HERPETOLOGICAL JOURNAL, Vol. 13, pp. 161-165 (2003)

HYPERBILIVERDINEMIA IN THE SHINGLEBACK LIZARD (*TILIQUA RUGOSA*)

MARCELLO PENNACCHIO[1], MICHAELA BOGYI[1], LIKHIM TEH[1] AND EMILIO L. GHISALBERTI[2]

[1]*Department of Environmental Biology, Curtin University of Technology, GPO Box U1987, Perth, WA 6845, Australia*

[2]*Department of Chemistry, The University of Western Australia, Nedlands, WA 6907, Australia*

Green pigmentation in the serum of shingleback lizards (*Tiliqua rugosa*) was the result of an excess of the bile pigment biliverdin (hyperbiliverdinemia). This was confirmed by comparing the absorbance spectrum of the affected serum with that of commercial biliverdin, using TLC and acidification with both nitric and sulphuric acid. The average content of biliverdin in animals with hyperbiliverdinemia was 2.52±0.15 mg/100 ml. Significant changes in the packed cell volume, haemoglobin content, blood glucose levels, body mass and levels of erythropoietin were also observed in animals with this form of green jaundice. Interestingly, significant erythrocyte degeneration, especially in the stroma area of the red blood cells, appears to result in a significant release of haemoglobin into the blood serum, which may account for the excess levels of biliverdin. Changes in the haematology of shingleback lizards are discussed along with the probable cause for hyperbiliverdinemia.

Key words: bile pigments, biliverdin, jaundice, shingleback lizards

INTRODUCTION

Green blood pigmentation has been reported for a variety of animals, including several species of fishes (see Fang & Bada, 1990 for a review); butterflies, moths (Kayser, 1985) and other insects (Law & Wells, 1989); frog eggs (Marinetti & Bagnara, 1983); lizards (Greer & Raizes, 1969), including skinks of the genus *Prasinohaema* of Papua New Guinea (Austin & Jessing, 1994); bird egg shell (Fox, 1976); dog placenta (Fox, 1976) and humans (Greenberg *et al.*, 1971). In all of these animals, the green colour was due to an excess of the bile pigment, biliverdin (hyperbiliverdinemia).

Biliverdin is a bilatriene compound produced during the metabolism of the haeme portion of haemoglobin (Britton, 1983). In most higher vertebrates, this transitory intermediate metabolite is rapidly oxidized into the more toxic bilirubin (Cowger, 1974). Reptiles, amphibians and birds lack the enzyme required for this process (biliverdin reductase) and therefore do not produce the latter compound. An increase in either of these bile pigments results in the pathological condition known as jaundice in most vertebrates.

While collecting blood from shingleback lizards (*Tiliqua rugosa*, Family Scincidae) for another study, it was noticed that the serum, normally pale yellow in colour, was green (Pennacchio, 2001). In this paper, we identify the pigment responsible for the abnormal coloration in the serum of *T. rugosa* (also referred to as *Trachydosaurus rugosus,* Cogger, 2000) and offer some explanation for the excess levels seen.

Correspondence: M. Pennacchio, Department of Environmental Biology, Curtin University of Technology, GPO Box U1987, Perth WA 6845. *E-mail:* M.Pennacchio@exchange.curtin.edu.au

MATERIALS AND METHODS

ANIMALS

Seven shingleback lizards, from the field trial area (FTA) at Curtin University of Technology's Department of Environmental Biology, were trapped using baited Sheffield traps and were housed together with shingleback lizards that once had hyperbiliverdinemia. A preliminary study had revealed that shingleback lizards kept with those previously affected by the condition also developed it. A permit to capture, collect and keep shingleback lizards was approved by the Department of Conservation and Land Management (CALM permit No. SF003566). The animals were fed and watered *ad libitum* and were maintained in an outdoor enclosure where they had access to sunlight.

BLOOD EXTRACTION

Blood (0.2 ml) from shingleback lizards was obtained directly from the ventricle of the heart (heart puncture). This method was approved by Curtin University of Technology's Animal Experimentation and Ethics Committee (Approval No. N12/2001) and has been used extensively and successfully elsewhere without harming the animals.

