Hepatic fibrosis induced zinc-deficient dermatosis in American alligators (*Alligator mississippiensis*)

Ilaria M. Piras[1], Annemarie Bezuidenhout[2], Josué Díaz-Delgado[3], Deirdre Slawski[2] and Pamela A. Kelly[1]

[1]School of Veterinary Medicine, University College Dublin, Belfield, Dublin, Ireland

[2]Padenga Ltd, 121 Borrowdale Road, Gunhill, Harare, Zimbabwe.

[3]Texas A&M Veterinary Medical Diagnostic Laboratory, College Station, USA

Corresponding author: Pamela Kelly, School of Veterinary Medicine, University College Dublin, Belfield, Dublin, Ireland; phone number 00353 (0)1 7166154, Pamela.Kelly@ucd.ie

Abstract

Crocodilian farming generates strong economic incentives for the conservation of several species previously endangered by intensive hunting. Ranching farms, in particular, are intimately connected to the natural crocodilian habitat and have a significant impact on wetland preservation. The financial sustainability of this industry relies on the production of first grade skins for the luxury leather market. Only flawless skins are considered of first grade by the stringent standards of the market, and even a single defect represents an economical loss. "Double scale" is one such defect that drastically reduces the appeal of crocodilian skin. Although double scale defects represent a threat to the economical sustainability of the farming industry, there is no scientific literature available on this topic. This study, carried out in a ranching farm of American alligators (*Alligator mississippiensis*), represents the first investigation into the pathogenesis of double scale. Our results indicate that double scale is a keratinization disorder associated with zinc deficiency. Furthermore, we found that portal hypertension due to liver fibrosis, underlies zinc deficiency in cases of double scale. Lastly, we found that chronic vitamin A toxicity can cause liver fibrosis in crocodilians. For the first time, we demonstrate a causal association between liver disease and skin quality in a crocodilian species. This study reveals the conserved role of zinc in the homeostasis of reptilian skin. Also, we show that, like mammals, reptiles may develop liver fibrosis following chronic vitamin A toxicity and through activation of hepatic stellate cells. Our results advance herpetological medicine and will translate into improved captive crocodilian welfare and husbandry.

Keywords: American alligator, *Alligator mississippiensis*, double scale, hepatic fibrosis, portal hypertension, vitamin A, zinc deficiency

Introduction

After decades of unregulated and intensive hunting for skin exploitation, most crocodilian species became endangered, and some were almost extirpated by the beginning of 1970s[1]. This dramatic decline of wild crocodilian populations induced several countries to protect crocodilians through legislation. In addition, the worldwide trade of wild species and their products became regulated by the Convention on International Trade in Endangered Species (CITES) from 1975. Crocodile farming gained momentum in this historical context as a valuable opportunity to provide skins for an ever-growing leather market and, at the same time, incentivize the conservation of crocodilians[2,3].

Ranching is the most common way of farming American alligators (*Alligator mississippiensis*), in the United States. Ranching farms are intimately connected to the natural alligator habitat and have a significant impact in wildlife conservation. This way of production is based on the collection of wild eggs, followed by regular reintroduction of juveniles and hatchlings born on farm, to maintain the wild crocodile population[4]. This practice incentivize the preservation of the wet land habitats, saves eggs that are often subject to predation or destruction in wild conditions[5] and boosts hatchlings survive until adulthood[5,6,7,8].

The main focus of crocodile farming is to produce high quality skins to supply the expanding demand for premium hides for the luxury leather market[7]. For this reason, care is taken during rearing to minimize damage to the belly skin, which is the most valuable part of the hide, either from abrasive surfaces, from interactions with other crocodiles, and from any dermatologic conditions that can produce a defect[4,9,10].

This study is a clinical and pathological investigation conducted on a ranching farm of American alligators following a year of poor skin gradings due to high incidence of “double

76 scale" (DS) defects in the population. This skin condition reduces leather quality and has an
77 important impact on the financial sustainability of the industry. The manifestations of this
78 condition remain uncharacterized, and the etiology is unclear. Multifactorial causes,
79 involving potential nutritional, metabolic and genetic factors have been considered
80 anecdotally, but none have been explored in the scientific literature so far.
81

82 **Results and discussion**

83 **Double scale is a disorder of keratinization**

84 The skin is a protective barrier composed of multiple layers of specialized epithelial
85 cells called keratinocytes, that offer protection against pathogen invasion, chemical, thermal,
86 and physical damage, and prevent body water loss. All these functions are provided by the
87 outermost layer of the epidermis composed of terminally differentiated keratinocytes, the
88 corneocytes. The constant balance of keratinocyte proliferation, differentiation and shedding
89 of corneocytes facilitates repair after external trauma and maintains the steady state of the
90 corneal layer[11]. Scales are skin appendages evolved by reptiles[12], which exquisite
91 arrangement pattern has made crocodilian leather one of the most demanded products of the
92 luxury fashion market since 1800[1,13].

93 DS defect undermines the appeal of the belly scale pattern with an array of defects. In
94 this study, DS presented as a combination of focal extensive pitting and roughening of the
95 cranial and medial edge of the belly scale (18/18 -100%), which was often accompanied by a
96 brown discoloration (Figure 1A). In the most severe cases, a linear, ring-shaped dent carving
97 the central portion of the scale was also present (7/18-38.8%) (Fig. 1A, arrow). The presence
98 of this dent is responsible for the duplicated appearance of the scale, from which this defect
99 derives its name.
100

