

## Article

# Linking *Caligus pageti* Infestation to Grey Mullet Mortality in a Mediterranean Lagoon: First Molecular Characterization

Margalida Vanrell-Valls <sup>1</sup>, José María Valencia <sup>1,2,\*</sup>, Samuel Pons <sup>3</sup>, Amalia Grau <sup>1,2</sup>  and Gaetano Catanese <sup>1,2</sup> 

<sup>1</sup> IRFAP LIMIA—Laboratorio de Investigaciones Marinas y Acuicultura, Govern de les Illes Balears, Av. Ing. G. Roca, 69, 07157 Port d'Andratx, Balearic Islands, Spain; mvanrell@irfap.es (M.V.-V.); amaliagrau@irfap.es (A.G.); gaetano.catanese@uib.es (G.C.)

<sup>2</sup> Agro-Environmental and Water Economics Research Institute (INAGEA-UIB), Complex Balear de Recerca, Desenvolupament Tecnològic i Innovació (Parc Bit), Carrer Blaise Pascal 6, 07120 Palma, Illes Balears, Spain

<sup>3</sup> Parc Natural de s'Albufera des Grau, Govern de les Illes Balears, Carretera Maó—es Grau Km 3.5 Desviació Llimpa, 07710 Maó, Balearic Islands, Spain; spons@dgmedinatural.caib.es

\* Correspondence: jmvalencia@irfap.es

## Abstract

Between November 2024 and February 2025, mass mortality events affecting grey mullets (*Liza ramada* and *Mugil cephalus*) were recorded in the Albufera des Grau, Menorca, resulting in approximately 5000 dead individuals. Gross examination revealed high levels of parasitic infestation and lesions, located on the skin and gills, typical of copepod infection. In addition, histological and molecular analyses were performed on selected potential pathogens to assess their possible contribution to fish mortality. This study focused on the molecular characterization of the ectoparasitic copepod *Caligus pageti*, providing the first genetic reference for the species. This enabled preliminary taxonomic and biological assessment despite the absence of previously available sequence data. Environmental measurements across the lagoon indicated conditions capable of sustaining the development, survival and proliferation of the copepod population. These findings highlight the importance of integrating morphological and molecular analyses to enhance understanding of the biology of *C. pageti*, providing a key reference for future studies on parasitic copepods in lagoon ecosystems.

**Keywords:** mortality; mullets; lagoon; parasitosis; copepods; *Caligus*; 28s rDNA; COI

**Key Contribution:** This study identifies a strong association between the infection of copepod *C. pageti* and Mass Mortality Events (MMEs) in a coastal lagoon under conditions of reduced hydrological exchange. Furthermore, it provides the first molecular characterization of the species (using mitochondrial and nuclear DNA markers), establishing a critical framework for future epidemiological and ecological monitoring.



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## 1. Introduction

Mass mortality events (MMEs) are characterized by rapid, large-scale die-offs of organisms occurring within a short time frame and exceeding baseline mortality rates by several orders of magnitude. In aquatic environments, these events can impact one or multiple species. In particular, fish MMEs are being reported with increasing frequency in rivers, lakes, and coastal wetlands, especially in semi-enclosed systems such as lagoons and estuaries, where environmental conditions can fluctuate markedly under the influence of both climatic variability and anthropogenic pressures [1–3].

These events are typically driven by a combination of biotic and abiotic stressors, including infectious disease outbreaks, extreme weather conditions, pollution, hypoxia, and harmful algal blooms. MMEs in fish are generally multifactorial in origin. They are often associated with shifts in key environmental variables, such as salinity, temperature, dissolved oxygen, the accumulation of organic matter and contaminants [4,5]. Prolonged environmental stress can compromise immune responses, favoring infections by pathogenic agents [6]. Pollution from organic and inorganic sources can impair fish health and destabilize aquatic ecosystems by promoting eutrophication, which drives phytoplankton blooms that alter dissolved oxygen dynamics and may release harmful toxins [7]. When coupled with hypoxic conditions, these processes can lead to substantial shifts in fish assemblages [8].

Additionally, MMEs affecting fish populations may be driven by pathogenic agents, including bacterial, viral and parasitic infections [9]. These include a broad range of parasites, including protozoans (e.g., *Ichthyophthirius multifiliis*), monogeneans (e.g., *Dactylogyrus* spp.), digeneans and parasitic copepods (e.g., *Caligus* spp. and *Lepeophtheirus* spp.), have been associated with disease outbreaks in both wild and cultured fish populations [10–12]. Parasitic copepods are particularly widespread across freshwater, brackish, and marine environments [13,14]. The taxonomic class comprises a total of 2296 species of parasitic copepods from 333 genera and 35 families, recorded as infecting 2690 fish species [15]. Some copepod species can negatively affect fish growth, body condition, and reproductive performance, while others are highly pathogenic and may cause severe infestations associated with mass mortality events in affected populations [16]. Many parasitic copepods are characterized by direct life cycles without intermediate hosts, meaning that each infected fish acts as an independent unit of transmission. Under such conditions, parasites can spread efficiently in environments where host density is high, particularly in marine and brackish aquaculture systems such as cages and ponds [17,18]. This transmission dynamic is primarily shaped by key ecological and biological factors, including high host density, increased host body size, and limited water exchange or retention, which collectively enhance parasite contact rates and persistence in the system [19,20]. Among caligid copepods, *Caligus pageti* Russell, 1925 is a parasite belonging to the family Caligidae predominantly infecting teleost fishes. In wild breeding mullets of the family Mugilidae (such as *Liza ramada* and *Mugil cephalus*), *C. pageti* has been associated with mortality events within the Mediterranean region [21]. It typically attaches to the anterodorsal region of the fish's body, inducing tegumentary lesions and rapid weight loss in the host, and may even cause fish mortality under conditions of high proliferation or overcrowding.

The “Parc Natural de s’Albufera des Grau”, located on the northeastern coast of Menorca (Balearic Islands, Spain), represents a typical semi-enclosed coastal lagoon ecosystem. In November 2024, a first MME affecting mullets was recorded in this intertidal coastal lagoon and a subsequent MME occurred in early 2025. In such systems, environmental stressors can be intensified by periodic disconnection from the sea, which restricts water exchange and increases the vulnerability of fish communities.

