Paper

Mass-mortality in green striped tree dragons (*Japalura splendida*) associated with multiple viral infections

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In spring 2011, high mortality in association with skin lesions, systemic haemorrhages and necrosis occurred in a group of green striped tree dragons (*Japalura splendida*) which were imported from southwestern China via Florida to Germany. Infections with various endoparasites were diagnosed in coprological examinations. Different antiparasitic and antibiotic treatments over a period of three months did not reduce the mortality rate. The remaining animals were therefore euthanased and submitted for additional testing. Predominant findings in pathological examination were granulomatous and necrotising inflammation of the skin, vacuolar tubulonephrosis of the distal renal tubules, hyperaemia and liver necrosis. Eosinophilic intranuclear and basophilic intracytoplasmic inclusion bodies were detected in the liver. Virological testing (PCR and virus isolation methods) demonstrated the presence of ranavirus, adenovirus and invertebrate iridovirus.

Introduction

Viruses infecting reptiles have been increasingly detected and studied during the last decades. As molecular methods used for the diagnosis of viral infections in these animals are becoming more available, coinfections with various infectious agents (including multiple viruses) have been documented (reviewed in Marschang, 2011). The interaction of concurrent infections remains speculative.

Adenoviruses (AdVs) (family *Adenoviridae)* are middle sized, icosahedral, non-enveloped, double-stranded DNA viruses, which infect ectothermic vertebrates, birds and mammals. All AdVs from squamates which have been characterised so far belong to the genus *Atadenovirus* (Wellehan and others 2004). In lizards, AdVs have been detected frequently in several species of bearded dragons (*Pogona* species) (Julian and Durham 1985, Frye and others 1994, Jacobson and others 1996, Kim and others 2002, Moormann and others 2009, Wellehan and others 2004, Hyndmann and Shilton 2011), as well as in a central netted dragon (*Ctenophorus nuchalis)* (Hyndmann and Shilton 2011), a common agama (*Agama agama*) (Ball and others 2012), Mexican beaded lizards (*Heloderma horridum*), Gila monsters (*Heloderma suspectum*), an emerald monitor (*Varanus prasinus)* (Papp and others 2009), a Savannah monitor (*Varanus exanthematicus*) (Jacobson and Kollias 1986), in chameleons (*Chamaeleo* species) (Jacobson and Gardiner 1990, Kinsel and others 1997, Wellehan and others 2004), in geckos (leopard geckos (*Eublepharis macularius*), fat-tail geckos (*Hemitheconyx caudicinctus*), tokay geckos (*Gekko gecko*)) and in a blue-tongued skink (*Tiliqua scincoides intermedia*) (Wellehan and others 2004).

The family *Iridoviridae* consists of five genera: *Iridovirus*, *Chloriridovirus*, *Ranavirus*, *Lymphocystivirus* and *Megalocystivirus*. Ranaviruses are large, icosahedral, enveloped, double-stranded DNA viruses that infect ectothermic vertebrates (fish, amphibians and reptiles). In amphibians, ranaviruses seem to be globally distributed and have caused multiple mass-mortality events (reviewed in Miller and others 2011). In reptiles, ranaviruses have been mostly found in chelonians (Heldstab and Bestetti 1982, Westhouse and others 1996, Mao and others 1997, Chen and others 1999, Marschang and others 1999, De Voe and others 2004, Allender and others 2006, Benetka and others 2007, Johnson and others 2007, Johnson and others 2008, Blahak and Uhlenbrok 2010, Allender and others 2011, Belzer and Seibert 2011, Uhlenbrok 2010), as well as once in snakes (green pythons (*Chondropython viridis*)) (Hyatt and others 2002). Only two case reports about ranaviruses in lizards have been published so far: one in a gecko (*Uroplatus fimbriatus*) (Marschang and others 2005) and one in a mountain lizard (*Lacerta monticola*) (Alves de Matos and others 2011).