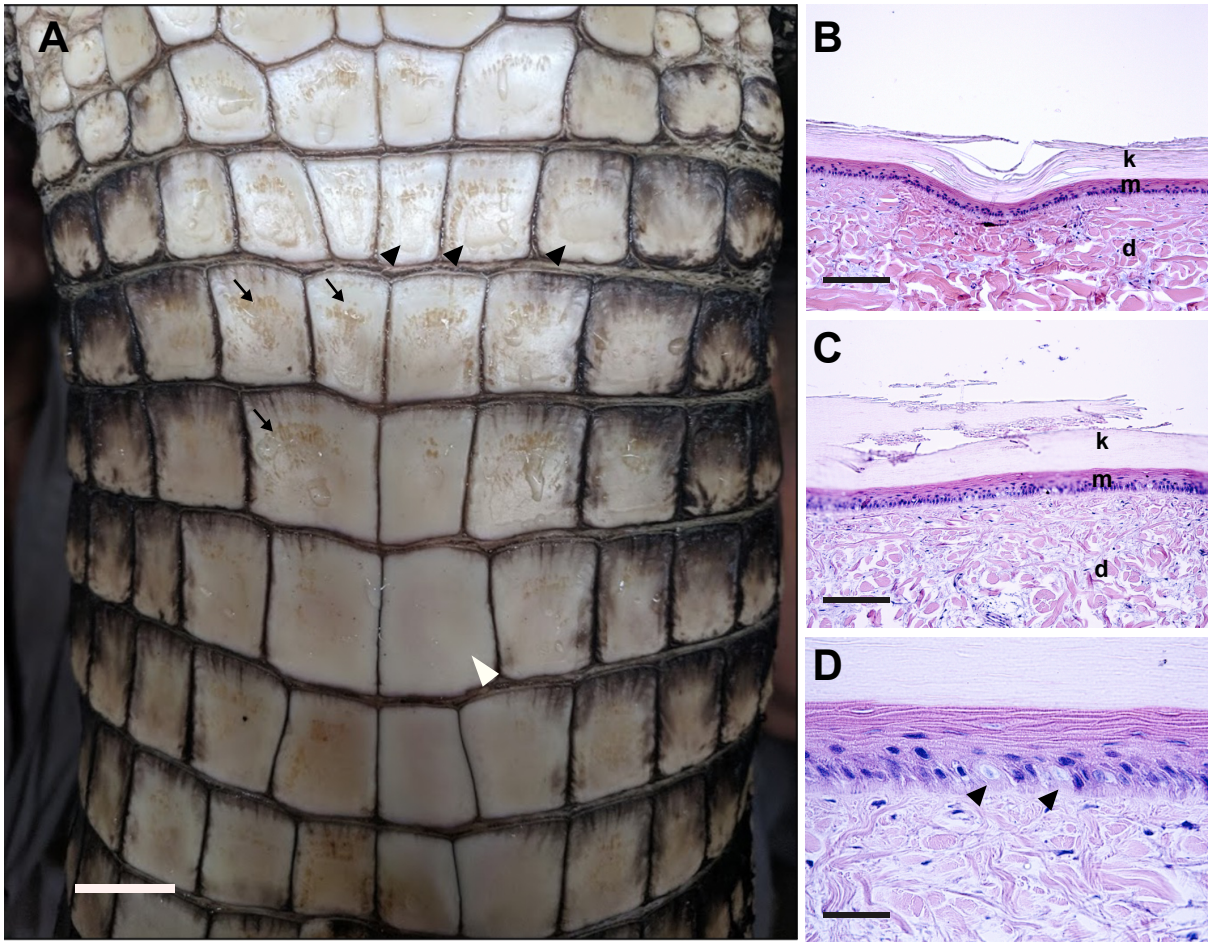

Figure 1. Belly skin with double scale defect, American alligator. Gross appearance of double scale defect (A) characterized by a rough and pitted surface (arrows) often associated with a linear dent (black arrowheads). Note the smooth and even surface of the normal scale (white arrowhead). Scale bar 1 cm. Microphotographs of a cross section of double scale (B-C-D). H&E, scale bar 200um (B-C), 50um (D). The grossly noticeable dent is histologically characterized by a focal depression of the epidermis and the dermis (B). At the gross level of the rough surface, histologically the corneal layer is disrupted and fragmented (C). Higher magnification of the epidermis shows frequent degeneration of the basal keratinocytes (arrowheads) (D). (k) corneal layer and (m) Malpighian layer of the epidermis, (d) dermis.

Histologically, this dent presented as a depression of the epidermis and superficial dermis (Figure 1B) giving the scale an irregular profile that resulted in downgrading of the processed skin (data not shown). DS also presented loss of the characteristic compactness of the

115 reptilian β-keratinized corneal layer, with frequent multifocal fragmentation (Figure 1C) that
116 corresponded grossly to the roughened pitted areas of the scale. Other changes evident at
117 histological examination were increased numbers of degenerating basal keratinocytes with a
118 mean of 3.7 per 10,000 cells, (SD=9.3, n19) (Figure 1D) compared to 0.21 per 10,000 cells in
119 normal scales (SD=0.39, n=9). β-keratin layer thickness was 94.6 µm on average in normal
120 scales (SD=11.6, n= 18) (figure 2A, 2B and 2G), which is consistent with measurements
121 reported in the literature for juvenile alligators of similar age[14]. The α-keratin, measured at
122 the level of the hinge, was 18.8 µm on average (SD=5.3, n=18) (Figure 2C and H). A marked
123 thickening of the keratin layer without retained nuclei, or orthokeratotic hyperkeratosis
124 (Figure 2), characterized all DS samples examined (18/18-100%) compared to normal scales
125 (n=9) (Figure 2A and 2B). This hyperkeratosis affected both, the β-keratin layering of the
126 scale plate (corneal layer, mean= 168.5µm; SD=35.4, n=36, *p* value <0.0001) (Figure 2D and
127 G), and the α-keratin in the hinge area (mean= 70.5 µm; SD=21.5, n= 36 *p* value <0.0001)
128 (Figure 2E and 2H). The thickness of the live strata of the epidermis, including the basal,
129 supra-basal and transitional layers (also referred to as Malpighian layer), was significantly
130 reduced in scales with hyperkeratosis (average of 33.1 µm, SD=9.9 µm, n=36, *p* value
131 <0.0001) compared to normal scales (mean 51, SD=11.9, n18) (Figure 2B, 2D and 2F). In
132 addition, changes to the dermis were evident. Underlying the epidermis, the dermis is the skin
133 stroma composed of collagen bundles, ground substance, blood vessels and nerves that
134 provides innervation, vascularization, and support to the organ.

