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Susceptibility of juvenile European lobster *Homarus gammarus* to shrimp products infected with high and low doses of white spot syndrome virus

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ABSTRACT: White spot syndrome virus (WSSV) is the most important pathogen known to affect the sustainability and growth of the global penaeid shrimp farming industry. Although most commonly associated with penaeid shrimp farmed in warm waters, WSSV is also able to infect, cause disease in and kill a wide range of other decapod crustaceans, including lobsters, from temperate regions. In 2005, the European Union imported US$500 million worth of raw frozen or cooked frozen commodity products, much of which originated in regions positive for white spot disease (WSD). The presence of WSSV within the UK food market was verified by means of nested PCR performed on samples collected from a small-scale survey of supermarket commodity shrimp. Passage trials using inoculum derived from commodity shrimp from supermarkets and delivered by injection to specific pathogen-free Pacific white shrimp *Litopenaeus vannamei* led to rapid mortality and pathognomonic signs of WSD in the shrimp, demonstrating that WSSV present within commodity shrimp was viable. We exposed a representative European decapod crustacean, the European lobster *Homarus gammarus*, to a single feeding of WSSV-positive, supermarket-derived commodity shrimp, and to positive control material (*L. vannamei* infected with a high dose of WSSV). These trials demonstrated that lobsters fed positive control (high dose) frozen raw products succumbed to WSD and displayed pathognomonic signs associated with the disease as determined by means of histology and transmission electron microscopy. Lobsters fed WSSV-positive, supermarket-derived commodity shrimp (low dose) did not succumb to WSD (no mortality or pathognomonic signs of WSD) but demonstrated a low level or latent infection via PCR. This study confirms susceptibility of *H. gammarus* to WSSV via single feedings of previously frozen raw shrimp products obtained directly from supermarkets.

KEY WORDS: White spot syndrome virus · WSSV · Commodity · Transmission · Risk assessment

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INTRODUCTION

White spot disease (WSD) caused by white spot syndrome virus (WSSV) has caused devastating losses in the global shrimp farming industry since its discovery in the 1990s. WSD is estimated to have caused at least US$10 billion in losses to the penaeid shrimp production systems since 1993 (Stentiford et al. 2012). Cultivation of marine shrimp accounts for a large proportion of global aquatic food production. In 2007, global shrimp production was over 3 million t, with a value of US$13 billion (www.fao.org/fishery/statistics/global-aquaculture-production/en). Annually, the European Union (EU) imports around

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US$500 million worth of farmed shrimp commodity products, the majority of which are raw frozen or cooked frozen penaeid shrimp (Stentiford et al. 2010). Owing to the wide global distribution of WSSV, large proportions of this product originate from regions in which WSSV is endemic (Lightner 2003, OIE Collaborating Centre for Information on Aquatic Diseases www.cefas.defra.gov.uk/idaad/).

Aquaculture production of crustaceans is limited within the EU and only accounts for around 200 t yr^{-1} (www.fao.org/figis). Conversely, the total fishery production of crustaceans from European waters totalled almost 400 000 t in 2004, with a large majority of this comprising marine prawns (ca. 200 000 t), lobsters (ca. 60 000 t) and crabs (ca. 85 000 t). In freshwaters, capture fisheries are solely composed of crayfish (ca. 6000 t). As a result, wild fisheries for marine crustaceans are considered key resources in the European maritime area, and in many countries (such as the UK) they rank above several important finfish species in terms of production quantity and value. Despite the high economic value of this fishery the exposure and consequent risks to wild populations of imported commodity products that may inadvertently enter the aquatic environment are largely unknown.

The European Commission (EC) Directive 2006/88 (on animal health requirements for aquaculture animals and products thereof and on prevention of and control of certain animal diseases; http://ec.europa.eu/fisheries/cfp/aquaculture/facts/index_en.htm) lists 3 notifiable crustacean diseases, all caused by viral pathogens and generally associated with farmed penaeid shrimp from tropical and subtropical regions. These are WSD, caused by WSSV, yellowhead disease, caused by yellowhead virus (YHV), and Taura syndrome, caused by Taura syndrome virus (TSV). All have caused severe economic losses in the global shrimp farming industry owing to their transmissible nature, their potential for socio-economic impact and their likelihood for spread via the international trade of animals and animal products. The listing of these diseases implies that their detection in crustaceans from European waters would be subject to compulsory control measures and formally recognises the potential for diseases traditionally associated with tropical penaeid shrimp to have an impact upon the sustainability of commercially and ecologically significant crustacean populations in the European aquatic network. YHV and TSV are listed as exotic to the EU, whereas WSSV is listed as non-exotic (Stentiford et al. 2010, Stentiford & Lightner 2011). In the European context, WSSV is considered to be the most significant threat owing to its wide host range, pathogenesis in temperate conditions and its potential for rapid spread (Stentiford et al. 2009, 2010).

EC Directive 2006/88 requires health certification for import of commodity products unless these products are destined for further processing, packaged in 'retail sale' packages and labelled in accordance with EC Regulation 853/2004. Therefore, products (live or frozen) imported directly for human consumption are not covered by the directive and do not need to originate from areas designated free from listed pathogens, even when imported to confirmed 'disease free' member states. Nunan et al. (1998) and Hasson et al. (2006) demonstrated that frozen commodity shrimp imported to the USA for human consumption tested positive for WSSV by PCR. Previous transmission trials with commodity shrimp have shown that sufficient viable virus remains in frozen commodity products to induce mortality of Pacific white shrimp *Litopenaeus vannamei* (Durand et al. 2000, Hasson et al. 2006). Oidtmann & Stentiford (2011, p. 479) highlighted the risk of these commodity products to naive crustacean populations by stating '...if such shrimp were introduced into a country free from the pathogen, and crustaceans in the receiving country were exposed to infected tissues per os, there is a considerable risk that such exposed crustaceans may become infected and the infection established in domestic populations of crustaceans'.

Stentiford & Lightner (2011) reported the presence of WSSV within European shrimp farms in 2000 after shrimp on these farms were exposed to WSSV-infected shrimp carcasses; however, the virus has not been discovered in wild European crustacean populations to date. WSSV can be successfully transmitted to a range of hosts from European marine and fresh waters. These include the marine crab species *Liocarcinus depurator* and *Necora puber*, the commercially significant marine crab *Cancer pagurus* and freshwater crayfish of the genera *Astacus* and *Pacifastacus* (Corbel et al. 2001, Jiravanichpaisal et al. 2001, 2004). These authors and others noted the unusually large host range for this virus, its potential for infection of freshwater, brackish and marine species and the potential sensitivity of naive European crustaceans to WSSV. These studies also demonstrated the high potential for spread to hosts from European waters, particularly from imported shrimp products and from imported brood stock or larvae for new cultivation ventures in the region and beyond. Furthermore, they demonstrated that temperature may affect host susceptibility and WSSV pathogenicity (Jiravanichpaisal et al. 2004).

In the present study, imported fresh and frozen shrimp products were screened for the presence of WSSV nucleic acid. Imported commodity products were also tested for the potential to act as a source for transmission of WSSV to a known WSSV-susceptible host, *Litopenaeus vannamei*, and an important species in the European crustacean fishery, the European lobster *Homarus gammarus*. Juvenile *H. gammarus* obtained from a commercial hatchery in the UK were exposed to known high-dose (high WSSV viral load) feed at 2 temperatures to determine initial susceptibility of this species. In addition, juvenile *H. gammarus* were fed high-dose WSSV-infected *L. vannamei* carcasses and low-dose (low WSSV viral load), supermarket-derived commodity product. Viral loading in feed was assessed before feeding using quantitative PCR. The susceptibility of juvenile lobsters to WSSV was assessed using histopathology, transmission electron microscopy (TEM) and nested PCR assays specific for WSSV.

MATERIALS AND METHODS

Market sampling and passage conditions

Frozen uncooked shrimp from various global production regions (Table 1, location as defined on the package) were purchased from supermarkets and from a large fish market in the UK. Products were tested for the presence of WSSV using a nested PCR assay recommended by the Office International des Epizooties (OIE 2009) with minor modifications, as described below. Products displaying positivity via PCR were used for production of inoculates and feeds for subsequent passage trials. All passage trials were conducted within the biosecure exotic diseases facility at the Centre for Environment, Fisheries and Aquaculture Science (Cefas) laboratory in Weymouth, UK, and used local, filtered and UV treated seawater. Day length was set at 14 h, night was at 10 h with a 30 min fade to simulate dusk and dawn. Temperature was regulated according to the experimental conditions required for *Litopenaeus vannamei* (Holthuis 1980) and for *Homarus gammarus* as appropriate.

