



Invasive fish disrupt host-pathogen dynamics leading to amphibian declines

Gonçalo M. Rosa^{a,b,c,*}, Gonçalo Ayala Botto^d, Amartya T. Mitra^{a,e}, João Simões de Almeida^f, Max Hofmann^{g,h}, William T.M. Leung^{a,i}, António Pedro Alves de Matos^j, Maria Filomena Caeiro^{k,l}, Elsa Froufe^m, Armando Loureiroⁿ, Stephen J. Price^{a,o}, Christopher Owen^{a,e}, Rui Rebelo^b, Claudia Soares^p

^a Institute of Zoology, Zoological Society of London, London, UK

^b Centre for Ecology, Evolution and Environmental Changes (cE3c) & Global Change and Sustainability Institute (CHANGE), Faculdade de Ciências da Universidade de Lisboa, Lisboa, Portugal

^c Medical Research Council Centre for Global Disease Analysis, Imperial College London, London, UK

^d Escola de Ciências e Tecnologia, Universidade de Évora, Portugal

^e University College London, London, UK

^f Mozambique Wildlife Alliance (MWA), Maputo, Mozambique

^g Institute of Biology, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany

^h German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, Leipzig, Germany

ⁱ Royal Veterinary College, Herts, UK

^j Centro de Investigação Interdisciplinar Egas Moniz (CiIEM), Egas Moniz - Cooperativa de Ensino Superior, Caparica, Portugal

^k Departamento de Biologia Vegetal, Faculdade de Ciências da Universidade de Lisboa, Lisboa, Portugal

^l Centre for Environmental and Marine Studies (CESAM), Universidade de Aveiro, Aveiro, Portugal

^m Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), Universidade do Porto, Matosinhos, Portugal

ⁿ Direção Regional Conservação da Natureza e das Florestas do Norte, Instituto da Conservação da Natureza e das Florestas, Senhora da Hora, Portugal

^o Oxfam, Coventry, UK

^p Centro de Investigação em Biodiversidade e Recursos Genéticos (CIBIO), Universidade do Porto, Vairão, Portugal

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ABSTRACT

Sudden disease outbreaks may not necessarily reflect a recent pathogen introduction but may instead arise from the disruption of a host-pathogen equilibrium. Together with invasive species, emerging pathogens pose significant threats to biodiversity. The dynamics of each stressor have been studied separately, yet rarely when interacting. Using a 40-year dataset, we tested the hypothesis that the introduction of an invasive fish leads to such a disruption, manifested by ranavirosis outbreaks on amphibian hosts. MCP sequencing revealed the historical presence of two major *Ranavirus* clades, with low prevalence. The introduction of fish was not followed by the emergence of new viruses, but rather by an increase in the prevalence of the strains already present, fitting the 'endemic pathogen hypothesis'. Two decades after the first die-offs, one amphibian species persists in extremely low numbers, but *Ranavirus* prevalence is closer to the enzootic phase that preceded the outbreaks. Models show that host population collapse and lack of recovery are best explained by the concerted interaction of *Ranavirus* and invasive fish. We provide robust evidence that invasive species can impact naïve communities by disrupting the host-pathogen balance, exacerbating health threats. This study emphasizes the importance of exploring the historical interactions between multiple stressors to understand population declines.

1. Introduction

Emerging infectious diseases are one of the most pressing issues of the century, recognized as a major driver of biodiversity loss worldwide (Daszak et al., 2000; Crowl et al., 2008; Schmeller et al., 2020). Disease

outbreaks are often perceived as resulting from pathogen introductions and/or geographical expansions (Barrett et al., 1998; Daszak et al., 2000; Santini et al., 2018). Yet, anthropogenic drivers can shift host-pathogen interactions leading to outbreaks affecting both wildlife and human health (Daszak et al., 2001; Gibb et al., 2020). These human-

* Corresponding author at: Imperial College London, Zoological Society of London, London, UK.

E-mail address: goncalo.m.rosa@gmail.com (G.M. Rosa).

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mediated changes create novel environmental opportunities for pathogen evolution (Daszak et al., 2001), likely resulting from the disruption of former host-pathogen equilibria. Such changes in host-pathogen ecology may be common and widespread, but are probably overlooked given the frequent lack of historical pre-outbreak data (Dieuliis et al., 2016).

Human-assisted introduction of invasive species is another well-studied source of ecological disruption and a leading cause of extinctions (Clavero and Garcia-Berthou, 2005). The introduction of exotic species into naïve ecosystems can have significant ecological impacts through either direct biotic interactions (e.g., predation, competition) or via indirect changes caused by habitat alteration (Crooks, 2002; Gallardo et al., 2016). Moreover, invasive species can also act as vectors and reservoirs for pathogens and have been implicated in pathogen spread and disease emergence (Peeler et al., 2011; Conn, 2014; Hulme, 2014). While the study of invasive species as ecological disruptors has been mostly focused on the impacts propagated along the trophic webs (Crooks, 2002; Gallardo et al., 2016), research on disease dynamics in invaded systems generally focus on pathogen introduction or on aspects of transmission and maintenance (e.g., van Helden et al., 2020). The effects of non-native species on existing host-pathogen systems are largely unexplored.

Animal trade as well as invasions has likely played roles in the emergence and spread of pathogens such as ranaviruses (Paperna et al., 2001; Hulme, 2014; Price et al., 2016). Ranaviruses are large, double-stranded DNA viruses with a wide range of hosts, able to infect ectothermic vertebrates including amphibians (Duffus et al., 2015; Price et al., 2017a; Rosa et al., 2017, 2019). Spill-over has been reported between different species and even classes (Bandín and Dopazo, 2011; Price et al., 2017a), with cases of sympatric freshwater fish and tadpoles infected by the same *Ranavirus* (Rv) strain (Mao et al., 1999). The long-term effects of ranavirosis on wild communities and ecosystems are of great concern worldwide (Rijks et al., 2016). Although often linked to die-offs in amphibians as well as wild and farmed fish populations worldwide, the pathogen does not always lead to population declines, even after recurring outbreaks (Price et al., 2017a).

In Europe, *Ranavirus* has been associated with amphibian mass mortality events and community collapses (Teacher et al., 2010; Miaud et al., 2016), particularly in Iberian Peninsula (Price et al., 2014; Rosa et al., 2017). Non-native fish and frogs are thought to have facilitated *Ranavirus* dispersal to uninfected sites (Peeler et al., 2011; Price et al., 2017b). A ranavirosis outbreak affected all amphibian species in a remote site in northern Portugal in the late 1990s (Froufe et al., 1999; Soares et al., 2003; Alves de Matos et al., 2008), but the history of the pathogen at this site remains unclear. The catastrophic events appeared to have been preceded by the introduction of the North American Pumpkinseed (*Lepomis gibbosus*), leading to the assumption that the pathogen was introduced with the fish (Soares et al., 2003; Arntzen et al., 2009). In addition to their direct impacts (e.g., Hartel et al., 2007), sunfishes of the genus *Lepomis* Rafinesque, 1819 have been suggested to be reservoirs of ranaviruses (Brenes et al., 2014). Yet, growing phylogenetic evidence supports the endemic status of some of the dominant *Ranavirus* strains linked to recent outbreaks in Europe (Price et al., 2017b).

