Nervous necrosis virus in wild Mediterranean Sea fish species: molecular characterization and prevalence of viral infection

Ran Berzak

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE MASTER'S DEGREE

University of Haifa

Faculty of Natural Sciences

Leon H. Charney School of Marine Science

The Department of Marine Biology

November, 2018

I

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Nervous necrosis virus in wild Mediterranean Sea fish species: molecular characterization and prevalence of viral infection

Ran Berzak

Abstract

Infectious diseases in marine organisms have ecological, socio-economic and environmental impacts. The RNA virus nervous necrosis virus (NNV) is the causative agent of viral nervous necrosis, a disease result in high mortality rates that affect fish species worldwide and is a major threat in Mediterranean aquaculture. Susceptibility of the host species to the virus depends on the viral genotype, some hosts have proven to be susceptible while others considered resistant to the virus and may serve as contagious host. However, despite their importance, there is lack of basic knowledge regarding the natural prevalence of this virus and other pathogens in wild fish species. Therefore, a pathogenic survey was conducted to quantify the prevalence level and to characterize the NNV genotypes in wild species from the Levantine basin. As part of the survey, the presence of *Streptococcus iniae* was analyzed as well. This bacterium is the etiological agent of streptococcosis in fish, a disease affect marine and freshwater species and has zoonotic potential.

Indigenous and Lessepsian species from different trophic levels and various biological niches were sampled from the Israeli Mediterranean coastline. Molecular amplification methods were used for the detection of both pathogens. A total of 174 fish and 32 crustaceans were tested for *S. iniae*, and a total of 195 fish and 33 crustaceans were tested for NNV.

I found an overall prevalence of 9.71% *Streptococcus* species and 21.49% NNV in selected marine fish and crustaceans with high variation between species. Prevalence of NNV in Lessepsian species was relatively higher than in the Mediterranean indigenous species. Co-infection of both pathogens was detected in only five specimens. In order to assess the possibility of horizontal pathogens transmission from wild fish to maricultured fish, I also examined gilthead sea bream (*Sparus aurata*) from an Israeli offshore marine farm during its grow-out period. Three out of 15 (20%) fish were found to be NNV positive after 120 days in the sea, suggesting a spontaneous transmission from wild to farm fish.

Follow this survey, an additional study was conducted in order to compare if there is a difference in the prevalence of NNV between indigenous and Lessepsian species. An additional group of 4 fish species were sampled from trawling surveys in the Israeli Mediterranean; the indigenous species *Sardinella aurita* and *Lithognathus mormyrus*, and the Lessepsian species *Nemipterus randalli* and *Saurida lessepsianus*.

I found that *N. randalli* displayed a significantly high level of NNV, suggesting that this species can serve as a carrier and might horizontally infect other susceptible species which live in proximity; this might be an additional mechanism for the fast establishment of this species in the Levantine basin. Based on the amplification and sequencing of partial sequences of both RNA1 and RNA2 of NNV in both surveys, the phylogenetic analysis revealed that all detected NNV's were of the red-spotted grouper nervous necrosis virus (RGNNV) genotype.

The results of my study highlight the necessity of molecular monitoring surveys in order to characterize the prevalence of pathogenic agents in wild fish populations. In addition, my findings of a relatively high viral prevalence in the Lessepsian migratory species, compared to native species, suggest that the tolerance or resistance of an invasive species to pathogens should be considered as an additional mechanism to support the successful establishment of invasive species.

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Introduction

Infectious diseases in marine organisms have ecological, socio-economic and environmental impacts. Disease outbreaks in the ocean may result in alteration of community structure and their function in the ecosystem. There has been a rise in reports of diseases affecting marine organisms in the last decades, but there is a lack of essential baseline data to confirm the hypothesis that marine diseases are increasing (Harvell et al. 1999; Ward & Lafferty 2004).

Marine organisms serve as hosts for a diversity of pathogens, including parasites, bacteria, viruses and fungi. A disease will occur when a susceptible host interacts with an infective agent under the right environmental conditions. Stressful conditions in the ocean, generated by climate changes and anthropogenic pressures as pollution, overfishing and introductions of new species, are likely to break the equilibrium between the pathogen and its host, result in more frequent disease outbreaks than in normal conditions (Moreno et al. 2014).

The Mediterranean Sea, the largest marginal sea on Earth, is considered one of the most impacted seas in the world which experiencing both climatic and anthropogenic pressures in recent years. In the last three decades the sea surface temperature of the Mediterranean Sea in general and in its easternmost basin the Levantine basin in particular has been increased in about 1°C, a 2-3 times the warming rate of the global ocean (Jeppesen et al. 2015; Marba et al 2015; Yeruham et al. 2015). The influence of this trend on the unique biodiversity of the Mediterranean Sea, consist with diverse cold and tropical marine fauna, can provide insights into global patterns of marine ecosystems (Lejeusne et al. 2010). The Mediterranean marine biodiversity keep changing as a result of new species introduction to its basins, either naturally through the Straits of Gibraltar or by different anthropogenic pathways. By 2012, 126 fish species out of more than 950 non-indigenous species were reported in the Mediterranean Sea (Zenetos et al. 2012). The main invasion pathway is the artificially corridor of Suez Canal. The opening of the canal in 1869 enabled the invasion and introduction of more than half of the total invasive species and result in significant decline of indigenous species in the fisheries catch. The mechanism behind the successful establishment of the invasive species is not absolutely clear yet, but several possible explanations have been suggested, include: favorable conditions in the new invaded area, available habitats or favorable food and reduced competition and predation stress. In addition, new species were introduced into the Mediterranean Sea by ballast water and as ship fouling and through introduction of new species to aquaculture (Galil 2009; Zenetos et al. 2012; Edelist et al. 2013).

As a result of the global growing demand for food, Mediterranean aquaculture production increased by 77% between 1999 and 2009 (Rosa et al. 2012). The Gilthead sea bream *Sparus aurata* and the European sea bass *Dicentrarchus labrax* are the main marine species cultured along the Mediterranean, mainly in Turkey and Greece (FEAP 2016). The aquaculture facilities in the sea, attracts wild species that tent to aggregate near the farming cages and interactions between wild and farmed populations may occur (Diamant et al. 2000). Small wild fish may invade into the cage through the mesh while larger fish will penetrate the cage only if they manage to find a larger hole in the cage net. Farmed fish can escape through holes caused by bites of the net by the farmed fish or predators, and may interact with wild populations. A study on the extent and costs of such escapes in European fish farming, evaluates that more than 7 million sea bream and sea bass fish escaped from Mediterranean aquaculture during 3 years period from 2007 to 2009 (Jackson et al. 2015). Interactions between wild and farmed fish may result in the exchange of pathogens in more than one direction. Infected escapees can serve as vectors of horizontal transmission and infect wild populations and neighbor cages. On the other hand, wild species may infect the farmed fish in the cages or escapees with native pathogens, and finally, both wild and escaped fish that moves between adjacent farms may contribute to the spread of pathogens to native coastal areas and other aquaculture facilities (Johansen et al. 2011; Arechavala-Lopez et al. 2013; Figure 1). Infectious disease and mortalities affect not only the host population but can cascade through ecosystems (Ward & Lafferty 2004). A specimen that manages to survive viral infection and overcomes the disease may become an asymptomatic carrier, exhibiting light viral loads and constantly excreting viruses (Moreno et al. 2014). In a study that investigated infection patterns of *Mycobacterium marinum* in wild Rabbit fish *Siganus rivulatus* associated with aquaculture facilities and from various sites along the Israeli Red Sea shoreline, higher prevalence level were detected within fish that were trapped inside the cages relatively to specimens sampled in a distant sites (Diamant et al. 2000).

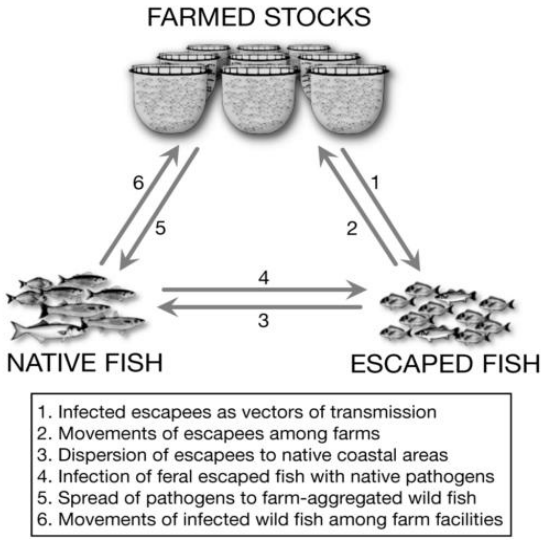

Figure 1: **Generalized flow chart of potential pathogen transmission through movements of wild fish and escapees**. (Arechavala-Lopez et al. 2013)

One indication for a pathogen transmission between wild and farmed fish is when both farmed fish and wild fish from the surroundings of the farm show similar infection from the

same pathogenic agent. For example, following mortalities events of cultured sea bream and sea bass in an Israeli Mediterranean fish farm occurred concurrently with mortalities of wild Rabbit fish *S. rivulatus* living in proximity to the aquaculture facility, an epidemiological study found that all fish were infected with a single clone of the bacteria *Streptococcus iniae* and it was suggested they were infected from the same source (Zlotkin et al. 1998). In another case from the Red Sea in Eilat, biochemical and molecular similarities between *S. iniae* isolated from 2 wild fish to those isolated from cultured fish species suggested a transmission of this pathogen between these populations (Colorni et al. 2002). Wild species can serve as a vector of transmission when they are caught from the wild and used for aquaculture purposes such as broodstock for hatcheries or as cleaner fish. In the Norwegian salmonids aquaculture, wild wrasses (Labridae) are extensively used as cleaner fish to remove sea lice from Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). A survey along the Norwegian and Swedish coastline reported that nervous necrosis virus is naturally present in wild wrasse. The total prevalence was 6.7% but in one out of three wrasse species sampled in this study, the prevalence was up to 18% with no geographic differences in the overall distribution of the virus suggesting they may be a potential risk for susceptible species in the Norwegian fish farming (Korsnes et al. 2017). In addition, it was previously suggested that the parasites affecting an organism may serve as a vector of transmission for bacterial and viral pathogens (Cusack & Cone 1986). Pathogens transmission within and between wild and cultured fish populations may result in the amplification of the pathogen in the environment and contribute to disease outbreaks within related marine ecosystems (Harvell et al. 2002; Gozlan et al. 2006).

Among pathogens, fish viruses are a major threat due to the high mortality rates of infected specimens. RNA viruses cause some of the most devastating diseases in aquaculture industry (Lang et al. 2009). The betanodavirus nervous necrosis virus (NNV), an RNA virus belonging to family *Nodaviridea*, is the causative agent of viral nervous necrosis (VNN) - also known as viral encephalopathy and retinopathy (VER). This disease, already been detected in more than

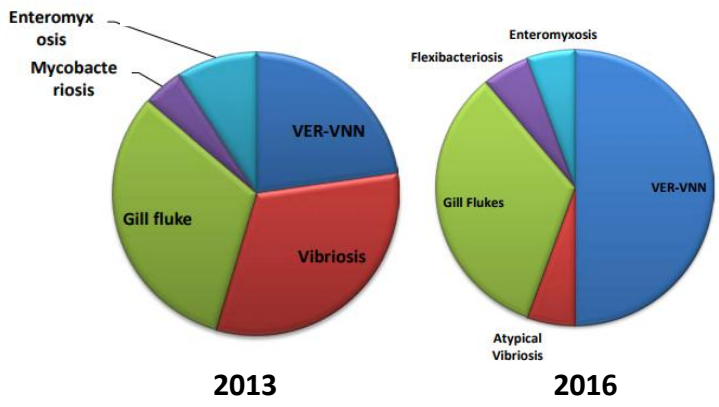

Figure 2: **Distribution of main diseases within Mediterranean marine aquaculture in 2013 compare to 2016**.
From *Annual Workshop of the National Reference Laboratories*, 2017

120 finfish species worldwide, is causing the highest ecological and socio-economical impacts in Mediterranean farmed marine fish (Gomez-Casado et al. 2011; Costa & Thompson 2016; Vendramin & Olesen 2017, Figure 2).

NNV is a neuro-tropic agent that causes pathology in neural organs (Figure 3). Infected fish show abnormalities of movement, swim bladder control, problems with sight and altered skin coloration. These clinical signs, caused by the viral replication, can lead to vocualation and necrosis of brain and retina cells (Vendramin et al. 2013). Fish affected at larval stages suffer from high mortality rates and VNN can cause significant losses in older fish (Crane & Hyatt 2011).

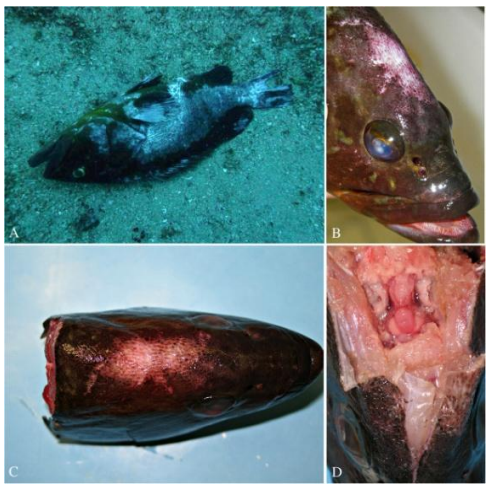

Figure 3: VNN-VER infected dusky grouper. A) Dead dusky grouper laying on the sea bottom, showing erosions of the fin likely associated with loss of swimming control and repeated trauma against rocks. B and C) Dusky grouper displaying head skin erosion and corneal opacity D) CNS hyperaemia in the same grouper of B and C. (Vendramin et al., 2013)

NNV is a small positive-RNA virus made of two single-stranded RNA segments: the RNA1 (3.1kb) segment encodes the viral replicase (protein A) and the RNA2 (1.4 kb) encodes the capsid protein. During the replication of RNA1, the additional sub-genomic RNA3 is transcribed from the 3' end of RNA1. It encodes the B2 protein that is an inhibitor of short interfering RNA (siRNA) silencing (Gomez et al. 2004). Based on a partial sequence of the coat protein named T4 region, NNV was classified into four different genotypes named after the fish species from which they were first derived, i.e. Striped Jack nervous necrosis virus (SJNNV), Tiger puffer nervous necrosis virus (TPNNV), Barfin flounder nervous necrosis virus (BFNNV) and Red-spotted grouper nervous necrosis virus (RGNNV) (Nishizawa et al. 1997). In addition, two reassortants have been described based on the donor genotype of the RNA1/RNA2 - RGNNV/SJNNV and SJNNV/RGNNV, respectively. Betanodavirus genotypes possess a distinct geographical distribution based on their optimal culture temperatures. BFNNV and TPNNV, cultured *in vitro* at 15-20°C, affect cold water species in the north Atlantic and Japan (Panzarin et al. 2012; Toffan et al. 2016), SJNNV reported in Japan and Spain in water temperature of 20-25°C and RGNNV with the widest optimal temperature range of 15-30°C is globally distributed and it is the most common detected genotype in the Mediterranean Sea (Mori et al. 1992; Nishizawa et al. 1997; Cutrín et al. 2007; Olveira et al. 2009).

Although many species including the European sea bass *D. labrax* have proven to be susceptible to NNV, until recently the gilthead sea bream *S. aurata*, a major species in the Mediterranean aquaculture, has been considered resistant to the disease and a potentially asymptomatic carrier of the virus (Castric et al. 2001). A clinical study of mass mortalities events in gilthead sea bream *S. aurata* from Mediterranean aquaculture found it was caused by VNN disease. The genotypic characterization of the virus revealed it was of the RGNNV/SJNNV reassortants and this reassortant was recognized as an emerging threat to this species in Mediterranean aquaculture (Toffan et al. 2017). Previous phylogenetic analyses on fish nodaviruses have been based solely on RNA2 sequences, but following these recent findings of reassortants pathogenicity it is important to analyze both genomic segments during genotypic characterization of the virus (Toffolo et al. 2007). Moreover, Ucko et al. (2004) reported that when using a set of primers targeting the RNA2 to detect NNV in *S. aurata*, a non specific bend was amplified and produced false-positive results. In contrast, an occasionally produced false-negative results were reported using the same reverse primer in other species (Ucko et al. 2004). Therefore, targeting highly conserved regions of both genomic segments and sequencing is important for validation of the molecular analysis.