In this context, the occurrence of repeated MMEs in this lagoon prompted a targeted investigation to determine the putative drivers of these episodic fish mortality events. Such knowledge is critical for informing effective management and conservation strategies aimed at mitigating mortality risks and preserving the ecological integrity of this vulnerable ecosystem.

The present study investigates the causes of detected fish mortality events through an integrative approach combining necropsy and targeted sampling for molecular and histological analyses.

## 2. Materials and Methods

### 2.1. Study Area: S'Albufera des Grau Lagoon

“S'Albufera des Grau” is located on the northeastern coast of Menorca (Balearic Islands, Spain), constitutes a representative example of a partially enclosed coastal lagoon ecosystem of high ecological value. The protected area covers a total of 5066.95 hectares, approximately one-third of which is marine, while the lagoon itself has a surface area of 78 hectares, an approximate volume of 1 hm<sup>3</sup>, and a mean depth of 1.4 m, reaching up to 3 m in its central zone (Figure 1). The lagoon's watershed is characterized by a mixed substrate, with impermeable soils near the lagoon and partially permeable formations in upland areas that contribute to the recharge of the lagoon aquifer. Hydrologically, the system is connected to the sea through a narrow channel known as “Gola channel”, which opens onto Es Grau beach and crosses a well-developed dune system (Figure 1). This connection is seasonal, typically remaining open during winter and closing in summer depending on rainfall and storm events.

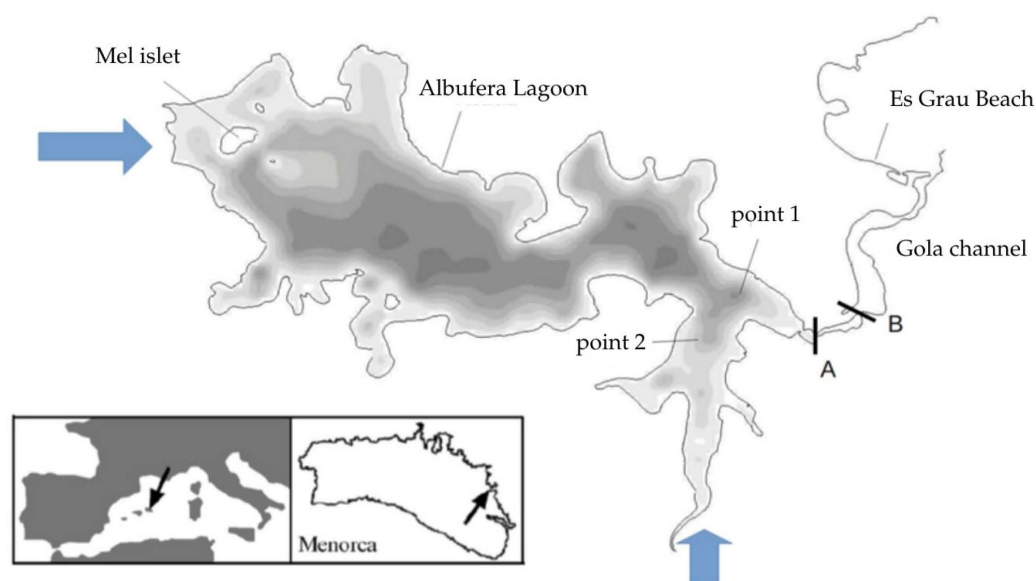


**Figure 1.** Bathymetric map of S'Albufera des Grau, provided by J. Ll. Petrus (contour lines are at 50 cm intervals, with sea level as the reference). The black arrows indicate the localization of the lagoon, while the blue arrows trace the water inflows from the streams. Line A points out the main sluice gate of the lagoon, whereas line B marks the secondary complementary sluice gate. Points 1 and 2 designate the water sampling locations for analysis.

The site is included in the Natura 2000 Network (site code: ES5310071) and provides critical habitat for numerous aquatic and migratory species. Due to its dynamic nature and exposure to multiple environmental pressures that may affect ecosystem stability and fish populations, it has been designated as a Natural Area of Special Interest under Law 4/1986 (BOE no. 180), which regulates human activities to ensure the conservation of its biodiversity and ecological integrity.

One of the main ecological challenges facing the lagoon is the seasonally variable but increasingly frequent reduction in freshwater inflow, which, combined with increased evapotranspiration associated with climate change, leads to episodes of high salinity. Although the lagoon water is considered brackish, physicochemical conditions show high variability, with salinity values historically ranging from 5 g/L (during periods of maximum freshwater input, such as in 2003) to over 50 g/L (recorded in 1995). Natural springs previously ensured a permanent freshwater flow, but they are currently depleted due to aquifer overexploitation and reduced cumulative rainfall in recent years.

The current management criteria of the Natural Park establish a minimum water level (between 45 cm and 60 cm), relative to sea level, to prevent the physical connection of “Illot d’en Mel” (Figure 1), habitat of an endemic subspecies of the Balearic lizard (*Podarcis lilfordi*), with the mainland.

Considering the environmental conditions of the lagoon, characterised by variable salinity, intermittent water exchange, and significant spatial heterogeneity, the fish community is dominated by euryhaline species. These include mosquitofish (*Gambusia holbrooki*), European eel (*Anguilla anguilla*), sand smelt (*Atherina boyeri*), and several mullet species: *Mugil cephalus*, *Chelon labrosus*, *Chelon ramada* (formerly *Liza ramada*), *Chelon auratus* (formerly *Liza aurata*), and *Chelon saliens* (formerly *Liza saliens*) [22].

2.2. Clinical History and Context of the Mortality Event

During 2024, the water level of the lagoon remained persistently below sea level with elevated salinity values. This situation required the main sluice gate to remain closed throughout the year (Figure S1 Supplementary Materials), hindering the migratory movements of fishes during the reproductive period. The lack of rainfall and freshwater inflow from streams led to the natural closure of the channel (“sa Gola”) in early 2024. In response to the increasing risk of hypoxia, mechanical openings of the channel were carried out on three occasions during the year, on a temporary basis and for short periods.

