Received: 22 July 2022 | Revised: 7 September 2022 | Accepted: 8 September 2022

DOI: 10.1111/jfd.13717

Journal of Fish Diseases WILEY

RESEARCH ARTICLE

# A fish kill at the Aposelemis dam (Crete, Greece) caused by heavy parasitism by *Ichthyobodo* sp.

Pantelis Katharios[1] | Constantina Kokkari[1] | Maria Chiara Cascarano[1] | Stylianos Gyparakis[2] | Chrissi Aggelidaki[2] | Ioanna Antonakaki[2] | Efthimia Vargiakaki[2]

[1]Institute of Marine Biology, Biotechnology & Aquaculture, Hellenic Centre for Marine Research, Greece, Former American Base of Gournes, Heraklion, Greece

[2]Organisation for the Development of Crete SA (OAK SA), Aposelemis WTP, Heraklion, Greece

**Correspondence**
Pantelis Katharios, Institute of Marine Biology, Biotechnology & Aquaculture, Hellenic Centre for Marine Research, Greece, Former American Base of Gournes, Heraklion, Crete 71500, Greece.
Email: katharios@hcmr.gr

## Abstract

A fish kill was recorded at the Aposelemis Dam, which is the main reservoir of drinking water for the island of Crete in Greece. Hundreds of goldfish were found dead at a side stream which provides water to the reservoir. The affected fish had been entrapped in a small pond at the side of the stream with practically zero water renewal as the event occurred in August which is a dry season for the island of Crete. The event was alarming for the local community since anthropogenic pollution was initially suspected which could pose a significant human health threat. Following examination of the fish, the mortality was attributed to heavy infection by the parasitic flagellate, *Ichthyobodo* sp., whilst no pollutants were detected. The parasite was studied through light and scanning electron microscopy and was identified molecularly.

**KEYWORDS**
fish kill, gill histopathology, *Ichthyobodo*, parasitism

## 1 | INTRODUCTION

Fish kills are events of massive and usually sudden mortality of fish populations in a localized area and are almost always causes of concern to human communities associated with the affected areas. Fish kills have been recorded worldwide in many different types of water bodies, including sea water, rivers, ponds and lakes (La & Cooke, 2011). There are many causes of fish kills with oxygen depletion and harmful algal blooms being the most commonly recorded. Causes related to anthropogenic activities are also often associated with these events and pollution and toxicity by hazardous wastes are usually suspected and investigated especially when these events occur in water bodies which are used by humans. Such an event was recorded in August 2019 at the artificial lake of the Aposelemis Dam which is the biggest dam in the island of Crete, Greece. The dam was constructed to provide drinking water to the urban areas of Norther Crete and particularly Heraklion city and Agios Nikolaos which are also major touristic centres.

The fish kill was recorded on the 20th of August 2019, when hundreds of goldfish, *Carassius auratus*, were found dead in a pond of a side stream which provides water to the dam (File S1). The mortality was reported to the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC) of the Hellenic Centre for Marine Research by the Organization for the Development of Crete (OAK S.A.) responsible for the management of Aposelemis Projects (dam, reservoir, pipelines network and water treatment plant). IMBBC is in close vicinity to the affected area and researchers of Institute visited the site to take samples and investigate the causes of the incidence with the cooperation of the OAK S.A. scientific staff.

In this paper, we present our findings regarding the causes of this fish kill and discuss its impacts on the ecosystem and on the local community.

## 2 | MATERIALS AND METHODS

### 2.1 | Sampling

Both freshly dead and moribund fish were collected ($n = 15$) during the incidence. Whole fish were placed inside an isothermic cooled box and transferred immediately to the Aquaculture Microbiology laboratory of IMBBC for examination. Water temperature and dissolved oxygen were measured in situ using portable oxygen metre. Water samples were taken from two locations including the pond where the fish were found (File S2: sampling point 1), and downstream at the water reservoir (File S2: sampling point 2). The water samples were placed in sterile polyethylene plastic bottles and in glass BOD bottles and processed immediately at the laboratory of the Water Treatment Plant of the Organization for the Development of Crete S.A. Water samples were also sent the same day at a private accredited analytical laboratory (Chemicotechniki SA) using isothermic cooled box for complementary analyses. Water analyses included the determination of the following parameters: turbidity, colour, odour, temperature, pH, conductivity, total hardness, alkalinity, chlorides, sulphates, fluorides, phosphates, nitrates, nitrites, ammonium, calcium, magnesium, potassium, sodium, biochemical oxygen demand (BOD), chemical oxygen demand (COD), oxidisability, total organic carbon (TOC), total suspended solids, total nitrogen, total cyanide, phenols, surfactants anionic, oil and grease, total petroleum hydrocarbons (TPH), substances extracted with chloroform, microbiological analysis (total coliforms, *E. coli*, intestinal enterococci, *Pseudomonas aeruginosa*, *Salmonella* spp., *Clostridium perfrigens*, aerobic colony count at 37 and 22°C), pesticide analysis (list of active compounds analysed is provided in the File S2) with GC and LC/MS–MS, and heavy metals analysis with ICP-OES.