Viral transmission of NNV can be either vertically, through sperm and eggs, or horizontally, through the surrounding water body; healthy fish were infected with the virus after 4 days of cohabitation with diseased fish, after exposed with infected tissues and when introduced with water with viral particles (Munday et al. 2002; Valero et al. 2015; Costa & Thompson 2016).

Despite the potential risk of NNV to wild and cultured fish species, only few studies in the Mediterranean area reported the prevalence levels of NNV in wild fish populations. In a survey of 22 wild species fished in the Adriatic Sea and were collected from Italian fish markets, Ciulli et al. (2007) reported a total prevalence level of 11.9%. When apparently healthy wild marine fish were sampled near fish cages in Tunisia the prevalence level was much higher with 62% of the samples positive to NNV (Cherif & Fatma 2017). Interestingly, a viral epidemiological study conducted around the Gulf of Cadiz (Atlantic Ocean) found that 63% of thicklip mullet (*Chelon labrosus*) from the river mouth, that is influenced by aquaculture, were positive to NNV compared to 19.6% obtained in 9/29 wild species sampled in the open sea in this area (Moreno et al. 2014).

This is the first study which focuses on detection and characterization of betanodavirus in wild species from the Levantine basin.

Research Objectives

General research hypothesis

Nervous necrosis virus (NNV) is prevalent in wild marine fish and it can infect cultured fish in the Levantine Basin of the eastern Mediterranean Sea.

General research questions

- What is the prevalence of NNV in wild fish species of economic importance?
- What are the different NNV genotypes in the selected wild and cultured fish species?
- Is there difference in prevalence of NNV between indigenous and Lessepsian species?
- Is there an NNV transmission between wild and cultured fish species?

Part I – Fish pathogens survey

Until recently, the only monitoring program of the Israeli marine ecosystem was the Israel National Monitoring Program in the Gulf of Eilat (NMP), initiated in 2003. This NMP is an annual survey covering the biological, ecological, chemical and physical aspects of the marine ecosystem in the Gulf of Eilat and an annual report, including all scientific data, are available online (www.iui-eilat.ac.il/NMP). In the Mediterranean Sea a first biodiversity survey was held from 2014 to 2016 as part of the Israel's National Nature Assessment Program – HaMaarag (www.hamaarag.org.il). In order to provide a comprehensive scientific data of the biodiversity, pollutants, water composition and pathogens in the Levantine basin, in 2016 The Morris Kahn Marine Research Station (MKMRS) at the University of Haifa initiated the Mediterranean long-term ecological research (LTER) program (www.med-lter.haifa.ac.il).

Disease outbreaks and pathogens from marine aquaculture are well documented and reported. Nevertheless, there is a lack of basic information regarding the prevalence of pathogenic agents in wild fish populations (Ward & Lafferty 2004; Arechavala-Lopez et al. 2013).

To close this knowledge gap and in order to establish the scientific baseline of the marine pathogens in the Levantine basin wild organisms, an annual pathogenic survey is being held as part of the LTER program. The focus of this survey is to quantify the prevalence levels of marine pathogens in wild indigenous and Lessepsian species from different trophic levels and biological niches with an importance to the Israeli fisheries.

In this part of my thesis I will present my work in the LTER pathogenic survey. Although NNV is the main subject of my work, the pathogenic survey includes the bacteria *Streptococcus iniae* as well and is discussed in this section. Both NNV and *S. iniae* are known to cause mortalities in wild and cultured marine populations worldwide.

The Gram-positive cocci *S. iniae* is the etiological agent of streptococcosis in fish, a disease occurred in marine and freshwater environments. This bacterium, first isolated from a freshwater dolphin *Inia geoffrensis*, were since being isolated from more than 27 species in three main regions – North America, Middle East and Asia-Pacific. Disease outbreaks may result in significant economic losses to aquaculture and can be devastating for the ecosystem when occurred in wild reef species as reported in the Caribbean and the Red Sea (Agnew & Barnes 2007; Chou et al 2014; Soto et al. 2015). Infection may occurred when the pathogen enters the blood of the host through gills and nares, skin injuries or through the guts follow an ingestion of a diseased fish, and then overcome the immune response of macrophages (Zlotkin et al. 2003). The possible transmission of *S. iniae* between wild and cultured fish species was suggested and discussed previously in the introduction of this work (Zlotkin et al. 1998; Evans et al. 2001; Colorni et al. 2002). Besides affecting animals, *S. iniae* was

identified as pathogen with zoonotic potential following several reports of infections by *S. iniae* in humans, after handling of fresh fish (Weinstein et al. 1997).

In order to assess the possibility for horizontal transmission of pathogens from wild fish to mariculture fish, we examined the presence of both pathogens in group of Gilthead sea bream (*Sparus aurata*) during their growth period in an Israeli marine fish farm.

Materials and Methods

Fish and tissue sampling

Ten wild species, eight fish and two crustacean species (Table 1), caught by fishermen were collected from four ports along the Israeli Mediterranean shoreline: Akko (Acre), Kishon, Yaffo (Jaffa) and Ashdod. *Sparus aurata* specimens were sampled from a single batch during three stages of their growth period at a fish farm located 12 km offshore. A group of 15 *S. aurata* specimens were sampled at each of the following stages - from the hatchery that supplied all fingerlings to the farm before introducing into the sea and at two different time points: 7 and 120 days after initial stocking in the sea cages. All wild specimens sampled during 2016 and the cultured *S. aurata* sampled during 2017.

Fish were placed on ice at the boat and transferred on ice to the laboratory where weight, total length and visual inspection were carried out. It should be mentioned that the fish were obtained at the ports and nearby fish markets but the exact definitive fish capture sites are not acknowledged in this study.

All specimens were aseptically dissected for tissue sample collection according to fish necropsy protocol (Yanong 2003). All samples were kept frozen at -80 °C until further analysis. From each fish specimen, liver and kidney tissues were used for the analysis of *S. iniae* and brain samples for NNV search. From both crustaceans, the hepatopancreas and muscle tissues were used for the analysis of S. iniae. Nerve tissues from the blue crab *Portunus pelagicus* only were used for the analysis of NNV.

Table 1: Organisms sampled and analyzed in this study (Part I). Total number of specimens tested and number of organisms sampled from each sampling site

			Number of sampled organisms per site					
Family, Species	Common name	Origin [i]	Akko	Kishon	Yaffo	Ashdod	Fish farm	Total
Mullidae								
Mullus surmuletus	Surmullet	M	3	13	3	3		22
Mullus barbatus	Red mullet	M	6	3				9
Upeneus moluccensis	Goldband goatfish	L	3	15	13	3		34
Sparidae								
Lithognathus mormyrus	Striped seabream	M	10	4	13	3		30
Sparus aurata	Gilthead seabream	M, F					45	45
Nemipteridae								
Nemipterus randalli	Randall's threadfin bream	L	3	13	10	3		29
Synodontidae								
Saurida lessepsianus	Brushtooth lizardfish	L	9	13	13	3		38
Clupeidae								
Sardinella aurita	Round sardinella	M	3	17	10			30
Dussumieriidae								
Dussumieria elopsoides	Slender rainbow sardine	M				3		3
Penaeidae								
Marsupenaeus japonicus	Kuruma prawn	M	7	3	13	3		26
Portunidae								
Portunus pelagicus	Blue crab	L	9	11	10	3		33

[i] M - Mediterranean natives , L - Lessepsian migrants, F - Fish farm

RNA extraction and cDNA synthesis by RT-PCR

NNV is an RNA virus; therefore total RNA must be extracted, followed by reverse transcription to cDNA for further molecular analysis. Extraction of total RNA from each brain tissue was done using the EZ-RNA total RNA isolation kit (Biological Industries, BH, IL) in accordance with the manufacturer's instructions. Tissue samples (30-50 mg) were homogenized and incubated for 5 min with 500µl of manufacturer's denaturation solution, followed by addition mix and incubation for 10 min with 500 µl of extraction solution. Then the samples were centrifuged for 12 min at 12,000 g at 4°C. For RNA precipitation, the aqueous colorless upper phase was transferred to fresh tube and incubated for 10 min with 500 µl Isopropanol, followed by centrifuge of 12,000 g for 8 min at 4°C. The supernatant was removed and the samples were washed with 1ml of 75% EtOH, centrifuged for 5 min at 8,000 g at 4°C and air-dried for 5 min after removal of supernatant. RNA was suspended in 100 µl nuclease-free water and incubated for 15 min at 55°C.

Concentration and quality of RNA were estimated using NanoDrop One (Thermo Scientific), and the extracted RNA was stored at -80°C until use.

RT-PCR carried out to generate cDNA from 4 µl (~3 µg) of total RNA using the GoScript™ Reverse Transcription System (Promega, WI, USA) with 0.5 µg of random primers according to the manufacturers' instructions.

NNV Real-time PCR (qPCR) and PCR amplification

Detection of NNV in sampled organisms performed in 2 steps (Figure 4):

- Screen for presence/absence of the virus by qPCR, targeting the RNA2 segment.
- Genotyping of NNV strain and confirmation of qPCR results by PCR, targeting both RNA1 and RNA2 genomic segments.

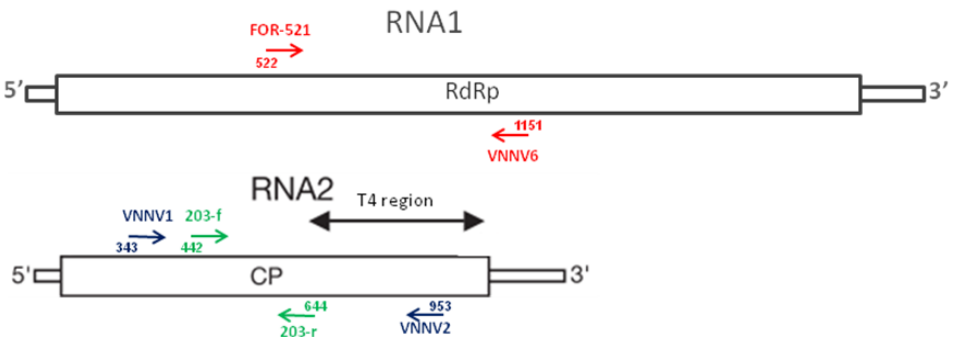

Figure 4: **Scheme of primers position on NNV genomic structure**. Open boxes and white lines represent ORFs and non-coding RNAs, respectively. Nucleotide positions of primers, presents below the arrows, are based on the Genbank accession numbers AB056571 for RNA1 and AB056572 for RNA2 (Iwamoto et al. 2001). Two-headed arrow directs the variable T4 region that has been used for the classification of betanodavirus by (Nishizawa *et al*. 1997). Modified from (Ito *et al*. 2008)

NNV specific gene amplification, targeting a 203nt fragment of the RNA2 segment was performed by qPCR using reaction tubes preloaded with 2 µl of cDNA template, 1 µl (10mM) of '203' primers (Table 2) (Kuo et al. 2011), 6 µl of ultra-pure PCR water, 10 µl GoTaq qPCR Master mix (Promega) on a AriaMx Real-Time PCR System (Agilent Technologies Inc., CA, USA). The qPCR thermal profile was 1 cycle of 95°C for 3 min, 45 cycles of 95°C for 5 s and 60°C for 30 s and a single melt curve cycle of 95°C for 30 s, 65°C for 30 s and 95°C for 30 s. In each run, 3 additional control wells were added – cDNA of NNV originally isolated from a diseased white grouper (*Epinephelus aeneus*) in Israel (GenBank accession number KP748520.1) used as positive control (PC), DDW used as non-template control (NTC) instead of cDNA and cDNA extracted from healthy fish, previously checked with different primers, was used as negative control (NC). Threshold level was determined by the qPCR instrument program. Due to the fact that sampled fish were apparently healthy with no clinical signs, low viral loads were expected to result in high C_t values relative to the positive control. Therefore, samples with C_t values of more than 10 cycles with similar melting curves and Tm values as the PC were defined as positive.

For genotyping the NNV strain and confirmation of qPCR results, positive samples were subjected to PCR targeting both genomic segments using the following primers (Table 2): 'FOR521' and 'VNNV6' target a 630nt fragment of the RNA1 segment, and 'VNNV1' and 'VNNV2' primers target a 605nt fragment of the RNA2 segment (Toffolo et al. 2007; Bovo et al. 2011). PCR reactions were performed in reaction tubes preloaded with 2 µl of cDNA template, 0.5 µl (10 mM) of each primer, 9.5 µl of ultra-pure PCR water and 12.5 µl GoTaq Green Master mix (Promega) on a SimpliAmp Thermal Cycler (Applied Biosystems, CA, USA). The PCR thermal profile for RNA1 was 1 cycle of 95°C for 4 min, 35 cycles of 95°C for 30 s, 57°C for 30 s, 72°C for 30 s and a final extension of 72°C for 10 min. PCR reaction thermal profile for RNA2 was 1 cycle of 95°C for 4 min, 35 cycles of 95°C for 30 s, 51°C for 30 s, 72°C for 30 s and a final extension of 72°C for 10 min. Positive, Negative and Non-template control were used as described above. A 6 µl of each amplicon were checked by 1.5% Agarose gel electrophoresis along with a 1kb BenchTop ladder (Promega).

DNA extraction

For the analysis of the bacteria *S. iniae*, DNA was extracted from liver and kidney of each analyzed specimen using the Wizard SV Genomic System (Promega) and the genomic DNA purification protocol following the manufacturer's instructions for tissue lysates. In brief, up to 20 mg of tissue sample were placed in 275 µl of manufacturer's Digestion Solution Master Mix, followed by an overnight incubation in 55°C, then a 250 µl of Lysis buffer was added.

For purification of DNA, the lysates were transferred to the manufacturer's minicolumn assembly, centrifuge for 3 min at 13,000 g and followed by 4 subsequent centrifuges at 13,000 g for 1 min with 650µl Wash Solution. DNA eluted using 2 min incubation with 250 µl nuclease-free water and 13,000 g centrifuge for 2 min.

DNA quantity and purity were estimated using NanoDrop One (Thermo Scientific).

Isolated genomic DNA was stored at −20 °C until use.

Streptococcus iniae PCR amplification

Detection of *Streptococcus iniae* in sampled organisms performed in 2 steps:

- Screen for presence/absence of the bacteria by PCR, targeting the 16S rRNA gene.
- Identification of *S. iniae* by specific PCR, targeting *rpoB* and *gyrB* genes.

PCR reactions were performed with reaction mix as described above. A 16S rRNA PCR amplification of 513bp fragment was performed using 'strp1' primers (Pourgholam et al. 2011) and the thermal profile was 1 cycle of 95°C for 4 min, 40 cycles of 95°C for 30 s, 57°C for 30 s, 72°C for 30 s and a final extension of 72°C for 10 min. Positive samples were subjected to further genetic characterization by PCR amplification of *rpoB* and *gyrB* genes using following primers and conditions : *rpoB* thermal profile was 1 cycle of 95°C for 4 min, 40 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and a final extension of 72°C for 10 min, *gyrB*: thermal profile was 1 cycle of 95°C for 4 min, 40 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 30 s and a final extension of 72°C for 10 min (Soto et al. 2017) (Table 2). In each run, 3 additional control wells were added – DNA of *S. iniae* isolated from infected fish used as PC, DDW used as NTC instead of DNA and DNA extracted from healthy fish, previously check with different primers was used as NC. A 6 µl of each amplicon were checked by 1.5% Agarose gel electrophoresis along with a 1kb BenchTop ladder (Promega).