Between November 2024 and February 2025, two episodes of MMEs of mullets were recorded within the lagoon involving a total of 4,811 dead individuals (predominantly *L. ramada* and *M. cephalus*) and marking an unprecedented occurrence in the history of the Natural Park (Table 1). However, these mortalities were not observed in the channel area, where deaths were anecdotal. Additionally, sporadic mortality events of considerably lower magnitude, involving only a few individuals, were observed during spring and summer, but considered as typical for the system.

**Table 1.** Variables measured from October 2024 to March 2025. Water temperature values represent the monthly means ± SD (standard deviation) calculated from daily measurements. Salinity values correspond to the specific sampling day (mean of all measurements taken at the different points indicated in Figure 1). Monthly values are reported for the remaining variables. Temperature anomalies refer to the whole of Menorca and are based on data from Agencia Estatal de Meteorología (AEMET). Rainfall values were obtained from the rain gauge installed in the sluice area of the lagoon.

|                               | October 2024 | November 2024 | December 2024 | January 2025 | February 2025 | March 2025  |
|-------------------------------|--------------|---------------|---------------|--------------|---------------|-------------|
| Lagoon level (cm a.s.l.)      | −44          | −38           | −35           | −35          | −32           | −27         |
| Salinity (g/L)                | 24.7         | 24.2          | 23.7          | 23.8         | 23.8          | 22.6        |
| Water temperature (°C)        | 22.5 ± 0.85  | 20.5 ± 2.86   | 11.5 ± 1.67   | 11.7 ± 1.62  | 12.4 ± 1.29   | 16.3 ± 0.91 |
| Rainfall (L/m²)               | 85           | 41            | 63            | 37           | 33            | 89          |
| Mean temperature (°C)         | 20.8         | 17.1          | 12.1          | 12.8         | 11.9          | 13.7        |
| Temperature anomaly (°C)      | +1.5         | +2.2          | −0.2          | +1.6         | +1            | +1.1        |
| Number of dead mullets        | 4            | 832           | 115           | 229          | 3091          | 540         |
| Oxygen saturation (%)—surface | 78.5         | 65.8          | 98.9          | 101.5        | 105.8         | 86.6        |
| Oxygen saturation (%)—bottom  | 64.2         | 57.4          | 97.9          | 89.2         | 99.8          | 85.3        |

The first warning sign occurred in October 2024, with the observation of unusually large aggregations of mullets near the sluice gates. The fish exhibited agitated behaviour, with frequent jumping and rapid movements at the surface. This pattern was repeated in November, when the first dead individuals were detected. From that point onwards, a continuous daily occurrence of mortality was recorded, with systematic removal of carcasses by park staff.

Dead individuals were large and did not show evident signs of emaciation, but they did present external lesions such as petechiae, ecchymoses, and skin desquamation. In January 2025, a worsening of clinical signs was observed, with fish showing reddened fins

and epithelial erosions localized in the dorsocranial region, culminating in a second MME in February 2025.

The lagoon was monitored daily for water temperature (°C), and monthly for salinity (g/L), rainfall (L/m<sup>2</sup>), mean air temperature (°C), temperature anomaly (°C), oxygen saturation (%) at both surface and bottom, as well as the number of dead mullets. These values represent the environmental and biological conditions observed during the event.

### 2.3. Sample Collection

In February 2025, seven freshly dead individuals belonging to two mullet species were collected from the affected area of s'Albufera des Grau: *L. ramada* ( $n = 6$ ) and *M. cephalus* ( $n = 1$ ). Specimens were individually placed in plastic bags, kept refrigerated, and transported to the IRFAP-LIMIA facilities, where necropsies and subsequent analyses using molecular biology and histological techniques were performed. Transport was completed within 24 h.

At the laboratory, necropsies were conducted on all specimens. A thorough macroscopic examination was first performed, both external and internal bodies.

Tissue samples were collected for histological and molecular analyses. Samples were fixed in 10% buffered formalin for histology or preserved in absolute ethanol for molecular studies. In addition, brain samples were preserved in RNAlater (Sigma-Aldrich, St. Louis, MO, USA) for RNA-based analyses.

Parasites were visually detected on the mullet's skin and examined under a stereomicroscope Olympus (Evident Scientific, Inc., State College, PA, USA). Fifteen parasite specimens were collected from the ventrolateral skin region using surgical forceps (Figure 2b,c). All the individuals were fixed in ethanol 70%. A subset ( $n = 5$ ) of these parasites was subjected to a brief morphological examination at IMEDEA (CSIC-UIB) to verify key diagnostic characters, while maintaining specimen integrity. Handling time during this initial assessment was strictly minimized to prevent DNA degradation. Immediately following examination, these five intact specimens were processed for DNA extraction alongside the remaining ten individuals using the same protocol.

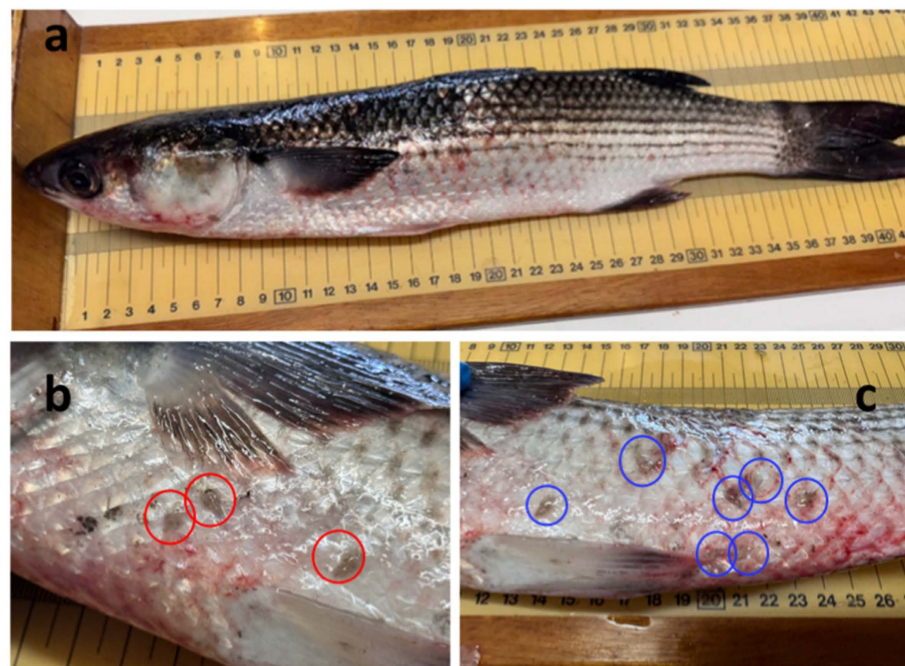


**Figure 2.** (a) Mullet showing lateral petechiae and ecchymosis, (b,c) Ventral image of the mullet showing hemorrhages and petechiae. Colored circles indicate the presence of copepod ectoparasites.