### 2.2 | Gross examination and microbiology

Wet mounts from gill samples and skin scrapings were examined using a compound light microscope (Nikon Eclipse 50i). Photographs were taken with a Nikon microscope camera (DS-Fi2). Maximum cell length and width of the parasite as described in (Isaksen et al., 2007) were measured with ImageJ using the microphotographs of live specimens in wet mounts.

### 2.3 | Histology

Samples ($n = 10$) fixed with 10% phosphate buffered formalin (PBF) were progressively dehydrated in higher concentrations of ethanol (from 70% to 96% EtOH), embedded in glycol methacrylate resin (Technovit 7100, Heraeus Kulzer) and cut in 4 μm sections with a microtome (RM 2245, Leica, Germany). Sections were mounted on slides and stained with methylene blue/azure II/basic fuchsin (polychrome stain) (Bennett et al., 1976).

### 2.4 | Scanning electron microscopy

Gill samples for SEM were washed with sodium cacodylate buffer, post-fixed with 1% $OsO_4$ and dehydrated in an ascending alcohol series, mounted on stubs, sputter-coated with gold palladium and examined using a JEOL JSM-6390LV scanning electronic microscope at 15 kV at the Electron Microscopy Laboratory of the University of Crete.

### 2.5 | Molecular analysis

Infected gill samples preserved in RNAlater were used for molecular analysis. DNA was extracted from mucus scrapped from gills with QIAamp® DNA Mini kit (Qiagen), using the procedure described in manufacturer's protocol. A primer set CosF1 and CosR6 (Isaksen et al., 2012) was used for the amplification of *Ichthyobodo* spp. partial SSU rDNA (18S rRNA gene) in a PCR with HotStar Taq Master Mix (Qiagen), following the procedure described by (Isaksen et al., 2012). PCRs were performed in T100 BIORAD thermal cycler. The amplified product of approximately 1800 bp was visualized in ethidium bromide-stained agarose gel, and purified with QIAquick PCR purification Kit (Qiagen), prior to sequencing. Sequencing was performed at CeMIA SA, Larissa, Greece with ABI3730xl sequencer (Applied Biosystems) according to the BigDye Terminators 3.1 protocol (Applied Biosystems, ThermoFisher Scientific Inc).

### 2.6 | Phylogenetic analysis

Small subunit rDNA sequences of *Ichthyobodo* species infecting fish were downloaded from GenBank and used in this study. The novel sequences were edited by eye, trimmed to 1726 bp with Geneious 9.1, and aligned with the GenBank sequences using ClustalW in Geneious 9.1 with default settings. Phylogenetic analysis was made by maximum-likelihood method in MEGA X (Kumar et al., 2018) using the Tamura-Nei model and pairwise deletion option. Genetic distances were calculated using MEGA X and the 'compute pairwise distances' option of the Tamura-Nei model.

## 3 | RESULTS

The gills and the skin of all fish examined ($n = 15$) were heavily infected with *Ichthyobodo* sp. (Figure 1c–e). Grossly, the gills appeared pale with mucous excess. Microscopically, both free swimming and attached forms of the parasite were found. The swimming form was oval, measuring $8.24 \pm 0.24 \times 11.39 \pm 0.27$ μm (W × L), and the attached form was more pyriform in shape. The parasite had two flagella protruding from a flagellar groove (Figure 1a,b).

