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Rising herpesvirus infections in a region with enigmatic declines of Common Toads (*Bufo bufo*)

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Abstract. Emerging infectious diseases are one of the major drivers of the current worldwide amphibian decline. Bufonid herpesvirus 1 (BfHV1) was recently described in 2018 in Switzerland following a mass-mortality episode in one Common Toad (*Bufo bufo*) population. Since then, the disease has been reported in the UK, Luxembourg and Germany. We studied the potential role of the emergence of BfHV1 in widespread, enigmatic toad declines in Belgium. During the spring migration of 2024 we compared body condition and herpesvirus compatible lesions in live Common Toads from 10 declining and 10 non-declining populations. We then compared BfHV1 infection prevalence in livers from road killed toads collected in 2011 and 2024. The proportion of populations with BfHV1 significantly increased between 2011 and 2014, with 19 out of 20 populations positive in 2024. Although no correlation was observed between viral presence and toad body condition or population status, the simultaneous rise in infections alongside declining toad population trends in the study region does not exclude herpesvirus as a potential contributor to toad declines.

Key words. Amphibia, Anura, Bufonidae, herpesvirus 1, emerging infectious diseases, population decline, disease prevalence, amphibian decline.

Introduction

Described for the first time by Origgi et al. (2018), the bufonid herpesvirus 1 (BfHV1) is a recently discovered pathogen affecting the Common Toad, *Bufo bufo* (Linnaeus, 1758) and likely *Bufo spinosus* Daudin, 1803 as well (Böning et al. 2025). The disease is caused by a herpesvirus belonging to the family of Alloherpesviridae, and phylogenetic analysis positions it in the *Batrachovirus* genus (Origgi et al. 2018, Boutier et al. 2021). Clinical signs include the manifestation of gross black-brownish skin lesions, sometimes accompanied by coalescing skin proliferations (Origgi et al. 2018). The lesions manifest usually on the back of the toads or on the belly, and can appear as small dots, spots or even as extensive patches (Origgi et al. 2021) (Supplementary material, Fig. 1). Sometimes, the lesions can scab and crack, leaving the flesh of the animal exposed and prone to secondary infections that can easily enter the bloodstream (Origgi et al. 2018). The migrating behavior of the toads and their mating strategy may play a key role in the transmission of the disease (Miller et al. 2011), as it is not unusual to witness males grappling each other (Marco & Linzana 2002), trying to get to females. This close contact facilitates the spreading of the pathogens (Brunner et al. 2007, Varga et al. 2019), especially those present on the skin (Brenes et al. 2014). In addition, toads undertake the migration shortly after coming out of hibernation (Peer et al. 2021, Blomme et al. 2025), which may leave them in a weakened state (Reading & Clarke 1995) and more prone to contract infections. Detected in Switzerland, the UK, Luxembourg and in Germany as well (Origgi et al. 2018, Eisenberg et al. 2021, Böning et al. 2025, Leineweber et al. 2025), sightings from volunteers involved in monitoring and toad crossing activities suggest that clinical signs consistent with BfHV1 may have appeared as early as 2014.

Common Toad populations are currently undergoing a slow but steady decline (Petrovan & Schmidt 2016, Herder 2021), which commenced around 20 years ago (Carrier & Beebee 2003, Bonardi et al. 2011). This negative trend is likely caused by a combination of environmental and anthropic factors, like habitat fragmentation, pollu-

tion, land management and pathogens (Beebee & Griffiths 2005, Bosch et al. 2018, Campbell Grant et al. 2020). However, while the prevalence and distribution of *Batrachochytrium dendrobatidis* and ranid herpesvirus 3 (RaHV3) in Common Toads are relatively well studied (Spitzen-van der Sluijs et al. 2010, Martel et al. 2012), little is known about BfHV1 in Belgium. This lack of information, coupled with the decline of a species so common and widespread warrants for more research and an in-depth analysis of the prevalence of the bufonid herpesvirus across the region of Flanders (Belgium) and its impact on the Common Toad's populations. While in Switzerland the disease was linked to a mass mortality episode (Origgi et al. 2018), no other similar events have been reported in Belgium or other countries where clinical signs consistent with herpesvirosis have been observed. Moreover, while some preliminary reports suggest that high BfHV1prevalence could be tied to the decline of toad populations, no strong evidence of this has been found yet (Origgi et al. 2018).

