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# The use of a multi disciplinary approach to investigate the etiology of clinical forms in an endangered avian species: the example of the Houbara Bustard

Manuela Crispo

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# Doctorat de l'Université de Toulouse

préparé à Toulouse INP

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Utilisation d'une approche multidisciplinaire pour déterminer  
l'étiologie de syndromes cliniques chez une espèce aviaire  
menacée : l'exemple de l'Outarde Houbara

---

Thèse présentée et soutenue, le 31 mai 2024 par

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## **ABSTRACT**

In the present work, we assessed the use of a multidisciplinary diagnostic approach to successfully investigate poorly characterized pathological entities using an endangered avian species as a model: the Houbara Bustard. This species is the object of several captive breeding operations located in North Africa, the United Arab Emirates and Central Asia. We focused our attention on three conditions that could potentially jeopardize conservation efforts: high pathogenicity avian influenza (HPAI), genital infections in artificially-inseminated breeders and a respiratory syndrome affecting outdoor birds destined to be released. For the first condition, we successfully validated an RNA scope in situ hybridization (ISH) assay for the detection of the avian influenza A virus matrix gene in formalin-fixed and paraffin-embedded (FFPE) tissues. We then provided the first comprehensive description of HPAI H5N8 natural infection in the Houbara, resulting in hyperacute and acute forms exhibiting marked tissue pantropism, endotheliotropism and neurotropism.

For the second condition we characterized a series of cases of peritonitis and salpingo-peritonitis. Chronic forms predominated and an ascending infection was highly suspected. Most of the cases were associated with the isolation of *Escherichia coli*. The identification of a variety of virulence profiles by molecular analysis of selected bacterial isolates suggested the involvement of multiple strains. Furthermore, histopathology allowed the identification of changes consistent with cystic oviductal hyperplasia, expanding the list of potential risk factors involved in the development of genital infections in the Houbara Bustard.

For the third condition, we were able to shed some light on a multifactorial respiratory syndrome, focusing on long-lasting, chronic forms. A variety of viral and bacterial pathogens were detected, including potentially a novel *Mycoplasma* species. Environmental conditions, such as heat stress and exposure to dust storms, were considered significant contributing factors. We showed that combining classical and novel diagnostic tools we were able to significantly improve the etiological diagnosis of emerging and re-emerging conditions in the Houbara. This approach should be promoted to study sanitary issues in other endangered species, characterized by a limited availability of samples.

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**KEYWORDS**

Bustard, Houbara, conservation, multidisciplinary, diagnostic, pathology

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**RÉSUMÉ**

Dans le travail présenté ici, nous avons évalué l'intérêt d'une approche diagnostique multidisciplinaire pour l'investigation d'entités pathologiques mal caractérisées en utilisant l'Outarde Houbara comme espèce modèle. Cette espèce fait l'objet de plusieurs projets d'élevages conservatoires en Afrique du Nord, au Moyen Orient et en Asie centrale. Nous nous sommes intéressés à trois entités pathologiques ayant toutes le potentiel de mettre en péril les efforts de conservation : l'influenza aviaire hautement pathogène (IAHP), les infections génitales chez les oiseaux inséminés artificiellement et un syndrome respiratoire affectant les oiseaux élevés à l'extérieur et devant à terme renforcer les populations sauvages. Pour la première entité, nous avons validé l'utilisation de l'hybridation in situ par RNAscope pour détecter le gène de matrice des virus influenza aviaries dans des tissus fixés au formol et inclus en paraffine. Cela nous a permis de décrire de manière exhaustive et pour la première fois un épisode infectieux à virus IAHP H5N8 chez l'Outarde Houbara, qui s'est manifestée par des formes cliniques suraigues à aigues de la maladie, associées à un pantropisme tissulaire, avec endothéliotropisme et neurotropisme viral. Pour la seconde entité pathologique, nous avons caractérisé une série de cas de péritonite et salpingo-péritonite associés à des infections par *Escherichia coli*. La diversité des profils de virulence de la bactérie, identifiée par biologie

moléculaire, suggère l'implication de nombreuses souches. Les formes chroniques étaient prédominantes et une infection ascendante a été suspectée. De plus, l'examen histologique a permis l'identification de modifications tissulaires compatibles avec une hyperplasie cystique endométriale, pouvant intervenir comme facteur de risque dans le développement des infections génitales chez l'Houbara. Enfin, pour la troisième entité pathologique, nous nous sommes concentrés sur un syndrome respiratoire multifactoriel d'évolution essentiellement chronique. Divers agents pathogènes ont été détectés chez les oiseaux affectés, y compris une espèce potentiellement nouvelle de Mycoplasme. Des facteurs environnementaux, comme le stress thermique et l'exposition à des tempêtes de sable sont considérés comme des facteurs contributifs importants. Nous avons montré dans ce travail que nous améliorions considérablement le diagnostic étiologique des affections émergentes et réémergentes chez l'Outarde Houbara par une approche multidisciplinaire. Cette approche devrait être encouragée pour étudier la santé d'autres espèces menacées, notamment lors d'une disponibilité limitée en échantillons.

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#### **MOTS-CLÉS**

Outarde, Houbara, conservation, multidisciplinaire, diagnostique, pathologie

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## TABLE OF CONTENTS

<b>List of figures .....</b>	<b>9</b>
<b>List of tables.....</b>	<b>11</b>
<b>List of abbreviations.....</b>	<b>12</b>
<b>CHAPTER 1: GENERAL INTRODUCTION .....</b>	<b>16</b>
A. The Houbara Bustard .....	16
1. Taxonomy and Geographical Distribution .....	16
2. Biology .....	17
3. Population trend and Conservation Strategy .....	20
4. Captive breeding.....	24
B. Sanitary issues encountered in the Houbara.....	29
1. Diseases affecting wild Houbaras.....	29
2. Diseases affecting captive Houbaras .....	31
C. Sanitary measures implemented in captive Houbaras flocks .....	32
1. Biosecurity.....	32
2. Disease surveillance .....	32
3. Vaccination.....	34
D. The multidisciplinary approach.....	34
1. Epidemiology.....	34
2. The post-mortem exam.....	35
3. Bacteriology.....	37
4. Cytology .....	37
5. Histopathology.....	38
6. In situ detection methods.....	40
7. Transmission electron microscopy .....	40
8. Molecular analysis .....	41
E. Purpose of research .....	42
<b>CHAPTER 2: RESEARCH STUDIES.....</b>	<b>44</b>
A. STUDY 1.....	44
1. Introduction .....	44
2. Article 1 .....	48
3. Conclusions and future perspectives .....	60
B. STUDY 2 .....	62
1. Introduction.....	62
2. Article 2.....	64

3. Conclusions and future perspectives .....	75
C. STUDY 3.....	77
1. Introduction .....	77
2. Article 3 .....	79
3. Conclusions and future perspectives .....	97
D. STUDY 4.....	100
1. Introduction .....	100
2. Article 4.....	104
3. Conclusions and future perspectives .....	121
<b>CHAPTER 3: FINAL DISCUSSION .....</b>	<b>126</b>
A. Limitations and points of strength of the study.....	126
1. Limitations.....	126
2. Strengths .....	126
B. General diagnostic guidelines .....	128
C. Diagnostic challenges in the modern era of captive breeding.....	131
<b>References .....</b>	<b>133</b>
<b>ANNEX.....</b>	<b>155</b>

### List of figures

<b>FIGURE 1. FEMALE AFRICAN HOUBARA (C. UNDULATA) (A) AND FEMALE ASIAN HOUBARA (C. MAQUEENII) (B).</b> .....	16
<b>FIGURE 2. MALE AFRICAN HOUBARA DISPLAYING.</b> .....	18
<b>FIGURE 3. TWO EGGS LAID IN A DEPRESSION ON THE GROUND BY AN ASIAN HOUBARA. NARC, SPRING 2017.</b> .....	19
<b>FIGURE 4. HOUBARA'S CHICKS.</b> .....	19
<b>FIGURE 5. GEOGRAPHICAL LOCATION OF THE DIFFERENT RENECO PROJECTS AND NATURAL DISTRIBUTION RANGE OF AFRICAN HOUBARAS AND ASIAN HOUBARAS.</b> .....	22
<b>FIGURE 6. AERIAL VIEW OF MISSOUR BREEDING CENTER, PART OF THE ECWP, MOROCCO.</b> .....	24
<b>FIGURE 7. OVERVIEW OF DIFFERENT HOUSING SYSTEMS FOR CAPTIVE HOUBARAS AND EXAMPLE OF INDIVIDUAL IDENTIFICATION NUMBERS. ECWP, SUMMER 2021.</b> .....	25
<b>FIGURE 8. OVERVIEW OF CAPTIVE BREEDING OPERATIONS, MOROCCO.</b> .....	27
<b>FIGURE 9. ANATOMY OF THE HOUBARA'S FEMALE REPRODUCTIVE TRACT.</b> .....	28

<b>FIGURE 10.</b> MYCOBACTERIOSIS IN A WOOD PIGEON, ORIGINALLY INTERPRETED AS A LYMPHOMA. .....	36
<b>FIGURE 11.</b> BLOOD SMEAR OBTAINED FROM AN AFRICAN HOUBARA.....	38
<b>FIGURE 12.</b> SEVERE CESTODIASIS IN AN ASIAN HOUBARA.....	39
<b>FIGURE 13.</b> THE MULTIDISCIPLINARY APPROACH. ....	43
<b>FIGURE 14.</b> SCHEMATIC COMPOSITION OF A SPHERICAL VIRION OF INFLUENZA A VIRUS. ....	44
<b>FIGURE 15.</b> OVERVIEW OF IAV REPLICATION CYCLE INCLUDING A SERIES OF SEQUENTIAL STEPS (1-7), FROM THE VIRAL ENTRY TO THE RELEASE OF PROGENY VIRIONS. ....	46
<b>FIGURE 16.</b> OVERVIEW OF HPAI H5Nx OUTBREAKS FROM 2005 TO 2022.....	62
<b>FIGURE 17.</b> NECROPSY FINDINGS IN CAPTIVE AFRICAN HOUBARA BUSTARDS WITH PERITONITIS/SALPINGO-PERITONITIS. ....	86
<b>FIGURE 18.</b> SIGNIFICANT HISTOPATHOLOGICAL FINDINGS IDENTIFIED IN CAPTIVE AFRICAN HOUBARA BUSTARDS WITH PERITONITIS/SALPINGO-PERITONITIS. ....	90
<b>FIGURE 19.</b> ORGANIZATION OF THE CONDUCTING AIRWAYS IN THE LEFT LUNG OF THE MUTE SWAN (CYGNUS OLOR).....	102
<b>FIGURE 20.</b> PATHWAYS OF AIR FLOW IN THE RESPIRATORY SYSTEM OF A CHICKEN (GALLUS GALLUS) DURING INSPIRATION (A) AND EXPIRATION (B), MEDIATED BY THE CHANGE OF VOLUME OF AIR SACS. ....	103
<b>FIGURE 21.</b> CLINICAL SIGNS AND POST-MORTEM FINDINGS IN CAPTIVE ASIAN HOUBARA BUSTARDS WITH CHRONIC RESPIRATORY DISEASE.....	110
<b>FIGURE 22.</b> PHOTOMICROGRAPH OF THE RESPIRATORY TRACT OF CAPTIVE ASIAN HOUBARA BUSTARDS WITH CHRONIC RESPIRATORY DISEASE.....	114
<b>FIGURE 23.</b> ULTRASTRUCTURAL CHANGES IDENTIFIED IN CAPTIVE ASIAN HOUBARA BUSTARDS WITH CHRONIC RESPIRATORY DISEASE BY TRANSMISSION ELECTRON MICROSCOPY (TEM). .....	118
<b>FIGURE 24.</b> INVESTIGATION OF THE MICROFLORA OF THE RESPIRATORY TRACT OF HOUBARAS. .....	125
<b>FIGURE 25.</b> NECROPSY FINDINGS IDENTIFIED IN CAPTIVE HOUBARAS DIAGNOSED WITH A LYMPHOPROLIFERATIVE DISORDER. ....	158
<b>FIGURE 26.</b> HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL FINDINGS IDENTIFIED IN HOUBARAS. DIAGNOSED WITH A LYMPHOPROLIFERATIVE DISORDER. ....	159

## List of tables

<b>TABLE 1.</b> TOTAL BIRDS PRESENT IN THE DIFFERENT CENTERS IN 2023, INCLUDING ADULTS, BREEDERS AND CHICKS PRODUCED .....	23
<b>TABLE 2.</b> OVERVIEW OF REPRODUCTIVE TRAITS OF HOUBARAS IN CAPTIVITY.....	26
<b>TABLE 3.</b> INFECTIOUS AGENTS REPORTED IN WILD, IMPORTED AND CONFISCATED HOUBARAS, DIVIDED BY ETIOLOGY AND DIAGNOSTIC METHOD .....	30
<b>TABLE 4.</b> OVERVIEW OF BIRDS EXHIBITING GROSS LESIONS CONSISTENT SALPINGITIS, WITH OR WITHOUT CONCURRENT OVIDUCTAL/CLOACAL IMPACTION.....	78
<b>TABLE 5.</b> OVERVIEW OF HISTOPATHOLOGICAL FINDINGS IDENTIFIED IN CAPTIVE AFRICAN HOUBARA BUSTARDS WITH GROSS LESIONS OF SALPINGITIS/SALPINGO-PERITONITIS.....	88
<b>TABLE 6.</b> BACTERIOLOGY RESULTS OF A TOTAL OF 14 AFRICAN HOUBARA BUSTARD FEMALE BREEDERS .....	90
<b>TABLE 7.</b> PREVALENCE OF APEC VIRULENCE-ASSOCIATED GENES (N=11) IN SELECTED E. COLI ISOLATES (N=9) RECOVERED FROM CAPTIVE AFRICAN HOUBARAS WITH LESIONS OF PERITONITIS, SALPINGO-PERITONITIS AND VAGINITIS. ....	93
<b>TABLE 8.</b> ANTIMICROBIAL SENSITIVITY RESULTS CONDUCTED ON E. COLI ISOLATES (N=24) RECOVERED FROM DIFFERENT ORGANS OF CAPTIVE AFRICAN HOUBARA BUSTARDS WITH GROSS LESIONS OF PERITONITIS/SALPINGO-PERITONITIS .....	98
<b>TABLE 9.</b> OVERVIEW OF HISTOPATHOLOGICAL FINDINGS IDENTIFIED IN THE UPPER AND LOWER RESPIRATORY TRACT OF CAPTIVE ASIAN HOUBARA BUSTARDS WITH CHRONIC RESPIRATORY DISEASE.....	113
<b>TABLE 10.</b> SPECIFIC NECROPSY AND SAMPLING RECOMMENDATIONS .....	129
<b>TABLE 11.</b> OVERVIEW OF IMMUNOHISTOCHEMICAL FINDINGS IDENTIFIED IN CAPTIVE ASIAN AND AFRICAN HOUBARA BUSTARDS DIAGNOSED WITH A LYMPHOPROLIFERATIVE DISORDER..	161

## List of abbreviations

**aCoVs:** Avian coronaviruses

**AI:** avian influenza

**AIV:** avian influenza virus

**AMC:** amoxicillin/clavulanic acid

**aMPV:** avian metapneumovirus

**AMX:** amoxicillin

**AOaV-1:** *avian orthoavulavirus-1*

**APEC:** avian Pathogenic E. coli

**API:** analytical Profile Index

**ARDC:** avian respiratory disease complex

**astA:** enteroaggregative heat-stable toxin

***Av. paragallinarum:*** *Avibacterium paragallinarum*

***B. avium:*** *Bordetella. avium*

***B. bronchiseptica:*** *Bordetella bronchiseptica*

***B. hinzii:*** *Bordetella hinzii*

**B.E.S.T:** Baculovirus Expression System Technology

**BAFF-R:** B-cell activating factor receptor

**BALT:** Bronchus-associated lymphoid tissue

**BCRS:** bustard chronic respiratory syndrome

**BRDC:** bovine respiratory disease complex

***C. macqueenii:*** *Chlamydotis macqueenii*

***C. undulata:*** *Chlamydotis undulata*

**CALT:** conjunctiva-associated lymphoid tissue

**CD3:** cluster of differentiation 3

**CD79:** cluster of differentiation 79

**CEH:** cystic endometrial hyperplasia

**CEX:** cephalixin

**CHL:** chloramphenicol

**CIRDC:** canine infectious respiratory disease complex

**CITES:** Convention on International Trade in Endangered Species of Wild Fauna and Flora

**COH:** cystic oviductal hyperplasia

**CR:** Congo red

**cRNAs:** complementary positive RNAs

**cva:** colicin V toxin

**DOX:** doxycycline

***E. coli:*** *Escherichia coli*

**ECCH:** Emirates Center for Conservation of Houbara

**ECWP:** Emirates Center for Wildlife Propagation

**ELISA:** enzyme-linked immunosorbent assay

**ENR:** enrofloxacin

**F gene:** Fusion gene

**FFPE:** Formalin-fixed and paraffin-embedded

**FTA:** Flinders Technology Associates

**gfCoV:** Guineafowl coronavirus

**Gs/Gd:** goose/Guangdong

**H&E:** Hematoxylin and eosin

**HA:** hemagglutinin

**HBCAD-AE:** Houbara Breeding Center Abu Dhabi - United Arab Emirates

**HI assay:** hemagglutination-inhibition assay

**HIER:** heat-induced epitope retrieval step

**HIV:** human immunodeficiency virus

***hlyF:*** putative avian hemolysin

**HPAI:** high pathogenicity avian influenza

**HPAIV:** high pathogenicity avian influenza viruses

**IAV:** Influenza A virus, *Alphainfluenzavirus influenzae*

**IBV:** Infectious bronchitis virus

**IFHC:** International Fund for Houbara Conservation

**IHC:** immunohistochemistry

**ILTv:** infectious laryngotracheitis virus

***iron:*** siderophore receptor

***irp-2:*** iron-repressible protein

**ISH:** in situ hybridization

***iss:*** increased serum survival protein

***iucD***: aerobactin

**IUCN**: International Union for Conservation of Nature

**LPAI**: low pathogenicity avian influenza

**M1**: matrix 1 protein

**M2**: matrix 2 ion channel protein

**MALDI-TOF MS**: matrix assisted laser desorption ionization-time of flight mass spectrometry

**MG**: *Mycoplasma gallisepticum*

**mRNA**: messenger RNA

**MS**: *Mycoplasma synoviae*

**MT**: Masson's Trichrome

**MUM-1**: multiple myeloma 1 protein

**NA**: neuraminidase

**NARC**: National Avian Research Center

**ND**: Newcastle disease

**NEO**: neomycin

**NGS**: next-generation sequencing

**NP**: nucleoprotein

**NWRC**: National Wildlife Research Center

***omp-T***: outer membrane protease

***P. multocida***: *Pasteurella multocida*

***papC***: P-fimbriae

**PAS**: Periodic acid-Schiff

**Pax 5**: pair box protein 5

**PCR**: polymerase chain reaction

**PhCoV**: Pheasant coronavirus

**PRDC**: Porcine respiratory disease complex

**qPCR**: quantitative polymerase chain reaction

**RNAscope-ISH**: RNAscope in situ hybridization

**rRNA**: ribosomal RNA

**RT-qPCR**: reverse transcription-quantitative polymerase chain reaction

**SKHBC-AD**: Sheikh Khalifa Houbara Breeding Center

**SKHBC-KZ**: Sheikh Khalifa Houbara Breeding Center - Kazakhstan

**SPS:** salpingitis/peritonitis/salpingo-peritonitis

**SXT:** sulfamethazole/trimethoprim

**TCoV:** Turkey coronavirus

**TE:** Tris-EDTA

***tsh:*** temperature-sensitive hemagglutinin

**UAE:** United Arab Emirates

***vat:*** vacuolating autotransporter toxin

**vRNA:** viral RNA

**vRNPs:** viral ribonucleoproteins

**ZN:** Ziehl-Neelsen

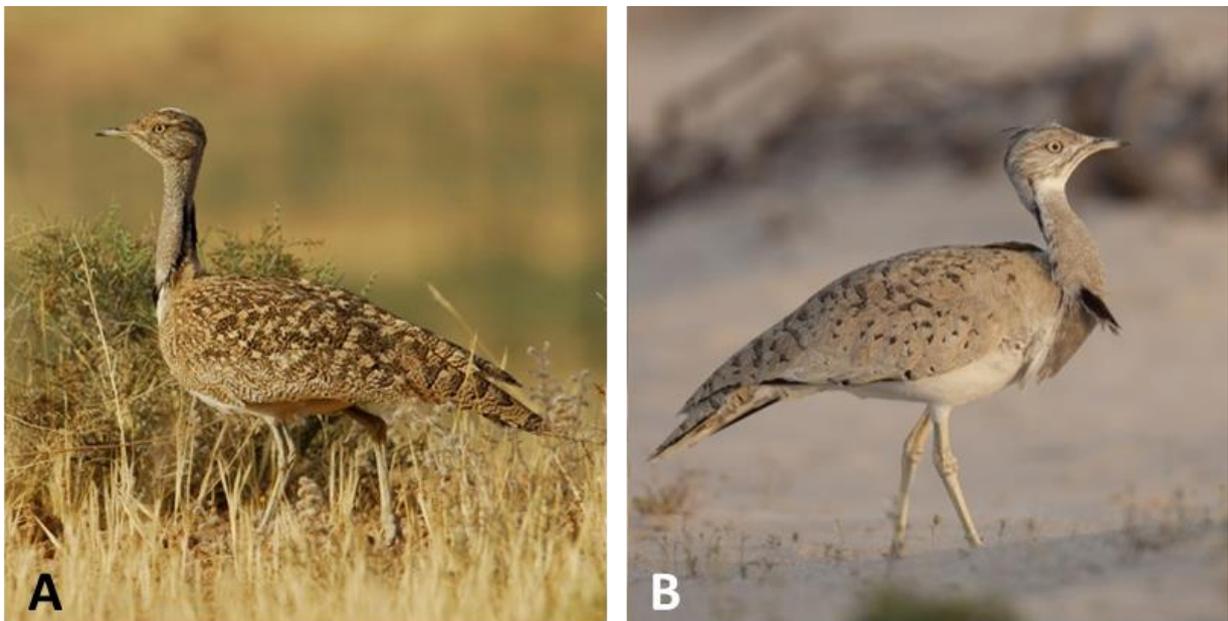
## CHAPTER 1: GENERAL INTRODUCTION

### The Houbara Bustard

#### 1. Taxonomy and Geographical Distribution

The Houbara Bustard is a secretive steppic bird belonging to the order *Otidiformes*, family *Otididae*, including 12 genera, 26 species and 29 subspecies (1,2). Based on recent studies (3–7), two different species are currently recognized in the genus *Chlamydotis*: the African Houbara Bustard (*Chlamydotis undulata*) further divided in two subspecies, *Chlamydotis undulata undulata* (8), residing in North Africa, from Mauritania to Egypt, and *Chlamydotis undulata fuerteventurae*, restricted to the Eastern Canary Islands of Lanzarote and Fuerteventura (9) and the Asian Houbara Bustard (*Chlamydotis maqueenii*) with a distribution area extending from the Nile Valley, in Egypt, through Palestine, Arabian Peninsula and Central Asia, to the Gobi Desert in China (10) (**Figure 1**).

In the present manuscript, the term « Houbara», without additional specifications, will be used to indicate either one of the two species.



**Figure 1.** Female African Houbara (*C. undulata*) (A) and female Asian Houbara (*C. maqueenii*) (B).  
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## 2. Biology

The Houbara is a terrestrial bird residing in poorly inhabited, semi-desertic and desertic regions of the world, including open unvegetated areas or areas with scattered shrubby vegetation, typically comprised of xerophytic and halophytic plants supported by sand dunes (10,11). Shrub-steppe habitats represent a rich source of food, and a shelter from danger and adverse weather conditions in winter (12,13). Unvegetated areas are favored at night, to limit the risk of ambush by predators (14) while flat areas with shorter shrub vegetation are exploited by males during the breeding season, as displaying grounds (15). The Asian Houbara is a latitudinal migratory species, with norther populations originating from western Kazakhstan and Mongolia wintering south in the Middle-East and South-Central Asia (16). Some resident populations can be also found in several parts of Central Asia, Middle East and Arabian Peninsula. The African Houbara, once considered to be sedentary and limitedly dispersive (17,18) is now classified as partial migratory. Seasonal movements between breeding and wintering areas have been identified, particularly in females (19–21).

The Houbara is an opportunistic omnivorous with an eclectic diet, comprised of a variety of plants (fruits, seeds, leaves, flowers) invertebrates (particularly ants and beetles, but also termites, locusts, grasshoppers, snails, spiders, scorpions, centipedes, caterpillars) and small vertebrates (rodents, lizards, snakes and birds). The diet's composition varies according to seasonality and availability of local resources. In general, plants predominate in winter while the consumption of vertebrates and invertebrates increases during the breeding season. Birds may also venture into farmlands and cultivated fields (cereals, alfalfa and other *Leguminosae*) when natural resources became scarce (22). Feeding can take place either at night or during the day (22,23).

The Houbara is a sexually dimorphic species with a promiscuous mating system (24,25). Males are larger than females and have long ornamental feathers. During the breeding season, from January to June, they congregate to specific areas known as « leks » (from the Swedish « lek », matrimonial affairs or from « lekställe », mating ground) (26) to perform in a spectacular courtship display to attract females. Each male engages in a circular running, maintaining the white ornamental feather on the neck and on the head fully erected, while tucking in the head. This activity results in a « snow ball » shape, conveniently visible from long distances in the semi-desertic landscape (27). A series of subsonic booming calls may accompany the dance (5,21) (**Figure 2**).



