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RESEARCH ARTICLE **ARAŞTIRMA MAKALESİ**

Investigation of the presence of nervous necrosis virus (NNV) in pikeperch (*Sander lucioperca*) and crayfish (*Pontastacus leptodactylus*)

Sudak (*Sander lucioperca*) ve kerevitte (*Pontastacus leptodactylus*) nervöz nekrozis virüsünün (NNV) varlığının araştırılması

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Abstract: In this study, samples were taken from pikeperch (*Sander lucioperca*) and crayfish (*Pontastacus leptodactylus*) living in Eğirdir, Beyşehir, and Suğla lakes, which are key stopovers for migratory birds. In 2022, crayfish and pikeperch were sampled from Eğirdir, Beyşehir and Suğla lakes at 3 different water temperature periods (15-20°C, 20-25°C and 25-30°C). A total of 540 samples (270 pikeperch and crayfish) were analyzed for the presence of NNV genome (RNA-1 and RNA2) by real-time RT-PCR and also inoculated in E-11 cell. No positive samples were recorded by any of the techniques. In this study, the presence of NNV was investigated for the first time in wild aquatic organisms living in freshwaters of Türkiye and NNV was not detected in the sampled pikeperch and crayfish. However, it is important to regularly examine these lakes, which are located on the migration routes of migratory birds, for NNV.

Keywords: Betanodavirus, wild fish, freshwater, RT-PCR, E-11 cell line

Öz: Bu çalışmada, göçmen kuşlar için önemli durak noktaları olan Eğirdir, Beyşehir ve Suğla göllerinde yaşayan sudak (*Sander lucioperca*) ve kerevitlerden (*Pontastacus leptodactylus*) NNV varlığı araştırılmıştır. 2022 yılında Eğirdir, Beyşehir ve Suğla göllerinden 3 farklı su sıcaklığı döneminde (15-20°C, 20-25°C ve 25-30°C) kerevit ve sudak örnekleri alınmıştır. Toplam 540 örnek (270 sudak ve kerevit) gerçek zamanlı RT-PCR ile NNV genomunun (RNA-1 ve RNA2) varlığı açısından analiz edilmiş ve ayrıca E-11 hücresine inokule edilmiştir. Hiçbir teknikle pozitif örnek kaydedilmemiştir. Bu çalışmada, Türkiye'nin tatlı sularında yaşayan yabani sucul organizmalarda NNV'nin varlığı ilk kez araştırılmış ve örneklenen sudak ve kerevitlerde NNV tespit edilmemiştir. Çalışmanın yapıldığı tarihte virusa rastlanılmamış olsada, göçmen kuşların göç yolları üzerinde bulunan bu göllerin NNV açısından düzenli olarak incelenmesinin önemli olduğu düşünülmektedir.

Anahtar kelimeler: Betanodavirus, yabani balık, tatlı su, RT-PCR, E-11 hücre hattı

INTRODUCTION

NNV is 25-30 nanometre (nm) in diameter (Sahul Hameed et al., 2019) non-enveloped, icosahedral (Chen et al., 2015), single-stranded RNA virus with positive polarity (Mori et al., 1992). The viral genome is segmented into RNA-1 and RNA-2. RNA-1 has been shown to play a role in temperature-dependent viral replication (Panzarin et al., 2014), while RNA-2 encodes the capsid protein responsible for virion structure (Mori et al., 1992). Analysis of the T4 variable region of the RNA-2 genome has led to the classification of NNV into four species: *Betanodavirus takifugui* (TPNNV), *Betanodavirus pseudocarangis* (SJNNV), *Betanodavirus verasperi* (BFNNV), and *Betanodavirus epinepheli* (RGNNV) (Nishizawa et al., 1997, Agrebi et al., 2025). Further genomic variability has resulted in the identification of reassortant strains between RGNNV and SJNNV genotypes in the forms, RGNNV/SJNNV and SJNNV/RGNNV (Olveira et al., 2009; Panzarin et al., 2012; Toffan et al, 2017).