Viability of WSSV in commodity products

Commodity shrimp from Ecuador and Vietnam that had been confirmed positive for WSSV via nested PCR were macerated using a sterile razor blade prior to homogenisation in sterile saline (4 ml of saline per gram of minced tissue) using a blender until tissues were liquefied. The homogenate was centrifuged at 5000 × *g* for 20 min at 4°C to pellet solid debris prior to the supernatant being diluted 1:20 with sterile saline and filtered (0.45 µm) to form the inoculum. Individual specific pathogen free (SPF) *Litopenaeus vannamei* (approximately 5 g in weight) obtained from the Centre for Sustainable Aquaculture Research at the University of Swansea, UK, were inoculated via intramuscular injection of the diluted viral homogenates at a rate of 10 µl g^{-1} shrimp weight. Water temperature was held constant at 24°C. Shrimp were monitored throughout the day for 5 d, and dead and moribund shrimp were removed

Table 1. Commodity shrimp tested for presence of white spot syndrome virus (WSSV) by nested PCR

Species	Origin	Source and description	Nested PCR (% positive)
Litopenaeus vannamei	Ecuador	Supermarket; headless, shell off	65
L. vannamei	Honduras	Supermarket; headless, shell off	80
Penaeus monodon	Indonesia	Supermarket; headless, shell off	0
L. vannamei	Thailand	Supermarket; headless, shell off	5
P. monodon	Thailand	Supermarket; headless, shell off	0
P. monodon	Vietnam	Supermarket; headless, shell off	100
P. monodon	Bangladesh	Market; whole animal	0
P. monodon	Bangladesh	Market; whole animal	0
P. monodon	Bangladesh	Market; headless, shell on	0
L. vannamei	Brazil	Market; whole animal	0
L. vannamei	China	Market; headless, shell on	0
P. monodon	India	Market; whole animal	0
P. monodon	Indonesia	Market; whole animal	0
L. vannamei	Indonesia	Market; whole animal	0
Farfantepenaeus notialis	Senegal	Market; headless, shell on	0
P. monodon	Vietnam	Market; headless, shell on	20

from the experimental tanks; pleopods were fixed in ethanol for molecular analysis, gills were taken for TEM and the carcass was fixed whole in Davidson's seawater fixative for histopathological confirmation of WSD.

Preparation of WSSV positive low- and high-dose feeds

For preparation of high-dose feeds, viral inoculates of WSSV were obtained from the OIE reference laboratory at the University of Arizona (UAZ), USA. The OIE isolate of WSSV (UAZ 00-173B) was generated in *Litopenaeus vannamei* from an original outbreak in *Fenneropenaeus chinensis* (Holthuis 1980) in China in 1995. Subsequent passages of this isolate into naive *L. vannamei* held at the Cefas Weymouth laboratory have demonstrated continued pronounced virulence of this isolate (data not reported here). High-dose WSSV-infected shrimp carcasses were prepared by direct injection of the OIE isolate into SPF *L. vannamei* as detailed above. The viral loading in high-dose feeds was assessed using quantitative PCR (qPCR). Abdominal tissues from *L. vannamei* that were confirmed positive for WSSV via histology and nested PCR were macerated into approximately 2 to 3 mm blocks using a sterile razor blade. For preparation of low-dose feeds, individual supermarket-derived shrimp (abdominal section) originating from Ecuador, Vietnam and Honduras, and confirmed positive for WSSV via nested PCR, were macerated. The viral loading in low-dose feeds was assessed using qPCR.

Lobster feeding trials with low- and high-dose products

Juvenile *Homarus gammarus* were obtained from the National Lobster Hatchery in Padstow, Cornwall, UK. Lobsters were at Moult Stage 4 and were approximately 2 mo of age. To prevent conflict, juvenile lobsters were housed individually in custom-made 'Orkney pots' that were suspended in the upper water column of each 30 l experimental tank. The pots contained a perforated base to allow for water circulation.

In Trial 1, water temperature was held constant at 15°C. Lobsters in Tank 1 (n = 20) received a single ration (~0.05 g) of high-dose WSSV-infected shrimp tissue on Day 0 and a further ration on Day 7. Lobsters in Tank 2 (n = 20) received a single ration (~0.05 g) of uninfected shrimp tissue on Day 0 and another on Day 7. Between these times lobsters were fed on 3 mm Royal Oyster pellets at a ration of approximately 3 to 4% body weight (bw) d^{-1} for 10 d.

In Trial 2, water temperature was held constant at 22°C. Lobsters in Tank 1 (n = 20) received a single ration (0.05 g) of high-dose WSSV-infected shrimp tissue on Day 0 and a further ration on Day 7. Lobsters in Tank 2 (n = 20) received a single ration (0.05 g) of uninfected shrimp tissue on Day 0 and another on Day 7. Thereafter, lobsters in both tanks were fed on 3 mm Royal Oyster pellets at a ration of approximately 3 to 4% bw d^{-1} for 10 d.

In Trial 3, water temperature was held constant at 20°C. On Day 0 lobsters in all tanks (n = 20 for each tank) received a single ration of feed (0.05 g). Lobsters in Tank 1 received high-dose WSSV-infected shrimp tissue, those in Tank 2 received uninfected shrimp tissue, those in Tank 3 received low-dose commodity shrimp originating from Ecuador, those in Tank 4 received low-dose commodity shrimp originating from Vietnam and those in Tank 5 received low-dose commodity shrimp originating from Honduras. Thereafter, lobsters in all tanks were fed on 3 mm Royal Oyster pellets at a ration of approximately 3 to 4% bw d^{-1} for 12 d.

In all trials, tanks were observed regularly throughout daylight hours. Dead and moribund animals were removed from each tank. Cheliped samples from all dead, terminally morbid and live (at the end of the trial period) animals were fixed in 100% ethanol for PCR. Remaining carcasses were prepared for histology and, selectively, for TEM. Juvenile lobsters were prepared for histology by making an incision along each side of the carapace, placing whole animals into histological cassettes and fixing them immediately in Davidson's seawater fixative. From selected juvenile lobsters, the abdomen was removed from the cephalothorax and the carapace opened as described for histology. The abdomen was fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at room temperature; cuticular epithelium and gills were dissected out of these fixed animals and processed for TEM.

Histology

For all tissues, fixation was allowed to proceed for 24 h before samples were transferred to 70% industrial methylated spirit. Fixed samples were dehydrated and infiltrated with paraffin wax in a vacuum infiltration processor using standard protocols. Sections were cut at a thickness of 3 to 5 µm on a rotary microtome and were mounted onto glass slides

before staining with haematoxylin and eosin (H&E) and Feulgen stains. Stained sections were analysed by light microscopy (Nikon Eclipse E800) and digital images and measurements were taken using the Lucia™ Screen Measurement System (Nikon UK).

Transmission electron microscopy

Tissues were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at room temperature and rinsed in 0.1 M sodium cacodylate buffer (pH 7.4). Tissues were post-fixed for 1 h in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. Samples were washed in 3 changes of 0.1 M sodium cacodylate buffer before dehydration through a graded acetone series. Samples were embedded in Agar 100 epoxy (Agar 100 pre-mix kit medium, Agar Scientific) and polymerised overnight at 60°C in an oven. Semi-thin (1 to 2 µm) sections were stained with toluidine blue for viewing under a light microscope to identify suitable target areas. Ultrathin sections (70 to 90 nm) of these areas were mounted on uncoated copper grids and stained with 2% aqueous uranyl acetate and Reynolds' lead citrate (Reynolds 1963). Grids were examined using a JEOL JEM 1210 transmission electron microscope and digital images captured using a Gatan Erlangshen ES500W camera and Gatan Digital Micrograph™ software.