**Figure 2.** Male African Houbara displaying. ©IFHC

Females visit display grounds for mating and start laying a few weeks later. The laying season is greatly affected by environmental factors, including altitude and rainfall occurrence. In north Africa, females may lay from February to June, while Asian Houbaras have a shorter laying season, ranging from March to May. In Oman the season may extend from October to May. Typically, a single clutch<sup>1</sup> is laid during the season. However, if the eggs are lost, for example due to predation, a replacement clutch<sup>2</sup> follows (19,20,28).

Grey-olive eggs, with brown spots, are laid in a depression in the ground (**Figure 3**). On average, the African Houbara and the Asian Houbara produce between 1-3 eggs and 1-4 eggs/clutch, respectively, on alternate days. They are incubated solely by the female for 22-24 days. The chicks are precocial and they are raised by the female alone (**Figure 4**). Fledging is completed around 35 days of age, but the chicks stay with the mother for a total of three months

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<sup>1</sup> Clutch: the total number of eggs laid by a bird per each nesting attempt.

<sup>2</sup> Replacement clutch: an additional set of eggs laid following the destruction or predation of the first clutch

(16,29). During the inter-breeding season, Houbaras tend to form small flocks of 10 individuals, including adults and juveniles of both genders, foraging together (10).



**Figure 3.** Two eggs laid in a depression on the ground by an Asian Houbara. NARC, spring 2017.

© J. Hirschinger



**Figure 4.** Houbara's chicks. © Reneco

### **3. Population trend and Conservation Strategy**

According to the Red List of Threatened Species of the International Union for Conservation of Nature (IUCN), Houbaras have been classified as vulnerable since 2014. They are included in the Appendix 1 of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (30) and they are part of the Appendix II of the Convention on the Conservation of Migratory Species of Wild Animals (31)

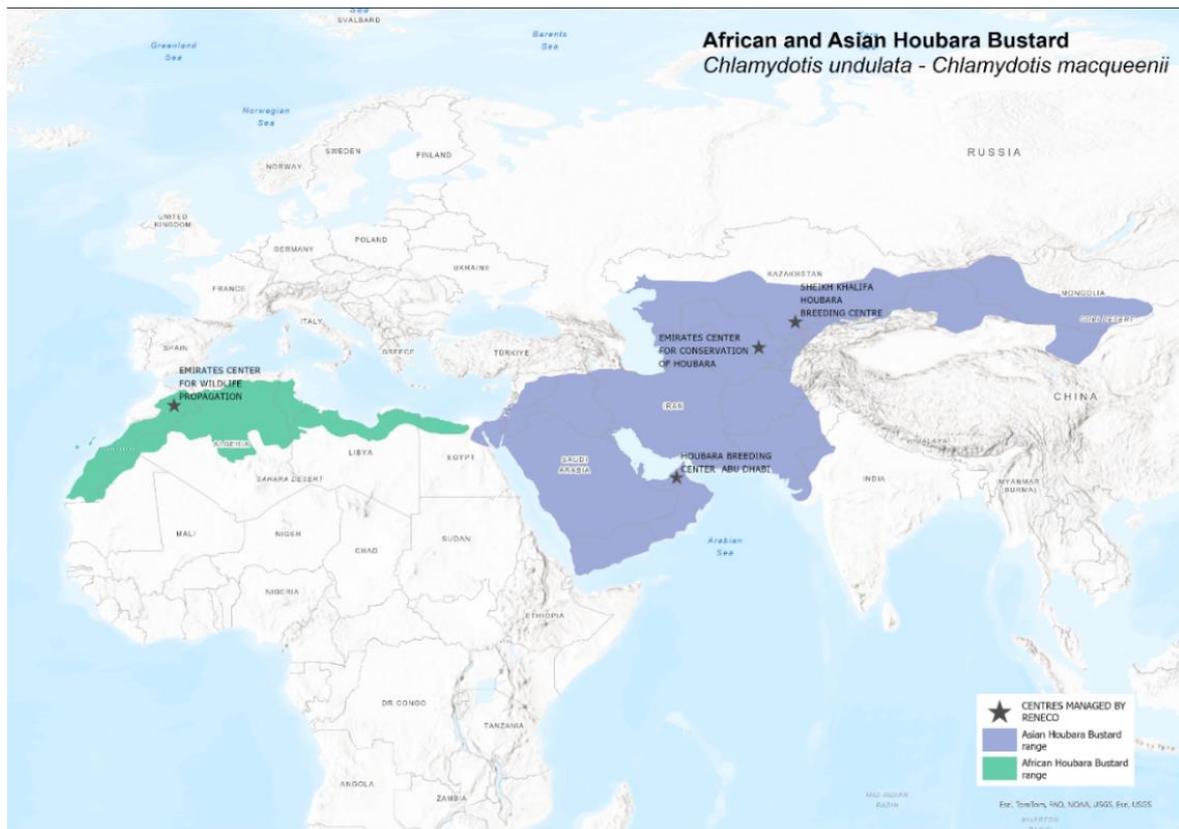
According to BirdLife International, at the end of 2014, the global population of Asian Houbaras was estimated to be between 78,960 and 97,000 individuals (32). From 2016, numbers were expected to fall between 50,000-99,999 individuals, including approximately 33,000-67,000 mature birds (32). Based on a 10-year assessment conducted between 2000 and 2009, the breeding population in Kazakhstan, which accounts for over 50% of the global population, was estimated at approximately 49,000, with a decline of 26-36% (33). The current population size of African Houbaras is unknown. In the mid-1990s it was estimated to be approximately of 10,550 individuals, mostly distributed in Algeria (50%) Morocco (20%) and Libya (10%) (17,34).

Factors contributing to the progressive decline of Houbaras include uncontrolled hunting, illegal harvesting and progressive habitat loss (35,36). Hunting and trapping, alone, accounted for 60% of total winter mortality of breeding populations of Asian Houbaras in central Uzbekistan (37). For centuries the Houbara has been an integral part of falconry in several countries, particularly the Middle East. The first descriptions of hunting Houbaras using falcons in Arabia dated more than 700 years ago and came from the legendary Oussama, the falconer poet (38). This practice was an essential part of the nomadic Bedouin culture, allowing to integrate a poor diet made of dates, locusts and camel milk, with nutritious meat during the winter period. Nowadays, the Houbara is still the favorite avian quarry of Arab falconers. Unfortunately, uncontrolled hunting, combined with the widespread availability of four-wheel vehicles, resulting in increased accessibility to the more remote and vulnerable populations, and the use of firearms, have contributed to the demise of this emblematic bird (39). Over-exploitation and degradation of Houbara's natural habitats associated with a variety of human activities, such as agricultural development, overgrazing, canal constructions, road building, oil and water pipelines, have also played a significant role (35).

The increasing concerns over the decline of Houbaras' populations over the years, prompted the creation of several captive breeding and reinforcement programs with the long-term goal of secure self-sustaining wild populations (40). The first project to be established was

the National Wildlife Research Center (NWRC) in Saudi Arabia, in 1986, which was soon followed by several other centers located in North Africa, Middle East and Central Asia. The same year, the Reneco International Wildlife Consultants LCC was founded to manage the different projects, providing scientific and technical support. In 2006, in order to optimize research and conservation efforts through a global conservation strategy, the Sheikh Mohammed bin Zayed al Nahyan, Crown Prince of Abu Dhabi, established the International Fund for Houbara Conservation (IFHC) (41). A total of four centers are currently under Reneco's management (**Figure 5**):

- The Houbara Breeding Center Abu Dhabi (**HBCAD-AE**) in the United Arab Emirates (UAE), including 2 sites: The National Avian Research Center (**NARC**) established in 1989 and managed by Reneco since 2010 and the Sheikh Khalifa Houbara Breeding Center (**SKHBC-AD**) established in 2012.
- The Emirates Center for Wildlife Propagation (**ECWP**) in Morocco, established in 1995 with the first breeding project located in Missouri. In 2006 a second breeding facility was created at Enjil, 50 km from Missouri.
- The Sheikh Khalifa Houbara Breeding Center in Kazakhstan (**SKHBC-KZ**), established in 2008.
- The Emirates Center for Conservation of Houbara (**ECCH**) in Uzbekistan, established in 2007.



**Figure 5.** Geographical location of the different Reneco projects and natural distribution range of African Houbaras and Asian Houbaras. © Reneco

Reneco’s activity is based on the implementation of an integrated, multidisciplinary approach combining fundamental and applied research in a variety of fields, such as ecology, physiology, nutrition and veterinary medicine. This strategy, in accordance with IUCN’s recommendations (42) relies on:

- ❖ **Conducting** basic and applied field research to gather essential information on wild Houbara populations, assess their distribution and status.
- ❖ **Maintaining** a healthy captive breeding population to produce individuals destined to be released, preserving genetic diversity, as well as birds for the training of falcons in countries with a strong falconry community.
- ❖ **Reinforcing** wild populations both directly, with translocations of captive-bred individuals and, indirectly, with falconry birds, to prevent illegal trapping and trading of wild Houbaras.
- ❖ **Creating** natural reserves in suitable areas and identify potential release sites to maximize the survival of released birds.

Since their creation, the two **ECWP**'s facilities in Morocco have produced more than 278,000 African Houbaras, with over 230,000 released birds. **NARC** and **SKHBC-AD** in UAE have raised more than 290,000 Asian Houbaras, including 224,000 released birds. **SKHBC-KZ** in Kazakhstan has released more than 28000 Asian Houbaras, reaching its full breeding capacity in 2021(43) (**Table 1**). Since 1997, a total of 656453 Houbaras have been produced in the collectivity of centers managed by Reneco.

**Table 1.** Total birds present in the different centers in 2023, including adults, breeders and chicks produced

<b>Project's Name</b>	<b>Geographical location</b>	<b>Breeding species</b>	<b>Total adults 2023</b>	<b>Total breeders 2023</b>	<b>Total chicks 2023</b>
<b>HBCAD-AE</b>	UAE	<i>C. maqueenii</i>	34868	7619	43887
		<i>C. undulata</i>	5081	4308	2148
<b>ECWP</b>	Morocco	<i>C. undulata</i>	24464	7642	21835
<b>ECCH</b>	Uzbekistan	<i>C. maqueenii</i>	3677	2184	5003
<b>SKHBC-KZ</b>	Kazakhstan	<i>C. maqueenii</i>	15705	8492	21382

The captive breeding centers have been strategically established in vast desertic or steppic areas, with sparse and rare vegetation (**Figure 6**) inhospitable for human activities. Specifically-organized areas are present in each center, including administrative and logistic quarters, residential and recreational buildings for the employees, breeding areas (indoor and outdoor housing systems, the hatchery and the rearing centers) life food production areas, the veterinary clinic, the laboratory and the necropsy room.



**Figure 6.** Aerial view of Missouri breeding center, part of the ECWP, in Morocco. © ECWP

#### **4. Captive breeding**

The founder birds of each center originated from wild eggs lawfully collected in different countries. Captive breeding relies completely on artificial insemination of females and artificial incubation of eggs, minimizing inbreeding, while ensuring a balanced representation of founder genes in the captive populations.

Birds released into the wild or destined to falconry training, must develop strong survival skills, including effective foraging strategies and tolerance to water deprivation. Contacts with humans are therefore limited and around 30 days of age these birds are moved to outdoor tunnels, in small groups comprised of 6 to 12 individuals (**Figure 7A**). On the other end, future breeders are housed individually, indoor or outdoor, and must become familiar with humans, as artificial insemination procedures require regular and frequent contacts and manipulations by technical personnel (44) (**Figure 7B-C**). Regardless the destination, each bird and its life (including production carrier, health status, hospitalizations, medical treatments, movements etc.) is strictly monitored with a unique identification number (leg band) assigned at hatch (**Figure 7D**). Data are electronically stored in a dedicated database.



**Figure 7.** Overview of different housing systems for captive Houbaras and example of individual identification numbers (ECWP, summer 2021). © IFHC and M. Crispo

Outdoor tunnels destined to release and falconry birds (A). Indoor (B) and outdoor (C) breeder cages. Plastic tags displayed at the entrance of an outdoor breeder cage (C10) including color-coded leg-bands also present on the birds (D).

In captivity, females may start laying as soon as 1 year of age. However, the majority of birds become sexually mature between 3 and 4 years of age (29,45). Males can be used as breeders since their first year of life, although the peak of fertility is reached around 4 years of age (46) (**table 2**). During the breeding season, between January and July, semen is collected from displaying, imprinted males using a dummy female and immediately transferred to the laboratory for analysis. Upon quality assessment (volume, spermatozoa concentration and motility and leucocytes content) semen is diluted in Lake 7.1 solution without added antibiotics (45). Subsequently, females are artificially inseminated within a few hours. Eggs are collected daily, to avoid brooding and induce the production of a replacement clutch, and artificially incubated (**Figure 8A-D**). The laying peak is usually reached between April and May. From the hatchery, chicks are transferred to dedicated facilities, where they are hand-fed following specific protocols according to their destination (44).

**Table 2.** Overview of reproductive traits of Houbaras in captivity.

\*data provided by Reneco and not published

**Reproductive traits of captive Houbaras**

<b>Breeding season</b>	January – end of July (29)
<b>Sexual maturity (males)</b>	as early as 1 year peak of fertility at 4 years (46)
<b>Sexual maturity (females)</b>	as early as 1 year (exceptionally) 2 year (25%) 3 year (65%) 4 year (85%) (29,45)
<b>Clutch size</b>	average 2 eggs, laid on alternate days*
<b>Number of eggs/female/season</b>	average 7.8 eggs (4 clutches/season)*
<b>Number of inseminations/female</b>	2-3 inseminations/clutch *

Females are inspected and weighted with a frequency varying according to their reproductive traits (clutch size, last egg laid and last insemination received). When deemed ready, upon visual inspection of the vaginal area, they are inseminated with fresh semen. Size, vascularization and mucous production of the vaginal area, are essential parameters to be considered while inspecting a female.



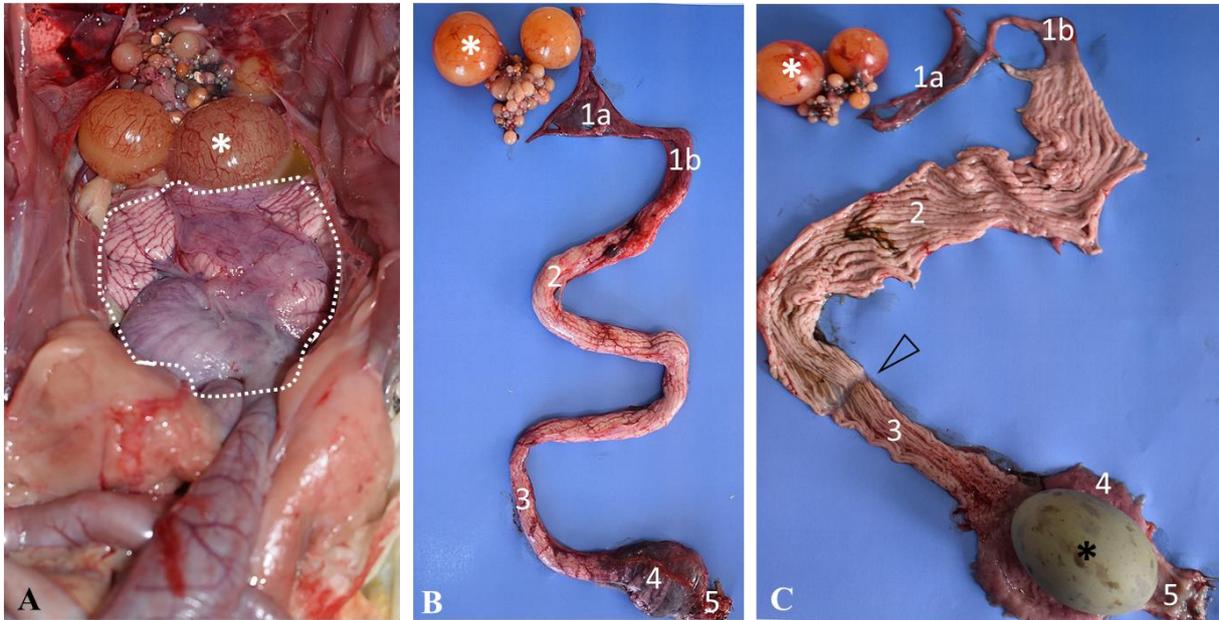
**Figure 8.** Overview of captive breeding operations (Morocco). © IFHC

Individual housing of breeders in outdoor cages (A). Collection of semen from an imprinted male using a dummy (B). Artificial insemination of a female (C). Artificial incubation of eggs (D).

Houbaras, as the majority of avian species, have a single ovary and oviduct, located on the left side of the coelomic cavity (**Figure 9**). Five morphologically and functionally distinct portions are recognized (47):

- ❖ **Infundibulum:** comprised of an upper portion (fimbria) (**Fig 9 B-C 1a**), responsible for capturing the ovum upon ovulation, and site of fertilization, and a lower portion (neck) (**Fig 9 B-C 1b**), where the chalazae are formed, a first layer of albumen is deposited and sperm is stored within the specific glands;
- ❖ **Magnum:** where the majority of albumen is produced (**Fig 9 B-C 2**);
- ❖ **Isthmus:** where the shell membranes are deposited and shell formation is initiated with the appearance of the mamillary cores (**Fig 9B-C 3**);
- ❖ **Uterus or shell gland:** where the egg acquires its final shape, through an osmotic process named « plumping », the eggshell calcification is completed and the pigments and cuticle are deposited (**Fig 9 B-C 4**) (48);

- ❖ **Vagina:** involved in the expulsion of the egg (oviposition) and in the storage of sperm within specific glands (**Fig 9 B-C 5**). It empties into the urodeum, which is part of the cloaca and it is separated from the shell gland by the uterovaginal-sphincter.



**Figure 9.** Anatomy of the Houbara's female reproductive tract. © Lena Meunier

In situ overview of the female reproductive tract of a healthy, sexually mature, African Houbara (A).

Upon isolation from the coelomic cavity (B-C), five different portions can be distinguished in the oviduct: infundibulum (1), including two portions (fimbria-1a and neck-1b), magnum (2), isthmus (3), uterus or egg chamber (4) and vagina (5). The passage between the mucosa of magnum and isthmus, is marked by a narrow band (arrowhead) (C). The ovary has large preovulatory follicles, with a prominent stigma (white asterisks) (A-C). There is a fully formed egg, olive green and with green-brown spots, in the egg chamber (black asterisk) (C).

For artificial insemination, the vaginal orifice is exposed using a vaginal speculum, with or without the aid of a light source. The speculum is inserted horizontally into the cloaca and gently rotated of 45° to open the oviduct. Subsequently, the semen dose is deposited to a depth of 1 cm, using a calibrated micropipette, with a disposable tip (45). Rough and/or improper manipulation, such as the over insertion of the speculum blades, may damage the oviductal mucosa, potentially leading to infection (49). Typically, a female receives one or two intravaginal inseminations at a 48-h interval, before laying the first egg of the clutch. However, a third insemination may follow, if the first egg takes longer than expected. At each insemination, the sperm originating from a single male, the same or a different one, is used (28).

## **B. Sanitary issues encountered in the Houbara**

### **1. Diseases affecting wild Houbaras**

Understanding disease susceptibility and pathogens prevalence in houbaras is essential to properly assess and manage sanitary issues and to minimize the risk of exposing free-ranging, naive populations to novel or emerging pathogens (50,51). Unfortunately, endangered avian species are poorly characterized from a sanitary point of view, compared to poultry.

Available data on wild populations are quite limited, mostly relying on individual observations from ecology expeditions or falconers' activity. The main factors complicating investigation of wild causalities include limited sample availability, due to geographical barriers, predators and scavengers' activity (52) and poor sample quality, following prolonged post-mortem delay and exposure to environmental factors.

Significant information originated from illegally-traded birds (**Table 3**). A comprehensive overview of clinico-pathological findings and disease prevalence in confiscated Asian Houbaras was provided by Bailey et al (53). Live and dead birds, for a total of 775 individuals, were examined at rehabilitation and quarantine centers located in UAE over a 8 year period. Poor nutritional status and traumatic lesions, secondary to trapping and transportation in overcrowded conditions, were typically observed on arrival. More than 50% of birds presented antibodies against avian orthoavulavirus-1 (AOaV-1) (formerly known as avian paramyxovirus-1) and *Chlamydia spp.*, while only 5% were positive for avian metaavulavirus 2 (formerly known as avian paramyxovirus 2). *Chlamydia* antigen was detected by enzyme-linked immunosorbent assay (ELISA) in 25% of birds exhibiting respiratory signs, in combination with other viral and bacterial pathogens. Aspergillosis, likely stress-related, was noticed at necropsy in 34% of cases, while 24% of birds had gross lesions consistent with pox. In terms of parasitic forms, seven different species of cestodes were identified in the digestive tract of 34% of birds while, *Haemoproteus* and *Trichomonas spp.* were detected in 55% in 15.5% of clinical specimens, respectively. Gram-negative bacteria, including *Salmonella spp.*, *Pseudomonas aeruginosa* and *Pasteurella multocida* predominated. In another study focusing on post-mortem findings identified in imported Asian Houbaras, euthanasia following the onset of neurological signs attributed to Newcastle disease (ND), caused by virulent strains of AOaV-1, due to the presence of a positive serology, was listed as the main cause of mortality, followed by aspergillosis, starvation and septicemia (54).

The illegal trade of wild bustards has also resulted in interspecies transmission of pathogens. In Saudi Arabia, falcons perished after exhibiting neurological signs and diarrhea,

following direct and indirect contacts with Houbaras naturally-infected with high pathogenicity avian influenza (HPAI) H5N1. Affected Houbaras were adult individuals illegally-acquired and transported in overcrowded and poor hygienic conditions. The viral agent involved was isolated and molecularly characterized by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and sequencing analysis (55).

**Table 3.** Infectious agents reported in wild, imported and confiscated Houbaras, divided by etiology and diagnostic method

<b>Category</b>	<b>Etiology</b>	<b>Diagnostic method</b>
<b>Viruses</b>	Avian Paramyxovirus-1 (53,54,56)	viral isolation, serology
	Avian Paramyxovirus-2 (53)	viral isolation serology
	High pathogenicity avian influenza H5N1 (55)	RT-qPCR, viral isolation, sequencing
	Avipox (56–58)	post-mortem exam, serology
	Reovirus (56)	viral isolation
	Adenovirus (58)	viral isolation
	Avian Leucosis (53,54)	post-mortem exam
<b>Bacteria</b>	<i>Chlamydia spp.</i> (53)	serology, antigen detection (ELISA)
	<i>Chlamydia psittaci</i> (59)	serology, cytology
	<i>Salmonella spp.</i> (53)	culture
	<i>Salmonella Arizonae</i> (53)	culture
	<i>Salmonella Typhimurium</i> (53)	culture
	<i>Salmonella reading</i> (53)	culture
	<i>Pseudomonas aeruginosa</i> (53)	culture
	<i>Pasteurella multocida</i> (53)	culture
	<i>Clostridium perfringens</i> (53)	culture
	<i>Clostridium botulinum</i> (53)	culture
	<i>Escherichia coli</i> (60)	culture
<b>Fungi</b>	<i>Aspergillus fumigatus</i> (53)	post-mortem exam, culture
	<i>Candida albicans</i> (53)	culture
	<i>Candida tropicalis</i> (53)	culture
<b>Parasites</b>	Lice (57)	clinical exam
	Ticks (57)	clinical exam

<i>Haemoproteus spp.</i> (53)	blood smear
<i>Leucocytozoon spp.</i> (53)	blood smear
<i>Trichomonas spp.</i> (53)	clinical exam
<i>Giardia spp.</i> (53)	not specified
Cestodes (53,61)	post-mortem exam
Nematodes (62)	post-mortem exam
Acanthocephalans (61)	post-mortem exam

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## 2. Diseases affecting captive Houbaras

Trauma-related injuries represent the most important cause of mortality and euthanasia in captive birds (54). Gizzard impaction, obstruction and/or perforation of the upper and lower digestive tract following the ingestion of foreign bodies is also a significant issue, particularly in outdoor juveniles (54). Metabolic and nutritional disorders, such as fatty liver syndrome (54,63) encephalomalacia secondary to vitamin E and Selenium deficiency, chronic bone disease and muscular-skeletal deformities have also been described (57,64).

Despite the continuous improvement of captive breeding conditions, infectious diseases remain a significant threat, that can lead to major outbreaks with significant losses, potentially nullifying conservation efforts. Juveniles are particularly at risk due to their incompletely developed immune system. For example, between 1987 and 1989 several cases of enteritis-peritonitis syndrome, due to *Chlamydia psittaci* were registered in Saudi Arabia (59). Other significant bacterial outbreaks, resulting in significant losses among chicks in the UAE, included salmonellosis associated with *Salmonella Arizonae* in 2011 and *Salmonella Enteritidis*, in 2012, and colibacillosis due to a highly pathogenic *Escherichia coli*, in 2013. In terms of viral agents, an outbreak of septicemic avian pox was registered in 2015, followed by LPAI H9N2 in 2015 and Newcastle disease in 2018 in the UAE (Reneco, unpublished data).

## **Sanitary measures implemented in captive Houbaras flocks**

In order to minimize the risk of introduction and subsequent spread of harmful pathogens in captive Houbaras flocks, stringent biosecurity measures are applied, following the example of commercial poultry productions. Biosecurity practices are integrated by disease surveillance, and systematic vaccination against significant pathogens.

