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Soft plastron, soft carapace with skeletal abnormality in juvenile tortoises

Histopathology and isolation of a novel picornavirus from *Testudo graeca* and *Geochelone elegans*

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Key words
Virus infection, nephropathy, osteodystrophy

Summary

Objective: A disease is described in juvenile tortoises (*Testudo graeca* and *Geochelone elegans*) consisting mainly of a soft carapace, soft plastron and deformed skeleton. The aim of this study was to determine histopathological lesions and the biological properties of the isolated viruses. **Materials and methods:** Clinical signs and gross pathology were determined on diseased and healthy appearing tortoises. Paraffin sections were stained with HE, PAS and Prussian Blue and histologically examined. Terrapene heart (TH-1) cell cultures served for virus isolations from 64 tissues and 104 swabs. One isolate (isolate 1243/37 tongue) was used in neutralization tests on 19 sera. **Results:** Retarded growth and increasingly soft plastron and carapace were the prominent signs in diseased tortoises. Pathological lesions consisted of dilated urinary sac, enlarged kidneys and livers. Histopathologically, hepatic hemosiderosis, hypoplastic anaemia, congestive glomerulonephrosis and osteodystrophy were seen. A novel virus ("virus X") was isolated from 64 organs and 79 of 104 swabs. The isolated viruses were identified as a novel chelonid picornavirus based on cytopathic effect, resistance to chloroform and stability at low pH. Co-cultivation with 5-iodo-2′-deoxyuridine and actinomycin D did not reduce virus titres. Electron microscopically, round, non-enveloped particles (25–30 nm) were detected. Neutralizing antibodies to the isolate 1243/37tongue were present in 17 of 19 sera from seven species of tortoises. **Conclusion and clinical relevance:** Nephropathy, osteodystrophy and virus isolations suggest a viral aetiology. Metabolic bone disease is the major differential diagnosis. Further investigations in vivo are needed to evaluate the likely effects of the picornavirus on tortoises.

Schlüsselwörter
Virusinfektion, Nephropathie, Osteodystrophie

Zusammenfassung

Gegenstand und Ziel: Beschreibung einer Erkrankung juveniler Landschildkröten (*Testudo graeca* und *Geochelone elegans*), die klinisch mit weichem Karapax und Plastron sowie Skelettdeformationen einhergeht. Ziele waren Dokumentation histopathologischer Befunde sowie Isolierung und Bestimmung der biologischen Eigenschaften der Virusisolate. **Material und Methoden:** An klinisch gesunden und erkrankten Schildkröten wurden klinische Symptome und pathologische Veränderungen erhoben. Histopathologisch wurden Paraffinschnitte nach HE-, PAS- und Berliner-Blau-Färbung untersucht. Terrapene-Heart(TH-1-)Zellkulturen dienten zur Virusisolierung aus 64 Organhomogenisaten und 104 Tupferproben. In Neutralisationstests wurden 19 Serumproben adulter, klinisch gesunder Schildkröten desselben Bestands auf Antikörper gegen das Isolat 1243/37 Zunge untersucht. **Ergebnisse:** Erkrankte Schildkröten zeigten Wachstumsverzögerung und eine zunehmende Erweichung von Plastron und Karapax. Pathologisch wurden ein erweiterter Harnsack sowie vergrößerte Nieren und Lebern festgestellt, histopathologisch hepatische Hämosiderose, hypoplastische Anämie, kongestive Glomerulonephrose und osteodystrophische Veränderungen. Ein Virus („Virus X") wurde aus allen 64 Organ- und aus 79 der 104 Tupferproben isoliert. Der zytopathische Effekt, die Resistenz gegenüber Chloroform, die Stabilität bei niedrigen pH-Werten, die Resistenz gegenüber 5-Jod-2′-Desoxiuridin und Actinomycin D sowie die elektronenmikroskopisch dargestellten kleinen (25–30 nm), rundlichen, unbehüllten Partikel weisen auf ein neuartiges, schildkrötenspezifisches Picornavirus hin. In 17 der 19 Seren ließen sich neutralisierende Antikörper gegen das Isolat 1243/37 Zunge nachweisen. **Schlussfolgerung und klinische Relevanz:** Der Nephrotropismus und der Nachweis von Nephropathie, osteodystrophischen Veränderungen und Virämie deuten auf eine virale Ätiologie hin. Wichtigste Differenzialdiagnose ist die Osteodystrophie. Weitere In-vivo-Untersuchungen sind nötig, um die Auswirkungen des isolierten Picornavirus auf juvenile Landschildkröten zu bestimmen.

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Erweichung von Karapax und Plastron mit Skelettdeformation bei juvenilen Landschildkröten. Histopathologie und Isolierung eines neuen Picornavirus aus *Testudo graeca* und *Geochelone elegans*
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Introduction

In recent years, increased health problems in juvenile tortoises were seen in a large, longstanding breeding collection. Both the Spur-thighed tortoise (*Testudo graeca*), and the Indian Star Tortoise (*Geochelone elegans*) were affected. The young tortoises of both species developed normally until 4–6 weeks after hatching. Later on growth was retarded and plastron and carapace softened severely. The uric acid concentration in the peripheral blood increased, and in a few animals respiratory disorders were noted. Most of the diseased tortoises died within the first year of life (9).

Nutritional deficiencies such as calcium and or phosphorous imbalances (1, 4) and deficits of vitamin D may result in soft carapace and plastron and skeletal abnormalities (15). Detrimental environmental conditions, in particular low levels of UV radiation, promote malformations of the external structures and bones (15). Such debilitating factors were excluded during repeated examinations of the tortoise collection. Also, parasites were rarely detected during post mortem examinations (20, 22, 25), pathogenic bacteria and invasive fungi were not cultured from carapace, plastron and internal organs of diseased tortoises (6).

The large order Picornavirales currently comprises five families which infect invertebrates (Dicistro- and Iflaviridae) and algae (Marnaviridae). Higher plants may be infected by viruses of the family Seciviridae that includes the genera *Como-*, *Faba-*, *Nepovirus*, but also *Sadwa-*, *Torrado-*, and *Waikavirus*. Most important are the members of the family Picornaviridae that infect and may cause severe diseases in mammals and man. The mammalian Picornaviridae include the genera *Aphthovirus, Enterovirus, Hepatovirus, Parechovirus, Erbovirus, Kobuvirus, Teschovirus, Sapelovirus, Senecavirus, Cardiovirus*, and *Megrivirus*. In birds, the genera *Avihepatovirus* and *Tremovirus* are of importance as disease causing agents (12, 13). So far, there have been only two descriptions of the isolation and partial characterization of picorna-like viruses in snakes of the species *Boa constrictor* (3) and the Aesculapian snake *Elaphe longissima* (8). In chelonians, a picornavirus has been reported in a single study (17). It was most frequently isolated from pharyngeal swabs of adult tortoises that presented variable clinical signs but never alterations of the carapace, plastron and skeleton.