The parasite had caused pronounced pathology to the gills of all goldfish examined. Histopathological analysis showed extensive hyperplasia and fusion of the secondary lamellae (Figure 1e) which

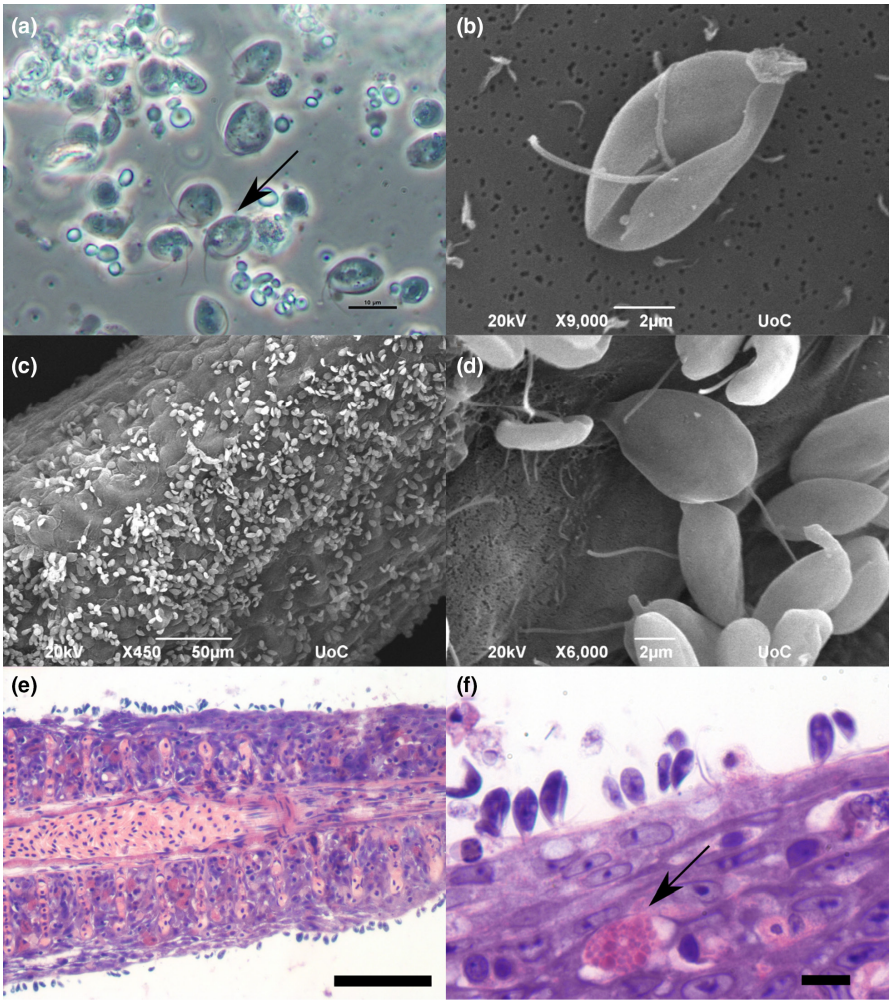


**FIGURE 1** (a) Free forms of *Ichthyobodo* sp. in a wet mount as observed with phase contrast under high-power magnification of a light microscope. (b–d). scanning electron micrographs of the parasite. (b) Ventral view of a biflagellated trophozoite showing the two flagella coming out of the groove. (c) Massive infection of the surface of the gill filament of goldfish by *Ichthyobodo* sp. (d) Higher magnification of attached trophozoites on the gills of goldfish. (e) Severe hyperplasia of the secondary lamellae caused by the attached parasites. Note the infiltration of the mast cells (eosinophilic granulocytes) in the area as a consequence of the *Ichthyobodo* parasitism (bar: 100 µm, polychrome stain). (f) High-power magnification of a gill histological section showing the attached trophozoites and the degranulating mast cell (arrow) attracted in the area (bar: 10 µm, polychrome stain).

resulted in significant loss of the available respiratory surface of the gills. Moreover, there was a marked infiltration of mast cells (eosinophilic granulocytes) in the gills (Figure 1e,f) which are effector cells that initiate the inflammatory response.

Sequencing of the PCR products resulted in identical sequences of approximately 1730 nt. The sequence was submitted to NCBI (Accession number: ON819563). Phylogenetic analysis using the small subunit rDNA gene sequence clustered the parasite in a subclade containing sequences from *Ichthyobodo* sp. obtained from goldfish from Singapore (99.83% similarity) and from common carp, *Cyprinus carpio* from Greece (99.93% similarity) (Figure 2).