Nervous necrosis virus is known to infect more than 177 fish species (Bandin and Souto, 2020), but the virus including freshwater fish: Australian catfish (*Tandanus tandanus*) and sleepy cod (*Oxyeleotris lineolatus*) (Munday et al., 2002), sturgeon (*Acipenser gueldestaedi* L.) (Xylouri et al., 2007), tilapia (*Oreochromis niloticus*) (Bigarré et al., 2009), largemouth bass (*Micropterus salmoides*), hybrid striped bass × white bass (*Morone saxatilis* × *Morone chrysops*), and pikeperch (*Sander lucioperca*) (Bovo et al., 2011). Other susceptible species include freshwater blenny (*Salaria fluviatilis*) (Vendramin et al., 2012), European eel (*Anguilla anguilla*), Chinese catfish (*Parasilurus asotus*) (Chi et al., 2003), mosquitofish (*Gambusia affinis*) (Praveenraj et al., 2018), and ornamental species such as zebrafish (*Danio rerio*) and goldfish (*Carassius auratus*) (Binesh, 2013). In addition, infections have been reported in bivalve molluscs, shrimp, and other crustaceans (Gomez et al., 2006). In addition to freshwater fish, RGNNV genotyping has been documented in other aquatic animals, including *Artemia* species (nauplii), copepods (*Tigriopus japonicus*), and shrimp (*Acetesinte medius*) (Chi et al., 2003).

Nervous necrosis virus, a neuropathogenic virus causing significant mortality in marine fish species, has been the focus of multiple studies in Türkiye aimed at mitigating its economic and ecological impact. The first identification of NNV in Türkiye

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occurred in 2011 in sea bass (*Dicentrarchus labrax*) from a Mediterranean aquaculture facility (Özkan Özyer et al., 2014). Subsequent molecular surveillance using real-time RT-PCR revealed asymptomatic infections in juvenile sea bass and sea bream (*Sparus aurata*) (Kalaycı et al., 2016). Between 2016 and 2023, the NNV genome was detected across various aquaculture operations and natural marine populations in the Mediterranean, Black Sea and Aegean regions, indicating a widespread and evolving epidemiological pattern (Kaplan and Karaoğlu, 2021; Kaplan et al., 2021, 2022a, 2023; Müftüoğlu, 2023). Notably, NNV was found in wild fish species such as garfish (*Belone belone*) and red mullet (*Mullus barbatus*), signifying potential reservoirs of infection. However, a recent investigation in three Mediterranean lagoons found no evidence of NNV presence in sampled populations of sea bass, sea bream, and mullet (Oğuz, 2024). Despite these findings, there are no published data on the occurrence of NNV in freshwater environments such as lakes or rivers in Türkiye. However, given that Türkiye hosts over 400 freshwater fish species (Yoğurtçuoğlu and Ekmekçi, 2018; Çiçek et al., 2020), and many freshwater ecosystems such as Eğirdir, Beyşehir, and Suğla lakes serve as resting sites for migratory birds potential vectors of NNV the monitoring of these environments has become increasingly relevant.

The main objective of this study was to investigate, for the first time in Türkiye, the presence of NNV genome segments RNA-1 and RNA-2 in two freshwater species of considerable economic and ecological importance: pikeperch (*Sander lucioperca*) and crayfish (*Pontastacus leptodactylus*) inhabiting Lakes Eğirdir, Beyşehir, and Suğla. These lakes are ecologically significant stopover sites for migratory birds, which raises the possibility of viral transmission into inland freshwater ecosystems via avian vectors. The selection of pikeperch and crayfish was based primarily on their use in breeding and aquaculture programs conducted by a publicly affiliated research institution, where broodstocks are routinely sourced from the wild, particularly from these lakes. Moreover, this study is the first to investigate the presence of nervous necrosis virus (NNV) in freshwater environments (lakes) in Türkiye.

MATERIALS AND METHODS

Collection of samples and processing

In 2022, to enable comprehensive detection of all four known genotypes of NNV, sampling was conducted at three different water temperature intervals: 15–20°C, 20–25°C, and 25–30°C. During each period, 30 crayfish (*Pontastacus leptodactylus*) and 30 pikeperch (*Sander lucioperca*) were collected from each of the three lakes: Eğirdir, Beyşehir, and Suğla. In total, 540 samples were obtained, consisting of 270 pikeperch and 270 crayfish (Table 1, Figure 1).