Some of these viruses cause epidemic diseases of major ecologic, economic and socio-economic relevance. A few of these diseases are effectively controlled by vaccination in combination with enhanced biosecurity and legal restrictions.

We report on histopathological lesions, on virus isolations from juvenile tortoises and characterization of biological properties of these isolates. Neutralization tests were performed on 19 sera derived from adult tortoises of several species using an isolate from the tongue of a diseased *T. graeca*.

Material and methods

Case history

A breeder maintained for many years a large collection of different species including European and tropical tortoises. The animals were kept outdoors with access to shelter in appropriate houses. Housing conditions, temperature regulation, food and water supply for all tortoises corresponded to common practice. Surplus offspring was usually sold and adult tortoises were occasionally introduced into the collection.

The juvenile tortoises of the species *Testudo graeca* and *Geochelone elegans* developed initially as expected. During the first weeks of life, body weight gain and behaviour of all tortoises equalled to normal values in all individuals. Later on *T. graeca* and *G. elegans* severely stagnated in growth, food intake and general health state. Beginning at an age of 4–6 weeks a softening of plastron and carapace was noticed only in *T. graeca* and *G. elegans* (▶Table 1). The softening (as determined by pressure with a finger) started in the plastron and extended subsequently to the carapace. The colour of the plastron changed from yellow to greyish-black. Morbidity and mortality was higher than generally expected in juvenile but not in adult tortoises (10, 11). Since the first virus isolations, the disease and losses persist until today. Severely diseased *T. graeca* and *G. elegans* (▶Table 1) were submitted to determine the cause of the disease and death.

Clinical and post mortem examination

Fifty adult tortoises and all of the available juvenile tortoises of a collection of land tortoises were visually examined in detail. Special attention was given to any alterations of the colour and firmness of the plastron, carapace and legs. Wet swabs were taken

Table 1 Species names and number of examined tortoises.
Tab. 1 Spezies und Zahl der untersuchten Schildkröten

Scientific name	Common name	Number of tortoises per species
Testudo hermanni	Hermann's tortoise	13
Testudo graeca	Spur-Thighed Tortoise	25
Testudo horsfieldii	Central Asian Tortoise	3
Chelonoidis carbonaria	Red-footed tortoise	3
Testudo marginata	Marginated Tortoise	6
Astrochelys radiata	Radiated Tortoise	1
Geochelone pardalis syn. *stigmochelys*	Leopard tortoise	2
Geochelone elegans	Indian Star Tortoise	3
Dipsochelys hololissa	Seychelles giant tortoise	2
Total		58

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from conjunctiva, pharynx and cloaca during physical examinations. Also, for bacteriological, mycological and parasitological examinations tissue samples were collected that correspond to samples for virological assays.

Seven out of 25 diseased juvenile *T. graeca* and one of three *G. elegans,* all showing pronounced softening of the plastron and carapace were pathologically examined according to the procedure described by Frye (5). Tissue samples were removed for bacteriological, mycological (18), parasitological (18, 19, 22) and virological tests (▶Table 2).

Virological examination

Tissue and swab sampling

From seven of 17 *T. graeca* and one *G. elegans* 64 tissue samples from eight different tissues (brain, tongue, heart, lung, liver, kidney, spleen and small intestine) were collected. In addition, 104 cotton swabs from pharynx, conjunctiva and cloaca (18 from conjunctiva, 43 from pharynx, and 43 from cloaca) from different species of tortoises were investigated for virus content.

Virus isolation

The 64 tissue samples and the 104 swab samples were immediately placed in basal medium Eagle (BME) with Earle's salt solution and supplemented with antibiotics (gentamycine and enrofloxacine) and antimycotics (nystatin). After sonication (Branson Sonifer, three pulses at 60%) and centrifugation, 200 µl of these homogenized tissue samples (Hettich Rotanta: 3000 x g for 10 minutes) were inoculated onto confluent monolayers of Terrapene heart cells ([2]; TH-1, ATCC CCL-50). All cells were maintained in Cellstar® tissue culture dishes (Greiner Bio-One GmbH, Frickenhausen, Germany). After absorption for 2 hours at 28 °C, 1.8 ml of complete Minimal Essential Medium (MEM) was added to each dish which was supplemented with 2% fœtal calf serum, antibiotics (50 mg/l gentamycine and 10 mg/l enrofloxacine plus 10,000 IU nystatin. Cultures were incubated at 28 °C and daily observed for cytopathic effects (CPE) using an inverted light microscope (Diavert, Leitz, Wetzlar). Cultures with CPE were harvested 5–7 days after inoculation of the cells and re-inoculated on the same type of cell culture for a passage and for subsequent characterization according to the procedure described by Mayr et al. (18).

Table 2 Data on eight necropsied juvenile tortoises used for virus isolation and microbiological examination.

Tab. 2 Angaben zu acht sezierten juvenilen Schildkröten, die der Virusisolierung und mikrobiologischen Untersuchung dienten.

Accession No.	Body weight	Sex	Investigated organs
402/1 T. g.	22 g	M	brain, tongue, lung, heart, small intestine, liver, kidney, spleen
402/2 T. g.	18 g	M	
1443/1 T. g.	25 g	F	
1243/62 T. g.	28 g	M	
1243/37 T. g.	23 g	M	
1705 T. g.	30 g	F	
1704 G. e.	29 g	F	
782 T. g. t.	27 g	M	

Species: T. g. = *Testudo graeca*; T. g. t. = *Testudo graeca terrestris*; G. e. = *Geochelone elegans;* sex: M = male; F = female

Virus characterization

Supernatant fluids from inoculated cell cultures with CPE infected either with one of the 64 organs or 104 swab samples were treated with chloroform and incubated in the presence of either 50 µg/ml 5-iodo-2'-desoxyuridin or 1 µg/ml actinomycin D (14). Three isolates with a titre about 10^7 $TCID_{50}$ per millilitre were used for electron microscopy. As a positive control, the herpesvirus isolate No. 302/04 previously isolated from a *T. hermanni* was used.

Assay of pH-stability of selected isolates was performed in Mellvaine buffer using the method that was recommended by Mayr et al. (18). For this purpose, three solutions were prepared that consisted of (a) 0.1 M citric acid, (b) 0.2 M Na_2HPO_4 x 2 H_2O and (c) 0.1 M NaOH. Solutions with pH 3.0, 5.0, 7.0 and 12.0 were produced by adding 1 N NaOH or 1 N HCl. The intended pH values were monitored using a calibrated pH meter (Mettler-Toledo, Seven Easy) according to the methods described by Mayr et al. (18).