We combined a long-term field dataset with molecular tools and population modelling, taking advantage of pre-outbreak archival reference collections to test the novel vs. endemic pathogen hypotheses (following Rachowicz et al., 2005). While the novel pathogen hypothesis (NPH) assumes a recent spread of the pathogen into naïve geographic areas, the endemic pathogen hypothesis (EPH) suggests an historical presence of the pathogen in the system that becomes more virulent owing to a change in the ecological, immunological, and/or behavioral parameters of the host or pathogen (Carey, 1993; Rachowicz et al., 2005). Thus, we investigated the role of an alien stressor (*L. gibbosus*) as either the vector of *Ranavirus* or as the disruptive agent that altered the existing native host-pathogen dynamics. In the first scenario we would

not expect pathogen detection prior to fish introduction, whilst the alternative would result in an increase of infection prevalence right after invasion. Yet, a third scenario could also emerge from the fish introducing a new *Ranavirus* strain, different from those already present in the system. Specifically, we 1) investigated the historical presence and genotypes of *Ranavirus* in the system and in relation to the fish introduction; 2) revisited the data that documented the decline, and assessed the post-epizootic response of the amphibian assemblage two decades after the first outbreaks; 3) modelled the cumulative effects of *Ranavirus* and *L. gibbosus* on host demography.

2. Materials and methods

All field-collection and application of protocols were performed in accordance with the relevant local guidelines, regulations and licensing. The project was approved after ethical review by the Centre for Ecology, Evolution and Environmental Changes (cE3c).

2.1. Study system

The Peneda-Gerês National Park (PNPG) is a mountain-dominated landscape in the north-western region of Portugal (Fig. 1). Lagoa dos Carris is a 1.5 ha lake located at an altitude of 1500 m in a remote area of the park with a “total protection” status. The lake historically held a diverse amphibian community including Bosca's newt (*Lissotriton boscai*), Midwife toad (*Alytes obstetricans*) and Perez's frog (*Pelophylax perezi*), being a particularly important breeding site for Marbled newt (*Triturus marmoratus*) (Caetano, 1982, 1988; Soares et al., 2005). Past records indicate a relatively high abundance of *T. marmoratus* in the 1980s (Caetano, 1982; Caetano et al., 1985; Malkmus, 1986), and the first die-off was reported in 1998 (Froufe et al., 1999). *Ranavirus*-driven mortality was observed annually until 2004, affecting all amphibian species, particularly *T. marmoratus* (Soares et al., 2003; Alves de Matos et al., 2008). Pumpkinseed was illegally released in several freshwater habitats within the park and is thought to have been introduced in Carris in the mid-1990s (Carvalho & Franco in Neves, 2002). Despite the intensive fieldwork in the 1980's and early 1990's, no fish was detected in the lake (e.g. Sostoa et al., 1987; Caetano, 1988; Santos et al., 1991; authors' personal observation).

2.2. Field sampling: post-epizootic response

We focused our study on *L. gibbosus* and *T. marmoratus* at Carris lake, as the latter has been the most visibly affected species, and its population was target of periodic surveys since the 1980s. We broke down our demographic and epidemiological analyses into three time windows: before the outbreaks (1980s); during the outbreaks (2001–2004); and after the outbreaks (2015–2020). Samples obtained from other amphibian species and sites within the park were also included in this study: Charco de Gontamil, Castro Laboreiro, Curveira, Fraga Escuro, Inverneira da Podre, Lagoa da Peneda, Lagoa do Batateiro, Portelinha, and São João do Campo (Fig. S1 in Supplementary Material, SM).

After the first reports of mass mortality in Carris lake, our team established a monitoring programme. The surveys took place between 2001 and 2004; Carris was visited annually in June/July to estimate *T. marmoratus* population size using the triple catch method (Heyer et al., 1994). Toe clips of marked animals were stored in 96 % ethanol. We also recorded visible lesions and/or signs of disease (see Price et al., 2014; Rosa et al., 2017).

To assess the long-term post-epizootic response of the amphibian assemblage we re-established the surveys in 2015 until 2020. We kept a standardized and comparable sampling effort. Individuals were sexed and a toe/tail clip was collected and stored in 96 % ethanol for later detection of *Ranavirus* DNA (St-Amour and Lesbarrères, 2007). Clipping also prevented re-sampling the same animals. Before release, we applied the antiseptic/analgesic Bactine (Bayer, USA) to the clipped toe/tail



Fig. 1. Study system at Peneda-Gerês National Park (Portugal). Ranavirosis outbreaks have been recorded in Lagoa dos Carris since 1998 affecting all amphibian species, particularly the marbled newt (*T. marmoratus*) at different life stages: **A.** Adult male exhibiting ulcerations in the head and limbs (Lagoa dos Carris, 2003); **B.** Dead larva with haemorrhages in the ventral side, including tail (Lagoa da Peneda, 2011); **C.** Dead female encountered with haemorrhagic lesions in the ventral side, particularly in the limbs (Fraga Escuro, 2015); **D.** Adult female with ulcerations in the buccal and gular regions (Lagoa dos Carris, 2015).

(Martin and Hong, 1991). Some carcasses were collected and a piece of liver was sampled and stored in 96 % ethanol.

Between 2015 and 2020, individuals of *L. gibbosus* were captured while dip-netting. Fish were euthanized through immersion in buffered MS-222 (AVMA Panel on Euthanasia, 2020) and a piece of liver was stored in 96 % ethanol.

All animals were processed individually using disposable vinyl gloves and previously sterilized tools. All equipment (including hiking boots) was immersed in 1 % Virkon (Antec International Ltd., UK) before and after sampling to prevent pathogen spreading (Phillott et al., 2010).

2.3. Historical sampling: assessing *Ranavirus* status

During the 1980s, MH Caetano conducted amphibian surveys throughout the PNPg, including Carris (Caetano et al., 1985; Caetano, 1988; Caetano and Castanet, 1993; Caetano and Leclair, 1999). As part of her studies, several specimens (*T. marmoratus* and *L. boscai*) were collected and deposited at the National Museum of Natural History and Science (MUHNAC, Portugal). We took advantage of this collection and past work conducted in the park over a decade before the first reports of mortality to assess the presence of *Rv* in the population.