The length of the pikeperch ranged from 8.7 cm to 88 cm, with weights varying from 6 g to 5.9 kg. Crayfish samples measured between 7.4 cm and 16 cm in length, weighing between 8 g and 126 g. Following capture, tissue samples were collected under aseptic conditions: brain and eye tissues from pikeperch, and brain and hepatopancreas tissues from crayfish. These were homogenized in Leibovitz's L-15 medium (Gibco, USA) supplemented with penicillin (1000 IU/ml), streptomycin (10 mg/ml), and amphotericin B (0.025 mg/ml) (Sigma Aldrich, USA).

Homogenized samples were centrifuged at 4000 × g for 15 minutes at 4°C. The resulting supernatants were filtered through 0.45 µm pore-size filters, and aliquots were stored at −80°C until virological analyses were performed.

Table 1. Sample type, lake and preferred water temperature for sampling

Water temperature	Lake Eğirdir	Lake Beyşehir	Lake Suğla
15-20°C	Pikeperch (n=30) Crayfish (n=30)	Pikeperch (n=30) Crayfish (n=30)	Pikeperch (n=30) Crayfish (n=30)
20-25°C	Pikeperch (n=30) Crayfish (n=30)	Pikeperch (n=30) Crayfish (n=30)	Pikeperch (n=30) Crayfish (n=30)
25-30°C	Pikeperch (n=30) Crayfish (n=30)	Pikeperch (n=30) Crayfish (n=30)	Pikeperch (n=30) Crayfish (n=30)
	90 Pikeperch 90 Crayfish	90 Pikeperch 90 Crayfish	90 Pikeperch 90 Crayfish
Total	540 Samples (270 Pikeperch 270 Crayfish)		

Virological analyses

Cell culture inoculation

For virus isolation, the supernatants of field samples, along with the positive control (NNV strain 475/198) and a negative control, were diluted at 1:10 and 1:100 and inoculated in duplicate onto 24-hour-old, semi-confluent E-11 cell monolayers. The cells were grown in 24-well plates using Leibovitz's L-15 medium (Gibco) supplemented with 5% fetal bovine serum (FBS). After inoculation, the plates were incubated at 25°C for 7 to 10 days and checked daily under a microscope for cytopathic effects (CPE). The reference strain (NNV 475/198) was used only as a positive control as part of the quality control process. At the end of the incubation, the cell monolayers from both the field samples and the control groups were scraped and transferred into fresh E-11 cells. These new cultures were incubated again at 25°C for another 10 days. NNV (475/198) strain obtained from Istituto Zooprofilattico Sperimentale delle Venezie, Dipartimento di Ittiopatologia by Izmir Bornova Veterinary Control Institute, the NNV Reference Laboratory of WOAH, was used as a positive control for Real Time RT-PCR and virus isolation.