Virus neutralization tests

One representative isolate obtained from a diseased tortoise (*T. graeca*) (acc. No. 1243/37tongue) was used in all neutralization tests to determine the antibody level in adult apparently healthy land tortoises of different species. Serum samples were diluted in 96-well microtitre plates and reacted with approximately 100 $TCID_{50}$ per well of the test virus for 2 hours. After that maintenance medium was added and all plates were incubated at 28 °C for 6 days. Neutralizing titres are expressed as $\log_2$ values of the highest serum dilution that prevented entirely the development of CPE. All titres are expressed as $\log_2$ values as seen on the plates.

Electron microscopy

For electron microscopy, two supernatant fluids of heavily infected cell cultures with an infectivity titre of 10^7 per millilitre from *T. graeca* (1243/37 tongue and 402/1 kidney) were absorbed onto carbon-coated parlodion films mounted on 300 mesh copper grids and negatively stained with phosphotungstic acid according to the methods described by Mayr et al. (18). Preparations were examined in an electron microscope (Zeiss, Oberkochem, Germany) at a primary magnification of 20,000 and 40,000.

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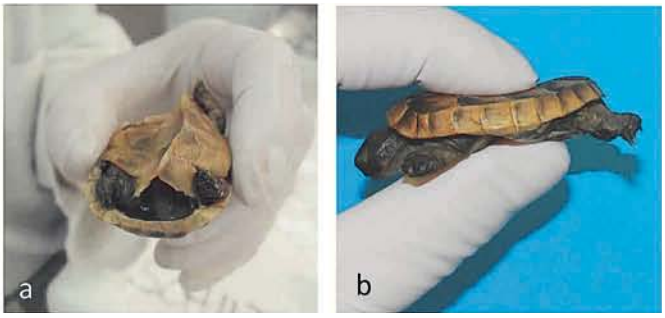

Fig. 1 Clinical picture: a) ventral view of a juvenile tortoise (*Testudo graeca*) displaying a soft, plicated and discoloured plastron. b) Lateral view of a 10-week-old tortoise (*Testudo graeca*) displaying a soft plastron and carapace. (Photo: S. Paries).

Abb. 1 Klinisches Bild. a) Ventralansicht einer juvenilen Schildkröte (*Testudo graeca*) mit weichem, eingefallenem und verfärbtem Plastron; b) seitliche Ansicht einer 10 Wochen alten Schildkröte (*Testudo graeca*) mit weichem Plastron und Karapax. (Foto: S. Paries).

Histopathology

For histopathologic evaluation, specimens from liver, kidney, bone marrow, carapace, and skeletal bone of various locations were embedded in paraffin blocks. Sections were stained with HE, PAS and Prussian Blue according to standard procedures and evaluated under a light microscope (Zeiss, Oberkochem, Germany).

Results

Clinical and post mortem examination

Only two of nine species (*T. graeca* and *G. elegans*) of tortoises, approximately 7 weeks of age, that lived in the collection displayed signs of disease. The growth of these animals was retarded. Body weights ranged between 18 and 30 grams (▶Table 2). All of them had a soft carapace as evidenced by low pressure with a finger. The colour of the plastron and carapace had changed from yellowish to dark brown and black (▶Fig. 1). These signs were not seen in any animals of the species *T. hermanni* and 17 tortoises of other species (▶Table 1) that lived intermingled in the same collection with *T. graeca* and *G. elegans*.

Post mortem examination of internal organs of seven juvenile *T. graeca* and one juvenile *G. elegans* resulted in only minor macroscopic lesions. The alterations in *T. graeca* consisted of dilated urinary sacs which were filled with whitish material and slightly enlarged livers and kidneys. The long bones and the mandibles appeared soft, bended and pliable. The intestines of some animals contained a few eggs of the pinworm (*Oxyuris* spp.), but *Hexamita parva*, a common protozoan parasite, known to cause shell softening, was never detected.

Histopathology

Histopathologic evaluation revealed hepatic hemosiderosis, hypoplastic anaemia, osteodystrophic changes in the carapace and the skeleton and congestive glomerulonephrosis in the kidney.

The bony layer of the carapace was characterized by a predominance of fibrous tissue containing thin discontinuous strands of osteoid. Physiologic bridging between the bone plates was absent

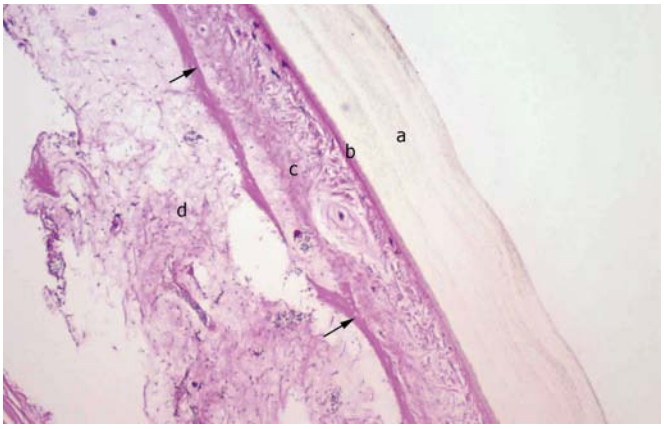

Fig. 2 Carapace, PAS stain, 40 x magnification. Keratin mantle (a), germinative layer (b), subepithelial connective tissue (c), osseous layer (d) with predominance of fibrous tissue containing thin strands of non reactive osteoid (arrows).

Abb. 2 Panzer, PAS-Färbung, 40fache Vergrößerung. (a) Keratinmantel, (b) Keimschicht (Stratum germinativum), (c) subepitheliales Bindegewebe, (d) Knochenschicht mit überwiegend fibrösem Gewebe, in dem dünne Streifen von nichtreaktivem Osteoid liegen (Pfeile).

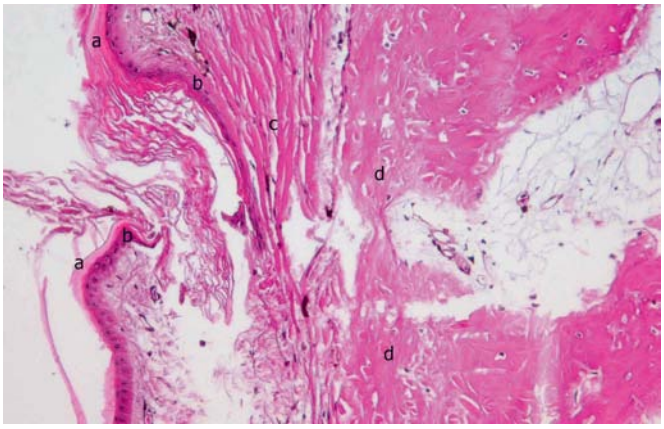

Fig. 3 Carapace, HE stain, 100 x magnification. Infraction of the keratinized mantle (a) with laceration of the Stratum germinativum (b) and impression of the subepithelial connective tissue (c), loss of firm connection to the underlying bony plates (d), the latter with signs of necrosis.