### **1. Biosecurity**

Access to vehicles is strictly regulated and directed through specific reception areas, equipped with washing and disinfection stations. All visitors are registered and required to follow specific biosecurity procedures. The hatchery, breeding and rearing facilities are located in different areas and managed as separated units. Specific epidemiological subunits are further recognized, according to birds age (chicks, juveniles, adults) and destination (release, falconry, breeding). Each point of access has a changing room with a hand washing area, dedicated personal protective equipment and material and disinfection foot mats. Personnel's activities and movements between areas are organized according to a logical flow, with a clear separation of dirty and clean zones. Other key routine practices include thorough cleaning and disinfection and downtime between flocks and application of pest, rodent and predator control programs (65).

### **2. Disease surveillance**

Disease surveillance, based on on-going and systematic collection of data, is an essential epidemiological tool. It allows to define the impact and epidemiology of specific diseases, monitor disease trends, detect outbreaks and the emergence of new pathogens in a timely manner, and assess the effect of specific sanitary and prophylactic measures (66,67). Captive bustards' flocks implement a surveillance strategy relying on the combination of active and passive activities.

Active surveillance is based on the continuous monitoring of epidemiological indicators (morbidity, mortality rates, disease prevalence and distribution) and routine testing for relevant conditions, such as avian influenza (AI), Newcastle disease (ND), salmonellosis, mycobacteriosis, aspergillosis and exposure to mycotoxins.

Non-vaccinated, sentinel bustards, residing in the same location of vaccinated birds, are serologically screened, relying on competitive enzyme-linked immunosorbent assay (ELISA) and inhibition of hemagglutination (HI) tests for *Alphainfluenzavirus influenzae* (IAV) and AOaV-1, every 3 months. Wild birds, captured nearby the centers, are also serologically and

molecularly tested (oropharyngeal and cloacal swabs) for the same pathogens, every month. For *Salmonella* screening, cultures are performed on environmental samples obtained from the different production units (hatchery, rearing etc.), fecal samples collected from adults and chicks, unhatched eggs, and mortality cases with suspicious lesions. General bacteriology and mycology are performed on environmental samples, feed and water. At necropsy, impression smears of lung, spleen, liver and intestinal mucosa are collected from all breeders of more than 1 year of age and non-breeder older than 3-4 years, stained with Ziehl-Neelsen (ZN) and examined for the presence of acid-alcohol-resistant bacteria. Positive cases are tested for *Mycobacterium avium* by molecular analysis (quantitative polymerase chain reaction - qPCR). Prior to be released into the wild, birds are tested for a variety of pathogens according to specific dispositions of the country involved, including IAV, AOaV-1, avian pox, *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae* (MS), *Chlamydia spp.* and *Salmonella spp.*

Passive surveillance is based on prompt investigation of morbidity and mortality events. Birds are monitored daily by the keepers and any abnormality is promptly reported to the veterinary service. Clinically affected birds are moved to the clinic for examination and a medical case is opened, tracking all diagnostic procedures and treatments provided. Depending on the case, birds may be hospitalized and followed individually. If deemed necessary, the entire tunnel or cage, including several birds, may receive treatment. Mortality is collected daily and recorded. Dead birds are examined as soon as possible, depending on the period of the year and the workload related with breeding activities. During the breeding season, post-mortem exams are usually conducted daily. All lesions, abnormal changes and findings of diagnostic significance are reported on individual forms, including diagnostic hypothesis (primary or secondary) and specific sampling for ancillary testing. Breeders are systematically sampled, while non-breeder individuals are selected according to necropsy findings. Subsequently, information is uploaded on a database and diagnostic hypotheses are accepted or discarded based on testing results and promptly updated.

In terms of diagnostics capacity, each breeding center has a necropsy room. NARC and ECWP are equipped for bacteriology (aerobic and microaerophilic cultures), serology (competitive enzyme-linked immunosorbent assay and inhibition of hemagglutination for IAV and AOaV-1), cytology (*Hemacolor*®, Gram, ZN stain) and hematology (leucogram, hematocrit, serum biochemistry, serum proteins electrophoresis) while SKHBC-KZ provides basic bacteriology. Molecular analysis, including molecular testing for influenza IAV, AOaV-1 and *Mycobacterium avium*, is performed in the UAE. At the end of 2023, a histopathology laboratory was created at NARC and the implementation of a second laboratory is under

development in ECWP. In addition, over the years, several partnerships have been established with national and international diagnostic and research facilities to promote diagnostic advancements, assess emerging and re-emerging sanitary issues and promote the dissemination and sharing of knowledge on Houbaras.

### **3. Vaccination**

Captive bustards are systematically vaccinated against AIV, AOaV-1 and avipox virus. Birds typically receive a polyvalent killed vaccine against low pathogenicity avian influenza (LPAI) H9N2 and ND (Ulster 2C strain) (Gallimune® 208 Merial), parenterally administered at 10 and 40 days or at 30 and 60 days, depending on the center, and a live attenuated vaccine against Canary Pox (KP1 strain, Poulvac ® Zoetis) provided at 30 and 60 days, by wing web inoculation. Birds residing in UAE are also inoculated with an inactivated subunit vaccine for H5 AIV (H5N1) and ND (Lasota strain) (Volvac B.E.S.T - Baculovirus Expression System Technology) at 30 and 60 days. Annual boosters are provided to all breeders and birds residing in outdoor tunnels for more than one year.

All birds are treated for intestinal parasites between 30 and 60 days of age, using different molecules according to the country's availability and the parasites' prevalence in the centers. Adult birds receive a treatment once a year.

### **The multidisciplinary approach**

When dealing with spontaneous field cases, the diagnostician is typically facing a complex reality, involving a variety of factors (infectious, environmental, host and management-related). The investigation of pathological entities is a process based on the collection, integration and interpretation of a set of data originating from different disciplines, presenting different advantages and/or limitations. In this chapter, we will focus our attention on epidemiology, the post-mortem exam, cytology, bacteriology, histopathology and in situ detection methods, transmission electron microscopy and molecular analysis.

### **1. Epidemiology**

According to the World Health Organization, epidemiology represents « the study of the distribution and determinants of health-related states or events (including disease), and the application of this study to the control of diseases and other health problems » (68). A variety of methods can be used to conduct epidemiological investigations, ranging from surveillance and descriptive studies to assess disease distribution, to analytical studies to identify disease determinants (69). The origin of this discipline trace back to 1854, during an outbreak of cholera

in London. At the time, this condition was thought to be transmitted by air, rather than contaminated water. However, Dr. John Snow, the father of epidemiology, analyzing the distribution of cholera cases was able to identify a single water source responsible for the contamination.

Epidemiology plays a key role in veterinary medicine. An increased incidence of morbidity and/or mortality in a population of interest should always trigger a diagnostic investigation. Surveillance programs are essential to promptly identify highly infectious conditions, potentially resulting in massive mortality events (for example highly pathogenic avian influenza), the re-emergence of a disease, the emergence of novel pathogens and also to assess the efficacy of vaccination and biosecurity practices (66,67). The identification of specific risk factors and transmission patterns is essential to select the most appropriate and effective diagnostic tools and to better understand the etiology and physiopathogenesis of a disease (69).

## **2. The post-mortem exam**

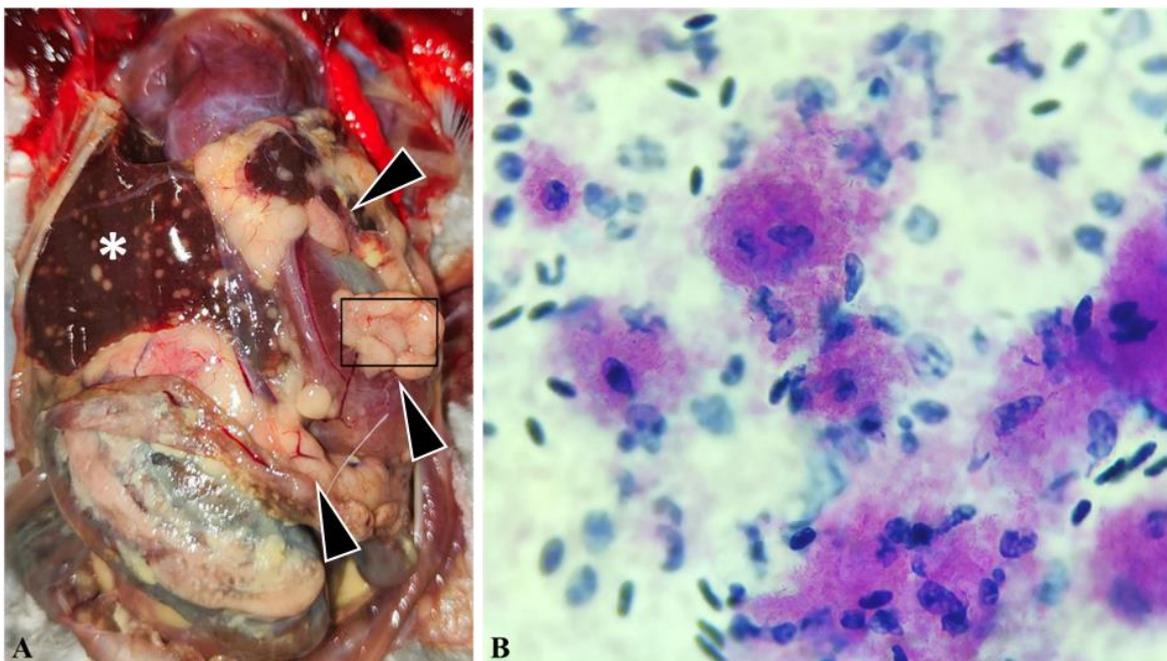
The necropsy room represents an important epidemiological observatory. A post-mortem exam can provide significant information on the cause/s of morbidity and mortality observed in the field, in a timely manner. It requires appropriate instruments, depending on the species involved, and should be conducted using a systematic approach, examining all organs (70,71). Interpretation of lesions may be complicated in case of advanced post-mortem changes due to delays in retrieving and examining the carcasses and/or exposure of the latter to high environmental temperatures, accelerating autolysis. Post-mortem predation may pose additional challenges (72).

Necropsy findings, combined with clinical signs and epidemiological data, are crucial to orient the diagnostic investigation, identify representative individuals and select the most appropriate ancillary testing. Relying on gross findings alone could lead to misdiagnosis, with significant implications from a therapeutic and prophylactic point of view. Since different conditions can induce similar gross lesions, a list of suitable diagnostic hypothesis (differentials) should always be provided. Subsequently, each hypothesis should be discarded or confirmed by additional testing.

For example, some cases of lymphomas in Houbaras may be misinterpreted as lipomas or liposarcoma, based on gross morphological traits alone. Lipomas are commonly reported in pet birds (73) and usually present as soft, encapsulated subcutaneous masses, with occasional necrotic-hemorrhagic areas. They result from a combination of poor nutrition, obesity,

endocrine disorders, and genetic factors and they can be treated by complete surgical excision (73). Liposarcomas, have been occasionally described in different avian species, including poultry, and tend to be locally invasive or metastasize to other organs (74–79). While an association between retroviruses and liposarcomas have been identified in cats, no evidence of a viral etiology has been proved in avian species so far (79–81). Lymphomas arise from the proliferation of B or T lymphocytes, and are one of the most common neoplasia encountered in avian species. Multicentric forms, leading to diffuse or multinodular infiltration of several organs, are commonly reported, followed by leukemic forms. They may have a viral etiology, such as in poultry, but they may occur spontaneously, like in parrots (82,83). In several avian species, including bustards, their exact etiology is still poorly understood (53,60,84,85). In similar cases, gross pathology is insufficient to reach a definitive diagnosis and cytology and/or histopathology, should follow.

A second example, involving a different avian species, was the identification of multiple white hepatic foci and nodular lesions with a lardaceous appearance on the serosa of the GI tract of a feral wood pigeon (*Columba palumbus*). This case was originally interpreted as multicentric lymphoma, based on gross morphological traits alone. However, subsequent cytology on isolated lesions revealed large multinucleated giant cells and epithelioid macrophages containing countless number of intracytoplasmic acid-alcohol bacilli (ZN stain) (**Figure 10A-B**). These findings were consistent with mycobacteriosis, discarding the previous diagnostic hypothesis.



**Figure 10.** Mycobacteriosis in a wood pigeon, originally interpreted as a lymphoma. © M. Crispo

At necropsy, pale pink nodules, with a lardaceous consistency were identified on the serosal surface of liver and gastrointestinal tract (arrowheads). Pale round foci were also scattered throughout the liver (white asterisk) (A). Cytological imprints were obtained from a serosal nodule and stained with ZN. Countless number of acid-alcohol resistant bacilli were detected within the cytoplasm of histiocytic cells and extracellularly (B) (ZN stain, 100X)

### **3. Bacteriology**

Culture remains the gold standard for investigating bacterial diseases (86). Bacterial isolates can be identified and typed based on phenotypic properties (colony and bacterial morphology, Gram staining, biochemical traits) matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), the use of specific antisera and molecular analysis, such specific PCR assays, 16S ribosomal RNA (rRNA) sequencing and restriction fragment length polymorphism (87–90). However, culture is time-consuming and labor-intensive, requiring appropriate equipment, protocols and experience. In addition, the involvement of slow-grown and fastidious pathogens or unculturable organisms, represents a major limitation (91). As a result, the implementation of molecular techniques, to directly detect infectious agents in clinical specimens, has been increasing over the years (92–95).

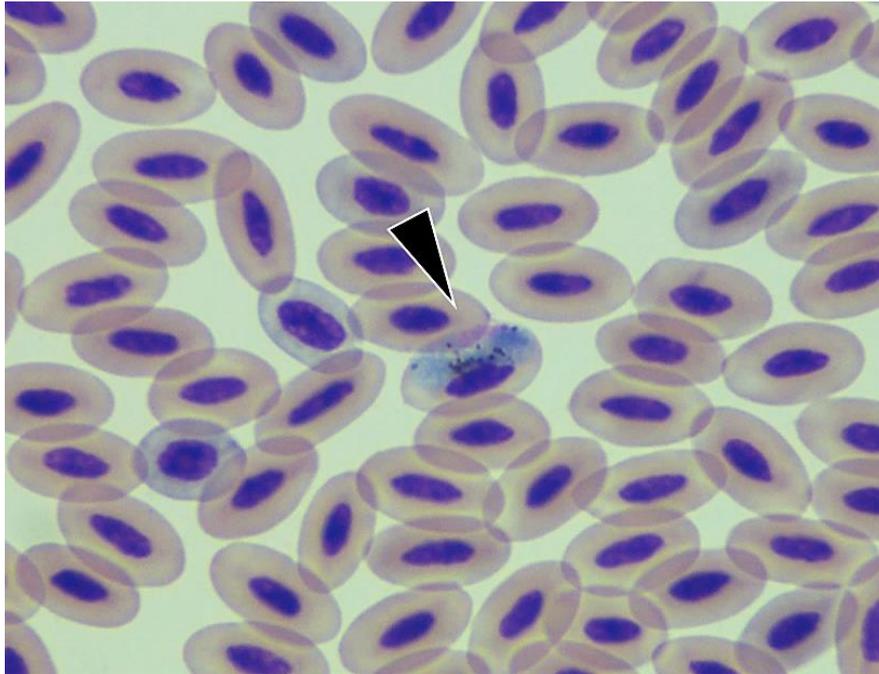
Selecting the most appropriate samples, based on affected species and clinico-pathological findings observed, combined with the application of a sterile sampling technique is essential. Advanced post-mortem changes and chronic forms can prevent isolation of clinically-significant pathogens, due to the overgrowth of contaminants (ex. *Proteus* spp.) or secondary opportunistic agents (ex. *E. coli*). Previous antibiotic treatment can also impair final results.

Understanding the composition of the normal microflora and the occurrence of specific primary pathogens in the different avian species is the first step to properly interpret bacteriological results. For example, *Bordetella avium* is commonly isolated from the respiratory tract of healthy chickens, in which it may cause opportunistic infections, following concurrent exposure to viral agents (96). The same bacterium is considered a primary pathogen in turkeys, and can result in a highly contagious disease known as turkey coryza (93).

### **4. Cytology**

Cytology/cytopathology is a versatile and cost-effective tool that can provide results in a timely manner. A variety of samples (fine needle aspirations, impression smears, mucosal scrapings, bronchoalveolar lavages, blood smears etc.) can be collected from live, symptomatic animals or dead specimens, at necropsy. It can be used as a screening tool, or in a follow-up context, but it can also provide important prognostic information and it can allow to reach a

definitive diagnosis (**Figure 10B**). A variety of staining, including immunocytochemistry, can be performed. Neoplastic forms may be identify based on cellular morphology, while infectious agents (Mycobacteria, *Chlamydia* spp. protozoan parasitic eggs, viral inclusions) (97,98) may be detected intracellularly or extracellularly (**Figure 11**). False negative/false positive results may originate from inadequate sampling (poor cellularity, poor representativity) inadequate sample fixation and preparation, cross-contamination, presence of extensive inflammatory, necrotic and/or hemorrhagic changes, obliterating cellular details (99,100).



**Figure 11.** Blood smear obtained from an African Houbara. © Reneco

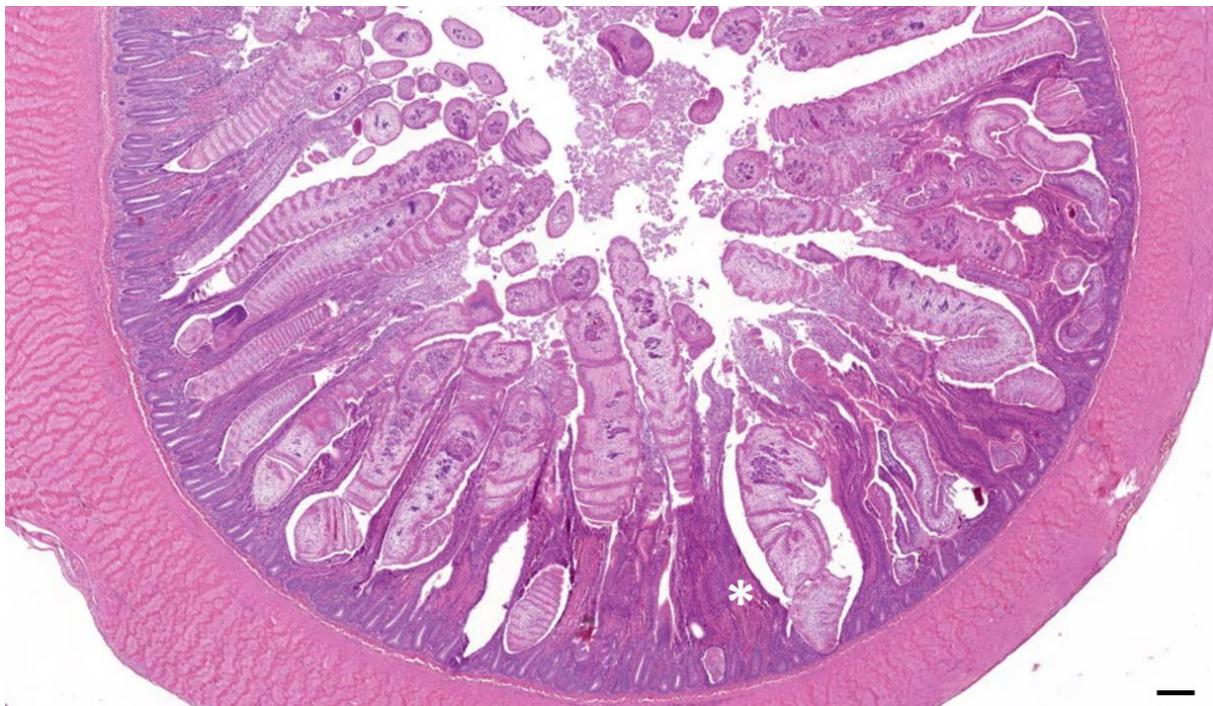
An oval structure, containing volutin (pink) and pigment (black, hemozoin) granules, consistent with a protozoan macrogamete of the genus *Haemoproteus*, is visible in the cytoplasm of an erythrocyte, perinuclearly-arranged (arrowhead) (Giemsa stain, 100X).

## 5. Histopathology

Histopathology can be conducted on tissue biopsies or surgical samples obtained from live birds, or lesional tissues collected at necropsy. Compared to cytology, this method is more labor-intensive/technically demanding, time consuming and costly. It requires proper fixation, to prevent autolysis and putrefaction and maintain tissue architecture, and processing of tissues for the production of paraffin blocks. The most widely used fixative is 10% formalin, at a ratio of 10:1 (formalin to tissue) (101). Technical issues may also originate from insufficient fixation time. In addition, samples shouldn't be stored for prolonged amount of time in formalin due to the occurrence over fixation artefacts and detrimental effects on tissue antigenicity (102).

Compared to cytology, the overall tissue architecture is preserved, allowing to assess severity and distribution of lesions and to recognize specific patterns and intralésional organisms (**Figure 12**). Several serial sections can be obtained from the same formalin-fixed and paraffin-embedded (FFPE) tissue block to perform special stains, in situ-detection methods and nucleic acids extraction for molecular analysis. FFPE blocks can be stored at room temperature and are easily shipped.

Limits may originate from poor representativity of samples, depending on the diagnostician's experience, presence of different lesions in different areas of the same organ (103) and the fact that different agents, infectious and non-infectious, may result in similar microscopic lesions. The absence of pathognomonic changes and intralésional pathogens (bacteria, viral inclusions, fungal or parasitic forms) as well as the stage of the pathological process (acute vs chronic) may complicate interpretation. For example, viral inclusions produced by infectious laryngotracheitis virus (ILTv) in chickens can be detected in the tracheal epithelium only between one and five days post-infection (104,105). Adequate assessment may be prevented by extensive post-mortem changes. Background and species-related changes, and incidental findings should be considered and properly interpreted.



**Figure 12.** Severe cestodiasis in an Asian Houbara. © M. Crispo

Numerous cestodes are present in the duodenum, among the intestinal villi and protruding into the lumen. Intestinal villi appear compressed and severely effaced (white asterisk). Hematoxylin and Eosin (H&E) stain (3X, scale bar 0.2 mm).

## **6. In situ detection methods**

Histopathology can be integrated by **in situ detection tools**, such as immunohistochemistry (IHC) and in situ hybridization (ISH). These techniques can be applied both in a diagnostic and a research setting. They allow to directly visualize a target of interest, represented by an antigen or nucleic acids (RNA or DNA) at a cellular and subcellular level (106–108) correlating it with the morphological changes present in the sample. Important applications in avian medicine include phenotyping of neoplastic forms, identification of specific infectious agents in lesions tissues, pathogenesis studies and characterization of emerging and reemerging diseases, including conditions involving fastidious organisms, difficult to isolate (83,105,109–119).

Improper sampling, in terms of selected tissues and timing during an infection process, advanced post-mortem changes and inadequate fixation can lead to false negative results. Some antigens are very sensitive to formalin fixation and may require frozen sections (86). Other IHC complications include lack of commercially available antibodies, and lack or variable cross-reactivity with avian tissues.

ISH combines immunohistochemical staining and molecular analysis at a cellular level, using specific probes. Probes can be custom-designed to allow the identification of specific agents/strains/viral subtypes, which is particularly useful in the absence of reliable IHC antibodies. Compared to IHC, it has a higher sensitivity for the detection of latent infections but it's more technically-demanding and expensive. Technical protocols should be validated and adapted to each laboratory, including suitable controls and, for ISH, nuclease-free reagents (120).

## **7. Transmission electron microscopy**

Transmission electron microscopy can be used to identify specific morphological changes, and infectious agents in a sample of interest (121–123). Since sensitivity and specificity are important limiting factors, it is advisable to judiciously interpret morphological details (size, level of electron density, shape, location etc.) and use this technique in conjunction with other diagnostic tools. For example, subcellular structures may be wrongly interpreted as viral particles (124). As regards false negative results, the concentration of the target of interest needs to be fairly high to be detected. The targets might be missed due to ununiform distribution within the sample and the morphological assessment alone doesn't always allow to differentiate different agents, such viruses belonging to the same family. Sensitivity can be increased by

immunolectron microscopy or immunolabelling with colloidal gold (86). This method is time consuming, technically demanding and requires specific instruments and experience.

## **8. Molecular analysis**

Nucleic acids (RNA or DNA) of pathogens of interest can be directly detected in a sample by several polymerase chain reaction (PCR)-based tests, followed by sequencing analysis, to specifically identify the microorganisms involved. Phylogenetic trees elaborated using sequencing data can provide useful information on the relationship of different organisms, based on the percentage of similarity. Multiplexing allows the simultaneous detection of several targets in the same sample (86).

A variety of samples can be used for molecular assessment, ranging from fresh swabs and tissue sections, Flinders Technology Associates (FTA®) cards and FFPE tissues.

FTA cards represent a paper-based, practical and safe option compared to fresh specimens, such as swabs and tissue sections. Upon application of the sample on the card, under sterile conditions, infectious agents are rapidly inactivated, while stabilizing and preserving nucleic acids. Nucleic acids are then collected using a punch biopsy tool and purified according to standard methods. FTA cards can be stored at room temperature, they are light and occupy less space and do not present an infectious risk, simplifying shipping procedures.

FFPE tissue blocks represent a safe alternative compared to tissue sections placed in formalin. As a general rule, to limit the DNA-protein/RNA-protein crosslinking induced by formalin, complicating molecular testing, tissue sections should be processed and paraffin embedded as soon as possible. The possibility of identify specific areas of interest and/or intralesional agents in FFPE blocks allows target sampling. The number of protocols for extracting nucleic acids from FFPE blocks has been increasing over the years and several commercial kits are currently available (125).

