock & Gose 1995; Spann, Cowley, Walker & Lester 1997; Pantoja & Lightner 2003).

The establishment of viral replication kinetics from early stages of infection is fundamental to understanding the infectious capacity of YHV including those strains that apparently do not affect cultured shrimp. A YHV strain (YHV-Lv) was isolated from *L.*

vannamei cultivated in Mexican farms without virus-associated mortalities or epizootics, and with the animals apparently being healthy (de la Rosa-Velez, Cedano-Thomas, Cid-Becerra, Mendez-Payan, Vega-Perez, Zambrano-Garcia & Bonami 2006). Therefore, the aim of this study was to sequence the genome of the YHV-Lv strain and compare it with the sequences of virulent strains to determine genomic differences that could explain the lack of infectivity and/or virulence in *L. vannamei*. In addition, the replication capability of the YHV-Lv strain was determined by monitoring the expression of genes involved in viral replication and those encoding structural proteins during development of the experimental infection.

Materials and methods

YHV isolates

The American YHV strain (YHV-Lv) was obtained from apparently healthy YHV-positive *L. vannamei* cultivated in shrimp farms from the Pacific coast of México (de la Rosa-Velez *et al.* 2006). The Asian YHV strain (YHV-Pm), recovered from *P. monodon* during a YHV epizooty in Thailand in 1996 and reproduced in *Litopenaeus setiferus*, was provided by the Ecosystemes Lagunaires Laboratory, Université Montpellier 2, France.

Sequencing

To determine genomic sequences, 11 primer sets were designed based on the GenBank sequences for YHV and GAV (GenBank accession nos. AF227196, AF148846, AY052786; DQ067891, AF540644). These regions correspond to genes associated with viral replication, such as the chymotrypsin-like protease 3CL^{PRO} (3CL^{PRO}), peptide 1 (PEP1), RNA-dependent RNA polymerase, helicase (HEL) and structural components, including motif 1 (M1), nucleocapsid protein p20 and the spike glycoprotein that contains glycoproteins GP116 and GP64, as well as the no-encoding ORF4. In addition, the overlapping region (ORF1a/1b) was amplified with primers published by Sittidilokratna *et al.* (2002) (Table 1).

Pleopods from animals infected with YHV-Lv or YHV-Pm were homogenized, total RNA was purified by TRIzol (Gibco BRL, San Francisco, CA, USA) and cDNA was synthesized using oligo-dT and reverse transcriptase SuperScriptTM II (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions.

Table 1 Primers used for PCR amplification and sequencing of YHV fragments

ORF	Codified protein	Primer name	Sequence	T _a (°C)	Location
1a	Protease 3CL ^{PRO}	3CL ^{PRO} -F ^{qPCR}	5'-TGCTCTCGGTTACACGCAGACC-3'	58	8321–8342
		3CL ^{PRO} -R ^{qPCR}	5'-AGGTCAGCAATACGGATCTGTG-3'		9466–9445
1a/1b	Replicative polyprotein (fragment)	ORF1a/1b-F*	5'-TGCTTTGACCGTGTGACGTCGATGAAGAC-3'	56	11 985–12 014
		ORF1a/1b-R*	5'-TGACGGTCTTCGTGTAGTTTGTGTATGCGACTGG-3'		13 605–13 571
1b	Peptide1	PEP1-F	5'-CGTATTGCATCGAACGTCACGTG-3'	56	12 945–12 966
		PEP1-R	5'-CAAGATCACTAATAACGCCTGATGC-3'		13 829–13 805
	Polymerase	POL-F ^{qPCR}	5'-GCATCGGCATCACTAACTCCC-3'	56	14 004–14 025
		POL-R ^{qPCR}	5'-CAATGGAAGAGAAGACTCGCTCG-3'		14 981–14 959
	Helicase	HEL1-F	5'-CTCCTTAGTTTCATTGCTGCTCG-3'	56	16 253–16 275
		HEL1-R	5'-ATGTGTGGACAGTGTAGATGGGG-3'		17 414–17 392
		HEL2-F	5'-CATCTTCGCTACTCTCCAGTCAACC-3'		16 894–16 918
		HEL2-R	5'-TTTTACCACCTTTACCGCCCCAG-3'	56	17 958–17 935
		HEL3-F	5'-ATGTAGCCGTCAGTCGTTTCCG-3'		17 493–17 514
		HEL3-R	5'-GAATGCTTGATGATACCGTCG-3'		18 556–18 535
2	Nucleocapsid	N-F	5'-AACGAAGTGACTATGCGCCTTCCA-3'	57	20 266–20 289
		N-R	5'-ATGATATGCGAGCCTGTCGAGAA-3'		21 316–21 293
3	Glycoprotein 116	GP116-F ^{qPCR}	5'-GCCCATGATAGACATAAGCTCACAC-3'	66	21 549–21 573
		GP116-R ^{qPCR}	5'-ACATCATGTTGTAACGGCAATCG-3'		22 629–22 607
	Glycoprotein 64	GP64-F ^{qPCR}	5'-CAGGTGCCGTTTGATTCTG-3'	56	24 847–24 867
		GP64-R ^{qPCR}	5'-CGTCGCATTGTGTGATAACTGG-3'		25 770–25 749
4	None	ORF4-F	5'-ACACAGGCACTACCGTTTCTC-3'	42	26 347–26 365
		ORF4-R	5'-TTTTTTTTTTTTTTTTTTTTTTT-3'		