Detection of WSSV in commodity shrimp via PCR and qPCR

Total shrimp DNA was extracted from the commodity product (tail muscle) using the High Pure PCR Template Preparation Kit (Roche Diagnostics) following the manufacturer's protocol for the isolation of nucleic acids from mammalian tissue. The quality and quantity of the DNA was determined with a Nanodrop spectrophotometer (Thermo Fisher Scientific). For use in the qPCR, prior to analysis, total DNA was standardised at 20 ng μl^{-1} to remove variations in viral load owing to variation in tissue weight or extraction efficiency between samples.

Nested PCR for high-dose fed lobsters

WSSV DNA presence was assessed by using the nested PCR assay of Lo et al. (1996) with minor modifications (B. Poulos, UAZ, pers. comm.). First, a product of 1447 bp was amplified using the primer pair 146F1 (5′-ACT ACT AAC TTC AGC CTA TCT AG-3′) and 146R1 (5′-TAA TGC GGG TGT AAT GTT CTT ACG A -3′), followed by an approximate 941 bp product in the nested reaction using primer pair 146F2 (5′-GTA ACT CCC CCT TCC ATC TCC A-3′) and 146R2 (5′TAC GGC AGC TGC TGC ACC TTG T-3′). For the first round of amplification (primer pair 146F1/146R1) each 25 µl PCR reaction contained the following: 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 200 µM of each dNTP, 0.31 µM of each primer, 2.5 U *Taq* polymerase and 1 µl genomic DNA (20 to 50 ng total). Amplifications were performed with an initial denaturation temperature of 94°C for 2 min, followed by 30 cycles at 94°C for 30 s, 62°C for 30 s and 72°C for 30 s, with a final elongation step at 72°C for 2 min. Reaction conditions and reagent concentrations were the same for the second round of amplification using the 146F2/146R2 primer pair; however, 0.5 µl of the first round of amplification was used as a template in place of genomic DNA. Amplified products were resolved on a 2% (w/v) agarose/TAE (40 mM Tris-acetate, pH 7.2, 1 mM EDTA) gel containing 1.0 lg ml^{-1} ethidium bromide and visualised under UV irradiation.

Nested PCR for commodity product and low-dose feed lobsters

The initial PCR method described above was modified to include an internal control to ensure false negatives did not occur without displaying any noticeable reduction in sensitivity (data not shown). To ensure validity of each sample, an internal control was included in each PCR sample. The internal control, consisting of a single primer set 5′-GTG GAC ATC CGT AAC CAC CTG TAC G-3′ (forward) and 5′-CTC CTG CTT GCT GAT CCA CAT CTG C-3′ (reverse), amplified a 201 bp product specific towards beta-actin, which is found in all eukaryotic cells. Both outer and inner nested PCR was performed in a 50 µl reaction volume containing 0.15 µM of primers, 0.25 µM dNTPs, 1.25 U GoTaq (Promega), 2.5 mM $MgCl_2$, 5× buffer and 2.5 µl of template. Amplification was performed as above.

qPCR

In individual shrimp demonstrating positivity for WSSV via nested PCR, and to ensure suitability for further utilisation for the feeding trial to naive *Homarus gammarus*, a DNA fragment of 69 bp representing the upstream primer plus the probe and the

downstream primer was cloned into the pGEM-T easy vector (Promega) and then transformed into JM109 competent cells (Promega) following the manufacturer's instructions. The plasmid was extracted using QIAprep spin Miniprep Kit (Qiagen) and the concentration was determined using a Nanodrop spectrophotometer. The copy number of the plasmid DNA containing the 69 bp insert was estimated and a series of dilutions were made to use as standards. The primers and probe used for the quantification of WSSV in study were developed by Durand & Lightner (2002). The primers were WSS1011F (5′-TGG TCC CGT CCT CAT CTC AG-3′) and WSS1079R (5′-GCT GCC TTG CCG GAA ATT A-3′). The TaqMan probe (5′-AGC CAT GAA GAA TGC CGT CTA TCA CAC A-3′) was synthesized and labeled with the fluorescent dyes 5-carboxyfluroscein (FAM) on the 5′ end and N,N,N′,N′-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3′ end. The TaqMan assay used was adapted from Durand & Lightner (2002). Briefly, 100 ng of total DNA was added to TaqMan Universal mastermix (Applied Biosystems) containing 0.3 µM of each primer and 0.15 µM of TaqMan probe in a final volume of 25 µl. Amplification and detection were performed using an ABI Biosystems TaqMan machine. The reaction mix was subjected to an initial temperature of 50°C for 2 min, then 1 cycle at 95°C for 10 min, 50 cycles at 95°C for 15 s and 60°C for 1 min. Quantification of the number of WSSV copies in samples to be used for feeding trials were determined by measuring C_t values and using the standard curve to determine the initial copy number per nanogram total DNA. Each unknown sample was analysed in triplicate and the mean calculated.

Sequencing

Selected reactions were analysed on an ABI 3130 Avant Genetic Analyser to confirm the specificity of the PCR. The final product was compared with known sequences using Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) to determine phylogenetic homology. This is in line with the OIE confirmatory technique for WSSV (Claydon et al. 2004).

RESULTS

Commodity shrimp product screening for WSSV

The prevalence of WSSV in commodity shrimp imported into the UK for human consumption ranged from 0 to 100%. Of the batches of shrimp purchased from supermarkets, 66% (4/6) tested positive for WSSV, and the within-bag prevalence ranged from 0 to 100%. Of the shrimp samples purchased from fish markets, 10% (1/10) were also positive for WSSV (Table 1).

Viability of WSSV in commodity shrimp products

Litopenaeus vannamei injected with homogenised WSSV positive commodity shrimp from either Ecuador or Vietnam experienced 100 and 40% mortality, respectively, within 3 d post-injection (Fig. 1). Nested PCR analysis (Fig. 2) indicated that all shrimp injected with commodity inoculate from Ecuador were positive for WSSV, and 4 of these animals displayed infection detectable in the first round of PCR, indicating a pronounced disease status (Fig. 2A). Shrimp injected with commodity inoculate from Vietnam were similarly all positive for WSSV (Fig. 2B), but only weak bands were present in the second round of PCR, suggestive of a lower level infection. Histological examination of these shrimp demonstrated the characteristic pathology associated with WSSV infection (Fig. 3); shrimp injected with commodity inoculate from Ecuador displayed signs of advanced WSD, whilst shrimp injected with commodity inoculate from Vietnam displayed lower grade, albeit characteristic, lesions associated with WSSV infection. Shrimp displaying advanced WSD identified from histopathology were further selected for TEM analysis. Electron microscopy revealed large