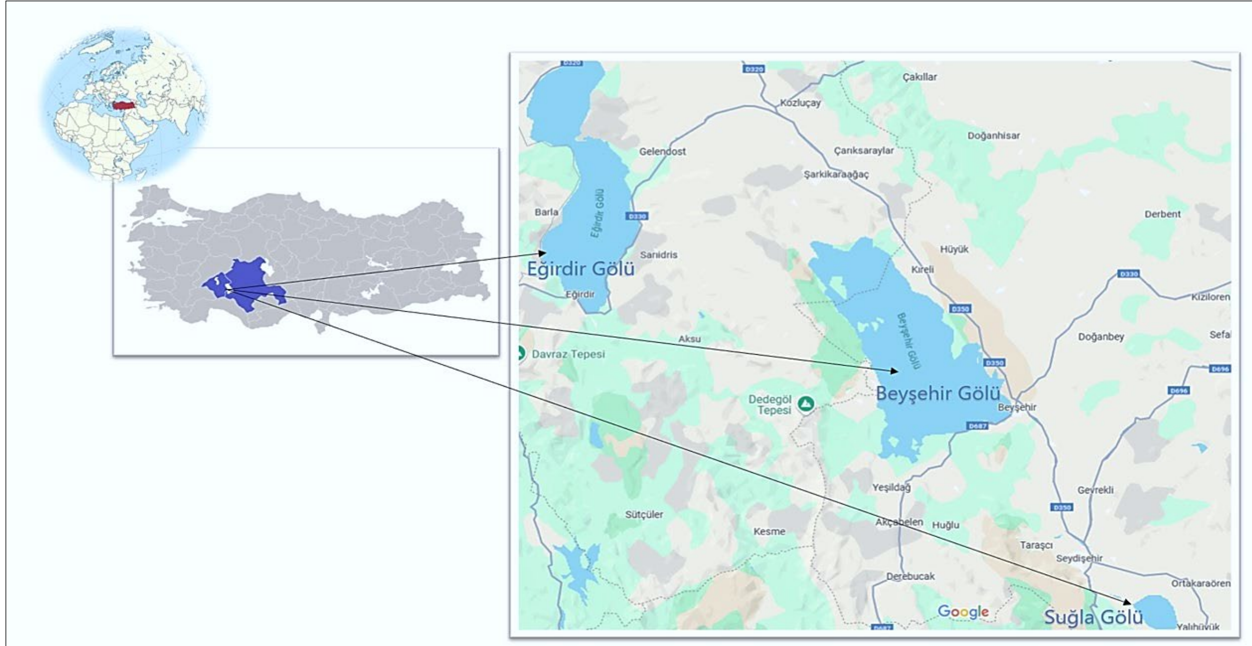

Figure 1. Map of Türkiye showing the lakes of Eğirdir, Beyşehir, and Suğla

Extraction of RNA, real-time polymerase chain reaction

For the extraction of total nucleic acids from homogenized tissues, 200 µl of supernatant was utilized and transferred to a 32-well extraction plate. A commercial extraction kit (MagNA Pure LC Total Nucleic Acid Isolation Kit, Roche, Germany) and an automated extraction apparatus (Roche MagNA Pure LC System, Germany) were utilized for extraction. A positive control and a negative control were employed during the extraction process to validate the procedure and identify any cross-contamination. The inoculum obtained from field material infected with NNV was used as a positive control (strain 475/198). Samples were analyzed using Real-Time RT-PCR in accordance with a published methodology outlined by Panzarin et al. (2010) based on the RNA-2 genome. A commercial kit (Real Time Ready Virus Master, Roche, Germany) and a real-time reverse transcription-polymerase chain reaction apparatus (Roche LightCycler® 480 Multiwell Plate 96, Germany) were utilized. In summary, 5 µl of template was combined with 15 µl of mastermix comprising: 7.6 µl of H_2O, 1 µl of Forward primer (10 pMol), 1 µl of Reverse primer (10 pMol), 1 µl of Probe (10 pMol), 4 µl of 5× buffer, and 0.4 µl of enzyme mix. Each plate received both positive and negative controls. The thermal amplification protocol included a 10-minute reverse transcription at 50°C, a 65-second initial denaturation at 95°C, followed by 45 cycles including 10 seconds of denaturation at 95°C, 30 seconds of annealing at 54°C, and 1 second of extension at 72°C.

PCR and genotyping of culture-passaged samples

Nucleic acid extraction from the third passage supernatants was carried out manually using the HP Virus Viral Nucleic Acid Kit (Roche, Germany) in accordance with the manufacturer's instructions. These samples were then analyzed for the presence of the RNA-1 genome.

Complementary DNA (cDNA) synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit in a SimpliAmp™ Thermal Cycler (Thermo Fisher). In each reaction, 9 µL of RNA was denatured with 200 nM random primers for 5 minutes at 95°C and cooled for 10 minutes at 4°C. The reverse transcription mix containing RevertAid enzyme was then added, followed by incubation at 25°C for 10 minutes and at 50°C for 50 minutes. The reaction was terminated at 85°C for 5 minutes.

Detection of the NNV RNA-1 genome was performed via real-time RT-PCR on a Rotor-Gene Q system (Qiagen), following the method described by Olveira et al. (2021). For genotyping, 2 µL of cDNA was amplified using the FastStart Essential DNA Green Master Mix kit (Roche, Germany) along with genotype-specific TaqMan probes and primer sets (20 mM each), resulting in a total volume of 20 µL. The amplification protocol included an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds, 57°C for 30 seconds, and 72°C for 20 seconds. The limit of detection was investigated by Real Time RT-PCR analysis for the RNA-1 genome according to $log10^4$ - $log10^{10}$ dilutions of the positive control.