False negative results may arise from improper sampling (poor representativity, selection should be based on tissue tropism, shedding routes), sample degradation due to extensive post-mortem delay, improper storage, prolonged formalin fixation (for FFPE samples), use of inadequate extraction protocols, inadequate primers (particularly in the presence of rapidly evolving agents, such as RNA viruses) and chronic stage of infection. False positive outcomes are due to improper handling of samples leading to cross-contaminations, and cross-reactivity between different pathogens (poor primers specificity). Results should always be interpreted in combination with clinico-pathological findings.

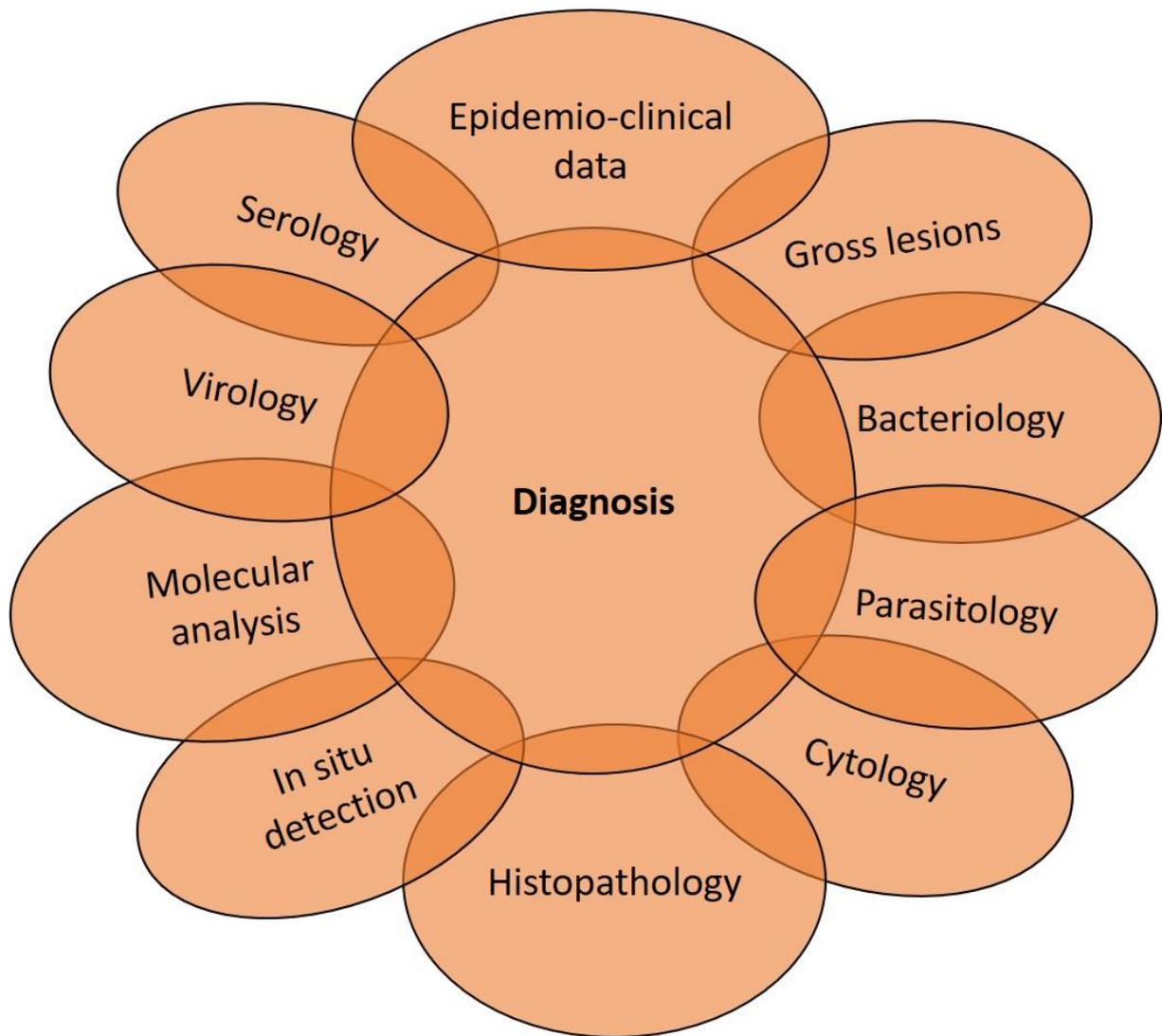
Several commercial PCR kits are currently available for a variety of avian pathogens and readily accessible. However, emerging and novel pathogens in non-poultry avian species, may be missed with conventional testing. The use of a broad-based, « Pan-PCR » approach may help to overcome these limits (126). In addition, metagenomics and next-generation sequencing have been showing promising results and applications for the unbiased detection of pathogens (127). With this method, all agents present in a sample are identified, defining the « pathobiome » of a specific tissue and unveiling complex intermicrobial interactions. This aspect is particularly important in case of complex, multifactorial syndromes (128,129). Specific primers can be designed based on sequencing data, and used to develop PCR assays for diagnostic and screening purposes in the field.

### **Purpose of research**

Accurate diagnosis is of pivotal importance for targeting the most appropriate management and control strategies. When dealing with field cases, the diagnostician is usually facing a complex scenario involving infectious and non-infectious factors. Additional challenges originate from working with endangered and exotic species, maintained in captivity. Species-specific traits (anatomical and physiological) must be considered and combined with management, environmental/geographical factors and epidemiological data (ex. endemic conditions in the areas, direct/indirect contact with poultry, wildlife, arthropod vectors). Sound epidemiological data represent the base.

The multidisciplinary approach is a dynamic concept, continuously evolving with the technological advancement. It aims at increasing diagnostic sensitivity and accuracy, overcoming the limits/gaps intrinsically present in the different diagnostic methods (130) but it often remains theoretical (**Figure 13**).

In the present work, we interrogated ourselves on the possibility of relying on the multidisciplinary approach, combining traditional and innovative tools, to effectively investigate poorly characterized pathological entities. In order to do this, we used the model of the Houbara Bustard, an endangered avian species object of several conservation projects. We focused our attention to three conditions that could potentially jeopardize conservation efforts: high pathogenicity avian influenza, genital infections in artificially-inseminated breeders and a respiratory syndrome affecting outdoor birds destined to be released into the wild.



**Figure 13.** The multidisciplinary approach.

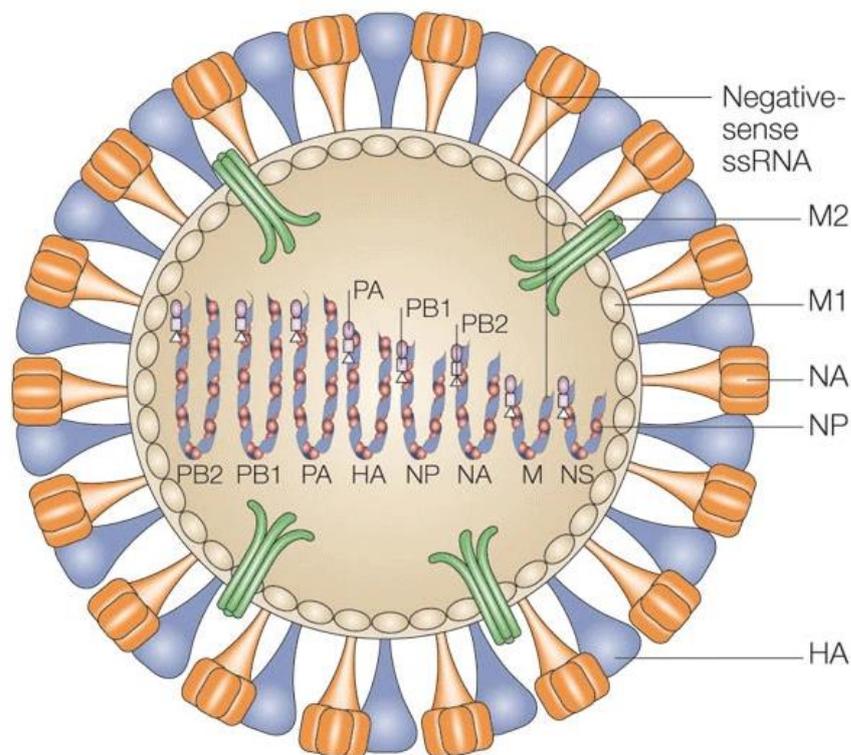
Integration of different disciplines (diagnostic synergism) to reach an accurate diagnosis. The different disciplines support and integrate each other, overcoming intrinsic limitations.

## CHAPTER 2: RESEARCH STUDIES

### A. STUDY 1

#### 1. Introduction

**Viral structure and morphology.** Avian influenza (AI) is a highly contagious disease caused by negative-sense, single stranded RNA viruses belonging to the *Alphainfluenzavirus* genus, within the *Orthomyxoviridae* family (131). Viral particles can be spherical to pleomorphic, approximately 100 nm in size, although filamentous forms exceeding several micrometers ( $\mu\text{m}$ ) have also been described (132). Influenza A viruses (IAVs) are characterized by a segmented genome, comprised of 8 segments encoding for at least 11 proteins, including structural and functional proteins. The viral genome is associated with the nucleoprotein (NP) and the RNA polymerase complex, including three subunits (PB1-PB2-PA), to form the viral ribonucleoproteins (vRNPs). The matrix 1 (M1) protein act as a bridge, enclosing the vRNPs and interacting with the host-derived lipid envelope, to provide structural support. The envelope is constellated by two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), and small amounts of a transmembrane protein, the ion-channel matrix 2 (M2) protein (**figure 14**).

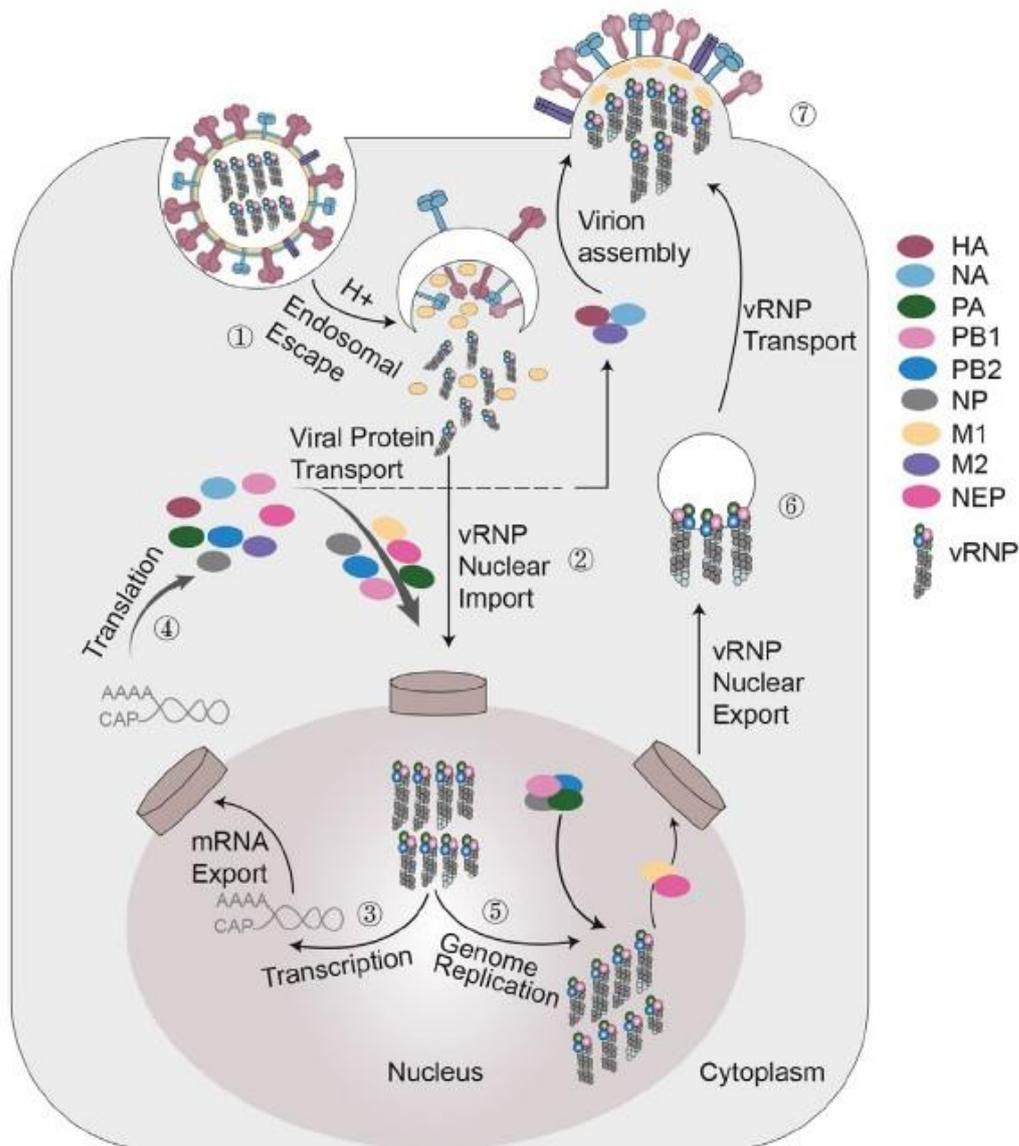


**Figure 14.** Schematic composition of a spherical virion of influenza A virus (133).

**Antigenicity and pathotypes.** HA and NA are major antigenic determinants, eliciting a protective antibody response following natural infection or vaccination, and based on their antigenic properties IAV are classified into different subtypes. To date, 16 HA (H1-16) and 9 NA (N1-9) subtypes have been identified in aquatic birds (134). Two additional subtypes, H17N10 and H18N11, have been recently detected in bats (135,136) Based on the ability of AIVs to produce disease in chickens two different pathotypes are recognized: high pathogenicity avian influenza viruses (HPAIV), resulting in systemic and rapidly lethal infections, with morbidity and mortality approaching 100%, and low pathogenicity avian influenza viruses (LPAIV) causing asymptomatic or milder forms, in the absence of secondary infections and other stress factors (134,143). To date, HPAI outbreaks have only been observed with H5 and H7 subtypes.

**Viral replication cycle.** The replication cycle of influenza A viruses (IAVs) involves a series of sequential steps, mediated by the different viral components (**Figure 15**). HA is responsible for **viral entry**, following the interaction with specific receptors present on the surface of susceptible cells, and enhancing the fusion of the viral envelope with the endosomal membrane (133,137). HA binds to sialic acids (SAs), such as N-acetylneuraminic acid, found as terminal residues of glycans linked to membranous glycoproteins, glycolipids and mucus (138,139). SAs are usually bound to galactose in an  $\alpha$ -2,3 or  $\alpha$ -2,6 configuration. SAs distribution, abundance and structural configuration vary according with the different species and tissues, affecting host susceptibility, viral tissue tropism and shedding patterns (138,140). Avian influenza viruses (AIVs) preferentially bind SAs in an  $\alpha$ -2,3 configuration, while human influenza A viruses recognize SAs in an  $\alpha$ -2,6 linkage (138). After cell attachment, the virus is **internalized** through receptor-mediated endocytosis in clathrin-coated vesicles or by macropinocytosis (141). Acidification of the endosome induces a conformational change in HA, promoting the fusion of viral and endosomal membranes, allowing the virus to access the cell cytoplasm. This step also activates the M2 ion channels, leading to the acidification of the interior of the virion, promoting uncoating and dissociation of the vRNPs from M1. As a result, vRNPs are released into the cytoplasm and transported to the cell nucleus, where they enter via the classical importin pathway, mediated by NP molecules, to initiate viral replication (142). Inside the nucleus, viral RNA segments are transcribed into complementary positive RNAs (cRNAs), acting as a template for the transcription of new viral RNAs, and into messenger RNAs (mRNAs). Viral mRNAs are transported into the cytoplasm and translated into viral proteins. HA, NA and M2 associate with the cell membrane, while the RNA dependent RNA polymerase

complex (PA, PB1 and PB2) NP, M1 and non-structural proteins (NS1, NS2) are imported into the nucleus promoting vRNA replication and the formation of vRNPs. Subsequently, vRNPs are transported into the cytoplasm, where progeny virions are **assembled** and **budded** at the plasma membrane (143). NA is involved in the release of newly formed virions from infected cells, thanks to its sialidase activity, promoting viral spread. It also facilitates viral access by removing decoy receptors present on cilia, mucins and glyocalix (144) and participates to viral motility, in collaboration with HA, enhancing the infection of susceptible cells (145).



**Figure 15.** Overview of IAV replication cycle including a series of sequential steps (1-7), from the viral entry to the release of progeny virions. Adapted from Du et al. 2023 (143)

**Proteolytic activation** is an essential requirement for viral infectivity and effective replication. The precursor of HA (HA0) must be cleaved into two functional subunits by the host's proteases (146): HA1, which contains the receptor binding domain and HA2, site of the fusion peptide. HA cleavability is affected by the amino acid composition of the cleavage site of HA, which represents one of the main determinants of pathogenicity and viral tissue tropism of AI (146,147). The HA cleavage site of the majority of LPAIV has a single basic amino acid, which means it can be only cleaved by extracellular, trypsin-like proteases, restricted to the respiratory and digestive tract. In addition, LPAI virions released from susceptible cells are not infectious, since HA is not cleaved. On the other end, the HA cleavage site of HPAIV contains multiple basic amino acids, and can be cleaved by intracellular, furin-like proteases, ubiquitously distributed. In addition, since HA is cleaved intracellularly, before viral assembly, HPAIV are already infectious at the time of release (146).

**In situ detection techniques.** In situ detection techniques have been used for investigating a broad range of non-infectious and infectious diseases, including AI. Among them, IHC represents a cost-effective and routinely used technique (148,149). The presence of an antigen of interest is demonstrated in selected tissues, using specific antibodies and different revelation methods. Since the original tissue architecture is maintained, a correlation can be made between antigen detection and the type and severity of histopathological lesions observed. Furthermore, significant information on the pathogenesis and tissue tropism of different viruses in different species can arise from studying antigen tissue distribution and subcellular localization (150). Unfortunately, several factors may negatively impact final results and assay reproducibility (150–152), including post-mortem delay and specimens handling, fixation protocol, paraffin blocks preparation, antigenic retrieval technique, antibody selection, and revelation system (153). Important limitations, impacting IHC's accuracy and reliability for the detection of IAVs, include the dependence on available antibodies, the type of antibody used (monoclonal or polyclonal) the occurrence of non-specific background staining preventing proper interpretation of findings, and the limited specificity due to antibodies cross-reactivity with different IAVs and strains (150,154,155). As a result, the need for developing additional reliable tools for the detection of avian influenza viruses (AIVs) in infected tissues has been increasing over the years.

The goal of this study was to validate a novel RNAscope ISH technique for the detection of AIV matrix protein gene in FFPE tissues obtained from several naturally-infected avian species and to compare it to the routinely-used anti-IAV nucleoprotein IHC.

## 2. Article 1

The present article was published in *Journal of Veterinary Diagnostic Investigation* under the following reference:

Nicolas Gaide\*, **Manuela Crispo\***, Adam Jbenyeni, Céline Bleuart, Maxence Delverdier, Timothée Vergne, Guillaume Le Loc'h, Jean-Luc Guérin. Validation of an RNAscope assay for the detection of avian influenza A virus. *J Vet Diagn Invest.* 2023. 35(5):500-506.

\* These authors contributed equally to this work and share first authorship.

## Validation of an RNAscope assay for the detection of avian influenza A virus

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**Abstract.** Highly pathogenic avian influenza (HPAI) is an acute viral disease associated with high mortality and great economic losses. Immunohistochemistry (IHC) is a common diagnostic and research tool for the demonstration of avian influenza A virus (AIAV) antigens within affected tissues, supporting etiologic diagnosis and assessing viral distribution in both naturally and experimentally infected birds. RNAscope in situ hybridization (ISH) has been used successfully for the identification of a variety of viral nucleic acids within histologic samples. We validated RNAscope ISH for the detection of AIAV in formalin-fixed, paraffin-embedded (FFPE) tissues. RNAscope ISH targeting the AIAV matrix gene and anti-IAV nucleoprotein IHC were performed on 61 FFPE tissue sections obtained from 3 AIAV-negative, 16 H5 HPAIAV, and 1 low pathogenicity AIAV naturally infected birds, including 7 species sampled between 2009 and 2022. All AIAV-negative birds were confirmed negative by both techniques. All AIAVs were detected successfully by both techniques in all selected tissues and species. Subsequently, H-score comparison was assessed through computer-assisted quantitative analysis on a tissue microarray comprised of 132 tissue cores from 9 HPAIAV-infected domestic ducks. Pearson correlation of  $r=0.95$  (0.94–0.97), Lin concordance coefficient of  $\rho_c=0.91$  (0.88–0.93), and Bland–Altman analysis indicated high correlation and moderate concordance between the 2 techniques. H-score values were significantly higher with RNAscope ISH compared to IHC for brain, lung, and pancreatic tissues ( $p \leq 0.05$ ). Overall, our results indicate that RNAscope ISH is a suitable and sensitive tool for in situ detection of AIAV in FFPE tissues.

**Keywords:** avian influenza A virus; immunohistochemistry; RNAscope; tissue microarray.

Avian influenza is a highly contagious disease caused by influenza A viruses (IAVs; *Orthomyxoviridae*, *Alphainfluznavirus*).<sup>16,24</sup> Wild birds, particularly aquatic and migratory species belonging to the orders *Anseriformes* and *Charadriiformes*, represent the natural reservoir of avian IAVs (AIAVs), contributing to viral circulation and transmission to commercial poultry.<sup>8,10,21</sup> Since 2016, clade 2.3.4.4b highly pathogenic avian influenza A(H5) viruses (H5 HPAIAVs) have been associated with several panzootics, resulting in high morbidity and mortality in both wild birds and poultry.<sup>10,17</sup> Between October 2021 and January 2023, 7,173 H5 HPAIAV outbreaks were reported worldwide and were responsible for massive die-offs; European and French outbreaks represented 69% and 22% of outbreaks, respectively.<sup>11</sup>

Prompt identification of infected birds, leading to the institution of effective control measures, is crucial to limit the spread of HPAIAVs within a susceptible population. Rapid diagnosis typically relies on molecular detection of AIAVs within clinical specimens and laboratory samples by reverse-transcription real-time PCR (RT-rPCR) and/or sequencing analysis.<sup>1,28</sup> In situ detection of viral antigens or nucleic acids within infected tissues, although more time-consuming and technically demanding, is important to understand the pathogenesis of AI, and to obtain valuable information on viral biodistribution, tissue tropism, and

interactions with host cell factors, in both experimentally and naturally infected birds.<sup>13,20,25</sup>

Immunohistochemistry (IHC) is a safe, economical, and effective method, widely used for the identification of AIAV antigens in formalin-fixed, paraffin-embedded (FFPE) tissues using specific antibodies. The most reliable and commonly used target, thanks to its highly conserved sequence and abundance within the virion, is the IAV nucleoprotein (NP).<sup>20</sup> Other targets include IAV hemagglutinin, matrix protein, and nonstructural protein 1.<sup>14,26,29</sup>

RNAscope in situ hybridization (ISH) is a novel and commercially available technique that allows the visualization of single RNA molecules in cytologic and histologic samples, at a single-cell level.<sup>27</sup> RNAscope ISH differs from conventional ISH by the use of a combination of RNA-specific oli-

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gonucleotide probes (Z) specifically designed to hybridize to the target RNA in pairs (ZZ).<sup>27</sup> Because a 1-kb region is commonly targeted by 20 probe pairs, sequential hybridizations with the preamplifier, amplifier, and label probes can theoretically yield up to 8,000 labels for each target RNA. Overall, RNAscope ISH has been reported to be more sensitive than conventional ISH and/or IHC, with a substantially similar specificity to both in situ detection tools, and it appeared to be fairly concordant with RT-PCR.<sup>2,7,23,27</sup>

RNAscope ISH has been widely used in humans, non-human primates, pigs, horses, cattle, cats, dogs, and mice for the identification of a variety of viral pathogens, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), porcine epidemic diarrhea virus, equine arteritis virus (*Alphaarterivirus equid*), bovine viral diarrhea virus (*Pestivirus bovis*), and feline and canine papillomaviruses (*Lambdapapillomavirus 1* and *2*, respectively).<sup>4-7,15,22,30</sup> This technique has been applied successfully for the detection of duck hepatitis A virus (*Avihepatovirus A*) genotype 3 in experimentally infected Pekin ducks, a novel orthoreovirus in American corvids, and AIAV NP in a mouse model comparing A(H7N9) and A(H9N2) infections.<sup>12,18,31</sup>

We aimed to validate an RNAscope ISH assay for the detection of the AIAV matrix protein gene (*M* gene) and to compare it to the routinely used anti-IAV NP IHC on selected FFPE tissues obtained from naturally infected birds.

## Methods

### Case selection

We selected 61 FFPE tissue sections from 20 birds sampled between 2009 and 2022, including 3 AIAV-negative commercial chickens (5 tissue sections) and 17 naturally AIAV-infected birds detected by RT-rtPCR, in compliance with official surveillance procedures (Suppl. Table 1). Positive birds included commercial poultry infected with clade 2.3.4.4b H5N8 HPAIAVs from the 2020–2021 and 2016–2017 epizootics (10 mule ducks, 1 quail, 1 goose;  $n=48$ ), commercial poultry infected with H5 HPAIAVs distant from Gs/Gd/96-like group HPAIAV from the 2015–2016 French outbreak (1 chicken, 1 guinea fowl;  $n=2$ ), 2 captive Houbara bustards infected with H5N8 AIAV ( $n=5$ ), and a commercial turkey infected with H6N1 low pathogenicity AIAV (LPAIAV;  $n=1$ ).