Once collected, the blood was immediately transferred to sterile, heparinized Vacutainer® vials and was subsequently divided into smaller samples. A small volume was centrifuged in a Hawksley micro-haematocrit centrifuge to determine the packed cell volume (% red blood cells and % white blood cells). The haemoglobin content in whole blood and serum was determined using two separate HemoCue haemoglobin analysers, (blood haemoglobin and plasma/low) with HemoCue self-filling micro-cuvettes. Blood glucose levels were measured using a MediSense glucose analyser while levels of erythropoietin (EPO) were measured with a commer-

cially-available kit (EPO.96) obtained through MD Biosciences. Blood smears were air-dried and stained using a commercially available kit (Harlequin DiffQuick). Photographs of relevant blood smears, to be used in determing the percentage of RBC as well as the extent and nature of the damage to them, were taken using an Olympus Vanox microscope (model AHBS-513). The degeneration of erythrocytes was compared to similar damage reported in De V. Pienaar (1962). The remainder of the blood was centrifuged at 3000 RPM for five minutes to separate the formed elements from the serum. The serum was retained for spectrophotometric analyses used to detect the presence of biliverdin.

SPECTROPHOTOMETRIC ANALYSES

The presence of biliverdin in serum was confirmed using four chemical tests (Fang, 1982). The first test involved the spectrophotometric analyses and comparison of green serum with normal pale yellow serum and pure biliverdin purchased from ICN Chemicals. Absorbance spectra, ranging from 350 nm to 800 nm, were measured using a Pharmacia Biochrom 4060 UV/Visible spectrophotometer. A standard curve, using commercial biliverdin, was prepared to determine the concentration of biliverdin in the green serum.

THIN LAYER CHROMATOGRAPHY

Retardation factors (R_f) of pure biliverdin and blue-green serum were compared using thin layer chromatography (TLC). Small volumes of serum and commercial biliverdin were placed onto an aluminium TLC plate coated with silica gel (Merck 60 F_{254}) and prewashed with methanol to remove water. Each TLC plate was then partially immersed upright in a chamber with a 2:1:1.5 butanol:methanol:water mixture until sufficient separation had occurred.

ACIDIFICATION

The third and fourth tests involved acidification of serum with concentrated nitric acid and sulphuric acid, respectively. In the first of these two tests, the Gmelin reaction, plasma proteins were removed from the serum by precipitation with ammonium sulphate (55 % w/v). This mixture was centrifuged at 3000 rpm for five minutes, after which the supernatant was recovered. Concentrated nitric acid was slowly added to the supernatant and observed for any visible changes in colour.

A small volume of concentrated sulphuric acid was then added to a separate sample of serum, which was gently heated. This process destroys biliverdin, but does not affect its isomer, mesobiliverdin (Fang, 1982).

DATA ANALYSIS

Paired *t*-tests were performed on the data to determine differences in means between the shingleback lizards with and without hyperbiliverdinemia, using SPSS (v. 10.0). All data in the form of percentages were arcsine transformed prior to comparing means to ensure normal distribution (Zar, 1984). All results are presented as means ± SE. The number of animals used was seven unless otherwise stated.

RESULTS

The green pigmentation in the serum of all seven of the shingleback lizards was the result of an excess accumulation of the bile pigment, biliverdin. A comparison of the absorbance spectra of sera with green pigmentation and with commercially available biliverdin revealed that the spectra were almost identical. Both sample types resulted in two bands, one of which occurred in the 380 and 450 nm range (normal for shingleback lizards), and a broader band at the 640-665 nm range (Fig 1). Normal pale- yellow shingleback- lizard serum lacks the broader band, which is characteristic of biliverdin (Fig. 1).

The absorbance spectra of sera with the green pigmentation were then compared to absorbances in the standard curve to determine the concentration of the bile pigment in each blue-green sample. The average concentration was 2.5±0.2 mg/100 ml during severe episodes of hyperbiliverdinemia (Table 1). The presence of biliverdin was also revealed by TLC. The R_f of the green sera and that of the pure biliverdin were identical. All plates were left overnight to allow for adequate separation.

The Gmelin reaction revealed that the supernatant of sera treated with ammonium sulphate immediately changed colour from green to yellow upon acidification with concentrated nitric acid. This also suggested that the green pigment in the supernatant was in fact biliverdin. Acidification of green serum with concentrated sulphuric acid resulted in complete discoloration, indicating that the pigment was indeed biliverdin and not its isomer mesobiliverdin.

