Antiviral immunity and protection in penaeid shrimp

Abstract
The global aquaculture of penaeid shrimp has recently undergone a huge expansion resulting in production near parity with quantities trawled from the wild. Despite this apparent success, the industry has been hindered by diseases, predominantly from virus infection, which result in losses that have been estimated at 40% of the global production capacity. An increased research focus on penaeid immune response to virus infection has ensued, with an emphasis on harnessing the immune system to protect cultured shrimp from virus infection. Here we review the current knowledge of the factors implicated in the penaeid shrimp immune response to viral infection and strategies based on these discoveries that have been examined as potential avenues for disease control. Immune priming has been observed in response to challenge with White spot syndrome virus following prior exposure to virus or viral components. We review the protection achieved following immune priming with these components, the specificity and duration as well as the generality of the response and discuss potential mechanisms that may facilitate immune priming. In addition we highlight challenges associated with future research directions.

Keywords
Immune priming • virus • Gill-associated virus • White spot syndrome virus • prawns • invertebrate immunity • invertebrate virus

1. Introduction
Over the past few decades the reliance on cultured shrimp for food production has increased greatly. From 1970 to 2006, production of cultivated shrimp increased 350-fold, while shrimp yields from capture fisheries increased only 3-fold [1]. By the end of this period, yields from both industries were close to parity [1]. Despite this remarkable growth, shrimp aquaculture has not been without problems and during the early 1990’s it was estimated that approximately 40% of the world production capacity was being lost due to newly emerging diseases. In economic value, this lost production equated to over US$3 billion [2,3]. Other estimates put the value of the economic loss in the fifteen years up to 2001 at around US$15 billion [4].

Viral diseases in cultured shrimp have been estimated to contribute up to 60% of the annual losses in global productivity [4]. Much of the loss has been the result of major epidemics and viral outbreaks. The first of these events occurred in the mid to late 1980’s in Taiwan, with outbreaks of Monodon baculovirus (MBV). Subsequent to this, in the late 1980’s and early 1990’s Asian shrimp farms were impacted by Yellow-head virus (YHV) and farms in the Americas were affected by infectious hypodermal and hematopoietic necrosis virus (IHHNV) and Taura syndrome virus (TSV) [4-9]. The largest impact of all were losses caused by white spot syndrome virus (WSSV), originating in the early 1990’s in Asia and spreading to the America’s by the end of that decade [5].

The economic and social impact of viral disease on aquaculture of penaeid shrimp has prompted concerted research efforts into understanding the immune capabilities and defense-responses of shrimp following challenge. It has also prompted many and varied control measures to be implemented at both hatcheries and farms, including for example, the molecular screening of hatchery broodstock and post-larvae for viral infection and the widespread transition from farming Penaeus monodon to farming Litopenaeus vannamei bred and certified to be specific pathogen free (SPF) [8,10]. Disease control by prophylactic methods is also being investigated (for review see Hauten, 2012 [30]) and understanding the crustacean immune system and how it interacts with viruses lays the platform for developing therapeutics. Despite these management-based responses for control of viral diseases, it has been suggested that disease including viral disease will continue to hinder the production of farmed crustaceans [11]. In a recent review, Stentiford and colleagues [11] proposed a list of guidelines that were developed to address the impact of disease on aquaculture related food security. Within these guidelines they suggest, “An increased focus is required on effective therapeutics for invertebrate pathogens and specifically those disease-causing agents affecting food production from aquaculture”, highlighting a need for a better understanding of the immune system of crustaceans and how viruses interact with their host.

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With this goal in mind, the following review focuses on antiviral immunity in penaeid shrimp and more specifically protection of shrimp from viral diseases by prior exposure to viral components. A brief background on penaeid shrimp antiviral immunity is provided before discussion of the literature on WSSV priming protection, what is known of the mechanism, the potential for further investigation and difficulties associated with such research.