In this study, we aim to clarify the impact of BfHV1 and assess its potential role in Common Toad population declines. We compared health characteristics and herpesvirus infection in toads from 20 breeding sites across Flanders in 2024, 10 with stable or increasing population trends and 10 experiencing declines (Blomme et al., in press). To estimate whether prevalence of BfHV1 has recently emerged or is increasing, we analysed toad samples from 2011 for the presence of the virus.

Materials and methods

Sites selection and locations across Flanders for the 2024 sampling

Based on a previous analysis conducted by Blomme et al. (in press), 20 ponds with known toad population dynamics were selected from over more than 100 locations with at least 10 years of historical data (represented on Fig. 1). Among these twenty ponds, ten had an overall decreasing trend (declining) while the other ten either had a stable or increasing population trend (non-declining). Only ponds for which no obvious reason for decline was present (e.g. loss of breeding habitat, migration routes blocked) were included in this study. A preliminary exploration of the selected sites was undertaken prior to the beginning of the field season (January 2024) to ensure that the ponds were accessible and to ascertain the general conditions of the surroundings. One additional pond was also sampled during the field season. However, due to the lack of historical data which made it impossible to ascertain its population status, it was excluded from most of the analyses. The samples from this pond were only used in determining prevalence of BfHV1in the population, as well as to test its connection with morphometrics factors like scaled mass index (SMI) and snout vent length (SVL) (Halliday & Verrell 1988, Lunghi et al. 2020).

Sample collection for the 2024 fieldwork

Fieldwork was conducted between the beginning of February 2024 and the first half of March 2024, during the peak of the annual toad migration. For every location, we aimed to sample at least 30 adult individuals. Toads were collected by volunteers using provided nitrile gloves and placed individually into pre-emptively prepared plastic containers for no longer than 2 hours before the sampling. All individuals were sexed, weighed and scaled pictures of both the belly and the back were taken. During the same period and after the toad migration peak, 191 road-killed toads found in the vicinity of the selected pond were collected and stored at -80 °C until further processing. Scaled pictures were taken of the back of the dead toads. When the condition of the

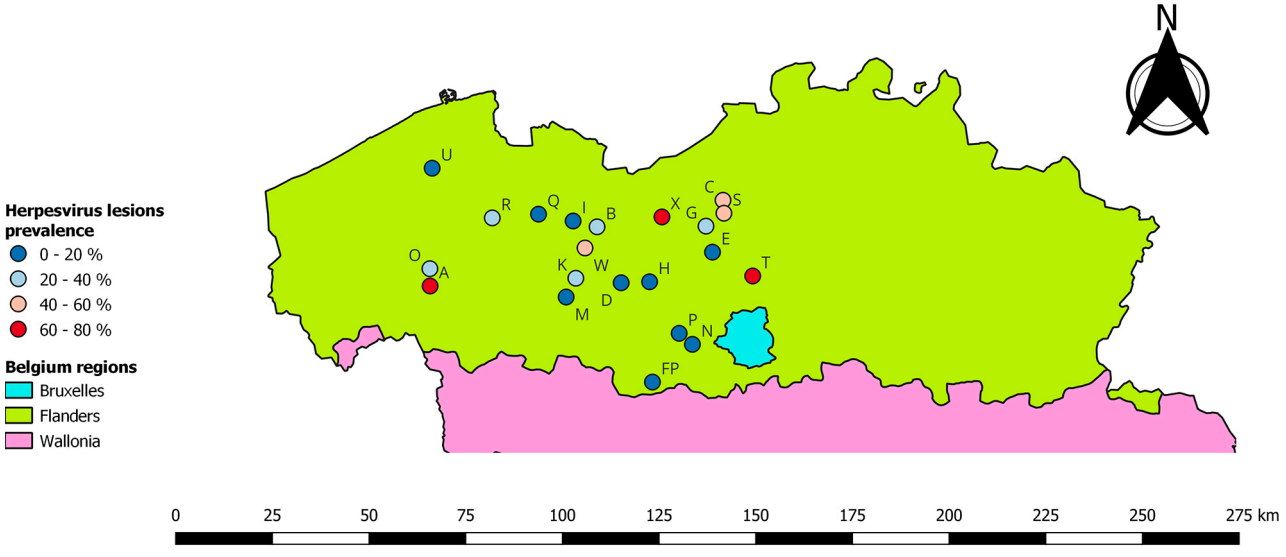

Figure 1. Geographical distribution and prevalence of skin anomalies consistent with herpesvirosis in living toads in 2024.

carcass allowed it, liver samples were collected from every individual for pathogen detection and stored at -20 °C for further processing. Due to the conditions of the majority of the carcasses it was impossible to visually detect any lesion on the skin of the road-killed toads.