Abb. 3 Panzer, HE-Färbung, 100fache Vergrößerung. (a) Einbruch des Keratinmantels mit Ruptur des Stratum germinativum, (b) Impression des subepithelialen Bindegewebes, (c) Verlust der festen Verbindung zu den darunter liegenden Knochenplatten, (d) in letzteren Anzeichen von Nekrose.

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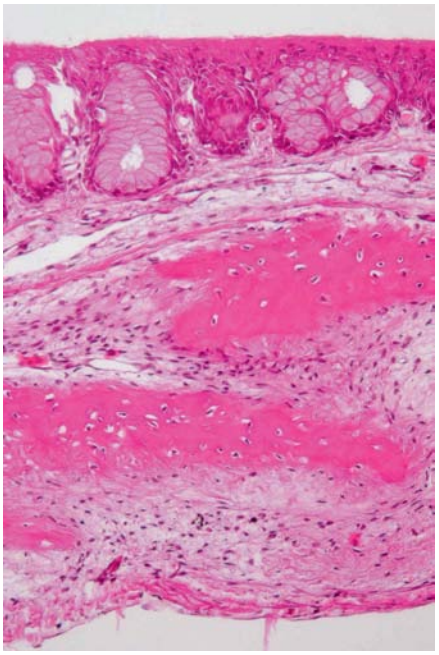

Fig. 4
Mandible, HE stain, 100 x magnification. Pharyngeal mucous membrane overlaying increased and distorted amounts of fibrous tissue containing two osteoid islets.

Abb. 4
Unterkiefer, HE-Färbung, 100fache Vergrößerung. Maulschleimhaut über vermehrt vorhandenem ungerichtet proliferierendem Bindegewebe mit zwei Osteoidinseln.

in many areas. Instead, single osteoid islets were embedded in fibrous tissue. Apart from few osteoclasts, remodelling activity could not be detected (▶Fig. 2). The often loosely aggregated layers of the keratinized mantle showed several folds. Infractions with laceration of the stratum germinativum and impression of the subepithelial connective tissue were also present. The underlying bony structures lacked a firm connection to the layers above and occasionally showed signs of necrosis (▶Fig. 3).

A picture similar to that of the carapace was seen in the facial bones (▶Fig. 4). The mandible and the humerus consisted of increased and distorted amounts of fibrous tissue containing isolated osteoid islets without signs of remodelling activity (▶Fig. 5).

In the growth plate of the humerus a hypo- to avascularity in the zones of proliferation, prehypertrophy and ossification was observed (▶Fig. 6). Fibroblastic cleft formation in the zone of proliferation and abundant amounts of fibrous tissue in the zone of ossification was accompanied by prominent osteoclastic activity and increased osteoid seams of the bony trabeculae. The zone of hypertrophy was characterized by a disorganization of chondrocytes, which were often binucleated. Columnar arrangement of the chondrocytes was only partially recognizable (▶Fig. 6). The transversal section of the humeral metaphysis showed a prominent proliferation and protrusion of fibroblastic tissue into the marrow

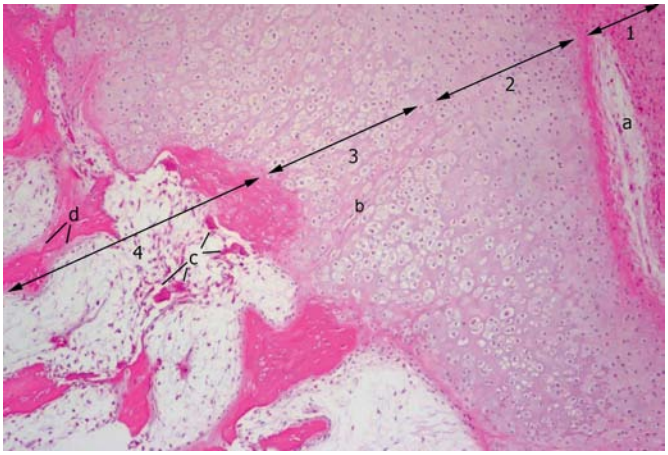

Fig. 5 Humerus, growth plate, HE stain, 100 x magnification.1 = zone of proliferation: avascularity and fibroblastic cleft (a) between epiphyseal cartilage and zone of proliferation; 2 = zone of prehypertrophy: avascularity; 3 = zone of hypertrophy: disorganization of chondrocytes with multiple binucleations (b), columnar arrangement only partially recognizable; 4 = zone of ossification: hypovascularity, osteoclastic activity (c) and fibrous replacement of trabeculae with partially enlarged osteoid seams (d).

Abb. 5 Humerus, Wachstumszone, HE-Färbung, 100fache Vergrößerung. 1 = Proliferationszone ohne Gefäßeinsprossung, fibroblastische Spaltenbildung (a) zwischen Epiphysenknorpel und Proliferationszone; 2 = prähypertrophe Zone ohne Gefäßeinsprossung; 3 = hypertrophe Zone mit Verlust der säulenartigen Anordnung der Chondrozyten und zahlreichen Doppelkernbildungen (b); 4 = Ossifikationszone: ungenügende Vaskularisierung, Fibroosteoklasie (typisch für renale Osteopathie): Aktivität von Osteoklasten (c) und bindegewebiger Ersatz der Trabekula mit teilweiser Verbreiterung der Osteoidsäume (Osteoidose, d).

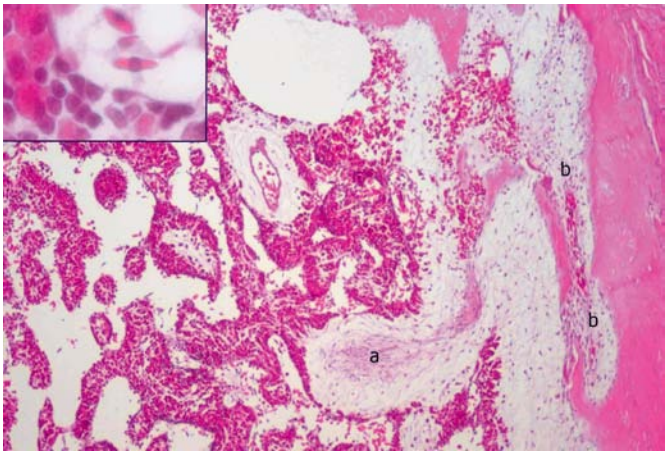

Fig. 6 Humerus, transversal section of the metaphysis, HE stain, 40 x magnification. Prominent proliferation and protrusion of fibroblastic tissue (a) into the marrow cavity, tunneling resorption of cortical bone by osteoclasts and replacement by fibrous tissue (b), bone marrow with left shift of the erythropoietic line (insert 400 x).

