

High Mortality Associated with Thyroid Hyperplasia in European Pond Turtles, *Emys orbicularis* (L., 1758) (Emydidae) in a Breeding Facility at the Ebro Delta, NE Spain

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Abstract: An outbreak of mortality in a breeding facility of European Pond Turtles (*Emys orbicularis*) in the Ebro Delta (Tarragona, Spain) occurred in 2014–2015. The breeding program began in 2006 and has generated up to 250 turtles every year. They are released when they are 4 to 5 years old. After a change of the diet of the turtles born during 2014–2015, a mortality of 91% (334 out of 366 animals) was calculated for the hatchlings from 2014. Clinical signs were soft shells, apathy and anorexia approximately 1–5 days before death in all affected turtles. Histologically, thyroid follicles were lined by hypertrophic columnar epithelium and follicular lumina were mostly devoid of colloid. Kidneys were also affected, showing mild to moderate acute degeneration and tubular necrosis with mineralization of tubular epithelium. *Stenotrophomonas maltophilia* was isolated in pure culture from tissue pools of tested animals. PCRs sensitive for detection of ranavirus, herpesvirus and coccidia (spp.) were all negative. Thus, we conclude that the high mortality outbreak was associated with a thyroid gland hyperplasia and renal malfunction due to a change in diet, complicated with an opportunistic secondary bacterial infection.

Key words: Emydidae, aquatic turtle, thyroïdal disease, outbreak, renal disease, mortality

Introduction

The European Pond Turtle, *Emys orbicularis* (Linnaeus, 1758) (Testudines: Emydidae) is a turtle widely distributed throughout Europe, North Africa, and Asia Minor. The population in Spain is considered endangered in several parts of its range and the species is protected by the Spanish law. Despite that in the whole range it is considered “Near Threatened”, the Spanish IUCN Category considers the species as “Vulnerable”, and the north-eastern populations are considered “Endangered” (AYRES 2015). Main threats are habitat loss, degradation and fragmentation, illegal capture of specimens for the pet market (VILARDELL 2015), as well as introduction of exotic species (MARTÍNEZ-SILVESTRE et al. 2006).

In 2006, a breeding facility focused on the recovery of this species was established in the Ebro Delta (Tarragona, northeastern Spain). The scope of this program has been breeding of turtles of haplotype V, after a genetic selection of individuals that followed the most recent data (STUCKAS et al. 2014). These animals are intended for reintroduction in their original distribution area, especially in the natural park of the Ebro Delta (MARTÍNEZ-SILVESTRE 1999). The breeding program started in April 2006 and has generated approximately 250 turtles every year (VILARDELL et al. 2013). Since the start of the project, the usual diet of these animals consisted of commercial pellets for aquatic turtles combined with mosquito larvae. For

economic and employment reasons, it was decided to make a change in diet in early 2014. The new diet consisted of food prepared in the facility, including various kinds of fish and shellfish and had originally been prepared for sea birds in the centre. No other diseases were detected in this facility before the change of the diet. Six months later, a case of mass mortality was observed, and after this outbreak the research started as is shown in the present article.

Materials and Methods

From late October 2014 to mid-February 2015, a progressive and increasing mortality among freshly hatched to two-year-old turtles started. When the mortality was very high (in rapport with the regular year baseline mortality), the park authorities decided to activate clinical measures in order to stop it. A mortality of 91% (334 out of 366 animals) was calculated for the 2014 hatchlings; 10 to 30 turtles died per week. Clinical signs were acute detection of soft shells, apathy and anorexia, approximately occurring 1–5 days before death.

Post-mortem examinations of four recently deceased turtles were performed. In each turtle the thyroid and parathyroid gland, thymus, heart, stomach and intestines as well as liver and kidney were collected and fixed in 4% neutral buffered formalin, trimmed, sectioned at 3–4 mm, and stained with haematoxylin and eosin. Von Kossa staining was used to demonstrate calcium deposits in the renal tubules.