Table 2: List of primers used for qPCR and PCR amplifications

Pathogen	Gene	Primer	Sequence 5'>3'	Amplicon size
NNV	RNA2 - capsid protein	*203*	Fw: GACGCGCTTCAAGCAACTC Rev: CGAACACTCCAGCGACACAGCA	203
	RNA1 - RdRp	*FOR-521* *VNNV6*	Fw: ACGTGGACATGCATGAGTTG Rev: ACCGGCGAACAGTATCTGAC	630
	RNA2 - capsid protein	*VNNV1* *VNNV2*	Fw: ACACTGGAGTTTGAAATTCA Rev: GTCTTGTTGAAGTTGTCCCA	605
Streptococcus spp.	16S rRNA	*strp1*	Fw: AACTAACCAGAAAGGGACGG Rev: CTCTGTCCCGAAGGAAAATC	513
	rpoB	*rpoB*	Fw: TTCCGTCGTTCAAACTCAGG Rev: TCTCACCAAAACGTTGTCCA	973
	gyrB	*gyrB*	Fw: ACATCGGCATCGGTCATKA Rev: GCGGAGGCGGYTATAAGGTT	1064

Sequencing and phylogenetic analysis

NNV qPCR (primer '203') and *Streptococcus* spp. PCR amplicons were purified by ExoSAP-IT (Affymetrix, Santa Clara, CA) and sequenced by Sanger sequencing method (HyLabs, Rehovot, IL). PCR amplicons of NNV larger fragments (RNA1 and RNA2) were purified and sequenced by Sanger sequencing method at the Macrogen Europe Laboratory (Amsterdam, The Netherlands). Sequences were aligned and compared to representative sequences available in GenBank by BLAST using the BioEdit Sequence Alignment Editor and MEGA7software (Kumar et al. 2016). Based on the complete genome of RGNNV and SJNNV available in GenBank, the similarity rate between the strains is 80-82%.

Genetic characterization of NNV positive isolates, based on RNA1 and RNA2, were inferred from maximum likelihood (ML) trees performed with the PhyML v.3.0 program (Guindon et al. 2010) by applying the following models of nucleotide substitution: K80+ G with four rate categories for RNA1 and K80 + I for RNA2, based on the Smart Model Selection (Lefort et al. 2017) available in the PhyML program. Robustness of nodes on the phylogeny was assessed by 1000 bootstrap replicates using the ML substitution model defined above. Phylogenetic trees were visualized with the FigTree v1.4.3 software (http://tree.bio.ed.ac.uk/software/figtree/).

Statistical analysis

Statistical significance between compared tissues, kidney and liver, of *Streptococcus* spp. positive samples and between sampled areas was determined by Chi-Square test of independence using IBM SPSS Statistics for Windows, version 20.0 Windows, version 20.0. For all tests, results were considered significant at $p < 0.05$.

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Title: **Prevalence of Nervous necrosis virus and *Streptococcus iniae* in wild marine fish and crustaceans from the Levantine Basin, Mediterranean Sea**

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Nadav Davidovich – molecular analysis and statistics

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Rei Diga – Sample collection and preparation, molecular work

Dan Tchernov – Study design and manuscript writing

Danny Morick – Study design, molecular analysis and manuscript writing

[1] DAO website - https://www.int-res.com/journals/dao

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DISEASES OF AQUATIC ORGANISMS
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Prevalence of nervous necrosis virus (NNV) and *Streptococcus* species in wild marine fish and crustaceans from the Levantine Basin, Mediterranean Sea

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ABSTRACT: Infectious diseases in marine animals have ecological, socio-economic and environmental impacts. Nervous necrosis virus (NNV) and *Streptococcus iniae* have become major threats to marine aquaculture and have been detected in morbid marine organisms worldwide. However, despite their importance, there is a lack of knowledge regarding the prevalence of these pathogens in wild fish species. Here we sampled indigenous and Lessepsian species from different trophic levels and different biological niches in the eastern Mediterranean. A total of 174 fish and 32 crustaceans were tested for *S. iniae* and a total of 195 fish and 33 crustaceans were tested for NNV. We found an overall prevalence of 9.71% *Streptococcus* spp. and 21.49% NNV in selected marine fish and crustaceans by PCR and qPCR. In fish, the zoonotic agent *S. iniae* was detected at a higher prevalence in kidney compared to liver tissue. Co-infection by both pathogens was detected only in 5 specimens. We also examined gilthead sea bream *Sparus aurata* from an Israeli offshore marine farm during the grow-out period, in order to assess the possibility of horizontal pathogen transmission from wild to maricultured fish. Three out of 15 (20%) fish were found to be NNV positive after 120 d in the sea, suggesting spontaneous transmission from wild to farmed fish. Our findings suggest that more surveys should be conducted, especially in areas were mariculture farms are planned to be established.

KEY WORDS: Betanodavirus · *Streptococcus iniae* · Pathogens · Marine fish · Wild fish

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1. INTRODUCTION

Marine organisms serve as hosts for a diversity of parasites, bacteria and viruses. In the last few decades, there has been a gradual increase in reports of diseases affecting marine organisms of different taxa (Harvell et al. 1999). Climate change and anthropogenic factors such as overfishing, eutrophication and the rapid expansion of mariculture can generate stressful conditions that are likely to break the fragile equilibrium between the pathogen and its host, resulting in increased pathogen expansion (Moreno et al. 2014). Horizontal and vertical transmission within and between wild fish populations may contribute to disease outbreaks, resulting in increased morbidity and mortality that may lead to reduced survival rates, increased pathogen transmission and increased host susceptibility within marine ecosystems (Harvell et al. 2002, Gozlan et al. 2006).

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The Mediterranean is an unusual sea, containing diverse marine fauna due to its variable geophysical and biogeographical properties in its different basins in different seasons; it could therefore serve as a 'test case' for the world's oceans and provide insight into larger-scale marine ecosystems (Lejeusne et al. 2010). Its easternmost basin, the Levantine Basin, is an area in which both air and water temperatures have increased in the last 2 decades (Jeppesen et al. 2015, Yeruham et al. 2015). The large upsurge of aquaculture in recent decades, in addition to urbanization of its coastlines, makes the Mediterranean one of the most impacted seas in the world (Lejeusne et al. 2010, Rosa et al. 2012); therefore, it is important to establish the extent of the resulting damage to the ecosystem.

Wild fish play an important ecological role in the ecosystem and an economical role as a major protein source for humans. Although global aquaculture production has increased dramatically in the last 2 decades, about half of the consumed fish is still based on wild fish and fisheries products (FAO 2016). Despite the fact that a wide range of marine pathogens from aquaculture are well documented, there is a lack of basic information regarding the prevalence of pathogenic agents in wild fish populations (Ward & Lafferty 2004, Arechavala-Lopez et al. 2013). The aim of this study was to establish a prevalence baseline of 2 important marine pathogens, namely nervous necrosis virus (NNV) and *Streptococcus iniae*, in wild marine fish in the Levantine Basin.

The gram-positive coccus *S. iniae* has caused documented mortalities in wild and cultured populations of marine and freshwater fish resulting from clinical streptococcosis (Chou et al. 2014). Besides affecting animals, this bacterium also has zoonotic potential (Weinstein et al. 1997). NNV, an RNA virus belonging to the family *Nodaviridae*, genus *Betanodavirus*, is the causative agent of viral nervous necrosis disease, also known as viral encephalopathy and retinopathy, a disease causing high ecological and economical losses in European farmed finfish (Olveira et al. 2009, Gomez-Casado et al. 2011, Vendramin & Olesen 2017). This virus is globally distributed and has already been detected in more than 120 finfish species (Costa & Thompson 2016).

In order to study the prevalence of these 2 important pathogens in the Levantine Basin of the Mediterranean Sea, we analyzed indigenous and Lessepsian wild species from different trophic levels and biological niches. To assess the possibility for horizontal transmission of pathogens from wild fish to mariculture fish, we also examined gilthead sea bream *Sparus aurata* from an Israeli marine fish farm, at each stage of somatic growth. This fish is an endemic species and is highly representative of farmed species in the Mediterranean.

2. MATERIALS AND METHODS

2.1. Sampling sites

Wild fish, including both indigenous and Lessepsian species, caught by fishermen were collected from 4 ports along the Israeli Mediterranean shoreline: Akko (Acre), Kishon, Yaffo (Jaffa) and Ashdod (Fig. 1). In addition, *Sparus aurata* specimens were sampled during their growth period from a fish farm located 12 km offshore in the southern area of the Israeli Mediterranean Sea (in the area of S2 in Fig. 1; the specific location of this farm is not provided following a confidentiality agreement with the farm that supplied the samples). Fish were placed on ice on the boat and transferred on ice to the laboratory, where weight and total length were measured and a visual inspection was carried out.

2.2. Tissue sampling for molecular analysis

Different tissues were sampled: liver and kidney tissues were used for the analysis of *Streptococcus iniae* and brain samples were used to screen for NNV. The following fish species were tested: *Mullus surmuletus* (22), *M. barbatus* (9 and 6 for NNV and

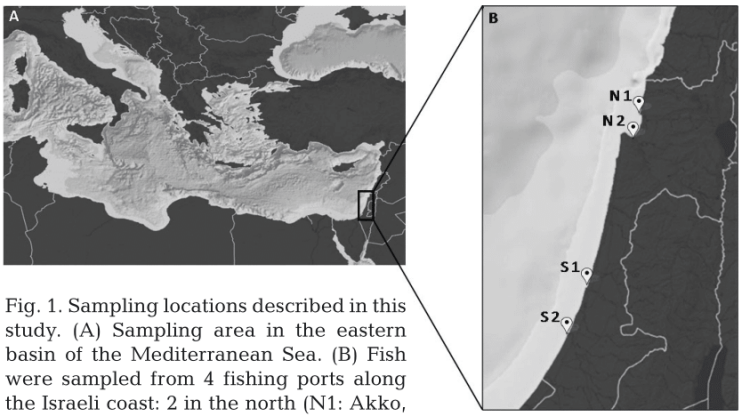

Fig. 1. Sampling locations described in this study. (A) Sampling area in the eastern basin of the Mediterranean Sea. (B) Fish were sampled from 4 fishing ports along the Israeli coast: 2 in the north (N1: Akko, N2: Kishon) and 2 in the south (S1: Yaffo, S2: Ashdod). Map originated using the ArcGIS software (www.arcgis.com)

Table 1. Fish and crustacean species sampled in this study. M: Mediterranean natives, L: Lessepsian migrants, F: fish farm, NNV: nervous necrosis virus

Order Family, species	Common name	Origin	Biological niche	Number of specimens tested *Streptococcus* spp.	 NNV
Perciformes					
Mullidae					
Mullus surmuletus	Surmullet	M	Demersal omnivorous	22	22
Mullus barbatus	Red mullet	M	Demersal omnivorous	6	9
Upeneus moluccensis	Goldband goatfish	L	Demersal omnivorous	34	34
Sparidae					
Lithognathus mormyrus	Striped seabream	M	Nectobenthic carnivorous	19	30
Sparus aurata	Gilthead seabream	M, F	Aquaculture	45	45
Nemipteridae					
Nemipterus randalli	Randall's threadfin bream	L	Benthopelagic carnivorous	29	29
Aulopiformes					
Synodontidae					
Saurida lessepsianus	Brushtooth lizardfish	L	Demersal piscivorous	38	38
Clupeiformes					
Clupeidae					
Sardinella aurita	Round sardinella	M	Pelagic zooplanktivorous	26	30
Dussumieriidae					
Dussumieria elopsoides	Slender rainbow sardine	M	Pelagic zooplanktivorous	-	3
Decapoda					
Penaeidae					
Marsupenaeus japonicus	Kuruma prawn	M	Benthic omnivorous	26	-
Portunidae					
Portunus pelagicus	Blue crab	L	Benthic omnivorous	6	33

S. iniae, respectively), *Upeneus moluccensis* (34), *Lithognathus mormyrus* (30 and 19 for NNV and *S. iniae*, respectively), *Nemipterus randalli* (29), *Saurida lessepsianus* (38), *Sardinella aurita* (30 and 26 for NNV and *S. iniae*, respectively) and 3 *Dussumieria elopsoides* were tested only for NNV. For crustaceans, the hepatopancreas and muscle were used for the analysis of *S. iniae* and nerve tissues for NNV. The following crustacean species were tested: *Portunus pelagicus* (33 and 6 for NNV and *S. iniae*, respectively) and *Marsupenaeus japonicus* (26, tested only for *S. iniae*, Table 1; see also Table S1 in the Supplement at www.int-res.com/articles/suppl/d133p007_supp.pdf). The fish were obtained at the ports and nearby fish markets, but the exact fish capture sites were not recorded in this study. A total of 45 farmed *Sparus aurata* from a single batch delivered to the farm were sampled in this study, with 15 fish analyzed at each of the 3 sampling time points: at the hatchery before being introduced into the sea cages (Day 0), and 7 and 120 d after initial stocking in the sea cages. All specimens were aseptically dissected for tissue sample collection according to a fish necropsy protocol (Yanong 2003). All samples were kept frozen at −80°C until further analysis.

2.3. RNA extraction and cDNA synthesis by RT-PCR

Extraction of total RNA from each brain tissue was done using the EZ-RNA total RNA isolation kit (Biological Industries) in accordance with the manufacturer's instructions. The RNA concentration and quality were estimated using NanoDrop One (Thermo Scientific), and the extracted RNA was stored at −80°C until use. RT-PCR (Reverse Transcription PCR) was carried out to generate cDNA of total RNA using the GoScript™ Reverse Transcription System (Promega, WI, USA) with random primers according to the manufacturer's instructions.

2.4. DNA extraction

DNA was extracted from liver and kidney of each specimen using the Wizard SV Genomic System (Promega) and the genomic DNA purification protocol following the manufacturer's instructions for tissue lysates. DNA quantity and purity were estimated as described in Section 2.3. Isolated genomic DNA was stored at −20°C until use.

2.5. NNV real-time PCR (qPCR) and PCR amplification

NNV-specific gene amplification, targeting a 203 nt fragment of the RNA2 segment, was performed by qPCR using reaction tubes preloaded with 2 µl of cDNA template, 1 µl (10 mM) of each primer (203f: 5′-GAC GCG CTT CAA GCA ACT C-3′; 203r: 5′-GAA CAC TCC AGC GAC ACA GCA-3′) (Kuo et al. 2011), 6 µl of ultra-pure PCR water and 10 µl GoTaq qPCR Master mix (Promega) on an AriaMx Real-Time PCR System (Agilent Technologies). The qPCR thermal profile was 1 cycle of 95°C for 3 min; 45 cycles of 95°C for 5 s and 60°C for 30 s; and a single melt curve cycle of 95°C for 30 s, 65°C for 30 s and 95°C for 30 s.

For genotyping the NNV strain and for confirmation of the qPCR results, all samples that were positive by qPCR were subjected to PCR targeting both genomic segments using primers previously described by Bovo et al. (2011) and Toffolo et al. (2007) targeting a 630 nt fragment of the RNA1 segment (FOR521: 5′-ACG TGG ACA TGC ATG AGT TG-3′ and VNNV6: 5′-ACC GGC GAA CAG TAT CTG AC-3′) and primers targeting a 605 nt fragment of the RNA2 segment (VNNV1: 5′-ACA CTG GAG TTT GAA ATT CA-3′ and VNNV2: 5′-GTC TTG TTG AAG TTG TCC CA-3′). PCRs were performed in reaction tubes preloaded with 2 µl of cDNA template, 0.5 µl (10 mM) of each primer, 9.5 µl of ultra-pure PCR water and 12.5 µl GoTaq Green Master mix (Promega) on a SimpliAmp Thermal Cycler (Applied Biosystems). The PCR thermal profile for RNA1 was 1 cycle of 95°C for 4 min; 35 cycles of 95°C for 30 s, 57°C for 30 s, 72°C for 30 s; and a final extension of 72°C for 10 min. The PCR reaction thermal profile for RNA2 was 1 cycle of 95°C for 4 min; 35 cycles of 95°C for 30 s, 51°C for 30 s, 72°C for 30 s; and a final extension of 72°C for 10 min.

2.6. *Streptococcus* species PCR amplification

The identification of *S. iniae* was performed by PCR amplification of a 513 bp fragment of the 16S rRNA gene using primer strp1-f (5′-AAC TAA CCA GAA AGG GAC GG-3′) and primer strp1-r (5′-CTC TGT CCC GAA GGA AAA TC-3′) (Pourgholam et al. 2011). The GoTaq master mix with cDNA samples were prepared as described in Section 2.5. The PCR thermal profile was 1 cycle of 95°C for 4 min; 40 cycles of 95°C for 30 s, 57°C for 30 s, 72°C for 30 s; and a final extension of 72°C for 10 min. Positive samples were subjected to further genetic characterization by PCR amplification of the *rpoB* and *gyrB* genes as previously described by Soto et al. (2017). For the *rpoB* gene, the primers used were 5′-TTC CGT CGT TCA AAC TCA GG-3′, 5′-TCT CAC CAA AAC GTT GTC CA-3′, and the thermal profile was 1 cycle of 95°C for 4 min; 40 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s; and a final extension of 72°C for 10 min; for the *gyrB* gene, we used the primers 5′-ACA TCG GCA TCG GTC ATK A-3′, 5′-GCG GAG GCG GYT ATA AGG TT-3′, and a thermal profile of 1 cycle at 95°C for 4 min; 40 cycles at 95°C for 30 s, 56°C for 30 s, 72°C for 30 s; and a final extension of 72°C for 10 min.

