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Dusky grouper massive die-off in a Mediterranean marine reserve

Diego K. KERSTING[1], Celia GARCÍA-QUINTANILLA[2], Nagore QUINTANO[1], Itziar ESTENSORO[1] and María del MAR ORTEGA-VILLAIZAN[2]

[1] Instituto de Acuicultura de Torre de la Sal (IATS, CSIC). Ribera de Cabanes, Spain
[2] Instituto de Investigación, Desarrollo e Innovación en Biotecnología Sanitaria de Elche (IDiBE), Universidad Miguel Hernández de Elche (UMH), Elche, Spain

Corresponding author: Diego K. KERSTING; diego.kersting@csic.es

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Abstract

The dusky grouper *Epinephelus marginatus* (Lowe, 1834) is a key predator in shallow Mediterranean rocky habitats. Due to fishing pressure and slow population dynamics, dusky grouper populations are declining across their distribution range. Here we document a massive die-off of *E. marginatus* in the Columbretes Islands Marine Reserve (NW Mediterranean Sea) during the summer and early autumn of 2023. The mortality event was triggered by a Betanodavirus infection. Genetic characterization of isolates from the affected fish confirmed that they belong to the RGNNV genotype. Our findings indicate that such disease outbreaks can be particularly severe in areas with dense dusky grouper populations, potentially undermining the historical conservation effectiveness of marine protected areas. Although our results do not determine whether the infection originated from wild or farmed fish, it is essential to implement monitoring and early detection systems for Betanodavirus infections in both wild and farmed fish to prevent cross-infections.

Keywords: dusky grouper; Betanodavirus; Mediterranean Sea; marine protected area; mortality.

Introduction

The dusky grouper *Epinephelus marginatus* (Lowe 1834) is a large-bodied fish species commonly found in rocky substrates and reefs (Condini *et al.*, 2018; Louisy, 2022). It plays an important role as a main predator occupying the highest trophic levels (Condini *et al.*, 2018; Reñones *et al*., 2002) and is considered a keystone species (Valls *et al.*, 2012). This species exhibits slow population dynamics, marked by its high longevity (up to ~60 years) and low growth rates (Reñones *et al*., 2007). Due to its high commercial value, *E. marginatus* is a fishing target for professional (Lloret & Riera, 2008) and recreational fishers, particularly spear fishers (Coll *et al*., 2004; Lloret *et al*., 2008). As a result of fishing pressure and the slow population dynamics of the species, dusky grouper populations are declining in most areas of its geographical distribution (Condini *et al*., 2018). The dusky grouper is currently included in the IUCN Red List as vulnerable (Pollard *et al*., 2018).

Mortalities of dusky groupers caused by Betanodavirus infections have been reported in several Mediterranean locations over the last two decades (Haddad-Boubaker *et al*., 2014; Kara *et al*., 2014; Marino & Azzurro, 2001; Valencia *et al*., 2019; Vendramin *et al*., 2013). Some of these mortalities have been detected within marine protected areas (MPAs) (Marino & Azzurro, 2001; Valencia *et al*., 2019), the main conservation tool which has allowed the species to recover in Mediterranean coasts (García-Rubies *et al*., 2013; Guidetti *et al*., 2014; Hackradt *et al*., 2014). The disease associated to these infections, viral nervous necrosis (VNN) disease, caused by nervous necrosis virus (NNV), is also known as viral encephalopathy and retinopathy (VER) (Munday *et al*., 1992). It is a destructive disease that specifically targets the central nervous system of infected fish, leading to noticeable aberrations in swimming behaviour, such as vertical positioning and spinning, body contortions, and muscle tremors. Furthermore, it causes hyperinflation of the swim bladder, leading sick fish to remain primarily at the water's surface (OIE - World Organisation for Animal Health, 2016).