Moreover, these turtles were analyzed for infectious agents through microbiological cultures, including antibiograms of two selected animals with clinical signs. Samples of three more animals were collected and were tested at Laboklin Laboratories (LABOKLIN GmbH & Co. KG, Germany) for the presence of coccidia, herpesvirus and ranavirus by means of PCR.

Pending the outcome of the results, a preventive antibiotic treatment was started with enrofloxacin (dose: 5 mg/Kg s.i.d.), isolation of the affected animals and return to the diet prior to the outbreak (commercial pellets and mosquito larvae).

Results

At necropsy, all turtles had enlarged thyroid glands (about 2–3 times the normal size; Fig. 1).

Histologically, similar changes were seen in all four animals. Thyroid follicles were lined by hypertrophic columnar epithelium and follicular lumina were mostly devoid of colloid (Fig. 2). In the kidneys, mild to moderate acute degeneration and tubular necrosis with mineralization of tubular epithelium was seen in all examined animals (Figs. 3, 4).

The conducted PCRs were all negative. *Stenotrophomonas maltophilia* was isolated in pure culture from the liver, kidney and intestines of two animals tested. Antibiogram (resistogram) showed that bacteria were (a) sensitive to quinolones (Enrofloxacin, Marbofloxacin, Pradofloxacin)



Fig. 1. One-year-old *Emys orbicularis* with enlarged thyroid glands (arrows). Photo courtesy of A. Martínez-Silvestre

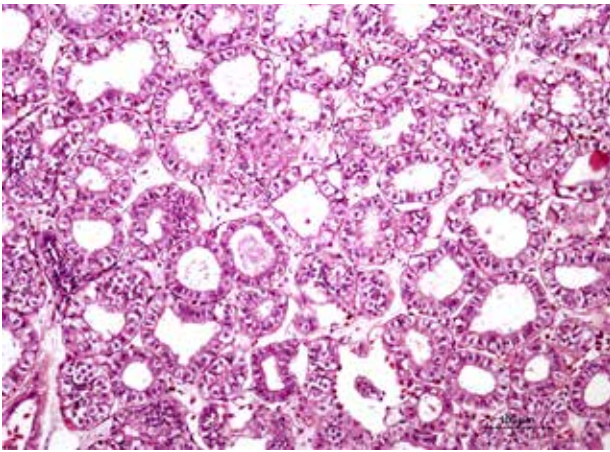


Fig. 2. Thyroid of *Emys orbicularis* hatchling, stained with Haematoxylin/Eosin. Diffuse follicular epithelial hypertrophy and hyperplasia are visible. Only a few follicles have colloid production in their lumens. Photo courtesy of Roser Velarde

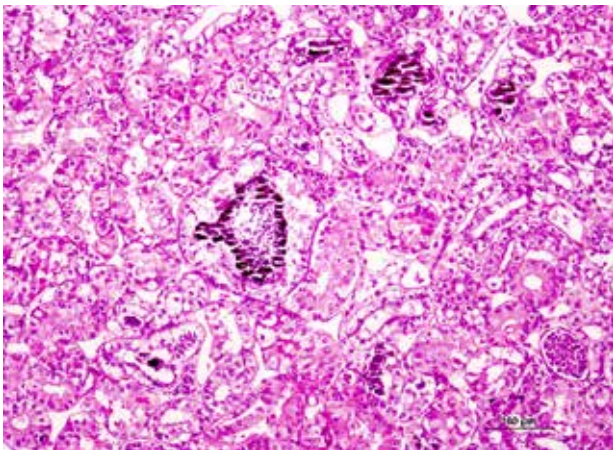


Fig. 3. Kidney. Haematoxylin/Eosin stain. Showing inflammatory tissue around the calcinosis. Photo courtesy of Roser Velarde

