

## **RH: REVIEW OF MOUNTAIN CHICKEN FROG NECROPSY FINDINGS**

### **A RETROSPECTIVE REVIEW OF POST-METAMORPHIC MOUNTAIN CHICKEN FROG (*LEPTODACTYLUS FALLAX*) NECROPSY FINDINGS FROM EUROPEAN**

#### **5 ZOOLOGICAL COLLECTIONS, 1998 TO 2018**

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Abstract: The mountain chicken frog (*Leptodactylus fallax*) is the largest endemic amphibian species in the Western Hemisphere. Since 1998, this Critically Endangered species has been maintained as a European Endangered Species Programme (EEP) but low breeding success and a high mortality rate threatens the sustainability of the captive frog population. In the current study, we analyzed gross and histopathological post-mortem information from 212 mountain chicken frogs which died in European zoological collections, 1998 - 2018. Thin body condition was the most commonly reported finding across all submissions, observed in 125 frogs. The gastrointestinal and urinary systems were reported to have the highest prevalence of pathological findings on gross and histopathological examination. Inflammatory disease was the most frequent diagnosis following histopathological examination of relevant tissues with intestinal inflammatory disease (n=76) followed by tubulointerstitial nephritis (n=26) most commonly reported. Neoplasia was reported in 42/212 (19.8%) frogs, all of which were adults. A defined cause of death, or reason for euthanasia, was proposed for 164/212 (77.4%) frogs with inflammatory diseases processes (74/212; 34.9%) most commonly implicated. Intestinal adenocarcinoma, seemingly restricted to the colon, caused the deaths of 31 adult frogs. Further investigations to determine factors contributing to the high incidence of inflammatory disease processes and neoplasia are advocated in order to help improve the health and sustainability of the captive mountain chicken frog population.

## INTRODUCTION

45 The mountain chicken frog (*Leptodactylus fallax*) is the largest endemic amphibian in the Western Hemisphere but its population has been decimated by habitat loss, hunting, introduced predators and, most notably, the infectious fungal agent, *Batrachochytrium dendrobatidis* (Bd).<sup>14</sup>

In recent times, mountain chicken frogs (MCFs) have been restricted to just two Caribbean islands, Dominica and Montserrat. Between 2002-2004, outbreaks of chytridiomycosis caused an 85% reduction in the MCF population on Dominica and, since the first detection of Bd on 50 Montserrat in 2009, the island's MCF population has experienced a catastrophic decline with the species now thought to be extinct on the island.<sup>14</sup>

In 1998, wild MCFs were first brought to Europe with the aim of founding a sustainable captive breeding population.<sup>11</sup> In 2009, during the chytrid outbreak on Montserrat, a further 50 55 wild frogs were brought to Europe from this island to form the core of a captive breeding population, maintained under strict biosecurity conditions, with the ultimate aim of future reintroduction to the wild.<sup>14</sup>

The long-term viability of the captive MCF population is uncertain due to low breeding success and high rates of mortality;<sup>11</sup> however, the precise causes and potential underlying 60 pathologies have not been determined. Mortality reviews relating to other wild and captive anuran species have been published; however, these are limited to non-Leptodactylid species and are unlikely to accurately reflect the situation facing the MCF.<sup>9,34</sup> Whilst case reports exist which describe the diagnosis of neoplasia and metabolic bone disease in individual captive MCFs<sup>20</sup>, the importance of these, or other, disease processes at a population level has not 65 previously been determined.

This study is the first comprehensive review of post-mortem data relating to captive MCFs from multiple European institutions. The results will help to identify possible disease risk factors

and guide future areas of investigation with the aim of improving the health and sustainability of the captive MCF population.

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## MATERIALS AND METHODS

### **Necropsy reports**

All European Endangered Species Programme (EEP) establishments holding MCFs were asked to supply necropsy reports relating to MCFs which died whilst housed at these institutions  
75 between 1<sup>st</sup> January 1998 and 31<sup>st</sup> August 2018. Information pertaining to each animal's age and sex was recorded. If known, the frog's birth status (wild born versus captive bred) and biosecure versus non-biosecure status was also recorded. Animals were categorized as juvenile (zero days to two years post-metamorphosis) or adult (>2 years). All wild born frogs were considered adults at the time of capture so, for the purposes of this study, the age at death for these individuals was  
80 estimated by adding two years to the time period they were held in captivity. Tadpoles were excluded from the study because *L. fallax* are not officially recorded in the EEP until they have metamorphosed and reliable data is not available. Details relating to environmental parameters and husbandry practices at different holding institutions were not requested.

Each necropsy report was individually reviewed and assessed for quality of information  
85 provided and the state of carcass preservation (no autolysis; mild autolysis; severe autolysis) at the time of the necropsy. Records which did not describe post-mortem examination findings and reports stating severe carcass autolysis were excluded from the study.

Body tissues were considered abnormal on gross examination if unusual macroscopic observations relating to shape, size, color or texture were reported; however, histopathology was

90 required to confirm and characterize disease processes further. All pathological findings were recorded.