Water quality analysis showed that all parameters assessed were within the acceptable limits with the exception of iron which was found at elevated concentration (1910 $\mu g\,L^{-1}$) at the affected pond, and there was no indication of anthropogenic pollution (File S2). Water temperature was 32°C and dissolved oxygen was 3 $mg\,L^{-1}$.

## 4 | DISCUSSION

The cause of the mass mortality of the goldfish at the Aposelemis dam was the heavy infection by the bodonid flagellate parasite, *Ichthyobodo* sp. The first and only report of *Ichthyobodo* sp. in Greece was in 1981 from a commercial rainbow trout farm in Peloponnese (Papatheodorou, 1981) where the parasite was considered as the etiological agent of severe mortality of rainbow trout fry. In addition, a Greek isolate was included in a phylogenetic analysis of *Ichthyobodo* sp. published in 2005; however, apart from its partial 18S sequence, the only other information provided is that it was isolated from mirror carp (*Cyprinus carpio*) from Greece (Callahan et al., 2005). The current work is the first case of feral fish mortality caused by *Ichthyobodo* sp. in Greece and the first report of this parasite in the island of Crete. The affected fish had been entrapped in a small pond at the side of the stream with practically zero water renewal as this occurred in August, which is a dry season for the island of Crete. Moreover, during the summer months, water consumption increases significantly since there is increased demand by the big numbers of tourists hosted in the area, which results in a substantial reduction in the water level in the reservoir. The static water, together with the very high water temperature created the ideal conditions for the proliferation of the parasite. More importantly, it has been shown that unfavourable environmental conditions intensify the pathological potential of this parasite by suppressing the capacity of the fish to withstand and confront the infection (Urawa, 1995). Gills of infected fish were completely obliterated due to the hyperplasia of the