### Tissue microarray construction

For computer-assisted quantitative (CAQ) analysis, a tissue microarray (TMA) was constructed using FFPE tissue blocks obtained from 9 commercial mule ducks naturally infected with clade 2.3.4.4b H5N8 HPAIAVs from the 2020–2021 epizootic (3 flocks, 3 birds/flock). To compare the 2 techniques on tissues with different degrees of viral expression, distribution, and lesion patterns, we selected brain, heart, pancreas, lung, and spleen. Upon histologic assessment, representative lesion areas (regions of interest, ROI) were identified and

marked on the blocks (3 ROI for each organ). Subsequently, 1-mm tissue cores were harvested from the donor blocks and transferred into a blank recipient paraffin block (MiniCore 3, Alphelys; Toulouse University Hospital, Toulouse, France). The TMA was designed asymmetrically, and tissues were distributed randomly; triplicates were placed successively.

### Histopathology

From the TMA and each FFPE tissue block, 3- $\mu$ m serial sections were produced and used for H&E staining, anti-IAV NP IHC, and RNAscope ISH targeting the AIAV *M* gene.

### Immunohistochemistry

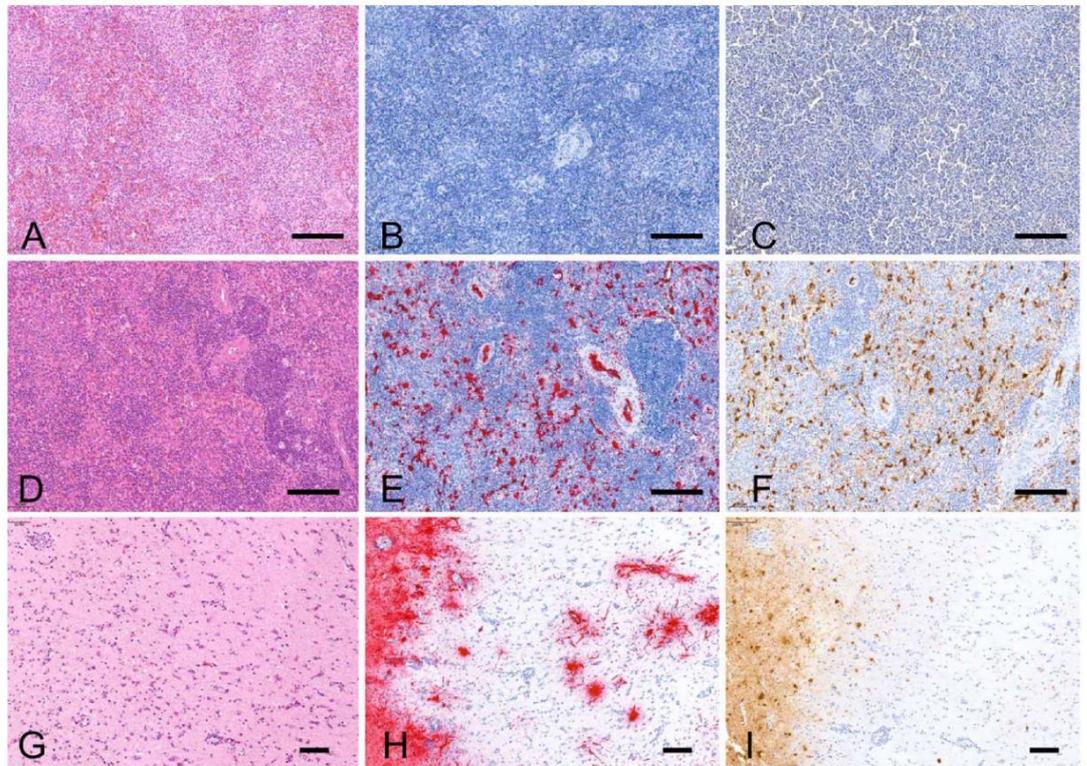
Immunostaining was performed using a monoclonal mouse antibody directed against IAV NP (HB65, FCG013; Kerabio). Specifically, the immunohistochemical protocol included an antigen retrieval step with pronase 0.05% applied for 10 min at 37°C, a peroxidase blocking step of 5 min at room temperature (S2023; Dako) followed by saturation of nonspecific binding sites with normal goat serum (X0907; Dako) applied for 25 min at room temperature, and overnight incubation with anti-AIAV NP antibody (1:800 dilution) at 4°C. For TMA-based quantitative comparison, immunohistochemical staining was visualized (ENVISSION FLEX HRP Magenta system; Dako Omnis) according to the manufacturer's recommendations; 3,3'-diaminobenzidine (DAB) chromogen was used for qualitative comparison.

### RNAscope ISH

The *M* gene RNAscope probe (V-H5N8-M1M2) was custom-designed (Advanced Cell Diagnostics) and consisted of 20 antisense oligopairs targeting the well-conserved IAV *M* genes 1 and 2 of clade 2.3.4.4b H5N8 HPAIAV (A/chicken/France/20P016448/2020; Suppl. File 1). The sequence submitted for probe design was selected to provide the highest homology with other AIAVs, H5 HPAIAVs in particular. RNAscope ISH assays were assessed using RNAscope 2.5 high-definition red assay according to the manufacturer's recommendations, including mild pretreatment conditions (15-min incubation with protease digestion for antigenic retrieval) and hematoxylin counterstaining. A probe targeting the dihydrodipicolinate reductase (*dapB*) gene from the *Bacillus subtilis* strain SMY, served as negative control; a probe directed against the peptidyl-prolyl cis-trans isomerase B (*PPIB*) gene from chicken and AIAV-positive tissues were used as positive controls for the validation steps (Advanced Cell Diagnostics).

### Quantitative comparison

Quantitative comparison was assessed on serial sections obtained from the TMA block stained with both *M* gene RNAscope ISH and anti-IAV NP IHC. Whole slides were scanned at 40 $\times$  magnification (3DHISTECH Panoramic scanner;



**Figure 1.** Avian tissues labeled with H&E, anti-influenza A virus (IAV) nucleoprotein (NP) immunohistochemistry (IHC), and RNAscope in situ hybridization (ISH) targeting the matrix (*M*) gene. Bars = 100  $\mu$ m. **A–C.** Spleen of an avian IAV (AIAV)-negative chicken. **A.** H&E. **B.** No *M* gene RNAs detected with RNAscope ISH. **C.** No NP antigens detected with anti-IAV NP IHC. **D–F.** Spleen of a chicken naturally infected with H5Nx highly pathogenic AIAVs (HPAIAVs; 2015–2016). **D.** Moderate multifocal lymphocytolysis. H&E. **E.** Widespread endothelial detection of *M* gene RNA. RNAscope ISH. **F.** Widespread endothelial detection of NP antigens. Anti-IAV NP IHC. **G–I.** Brain of a duck naturally infected with H5N8 Gs/Gd clade 2.3.4.4b HPAIAV (2020). **G.** Nonsuppurative perivascular and parenchymal infiltrates. H&E. **H.** Widespread detection of *M* gene RNAs within neurons and neuropil. RNAscope ISH. **I.** Widespread detection of *M* gene RNAs within neurons and neuropil. Anti-IAV NP IHC.

epredia) and analyzed using open-source software for digital image analysis (QuPath, <https://qupath.github.io/>). Color deconvolution and automatic cell detection were adjusted for each TMA and each type of organ examined, respectively. Subcellular detection was determined for each core through script-based batch analysis. RNAscope ISH and immunohistochemical detection expression was expressed as an H-score according to the manufacturers' recommendations.

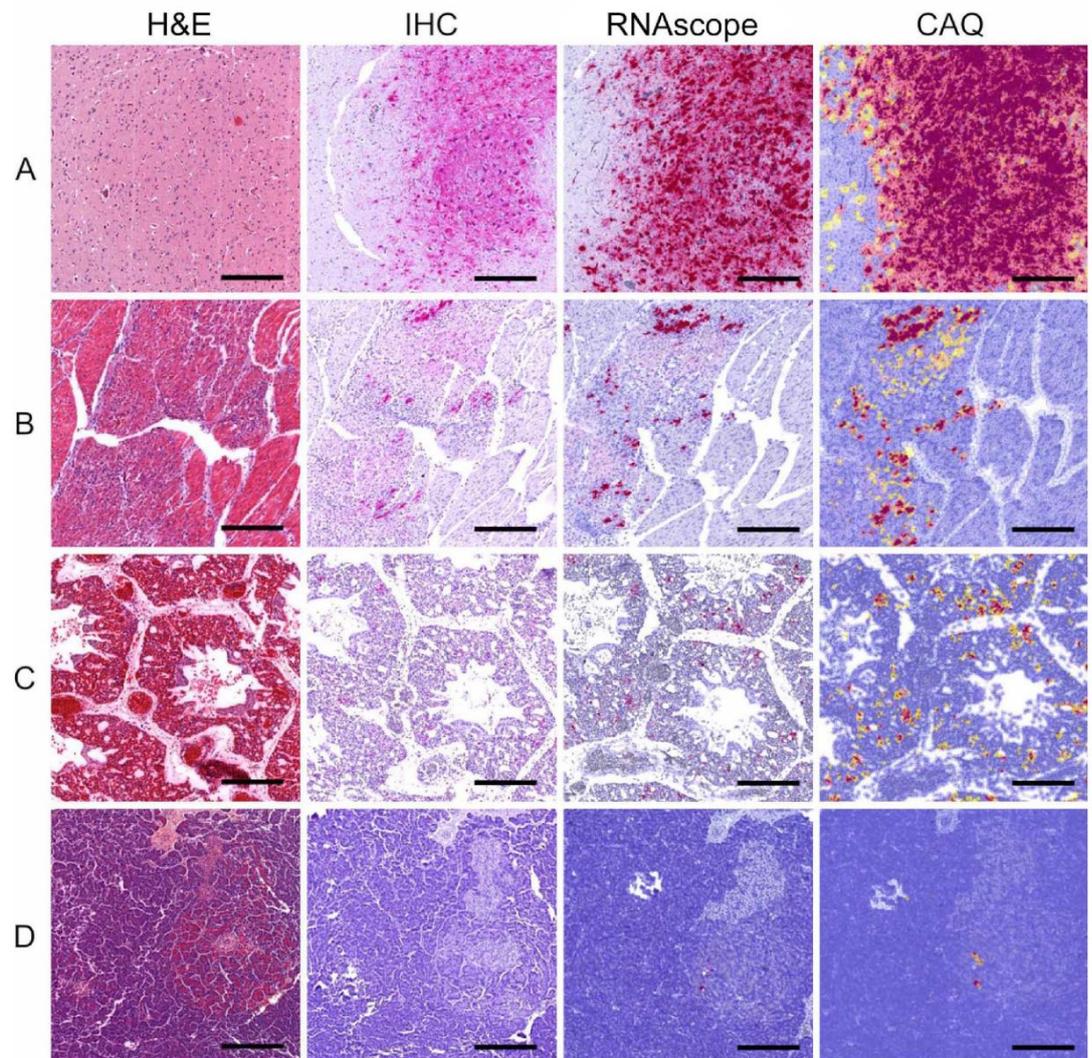
#### Statistical analysis

For CAQ analysis, Pearson correlation coefficient ( $r$ ) and Lin concordance coefficient ( $\rho$ ) were used to determine correlation and concordance between *M* gene RNAscope ISH and anti-IAV NP IHC H-score measurements.<sup>19</sup> Additionally, Bland–Altman analysis was performed, and limits of agree-

ment were built after assessing the normality of difference scores. If normality was not observed, limits were built using 2.5 and 97.5 centiles.<sup>3</sup> A Wilcoxon–Mann–Whitney test was used to compare H-score measurements for each organ;  $p \leq 0.05$  was considered to be statistically significant. All measurements were performed using R software (<https://www.R-project.org/>).

#### Results

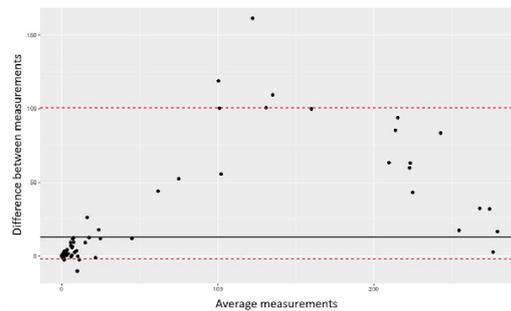
All tissues obtained from AIAV-negative birds were negative with both techniques (Fig. 1A–C). All tested AIAV strains, including H5 HPAIAVs distant from Gs/Gd/96-like group (Fig. 1D–F), clade 2.3.4.4b H5N8 HPAIAV (Fig. 1G–I), and H6N1 LPAIAV, were detected by both techniques in all selected tissues and avian species.



**Figure 2.** H&E sections and tissue microarray–based computer-assisted quantitative (CAQ) comparison of anti-IAV NP IHC and *M* gene RNAscope ISH performed on 4 tissue cores of mule ducks naturally infected with H5N8 clade 2.3.4.4b HPA1AV (2020–2021). Bars = 500  $\mu$ m. **A.** Brain. RNAscope ISH positivity is more widespread compared to IHC. **B.** Heart. Positive labeling within areas of nonsuppurative myocarditis with both techniques. Positive clusters appear wider and more numerous with RNAscope ISH compared to IHC. **C.** Lung. Positive labeling is visible at low magnification with RNAscope ISH. **D.** Pancreas. Sparse positive cells can be detected with RNAscope ISH.

The distribution pattern of positive cells was similar among techniques. Tissues originating from chickens and guinea fowl naturally infected with H5 HPA1AVs had widespread endothelial expression of both viral NP antigens and *M* gene RNAs (Fig. 1E, 1F). In ducks, positivity was demonstrated in neurons (Figs. 1H, 1I, 2A), cardiomyocytes (Fig. 2B), pulmonary

capillary bed (Fig. 2C), and pancreatic acinar cells (Fig. 2D). In Houbara bustards, mild autolysis of intestinal sections resulted in nonspecific background staining that appeared substantially higher with anti-IAV NP IHC compared to *M* gene RNAscope ISH. However, positive detection occurred within endothelial cells, myocytes, and mesothelial cells with both



**Figure 3.** Bland–Altman analysis of *M* gene RNAscope ISH and anti-IAV NP IHC H-scores. The black line indicates the bias between the 2 techniques, the red dashed lines are the limits of agreement built using the percentiles, and the black dots are the individual tissue core measurements.

techniques. In the turkey naturally infected with H6N1 LPAIAV, both viral antigens and RNAs were detected within tracheal epithelial cells.

Subcellular distribution appeared slightly different between the 2 techniques. NP antigens could be detected both at a nuclear and a cytoplasmic level by anti-IAV NP IHC. In contrast, *M* gene RNAscope ISH highlighted viral RNAs mainly as coalescing-to-diffuse cytoplasmic clusters or a mix of cytoplasmic and nuclear dots. On the other hand, *M* gene RNAscope ISH was able to highlight positive cells, as cytoplasmic and nuclear dots, within IHC-negative areas of brain, heart, and lung tissue sections.

Of 132 tissue cores included in the TMA, 126 were used for CAQ comparison (Fig. 2), and 6 were lost (index core loss: 4.5%). The histologic lesions originally detected in the recipient blocks could also be observed in the tissue cores. Specifically, nonsuppurative inflammation and parenchymal necrosis were invariably present in brain tissue cores, more variably identified in heart tissue cores, and only rarely seen in spleen, lung, and pancreas tissue cores. Spleen tissue cores were unremarkable. The 2 techniques were correlated, with a Pearson correlation coefficient of  $r=0.95$  (0.94–0.97). The concordance between the 2 techniques was moderate, with a Lin concordance coefficient of  $\rho_c=0.91$  (0.88–0.93).<sup>16</sup> Bland–Altman analysis indicated that the 2 techniques had a bias (median difference H-score RNAscope – H-score IHC) of 12.7 (Fig. 3). The limits of agreement appeared wide, ranging from 0 to 102. Disagreement was higher between the 2 techniques for average H-scores of 50–250. Brain tissue cores provided the highest H-score values, with a median of 226 (interquartile range [IQR]: 146–262) and 139 (IQR: 47–202) for *M* gene RNAscope ISH and anti-IAV NP IHC, respectively ( $p<0.05$ , Fig. 4); heart tissue cores had lower H-scores with median values of 6 (IQR: 3–17) and 5 (IQR: 3–12), respectively. For lung, spleen, and pancreas tissue cores, detection measurements

appeared low, with H-scores  $<1$ , and a significant difference between techniques was found for pancreas and lung ( $p<0.05$ ; Suppl. Table 2).

## Discussion

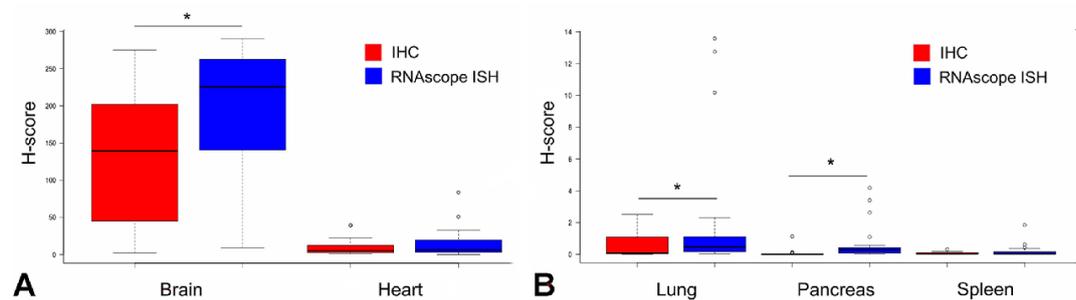
We designed our RNAscope probe to target the AIAV *M* gene RNAs. Overall, *M* gene RNAscope ISH appeared to be as specific as anti-IAV NP IHC and was able to successfully detect all selected AIAVs, including clade 2.3.4.4b H5 HPAIAVs, H5 HPAIAV distant from Gs/Gd/96-like H5 HPAIAV, and H6N1 LPAIAV. Quantitative comparison between the 2 techniques indicated high correlation and moderate concordance, explained by higher detection rates with *M* gene RNAscope ISH, particularly for brain, lung, and pancreatic tissues.

Performance comparison between RNAscope ISH and IHC has been performed in the fields of infectious diseases and oncology.<sup>9</sup> Similar to our results, RNAscope ISH has been reported to be a highly sensitive in situ detection technique that had high concordance with molecular detection (qPCR, RT-PCR, and DNA ISH) and lower concordance with IHC, mostly because of different target molecules (RNAs vs. proteins).<sup>2,7,23</sup> In work assessing the use of RNAscope ISH for the detection of SARS-CoV-2 in FFPE human lung tissues, substantial agreement was found between RNAscope ISH and IHC ( $\kappa=0.75$ ), with a specificity of 100% for both techniques<sup>23</sup>; sensitivity was higher for reverse-transcription droplet digital PCR (96%) compared to RNAscope ISH (36%) and IHC (31%). Another comparative study found that RNAscope ISH was more sensitive than conventional ISH, but not IHC, for the detection of equine arteritis virus.<sup>7</sup>

On the one hand, we found that additional positive cells were highlighted by *M* gene RNAscope ISH compared to anti-IAV NP IHC, suggesting a substantially higher sensitivity of detection within positive tissues. This result can be partially explained by the combination of strong signal amplification and the use of 20 oligopairs specifically designed to hybridize to the targeted RNA. On the other hand, it was occasionally difficult to differentiate nuclear positivity from cytoplasmic positivity with *M* gene RNAscope ISH, given intense and widespread signal amplification.

Improved viral detection was observed with *M* gene RNAscope ISH compared to anti-IAV NP IHC in tissues obtained from Houbara bustards, thanks to reduced nonspecific staining. These semi-desert birds reside in geographic areas characterized by high environmental temperatures that can accelerate autolysis, which is a well-recognized cause of background signal. A similar improvement was also observed in tissues originating from wild and captive exotic birds, naturally infected with H5N1 HPAIAVs during the most recent outbreaks registered in France in 2022–2023 (data not shown).

Although the anti-IAV NP IHC can detect a wide range of IAVs, including those affecting humans, equids, and swine, *M* gene RNAscope ISH is specifically designed to detect



**Figure 4.** Box plot of *M* gene RNAscope ISH and anti-AIV NP IHC H-scores. **A.** Brain, heart. **B.** Lung, pancreas, spleen. The central line in each box is the median. The bottom and top box margins are the 25th and 75th interquartiles, respectively. The whiskers are the range of data, and the unfilled dots are the outliers. Asterisk (\*) indicates statistical significance ( $p < 0.05$ ), Wilcoxon Mann Whitney test.

AIAV RNAs. A remarkable application of such specificity is the use of *M* gene RNAscope ISH by our laboratory in lesional tissues obtained from mammals naturally infected with AIAVs, supporting the occurrence of spillover events (data not shown).

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#### Availability of data and materials

Our datasets are available from the corresponding author.

#### Declaration of conflicting interests

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

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#### Supplemental material

Supplemental material for this article is available online.

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**Supplemental Table 1.** Overview of the 61 formalin-fixed, paraffin-embedded tissue sections included in our study.

	Species	Tissue	Flock status (PCR/sequencing analysis)
1	Turkey	Trachea	Positive H6N1 LPAIAV (2009)
2	Guinea fowl	Lung	Positive H5N9 HPAIAV
3	Chicken	Spleen	Positive H5Nx HPAIAV (2015–2016)
4	Mule duck	Brain	Positive H5N8 clade 2.3.4.4b HPAIAV (2016–2017)
5	Mule duck	Lung	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
6	Mule duck	Heart	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
7	Mule duck	Spleen	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
8	Mule duck	Pancreas	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
9	Mule duck	Brain	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
10	Mule duck	Spleen	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
11	Mule duck	Heart	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
12	Mule duck	Pancreas	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
13	Mule duck	Brain	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
14	Mule duck	Brain	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
15	Mule duck	Lung	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
16	Mule duck	Heart	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
17	Mule duck	Spleen	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
18	Mule duck	Pancreas	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
19	Mule duck	Brain	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
20	Houbara bustard	Pancreas	Positive H5N8 (2020–2021)
21	Houbara bustard	Liver	Positive H5N8 (2020–2021)
22	Houbara bustard	Intestine	Positive H5N8 (2020–2021)
23	Houbara bustard	Spleen	Positive H5N8 (2020–2021)
24	Houbara bustard	Intestine	Positive H5N8 (2020–2021)
25	Mule duck	Lung	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
26	Mule duck	Heart	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
27	Mule duck	Spleen	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
28	Mule duck	Pancreas	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
29	Mule duck	Brain	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
30	Mule duck	Lung	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)

31	Mule duck	Heart	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
32	Mule duck	Spleen	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
33	Mule duck	Pancreas	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
34	Mule duck	Brain	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
35	Mule duck	Lung	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
36	Mule duck	Heart	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
37	Mule duck	Spleen	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
38	Mule duck	Pancreas	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
39	Mule duck	Brain	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
40	Mule duck	Lung	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
41	Mule duck	Heart	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
42	Mule duck	Spleen	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
43	Mule duck	Pancreas	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
44	Mule duck	Brain	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
45	Mule duck	Lung	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
46	Mule duck	Heart	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
47	Mule duck	Spleen	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
48	Mule duck	Pancreas	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
49	Mule duck	Brain	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
50	Mule duck	Lung	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
51	Mule duck	Heart	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
52	Mule duck	Spleen	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
53	Mule duck	Pancreas	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
54	Mule duck	Brain	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
55	Goose	Heart	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
56	Quail	Pancreas	Positive H5N8 clade 2.3.4.4b HPAIAV (2020–2021)
57	Chicken	Spleen	AIAV negative
58	Chicken	Lung	AIAV negative
59	Chicken	Liver	AIAV negative
60	Chicken	Liver	AIAV negative
61	Chicken	Lung	AIAV negative

AIAV = avian influenza A virus; chicken = *Gallus gallus*; goose = *Anser anser*; guinea fowl =

*Numida meleagris*; mule duck = *Anas platyrhynchos domesticus* × *Cairina moschata domestica*;

Houbara bustard = *Chlamydotis undulata undulata*; HPAIAV = highly pathogenic AIAV;

LPAIAV = low pathogenicity AIAV; NA = not available; quail = *Coturnix coturnix*; turkey =

*Meleagris gallopavo*.

**Supplemental Table 2.** *M* gene RNAscope in situ hybridization (ISH) and anti-influenza

A virus nucleoprotein immunohistochemistry (IHC) H-score values on tissue microarray.

Tissue core	RNAscope ISH	IHC
Brain ( <i>n</i> = 24)	226 (146–262)*	139 (47–202)
Heart ( <i>n</i> = 26)	6 (3–17)	5 (3–12)
Lung ( <i>n</i> = 24)	0.45 (0.16–0.96)*	0.08 (0.03–1.08)
Pancreas ( <i>n</i> = 25)	0.29 (0.07–0.43)*	0 (0–0.02)
Spleen ( <i>n</i> = 27)	0.03 (0.01–0.15)	0.03 (0.02–0.11)

H-scores expressed as median (1st–3rd quartiles) of brain, heart, lung, pancreas, and spleen tissue cores of 3 flocks (3 ducks/flock, 3 cores/organ/duck; total of 126 tissue cores; index core loss: 4.5%).

\* Significant difference at  $p \leq 0.05$ .

**Supplemental File 1.** Sequence from A/chicken/France/20P016448/2020 provided for the design of matrix gene avian influenza A virus probe V-H5N8-M1M2.