Invertebrate Iridoviruses (IIV) are known pathogens in invertebrates. The infection causes high mortality, reduced fertility and reduced life span in affected insects (Kleespies and others 1999). During the last years, IIV have been detected repeatedly in reptilian hosts. In lizards, IIV have been detected in different organs in bearded dragons (*Pogona vitticeps*), a four-horned chameleon (*Chamaeleo quadricornis*), a high casqued chameleon (*Chamaeleo hoehnelii*), a frilled lizard (*Chlamydosaurus kingii*), a green iguana (*Iguana iguana*) and a spiny-tailed lizard (*Uromastyx* species) (Just and others 2001, Weinmann and others 2007, Papp and others 2011). It has been demonstrated that an iridovirus isolated from a chameleon caused disease in crickets (Weinmann and others 2007). These findings support the hypothesis that IIV from insects are able to infect reptiles (reviewed in Marschang 2011).

This case report describes a mass mortality event in a group of green striped tree dragons (*Japalura splendida*) imported into Germany.

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Virological testing of several animals revealed mixed infections with AdV, ranavirus and IIV, as well as with a number of endoparasites. This is the first description of AdV, ranavirus and IIV infection in this species.

Materials and methods

History

In February 2011, a group of 207 green striped tree dragons were imported from southwestern China via Florida to Germany. On arrival, the animals were moderately dehydrated. The animals were housed in groups of 50 animals in screen cages (110×200×90 cm). During the day, the temperature was 27–28°C, 29°C at local sunning spots. At night, the temperature decreased to 22–23°C. The relative humidity varied between 45–55 per cent. Coprological examination (flotation methods and native smear) of pooled faecal samples demonstrated various parasitological infections: a high load of flagellates (incidence 80–100 per cent), various nematode species (*Heterakis* species, *Spirurida* species; incidence 80–100 per cent), and several infections with coccidia (*Choleoeimeria* species; incidence 20–30 per cent). Many animals were infected with larval ascarids (probably *Hexametra* species; incidence 60–80 per cent) in the pharynx, in some cases leading to a severe purulent, abscess-forming pharyngitis. All animals were treated via drinking water using ronidazole (Ridzol, 10 per cent 200 mg/l H_2O) and levamisole (Concurat, 200 mg/l H_2O) over five days.

Soon after arrival, 21 animals showed central nervous disorders (incoordination, running in circles, inability to climb), became lethargic, and died within 24–36 hours after the first signs were recorded. A short pathological examination demonstrated a haemorrhagic, oedematous gastrointestinal tract (Fig 1), and ecchymotic haemorrhages in the fatty tissue and the liver. A low infestation with nematodes was detected. Pustular-encrusting dermatitis appeared occasionally during the course of disease (Fig 2).

Antibiotic treatment with enrofloxacin (Baytril 10 per cent oral solution, 150 mg/l drinking water over five days) as well as modified housing conditions to create similar environmental temperatures as in the wild (reducing the size of animal groups to 5–7 individuals placed in gauze terrariums and decreased environmental temperature, 24–26°C during the day and 19°C at night) were initiated on 25 February 2011. These measures did not show any positive impact on the diseased animals and did not reduce the numbers of newly affected animals (Fig 3). On 25 March 2011, the remaining animals were moved to a greenhouse. The environmental temperature in the gauze terrariums varied between 25°C and 38°C during the day with direct exposure to the sun, and at night the temperature decreased to 11°C. The terrariums were sprayed with water twice a day to reach a relative humidity of 90 per cent temporarily. As no positive effect was observed during the next weeks, the remaining green striped tree dragons were euthanased on 23 May 2011 using pentobarbital-natrium (Narcoren 0.2 ml (32 mg/animal) intracoelomically) to avoid the spread of disease to other animals. Animals were frozen for further examination.

