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TITLE OF CASE ***Do not include "a case report"***

Disease Surveillance and Risk Factors Affecting Mortality of Captive Cirl Buntings (*Emberiza cirlus*) in a Translocation for Conservation Purposes

PLEASE NOTE CORRECT LIST OF AUTHORS Fountain K, C Laven, J Gregson, C Jeffs, Molenaar F, Vaughan-Higgins RJ, Carter I, Evans A, Chang YM, Sayers G, A Pocknell, Sainsbury AW

SUMMARY ***Up to 150 words summarising the case presentation and outcome (this will be freely available online)***

Cirl buntings in the UK were translocated over a five-year period by collecting chicks from the residual population, hand-rearing and releasing them at a site in Cornwall with the aim of establishing a second breeding population. Because mortality and morbidity during captivity restricts the number and fitness of individuals available for release, selected parasites were monitored in the captive chicks, and all deaths were investigated by autopsy, histopathology and bacteriology. Risk factors associated with captive deaths were analysed. Annual mortality during captivity ranged from 4% (3 of 75 chicks in 2006) to 42% (26 of 73 in 2007) of chicks collected. Infectious disease associated with immunosuppression was an important factor in many deaths, and chicks collected with lower body weight were at greater risk of mortality. These findings emphasize the need for rigorous monitoring of all aspects of captive care during passerine translocations and provide evidence-based recommendations for future projects.

BACKGROUND ***Why you think this case is important – why did you write it up?***

Introduction

The cirl bunting (*Emberiza cirlus*) was once a common farmland bird in southern Britain but changes in agricultural methods in the 20th Century led to a reduction in both range and numbers. The species became concentrated in a residual population in Devon estimated at 118 pairs in 1989.[1] Cirl buntings are a sedentary species so although action to provide suitable habitat increased the population to around 860 pairs by 2010, there was little increase in range. To increase the species range a plan was formulated by a consortium of organisations to establish a second population at a different site and cirl buntings were translocated to Cornwall between 2006 and 2011.[2] It was anticipated from the results of monitoring of cirl buntings through mist-netting, that capture and translocation of adult cirl buntings would result in unacceptable mortality (Evans, personal communication), probably due to capture myopathy. Therefore it was decided to collect chicks from nests in Devon, hand-rear and delayed-release them at a suitable site in Cornwall as described by Jeffs et al.[2]

The reported success of programmes which release captive reared animals for conservation is variable,[3] and there is a lack of studies to inform evidence-based decisions about which rearing and release methods would increase success.[4] In order to improve the usefulness of translocation as a conservation tool, detailed planning and monitoring of projects is advocated and an analysis of the risks to health should be included in this planning.[5-9] Disease and parasite management are of particular concern as they can have a profound effect on survival and ultimately the numbers of animals available for release and on the ability of released individuals to survive and breed.[8] Each species carries its own suite of parasites and it has been proposed that the animal is released together with this suite of parasites,[10] provided these are native to the release area,[11] in order to maintain biodiversity. However, parasites which are considered harmless commensal organisms in the healthy animal may become pathogenic in animals subjected to the immunosuppressive stresses of captivity and translocation, compounded by the effect of crowding, poor hygiene, and a suboptimal diet,[7] and so careful evaluation of the risks from disease is recommended.[12]

A disease risk analysis (DRA) was part of the planning of the cirl bunting reintroduction project.[13] The major risk of disease identified was the potential for infection of cirl buntings with alien parasites from exotic species in Paignton Zoo. As a result the captive rearing element of the project was moved to an alternative location distant from the zoo, and dedicated staff, housing and equipment were used for the translocated birds. Critical control points (CCP) were identified at each of five stages of the project, and disease mitigation measures were suggested for each CCP.[14] These included recommendations for parasite screening, quarantine, hygiene, handling and stocking density restrictions. Benefit-cost analysis of each measure included, for example, assessing the risk to the health of the birds by handling for sampling, and as a result blood sampling for a parasite (*Haemoproteus* sp) was omitted from the testing protocol, despite the potential for depression of performance.
The DRA identified the feeding of exotic invertebrates to the buntings as a possible source of exotic parasites to native invertebrates at the release site. Since native invertebrates were not always available, as a compromise only native invertebrates were fed to the cirl buntings for at least three days prior to release.

In healthy free-living cirl buntings in Italy coccidial oocysts, *Isospora normalevinei* and *I. coluzzi*, were identified from faeces samples.[15] During a trial translocation in 2004 captive cirl buntings suffered a disease outbreak associated with an isosporoid coccidial infection.[16] In order to release the cirl buntings in this project with some of their commensal coccidial parasites and enable the birds to retain protection against coccidial parasites in the release area,[7] a preventative medication protocol was devised to balance the development of immunity against the need to prevent disease in captivity.[17]

To increase the evidence base for future translocations of cirl buntings and other passerine birds, this study sought to examine the effect of specific capture and rearing risk factors on the health and survival of the cirl buntings during their rearing in captivity between 2006 and 2011, and to assess the impact of

the preventive medicine protocols developed during the disease risk analysis. A second paper analyses the effect of rearing factors on post-release survival.[18]

CASE PRESENTATION ***Presenting features, clinical and environmental history***

INVESTIGATIONS ***If relevant***

Materials and Methods

Chicks were collected from free-living cirl bunting nests, at an estimated five to seven days of age, from up to ten different sites in Devon. Between twenty and thirty complete broods were collected each year. Chicks were placed into a single-use cardboard travel box and transported to a dedicated rearing facility in Cornwall.