Pictures processing and SMI calculation

All pictures collected during the field season were assessed for lesions compatible with herpesvirus infection by a blinded observer and toads were measured using the software ImageJ (Schneider et al. 2012). An individual was considered "presumably infected" if one or more pictures showed any gross lesions of herpesvirus infection, as shown in Supplemetary Figure 1. The SMI was chosen as a proxy for the body condition of the individuals measured in the field (Peig & Green 2010). SMI was calculated as described by Peig & Green (2009). To calculate the SMI, only males where included when calculating the coefficient as females might have skewed the measure depending on the amount of eggs they contained. SVL was also measured using the ImageJ software and used as a proxy for age (Lunghi et al. 2020)

PCR for bufonid herpesvirus 1(BfHV1)

DNA from the liver samples collected from the road-killed toads was extracted following the protocol's instructions present in the Isolate II Genomic DNA kit from Meridian Bioscience®. Tissue samples were first cut into smaller pieces (~ 25 mg), to ensure that the same amount of tissue was processed for each individual. During the same passage, the tissue samples were also partially homogenized by using a scalpel blade. After that, samples were processed according to the manufacturer protocol for cultured cells and human or animal tissue. The only adjustment implemented was to leave the sample to lysate overnight instead of the 1–3 hours recommended at the pre-lysis step.

Based on the work of Origgi et al. (2018) we used a PCR protocol specific to the DNA polymerase gene of the BfHV1. We used two primers with the following composition: BfHV-1F: CAAACTGAAGATACAGAAGGTTGGGG; BfHV-1R: GCGCGAGTGCTTTGTACGCAACC. The samples were then run on a PCR mastercycler using the following amplification program: 3' at 94 °C, followed by 40 cycles of 30" at 95 °C, 30" at 55 °C and 30" at 72 °C; this was followed by 10' at 72 °C and finally the samples were kept at a stable temperature of 15 °C until stored away or ready to be used.

Visual detection rate

Based on the data collected, we formulated the index "visual detection rate" (VDR). It was defined as the proportion of sampled live animals with gross skin lesions consistent with herpesvirosis, relative to the proportion of dead animals (road-killed) from the same location that tested PCR-positive. This measure provides an index of how often infection in the population is clinically apparent, assuming that PCR prevalence in dead animals approximates infection prevalence in the population.

Analysis of toad samples, collected in 2011

We tested samples, collected in a previous study (Martel et al. in 2011), for BfHV1. Carcasses from road-killed toads were collected during the 2011 spring migration from 97 sites across all five Flemish provinces. Six sites were re-included in the sampling effort of 2024. All liver samples were tested for BfHV1 using the PCR described above.

Statistics

All statistical analysis were performed using the statistical software R (version 4.3.2) (R Core team 2023) and all graphs were realized using either the ggplot2 R package (Wickham 2016). Models' assumptions were checked using the R package 'performance'. We considered a test statistically significant when the p-value was below the significance level of 0.05. Confidence intervals for prevalence values were calculated using Wilson score confidence interval with a 95% confidence (Wilson 1927, Newcombe 1998).