Betanodaviruses are distributed worldwide, and the geographical distribution of their genotypes seems to be related to their thermotolerance (Bandín & Souto, 2020). The red-spotted grouper nervous necrosis virus (RGNNV) genotype, with optimal growth temperature of 25-30 °C, is the most widely distributed, affecting temperate and tropical fish species. The striped jack nervous necrosis virus (SJNNV) genotype, with optimal growth tem-

perature of 20-25 °C, is also present in temperate areas and has been detected in fish reared in Japanese waters and farmed fish on the Iberian Peninsula. Natural SJNNV/RGNNV reassortants have been isolated from sea bass on the Italian coast, whereas RGNNV/SJNNV reassortants are widespread in Southern Europe.

Several environmental factors, such as temperature, fish density, and stress have been suggested to influence VER outbreaks (Munday *et al.*, 2002). Since the first descriptions in the 1990s, VER outbreaks have been consistently reported, predominantly in marine fish reared in Asian, Australian and European waters. The most affected species include grouper, Japanese and barfin flounder, Asian seabass/barramundi, European sea bass, gilthead sea bream, Atlantic and Pacific cod and Atlantic halibut (as reviewed by Bandín & Souto, 2020). The RGNNV genotype is highly prevalent in farmed fish in Asia and the Mediterranean, leading to disease outbreaks when temperatures and/or fish densities rise (Bandín & Souto, 2020). Additionally, many asymptomatic species and individuals have been reported, potentially acting as carriers and increasing outbreak occurrences (Bandín & Souto, 2020). NNV infections are particularly difficult to control on account of the high stability of the NNV particle in the environment (Frerichs *et al*., 2000). Current control strategies focus on good husbandry, biosecurity and sanitation practices (Frerichs *et al*., 2000; Costa & Thompson, 2016; Doan *et al*., 2017).

We document a massive die-off of *E. marginatus* caused by Betanodavirus infection in the Columbretes Islands Marine Reserve (NW Mediterranean Sea) during the summer and early autumn of 2023. This serves as a warning to other regions in the area regarding the potential spread of the disease.

Material and Methods

Study site

The Columbretes Islands emerge at 30 nautical miles off the coast of Castelló, Spain (NW Mediterranean) (39.896869° N, 0.686339° E). This volcanic archipelago is formed by four main islet groups and is surrounded by a 5,500 ha no-take marine reserve established in the year 1990. The Illa Grossa, the largest islet of the archipelago, is a semi-submerged volcanic caldera that forms a characteristic bay (Fig. 1). Sea surface temperature (SST) is recorded daily in the Illa Grossa Bay (Kersting *et al*., 2013).

Fish mortality

Dead and moribund individuals were recorded by the wardens of the Columbretes Islands Marine Reserve during their daily surveillance at sea. Whenever possible, the wardens collected the following data for every individual encountered: date, health status (moribund or deceased), size (total length), and location.

Fish censuses

Fish censuses, specifically targeting dusky groupers, were carried out in November 2016, unrelated to any mortality event, and once again in November 2023, following the mortality event earlier that year. Censuses were carried out in 5-minute transects, in which divers swim at a constant velocity to cover a transect of 50 m in length and 5 m in width (250 m^2 total area) (Grane-Feliu *et al*., 2019). In each transect the number of groupers