Bodyweight at collection
Chicks were weighed at collection from the nest using spring balance scales. Following a review, no chicks below 10g bodyweight were collected after July 2009.

Rearing
Chicks were placed in a heated brooder cage held at 28^{o}C and individually hand-fed every two hours with a pelleted diet (Diet A; Mazuri Zoo Foods) mixed with boiled eggs and banana, and locusts (of unknown species) and mealworms (*Tenebrio spp*.). They were transferred to a box cage (canary cage) (in a different rearing room between 2008 and 2011 inclusive), once sufficiently developed and hand feeding was continued until the birds were feeding themselves. Mixed millet was added to the food at this stage. Birds were not handled after fledging in an effort to reduce levels of stress. Chicks were transferred from the box cages to pre-release aviaries for several days before being 'delayed-released' with food being provided at the release site. The time spent in each type of housing was noted. Numbers of cirl bunting broods in brooders and canary cages were restricted to a maximum of eight broods in total.

Quarantine
Each brooder, box cage and aviary was held in quarantine from every other brooder/cage/aviary. Dedicated tools and equipment were available for each brooder, box cage and aviary. Following an increase in mortality rate in 2007, a second rearing room was set up which housed exclusively canary cages, and the first room housed only brooders from 2008 onwards. Personnel changed footwear and walked through a disinfectant footbath into the brooder room, and again, when working in the box cage room. Separate footwear was worn in each aviary and a footbath used. Overalls were changed between each brooder, box cage and aviary. F10 wipes and disinfectant (QAC and Biguanide, Health and Hygiene Ltd, S. Africa) was used to maintain hygiene: the brooder was wiped with F10 after every feed. Clean feeding receptacles were used in the box cages and aviaries for every feed and feeding areas cleaned.

Faecal examination for parasites
Pooled faecal samples were collected (i) from the nest when the chicks were removed, (ii) from the box used for transport to the rearing site, (iii) on day 3 in the brooders, (iv) on day 10 in the canary cages, (v) on days 17 and 24 in the pre-release aviaries, and (vi) post-release from any birds returning to aviaries to roost. Samples collected in 2006 were examined by phase-contrast light microscopy. After January 2007 samples were examined either by light microscopy or by salt flotation as set out by McGill and Sainsbury.[17] Parasites detected included coccidial oocysts which were not identified to species level, *Hymenolepsis*-like ova, or strongyle-type ova. During 2009 the frequency of faecal sampling was reduced (n=28), and in 2010 and 2011 no faecal screening was undertaken. Birds were noted as parasite positive

or negative on the basis of the pooled samples.

Faecal Culture
Faecal samples (n=43) were collected opportunistically from nests when chicks were collected, from transport boxes or from birds in captivity for monitoring or clinical reasons and cultured for bacteria and fungi as described by McGill et al.[16]

Medication
Toltrazuril (Baycox, Bayer) was administered orally to all birds at a dose rate of 12.5mg/kg bodyweight as a protozoal prophylaxis on days 5 & 6, 12 & 13, 19 & 20, and 26 & 27. From July 2006 until July 2007 the dosing frequency was reduced to days 5, 12, 19 and 26, because coccidia were not detected in routine faecal samples using microscopy. The introduction of salt-flotation demonstrated the presence of the parasite, so the original dosing protocol was reinstated after July 2007. The medication was given in food to birds at the brooder stage and thereafter in the drinking water at a rate of 1.8ml of 2.5% solution per 1 litre of water. Only birds receiving additional toltrazuril, over and above both these two therapeutic regimes, were included in a 'medication' category for the purposes of the analysis. Other medication was administered to birds showing signs of illness or following trauma, or on a prophylactic basis to broods following disease outbreaks. Medication included enrofloxacin (Baytril, Bayer, 20 mg/kg body weight four times per day by mouth), meloxicam (Metacam, Boehringer, 0.2 mg/kg body weight four times per day by mouth) or toltrazuril where it was additional to the normal prophylactic protocol. Where the information was available the medication of individual birds was noted, otherwise the whole brood was marked as medicated.

Post Mortem Examination
Captive birds which died were transported by post to the Institute of Zoology, incorporating a freezer pack in the parcel to keep the carcase cool, and examined according to a standard avian post-mortem examination procedure. The degree of carcase autolysis was graded as 'fresh', 'mildly autolysed', 'autolysed' or 'mummified'. Tissues obtained were preserved and examined as appropriate by histology, parasitology, mycology, bacteriology and virology. Frozen lung tissue from 17 birds which died during 2011 was examined retrospectively by PCR for *Chlamydia psittaci* and *Avipoxvirus.*

Statistical analysis
The information was analysed using statistical software package 'R' version 3.0.2 (2013-09-25, "Frisbee Sailing"). Generalized estimating equations modelling was used to account for the clustering of birds from the same brooder in the analysis; exchangeable correlation structure was used. Univariable logistic models were used to assess risk factors individually, and those with p-values < 0.1 were then included in a multivariable model followed by a backward elimination model selection approach. Type I error rate was set at 5%.