#### 2.4. Histological Processing and Staining

After gross examination, samples of liver, kidney, spleen and gill were collected for histological analysis, dehydrated through an ascending ethanol gradient to absolute ethanol, cleared with X-free (Bio Optica, Milan, Italy), and embedded in paraffin wax. Sections (3–4 µm) were cut using a rotary microtome and mounted on glass slides. Staining was performed using hematoxylin–eosin [23] and hematoxylin–VOF [24] for morphological assessment. Additionally, Ziehl–Neelsen staining was applied for the detection of acid-fast bacteria, and Gram staining [25] for bacterial characterization. Finally, slides were mounted in Eukitt(ORSAtec GmbH, Freiburg, Germany) for examination under a light microscope.

#### 2.5. Molecular Biology Analyses

DNA extraction was carried out from muscle, liver, skin and kidney tissues of mullets using the Nucleospin DNA Tissue Extraction kit (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions. DNA quality and concentration were assessed by NanoDrop 2000 spectrophotometry (Thermo-Fisher, San Jose, CA, USA).

For the detection of bacterial pathogens, PCR protocols targeting *Vibrio mediterranei* and species of the genus *Photobacterium* were applied. For *V. mediterranei* detection, primers described by [26] were used. For *Photobacterium* spp., a duplex PCR was performed using primers Ure-5'/Ure-3' (subsp. *damselae*) [27] and 76a/76b (subsp. *piscicida*) [28].

Additionally, DNA extracted from fifteen parasites were used as template for polymerase chain reaction (PCR) to enable molecular identification. A partial fragment of the 28S rRNA gene was amplified using primers LSU5 and 1500R [29], and a fragment of the cytochrome c oxidase subunit I (COI) gene was amplified using primers LCO1490 and HCO2198 [30].

Moreover, to investigate the putative presence of nodavirus, total RNA was extracted from brain samples preserved in RNAlater using the NucleoSpin® RNA II kit (Macherey-Nagel, Düren, Germany). The concentration of obtained RNA was measured with NanoDrop 2000. Complementary DNA (cDNA) was then synthesised using the SensiFAST® kit (Bioline Meridian Bioscience, Memphis, TN, USA) from 500 ng of RNA. Specific primers for nodavirus [31,32] were used to amplify regions of RNA1 and RNA2, following the PCR protocols described by the authors.

All PCR reactions were performed in a total volume of 20 µL, containing 10 L KAPA Taq Ready Mix PCR kit (Sigma-Aldrich, Burlington, MA, USA), 0.4 L of each primer (stock 20 M) and 1 L of DNA (20 up to 80 ng/L) and water to make up the final volume. Annealing conditions followed those specified in the original studies for each primer pair. Positive and negative controls were included in all PCR reactions. Amplified products were separated by electrophoresis on 1% agarose gels (1× TAE), stained with GelRed (Biotium, Fremont, CA, USA), and visualised under UV transillumination. Positive bands were purified using MiGel Extraction kit (Metabion International, Planegg, Germany) and, when necessary, bidirectionally sequenced using an ABI 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA), at Secugen S.L. service (Madrid, Spain). All the resulting sequences were edited and aligned using BioEdit 7.1.3.0 software [33]. Sequences were compared against GenBank and BOLD databases using BLAST.

To determine the best fit-mode substitution model of nucleotide substitution for phylogenetic reconstruction, five different model selection strategies were implemented using jModelTest2 [34]. Phylogenetic relationships were analyzed using Neighbor-Joining (NJ), Maximum Likelihood (ML), and Bayesian Inference (BI) methods. Genetic distances were calculated based on the Tamura-Nei model [35] in MEGA 12 software [36]. Tree reliability for NJ and ML was assessed with 1000 bootstrap replicates, while BI analysis was performed using MrBayes 3.2 with 10,000 generations [37].

The dataset comprised sequences generated in this study integrated with reference sequences for *Caligus* spp. and related genera retrieved from GenBank. *Monstrillopsis* spp. sequences (Acc. No. KY553229 for COI and KR048927 for 28S rRNA) were utilized as the outgroup to root the phylogeny.

## 2.6. Water Sampling and Analysis

To evaluate potential environmental drivers related to mortality, water quality analyses were conducted. Two sampling campaigns were performed at different sites within the water body of the lagoon on 3 February 2025 and 22 April 2025 (Figure 1). At each sampling point, surface water samples were collected using sterile 500 mL containers.

Samples for biochemical assays were stored at 4 °C during transport until delivery to two separate laboratories for analysis: Laboratori de l'Aigua and IRFAP LIMIA (Govern de les Illes Balears, Spain). The following physicochemical parameters were determined: pH, electrical conductivity, concentrations of nitrates ( $\text{NO}_3^-$ ), nitrites ( $\text{NO}_2^-$ ), phosphates ( $\text{PO}_4^{3-}$ ), ammonium ( $\text{NH}_4^+$ ), total nitrogen, total phosphorus, non-purgeable organic carbon (NPOC), turbidity, and fluorides ( $\text{F}^-$ ).

Salinity was measured in situ using a Hanna HI989192 salinometer (Hanna Instrument, Woonsocket, RI, USA) and surface temperature was monitored via a HOBO TidbiT MX data logger (LI-COR, Lincoln, NE, USA).

For phytoplankton analysis, two water samples collected at the same time were fixed with acidic Lugol's solution [38] and then quantitative and qualitative analyses were performed following the Utermöhl method [39], using an inverted light microscope (Olympus IX70, Tokyo, Japan) at IRFAP LIMIA laboratory.