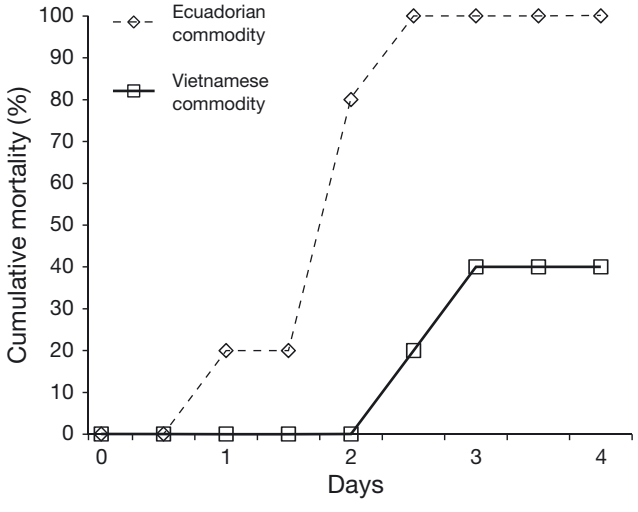


Fig. 1. *Litopenaeus vannamei*. Mortality data of shrimp exposed to WSSV

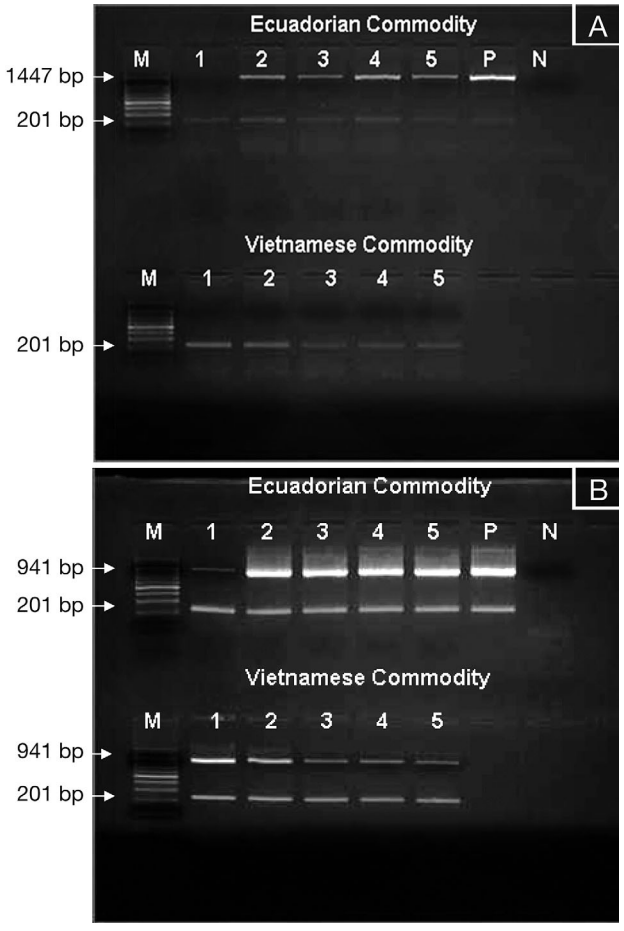


Fig. 2. PCR results from bioassay of *Litopenaeus vannamei* injected with homogenates of WSSV-infected commodity shrimp. (A) First step of nested PCR. Ecuadorian commodity: 4/5 weak positive for WSSV; Vietnamese commodity: 0/5 positive for WSSV. (B) Second step of nested PCR. 201 bp represents decapod-specific DNA; 941 bp represents WSSV. Ecuadorian commodity: 4/5 strong positive for WSSV; Vietnamese commodity: 5/5 weak positive for WSSV. M: 100 bp DNA ladder; P: positive control for WSSV; N: negative control for WSSV

numbers of rod-shaped virions synonymous with WSSV in epithelial tissues (Fig. 3E,F). Manifestation of WSD in SPF shrimp injected with commodity products derived from supermarkets and fish markets confirmed the viability of WSSV in such products.

Viral loading in low- and high-dose feeds

The WSSV-infected shrimp used in the high-dose challenge had an equivalent viral loading of 3.65×10^5 copies ng^{-1} total DNA. The supermarket-derived commodity shrimp used in the low-dose feed challenge had equivalent viral loadings of 5.16×10^2, 4.68×10^1 and 1.04×10^2 copies ng^{-1} total DNA in the Honduran, Ecuadorian and Vietnamese shrimp, respectively.

Lobster feeding trials: high-dose products

Cumulative mortality of WSSV-fed lobsters in Trial 1 (15°C) reached 5% on Day 1 and 10% by Day 10 of the trial. In Trial 2 (22°C), cumulative mortality of WSSV-fed lobsters reached 40% by Day 3 and 55% by Day 6. No further mortalities occurred between Day 6 and Day 10. Cumulative mortalities in control tanks in Trials 1 and 2 reached 15% by the end of the 10 d trial (Fig. 4).

Two moribund or recently dead lobsters in Trial 1 (15°C) and 8 in Trial 2 (22°C) displayed histopathological lesions typical of WSD in other crustacean species, including penaeids. WSD-associated lesions were most apparent in the gill, haemopoietic centres, haemocytes, connective tissues, stomach epithelium and particularly the cuticular epithelium. Cellular changes in each of these target sites included nuclear hypertrophy, margination of chromatin at the nuclear periphery and the presence of a single basophilic to strongly eosinophilic inclusion that occupied a large proportion of the host nucleoplasm. In some cases, this inclusion was separated from the nuclear periphery by an unstained zone (Fig. 5A–E). Feulgen-positive staining of affected nuclei demonstrated the DNA composition of the inclusion and of the darkly staining nuclear periphery (Fig. 5F). The connective tissues and cuticular epithelium of the limbs appeared to be particularly heavily affected with an apparent loss of tissue mass in these regions and the presence of necrotic cellular debris and remnant WSSV inclusions. The hepatopancreas, midgut and skeletal muscle of infected lobsters appeared to be unaffected. Of those lobsters that were fed WSSV-infected material and survived the experimental period, none exhibited the pathologies described above.

Semi-thin sections (Fig. 6A) and TEM sections (Fig. 6B–F) of lobsters displaying the aforementioned pathologies confirmed the presence of intranuclear inclusions and oval-shaped viral particles, typical of WSSV, within epithelial cell nuclei. Viral particles were elliptical to short-rod shaped in longitudinal sections and round in transverse sections (Fig. 6C,D). Virions consisted of an electron dense capsid, bound by a clear envelope, surrounded by a double membrane. Virions at various stages of development and apparent assembly could be observed embedded within a granular matrix that corresponded to a viral