The risk factors used were: capture body weight (g), days in brooder, days in canary cage, days in aviary, year of capture, month captured (May, June, July, August), parasite positive and medicated birds. For the risk factor 'Year' the year '2006' is used as the baseline in the multivariable model. For 'Month Captured' the month 'August' is used as the baseline. Odds ratios are quoted relative to these factor levels. For 'Parasite Positive', data was only available for years 2006-2009.

In 2009 data on capture body weight between 2006 and 2009 was analysed using odds ratios to compare the likelihood of mortality in the groups: less than 10g, 10-11g, 11-12g, 12-13g and greater than 13g. Statistical software package SPSS® (IBM) was used for this analysis.

DIFFERENTIAL DIAGNOSIS ***If relevant***

TREATMENT ***If relevant***

OUTCOME AND FOLLOW-UP

Results

Of 455 cirl bunting chicks collected during the course of the project, 79 chicks died in captivity and 376 were released (Table One).

Year	Number of Birds Collected	No of birds which died in captivity (% of captive chicks)	Died in Brooder (% of chicks which died)	Died in Canary Cage (% of chicks which died)	Died in Aviary (% of chicks which died)	Number of Birds Released
2006	75	3 (4)	2 (67)	1 (33)	0 (0)	72
2007	73	26 (42.5)	4 (15)	15 (58)	7 (27)	47
2008	75	7 (9.3)	3 (43)	3 (43)	1 (14)	68
2009	80	13 (16.2)	5 (38)	4 (31)	4 (31)	67
2010	76	6 (7.9)	0 (0)	4 (67)	2 (33)	70
2011	76	24 (31.6)	1 (4)	16 (63)	7 (33)	52
Total	**455**	**79 (17.4)**	**15 (19)**	**43 (53)**	**21 (28)**	**376**

Table One. The number of cirl buntings collected from nests in Devon, and the number of these which died in captivity including the number of deaths in each type of housing: brooder, canary cage and aviary, and number of birds released during the years 2006 to 2011.

A total of 145 broods (mean 3.11 (range 2-5) chicks per brood) were collected and chicks from 50 of these broods died in captivity, however the deaths were not uniformly distributed. In 35 broods one chick died, in five broods two died, in six broods three chicks died and in four broods four chicks died. Thus 10% of the broods contained 56% of the chicks which died in captivity.

Risk Factor	Birds which Died in Captivity	Birds Released
Mean Capture Body Weight (g) (SD)	13.7 (3.52)	14.7 (2.33)
Mean Days in Brooder (SD)	8.48 (3.52)	8.26 (1.73)
Mean Days in Canary Cage (SD)	7.5 (3.67)	8.84 (2.89)
Number Parasite Positive (% of total birds died or released)	6 (8)*	37 (10)*
Number Medicated Birds (% of total birds died or released)	48 (61)	50 (13)
Month Captured May (% of birds captured in May)	6 (16)	32 (84)
Month Captured June (% of birds captured in June)	41 (25)	121 (75)
Month Captured July (% of birds captured in July)	22 (11)	172 (89)
Month Captured August (% of birds captured in August)	10 (16)	51 (84)

Table Two. Summary data for the risk factors capture body weight, days in brooder, days in canary cage, parasite positive, medicated and month captured. Percentages in brackets – for number parasite positive and medicated this is a percentage of the total birds which died or were released, for month captured

this is a percentage of the birds captured in each month. *Number of parasite positive birds in years when testing was undertaken.

The multivariable logistic regression analysis of risk factors found that 'medication' significantly increased the risk of mortality, and that there was an increased risk in 2011 (Table Three). The model did not return a result for 'days in canary cage or aviary' due to missing values for birds which died at these stages.

Risk Factor	Odds Ratio	95% Confidence Intervals
Year 2011	4.54	1.2-16.9 *
Medication	5.40	2.8-10.3 *

Table Three. Results of Multivariable Logistic Regression of Risk Factors for Mortality of Cirl Buntings in Captivity. * Indicates variables having a significant effect on mortality.

Capture Body Weight	Total number of chicks in category	Number dead (%)	Odds Ratio (95% CI)
Less than 10g	15	7 (47)	5.61 (1.89-16.65)
10-11g	17	6 (35)	3.5 (1.2-10.19)
11-12g	21	6 (29)	2.57 (0.92-7.15)
12-13g	33	4 (12)	0.88 (0.29-2.7)
Greater than 13g	215	29 (13)	1
Total	301	52	

Table Four. Odds ratios for comparison of captive mortality in cirl buntings collected at bodyweights of less than 10g, 10-11g, 11-12g, 12-13g and greater than 13g during 2006 - 2009.

