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Short Communication

Ranavirus-associated mass mortality in wild amphibians, The Netherlands, 2010: A first report

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ABSTRACT

In 2010, a mass die-off of over 1000 wild water frogs (*Pelophylax* spp.) and at least 10 common newts (*Lissotriton vulgaris*) occurred in a pond in The Netherlands. Haemorrhagic disease with hepatomegaly and splenomegaly was evident. Microscopically, multiple organs presented cells with multifocal intracytoplasmic inclusion bodies, in which ranavirus-like particles were demonstrated ultrastructurally. All specimens examined tested positive for ranavirus by PCR. The sequence obtained showed a 100% identity with the one deposited for common midwife toad virus (CMTV). This is the first report of ranavirus-associated mortality in wild amphibian populations in The Netherlands. It is also the first time CMTV or a CMTV-like virus has been reported in these two species in the adult stage and outside of Spain.

Ranavirus infections have been associated with amphibian mass mortalities and as they are considered to be emerging diseases the infection has been listed as notifiable by the World Organization for Animal Health (Duffus and Cunningham, 2010; Schoegel et al., 2010). In the United Kingdom, where the infection emerged in the 1980s, significant local declines in numbers of adult common frogs (*Rana temporaria*) have been recorded (Teacher et al., 2010). In mainland Europe, ranavirus disease and mortality in wild amphibians have been reported unambiguously only in Spain in common midwife toads (CMT, *Alytes obstetricans*) and alpine newts (*Ichthyosaura alpestris cyreni*) and in Denmark in wild edible frogs (*Pelophylax kl. esculentus*) (Ariel et al., 2009; Balseiro et al., 2010a,b).

We report here the first ranavirus outbreak associated with mass mortality in wild amphibians in The Netherlands. The mortality occurred in a shallow, 3000 m^3 artificial pond adjacent to the visitor centre of National Park Dwingelderveld (52°78′15″N, 6°37′25″E). The park staff and a local volunteer reported sudden mortality in September 2010, and estimated that over 1000 young, incompletely metamorphosed and adult water frogs (edible frogs and pool frogs *P. lessonae*), and at least 10 common newts (*Lissotriton vulgaris*) had died. None of the stickleback fish (*Gasterosteus aculeatus*) present in the pond were found dead. The reported clinical signs in the frogs were variable. A number of live and dead animals showed haemorrhages in the skin from the eardrums and mouth; tadpoles showed severe oedema and erythema (Figs. 1A and B). Some frogs lay dead in the pond, but many were found dead sitting upright on land, as if ready to jump away.

Necropsy was performed on eight adult frogs and one common newt. Other animals were not available for pathological examination. The skins of the frogs showed greyish foci, and focal erythema. The livers were enlarged, mottled, pale brown and friable. The spleens were enlarged. The kidneys were beige. Tissue samples from the skin, liver, spleen, kidney and pancreas were fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with haematoxylin and eosin (H & E).

On histological examination the skins were found to be covered with mucous material, bacteria and large mycotic elements consistent with post mortem overgrowth. The epithelium was unrecognizable. The livers of the frogs and the newt showed heterophilic granulocytes in the sinusoids and microvacuolation of hepatocytes. Focal apoptotic hepatocytes were present. Necrosis was detected in the spleen. The kidneys were autolytic, but signs of necrosis in glomeruli and tubular epithelium with pyknotic cell nuclei were seen. The exocrine pancreas showed pyknotic cell nuclei. Further multifocal basophilic intracytoplasmic inclusion bodies suggestive of viral infection were evident in the hepatocytes (Fig. 2), the endothelial cells of the ellipsoidal capillaries in the spleen, the exocrine