We sexed and sampled a total of 161 specimens: *T. marmoratus* ($n = 89$) and *L. boscai* ($n = 72$). A toe clip or, in more degraded specimens ($n = 82$) who already had a ventral incision, a small sample of liver tissue was collected to increase the likelihood of pathogen detection (and potentially systemic infection) (Miller et al., 2015). All specimens were processed individually using disposable gloves and previously sterilized equipment to avoid cross-contamination.

2.4. Disease screening, *Ranavirus* sequencing and phylogenetics

We extracted DNA from tissue samples using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA extractions were screened for the presence of *Ranavirus* using qPCR following the protocol by Leung et al. (2017). All qPCR assays were run in duplicate with a negative control (HPLC water) and plasmid standards containing the viral MCP target (amplified from an FV3-like virus isolate, RUK13; KJ538546). Samples were considered positive when a sigmoidal amplification occurred in both duplicates and a cycling threshold quantity >3.26 standard copies between the two runs was obtained (Leung et al., 2017).

Positive qPCR samples obtained from all the different species across

all the surveyed sites were then subjected to a conventional PCR assay which also targeted the MCP gene, using primers 4 and 5 from Mao et al. (1997) according to the adapted protocol by Price et al. (2017a). The MCP PCR amplicons were submitted to Beckman Coulter Genomics for Sanger sequencing of both DNA strands. Additional MCP sequences from representative species of the *Ranavirus* genus were downloaded from NCBI GenBank (see SM). Sequence electropherograms were viewed in 4Peaks (Griekspoor & Groothuis, Nuclobytes B. V.) to check the quality of base calls, and forward sequences were reverse complemented using the 'revseq' tool of the EMBL v6.6.0 software suite (Rice et al., 2000). The forward and reverse sequences for each sample were viewed in Unipro UGENE v3.6 (Okonechnikov et al., 2012), and ambiguous base calls were corrected with reference to the electropherograms. All study-generated and published MCP sequences were then aligned using MUSCLE v3.8.1551 (Edgar, 2004) with the default settings. All gaps in ≥ 20 % of sequences were removed from the resulting alignment with trimAl (Capella-Gutiérrez et al., 2009). A Maximum-Likelihood (ML) phylogenetic tree was then constructed using RAxML v8.2.12 (Stamatakis, 2014), with the GTR model of nucleotide substitution and rate variation among sites modelled by a discrete gamma distribution with four categories. The most parsimonious tree was selected after 100 ML iterations generated with distinct starting trees, which was then annotated with node support values based on 1000 bootstrap replicates. The tree was then rescaled from substitutions⁻¹/site⁻¹ to absolute number of SNPs, and plotted using the R package ggtree (Yu et al., 2018).

2.5. Modelling the interaction of multiple stressors

Stochastic simulation tools are typically used to estimate the probability of extinction of a certain population (Beissinger and Westphal, 1998; Brook et al., 2000). To test the cumulative vs. non-cumulative effects of the pathogen (*Ranavirus*) and the competitor/predator (*L. gibbosus*) on *T. marmoratus* population, we modelled six scenarios for a 40-year population projection (1980 to 2020) based on the Carris system. The effects of disease and fish were modelled after the 18th year of the simulation to coincide with the detection of fish and effects of *Rv* in the lake. As the precise effects of *L. gibbosus* on this newt system are not known, scenarios with mild and strong effects were considered based on available literature (Smith et al., 1999; van Kleef et al., 2008):

Scenario 1: Healthy population unaffected by disease outbreaks or fish.

Scenario 2: Population mildly affected by fish in the absence of disease.

Scenario 3: Population strongly affected by fish in the absence of disease.

Scenario 4: Population affected by disease in the absence of fish.

Scenario 5: Population affected by both disease and mild effects of fish.

Scenario 6: Population affected by both disease and strong effects of fish.

Given the lack of successful treatments to remove *Ranavirus* from any system, we created a new model to test the effect of fish removal after the severe decline as a potential conservation action. Scenario 7 simulated the removal of the fish impacts using parameters from Scenario 6 in a 100-year population projection. ‘Fish removal’ was modelled to take place when the population reached a value ≤ 50 individuals (the estimated newt population size in Carris in 2020).

Two separate model types were developed across all scenarios: 1) a stochastic age and sex-structured demographic model of *T. marmoratus* in VORTEX v10.5.0 with fish impacts modelled through changes in demographic processes (Lacy et al., 2020); 2) an individual-based epidemiological model in OUTBREAK v2.11.0 simulating the *Ranavirus* dynamics when the first outbreaks were detected at the site (Pacioni et al., 2019). These simulation models were linked and run simultaneously on METAMODEL MANAGER for 100 iterations and averaged (Lacy et al., 2013; Raboy et al., 2018). A detailed software description and all parameters are provided in the SI. Sensitivity testing was used to uncover particularly uncertain parameters associated with fish impact that could significantly alter the outputs derived from the models (see SM). Combined with available data from closely related systems, the results from sensitivity testing, helped better parameterise the different scenarios.

2.6. Statistical analysis

2.6.1. Species abundance over time

We combined *T. marmoratus* abundance data retrieved from pre-outbreaks (Caetano et al., 1985; Caetano, 1988; Caetano and Castanet, 1993; Caetano and Leclair, 1999; MUHNAC collection data), as well as during epizootic and enzootic phases. Abundance was calculated by dividing the total number of individuals captured in a year by sampling effort (n persons*time (h)). Population trends were estimated using R package *rtrim* (Bogaart et al., 2020; see SI for details). We used the linear trend model considering the year 2001 – the first year of outbreaks with count data – as changepoint (i.e. the time point where the slope parameter changes). We plotted overall trend estimates for *T. marmoratus* adults, calculated as the slope of the regression line through the logarithms of the indices over the entire study period. We used 95 % confidence intervals of the overall trend estimate to test for significant population trends (Pannekoek et al., 2018). We followed the trend classification proposed by van Strien et al. (2001) (Bogaart et al., 2020; see SI for details).

2.6.2. Sex ratio

During the first outbreaks in Carris, Froufe et al. (1999) noted that females were particularly affected. To test whether the phase of the disease invasion affected the sex ratio of the population we performed a generalized linear mixed model (GLMM). We assumed a binomial distribution with a logit link function, with ‘year’ included as a random effect. Sex ratio was expressed as: males/(males + females).

2.6.3. Prevalence of *Ranavirus* infection

We calculated the prevalence of *Ranavirus* infection as the number of infected / total number of individuals sampled (in %). We fitted a similar GLMM with a binomial distribution to test if the phase of the disease invasion could predict the prevalence of Rv infection.