Between 2006 and 2009 chicks collected with a bodyweight less than 10g were 5.61 times more likely to die than chicks over 13g at collection (Table Four**)**. As a result of this finding, chicks less than 10g in bodyweight were not collected in the years 2010 and 2011.

Post Mortem Examination Findings

The majority of carcases were submitted unfrozen for post mortem examination, but in 2007/8 a total of 11 birds were frozen for transport which reduced the value of histopathology. Transport was by post and the mean time from death to post mortem examination varied each year, (2006-2011: 3 days, 2.8 days, 1.4 days, 3.2 days, 2.2 days, 2 days) with a range of 1-6 days. Postal strikes caused delays in 2009. Sixty eight (91%) carcases had evidence of autolysis by the time of examination and this hampered interpretation of the histopathology, particularly of the gastrointestinal system, and associated bacterial overgrowth hampered bacteriological interpretation. As a result many of the descriptions of the diseases detected were limited to gross findings.

Multiple pathological changes were found on examination of many birds and it was difficult to establish the primary cause of death because the chronology of the pathogenesis was unclear. The pathological findings have been grouped where appropriate and are summarised in Tables Five and Six. Bacteriological findings have been reported where isolates were in pure culture and associated with appropriate pathological findings.

Pathological Finding	**Number of Cirl Buntings Affected (%)**			
	Brooder (%) n=15	*Canary Cage (%) n=43*	*Aviary (%) n=21*	**At all management stages (%) n=79**
Respiratory Disease (respiratory disease, pulmonary	5 (33)	12 (28)	7 (33)	**24 (30)**

atelectasis, bronchitis, aspiration pneumonia, asphyxiation)				
Hepatic Changes (hepatomegaly, necrosis, lipidosis)	8 (53)	19 (44)	9 (43)	**36 (46)**
Enteric Changes (enteritis, enteropathy, oesophageal necrosis)	1 (7)	20 (47)	8 (38)	**29 (37)**
Immunosuppressive changes (bursal, thymic or lymphoid atrophy)	8 (53)	18 (42)	5 (24)	**31 (39)**
Trauma	1 (7)	7 (16)	6 (29)	**14 (18)**
Ocular Disease (conjunctivitis, keratitis)		4 (9)		**4 (5)**
Renal Disease (inc tubulonephritis)	2 (13)	1 (2.3)		**3 (4)**
Suspected septicaemia and bacteraemia.	2 (13)	5 (12)	2 (10)	**9 (11)**
Ascites	1 (7)			**1 (1.2)**
Cardiac Changes (pericarditis, endocarditis, cardiomyopathy, epicardial infarction)	1 (7)	2 (5)	1 (5)	**4 (5)**
Musculoskeletal Changes (myonecrosis, osteomyelitis, pododermatitis, toe necrosis)	1 (7)	4 (9)		**5 (6)**
Developmental Abnormalities (rotational leg deformity, feather abnormality)	1 (7)	1 (2.3)	1 (5)	**3 (4)**

Table Five. Summary of pathological findings at post mortem examination in cirl buntings reared for reintroduction which died in captivity between 2006 and 2011, and the number of birds which were affected by each disease at each housing stage. The proportion of cirl buntings with each disease at each housing stage is shown in brackets.

Pathological Finding	**Number of cirl buntings affected (%)**						
	2006 (%) n=3	*2007 (%) n=26*	*2008 (%) n=7*	*2009 (%) n=13*	*2010 (%) n=6*	*2011 (%) n=24*	**All years (%) n=79**
Respiratory Disease (respiratory disease, pulmonary atelectasis, bronchitis, aspiration pneumonia)			2 (29)	4 (31)	1 (17)	17 (71)	**24 (30)**
Hepatic Changes (hepatomegaly, necrosis, lipidosis)		14 (54)	1 (14)	7 (54)		17 (71)	**36 (46)**

Enteric Changes (enteritis, enteropathy, oesophageal necrosis)		9 (35)	1 (14)	3 (23)	2 (33)	14 (58)	**29 (37)**
Immunosuppressive changes (bursal, thymic or lymphoid atrophy)		21 (81)	2 (29)	4 (31)	1 (17)	3 (12)	**31 (39)**
Trauma	1 (33)	2 (8)	1 (14)	3 (23)		7 (29)	**14 (18)**
Ocular Disease (Conjunctivitis, keratitis)						4 (17)	**4 (5)**
Renal Disease (inc tubulonephritis)		1 (4)				2 (8)	**3 (4)**
Suspected septicaemia and bacteraemia.	2 (67)	3 (12)		1 (8)		3 (13)	**9 (11)**
Ascites			1 (14)				**1 (1.2)**
Cardiac Changes (pericarditis, endocarditis, cardiomyopathy, epicardial infarction)		1 (4)		2 (15)		1 (4)	**4 (5)**
Musculoskeletal Changes (myonecrosis, osteomyelitis, pododermatitis, toe necrosis)		1 (4)	1 (14)	1 (8)	1 (17)	1 (4)	**5 (6)**
Developmental Abnormalities (rotational leg deformity, feather abnormality)			1 (14)	2 (15)			**3 (4)**

Table Six. Summary of pathological findings in cirl buntings reared for reintroduction which died in captivity between 2006 and 2011 and number of birds with each disease which died in each year. The proportion of cirl buntings with each disease in each year is shown in brackets.