## 3. Results

### 3.1. Mortality Event and Physicochemical Context

The mortality events resulted in an extensive die-off of individuals, representing an unprecedented occurrence in the s'Albufera des Grau lagoon.

A comprehensive set of environmental and biological parameters was recorded in the lagoon during the period corresponding to the observed mullet mortality. These measurements allowed an integrated assessment of the lagoon's physicochemical and biological conditions during the mortality event, providing a baseline for comparison and interpretation of potential environmental drivers. Environmental and biological monitoring from October 2024 to March 2025 revealed distinct fluctuations in the lagoon's conditions during the mortality event (Table 1). Water temperature exhibited a seasonal decline, reaching a minimum in December (11.5 °C), accompanied by consistent positive temperature anomalies throughout the study period, with the exception of December. Salinity remained relatively stable, ranging from 22.6 to 24.7 g/L. Dissolved oxygen saturation showed marked variability; surface and bottom values were lower during the initial months (October–November 2024) and significantly increased from December 2024 onwards, peaking in February 2025 (105.8% and 99.8%, respectively). Notably, the highest mortality event comprising 3091 dead mullets coincided with the period of lower rainfall and relatively stable salinity and temperature conditions in February 2025.

### 3.2. Anamnesis and Gross Examination

Gross examination revealed adult specimens presenting evident clinical signs. The mean total length of necropsied fish was  $47.9 \pm 4$  cm, while the mean body weight was  $680 \pm 70$  g. Of the seven specimens analyzed, four were female and three were male. Overall, body condition was considered moderately adequate (score 2–2.5/5).

Externally, scale loss was observed in the lateral and ventral regions. Generalized ecchymoses and petechiae were recorded, particularly in the ventral area (Figure 2a). Notably, two specimens exhibited severe hemorrhages at the base of the caudal fin and in the ventral region. In the context of these findings, the most notable gross feature was the presence of generalized external parasitosis by copepods in all specimens (Figure 2b,c).

Additionally, several nonspecific alterations were identified, including branchial congestion, swim bladder hyperinflation, and hepatic congestion, accompanied by focal blood extravasation (Figures S1 and S2, Supplementary Information) and yellowish discoloration of the liver. The yellowish areas may be associated with bile leakage, as the gallbladder was distended. Importantly, focal hepatic blood extravasation is interpreted as a post-mortem artifact related to autolytic changes given that necropsies were performed more than 24 h after death.

Regarding the digestive and associated organs, the intestines were empty and contained only small amounts of whitish mucilaginous material, suggesting reduced recent feeding activity. This is consistent with bile retention, a finding commonly associated with starvation and/or anorexia. The kidneys showed no apparent alterations. The gonads were in a resting phase in all specimens, although one individual (Individual 4) exhibited ovarian congestion. Additionally, unilateral corneal opacity was observed in a subset of individuals ( $n = 2/7$ ).

### 3.3. Water Analysis

Despite minor field observations suggesting localized disturbances (e.g., slight greenish coloration and foam accumulation), laboratory analyses of the two water samples collected during the mortality period did not detect phytoplankton blooms and indicated generally good water transparency.

Physicochemical parameters remained within normal ranges, with pH and conductivity reflecting expected spatial variation due to marine influence. Nutrient concentrations (nitrate, nitrite, phosphate, phosphorus, and fluoride) were below detection limits, while ammonium showed some variability and total nitrogen remained stable (Table S1, Supplementary Information). Higher turbidity and NPOC values were also recorded in the inlet area (Table S1).

### 3.4. Histological Analysis

Histological examination of the tissue samples revealed early autolytic changes, characterized by poorly defined cell boundaries, likely attributable to the time elapsed between fish dead and sample fixation. The specimens were transported under refrigeration in individual airtight bags shortly after death.

Within this context, no significant histopathological lesions were detected in the gill, liver, kidney, or spleen samples using standard hematoxylin–eosin (H&E) and Hart-VOF (H/VOF) staining protocols. Furthermore, histological sections were consistently negative for Gram and Ziehl–Neelsen staining. Consequently, there was no histological evidence of systemic bacterial infection, and no intra- or extracellular protozoan or metazoan parasites were observed. Consistent with these findings, none of the analyzed tissues exhibited areas of focal necrosis, supporting the absence of acute systemic pathological processes.

### 3.5. Copepod Morphological Identification

The specific identification of the taxon recorded in the Albufera des Grau was achieved by combining information on its geographical distribution (Mediterranean Sea) and host group (Mugilidae). Above all, the morphology of the fourth pair of swimming legs (Figure 3), whose number and proportions of armature elements have proven diagnostic, confirmed its identification as *Caligus pageti* Russell, 1925. This species was originally described from material collected from *Mugil capito* Cuvier, 1829 maintained in experimental aquaculture tanks at Lake Maryût, at Mex, near Alexandria (Egypt) [40]. The

description of this species has recently been supplemented by an additional morphological study of the type material preserved in the crustacean collection of The Natural History Museum (London) by [41], our specimens showing complete agreement with the diagnostic fourth leg described by these authors. Following its original description, *C. pageti* has been recorded from other species of Mugilidae along the North African coast, from Egypt to Algeria [42–44], and also along the French Mediterranean coast [45]. The Menorcan locality therefore falls within the general distribution range of the species.