2.7. Sequencing and phylogenetic analysis

NNV qPCR, NNV genotyping PCR and *Streptococcus* spp. PCR amplicons were purified by ExoSAP-IT (Affymetrix). NNV genotyping PCR amplicons were sequenced by the Macrogen Europe Laboratory (Amsterdam, the Netherlands), while the remaining amplicons were sent to HyLabs (Rehovot, Israel) for sequencing. Sequences were aligned and compared to representative sequences available in GenBank by BLAST using the BioEdit Sequence Alignment Editor and MEGA7software (Kumar et al. 2016).

Genetic characterization of positive isolates based on RNA1 and RNA2 were inferred from maximum likelihood (ML) trees performed with the PhyML v.3.0 program (Guindon et al. 2010), with the Kimura 2-parameter model of nucleotide substitution, K80+G with 4 rate categories for RNA1 and K80+I for RNA2, based on the Smart Model Selection (Lefort et al. 2017) available in the PhyML program. Robustness of tree nodes was assessed by 1000 bootstrap replicates using the ML substitution model defined above. Phylogenetic trees were visualized with FigTree v1.4.3 software (http://tree.bio.ed.ac.uk/software/figtree/).

Sequences were deposited in GenBank and accession numbers are provided in Table S1.

2.8. Statistical analysis

Statistical significance between compared tissues of *Streptococcus* spp. positive samples and between sampled areas was determined by a chi-squared test of independence using IBM SPSS Statistics for Windows, version 20.0. For all tests, results were considered significant at $p < 0.05$.

3. RESULTS

A total of 195 wild fish and 33 wild crustaceans were tested for NNV, and a total of 174 wild fish and 32 wild crustaceans were tested for *Streptococcus* spp. (Table 1). Based on the sequencing of qPCR amplicons, NNV was detected in fish from all species examined in this study, except from *Sardinella aurita* and *Dussumieria elopsoides* (Order: Clupeiformes) (Table 2). Brain samples of the following fish species were positive for NNV: *Mullus surmuletus* (1/22, 4.55%), *M. barbatus* (7/9, 77.78%), *Upeneus moluccensis* (9/34, 26.47%), *Lithognathus mormyrus* (4/30, 13.33%), *Nemipterus randalli* (15/29, 51.72%) and *Saurida lessepsianus* (11/38, 28.95%). In nerve tissue of the crustacean *Portunus pelagicus*, 2/33 samples (6.06%) were found to be positive for NNV. In farmed *Sparus aurata*, 3 NNV positive brain samples were detected from the fish group (n = 15, 20%) sampled after 120 d at the farm. In the groups sampled at the hatchery and 7 d after populating the farm, all tissue samples were negative for NNV (Table 2).

Positive results for *Streptococcus* spp. were obtained in 20 out of 206 total wild specimens, and in 5 out of 7 wild fish species (Table 3). *Streptococcus* spp. were detected in *M. surmuletus* (2/22, 9.1%), *M. barbatus* (4/6, 66.6%), *L. mormyrus* (1/19, 5.3%), *N. randalli* (9/29, 31.03%) and *Saurida lessepsianus* (4/38, 10.53%). Out of the same samples, 13 were positive for *S. iniae*, and 3 *S. lessepsianus* were positive for *Carnobacterium inhibens*. Negative results were obtained in all samples from the remaining 2 wild fish species, i.e. *Sardinella aurita* and *Upeneus moluccensis*, the mariculture *Sparus aurata* and from both crustacean species, i.e. *Marsupenaeus japonicus* and *P. pelagicus*. Within *Streptococcus* spp. and *S. iniae* positive samples, a significantly higher prevalence ($\chi^2 = 6.75$, $p < 0.05$) was found in kidney compared to liver tissues. From kidney samples, positive results were obtained in 7 *N. randalli*, 2 *M. surmuletus* and 4 *M. barbatus*, for a total of 13 out of 107 samples (12.1%). From liver samples, positive results were obtained in 1 sample each of *S. lessepsianus, N. randalli* and *L. mormyrus*, for a total of 3 out of 107 samples (2.8%, Table 4).

The phylogenetic analysis of NNV RNA1 and RNA2 segments from wild and cultured species revealed that all samples were of the RGNNV genotype and no reassortants were found (Figs. 2 & 3). All samples formed a monophyletic cluster (bootstrap values ≥70%) similar to the RGNNV reference sequences from GenBank. The virus RNA2 partial sequence detected in the cultured *S. aurata* showed high similarity to the virus sequence detected in the wild species (Fig. 3). Co-infection of *Streptococcus* spp. and NNV was detected in 5 specimens: 2 *M. barbatus* and 3 *N. randalli*. All but 1 *M. barbatus* were positive for *S. iniae*. There was a significant difference ($\chi^2 = 4.84$, $p < 0.05$) in the prevalence of NNV positive specimens between the 2 sampled areas, i.e. 27.4% in the north and 15.1% in the south. No spatial significant difference was found for *S. iniae* ($p = 0.11$).

Table 2. Detection of nervous necrosis virus (NNV) in wild and cultured fish and crustaceans with amplification of a 203 nt RNA2 amplicon by qPCR. All positive samples were identified as the RGNNV genotype based on sequencing of the RNA1 and RNA2 amplicons

Host species	Total n	Positive for NNV n	Positive for NNV %
Saurida lessepsianus	38	11	28.95
Nemipterus randalli	29	15	51.72
Mullus surmuletus	22	1	4.55
Mullus barbatus	9	7	77.78
Lithognathus mormyrus	30	4	13.33
Sardinella aurita	30	0	0.00
Dussumieria elopsoides	3	0	0.00
Upeneus moluccensis	34	9	26.47
Portunus pelagicus	33	2	6.06
Total wild	228	49	21.49
Sparus aurata - hatchery	15	0	0.00
- 7 d	15	0	0.00
- 120 d	15	3	20.00

4. DISCUSSION

In this study, we report the prevalence of 2 important aquatic pathogens, *Streptococcus* spp. and NNV, in several marine wild fish and crustaceans in the eastern Mediterranean Sea. Both pathogens were detected in indigenous and Lessepsian species, and their prevalence varied vastly between fish species. The prevalence of *Streptococcus* spp. ranged between 5.26 and 66.67% and that of NNV was between 4.55 and 77.78% among different species, with an overall prevalence of 9.71 and 21.49%, respectively. To our knowledge, this is the first report of NNV and *S. iniae* in all analyzed species except *Mullus barbatus* and *M. surmuletus* (Agnew & Barnes 2007, Ciulli et al. 2007, Panzarin et al. 2012, Moreno et al. 2014, Costa & Thompson 2016). Moreover, this is the first survey of *S. iniae* prevalence in asymptomatic wild fish. Most previous reports of *Streptococcus* spp. were from morbid fish associated with proximity to fish

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Table 3. Detection of *Streptococcus* species by PCR with primers targeting 16s, *gyrB* and *rpoB* genes in wild fish. Calculation of percentage is based on the ratio of positive with 16s to the total analyzed fish. Positive results are indicated with '+'. Samples co-infected with nervous necrosis virus (NNV) are highlighted in **bold** and marked with '+' in the NNV co-infection column

Host species	Total n	Positive for 16s gene n	%	Sample ID	16s gene	*gyrB*	*rpoB*	NNV co-infection	Pathogenic strain
Wild fish									
Mullus surmuletus	22	2	9.09	16.181.MS.226	+	+	+	–	*Streptococcus iniae*
				16.182.MS.227	+	+	+	–	*Streptococcus iniae*
Mullus barbatus	6	4	66.67	**16.185.MB.216**	+	+	+	+	*Streptococcus iniae*; RGNNV
				16.186.MB.217	+	+	+	–	*Streptococcus iniae*
				16.187.MB.218	+	+	+	–	*Streptococcus iniae*
				16.188.MB.219	+	–	–	+	*Streptococcus* spp.; RGNNV
Upeneus moluccensis	34	0	0.00						
Lithognathus mormyrus	19	1	5.26	16.162.LM.052	+	+	–	–	*Streptococcus iniae*
Nemipterus randalli	29	9	31.03	16.392.NR.089	+	+	–	–	*Streptococcus iniae*
				16.079.NR.024	+	+	–	+	*Streptococcus iniae*; RGNNV
				16.199.NR.240	+	+	+	+	*Streptococcus iniae*; RGNNV
				16.200.NR.241	+	+	+	+	*Streptococcus iniae*; RGNNV
				16.201.NR.242	+	+	+	–	*Streptococcus iniae*
				16.202.NR.243	+	–	+	–	*Streptococcus iniae*
				16.203.NR.244	+	–	–	–	*Streptococcus* spp.
				16.205.NR.246	+	+	+	–	*Streptococcus iniae*
				16.206.NR.247	+	–	–	–	*Streptococcus* spp.
Saurida lessepsianus	38	4	10.53	16.113.SL.110	+	–	–	–	*Streptococcus* spp.
				16.380.SL.150	+	–	+	–	*Carnobacterium inhibens*
				16.381.SL.151	+	–	+	–	*Carnobacterium inhibens*
				16.382.SL.152	+	–	+	–	*Carnobacterium inhibens*
Sardinella aurita	26	0	0.00						
Wild crustaceans									
Marsupenaeus japonicus	26	0	0.00						
Portunus pelagicus	6	0	0.00						
Total wild	206	20	9.71						
Mariculture species									
Sparus aurata - hatchery	15	0	0.00						
- 7 d	15	0	0.00						
- 120 d	15	0	0.00						

Table 4. Relative distribution and percentage of *Streptococcus* spp. in kidney and liver tissues of wild fish. A significantly higher prevalence ($\chi^2 = 6.75$, $p < 0.05$) was found in kidney than in liver tissue. Only specimens tested for both tissues were included in the statistical analysis

Species	Total n	Positive for *Streptococcus* spp. Kidney n	Kidney %	Liver n	Liver %
Mullus surmuletus	22	2	9.1	0	0.0
Mullus barbatus	6	4	66.7	0	0.0
Lithognathus mormyrus	19	0	0.0	1	5.3
Nemipterus randalli	25	7	24.1	1	4.0
Saurida lessepsianus	35	0	0.0	1	2.9
Total	107	13	12.1	3	2.8

farming, with few exceptions describing infection of diseased wild reef fish (Zlotkin et al. 1998, Colorni et al. 2002, Agnew & Barnes 2007, Chou et al. 2014, Soto et al. 2015).

Streptococcosis has significant impacts on fish health and global aquaculture productivity, and the most pathogenic *Streptococcus* species in worldwide aquaculture are *S. iniae*, *S. agalactiae*, *S. dysgalactiae* and *Lactococcus garviae*. While *S. iniae* has been implicated in significant economic losses in global aquaculture, equally important is the high mortality that *S. iniae* can cause in wild fish stocks. In some regions of the world, like the Caribbean and Red Seas, outbreaks in wild fish populations can have major effects on the ecosystem (Colorni et al. 2002,

Chou et al. 2014). Since the first report of *S. iniae* in 1976, there have been many confirmed outbreaks in numerous fish species in temperate and warm water regions across the globe (Agnew & Barnes 2007), including a very recent outbreak in the northern part of the Red Sea along the Israeli coast during 2017 (A. Diamant pers. comm.). Beside the ecological importance and potential economic consequences, *S. iniae* can be dangerous to humans due to its zoonotic features, and human populations working with or in proximity to infected fish are considered to be at higher risk (Chou et al. 2014). The increasing number and spread of cases supports that *S. iniae* is considered a re-emerging infection concern in certain geographic locations, like North America, the Middle East and the Asian-Pacific region, possibly due to new variants and increased antibiotic resistance (Bachrach et al. 2001, Miller & Neely 2005, Pridgeon & Klesius 2012).

In all but 3 cases, *Streptococcus* detected in this study was highly similar to *S. iniae.* Although *S. iniae* can be isolated from different organs, it has been suggested that *S. iniae* has an affinity for, or is more readily detected in, kidney tissue (Bromage et al. 1999, Evans et al. 2001, Agnew & Barnes 2007); this is consistent with our results that the prevalence of *S. iniae* was higher in kidney than in liver tissues, except in *Saurida lessepsianus* and *Lithognathus mormyrus*, where positive results were only obtained in liver tissue. In this study, we also detected *Carnobacterium inhibens* in 3 *S. lessepsianus* collected from the port of Ashdod during December 2016. Most *Carnobacterium* species belong to a group that was originally isolated from biological sources such as fish or other farmed animals (Leisner et al. 2007). *C. inhibens* was also detected in apparently healthy Atlantic salmon *Salmo salar* more than 25 yr ago (Jöborn et al. 1999), and the meaning of its anecdotal finding in this study needs further investigations.

Previous studies in the Mediterranean area reported the prevalence of viruses belonging to the *Betanodavirus* genus in wild and cultured fish (Castric et al. 2001, Ucko et al. 2004, Vendramin et al. 2013, Kara et al. 2014) and invertebrates (Gomez et al. 2008, Panzarin et al. 2012, Fichi et al. 2015, Volpe et al. 2018). NNV strains were isolated from fish samples belonging to several distinct orders, including Clupeiformes and Perciformes. Phylogenetic analysis of viral isolates from fish along the European Medi-

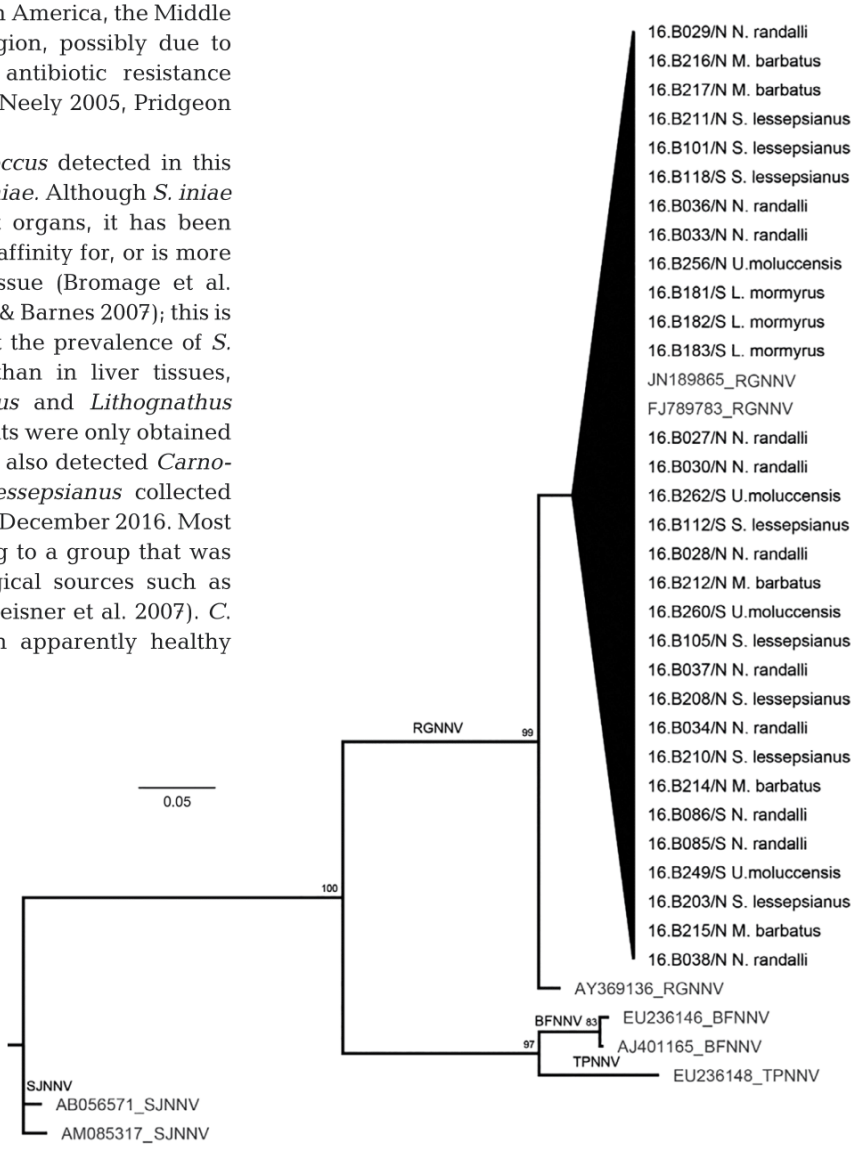

Fig. 2. Maximum likelihood (ML) phylogenetic tree of the RNA1 partial sequences of nervous necrosis virus (NNV). The sequence name includes the identification number, area of collection (N: north, S: south; Fig. 1) and host species. Positive samples from this study are shown in black, and reference sequences from GenBank are in grey. NNV genotype subdivision is displayed on the branches. The numbers at the branch nodes represent bootstrap values (only values ≥70% are shown). The scale bar represents 0.05 nucleotide substitutions per site