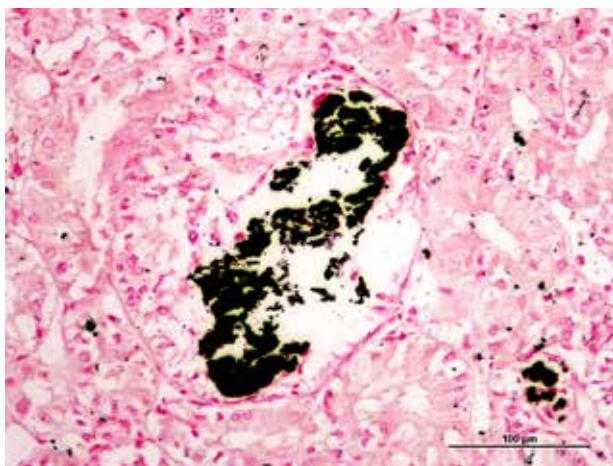


Fig. 4. Kidney. Von Kossa stain showing multifocal tubular calcinosis (black). Photo courtesy of Roser Velarde

and sulfonamide + trimethoprim; (b) intermediate to: Orbifloxacin, Ibafloracin; and (c) resistant to: Penicillin G, Ampicillin, Amoxicillin, Amox + clavulanic acid, Cefalexin, Cefoxitin, Cefoperazone, Cefovecine, Cefquinome, Erythromycin, Spiramycin, Clindamycin, Lincomycin, Difloxacin, Tetracycline, Doxycycline, Nitrofurantoin and Rifampicin.

After a change from the homemade diet to the commercial diet, providing fluid therapy and antibiotic therapy, the problem started to resolve in several weeks. Enrofloxacin was applied intramuscularly, once a day, for 2 weeks to 20 sick turtles. The outbreak was resolved five weeks after the diagnosis. One year after the treatment of the causes of this high mortality event, with 30 survivors and with a current population of 219 newborns, mortality due to this cause has not been repeated.

Discussion

The high mortality outbreak was associated with a thyroid gland hyperplasia, renal malfunction and opportunistic secondary bacterial infection as a result of change in diet and high concentrations of turtles in the enclosures, which facilitated the spread of bacteria.

It is also important to have in mind that high nitrogen levels are very well described as goitrogenic for most animals (RIBERA & LOCK 2008), and a low exchange rate of the water within the tanks could be involved. Turtles may have problems when they are exposed for prolonged time to an environment with high nitrogen and ammonia levels especially when raised in captivity.

However, in our case water exchange and maintenance followed strict sanitary protocols already described (JACOBSON 1999) and no previous symptom of mortality was detected since the operation of the breeding facility after the protocol was implemented.

Although we would have greatly benefited by being able to determine iodine or sodium content in the blood or tissue of the diseased individuals in order to detect the renal or thyroid disease *in vivo*, the really small size and weight of turtles impeded the extraction of the minimum amount of blood required for the procedure. Weight of the turtles was no more than 4 g and following the general rule for a single blood extraction (not to take more than 5–10% blood volume) the sampling could not be performed (MACEDA et al. 2015).

Excessive iodine causes malfunction of the digestive tract, the kidneys and the skin in animals

such as mammals, but we did not notice these problems. The only symptom was the soft shell 1–5 days before death. Actually, due to the low mineralization of the carapace in turtle hatchlings, the hardness of it is actually just a feeling of the internal pressure. The detection of soft shell in hatchlings less than two years of age is a feeling of softening by decreasing the internal pressure (due to the disappearance of the yolk sac and internal fat) after long periods of weakness and thinning. In aquatic turtles, when ambient and internal nitrogen levels reach a certain threshold the animals may use minerals from the body to create urates (potassium, calcium) internally to protect their organs, which can contribute to weight loss and mineral deficiency and subsequently to the soft shell syndrome (DANTZLER 1976, WANG & NIU 2008). In terrestrial tortoises, the soft shells of juveniles are also involved in their more rapid loss of body water and increased susceptibility to dehydration during droughts, compared to adults (ZNARI et al. 2012). Thus, the soft shell is a nonspecific sign that is not directly related to any particular pathology (NAGY et al. 2011). Nutritional, congenital, metabolic, bacterial or even viral diseases can be related to the soft shell syndrome in terrestrial tortoises (HEUSER et al. 2014). Accordingly, in all cases with soft shell, additional examinations to identify the origin of that multi-factorial syndrome must be performed. In our case, thyroid and renal histopathology were determinant in diagnosing the problem.