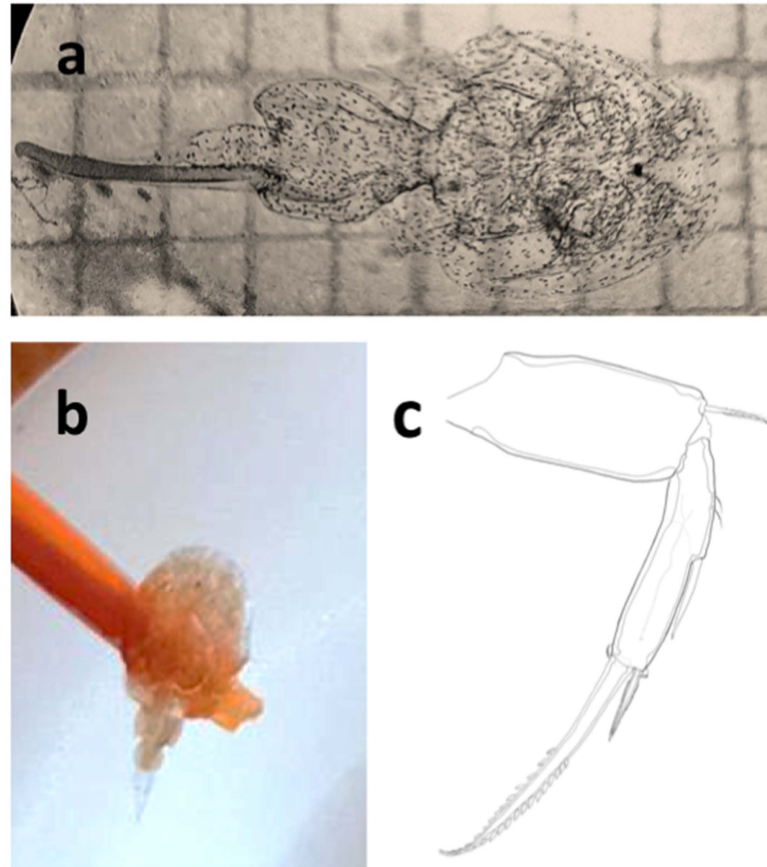


**Figure 3.** (a,b) Photographs of one of *Caligus pageti* specimens; (c) detailed illustration of a female's fourth leg of *Caligus pageti* from Albufera des Grau, showing diagnostic features.

### 3.6. Molecular Analyses

Molecular analyses were mainly performed to reject the presence of relevant bacterial and viral pathogens in sampled tissues.

In this context, PCR assays targeting *Vibrio mediterranei* and both subspecies of *Photobacterium damsela* resulted in negative PCR amplification for all specimens. Moreover, no amplification for *Betanodavirus* (VER) was obtained.

Conversely, PCR amplifications from DNA of the external copepod samples were successfully obtained, enabling subsequent sequencing for taxonomic identification. All 15 sequenced specimens yielded identical sequences for both markers, revealing a single unique haplotype for COI and a single unique haplotype for 28S rDNA. Consequently, a single representative sequence for each marker was deposited in GenBank under accession numbers LC872583 (COI) and LC872584 (28S rDNA). Subsequent BLAST analysis of the mitochondrial COI gene revealed 82.6% identity with *Caligus minimus* (PQ166295) and 81.6% identity with *Caligus lacustris* (MT920724). Furthermore, analysis of the 28S rDNA gene showed a higher sequence identity (97.61%) with *Caligus curtus* (DQ180338) and 96.5% *C. lacustris* (MT937093).

**Figure 4.** Phylogenetic reconstructions of copepod taxa based on (a) mitochondrial COI and (b) nuclear 28S rDNA sequences. Trees were inferred using Neighbor-Joining (NJ), Maximum Likelihood (ML), and Bayesian Inference (BI) methods. Support values (NJ/ML bootstrap percentages and BI posterior probabilities) are shown at each node.



In the COI-based phylogenetic trees, *C. pageti* nested within the genus *Caligus*, clustering as a sister taxon to *C. lacustris*. However, this terminal node was weakly supported, with bootstrap values falling below the resolution threshold for both NJ and ML (<50%), and yielding a low posterior probability in the Bayesian analysis (0.62 BI). A slightly broader cluster grouping these two species with *C. minimus* was recovered with low-to-moderate support (56% NJ/-- ML/0.83 BI).

In contrast, the topology inferred from 28S rRNA sequences placed *C. pageti* within the Caligidae clade and further resolved it within a more defined subgroup of *Caligus* species. Notably, Bayesian Inference significantly enhanced the phylogenetic resolution of this subgroup, providing maximum node support (Bayesian posterior probability = 1.00) for the key diagnostic branches. Within this clade, *C. pageti* was recovered as a sister taxon to *C. curtus* (73% NJ/72% ML/1.00 BI), and this sister pair clustered with *C. lacustris* under exceptionally high support values (99% NJ/95% ML/1.00 BI). Within this well-resolved framework, *C. pageti* formed a distinct, clearly differentiated lineage. Consequently, while the COI dataset successfully confirms the generic assignment of the specimens to *Caligus*, it exhibited lower phylogenetic resolution and weaker node support for these shallow interspecific relationships compared to the highly resolved 28S rRNA topology.

Overall, despite some differences in tree topology between markers, both datasets were consistent with the assignment of the analyzed specimens to *C. pageti*, supporting the morphological identification and phylogenetic placement within the genus *Caligus*, while indicating detectable molecular divergence from related species.

In confirmation of this, genetic distances based on comparisons between *C. pageti* and other species within the genus *Caligus*, ranged from 0.204 (with *C. minimus*) to 0.268 (with *C. elongatus* and *C. fugu*) for the COI marker. For the 28S rDNA sequences, genetic distances of *C. pageti* ranged from 0.023 (with *C. curtus*) to 0.122 (with *C. sclerotinosus*) (Tables S2 and S3, Supplementary Information).

This dual-marker approach confirms the morphological identification and establishes the first comprehensive molecular profile for *C. pageti*.

#### 4. Discussion

The mortality event recorded in s'Albufera des Grau represents an unprecedented episode in terms of magnitude, duration, and number of individuals affected, primarily involving mugilids. This die-off is a first for the Balearic Islands, as no large-scale events had previously been documented in its brackish ecosystems.

Our findings suggest that this mortality event could be associated with the presence of the copepod *C. pageti*. Furthermore, the first molecular characterization of this species provided here not only confirms its taxonomic identity but also establishes a critical baseline for monitoring this parasite in the Mediterranean. While previous mortality events in the Balearic Islands have been largely restricted to fully marine environments, this study shifts the focus toward the vulnerability of coastal lagoons.