Abb. 6 Humerus, Transversalschnitt in der Metaphyse, HE-Färbung, 40fache Vergrößerung. Auffallende Proliferation und Vorwölbung des Bindegewebes (a) in die Markhöhle, tunnelförmige Resorption des kortikalen Knochens und Ersatz durch Bindegewebe (b), Knochenmark mit Linksverschiebung der erythropoetischen Linie (kleines Bild: 400fache Vergrößerung).

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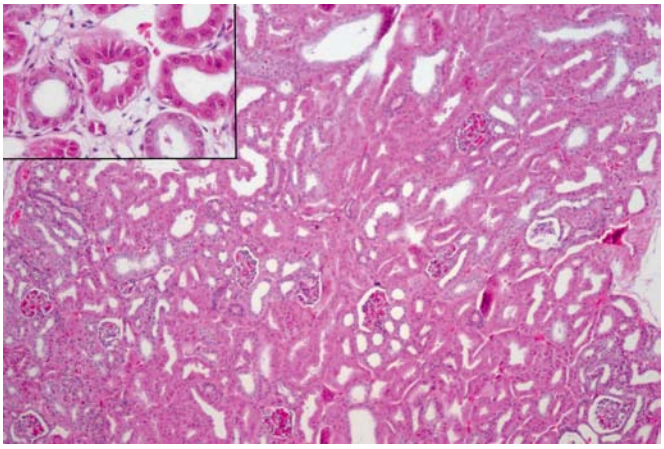

Fig. 7 Kidney, HE stain, 100 x magnification. Dilatation of dark staining proximal and light staining distal tubules, some areas with interstitial delicate fibrous tissue, oedema, and haemorrhage (insert 400 x).

Abb. 7 Niere, HE-Färbung, 100fache Vergrößerung. Vergrößerung und Erweiterung der dunkel gefärbten proximalen und der hell gefärbten distalen Tubuli, einige Gebiete mit interstitiellem, zartem fibrösem Gewebe, Ödem und Blutungen (kleines Bild: 400fache Vergrößerung).

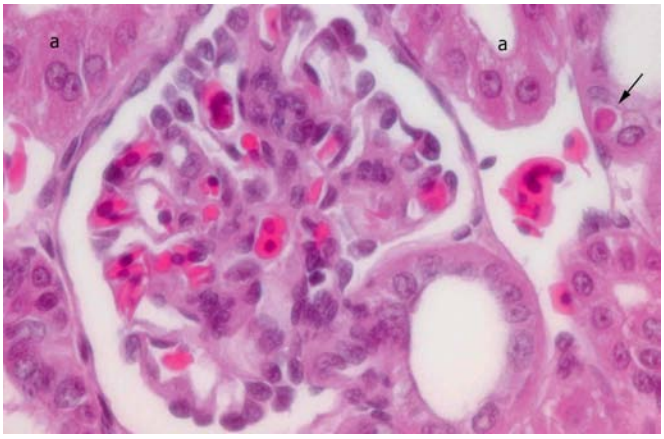

Fig. 8 Kidney, HE stain, 400 x magnification. Glomerulus with reactive (round instead of flat) nuclei of the visceral surface of Bowman's membrane (sign of irritation), proximal tubular cells (a) with delicate cytoplasmic vacuolation (tubulonephrosis) and binucleation (regenerative attempt), one cell with acidophilic intracytoplasmic granule (arrow).

Abb. 8 Niere, HE-Färbung, 400fache Vergrößerung. Glomerulus mit reaktiven (runden statt flachen) Zellkernen des viszeralen Blattes der Bowman'schen Kapsel (Anzeichen für eine Störung), proximale Tubuluszellen (a) mit feiner zytoplasmatischer Vakuolierung (Tubulonephrose) und Doppelkernigkeit (Regenerationsversuch), eine Zelle mit azidophilem intrazytoplasmatischem Granulum (Pfeil).

cavity. Tunnelling resorption of cortical bone by osteoclasts and replacement by fibrous tissue was seen in several areas (▶Fig. 6). Many bone marrow flecks presented with hypoplasia and left shift of the erythropoietic line, the medullary sinusoids appeared empty in many cases (▶Fig. 6).

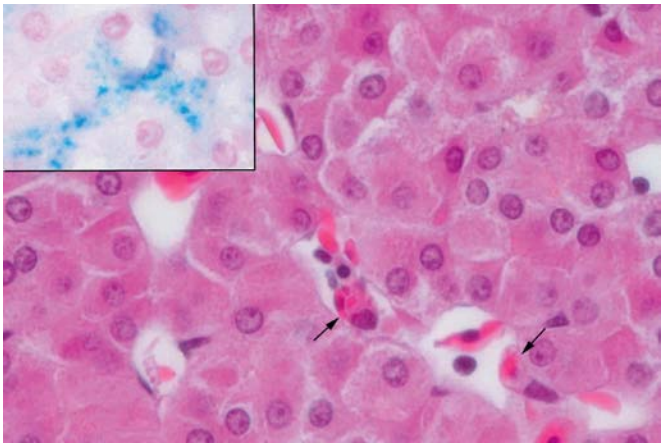

Fig. 9 Liver, HE stain, 400 x magnification. Disseminated degeneration and necrosis of liver cells, hemosiderosis (insert: Prussian blue), erythrophagocytosis (arrow).

Abb. 9 Leber, HE-Färbung, 400fache Vergrößerung. Disseminierte Degeneration und Nekrose der Hepatozyten, Hämosiderose (kleines Bild: Preußisch-Blau-Färbung), Erythrophagozytose (Pfeil).

Findings in the kidney (▶Fig. 7, ▶Fig. 8) included a dilatation of both proximal, distal and collecting tubules. Additionally, in some areas an increased amount of interstitial delicate fibrous tissue with oedema and haemorrhage was present (▶Fig. 7). Many glomeruli showed reactive (round instead of flat) nuclei of the visceral surface of the Bowman's membrane. Tubulonephrosis with delicate cytoplasmic vacuolation and signs of regeneration (binucleated cells) was present in the proximal tubules. Very few cells contained acidophilic intracytoplasmic granules (▶Fig. 8). In the liver disseminated degeneration and necrosis of hepatocytes was accompanied by increased erythrophagocytosis and moderate hemosiderosis (▶Fig. 9). Many vessels including the renal capillary loops appeared empty or showed an aniso- and poikilocytosis of the red cell line.

Bacteriological and mycological examination of organs

Only a few bacteria grew on blood agar under aerobic conditions out of 64 organ samples. These were a few *Staphylococcus* spp., *Streptococcus* spp. and a few bacillae. The mycological examination failed to recover any yeasts or fungi.