Both GLMMs were constructed using the R package *lme4* (Bates et al., 2015), and p values were obtained using the function *Anova* (type II) from the R package *car* (Fox and Weisberg, 2019). For both models, post hoc pairwise comparisons with Bonferroni adjustment were conducted to determine differences among the fixed effect using the R package *emmeans* (Lenth, 2020).

2.6.4. Population viability analysis

To detect differences in the probability of extinction across the different scenarios we used Friedman’s test. Following a significant result, post hoc pairwise comparisons with Wilcoxon signed-rank were then applied with Bonferroni correction.

3. Results

3.1. Demography over time

Despite intensive year-round sampling throughout the 1980s (Caetano, 1982, 1988; Caetano et al., 1985; Caetano and Leclair, 1999) and periodic visits during the 1990s, no unusual amphibian mortality was ever observed until the outbreak. In 1997 and prior to the first die-offs of 1998, we did not observe signs of mortality in Carris (AL personal observation).

We found a highly significant effect of time on the abundance of *T. marmoratus* at Carris: while the population was ‘stable’ from early 1980s until the late 1990s (model slope = -0.0091 ; SE = ± 0.013), the *Ranavirus*-driven die-offs that started in 1998 coincide with a turning point to a ‘strong decrease’ in host abundance ($p < 0.05$; model slope = -0.1937 ; SE = ± 0.061). There was a significant change in trend (Wald test = 13.69, df = 1, $p < 0.001$) with *T. marmoratus* experiencing a decline of 96.6 % (in relation to the average population abundance between 2001 and 2004), with no signs of recovery two decades after the first outbreaks (Fig. 2A, Fig. S2).

Higher mortality in females seems to have prevailed in subsequent years following the first die-offs, explaining the significant effect of the phase of the disease progression on the sex ratio of the host population (Wald $\chi^2 = 26.793$; df = 2; $p < 0.001$). This trend led to a consequent reversal in the sex ratio during the epizootic phase (Fig. 2B). The sex ratio shifted from up to 44 % males in late spring/summer during the 1980s (enzootic phase) to an average of over 79 % in the epizootic (pairwise: $p < 0.001$; Fig. 2B). In recent years the proportion of females has increased, approaching pre-epizootic values ($p = 0.223$) and, although the overall sex ratio remained above 50 % (except for 2020), the difference is significant in relation to the epizootic phase ($p = 0.032$; Fig. 2B).

All other species were not so intensively monitored, however, we observed steep declines in their relative abundance, namely *Lissotriton boscai*, *Alytes obstetricans* and even the formerly very common *Pelophylax perezi* (see Caetano et al., 1979; Caetano, 1982). A single *A. obstetricans* tadpole was found in 2004 and the species has not been recorded in Carris since then.

Species demography data for other affected sites in Peneda-Gerês is not available prior to the *Ranavirus* outbreaks, but both newts (*T. marmoratus* and *L. boscai*) and *A. obstetricans* were regarded as common and abundant (Caetano, 1982; Malkmus, 1986; Soares et al., 2005). In Lagoa do Batateiro and Lagoa da Peneda, the first mortality events were recorded in June 2003. Recent surveys revealed a relatively low abundance of all species (Fig. S1). Both sites were also stocked with river trout (*Salmo trutta fario*) during the 1990s, a fish that predares upon amphibians and is not native to pond habitats.

3.2. Prevalence of infection with *Ranavirus*

Amphibians found dying or dead at Carris were confirmed to be infected with *Ranavirus* between 1998 and 2020 (Alves de Matos et al., 2008; this study). Sick/moribund and dead *T. marmoratus* exhibited skin

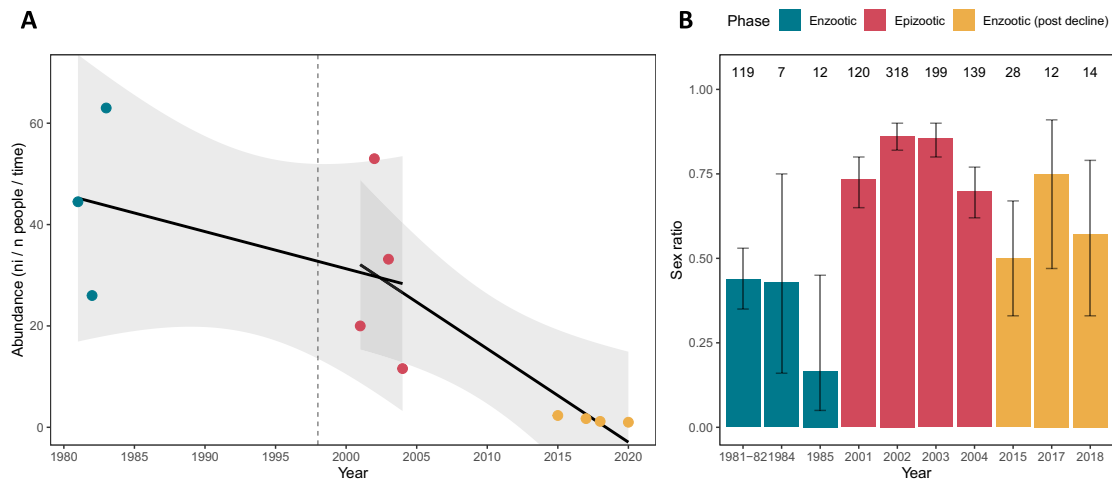


Fig. 2. Demography of the adult population of Marbled newt (*Triturus marmoratus*) from Lagoa dos Carris, Peneda-Gerês (Portugal) across three phases of the host-pathogen (*Ranavirus*) dynamics: enzootic, epizootic and a recent enzootic stage post host population decline. **A.** Population abundance and overall trends; linear regression lines (with respective shaded standard errors) correspond to the periods before and after the outbreaks of ranavirosis; Vertical dashed line indicates the first ranavirosis outbreak in 1998; A severe decline in *T. marmoratus* abundance followed the outbreaks and introduction of invasive fish in the late 1990s. **B.** Sex ratio over time: while the historical sex ratio was female-dominated, there was an overall increase in the proportion of males after the first outbreaks of ranavirosis. The sex ratio is expressed as the proportion of males/(males + females), with error bars indicating the 95 % confidence intervals. Top numbers reflect the total sample size (N). In 2020, a single female *T. marmoratus* was captured (not depicted).

haemorrhages and ulcerations on their mouth, ventral body surfaces and, in a few cases, limb necrosis – all gross signs typical of lethal ranavirosis (Fig. 1; Gray et al., 2009; Rosa et al., 2017). The necropsied fresh carcasses presented focal erythema associated with enlarged, mottled pale brown and friable livers. Mortality was recorded across all life stages and ages of amphibians making use of the aquatic environment, including adult and larval caudates (*L. boscai*, *T. marmoratus*) and anurans (*A. obstetricans*, *P. perezi*) (Fig. S1).