terranean area revealed the presence of RGNNV, SJNNV and RGNNV/SJNNV reassortants (Cutrín et al. 2007, Toffolo et al. 2007, Olveira et al. 2009). The reassortants were found only in cultured fish and revealed a strong clustering of NNV according to their farming status, i.e. wild or cultured (Panzarin et al. 2012). The phylogentic analysis in our study suggested that NNV detected in wild species in the sampled area is of the RGNNV strain. In our study, the prevalence of NNV was similar to different areas of the Mediterranean Sea, where previous studies surveying the presence of *Betanodavirus* in wild fish populations reported a prevalence of 11.9% in the Adriatic Sea (Ciulli et al. 2007), 19.6% in the Gulf of Cadiz in the Atlantic Ocean, near the Strait of Gibraltar (Moreno et al. 2014), and higher levels of up to an average of 62% in wild fish adjacent to fish farms in Tunisia (Cherif & Fatma 2017). The relatively high prevalence of NNV that we detected in some of the fish species may indicate that those species are resistant or have low susceptibility to NNV, that the positive fish can act as carriers and that they could serve as a virus reservoir. In contrast, NNV was not detected in both clupeiform species, which may suggest either high susceptibility, resulting in death of any infected specimens, or a high resistance to infection by NNV. NNV was present in all 3 species of the family Mullidae tested in this study, whereas previous surveys reported the presence of NNV only in *M. barbatus* and *M. surmuletus* (Ciulli et al. 2007, Panzarin et al. 2012, Moreno et al. 2014). We detected higher total NNV prevalence in fish sampled in the northern area; however, this might be due to the higher prevalence of NNV detected in *M. barbatus*, a species that was not sampled in the southern area. Further larger-scale surveys should be performed for better spatial accuracy.

Interestingly, we detected NNV in all Lessepsian species, i.e. *S. lessepsianus*, *Nemipterus randalli*, *Upeneus moluccensis* and the blue crab *Portunus pelagicus*, while *Streptococcus* spp. were detected only in *N. randalli* and in a single *S. lessepsianus* specimen. Invasive species from the Red Sea have been successfully established in the new environment, with vast implications for local biodiversity, changes in food webs and the ecosystem. The reasons for this successful establishment are not fully understood, but it is possible that mechanisms such

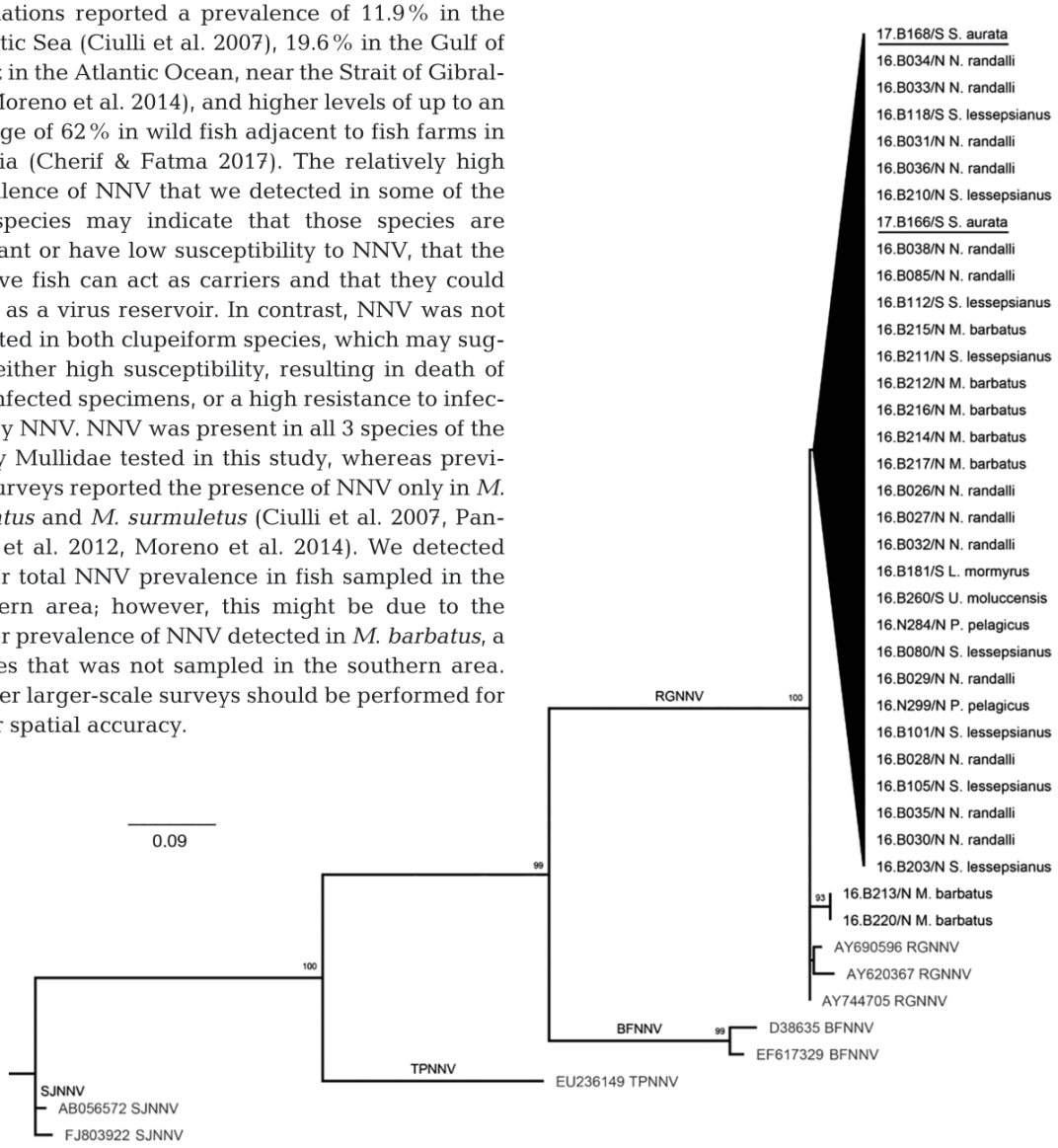

Fig. 3. Maximum likelihood (ML) phylogenetic tree of the RNA2 partial sequences of nervous necrosis virus (NNV). Sequences of cultured *Sparus aurata* are underlined. The scale bar represents 0.09 nucleotide substitutions per site. All other details as in Fig. 2

as favorable temperature, foraging activities and partitioning of biological niches might be involved (Edelist 2013, Stern et al. 2014). It has been suggested that *N. randalli* is responsible for the disappearance of the indigenous *Pagellus erythrinus* (Edelist et al. 2013). In our study, *N. randalli* specimens sampled showed a prevalence of 51.72% (NNV) and 31.03% (*S. iniae*). A high prevalence of a specific pathogen in a species may suggest its resistance to the pathogen and should be considered as an additional factor when predicting the successful invasion of an alien species and its establishment. The blue crab *P. pelagicus* became a common species after its establishment in the Mediterranean and has become a significant species for fisheries (Edelist 2013). Nodaviruses, including *Macrobrachium rosenbergii* nodavirus (MrNV) and *Penaeus vannamei* nodavirus (PvNV), affect both freshwater and marine crustaceans (Arcier et al. 1999, Tang et al. 2007), and the genus *Betanodavirus* has been detected in wild invertebrates and may be a vector for infection of wild and cultured marine species (Panzarin et al. 2012, Fichi et al. 2015, Volpe et al. 2018). Different invertebrate species, including crabs, are asymptomatic carriers for viruses such as the white spot syndrome virus (WSSV) affecting shrimp species (Kanchanaphum et al. 1998, Supamattaya et al. 1998, Hossain et al. 2001), thus further studies are necessary for understanding if *P. pelagicus* might also be a carrier and a vector of betanodavirus.

The farmed *Sparus aurata* tested in this study were negative for *Streptococcus* species in all 3 sampling events, but 3 out of 15 specimens sampled after 120 d in the farm were found to be positive for NNV. The isolates were genotyped and belong to the RGNNV strain, and are similar (bootstrap values >70%) to the betanodavirus detected in the wild species (Fig. 3). This offshore farm is located far from any other marine farm, and *S. aurata* is the sole species cultured there. Moreover, it is a new farm, operating only since 2017, and the fish tested in our study are the first batch of fish stocked in the facilities. Therefore and although only 45 specimens were tested, this finding together with the phylogenetic similarity of the virus from wild species, suggests spontaneous transmission of NNV from wild to cultured fish in that selected time frame.

Due to the expected low pathogenic loads of infectious agents in wild populations, it is hard to perform an epidemiological surveillance on wild fish populations. Nevertheless, a baseline for a pathogen database will provide the starting point for future studies on the relationship and interactions between wild and cultured fish and the mechanisms of infection. The presence of pathogens in wild fish populations is, of course, predicted; understanding the prevalence of each pathogen in different hosts and the meaning of different prevalences is challenging and important from both ecological and agricultural aspects.

The transmission of pathogens between different hosts in the ocean is fascinating, but difficult to study and to prove. There are several ways for novel pathogens to be introduced into a naïve population. For example, a benign organism can undergo a genetic change that renders it more or less pathogenic and more or less susceptible to different hosts. A pathogen can also be introduced via the range expansion of wild fish to new geographic areas as a result of global warming or removal of natural barriers (i.e. canals, habitat management). It is possible that high stocking densities, accumulation of waste, handling and poor water quality all serve to compromise immunity of fish and favor disease emergence. The influence of infectious diseases on global biodiversity loss in the ocean and the impact of mariculture on pathogen transmission in the aquatic environment are yet to be assessed; surveillance studies describing infectious agents in aquatic animals should be conducted more often and in multiple seas around the globe for a better understanding of marine disease dynamics and epidemiology. All fish and crustacean species sampled in this study are important species in the eastern Mediterranean fishing industry (Edelist 2013), and very limited data are available regarding the presence and prevalence of these pathogens in wild fish in this survey area. Our findings highlight the potential of multiple fish and crustacean species to serve as pathogen reservoirs that might, in turn, transmit diseases to other species. As mariculture farming expands, multiple factors should be considered, and a long-term epidemiological study of wild fish populations should serve as a critical component of marine fish farm site planning.

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LITERATURE CITED

Agnew W, Barnes AC (2007) *Streptococcus iniae*: an aquatic pathogen of global veterinary significance and a challenging candidate for reliable vaccination. Vet Microbiol 122:1–15

Arcier JM, Herman F, Lightner DV, Redman RM, Mari J, Bonami JR (1999) A viral disease associated with mortal-

ities in hatchery-reared postlarvae of the giant freshwater prawn *Macrobrachium rosenbergii*. Dis Aquat Org 38:177–181

Arechavala-Lopez P, Sanchez-Jerez P, Bayle-Sempere JT, Uglem I, Mladineo I (2013) Reared fish, farmed escapees and wild fish stocks — a triangle of pathogen transmission of concern to Mediterranean aquaculture management. Aquacult Environ Interact 3:153–161

Bachrach G, Zlotkin A, Hurvitz A, Evans DL, Eldar A (2001) Recovery of *Streptococcus iniae* from diseased fish previously vaccinated with a *Streptococcus* vaccine. Appl Environ Microbiol 67:3756–3758

Bovo G, Gustinelli A, Quaglio F, Gobbo F and others (2011) Viral encephalopathy and retinopathy outbreak in freshwater fish farmed in Italy. Dis Aquat Org 96:45–54

Bromage ES, Thomas A, Owens L (1999) *Streptococcus iniae*, a bacterial infection in barramundi *Lates calcarifer*. Dis Aquat Org 36:177–181

Castric J, Thiéry R, Jeffroy J, de Kinkelin P, Raymond JC (2001) Sea bream *Sparus aurata*, an asymptomatic contagious fish host for nodavirus. Dis Aquat Org 47:33–38

Cherif N, Fatma A (2017) Nodaviruses in wild fish population collected around aquaculture cage sites from coastal areas of Tunisia. Fish Aquacult J 08:2–7

Chou L, Griffin MJ, Fraites T, Ware C and others (2014) Phenotypic and genotypic heterogeneity among *Streptococcus iniae* isolates recovered from cultured and wild fish in North America, Central America and the Caribbean islands. J Aquat Anim Health 26:263–271

Ciulli S, Galletti E, Grodzki M, Alessi A, Battilani M, Prosperi S (2007) Isolation and genetic characterization of *Betanodavirus* from wild marine fish from the Adriatic Sea. Vet Res Commun 31:221–224

Colorni A, Diamant A, Eldar A, Kvitt H, Zlotkin A (2002) *Streptococcus iniae* infections in Red Sea cage-cultured and wild fishes. Dis Aquat Org 49:165–170

Costa JZ, Thompson KD (2016) Understanding the interaction between *Betanodavirus* and its host for the development of prophylactic measures for viral encephalopathy and retinopathy. Fish Shellfish Immunol 53:35–49

Cutrín JM, Dopazo CP, Thiéry R, Leao P, Olveira JG, Barja JL, Bandín I (2007) Emergence of pathogenic betanodaviruses belonging to the SJNNV genogroup in farmed fish species from the Iberian Peninsula. J Fish Dis 30: 225–232

Edelist D (2013) Fishery management and marine invasion in Israel. PhD dissertation, University of Haifa

Edelist D, Rilov G, Golani D, Carlton JT, Spanier E (2013) Restructuring the sea: profound shifts in the world's most invaded marine ecosystem. Divers Distrib 19:69–77

Evans JJ, Shoemaker CA, Klesius PH (2001) Distribution of *Streptococcus iniae* in hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) following nare inoculation. Aquaculture 194:233–243

FAO (2016) The state of world fisheries and aquaculture 2016. Contributing to food security and nutrition for all. FAO, Rome

Fichi G, Cardeti G, Perrucci S, Vanni A and others (2015) Skin lesion-associated pathogens from *Octopus vulgaris*: first detection of *Photobacterium swingsii, Lactococcus garvieae* and betanodavirus. Dis Aquat Org 115:147–156

Gomez DK, Baeck GW, Kim JH, Choresca CH, Park SC (2008) Molecular detection of betanodaviruses from apparently healthy wild marine invertebrates. J Invertebr Pathol 97:197–202

Gomez-Casado E, Estepa A, Coll JM (2011) A comparative review on European-farmed finfish RNA viruses and their vaccines. Vaccine 29:2657–2671

Gozlan RE, Peeler EJ, Longshaw M, St-Hilaire S, Feist SW (2006) Effect of microbial pathogens on the diversity of aquatic populations, notably in Europe. Microbes Infect 8:1358–1364

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0′. Syst Biol 59:307–321

Harvell CD, Kim K, Burkholder JM, Colwell RR and others (1999) Emerging marine diseases — climate links and anthropogenic factors. Science 285:1505–1510

Harvell CD, Mitchell CE, Ward JR, Altizer S, Dobson AP, Ostfeld RS, Samuel MD (2002) Climate warming and disease risks for terrestrial and marine biota. Science 296: 2158–2162

Hossain MS, Chakraborty A, Joseph B, Otta SK, Karunasagar I, Karunasagar I (2001) Detection of new hosts for white spot syndrome virus of shrimp using nested polymerase chain reaction. Aquaculture 198:1–11