2. Penaeid immune responses

Like vertebrates, invertebrates require effective immune responses to protect themselves against potentially pathogenic viruses, bacteria or fungi. In vertebrates, both innate and adaptive immune systems are utilized to defend against pathogens and these systems are comparatively well understood [12]. Invertebrates, however, do not possess an adaptive immune response that utilises immunoglobulin [13]. Despite this, they have well developed innate defense mechanisms reliant on non-self recognition that provide a non-specific, yet highly effective, defense response to pathogen invasion [14-20]. As invertebrates are highly diverse, it is important to examine immune responses in diverse phyla and species within these to identify what common and unique mechanisms are employed [19]. Such knowledge across diverse invertebrates will also instruct as to what immune strategies can be applied universally or otherwise to control disease. While this is important for the many farmed crustacean and mollusk species that command high commercial values, understanding host responses to infection and disease is also important in invertebrates that are agricultural pests or disease vectors for humans [19].

The innate pathogen defense system of invertebrates encompasses various defense systems responsible for a rapid response to bacterial, fungal and viral pathogens; many of which are conserved in the innate immune response of vertebrates [21,22]. The major innate host-defense systems of invertebrates include: haemolymph coagulation, pro-phenoloxidase (proPO) activation, lectin-complement, agglutinin-lectin, reactive oxygen species, phagocytosis and the antimicrobial peptides (AMPs) that are mediated by Toll-like receptors and the immune-deficiency (IMD) pathways [14,15]. However, how invertebrates respond to viral infections is not as well understood as how they respond to bacterial or fungal infections.

2.1 Penaeid antiviral immune responses

2.1.1 Pattern recognition proteins

Stimulation of the innate invertebrate immune response by recognition of pathogen-associated molecular patterns (PAMPs) is well characterised in response to bacterial and fungal pathogens [15,16,23-30]. The recognition of PAMPs by a variety of pattern recognition proteins (PRPs) such as peptidoglycan- or β-glucan-binding proteins is conserved amongst invertebrates including penaeid shrimp [31-33]. These PRPs induce the major innate host-defense systems of invertebrates. The PAMPs and PRPs associated with the recognition of viruses by penaeid shrimp are not as well defined.

Despite this, genes encoding lectin-like proteins with proposed roles in pathogen recognition, as well as small GTPases expected to be involved in virus infection and cellular trafficking, have been identified to have antiviral defense roles in penaeid shrimp. Most data has emanated from studies of gene expression changes in response to WSSV infection [34-47]. Lectins are believed to have roles in non-self recognition in invertebrates [48,49]. However, the range of immune functions undertaken by the superfamily of proteins containing C-type lectin-like domains (CTLDs) is highly diverse [50].

Proteins containing CTLDs are grouped by their differing recognition domains which include carbohydrate recognition domains (CRDs) and domains recognizing non-sugar ligands [51]. Various penaeid shrimp proteins with CTLDs have been studied including those characterised by CRDs [52-54]. PmAV and PmLT are among the CTLD-containing proteins regulated differentially in response to WSSV [53,54]. While not possessing agglutination activity, PmAV has been shown to have antiviral properties as demonstrated in fish, in which a recombinant PmAV protein strongly inhibited iridovirus replication [53]. In shrimp, PmAV expression levels drop 12 hours post-WSSV infection before becoming elevated 3 to 4 days later [55]. PmLT expression levels also drop by 2 hours post-WSSV infection but return to normal levels by 4 hours post-infection [54].

GTPase Ras superfamily members function as molecular switches [56] and have been grouped into five major branches by differences in structure and function [57]. Much of the experimental data reported in the literature has focused on proteins in the Rho family (Ras homologs) that control many signal transduction pathways, have an integral role in actin cytoskeleton regulation [58] and are in the Rab family, the largest within the Ras superfamily [57]. Rab GTPases have roles in vesicular transport and protein trafficking and thus in the endocytosis and secretory pathways [59,60]. In humans, Rab7 trafficking has been localised to late endocytosis pathways and Rab6a localised to Golgi traffic [61].