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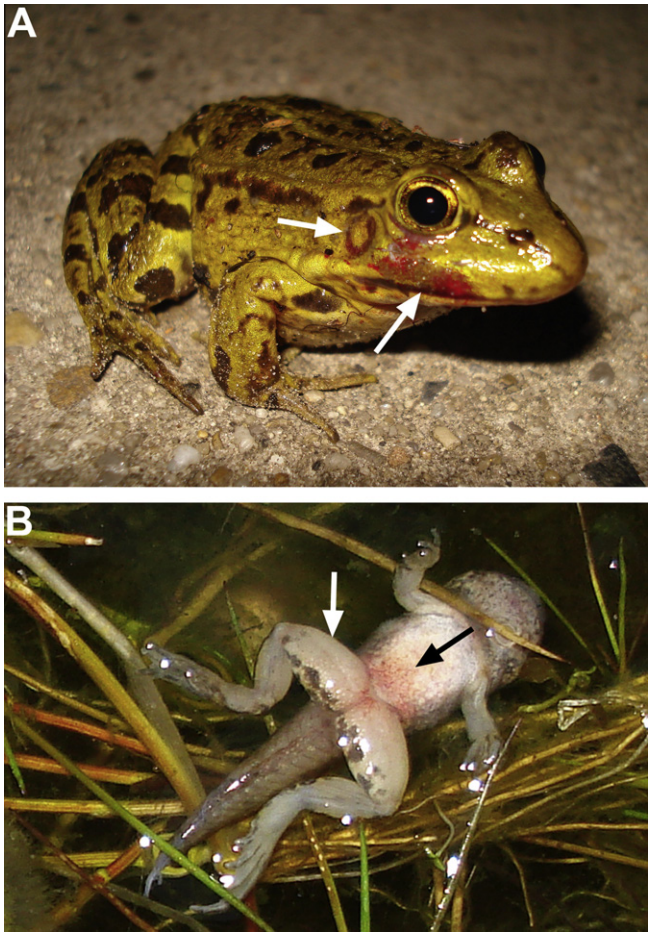

Fig. 1. (A) Live frog with multiple haemorrhages in the skin, including around the eardrum (arrows). (B) Dead metamorphosing tadpole with haemorrhages within the skin (black arrow) and subcutaneous oedema of the legs (white arrow).

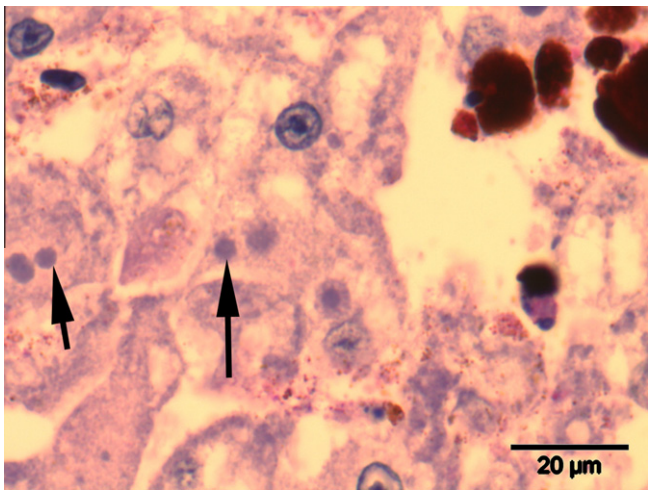

Fig. 2. Liver of one of the frogs showing multifocal intracytoplasmic inclusion bodies in the hepatocytes indicated by arrows, H.E. Bar = 20 µm.

pancreas, the endothelial cells of the glomeruli and the renal tubular epithelium of the frogs, and in the liver of the newt.

For ultrastructural examination, the area with inclusions was selected by light microscopy. A 5 µm paraffin slide was deparaffinised, rehydrated through a series of ethanol and washed in phosphate buffered saline (PBS). The specimen was subsequently fixed in Karnovsky's, rinsed with PBS, post fixed in osmium tetroxide 2%, dehydrated in acetone and covered with a Durcupan filled beam capsule. After polymerization the glass slide was taken off in liquid nitrogen. Ultrathin sections were cut and stained with lead citrate. Particles consistent with iridovirus (in this case ranavirus) were demonstrated ultrastructurally in the hepatocyte inclusions of the frogs and the newt (Fig. 3) (Chinchar et al., 2005).