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> M_A/chicken/France/20P016448/2020
ATGAGTCTTCTAACCGAGGTCGAAACGTACGTTCTCTCTATCGTCCCGTCAGGCCCC
CTCAAAGCCGAGATCGCGCAGAGACTTGAAGATGTCTTTGCAGGGAAGAACACCG
ATCTTGAGGCTCTCATGGAATGGCTAAAGACAAGACCAATCCTGTACCTCTGACT
AAGGGGATTTTGGGATTTGTGTTACGCTCACCGTGCCAGTGAGCGGGGACTGCA
GCGTAGACGCTTTGTCCAAAATGCTCTAAATGGAATGGAGACCCAAACAACATGG
ACAGGGCAGTCAAACGTACAGGAAATTGAAGAGAGAGATAACATTCCATGGGGC
TAAAGAAGTTGCACTCAGTTACTCAACCGGTGCACTTGCCAGTTGTATGGGTCTCAT
ATACAACAGGATGGGGACGGTGACCGCAGAAGTGGCATTGGGCCTAGTGTGTGCC
ACCTGTGAGCAGATTGCTGATTCACAGCATCGGTCTCACAGACAGATTGCAACCAC
CACCAATCCACTAATCAGACATGAAAACAGAATGGTGTGGCCAGTACTACAGCTA
AGGCTATGGAGCAGATGGCTGGATCGAGTGAGCAAGCAGCGGAAGCCATGGAGGT
TGCTAGTCAGGCTAGGCAGATGGTGCAGGCGATGAGGACCATTGGAACATCATCCTA
GCTCCAGTGCCGGTCTGAGAGATGATCTCCTTGAAAATTTGCAGGCCTACCAAAAA
CGGATGGGAGTGCAACTGCAGCGATTCAAGTGATCCTCTCGTTATTGCCGCAAGTAT
CATTGGGATCTTGCACTTGATATTGTGGATTCTTGATCGCCTTTTCTTCAAATGCGTT
TATCGTCGCCTTAAATACGGTTTGAAGAGAGGGCCTTCTACGGAAGGAGTACCTGA
GTCCATGAGGGAAGAGTACCGGCAGGAACAGCAGAGTGCTGTGGATGTTGACGAT
GGTCATTTTGTCAACATAGAGCTGGAGTAA
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### 3. Conclusions and future perspectives

Our study supports the use of RNAscope ISH as a reliable tool for the detection of AIVs in FFPE tissues. The first aspect to consider when choosing to rely on RNAscope ISH is its ability to clarify inconclusive or conflicting results provided by IHC (156). A similar outcome may originate from a variety of factors, including sample degradation, improper application of technical protocols, and poor test performances (151). For this reason, the association of IHC and RNAscope ISH, targeting two different molecules (RNA and protein) belonging to the same infectious agent would be beneficial.

RNAscope ISH's probes can be custom-designed and made available quickly compared to the production of antibodies for immunohistochemical staining. While the development of a new antibody requires between 6 months and 1 year, probe design can be completed in a matter of weeks (156). In addition, customized probes represent an encouraging option when dealing with targets for which IHC can't be pursued due to lack of cross-reactivity, a well-known immunohistochemical challenge in avian species. IHC against the nucleoprotein (NP) of influenza A virus (IAV) lacks specificity, detecting avian but also human, equine and swine viruses (150–152). On the other end, our RNAscope ISH assay targeting the matrix gene (M1/M2) was designed to detect avian influenza RNAs, with significant diagnostic implications, such as supporting the occurrence of spillover events from avian to mammalian species (157).

Despite the higher costs, compared to IHC, hampering its use in a daily-basis diagnostic routine, RNAscope ISH could be essential in a research setting. The simultaneous detection of several targets, through duplex or multiplex analysis, makes RNAscope ISH suitable for pathogenesis studies involving specific viral subtypes, coinfection studies and host immune response studies. RNAscope ISH could support the etiological role played by emerging or poorly characterized infectious agents within lesions of interest, helping fulfilling the Koch's postulates, especially in case of endangered species for which challenge studies would be difficult or impossible to perform due to ethical reasons. For example, RNAscope ISH was able to support the role of *Felis catus papillomavirus 2* (FcaPV-2) in the development of cutaneous squamous cell carcinoma in cats, highlighting the presence of FcaPV-2 E6/E7 viral oncogenes mRNAs within neoplastic skin samples, with a hybridization pattern similar to that reported in cervical cancers induced by human papillomavirus. (158).

The well-known RNA's sensitivity to natural post-mortem changes and formalin fixation may negatively affect sensitivity, particularly when dealing with spontaneous field cases. However, in our study, RNAscope ISH proved to be more sensitive compared to IHC

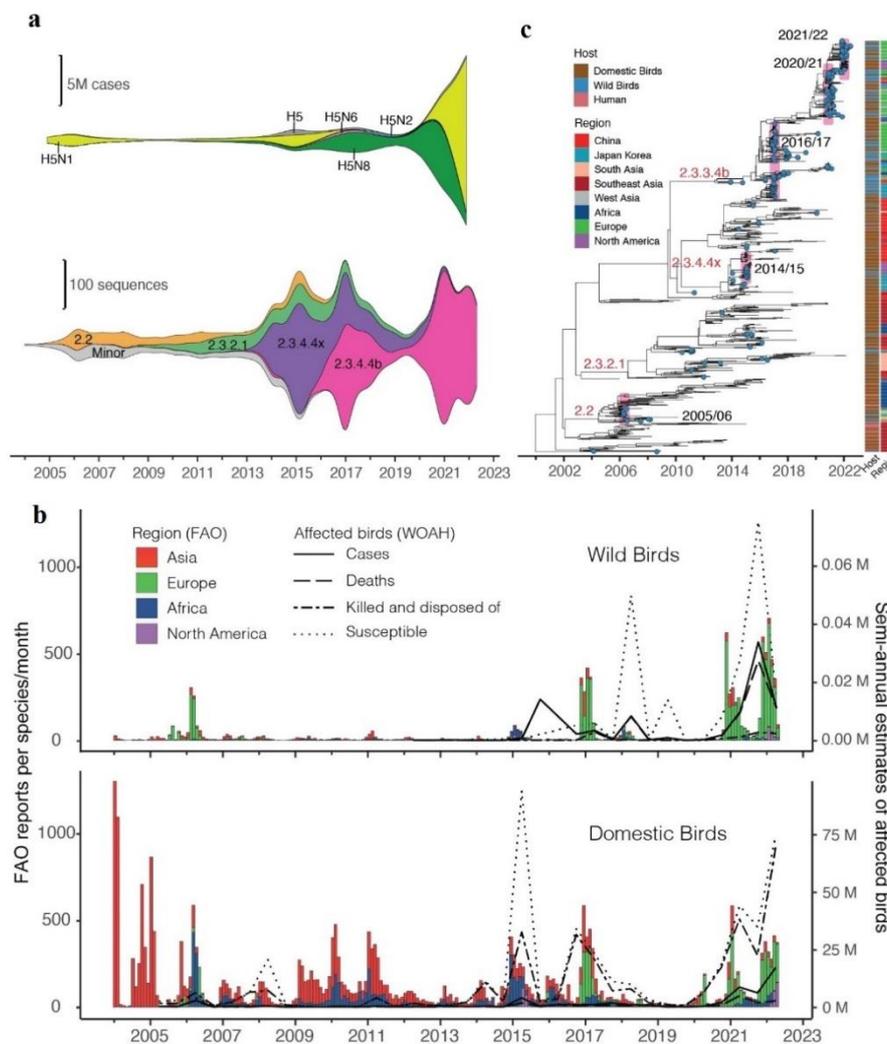
and less affected by autolysis, with little to no background staining. This aspect is important when working with samples originating from Houbaras, a semi-desertic bird residing in geographical areas characterized by extreme environmental conditions, accelerating post-mortem changes.

In our study we were able to successfully detect AIV in FFPE blocks stored for more than 10 years old. Considering the high value of Houbaras and the limited availability of samples that characterized endangered species, the possibility of relying on archive samples is of great interest. RNA scope ISH could become a game changer, allowing to conduct a variety of retrospective studies on «cold cases », and promoting collaborations with other diagnostic laboratories, research facilities and zoos equipped with an archive of FFPE's blocks. A similar approach could be easily translated to other endangered species, using the Houbara as a model.

## 4. B. STUDY 2

### 1. Introduction

Before the emergence *A/goose/Guangdong/1/1996*(Gs/Gd) H5N1, HPAI outbreaks were mostly confined to poultry, following the evolution of LPAI viruses (H5 and H7 subtypes) originating from asymptomatic wild aquatic birds, representing the natural reservoir of AI viruses. Since then, mortality events associated with Gs/Gd HPAI H5 viruses, especially from clade 2.3.4.4b and involving multiple NA subtypes, have been increasingly reported in both wild and domestic birds. Four major HPAI H5 epizootics were registered, with H5N8 being involved in 2014/15 (Asian origin), 2016/17 (Asian origin) and 2020/21 (African origin). H5N6 viruses played a role in 2016/17, while 2021/22 outbreaks were associated with H5N1, which emerged from H5N8 viruses in wild birds in Europe, becoming the most prevalent subtype across the globe (159) (**Figure 16**).



**Figure 16.** overview of HPAI H5Nx outbreaks from 2005 to 2022. Modified from Xie et al. 2023(159)

H5N1 has become a global threat for domestic, captive and wild birds due to its widespread circulation and high mortality rates (160). Besides the devastating economic losses resulting from the infection of commercial poultry flocks, the recent unprecedented spread to a variety of avian species, including endangered ones, is particularly concerning. The alarming expansion of H5N1 host range (160), accompanied by an increased occurrence of spillover events from the avian to the mammalian compartment, calls for the improvement of our knowledge in terms of clinico-pathological findings and target (“hot”) tissues for diagnostic sampling, to maximize surveillance efforts.

Pathobiological features of AI can vary significantly due to a variety viral and host-related factors (146). From this point of view, studies focusing on the pathology and tissue distribution of AIV are essential to define viral tropism, unveil transmission dynamics and explain clinico-pathological findings observed. They can also guide and support the diagnostic investigation, highlighting key necropsy and histopathological lesions and identifying the most representative/significant tissues for sampling (161). This aspect is particularly important when dealing with highly valuable and endangered species, with limited information in terms of disease susceptibility, and clinico-pathological presentation.

Available literature on AI in Houbaras is still scarce, including a few reports on H5N2 LPAI and H5N1 HPAI (55,162). H5N2 LPAIV was detected African Houbaras exhibiting neurological and/or respiratory signs, alone or in combination with other viral and bacterial pathogens (162,163). Natural infection with HPAI H5N1 was reported in falcons in Dubai, following the contact with imported Asian Houbaras. The virus was molecularly characterized, supporting interspecies transmission. Bustards presented with neurological signs, swollen head, nasal discharge, cyanotic shanks as well as diarrhea. However histopathological and immunohistochemical assessments were not attempted.

The goal of this study was to provide the first comprehensive pathological description of H5N8 HPAI natural infection in captive African Houbaras, including an overview of viral tissue distribution based on two different in situ detection techniques and the use of FFPE tissue samples for molecular analysis

## 2. Article 2

The present article was published in *Scientific Reports* under the following reference:

**Manuela Crispo**, Mar Carrasco Muñoz, Frédéric Lacroix, Mohamed-Reda Kheyi, Maxence Delverdier, Guillaume Croville, Malorie Dirat, Nicolas Gaide, Jean Luc Guerin & Guillaume Le Loc'h. Pathological investigation of high pathogenicity avian influenza H5N8 in captive houbara bustards (*Chlamydotis undulata*), the United Arab Emirates 2020. (2024) 14:4235. <https://doi.org/10.1038/s41598-024-54884-2>



## OPEN Pathological investigation of high pathogenicity avian influenza H5N8 in captive houbara bustards (*Chlamydotis undulata*), the United Arab Emirates 2020

Manuela Crispo<sup>1,✉</sup>, Mar Carrasco Muñoz<sup>2</sup>, Frédéric Lacroix<sup>2</sup>, Mohamed-Reda Kheyi<sup>2</sup>, Maxence Delverdier<sup>1</sup>, Guillaume Croville<sup>1</sup>, Malorie Dirat<sup>1</sup>, Nicolas Gaide<sup>1</sup>, Jean Luc Guerin<sup>1</sup> & Guillaume Le Loc'h<sup>1</sup>

At the end of 2020, an outbreak of HPAI H5N8 was registered in captive African houbara bustards (*Chlamydotis undulata*) in the United Arab Emirates. In order to better understand the pathobiology of this viral infection in bustards, a comprehensive pathological characterization was performed. A total of six birds were selected for necropsy, histopathology, immunohistochemistry, RNAscope in situ hybridization and RT-qPCR and nanopore sequencing on formalin-fixed and paraffin-embedded (FFPE) tissue blocks. Gross lesions included mottled and/or hemorrhagic pancreas, spleen and liver and fibrinous deposits on air sacs and intestine. Necrotizing pancreatitis, splenitis and concurrent vasculitis, hepatitis and fibrino-heterophilic peritonitis were identified, microscopically. Viral antigens (nucleoprotein) and RNAs (matrix gene) were both detected within necro-inflammatory foci, parenchymal cells, stromal cells and endothelial cells of affected organs, including the myenteric plexus. Molecular analysis of FFPE blocks successfully detected HPAI H5N8, further confirming its involvement in the lesions observed. In conclusion, HPAI H5N8 in African houbara bustards results in hyperacute/acute forms exhibiting marked pantropism, endotheliotropism and neurotropism. In addition, our findings support the use of FFPE tissues for molecular studies of poorly characterized pathogens in exotic and endangered species, when availability of samples is limited.

**Keywords** Influenza A virus, H5N8 subtype, Endangered species, United Arab Emirates, Immunohistochemistry, Nanopore sequencing

The concept that high pathogenicity avian influenza (HPAI) outbreaks were confined mostly to poultry flocks, as a result of the introduction of low pathogenicity viral subtypes H5 and H7 originating from wild aquatic birds, was shattered by the emergence of the A/goose/Guangdong/1/1996(Gs/Gd) H5N1 influenza A virus<sup>1,2</sup>. Since 2002, mortality events associated with Gs/Gd HPAI viruses, especially from clade 2.3.4.4b, have been reported in a variety of avian species, including endangered ones<sup>1</sup>. A few recent examples include the demise of more than 6500 Cape cormorants (*Phalacrocorax capensis*) and 350 African penguins (*Spheniscus demersus*) in Namibia, the loss of 750 great white pelicans (*Pelecanus onocrotalus*) belonging to the Djoudj National Bird Sanctuary, near the Senegal–Mauritania border and massive die-offs involving breeding populations of great skuas (*Stercorarius skua*) in Great Britain<sup>3–7</sup>.

Numerous avian species are the object of captive breeding and reinforcement programs, actively engaged in preserving and restoring wild populations. From this point, a remarkable example is provided by the African houbara bustard (*Chlamydotis undulata*) a terrestrial bird inhabiting the semi-desertic regions of North Africa and Canary Islands, classified as vulnerable by the International Union for the Conservation of Nature<sup>8</sup>. As a result, several conservation breeding projects have been established in North Africa and United Arab Emirates. Infectious diseases represent a significant threat for captive-breeding and release programs, potentially impairing

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repopulation efforts on multiple levels: loss of valuable individuals, including breeding adults, reduced breeding success and survival rates, local spread of harmful pathogens following the exposure of native wild populations to harmful pathogens associated with the translocation of infected individuals for restocking purposes<sup>9–12</sup>. Predator and scavenger species may also be threatened, due to the consumption of infected animals<sup>13–15</sup>. Additional risks arise from the rehabilitation of illegally traded birds into the wild or their introduction within captive breeding projects<sup>16</sup>. Quarantine measures, routine health monitoring and screening, are essential to properly understand sanitary issues and implement the most effective control measures, especially in species for which the presentation of a variety of pathogens is still poorly described<sup>16</sup>. From this point of view, necropsy findings can provide useful insights, guiding the diagnostic process, through target sampling<sup>17–19</sup>.

The clinico-pathological picture of HPAI can vary significantly, due to both virus-related and host-related factor<sup>20,21</sup>. While our knowledge on the pathobiological features of HPAI infection in captive and, to a lesser extent, wild bird species have been expanded by a variety of research works, available literature on bustards is still limited<sup>7,13,15,18,21–24</sup>.

To the authors' best knowledge, this is the first pathological description of H5N8 HPAI natural infection in captive African houbara bustards in the United Arab Emirates, including an overview of viral tissue distribution based on two different *in situ* detection techniques and the use of formalin-fixed and paraffin-embedded (FFPE) tissue samples for molecular analysis.

## Materials and methods

### Selection of birds belonging to a H5N8 HPAI outbreak

In November 2020, an outbreak of H5N8 HPAI was registered in a flock of captive African houbara bustards, part of a breeding conservation project established in the United Arab Emirates (International Fund for Houbara Conservation)<sup>25</sup>. Affected birds, all vaccinated against H5N2 (inactivated vaccine) 4.5 months before, ranged between 18 to 32 months of age (average 22 months) and were housed in outdoor pens, including 4–7 bird each, for a total of 314 individuals. Starting from November 25th, over a 7 days period, 8 birds belonging to 6 different pens died. Of these, 6 were found dead without exhibiting clinical signs, while 2 appeared mildly lethargic 24–48 h prior to the *exitus*. An additional bird exhibited similar signs for 24 h. Due to the drastic decline of its conditions, a decision was made by the veterinarian and the bird was humanely euthanized by intravenous injection of T-61.

H5N8 HPAI infection was confirmed by real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) targeting the subtype H5 of hemagglutinin (HA) and by HA and neuraminidase (NA) sequencing on swabs (oropharyngeal and cloacal) and fresh tissue sections (lung and spleen). Among the eight birds that died spontaneously, six birds were randomly selected for pathological investigation, including four birds experiencing hyperacute death (three males and one female) and two symptomatic individuals (one male and one female).

### Pathology and viral tissue distribution

A complete necropsy was performed within 48 h after the *exitus*. For histopathology, sections of trachea, lung, heart, liver, spleen, pancreas, gizzard, small and large intestine, cecal tonsils, kidney and ovary were fixed in 10% neutral buffered formalin, paraffin-embedded and sectioned at 3 µm. Slides were stained with hematoxylin and eosin (H&E) and examined by light microscopy. Necrotic-inflammatory lesions were scored according to severity<sup>26</sup>.

For viral tissue distribution, serial sections, 3 µm-thick, were obtained FFPE tissues, mounted on charged slides and stained with immunohistochemistry (IHC) and RNAscope *in situ* hybridization (ISH). The immunohistochemical assay was performed using a monoclonal mouse antibody directed against influenza A virus (IAV) nucleoprotein (NP) (Clone HB65, FCG013, Kerastat, Boston). Sections of IAV positive tissues were used as positive controls. Negative controls included sections incubated without the primary antibody or with another monoclonal antibody belonging to the same isotype (IgG2). The RNAscope ISH assay relied on a custom-designed probe (V-H5N8-M1M2) targeting the well-conserved influenza matrix protein genes (M gene) 1 and 2 of HPAIV (H5N8) Clade 2.3.4.4b (A/chicken/France/20P016448/2020) following the RNAscope® 2.5 HD Assay RED (Advanced Cell Diagnostics, Hayward, CA)<sup>27,28</sup>. A probe targeting the dihydrodipicolinate reductase gene from the *Bacillus subtilis* strain SMY, served as negative control, while sections of AIV positive tissues were used as positive controls. Parenchymal and endothelial immunoreactivity of each organ were scored according to distribution for both techniques<sup>26</sup>.

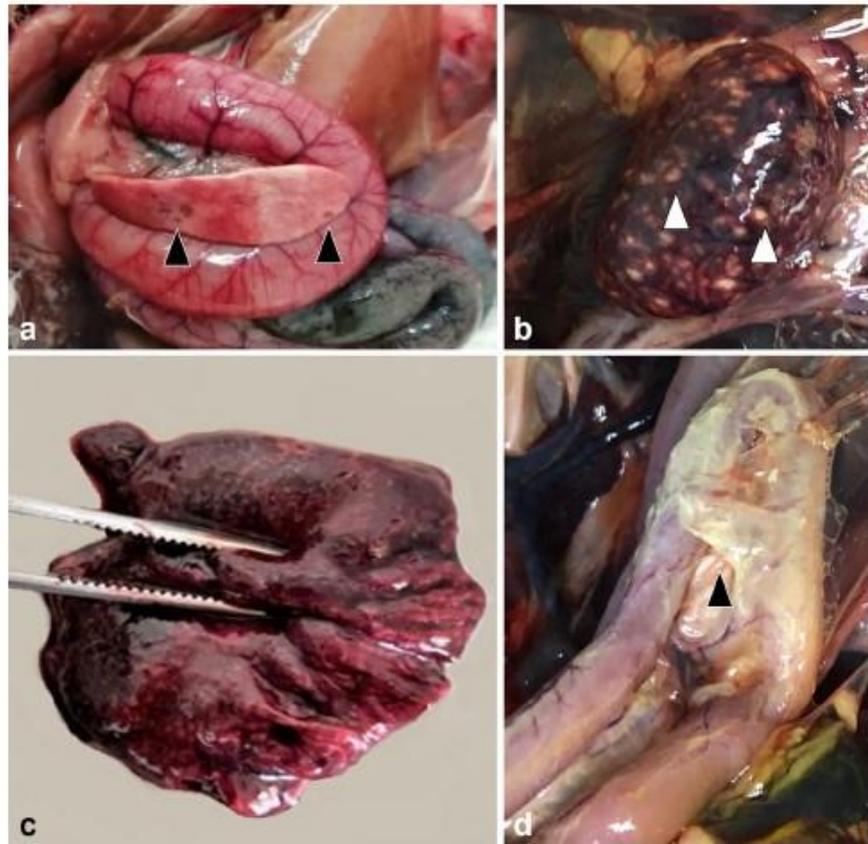
### Molecular analysis on FFPE tissue blocks

A total of 4 FFPE tissue blocks, obtained from 3 birds and including sections of liver, spleen, pancreas and intestine exhibiting necrotic-inflammatory lesions and positive immunostaining, were selected for molecular analysis. RNA was extracted using the Formapure XL RNA Kit (Beckman Coulter, Indianapolis, IN, USA), following manufacturer's recommendations. Qualitative and quantitative analysis of extracted RNA and DV200 assessment were conducted using high-throughput, automated electrophoresis (Tapestation 4200, Agilent). Subsequently, RT-qPCR targeting AIV H5 was performed (Influenza H5/H7 ID Gene PCR kit, IDVet), followed by HA and NA sequencing using the MinION Mk1C device (Oxford Nanopore Technologies, Oxford, UK) according to the protocol detailed by Croville et al.<sup>29</sup>.

## Results

### Gross pathology

Selected birds were mostly in good nutritional status (5/6 birds). The pancreas was diffusely enlarged, firm and mottled (4/6) or hemorrhagic (2/6) (Fig. 1a). The liver was diffusely enlarged, pale and firm (5/6) or friable (1/6).



**Figure 1.** Necropsy findings in African houbara bustards (*Chlamydotis undulata*) naturally-infected with H5N8 HPAIV. Mottled pancreas with multiple hemorrhagic foci (arrowheads) (a). Diffusely enlarged and mottled spleen with multiple necrotic foci (arrowheads) (b). Diffusely congested and edematous lungs (c). Accumulation of yellow fibrinous exudate on the surface of the small intestine (duodenum) (d).

The spleen was enlarged and mottled with a gritty consistency (5/6) or hemorrhagic (1/6) (Fig. 1b). Multiple, pale foci, 1 to 3 mm (mm) in diameter, were visible on the surface of liver (5/6) and spleen (3/6). Kidneys were diffusely congested (4/6) or pale and swollen (2/6). The glottis (2/6), tracheal mucosa (4/6) and lungs (3/6) were markedly congested and edematous (Fig. 1c), while white fibrinous plaques were present in the oropharynx (3/6). Yellow fibrinous exudate was covering the thoracic and abdominal air sacs, extending to the surface of the small intestine and pancreas (3/6) (Fig. 1d). Multifocal to coalescing petechial hemorrhages were identified in the ovary (1/6), gizzard's mucosa and underneath the koilin (1/6), the mucosa of the small intestine and cecal tonsils (2/6). Occasional ulcers were noticed in the cecal tonsils and colon mucosa (1/6). Mild hydropericardium was present in one bird.

