

## Pyrrolizidine alkaloid toxicosis and hepatic encephalopathy in horses in Easter Island, Chile

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**ABSTRACT.** A group of horses died in Easter Island following anorexia, weight loss and neurological signs. Similar cases have occurred since the introduction of a plant containing pyrrolizidine alkaloid to the island. This study describes the grazing behaviour of the horses and the potential correlation with the development of these horses clinical signs.

*Key words:* pyrrolizidine alkaloid, *Crotalaria* spp., hepatopathy, monocrotaline.

Easter Island (Rapa Nui) is located at 3,700 km west of the Chilean Pacific Coast (27°7'10''S 109°21'17''O), it has an area of 16,628 hectares and a population of over 2,300 horses (Montoya 2011). Extensive soil erosion was noticed in the late 1970's, and *Crotalaria grahamiana* was introduced by National Forest Corporation to prevent further damage to the landscape (Etienne *et al* 1982, Dubois *et al* 2013). The plant thrived and invaded the island, being described in the areas of Mataverí, Rano Kau, Vinapu, Rano Raraku and Poike (Dubois *et al* 2013), and since the early 1980's the islanders observed that horses (any age and sex) that consumed *C. grahamiana* eventually died from anorexia, weight loss and neurological signs, with locals referring to them as "crazy horses" (CH). Cases occur all year round with a peak of mortality rate during the summer (December to February) (Artz and Mount 1999). A case report on pyrrolizidine alkaloid (PA) toxicity in a horse in this island was reported by Artz and Mount (1999).

The consumption of plants containing PAs can cause intoxication and the development of clinical signs due to liver damage, neurotoxicity, cardiac and pulmonary problems in livestock and wildlife (Fu *et al* 2002). Also, genetic mutation and embryotoxicity were reported (Fu *et al* 2002, Stegelmeier 2011). In humans, veno-occlusive hepatic disease has been reported (Stegelmeier *et al* 1999, Fu *et al* 2002, Stegelmeier 2011).

The mechanisms of hepatotoxicity due to PA include multiple pathways such as activation of pyrrolic ester, which in turn is catalysed by the hepatic cytochrome P450 enzyme

system (CYP450), production of reactive oxygen species (ROS) and GSH-metabolising enzymes (Stegelmeier *et al* 2016, Yan *et al* 2016). PAs have been identified in over 6,000 plants of the genus *Senecio*, *Crotalaria*, *Heliotropium* and *Echium* (Stegelmeier 2011). *C. grahamiana* and *C. pallida* have been identified in Easter Island, and it has been suggested that CH cases might be the consequence of the animals consuming these plants (Artz and Mount 1999). *C. pallida* was described in the island since 1913 (Dubois *et al* 2013).

The role of PAs as the causative agent of CH in Easter Island has not been thoroughly investigated. The objective of this study is to record the distribution of *Crotalaria* spp. in this island, the grazing behaviour of horses and to establish any potential correlation between the consumption of those plants and the appearance of the clinical signs described as CH. This prospective study was performed between the years 2010 and 2015, and was approved by the Ethics Committee of Universidad Viña del Mar (11/13/2012). The distribution of *C. grahamiana* and *C. pallida* across the Easter Island was recorded by semi-systematic observation on each trip. Grazing habits were monitored following a group of 10 horses during 10 days chosen randomly on each trip by one researcher, during the summer of 2010 and 2011, autumn 2013 and summer 2014, due to the islanders tradition of letting horses roam freely through the Island. Eight horses (H1 to H8) (6 geldings and 2 mares), ponies, aged between 2-15 years (mean 10 years) and body weight ranging from 250-320 kg, were selected through nonprobability sampling with the neurological clinical signs described as CH by Artz and Mount (1999), these animals had been observed consuming *Crotalaria* spp. Clinical examination, complete blood cell count<sup>1</sup> and serum biochemistry profiles<sup>2</sup> (LQCE laboratory, Santiago) were performed in 2014 (H1 to H8). Ammonia blood levels were measured in plasma samples

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<sup>1</sup> Wiener Lab Group. Rosario, Argentina.