Respiratory Disease

Findings in this category included atelectasis of the lungs, erosive bronchitis, congestion and production of yellow-coloured exudate from the lungs. *Enterococcus faecium* was isolated in pure culture from the lung, liver and heart in one bird, and *Enterococcus faecalis* from the lungs of a second. *Stenotrophomonas maltophilia*, *Micrococcus lutea*, *E. coli* and *Pseudomonas* sp were cultured from the lungs in other cases. Atelectasis was noted in eight chicks but the cause of the air capillary collapse was not apparent in any of these cases. One bird died while being fed and was found to be obese at post mortem examination with food covering the cranial trachea. Three other birds had impaction of the crop, proventriculus and ventriculus. White frothy fluid was present in the trachea in all three cases and asphyxiation was suspected to be the proximate cause of death. All of the birds were of low body weight (7.6g, 8.4g and 10g) at collection from the nest and had experienced a period of cold weather which may have reduced the rate of intestinal peristalsis and led to impaction. Frozen lung tissue from 17 birds which died during 2011 with respiratory findings was negative by PCR for *Chlamydia psittaci* and *Avipoxvirus*.

Hepatic Changes

In 44% of cirl buntings found dead the liver was grossly enlarged (n=35), in six of these birds the liver was an abnormal orange-beige colour, and in 21 cases the liver was friable. Hepatic lipidosis was confirmed through histopathology in one case where the liver was enlarged and orange-beige and suspected in another. Hepatic necrosis was confirmed in one case and suspected in two others. Virological examination by tissue culture, passage through embryonated eggs and electromicroscopy failed to identify any viral involvement.

Enteric changes

Lesions were seen in the bursa, cloaca, ventriculus and intestines of birds. Five cases were associated with *Campylobacter* sp., and *Salmonella enterica subsp arizonae* was also isolated from one of these five. *Hafnia alvei* was isolated from a bird with gross changes consistent with gastroenteritis. Coccidia were associated with enteritis in six cases although microscopic lesions were not visible due to autolysis of tissues. In two cases there was concurrent splenic enlargement which might be expected in systemic coccidiosis.

Disease associated with immunosuppression.

Reduced amounts of thymic tissue or a smaller Bursa of Fabricius than would be expected in a young bird were found in 31 cirl buntings. In 18 cases both tissues were reduced and lymphoid atrophy was confirmed by histology. In 10 cases only bursal atrophy and in 2 cases only thymic atrophy was seen. 80% (n=25) of cases were seen in birds which were underweight or had signs of enteritis, hepatomegaly, tubulonephritis, septicaemia, coccidiosis or other infectious disease such as disease associated with *Campylobacter* sp infection.

Trauma

In this category multifocal haemorrhages of the shoulder, wings, cere and crown, a jugular haematoma, a blood clot at the heart base, a torn crop, skull fractures and injuries to feet and legs were among changes noted. One nestling cirl bunting suffered severe head trauma following an accident.

Renal Disease

One nestling had histological findings of multifocal acute tubulonephritis and a pure culture of *Serratia odorifera* was isolated from the kidneys, heart and liver. A second nestling had histological evidence of tubulonephritis but no agent suspected to be causal was identified.

Septicaemia

Escherichia coli was isolated in pure culture from the liver of one bird and the heart and lungs of another bird. These two birds died within 24 hours of each other but in different brooders. *Enterobacter* sp was isolated in pure culture from the lungs, heart and liver of another bird, and *Staphylococcus* sp was isolated in pure culture from the liver and heart of another. *E. coli* was isolated in pure culture from the liver, gizzard and pectoral muscle of a bird with gross and histological changes of acute, fibrinous pericarditis in which a pure population of Intralesional gram-negative rods was seen. This last bird was under expected body weight and died at 30 days of age after 20 days in captivity, in the aviaries after a stormy night.

Cardiac Disease

Gross lesions included epicardial necrosis and cardiomyopathy, but the lesions were not confirmed histologically. One case was a suspected cardiac arrest and no significant gross or histological lesions

were seen.

Developmental Abnormalities

Two birds showed rotational deformity of the tarsometatarsal joints and one also had a lateral rotation of the hip joint with secondary necrosis of the distal limb due to constriction by the leg ring. A third bird was a slow feeder, had poor wing feather development, and was euthanased as it was considered unsuitable for release. Other congenital defects noted as incidental findings included the absence of one testis in one bird, and missing secondary feathers on one wing of another.

Osteomyelitis

One bird developed a swelling between the right eye and the middle of the right side of the neck which appeared to prevent normal ingestion. At post mortem examination a yellow, well-circumscribed mass was noted which had infiltrated the quadrate bone of the skull and apparently compressed the brain tissue. Severe chronic osteomyelitis of the skull with intralesional bacteria and accompanying cellulitis was seen histologically, and *E. coli* was cultured pure from the lesion.

Bacteriology and Mycology

Those bacteria which were considered to be associated with disease are summarised in Table Seven.