RESULTS

Macroscopic examination revealed no visible lesions or clinical signs of disease in any of the sampled pikeperch and crayfish. The average water temperatures during the sampling periods were recorded as follows: 18.3°C for the 15–20°C range, 22.5°C for the 20–25°C range, and 26.3°C for the 25–30°C range.

All homogenized brain and eye tissues from pikeperch, and brain and hepatopancreas tissues from crayfish, were initially screened using real-time RT-PCR targeting the RNA-2 segment. While the positive control samples (strain 475/198) yielded Ct values ranging from 25 to 27, all test samples from both pikeperch (n = 270) and crayfish (n = 270) were negative.

Subsequently, the same tissue samples were inoculated onto E-11 cell lines. No cytopathic effects (CPE) were observed in the inoculated samples during a 10-day incubation period. Positive control samples, however, exhibited CPE by day 5 (Figure 2).

Following three blind passages, RNA was extracted from culture supernatants and analyzed for the RNA-1 genome using real-time RT-PCR. The detection limit of Real Time RT-PCR analysis of log10^4 to log10^{10} dilutions of the positive control was calculated between 11 and 32 Ct. Although the detection limit of Real Time RT-PCR analysis based on the RNA-1 genome is between 12 and 32 Ct, again, the NNV genome was not detected in any of the samples tested (Figure 3).

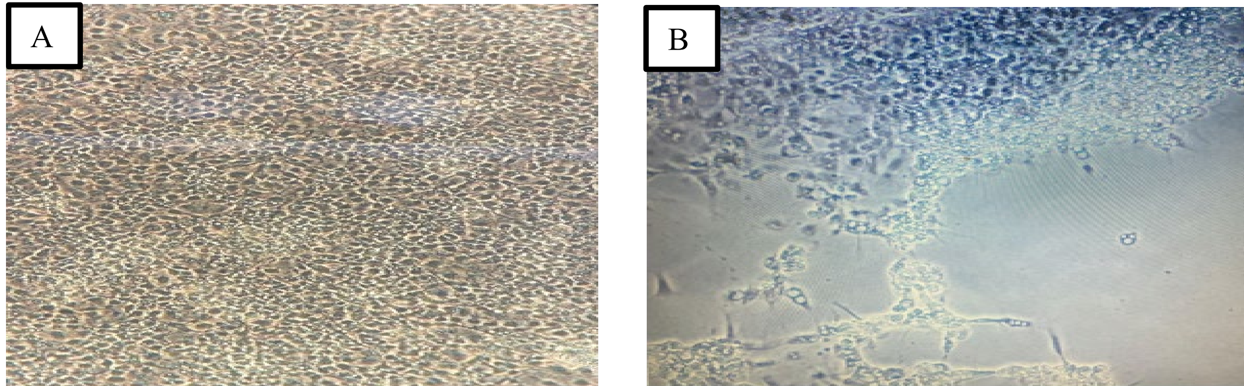

Figure 2. A) E-11 cell control, B) NNV positive control/ CPE

Figure 3. Real-time PCR results of serial dilutions of RNA-1 Positive sequence prepared according to log10 base

DISCUSSION

Viral diseases remain a significant obstacle to sustainable development in the aquaculture industry (Padrós et al., 2022). Among these, nervous necrosis virus (NNV) has been responsible for substantial losses in both marine and freshwater species over the past three decades (Costa and Thompson, 2016; Valero and Cuesta, 2023). NNV can infect not only farmed fish but also wild-caught species, leading to serious economic and ecological consequences (Fernández-Sánchez et al., 2022; Zrnčić et al., 2022; Kaplan et al., 2023; Vázquez-Salgado et al., 2024).

Although studies on NNV have been widely conducted in marine fish species in Türkiye (Özkan Özyer et al., 2014; Kalaycı et al., 2016; Kaplan and Karaoğlu, 2021; Kaplan et al., 2021; 2022a; 2023; Müftüoğlu, 2023), investigations in freshwater ecosystems remain extremely limited. Lakes are often considered to be more geographically isolated and environmentally contained than marine environments, which may lead to the assumption that they are less susceptible to viral transmission compared to marine environments. However, this assumption does not eliminate the possibility of virus introduction via migratory birds or other vectors.