<sup>2</sup> BioSystems BA 400®. BioSystems S.A., Barcelona. España.

by enzymatic assay<sup>3</sup> (UC-Christus Laboratory). Urine samples were analysed using Multistix strips<sup>4</sup>, and specific gravity was determined using a refractometer (ATC model 311). Liver ultrasonography was performed in all horses using convex 2.5-3.5 MHz transducer (Mindray)<sup>5</sup>. Liver biopsies were obtained using a Tru-cut 14G -15 cm needle biopsy under ultrasound guidance (n=5). Three horses were euthanised due to severe clinical signs consistent with CH, and postmortem examination was performed<sup>6</sup>. Tissues from liver, lung, brain, spleen and kidney were collected, fixed in 10% buffered formalin and processed for routine histological analysis. Total of liver samples (n=8) were scored by a pathologist using a semi-quantitative scale as previously described (Universidad Austral de Chile, Valdivia). Additionally, immunohistochemical staining for proliferating cell nuclear antigen (PCNA), a marker of cell proliferation, was performed in liver tissues according to the manufacturer's instructions<sup>7</sup>. A terminal deoxynucleotidyl transferase dUTP Nick End Labeling assay (TUNEL)<sup>8</sup> for the detection of apoptosis was performed. *Crotalaria* spp. samples were collected from areas with occurrence of CH cases and submitted for toxicological analysis. Gas chromatography mass spectrometry analysis (GC/MS)<sup>9</sup> for qualitative analysis of PAs in *Crotalaria* spp. samples from this island, quantitative of Monocrotaline (MCT)<sup>10</sup> in *Crotalaria* spp., liver biopsies and urine from the studied horses, were carried out. Descriptive statistical analysis was performed, and Spearman's correlation coefficient was used to determine the correlation between MCT's concentrations in *Crotalaria* spp. and in liver biopsies ( $P < 0.01$ ).

*C. grahamiana* was found throughout the Island including the areas Puna Pau, Ahu Akivi, Ahu Te Peu, Ahu Nau Nau and Rano Aroi in addition to the areas reported by Dubois *et al* (2013), except for the north part (Maunga Tere Vaka). This current distribution is larger than previously reported (Artz and Mount 1999, Dubois *et al* 2013). *C. pallida* was observed mainly in some areas of Vinapu and Rano Raraku. The factors responsible for the propagation of *C. grahamiana* remain unknown.

During our study in Easter Island between 2010 and 2014, horses were observed grazing *C. grahamiana* all year

round, this is contrary to the reports of Artz and Mount (1999) who mention that it is only consumed during the period of food shortage in the summer.

On physical examination, horses had poor body condition scores (Henneke system), jaundice and congested mucous membranes (7/8), mild to moderate dehydration (6-8%) (5/8), in addition to anorexia, tachycardia, secondary photosensitization or hepatogenous, ataxia (3-5 Mayhew's grading system), obtundation and negative menace response (4/8) (table 1). The serum biochemistry profile and urinalysis revealed an increase of AST, GGT, ALP, hypoalbuminemia, plasma hyperammonemia and bilirubinuria (table 2). These clinical signs and all of these changes in the reference values suggest liver alterations (Botha *et al* 2012, Lecocq and Moreira 2006, Stegelmeier *et al* 2016). The liver sonography showed increased heterogeneity, echogenicity and bile duct dilatation in all horses.

On necropsy, jaundice, petechiae and ecchymosis of subcutaneous tissue was observed. The liver was enlarged, congested, firm with rounded edges and hemorrhagic areas with an irregular yellowish-brown surface and white foci proliferation on the diaphragmatic surface (H1, H5, H6). The spleen had multiple ecchymosis and the mucous membranes of the stomach were edematous with moderate distension (H1, H5, H6). All these findings are consistent with PAs intoxication in horses (Artz and Mount 1999, Santos *et al* 2008, Stegelmeier *et al* 2016). Liver histological analysis (H1-H8) revealed a mild to moderate fibrosis (8/8), mild to moderate hepatocellular megalocytosis (7/8), mild to moderate bile duct hyperplasia (6/8), mild to severe pigment deposition (6/8), mild to moderate degenerative damage and inflammatory cell infiltration (5/8), and mild to severe tissue necrosis (3/8). All of these alterations show hepatopathy by PAs toxicosis, characteristic and highly suggestive of the condition but not pathognomonic as described in previous reports (Artz and Mount 1999, Santos *et al* 2008, Stegelmeier *et al* 2016). Brain histopathology revealed congestion and presence of enlarged astrocytes with enlarged core and vesicular appearance, probably induced by hyperammonemia (Albrecht and Norenberg 2006).