Recent years have seen a rise in mass mortality events (MMEs) and newly detected pathogens across the Balearic Sea, affecting both fish and marine invertebrates. Consequently, these episodes have heightened concern about the conservation status of the region among the scientific community, non-governmental organizations (NGOs), and other marine stakeholders. These events have raised increasing concern about the conservation status of the Balearic Sea among the scientific community, non-governmental organisations (NGOs) and other marine conservation stakeholders. Notable cases include recurrent outbreaks of viral encephalopathy and retinopathy (VER), caused by betanodavirus, impacting commercially important species such as the dusky grouper (*Epinephelus marginatus*) and the Mediterranean moray (*Muraena helena*). In addition, the widespread mass mortality of the

fan mussel (*Pinna nobilis*), primarily attributed to the haplosporidian *Haplosporidium pinnae*, represents one of the most severe recent ecological crises in the region [46–49]. Parasitic diseases have also been increasingly documented. Hyperpigmentation syndromes (“black spot disease”) have been reported in species such as the pearly razorfish (*Xyrichtys novacula*) and the velvet belly lanternshark (*Etmopterus spinax*), associated with cestodes of the genera *Scaphanocephalus* [50] and *Grillotia* [51], respectively. Moreover, digenean infections (e.g., sanguinicoliasis) have been described in cultured greater amberjack (*Seriola dumerili*) [52], along with infestations by the monogenean *Zeuxapta seriolae* in early life stages (0-group individuals) [53,54]. Mortality events have also been reported in captive species in the Balearic Islands, such as the nursehound (*Scyliorhinus stellaris*), associated with infections by bacterial pathogens, including *Vibrio* spp. and *Photobacterium* spp. [55]. Both groups are widely distributed in Mediterranean marine environments and include strains with recognized pathogenic potential, associated with disease outbreaks and mortality events in marine organisms. Furthermore, obligate intracellular pathogens (e.g., chlamydia-like organisms causing epitheliocystis) have been detected in fish, including *Seriola dumerili* [56], as well as in cephalopods [57] and bivalves [58]. However, to date, these pathogens have not been linked to significant mortality events in the study area.

From an environmental perspective, physicochemical parameters remained largely within expected ranges during the sampling period, and no evidence of phytoplankton blooms or eutrophication processes was detected. Nutrient concentrations were consistently low and oxygen saturation values at both surface and bottom did not indicate hypoxic stress. These findings argue against classical drivers of fish kills in coastal lagoons, such as harmful algal blooms or acute oxygen depletion. However, field observations (e.g., localized greenish coloration and foam accumulation), together with higher turbidity and NPOC values in the inlet area, suggest localized organic enrichment processes. These patterns likely reflect reduced water renewal and progressive confinement of the lagoon, consistent with previous descriptions of Mediterranean coastal systems [59,60]. Species such as *L. ramada* and *M. cephalus*, can tolerate high salinities if acclimation is gradual, although tolerance can vary by species and other biological factors [61]. In this context, even moderate environmental fluctuations, potentially not captured at the temporal resolution of sampling, may have acted as chronic stressors.

The prolonged reduction in hydrological connectivity between the lagoon and the sea, persisting for approximately two years, likely played a central role in shaping these conditions (S. Pons, personal communication). Such confinement can enhance host density, reduce environmental buffering capacity, and promote the accumulation of organic matter, thereby creating favorable conditions for parasite proliferation. In addition, environmental stressors such as anomalously high seasonal temperatures and episodic rainfall events may have further contributed to ecosystem instability and increased host susceptibility.

Pathological findings consistently revealed generalized external parasitosis in all examined individuals, accompanied by hemorrhagic lesions, scale loss, and clear indicators of physiological stress, including empty intestines and distended gallbladders. The absence of significant internal lesions in histological examinations, together with consistently negative molecular screening for bacterial and viral pathogens (including *V. mediterranei*, *P. damsela*, and betanodavirus), provides little support for a primary infectious disease etiology.

All these results suggest that mortality was most likely associated with a chronic or subacute stress condition, in which severe ectoparasitic infestation played a central role through sustained impairment of host condition and physiological balance. While external lesions may have increased susceptibility to opportunistic secondary infections, as previously reported in parasitized fish [62,63], the lack of corroborating internal pathology

indicates that such infections were, at most, a secondary component rather than a driving factor in mortality dynamics.

In the present study, the identification of the ectoparasite copepods belonging to the order Siphonostomatoida, genus *Caligus* Müller, 1785 (Copepoda: Caligidae), were identified through combined morphological and molecular approaches, confirming their taxonomic assignment and relevance as pathogenic agents in marine fish. Members of the genus *Caligus* which comprises approximately 277 described species [64], are widely recognized as pathogenic ectoparasites of teleost fishes. They attach to the host's skin, gills, or oral cavity using specialized structures and feed on epithelial tissues, causing mechanical damage ranging from superficial abrasions to severe ulcerations. These injuries disrupt osmoregulatory and respiratory functions, enhance mucus production, and increase vulnerability to secondary opportunistic infections [65–67]. Accordingly, *Caligus* spp. have been consistently associated with reduced host fitness, increased susceptibility to disease, and, under severe infestations, mortality events in both wild and cultured populations [68–70].

The proliferation of caligid copepods is typically favored in semi-enclosed or confined environments with high host density and limited water exchange, as described for aquaculture systems affected by *Lepeophtheirus salmonis* or *Caligus elongatus* [71,72]. Furthermore, *C. curtus* have been linked to epizootic events in marine fish farms, producing skin lesions, ulcerations, and significant health deterioration in affected fish populations [14,73].

The observed infestation patterns suggest that *C. pageti* could be a relevant factor in the overall condition of the individuals. Clinical signs observed in affected mullets, including hemorrhagic lesions, skin opacity, fin erosion, excessive mucus secretion and abnormal behavior such as surface aggregation and rubbing, closely match those reported in other *Caligus* infections [74]. Comparable pathological outcomes have been documented in lagoon and aquaculture systems, where mixed infestations involving multiple *Caligus* species and co-infecting parasites have resulted in severe clinical deterioration and mortality [75].

Consistent with the typical caligid life cycle, previous studies on *C. pageti* described a direct development that alternates between free-swimming and parasitic stages []. This strategy inherently enhances host-finding efficiency and transmission potential in confined environments. Prior to reaching maturity, the life cycle encompasses four distinct phases: a two-stage nauplius, a single-stage copepodid that progresses through three behavioral and attachment states, four obligate chalimus stages, and a preadult phase consisting of one stage in females and two in males [76].