To investigate the relationship between the prevalence of gross lesions in living toads and the populations trend, we employed a Fisher's Exact Test for Count Data. To inspect the association between the BfHV1 infection and SMI, statistical analysis were performed using a linear mixed model (lmm) (formula: lmer(log(SMI) ~ herpes + (1 | label)), adding the sampling location as a random effect. All the assumptions were checked and the log transformation applied to the SMI values. For the SMI we restricted the dataset to only male individuals to exclude any confounding of the presence of eggs in females. Similarly, we tested whether SVL, a measure employed as a proxy for age class in other amphibians (Halliday & Verrell 1988, Lunghi et al. 2020) was correlated with the sex and the presence of BfHV1 using a glmm with a binomial distribution (formula: glmer(herpes ~ SVL × sex + (1 | label)), again using the sampling location as the random effect. A generalized linear model was also to investigate both the E–W gradient and the N–S gradient in relation to the infection prevalence (formula: glm(fraction ~ lat + lon)). Finally, a linear model was used to check for any correlation between the presence/absence of BfHV1 in the sampled locations between the years (formula: glm(presence ~ as.factor(year)), and another linear model was employed to test for differences in BfHV1 prevalence between 2011 and 2024 (formula: glm(tot.pos/tot.buf~ as.factor(year)), setting a threshold to select locations with at least 14 sampled individuals (Vittinghoff & McCulloch 2007, Riley et al. 2019).

Relatively to the VDR, after checking the groups for normality using Shapiro test (Shapiro & Wilk 1965) as well as the heteroscedasticity, linearity and independence, a linear model (lm) (formula: lm (visual_detection_rate ~ trend)) was performed to determine whether populations in decline are characterized by a higher VDR, reflecting a larger fraction of infected animals with visible clinical signs.

Results

High prevalence of herpesvirus associated skin lesions in living toads across Flanders in 2024

A total of 526 living toads (186 females, 340 males) were sampled across 20 locations in 2024. Not all locations had 30 individuals sampled at the end of the season, with 5 out of 20 having a number of sampled toads ranging from 12 to 18 (Supplementary material Table 1). An additional 125 living toads were sampled in an additional location (labeled "FP" in Supplementary material tables; Fig. 1) where no population status data were available. Visual inspection for external herpesvirus-associated gross lesions suggested a high prevalence of bufonid herpesvirus in 19/20 locations across Flanders, with a mean prevalence of 28.3% (CI 19.0–37.7%, ranging from 9% to 69%; Fig. 2). Lesions were absent in only one location, where the sample size was limited to 16 animals compared to the target of 30. Of the 125 toads from the additional location, eleven toads presented gross herpesvirus lesions (8.9% prevalence). No geographical pattern was visually detected, as presented in Figure 1, but statistical analysis detected an impact of latitude (estimate 3.136; SE 0.727; p-value < 0.001) and longitude (estimate -0.728; SE 0.255; p-value 0.004) in the BfHV1 distribution. This shows a spatial gradient in the prevalence of BfHV1 compatible lesions across sampled sites, which increases with latitude and decreases with longitude, thus indicating a higher prevalence of BfHV1 in the North-West of Flanders. Fisher's Exact Test showed no relationship between the prevalence of gross lesions suggestive of BfHV1 in living toads and the population trend, with a p-value of 0.1849. Further analysis also showed no significant associations between the prevalence of BfHV1 compatible lesions and the SMI of the male individuals suggesting that the presence or absence of lesions is not associated with the body condition of the individuals (estimate 0.130 ± 0.416 SE; p-value 0.754). Results relative to the interaction between SVL, sex and BfHV1 infection showed no significant interaction (estimate 0.052 ± 0.083 SE; p-value: 0.533), nor effect of BfHV1 infection (estimate -0.016 ± 0.068 SE; p-value 0.818), but a significant effect of sex, with males having a lower SVL (estimate -0.985 ± 0.057 SE; p-value < 0.001).

Figure 2. Boxplots representing the percentage of individuals sampled in 2024 across populations showing clinical signs compatible with bufonid herpesvirus infection per population trend (non-declining or declining). The black lines represent the mean of each boxplot.

PCR results for bufonid herpesvirus 1(BfHV1)

Liver samples from 210 road-killed toads were analyzed from 13 out of the 21 locations (Supplementary material Table 2) where living toads were sampled in 2024. The numbers of individuals across locations varied significantly, with some of them yielding up to 53 toads, while others had few (1–4). Lowest numbers were available from locations with toads' populations in decline, showing a negative trend and a low population to begin with, or low traffic. All samples were tested for BfHV1 in randomized order. Overall, 47 toads (24.6%) were positive. Prevalence per location ranged from 0 to 100%, as shown in Figure 3, with an average of 36.4% (CI 17.3–55.5%) infected toads per location. The prevalence varied widely across locations. No geographical trend was detected by the aforementioned spatial model, neither for latitude (estimate 0.11987; SE