#### Histopathology

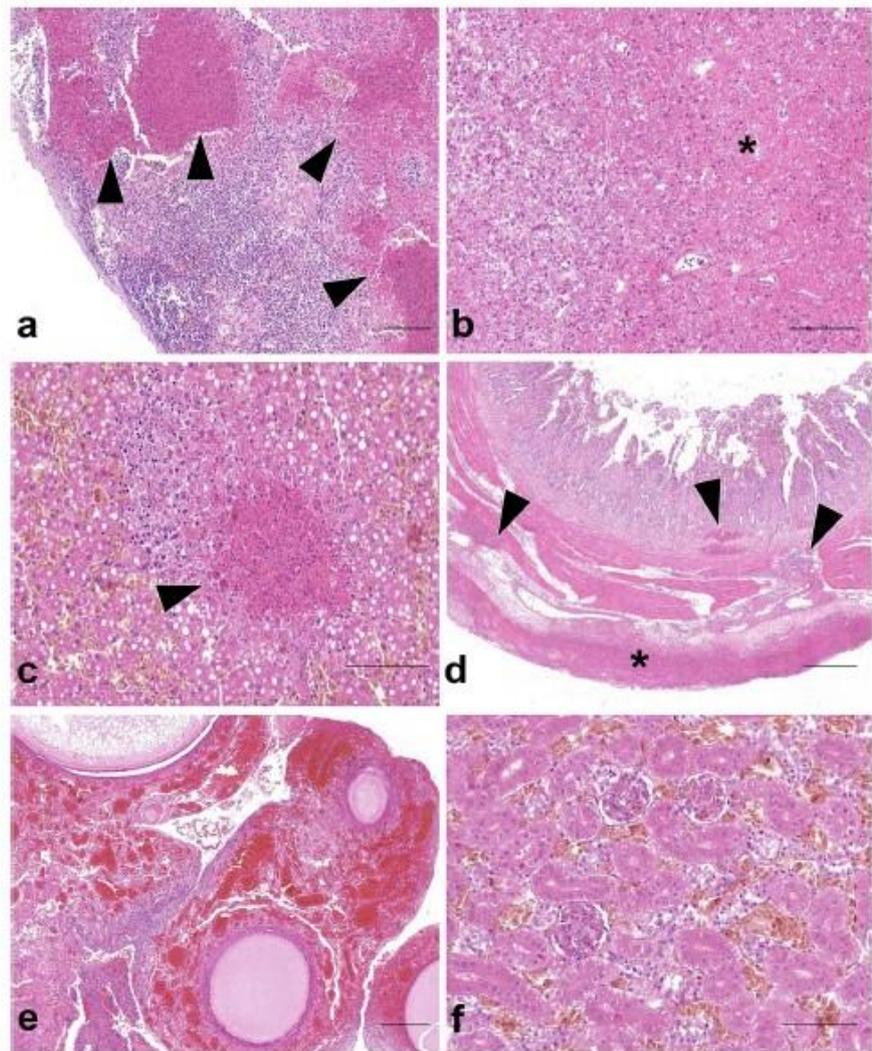
Results are summarized in Table 1. The spleen revealed severe necrotizing vasculitis with fibrinoid necrosis (4/4) and concurrent fibrino-heterophilic inflammation (3/4) (Fig. 2a). The pancreas exhibited mild to severe, parenchymal degeneration and lytic necrosis, with no to minimal mononuclear inflammation (5/5) (Fig. 2b). Hepatic lesions included diffuse macrovesicular steatosis (2/3), mild to moderate, multifocal lytic necrosis, with no to minimal mononuclear inflammation (3/3) and occasional fibrino-heterophilic exudation (1/3) (Fig. 2c). In the small intestine, the serosa was covered with large amounts of fibrino-heterophilic exudate, extending to the mesentery (1/2) (Fig. 2d). Several necrotic areas were scattered in the mucosa and submucosa, extending to the tunica muscularis (1/2). The tunica muscularis was also markedly expanded by lymphoplasmacells, occasionally organized in follicular structures exhibiting necrotic changes (1/2). Rare ulcers, were identified in the mucosa of ceca and colon-rectum (1/2). Moderate to marked generalized congestion was the only remarkable finding in the urogenital system (1/1) (Fig. 2e–f) and respiratory tract (3/3), while no lesions were observed in gizzard and heart sections (1/1).

Bird	Findings	Liver	Pancreas	Spleen	Gizzard	Intestine	Kidney	Gonad	Trachea	Lung	Heart
D1	Gross	Yes	Yes	Yes	No	Yes	Yes	Yes	No	Yes	Yes
	HE	+	+	na	-	-	-	-	-	-	na
	IHC	+++	+++	na	+	++	+++	+++	+	+++	na
	ISH	+++	+++	na	++	++	+++	+++	+	+++	na
S2	Gross	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes	No
	HE	++	+++	+++	na	+	na	na	-	-	-
	IHC	+	++	++	na	+	na	na	+	+	-
	ISH	+	+++	+++	na	++	na	na	+	+	-
S3	Gross	Yes	Yes	Yes	No	No	Yes	No	Yes	No	No
	HE	+	++	+++	na	-	na	na	-	-	na
	IHC	++	+++	+++	na	+	na	na	+++	+	na
	ISH	++	+++	+++	na	++	na	na	+++	+	na
D4	Gross	Yes	Yes	Yes	No	Yes	Yes	No	Yes	Yes	No
	HE	na	+++	na	na	na	na	na	na	na	na
	IHC	na	+++	na	na	na	na	na	na	na	na
	ISH	na	+++	na	na	na	na	na	na	na	na
D5	Gross	Yes	Yes	Yes	No	No	Yes	No	Yes	No	No
	HE	na	na	+++	na	na	na	na	na	na	na
	IHC	na	na	+++	na	na	na	na	na	na	na
	ISH	na	na	+++	na	na	na	na	na	na	na
D6	Gross	Yes	Yes	Yes	No	Yes	Yes	No	Yes	No	No
	HE	na	++	+++	na	+++	na	na	na	na	na
	IHC	na	++	+++	na	+++	na	na	na	na	na
	ISH	na	++	+++	na	+++	na	na	na	na	na

**Table 1.** Clinico-pathological findings and viral tissue distribution by immunohistochemistry (IHC) and RNAscope in situ hybridization (ISH) in African houbara bustards (*Chlamydotis undulata*) naturally-infected with H5N8 HPAIV (n = 6). D: found dead; S = symptomatic 24–48 h prior to the *exitus*; Gross: presence (yes) or absence (no) of lesions at the necropsy exam; HE: necrotic-inflammatory changes identified in histopathological slides routinely stained with hematoxylin and eosin, scored as -, +, ++, +++\*; IHC: positivity for IAV antigen (nucleoprotein) scored as -, +, ++, +++\*; ISH: positivity for AIV RNA (matrix gene) scored as -, +, ++, +++\*; na: not available; \*Semi-quantitative scoring according to Landmann<sup>26</sup>.

### In situ detection of avian influenza virus

Results of viral tissue distribution and immunoreactivity are summarized in Tables 1, and 2. Viral antigens and RNAs were detected as intranuclear and intracytoplasmic staining in all tissues examined, except for the heart. For IHC, positive immunostaining was identified in liver sections within the hepatocytes (3/3), endothelial cells (3/3), cholangiocytes (2/3), periportal mononuclear leukocytes (2/3), stromal cells and mesothelial cells lining the Glisson's capsule (1/3). In the pancreas, intact or necrotic acinar cells (5/5) followed by Langerhans' islets cells and ductal epithelial cells (2/5) and occasional endothelial cells (4/5) appeared immunoreactive (Fig. 3a). Splenic sections showed multifocal to widespread positivity within the necrotic and fibrino-heterophilic foci (4/4), endothelial cells in both arterioles and penicillar capillaries (4/4), mononuclear leukocytes located in the white and red pulp (4/4), germinal centers (2/4), capsular smooth muscle cells (2/4) and ellipsoidal reticular cells (1/4) (Fig. 3b). In the intestine, viral antigen detection ranged from multifocal positive areas scattered within the lamina propria and neuronal cells of the myenteric plexus (3/4) to widespread immunoreactivity involving several compartments (1/4). In this case, positivity was recorded in smooth muscles cells in the lamina propria, muscularis mucosae and tunica muscularis, neuronal cells in the myenteric plexus, necrotic-inflammatory areas, as well as mononuclear leukocytes, endothelial cells and enterocytes (Fig. 3c–d). In the gizzard, the myenteric plexus was also positive, together with occasional endothelial cells in the lamina propria, and the interstitium of submucosa and tunica muscularis (1/1). In the ovary, endothelial and stromal cells of the vascular zone, followed by thecal cells and mesothelial cells, were highly positive (1/1) (Fig. 3e). Renal epithelial cells, in both cortical regions and medullary tracts, revealed large amounts of viral NP, while occasional positive cells were detected within glomerular tufts and interstitial blood vessels (1/1) (Fig. 3f). Viral antigen was also present in the ciliated epithelium of trachea (3/3) and secondary bronchi (1/3), tracheal intraluminal debris (1/3) pulmonary capillary bed (3/3) and endothelium of blood vessels in the interparabronchial septa (3/3) (Fig. 3g–h). Positive detection of viral RNA (M gene) by RNAscope ISH exhibited a tissue distribution equivalent to IHC (Fig. 4a–d). Additional positive areas were identified in gizzard (1/1) (mucosal epithelium and endothelial cells in the lamina propria) intestine (3/4) (mesothelial cells, mucosal epithelium, endothelial cells in the lamina propria) pancreas (1/5) (ductal epithelium and necrotic foci) and liver (2/3) (endothelial cells) (Fig. 4e–f).



**Figure 2.** Histopathological lesions identified in African houbara bustards (*Chlamydotis undulata*) naturally-infected with H5N8 HPAIV. Tissue sections are stained with hematoxylin and eosin stain (H&E). Necrotic-inflammatory changes, ranging from multifocal to diffuse (arrowheads and asterisks) are present in spleen (a) pancreas (b) liver (c) and intestine (d). Generalized congestion predominated in ovary (e) and kidney (f). Scale bars: 100  $\mu\text{m}$  (a–c, f) and 300  $\mu\text{m}$  (d, e).

#### Molecular analysis on FFPE tissue blocks

Between 17.2 and 55.5 ng/ $\mu\text{l}$  of RNA (average 30.07 ng/ $\mu\text{l}$ ) were obtained from 4 selected FFPE blocks. RNA integrity number (RIN) ranged from 5.5 to 7 (average 6.4), while DV200 score varied from 54.62 to 68.08 (average 60.59). AIV H5 was successfully detected by RT-qPCR in all 4 FFPE blocks, with a cycle threshold (Ct) value ranging between 21.23 and 25.05 (average 23.34). Partial sequencing of HA and NA highlighted a cleavage site consistent with HPAI and confirmed the involvement of N8 subtype, respectively (Accessions OR699288 and OR699289).

#### Discussion

We provided the first comprehensive characterization of H5N8 HPAI natural infection in captive African houbara bustards, enriching available literature on the pathology of HPAI in an endangered avian species. A single report described an outbreak of H5N1 HPAI in Asian houbara bustards (*Chlamydotis maqueenii*) and falcons, exhibiting neurological signs and diarrhea<sup>13</sup>. The virus was isolated and molecularly characterized but, besides clinical signs, pathological findings were not provided and viral tissue distribution was not assessed. In another

Bird	Findings	Liver	Pancreas	Spleen	Gizzard	Intestine	Kidney	Gonad	Trachea	Lung	Heart
D1	IHC	+	+	na	+	++	++	+++	+	+++	na
	ISH	+	+	na	++	++	++	+++	+	+++	na
S2	IHC	-	-	+	na	-	na	na	+	++	-
	ISH	+		+	na	+	na	na	+	++	-
S3	IHC	-	+	++	na	-	na	na	+	++	na
	ISH	+	+	++	na	+	na	na	+	++	na
D4	IHC	na	+	na	na	na	na	na	na	na	na
	ISH	na	+	na	na	na	na	na	na	na	na
D5	IHC	na	na	+	na	na	na	na	na	na	na
	ISH	na	na	+	na	na	na	na	na	na	na
D6	IHC	na	+	+++	na	++	na	na	na	na	na
	ISH	na	+	+++	na	+++	na	na	na	na	na

**Table 2.** Endothelial viral detection by immunohistochemistry (IHC) and RNAscope in situ hybridization (ISH) in African houbara bustards (*Chlamydotis undulata*) naturally-infected with H5N8 HPAIV (n = 6). D: found dead; S = symptomatic 24–48 h prior to the *exitus*; IHC: endothelial positivity for IAV antigen (nucleoprotein) scored as -, +, ++, +++\* ISH: endothelial positivity for AIV RNA (matrix gene) scored as -, +, ++, +++\*; na: not available; \*Semi-quantitative scoring according to Landmann<sup>26</sup>.

study, necrotizing pancreatitis was mentioned as a hallmark lesion in houbara bustards infected with H7N1 and H5N1 HPAI viruses but no additional details were provided<sup>30</sup>.

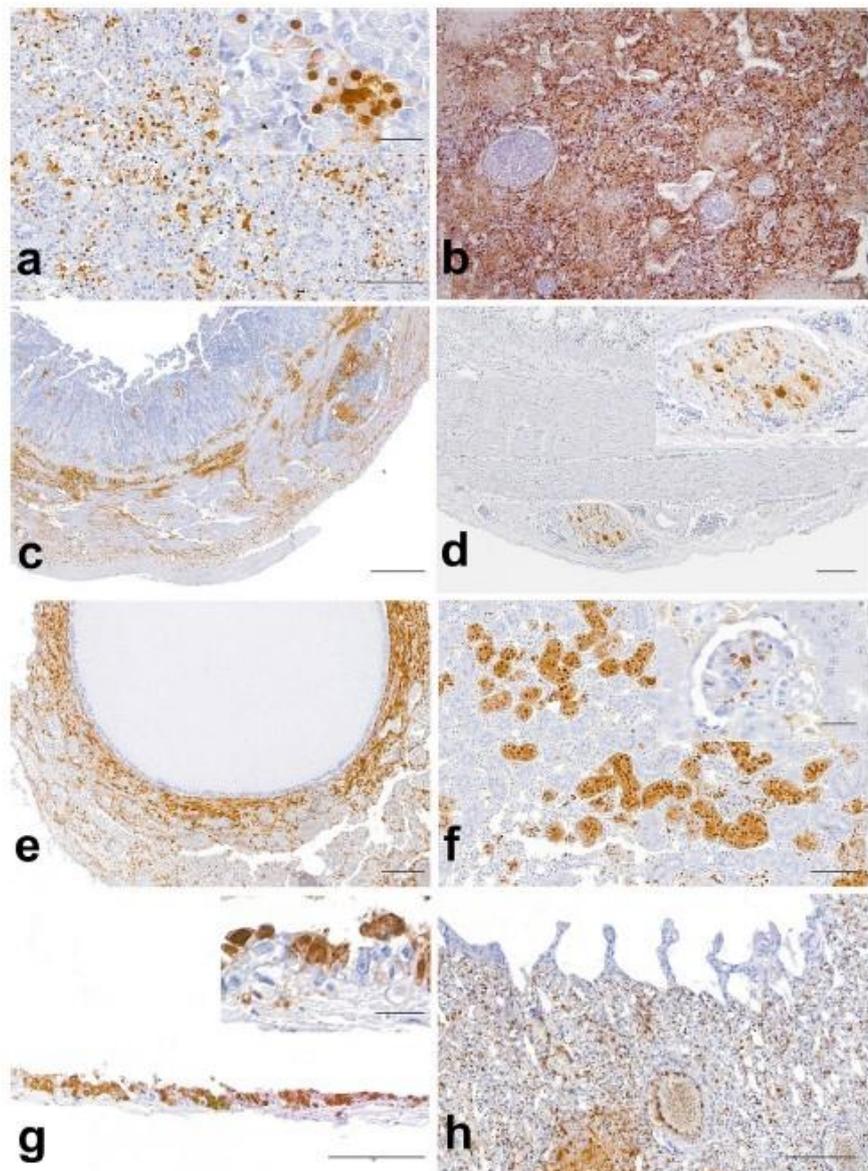
The birds included in our study exhibited hyperacute death, or presented with listlessness between 24 and 48 h prior to the *exitus*. Spleen, liver and pancreas were more commonly affected at necropsy, showing various degrees of degenerative changes, further confirmed at histopathology. Spleen sections also revealed marked fibrinoid necrosis, consistent with vasculitis. IHC and RNAscope ISH successfully highlighted the presence of viral antigen and RNA in the vast majority of the tissues examined. However, compared to IHC, RNAscope ISH appeared to be less affected by autolytic changes, and more sensitive. Positive detection was specifically recorded and consistent with multi-visceral, parenchymal and endothelial cells involvement, supporting a systemic infection and explaining the rapid course of the disease.

Interestingly, endothelial immunolabelling varied, in terms of frequency and severity, among affected bustards. A possible explanation for this finding is the fact that, in some individuals, death may have occurred before extensive viral replication within the vascular endothelium<sup>20</sup>. Compared to Anseriformes, with the exception of the black swan (*Cygnus atratus*), systemic endotheliotropism with necrotizing vasculitis and hyperacute/acute forms, are considered hallmarks of HPAI virus infection in domestic Galliformes<sup>20,31</sup>. In chickens, endothelial cells are reported to be more permissive to HPAI virus infection, compared to ducks, due to a lower antiviral response<sup>31</sup>. An acute course of disease (short mean death time) in chickens is also associated with a reduced viral excretion time. On the other end, ducks have a longer mean death time and shed more virus over the course of the infection<sup>32,33</sup>. Overall, since several factors may have been involved, including species affected, host genetic polymorphism, course of the disease and concurrent infections, additional studies are needed to properly define the role played by endothelial cells in HPAI infection in bustards<sup>34</sup>.

In terms of tissue distribution, large amounts of AIV were detected in the urogenital tract of bustards, despite the absence of significant lesions, except for generalized congestion. Similarly, H5N1 HPAI was able to replicate in the renal tubular epithelium of both naturally and experimentally-infected waterfowl, without inducing significant changes<sup>35,36</sup>. However, renal lesions with concurrent positive immunolabelling were reported in wild Baikal teals (*Anas Formosa*), bean geese (*Anser fabalis*), and whooper swans (*Cygnus Cygnus*) and backyard chickens naturally-infected with H5N8 HPAI<sup>37,38</sup>. The involvement of the reproductive tract was mentioned in different avian species exposed to several HPAI subtypes. H5N8, H5N2 and H5N1 viruses were detected by RT-qPCR and/or IHC in the oviduct, eggshell and internal egg contents of experimentally-infected white leghorn chickens (*Gallus gallus domesticus*)<sup>39,40</sup>. IHC revealed IAV antigen in the ovarian stroma of commercial meat-type turkeys (*Meleagris gallopavo*) naturally-infected with H5N8 HPAI and in several tissues, including the reproductive tract, of Passeriformes and budgerigars (*Melopsittacus undulatus*) experimentally-infected with H5N1 HPAI<sup>41,42</sup>.

A limitation of this study was the lack of systematic tissue sampling, regardless the presence of specific clinical signs and/or macroscopic lesions. Non-suppurative, necrotizing encephalitis and myocarditis have been commonly described in a variety of commercial, captive and wild birds, naturally and experimentally-infected with H5N8 HPAI<sup>14,15,24,42–44</sup>. In our case, the presence of cerebral lesions couldn't be assessed because the nervous system was not collected due to the lack of neurological signs. However, immunoreactivity of the myenteric plexus in the gastrointestinal tract, confirmed by both IHC and RNAscope ISH, still supported a neuronal tropism. Cardiac sections appeared unremarkable although, false negative results may have originated from a non-uniform distribution of lesions and from the fact that only 1/6 birds was sampled.

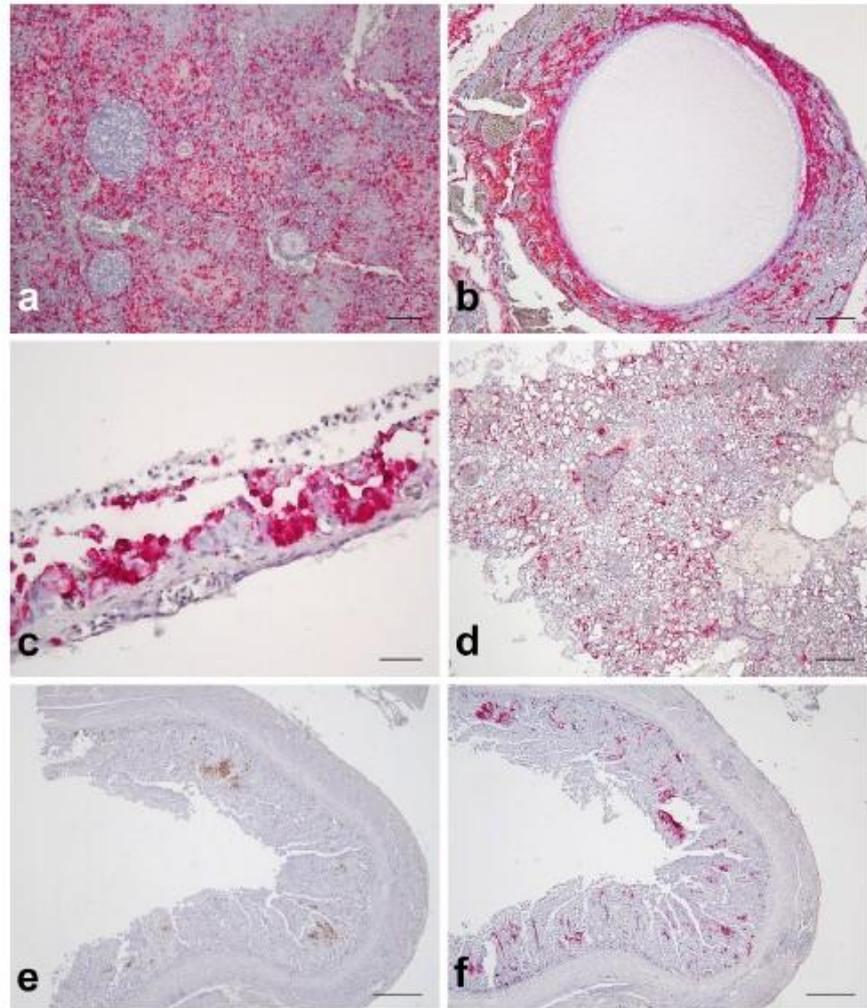
In birds infected with HPAI viral shedding can occur through the respiratory, digestive and urogenital tract<sup>20</sup>. In addition, due to systemic infection, large amounts of virus are present within a variety of tissues, including musculoskeletal and integumentary systems, making scavenging birds particularly at risk of being exposed following the consumption of infected carcasses<sup>13–15,20,45,46</sup>. Non-respiratory particles, such as dust originating from



**Figure 3.** Viral antigen tissue distribution in African houbara bustards (*Chlamydotis undulata*) naturally-infected with H5N8 HPAIV. Tissue sections are stained for IAV nucleoprotein by immunohistochemistry. Multifocal immunolabelling of pancreatic acinar cells (a); widespread positivity of white and red pulp and necrotic foci in the spleen (b); multifocal transmural positivity of the intestine (c), including neuronal cells of the myenteric plexus (insert) (d); positive endothelial and mesenchymal cells in the ovary (e); multifocal to coalescing immunolabelling of the renal tubular epithelium and occasional glomeruli (insert) (f); diffuse positivity of the tracheal respiratory epithelium (insert) (g); multifocal positivity of the pulmonary capillary bed and endothelial cells lining the interlobular blood vessels (h). Scale bars: 100 µm (a, b, d–h), 300 µm (c) and 20 µm (inserts a, d, f, g).

infected poultry houses, have also been recognized as an important source of infection<sup>47–49</sup>. In our case, two nests belonging to an unidentified waterfowl species, were discovered nearby the first positive pen, making the exposure to infectious aerosol, fecal particles as well as infected feathers and dust, a plausible event.

Molecular analysis conducted on FFPE tissue blocks, further confirmed the involvement of H5N8 HPAI in affected bustards. Nucleic acids fragmentation, resulting from formalin-fixation makes short-read sequencing technologies more suitable for FFPE tissues. However, we chose to assess the feasibility of the Oxford Nanopore



**Figure 4.** Viral RNA tissue distribution in African houbara bustards (*Chlamydotis undulata*) naturally-infected with H5N8 HPAIV. The first two rows include tissue sections stained for AIV matrix gene (M gene) RNA by RNAscope in situ hybridization (ISH). The third row includes serial intestinal sections stained for IAV nucleoprotein by immunohistochemistry (IHC) and M gene RNA by RNAscope ISH. Widespread positivity of splenic red and white pulp (a), mesenchymal and endothelial cells in the ovary (b), tracheal respiratory epithelium (c) and pulmonary capillary bed and blood vessels in the interparabronchial septa (d). Compared to IHC (e), RNAscope ISH revealed additional positive areas in the intestinal lamina propria (f). Scale bars: 100  $\mu\text{m}$  (a, b, d), 300  $\mu\text{m}$  (e–f) and 20  $\mu\text{m}$  (c).

MinION, a long-read technology, since it can generate real-time results, is economically affordable and portable<sup>50</sup>. To the author's best knowledge, this is the first time nanopore sequencing has been used on FFPE samples for veterinary viral investigation. FFPE blocks represent practical samples that can be stored at room temperature and can be easily shipped. They are an invaluable resource of information for retrospective studies, and diagnostic investigation of poorly characterized conditions and unsolved archived cases, which is particularly interesting for exotic and endangered species with a limited availability of samples. In addition, preserved tissue architecture and histomorphological features allow target and custom-designed testing, increasing sensitivity. An example is provided by the molecular identification of *Toxocara cati* in FFPE blocks obtained from brown kiwis (*Apteryx mantelli*) with a histopathological diagnosis of visceral and neural *larva migrans*, between 2004 and 2017<sup>51</sup>. Nevertheless, Gaide et al.<sup>28</sup> successfully detected AIVs by RNAscope ISH and IHC in FFPE blocks stored at room temperature between 2009 and 2022, with no detrimental effects on final results.

In conclusion, our results show that natural infection with H5N8 HPAI virus in African houbara bustards is characterized by hyperacute/acute forms exhibiting pantropism, as well as endotheliotropism and neurotropism. Significant pathological indicators to be considered at necropsy include necrotizing splenitis, pancreatitis and

hepatitis and fibrinous peritonitis. FFPE samples represent a valuable source of nucleic acids for in situ detection and molecular analysis of emerging and re-emerging avian pathogens in poorly characterized species.

### Data availability

The datasets generated and/or analyzed during the current study are available in the GenBank repository, Accession Numbers OR699288 and OR699289.

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### Author contributions

M.C. and G.L.L. conceived and designed the study. M.C., N.G., M.D.I. and M.D. analysed data. M.R.K. and M.C.M. collected data. M.C. wrote the paper, prepared all figures and tables. G.L.L., J.L.G., F.L. and M.C.M. oversight the study and supervised the research group. All authors contributed to the critical analysis. All authors reviewed and approved the final manuscript.

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### Competing interests

The authors declare no competing interests.

