Course of an Isolated Ranavirus Outbreak in a *Pelobates fuscus* Population in The Netherlands

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Abstract: Viruses in the genus *Ranavirus* (family *Iridoviridae*) are known to have the potential to adversely affect fish, amphibians, and reptiles. Ranaviruses are associated with large-scale die-offs and rapid population declines in amphibians. The development and progression of an outbreak, however, vary greatly depending on the host species and geographic location. We describe the recurrent course of an outbreak of common midwife toad virus in an isolated population (Staphorst) of common spadefoot toads (*Pelobates fuscus*) in The Netherlands from 2012 to 2015. After initial mass mortality of toad larvae in 2012, no mass mortality was recorded in 2013 and 2014. In 2015, however, a recurrent outbreak of the virus is believed to have caused high mortality rates among this species in the Staphorst population.

Key Words: amphibian, common midwife toad virus, *Pelobates fuscus*, population dynamics, ranavirosis.

Ranaviruses are an emerging group of viruses that can cause infections in amphibian, fish, and reptile species (Gray and Chinchar, 2015). Generally, die-offs due to ranavirus progress rapidly, causing high initial mortality rates. The outcomes of ranavirus epidemics, however, vary greatly between species, populations, and geographical locations (Brunner *et al*., 2015). The knowledge on the course of a ranavirus-induced mass mortality event is valuable for the assessment of disease-related effects on wildlife populations, which can be considerable (Price *et al*., 2014). However, few longitudinal studies on populations with recurring ranavirus-associated die-offs have been published (Gray and Chinchar, 2015). The first outbreak of ranavirus in The Netherlands occurred in 2010 in Dwingelderveld National Park when thousands of amphibians died as a result of an outbreak of common midwife toad virus (CMTV) (Kik *et al*., 2011). Geographically, this first outbreak site is relatively close (~20 km in a straight line; 52°47′22.61″N, 6°22′09.82″E) to one of the only 41 remaining Dutch populations of the common spadefoot toads (*Pelobates fuscus*), namely, the Staphorst population (52°37′28.61″N, 6°16′23.61″E). The common spadefoot toad is one of The Netherlands' rarest amphibian species and is listed as "threatened" on the International Union for Conservation of Nature's Red List of Threatened Species. Between 1950 and 2006, the distribution of common spadefoot toads has decreased in area by 74%. The most important reasons identified previously for this decrease were the changes in land use through which both the aquatic and terrestrial habitat of the species were destroyed (Van Delft *et al*., 2007).

In June and July 2012, an outbreak of CMTV-like ranavirus occurred at this Staphorst site, killing hundreds of common spadefoot toad larvae. In this case report, we describe the patterns of disease development during the 2012–2015 epidemic and its impacts on an isolated population.

The distribution of common spadefoot toads in The Netherlands is restricted to Pleistocene sandy soils. The Netherlands forms the westernmost distribution range for this species. From October to April the species hibernates underground and reproduces mainly in April and May in relatively eutrophic water bodies. In 2010, 71% of the remaining populations consisted of choruses smaller than 10 individuals; therefore, a reintroduction and restocking program commenced, combined with the improvement of the species' habitat (Bosman *et al*., 2010).

The focus of our study is an isolated population of common spadefoot toads at Staphorst that can only reproduce in a single water body (surface 0.012 km^2) with a sloping sandy shore. The water is 2.5 m at its maximal depth and is used as a recreational

swimming pool during summer. Because of the excessive growth of waterweeds (*Elodea* spp.), every 2 years the water body is vigorously cleaned. In the autumn all of the water is pumped out, allowing the removal of vegetation. The water is left to sink in nearby grasslands or pumped into a nearby ditch. Water quality is measured nine times a year during the summer by the local water board to ensure the water quality complies with swimming water standards. Tests include *Escherichia coli* and intestinal enterococci testing and measuring temperature, acidity, conductivity, and oxygen levels. Other amphibian species present that are currently not being monitored include smooth newts (*Lissotriton vulgaris*), common frogs (*Rana temporaria*), common toads (*Bufo bufo*), and water frogs (*Pelophylax* spp.).