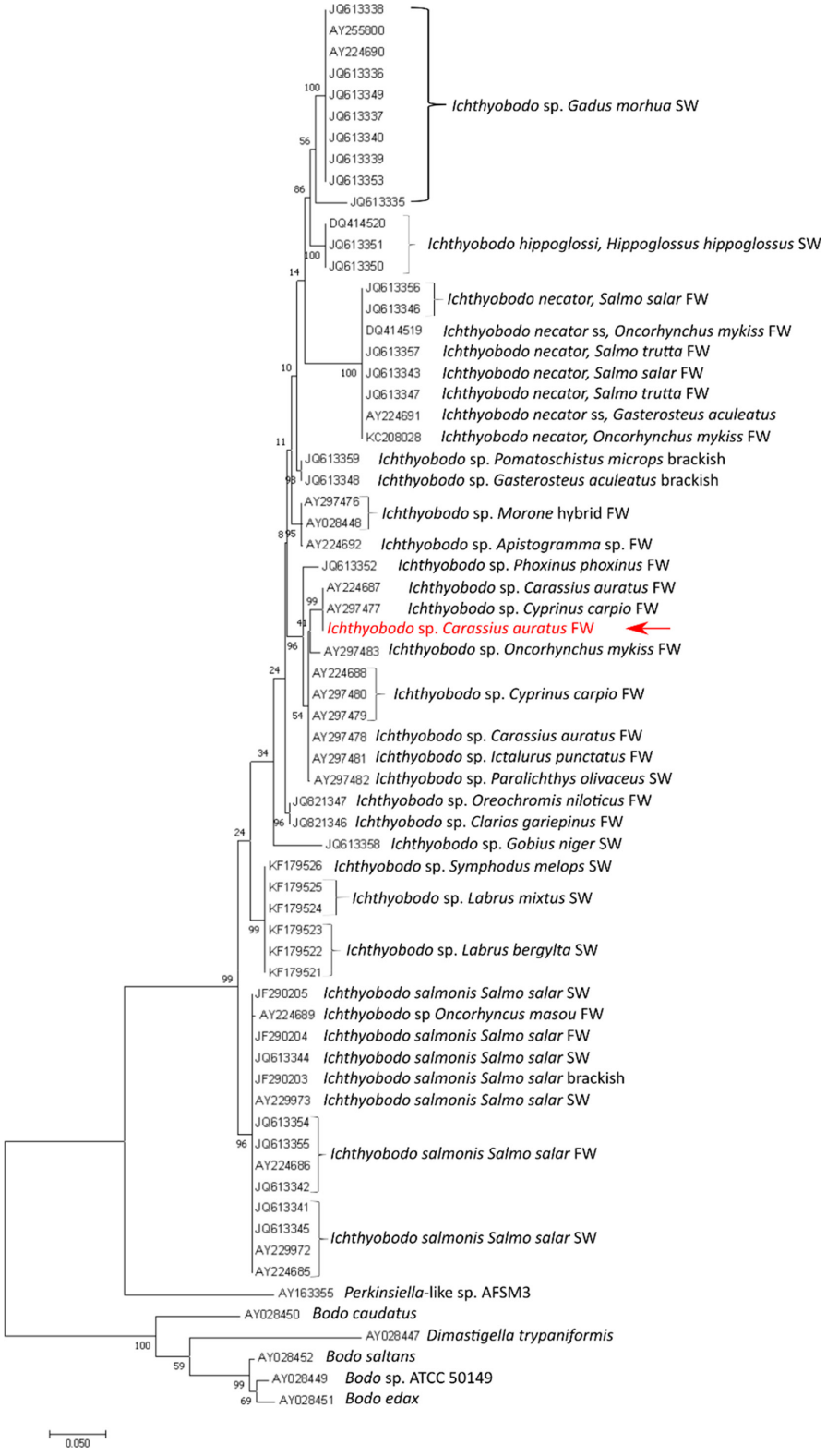


**FIGURE 2** Phylogeny of the *Ichthyobodo* genus inferred by using the maximum-likelihood method and Tamura-Nei model. The tree with the highest log likelihood (−3396.99) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 66 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 555 positions in the final data set. Evolutionary analyses were conducted in MEGA X. the sequence of the isolate from the current study is in red (arrow).

secondary lamellae. Gill hyperplasia and fusion of the lamellae is a common response to parasitic infection and has been observed in infections by *Ichthyobodo* sp. (Ellis & Wooten, 1978). Another interesting pathological finding of this study is the infiltration of the mast cells (or eosinophilic granulocytes). There is a marked increase of these cells in the gills, which play a proinflammatory role. They release effectors such as histamine or histamine-like substances that are attractants of leucocyte in the area, but they also contain antimicrobial peptides like piscidins (Alesci et al., 2022; Mulero et al., 2007; Reite & Evensen, 2006). Infiltration of mast cells and degranulation in response to parasitic infection has been observed in fish (Katharios et al., 2011).

Mass mortalities due to *Ichthyobodo* spp. infections, which may reach 100% of the affected population, are not uncommon both in reared and wild fish (Urawa, 1995). The parasite has a worldwide distribution and infects fish in freshwater, brackish and marine environments. Until recently, the only valid species was *Ichthobodo necator*, a parasite of freshwater fishes. However, the observation of the parasite in strictly marine fish species but more importantly the use of molecular tools has led to the identification of new species of the genus and various genotypes of *I. necator* (Isaksen, 2013; Isaksen et al., 2007, 2011, 2012; Todal et al., 2004). Our isolate was phylogenetically clustered with isolates from common carp from Greece and goldfish from Singapore (NCBI accession numbers AY297477 and AY224687, respectively) which form genotype VIII of *Ichthyobodo* sp. (Isaksen et al., 2012).

The only fish species involved in this event was goldfish. Apart from goldfish, the reservoir of Aposelemis Dam has carps (*Cyprinus carpio*), eels (*Anguilla anguilla*) and mosquito fish (*Gambusia holbrooki*). The origin of these fish populations and their abundances are not known since stocking of the reservoir was not done or supervised by the local authority which is the Fisheries Department of the Veterinary Directorate of the Prefecture of Crete (personal communication). *Ichthyobodo* sp. is not considered a host-specific parasite; however, the phylogenic analysis clustered the current strain with isolates obtained from goldfish and common carp (also from Greece). Therefore, it could be hypothesized that the parasite was introduced to the reservoir together with its host (most likely goldfish or carp) probably pet fish released by private citizens. Despite only goldfish were detected entrapped in the specific pond, it cannot be excluded that other fish species might have been affected by the parasite as well. Underestimation of the number of fish but also the fish species involved is not unusual in fish kills. Factors contributing to this include the activity of scavengers, inaccessibility of affected areas and the small size of fish that may go undetected (Labay & Buzan, 1999). Goldfish is an allochthonous species in Greece. Due to its great adaptability, it often becomes dominant in the ecosystems introduced (Dal Bosco et al., 2012). This could also explain why only goldfish were found in the affected area. A fish kill affecting the dominant fish population may be detrimental for the health of the ecosystem. Unfortunately, the ecosystem of the Aposelemis reservoir has not been studied yet; therefore, we cannot draw any conclusion about the impacts of this fish kill at a larger scale.