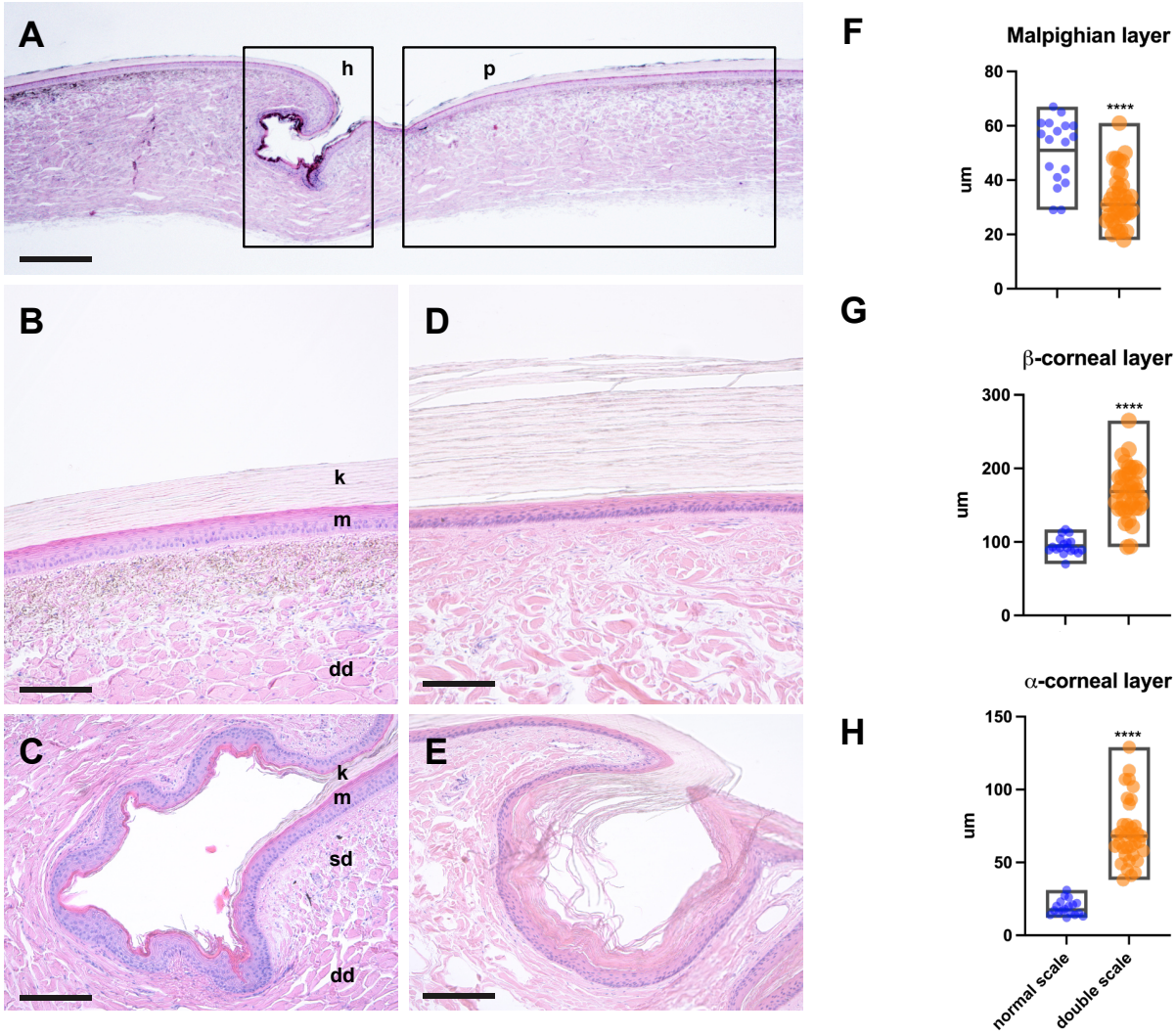

Figure 2. Hyperkeratosis characterizes double scale in American alligator. Microphotographs of a normal scale (A-B-C) and double scale (C-D). H&E. Normal anatomy of alligator scales (A) showing the plate region (p box) constituting most part of the scale and the hinge region (h box) which connects the cranial (left) to the caudal (right) scale. Scale bar 1mm. Compared to normal scale (B and C), the keratin layer of skin affected with double scale is markedly thickened both at the level of the plate (D) and the hinge (E). Note the β-keratin covering the plate of the scale stains negatively (B and D) whilst the α-keratin at the level of the hinge dyes pink with eosin (C and E). The live strata of the epidermis, also called Malpighian layer, is instead thinner (E). Scale bar 200μm. (k) Corneal layer, (m) Malpighian layer of the epidermis, (sd) superficial dermis, and (dd) deep dermis. The box graphs show measurements of Malpighian layer (F) beta (G) and alpha (H) corneal layers of normal (n=18 measurements) and double scales (n=38 measurements). Central line in the box indicates mean. Difference of means calculated with Student *t* test was significant with *p* value <0.0001 (****).

148 A disarray in the orientation of collagen fibers was evident in the superficial and deep
149 dermis of several DS individuals (9/19; 47%), accompanied by a multifocal increase of the
150 ground substance (Figure 3). Disarray and loss of collagen fibers could be considered
151 responsible for the altered staining properties noted in processed alligator skin with DS defect
152 (data not shown).
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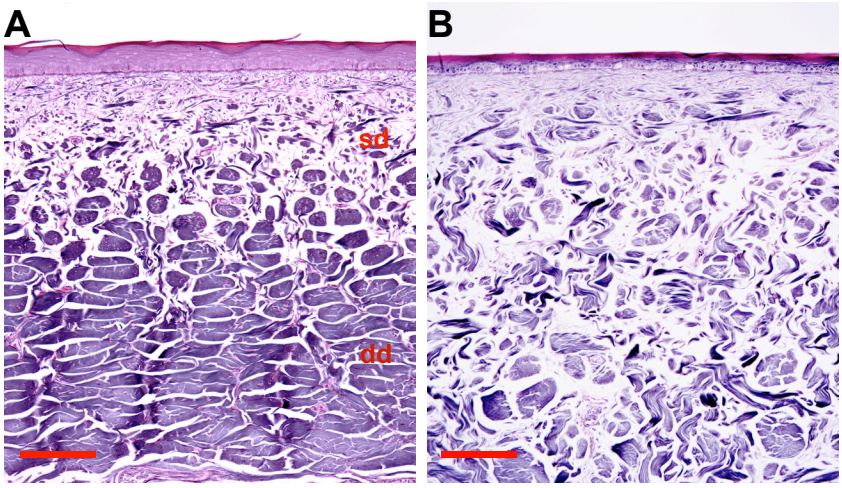