The location in the YHV genome (GenBank accession no. EU987200) is indicated.

*Sittidilokratna *et al.* (2002).

T_a, primer annealing temperature; ^{qPCR}, quantitative PCR; PCR, polymerase chain reaction; ORF, open reading frame.

Viral genes were amplified by polymerase chain reaction (PCR) (iCyclerTM thermocycler, Bio-Rad, Carlsbad, CA, USA) using specific primers. The final reaction volume of 25 µL contained 2.75 µL 10 × PCR buffer (50 mM KCl, 10 mM Tris-HCl; pH 8.3), 1 µL of 10 mM dNTPs, 0.75 µL of 50 mM MgCl₂, 1 µL of 10 mM each primer, 0.25 µL of Taq DNA polymerase 5 U µL⁻¹ (Go-Taq[®], Madison, WI, USA) and 2 µL cDNA. The optimal annealing temperature for each primer pair was determined previously by gradient temperature, and the PCR conditions were: one cycle of 94 °C/5 min; 30 cycles of 94 °C/30 s, primer annealing temperature/30 s and 72 °C/1 min; followed by extension at 72 °C for 7 min. Polymerase chain reaction products were separated by electrophoresis in a 1% agarose gel, stained with ethidium bromide (1 µg mL⁻¹) and visualized under UV light.

Polymerase chain reaction products were purified using the Wizard[®] PCR Preps DNA Purification System (Promega, Madison, WI, USA), and thoroughly sequenced in both directions by SeqXcel[®] (DNA Sequencing Services, San Diego, CA, USA). The sequences from each strain were edited using Chromas Pro V 1.14 before comparison using discontinuous megablast against the nr/nt nucleotide

database (NCBI, <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). Fragments amplified with primer pairs HEL1, HEL2 and HEL3 were assembled using the program BIOEDIT V7.0.9 (Hall 1999).

Sequences comparison

All sequences from YHV-Lv and YHV-Pm were aligned using CLUSTAL X (Thompson, Gibson, Plewniak, Jeanmougin & Higgins 1997) and compared individually with the corresponding viral region from other YHV strains (Cholburi, Thailand and Chachoengsao) isolated from Thailand farms (GenBank accession nos. EU487200, AF148846, AY052786, EF156405, AF148846, DQ067891 and AF540644) and sequences from GAV (GenBank accession no. AF227196), another member of the *Nidovirales* family. Sequences from the severe acute respiratory syndrome (SARS) virus (GenBank accession nos. DQ898174 and AY502924) and transmissible gastroenteritis virus (TGV) (GenBank accession nos. AY335549, DQ201447 and Y00560) were also included as external groups. Genetic distances were calculated using the program MEGA 4.1 (Kumar,

Tamura & Nei 2004), and phylogenetic trees were generated for each amplified region following the neighbour-joining method (Saitou & Nei 1987) with a bootstrap of 1000 repeats.

Inoculum doses and detection in different tissues

To estimate the adequate viral inoculum for 100% survival up to 96 h, different dilutions (1:2–1:50) of homogenized pleopods were prepared from YHV-Lv-infected shrimp (*L. vannamei*) as described previously (de la Rosa-Velez *et al.* 2006). Groups of 10 juvenile shrimp *L. vannamei* were inoculated in the third abdominal segment with 20 µL of different viral dilutions of YHV-Lv. As a control, 10 animals were inoculated with negative-YHV shrimp tissue. Typical symptoms of YHV infection and mortality were monitored up to 125 h postinoculation.