Virological examination

Virus isolation

In inoculated TH-1 cell cultures the cytopathic effect (CPE) consisted of round to fusiform cells. Such alterations were observed in all TH-1 cell cultures that were inoculated with 64 tissue homo-

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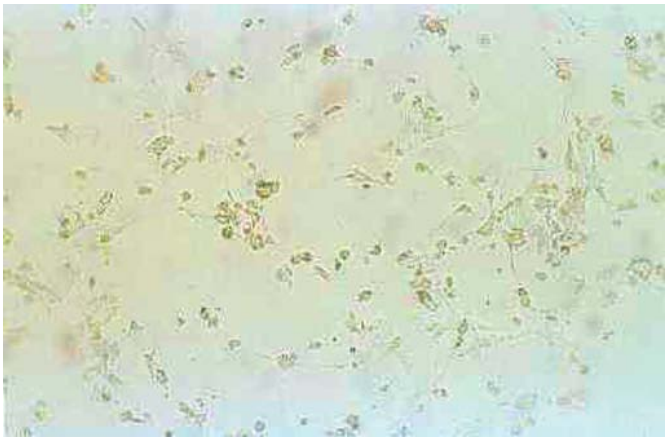

Fig. 10 TH-1 cell culture, 100 x magnification, unstained. Lytic cytopathic effect at 7 days post inoculation induced by isolate 1243/37tongue.

Abb. 10 TH-1-Zellkultur, 100fache Vergrößerung, nicht gefärbt. Lytischer zytopathischer Effekt 7 Tage nach Inokulation mit Isolat 1243/37Zunge.

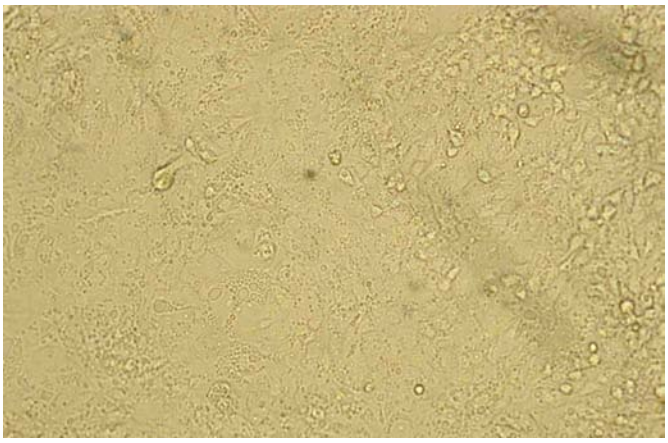

Fig. 11 TH-1 culture not inoculated; unstained, 100 x magnification.

Abb. 11 TH-1-Zellkultur, 100fache Vergrößerung. Nicht inokulierte, ungefärbte Kontrolle.

genates and in 79 of 104 swab samples. Initial cellular alterations were already seen 1–2 days after inoculation. The monolayers were completely destroyed and most of the cells were lysed at 5–7 days after inoculation (▶Fig. 10). No CPE was observed in mock inoculated control cell cultures (▶Fig. 11).

Titrations in TH-1 cell cultures of eight selected isolates yielded titres in the range between 4.0 and 7.3 $\log_{10}$ $TCID_{50}$ per millilitre. Highest titres were obtained in titrations of brain, tongue and kidney (▶Table 3). Slightly lower titres were obtained from lung, intestine and liver and the lowest from the heart. The species of the animal seems to have no major effect on the obtained titres (▶Table 3).

Biological characteristics of the isolated viruses

All five examined isolates were resistant to chloroform treatment (▶Table 4). The cultivation of the isolates in the presence of 5-iodo-2'-deoxyuridine (IUdR) did not alter the titres as compared to the control. It appears that virus cultivation in the presence of actinomycin D resulted in an increment of titres up to $\log_{10}$ = 2.7 in comparison to titres obtained in the control, chloroform and IUdR. In contrast, the development of CPE of the herpesvirus 302/04 (16) that was used as a positive control was markedly inhibited in the presence of IUdR. Low pH values (pH = 3 and pH = 5) did not affect virus titres as compared to the not treated control. In contrast, the pH = 12 drastically reduced the obtained titre (▶Table 5).

Electron microscopy

The viral particles detected in examined samples were small (25–30 nm in diameter), non-enveloped and had a round shape with icosahedral capsid structure (▶Fig. 12). Viral particles of other size and shape were never observed.

Table 3 Virus titres in TH-1 cell cultures of selected organs derived from eight tortoises.

Tab. 3 Virustiter in TH-1-Zellkulturen ausgewählter Organe von acht Landschildkröten

Accession No. and species[1]	Virus titres ($\log_{10}$ $TCID_{50}$ per ml)							
	Brain	Tongue	Lung	Heart	Intestines	Kidney	Liver	Spleen
402/1 T. g.	6.5	6.5	5.5	4.0	4.5	**7.5**	5.0	5.5
402/2 T. g.	6.1	**7.3**	5.2	4.5	5.1	6.5	5.8	5.5
1243/37 T. g.	6.5	**7.1**	6.0	5.5	5.5	6.0	5.5	5.0
1243/62 T. g.	**7.1**	6.5	5.5	5.0	5.5	6.0	5.5	6.0
1443/1 T. g.	**7.1**	6.5	5.0	4.5	5.5	6.0	5.0	5.5
1704 G. e.	5.9	5.5	4.1	3.0	5.5	5.5	5.0	5.0
1705 T. g.	6.5	6.0	4.5	3.5	5.5	6.0	4.5	6.1
782 T. g.	6.5	6.5	5.0	5.5	5.0	5.8	5.5	4.0

[1] T. g. = *Testudo graeca*, G. e. = *Geochelone elegans.* Titres in bold letters indicate isolates that were used for further virus characterization.

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Table 4
Virus titres after chloroform treatment and cultivation with 5-iodo-2′-deoxyuridine (IUdR) and actinomycin D in comparison to the control.

Tab. 4
Virustiter nach Chloroformbehandlung und Kultivierung in Anwesenheit von 5-Jod-2′-deoxiuridin (IUdR) und Actinomycin D im Vergleich zur Kontrolle

Isolate No.	Virus titres (log_{10} KID_{50} per ml)			
	Chloroform treatment	Cultivation with IUdR	Cultivation with Actinomycin D	Untreated control
T. g. 1243/62 brain	5.0	5.0	6.8	6.0
T. g. 402/1 kidney	5.0	5.0	7.0	5.5
T. g. 402/2 tounge	4.0	4.0	6.7	4.0
T. g. 1443/1 brain	5.0	5.0	6.8	5.0
T. g. 1243/37 tongue	5.5	5.5	6.7	6.0
Herpesvirus 302/04 (positive control)	0.8	0.8	0.7	5.2

Neutralization tests

Neutralizing titres (equal or higher than $log_2 = 3$) were obtained in 17 of 19 healthy appearing respectively recovered tortoises of different species. The positive titres of sera varied between $log_2 = 3$ and $log_2 = 8$ (▶Table 6).