Pathological examination

Five formalin-fixed animals were submitted for pathological examination in July 2011. One animal was in poor body condition, the others were well nourished. Multifocal skin lesions (submiliary to miliary light coloured nodules (Fig 4), partially associated with muscle necrosis) were observed in four animals. Based on histological examination, the skin lesions were characterised as granulomatous and necrotising inflammation with a moderate to marked number of intralesional filiform bacteria in some lesions. A catarrhal purulent to granulomatous sinusitis was detected in two animals. Pathological changes in the liver (hyperaemia, haemorrhages and multiple necroses) were found in three animals; eosinophilic intranuclear inclusion bodies of Cowdry type B

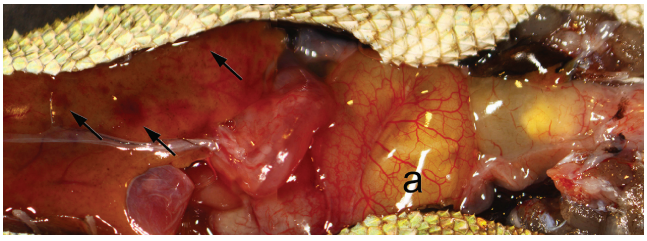

FIG 1: Postmortem examination. Coelom of an affected green striped tree dragon. Note the oedema of the intestine (a) and the ecchymosis of the liver (arrows)

FIG 2: Green striped tree dragon (*Japalura splendida*): granulomatous necrotising skin lesion (black arrows), open wound following removal of the crust (red arrow)

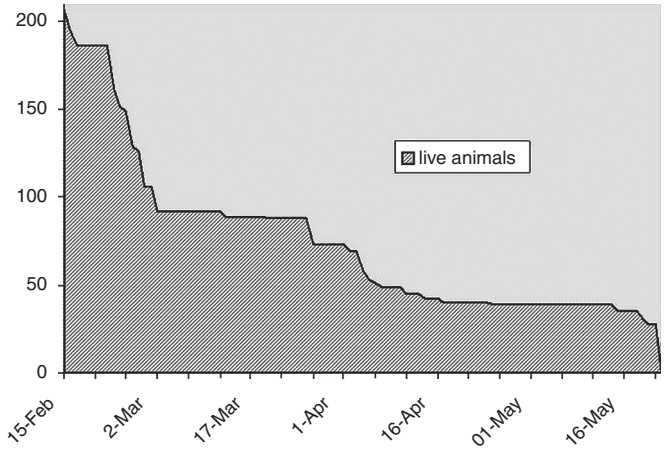

FIG 3: Mortality rate in a group of newly imported green striped tree dragons (*Japalura splendida*) over a period of three months in 2011. The number of animals is presented in the y axis, time is presented in the x axis

FIG 4: Formalin-fixed cachectic green striped tree dragon (*Japalura splendida*). Note the miliar bright nodules on the legs, tail and back

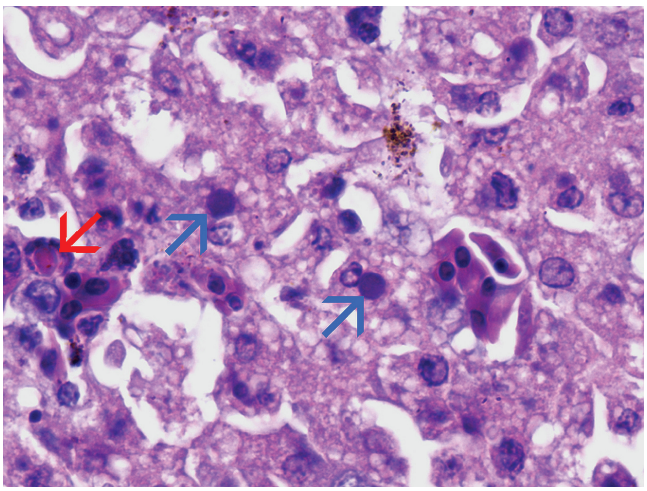

FIG 5: Liver of a green striped tree dragon (*Japalura splendida*). Note the the eosinophilic intranuclear Cowdry type B inclusion body (red arrow) and the basophilic intracytoplasmic inclusion bodies (blue arrows) (H&E – staining, x 1000)

and basophilic intracytoplasmic inclusion bodies were detected in one animal (Fig 5). Pathohistologically, vacuolar tubulonephrosis of the distal renal tubules was diagnosed in the kidneys from two animals. Parasites were detected in the oral cavity in one animal (nematodes), and in the intestine of two animals (trematodes).