Jeppesen E, Brucet S, Naselli-Flores L, Papastergiadou E and others (2015) Ecological impacts of global warming and water abstraction on lakes and reservoirs due to changes in water level and related changes in salinity. Hydrobiologia 750:201–227

Jöborn A, Dorsch M, Olsson JC, Westerdahl A, Kjelleberg S (1999) *Carnobacterium inhibens* sp. nov., isolated from the intestine of Atlantic salmon (*Salmo salar*). Int J Syst Bacteriol 49:1891–1898

Kanchanaphum P, Wongteerasupaya C, Sitidilokratana N, Boonsaeng V and others (1998) Experimental transmission of White Spot Syndrome Virus (WSSV) from crabs to shrimp *Penaeus monodon*. Dis Aquat Org 34:1–7

Kara HM, Chaoui L, Derbal F, Zaidi R, de Boisséson C, Baud M, Bigarré L (2014) Betanodavirus-associated mortalities of adult wild groupers *Epinephelus marginatus* (Lowe) and *Epinephelus costae* (Steindachner) in Algeria. J Fish Dis 37:273–278

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for bigger datasets. Mol Biol Evol 33:1870–1874

Kuo HC, Wang TY, Chen PP, Chen YM, Chuang HC, Chen TY (2011) Real-time quantitative PCR assay for monitoring of nervous necrosis virus infection in grouper aquaculture. J Clin Microbiol 49:1090–1096

Lefort V, Longueville JE, Gascuel O (2017) SMS: Smart Model Selection in PhyML. Mol Biol Evol 34:2422–2424

Leisner JJ, Laursen BG, Prévost H, Drider D, Dalgaard P (2007) *Carnobacterium*: positive and negative effects in the environment and in foods. FEMS Microbiol Rev 31: 592–613

Lejeusne C, Chevaldonné P, Pergent-Martini C, Boudouresque CF, Pérez T (2010) Climate change effects on a miniature ocean: the highly diverse, highly impacted Mediterranean Sea. Trends Ecol Evol 25:250–260

Miller JD, Neely MN (2005) Large-scale screen highlights the importance of capsule for virulence in the zoonotic pathogen *Streptococcus iniae*. Infect Immun 73:921–934

Moreno P, Olveira JG, Labella A, Cutrín JM, Baro JC, Borrego JJ, Dopazo CP (2014) Surveillance of viruses in wild fish populations in areas around the Gulf of Cadiz (South Atlantic Iberian Peninsula). Appl Environ Microbiol 80: 6560–6571

Olveira JG, Souto S, Dopazo CP, Thiéry R, Barja JL, Bandín I (2009) Comparative analysis of both genomic segments of betanodaviruses isolated from epizootic outbreaks in farmed fish species provides evidence for genetic reassortment. J Gen Virol 90:2940–2951

Panzarin V, Fusaro A, Monne I, Cappellozza E and others (2012) Molecular epidemiology and evolutionary dynamics of betanodavirus in southern Europe. Infect Genet Evol 12:63–70

Pourgholam R, Laluei F, Saeedi AA, Zahedi A and others (2011) Distribution and molecular identification of some causative agents of streptococcosis isolated from farmed rainbow trout (*Oncorhynchus mykiss*, Walbaum) in Iran. Iran J Fish Sci 10:109–122

Pridgeon JW, Klesius PH (2012) Major bacterial diseases in aquaculture and their vaccine development. CAB Rev 7: 048

Rosa R, Marques A, Nunes ML (2012) Impact of climate change in Mediterranean aquaculture. Rev Aquacult 4: 163–177

Soto E, Wang R, Wiles J, Green C, Plumb J, Hawke J (2015) Characterization of isolates of *Streptococcus agalactiae* from diseased farmed and wild marine fish from the U.S. Gulf coast, Latin America, and Thailand. J Aquat Anim Health 27:123–134

Soto E, Richey C, Stevens B, Yun S and others (2017) Co-infection of *Acipenserid herpesvirus 2* (AciHV-2) and *Streptococcus iniae* in cultured white sturgeon *Acipenser transmontanus*. Dis Aquat Org 124:11–20

Stern N, Levitt Y, Galil BS, Diamant A, Yoke MB, Goren M (2014) Distribution and population structure of the alien Indo-Pacific Randall's threadfin bream *Nemipterus randalli* in the eastern Mediterranean sea. J Fish Biol 85: 394–406

Supamattaya K, Hoffmann RW, Boonyaratpalin S, Kanchnaphum P (1998) Experimental transmission of white spot syndrome virus (WSSV) from black tiger shrimp *Penaeus monodon* to the sand crab *Portunus pelagicus*, mud crab *Scylla serrata* and krill *Acetes* sp. Dis Aquat Org 32:79–85

Tang KFJ, Pantoja CR, Redman RM, Lightner DV (2007) Development of *in situ* hybridization and RT-PCR assay for the detection of a nodavirus (*Pv*NV) that causes muscle necrosis in *Penaeus vannamei*. Dis Aquat Org 75: 183–190

Toffolo V, Negrisolo E, Maltese C, Bovo G, Belvedere P, Colombo L, Dalla Valle L (2007) Phylogeny of betanodaviruses and molecular evolution of their RNA polymerase and coat proteins. Mol Phylogenet Evol 43: 298–308

Ucko M, Colorni A, Diamant A (2004) Nodavirus infections in Israeli mariculture. J Fish Dis 27:459–469

Vendramin N, Olesen NJ (2017) Overview of the disease situation and surveillance in Europe in 2016. 21st Annual Workshop of the National Reference Laboratories for Fish Diseases, Kgs. Lyngby, Denmark

Vendramin N, Patarnello P, Toffan A, Panzarin V and others (2013) Viral encephalopathy and retinopathy in groupers (*Epinephelus* spp.) in southern Italy: a threat for wild endangered species? BMC Vet Res 9:20

Volpe E, Grodzki M, Panzarin V, Guercio A, Purpari G, Serratore P, Ciulli S (2018) Detection and molecular characterization of betanodaviruses retrieved from bivalve molluscs. J Fish Dis 41:603–611

Ward JR, Lafferty KD (2004) The elusive baseline of marine disease: Are diseases in ocean ecosystems increasing? PLOS Biol 2:E120

Weinstein MR, Litt M, Kertesz DA, Wyper P and others (1997) Invasive infections due to a fish pathogen, *Streptococcus iniae*. New Engl J Med 337:589–594

Yanong RPE (2003) Necropsy techniques for fish. Semin Avian Exotic Pet Med 12:89–105

Yeruham E, Rilov G, Shpigel M, Abelson A (2015) Collapse of the echinoid *Paracentrotus lividus* populations in the Eastern Mediterranean — result of climate change? Sci Rep 5:13479

Zlotkin A, Hershko H, Eldar A (1998) Possible transmission of *Streptococcus iniae* from wild fish to cultured marine fish. Appl Environ Microbiol 64:4065–4067

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Part II – Prevalence of NNV in indigenous and Lessepsian species of the Levantine Basin

Introduction

In the 2016 annual survey (Part I), I discovered that NNV is a widespread pathogen that can become a threat to wild and cultured fish populations. The prevalence rate of the virus varied among different species and a relatively high percentage of the positive results were detected in the Lessepsian species compared to the Mediterranean indigenous species. Following this survey, I decided to compare if there is a difference in the prevalence of NNV between indigenous and Lessepsian species. Two representative species of each group were selected for further analysis - the indigenous species *Sardinella aurita* and *Lithognathus mormyrus*, and the Lessepsian species *Nemipterus randalli* and *Saurida lessepsianus*. All four species are a major species in the Israeli Mediterranean fisheries and fish were sampled from fisheries trawling surveys. Positive results were compared to available sequences of NNV isolated from wild and farmed fish from Israel and along the Mediterranean Sea, after previous reports revealed a clustering of NNV sequences according to their geographical origin and farming status – wild or cultured (Ucko et al. 2004; Panzarin et al. 2012). The results of this study will help to further understand the natural distribution of leading pathogen (to date) in Mediterranean aquaculture and will provide additional insights of the relationship between indigenous to migrant species.

Materials and Methods

Fish and tissue sampling

A total of 109 specimens from four wild fish species – 20 *Lithognathus mormyrus*, 28 *Sardinella aurita*, 31 *Nemipterus randalli* and 30 *Saurida lessepsianus* were caught and brought on ice to the lab. All specimens were caught along the Israeli Mediterranean shoreline by trawling during the semiannual ecological survey conducted at November 2017 by the Israel Oceanographic and Limnological Research (IOLR) and during the surveys conducted during October to December 2017 by the Fishery and Aquaculture Unit of the Ministry of Agriculture. General measurements of weight, total length and visual inspection were carried out. All specimens were aseptically dissected for brain tissue sample collection according to the fish necropsy protocol of Yanong (2003). All samples were kept frozen at -80 °C until further analysis.

RNA extraction and cDNA synthesis by RT-PCR

Total RNA extracted, and cDNA was synthesized as described in Part I.

RNA1 and RNA2 PCR amplification

For detection and genotyping of NNV, all samples were subjected to PCR targeting of both genomic segments using the following primers as described in Part I: – 'FOR521' and 'VNNV6' targeted a 630nt fragment of the RNA1 segment, and 'VNNV1' and 'VNNV2' primers targeted a 605nt fragment of the RNA2 segment (Table 2 and Figure 4).

Sequencing and phylogenetic analysis

PCR amplicons were purified and sequenced by Sanger sequencing method at the Macrogen Europe Laboratory (Amsterdam, The Netherlands). Sequences were aligned using the MEGA7software (Kumar et al. 2016) and compared by BLAST to representative sequences available in GenBank. Sequences of NNV isolates from wild and farmed fish from the Mediterranean Sea region, including two from the Israeli Mediterranean and Red Sea (only RNA2 sequences were available), were included in the phylogenetic analysis (Table 3). In addition, a representative RNA1 and RNA2 positive sequence from Part I of this work were included as well.

Genetic characterization of NNV positive isolates, based on RNA1 and RNA2, were inferred from maximum likelihood (ML) trees performed with the PhyML v.3.0 program (Guindon et al. 2010) by applying the K80 + I model of nucleotide substitution for RNA1 and RNA2, based on the Smart Model Selection (Lefort et al. 2017) available in the PhyML program.

Robustness of nodes on the phylogeny was assessed by 1000 bootstrap replicates using the ML substitution model defined above. Phylogenetic trees were visualized with the FigTree v1.4.3 software (http://tree.bio.ed.ac.uk/software/figtree/).

Table 3: List of NNV isolates used in the phylogenetic analysis.
The following information is reported: Host fish species or isolate number, country and origin, wild or farmed, viral strain, and accession numbers in GenBank. n.a.: not available

			Accession number	
Host species / Isolate	**Origin**	**NNV strain**	**RNA1**	**RNA2**
Epinephelus akaara	China	RGNNV	-	AY744705
G9508KS	n.a	RGNNV	-	AY690596
Dicentrarchus labrax	Tunisia	RGNNV	FJ789783	-
Sardina pilchardus	Italy, wild	RGNNV	JN189868	JN190021
Epinephelus spp.	Greece, wild	RGNNV	JN189823	JN189975
Mullus barbatus	Croatia, wild	RGNNV	JN189808	JN189962
Epinephelus aeneus	Israel (Med. Sea [b]), wild	RGNNV	-	AY284965
Lates calcarifer	Israel (Red Sea), farmed	RGNNV	-	AY284973
Sparus aurata	Portugal, farmed	RG/SJ [a]	JN189844	JN189916
Pseudocaranx dentex	Japan	SJNNV	AB056571	AB056572
Verasper moseri	Japan	BFNNV	EU826137	D38635
TPKag93	Japan	TPNNV	EU236148	EU236149

[a] Genotyping of RNA 1/RNA2 of NNV
[b] Mediterranean Sea

Statistical analysis

Initially, I tested if there was a significant difference in the prevalence of NNV per each species between the sampled years – 2016 in Part I and 2017 in Part II. In addition, the significant difference of NNV prevalence between indigenous and Lessepsian species was tested using the results of both sampled year's results. Finally, the significant difference of the virus prevalence within each of the species was tested in comparison to all other species.

Statistical significance was determined by Chi-Square test of independence using IBM SPSS Statistics for Windows, version 20.0. For all tests, results were considered significant at $p < 0.05$.

Results

A total of 109 wild fish were tested for NNV by PCR amplification of both genomic RNA1 and RNA2 segments. Based on the sequencing of PCR amplicons, in the 2017 study the total prevalence of NNV was 22.94%. NNV was detected in all wild fish species examined in this study: *Lithognathus mormyrus* (3/20, 15.0%), *Sardinella aurita* (3/28, 10.7%), *Nemipterus randalli* (16/31, 51.6%) and *Saurida lessepsianus* (3/30, 10.0%). There was no significant difference ($p > 0.05$) in total prevalence of NNV between the species sampled in 2017 to the same species sampled in 2016 (Table 4).

Table 4: Summary of results of the 2016 and 2017 surveys for NNV prevalence in wild fish species from the Mediterranean Sea. Results based on the PCR targeting RNA1 and RNA2 segments. No significant difference was found between the consecutive years ($p > 0.05$).

	2016			2017			Total		
Fish species	n	positive	% positive	n	positive	% positive	n	positive	% positive
Lithognathus mormyrus	30	4	13.33%	20	3	15.00%	50	7	14.00%
Sardinella aurita	30	0	0.00%	28	3	10.71%	58	3	5.17%
Nemipterus randalli	29	15	51.72%	31	16	51.61%	60	31	51.67%
Saurida lessepsianus	38	11	28.95%	30	3	10.00%	68	14	20.59%
Total wild	127	30	23.62%	109	25	22.94%	236	55	23.31%

Specific prevalence per species showed no significant difference between the consecutive years as well (Figure 5). The total prevalence of NNV in *N. randalli*, relative to the other three species, was significantly higher in the 2017 sampling ($\chi^2 = 20.15$, $p < 0.001$) and in both consecutive years together ($\chi^2 = 36.2$, $p < 0.001$).

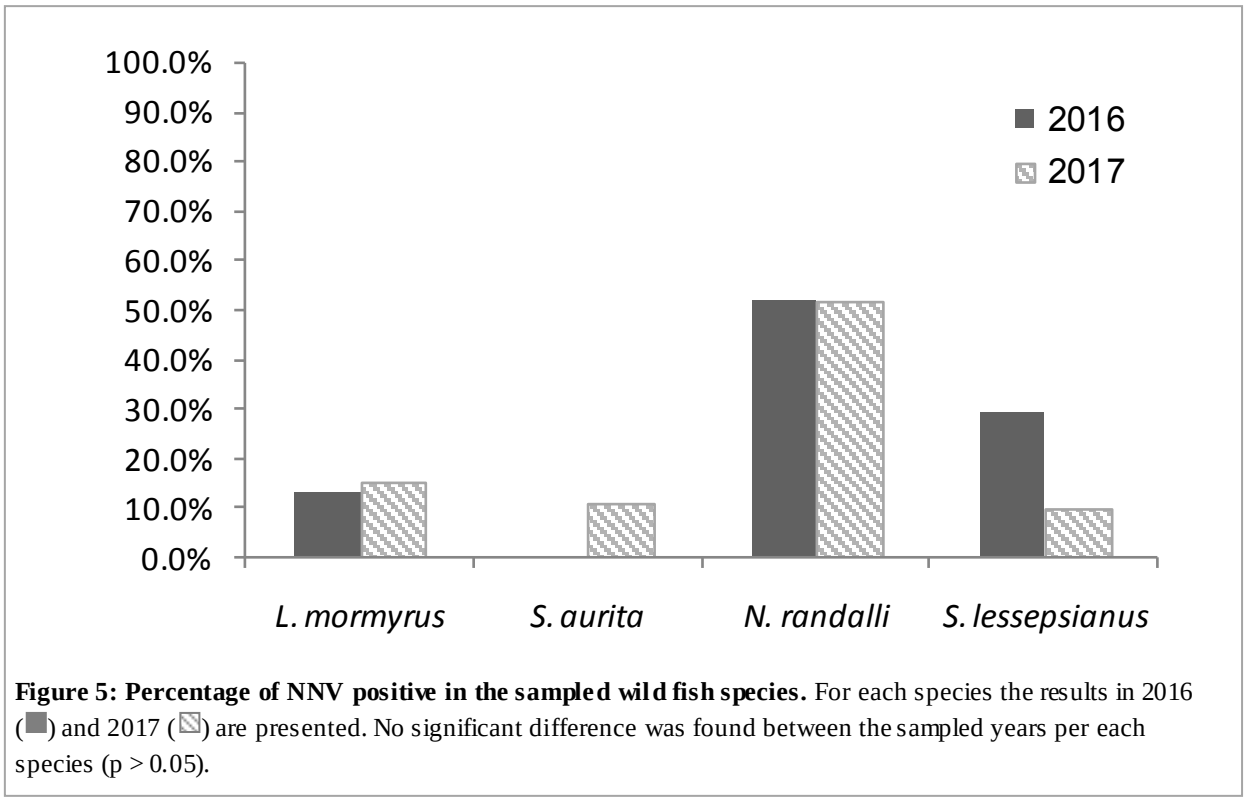

Figure 5: Percentage of NNV positive in the sampled wild fish species. For each species the results in 2016 (■) and 2017 (▨) are presented. No significant difference was found between the sampled years per each species ($p > 0.05$).