The elevated iron concentration in the water of the pond could have also played a role in the physiology of the fish. Iron dissolved in the water can impact the integrity of the gills but in much higher concentrations than the concentration measured here (Dalzell & MacFarlane, 1999). It should be noted that an important factor is the speciation of the total iron in the water since it is known that divalent iron may result in cellular oxidative stress and trivalent iron may precipitate and cause damage to the gills (Teien et al., 2008). Nevertheless, 14-day exposure of brown trout to 12 $mg\,L^{-1}$ of iron did not cause a significant alteration in the gills of the exposed fish (Dalzell & MacFarlane, 1999). Low dissolved oxygen may have also played a role in the observed mortality, however this parameter was measured in situ after the occurrence of the event and had probably been influenced by the large number of dead fish.

The incidence of this fish kill had a significant impact to the local communities since it occurred in the main water body that supplies drinkable water to the largest city of Crete, Heraklion and raised the public interest even outside the island of Crete. Due to the concerns raised by the picture of the dead fish, there were many speculations regarding the underlying causes with the main scenarios involving anthropogenic activities and pollution. Moreover, the Aposelemis dam was one of the biggest rural developmental plans for the island of Crete and it created many controversies amongst the local communities (Chifosa et al., 2019). The investigation of the actual cause of a fish kill is thus important as it can be misleading if it remains unresolved. Few countries have a systematic protocol for investigating fish kills and this is an emerging requirement as fish kills are usually recurrent events and the scientific knowledge about their causes is essential for the improvement of policy and decision-making regarding water management (La & Cooke, 2011).

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The partial sequence of the 18S gene of the parasite reported in this study is openly available in NCBI at https://www.ncbi.nlm.nih.gov/nuccore/ON819563, accession number ON819563.

## ORCID

*Pantelis Katharios* https://orcid.org/0000-0001-9905-5068

## REFERENCES

Alesci, A., Pergolizzi, S., Fumia, A., Calabrò, C., Lo Cascio, P., & Lauriano, E. R. (2022). Mast cells in goldfish (*Carassius auratus*) gut: Immunohistochemical characterization. *Acta Zoologica*, 1–14. https://doi.org/10.1111/azo.12417

Bennett, H. S., Wyrick, A. D., Lee, S. W., & McNeil, J. H. (1976). Science and art in preparing tissues embedded in plastic for light microscopy, with special reference to glycol methacrylate, glass knives and simple stains. *Stain Technology*, *51*, 71–97.

Callahan, H. A., Litaker, R. W., & Noga, E. J. (2005). Genetic relationships among members of the *Ichthyobodo necator* complex: Implications for the management of aquaculture stocks. *Journal of Fish Diseases*, *28*, 111–118.

Chifosa, C., Doxastakis, Z., & Romanos, M. C. (2019). Public discourse and government action in a controversial water management project: The damming of the Aposelemis River in Crete, Greece. *Water Policy*, *21*(3), 526–545. https://doi.org/10.2166/wp.2019.140

Dal Bosco, A., Mugnai, C., Mourvaki, E., & Castellini, C. (2012). Seasonal changes in the fillet fatty acid profile and nutritional characteristics of wild Trasimeno Lake goldfish (*Carassius auratus* L.). *Food Chemistry*, *132*(2), 830–834. https://doi.org/10.1016/j.foodchem.2011.11.043

Dalzell, D. J. B., & MacFarlane, N. A. A. (1999). The toxicity of iron to brown trout and effects on the gills: A comparison of two grades of iron sulphate. *Journal of Fish Biology*, *55*(2), 301–315. https://doi.org/10.1006/jfbi.1999.0994

Ellis, A. E., & Wooten, R. (1978). Costiasis of Atlantic salmon, *Salmo salar* L. smelts in seawater. *Journal of Fish Diseases*, *1*(4), 389–393. https://doi.org/10.1111/j.1365-2761.1978.tb00043.x

Isaksen, T. E. (2013). *Ichthyobodo infections on farmed and wild fish*. The University of Bergen.