Bacterium Isolated	**Associated Disease (number of cases in brackets)**
Escherichia coli.	Septicaemia (1), fibrinous pericarditis (1), osteomyelitis (1), respiratory disease (1)
Campylobacter sp. In one case *C. jejuni* phage type UT, HS untypable.	Enteritis (4).
Aeromonas hydrophila/caviae.	Hepatic changes.
Staphylococcus aureus.	Necrotic toe.
Serratia odorifera.	Tubulonephritis.
Enterobacter sp.	Septicaemia.
Enterobacter amnigensis.	Enteritis, hepatomegally.
Klebsiella pneumoniae.	Isolated from the lungs, muscle and liver in a case with concurrent trauma.
Hafnia alvei.	Enteritis.
Enterococcus sp	Respiratory disease (1).
Enterococcus faecalis	Respiratory disease (1)
Enterococcus faecium	Respiratory disease (1)
Stenotrophomonas maltophilia.	Respiratory disease (1), isolated from the eye, lungs and liver in a concurrent trauma case (1).
Pseudomonas sp.	Bilateral limbal keratitis.
Micrococcus luteus.	Isolated from lung, heart and liver associated with gross pathological changes, in a case of concurrent trauma.
Candida sp.	Associated with gross pathological intestinal changes in a case with concurrent trauma.

Table Seven. Bacteria isolated from cases associated with significant gross or microscopic disease.

Parasitology

Isospora-type, and *Eimeria*-type coccidial oocysts were detected in 23 faecal samples from captive birds from a total of 15 broods between 2006 and 2009. *Hymenolepsis* sp. ova were detected in one faeces sample. *Isospora*-type oocysts were detected in the faeces of free-living birds at the release site in 2006 and 2009.

Coccidial oocysts were detected in the intestinal contents of six birds examined post mortem, and necrotic parasites were found in the impression smear of the spleen of two birds. Structures resembling protozoal oocysts were seen on histology of the small intestine in six cases. Autolysis precluded identification of the species or confirmation of an association with coccidial disease in all cases.

A single *Trypanosoma* sp. was detected in a heart blood impression smear from one bird in 2009.

Faecal Culture

In 2007 *Campylobacter* sp. were detected in post-mortem samples from four cases, in a sample from an RSPB staff member (cultured at a medical laboratory) and in 2009 from a faecal sample from a captive bird. *Salmonella enterica subsp. arizonae* was also cultured from one of the same cases, associated with enteritis.

DISCUSSION *Include a very brief review of similar published cases*

Discussion

Mortality of captive cirl buntings was greatest in the years 2007 and 2011 with the deaths of 26 (33%) and 24 (30%) birds respectively (Table One). There were 13 deaths (16%) in 2009 when a larger number of chicks (n=80) were collected, however the recommended stocking density at each housing stage was never exceeded. The majority of deaths during captive rearing of cirl buntings were in the canary cage housing (n = 43; 54% of all deaths), followed by the aviary (n = 21; 27%) and the brooder stage (n = 15; 19%).

The multivariable logistic regression of the risk factors for mortality demonstrated that 'medication' was associated with a significantly greater risk of mortality as was capture during 2011 (Table Three). We have used generalized estimating equations modelling to account for the clustering of birds from the same brood, and this has allowed us to correct for the possible lack of independence among cirl bunting brood-mates.

The most prevalent pathological changes in the 15 cirl buntings which died at the brooder stage were hepatic changes (n=8, 53%) and immunosuppressive changes (n=8, 53%), with aspiration pneumonia (n=3, 20%) and suspected bacteraemia and septicaemia (n=3, 20%) also being common. At the canary cage stage hepatic changes (n=22, 51%) and enteric changes (n=19, 44%) were common, while in the aviaries hepatic changes (n=9, 43%), enteric changes (n=9, 43%) and respiratory changes (n=8, 38%), followed by trauma (n=7, 35%) were all frequently seen (Table Five). When pathological changes are analysed by year (Table Six) it is of note that in 2007 the most common pathological changes were immunosuppressive changes (n=21, 81%), hepatic changes (n=14, 54%) and enteric changes (n=9, 35%). In 2009 hepatic changes were common (n=7, 54%) along with immunosuppressive changes (n=4, 31%) while in 2011 hepatic changes (n=17, 71%), respiratory disease (n=17, 71%) and enteric changes (n=14, 58%) were common.

The gross and microscopic post-mortem examinations were hampered by autolysis of tissues due to the time which had elapsed between death and examination. Every effort was made by the aviculturists to dispatch cadavers on the day of death, and by pathologists to examine the cirl buntings immediately on arrival, but in most years the mean time from death to post-mortem examination was 2-3 days. This

resulted in a reliance on gross changes to assess for the presence of disease. In a study of mortality and disease in marine turtles, there was a poor association between the gross and microscopic findings post-mortem.[19] It would be of value in future projects to have a pathologist on-site where animals are reared to conduct post-mortem examinations and gather samples as soon as practical after death to improve information on the causes of morbidity and mortality.