Türkiye is home to 409 documented freshwater fish species (Yoğurtçuoğlu and Ekmekçi, 2018; Çiçek et al., 2020). Due to the fact that the studies conducted in Türkiye have been focused on fish species living in the seas, the presence of NNV was investigated in pikeperch and crayfish, which are of economic importance. The results of this study are the first data obtained in Türkiye on the status of NNV in freshwater aquatic species, such as pikeperch and crayfish.

The genetic similarity of the NNV isolates detected by Kaplan and Karaoğlu (2021) and Kaplan et al. (2021) in the studies conducted in sea bass with the isolate detected from pikeperch (*Sander lucioperca*) in Italy (Bovo et al., 2011) and the fact that pikeperch have a very wide habitat in Türkiye constituted the rationale for this study.

In addition, it has been reported that the nervous necrosis virus has been detected in aquatic animals such as crustaceans, bivalve molluscs and gastropods other than fish (Gomez et al., 2006; 2008; 2010; Kim et al., 2016; Volpe et al., 2018; Bitchava et al., 2019). The close genetic similarity of strains obtained from cultured and wild fish and bivalves suggests that bivalve molluscs transmit the virus to fish and vice versa (Volpe et al., 2018), and invertebrates have been reported as carriers in the spread of NNV (Gomez et al., 2010; Panzarin et al., 2012). In a study conducted in the UK on crayfish, specifically *Pontastacus leptodactylus*, using the metatranscriptomics method, various RNA viruses such as Hepeviridae, Nodaviridae, Dicistroviridae, and Tombusviridae were reported to be detected. According to a sequence showing more than 80% similarity with a Nodavirus, which belongs to the same family as NNV (Nervous Necrosis Virus), crayfish nodaviruses were reported to phylogenetically cluster within a lineage of the *Alphanodavirus* genus and group on the same branch with "Flock House Virus" *(Alphanodavirus flockense*), which is used in control applications due to its insecticidal properties (Harwood et al., 2025). This study demonstrates that *P. leptodactylus* is not only a host but also a vector or carrier for numerous RNA viruses. The coexistence of aquatic organisms such as pikeperch and crayfish in the freshwaters of Türkiye and the idea that there may be interspecies viral transmission have revealed the necessity to investigate the presence of NNV in crayfish as well as pikeperch.

In the study, no presence of NNV was detected in any of the analyzed individuals. The presence of NNV was detected in 60-day-old pike-perch on a freshwater farm in northern Italy. It was reported that the disease was observed after the introduction of 30-day-old hybrid striped bass (*Morone saxatilis* × *Morone chrysops*) imported from Israel (Bovo et al., 2011). In our study, pike perch living in the wild were examined and there was no entry of fish from outside compared to the study of Bovo et al. (2011).

In previous studies, horizontal transmission has been emphasized as the primary route for NNV spread in freshwater systems, often linked to marine water inflow or farmed marine species being temporarily held in freshwater environments (Chi et al., 2003; Athanassopoulou et al., 2004; Bigarré et al., 2009; Bovo et al., 2011; Vendramin et al., 2012; Gherbawy et al., 2024). In contrast, the lakes included in this study are relatively isolated, limiting such transmission mechanisms.

Research undertaken in Türkiye indicates that the virus is spread horizontally by water, rather than vertically (Kaplan and Karaoğlu, 2021; Kaplan et al., 2022a; Kaplan et al., 2023; Müftüoğlu, 2023). In 2015, the Suez Canal underwent significant expansion, which facilitated the entry of several fish species into Türkiye via the Mediterranean Sea (Galil et al., 2015). It has also been reported that the average seawater temperature has increased by 1.2 °C in the Black Sea, 0.9 °C in the Aegean Sea, and 1.5 °C in the Marmara Sea, creating favorable conditions for the establishment of these alien species (Kalıpcı et al., 2021). The prevalence of the NNV in Türkiye has been associated with fish migration through the Suez Canal and the Strait of Gibraltar, as well as rising seawater temperatures linked to climate change (Eren et al., 2024).

There are studies in which the presence of NNV genome could not be detected (Zrnčić et al., 2021; Kaplan et al., 2022b; Oğuz et al., 2023; Oğuz, 2024). They reported that no positive samples were found in sea bream, sea bass and mullet in Akyatan, Akgöl-Paradeniz and Beymelek lagoons, especially in the Mediterranean region. They stated that the reason for the lack of positive detection was that the lagoons were a more limited area compared to the seas, there were not as many fish species as in the seas, possible fish migrations were limited and the transmission routes were limited (Oğuz, 2024).