The immunohistochemistry of liver biopsies were positive to PCNA in 5/8 horses (H2, H3, H4, H7, H8), suggesting that the hepatocyte proliferation was an attempt to restore the liver mass. Increase of apoptosis, indicated by a positive to TUNEL assay, was only observed in horses that were euthanised. The potential role of PAs to induce apoptosis by a mitochondrial pathway has been suggested (Yang *et al* 2017). The qualitative analysis of PAs in *Crotalaria* spp. with GC/MS were: 93% indolizidine, 89% MCT, 74% jacozone and 72% to jacobine, seneciphiline and integerrimine; further investigations are needed to quantify them. The quantitative analysis revealed a concentration of 1.04 to 310.17 µg/g in *C. grahamiana* and 0.06 to 1.04 µg/g in *C. pallida* plants, 0.014 to 0.137 µg/g in the liver, and 2.47 ppm in the urine. These concentrations were

<sup>3</sup> Glutamate dehydrogenase/Cobas c311, Roche Diagnostics GmbH, Mannheim, Germany.

<sup>4</sup> Multistix 10 SG Reagent Strips. Siemens Healthcare Diagnostics Inc. USA.

<sup>5</sup> Shenzhen Mindray Bio-Medical Electronics Co., Ltd.

<sup>6</sup> Euthanasia solution T-61TM, Intervet Chile Ltda. Previously Xilazine 0.5mg/kg and Ketamine 2.2mg/Kg

<sup>7</sup> ImmPRESS®. Vector Lab, Burlingame, Ca. USA.

<sup>8</sup> ApopTag®. Merck Millipore, Darmstadt, Germany.

<sup>9</sup> Shimadzu Corporation. Tokyo, Japan.

<sup>10</sup> Crotaline C2401 Sigma-Aldrich Co. Llc. TM St. Louis, MO, USA.

**Table 1.** Clinical signs of Crazy Horse disease in horses of Easter Island (n=8).

Variable	H1	H2	H3	H4	H5	H6	H7	H8	RI
Heart rate (bpm)	36	28	64	60	28	68	68	30	25-50
Respiratory rate (breaths/min)	8	12	8	10	10	8	14	12	8-18
Temperature °C	37.2	36.9	37.8	37.3	37	38.1	37.6	37.3	37.2-38.3
Body condition	2	3	2	2	3	3	3	2	1-9
Anorexia-hyporexia	X	-	X	-	X	X	-	-	
Photosensitivity	-	-	X	-	X	X	-	X	
Hyperemic mucosae	X	X	X	-	X	X	X	X	
Jaundice	X	X	X	X	-	X	X	X	
Dehydration	X	-	-	X	X	X	X	-	
Ataxia	X	-	-	-	X	X	X	-	
Obtundation	X	-	-	X	X	-	X	-	

H: Horse; RI=Reference interval; LRL=Low RI; URL=Upper RL; “-”: negative; “X”: positive.

**Table 2.** Results of blood analysis, urinalysis and liver ultrasound associated with pyrrolizidine alkaloid ingestion from *Crotalaria grahamiana* in horses of Easter Island (n=8).