Based on relevant gross and histopathological information provided by pathologists within submitted reports, a primary cause of death or reason for euthanasia (hereafter “cause of death”) was assigned to each frog. Pathological findings and cause of death were categorized by main  
95 body system affected (body as a whole; cardiopulmonary; celomic cavity; gastrointestinal (GI); integumentary; hepatobiliary; musculoskeletal; reproductive; special senses; urinary; multisystemic; not determined) and primary disease process (inflammatory; neoplasia; other-determined; not determined).

## 100 **Statistical analysis**

For any particular pathological finding, prevalence was calculated using the number of animals for which that particular organ, tissue or specific finding was reported as screened for, rather than using the total number of post-mortem reports. This was done to prevent bias caused by variation in the breadth and depth of information contained within submitted necropsy reports.

105 All further analyses were performed using a statistics program (SPSS Statistics Version 24; IBM, Armonk, NY 10504, USA). Statistical significance was set at  $P < 0.05$ . Analyses were performed to determine whether significant differences existed between pathologic findings and sex, age, wild born versus captive bred status and biosecure versus non-biosecure status.

Pearson’s chi-square test was performed to examine relationships between mutually exclusive  
110 categorical data. The Cramer’s V strength test was used to test the data when a significant Chi-square result had been obtained. If the assumptions of the adequate expected cell count of 5 or more had been violated, the Fisher’s Exact test was used. Two sample t-tests were used to analyze differences between the occurrence of pathologic alterations and age.

Information relating to post-mortem microbiological testing was limited and not standardized  
115 and was therefore not considered suitable for statistical analysis.

## RESULTS

### Study Population

Reports were received for 343 post-metamorphic MCF from eleven different European  
120 institutions. Due to poor carcass preservation and or lack of information relating to post-mortem  
examination findings, 131 reports were excluded from the study.

Of the 212 records included in the statistical analysis, 53 post-mortem reports contained only  
gross necropsy findings whilst 149 reports contained information from both gross and  
histopathological examinations. A further ten reports contained information relating to  
125 histopathological examination only. The breadth and depth of information provided varied  
between reports.

One-hundred-and-fifty-eight reports were for captive bred frogs and 54 were from wild born  
animals. Sixty-one frogs (28.8%) were from the biosecure captive population whilst 136 frogs  
(64.1%) were held under non-biosecure conditions. The status of 15 frogs was not reported.  
130 Adult frogs were most represented (148/212; 70.1%) with 62 juveniles and two frogs of unknown  
age also included. Wild born frogs lived, on average, significantly longer (7y2m) than captive-  
bred frogs which had reached adult status (3y7m) ( $t=6.08$ ,  $p<0.001$ ). Sex was confirmed as  
female in 86 frogs (40.6%) and male in 78 animals (36.8%) whilst the sex of 48 frogs was not  
recorded, 44 (91.2%) of which were juveniles.

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### Post-mortem findings

The most prevalent findings observed on gross and histopathological examination are shown in Table 1.

140 Body condition was reported in 200 out of 212 submitted reports (94.3%) with thin body condition, often to the point of suspect emaciation, commonly described (125/200; 62.5%). This finding was significantly more prevalent in adult ( $\text{Chi}^2=31.9$ ,  $p<0.001$ ) and captive bred frogs ( $\text{Chi}^2=14.1$ ,  $p<0.001$ ). An underlying disease process was reported in 98 of these individuals.

The gastrointestinal tract was the organ system with the highest frequency of lesions reported. Gross examination of the gastrointestinal tract was performed in 202 animals and lesions were 145 observed in 82 (40.6%) of these animals, 77 of which were adult frogs. Adhesions between the colon and the urinary bladder was the most commonly reported lesion involving this body system (45/202; 22.3%) with concurrent fistula formation seen in 11 cases. These adhesions were more commonly reported in adult frogs ( $F$ ,  $p<0.001$ ). Soft tissue intestinal masses (31/202; 15.3%) (Figure 1), intestinal distension (29/202; 14.1%) and intestinal impaction (18/202; 8.9%) were 150 also commonly reported.

Histopathological examination was performed on GI tissues from 28 juveniles and 114 adult frogs plus two animals of unrecorded age. GI tract lesions were identified on histopathological examination of tissues from 86/144 (59.7%) specimens of which 79/86 (91.9%) were adults. The most frequently occurring pathological process was intestinal inflammatory disease, reported in 155 76/144 (52.3%) frogs, and was defined by the presence of inflammatory cell infiltrates within intestinal tunics, most often effacing the lamina propria and submucosa. This disease process was identified in 66/114 (57.9%) adult frogs screened. The majority of these cases were restricted to the colon ( $n=41$ ) whilst eleven cases appeared restricted to the small intestine. Histological descriptions of inflammatory infiltrates varied in detail; however, mixed cellular infiltrates 160 involving lymphocytes, plasma cells, histiocytes and or granulocytes were commonly described.

Intralesional bacteria were often observed; however, microbiological analysis was inconsistent and a specific etiology was never reported.