The phylogenetic analysis of NNV RNA1 and RNA2 segments revealed that all samples were of the RGNNV genotype (bootstrap values ≥ 70%) and no reassortants were found. Within the RGNNV genotype in the RNA1 phylogenetic tree (Figure 6), all samples of Mediterranean origin clustered differently than the sample from Portugal (Atlantic Ocean).

In the RNA2 phylogenetic tree (Figure 7), the sequences of this study were subclustered within the RGNNV genotype but with bootstrap support values lower than 70%.

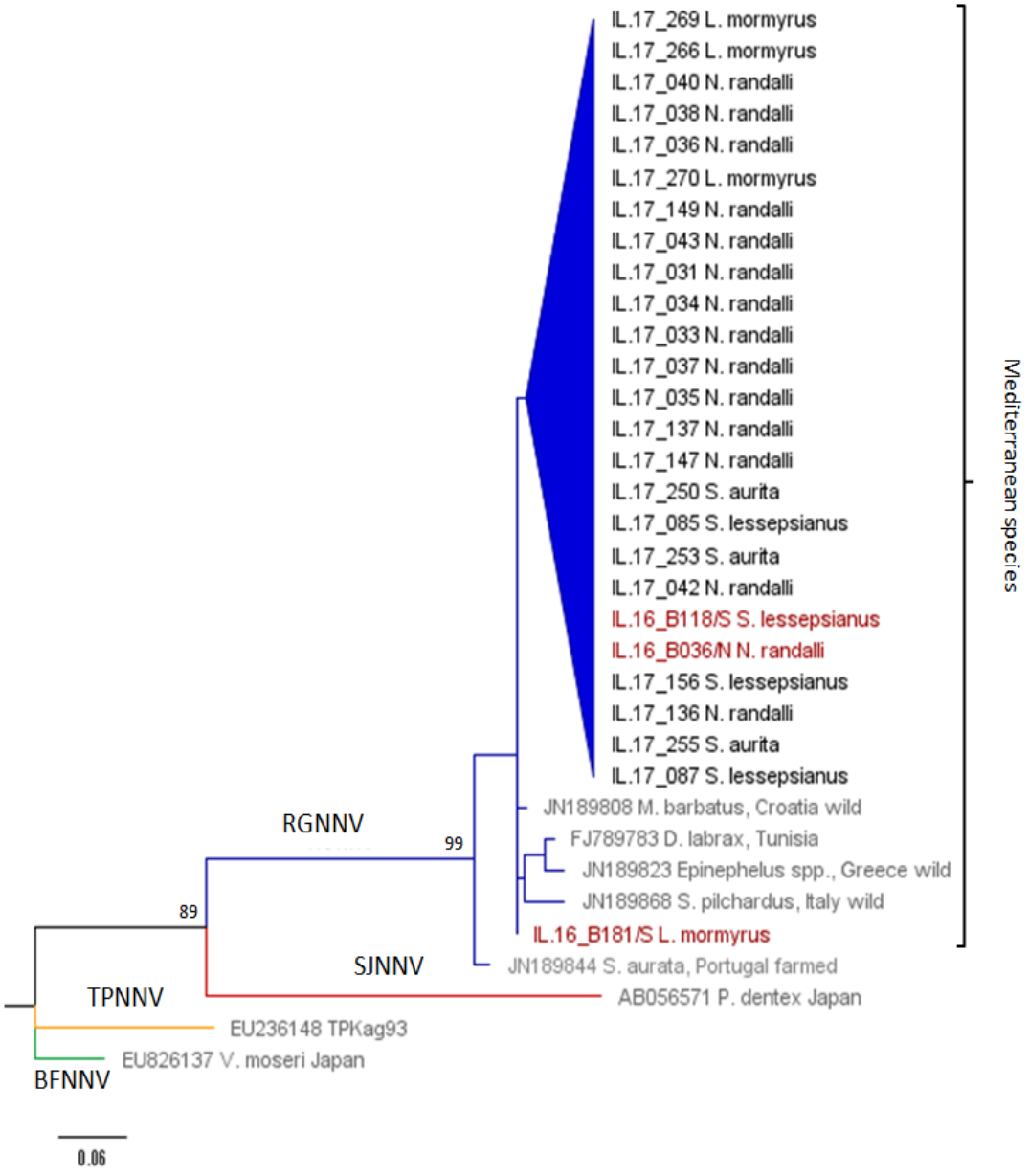

Figure 6: RNA1 phylogenetic tree. Maximum likelihood (ML) phylogenetic tree of the RNA1 partial sequences. The sequence name includes the identification number and host species. Positive samples of this study (those beginning with IL) are in black, reference sequences from GenBank are in grey, and representative sequences from 2016 (Part I) are in red. NNV genotype subdivision is displayed on the branches (blue: RGNNV; green: BFNNV; yellow: TPNNV; red: SJNNV). The vertical brace designates subclustering of samples isolated from fish of Mediterranean Sea origin. The numbers at the branch nodes represent bootstrap values (only values ≥ 70% are reported). The scale bar represents 0.06 nucleotide substitution per site.

Figure 7: RNA2 phylogenetic tree. Maximum likelihood (ML) phylogenetic tree of the RNA2 partial sequences. The sequence name includes the identification number and host species. Positive samples of this study (begins with IL) are in black, reference sequences from GenBank are in grey or purple for isolates from Israel and representative sequences from 2016 (Part I) are in red. NNV genotype subdivision is displayed on the branches (blue: RGNNV; green: BFNNV; yellow: TPNNV; red: SJNNV). The numbers at the branch's nodes represent bootstrap values (only values ≥ 70% are reported). The scale bar represents 0.2 nucleotide substitution per site.

Discussion

Following my first findings during the 2016 fish pathogen surveys (LTER) at Morris Kahn Marine Research Station (MKMRS), which demonstrated varied prevalence between wild species and relatively higher prevalence in Lessepsian species, an additional survey for NNV prevalence was conducted during the consecutive year (2017). Detection of the pathogens in this study was based on amplification methods, PCR and qPCR, that are capable to detect genomic fragments in a given tissue. Confirmation of the actual infection by a vital pathogen require an additional method such as histology, pathogen isolation and infection trials. Nevertheless, the results of this work can provide the first and basic indication of the pathogen abundance in the sampled species.

In this study, I report and compare the prevalence of NNV in two indigenous and two Lessepsian wild fish species from the eastern Mediterranean Sea. Since different strains of NNV were previously reported, including the presence of reassortants (Toffolo et al. 2007), both genomic segments have been characterized. In the four sampled species, the prevalence range was 0%-51.72% (mean 23.62%) in 2016 and 10%-51.61% (mean 22.94%) in 2017. The total mean prevalence of both years in the 4 species was 5.17%-51.67% (mean 23.31%).

The mean prevalence found in this study is higher than previous studies in wild fish populations (11.9% in the Adriatic Sea (Ciulli et al. 2007) and 19.6% at the Gulf of Cadiz (Moreno et al. 2014)), but this should be consider with caution due to the variation in prevalence levels between species. High variability in prevalence levels between different species, as obtained in both part of my study, highlights the difficulty to rely on total mean values as an indication measure in pathogenic survey. Therefore, prevalence level of a pathogen should be analyzed per species and interpretation of the results should be done accordingly.

When comparing both years, it interesting to notice that while in *Lithognathus mormyrus* and in *Nemipterus randalli* there was minor difference, in the 2 others species an opposite trends were obtained; prevalence in *Sardinella aurita* increased from total negative in 2016 to 10.7% in 2017, but in *Saurida lessepsianus* it decreased from 28.9% in 2016 to 10% in 2017.

In addition to the explanations that were suggested in part 1, another possible explanation to the low prevalence in *S. aurita* may be related to the lower probability of this pelagic fish to encounter with the virus in the open sea compare to other three benthic fish. Based on this study it is impossible to determine whether there is or not any correlation between the hosts' biological niche and level of infection and further investigation is required. This highlights the importance of long term monitoring in order to be possibly obtaining an actual trend between years, species and biological niche. This study reveals a significantly higher prevalence level

of NNV in the Lessepsian species *N. randalli* with more than half of the sampled fish obtained positive to the virus. It is possible that the virus has high capability to infect *N. randalli*, but with a low ability to produce lethal disease due to different host immune mechanisms (Ito et al. 2008). High prevalence of the virus in an asymptomatic fish may indicate they can serve as carriers and horizontally infect other susceptible species lives in proximity (Castric et al. 2001; Gomez et al. 2004; Korsnes et al. 2017). The invasive species *N. randalli* was first reported in the Mediterranean in 2005 (Golani & Sonin 2006), and become one of the dominant fish species in the Israeli ichthyofauna within 5 years only (Stern et al. 2014). It was previously suggested that this fish is responsible for the abundance drop of the indigenous *Pagellus erythrinus*, but the reasons for its successful establishment are unknown (Edelist 2013). Establishment of invasive species may result with shifts in local biodiversity, altered disturbance regimes and basic ecosystem processes, changes in food webs and with the introduction of new pathogens. The reasons for this successful establishment are not well understood, but few possible mechanisms, such as favorable temperature, foraging activities and partitioning of biological niche, have been suggested (Ben Rais Lasram & Mouillot 2009; Edelist 2013; Stern et al. 2014). Based on this study results, the sensitivity level of a species to pathogens should be considered as an additional mechanism to support the establishment of exotic species. Pathogenicity of NNV genotypes is influenced by combination of host specificity and environmental conditions that depends on the genomic segments of the virus (Toffan et al. 2016). Proteins and molecules presented on the capsid protein, encoded by RNA2 and differ between genotypes, interact with receptors on the surfaces of target cells and facilitate the entry of the virus (Ito et al. 2008; Chang & Chi 2015). After the viral infection, the rate of its replication depends on temperature influence the RNA-dependent RNA polymerase, encoded by RNA1. The hosts' siRNA, antagonized by the B2 protein, can limit the accumulation of the virus (Costa & Thompson 2016).

The phylogenetic analysis in my study showed that all detected NNV in the wild fish species from the Levantine basin were of the RGNNV genotype. This result agrees with previous studies reporting that in wild species no reassortants were detected and RGNNV is the main genotype (Ucko et al. 2004; Panzarin et al. 2012; Moreno et al. 2014). The different clustering of Mediterranean origin samples relative to the sample from Portugal within the RGNNV in the RNA1 phylogenetic tree is in agreement with the report of Panzarin et al. (2012), which revealed there is a strong clustering of betanodavirus based on their geographic distribution. Interestingly, the phylogenetic similarity of RGNNV sequences in the RNA2, which includes a sequence of an isolate from a grouper *Epinephelus aeneus* that brought from the

Mediterranean to the Red Sea, support the hypothesis of Ucko et al. (2004) that RGNNV were introduced into the Red Sea with specimens from the Mediterranean.

Conclusions

This study reports, for the first time, a fish pathogens survey in wild marine species in the Levantine Basin of the eastern Mediterranean and provides a scientific baseline of marine fish pathogens in this area. The high prevalence of NNV in the Lessepsian species *N. randalli* suggest that viral resistance may be an additional mechanism for the establishment of invasive species that should be taken into account. Further infection trials, either with indigenous and Lessepsian, or with wild and cultured species, are essential to complete the understanding of this possible mechanism.

The results of my study presented here highlight the importance of the molecular monitoring surveys in order to close the knowledge gap of pathogenic agents' prevalence in wild fish populations.

References (Thesis)

Arechavala-Lopez, P., Sanchez-Jerez, P., Bayle-Sempere, J.T., Uglem, I., Mladineo, I. (2013). Reared fish, farmed escapees and wild fish stocks - A triangle of pathogen transmission of concern to Mediterranean aquaculture management. *Aquaculture Environment Interactions*, *3*, 153–161.

Ben-Rais Lasram, F., Mouillot, D. (2009). Increasing southern invasion enhances congruence between endemic and exotic Mediterranean fish fauna. *Biological Invasions*, *11*, 697–711.

Bovo, G., Gustinelli, A., Quaglio, F., Gobbo, F. and others (2011). Viral encephalopathy and retinopathy outbreak in freshwater fish farmed in Italy. *Diseases of Aquatic Organisms*, *96*, 45–54.

Castric, J., Thiéry, R., Jeffroy, J., de Kinkelin, P., Raymond, J.C. (2001). Sea bream *Sparus aurata*, an asymptomatic contagious fish host for nodavirus. *Diseases of Aquatic Organisms*, *47*, 33–38.

Chang, J.S., Chi, S.C. (2015). GHSC70 is involved in the cellular entry of Nervous necrosis virus. *Journal of Virology*, *89*, 61–70.

Cherif, N., Fatma, A. (2017). Nodaviruses in wild fish population collected around aquaculture cage sites from coastal areas of Tunisia. *Fisheries and Aquaculture Journal*, *08*, 2–7.

Chou, L., Griffin, M.J., Fraites, T., Ware, C. and others. (2014). Phenotypic and genotypic heterogeneity among *Streptococcus iniae* isolates recovered from cultured and wild fish in north America , central America and the Caribbean islands. *Journal of Aquatic Animal Health*, *26*, 263–271.

Ciulli, S., Galletti, E., Grodzki, M., Alessi, A., Battilani, M., Prosperi, S. (2007). Isolation and genetic characterization of betanodavirus from wild marine fish from the Adriatic Sea. *Veterinary Research Communications*, *31*, 221–224.

Colorni, A., Diamant, A., Eldar, A., Kvitt, H., Zlotkin, A. (2002). *Streptococcus iniae* infections in Red Sea cage-cultured and wild fishes. *Diseases of Aquatic Organisms*, *49*, 165–170.

Costa, J.Z., Thompson, K.D. (2016). Understanding the interaction between betanodavirus and its host for the development of prophylactic measures for viral encephalopathy and retinopathy. *Fish and Shellfish Immunology*, *53*, 35–49.

Crane, M., Hyatt, A. (2011). Viruses of fish: An overview of significant pathogens. *Viruses*, *3*, 2025–2046.

Cusack, R., Cone, D.K. (1986). A review of parasites as vectors of viral and bacterial diseases of fish. *Journal of Fish Diseases*, *9*, 169–171.

Cutrín, J.M., Dopazo, C.P., Thiéry, R., Leao, P., Olveira, J.G., Barja, J.L., Bandín, I. (2007). Emergence of pathogenic betanodaviruses belonging to the SJNNV genogroup in farmed fish species from the Iberian peninsula. *Journal of Fish Diseases*, *30*, 225–232.

Diamant, A., Banet, A., Ucko, M., Colorni, A., Knibb, W., Kvitt, H. (2000). Mycobacteriosis in wild rabbitfish *Siganus rivulatus* associated with cage farming in the gulf of Eilat, Red Sea. *Diseases of Aquatic Organisms*, *39*, 211–219.

Edelist, D. (2013). *Fishery management and marine invasion in Israel*. PhD. dissertation, University of Haifa.

Edelist, D., Rilov, G., Golani, D., Carlton, J.T., Spanier, E. (2013). Restructuring the sea: Profound shifts in the world's most invaded marine ecosystem. *Diversity and Distributions*, *19*, 69–77.

Evans, J.J., Shoemaker, C.A., Klesius, P.H. (2001). Distribution of *Streptococcus iniae* in hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) following nare inoculation. *Aquaculture*, *194*, 233–243.