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155 **Figure 3. Dermal collagen disarray characterizes the skin with double scale.** Microphotographs
156 of a normal scale (A) and double scale (B). Reticulin Stain. Scale bar 200μm. Normal anatomy of
157 alligator dermis (A) showing and orderly arrangement of collagen bundles (stained black) that became
158 larger in the deep dermis. Note that all bundles are oriented perpendicular to the cut section the
159 separation between superficial and deep dermis is well-defined. In double scales (B) collagen bundles
160 are reduced in size and are separated by an increase of clear spaces (ground matter). The bundles
161 are arranged haphazardly and a clear definition between superficial and deep dermis is lost.
162

163 **There is a correlation between skin lesions and liver injury**

164 A further, systematic histopathological examination of intestine, lung, heart, brain,
165 spleen, liver, and kidneys from 86 alligators revealed a high incidence of liver disease (47/86;
166 54%); 70% of pathologic livers (33/47) presented fibrosis with variable severity (Figure 4A
167 and Supplementary Figure 1). Most interestingly, liver fibrosis was always present in

168 alligators with DS (18/18; 100%). No other related histopathological findings were observed
169 in other tissues. There is still little information available on normal liver histology of reptiles,
170 and to the best of our knowledge, liver fibrosis has not been described in alligators. Hepatic
171 fibrosis represents a scarring response to chronic liver injury after a variety of insults[15]. The
172 most advanced stage of fibrosis, called cirrhosis, defines the distortion of the liver
173 parenchyma with bridging scars and nodule formation, accompanied by altered blood flow[15].
174 In the alligator livers examined, fibrosis formed within and around periportal tracts and the
175 liver capsule. In the most severe cases, fibrosis bridged several portal areas resulting in
176 complete loss of normal lobular architecture with evidence of nodular regeneration, as
177 described in cirrhosis of mammalian species (Figure 4C). Other liver changes consistently
178 associated to fibrosis in alligators were bile duct proliferation (Figure 4A), which is
179 considered a nonspecific reaction to liver injury described in humans and several domestic
180 species,[16] glycogen accumulation (figure 4E), causing hepatocytes vacuolation and swelling
181 and increase of periportal melanomacrophage numbers (Figure 4A). Except for the expected
182 accumulation prior to hibernation, glycogen accumulation is largely considered a
183 consequence of hepatocytes metabolic pathways disturbance[17]. An increase of melanin laden
184 macrophages (melanomacrophages) suggests an enhanced phagocytic activity in reptilians[18]
185 and is likely due to an increased rate of cell debris clearance, resulting from hepatocyte loss
186 in these cases. Myofibroblasts are considered the main source of collagen production in liver
187 fibrosis in humans, mice, and dogs[19,20]. They are fibroblast-like cells that express α-smooth
188 muscle actin which confers them contractile properties[19,17]. As expected, immunolabelling of
189 alligator livers showed α-SMA-positive cell numbers were markedly increased around
190 periportal areas, where the collagen accumulated (Figure 5A).
191

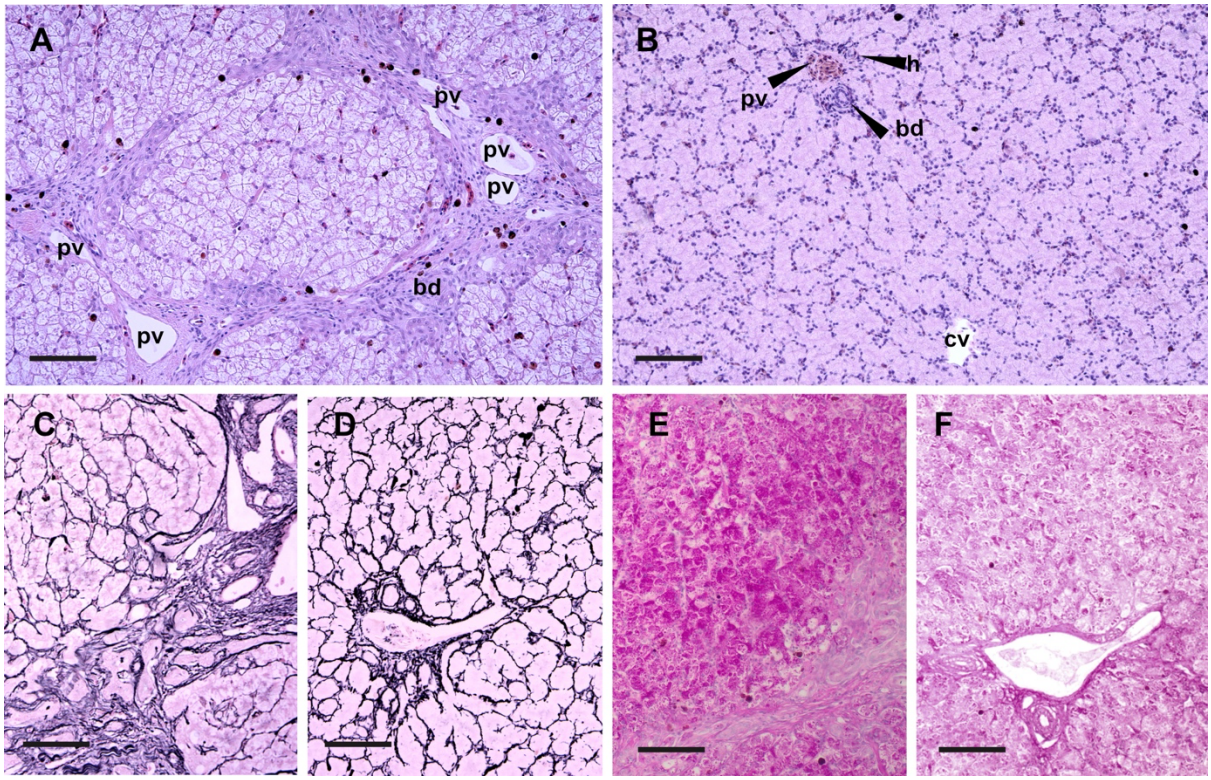
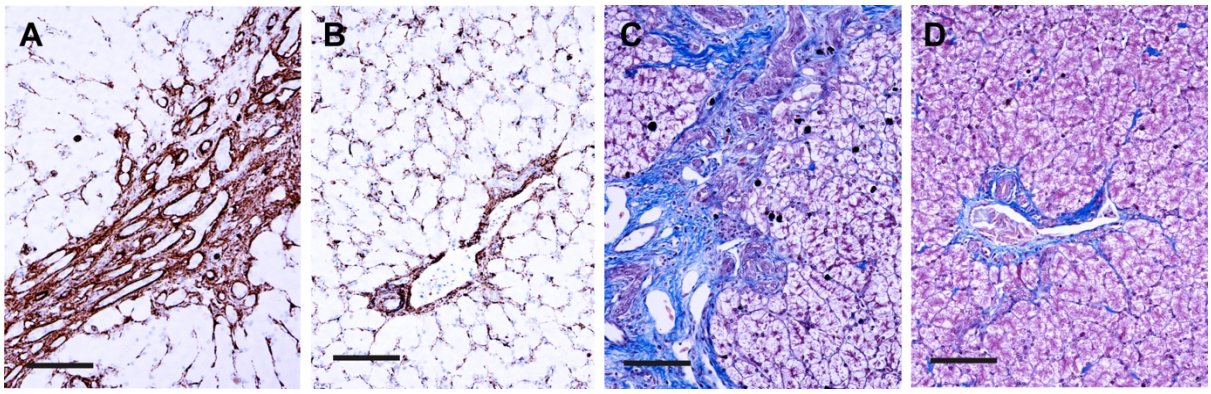