Thyroid function is very important particularly in young reptiles during their growing (RIVERA & LOCK 2008; BOYER 2011). Thyroid diseases are especially well documented in tortoises attaining large size such as the African Spurred Tortoise *Centrochelys sulcata*, often due to hypothyroidism (FRANCO et al. 2009, FRANCO & HOOVER 2009). However, in our case the observed lesions can also be explained by hyperthyroidism. The histological lesions were consistent with hyperplastic goitre and included narrowing of follicular lumen, reduction or absence of colloid, and both hypertrophy and hyperplasia of follicular epithelium. The possible pathogenic mechanism include iodine-deficient diet, goitrogenic compounds that interfere with thyroid hormone synthesis, dietary iodide excess, and genetic enzyme defects in the biosynthesis of thyroid hormones (CAPPEN 1993). Similar outbreaks have been described in young captive born mammals and birds breeding programs (GARNER et al. 2002, LOUKOPOULOS et al. 2015). In *E. orbicularis*, the only European turtle whose habitat extends to brackish waters (AYRES 2015), iodine deficiency problems have not been observed until today, because this element is provided in their usual diet. However, in captive

conditions the delicate balance between food source and growth needs cannot always be maintained. In the present case, considering the recent changes in diet, we have enough evidence to believe that it was a problem with excessive amount of iodine, which allowed the emergence of the problem. The received diet was composed by a homemade cake with fish and mussels used in feeding sea birds recovering in the same centre. While the diet did not contain goitrogenic plants as broccoli, mussels have high iodine content (SANTOS et al. 2014). To correct the problem, this homemade diet was replaced by a diet based on commercial feed (pellets) for aquatic turtles.

Local bacterial infections, parasitic diseases and systemic illnesses are described in other aquatic turtles and in the European Pond Turtle even in natural habitats (AYRES & ACUÑA 2013, ALEKSIC-KOVACEVIC et al. 2014, MERCHAN & MARTÍNEZ-SILVESTRE 1999).

Histologically, no evidence of a systemic bacterial infection was detected. However, this cannot be ruled out because acute infections can lack histological lesions. In our case, in order to confirm the presence of sepsis, the culture of three organs was fulfilled in both animals analyzed. The systemic infection present in these animals can be considered as an opportunistic infection complicating a previous pathological underlying condition. Secondary infections to a primary disease are common in turtles and they should be treated preventively to avoid aggravating the main pathology (JACOBSON 1999). The bacteria isolated in this study had been described as possible complications in cases of sepsis in immunocompromised patients (ABBOTT et al. 2011). These bacteria have been described as a cause of septicaemia in the Dwarf Crocodile (*Osteolaemus tetraspis*) (HARRIS et al. 2001). Accordingly, after the isolation of *Stenotrophomonas maltophilia* in the organs of two captive dead animals, the application of a preventive treatment of live animals was carried out. The enrofloxacin dosage remained at low doses to avoid the risk of complicating renal malfunction diagnosed in necropsies.

Despite *E. orbicularis* being a species that frequents brackish environments, affected turtles were introduced in isolated containers with fresh and clean water to promote proper hydration and rapid renal recovery, as this is one of the best hydration systems in aquatic turtle.

We can conclude that the European Pond Turtle has strict feeding requirements in the first two years of life and variations in the composition of its food can provoke outbreaks of mortality. More research is necessary to know the first years' growth and the nutritional and thyroid requirements of *E. orbicularis*.

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