The common spadefoot toad population has been monitored annually since 2009 within the Network Ecological Monitoring framework (Goverse *et al*., 2006). Counts were conducted according to the nationally standardized protocol of noting the number of vocalizing adults (Groenveld *et al*., 2011). In addition, all sightings of subadults and adults were recorded (see Supplementary Material). Monitoring occurred by visiting the site between one and five times per year (Table 1). The common spadefoot toad is difficult to detect because both sexes call for mates under water; hence, a hydrophone (DolphinEar DE200) was used from 2010 onward to detect their underwater choruses.

We recorded the number of living and dead amphibians. We were only permitted to bring the dead animals to the laboratory (permit FF/75A/2008/075) to undergo necropsy and be tested for ranavirus infection as described by Kik *et al*. (2011). The findings upon external inspection of live amphibians ranged from no evident cutaneous lesions to severe hemorrhages, with or without signs of emaciation and lethargy (Duffus and Cunningham, 2010).

Ranavirus infection was confirmed by histological examination and polymerase chain reaction (PCR) with primers for the major capsid protein gene (MCP) as described by Mao *et al*. (1997). The PCR was performed on extracted DNA samples from the liver in adults and subadults (Martel *et al*., 2013). Extracted DNA samples from larvae were obtained from one half of a longitudinally sliced larva. The other half was used for histological examination. The PCR yielded a product of approximately 500 base pairs, consistent with a portion of the MCP. Sequencing of this product from one of the specimens was done on both ends by using the Sanger method. After the sequences were analyzed, the primers were trimmed with the aid of Lasergene Core Suite 9.1. Then, the sequence was blasted in GenBank (Megablast) and revealed a 100% identity with the sequence of CMTV-NL (GenBank accession KP056312; Van Beurden *et al*., 2014). The histopathological criteria for a positive diagnosis of ranavirus included the presence of characteristic intracytoplasmic basophilic inclusion bodies in multiple organs (liver, kidney, spleen, intestine, and others) associated with severe areas of necrosis.

To minimize the chances of pathogen transmission by researchers between study sites, we handled animals while wearing a fresh pair of nonpowdered, disposable, vinyl gloves. In addition, equipment and field clothing were cleaned and disinfected between the sampling locations with a broad spectrum disinfectant (Virkon®S, potassium peroxymonosulfate, 1% solution) according to the manufacturer's guidelines (DuPont Animal Health Solutions, Hertfordshire, U.K.) (Bryan *et al*., 2009).

Table 1. Monitoring results per year, indicated as the maximum number of calls heard from common spadefoot toads (*Pelobates fuscus*) per year per site visit and the mean and range of the number of common spadefoot toads heard per visit.

Year	No. of site visits	Maximum no. calling toads/visit	Mean no. (range) toads/visit
2009	1	1	NA
2010[H]	1	12	NA
2011[H]	1	6	NA
2012[H]*	3	5	4.7 (4–5)
2013[H]	4	15	9.3 (1–15)
2014[H]	4	20	12 (3–20)
2015[H]	5	20	10 (3–20)

NA = not applicable.
* year of the initial ranavirus outbreak.
[H] listened with a hydrophone.

Monitoring intensity and methods were inconsistent between the years, thereby preventing the calculation of a trend for this population, but in the period 2009–2015 (Table 1) no obvious decline was observed in the maximum number of calling common spadefoot toads. The die-off was first noted on 23 June 2012, affecting hundreds of common spadefoot toad larvae in the late developmental stages, over a short time span of approximately 3 wk (Table 2). In the period preceding the outbreak, the site was visited two to three times per week, thus the start of the outbreak is known with great certainty (see Supplementary Material). The dead common spadefoot toads were not only sighted on the shores but also observed floating in the middle of the water body as described by the public.