Despite the limited number of records of *C. pageti*, its occurrence has been previously associated with episodic outbreaks in mugilids, including a skin infection epizootic in *Mugil capito* from Lake Maryut [40] and its detection in *M. cephalus* in a seawater pond in Israel [77], suggesting a capacity for opportunistic proliferation particularly under restricted environmental conditions. In s'Albufera des Grau, reduced hydrological connectivity likely enhanced transmission efficiency by increasing host encounter rates and limiting parasite dispersal. Under such constrained conditions, the ecological and biological characteristics of *C. pageti* would favor rapid population build-up and sustained reinfection cycles, ultimately intensifying infection pressure on the host population and exacerbating disease outcomes.

In our work, although a multifactorial origin cannot be entirely excluded, the evidence consistently points to *C. pageti* as the most likely biological agent associated with the observed mortality event. The presence of the copepod observed in all examined *Mugilidae* specimens, together with pathological signs consistent with parasitic infestation, including cutaneous hemorrhages, fin erosion, ocular opacity, and excessive mucus production, strongly suggest this ectoparasite associated with the decline of host fitness and the eventual mortality of affected individuals. While environmental stressors such as reduced water renewal, localized organic enrichment, and anomalously high seasonal temperatures may

have contributed to the vulnerability of the fish, the consistent presence of *C. pageti* and the severity of lesions suggest that parasitism was directly associated with the mortality event. Despite these consistent findings, it is important to acknowledge certain limitations associated with the relatively small number of fish specimens examined in comparison to the scale of the mortality event. However, while the pathological and parasitological patterns were uniform across all analyzed individuals, this sample size should be considered when interpreting the strength of the association between *C. pageti* infestation and the overall mortality dynamics.

Crucially, this work provides the first molecular characterization of *C. pageti*, integrating mitochondrial (COI) and nuclear (28S rDNA) markers with classical morphological identification, and confirming its taxonomic status within the genus *Caligus*. The congruence between morphological diagnostic features, particularly the fourth pair of swimming legs, and phylogenetic placement in the *Caligus* clade validates both approaches and highlights the reliability of molecular barcoding for the detection of understudied ectoparasites. This molecular description not only establishes a reference for future studies but also represents the first direct association of *C. pageti* with a mass mortality event in a wild brackish ecosystem, demonstrating its potential pathogenic significance.

Molecular analyses corroborated the morphological identification and provided the first genetic characterization of *C. pageti*. Both molecular markers consistently placed the sequences within a well-supported *Caligidae* clade. Although COI sequences exhibited relatively high similarity to *Caligus* spp. available in public databases, phylogenetic analyses based on the 28S rDNA marker revealed higher sequence identity with *C. curtus* and *C. lacustris*, while still maintaining clear genetic distinction. This discrepancy is most likely attributable to differences in evolutionary rates between mitochondrial (COI) and nuclear (28S rDNA) markers. Notably, the phylogenetic reconstruction also highlighted a substantial gap in the currently available molecular data for this genus.

Genetic distance analyses further supported the distinctiveness of *C. pageti*. Based on 28S rDNA, the genetic distances between *C. pageti* and both *C. curtus* and *C. lacustris* were greater than those observed between *C. fugu* and *C. punctatus*, and were comparable to the distances between *C. fugu* and *C. elongatus* or between *C. curtus* and *C. lacustris*. Similarly, for the COI marker, the genetic distance between *C. pageti* and *C. minimus* exceeded that observed between *C. curtus* and *C. minimus* or *C. hoplognathi*, as well as between *C. minimus* and *C. lacustris*. Notably, the genetic distances involving *C. pageti* were similar or consistently higher than the interspecific values reported by other authors, including those reported for other *Caligus* and/or *Lepeophtheirus* species [78–81].

The combined use of mitochondrial (COI) and nuclear (28S rDNA) markers proved to be robust and is consistent with previous studies highlighting their complementarity for species delimitation and phylogenetic inference [82,83]. These results not only confirm the taxonomic identity of the parasite but also contribute valuable reference data for future studies.

This study underscores the importance of combining taxonomic, molecular, ecological and pathological data to identify the causative agents of mortality events. While multifactorial stressors cannot be completely ruled out, the data strongly support the interpretation that *C. pageti* infestation was the dominant biological factor contributing to the unprecedented mortality observed in s'Albufera des Grau.

In summary, we cannot discard a multifactorial scenario in which environmental stressors and parasitic infestation acted synergistically. While environmental conditions were not extreme, the combination of lagoon confinement, potential organic enrichment, and seasonal anomalies likely reduced host resilience. Under these conditions, the high burden of *C. pageti* infestation appears to have been a critical factor driving morbidity and mortality.

## 5. Conclusions

This study also highlights the vulnerability of semi-enclosed coastal lagoons to ecological imbalance, particularly under scenarios of reduced hydrological exchange and climate-driven environmental variability. The MMEs were primarily associated with an infestation of *C. pageti*, which was associated with the mortality of the affected fish population. A key scientific advancement of this study was the first molecular characterization of *C. pageti*, combining COI and 28S rDNA markers. This provides a valuable reference for future research and epidemiological monitoring.

It also underscores the importance of integrated monitoring approaches combining environmental assessment, pathology, and molecular tools for the investigation of fish mortality events.

Future research should focus on the ecology, genomic and pathogenicity of *C. pageti*, as well as on expanding molecular reference databases for caligid copepods. Continuous monitoring of lagoon systems will be essential to detect early warning signals and support effective management strategies aimed at preventing similar events.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fishes11060344/s1>, Figure S1: Gross lesions in the gills of an affected mullet, characterized by marked branchial congestion. Figure S2: Gross lesions in the liver of an affected mullet, characterized by sever hepatic congestion. Table S1: Results of the chemical analyses of the water samples. Table S2: Pairwise Tamura-Nei genetic distances based on the COI marker. Table S3: Pairwise Tamura-Nei genetic distances based on the 28S rDNA marker.

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**Institutional Review Board Statement:** Ethics Committee or Institutional Review Board approval is not required for our manuscript since the research carried out was based on the detection of the pathogens that have caused the death of the studied specimens and no experimentation with live animals or specific sacrifices for research has been done. In summary, the individuals analyzed are not animals used for experimentation, and therefore no approval from the ethics committee is necessary.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** All relevant data relative to the DNA sequences presented in the study are included at DDBJ/GenBank under accession numbers LC872583 and LC872584.

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