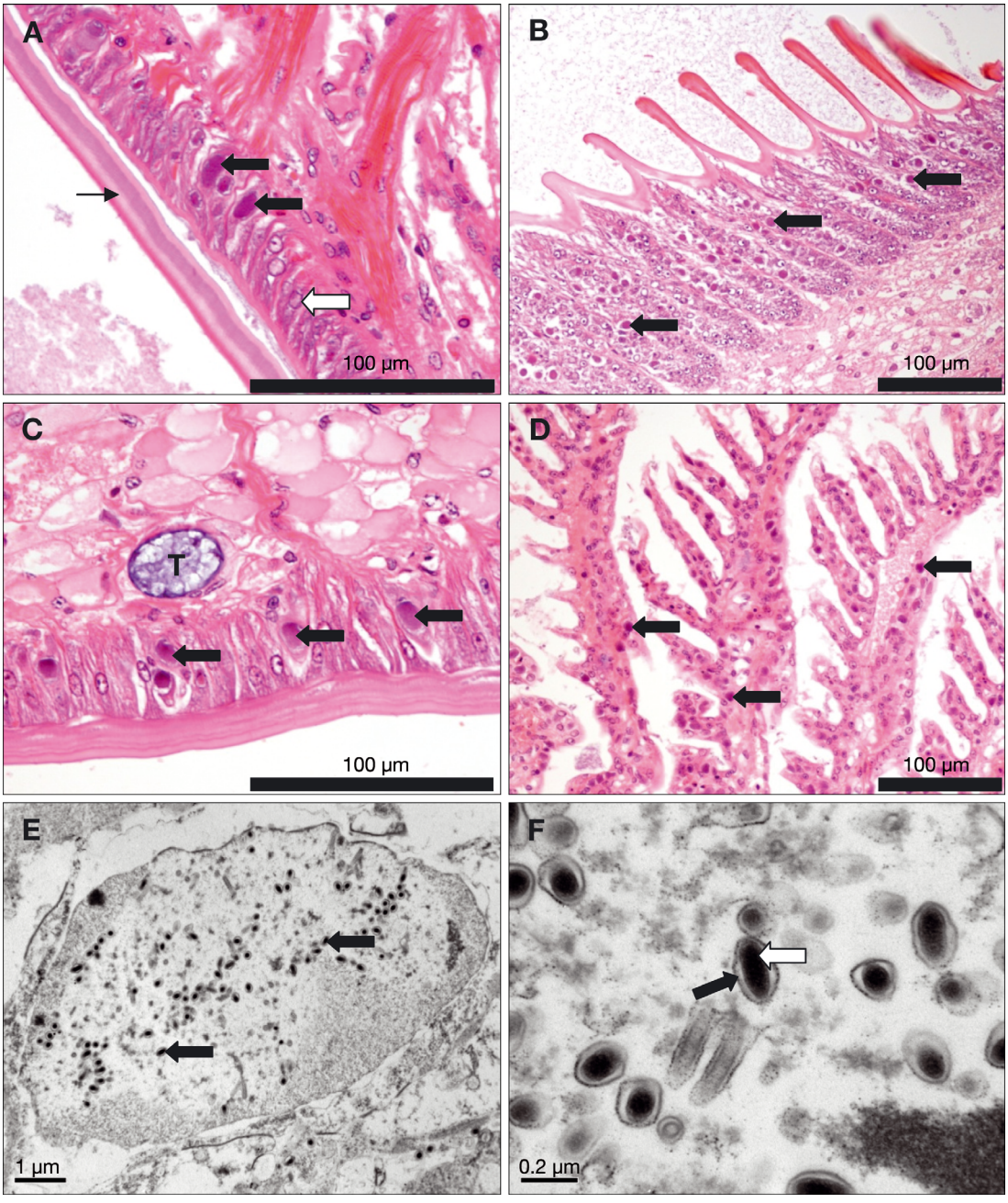


Fig. 3. *Litopenaeus vannamei*. Shrimp infected with WSSV. (A) Carapace (narrow black arrow) and columnar epithelial cells (white arrow) showing enlarged nuclei with dense staining (wide black arrows). (B) Stomach epithelium showing hypertrophied nuclei with eosinophilic staining (arrows). (C) Enlarged infected nuclei (arrows) within epithelial cells, also showing tegmental gland (T). (D) Gills with enlarged, infected nuclei (arrows) distributed throughout the cuticular epithelium. (A–D) Sections stained with H&E. (E) Transmission electron (TE) micrograph showing infected cell within the gills. Numerous viral particles can be seen within the nucleus of the cell (arrows). (F) TE micrograph of longitudinal and transverse sections of viral particles within the nucleus. Electron dense nucleocapsid (white arrow) is surrounded by a trilaminar envelope (black arrow)

stroma within host nuclei. The presence of free capsid material (Fig. 6E) and a long rod-shaped structure (Fig. 6F), corresponding to the viral nucleosome observed in other studies of WSSV, was also noted. Completed virions measured 280 ± 5.5 nm (mean ± SE) in length and 116 ± 1.4 nm in width (n = 30), consistent with measurements of 270 to 300 nm × 90 to 120 nm previously described for WSSV.

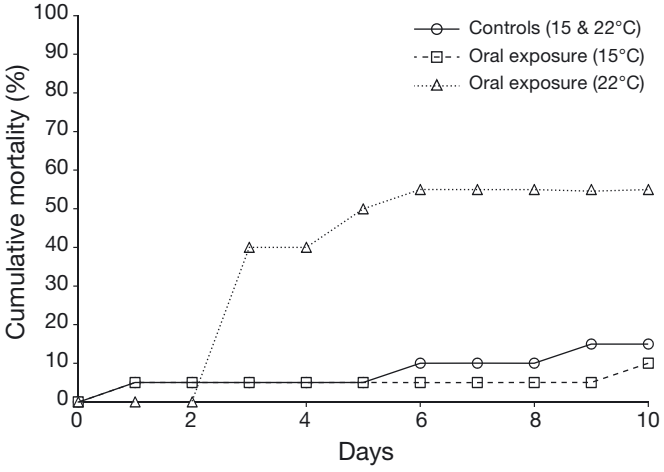


Fig. 4. *Homarus gammarus.* Mortality data of lobsters held at different temperatures after oral exposure to WSSV

Lobster cheliped tissues used for DNA extraction and nested PCR yielded high quality host genomic DNA as indicated by a characteristic 1800 bp amplification product using the universal SSU rRNA gene primers. All WSSV-challenged lobsters (n = 18) held at 15°C were positive for WSSV by nested PCR. Of the WSSV-challenged lobsters held at 22°C, 80% (16/20) were positive for WSSV by nested PCR. No WSSV-positive results were obtained from the negative control group samples.

Lobster feeding trials: low-dose products

Cumulative mortality of lobsters in all treatment tanks varied between groups. Lobsters fed the positive control diet (high-dose WSSV-infected *Litopenaeus vannamei*) underwent 41% mortality while lobsters in the negative control tank (fed with SPF *L. vannamei*) underwent 17% mortality. All negative control lobsters that died during the experiment were undergoing moult at the time of death. Mortality rates in lobsters fed low-dose, supermarket-derived commodity shrimp were 0, 20 and 22% for the feed prepared from Honduran, Ecuadorian and Vietnamese shrimp, respectively. Histological examination of low-dose fed lobsters did not reveal any of the characteristic signs of WSD as observed in penaeid shrimp or in high-dose fed lobsters. However, characteristic signs of WSD were observed once more in the positive control high-dose fed lobsters, particularly in the antennal gland and gill epithelium (Fig. 7). TEM of tissues from positive control lobsters once again revealed the rod-shaped viral particles typical of WSSV within the nucleus of infected cells (Fig. 7G,H). Despite the lack of cellular pathology associated with WSD, nested PCR carried out on samples from the low-dose study revealed that almost 100% (16 of 17) of lobsters fed positive control shrimp were positive for WSSV (Fig. 8A). In addition, 70% of the lobsters fed supermarket-derived commodity shrimp from Honduras displayed positivity in the second round of PCR (sub-patent infection) whilst lobsters fed with supermarket-derived commodity shrimp from Ecuador and Vietnam displayed 30 and 45% positivity for WSSV in the second round of PCR, respectively (Fig. 8B–D). Sequencing of the nested PCR amplicon from one lobster in each of the treatment tanks followed by analysis using BLAST confirmed with at least 99% homology that the amplicons were from WSSV (GenBank accession AF332093.1) in all cases tested.

DISCUSSION

As expected from similar surveys in the USA and Australia, this small-scale survey of imported penaeid shrimp commodity products from UK supermarkets and fish markets has revealed an apparent widespread prevalence of WSSV-contaminated products. This study demonstrated that frozen commodity shrimp is a route of entry for WSSV into European member states. We have also demonstrated that the WSSV contaminated shrimp tissues contain viable virions that can be transmitted to naive susceptible hosts via injection into *Litopenaeus vannamei* and by feeding these shrimp to *Homarus gammarus*. Despite the limitations of the survey design, the underlying within-batch prevalence of the WSSV was relatively high, though in individual components of these batches (e.g. single abdominal sections), the virus was present at a relatively low viral titre and was certainly lower than positive control material generated by passage bioassays to shrimp within our laboratory. The present study has also revealed relatively high batch prevalence for WSSV in supermarket-derived commodity shrimp tested using PCR. The prevalence was apparently lower in shrimp sampled from a large fish market, possibly owing to the larger size of specimens obtained from this location. Albeit limited, these preliminary results demonstrate a potentially large variation in the presence of WSSV in batches of commodity shrimp between type of importer and country of origin as demonstrated in Table 1. For example, the batch of shrimp imported from Vietnam for sale in supermarkets had a within-batch prevalence of 100% while shrimp imported from the same country but sold at the