To determine the best tissue for the detection of viral genes, 10 juveniles (18–20 g) were infected with YHV-Lv, and samples from haemocytes, the hepatopancreas, muscle, gill, intestine and pleopods were taken at different times post-inoculation. Total RNA was isolated from each tissue, and cDNA was synthesized as above. Peptide 1, as a representative viral replication gene, was amplified by PCR, using the following conditions: one cycle of 94 °C/5 min; 30 cycles of 94, 56 °C/30 s and 72 °C/1 min; followed by extension at 72 °C for 7 min. Polymerase chain reaction products were separated by electrophoresis in 7.5% acrylamide gels and silver stained (Sanguinetti, Dias Neto & Simpson 1994).

YHV-Lv gene expression kinetics

Amplified and sequenced fragments corresponding to the polymerase, the protease 3CL^{PRO} and two glycoproteins (GP116 and GP64) were cloned into pCR2.1⁺-TOPO⁺ vector, and these vectors were used to transform One Shot⁺ Mach1TM-T1R Competent Cells (TOPO TA Cloning⁺, Invitrogen). After being expanded in LB medium, the plasmid was purified using the Wizard⁺ SV 96 Plasmid DNA Purification System (Promega) and its concentration and quality were estimated by optical density at 260 and 280 nm. To confirm, each insert was sequenced in both directions by Millegen⁺ Biotechnologies (Labège, France), and was then used to prepare standard curves for quantitative real-time polymerase chain reaction (qPCR).

To follow viral infection, 150 shrimp (average weight, 20 g) were inoculated in the third abdominal segment with 20 µL of a 1:25 viral dilution. Pleopods from 10 *L. vannamei* were sampled at different times (1.5, 3, 6, 12, 24, 48, 72 and 96 h) and pooled, and total RNA was purified. In addition, pleopods from five animals were sampled at 24, 48, 72 and 96 h, and the total RNA was individually purified. In both cases, cDNA was synthesized by reverse transcriptase, as described above.

Quantitative real-time polymerase chain reaction was performed using a Lightcycler[®] system (Roche Molecular Biomedicals, Indianapolis, IN, USA). 0.5 µL of each primer (3CL^{PRO}, POL, GP116 and GP64) was separately mixed with 2 µL of Master Mix SYBR Green (Qiagen, Valencia, CA, USA) and 1 µL of cDNA, in triplicate. The PCR protocol was: one denaturing cycle (95 °C/600 s), 40 amplification cycles (95 °C/15 s, 55 °C/20 s for 3CL^{PRO}, POL and GP64; or 68 °C/20 s for GP116) and one elongation cycle (72 °C/20 s). Melting curve analysis was performed using 0.5 °C increments from 65 to 95 °C.

Results and discussion

Yellow head virus has been responsible for devastating mortalities in cultured shrimp, causing a negative impact on this industry. Although *P. monodon* is the most affected shrimp, other penaeids have also been reported to be susceptible to different grades of virulence. For example, when a mixed culture of *P. monodon*, *Pterocarpus indicus* and *Penaeus merguensis* was affected by YHV, significant mortalities were observed only in *P. monodon*, and this phenomenon was explained as a host specificity (Mohan, Shankar, Kulkarni & Sudha 1998). In addition, YHV has been found in non-cultivable shrimp (*P. merguensis*, *Acetes* sp., *P. styliiferus* and *P. ensis*) without typical disease symptoms (Durand *et al.* 2000). This apparent host specificity and wide distribution in natural non-cultivable shrimp populations is indicative of the immense natural reservoirs and their role in YHV dissemination. An apparently non-virulent strain of YHV, YHV-Lv, was recovered from healthy *L. vannamei* cultivated in Mexican farms, where YHV-associated epizootics have not been reported (de la Rosa-Velez *et al.* 2006), leading to questions about the different virulence of YHV-Lv and other YHV strains. Also, the Asian strain, YHV-Pm, was recovered from *P. monodon* during a YHV epizooty in Thailand, in 1996, and it has been experimentally reproduced in *L. setiferus*.

Table 2 Genetic distance of YHV-LvA strain isolated from Mexican farms and reported YHV strains, GAV and coronavirus by Jukes–Cantor method

Genomic region	YHV Chachoengsao	YHV Cholburi	GAV	Coronavirus
3CL ^{PRO}	0.0231	NA	0.2242	1.0273
ORF1a/1b	0.0244	NA	0.2117	1.1079
PEP1	0.0179	NA	0.2352	NA
POL	0.0149	NA	0.1523	0.9304
HEL-M1	0.0208	NA	0.1999	0.9778
N	0.0150	0.0075	0.2232	1.0573
GP116	0.0198	0.0111	0.4908	1.1350
GP64	0.0104	0.0069	0.2614	NA
ORF4	0.0041	NA	0.1501	NA

NA, not available sequence; YHV, yellow head virus; ORF, open reading frame; PEP1, peptide 1.