Intestinal adenocarcinoma was diagnosed only in adult frogs from which relevant tissues were submitted for histopathological review (31/114; 27.2%) (Figure 2). A soft tissue intestinal mass was reported on gross examination in only 21/31 (67.7%) of these cases. Neoplasia was not diagnosed in any juvenile frogs. Twenty-seven cases of intestinal adenocarcinoma were reportedly restricted to the colon whilst the location was not specified in four cases. In no instances was adenocarcinoma reported as occurring in the small intestine. Histologically, these primary neoplasms were most often described as focally extensive and composed of large, pleomorphic neoplastic epithelial cells with moderate-to-high amounts of eosinophilic cytoplasm and high mitotic activity. Six of these frogs showed histopathological evidence of metastatic spread with similar neoplastic cell-types identified within the kidneys, liver and or lungs. Intestinal dysplasia or metaplasia was identified in the colon of a further eleven adult frogs.

A significant association was identified between the presence of colonic adenocarcinoma and that of adhesions or fistula formation between the urinary bladder and distal colon ( $\text{Chi}^2=26.17$ ,  $p<0.001$ ). Significant differences existed between mean age and the presence of intestinal lesions: frogs with intestinal adenocarcinoma were significantly older at time of death (mean age=2943d,  $t=3.86$ ,  $p<0.001$ ) than animals with intestinal inflammatory disease alone (mean age=1825d) and these were significantly older than animals with no reported GI lesions (mean age=1244d,  $t=2.28$ ,  $p=0.03$ ). No significant differences were identified between the presence of inflammatory intestinal disease or neoplasia in male versus female frogs, wild born versus captive bred frogs or between frogs from the biosecure versus non-biosecure captive populations.

Enteric nematodiasis was reported in 77/144 (53.5%) frogs, based on identification of intestinal nematodes on gross or histologic examination of the GI tract or post-mortem analysis of

185 GI contents. Twenty-four out of 67 adult frogs (35.8%) with enteric nematodiasis showed  
histologic evidence of accompanying inflammatory disease. Nematode species identification was  
rarely performed; however, adult worms, larvae and ova of multiple roundworms were reported.  
Oxyurid pinworms, *Strongyloides* spp. and trichostrongyle-type parasites were mentioned in the  
submitted reports. Suspect *Rhabdias* spp. larvae and flagellated protozoa were infrequently  
190 observed in the gastrointestinal tract of individual frogs; however, no evidence of accompanying  
disease was reported.

The urinary tract presented a high prevalence of abnormalities on gross examination (55/197;  
27.9%) with abnormal kidney appearance reported in 38/197 (19.3%) frogs (Figure 3). Kidney  
samples from 32 frogs with gross kidney abnormalities were submitted for histological review, of  
195 which 26 showed histologic evidence of nephropathy. Conversely, of 48 reports where  
nephropathy was identified following histopathological examination, only 26 animals (54.2%)  
were described as having abnormal kidney appearance on gross observation.

Histological abnormalities affecting the urinary tract were reported in 59/120 (49.2%) adult  
frogs and 6/31 (19.4%) juvenile frogs. Tubulointerstitial nephritis containing mixed infiltrates of  
200 lymphocytes, histiocytes, plasma cells and granulocytes was reported in 26 adult frogs whilst  
tubular dilation (n=33), oxalate crystal formation (n=19) and renal fibrosis (n=8) were also  
reported, often concurrently. Acid-fast staining of lesions was performed in 18 cases of nephritis  
but was negative in each case. Intralesional Gram-negative rod-shaped bacteria were observed in  
13 frogs. Primary renal carcinoma (n=2) and suspect metastatic neoplasia (n=6) were reported in  
205 adult frogs. Cystitis was reported in 26 of 76 (34.2%) urinary bladder samples submitted for  
histopathology with mixed inflammatory cell infiltrates described.

Hepatitis was diagnosed on histological examination of 14/153 (9.2%) liver samples reported.  
Lesions were most often categorized as necrotizing with the accumulation of cellular debris,

210 fibrin and inflammatory cells within the necrotic defects. Gall bladder distension (n=11) and  
cholelithiasis (n=9) were observed on gross examination of the hepatobiliary system with both  
findings occurring concurrently in six cases. Suspect fibrous osteodystrophy ('metabolic bone  
disease') (n=19) and generalized underdevelopment (n=13), were observed on gross examination  
of non-biosecure captive bred juvenile animals from two separate clutches.

215 Granulomatous inflammatory infiltrates were confirmed within various viscera of 35 frogs, 31  
of which were adult. Ziehl-Nielsen staining was performed on samples from each of these  
individuals and acid-fast organisms, suspect *Mycobacterium* spp., were identified in four cases.  
Intralesional nematodes were observed in ten frogs with granulomatous enterocolitis. Encysted  
acanthocephalan parasites were noted within the skeletal muscle of 39 frogs, including 33/52  
(63.4%) of screened wild born frogs.

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### **Causes of death**

Based on the information submitted, a cause of death was identified for 26/53 (49.1%) frogs  
for which only gross examination details were available, compared to 138/159 (86.8%) frogs for  
which tissues were submitted for histopathological examination.

225 Amongst adult frogs, gastrointestinal tract disease was considered the most likely cause of  
death in 78/148 (52.7%) cases, followed by disease affecting the urinary system (22/148; 14.9%).