In response to WSSV infection, the PmRab7 protein has been shown to bind to the virion envelope protein VP28 and to lead to a decrease in R. monodon mortality [62]. Despite PmRab7 expression levels remaining stable in response to WSSV infection, injection of shrimp with dsRNA specific to PmRab7 results in reduced WSSV replication, and also reduced YHV replication [63,64]. Another study found PmRab7 expression to increase in M. japonicus in response to injection of DNA-based vaccine to WSSV [65]. Also in M. japonicus, WSSV infection elevates expression of PjRab, a Rab6-like gene, between 8 and 72 hours post-injection [66] and alters expression of a Ras-like gene as determined by a suppression subtractive hybridization (SSH) analysis [39]. PjRab is also involved in haemocytic phagocytosis and silencing of its expression causes WSSV infection loads to increase [67].

2.1.2 DSCAM

Recently, the Down syndrome cell adhesion molecule (DSCAM) receptors have been implicated in the invertebrate immune recognition of WSSV.
response. Initially identified in Drosophila melanogaster, the hyper-variable gene encoding the receptors was found to be capable of translating thousands of protein isoforms via alternative splicing of precursor mRNAs [68]. DSCAM receptors are believed to have roles in immunity through a process where the different isoforms act to variably identify PAMPs [69]. The finding that D. melanogaster DSCAM loss of function mutants have inhibited phagocytic uptake of bacteria [70] supports this hypothesis. In shrimp, DSCAM receptors have been identified in P. monodon and L. vannamei [71,72] and it is hypothesized that different PAMPs (including those derived from viruses) result in alteration of the variable domains [30,72,73].

2.1.3 Antimicrobial peptides

In response to the recognition of PAMPs the humoral immune response of invertebrates is mediated by antimicrobial peptides. In Drosophila sp., AMPs are synthesized primarily in the fat body for secretion into the haemolymph [28]. Of the AMPs, seven distinct families have been studied extensively. These families include: (i) Drosomycin and (ii) Metchnikowin, which are produced predominantly in response to fungi, (iii) Attacins, (iv) Cecropins, (v) Diptericins and (vi) Drosocin, produced predominantly in response to Gram-negative bacteria, as well as (vii) Defensin, which is produced predominantly in response to Gram-positive bacteria [74,75].

These AMPS have been characterized in most detail in Drosophila sp., but many related peptides have been found across diverse invertebrate species [16,23,74,76,77]. In penaeid shrimp AMPs have been reported with activity against a wide range of pathogens including Gram-positive and Gram-negative bacteria, fungi and viruses. Some important AMP families include: crustins, which have been identified in a wide range of crustaceans. The haemocyte expressed 7-14 kDa proteins have a characteristic whey acidic protein (WAP) domain and have varying reports on antimicrobial activity in penaeid prawns [47,78]. Penaedins, a family of four classes of AMPs, are characterised by an N-terminal proline-rich domain and a C-terminal cysteine rich domain. The expression of these small proteins (5-7 kDa) differs between species and as with crustins, differing antimicrobial activity has been reported in various studies [30,79]. Along with penaedins and crustins, important immune roles are associated in shrimp with Anti-Lipopolysaccharide Factor (ALF). ALFs are AMPs that can provide broad-spectrum pathogen defense in shrimp [47,80,81]. Hauten [30] and Rowley and Pope [82] have reviewed the literature on these AMPs extensively, including details about which AMPs are active against which pathogens in penaeid shrimp.

The implication of various AMPs in antiviral responses is established, however the activity does not always appear consistent depending on the pathogen and host species. For example, activity of crustins against WSSV and YHV infection has been reported in L. vannamei and P. monodon [47,78], however no activity was observed in another study of WSSV infection in M. japonicus [65]. A similar pattern is seen with ALFs; one study reporting activity against YHV [47], while another reports no activity against the virus [80]. Various penaeedins have also been demonstrated to have activity against viruses [65,78,83] in the penaeid shrimp innate immune response. The determination of why various AMPs appear to differ in activity among differing shrimp species warrants further investigation.