The first step was to sequence several portions of the viral genome. The genomic regions (3CL^{PRO}, POL, HEL, GP64, GP116, N, PEP1, ORF1a/1b and M1) to be sequenced were selected based on the importance of these genes in viral metabolism, the availability of similar sequences and/or similarity to related viruses (Cowley *et al.* 2000, 2002; Jitrapakdee *et al.* 2003; Ziebuhr, Bayer, Cowley & Gorbalenya 2003; Sittidilokratna *et al.* 2006). In addition, primers published by Sittidilokratna *et al.* (2002) were used for amplification and sequencing of the region containing the slippage and involving an RNA pseudo knot located immediately downstream of this slippage sequence. The optimal annealing temperature was determined by temperature gradient, for each primer pair. Most annealed between 56 and 58 °C, while temperatures of 42 and 66 °C were used for ORF4 and GP116 respectively (Table 1). After PCR, the corresponding fragments were sequenced as many times as necessary to obtain complete and error-free sequences. The sequences amplified with primer pairs HEL1, HEL2 and HEL3 were assembled by overlapping extremes. The sequences were deposited in GenBank with accession nos. DQ978355 to DQ978363 and EU977577 to EU977584. The amplified sequences corresponding to GP116, N, POL, 3CL^{PRO} and ORF4 were identical in both Lv and Pm strains and only slight differences were observed in GP64 (0.1%), ORF1ab (0.43%) and HEL-M1 (0.04%).

Sequence analysis and phylogenetic comparison

Each sequence from YHV-Lv and YHV-Pm was compared with those reported for other strains (Cholburi, Thailand and Chachoengsao), and sequences of GAV were included as references. When available, se-

quences from either the SARS virus or TGV were included as an external group. After CLUSTAL alignment, genetic distances were determined (Table 2) and phylogenetic trees (Fig. 1) were generated using neighbour joining and bootstrap. In both cases, a close phylogenetic relationship could be established among YHV strains, forming a compact group (genetic distances 0.0085–0.0182), which is associated with GAV but different from the external groups. In sum, all determined sequences from YHV-Lv and YHV-Pm were identical (99.93% similar), and both showed high similarity (98%) to the corresponding sequences reported for other strains of YHV (Cholburi and Chachoengsao). There is also a moderate similarity to GAV, another *Nidovirales*. Thus, the high similarity found in a large portion of the virus genome confirms the identity of the YHV strain isolated in Mexican farms.

The *Nidovirales* glycoproteins (GPs) have a central role in the induction of the host immune response and are targets for protective immunity (Spaan, Cavanagh & Horzinek 1988). They have also been demonstrated to determine virulence (Phillips, Chua, Lavi & Weiss 1999; Almazan, Gonzalez, Penzes, Izeta, Calvo, Plana-Duran & Enjuanes 2000) because they are key proteins in the recognition of host cells and the fusion of the virus to the cellular membrane (de Vries *et al.* 1997; González *et al.* 2003; Gorbalenya *et al.* 2006). Similar functions have been reported for the surface glycoproteins GP64 and GP116 of YHV and GAV (Cowley *et al.* 2002; Jitrapakdee *et al.* 2003). Focusing the genomic analysis on the GP116-encoding region, significant differences could be observed between any YHV strain and the less aggressive pathogen GAV, suggesting the role of this protein in virulence. The YHV-Lv GP116-encoding region seems to be more similar to the Cholburi than the Cha-

Figure 1 Phylogenetic analysis. Sequenced genomic regions of YHV-Lv were compared with available sequences of YHV strains and the sequence of GAV. Coronavirus was included as an external group. The tree was constructed using the neighbour-joining method (bootstrap = 1000). YHV, yellow head virus.

choengsao corresponding genomic region. Interestingly, the Chachoengsao strain was responsible for epizootics in Thailand (Wijegoonawardane, Cowley, Phan, Hodgson, Nielsen, Kiatpathomchai & Walker 2008), while there have not been reports of extended damage associated with the Choburi strain. Additionally, several low pathogenic genotypes of YHV have been identified in India, Malaysia, Mozambique, Thailand and Philippines (Sittidilokratna *et al.* 2008), and in an earlier work, the authors associated such a behaviour with detectable differences in the GP116 N-terminal region (Sittidilokratna, Chotiwattanakun, Wijegoonawardane, Unajak, Boonnad, Wangnai, Jitrapakdee, Cowley & Walker 2009).