Respiratory disease was seen in 29% of cirl buntings found dead during captive rearing (n=33) with 17 cases in 2011. This unusual clustering of deaths must raise the suspicion of an infectious aetiology, and all the deaths occurred within one to two days of a move to the next housing stage suggesting that stress may have played a role. Herpesviruses, paramyxoviruses, avipoxviruses, cytomegaloviruses,[20,21] *Mycoplasma gallisepticum* and *Chlamydia psittaci* have been reported to cause mortality and respiratory disease in passerines.[22,23] No histopathological or cultural evidence of viral involvement was detected in these cases, however autolysis may have precluded identification of characteristic lesions. PCR testing for *Chlamydia psittaci* and *Avipoxvirus* on frozen lung tissue from these 17 cases was negative. *Enterococcus faecium* and *E. faecalis* were cultured from one case each and *Stenotrophomonas maltophilia* from another. *E. faecalis* has been reported associated with tracheitis, pneumonia and air sac infections in canaries.[24] In six separate chicks atelectasis of the air capillaries was seen which can result from pneumocoelom or compressive coelomic effusion, trauma, upper airway obstruction, inhaled gases and certain infections.[21] Four birds were thought to have died due to asphyxiation. Three of these birds were from broods which had experienced poor growth and delayed crop emptying. At post mortem examination the proventriculus and ventriculus were found to be impacted. Delayed crop emptying was ascribed to the low environmental temperatures at the time, however infection with *Candida albicans* and *Macrorhabdus ornithogaster* (Megabacteria) can both be associated with similar clinical signs although no fungi were detected on culture of one case, and histopathology was not undertaken in any case.[21] Respiratory lesions occur in aspergillosis in captive passerine birds, but this was not detected in any captive birds in this project, however a bird which died shortly after release was found to have chronic aspergillosis.[18]

Hepatic changes were the most frequent pathological finding in birds which died at all three housing stages, however a greater percentage of birds were affected by hepatic changes in those years with the highest mortality, 2007 (54%), 2009 (54%) and 2011 (71%) (Table Six). The changes seen were non-specific and included enlargement, abnormal colouration and necrosis which have all been reported in passerines following infection with *Haemoproteus* spp, avian polyomavirus, Buggy Creek virus, avian paramyxovirus, *Plasmodium relictum*, *Salmonella* spp, *Yersinia pseudotuberculosis* and as a result of aflatoxicosis.[25-31] While no viral agents were identified, autolysis of hepatic tissues may have hampered histopathological identification of suggestive microscopic lesions and reduced the likelihood of live virus culture. Hepatic lipidosis was confirmed in one bird and in passerines is associated with feeding high energy diets such as mealworms and with inadequate exercise.[28]

Enteric changes were seen in 37% (n=29) of all cirl buntings with lesions seen grossly in all of the intestine. *Campylobacter* sp. were isolated from five cases and in one of these birds was typed as C. *jejuni* phage type UT. *Campylobacter* sp. associated disease is a zoonosis and this bacterium was isolated from a staff member of the nest finding team in 2007. *Campylobacter* sp. can be carried asymptomatically by passerine birds, but young or immunosuppressed individuals and some species such as tropical finches may be more susceptible to disease.[21] *Salmonella enterica subsp arizonae* was isolated from one case in association with enteritis. It is most commonly associated with reptiles but causes enteritis and mortality in turkeys in the USA (avian arizonosis).[32] *Hafnia alvei* is another zoonotic species which was isolated in one case of enteritis. The pathogenic potential of this organism is debated, however it has been associated with catarrhal enteritis in laying hens, and diarrhoea in pullets.[33]

Enteritis was associated with coccidia in six cases and in two of these cases splenic enlargement was noted. *Isospora* sp are directly transmitted intestinal parasites which are usually host specific and can be carried in the intestine of healthy birds. Disease due to coccidiosis can occur in passerines under conditions of stress, overcrowding and poor hygiene, and can result in oedema and haemorrhage of the

intestine, hepatic and splenic enlargement and focal necrosis.[21] During 2007 many of the birds which died showed some features consistent with isosporoid coccidiosis, including the demonstration of the parasite in impression smears of the spleen, however a definitive diagnosis was lacking in most cases due to autolysis of tissues which precluded histological confirmation. The dosing frequency of prophylactic toltrazuril was reduced in 2006/7 because no parasites were seen in routine faecal samples, and while we cannot rule this out as a contributing factor to some of the deaths in 2007, we believe other factors leading to immunosuppression were more important.