Neoplasia was frequently proposed as the cause of death but was only identified in adult frogs,  
accounting for the death of 42/148 animals (28.4%) in this age group. Neoplasia was suspected  
grossly in 27/42 (64.3%) of these cases. The majority of neoplasia cases (31/42; 73.8%) were  
230 confirmed as intestinal adenocarcinoma with lesions identified only within the colon. Intestinal  
neoplasia was reported in a further four adult frogs, based on gross examination alone; however,

tissues were not submitted for histopathological examination and so this diagnosis could not be categorized further. Renal carcinoma (n=2), large intestinal squamous cell carcinoma (n=1), urinary bladder carcinoma (n=1), gastric adenocarcinoma (n=1), ovarian neoplasia (n=1) and mesothelioma (n=1) were also identified as primary neoplasms.

Across all age groups, inflammatory disease processes caused the highest number of frog deaths (74/212; 34.9%) with adults more frequently affected (63/148; 42.6%) than juveniles (10/62; 16.1%) (Table 2). Inflammatory disease affecting the GI tract was the most commonly diagnosed inflammatory process leading to death in adult frogs (n=32) followed by inflammatory disease affecting the urinary system (n=18).

Foreign body ingestion with subsequent intestinal impaction or perforation resulted in the death of twelve frogs, nine of which were adult. Cataract formation was noted in six adult frogs and, in four of these cases, loss of vision and an inability to locate food items and maintain body condition was considered the main cause of death or euthanasia.

Amongst juvenile frogs, disease affecting the musculoskeletal system was the most frequently reported cause of death (23/62; 37.1%) with nineteen of these animals being euthanized due to severe fibrous osteodystrophic abnormalities. Other determined causes included foreign body ingestion and subsequent gastrointestinal perforation (n=2), suspect cardiovascular disease (n=1) and injuries suffered from conspecific aggression (n=1). A cause of death was not determined for 25/62 (40.3%) juvenile frogs.

No statistically significant differences were identified between causes of death affecting wild born versus captive bred frogs or between frogs held under biosecure versus non-biosecure conditions.

This study has identified the most prevalent pathologies affecting the European population of captive MCFs. This data will help guide future research and inform future management of the species in captivity. That said, given the inconsistency between necropsy protocols, pathologists' interpretation of findings and completeness of submitted reports inherent in this type of study, a degree of bias may have resulted in over or under reporting of different findings.

Ideally, all major body systems should be observed on gross examination with representative samples from each, plus samples from all suspect gross lesions, then submitted for histopathological review;<sup>28</sup> however, information within the necropsy reports received suggests that this was not always the case. If grossly abnormal tissue alone is submitted for histology, this risks under-diagnosing disease processes which are only visible microscopically. In addition, gross examination of the endocrine system (65/212; 30.7%) and the brain (58/212; 27.4%), for instance, were infrequently reported on with samples from these tissues rarely submitted for histological examination. It is therefore difficult to determine the prevalence of disease affecting certain tissues. Although some caution is required when considering the prevalence for different necropsy findings, the results provide an overall reliable estimate of the conditions which appear most relevant to the captive population of MCFs.

This study has shown colonic adenocarcinoma to be a major cause of disease in adult MCFs. Based on our findings, the MCF appears to be more susceptible to inflammatory and neoplastic disease processes affecting the GI and urinary systems than non-Leptodactylid frog species reported in previous studies.<sup>9,34</sup>

In this study, wild born frogs showed a significantly longer life expectancy than captive-bred frogs, even after juvenile mortality was accounted for. Furthermore, the likelihood that many wild born frog were older than two year at the time of capture means this difference is likely

280 under-estimated. Why captive bred frogs are dying at a younger age is not apparent but this finding suggests there are fundamental issues with how MCFs are being reared and developed in captivity or that captive bred individuals are exposed to disease risk factors from an earlier age and are more susceptible to terminal disease processes than wild born individuals.

285 Neoplasia and especially colonic adenocarcinoma are amongst the most frequent causes of death in captive adult MCFs. Intestinal adenocarcinoma has previously been reported in a captive wild born adult female MCF.<sup>15</sup> Based on the lack of pathological findings on clinical examination immediately post-capture, it is thought the neoplasm developed during the animal's two years in captivity. This condition was not observed in necropsy examinations of wild free-ranging MCFs (López pers. comm.).

290 Neoplasia has previously been described in amphibians.<sup>2,3,8,10,13,33</sup> With the exception of Lucke's renal carcinoma in the Northern leopard frog (*Rana pipiens*) and a high prevalence of intestinal adenocarcinoma reported in a single group of Amazon milk frogs (*Trachycephalus resinifictrix*), most reports of neoplasms in anurans have been stochastic events in individual animals.<sup>21,25,29,39</sup>

295 Intestinal neoplasia in humans has been associated with multiple processes, including infection with oncogenic pathogens, exposure to carcinogenic substances, genetic predisposition and lack or excess of certain components within the diet.<sup>5,22,26,27,30,36</sup> In mammalian species, high dietary fiber has been associated with decreased risk of GI disease, particularly colorectal neoplasia, and human studies suggest that slow GI transit time is associated with an increased risk of developing large bowel cancer.<sup>23,24</sup> Wild MCFs show a highly varied invertebrate diet with Orthoptera, Coleoptera, Opilionida and millipedes well represented.<sup>4,16</sup> In contrast, diets fed to captive MCFs are limited to a very small number of commercially available invertebrate species. As a consequence, captive MCF diets are less varied and higher in energy and crude fat