Experimental infection

Because the YHV-Lv strain shows low infectivity towards *L. vannamei* under natural conditions, experimental inoculation was used to confirm the occurrence of the viral replication. To determine viral gene expression through an experimental infection, the viral doses for inoculation were established as the maximal viral dilution able to induce all disease symptoms, but still showing 100% survival of animals up to 96 h. This assay was performed by inoculation of 90 apparently healthy animals with different viral dilutions (10 animals per dilution) and the time for maintaining 100% of living animals was recorded. As expected, with high doses (up to dilution 1:10), mortality was observed in the first 36 h; however, when dilutions 1:20 and 1:30 were used, the first death of an animal was recorded at 84 and 108 h respectively. By non-linear regression analysis, the 1:24 dilution was determined to be the highest viral concentration where 100% survival could be observed at 96 h after inoculation (Fig. 2), and then this dilution was used in all subsequent experiments.

Next, the best tissue for sampling was determined by inoculation of 10 animals with 20 µL of adequate viral dilutions, and the amplification of the PEP1 gene was performed in haemocytes, the hepatopancreas, muscle, gill, gut and pleopods obtained at 24 and 96 h postinoculation. Polymerase chain reaction products were separated on acrylamide gels and silver stained, which is a fast, inexpensive and sensitive method (Fig. 3a). Amplification was not observed in the hepatopancreas and gut (Fig. 3b), while a moderate signal was obtained in the muscle and gill. However, intense amplification of the PEP1 gene was observed in pleopods from 36 to 96 h (Fig. 3c) and

Figure 2 Determination of viral doses. Different viral dilution was inoculated and the time for maintaining 100% of living animals was recorded. The maximal viral dilution to reach 100% survival up to 96 h postinoculation (discontinue line) was estimated from a non-linear regression (solid line).

haemocytes at 24 and 96 h (Fig. 3d). Although haemocytes and pleopods showed the most intense signal, the isolation of total RNA from haemocytes requires a careful manipulation of the sample to avoid degranulation or cellular lysis. Thus, pleopods were selected as the sampling tissue for monitoring the expression of 3CL^{PRO}, POL, GP116 and GP64 as a measurement of the replicative capability of the YHV-Lv.

The selected genes, 3CL^{PRO}, POL, GP116 and GP64, are relevant in viral replication and interaction with the host cell. In *Nidovirales*, 3CL^{PRO} is responsible for the post-translational release of key proteins for replication (Ziebuhr, Snijder & Gorbalenya 2000; Cowley & Walker 2002; Ziebuhr *et al.* 2003; Gorbalenya *et al.* 2006; Sittidilokratna *et al.* 2008), while the polymerase is the most conserved gene and thus has been used to propose a common ancestor for all *Nidovirales* (de Vries *et al.* 1997; Cowley *et al.* 2000; Sittidilokratna *et al.* 2002). As in all *Nidovirales* studied, the structural glycoproteins, GP116 and GP64, are involved in the interaction with host cells (González *et al.* 2003; Jitrapakdee *et al.* 2003; Gorbalenya *et al.* 2006), and specifically, GP116 has been associated with the pathogenic behaviour of several YHV strains (Sittidilokratna *et al.* 2008). According to the results shown in Fig. 4, three phases could be defined: (a) the first

Figure 3 Detection of yellow head virus (YHV) genes in shrimp tissues. Polymerase chain reaction products were separated by electrophoresis in 7.5% acrylamide gels and silver stained. (a) Summary of YHV detection in different tissues by amplification of peptide 1; band intensity is represented by '+'. (b) M, muscle; I, Gut; G, gill and H, haepatopancreas. (c) Pleopods at 4–96 h. (d) Haemocytes at 24, 36 and 96 h.

phase from 1.5 to 24 h postinoculation; (b) the second phase from 24 to 72 h postinoculation, is characterized by a fast increment in gene expression (> two orders); and (c) the third phase from 72 to 96 h postinoculation, shows a slow increment in gene expression. The gene expression profile is compatible with the viral replication model, initially proposed by Ellis and Delbruck (1939). The first phase, associated with virus adsorption and/or penetration, and the third phase, related to virus assembly and release, characteristically show low gene expression, while the second phase is differentiated by a fast increment in gene expression probably due to virus replication.

In addition, five shrimp were sampled during the second phase (24, 48 and 72 h postinoculation) and analysed individually. Although comparable expression profiles could be established, quantitative differences were detected (Fig. 4) among individuals and, consequently, high dispersion was obtained. For example, at 96 h postinfection, the number of copies of GP64 ranged from 10^5 to 10^8 , although discrete dispersion was observed for GP116 (10^5 – 10^6) and $3CL^{PRO}$ (10^7 – 10^8). Then the curve slope from 24 to 72 h, representing the expression rate, was calculated for each gene and each shrimp. Equimolecular concentrations of $3CL^{PRO}$ and POL coding sequences are expected because they are transcribed together as the replicase polyprotein 1ab, which is encoded by two large overlapping ORFs (ORF1a/1b) that contain the –1 ribosomal frame shift. After transcription, $3CL^{PRO}$ and POL functional proteins are produced by site-specific cleavage of the replicase polyprotein 1ab.