Isaksen, T. E., Karlsbakk, E., & Nylund, A. (2007). *Ichthyobodo hippoglossi* n. sp. (Kinetoplastea: Prokinetoplastida: Ichthyobodonidae fam. nov.), an ectoparasitic flagellate infecting farmed Atlantic halibut *Hippoglossus hippoglossus*. *Diseases of Aquatic Organisms*, *73*(3), 207–217. https://doi.org/10.3354/dao073207

Isaksen, T. E., Karlsbakk, E., Repstad, O., & Nylund, A. (2012). Molecular tools for the detection and identification of *Ichthyobodo* spp. (Kinetoplastida), important fish parasites. *Parasitology International*, *61*(4), 675–683. https://doi.org/10.1016/j.parint.2012.07.006

Isaksen, T. E., Karlsbakk, E., Watanabe, K., & Nylund, A. (2011). *Ichthyobodo salmonis* sp. n. (Ichthyobodonidae, Kinetoplastida), an euryhaline ectoparasite infecting Atlantic salmon (*Salmo salar* L.). *Parasitology*, *138*(9), 1164–1175. https://doi.org/10.1017/S0031182011000916

Katharios, P., Rigos, G., & Divanach, P. (2011). Enteromyxum leei (myxozoa), a lethal intruder of tropical pet fish: First case in Humphead wrasse, Cheilinus undulatus (Rüppell, 1835). *Journal of Exotic Pet Medicine*, *20*(2), 138–143.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, *35*(6), 1547–1549. https://doi.org/10.1093/molbev/msy096

La, V. T., & Cooke, S. J. (2011). Advancing the science and practice of fish kill investigations. *Reviews in Fisheries Science*, *19*(1), 21–33. https://doi.org/10.1080/10641262.2010.531793

Labay, A. A., & Buzan, D. (1999). A comparison of fish kill counting procedures on a small, narrow stream. *North American Journal of Fisheries Management*, *19*(1), 209–214. https://doi.org/10.1577/1548-8675(1999)019<0209:acofkc>2.0.co;2

Mulero, I., Sepulcre, M. P., Meseguer, J., García-Ayala, A., & Mulero, V. (2007). Histamine is stored in mast cells of most evolutionarily advanced fish and regulates the fish inflammatory response. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(49), 19434–19439. https://doi.org/10.1073/pnas.0704535104

Papatheodorou, V. (1981). The parasitic disease of costiasis as the etiology of losses of rainbow trout fry. *Bulletin of the Hellenic Veterinary Medical Society*, *32*(3), 243–244.

Reite, O. B., & Evensen, Ø. (2006). Inflammatory cells of teleostean fish: A review focusing on mast cells/eosinophilic granule cells and rodlet cells. *Fish and Shellfish Immunology*, *20*(2), 192–208. https://doi.org/10.1016/j.fsi.2005.01.012

Teien, H. C., Garmo, Ø. A., Åtland, Å., & Salbu, B. (2008). Transformation of iron species in mixing zones and accumulation on fish gills. *Environmental Science and Technology*, *42*(5), 1780–1786. https://doi.org/10.1021/es071965m

Todal, J. A., Karlsbakk, E., Isaksen, T. E., Plarre, H., Urawa, S., Mouton, A., Hoel, E., Koren, C. W. R., & Nylund, A. (2004). *Ichthyobodo necator* (Kinetoplastida)–a complex of sibling species. *Diseases of Aquatic Organisms*, *58*(1), 9–16. https://doi.org/10.3354/dao058009

Urawa, S. (1995). Effects of rearing conditions on growth and mortality of juvenile chum salmon (Oncorhynchus keta) infected with *Ichthyobodo necator*. *Canadian Journal of Fisheries and Aquatic Sciences*, *52*(Suppl. 1), 18–23. https://doi.org/10.1139/f95-504

## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Katharios, P., Kokkari, C., Cascarano, M. C., Gyparakis, S., Aggelidaki, C., Antonakaki, I., & Vargiakaki, E. (2022). A fish kill at the Aposelemis dam (Crete, Greece) caused by heavy parasitism by *Ichthyobodo* sp.. *Journal of Fish Diseases*, *00*, 1–6. https://doi.org/10.1111/jfd.13717