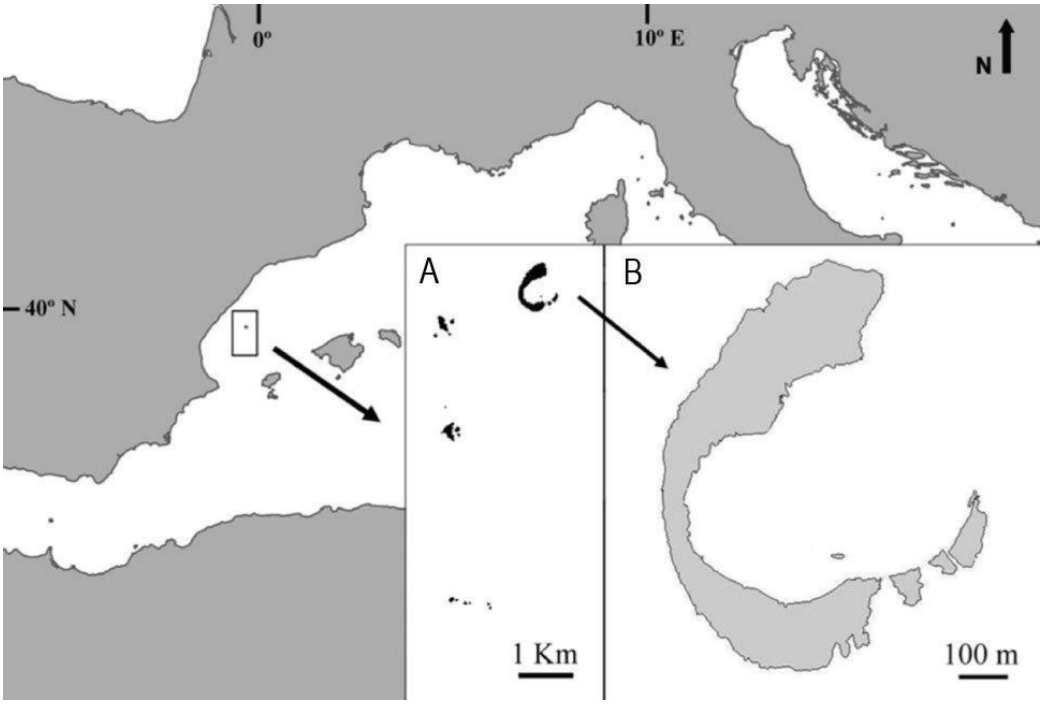

Fig. 1: The Columbretes Islands (A) and the Illa Grossa (B).

and their sizes were recorded. A total of 24 transects in 2016 and 14 transects in 2023 were conducted, covering a depth range of 10-25 m. The transects were conducted at the same locations within the bay formed by Illa Grossa, the largest islet in the archipelago and where most of the sightings of affected groupers were recorded.

A Mann-Whitney U test was conducted to compare fish density between 2016 and 2024. Statistical analyses were performed using R (R Core Team, 2024).

RNA extraction, cDNA synthesis and quantitative RT-qPCR

Two recently deceased individuals, measuring 70 and 57 cm, were sampled in the Illa Grossa Bay on the 26th and 31st of July 2024. Brain and retina samples were stored in RNAlater and kept at -80 °C. Subsequently, RNA extraction was carried out using the E.Z.N.A. total RNA extraction kit I (Omega Bio-tek, Inc.) as indicated by the manufacturer. Genomic DNA was then removed using the TURBO Dnase kit (Ambion, Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. cDNA was obtained using M-MLV reverse transcriptase (Invitrogen, Thermo Fisher Scientific Inc.), as previously described (Chico *et al.,* 2006). To do this, RNA was incubated with dNTPs (10 mM) and Random hexamers (50-250 ng) for 5 minutes at 65 °C in the thermal cycler (GeneAmp PCR System 2700, Applied Biosystems). First Strand Buffer RT 5x (Invitrogen & Thermo Fisher Scientific Inc.), DTT (Invitrogen), RNAse Inhibitor (Invitrogen) and MMLV-RT [200 U] (Invitrogen) were added to each sample. Thermal cycling conditions were: 25 °C for 10 minutes, 37 °C for 50 minutes, and 70 °C for 15 minutes. Finally, real time quantitative PCR (RT-qPCR) was performed using the QUANTSTUDIO 3 system (Applied Biosystems, Thermo Fisher Scientific Inc.). The reactions were performed in a total volume of 20 µl with 24 ng of cDNA, 900 nM of each primer (Table S1) and 10 µl of SYBR Green PowerTrack PCR buffer (Applied Biosystems, Thermo Fisher Scientific Inc.). Thermal cycling conditions were: 1 cycle for 2 minutes at 50 °C and 10 minutes at 95 °C, followed by 40 cycles at 95 °C for 15 seconds and 60 °C for 1 minute.