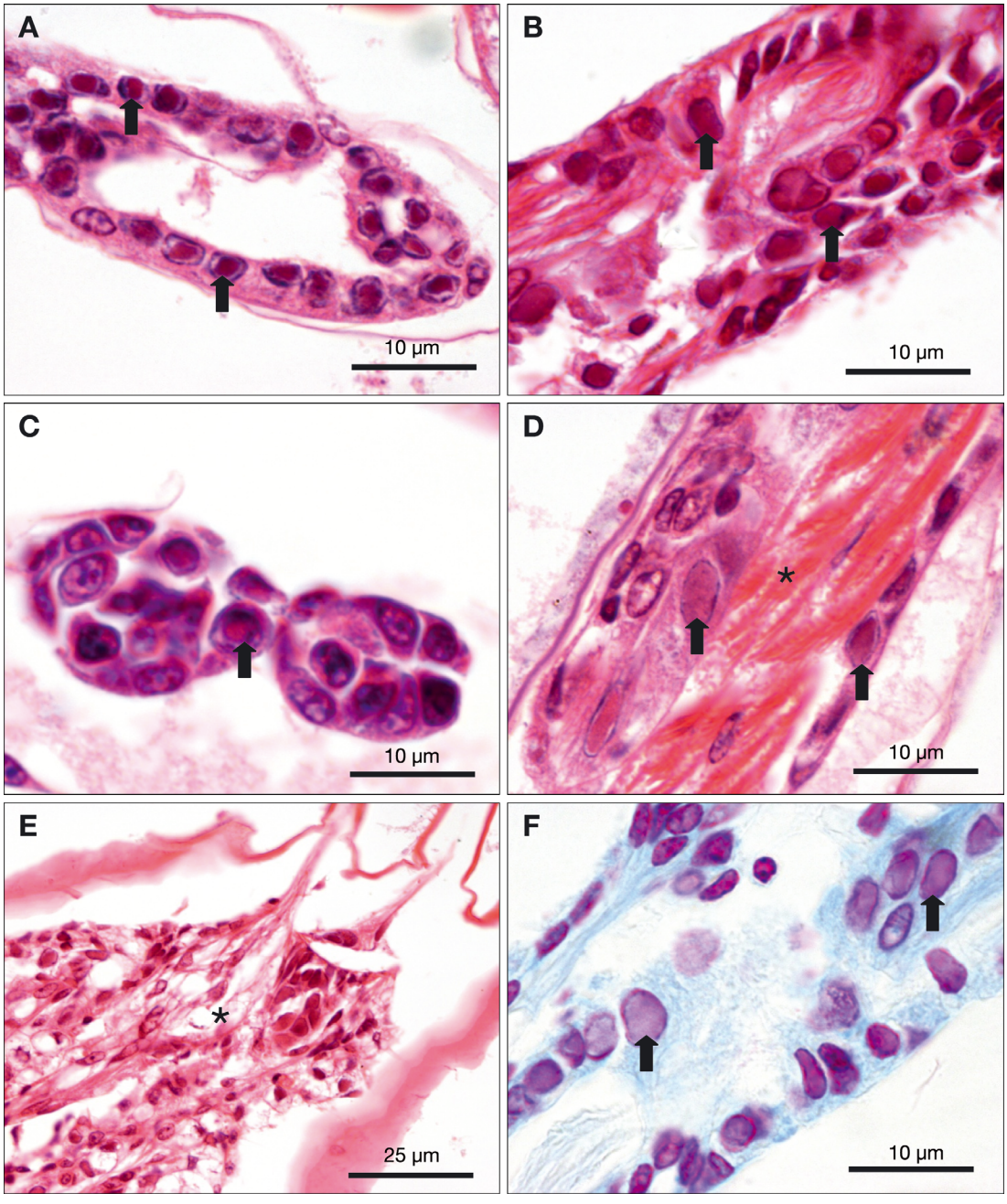


Fig. 5. *Homarus gammarus.* Histopathology of WSSV infection. (A) Transverse section of gill secondary lamellae. Infected epithelial cell nuclei containing eosinophillic inclusions (arrows). (B) Epidermis and connective tissue at extremity of walking limb. Hypertrophic nuclei with marginalized chromatin (arrows). (C) Haemopoeitic cluster with infected germinal cells (arrow). (D) Mid section of walking limb with infected connective tissue and epidermal cells (arrow) but absence of infected nuclei within skeletal muscle (*). Scale bar = 10 µm. (E) Extremity of telson containing infected connective tissue and epidermal cells (asterisk). (F) Hypertrophic nuclei borders and nucleoplasm of epidermal and connective tissue cells within walking limb (arrows). Sections stained with (A–E) H&E or (F) Feulgen stain

fish market demonstrated a within-batch prevalence of 20%. The variation in WSSV batch and within-batch prevalence may reflect a focus on sale of relatively small shrimp (bags containing shrimp of approximately 10 g original size) by supermarkets and a contrasting focus on larger, whole animals (15 to 20 g) with head and shell on at fish markets. Whether the presence of smaller animals in batches from super-

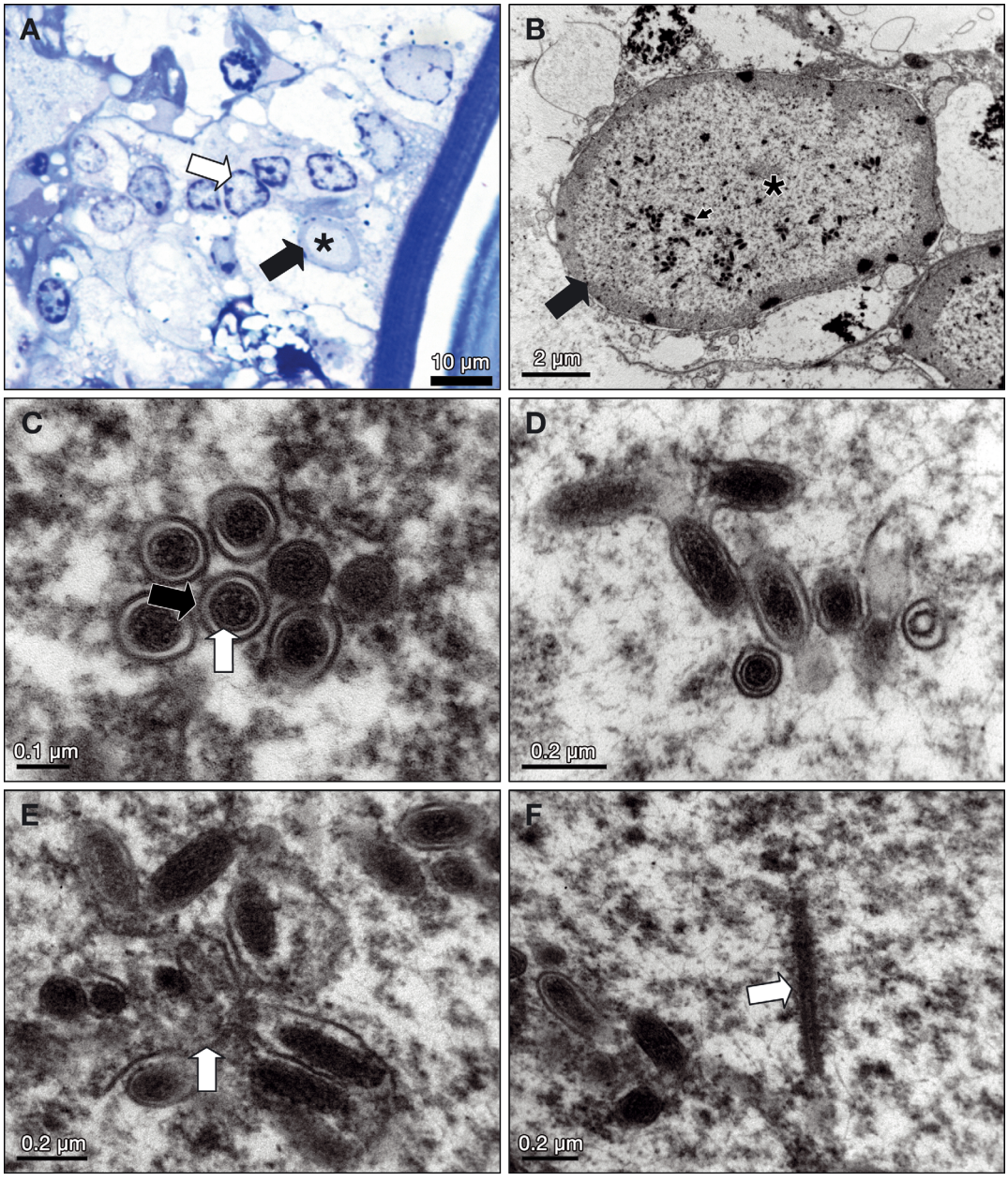
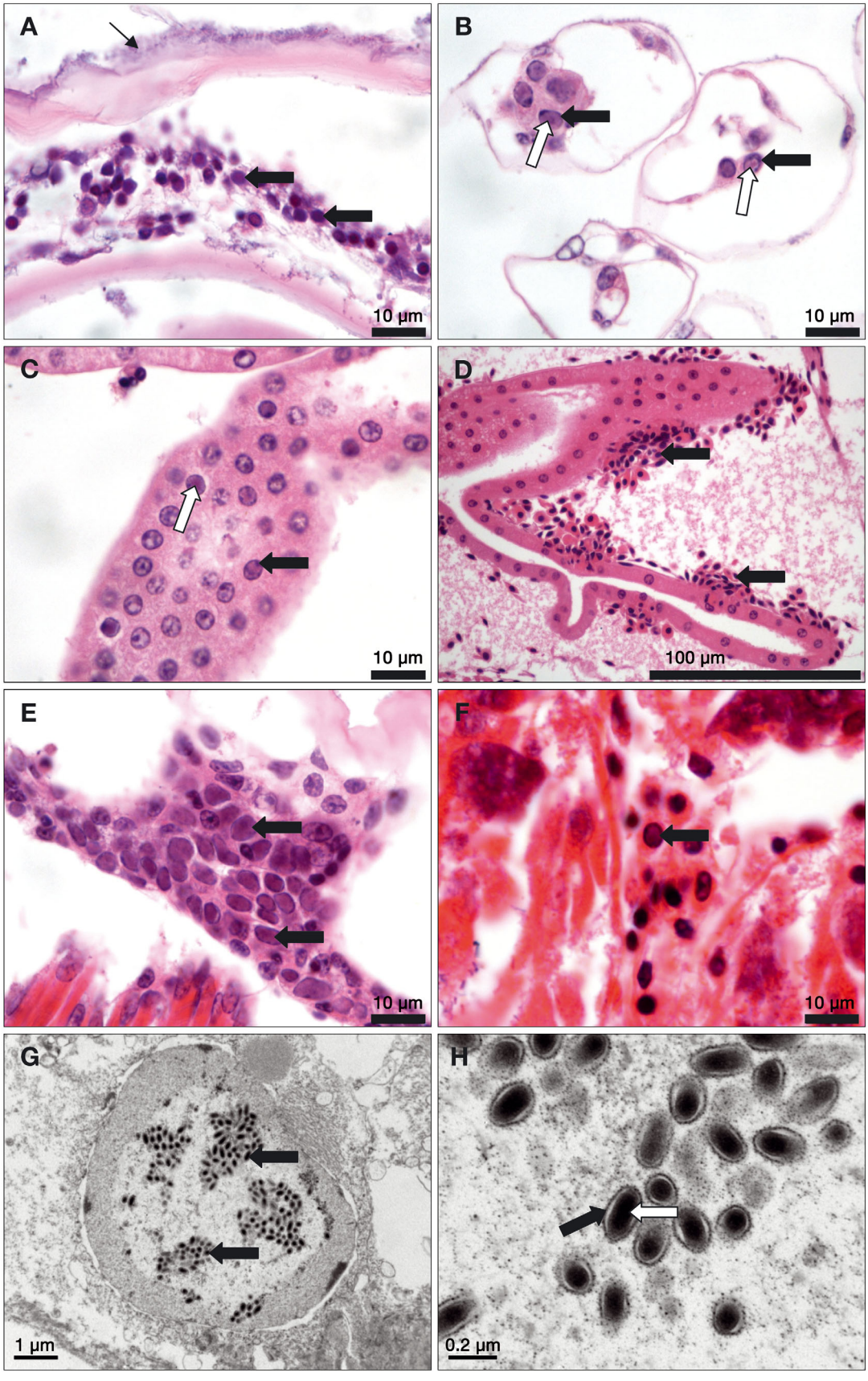