Historical sampling of museum specimens revealed an absence of overt signs of ranavirosis. Yet, molecular analyses determined the presence of *Ranavirus* in the amphibian assemblage (Figs. 3, 4). In Carris, the prevalence was relatively low before the epizooty, averaging 23.5 % in *T. marmoratus* (Fig. 3; Table S1).

We found the phase of the disease invasion to be a predictor of the prevalence of Rv infection in *T. marmoratus* (Wald $\chi^2 = 7.948$; df = 2; $p = 0.019$). The peak recorded during the epizootic phase in Carris showed a high contrast with the historical *Ranavirus* prevalence ($p = 0.015$) with 90 % of all live and dead *T. marmoratus* infected (100 % in all dead *L. boscai*; Fig. 3; Table S1). The peak was then followed by a

significant decrease ($p = 0.028$), with the overall Rv prevalence in recent times closer to values of the 1980s ($p = 1.000$), except for 2020, where the single individual found in the lake was infected (Fig. 3; Table S1). This pattern seems to be replicated in other amphibian species, with a recent prevalence closer to the values before the die-offs (enzootic phase; see Fig. 3). The introduced Pumpkinseed had an average prevalence of infection of 24 % ($n = 57$) over the years when it was sampled (all post-epizootic) (Fig. 3; Table S1).

3.3. *Ranavirus* phylogeny

Sequencing data showed the presence of strains belonging to two major *Ranavirus* clades/species: *Common midwife toad virus* (CMTV)-like and *Frog virus 3* (FV3)-like (Fig. 4; Table S2). Within both clades, we found that overall sequences do not differ in the SNPs within their MCP sequence, spanning from the 1980s until now. Both CMTV and FV3-like strains were able to infect several amphibian host species across the park a decade before the observed disease outbreaks and have been infecting the same amphibian hosts since. By contrast, all Rv-positive *L. gibbosus*

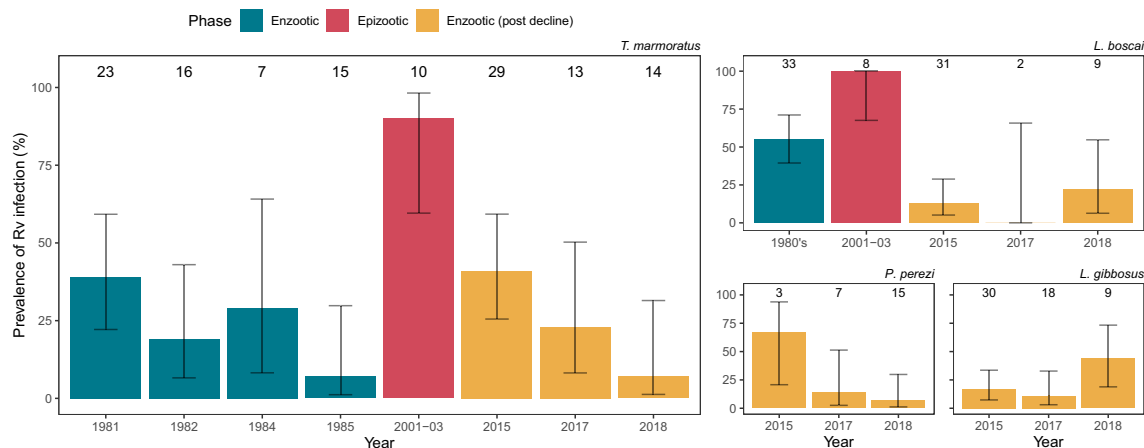


Fig. 3. Prevalence of *Ranavirus* infection in the amphibian assemblage and invasive Pumpkinseed (*L. gibbosus*) from Lagoa dos Carris, Peneda-Gerês (Portugal) across three phases of the host-pathogen (*Ranavirus*) dynamics: enzootic, epizootic and a recent enzootic stage post host population decline. Top numbers reflect the total sample size (N) and error bars indicate the 95 % confidence intervals. In 2020, a single infected *T. marmoratus* was captured (not depicted).