Reverse transcriptase PCR (RT-PCR), sequencing and phylogenetic analysis

RNA extraction and reverse-transcription were performed with NNV positive samples, as described in the previous section. PCR amplification was carried out using the GoTaq G2 Flexi DNA Polymerase kit (Promega Corporation), following the manufacturer's instructions. Semiquantitative PCR was performed with primer pairs shown in Table S2, following Bovo *et al*. (2011). For semiquantitative PCR, the GoTaq G2 Flexi DNA Polymerase kit (Promega Corporation) was used, following the manufacturer's instructions. Primers were used at a final concentration of 0.2 mM. The FOR 521–VNNV6 primer pair amplified a region within the viral RNA1 molecule, while the VNNV1–VNNV2 primer pair targeted a variable region within the viral RNA2 segment (Table S2) (Bovo *et al*., 2011). PCR was carried out in the GeneAmp PCR System 2700 (Applied Biosystems). The thermal cycling conditions were: 50 °C for 30 minutes, 95 °C for 15 minutes and 40 cycles of 30 seconds denaturation at 94 °C, 30 seconds banding at 60°C and 45 seconds elongation at 72 °C followed by a final cycle of 10 minutes at 72 °C. PCR products were analyzed for purity and size by electrophoresis on a 2% agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen). DNA was then purified from the gel using the NZYGelpure kit (Nzytech), following the manufacturer's instructions. Purified amplicons were then sequenced at SECUGEN S.L. (Centro de Investigaciones Biológicas, CSIC, Spain) using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) in an ABI 3730 DNA Analyzer (Thermo Fisher Scientific). Sequence data were edited with CHROMAS LITE (http://www.technelysium.com.au/chromas_lite.html). The partial RNA1 and RNA2 sequences obtained were aligned by CLUSTALW with Betanodavirus representative genotypes isolates retrieved from GenBank (http://www.ncbi.nlm.nih.gov) (Table S3) using the MEGA 11 software (Tamura *et al*., 2021). Phylogenetic trees were developed using the Maximum-Likelihood method, with 100 bootstrap resamplings, using MEGA 11 software. Nucleotide similarities between investigated samples and Betanodavirus representative isolates were also determined.

No additional bacteriological or parasitological analyses were conducted in order to rule out the involvement of other pathogens in the current field cases. However, no macroscopic external or behavioural signs suggesting coinfections were detected.

Results

Fish mortality

The first disease-affected dusky grouper was observed on 19th May 2023. Sightings increased gradually and peaked during the first two weeks of October 2023 (Fig. 2). The last affected individual was recorded on November 8th 2023. The drop in the sightings of affected groupers coincided with a drastic decrease in SST, which dropped by almost 10 °C in about two weeks, between the end of October and beginning of November (Fig. 2). On the whole, 45 moribund or dead individuals were recorded throughout this period in 2023. The average size of the affected individuals was 67 ± 23 cm (± SD) and ranged from 19 to 110 cm (Fig. 3). Moribund individuals were always found floating, showing an erratic swimming behaviour and loss of swim bladder control, which was hyperinflated and prevented them from submerging (Fig. 4).

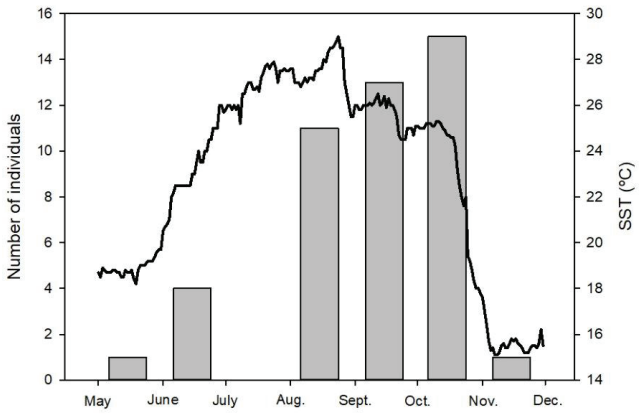

Fig. 2: Number of sighted moribund and dead groupers in the Columbretes Islands in 2023 (bars) and daily sea surface temperature (SST) in Illa Grossa Bay (line).