Discussion

Soft carapaces in juvenile tortoises

Captive juvenile tortoises of *T. graeca* and *G. elegans* displaying a soft and discoloured plastron and carapace were frequently seen in recent years. Apart from nutritional and environmental causes, endoparasites, renal disease, and microbial infections have been suggested as aetiologic causes (4–8, 15, 21, 25, 26). In our case, a yet undetermined infectious agent was suspected as breeders and keepers reported a spreading pattern of the development of soft carapace and plastron. Bacteria, parasites, and fungi were conclusively excluded. History did not reveal any indications for a nutritional or environmental cause. All tortoises had free access to a large outdoor run covered with green vegetation, used the same containers for food and water and stayed in the same large enclosure at winter time. Only two species (*T. graeca* and *G. elegans*) of a multi-species collection (co-mingled with *Geochelone pardialis, Testudo hermanni, Testudo marginata*) were affected making nutritional and management factors unlikely as those would have probably affected a larger variety of species.

Virus characterization

The frequently isolated virus from organs and swabs possesses properties that are indistinguishable from viruses of the family Picornaviridae (11–13, 23).

In our study it was possible to isolate a virus with characteristics of Picornaviridae (provisionally named "virus X") from all 64 investigated organ homogenates and 79 of 104 swab samples of all eight examined tortoises (seven *Testudo graeca* and one *Geochelone elegans*) displaying a soft plastron and carapace. Chloroform treatment of infectious supernatant fluids did not prevent virus replication in TH-1 cell cultures demonstrating a non-enveloped virus (12, 17, 18). Incorporation of IUdR in the cell culture medium did not inhibit the replication of our viruses. Addition of actinomycin D to the cell culture medium seems to enhance the obtained virus titres by an unknown pathway. However, these numerical differences are not statistical significant (t test, data not shown). Taken these results together, we conclude that virus X is a single-stranded RNA virus (12, 16).

To our knowledge, a virus possessing these attributes was never described as a cause of softening of carapace and plastron. Therefore it can be considered as a novel virus of the family Picornaviridae. However, Marschang (16) and Marschang and Rümenapf (17) described a virus that was isolated from pharyngeal, conjunctival and cloacal swabs of 59 adult chelonians. It is currently not clear if this virus is different from the one described above, because direct comparisons were not yet possible. Cross neutralization tests performed in the future will provide information on antigenic homologies or differences.

Correlation renal disease – metabolic bone disease – viral infection

Metabolic bone disease, also called osteodystrophy, is an umbrella term for systemic defective bone formation. According to their characteristic features and causes, four classic subtypes are distinguished: osteoporosis, osteomalacia (adult individuals), rickets (juvenile individuals), and fibrous osteodystrophy. Clinically, mixed

Table 5
Tests for pH-stability of two selected isolates.

Tab. 5
Ergebnisse der pH-Stabilitätsprüfung von zwei ausgewählten Isolaten

Isolate Accession No. and organ	Titres (log_{10} KID_{50} per ml) following exposure at				
	pH = 3	pH = 5	pH = 7	pH = 12	Control
Acc. No. 1243/37 tongue	6.5	6.5	6.5	2.5	7.1
Acc.-No. 402/1 kidney	7.0	7.0	7.0	3.0	7.3

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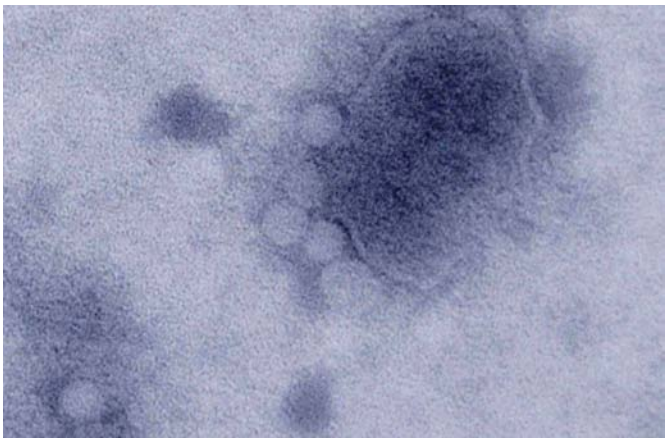

Fig. 12 Electron microscopic appearance of a group of small, round viral particles of isolate 1243/37 obtained from a tongue of *Testudo graeca*; on the left side viral particles close to cellular debris (right).

Abb. 12 Elektronenmikroskopisches Bild einer Gruppe kleiner, runder Viruspartikel des Isolats 1243/37 aus der Zunge von *Testudo graeca*. Links Viruspartikel, rechts dicht daneben Zelldebris.

Table 6 Results of virus neutralisation tests in TH-1 cell cultures with isolate 1243/37 and blood serum from 19 adult healthy tortoises.

Tab. 6 Ergebnisse der Neutralisationstests in TH-1-Zellkulturen mit dem Isolat 1243/37 und Serumproben von 19 erwachsenen, gesunden Landschildkröten

Accession Code	Species	Sex	Titre*
Z-06	*Testudo marginata*	F	5
OD-06	*Testudo marginata*	M	< 2
Gl-06	*Testudo marginata*	F	4
A-06	*Testudo graeca*	F	5
S-06	*Testudo graeca*	F	3
L-06	*Testudo graeca*	M	8
R-06	*Testudo graeca*	F	3
P-06	*Testudo graeca*	M	8
Qu-06	*Testudo hermanni (h.) boettgeri*	M	< 2
Joh-06	*Testudo h. boettgeri*	M	3
Iv-06	*Testudo h. boettgeri*	F	4
B-06	*Testudo h. boettgeri*	F	4
E-06	*Geochelone elegans*	F	5
M-06	*Geochelone elegans*	M	4
K-06	*Geochelone sulcata*	M	6
ET-06	*Dipsochelys hololissa*	M	4
881/1	*Geochelone carbonaria*	M	3
881/2	*Geochelone carbonaria*	F	4
881/3	*Geochelone carbonaria*	F	5

* All titres are expressed as $\log_2$ values.
M = male; F = female

forms of all four forms may occur making a clear diagnosis often difficult (15, 26).

Osteoporosis is characterized by a reduced bone mass resulting in thin, fragile bones. In contrast, the key feature of osteomalacia and rickets are soft bones caused by decreased bone mineralisation due to vitamin D and phosphorous deficiency. Predominant histologic findings include avascularity, accumulation of osteoid seams, thickening of the growth plates and failure of enchondral ossification with broadening and disorganization of the zone of proliferation (▶Fig. 5, ▶Fig. 6).

The term fibrous dystrophy refers to decreased bone mass due to resorption and replacement by fibrous tissue resulting in a rubber-like consistency of the bone. Hyperparathyroidism of various etiologies is the underlying pathophysiology of fibrous dystrophy (19).

The concurrent findings of a nephropathy (▶Fig. 7, ▶Fig. 8) and fibrous osteodystrophic changes in the skeleton and carapace suggest renal fibrous osteodystrophy as underlying cause. The observed hypoplastic anaemia substantiates renal dysfunction, as it likely represents a direct consequence of impaired erythropoietin synthesis. Just as in mammals, erythropoietin production in reptiles takes place in the kidney (24). Decreased red blood cell production is the most common etiology of hemosiderosis, as seen in the liver of the affected tortoises (21) (▶Fig. 9).