However, $3CL^{PRO}$ was highly expressed almost immediately after viral inoculation, increasing to 2.08×10^5 copies μL^{-1} at 1.5 h, while POL showed a modest expression ($\cong 10^2$ copies μL^{-1}). Afterwards both transcripts displayed similar increasing rates, but maintained the $3CL^{PRO}/POL$ ratio around 10^3 . Likewise, GP116 and GP64 are encoded by ORF3, but different concentrations of their corresponding transcripts were detected, mainly between 24 and 48 h, when the concentration of the GP64 transcript rapidly increased (Fig. 4). These disparities could not be explained due to the poorly understood replication and transcription mechanisms in *Nidovirales* where sub-genomic mRNA and double-stranded RNA intermediates, as well as the poorly described non-structural proteins seem to be implicated (Pasternak *et al.* 2006; Enjuanes, Sola, Zuñiga & Moreno 2007). The importance of $3CL^{PRO}$ for *Nidovirales* replication has been corroborated by the silencing of this gene in YHV-infected *P. monodon*, which caused a strong inhibition of viral replication, whereas silencing GP116 and GP64 was less efficient (Ziebuhr *et al.* 2003; Tirasophon, Roshorm & Panyim 2005; Yodmuang, Tirasophon, Roshorm, Chinnirunvong & Panyim 2006; Tirasophon, Yodmuang, Chinnirunvong, Plongthongkum & Panyim 2007). As shown in Fig. 4, $3CL^{PRO}$ is highly expressed early, perhaps because this protein is required for creating other functional viral proteins by proteolytic cleavage (Ziebuhr *et al.* 2000). On the other hand, transcripts of the structural glycoproteins GP116 and GP64 also showed different concentrations. This could be due to differences in

Figure 4 Expression of 3CL^{PRO}, POL, GP116 and GP64 transcripts at different times (1.5–96 h postinoculation), quantified by SYBR Green qPCR in 10 animal-pool samples. The inserts show the quantification of five individual shrimp samples, evaluated from 24 to 96 h.

the hydrolytic action of RNases where polyA tails have a degradation-protective role (de Vries *et al.* 1997; Pasternak *et al.* 2006).

Although each gene showed different expression rates, the mean of the five genes expression rate is the sum of the events that occur during the viral replication. In this way, two shrimp (Org 3 and Org 5)

showed a lower viral expression rate (Fig. 5) and this could be associated with individual resistance to virus attachment. Expression of viral genes is associated with viral replication and disease severity; hence, organisms with high viral replication should be more susceptible than those with lower rates of virus replication. Considering that viral replication

Figure 5 Expression rates of 3CL^{PRO}, POL, GP116 and GP64 monitored in five shrimp during the viral replication phase (24–72 h). The mean of the expression rates is also graphed.

is complete 24–72 h after inoculation, the slope in this time frame is representative of the gene expression rate and could be used as a measurement of viral susceptibility. Although further experiments are required, viral replication analysis could be used to establish shrimp infection status and expression levels associated with disease.

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References

- Almazan E., Gonzalez J.M., Penzes Z., Izeta A., Calvo E., Planaduran J. & Enjuanes L. (2000) Engineering the largest RNA virus genome as an infectious bacterial artificial chromosome. *Proceedings of the National Academy of Sciences of the United States of America* **9**, 5516–5521.
- Boonyaratpalin S., Supamattaya K., Kasornchandra J., Dirakbusaracom S., Aekpanithanpong U. & Chantanachookin C. (1993) Non-occluded baculo-like virus, the causative agent of yellow-head disease in the black tiger shrimp (*Penaeus monodon*). *Fish Pathology* **28**, 103–109.
- Cowley J.A. & Walker P.J. (2002) The complete genome sequence of gill-associated virus of *Penaeus monodon*

prawns indicates a gene organisation unique among nidoviruses. *Archives of Virology* **147**, 1977–1987.