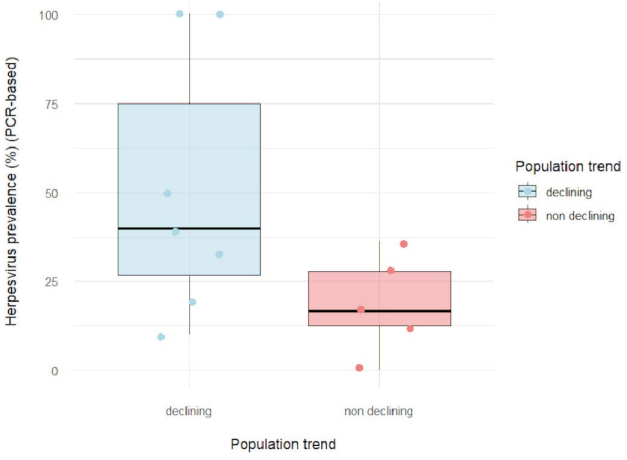

Figure 3. Boxplots representing the percentage of bufonid herpesvirus 1 PCR-positive road-killed individuals sampled across locations in 2024, grouped by population trend (declining vs. non-declining). The black lines represent the mean of each boxplot.

0.33504; p-value 0.725) nor for longitude (estimate 0.05715; SE 0.10607; p-value 0.597).

A total of 234 individuals of the 1707 toads sampled during 2011 were positive for BfHV1, with an overall mean prevalence of 13.7% (CI 12.1–15.4%) and 38 sites infected out of 97. Across provinces, the one with the highest prevalence was Antwerpen (20.9%; CI 17.4–25.0%), followed by Limburg (15.9%; CI 12.4–20.1%), West-Vlaanderen (13.5%; CI 8.6–20.5%), Oost-Vlaanderen (12.8%; CI 9.6–16.9%), and Vlaams Brabant (6.1%; CI 4.3–8.7%). We found a significant difference in the presence of herpesvirosis across the years, with BfHV1 occurring at more sites in 2024 than in 2011 (estimate 1.579 ± 0.456 SE; p-value 0.0005), and a tendency suggesting a higher prevalence of BfHV1 in 2024 (estimate 0.383 ± 0.220 SE; p-value 0.082). Regarding BfHV1 presence in the six sites sampled in both 2011 and 2024, the number of infected sites increased from two in 2011, to five in 2024, as shown in Figure 4.

Visual Detection Rate across populations was highly variable, even when discarding locations with a low number of samples (n < 4) to reduce any stochastic effect, with values ranging from 0 to 2.3 and an average rate of 0.98 ± 0.8 across 8 locations. The results (estimate 0.732 ± 0.589 SE; p-value 0.236) indicated no significant difference between trends, as shown in Figure 5.

Discussion

Previously reported in Switzerland, UK, Luxembourg and Germany (Origgi et al. 2018, Allain & Duffus 2019, Eisenberg et al. 2021), our study is the first to confirm and characterize the presence and distribution of the BfHV1in Flanders, Belgium, as suggested in Böning et al. (2025). Despite being described less than a decade ago, this pathogen appears to be widespread across Flanders. Our findings confirm that BfHV1 has been affecting Common Toad populations in Flanders for several years and its prevalence is higher than initially suspected. One of the possible reasons for this underreporting is that the virus does not appear to have an obvious negative impact on affected toads, both at the individual (obviously sick animals, besides the presence of colour anomalies) and at the population level (no mass mortality events reported).