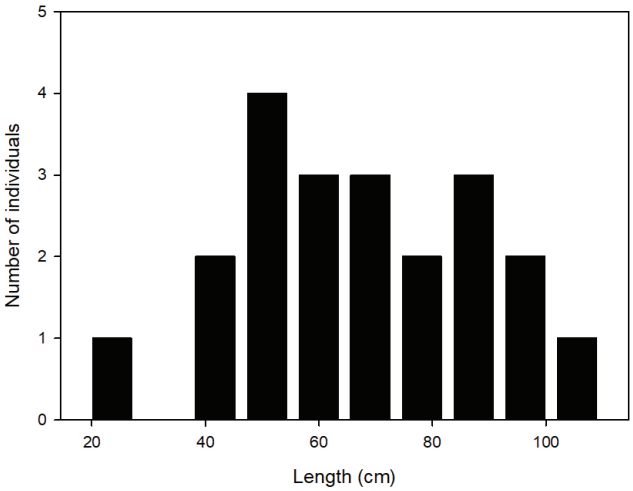

Fig. 3: Size-frequency distribution (total length) of sighted sick dusky groupers.

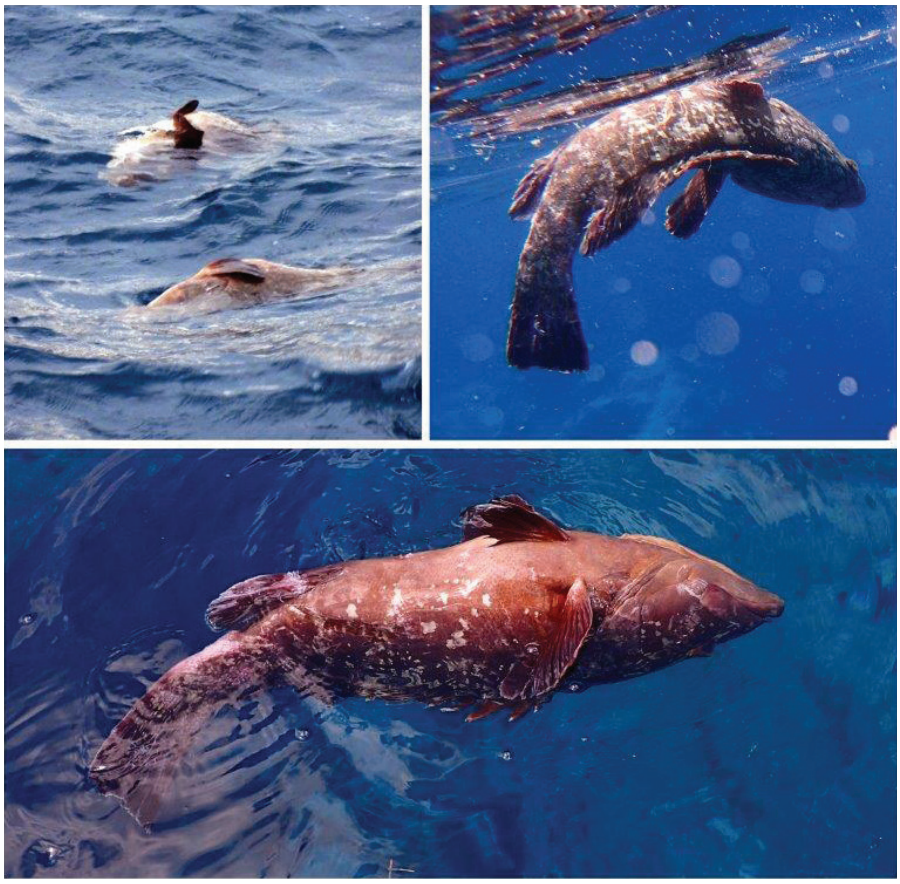

Fig. 4: Moribund and dead dusky groupers floating in the Illa Grossa Bay.