- Cowley J.A., Dimmock C.M., Wongteerasupaya C., Boonsaeng V., Panyim S. & Walker P.J. (1999) Yellow head virus from Thailand and gill-associated virus from Australia are closely related but distinct prawn viruses. *Diseases of Aquatic Organisms* **36**, 153–157.
- Cowley J.A., Dimmock C.M., Spann K.M. & Walker P.J. (2000) Gill-associated virus of *Penaeus monodon* prawns: an invertebrate virus with ORF1a and ORF1b genes related to arteri- and coronaviruses. *Journal of General Virology* **81**, 1473–1484.
- Cowley J.A., Dimmock C.M. & Walker P.J. (2002) Gill-associated nidovirus of *Penaeus monodon* prawns transcribes 3H-coterminal subgenomic mRNAs that do not possess 5H-leader sequences. *Journal of General Virology* **83**, 927–935.
- Cowley J.A., Cadogan L.C., Spann K.M., Sittidilokratna N. & Walker P.J. (2004) The gene encoding the nucleocapsid protein of gill-associated nidovirus of *Penaeus monodon* prawns is located upstream of the glycoprotein gene. *Journal of Virology* **78**, 8935–8941.
- de la Rosa-Velez J., Cedano-Thomas Y., Cid-Becerra J., Mendez-Payan J.C., Vega-Perez C., Zambrano-Garcia J. & Bonami J.R. (2006) Presumptive detection of yellow head virus by reverse transcriptase-polymerase chain reaction and dot-blot hybridization in *Litopenaeus vannamei* and *L. stylirostris* cultured on the Northwest coast of Mexico. *Journal of Fish Diseases* **29**, 717–726.
- de Vries A.A.F., Horzinek M.C., Rottier P.J.M. & de Groot R.J. (1997) The genome organization of the Nidovirales: simi-

- rities and differences between arteri-, toro-, and coronavirus. *Seminars in Virology* **8**, 33–47.
- Durand S.V., Tang K.F.J. & Lightner D.V. (2000) Frozen commodity shrimp: potential avenue for introduction of white spot syndrome virus and yellow head virus. *Journal of Aquatic Animal Health* **12**, 128–135.
- Ellis E.L. & Delbruck M. (1939) The growth of bacteriophage. *Journal of General Physiology* **22**, 365–384.
- Enjuanes L., Sola I., Zuñiga S. & Moreno J.L. (2007) Coronavirus RNA synthesis: transcription. In: *Coronaviruses: Molecular and Cellular Biology* (ed. by V. Thiel), pp. 87–107. Caister Academic Press, Switzerland.
- González J.M., Gomez-Puertas P., Cavanagh D., Gorbalenya A.E. & Enjuanes L. (2003) A comparative sequence analysis to revise the current taxonomy of the family *Coronaviridae*. *Archives of Virology* **148**, 2207–2235.
- Gorbalenya A.E., Enjuanes L., Ziebuhr J. & Snijder E.J. (2006) *Nidovirales*: evolving the largest RNA virus genome. *Virus Research* **117**, 17–37.
- Hall T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95–98.
- Jitrapakdee S., Unajak S., Sittidilokratna N., Hodgson R.A., Cowley J.A., Walker P.J., Panyim S. & Boonsaeng V. (2003) Identification and analysis of gp116 and gp64 structural glycoproteins of yellow head nidovirus of *Penaeus monodon* shrimp. *Journal of General Virology* **84**, 863–873.
- Kumar S., Tamura K. & Nei M. (2004) MEGA 3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics* **5**, 150–163.
- Longyant S., Sithigorngul P., Chaivisuthangkura P., Rukpratanporn S., Sithigorngul W. & Menasveta P. (2005) Differences in susceptibility of palaemonid shrimp species to yellow head virus (YHV) infection. *Diseases of Aquatic Organisms* **64**, 5–12.
- Longyant S., Sattaman S., Chaivisuthangkura P., Rukpratanporn S., Sithigorngul W. & Sithigorngul P. (2006) Experimental infection of some penaeid shrimps and crabs by yellow head virus (YHV). *Aquaculture* **257**, 83–91.
- Lu Y., Tapay L.M., Brock J.A. & Loh P.C. (1994) Infection of the yellow head baculo-like virus (YBV) in two species of penaeid shrimp, *Penaeus stylirostris* (Stimpson) and *Penaeus vannamei* (Boone). *Journal of Fish Diseases* **17**, 649–656.
- Lu Y., Tapay L.M., Loh P.C., Brock J.A. & Gose R. (1995) Development of a quantal assay in primary shrimp cell culture for yellow head baculovirus (YBV) of penaeid shrimp. *Journal of Virology Methods* **52**, 231–236.
- Ma H., Overstreet R.M. & Jovonovich J.A. (2009) Daggerblade grass shrimp (*Palaemonetes pugio*): a reservoir host for yellow-head virus (YHV). *Journal of Invertebrate Pathology* **101**, 112–118.
- Mohan C.V., Shankar K.M., Kulkarni S. & Sudha P.M. (1998) Histopathology of cultured shrimp showing gross signs of yellow head syndrome and white spot syndrome during 1994 Indian epizootics. *Diseases of Aquatic Organisms* **34**, 9–12.
- Nadala E.C.B. Jr, Tapay L.M. & Loh P.C. (1997) Yellow-head virus: a Rhabdovirus-like pathogen of penaeid shrimp. *Diseases of Aquatic Organisms* **31**, 141–146.
- Pantoja R.C. & Lightner D.V. (2003) Similarity between the histopathology of the white spot syndrome virus and yellow head syndrome virus and the relevance to diagnosis of YHV diseases in the Americas. *Aquaculture* **218**, 47–54.
- Pasternak A.O., Spaan W.J.M. & Snijder E.J. (2006) Nidovirus transcription: how to make sense . . .? *Journal of General Virology* **87**, 1403–1421.
- Phillips J.J., Chua M.M., Lavi E. & Weiss S.R. (1999) Pathogenesis of MHV4/MHV-A59 recombinant viruses: the murine coronavirus spike protein is a major determinant of neurovirulence. *Journal of Virology* **73**, 7752–7760.
- Saitou N. & Nei M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**, 406–425.
- Sanguinetti C.J., Dias Neto E. & Simpson A.J. (1994) Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. *BioTechniques* **17**, 914–921.
- Sittidilokratna N., Hodgson R.A., Cowley J.A., Jitrapakdee S., Boonsaeng V., Panyim S. & Walker P.J. (2002) Complete ORF1b-gene sequence indicates yellow head virus is an invertebrate Nidovirus. *Diseases of Aquatic Organisms* **50**, 87–93.
- Sittidilokratna N., Phetchampai N., Boonsaeng V. & Walker P.J. (2006) Structural and antigenic analysis of the yellow head virus nucleocapsid protein p20. *Virus Research* **116**, 21–29.
- Sittidilokratna N., Dangtip S., Cowley J.A. & Walker P.J. (2008) RNA transcription analysis and completion of the genome sequence of yellow head nidovirus. *Virus Research* **136**, 157–165.
- Sittidilokratna N., Chotwiwatthanakun C., Wijagoonawardane P.K., Unajak S., Boonnad A., Wangnai W., Jitrapakdee S., Cowley J.A. & Walker P.J. (2009) A virulent isolate of yellow head nidovirus contains a deformed envelope glycoprotein gp116. *Virology* **384**, 192–200.
- Spaan W., Cavanagh D. & Horzinek M.C. (1988) Coronavirus structure and genome expression. *Journal of General Virology* **69**, 2939–2952.
- Spann K.M., Cowley J.A., Walker P.J. & Lester R.J.G. (1997) A yellow-head-like virus from *Penaeus monodon* cultured in Australia. *Diseases of Aquatic Organisms* **31**, 169–179.
- Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F. & Higgins D.G. (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **24**, 4876–4882.
- Tirasophon W., Roshorm Y. & Panyim S. (2005) Silencing of yellow head virus replication in penaeid shrimp cells by dsRNA. *Biochemical and Biophysical Research Communications* **334**, 102–107.
- Tirasophon W., Yodmuang S., Chinnirunvong W., Plongthongkum N. & Panyim S. (2007) Therapeutic inhibition of yellow head virus multiplication in infected shrimps by YHV-protease dsRNA. *Antiviral Research* **74**, 150–155.