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which may be a factor contributing to the high prevalence of gastrointestinal disease in this  
305 species.<sup>17</sup>

Chronic GI inflammation has also been shown to increase the likelihood of localized cancer  
development in human patients.<sup>22</sup> Chronic GI inflammation was a very common finding in this  
study and might have predisposed to the development of intestinal neoplasia. Statistical analysis  
did not show an obvious correlation between the presence of inflammatory intestinal processes  
310 and neoplasia; however, many of the affected frogs presented with long standing neoplastic  
changes which may have obscured evidence of a previous inflammatory disease process.  
Furthermore, the frequent concomitant presence of inflammatory intestinal disease and intestinal  
hyperplasia, metaplasia and neoplasia could suggest that these processes are progressive, as has  
been observed in human studies.<sup>12</sup>

315 Neoplastic disease was detected only in adult frogs. This may be a reflection of increased  
exposure over time to previously discussed risk factors (diet; GI tract irritants; unknown  
environmental factors) which may predispose to or induce primary neoplasia or result in chronic  
inflammatory disease processes which may, in turn, lead to neoplasia. One particular risk factor  
that warrants further research is the type of substrate used in the enclosures of captive frogs as it  
320 is possible that some commercially available products may contain irritant or even carcinogenic  
substances. Since the feeding technique of the MCF often results in particles of substrate being  
ingested alongside the prey item, the possibility of exposure to noxious substances via this route  
cannot be ruled out and should be further investigated.

The relevance of observed parasite burdens in the necropsied frogs is equivocal. Whilst a high  
325 proportion of reports described GI parasite burdens, evidence of associated disease was less  
frequently diagnosed on histopathological examination. Granulomatous lesions associated with  
migrating nematodes were not considered to be severe and so were unlikely to be a direct cause

of death. Based on these findings, it seems less likely that prolonged parasite infestation may have led to chronic intestinal irritation and inflammation in these frogs, although it cannot be ruled out. In the absence of consistent disease associated with helminth infestation in the study population, anthelmintic treatment should be carried out on a case by case basis depending on the clinical concerns presented. The ethics of eradicating parasites which may be host-specific to endangered species and the need to maintain some level of immunity within a population should also be considered, especially when there is the possibility of these animals being reintroduced to the wild.<sup>35,36,38</sup>

The role of GI microbiomes in the development of GI disease has received much attention across several mammalian species.<sup>1</sup> Studies to examine and compare the microbiota of MCFs in various states of health are underway. It is hoped the results of such investigations may provide information regarding the significance and pathogenicity of different organisms in the development of GI disease in this species.

Overall, inflammatory disease was the most commonly reported disease process across all frogs. Often, despite the cellular infiltrates observed on histopathology, it was not possible to determine an etiology. Intralesional bacteria were frequently reported in cases of enterocolitis or nephritis; however, specific microbiological testing was inconsistently performed and could not attribute causation to micro-organisms isolated as these may have represented secondary pathogens or even post-mortem contamination. Multiple other factors may contribute to the high prevalence of inflammatory disease identified in MCFs, particularly in adult frogs. Besides the potential effect of substrate components, as mentioned previously, sub-optimal husbandry and management factors, as well as likely poor genetic variability amongst members of this critically endangered species, may increase the risk of chronic immunosuppression and infection by opportunistic pathogens.<sup>6</sup> Poor water quality or enclosure hygiene may have been associated with

an increased risk of infectious disease and may also contribute to the development of observed disease processes.<sup>7,19</sup> In this study, inflammatory disease was most frequently reported in the GI, urinary and hepatobiliary systems. If these pathological findings were demonstrated to be of bacterial origin, it could be hypothesized that a primary enteric infection may have resulted in other organs being affected via ascending infection. Investigating this potential pathogenesis would require methodical collection of bacteriological information whilst identifying an association between risk factors and development of disease would require analysis of husbandry and management conditions across different collections, which was beyond the scope of this study. Such investigations would be further complicated by the movement of individual frogs between institutions and the chronic nature of many observed lesions making it difficult to determine exactly when certain disease processes were initiated.

Urinary tract disease was frequently reported in adult frogs. Disease was often diagnosed on histopathological examination of the kidneys in the absence of gross lesions. Renal inflammatory disease was most frequently identified within the tubulointerstitium with tubular dilation and mixed cellular infiltrates reported. Whilst intralésional bacteria, often containing Gram negative rods, were commonly observed, their importance could not reliably be determined in this study. The presence of oxalate crystals within the renal parenchyma was considered equivocal and likely secondary to ingestion of prey items which had been fed on an oxalate-rich diet.

Whilst the MCF is reported to be highly susceptible to chytridiomycosis, which has decimated the species in the wild, this condition was not diagnosed in any of the captive frogs included in this study. Routine testing for infection with Bd is advised for all dead captive MCFs; however, it is unclear from the reports received how many of the MCF examined post-mortem were explicitly tested for Bd infection. No evidence for chytrid fungus infection was noted on histological examination of submitted skin samples (n = 99). Furthermore, the lack of suspicious

skin lesions on gross examination nor histologic diagnosis of chytridiomycosis suggests that the strict biosecurity and management protocols implemented when wild MCFs were first brought into captivity have been effective at minimizing the risk of this disease within the European MCF population.