Fish censuses

The fish censuses carried out in Illa Grossa Bay in November 2016 showed a dusky grouper density of 0.018 ± 0.010 individuals·m^{-2}, whereas in November 2023 the density was of 0.002 ± 0.003 individuals·m^{-2}. Fish densities were significantly different between 2016 and 2023 (Mann-Whitney U test, W=319, $p < 0.001$).

RT-qPCR

Betanodavirus was detected in the two samples examined, both speciments were positive by RT-qPCR of the brain and retinal samples (Table S4). Positive control brain samples from a sea bass individual infected with RGNNV strain it/411/96 by intramuscular injection were used.

Phylogenetic characterization

Partial coat protein and RdRp gene sequences for the 2 isolates of NNV from dusky grouper were obtained. Sequences were deposited in GenBank under the Accession Nos. PP814714, PP814715, PP814716, and PP814717 (Table S3). The nucleotide sequences were almost identical between individuals (99.9% similar). Maximum-likelihood analyses of the viral RNA1 segment revealed that the two isolates from dusky grouper affected with VNN fell within the RGNNV genogroup (Table S3, Fig. 5A), forming a well-defined cluster supported by high bootstrap values (Fig. 5A). RNA1 segment (RdRp) closest sequence retrieved using BLAST similarity search on GenBank was from Italy (Accession No. JN189865.2), with a nucleotide homology of 97%, corresponding to a *Dicentrarchus labrax* Betanodavirus 283.2009 isolate, which belongs to RGNNV genotype (Fig. 5A). Maximum-likelihood analyses of the viral RNA2 did not cluster the dusky grouper isolates sequences within any genogroup (Fig. 5B). However, RNA2 segment (coat protein) closest sequence retrieved by BLAST was from Japan (Accession No. AY324870.1), with a nucleotide homology of 99%, corresponding to a sevenband grouper nervous necrosis virus (SGWak97) isolate, which belongs to RGNNV genotype (Table S3, Fig. 5B).

Discussion

We show that the Betanodavirus-related mortality detected in the Columbretes Islands in 2023 has significantly affected the dusky grouper population in the marine reserve. The genetic characterization of the isolates from the affected fish confirmed that they belong to the RGNNV genotype, which is the most widespread in the Mediterranean region. Dusky grouper density in Illa Grossa Bay was significantly lower after the disease episode compared to 2016. The Columbretes Islands Marine Reserve is a well-enforced MPA, where fishing is prohibited and *E. marginatus* density in 2016 ranked amongst the highest reported for the species (Hackradt *et al*., 2014). Although the fish density data utilized for comparison originates from 2016, it is reasonable to infer that the fish population in 2023, prior to the mortality event, would have been similar. This inference is supported by the constant increase in populations of *E. marginatus* observed over decades in other well-enforced Mediterranean MPAs (Astruch *et al*., 2018). These findings highlight the potential impact of this disease on well-preserved populations of *E. marginatus*. Considering the slow dynamics of the species (Reñones *et al*., 2007), full recovery is expected to take a significant amount of time. This is particularly true for the recovery of large individuals, which have been widely affected by the mortality in Columbretes.

Valencia *et al*. (2019) also reported a VNN disease outbreak in several MPAs in the Balearic Islands. How-