Fig. 6. *Homarus gammarus.* WSSV infection in epidermis. (A) Semi-thin section of epidermis. Viroplasm (*) can be seen within the hypertrophied WSSV-infected nuclei (black arrow). Uninfected nuclei (white arrow) are also present. Toluidine blue stain. (B) Transmission electron (TE) micrograph of nucleus within the epidermis infected with WSSV. Nuclei were enlarged with marginalised chromatin (arrow) and contained viral particles (arrowhead) within the viroplasm (*). (C) TE micrograph of a cross section of WSSV virions within the nucleus. Virions consist of an electron-dense capsid within an envelope (white arrow) that is surrounded by a double membrane (black arrow). (D) TE micrograph of longitudinal and cross sections of WSSV virions in various stages of development within the nucleus. (E) TE micrograph showing capsid material free within the viroplasm (white arrow) surrounding viral particles in various stages of development. (F) TE micrograph of long rod-shaped structure (white arrow) within the viroplasm of WSSV-infected nucleus

markets represents a higher propensity for supermarket shrimp to be derived from so-called 'emergency harvest' (rapid harvesting of shrimp from culture facilities when it is suspected that a mortality event may be impending), remains to be ascertained. The rapid harvesting of animals essentially averts financial losses but may lead to higher viral loading in harvested animals (Flegel 2009).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H
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Fig. 7. *Homarus gammarus*. Lobster infected with WSSV. (A) Carapace (narrow black arrow) and epithelial cells with marginated chromatin and eosinophilic staining (wide black arrows). (B) Transverse sections of secondary gill filaments showing marginated chromatin (black arrows) and eosinophillic inclusions (white arrows) within nuclei. (C) Antennal gland showing infected nuclei with marginated chromatin (black arrow) and eosinophillic inclusions (white arrow). (D) Antennal gland with haemocyte aggregations (arrows). (E) Haematopoietic tissue with numerous enlarged nuclei showing marginated chromatin and dense, uniform staining (arrows). (F) Connective tissues with infected nuclei displaying dense uniform staining and marginated chromatin (arrow). (A–F) Sections stained with H&E. (G) Transmission electron (TE) micrograph of infected cell within the gills. Numerous viral particles can be seen within the nucleus of the cell (arrows). (H) TE micrograph of viral particles within the nucleus. Electron-dense nucleocapsid (white arrow) is surrounded by a trilaminar envelope (black arrow)