DNA was extracted from stored frozen (−80 °C) tissues harvested from nine whole tadpoles and samples of skin, liver, kidney and intestine from the eight adult frogs, as well as from a paraffin-embedded liver sample of the common newt using the DNeasy Blood and Tissue kit (Qiagen). The ranavirus PCR was performed as described by Mao et al. (1997), without modifications, using the forward primer 5′-GACTTGGCCACTTATGAC-3′ and the reverse primer 5′-GTCTCTGGAGAAGAAGAA-3′. A product of 530 bp was generated from all samples. Sequencing of the fragment was performed using the BigDye Terminator Cycle Sequencing kit (PE Biosystems) on an ABI Prism 3100 Genetic Analyzer. The electropherogram was exported and converted to Kodon (Applied Maths). The sequence was blasted in GenBank (Megablast) and revealed a 100% identity with the sequence of ranavirus isolated from the common midwife toad in Spain (GenBank Accession No.: FM213466.1).

All frog samples tested negative in a qPCR for *Batrachochytrium dendrobatidis* (Boyle et al., 2004). Considering all the above data, we concluded that the die-off was caused by ranavirus, most likely common midwife toad virus (CMTV), or a CMTV-like virus. To date, CMTV is known to infect tadpoles of the CMT and juvenile alpine newts (Balseiro et al., 2010a,b). In the current outbreak adult water frogs and common newts were also affected suggesting that both of these species are susceptible to CMTV. The necrotic lesions with intracytoplasmic inclusion bodies and the presence of ultrastructural virus particles are consistent with those described in CMT and alpine newts in Spain. The origin of the infection could not be determined.

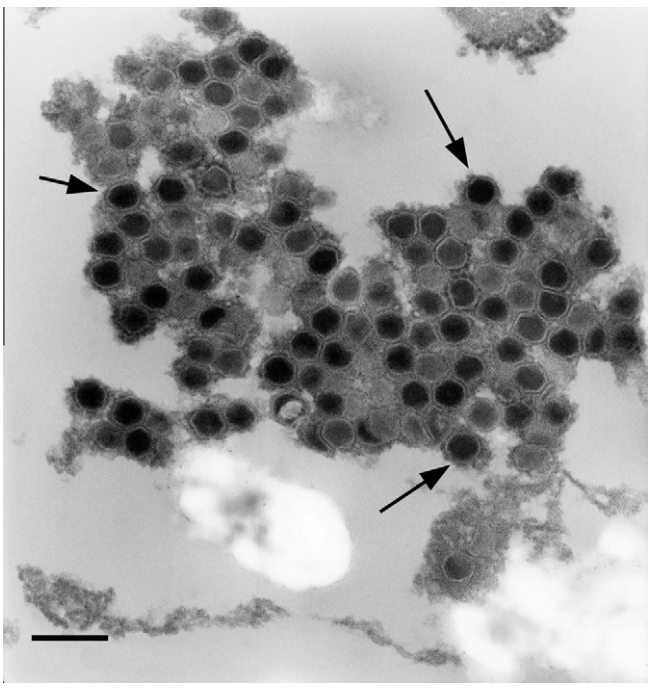

Fig. 3. Transmission electron micrograph of paraffin embedded liver of one of the frogs, showing icosahedral virus particles consistent with an iridovirus in this case ranavirus (indicated by arrows) in inclusion bodies located in the hepatocytes. Original magnification 40,000×. Bar = 250 nm.

Ranavirus infection has been previously detected in captive red tailed knobby newts (*Tylototriton kweichowensis*) retailed in The Netherlands and Belgium (Pasmans et al., 2008), but never in wild amphibians. In The Netherlands, this may be due to under-surveillance (Duffus and Cunningham, 2010) but in neighbouring Belgium episodes of amphibian mortality in the wild have been systematically tested for the presence of ranavirus since 2007 without any confirmation of ranavirus infection (F. Pasmans, unpublished data).

Shortly after the onset of the present outbreak, the pond was temporarily closed to the public. However, transmission of ranavirus from this outbreak to other populations of wild amphibians in The Netherlands and adjacent countries is a threat that cannot be ignored. Therefore, broad-scale surveillance for ranavirus infection and implementation of control measures with education of the public to prevent its spread is warranted.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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