In addition, it has been reported that NNV is resistant to high water temperatures, including up to 37 °C, and can survive in acidic environments (Frerichs et al., 2000). The World Organisation for Animal Health (WOAH, 2019) indicated that migratory water birds feeding on infected fish may act as potential vectors in the spread of the virus through their feces. Supporting this, Kuo et al. (2012) reported that the NNV genome was detected by PCR in the feces of waterfowl (4 out of 10 samples), suggesting that the virus can persist in the digestive tract and be transmitted to other organisms via fecal shedding.

In Türkiye, the 2024 "Mid-winter Census of Waterbirds" reported the species composition and total numbers of migratory birds across the country (KOSKS, 2024). According to the report, 1,684,756 migratory waterbirds representing 106 species were recorded nationwide, including those observed at Hazar, Bafa, Manyas, and Uluabat lakes. Specifically, 83,382 individuals from 32 species were recorded in Lake Eğirdir, while 249,113 individuals from 38 species were observed in Lake Beyşehir. Although the report did not include specific data for Lake Suğla, the presence of migratory waterbirds at the time of sampling was confirmed through field observation. As a result of all these reasons, it is important to carry out a study on the presence of NNV in aquatic organisms in lakes hosting

migratory waterfowl, which are likely to transmit the virus by carrying it in their faeces. Thus, it may provide an idea about the role of migratory birds in the spread of the virus.

NNV has been reported to spread through migrating wild fish, fish originating from breeding farms, or via fish transported across borders from neighboring countries (Boukedjouta et al., 2020). In a study conducted in the Persian Gulf, the presence of *Betanodavirus* was detected in groupers and sea bass, and the infection was attributed to groupers imported from neighboring countries. The same study also documented horizontal transmission of NNV between wild and farmed fish populations (Ziarati et al., 2020). Similarly, Kaplan and Karaoğlu (2021) reported that sea bass farms and hatcheries in their study exchanged fish stock with each other, which may have contributed to the spread of the virus.

In contrast, the lakes included in the present study are not used for aquaculture activities, which eliminates a key risk factor for viral transmission observed in other regions. This factor likely contributes to the absence of NNV detection in the sampled populations.

CONCLUSION

This study presents the first assessment of nervous necrosis virus in Türkiye' freshwater ecosystems, focusing on two ecologically and economically important species pikeperch (*Sander lucioperca*) and crayfish (*Pontastacus leptodactylus*) sampled from Lakes Eğirdir, Beyşehir, and Suğla. Molecular analyses targeting RNA-1 and RNA-2 segments, along with virological tests via cell culture, did not reveal any evidence of NNV infection in the collected samples.

The lack of NNV detection may reflect the relatively isolated nature of these lakes, limited human-mediated fish movement, and the absence of aquaculture operations in the region. Still, considering the ecological significance of these water bodies and the seasonal presence of migratory birds' potential vectors for viral pathogens regular monitoring remains important.

The fishing ban during the breeding period and the inability to sample early life stages were the limiting factors of the study. Future investigations should include sampling of larvae and juveniles, expand the geographic and seasonal scope of sampling, and incorporate additional susceptible aquatic species and potential invertebrate hosts. Such efforts will be crucial for a better understanding of NNV transmission routes and risk factors in inland aquatic environments.

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AUTHOR CONTRIBUTIONS

This study summaries the doctoral dissertation of Hakan EREN. Hakan Eren: Investigation, writing. Sibel Yavru: Methodology, writing, review and editing, supervision.

CONFLICT OF INTEREST

The authors assert that they have no conflicts of interest.

ETHICAL APPROVAL

This study was conducted in accordance with the principles outlined in the Directive of the Animal Experiments Local Ethics Committee of the Eğirdir Fisheries Research Institute Directorate, affiliated with the General Directorate of Agricultural Research and Policies (TAGEM), Ministry of Agriculture and Forestry of the Republic of Türkiye. Ethical approval was granted by the committee on 28 April 2021 (Decision No: 01).

DECLARATION OF AI USE

We did not use AI-assisted technologies to create this article.

DATA AVAILABILITY

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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