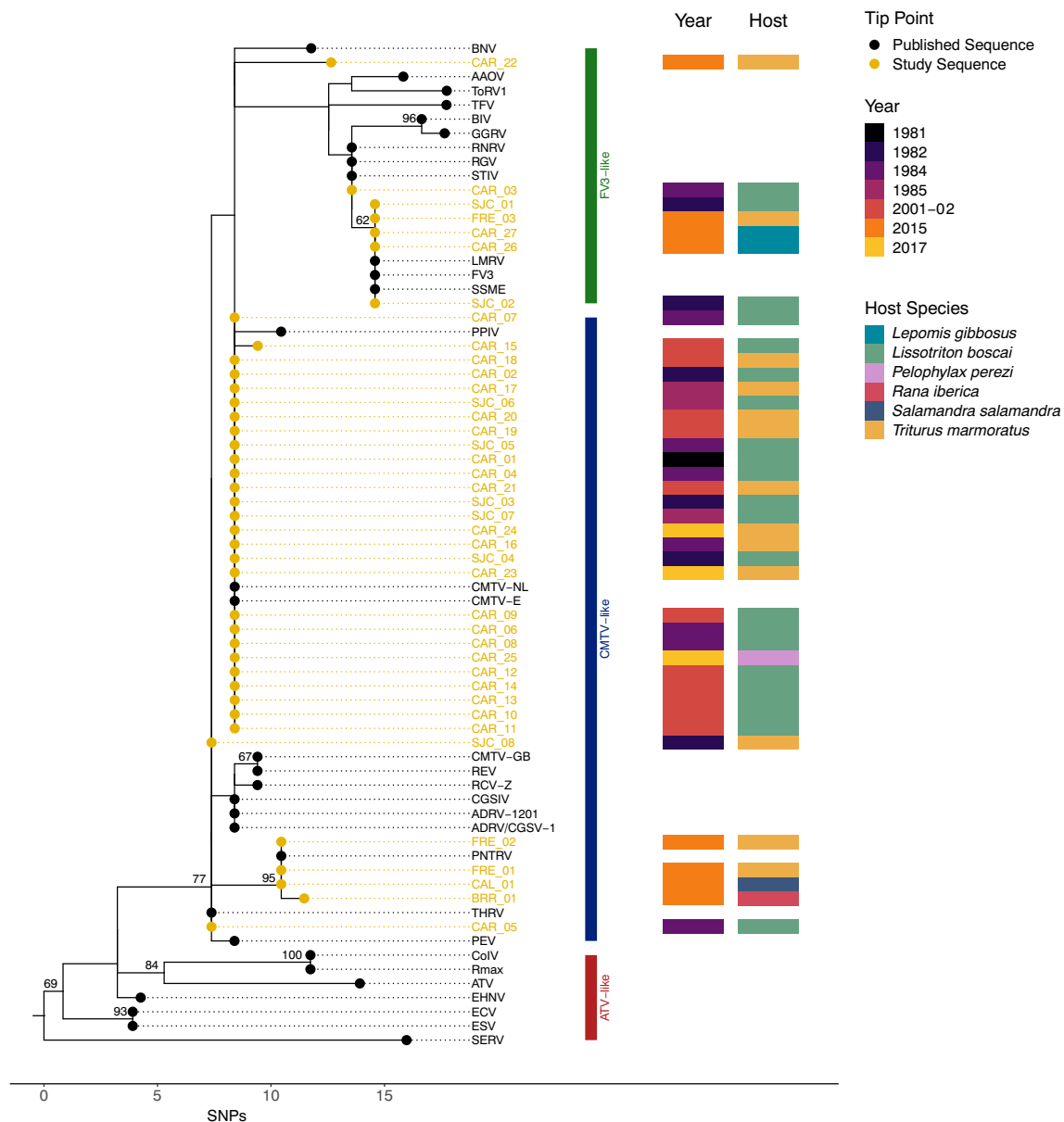


Fig. 4. Major Capsid Protein (MCP) Maximum-Likelihood phylogeny of Peneda-Gerês ranaviruses and known representatives of the three major clades: Vertical bars indicate FV3-like ranaviruses (green), CMTV-like ranaviruses (blue), and ATV-like ranaviruses (red). Yellow tip points denote sequences generated from this study, whereas black tip points indicate previously published clade-representative ranaviruses (see Methods and Results in SM). Included in the tree are samples from three distinct time windows: before the outbreaks (1980's), during the outbreaks (2001–2004), and after the outbreaks (≥ 2015). The sites within the park from where samples were obtained are reflected in the name coding: CAR, Lagoa dos Carris; CAL, Castro Laboreiro; FRE, Fraga Escuro; SJC, S. João do Campo; GTM, Charco de Gontamil. Amphibian hosts were infected with ranaviruses from both the FV3-like and CMTV-like clades, while the ranavirus strains from the invasive *L. gibbosus* are limited to the FV3-like group. The phylogeny was constructed from a multiple sequence alignment of the MCP gene (see main text), which was 464 bp in length and contained 74 SNPs. Node support values are based on 1000 bootstrap replicates, with support values $>60\%$ displayed. Branch lengths are scaled by absolute number of SNPs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were infected with a single FV3-like strain identical to the sequences detected in infected *L. boscai* sampled in the early 1980s, and present in other sites with no fish ever recorded (e.g. S. João do Campo) (Fig. 4).

There was at least one CMTV-like strain overrepresented across all time points and amphibian hosts, sharing 100 % MCP homology with CMTV-E (JQ231222). A smaller number of samples grouped with Portuguese newt and toad ranavirus (PNTRV), a strain previously described to Serra da Estrela (north-central Portugal; Rosa et al., 2017). These closely related sequences were only detected during the post-epizootic sampling in three different amphibian species, but not at Carris lake.

3.4. Modelling marbled newt decline

Scenario 1 involved a healthy *T. marmoratus* population. The simulated effective population size stabilised below carrying capacity at an average of about 3600 individuals, ranging from ca. 2280 to 5050 (Fig. 5; Table S6). Scenarios 2 and 3 incorporated increased larval mortality and reduced carrying capacity due to mild (Scenario 2) or strong competition/predation (Scenario 3) following the introduction of *L. gibbosus*. Average population sizes declined steadily and stabilised at 921 and 473 individuals with decreased average growth rates of 0.099 and 0.095 respectively (Fig. 5A; Table S6). Ranaviruses outbreaks alone (Scenario 4) resulted in an initial sharp decrease in the average