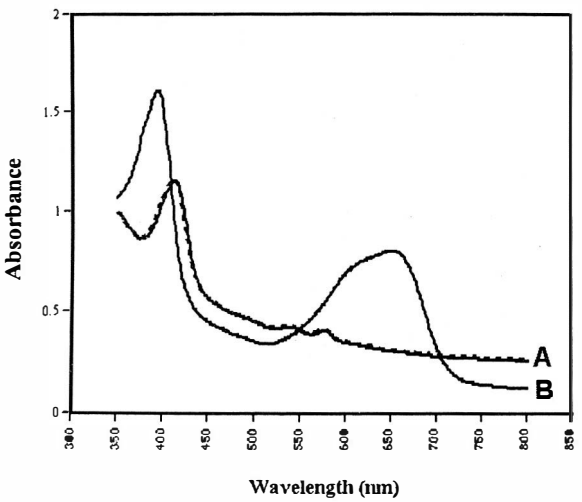

FIG. 1 Absorbance spectra of normal shingleback lizard serum (A) and green-pigmented serum with hyperbiliverdinemia (B)

TABLE 1. Changes in the haematology and other parameters of shingleback lizard with and without hyperbiliverdinemia. Key to table: Hyper, hyperbiliverdinemia; Damage, percentage of damage to erythrocytes seen in blood smears; RBC, red blood cells (erythrocytes); WBC, white blood cells (leucocytes); Hb, haemoglobin content of whole blood; LHb, low haemoglobin content of serum; EPO, erythropoietin.

	Damage (% RBC)	Biliverdin (mg/100 ml)	RBC (%)	WBC (%)	Hb (g/dL)	LHb (g/L)	EPO (mU/L)	Glucose (mmol/L)	Body Mass (g)
Normal	0.0±0.0	0.0±0.0	18.6±1.4	1.0±0.3	7.8±4.5 (n=4)	0.0±0.0	1.4±2.2 (n=4)	7.9±2.4	378.1±126.0
Hyper	31.8±11.2	2.52±0.2	11.3±1.6	3.1±0.9	3.9±1.5 (n=4)	1.8±0.6	32.3±0.0 (n=4)	5.7±1.7	327.7±103.6

The packed cell volumes of both the normal and green-pigmented blood were also compared (Table 1). There was a significant difference (P=0.000) in the percentages of white blood cells (WBC; leucocytes) between the two groups. Up to five times as many WBC were observed in the blood of some affected animals. The average percentage of leucocytes was, however, 1.0±0.3% at the start and 3.1±0.9% during bouts of severe hyperbiliverdinemia. Erythrocytes, which averaged 18.6±1.4% in normal animals, decreased significantly (P=0.003; Table 1) to 11.3±1.6% in animals with hyperbiliverdinemia. Interestingly, there was a significant (P=0.008) increase in the RBC percentage during the first two weeks, when it increased to 24.2±1.7%. This occurred prior to the animals developing hyperbiliverdinemia.

Shingleback lizards with hyperbiliverdinemia also exhibited a significant (P=0.002) degeneration of erythrocytes (Table 1). An average of 31.8±11.2% of all erythrocytes seen in blood smears derived from affected animals was associated with intraerythrocytic inclusions (albuminoid vacuoles). These included extensive aniso- and poikilo-cytosis, as well as cellular distortion and cytolysis of the stroma. Coinciding with the destruction of erythrocytes were significant increases in biliverdin levels (P=0.001), as well as significant decreases in haemoglobin content in the sera (P=0.003; Table 1) and EPO levels (P=0.019; Table 1). The haemoglobin content in whole blood decreased significantly (P=0.016; Table 1). A number of phagocytic lymphocytoid azurophils (WBC), which had ingested damaged erythrocytes, were clearly visible in some blood smears.

Finally, there was a significant decrease in both body mass and blood glucose levels of shingleback lizards with excess biliverdin. These decreased from 378±126.0 g to 327.7±103.6 g (P=0.034) for body mass and 7.9±2.4 mmol/L to 5.7±1.7 mmol/L for blood glucose levels (P=0.002). Most shingleback lizards presented normal haematology after developing hyperbiliverdinemia, but it was not clear precisely how long the condition lasted and why it soon recurred in some of our animals. This is currently the focus of another study.