On 25 June 2012 hundreds of sick or dead common spadefoot toad larvae were found. Twenty common spadefoot toad larvae and four smooth newt larvae were collected for further analysis. Simultaneously, approximately 60 healthy-looking common spadefoot toad larvae were seen, and one larva had focal hemorrhages on the tail. All 24 animals were inspected for ranavirus infection, both by PCR and histological examination. From these specimens, 19 (3 smooth newts and 16 common spadefoot toads) tested positive for the histological examination (n = 10), the PCR analysis (n = 14), or both (n = 6). On follow-up visits, vast numbers of dead common spadefoot toad larvae were found, as well as live larvae with hemorrhages, and a single dead adult smooth newt (Table 2). None of these specimens were collected. On 18 August 2012, the last visit took place, and no dead or living amphibians with external signs were sighted.

No dead amphibians were reported in 2013. During the periodic maintenance work at the pond in 2014, a single dead adult smooth newt was found with ranavirus infection by histological examination and PCR. Upon sequencing of the product, we identified the virus as CMTV-NL. In April and May 2015 (see Supplemental Material), no animals with signs of disease were seen during the site visitations. On 22 June 2015, two common spadefoot toad larvae with hemorrhages and one larva that was swimming erratically were sighted. Two days later CMTV-NL

Table 2. Timeline of ranavirus monitoring 2009–2015.

				Larvae					Adults and subadults				
Year	Month	nr site visits	Species	Dead with lesions[a]	Alive with clinical signs	Alive without clinical signs	nr cmtv+	nr collected	Dead with lesions[a]	Alive with clinical signs	Alive without clinical signs	nr cmtv+	nr collected
2009	April	1	Pf	0	0	0	-	-	0	0	1	-	-
2010	April	1	Pf	0	0	0	-	-	0	0	12	-	-
2011	April	1	Pf	0	0	0	-	-	0	0	6	-	-
2012	March	2	Pf	0	0	0	-	-	0	0	5	-	-
	April	1	Pf	0	0	0	-	-	0	0	5	-	-
	June[b]	3	Pf;Lv	>200;4	1;0	60;0	16;3	20;4	0;0	0;0	0;0	-	-
	July	2	Pf;Lv	~250;0	~80;0	46;0	-	-	0;1	0;0	0;0	-	-
2013	April	3	Pf	0	0	0	-	-	0	0	15	-	-
	May	1	Pf	0	0	0	-	-	0	0	6	-	-
2014	April	3	Pf	0	0	0	-	-	0	0	20	-	-
	May	1	Pf	0	0	0	-	-	0	0	5	-	-
	October	1	Lv	0	0	0	-	-	1	0	0	1	1
2015	April	3	Pf	0	0	0	-	-	0	0	20	-	-
	May	3	Pf	0	0	0	-	-	0	0	7	-	-
	June	5	Pf;Lv	61;4	19;0	13;0	5;4	5;4	1;1	0;0	5;0	1;1	1;1
	July	4	Pf	12	8	20	-	-	0	0	13	-	-
	August	4	Pf	0	2	13	-	-	0	0	17	-	-

The number of site visits per month per year are given. Pf = *Pelobates fuscus* (common spadefoot toad), Lv = *Lissotriton vulgaris* (smooth newt). The numbers of sighted larvae and adults/subadults are presented per category (dead specimens with lesions; alive specimens with clinical signs and alive specimens without clinical signs). The number of alive sighted adults and subadults totals the maximum number of adults/subadults seen or heard per monitoring moment in that month to prevent double countings. Nr cmtv+ = the number of specimens that tested positive for ranavirus; nr collected = total number of collected dead specimens. A hyphen (-) means no animals were collected, hence no ranavirus was found. More detailed information can be found in the Supplementary Material.

[a]Sighted lesions and clinical signs consisted of focal hemorrhages.