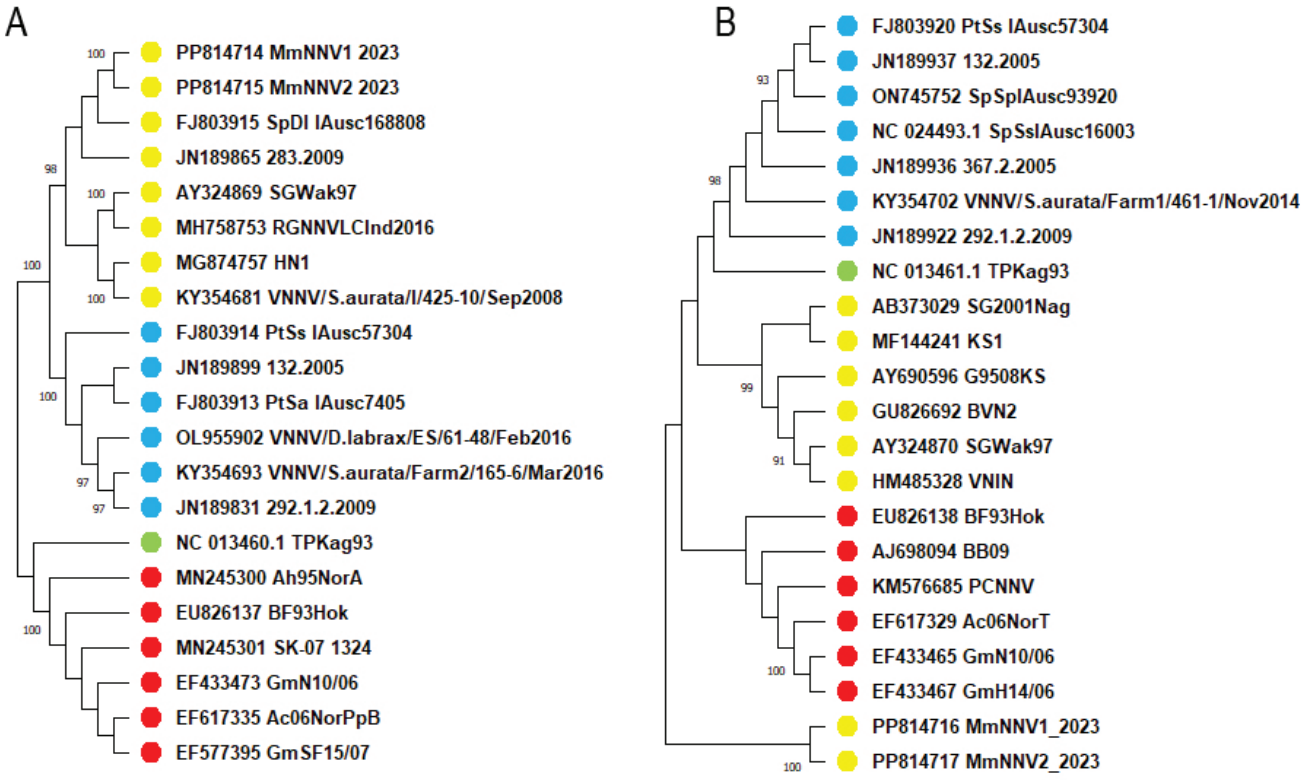

Fig. 5: Maximum-likelihood phylogenetic tree of the two dusky grouper positive samples and 20 virus isolates representing four Betanodavirus genotypes (RGNNV, SJNNV, BFNNV, TPNNV), based on the RNA1 segment (A) and the RNA2 segment (B). Nodes are coloured according to the genotypes (RGNNV, yellow; RGNNV/SJNNV, blue; BFNNV, red; TPNNV, green). Nodes are labelled with bootstrap values expressed as percentages. Only values ≥90 are reported.

ever, the impact on dusky grouper populations could not be quantified due to the lack of abundance estimates. Nevertheless, given the significant fish density within these protected areas, it is reasonable to expect that such disease outbreaks could have noticeable impacts on MPAs, potentially undermining their effectiveness as the most successful tool for conserving the species. Disease outbreaks originating outside MPAs are particularly challenging to prevent and manage. For instance, following the 2023 event in the Columbretes Islands, the MPA management authority acted to prevent the spread of the virus in potential future outbreaks by ensuring the removal of dead individuals from the water. Furthermore, such drastic reductions in apex predators not only compromise MPA effectiveness but can also trigger extensive cascading effects, leading to ecosystem-wide impacts (Myers *et al.,* 2007; Estes *et al.*, 2011).