Disease associated with immunosuppression is a frequently cited cause of mortality in captive wild birds and 'stress' is often cited as a factor affecting success in avian conservation activities such as translocations.[16,28,34] However, because immunity to infectious disease agents involves multiple, complex mechanisms including physical, chemical and cell-mediated elements it is challenging to demonstrate immunosuppression in an individual, particularly post-mortem.[35] In this study the physical measurement of the organs of immunity, the thymus, spleen and Bursa of Fabricius, and histological assessment of the lymphoid tissue therein were undertaken in order to attempt to provide an objective measure of 'immunosuppression'. Of all birds which died, 39% had atrophy of one or more of these tissues with 53% (n = 15) and 42% (n = 43) of birds which died at the brooder and canary cage stage respectively being affected. These findings suggested that younger cirl buntings under these management conditions were more likely to be affected by immunosuppressive changes. In 2007 81% of birds which died showed these changes compared to between 0 and 31% in other years. The difference in the rate of these changes between the years 2007 (81%) and 2011 (12%) is unexpected since both years had similarly high rates of mortality. However in 2011 there were a greater number of respiratory changes and of trauma. This difference might suggest that either the respiratory changes seen did not have an infectious aetiology, or demonstrate the limitation of using physical changes in the immune system to assess overall immune function. In a study examining the effect on the stress physiology of birds undergoing capture, transport, handling, captivity and release, Dickens et al (2009) found that captivity was the critical factor which induced a significant and prolonged loss of the negative feedback mechanism of the stress response axis.[36] Many of the diseases associated with pathological changes in this project have been associated with immunosuppression in other contexts. Coccidiosis, *Campylobacter* associated diarrhoea, aspergillosis, systemic infection with *Staphylococcal* sp., *Aeromonas hydrophila, Serratia odifera* and *E. coli* were all seen in this project and would be considered unusual in healthy birds.[37,38] *Stenotrophomonas maltophilia* was isolated from two birds and has been identified as a potential pathogen in immunocompromised humans.[39]

Trauma was a leading cause of death in this project with 15% of birds thought to have died as a direct result of trauma. Other birds suffered minor trauma which may have contributed to debility and the onset of other disease as these physical findings of trauma are likely to be accompanied by activation of the physiological stress response.[36] In 2011 four birds died in the canary cages with evidence of trauma at post mortem examination, possibly as a result of flying into the bars. This flighty behaviour seemed to be associated with a longer feeding interval (J.Gregson, personal communication), and when birds were fed more frequently this behaviour was reduced. In the aviaries five birds showed injuries at post mortem such as bruising of the cere which are typical of flying into mesh. It was suspected this was as a result of predator activity, and following these deaths additional screening between the cirl buntings and external birds was added to the aviaries and physical barriers were used to prevent predatory birds perching on the aviary roofs.

Coccidial oocysts were detected in faeces during routine monitoring. It is known that there is a regular diurnal rhythm in the shedding of coccidial oocysts in free-living wild passerines, with oocyst shedding being highest during the afternoon.[40] The time of day that faeces were collected in this project was not recorded and it is possible that more positive samples may have been found by restricting sampling to the afternoon. Faecal testing was reduced in 2009 and stopped in 2010-2011 as the prevention protocol seemed to be working well. In general the protocol used in the project seems to have fulfilled the objectives, provided strict attention to hygiene and quarantine was maintained.

The logistic regression identified that in 2011 birds were at significantly greater risk of death. There was an increased incidence of respiratory disease in that year, however further investigations, including PCR for *Chlamydia psittaci* and *Avipoxvirus* did not identify a primary pathogen. 'Medication' had a significant negative effect on the risk of mortality. This is not surprising given that birds in broods in which a bird died or showed signs of ill health were all medicated, and medication was given to all broods at the times of disease outbreaks. In addition the records sometimes did not note which individual bird was medicated which will artificially amplify the effect of 'medication' in the logistic regression. Of greater interest are the risk factors which were found to have no significant effect such as capture body weight, parasite positive and month of capture. Epidemiological analysis during the course of the project identified a negative effect of harvesting chicks under 10g bodyweight from the wild, but the effect of lower bodyweight seemed to continue in birds of 11g and 12g when compared with birds over 13g (Table Four). It is possible that this effect became statistically insignificant in the logistic regression due to cessation of harvesting of smaller chicks in the later stages of the project, although pre-post univariable analysis for this factor did not identify a significant difference.

Environmental factors such as temperature and humidity were not measured routinely except in the rearing room containing the brooder cages, and in the barn containing the canary cages temperature was recorded in 2008, so it is not possible to assess whether these factors might have had an impact on the apparent spikes of mortality in 2007 and 2011. Weather factors may have affected the captive birds during rearing by producing extremes of temperature or humidity in the rearing rooms. 2007 was notable for prolonged rainfall during June and July, which might have increased the humidity and the likelihood of persistence and transmission of infectious agents such as coccidia and *Campylobacter* sp.[41,42] Given the potential for temperature and humidity to affect the health of the captive birds, routine monitoring in all the housed stages of this project would have been advantageous.

Conclusion

The pathological findings, risk factor analysis and pattern of deaths seen in this project suggest that infectious disease associated with immunosuppressive stressors was a major factor and highlight the need for strict application of CCP and control measures suggested such as timing of housing changes, restriction of stocking density and rigorous hygiene. Reducing the time interval from death to post mortem examination would improve the information available to investigate the cause of any deaths.

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LEARNING POINTS/TAKE HOME MESSAGES ***3 to 5 bullet points – this is a required field***

- Infectious disease associated with immunosuppression was a major factor in mortalities.
- Strict application of control measures is essential for success.
- Reduced time from death to post-mortem would improve the quality of investigation of mortalities.

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