Figure 4. Liver fibrosis in American alligators with double scale. Microphotographs of alligator livers (A-B) H&E. (C-D) Reticulin stain. (E-F) PAS. Scale bar 200μm. Liver affected by severe fibrosis (A) showing bands of collagen fibers bridging between several triads associated with increased numbers of melanin laden macrophages. Biliary ducts are increased in number and are tortuous. Portal veins were tortuous and irregularly dilated, suggestive of shunting. Hepatocytes show diffuse feathery vacuolation and swelling. Central vein is collapsed and undetectable. Normal liver lobule architecture (B) showing a triad (upper left) composed of one portal vein, one hepatic artery and one biliary duct. A central vein (lower right) is seen at the center of the lobule. Reticulin stain accentuates the nodular deformation of the lobule and distortion of hepatocellular plates and nodular deformation of hepatic parenchyma (c) in cirrhotic liver compared to a normal one (D). Periodic acid Schiff stain highlights glycogen accumulation is causing hepatocyte vacuolation of livers with fibrosis (E) compared with normal livers (F). Biliary ducts (bd), portal vein (pv) and (h) hepatic artery and (cv) central vein.

Myofibroblasts in livers can be recruited by activation of hepatic stellate cells (or Ito cells), liver resident fibroblasts (portal or centrilobular), epithelial cells that undergo epithelial-to-

209 mesenchymal transition, bone marrow-derived fibrocytes, and smooth muscle cells that
210 surround blood vessels[21]. Comparison of α-SMA and desmin immunolabelling with normal
211 alligator livers indicated hepatic fibrogenic cells derived from proliferation of periportal and
212 perivascular fibroblasts but also recruitment of desmin positive cells from the sinusoids,
213 which are most likely hepatic stellate cells (Figure 5 and 6).
214



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216 **Figure 5. Myofibroblasts are the fibrogenic cells in American alligator's livers**.
217 Microphotographs of alligator livers. (A-B) Anti-α-smooth muscle (SMA) immunohistochemistry. (C-D)
218 Masson's Trichrome. Scale bar 200μm. In livers with fibrosis α-SMA-positive myofibroblasts increase
219 within periportal areas (A) in association with collagen deposition (blue stain, C). Note the numbers of
220 myofibroblasts in the perisinusoidal spaces are reduced compared to normal livers (B). Collagen
221 distribution in normal livers (D).
222

223 **Vitamin A toxicity underlies liver injury**

224 Progressive liver fibrosis can be caused by chronic viral infection, prolonged use of
225 hepatotoxic drugs, longstanding exposure to aflatoxin, iron, and copper accumulation,
226 chronic biliary cholangitis, autoimmune hepatitis, and chronic vitamin A toxicity among
227 others[19,22,23]. Alcohol abuse, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis
228 are largely reported in humans[16].
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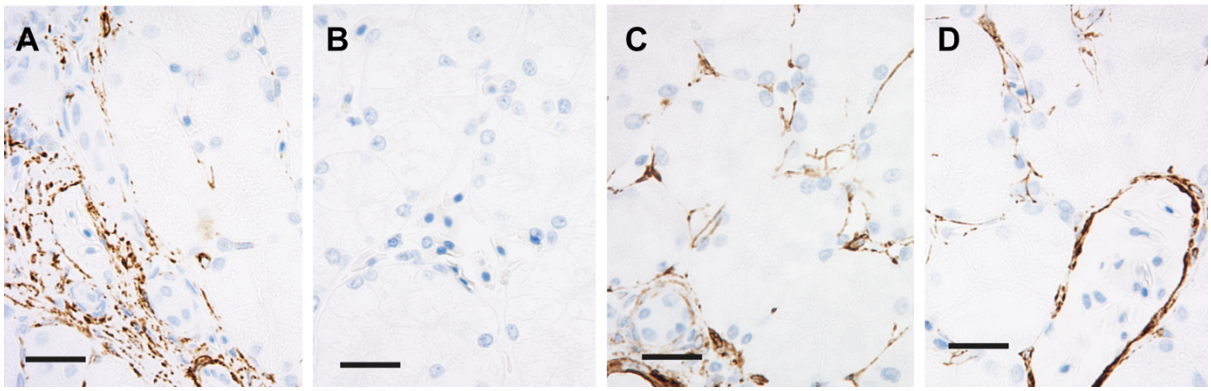

Figure 6. Migration of desmin-positive cells from perisinusoidal spaces to periportal areas in association to liver fibrosis in American alligator. Microphotographs of alligator livers. Anti-desmin immunohistochemistry. Scale bars 50μm. When fibrosis is present immunolabelled cells are concentrated in the periportal areas (A), leaving the sinusoids depleted of desmin-positive cells in the midzonal and centrilobular areas (B). In normal livers, desmin-positive cells have cytoplasmatic processes that diffusely and evenly stretch into the perisinusoidal spaces in both periportal (C) and centrilobular areas (D).