2.1.4 RNAi

The RNAi pathway was first discovered in the nematode Caenorhabditis elegans [84] and has since been found to exist in most invertebrate and plant species [85,86].

RNA interference (RNAi) responses in shrimp can be mounted in three ways, with one of these, the small interfering RNA (siRNA) pathway, playing a major role in viral defense [85,87-91]. In the siRNA pathway, Dicer-2 cleaves long, virus-specific double-stranded RNA (dsRNA) into ~21 bp siRNAs that are incorporated into the RNA-induced silencing complex (RISC) as the Dicer-2 enzyme forms a heterodimer with R2D2. One strand of the siRNA is used as a template to associate with viral RNA in a sequence specific manner and the Argonaute-2 (Argo-2) enzyme facilitates cleavage of the complementary viral RNA, thus interfering with viral replication and protein expression [84,92-94].

The RNAi pathway plays a significant role in antiviral immunity of penaeid shrimp and various responses induced by dsRNA have been demonstrated [85]. Components critical to the functioning of the RNAi pathway including the Dicer and Argonaute enzymes have also been identified in shrimp [96-98]. There is evidence that both specific as well as non-specific stimulation of the RNAi pathways can have antiviral affects, and when delivered exogenously, long dsRNAs are far more effective and specific in their action compared to siRNAs [95,99]. This non-specific innate antiviral action induced by dsRNA was long thought to be restricted to vertebrates; it’s action in invertebrates likely acts through a different pathway to the sequence specific RNAi response.

2.1.5 JAK/STAT

In Drosophila sp., the JAK/STAT pathway is well characterized, with genes involved in the JAK/STAT pathway activated in response to viral infection but not to bacterial or fungal infection [100]. To stimulate the pathway, various unpaired ligands (ie. UPD, UPD2 and UPD3) bind the Domeless receptor, a transmembrane protein responsible for signaling through Hopscotch and STAT92E, and phosphorylated STAT is transported to the nucleus with genes involved in the JAK/STAT pathway activated in response to viral infection but not to bacterial or fungal infection [100]. To stimulate the pathway, various unpaired ligands (ie. UPD, UPD2 and UPD3) bind the Domeless receptor, a transmembrane protein responsible for signaling through Hopscotch and STAT92E, and phosphorylated STAT is transported to the nucleus where it promotes expression of genes like tep1 and tolA which are involved in humoral immune responses. The tep1 gene encodes one of a family of thioester-containing proteins (TEPs) with similarities to the superfamily of complement proteins. The tolA gene also encodes a protein suggested to promote phagocytosis in a complement-like manner [101].

The JAK and STAT components critical to functioning JAK/STAT pathways used by Drosophila sp. for pathogen protection have been found to be stimulated in shrimp in response to viral challenge [102,103]. Components of the JAK/STAT pathway have been described from multiple species of penaeid shrimp, including a STAT from the Chinese white shrimp Fenneropenaeus
chinesis [104]. JAK and STAT have also both been identified in the Brine shrimp, Artemia franciscana [105]. In P. monodon, WSSV has been shown to activate STAT [102,103].

2.1.6 Apoptosis
Another cellular response associated with viral infection in shrimp is apoptosis: the mechanism of programmed cell death, with roles in eliminating unhealthy or unnecessary cells. Genes related to apoptosis are often reported to be up- or down-regulated in response to viral infection, however it is not well established whether apoptosis plays a role in viral immunity and conflicting results have resulted in this distinction becoming controversial. Some studies report apoptosis playing a major role in defense by removal of dangerous cells, such as those that may be viral infected [106]. Contrary to another study showing that knockdown of caspase-3, a key gene in the apoptosis pathway, resulted in reduced mortality in P. vannamei [107]. Another study found that apoptosis was not important in the protection of P. japonicus that were resistant to subsequent WSSV infection after previous exposure [108].