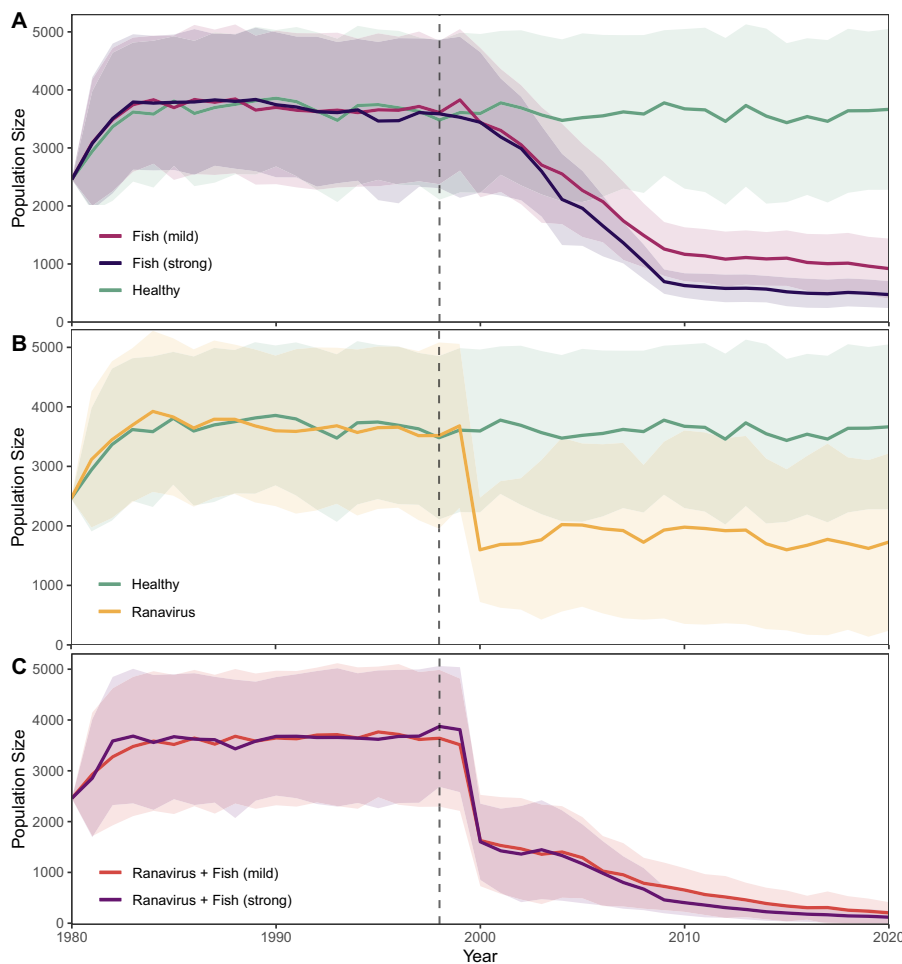


Fig. 5. Forty-year population projections of *Triturus marmoratus* in METAMODEL MANAGER under six different scenarios reflecting different effects of invasive Pumpkinseed (*L. gibbosus*) and disease. Vertical dashed line indicates both the first detected ranavirus outbreak (1998 and 18th year of the simulation) and the presumed introduction of invasive fish at Carris: **A.** Strong and mild effects of fish alone (Scenarios 2 and 3) contrasted with a healthy population (Scenario 1, no fish or *Ranavirus*); **B.** Impacts of *Ranavirus* alone (Scenario 4) contrasted with a healthy population (no fish); **C.** The cumulative effects of *Ranavirus* and fish (Scenarios 5 and 6 for mild and strong effects of fish respectively) leading to a steep and persistent decline in population size. Ribbons represent standard deviation.

population size to 1728 individuals with a lower average growth rate of 0.053 (Fig. 5B; Table S6), but the greatest, most persistent declines were observed when the amphibian population was exposed to the combined effects of *L. gibbosus* and *Rv*. The interaction of these two factors led to final average population sizes of 204 and 118 individuals with average growth rates of -0.008 and -0.015 in Scenarios 5 (mild competition/predation) and 6 (strong competition/predation) respectively (Fig. 5C; Table S6). The pattern of decline and final population size (year 40 of the simulation) in Scenario 6 of 118 ± 128 ($N \pm SD$) (already within the range of probable extinction) was closest to what was observed in the *T. marmoratus* population of Carris, with an estimated population size of 55 individuals in 2020 (after correcting for sampling effort).

The probability of extinction was significantly different across the scenarios for the 40-year (Friedman $\chi^2 = 41.6$; $df = 5$; $p < 0.0001$) as well as for the 100-year projections (Friedman $\chi^2 = 318$; $df = 5$; $p < 0.0001$). Pairwise comparisons were non-significant for 40-year population projections but showed that the proportion of extant populations per year was significantly different across all scenarios except between Scenarios 5 and 6 (Table S7) for 100-year projections. Thus, although the probability of extinction was higher in models with strong fish impact than with mild fish impact (and no disease) there was no significant difference in extinction probability between models when the disease is combined with impacts of invasive fish (Table S7).

3.5. Modelling the impacts of fish removal

Removing the effects of invasive fish (competition, predation) today resulted in the persistence of the *T. marmoratus* population (Scenario 7; Fig. 6). When projected over 100 years, the population had an average

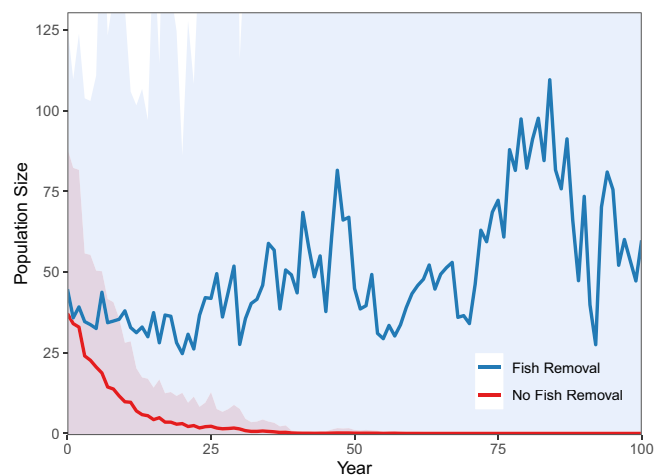


Fig. 6. Hundred-year population projections of *Triturus marmoratus* in METAMODEL MANAGER under two different scenarios. The population persists, but with erratic oscillations, when invasive fish (*Lepomis gibbosus*) is removed from a simulation with strong effects of fish and *Ranavirus*; no removal of fish leads to host population extinction. Year “0” of the simulation corresponds to 2020. Ribbons represent standard deviation.

growth rate of -0.029 ± 0.720 (SD) with a final population size of 60 ± 326.3 (SD) individuals. Yet, the probability of extinction never exceeded 0.07, overall averaging 0.0065 ± 0.0045 mean SE. This was compared to Scenario 6 with sustained strong impacts of fish and disease (starting

population of ≤ 50 individuals), which had a significantly higher probability of extinction (Friedman $\chi^2 = 58.7$; $df = 2$; $p = 0.0001$) with a lower average growth rate of -0.060 ± 0.271 and certain extinction (Fig. 6).

4. Discussion

Some important wildlife population declines remain enigmatic (e.g., Blackburn et al., 2010; Caruso and Lips, 2013; Lukoschek et al., 2013; Blake and Loisel, 2015). Yet, for others, it is now known that multiple causal factors coincide (e.g., Daszak et al., 2001; Wake and Vredenburg, 2008; Zhan et al., 2018). This study illustrates how the introduction of an invasive species very probably disrupted a stable host-pathogen system leading to a steep decline of the host. Such studies are scarce and often limited to speculation about the origin of the pathogen and possible outcomes.

4.1. From enzootic to epizootic and back: host-pathogen co-existence

The historical detection of Rv in our study system suggests the endemic status of the pathogen. Moreover, all Rv strains currently found were detected in multiple hosts in the 1980s, well before the first outbreaks. Historical pathogen presence was accompanied by a relatively low prevalence pattern, which is characteristic of an enzootic disease system. CMTV-like strains, among the most virulent of the genus (see Balseiro et al., 2009; Price et al., 2014; Rosa et al., 2017), were dominant in the system. Despite the limited resolution, our phylogeny implies the presence of a strain closely related to the highly virulent PNTRV, linked to the community collapses described for Serra da Estrela, Portugal (Rosa et al., 2017). However, the presence of all the strains with no detected mortality events up until the late 1990s suggests that it was a stable host-pathogen system (DiRenzo et al., 2018), providing additional support to the 'endemic pathogen hypothesis' (Rachowicz et al., 2005).