- Wijegoonawardane P.K.M., Cowley J.A., Phan T., Hodgson R.A.J., Nielsen L., Kiatpathomchai W. & Walker P.J. (2008) Genetic diversity in the yellow head nidovirus complex. *Virology* **380**, 213–225.
- Wongteerasupaya C., Sriurairatana S., Vicker J.E., Akrajamorn S., Boonsaeng V., Panyim S., Tassanakajon A., Withayachumnarnkul B. & Flegel T.W. (1995) Yellow-head virus of *Penaeus monodon* is an RNA virus. *Diseases of Aquatic Organisms* **22**, 45–50.
- Yodmuang S., Tirasophon W., Roshorm Y., Chinnirunvong W. & Panyim S. (2006) YHV-protease dsRNA inhibits YHV replication in *Penaeus monodon* and prevents mortality. *Biochemical and Biophysical Research Communications* **341**, 351–356.
- Ziebuhr J., Snijder E.J. & Gorbalenya A.E. (2000) Virus-encoded proteinases and proteolytic processing in the *Nidovirales*. *Journal of General Virology* **81**, 853–879.
- Ziebuhr J., Bayer S., Cowley J.A. & Gorbalenya A.E. (2003) The 3C-Like proteinase of an invertebrate nidovirus links coronavirus and potyvirus homologs. *Journal of Virology* **77**, 1415–1426.