The observed viraemia in the course of clinical disease and the fact that kidney homogenizates besides those from brain and tongue produced high titres on TH-1 cell cultures support a direct correlation between viral infection and nephropathy with subsequent osteodystrophic changes. Further studies have to prove, whether the intracytoplasmic eosinophilic granules found rarely in the proximal tubular epithelial cells are directly correlated with the viral infection (▶Fig. 7, ▶Fig. 8). The histologic findings correspond well to the typical signs of renal secondary hyperparathyroidism, which are described in literature as a mixture of both osteodystrophia fibrosa and rickets/osteomalacia (4, 15).

Chronic kidney disease as cause of secondary hyperparathyroidism is a well known condition both in human and veterinary medicine (15). Subsequent metabolic bone disease consists of fibrous osteodystrophy, osteomalacia/rickets or a combination of both. In small animal medicine it is most commonly seen in dogs (clinically) presented with bone pain, loss of teeth and deformities of facial bones. The pathogenesis of renal osteodystrophy is still not fully understood, but thought to be based on two main pathways. Firstly, reduced production of 1-25-dihydroxy-cholecalciferol (the active metabolite of vitamin D) and impaired mineralization due to uremic and uricemic acidosis are responsible for the onset of rickets/osteomalacia. Clinical manifestation varies with the availability of dietary vitamin D and the severity and type of renal disease. Secondly, the pathogenesis of osteodystrophia fibrosa is initiated by phosphate retention due to decreased renal excretion. Subsequent hyperphosphatemia leads to an increased level of calcium-phosphor compounds. At the stage of saturation calcium is precipitated in soft tissue resulting in hypocalcemia, which

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triggers production and release of parathyroid hormone (PTH) (26). In bone tissue, PTH stimulates osteoblasts to increase osteoclast differentiation factor synthesis and to decrease the production of osteoprotegerin. Additionally, bone marrow stromal cells have receptors for PTH and transform into fibroblasts under permanently high PTH levels. Therefore, the key histologic feature of chronically high PTH levels is proliferation of fibrous tissue into the marrow cavity and vascular spaces in the bone (▶Fig. 5, ▶Fig. 6). Sometimes this proliferation obliterates the original cavities completely (26). Additionally, fibroblastic activity can be seen in germinal layers such as periost and the zone of proliferation in the growth plate (▶Fig. 5, ▶Fig. 6).

Changes in the cortical bones are characterized by osteoclastic bone resorption, usually starting at the endocortical bone surface, and replacement by fibrous tissue (▶Fig. 6). In areas with increased mechanical stress such as maxilla and mandible (i.e. chewing stress) fibrous proliferation can exceed normal dimensions of the original bone resulting in conspicuous deformations and enlargements (▶Fig. 4).

The carapace of juvenile terrestrial turtles should have reached full strength and stability by the age of approximately 6 months (7). As the animals in this study were only several weeks of age, it is conceivable, that embryonic fibrous tissue never had undergone proper ossification from the very beginning. This would be consistent with a juvenile hypoplastic osteoporosis (7). However, the clinical findings of a softening after a period of normal development and the histologic findings of prominent fibrous proliferation suggest a different pathogenesis of remodelling in our case. Dilated urinary sacs and kidney congestion have also been described (7). It was assumed that lowering of the soft carapace and approximation to the plastron constricted the cloacal area and resulted in a postrenal obstructive uricemia. It is conceivable, that these mechanisms also occurred in the tortoises of this study exacerbating the already existing nephropathy.

No evidence was obtained during the extensive histopathological examinations for a likely presence of intranuclear coccidia in the kidney that can cause lesions and dysfunctions.

By excluding other possible factors as cause of the clinical symptoms in young tortoises, it seems likely that the isolated picornavirus is indeed the causative agent of the described disease in young tortoises by damaging the kidney and jeopardizing the calcium-phosphor metabolism. The type of lesions, the preference of young age and the restricted host range for only two chelonian species make this "syndrome" unique among tortoise diseases. Heavy, but unspecified losses among young tortoises were recorded by the breeder. It is noteworthy that a few *T. graeca* and *Geochelone elegans* contained neutralizing antibodies in their circulating blood (▶Table 6). Unfortunately, the time of first infection of the adult seropositive tortoises remains unknown to us. Therefore, firm statements are not possible whether these animals recovered post early infection or were infected at later life.

Conclusion for practice

Soft carapace, plastron and skeletal abnormalities can be the result of inadequate nutrition and poor husbandry conditions. However, similar lesions observed in our study most likely developed due to an infection by a hitherto unrecorded picornavirus. Therefore, in the absence of non-infectious causes virological investigations are required to detect or to exclude a viral aetiology of the described disease in juvenile tortoises of *Testudo graeca* and *Geochelone elegans*.

Additionally, some individuals of species other than *T. graeca* and *G. elegans* were obviously infected, did not develop signs but contained neutralizing antibodies (▶Table 6). Further studies into the prevalence of this virus in different collections of tortoises and transmission experiments fulfilling Henle-Koch's postulates are necessary to substantiate our assumption.

Declaration of conflict of interests

The authors declare the absence of any form of conflict of interests. No financial or any other external resources were involved in the conduct of this study.

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Rezension

Taschenatlas Akupunktur bei Hund und Katze

Mit diesem Buch gibt die Autorin Christina Eul-Matern bereits die 2. Auflage des 2010 erstmals erschienenen gleichnamigen Werkes heraus. Die Ausgabe wurde vollständig überarbeitet. Kleinere Fehler gegenüber der ersten Auflage wurden ausgeräumt.

In neun Kapiteln werden in einer übersichtlichen Einführung die Grundlagen der Akupunktur besprochen. Wichtige Kapitel wie Prinzipien der TCM, die Meridiane, Diagnostik, Akupunkturpunkte, Punktauswahl, Punktfindung und Nadelung sowie Arten der Akupunktur geben dem Therapeuten einen kurzen, effizienten Überblick. Der Hauptteil des Buches, der Punkteatlas, bietet eine ausführliche und vollständige Übersicht über alle Punkte der Meridiane. Hierbei werden systematisch und sehr übersichtlich Lokalisation, Indikation und vor allem auch die Wirkung nach TCM erklärt.

Das Buch eignet sich sowohl für den Einsteiger als auch für den routinierten Anwender. Ein praktisches Nachschlagewerk für Akupunkturpunkte beim Kleintier. Alles in allem, ein hervorragendes, gut strukturiertes, empfehlenswertes Werk, das jeder besitzen sollte, der Akupunktur an Hund und Katze durchführt.

Angelika Dura, Langgöns

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