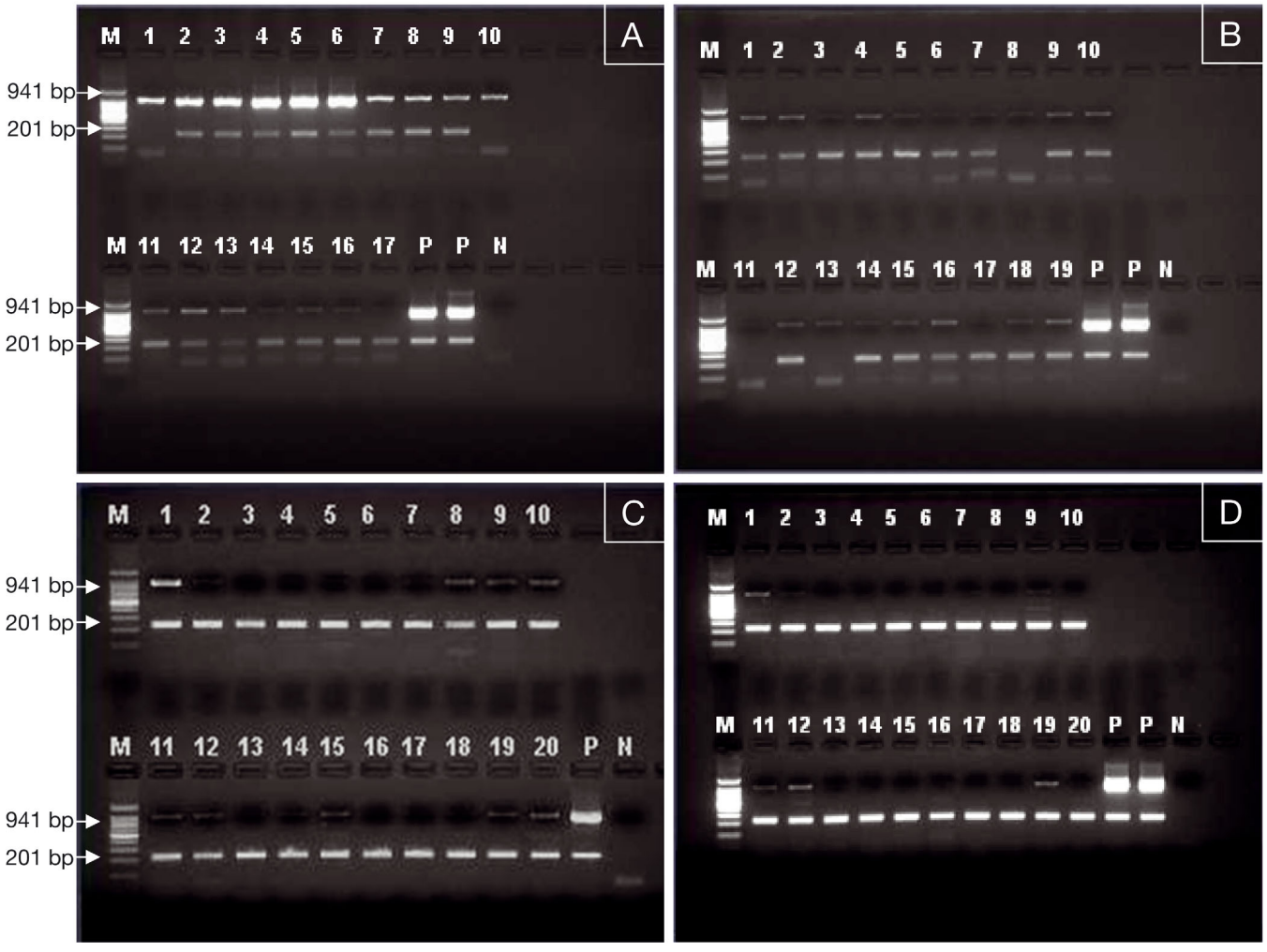

Fig. 8. Nested PCR results from bioassay of *Homarus gammarus* fed on WSSV-infected commodity shrimp. (A) Positive control tank; 16 of 17 positive for WSSV. (B) Honduran commodity-fed tank; 13 of 20 positive for WSSV. (C) Ecuadorian commodity-fed tank; 5 of 20 positive for WSSV. (D) Vietnamese commodity-fed tank; 10 of 20 positive for WSSV. M: 100 bp DNA ladder; P: positive control for WSSV; N: negative control for WSSV. 201 bp represents decapod-specific DNA; 941 bp represents WSSV DNA

Central to any national import risk assessment for commodity products contaminated with pathogens such as WSSV, is an assessment of the likelihood for a pathogen to establish in naive susceptible hosts if the pathogen is released in the receiving location. Considering normal shrimp-farming scenarios (i.e. non-emergency harvest), it has been reported that cultivated shrimp can carry viruses (such as WSSV) as a low level (latent) infection without showing gross signs of disease for relatively long periods of time, provided that rearing conditions are favourable (Flegel et al. 2004). However, a change in

physiological conditions, often due to environmental changes, can allow for rapid replication of the virus and the onset of disease. Whilst the loading of WSSV in commodity shrimp products analysed in the present study was apparently low in most cases, suggesting latent or sub-patent infection in those hosts, the virus remained viable and was able infect and kill SPF *Litopenaeus vannamei* within a few days of exposure (albeit by a non-natural route of transmission). However, feeding trials in which lobsters were fed with the same low-dose commodity products also demonstrated that passage of WSSV could occur via this natural route and that infection could establish, albeit in latent form, in naive hosts (lobsters) held at temperatures experienced within Europe. Since infection progressed rapidly to disease in lobsters fed with high-dose products (positive control), particularly at higher temperatures, the limiting factor in the rapid appearance of WSD in lobsters is therefore the initial dose; a low-level infectious dose establishes latent infection and a high-level dose progresses more rapidly to disease. Fundamental studies are now required to assess the potential for latent infections to progress to a disease state and cause mortality in lobsters retained at European temperatures. Such data are vital for accurate consequence assessment following release and establishment of WSSV infections in wild populations.

Results from the high-dose WSSV feed trial confirm the susceptibility of juvenile European lobsters to WSSV. Lobsters were fed with WSSV-infected carcasses of *Litopenaeus vannamei* and were shown to succumb to the disease within the time course of the experiment (10 d). However, not all of the lobsters fed WSSV-positive commodity shrimp products became infected with the virus. This could be due to a number of reasons. Firstly, although the lobsters were provided with the same sized ration originating from the same individual shrimp (per treatment tank), some animals did not consume all of the food provided. In addition, since the shrimp were not homogenised prior to feeding but rather, fed as blocks of tissue, the concentration of virus may also have varied in specific meals (Soto et al. 2001). However, the result may also indicate an inherent variation in susceptibility to viral infection between individuals of the same species. The concept of 'susceptibility' in hosts should therefore not only be considered at the species level but also at the level of the individual within that species; this is particularly important in animals that display non-continuous growth and that must moult at relatively short intervals. Differential pathogen prevalence related to the crustacean moult cycle has previously been demonstrated in field studies (Stentiford et al. 2001). In such cases, differential susceptibility probably relates directly to immune suppression around the time of ecdysis and in relation to other stressful events (Le Moullac & Haffner 2000). Further, it is clear that European species such as *Homarus gammarus* can become infected with WSSV following ingestion of a single contaminated meal, though factors such as viral loading and host age or condition appear to be key variables in assessing the likelihood of progression from an infection to a disease state. We have also shown that in *H. gammarus*, the development of WSD and associated cumulative mortality is enhanced when animals are held at 22°C rather than 15°C. Temperature can also affect the development of WSD in penaeid shrimp and in crayfish, causing reduced mortality rates and lower viral loads when hosts are retained in both hypothermic and hyperthermic conditions. Interestingly, in both scenarios disease developed rapidly when infected individuals were returned to optimal conditions for viral replication (Vidal et al. 2001, Granja et al. 2003, 2006, Jiravanichpaisal et al. 2004, Du et al. 2006, 2008, Rahman et al. 2006, 2007a,b). Whether infection would continue in a latent state in lobsters retained at 15°C for longer periods of time, or would progress to disease via exposure of infected hosts to external stressors, will govern the potential for WSSV to sustain a presence in natural aquatic environments of temperate regions.

Only one naturally occurring virus infection has been documented in lobsters. *Panulirus argus* virus 1 (PaV1) infects the spiny lobster species *P. argus* from the Florida Keys, USA, and the Caribbean (Shields & Behringer 2004). To date, no viral infections have been described from homarid lobsters. Whilst this may be due to a general lack of comprehensive disease surveys or a sampling bias away from juvenile life stages (as suggested in studies on crabs by Bateman & Stentiford 2008 and Bateman et al. 2011), it also suggests some inherent non-susceptibility to viruses in this group. The latter is now apparently unlikely owing to the rapid pathogenesis of WSSV in juvenile *Homarus gammarus* demonstrated during this study. Pathogenicity of WSSV has also been investigated in the spiny lobster species *P. versicolor*, *P. penicillatus*, *P. ornatus*, *P. longipes*, *P. homarus* and *P. polyphagous* (Chang et al. 1998, Rajendran et al. 1999, Musthaq et al. 2006). Chang et al. (1998) reported that lobsters fed WSSV-

contaminated feed did not succumb (although WSSV was detected within their tissues), while Rajendran et al. (1999) provided some evidence that WSSV infection via feeding and ingestion could result in mortalities of *P. polyphagous* and *P. ornatus*. However, in a follow-up study by Musthaq et al. (2006), *P. homarus* and *P. ornatus* fed WSSV-contaminated feed did not die, while those that were injected with WSSV did. These studies demonstrate the likelihood that spiny and homarid lobsters are susceptible to viral infections and that investigations into the presence of natural viral infections in field-caught lobsters, particularly juvenile life stages, may prove fruitful.

In terms of import risk assessment for the trading of shrimp commodity products, the risks associated with viral release through shrimp packaging and reprocessing plants in importing countries has previously been highlighted (Nunan et al. 1998, Durand et al. 2000). Shrimp imported for human consumption can also be diverted into alternative uses. The use of raw, frozen shrimp products as angling bait has been identified as a relevant risk for introduction of viral crustacean pathogens in Australia (Biosecurity Australia 2006). Furthermore, there is anecdotal evidence (e.g. via online forums) that this practice is also relatively commonplace in the UK (and probably other parts of Europe). A recent questionnaire sent to a subset of the UK angling community (thought to exceed 4 million individuals) suggested that up to 7% of these may use frozen shrimp products as bait (B. Oidtmann pers. comm.). This increased use appears to be directly associated with the current price competitiveness between frozen, imported shrimp commodity and other types of specialist angling bait, as well as the ease of purchase through supermarkets.

Since per os feeding represents a realistic route of entry for pathogens (such as WSSV), further work is now required to investigate the likelihood for pathogen transmission between latently infected lobster conspecifics and also between infected lobsters and other non-penaeid decapod hosts. Only when such studies are carried out will it be possible to determine the potential for the establishment of WSSV in wild populations of decapods residing in aquatic habitats of temperate regions. Further work is also required to assess the potential for differential risk associated with commodity imported from particular regions, particularly where approaches to within-country biosecurity or emergency harvesting of infected shrimp ponds are likely to generate products with high disease status.

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