Results from hepatic vitamin and mineral level analysis (table 1) indicated the average concentration of vitamin A in alligator livers with fibrosis was 880,000 mg/100g (n= 27). This value is significantly higher than vitamin A concentration found in normal alligator livers (389,000 mg/100g; n=22) examined in this study. In our alligators, hypervitaminosis A resulted from a longstanding diet with vitamin A concentration ranging from 23,300 IU/Kg to 38,200 IU/Kg. The tolerable range of dietary vitamin A levels for crocodilians is currently unknown. Vitamin mix supplementation is generally used in excess in commercial crocodilian diets as no toxicities have been reported in the literature, so far[24].The maximum tolerable dose of dietary vitamin A for chicken broiler breeders appears to be 35,000 IU/kg, corresponding to 195.2 mg/100 g of retinyl esters in the liver whilst higher supplementation has been shown to impair liver function[25]. Compared to chickens and turkeys, which are the closest phylogenetically related domestic species, it seems that alligators have a much higher capacity to accumulate vitamin A in the liver[25,26]. Considering that 70% of alligators fed with

252 vitamin A concentration above 23,300 IU/Kg developed liver fibrosis, we suggest these
253 levels may be toxic. Liver damage related to chronic hypervitaminosis A is a rare, but well
254 described condition in humans[27] and has only been reported once in a cat[28]. Although
255 occasionally suspected in pet reptiles, it has not been characterized[29]. The pathologic changes
256 in the liver are related to the increased retinyl storage in the organ and may result in portal
257 hypertension, secondary to sinusoidal compression, and hyperplasia of Ito cells followed by
258 fibrosis[16,30]. Toxicological studies are warranted to confirm the toxic ranges of vitamin A in
259 crocodilians.

260 Hepatic iron levels were also increased in alligator livers with fibrosis (Table 1).
261 Histologically, iron increase and pattern of accumulation occurred exclusively within
262 periportal macrophages (Supplementary figure 2). The mesenchymal pattern of iron
263 accumulation together with the lack of stainable iron in hepatocytes and other tissues
264 indicates the origin of iron loading is secondary to hepatocyte loss rather than chronic,
265 augmented intake[31]. Other causes of liver disease leading to fibrosis such as copper loading,
266 aflatoxin and CCl_4 exposure were excluded based on the result of liver oligoelements analysis
267 and toxicologic test of feed and water. As histological evidence of cholangiohepatitis and
268 bacterial infection were absent in the livers examined, they were discarded as possible
269 underlying causes of liver disease.

270

271 **Zinc deficiency due to portal hypertension is the cause of skin lesions**

272 Mineral and vitamin analysis of the serum and livers revealed an imbalance between
273 hepatic accumulation and systemic distribution of several elements in alligators with liver
274 fibrosis (Table 1). Specifically, vitamin E, copper and zinc were significantly decreased in
275 peripheral blood, although their hepatic concentration remained steady in the liver or, like

276 zinc, slightly increased (Table 1). These imbalances suggested portal hypertension had
277 developed in these individuals, which is supported by histopathological observations of
278 sinusoidal compression, myofibroblast hyperplasia, sinusoidal and periportal fibrosis. All
279 these changes in alligator livers, most likely contributed to increased intrahepatic blood flow
280 resistance, impairing the systemic availability of nutrients absorbed by the gut and delivered
281 to the liver through the portal vein.
282
283

	Controls		Liver Fibrosis					
	Liver	Serum	Liver	*p* value	Trend	Serum	*p* value	Trend
vitamin A	389		880	****	↑			
Palmate	362		855	****	↑			
retinol	28		41					
Iron	388	136	734	****	↑	182		
Zinc	81	1	96	*	↑	0.6	****	↓
Copper	39	0.67	57			0.55	*	↓
Selenium	2.9	324	3.3	*	↑	353		
Manganese	5.4	8	3.4	****	↓	10		
Molybdenum	0.29	19	0.13	****	↓	22		
Cobalt	0.02	0.2	0.02			0.7		
Arsenic	0.09	0.017	0.1			0.025	**	↑
Cadmium	0.02	0.1	0.02			0.1		
Thallium	0.02	0.1	0.02			0.1		
Lead	0.046	0.1	0.023			0.1		
Vitamin E	69	4.9	57			3.4	**	↓

286 **Table 1. Micronutrient concentration in liver and serum of alligators with liver fibrosis**
287 **compared to alligators with normal livers**. Micronutrients in the liver and serum are indicated as
288 mean value in *μ*g/g and *μ*g/mL respectively. Means are calculated on 22 samples from animals with
289 normal livers and 28 with liver fibrosis. Difference of means was calculated with Student *t* test.
290 Significant results with *p* value <0.0001 are marked as ****, *p* value 0.0021 as **, and *p* value <0.05
291 as*.
292

293 The venous shunting seen in histological sections as tortuous and irregularly dilated portal
294 veins in livers with fibrosis (Figure 4A, asterisk) is an attempt to correct the circulatory
295 disturbances present in the organ and is consistently present in human livers with portal
296 hypertension[32].