3. Immune priming
Invertebrates rely solely on the innate immune system for pathogen defense which has long been considered to lack memory or inducible elements related to those mediating the adaptive immune response of vertebrates. Contrary to this belief, it has now become accepted that some form of inducible response which may include elements of memory, exists in invertebrates and this response can be primed for pathogen defense [15,16,76,109-112]. Past exposure to a pathogen, or pathogen components has been shown to result in immune memory or 'priming' (reviewed by Little and Kraaijeveld, [113]; Sadd and Schmid-Hempel, [114] and Johnson et al. [115]). Since invertebrates lack the necessary machinery, the factors and mechanisms involved must be different to the conventional mechanisms of immune priming. While the mechanisms of immune priming in invertebrates remain unknown, it is important that the distinction between these factors utilizing different methods of priming and varied penaeid species. For the purpose of this review, we will refer to these studies as "immune priming assays" to avoid any confusion with vaccination relying on antibody mediated immune priming. Protection against WSSV challenge was first demonstrated by pre-exposure of M. japonicus to a sub-lethal dose of WSSV [124]. Pre-exposure to WSSV led to a significant increase in survival rates 3 to 4 weeks post-challenge compared to not-exposed control shrimp and protection persisted for a further month [124]. However, when re-challenged 3 months after immune priming, the immune primed shrimp died at a rate similar to the control shrimp, suggesting that the protective mechanism

3.1 WSSV immune priming
At least for WSSV, which has been investigated in most detail as a model challenge system, immune priming induced through prior exposure to virus or viral components has been shown to be effective in protecting penaeid shrimp from developing disease following challenge with a normally lethal dose of virus. Pre-exposure to either virus particles or envelope glycoproteins can provide protection in multiple different penaeid species [120-124]. The amount and longevity of protection can vary depending on pre-exposure variables and multiple studies have investigated these factors utilizing different methods of priming and varied penaeid species. For the purpose of this review, we will refer to these studies as "immune priming assays" to avoid any confusion with vaccination relying on antibody mediated immune priming.

![Figure 1](image1.png)

**Figure 1.** Schematic representation of the WSSV virion showing envelope glycoproteins (circles) and tail-like projection (black tail) and a cut away diagram showing the virus envelope, tegument and nucleocapsid (white cylinder). The major structural proteins and WSSV proteins used in vaccination trials and their location in the virion are noted. [136].
<table>
<thead>
<tr>
<th>Reference</th>
<th>Vaccine Type</th>
<th>Details</th>
<th>Response (RPS) *</th>
</tr>
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<td>Wu et al. [66]</td>
<td>Live virus</td>
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<td>1 month = 67%</td>
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<td></td>
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<td>2 months = 54%</td>
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<td></td>
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<td>3 months = 6%</td>
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<td>Inactivated</td>
<td>Single injection of heat-inactivated virus</td>
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<td>[120]</td>
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<td>Injection and booster of VP26 tegument protein</td>
<td>10 days = 55%</td>
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<td>Injection and booster of VP28 tegument protein</td>
<td>30 days = 5%</td>
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<td>Injection and booster of VP28 envelope protein</td>
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<td>10 days = 60%</td>
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<td>10 days = 95%</td>
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<td>Single injection of VP19 + VP28 envelope protein mix</td>
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<td>Single injection of VP28 envelope protein</td>
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<td>Injection of DNA vaccine from nucleocapsid VP35</td>
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<td>[126]</td>
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<td>2 weeks = 24% (Avg)</td>
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<td>Oral vaccination of VP28 envelope protein: injection challenge</td>
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<td>14 days = 20%</td>
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* Response is calculated as a relative percent survival (RPS): (1 – treatment group mortality/control group mortality) x 100
was transient. In another study protection was also achieved when M. japonicus were injected with formalin-inactivated WSSV, with and without immunostimulants, prior to challenge. This showed that the protective mechanism was not reliant on virus replication [120]. However, even in the best-case scenario of 30% survival over a 1-month period using heat-inactivated WSSV as an immune primer, the protection levels achieved were lower than using prior infection with a sub-lethal dose of WSSV [120,124]. Protection has also been achieved by immune priming using recombinantly expressed WSSV structural proteins. These include VP26, which is known to be associated with the virus tegument, and VP28, which is an envelope glycoprotein [137,138]. Significantly lower mortalities were observed after 20 days post-challenge in shrimp exposed to protein subunits VP26 and VP28 and challenged with WSSV compared to non-exposed controls [120]. Furthermore shrimp that survived subsequent challenge after WSSV component immune priming had reduced infection levels of WSSV, low enough that they were undetectable by one-step PCR [121-123].