FAO. (2016). *The state of world fisheries and aquaculture 2016. Contributing to food security and nutrition for all. Rome. 200*.

FEAP. (2016). *European Aquaculture Production Report 2007-2015*. Federation of European Aquaculture Producers, Liege, Belgium

Galil, B.S. (2009). Taking stock: Inventory of alien species in the Mediterranean sea. *Biological Invasions*, *11*, 359–372.

Golani, D., Sonin, O. (2006). The Japanese threadfin bream *Nemipterus japonicus*, a new Indo-Pacific fish in the Mediterranean Sea. *Journal of Fish Biology*, 940–943.

Gomez-Casado, E., Estepa, A., Coll, J.M. (2011). A comparative review on european-farmed finfish RNA viruses and their vaccines. *Vaccine*, *29*, 2657–2671.

Gomez, D.K., Sato, J., Mushiake, K., Isshiki, T., Okinaka, Y., Nakai, T. (2004). PCR-based detection of betanodaviruses from cultured and wild marine fish with no clinical signs. *Journal of Fish Diseases*, *27*, 603–608.

Gozlan, R.E., Peeler, E.J., Longshaw, M., St-Hilaire, S., Feist, S.W. (2006). Effect of microbial pathogens on the diversity of aquatic populations, notably in Europe. *Microbes and Infection*, *8*, 1358–1364.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O. (2010). New algorithms and mehtods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology*, *59*, 307–321.

Harvell, C.D., Kim, K., Burkholder, J.M., Colwell, R.R. and others (1999). Emerging marine diseases-climate links and anthropogenic factors. *Science*, *285*, 1505–1510.

Harvell, C.D., Mitchell, C.E., Ward, J.R., Altizer, S., Dobson, A.P., Ostfeld, R.S., Samuel, M.D. (2002). Climate warming and disease risks for terrestrial and marine biota. *Science*, *296*, 2158–2162.

Holmlund, C.M., Hammer, M. (1999). Ecosystem services generated by fish populations. *Ecological Economics*, *29*, 253–268.

Ito, Y., Okinaka, Y., Mori, K.I., Sugaya, T., Nishioka, T., Oka, M., Nakai, T. (2008). Variable region of betanodavirus RNA2 is sufficient to determine host specificity. *Diseases of Aquatic Organisms*, *79*, 199–205.

Iwamoto, T., Mise, K., Mori, K.I., Arimoto, M., Nakai, T., Okuno, T. (2001). Establishment of an infectious RNA transcription system for Striped jack nervous necrosis virus, the type species of the betanodaviruses. *Journal of General Virology*, *82*, 2653–2662.

Jackson, D., Drumm, A., Mcevoy, S., Jensen, Ø. and others. (2015). A pan-European valuation of the extent, causes and cost of escape events from sea cage fish farming. *Aquaculture*, *436*, 21–26.

Jeppesen, E., Brucet, S., Naselli-Flores, L., Papastergiadou, E. and others (2015). Ecological impacts of global warming and water abstraction on lakes and reservoirs due to changes in water level and related changes in salinity. *Hydrobiologia*, *750*, 201–227.

Johansen, L. H., Jensen, I., Mikkelsen, H., Bjørn, P.A., Jansen, P. A., Bergh, O. (2011). Disease interaction and pathogens exchange between wild and farmed fish populations with special reference to Norway. *Aquaculture*, *315*, 167–186.

Korsnes, K., Karlsbakk, E., Skår, C.K., Sælemyr, L., Nylund, A., Kvamme, B.O., Mortensen, S. (2017). High nervous necrosis virus (NNV) diversity in wild wrasse (*Labridae*) in Norway and Sweden. *Diseases of Aquatic Organisms*, *126*, 43–50.

Kumar, S., Stecher, G., Tamura, K. (2016). MEGA7 : Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Evolutionary Genetic Analysis*, *33*, 1870–1874.

Kuo, H.C., Wang, T.Y., Chen, P.P., Chen, Y.M., Chuang, H.C., Chen, T.Y. (2011). Real-time quantitative PCR assay for monitoring of nervous necrosis virus infection in grouper aquaculture. *Journal of Clinical Microbiology*, *49*, 1090–1096.

Lang, A.S., Rise, M.L., Culley, A.I., Steward, G.F. (2009). RNA viruses in the sea. *FEMS Microbiology Reviews*, *33*, 295–323.

Lefort, V., Longueville, J.E., Gascuel, O. (2017). SMS: Smart Model Selection in PhyML. *Molecular Biology and Evolution*, *34*, 2422–2424.

Lejeusne, C., Chevaldonné, P., Pergent-Martini, C., Boudouresque, C.F., Pérez, T. (2010). Climate change effects on a miniature ocean: the highly diverse, highly impacted Mediterranean Sea. *Trends in Ecology and Evolution*, *25*, 250–260.

Marbà, N., Jordà, G., Agustí, S., Girard, C., Duarte, C.M. (2015). Footprints of climate change on Mediterranean Sea biota. *Frontiers in Marine Science*, *2*, 1–11.

Moreno, P., Olveira, J.G., Labella, A., Cutrín, J.M., Baro, J.C., Borrego, J.J., Dopazo, C.P. (2014). Surveillance of viruses in wild fish populations in areas around the gulf of cadiz (South Atlantic Iberian Peninsula). *Applied and Environmental Microbiology*, *80*, 6560–6571.

Mori, K.I., Nakai, T., Muroga, K., Arimoto, M., Mushiake, K., Furusawa, I. (1992). Properties of a new virus belonging to nodaviridae found in larval striped jack (*Pseudocaranx dentex*) with nervous necrosis. *Virology*, *187*, 368–371.

Munday, B.L., Kwang, J., Moody, N. (2002). Betanodavirus infections of teleost fish: A review. *Journal of Fish Diseases*, *25*, 127–142.

Nishizawa, T., Furuhashi, M., Nagai, T., Nakai, T., Muroga, K., Asia, S. (1997). Genomic classification of fish nodaviruses by molecular phylogenetic analysis of the coat protein gene. *Microbiology*, *63*, 1633–1636.

Olveira, J.G., Souto, S., Dopazo, C.P., Thiéry, R., Barja, J.L., Bandín, I. (2009). Comparative analysis of both genomic segments of betanodaviruses isolated from epizootic outbreaks in farmed fish species provides evidence for genetic reassortment. *Journal of General Virology*, *90*, 2940–2951.

Panzarin, V., Fusaro, A., Monne, I., Cappellozza, E. and others (2012). Molecular epidemiology and evolutionary dynamics of betanodavirus in southern Europe. *Infection, Genetics and Evolution*, *12*, 63–70.

Rosa, R., Marques, A., Nunes, M.L. (2012). Impact of climate change in Mediterranean aquaculture. *Reviews in Aquaculture*, *4*, 163–177.

Soto, E., Richey, C., Stevens, B., Yun, S. and others (2017) 'Co-infection of *Acipenserid herpesvirus 2* (AciHV-2) and *Streptococcus iniae* in cultured white sturgeon *Acipenser transmontanus*', *Diseases of Aquatic Organisms*, *124*, 11–20.

Soto, E., Wang, R., Wiles, J., Green, C., Plumb, J., Hawke, J. (2015). Characterization of isolates of *Streptococcus agalactiae* from diseased farmed and wild marine fish from the U.S. Gulf coast, Latin America, and Thailand. *Journal of Aquatic Animal Health*, *27*, 123–134.

Stern, N., Levitt, Y., Galil, B.S., Diamant, A., Yokeş, M.B., Goren, M. (2014). Distribution and population structure of the alien indo-pacific randall's threadfin bream *Nemipterus randalli* in the eastern Mediterranean sea. *Journal of Fish Biology*, *85*, 394–406.

Toffan, A., Panzarin, V., Toson, M., Cecchettin, K., Pascoli, F. (2016). Water temperature affects pathogenicity of different betanodavirus genotypes in experimentally challenged *Dicentrarchus labrax*. *Diseases of Aquatic Organisms*, *119*, 231–238.

Toffan, A., Pascoli, F., Pretto, T., Panzarin, V. and others (2017). Viral nervous necrosis in gilthead sea bream (*Sparus aurata*) caused by reassortant betanodavirus RGNNV/SJNNV: an emerging threat for Mediterranean aquaculture. *Scientific Reports*, *7*, 1–12.

Toffolo, V., Negrisolo, E., Maltese, C., Bovo, G., Belvedere, P., Colombo, L., Dalla Valle, L. (2007). Phylogeny of betanodaviruses and molecular evolution of their RNA polymerase and coat proteins. *Molecular Phylogenetics and Evolution*, *43*, 298–308.

Ucko, M., Colorni, A., Diamant, A. (2004). Nodavirus infections in Israeli mariculture. *Journal of Fish Diseases*, *27*, 459–469.

Valero, Y., Arizcun, M., Esteban, M.Á., Bandín, I. and others (2015). Nodavirus colonizes and replicates in the testis of gilthead seabream and European sea bass modulating its immune and reproductive functions. *PLoS ONE*, *10*, 1–24.

Vendramin, N., Patarnello, P., Toffan, A., Panzarin, V. and others (2013). Viral Encephalopathy and Retinopathy in groupers (*Epinephelus spp*.) in southern Italy: a threat for wild endangered species? *BMC Veterinary Research*, *9*, 20.

Vendramin, N., Olesen, N.J. (2017) Overview of the disease situation and surveillance in Europe in 2016, in *21 st Annual Workshop of the National Reference Laboratories for Fish Diseases*, Kgs. Lyngby, Denmark.

Ward, J.R., Lafferty, K.D. (2004). The elusive baseline of marine disease : Are diseases in ocean ecosystems increasing ? *PLoS Biology*, *2*, 0542–0547.

Weinstein, M.R., Litt, M., Kertesz, D.A., Wyper, P. and others (1997). Invasive infections due to a fish pathogen, *Streptococcus iniae*. *New England Journal of Medicine*, *337*, 589–594.

Yanong, R.P.E. (2003). Necropsy techniques for fish. *Seminars in Avian and Exotic Pet Medicine*, *12*, 89–105.

Yeruham, E., Rilov, G., Shpigel, M., Abelson, A. (2015). Collapse of the echinoid Paracentrotus lividus populations in the Eastern Mediterranean-result of climate change? *Scientific Reports*, *5*, 13479.

Zenetos, A., Gofas, S., Morri, C., Rosso, A. and others (2012). Alien species in the Mediterranean Sea by 2012 . A contribution to the application of European union's Marine Strategy Framework Directive (MSFD). Part 2. Introduction trends and pathways. *Mediterranean Marine Science*, *13*, 328–352.

Zlotkin, A., Chilmonczyk, S., Eyngor, M., Hurvitz, A., Ghittino, C., Eldar, A. (2003). Trojan horse effect : phagocyte-mediated *Streptococcus iniae* infection of fish. *Infection and Immunity*, *71*, 2318–2325.

Zlotkin, A., Hershko, H., Eldar, A. (1998). Possible transmission of *Streptococcus iniae* from wild fish to cultured marine fish. *Applied and Environmental Microbiology*, *64*, 4065–4067.

איפיון מולוקולארי ורמת נגיעות של וירוסNNV בדגי בר בים תיכון

רן ברזק

תקציר

התפשטות גורמי תחלואה (פתוגנים) ומחלות בבעלי חיים ימיים משפיעים רבות על הסביבה , על הכלכלה ועל כלל המערכת האקולוגית הימית . וירוס NNV הוא וירוס RNA המהווה את גורם המחלה המשפיע והמזיק ביותר בחקלאות הימית בים תיכון בכלל ובאגן המזרחי בפרט . הוירוס והמחלה הגורמים לאחוזי תמותה גבוהים , זוהו במספר רב של מיני דגים ברחבי העולם אך הרגישות של מין מאכסן לוירוס תלויה בזן הוירוס . דגים מסויימים נמצאו רגישים ואילו אחרים נמצאו עמידים עם יכולת הדבקה.

למרות החשיבות הרבה בזיהוי, איפיון וכי מות רמת הנגיעות של פתוגנים, קיים פער משמעותי במידע על שכיחותם של פתוגנים שונים באוכלוסיות דגי בר . על מנת לייצר את בסיס המידע בנוגע לפתוגנים שונים באגן המזרחי של הים התיכון, בוצע סקר פתוגנים שנועד לכמת ולאפיין את רמת הנגיעות של וירוס NNV בדגי בר נבחרים מחופי הים התיכון הישראלי. כחלק מהסקר נבדקה רמתהנגיעות של חיידק ימי נוסף *Streptococcus iniae* הידוע גם כן כגורם מחלה משמעותי הן בדגי ים והן בדגי מים מתוקים ובעל יכולת להדביק ולחולל מחלה גם בבני אדם.

בסקר ראשוני מסוגו שבוצע כחלק מתכנית הניטור ב "תחנת מוריס קאהן לחקר הים " , נדגמו 10 מיני דגים וסרטנים, מינים מקומיים לים תיכון ומינים מהגרים שמקורם בים סוף הידועים כ"מינים לספסיים", מרמות טרופיות ומנישות ביולוגיות שונות בעלי חשיבות מסחרית וסביבתית לצורך זיהוי ואיפיון רמת נגיעות הוירוס NNV והחיידק *S. iniae* באמצעות שיטות מולקולאריות . מקטעים ספציפים לכל פתוגן הוג ברו ע"י PCR ו-qPCR ונשלחו לריצוף לצורך איפיון גנוטיפי ופילוגנטי . בסקר זה מצאתי נגיעות של 21.49% ב-NNV ו-9.71% במיני *Streptococcus* עם שונות גדולה מאוד ברמת הנגיעות של NNV בין מיני הדגים שנבדקו ו נגיעות גבוהה יחסית נמצאה במינים הלספסיים לעומת המינים המקומיים. נגיעות משותפת של שני הפתוגנים יחד נמצאה במספר פרטים בודד. כמו כן , לצורך הערכת אפשרות הדבקה בין אוכלוסיות דגי בר לדגי חקלאות , דגמתי ובדקתי דגי דניס (*Sparus aurata*) מחוות דגים ישראלית הממוקמת בים הפתוח לאורך תקופת הגידול של קבוצת דגים נתונה. מצאתי שבקבוצת ה דגים, אשר נמצאו נקיים מפתוגנים בתחילת הגידול , 20% מהדגים שנדגמו לאחר 120 ימי גידול בים היו חיוביים ל-NNV. תוצאה זו מרמזת על העברה פתוגנית של הוירוס מהסביבה לדגי החקלאות בעקבות תוצאות הסקר , ביצעתי סקר נוסף על מנת לבדוק האם אכן קיים הבדל ברמת הנגיעות של NNV בין מינים מקומיים למינים לספסיים . לשם כך פרטים נוספים נדגמו מסקרים של דיג מכמור ות - שני מינים מקומיים *Sardinella aurita* ו-*Lithognathus mormyrus* , ושני מינים מהגרים *Nemipterus randalli* ו- *Saurida lessepsianus*. מצאתי ש-51.6% מדגי המין הלספסי *N. randalli* שנדגמו היו חיוביים לוירוס , ערך גבוה באופן מובהק ביחס לשאר המינים שנבדקו ולכן מין דג זה יכול להוות נשא וגורם הדבקה של מינים נוספים באותה סביבת הגידול וייתכן ויש קשר בין הרמה הגבוהה להתבססות המהירה של מין זה בחופי ישראל.

עפ"י האיפיון הפילוגנטי המבוסס על הגברה ו ריצוף של מקטעים חלקיים של שני הסגמנטים הגנומיים של הוירוס , כל הדוגמאות החיוביות שייכות לגנוטיפ RGNNV.

מחקר זה מדגיש את החשיבות של סקרי ניטור על מנת לענות על המחסור הקיים במידע על שכיחות פתוגנים באוכלוסיות דגי הבר . מידע זה יאפשר להבין טוב יותר את הדינמיקה ש ל העברת פתוגנים שונים בין אוכלוסיות שונות בבר ובינם לבין מינים של חקלאות ימית . כמו כן, תוצאות המחקר מעלות השערה כי רמת הרגישות להדבקה פתוגנית של מינים מהגרים יכולה להוות גורם נוסף ביכולת של מינים אלו להתבסס בסיבה החדשה אליה היגרו.

איפיון מולוקולארי ורמת נגיעות של וירוסNNV בדגי בר בים תיכון

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