Parameters	H1	H2	H3	H4	H5	H6	H7	H8	Mean ± SD	CV (%)	RI LRL-URL
Blood analysis*											
RBC (x10 <sup>6</sup> /μl)	5.7	5.5	6.6	6.6	7.7	7.9	6.1	6.2	6.6 ± 0.9	0.1	6-12
Haemoglobine (%)	11.1	9.3	11.4	11.4	14.2	13.9	10.4	10.7	11.6 ± 1.7	0.1	10-18
Haematocrit (%)	31.7	25.7	32.7	31.7	40	39.7	30.4	30.8	32.8 ± 4.8	0.1	32-48
WBC (x10 <sup>9</sup> /L)	12.6	5.0	7.1	7.5	8.3	13.4	6.4	7.3	8.5 ± 3.0	0.3	5.3-11.0
S. neutrophils (x10 <sup>9</sup> /L)	6.6	2.7	4.0	3.5	6.6	12.3	3.2	4.7	5.5 ± 3.1	0.5	2.1-6.0
Lymphocytes (x10 <sup>9</sup> /L)	4.1	1.5	1.9	3.0	1.2	0.6	2.5	1.8	2.1 ± 1.1	0.5	1.5-5.0
Eosinophils (x10 <sup>9</sup> /L)	0.3	0.1	0	0.7	0	0	0.2	0.7	0.3 ± 0.3	1.1	0-0.8
Monocytes (x10 <sup>9</sup> /L)	0.2	0	0.1	0	0	0	0.1	0.1	0.1 ± 0.1	1.1	0-0.6
Thrombocyte (x10 <sup>9</sup> /L)	210	146	167	117	133	158	131	119	147.6 ± 30.8	0.2	90-210
BUN (mg/dl)	7	10	7.9	9.1	6.9	7	7.5	7.9	7.9 ± 1.1	0.1	8-27
Creat (mg/dl)	1.3	1.2	1.2	0.9	1.0	1.2	1.6	1.7	1.3 ± 0.3	0.2	1.2-1.9
ALP (U/L)	903	1000	926	2046	1060	3283	2077	935	1528.8 ± 866.2	0.5	70.1-226.8
AST (U/L)	516	670	453	527	399	525	423	623	517 ± 93.9	0.2	226-366
GGT (U/L)	1368	50	269	1368	199	77	47	201	447.4 ± 573.8	1.2	4.3-13.4
CK (U/L)	1903	1785	261	375	621	527	923	243	829.8 ± 663.6	0.7	160-330
Proteins (g/dL)	6.1	8.5	6.9	8.2	7.5	8	7.3	7.9	7.6 ± 0.8	0.1	5.2-7.9
Albumin (g/dL)	1.9	1.5	2.2	1.9	3.1	2	2.1	2.2	2.1 ± 0.5	0.2	2.6-3.7
Globulin (g/dL)	4.2	5.2	4.7	5.4	4.4	4.8	5	5.7	4.9 ± 0.5	0.1	2.6-4.0
Total bilirubin (mg/dL)	1.0	0.8	3.6	1.0	2.9	4	4.3	3.2	2.6 ± 1.5	0.5	0.3-3.0
Ammonia (umol/L)	363	40	33	19	431	345	NO	NO	153.9 ± 189.1	1.1	4-49
Urinalysis**											
Protein	L	L	-	-	-	-	NO	NO			-/T
Occult Blood	M	-	-	-	-	M	NO	NO			-
Glu(mg/dL)	1000	250	-	-	-	1000	NO	NO			-
pH	7.5	7.5	7	8	8	8	NO	NO			7.0-9.0
Bilirubin	M	L	-	-	-	-	NO	NO			-
Density	1.05	1.02	1.04	1.05	1.02	1.03	NO	NO			1.02-1.05
Ultrasound	Cho Fib	Cho Fib	Cho Fib	Cyst Fib	Cho Fib	Cho Fib	Cho Fib	Cho Fib			

The data are shown as mean ± SD. Mean = Mean of the eight measurements, SD = Standard deviation, CV = Coefficient of variation. RBC: Red Blood Cells; ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transpeptidase; Creat: Creatinine; BUN: blood urea nitrogen; CK: Creatine phosphokinase; Glu: Glucose; -: negative; -/T=negative/trace; NO: Not obtained; Cho: Cholestasis; Fib: Fibrosis; RI=Reference interval; LRL=Low RI; URL=Upper RL; \*RI, Robinson (2003); \*\*RI, Wittwer (2012).

higher than those described by Artz and Mount (1999). Other plants usually consumed by horses in Eastern Island tested negative for the presence of PAs (Börgel *et al* 2013). A significant and strong positive correlation between the MCT concentrations in *C. grahamiana* and liver biopsies ( $r=1.0$ ) (H1-H8) was found. There is a strong suspicion of a causative relationship between *C. grahamiana* and hepatic encephalopathy in CH, although this has not been definitively proven.

Based on the evidence presented in this study, including the observation of horses with CH ingesting *C. grahamiana*, clinical signs of hepatic encephalopathy, livers enlarged and congested, hepatic histopathological changes and strong positive correlation between MCT concentrations in *C. grahamiana* and liver biopsies, it is concluded that the most reasonable cause of CH is due to consumption of pyrrolizidine alkaloid-containing plants.

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