297 Of all serum micronutrient imbalances in alligators, zinc reduction was the most
298 striking for its severity (0.6 ug/mL; n= 20) compared to controls (mean 1µg/mL; n= 22)
299 (Figure 7D). These results are highly suggestive of zinc deficiency as a primary cause of
300 alligator skin lesions, specifically DS. Zinc deficiency is a common finding in people
301 suffering of liver cirrhosis and is due to portal hypertension or decreased serum levels of
302 albumin[33]. In this case albumin serum levels of alligators with liver disease were comparable
303 to the controls (1.77 and 1.8 g/dL, respectively). Cutaneous lesions are common
304 manifestations of zinc deficiency both in humans and in animals, but the pathogenic
305 mechanisms are unclear[34]. Zinc in the skin is regulated by transporters (ZnTs and ZIPs) and
306 metallothioneins (MTs) which regulate epidermal proliferation and differentiation. MTs are
307 cysteine-rich, zinc-binding proteins synthetized in response to tissue zinc levels and
308 essentially control its availability[35]. MTs also act as free radical scavengers[36]. Several authors
309 suggest that zinc antioxidant effects explain its inhibitory effect on apoptotic pathways,
310 which is considered a key aspect of the pathology of zinc-deficient skin[37].

311 MTs expression may be used for subjective determination of zinc concentration in
312 skin samples[34]. Anti-metallothionein immunolabelling of alligator skin revealed a drastic
313 reduction of MTs in alligators with liver fibrosis, confirming zinc deficiency is a primary
314 underlying cause of skin lesions in these animals (Figure 7).
315

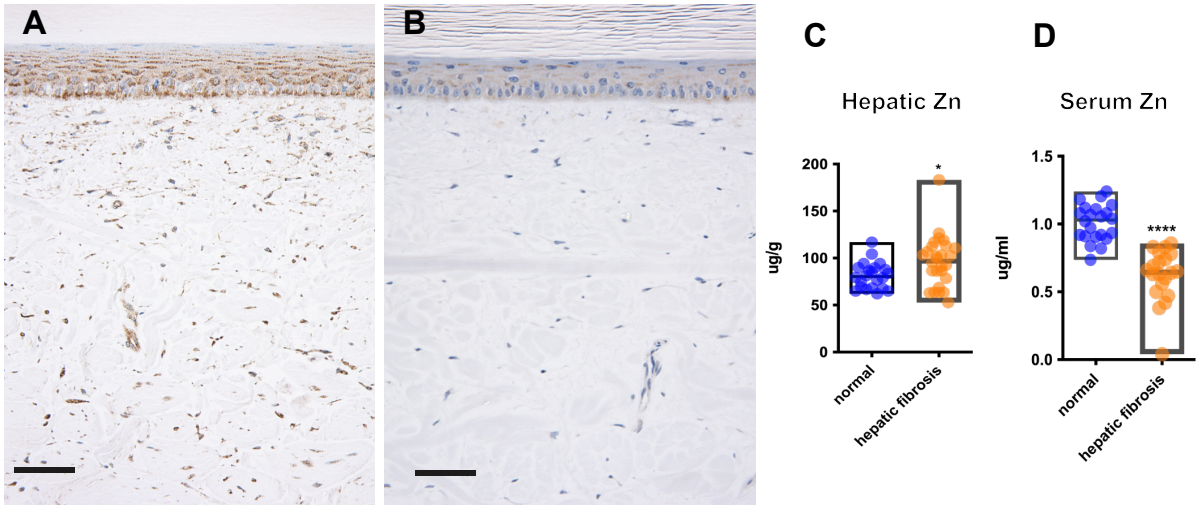

Figure 7. Zinc deficiency and metallothionein depletion in the epidermis of alligators with liver fibrosis. (A-B) Microphotographs of belly scale. Anti-metallothionein immunohistochemistry, scale bar 50μm. Metallothionein is labelled (granular cytoplasmic pattern) diffusely in all layers of the live epidermis and dermal fibroblasts in normal scales (A). Very scant metallothionein immunolabelling is detectable in the epidermis and dermis of animals with double scale (B). Box graphs show levels of hepatic (C) and serum (D) zinc in alligators with liver fibrosis (n=28) compared to alligators with normal livers (n=22). Central line in the box indicates mean. Difference of means calculated with Student *t* test. Significant differences are indicated with **** for with *p* value <0.0001 and * for *p* value <0.05.

The presence of increased numbers of degenerated basal keratinocytes in the skin samples examined seem to support the presence of an increased oxidative stress in the epidermis of alligators. Interestingly, the histopathology of zinc-deficient dermatosis in alligators seems to differ from humans and domestic animals. In mammalian species the characteristic thickening of the corneal layer is accompanied by the retention of nuclei in corneocytes (parakeratosis) and the layers of live strata of the epidermis are also increased (hyperplasia or acanthosis)[34]. These morphological differences are likely due to the different mechanism of keratinization in crocodilian skin, where terminally differentiated keratinocytes produce β-keratin, which is exclusive of reptilian and avian appendages and is responsible for the

336 rigidity of scales and feathers. Another important difference concerns keratohyalin granules,
337 which are essential in mammalian keratinization process and absent in crocodilian
338 keratinocytes. Consistent to all species with zinc-deficient dermatosis, is the severe
339 thickening of the corneal layer which is considered secondary to disorders of proliferation,
340 differentiation, and exfoliation of the epidermis[34], although the precise pathogenesis remains
341 unknown. Regarding the collagen disarray present in the dermis of scales with DS defect, we
342 suggest an altered function of zinc-dependent matrix metalloproteinases could be involved.
343 Matrix metalloproteinases belong to a family of endopeptidases involved in the cleavage of
344 extracellular matrix proteins, required for tissue remodeling and wound healing[38]. We
345 hypothesize that a lack of tissue remodeling capability would cause the collagen disarray in
346 alligator skin with zinc deficiency. Further research is warranted to assess this hypothesis.
347

348 **Conclusions**

349 This study reveals how liver disease can affect skin quality in farmed alligators, highlighting
350 the importance of animal health and welfare for the economical sustainability of crocodilian
351 farming. Double scale is considered anecdotally as a multifactorial skin condition; here we
352 demonstrate how zinc deficiency due to hepatic portal hypertension plays a main role. This
353 finding indicates a conserved role of zinc in reptilian skin homeostasis, suggesting that
354 acquired nutritional and metabolic disturbances can lead to skin disease in this species. There
355 is limited knowledge on nutritional needs of captive crocodilians and literature lacks
356 reference ranges. The liver disease in this case was likely due to chronic vitamin A toxicity.
357 Based on histopathological examination of livers, we suggest dietary concentrations of
358 vitamin A higher than 23,000 IU/kg are potentially hepatotoxic, whilst an average of 15,000
359 IU/kg seem to be safe for captive alligators.
360

361 **Methods:**

362 **Ethic statement**

363 This study was ethic review exempt (AREC-E-22-20-KELLY) as it involves samples
364 collected exclusively during normal husbandry or veterinary clinical procedures. No
365 procedures were carried out on live animals and no animal was euthanized for the sole
366 purpose of this study.