VNN disease, caused by Betanodavirus, has been reported in the Mediterranean Sea since 1991, and is a major concern for reared marine fish (Breuil *et al*., 1991; Chérif *et al*., 2009; Ucko *et al*., 2004) and wild species (Ciulli *et al*., 2007; Panzarin *et al*., 2012). According to published reports, the incidence of the disease in groupers appears to have peaked in the 2010's (Haddad-Boubaker *et al*., 2014; Kara *et al*., 2014; Valencia *et al*., 2019; Vendramin *et al*., 2013), with the most recent report from Algeria dating to 2019 (Boukedjouta *et al*., 2020). As reported in other studies (Boukedjouta *et al*., 2020) and based on the obtained phylogenetic results, the origin of the infection in the Columbretes Islands cannot be determined. The hypotheses that could explain such a disease outbreak are: i) disease transmission originating in farmed fish populations (Munday *et al.*, 2002; Vendramin *et al.,* 2013), and ii) transmission among wild fish, either by means of migrating individuals (Berzak *et al.,* 2019; Chérif *et al*., 2009; Lampert *et al*., 2020) or through the activation of the disease in carriers as a result of environmental conditions (Kara *et al*., 2014). It is worth mentioning that, so far, the disease has been documented in more than 50 fish species (Munday *et al*., 2002; OIE - World Organisation for Animal Health, 2019; Sano *et al*., 2011). The Columbretes Islands are located in the open sea, at around 60 km off the eastern coast of Spain, where the nearest farmed fish infrastructures are located. Although a direct transmission through farmed fish might seem improbable at first due to the distance, recent reports of fish movements using acoustic telemetry show that some species are able to cover such distances effortlessly (www.marcatgemari.cat). On the other hand, the only case of a diseased *E. marginatus* in the Columbretes Islands prior to the 2023 episode was a single moribund individual reported in November 2022, which tested positive to Betanodavirus (unpublished data from the Secretaría General de Pesca, the Columbretes Islands Marine Reserve management authority). This shows that the pathogen was already present in the area prior to the 2023 outbreak. Further studies are needed to determine the origin of such disease outbreaks in *E. marginatus* populations.

Following a similar pattern to other VNN disease outbreaks in dusky groupers in the Mediterranean Sea (Haddad-Boubaker *et al*., 2014; Kara *et al.*, 2014; Valencia *et al*., 2019; Vendramin *et al*., 2013), mortality in the Columbretes Islands started in summer and peaked in autumn. The development of VNN disease has been associated to increased seawater temperatures (Munday *et al.*, 2002; Vendramin *et al*., 2013). Our findings indicate that the onset of the disease initiated in concurrence with the rise in SST at the beginning of the summer, whereas a sharp drop in disease incidence was observed as water temperatures sharply declined in November.

As evidenced in this study, the mortality rate attributed to this disease can be particularly severe in areas with a high density of dusky groupers, posing a significant additional risk to this endangered species. We also demonstrate that wild fish in locations far from aquaculture facilities can still be impacted by severe Betanodavirus disease outbreaks. Locations nearby, especially other MPAs, should be vigilant for potential disease outbreaks in upcoming summers. It is crucial to closely monitor *E. marginatus* populations to detect any signs of mortality and evaluate the impact on their populations. In the case of a disease outbreak, dead groupers should be removed from the water to prevent further spread of the virus. Betanodavirus in the natural environment pose a continuous threat to both wild and farmed fish species, hence the importance of the implementation of monitoring and early detection systems to prevent transmissions in both directions.

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Supplementary Material

The following supplementary material is available for this article:

Table S1: Primer pairs used for RT-qPCR.

Table S2: Primer pairs used for RT-PCR and sequencing. The table shows the size of the resulting amplicons, the fragment of the viral genome they amplify and the position in the genome.

Table S3: Betanodavirus isolates used for phylogenetic analysis, genotype, host species, country and year of isolation, GenBank accession number for RNA1 and RNA2 segments.

Table S4: RT-qPCR results showing the Ct value obtained for the samples analyzed.