[b]The first report of dead common spadefoot toads dates from 23 June 2012.

was diagnosed in an adult and four smooth newt larvae, and another few days later on 29 June 2015, 60 dead common spadefoot toad larvae and a dead adult common spadefoot toad were found. Five larvae and the adult were collected and all tested positive for ranavirus (by both PCR and histology). Simultaneously, 24 living common spadefoot toad larvae were sighted, 12 of which showed multiple hemorrhages. In July and August 2015, several site visits were made. In this period 12 dead common spadefoot toad larvae, 33 living common spadefoot toad larvae with clinical signs, and 10 recently metamorphosed common spadefoot toads that seemed to be in good health were sighted. None were collected (see Supplemental Material). The observed clinical signs were variable, but similar to those described in Duffus and Cunningham (2010), Kik *et al.* (2011), and Miller *et al*. (2011) and included multiple hemorrhages in metamorphosed individuals on their legs, eardrums, mouth, and ventrum and on the tail and ventrum of larvae. Animals were also found to be lethargic and emaciated. Water quality was assessed by the local water board and deemed "excellent" as bathing water throughout 2012–2015.

With regard to the national reintroduction and restocking project, one egg strand was collected in 2011 from the Staphorst site. These larvae were distributed over three populations in the south of The Netherlands (province of Limburg) and over three populations in the province of Overijssel (including Staphorst) in the same year. In the period 2011–2013, 38 common spadefoot toad larvae in total were introduced in Staphorst from a site in the province of Gelderland (Soerel). From 2014 onward, the organization responsible for these movements started testing the larvae for ranavirus (Crombaghs *et al*., 2015). In 2014, a ranavirus outbreak caused by CMTV-NL occurred in one of the populations where in 2011 common spadefoot toad larvae from Staphorst were introduced (Rijks *et al*., 2016). The translocation of infected amphibians can contribute to the spread of ranaviruses (Miller *et al*., 2011); therefore, it is of great importance that all amphibians in restocking projects are tested for pathogens before they are used for reintroduction purposes (Rijks *et al.*, 2016).

We cannot confirm that all living animals with lesions as well as the sighted dead animals that were not collected were actually infected with ranavirus, nor that any of the living and healthy-looking animals were subclinically infected. Although there was occasional presence of intestinal parasites in a few of the specimens, the severity of lesions and cause of death in all animals were only attributed to ranavirus infection. Our data contribute insight into the long-term effects of CMTV-NL infection on common spadefoot toad in The Netherlands. Only during 2012 and 2015 was the site regularly visited during the summer (June–September), and it was during the summer that the most infected animals were found. Hence, the timing of the site visits may explain the observations of infection and disease. Perhaps a ranavirus infection went unnoticed in other years, which is an omission in this study. Nonetheless, the monitoring data (Table 1) do not show a decline in population size of common spadefoot toad in this water body, despite the continuous presence (2012, 2014, 2015) of CMTV-NL. This could be because mostly young, nonreproducing animals were affected, and as a result the populations' chorus size in the immediate succeeding year remained unaffected. Unfortunately, in the long term, this isolated population of a vulnerable species may decline due to this additional mortality in its early life stages. Hence, with regard to the conservation of this species, and of this population specifically, ranavirus should be seen as an additional, novel challenge that negatively impacts the sustainability of common spadefoot toad. Because both the intensity and the methodology of monitoring varied between the years, population trend calculations for common spadefoot toad could not be determined. Remarkably, during the monitoring, no infected *Pelophylax* spp. were observed, although these frogs have proven to be highly susceptible to CMTV-NL (Kik *et al*., 2011; Spitzen-van der Sluijs *et al*., in press).

This longitudinal study provides insight into the course of a ranavirus-associated mortality event over multiple years. We did not observe a short-term effect on the common isolated population; however, although the actual long-term impacts of the virus on this isolated common spadefoot toad population are likely to be considerable, for the moment the impacts remain uncertain.

Acknowledgments: The authors thank Edo Goverse and Lonneke van Lieshout from Reptile, Amphibian & Fish Conservation Netherlands, Ruud Jonker from Staatsbosbeheer, and the two anonymous reviewers of the manuscript. This work was supported by Province Drenthe.

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