367 **Animals**

368 All alligators included in this study were reared on the same farm. At the time of
369 investigation, the farm produced over 12,000 animals per annum on a single site. Animals
370 were housed in groups of 20 to 80 depending on their size. These groups consisted of a mix
371 of both male and female alligators. The houses were insulated and were divided into pools
372 and feeding and basking decks. Diet consisted of a commercially available complete
373 crocodilian pelleted feed purchased from a single manufacturer that was tested routinely for
374 mycotoxins.

375 **Sample Collection**

376 Between April 2019 and June 2020, during routine abattoir procedures, samples of
377 liver, lung, kidney, heart, spleen, intestine, brain, and serum were collected from 86 randomly
378 selected alligators (Supplemental table 1). From the same animals we collected 18 samples of
379 belly skin with double scale and 9 samples of normal belly skin (Supplemental table 1). Age
380 range for selected animals was between 9 and 19 months; sex and farming pen of origin were
381 mixed. The average length and belly width for each age group of alligator at harvest is
382 presented in Supplemental table 2.

383 **Histopathology**

384 Following fixation in 10% neutral buffered formalin, tissues were processed and then
385 embedded in paraffin-wax, sectioned at 4 µm, and stained with hematoxylin and eosin

386 (H&E). Histological sections were assessed for the presence of histopathological changes by
387 pathologists P.A.K (ECVP board certified), J.D.D. (ACVP board certified) and I.M.P.
388 (anatomic pathology resident).
389 3 representative samples of liver with fibrosis and 3 with normal histology were stained with
390 Masson trichrome, Gordon and Sweet's reticulin, Perls' Prussian blue stains to highlight
391 collagen, reticular fibers and iron respectively. To characterize the vacuolar hepatopathy
392 further, Periodic acid Schiff (PAS) staining was also carried out on these samples.
393 The histopathological examination of skin was based on 61 scales with defects
394 sampled from 18 alligators and 32 normal scales sampled from 9 individuals, for a total of 93
395 scales. Skin measurements were carried out on 18 normal and 36 scales with DS (2 scales per
396 alligator) using imageJ on microphotographs of 20x magnification with scale set to 15 µm
397 equivalent to alligator erythrocyte wide diameter.
398 Six representative samples of skin, three normal and three with DS defect, were also stained
399 with Gordon and Sweet's reticulin, Masson trichrome, PAS, Gram and used for
400 immunohistochemical labelling.
401 **Immunohistochemistry**
402 Fibrogenic cells were assessed by immunohistochemistry (IHC) on six samples of
403 liver, three with fibrosis and three normal, using an anti-desmin antibody at concentration of
404 1/25 (a-desmin clone D33, M0760; Denmark A/S) and a monoclonal antibody directed
405 against the alpha isoform of smooth muscle actin at a working dilution of 1/100 (a-SMA,
406 clone 1A4, n M0851; Dako, Denmark A/S). Alligator skeletal muscle was used as positive
407 control for anti-desmin antibody (Supplemental figure 3A). The tunica muscularis of alligator
408 intestines served as positive control for a-SMA antibody (Supplemental figure 3C).
409 The presence of zinc deficiency was assessed targeting metallothionein using a rabbit
410 polyclonal anti-metallothionein at 1/400 dilution (ab192385, abcam) on six samples of

411 alligator skin, three with double scale defect and three normal. Mouse skin was used as
412 positive control for the primary antibody (Supplemental figure 3E). For all antibodies, one
413 reaction without primary antibody was included as a negative control (Supplemental figure
414 3B, D and F).

415 **Vitamin A, vitamin E and mineral concentration in liver and serum:**

416 Copper, iron, zinc, manganese, molybdenum, cobalt, arsenic, cadmium, lead,
417 thallium, selenium, vitamin E, retinol, palmate and total vitamin A concentrations were
418 determined on 49 of the 86 frozen liver samples by inductively coupled plasma mass
419 spectrometry (ICP-MS) and chromatography at Texas A&M Veterinary Medical Diagnostic
420 Laboratories. The same laboratory determined copper, iron, zinc, manganese, molybdenum,
421 cobalt, arsenic, cadmium, lead, thallium, selenium, vitamin E concentration on 42 samples of
422 frozen serum. Metals and minerals were all assayed on ICP-MS, each element with its own
423 standard curve of at least 5 points with correlation coefficient greater than 0.995. Vitamin A,
424 retinol, palmate and vitamin E were measured by chromatography performed on a Waters
425 system using a C18 Cosmosil PBr column (4.6x250mm). Detailed protocols for ICP-MS and
426 chromatography are provided in the supplementary material.

427 **Statistical analysis**

428 Equality of means between the two groups of data was assessed using *t* test and
429 PRISM 9 was used for statistic calculations and graphs design.

430

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Author contributions

P.A.K. and D.S. and A.B. conceived the study. P.A.K and I.M.P designed the study with the support of D.S and A.B. P.A.K., I.M.P and J.D.D carried out the histopathological examination and interpretation of tissue samples. A.B. and D.S. examined the alligators on farm, collected samples, contributed photos and data from the animals included in this study. I.M.P. analyzed the data with the support of D.S and A.B. I.M.P. wrote the manuscript. J.D.D and P.A.K contributed to the writing and revised the manuscript. All authors reviewed the manuscript and consented to its submission and publication.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.