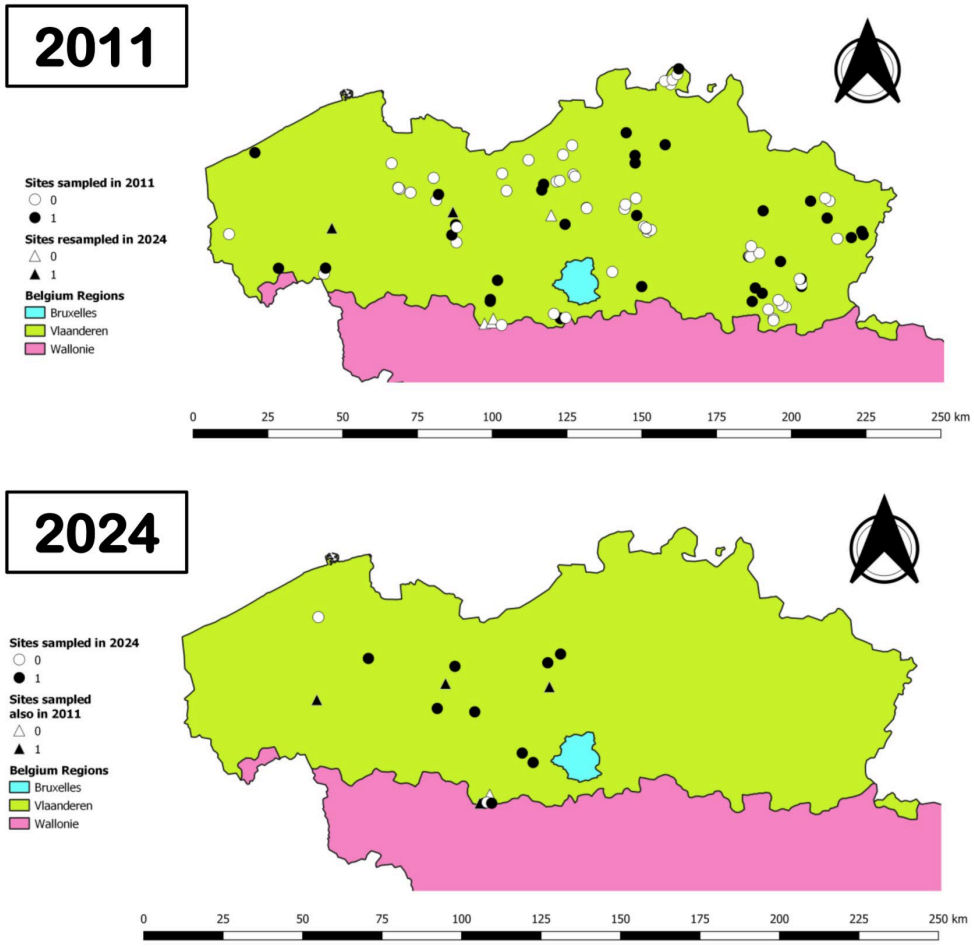

Figure 4. Geographical distribution of Bufonid herpesvirus 1 across Flanders, in 2011 (up) and 2024 (down), based on PCR analysis from road-killed individuals. White symbols represent absence of detection, black symbols detection of herpesvirus.

Nonetheless, despite the lack of an obvious adverse effect on the Common Toads, our results indicate an increase over the years in the occurrence of BfHV1 in Flanders. Over a 13-year period, the number of infected sites rose from 38 out of 97 in 2011, to 20 out of 21 in 2024, demonstrating a significant increase in the presence of the infection within the toad populations. In addition, although not statistically significant ($p = 0.082$), our results suggest a possible increase over time in the prevalence of BfHV1, indicating a tendency toward a higher proportion of infected individuals per site in 2024. Concerning the geographical distribution of the infected animals and BfHV1 prevalence, the infection appears to be widespread across Flanders. While our analysis suggests the existence of an E–W and a S–N gradient, the results are likely driven by the cluster located in the North of Flanders, as shown in Figure 1, and results may be skewed by the low number of locations. The prevalence of symptomatic animals, presenting black-brownish skin lesions, also varies greatly (average site prevalence of 28.3%; CI 19.0–37.7%). The proportion of animals carrying the virus (PCR-positive) appears to be similar, with an average of 36.4% (CI 17.3–55.5%) infected animals per site. The virus appears to affect post-metamorphic adult toads of all ages (using SVL as a proxy) and both sexes equally, while the impact on early life stages and thus recruitment remains unknown. However, if herpesvirosis significantly decreased recruitment, this should be reflected in the population dynamic. However, despite the widespread distribution and high prevalence, we did not observe any impact of the disease at population (population trend) or individual animal (health indicators) level. In fact, no significant correlations were found between BfHV1 presence and population trends, nor between BfHV1 infection and individual-level variables such as sex or body condition. While proxies for age and body condition can be useful, they may lack precision. More accurate methods, like toe-clipping for age estimation, could improve reliability but are also more invasive, time-consuming, and resource-intensive. In contrast with the mass mortality episodes reported in Switzerland (Origgi et al. 2018), no outbreaks were detected in our study region, despite the presence of an early warning system that examines amphibian die-off events. Our results suggest infections in Flanders with bufonid herpesvirus to be relatively benign, probably posing a far lower risk than for example ranavirus (Grey et al. 2009, Price et al. 2014, Rijks et al. 2016, Hartmann et al. 2022) or chytridiomycosis (Laurence et al. 1996, Bosch et al. 2001, Scheele et al. 2019). Within population transmission could be promoted during toad conservation activities such as toad patrols. During such activities, toads are collected in buckets and handled, facilitating pathogen spread. Widespread occurrence of infections in amphibians stresses the importance of biosecurity measures, also during such activities. For example, the use of materials should be restricted to a single location, unless thorough disinfection protocols are applied.