380 The majority of deaths in juvenile frogs were due to non-infectious husbandry-related processes such as fibrous osteodystrophy, which has been documented previously in captive MCFs.<sup>20</sup> Although the numbers of affected frogs were significant, they represent two independent and isolated episodes in which the conditions provided for the developing juvenile frogs would not have been adequate. Further studies are required to ascertain normal Vitamin D levels for  
385 MCFs. In the meantime, juvenile frogs should be provided with a balanced diet and sufficient UVB provision to maintain calcium homeostasis.<sup>37</sup>

Many frogs in this study were reported as thin or emaciated; however, an underlying cause was not determined for 24/122 (19.7%) of these cases. Tissues were only submitted for histopathological review from 13 of these animals. The thin body condition noted in these cases  
390 may have resulted from an underlying pathological process or as a consequence of management related factors, such as inappropriate food provision or competition from conspecifics.

Routine health examinations of adult frogs (>2 years old) may help to identify disease processes early enough to allow effective medical intervention; however, handling and restraint have been shown to increase corticosterone secretion in anurans, suggesting frequent handling  
395 and restraint is stressful with possible negative welfare implications.<sup>31,32</sup> An objective visual body scoring system for MCF has recently been developed which does not require handling of the animal.<sup>18</sup> Frequent visual assessments might prove a useful non-invasive method for identifying health issues and monitoring response to treatment.

As mentioned above, the lack of consistency between necropsy protocols reduces the accuracy  
400 of the analysis of studies such as this one and limits to the capacity to confirm potential  
etiologies. A standardized necropsy protocol is now recommended across participating European-  
holding collections in order to ensure that MCF carcasses are examined thoroughly and that  
maximum information is obtained. Examination of a larger dataset, including captive MCFs  
maintained within U.S. institutions might help to determine whether the disease processes  
405 identified are similar or significantly different to those affecting the European population.

## CONCLUSIONS

The mountain chicken frog is Critically Endangered and is considered functionally extinct  
across almost all of its former range. This study shows that inflammatory disease processes and  
410 neoplasia, particularly affecting the gastrointestinal and urinary systems, are prevalent within  
European-held mountain chicken frogs. Whilst inciting etiologies have not been confirmed, this  
study informs further research into possible environmental, management and frog-related risk  
factors so that these may be identified and assuaged in order to maintain a sustainable captive  
population.

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565 **Table 1.** Prevalence of the most frequently reported findings following gross (A) and  
histopathological (B) post-mortem examination of mountain chicken frogs (MCF) (*Leptodactylus*  
*fallax*, n = 212) categorized by age group, sex and birth status across the captive biosecure and  
non-biosecure European Endangered Species Programme populations, 1998 to 2018. Individual  
frogs may be represented more than once.

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N refers to the number of reports which contained relevant information allowing the presence or  
absence of disease to be determined

J represents juvenile frogs defined as 30 days to 2 years post-metamorphosis

575 A represents adult frogs greater than 2 years of age

NR represents frogs where the relevant information was not reported

WB refers to wild born MCFs

CB refers to captive bred MCFs

B refers to MCFs maintained as part of the biosecure captive population

580 NB refers to MCFs maintained as part of the non-biosecure captive population

Total represents the total number of cases in which the pathological finding was reported

% represents the percentage prevalence of each post-mortem finding across all categories

Bracketed numbers represent the percentage of affected frogs within each category

**Table 2.** Disease processes and main body systems associated with primary cause of death in  
585 mountain chicken frogs (MCFs) (*Leptodactylus fallax*, n = 212) categorized by age group, sex  
and birth status across the biosecure and non-biosecure European Endangered Species  
Programme populations, 1998 to 2018.

590 J represents juvenile frogs defined as 30 days to 2 years post-metamorphosis  
A represents adult frogs greater than 2 years of age  
NR represents frogs where the relevant information was not reported  
WB refers to wild born MCFs  
595 CB refers to captive bred MCFs  
B refers to MCFs maintained as part of the biosecure captive population  
NB refers to MCFs maintained as part of the non-biosecure captive population  
T represents the total number of cases in which the disease process was considered the cause of  
death  
600 Z represents the percentage prevalence of each disease process as the cause of death across frogs  
in all categories  
Bracketed numbers indicate the percentage of affected frogs within individual categories

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### **Figure Legends.**

**Figure 1.** Post-mortem image of an adult mountain chicken frog (*Leptodactylus fallax*) showing significant intestinal distension (small arrow) caused by an obstructive soft tissue mass (large arrow). Colonic adenocarcinoma was diagnosed on histopathological examination.

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**Figure 2.** Photomicrograph showing transverse section of mountain chicken frog (*Leptodactylus fallax*) colon with severe effacement of the intestinal wall by neoplastic tissue (black arrows). The intestinal lumen (star) is labelled to aid tissue orientation and the presence of poorly-differentiated mucus-secreting cells confirms the diagnosis of adenocarcinoma. H&E, 20x.