Immune priming using a recombinant form of the WSSV VP19 envelope glycoprotein fused to maltose binding protein (MBP) also protects shrimp against disease and mortality when challenged either 2 days or 25 days post-exposure [123]. In contrast, recombinant WSSV VP28 envelope glycoprotein fused to MBP only provided similar levels of protection in shrimp challenged 2 days post-exposure, with no obvious protection provided to shrimp challenged 25 days post-exposure [123]. A mix of the recombinant VP19 and VP28 proteins again provided protection from challenge at 2 days post-exposure but not 25 days [123].

With the potential for immune priming of shrimp to control viral infection being demonstrated by injection, other means of delivery more logistically feasible to shrimp aquaculture have been investigated. In a trial to examine whether oral delivery of antigens might result in immune priming, shrimp were fed commercial feed pellets coated with inactivated bacteria in which recombinant VP19 and VP28 had been expressed prior to WSSV challenge [122]. As it is possible that a general immune response could be achieved from the presence of bacteria alone [139,140], the positive control shrimp were fed bacteria containing empty plasmid. Shrimp fed VP28 bacteria were protected against WSSV challenge; in contrast, shrimp fed VP19 bacteria were not protected. When challenged 3, 7 and 21 days after feeding on VP28 bacteria was ceased, survival rates of 64%, 77% and 29%, respectively, were obtained relative to controls fed bacteria containing the empty plasmid. Although the difference in survival was not statistically significant when challenge was undertaken 21 days after feeding, survival rates when challenged earlier were consistent with oral delivery of VP28 expressed in bacteria being capable of inducing an anti-WSSV immune response.

As an alternative strategy, to potentially increase the duration of the protective response, DNA vaccination based on intracellular protein delivery from a eukaryotic expression plasmid expression has been investigated [121]. Plasmids designed to express the VP15 and VP35 nucleocapsid-associated proteins and the VP28 and VP281 envelope proteins were each injected into the shrimp and intracellular gene expression from each plasmid was confirmed by RT-PCR amplification of the expected mRNA. Expression of only the VP28 and VP281 envelope proteins resulted in significantly lower shrimp mortality following WSSV challenge, with protection lasting for up to 7 weeks. Which is far longer than observed using other immune priming approaches. While the mechanisms involved remain to be determined, this prolonged protection is likely to be due to the extended ability of the vectors to continue expressing viral protein in host tissues [121]. A summary of the duration and protection provided by different viral components utilized in immune priming trials is presented in Figure 2 [120,121,124,135].