Mass mortality was accompanied by a sharp escalation in the prevalence of infection, both characteristic of an epizootic disease (Ostfeld et al., 2008). Additionally, sex ratio reversal has also been noted in newt populations affected by ranavirosis (Rosa et al., 2019). While adult newts start leaving the pond after breeding, females usually become more abundant than males towards the end of the aquatic season (Caetano et al., 1985; Arntzen, 2002; and see also Díaz-Paniagua, 1998), which could explain disproportional mortality affecting females. The steep decline in the abundance of *T. marmoratus* following the ranavirosis outbreaks seems transversal to the whole amphibian community, where previously abundant species (Almaça et al., 1976; Caetano et al., 1979, 1985) are now undetected or sporadically observed. This is not surprising, considering the high susceptibility of these species across all life stages to the particularly virulent CMTV-like ranaviruses (Rosa et al., 2017). Despite the prevalence of infection being closer to historical levels, mortality currently still occurs with no signs of population recovery, which suggests the presence of additional stressor(s) preventing host recovery. Multi-stressor studies and theoretical models have predicted reduced population viability in such cases (e.g. Bårdsen et al., 2018; Cervin et al., 2020).

4.2. Invasive fish as a disruptor of host-pathogen balance

The enzootic nature of the Rv infection during the 1980s suggests that invasive *L. gibbosus* may have been responsible for changing host-Ranavirus dynamics, leading to the outbreaks. Previous studies indicate that the presence of a potential predator (i.e., an additional stressor) leads to weakened host immune system and increased disease susceptibility (Carey et al., 1999; Hayes et al., 2010). Both the sighting of predators (Narayan et al., 2013) and the aggressive nature of *L. gibbosus* (inducing changes in newt foraging behaviour; Winandy and Denoël, 2015) may lead to increased levels of stress (Warne et al., 2011). These may cause stable host-pathogen systems to fall out of equilibrium

(Rollins-Smith and Woodhams, 2012). This scenario seemed consistent across all sites where outbreaks of ranavirosis (with subsequent declines) were recorded (i.e., Lagoa dos Carris, Lagoa do Batateiro, Lagoa da Peneda), following the introduction of non-native fish.

The role of invasive species in disease is often limited to their function as vectors. This was the case of the local disappearance of the frog *Atelognathus patagonicus* following the detection of both Rv and the invasive fish *Pericichthys colhuapiensis* (Fox et al., 2006). Here we showed that the outbreaks of ranavirosis overlapped with the introduction of invasive fish but were not linked to an increase in Rv diversity, ruling out fish as the responsible for the pathogen introduction. Moreover, while pumpkinseed was recorded with an amphibian-associated Rv (FV3 clade), no strains typically infecting fish were detected (mostly in the ATV clade; Duffus et al., 2015).

4.3. Invasive species and disease exert a cumulative effect on host populations

Our study supports the hypothesis that the decline in the *T. marmoratus* population was due to the cumulative effects of invasive fish and Rv. The pattern of steep decline was best captured when incorporating the concerted effect of disease and predation/competition from *L. gibbosus*. Even a strong-pressure scenario from the invasive fish alone would be unlikely to cause such a devastating impact (e.g., Préau et al., 2017).

Lepomis gibbosus may still play a role in escalating disease by acting as an alternative host for Rv, causing 'spill back' to the native amphibian community. This is supported by their population increase over the last two decades (authors' personal observation) together with a lack of mortality and/or ranavirosis, allowing the pathogen to persist regardless of amphibian densities. Experimental work has shown Rv transmission between vertebrate classes (Brenes et al., 2014), which supports this hypothesis. Likewise, our observations suggest transmission may happen in both directions: while *L. gibbosus* diet mainly consists of macroinvertebrates (Godinho and Ferreira, 2014) with adult fish having difficulties in preying on adult amphibians due to gape limitations (feeding preferably on amphibian eggs and larvae; Hartel et al., 2007), adult *T. marmoratus* have been recorded regurgitating juvenile fish on multiple occasions (author's personal observation).

4.4. Invasive species removal reduces extinction risk in the presence of disease

Ranavirus has high environmental persistence (Brunner and Yarber, 2018) and successful treatment in amphibians has not been recorded (O'Rourke and Rosenbaum, 2015). On the other hand, invasive fish removal from lakes is a practical conservation strategy that can lead to the recovery of newt populations (Denoël and Winandy, 2015). Our modelling approach suggests that this may be true even in multi-stressor systems; the removal of invasive fish from the system when the host population reaches a critically low density can still be a viable way to preserve the population, especially if the amphibian immune system is able to recover. Despite ranaviruses meeting the conditions required to cause host extinction (Miller et al., 2011; Earl and Gray, 2014), recovery after declines due to ranavirosis can still occur (Greer et al., 2008; Rosa et al., 2019). This strengthens the hypothesis that amphibian populations may be able to afford recurring disease-driven mortality and morbidity in the absence of additional environmental challenges (Carey et al., 2003; Brunner et al., 2004).

The fate of amphibian populations afflicted by ranavirosis is variable with some populations either recovering or maintaining population size after outbreaks and others going extinct (Teacher et al., 2010; Sutton et al., 2015; Rosa et al., 2017). This study provides support to the hypothesis that, in addition to pathogen genotype (Price et al., 2014), additional anthropogenic stressors, such as the introduction of invasive species, may be contributing to the extirpation of systems afflicted by

Ranavirus (Teacher et al., 2010; North et al., 2015).

4.5. Final considerations

Our study provides robust evidence that invasive species can impact naïve communities not just through direct competition/predation or the introduction of new pathogens, but also by disrupting host-pathogen equilibria, exacerbating health threats. This contrasts with the general perception of human-mediated spread as the main cause of ranaviruses emergence (Jancovich et al., 2005) and is an equally likely hypothesis in many multi-stressor interaction systems.

Sudden and, at times, catastrophic emergences of ranavirus disease in European amphibian populations were consistent with recent introductions of novel viruses (Price et al., 2017a). Increasingly, however, phylogenetic evidence suggests that the CMTV group of ranaviruses may have originated in Europe and have an ancient association with its amphibians (Storfer et al., 2007; Mavian et al., 2012; Price et al., 2017b). Our results support this view while emphasizing the importance of exploring the historical interactions between multiple stressors to understand population decline in a complex host-pathogen-invasive species system.

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CRediT authorship contribution statement

GMR designed the study, led the data collection and compilation. GMR, GAB, JSA, MH, EF, AL, CS performed sampling. GMR, GAB, JSA, WTML, APAM, MFC, SJP carried out molecular screening. SJP, GAB, WTML performed sequencing. CO, SJP carried out phylogenetic analyses and advised on interpretation. ATM, GMR performed statistical analyses and modelling. GMR wrote the manuscript with input from all authors. All authors gave final approval for publication. The authors declare that they have no competing interests.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Any computer code used to generate results reported in the manuscript as well as raw data that support the findings of this study are available on request from the corresponding author, without undue reservation. Additional detailed data are available in Supplementary Material (SM). DNA sequences are deposited in the GenBank database (accession numbers available in SM).

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Appendix A. Supplementary Material

Supplementary Material (SM) to this article can be found online at <https://doi.org/10.1016/j.biocon.2022.109785>.

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