Virological testing

Five dead adult *J. splendida* were sent for virological testing in July 2011. Small tissue samples from the skin, lungs, intestine and pooled samples from liver and kidneys were taken from each animal separately and submitted in cell culture medium (Dulbecco's modified Eagle medium (DMEM) (Biochrom AG, Berlin, Germany) supplemented with antibiotics). The samples were sonified, centrifuged at low speed, inoculated onto iguana heart cells (IgH-2, ATCC: CCL-108) and incubated at 28°C. Tissue cultures were observed twice a week for cytopathic effects (CPE). Dishes showing no CPE after two weeks of incubation were frozen, thawed and reinoculated for a second passage.

DNA was extracted from the original sample or from the cell culture supernatant using a DNeasy Kit (Qiagen, D-40724 Hilden, Germany) and PCRs for the detection of AdVs, ranaviruses and IIV were done as described previously (Mao and others 1997, Marschang and others 1999, Wellehan and others 2004, Weinmann and others 2007). Obtained PCR products were agarose gel purified (peqGOLD gel extraction kit, Peqlab Biotechnologie GmbH, D-91052 Erlangen, Germany) and submitted to MWG Biotech GmbH for sequencing from both directions. The sequences were edited and compared using the STADEN Package V.2003.0 Pregap4 and Gap4 programmes. The sequences were then compared to those in GenBank (National Center for Biotechnology Information, Bethesda, Maryland 20894, USA) online (http://www.ncbi.nih.gov) with the Basic Local Alignment Search Tool (BLAST) using BLASTN and BLASTX options and to the local iridovirus database of the Fachgebiet für Umwelt und Tierhygiene at Hohenheim University (Papp, personal communication).

TABLE 1: Results of virological testing of samples from five animals from a group of green striped tree dragons (*Japalura splendida*): The results of three different PCRs (for the detection of adenovirus (AdV), ranavirus (RV), and invertebrate iridovirus (IIV)), as well as virus isolation on cell culture (IgH-2)

Samples	PCR from original samples			Virus isolation	PCR from virus isolates		
	AdV	RV	IIV		AdV	RV	IIV
1: skin	–	–	+	–			
lungs	–	–	+	–			
liver and kidney	–	–	–	–			
intestine	+*	–	–	–			
2: skin	–	–	+	+	–	–	+
lungs	–	–	+	+	–	–	+*
liver and kidney	–	–	+	+	–	–	+
intestine	+	–	+	+	–	–	+
3: skin	–	–	+	+	–	+	+
lungs	–	+	+	+	–	+*	+*
liver and kidney	–	–	+	+	–	+	+
intestine	–	+	+	+	–	+	+
4: skin	–	–	+	–			
lungs	–	–	+	–			
liver and kidney	–	–	+	–			
intestine	–	–	–	–			
5: skin	–	–	+	–			
lungs	–	–	+	+	–	–	+
liver and kidney	–	–	–	+	–	–	+
intestine	+*	–	+	+	–	–	+

*PCR product sequenced
+, positive; –, negative

Results

Results from virological testing are listed in Table 1. AdV was detected in the intestines of three animals by PCR. Lungs and intestine from another animal tested positive for ranavirus. IIV was detected in all samples except liver and kidneys from two animals and intestine from two animals. Viruses could be isolated from three animals: in two animals, IIV was isolated from various samples, in one animal, ranavirus and IIV were isolated from all tissues. AdV could not be isolated on IgH-2 cells. Comparison of the obtained partial DNA-dependent DNA polymerase nt sequence from the AdV (272nt) with virus sequences available from GenBank demonstrated that this virus clearly clusters with members of the genus *Atadenovirus*, with 61.7 per cent identity to the corresponding region of eublepharid AdV-1 (Accession No: AAS89693) and 68.7 per cent to the corresponding region of helodermatid AdV-1 (AAS89696). Comparison of aa sequence (90 aa) demonstrated 71.1 per cent identity to the corresponding regions of both eublepharid and helodermatid AdVs. Based on the obtained 500 bp portion of the MCP gene, the detected ranavirus showed 100 per cent identity to nucleotide sequences from frog virus 3 (FV3) (AY548484) and Terrapene carolina ranavirus (U82553, TCU82553). Partial nucleotide sequences of MCP gene from the obtained IIV (222nt) were 98 per cent identical to Chilo iridescent virus (AF303741) and 100 per cent identical to the cricket iridovirus isolates found in crickets and lizards (Kleespies and others 1999, Just and others 2001, Papp and others 2011).