![Figure 2](image-url) Immune priming assay summary. Summary of the time and amount of protection afforded by exposure to various WSSV components prior to challenge. Time line showing the level of protection (RPS) in immune priming assays where challenge was administered at the longest period post-exposure. Immune priming by injection of a low-dose of live WSSV provided an RPS of 67% when challenged after 1 month, 54% when challenged after 2 months and 6% when challenged after 3 months [124]. Immune priming by injection of heat-inactivated WSSV resulted in an RPS of 15% when challenged after 10 days and 30% when challenged after 30 days [120]. Recombinant VP292 injection and booster injection resulted in an RPS of 52% when challenged at 30 days post-booster [135] and DNA vaccination of recombinant VP281 provided an RPS of 46% when subjected to challenge after 25 days and 34% when challenged with WSSV after 50 days [121]. No protection has been elucidated with recombinant nucleocapsid protein or DNA vaccine (as represented by red cross).
3.2 Specificity and generality of protection

Until recently, WSSV was the only shrimp virus for which immune priming had been examined. The focus of the investigations using WSSV has been on achieving protection, with very little done to understand the underlying mechanisms [141]. Indeed the mechanisms involved in generating specific immunity, even if relatively short-lived compared to the memory responses of vertebrates, remain to be elucidated for any invertebrate species. Important questions for which answers are needed include whether protection afforded by the various immune priming strategies described earlier are WSSV-specific or generalized, and whether similar immune priming can protect shrimp against other viruses.

With WSSV, shrimp have only been protected against challenge when viral envelope glycoproteins have been included as a vaccine component. Therefore, to help discover the mechanism involved in protection, it will be useful to determine whether pre-exposure to envelope proteins or surface-exposed proteins of viruses with differing methods of cell attachment, entry and infection might also provide protective responses. One study addressed this question using Gill-associated virus (GAV), an enveloped shrimp virus containing a positive-sense single-stranded RNA genome, which is related to members of the Nidovirales. However, no protection was observed following immune priming of shrimp with GAV envelope glycoprotein components expressed and purified from bacteria [142]. Further research is needed to determine if protection can be achieved against other viruses or if other methods can be used to successfully protect against GAV.

3.3 Mechanisms of protection

The longest duration of protection has been achieved through non-lethal exposure to live virus and intracellular expression of envelope glycoproteins from expression plasmids, suggesting persistent exposure to viral protein may be required for continued protection [120-124,126,143]. The molecular mechanisms of this novel immune response are not fully understood. A model of viral accommodation where the virus is sustained at low levels has been put forward, where the host is not resistant to re-infection but provided with a specific memory response capable of interfering with pathogenicity upon viral reinfection [144]. Another model proposed by Johnson et al. postulates that cell surface receptors required for viral uptake may bind specific viral proteins, blocking virus attachment and entry [115].

The specificity of immune priming responses remains unclear and as yet, cross-protection in shrimp has only been identified through sub-lethal infection of an unrelated virus. In cultured Penaeus stylirostris infected with IHHNV, a single-strand DNA virus, protection against WSSV, a double-stranded DNA virus, has been noted [145,146]. Why this occurs remains unknown, but it would be useful to determine whether the viruses can infect the same cells and thus compete with each other, or whether prior cell infection by IHHNV might result in the withdrawal of cell surface receptors needed for attachment and entry of WSSV [1]. To develop this research and gain further understanding of the mechanisms, immune priming assays into cross-protection should incorporate viral proteins or DNA vaccines, as it is possible the mechanisms are different to those involved in response to live virus cross-protection.

In a recent study in which L. vannamei were exposed to the live bacterium Bacillus subtilus carrying recombinant VP28 envelope glycoprotein, protection against WSSV challenge was found to be associated with elevated haemocytes phagocytosing Taura syndrome virus (TSV), suggesting a selective or specific activation of the haemocyte response to VP28 [82]. Thus shrimp might use multiple mechanisms involving a specific haemocyte response quasi-comparable to the antibody-based memory response of vertebrates, and cell-specific response involving either the blocking or withdrawal for receptors essential for virus attachment and entry, as suggested previously [115].