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**Figure 3.** Gross post-mortem image showing the kidneys of a mountain chicken frog (*Leptodactylus fallax*) *in situ*. Both kidneys are enlarged, asymmetrical and nodular (white arrows) indicative of diffuse nephropathy. Histopathological examination revealed a chronic tubulointerstitial nephritis. Intralesional bacteria were noted; however, their significance in this case was undetermined.

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Body system (N)	Age			Sex			Birth status		Population			Total (%)
	J	A	NR	Male	Female	NR	WB	CB	B	NB	NR	
Body as a whole (200)												
Thin body condition	19 (15.2)	106 (84.8)		59 (47.2)	56 (44.8)	10 (8.0)	45 (36.0)	80 (64.0)	43 (34.4)	71 (56.8)	11 (8.8)	125 (62.5)
Gastrointestinal tract (202)												
Adhesions between colon and urinary bladder	1 (2.2)	44 (97.8)		14 (31.1)	30 (66.7)	1 (2.2)	14 (31.1)	31 (68.9)	10 (22.2)	31 (68.9)	4 (8.9)	45 (22.3)
Gastrointestinal foreign body or impaction	1 (5.5)	17 (94.5)		7 (38.9)	10 (61.1)	1 (5.5)	5 (27.8)	13 (72.2)	6 (33.3)	10 (55.5)	2 (11.1)	18 (8.9)
Gastrointestinal perforation	1 (6.7)	13 (86.6)	1 (6.7)	7 (46.7)	7 (46.7)	1 (6.6)	6 (40.0)	9 (60.0)	4 (40.0)	9 (60.0)	2 (13.3)	15 (7.4)
Soft tissue intestinal mass		31 (100)		11 (35.5)	20 (64.5)		13 (41.9)	18 (58.1)	9 (29.0)	19 (61.3)	3 (9.7)	31 (15.3)
Intestinal distension	1 (3.4)	28 (96.6)		10 (34.5)	19 (65.5)		10 (34.5)	19 (65.5)	9 (31.0)	17 (58.6)	3 (10.3)	29 (14.1)
Fistula between colon and urinary bladder		11 (100)		3 (27.3)	8 (72.7)		3 (27.3)	8 (72.7)	1 (9.1)	10 (90.0)		11 (5.4)
Intestinal nematodes (144)	10 (13.0)	67 (87.0)		34 (44.2)	38 (49.4)	5 (6.5)	27 (35.1)	50 (64.9)	24 (31.2)	50 (64.9)	3 (3.9)	77 (53.5)
Urinary tract (194)												
Abnormal soft tissue mass within urinary system		6 (100)		3 (50.0)	3 (50.0)		2 (33.3)	4 (66.7)	1 (16.7)	5 (83.3)		6 (3.1)
Musculoskeletal (198)												
Acanthocephalan parasites within skeletal muscle	2 (5.1)	37 (94.9)		19 (48.7)	20 (51.3)		33 (84.6)	6 (15.4)	20 (51.3)	13 (33.3)	6 (15.3)	39 (19.7)
Fibrous osteodystrophy	19 (100)			1 (5.3)	1 (5.3)	17 (89.4)		19 (100)		19 (100)		19 (9.6)
Long bone fracture	2 (40.0)	3 (60.0)		3 (60.0)	1 (20.0)	1 (20.0)	2 (40.0)	3 (60.0)	2 (40.0)	3 (60.0)		5 (2.5)
Hepatobiliary (199)												
Distended gall bladder		11 (100)		5 (45.5)	6 (54.5)		3 (27.3)	8 (72.7)	4 (36.4)	6 (54.5)	1 (9.1)	11 (5.5)
Cholelithiasis		9 (100)		2 (22.2)	7 (77.8)		6 (66.7)	3 (33.3)	7 (77.8)	2 (22.2)		9 (4.5)
Integumentary (199)												
External wounds	4 (57.1)	3 (42.9)		4 (57.1)	2 (28.6)	1 (14.3)	2 (28.6)	5 (71.4)	4 (57.1)	2 (28.6)	1 (14.3)	7 (3.5)
Special senses (151)												
Cataract/s		6 (100)		4 (66.7)	2 (33.3)		5 (83.3)	1 (16.7)	4 (66.6)	1 (16.7)	1 (16.7)	5 (3.3)