Discussion

In the current study, multiple infections were detected in a group of green striped tree dragons. None of the detected viruses (AdV, ranavirus, and IIV) has been found in this species before. A maximum of two viruses could be detected in any single animal tested. Unfortunately, it was not possible to test those animals which were sent for pathohistological examination virologically.

Neurologic disease and hepatic necrosis have been described in association with AdV infections in lizards; in several cases, basophilic and eosinophilic intranuclear inclusion bodies have been detected in hepatocytes, enterocytes, renal tubules, pancreatic acini and oral mucous membranes (Jacobson and Kollias 1986, Frye and others 1994, Jacobson and others 1996, Kim and others 2002, Wellehan and others 2004, Moormann and others 2009). Ranavirus infections in lizards have been found in association with granulomatous lesions on the tongue and hepatitis (Marschang and others 2005). In chelonians, basophilic intracytoplasmic inclusion bodies in hepatocytes have been described in association with ranaviral disease (Heldstab and Bestetti 1982, Westhouse and others 1996, Johnson and others 2008). Skin lesions have also been detected in several lizards which tested positive for the presence of IIV: a frilled lizard showed pox-like skin lesions (Just and others 2001); hyperkeratosis has been observed in a green iguana and a spiny-tailed lizard with IIV (Papp and others 2011).

Many clinical and pathological findings in the diseased green striped tree dragons correspond with previous reports associated with the detected viruses, but infection without signs of concurrent disease have also been reported for each virus (reviewed in Marschang 2011). A marked number of filiform bacteria were found in some skin

lesions, but the initial antibiotic treatment with enrofloxacin did not reduce the mortality rate of the diseased animals. We therefore speculate that the causative agent for the disease was not the bacterial infection, although it is not possible to clearly define a primary pathogen in this case. It also remains unclear whether all the animals were infected with the same viruses. It is probable that the animals were wild-caught at different locations. They may also have come into contact with other species during transport. The various pathogens detected in this group of green striped tree dragons could therefore have been introduced into the group by different animals. Interestingly, the detected AdV showed relatively low identity values to all other AdVs detected in lizards, indicating that it may represent a distinct atadenovirus species. It has been demonstrated previously that the same ranavirus can infect different species (Johnson and others 2007, Blahak and Uhlenbrok 2010, Ariel and Owens 2011). The detected ranavirus shows highest similarity to other ranaviruses from the USA (FV3, Terrapene carolina ranavirus) in the analysed partial sequences of the MCP gene. Based on the sequencing results, it is therefore possible that the animals were infected in the USA. Nevertheless, the origin of the different viruses remains speculative.

In squamates, coinfections with viruses and other infectious agents have been detected previously (see for example, Kim and others 2002, Ball and others 2012). Concurrent infections with multiple viruses have also been described (see for example, Raynaud and Adrian 1976, Papp and others 2010, Abbas and others 2011) but their interactions are not well understood. Based on the current state of scientific knowledge, both host and viral factors influence the clinical outcome of viral diseases (reviewed in Marschang 2011). We therefore hypothesise that the dramatic course of disease observed in this group of lizards was caused by an interaction of the concurrent infections (viruses, parasites, bacteria) and suboptimal environmental factors (stress during transport, overcrowding, dehydration) which may have suppressed the animal's immune systems.

This case report is an example for the emergence of pathogen pollution. It underlines the need to put newly transported animals under strict quarantine and the importance of carrying out multiple tests when diagnosing a diseased or new animal before introducing it to an existing collection.

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