DISCUSSION

All four of our tests confirmed that the distinct green serum of the seven shingleback lizards was in fact due to excess biliverdin. Researchers have proposed a number of hypotheses to account for green jaundice in animals. Yamaguchi & Hashimoto (1968), for example, reported that hyperbiliverdinemia appears to help with lipid transport in some species and may protect them from UV rays (Yamaguchi *et al.*, 1976). Low & Bada (1974) reported that the condition assists with cryptic coloration in some animals, as it may manifest itself in the green coloration of their exteriors. Schwalm *et al.* (1977) and Emmerson *et al.*, (1990), in contrast, have suggested that the green serum appears to confer advantages to thermoregulation for animals with excess

biliverdin. High levels of biliverdin may even make the animals "distasteful" to their predators (Austin & Jessing, 1994). Most of these hypotheses have not been seriously tested.

In contrast, hyperbiliverdinemia in shingleback lizards appears to be the result of erythrocyte degeneration (erythrolytic jaundice), resulting in a significant increase in the content of haemoglobin in the serum of affected animals. Similar findings were reported by Maeno *et al.* (1995) who recently suggested that erythrocyte destruction was responsible for increases in serum haemoglobin and bilirubin concentrations in jaundiced yellowtail fish (*Seriola quinueradiata*). Maeno *et al.* (1995) suggested that a bacterium was the likely causative agent.

The increase in WBC seen during episodes of shingleback-lizard hyperbiliverdinemia suggests that an immune response had taken place and that possibly a contagious parasite may be responsible for the destruction seen in their erythrocytes. This may also be inferred from the fact that the animals developed the condition upon contact with previously affected animals.

At first, Pirhemocyton-like infection of erythrocytes by viruses was suspected. This had previously been reported for a number of Australian lizards (Paperna & Alves de Matos, 1993) and other reptiles (Daly *et al.*, 1980; Alves de Matos & Paperna, 1993; Telford & Jacobson, 1993), as well as for frogs (Alves de Matos *et al.*, 1995) and ornamental fish (Paperna *et al.*, 2001). It was not clear what type of pathogen was responsible for the degeneration of shingleback- lizard erythrocytes.

The elevated EPO levels observed during the development of hyperbiliverdinemia may also be due to erythrocyte degeneration. It was not clear at this stage, but it is thought that EPO plays a role in the haematopoietic recovery of shingleback lizards with excess biliverdin levels. The kit used in these experiments was, however, for human EPO and therefore provides only limited evidence that the serum of shingleback lizards with hyperbiliverdinemia contains an immunoreactive erythropoietin-like molecule that,with an increase in damage to erythrocytes, results in the higher levels. A similar kit was used by Wickramasinghe *et al.* (1994) to provide evidence that teleost kidneys are erythropoietic-producing organs.

The significant decreases in body mass and blood-glucose levels are interesting but cannot be explained based on the data gathered in this study. The intake of food and blood glucose level decreases in shingleback lizards affected by hyperbiliverdinemia is currently the subject of a more detailed study into the long-term effects of the condition on the animals. The study also aims to determine the time it takes for animals to recover from the condition and why some later relapse. It is hoped that studies of this type will help with our understanding of hyperbiliverdinemia in shingleback lizards and with the pathology of jaundice in humans and other animals (Colleran & O'Carra, 1977; Fang & Bada, 1990).

ACKNOWLEDGMENTS

The authors wish to thank the Department of Conservation and Land Management (CALM) for giving us permission to collect, and take blood from, shingleback -lizard. E. G. Cockett is thanked for assisting with the capture of shingleback lizard and for extracting blood from them. Mr Ian Abercrombie, Mr Peter Mioduszewski, Mr Charles LaCoste, Ms Lydia Kupsky, Mr William Parkinson and Dr M. Monique Gagnon are also thanked for their assistance, as are Ms Ee Mok, Mr Jalal Volker and Mr Rowan Dawson. Finally, we would like to thank Mr George Newland for reviewing this manuscript and for providing useful comments.

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Accepted: 8.11.02