Body system (N) Histopathological diagnosis	Age			Sex			Birth status		Population			
	J	A	NR	Male	Female	NR	WB	CB	B	NB	NR	Total (%)
Gastrointestinal (144)												
Intestinal inflammatory disease	9 (11.8)	66 (86.8)	1 (1.4)	33 (43.4)	39 (51.3)	4 (5.3)	23 (30.3)	53 (69.7)	24 (31.6)	47 (61.8)	5 (6.6)	76 (52.3)
Small intestine only	1 (7.7)	11 (84.6)	1 (7.7)	7 (53.8)	5 (38.5)	1 (7.7)	7 (53.8)	6 (46.3)	5 (38.5)	6 (46.2)	2 (15.4)	13 (9.0)
Large intestine only	5 (10.9)	41 (89.1)		19 (41.3)	25 (54.3)	2 (4.3)	11 (23.9)	35 (76.1)	13 (28.3)	31 (67.4)	2 (4.3)	46 (31.9)
Colonic adenocarcinoma (137)		31 (100)		13 (41.9)	18 (58.1)		12 (38.7)	19 (61.3)	10 (32.2)	18 (58.1)	3 (9.7)	31 (22.6)
Parasitic enterocolitis (144)	5 (20.8)	19 (79.2)		10 (41.7)	12 (50.0)	2 (8.3)	6 (25.0)	18 (75.0)	9 (37.5)	13 (54.2)	2 (8.3)	24 (16.7)
Colonic hyperplasia/ metaplasia/ dysplasia (137)		11 (100)		2 (18.2)	9 (81.2)		1 (9.1)	10 (90.9)	2 (18.2)	8 (72.3)	1 (9.1)	11 (8.0)
Urinary (153)												
Nephropathy	2 (4.2)	45 (93.7)	1 (2.1)	19 (39.6)	26 (54.2)	3 (6.2)	20 (41.7)	28 (58.3)	13 (27.1)	31 (64.6)	4 (8.3)	48 (31.4)
Tubulointerstitial nephritis		26 (100)		12 (46.2)	14 (53.8)		13 (50.0)	13 (50.0)	7 (26.9)	17 (65.4)	2 (7.7)	26 (17.0)
Renal granuloma formation		23 (100)		13 (56.5)	10 (43.5)		13 (56.5)	10 (43.5)	9 (39.1)	11 (47.8)	3 (13.0)	23 (15.0)
Oxalate crystal present		19 (100)		5 (26.3)	13 (68.4)	1 (5.3)	8 (42.1)	11 (57.9)	3 (15.8)	14 (73.7)	2 (10.5)	19 (12.4)
Renal neoplasia (primary and metastases)		8 (100)		3 (37.5)	5 (62.5)		4 (50.0)	4 (50.0)	2 (25.0)	5 (62.5)	1 (12.5)	8 (5.2)
Cystitis	3 (12.0)	22 (88.0)		9 (36.0)	13 (52.0)	3 (12.0)	7 (28.0)	18 (72.0)	6 (24.0)	18 (72.0)	1 (4.0)	25 (16.3)
Hepatobiliary (153)												
Hepatitis	1 (7.1)	12 (85.8)	1 (7.1)	6 (42.9)	7 (50.0)	1 (7.1)	8 (57.1)	6 (42.9)	6 (42.9)	7 (50.0)	1 (7.1)	14 (9.2)
Body as a whole												
Acid-fast bacteria identified (any tissue) (N=48)		4 (100)		3 (75.0)	1 (25.0)		1 (25.0)	3 (75.0)	1 (25.0)		3 (75.0)	4 (8.3)
Metastatic neoplasia (excl. renal tissue) (N=159)		7 (100)		1 (14.3)	6 (85.7)		2 (28.6)	5 (71.4)	2 (28.6)	5 (71.4)	1 (14.3)	7 (4.4)
Integumentary (99)												
Dermatitis	2 (28.6)	5 (71.4)		3 (42.9)	4 (57.1)		3 (42.9)	4 (57.1)	4 (57.1)	3 (42.9)		7 (7.1)
Endocrine (64)												
Thyroid hyperplasia		2 (100)		1 (50.0)	1 (50.0)		1 (50.0)	1 (50.0)		2 (100)		2 (3.1)

Disease process Body system	Age group			Gender			Birth status		Population			Total (%)
	J	A	NR	Male	Female	NR	WB	CB	B	NB	NR	
Inflammatory	10 (14)	63 (85)	1 (1)	29 (39)	38 (52)	7 (9)	24 (32)	50 (68)	20 (27)	49 (66)	5 (7)	74 (35)
GI	4	32		16	18	2	11	25	12	23	1	36
Urinary	5	18		9	10	4	6	17	4	18	1	23
Hepatobiliary	1	5		2	4		4	2	4	1	1	6
Multisystemic		4	1	2	2	1	3	2		4	1	5
Celomic cavity		2			2			2		1	1	2
Integumentary		2		1	1			2		2		2
Neoplasia		42 (100)		16 (38)	26 (62)		16 (38)	26 (62)	12 (29)	27 (64)	3 (7)	42 (20)
GI		37		15	22		14	23	10	24	3	37
Urinary		3		1	2		1	2	1	2		3
Reproductive		1			1			1		1		1
Celomic cavity		1			1		1		1			1
Other-determined	27 (56)	20 (42)	1 (2)	16 (33)	10 (21)	22 (46)	9 (19)	39 (81)	10 (21)	34 (71)	4 (8)	48 (23)
Musculoskeletal	23	2		3	3	19	24	1	1	22	2	25
GI	2	9	1	6	4	2	3	9	4	7	1	12
Body as a whole	1	3		3	1		1	3	2	2		4
Special senses		4		2	2		4		3		1	4
Cardiopulmonary	1			1				1		1		1
Integumentary		1		1				1		1		1
Urinary		1				1		1		1		1
Not determined	25 (52)	23 (48)		17 (33)	12 (25)	19 (40)	5 (10)	43 (90)	19 (40)	26 (54)	3 (6)	48 (23)