4. Research directions

Research effort to identify and develop strategies to protect shrimp against virus disease has been largely reactionary due to the importance of viral disease to the aquaculture industry. With WSSV having the greatest impact on the industry worldwide [1,9,148], it is not surprising that protection strategies and immune responses to WSSV have been investigated in the most detail. Much has been learned from this research including: what immune-related genes are up- or down-regulated in response to infection and these may provide useful targets to promote protection; the impact of dsRNA-based interference on viral replication and potential use of this to control virus infection; and similarly the phenomenon of protein-based immune priming which can be harnessed to protect against virus challenge. However, while such information might result in commercial applications benefiting aquaculture, there remains much academic interest in unraveling the molecular mechanisms involved. Research on shrimp responses to viruses other than WSSV should help identify which immune mechanisms are generic or unique to WSSV. Moreover, while gene expression and bioinformatics analyses have identified many shrimp proteins with predicted immune function, confirming the function of these proteins using RNAi and other approaches is still in its infancy.

4.1 Persistent viral infections

As occurs in many invertebrates, persistent viral infection is common in shrimp. Many shrimp viruses are observed to persist where the host is tolerant to the virus and shows no overt symptoms of infection. The mechanism(s) for such tolerance are unknown (for review see Flegel, [1]). Most of the immune priming assays carried out to date have not investigated whether animals that are protected from challenge after prior-exposure to viral components show clearance of the virus or remain infected but are tolerant. This is a very important distinction in determining the mechanisms of protection, and in utilizing the response for practical applications. For some viruses like GAV that commonly
exist as persistent infections \cite{149,150}, tolerance of any pre-existing infection might also be important to and interfere with the outcome of disease protection strategies based on immune priming. There is also potential for experimental outcomes of immune priming or immune response analysis to be confounded by persistent virus infection in experimental animals. The use of shrimp certified to be SPF or screened to be free of infection in such bioassays might thus be beneficial, as would analysis of protected shrimp to determine whether a similar tolerance state is established post-challenge.

4.2 Specificity and duration of protection and logistics of immune priming

As stated earlier, understanding the specificity, efficacy and longevity of various immune priming strategies will be important in their commercial application in shrimp aquaculture to control disease. Understanding the mechanisms allows the determination of how long it is possible to protect animals from disease and whether it is logistically feasible. This should be taken into account along with the efficacy of different methods of immune priming. As demonstrated in Table 1, many of the immune priming assays resulting in significant protection have relied on injection. There are logistical problems associated with using this method in aquaculture and it also raises concerns in regards to animal health following handling stress. Shrimp are very susceptible to compromised immunity from stress, with examples of this from multiple species. Sub-optimal levels of nitrate and ammonia and other water quality factors can suppress immune capability of *P. japonicas* \cite{151}, changes in salinity can similarly affect *L. vannamei* \cite{152}, temperature fluctuations can elevate heat shock protein expression in *P. monodon* \cite{153} as well as cause increased susceptibility to bacterial infection \cite{154}.

In wild-captured *P. monodon* broodstock, repeated handling stress associated with bleeding has also been noted to markedly increase GAV infection levels in haemocytes \cite{155}.

The potential for immune priming via oral delivery has also been investigated with promising results \cite{122,123,128}. Generally utilizing feed coated with killed bacteria expressing recombinant viral proteins \cite{122,123} and recently utilizing live bacteria \cite{127,147}. Alternatively, enrichment and feeding of artemia in smaller animals may result in transfer of live probiotic bacteria into the gut \cite{156}. Potentially, recombinant proteins could also be transferred into the gut this way. The fate of antigens in the gut remains uncertain and again, further investigation is required to determine if this is a feasible delivery method.

4.3 Conclusions

To understand the immune priming response described in the WSSV-shrimp model a continued focus on determining the underlying mechanisms is required. This includes determination of whether a similar response is achieved in shrimp challenged with other viruses and whether protection displays cross-specificity. It is interesting to note that almost 10 years have passed since the initial immune priming trials on WSSV and we are yet to see evidence of this type of response against any other virus. However, it is currently unclear whether this is indicative of a WSSV-specific mechanism or simply an outcome of the prior research focus. Future research driven by fundamental questions of penaeid response to virus infection will hopefully lead to a mechanistic understanding of this important phenomenon.

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