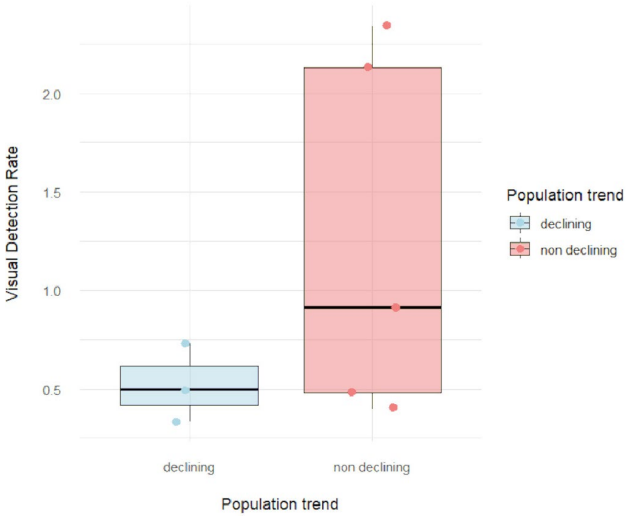

Figure 5. Boxplots representing the visual detection rate of bufonid herpesvirus across locations sampled in 2024 per population trend relative to bufonid herpesvirus 1. The visual detection rate (VDR) was defined as the proportion of live animals with gross skin lesions consistent with herpesvirosis, relative to the proportion of dead animals from the same location that tested PCR-positive. The black lines represent the mean of each boxplot.

VDRs did not differ between stable and declining populations, suggesting the proportion of clinically apparent infections is comparable across population trends. However, some caution is necessary in the interpretation of the results due to the low number of locations employed in its calculation ($n = 8$). Both the presence of visible lesions and PCR identified similar proportions of infected animals in the populations. While it is important to consider that the visual detection of lesions may not be sufficiently specific and might overlook cases where the infection is not clinically evident, skin lesions may be used as a quick and effective field technique for a presumptive herpesvirus diagnosis. The ability to estimate the occurrence of the infection in a population without the need for laboratory techniques would promote early detection.

Disease prevalence and distribution are key components to assess the impacts and effects of a pathogen on its host (Zheng 2023). This is especially true for newly discovered pathogens such as BfHV 1, where baseline data are limited. Despite the lack of obvious impact on toad individual health or population status, the marked increase of herpesvirus infections over the 13-year period and high prevalence of herpesvirus associated skin anomalies coincides with a general trend of toad declines in the study region (Blomme et al. 2025). We thus cannot exclude that BfHV1 contributes to the decline, perhaps as one of several stressors in a changing environment (Wilkinson et al. 2018, Smith et al. 2009, Brearley et al. 2013). The interplay of a widespread herpesvirus infection with pollutants, a shifting climate, and fragmented and altered habitats could produce a cumulative impact, amplifying the decline of this species.

Acknowledgments

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Supplementary data

The following data are available online:

Supplementary Figure 1. Picture collected in Flanders during spring 2024 of the back of a toad heavily infected by bufonid herpesvirus 1 showing gross black-brownish lesions.

Supplementary Table 1. Fieldwork results of living toad sampling during spring 2024.

Supplementary Table 2. Labwork results of PCR conducted on liver samples from road-killed toads collected during spring 2024.

Supplementary Table 3. Visual detection rate (VDR) of bufonid herpesvirus 1 across locations sampled during spring 2024.