### Additional information

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### **3. Conclusions and future perspectives**

Our results provide significant insights on the pathobiology of HPAI H5N8 natural infection in captive African Houbaras, supporting diagnostic procedures and surveillance activities. Sudden death, consistent with an hyperacute form, was the most common clinical presentation observed. Besides generalized visceral congestion, mottled and/or hemorrhagic liver, spleen and pancreas, and fibrinous peritonitis should be regarded as pathological indicators or “red flags” of H5N8 HPAI infection in Houbaras. Ulcerative lesions and petechial hemorrhages were also observed in the intestinal mucosa and cecal tonsils. However, similar changes may be missed due to the rapid occurrence of autolytic changes that characterized the digestive tract. Bacteriological culture of fibrinous lesions should also be conducted, to assess the involvement of bacterial pathogens. Overall, considering the continuously evolving landscape of HPAIv worldwide (159,160), and since different pathological pictures may originate with different viral subtypes in the same host (146), unexplained deaths should always be promptly investigated through a complete necropsy exam and molecular testing for avian influenza.

Both IHC and RNAscope ISH were able to successfully detected AIV antigens and RNAs in the majority of tissues examined, supporting marked pantropism, endotheliotropism as well as neurotropism. Based on the level of positivity and cellular distribution observed, tracheal and cloacal swabs, and a set of fresh tissue sections of lung, liver, spleen, pancreas, kidney and gonads represent suitable samples for diagnostic investigations by molecular analysis.

This study supports the suitability of FFPE tissue samples as a valuable source of nucleic acids for in situ detection and molecular analysis, including nanopore sequencing. This aspect is particularly promising when dealing with unresolved cases, affecting poorly characterized species such as Houbaras, allowing the identification of new pathogens (113). As an example, it is worth mentioning that the RNA extracted from archived FFPE lung tissue belonging to an influenza victim, contributed to the reconstruction and characterization of the 1918 Spanish Influenza A/H1N1 (164). FFPE samples have also been the object of several metagenomic studies with promising results. As regards avian influenza, metagenomic analysis of FFPE tissues obtained from a wild gull and a captive bear part of a HPAI outbreak registered in a French zoo in 2022, allowed to identify an epidemiological link between the two animals, supporting the involvement of the same virus (clade 2.3.4.4b H5N1) and the emergence of specific mammalian-adapting mutations (157). Recently, a whole genome sequencing method was validated for the unbiased detection of bacterial pathogens in human lung FFPE tissues

obtained during the first and second waves of the Covid-19 pandemic (165). A shotgun metagenomic analysis successfully characterized the microbiota of normal and neoplastic colon-rectal FFPE samples, identifying significant differences in terms of bacterial prevalence and composition (166). Another promising application of FFPE samples is laser capture microdissection, which allows to precisely collect areas of interest, directly identified by microscopic observation, for molecular analysis (167–169). This would translate into target sampling of poorly characterized lesions, intralesional organisms, including fastidious pathogens difficult to culture, or unknown infectious agents, and specific neoplastic cell populations (170–175).

At the end of 2023, a histopathological laboratory was established in UAE, as part of the diagnostic complex at NARC and second laboratory is about to be completed in Morocco (ECWP). The aim of these additions, coupled with the implementation of specific protocols for samples fixation, tissue processing and production of FFPE blocks, is to ensure rapid on-site assessment of necropsy and biopsy samples, significantly reducing the turnaround time of histopathological diagnoses. The systematic production of FFPE blocks will also reduce issues related with the long-term storage of formalin-fixed samples, such as limited storage capacity, safety issues and the occurrence of over fixation's artefacts and reduced immunoreactivity. FFPE tissues can be stored at room temperature and are easily shipped. The creation of FFPE-tissue archives will enrich the overall knowledge of infectious and non-infectious diseases in captive houbara bustards, enhancing collaborations with international research centers, diagnostic laboratories and pathologists.

## C. STUDY 3

### 1. Introduction

**Infectious salpingitis/peritonitis/salpingo-peritonitis (SPS)** are well known conditions in poultry (176–178). From an etiological point of view, a variety of agents have been recognized depending on the species affected (179). For example, in ducks, salpingitis has been commonly reported with *E. coli*, *P. multocida*, *Riemerella anatipestifer* as well as *Tetratrichomonas* spp., with or without previous episodes of necrotic enteritis (177,180). Among bacterial causes in chickens, a major role is played by avian pathogenic *E. coli* (APEC), resulting in systemic or localized extra-intestinal forms, acting as primary or opportunistic agents (181).

Three main routes are recognized in the development of bacterial salpingitis: **(i)** a descending infection from the respiratory tract, due to the anatomical proximity of abdominal sacs to the oviduct, particularly in case of previous exposure to viral agents damaging the respiratory epithelium; **(ii)** an ascending infection from the cloaca, **(iii)** and hematogenous spread in the event of septicemia (177,181,182). Salpingitis may also develop secondary to egg binding, although the latter could result from oviductal infections due to scarring and luminal constrictions. Peritonitis represents a common sequela of salpingitis, following the retrograde passage of oviductal contents into the coelomic cavity, and/or the translocation of bacteria from the impaired oviductal walls. Additional predisposing factors include enteric disorders leading to increased intestinal, respiratory infections, septicemia and complicated internal ovulation or egg-yolk peritonitis (176,181,183,184).

Stress, related with the onset of egg production and sustained oviposition, increases susceptibility to bacterial infections due to the detrimental effects on the immune system (181). Inadequate rearing conditions and management factors, particularly poor hygienic levels and overcrowding, can lead to overwhelming bacterial exposure (181). Nutritional deficiencies, such as hypocalcemia, obesity and senescence can also negatively affect oviductal function and oviposition (185,186).

SPS is typically detected in adult laying birds, although salpingitis may also occur in young individuals following airsacculitis involving the left abdominal air sacs, impacting future reproduction (178,179). Commercial layer chickens may experience significant losses due to egg production loss, mortality and culling as well as serious repercussions on animal welfare (181,187). Backyard flocks are also affected. In a 5-year retrospective study focusing on the causes of mortality in backyard chickens in Northern California, *E. coli* SPS accounted for 12%

of cases examined (188). Similar disorders have also been reported in domesticated cage birds, particularly psittacines and passerines (canaries), as well as non-domesticated species originating from the wild or belonging to aviaries and zoos (189). A 10-year study evaluated the occurrence of reproductive disorders in a total of 1666 mature females, including 650 non-domesticated species divided into 24 orders in the class Aves. Based on necropsy findings, oviductal and/or cloacal impaction, almost consistently associated with salpingitis, was observed in 28.6% of cases (n=46). Salpingitis, without concurrent oviductal impaction, accounted for 10.6% of cases (n=17) (**Table 4**). *E. coli* was considered the most significant pathogen involved, although its role couldn't always be confirmed due to the variable extent of autolytic changes, preventing proper bacteriological (189).

**Table 4.** Overview of birds exhibiting gross lesions consistent salpingitis, with or without concurrent oviductal/cloacal impaction. Modified from Keymer et al. (189).

<b>Necropsy findings</b>	<b>Order</b>	<b>Examples of Species</b>	<b>Total females</b>	<b>Affected females</b>
<b>Oviductal/cloacal impaction with/without salpingitis</b>	Tinamiformes	Tinamous	2	1 (50%)
	Gruiformes	Rails, waterhens, crakes, cranes	32	3 (9.4%)
	Columbiformes	Pigeons, doves	91	7 (7.7%)
	Sphenisciformes	Penguins	33	2 (6.1%)
	Galliformes	Quails, pheasants, partridges, peafowl, jungle fowl, capercaillies	120	6 (5%)
	Falconiformes	Eagles, hawks, falcons, harrier, secretary birds	41	2 (4.9%)
	Psittaciformes	Budgerigars, cockatiels, lovebirds, cockatoos, lorys, parakeets , parrots	365	12 (3.3%)
	Strigiformes	Owls	38	1 (2.6%)
	Anseriformes	Ducks, geese , swans	161	4 (2.5%)
	Ciconiformes	Hérons, egrets, storks, spoonbills	61	1 (1.6%)
	Passeriformes	Canaries, zebra and other finches, weavers, mynahs, starlings, bulbuls, cardinals, pittas, magpies, mesias, whydahs	504	7 (1.4%)
<b>Salpingitis without oviductal impaction</b>	Rheiformes	Rheas	3	2 (66.7%)
	Galliformes	Quails, pheasants, partridges, peafowl, jungle fowl, capercaillies	120	5 (4.2%)
	Anseriformes	Ducks, geese , swans	161	6 (3.7%)
	Gruiformes	Rails, waterhens, crakes, cranes	32	1 (3.1%)
	Sphenisciformes	Penguins	33	1 (3%)
	Psittaciformes	Budgerigars, cockatiels, lovebirds, cockatoos, lorys, parakeets , parrots	365	2 (0.6%)

Houbaras have been the object of captive breeding programs, relying on artificial insemination and artificial incubation of eggs, for many years (41). While reproductive disorders, including genital infections, have been regularly observed in the field, they were rarely documented. In order to enrich the overall knowledge of reproductive pathology of captive African Houbaras and to help securing conservation efforts, a series of cases of salpingitis/salpingo-peritonitis were selected for characterization, providing significant insights in terms of pathological presentation, etiology, and pathogenesis. We also discussed potential predisposing and risk factors.

## 2. Article 3

The present article, currently under preparation, will be submitted to *Avian Pathology* with the following reference:

### **Salpingo-peritonitis in captive houbara bustards (*Chlamydotis undulata*)**

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## **Introduction**

Since 2014, the African houbara bustard (*Chlamydotis undulata*) a steppic bird residing in North Africa, has been included in the Red List of the International Union for the Conservation of Nature (IUCN) (34) following the drastic decline of wild populations due to progressive habitat loss and overhunting. As a result, several captive breeding projects have been established over the years (29,45). Captive breeding of endangered species is an essential conservation tool that aims at producing healthy individuals, capable of surviving into the wild, to reinforce endangered wild populations and secure captive flocks in case of catastrophic events leading to population collapse (190–193). Maintaining a healthy breeder flock is

essential for a successful *ex-situ* conservation strategy. However, several factors can negatively impact the health and reproductive performances of captive individuals, including genital infections. Salpingitis and salpingo-peritonitis are well known conditions in poultry, with *Escherichia coli* being one of the pathogens most commonly involved (176,181,194,195). Commercial layer and broiler breeder flocks may experience significant losses due to decreased egg production, increased mortality and culling as well as serious repercussions on animal welfare (176,181,187). Significant predisposing factors include poor hygiene levels and inadequate environmental conditions leading to overwhelming bacterial exposure, nutritional deficiencies, viral infections damaging the respiratory and reproductive tract and immunosuppression, either viral-induced or stress-related (181).

Available literature on salpingitis/salpingo-peritonitis in captive Houbaras is still quite limited. While reproductive disorders, including genital infections, have been regularly observed in the field, they were rarely characterized and documented. For example, salpingitis with/without concurrent oophoritis was identified at necropsy in three Asian Houbara bustards (*Chlamydotis macqueenii*) but diagnostic investigation was not attempted (60,196).

In order to improve the overall knowledge of reproductive pathology in captive African Houbaras, we characterized a series of cases of salpingitis/salpingo-peritonitis registered in a conservation breeding center in Morocco, providing significant insights in terms of pathological presentation, etiology, and pathogenesis. Potential predisposing and risk factors were also discussed.

## **Materials and Methods**

### **Captive breeding**

The object of this study was a conservation breeding project in eastern Morocco, the Emirates Center for Wildlife Propagation (ECWP) (41), organized in two different centers located 50 km from each other (Missour and Enjil) and relying exclusively on artificial insemination and artificial incubation of eggs.

During the breeding season, from January to the end of July, sperm is collected from imprinted males, transported to the laboratory for analysis (volume, spermatozoa concentration and motility and leucocytes content) and diluted in Lake 7.1 solution without added antibiotics (45) before being used within a few hours. Females deemed ready after inspection, are inseminated with fresh semen. The vaginal orifice is exposed using a speculum, combined with the help of a light source. Subsequently, the semen dose is deposited to a depth of 1 cm, using a calibrated

micropipette with a disposable tip (45). Typically, females receive between one and two intravaginal inseminations at a 48-h interval, before laying the first egg of the clutch. A third insemination may follow, if the first egg takes longer than expected. Between 1-4 eggs/clutch (average 2 egg/clutch) are laid on alternate days. Eggs are collected daily, to avoid brooding and induce the production of replacement clutches (average 4 clutches/season), and artificially incubated. The laying peak is usually reached between April and May (Reneco, unpublished data).

Selected breeders were housed in individual, outdoor cages, lined with gravel. Food (specifically formulated “houbara” pellets, alfalfa and mealworms) and water were provided in individual plastic caps. All birds received a polyvalent killed vaccine against low pathogenic avian influenza (LPAI) subtype H9N2 and avian Orthoavulavirus 1 virus (AOaV-1) Ulster strain (Gallimune® 208 Merial) and a live attenuated canarypox vaccine (KP1 strain, Poulvac® Zoetis) at 30 and 60 days of age, followed by annual boosters. Deworming treatment for intestinal parasites was provided once a year.

### **Epidemiology and selection of cases**

Over a 14-year period, between 2010 and 2023, a total of 439 cases of salpingitis/salpingo-peritonitis mostly associated with *E. coli*, based on post-mortem lesions and bacteriological analysis, were registered in a flock of average 4922 female breeders. The annual prevalence ranged from a minimum of 0,07%, in 2013 and a maximum of 1.64%, in 2020 (average 0.54%), with cases occurring mostly in April and May. Affect birds ranged between 1 and 25 years of age (median 9.7 years).

A representative sample of 14 cases, including 7 cases originating from Enjil and 7 cases from Missouri, registered between the beginning of March and the end of May 2022, was selected for pathological assessment and diagnostic investigation.

### **Clinico-pathological exam, cytology and histopathology**

Clinical signs were recorded and a complete necropsy was performed within 2h and 48h after natural death or euthanasia. For cytology, imprints of liver, spleen and lung tissue samples and intestinal scrapings (n=14) were collected at necropsy and stained with Ziehl-Neelsen (ZN). For histopathology, sections of brain (n=14), sciatic nerves (n=8), nasal cavity (n=13), trachea (n=13), abdominal air sacs (n=7), lung (n=12), heart (n=13), liver (n=14), spleen (n=14), pancreas (n=14), proventriculus (n=14), gizzard (n=9), small and large intestine (n=14), kidney (n=13), ovary (n=14), oviduct (n=14), including infundibulum, magnum, shell gland or uterus

(n=14) and vagina (n=2) were collected and fixed in 10% neutral buffered formalin, paraffin-embedded and sectioned at 3 µm. Slides were routinely stained with hematoxylin and eosin (H&E) and examined under bright light microscopy. Gram, Perl's, Periodic acid-Schiff (PAS), Ziehl-Neelsen (ZN) and Masson's Trichrome (MT) special stains were performed on air sacs, lung, liver, spleen, intestine, kidney, ovary and oviduct tissue sections from selected cases.

### **Bacteriology**

Amie's swabs were aseptically collected from the oviduct (magnum/uterus) (n=13), ovary (n=5), coelomic cavity (n=6), liver (n=8), abdominal air sacs (n=2), spleen (n=1), trachea (n=1), lung (n=1) and heart (n=1) at necropsy. Oviduct (n=3) and vagina (n=1) were also sampled at surgery. Samples were plated on general and differential media (nutrient agar, eosin methylene blue agar, hektoen enteric agar and chapman/mannitol salt agar) incubated aerobically at 37°C and examined at 24 and 48h post-incubation. Bacterial isolates were identified based on colony's morphology, biochemical tests and Analytical Profile Index (API) 20E test strips (bioMerieux, Inc.). For *Salmonella*, swabs were placed into an enrichment media (Selenite broth) prior to inoculation in hektoen enteric agar. Colonies consistent with *Salmonella* spp. were confirmed by API-20E test strips (bioMerieux, Inc.) and serotyped using specific antisera (Biorad®) according to the White-Kauffmann-Le Minor scheme (197).

### **Molecular biology**

Tracheal and cloacal swabs, and sections of lung, ovary, oviduct and cecal tonsils (n=14) were aseptically collected at necropsy and applied on Flinders Technology Associates (FTA®) cards. After suspension of FTA cards in Tris-EDTA (TE) buffer (198), nucleic acids were extracted using Mag Fast Extraction Kit (MAGFAST384) and IDEAL-96HP extraction robot (Innovative diagnostics, France) and stored at -80°C until analysis.

For viral pathogens, RT-qPCR targeting alphainfluenzavirus influenzae (IAV) (matrix gene), avian orthoavulavirus-1 (AOaV-1) and infectious bronchitis virus (IBV) were performed (Innovative diagnostics, France). For bacterial agents, qPCR targeting *Chlamydia* spp. (199), RT-qPCR for *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) (Innovative diagnostics, France) were conducted.

A total of nine *E. coli* isolates, recovered from the reproductive tract, were selected for molecular characterization using an Avian Pathogenic *E. coli* (APEC) multiplex qPCR targeting eleven genes encoding for specific virulence factors: increased serum survival protein (*iss*), P-fimbriae (*papC*), aerobactin (*iucD*), temperature-sensitive hemagglutinin (*tsh*), iron-

repressible protein (*irp-2*), outer membrane protease (*ompT*), putative avian hemolysin (*hlyF*), siderophore receptor (*iron*), colicin V toxin (*cva*), vacuolating autotransporter toxin (*vat*), enteroaggregative heat-stable toxin (*astA*) (Innovative diagnostics, France).

## Results

### History and clinical signs

Selected bustards ranged between 4 and 17 years of age (average 11 years). Spontaneous death occurred in 4 individuals (4/14) including one bird found dead without clinical signs and three birds dying soon after the onset of lethargy and anorexia. In terms of production, 3 birds had laid an egg between 1 and 10 days prior to the *exitus*, while one bird did not lay in 2022. The rest of the bustards (10/14) was humanely euthanized by means of intravenous injection of T61 (200) after exhibiting reduced activity, anorexia/dysorexia and lack of oviposition for a variable amount of time, ranging from a minimum of 3 weeks to a maximum of 3 consecutive seasons. A palpable coelomic mass was detected in 9/10 birds, while egg binding associated with the presence of a vaginal mass, identified upon visual inspection of the cloacal region, was mentioned in one case. Cloacal prolapse, (1/10), poor body conditions (1/10), tenesmus (1/10), coelomic distension and lameness (1/10) were occasionally mentioned. A cesarian section was performed in 4/10 birds, revealing an abnormally enlarged oviduct, impacted with abundant caseous exudate (3/4) and confirming the presence of a retained egg in the egg chamber, together with a focal caseous nodule in the vagina (1/4).

### Necropsy findings.

Post-mortem conditions ranged from excellent to acceptable. The vast majority of birds (13/14) was in good nutritional status, with large amounts of subcutaneous and coelomic fat. A single animal was moderately emaciated, and had a pasted vent with dry urates. Weight ranged between 1223 and 1930 g, with an average of 1485 g (expected normal range between 900 and 1500g).

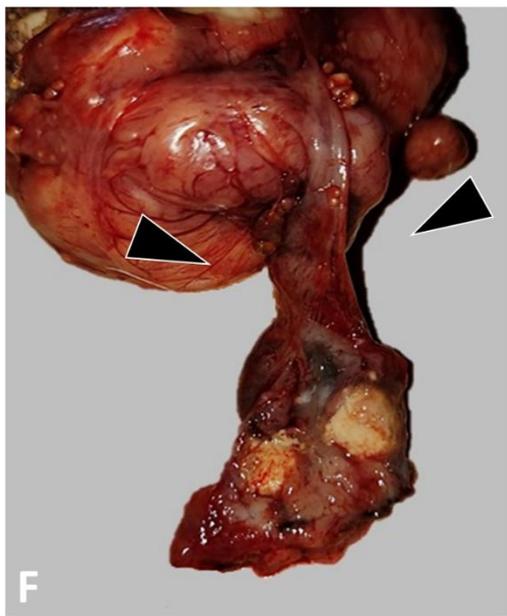
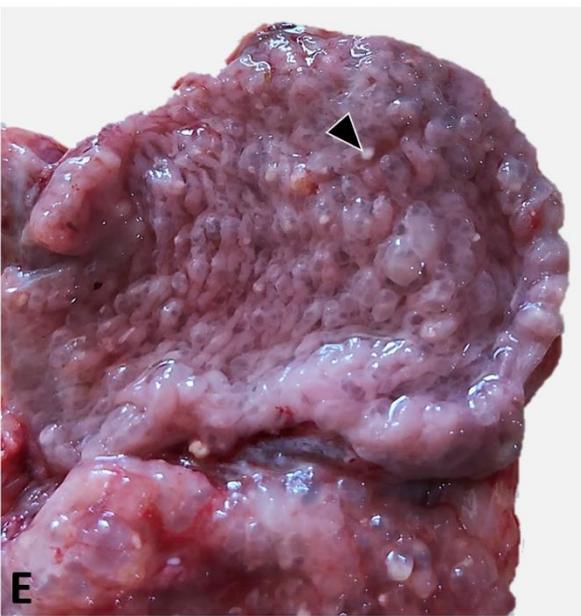
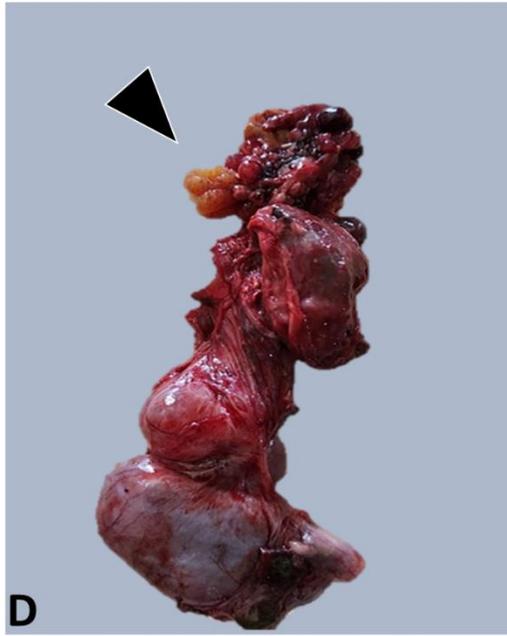
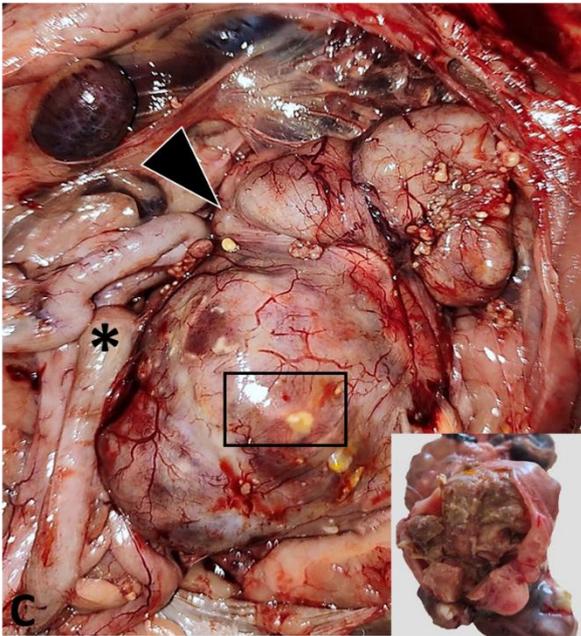
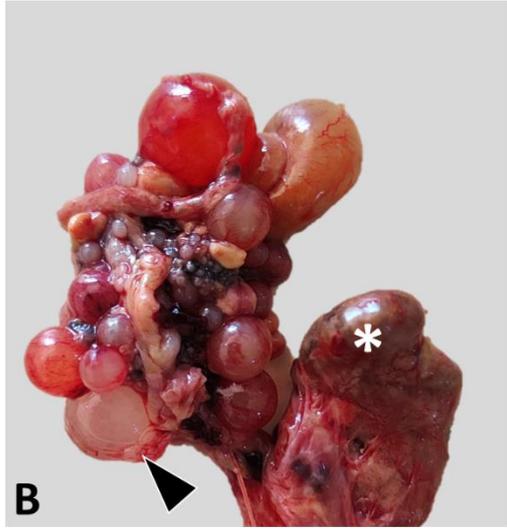
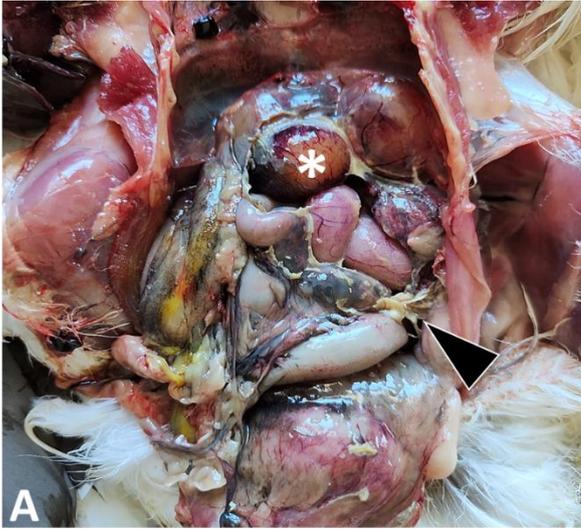
Well-developed, pre-ovulatory ovarian follicles, diffusely congested, were present in 4/14 birds (**Fig. 17A**). The rest of the bustards (11/14) exhibited various degree of ovarian regression, ranging from the presence of good-size, wrinkle and collapsed ova admixed with small to medium size follicles (9/11), to a fully regressed organ, barely detectable (2/11). A single ovarian follicle, 6 mm in size, had a cystic appearance with clear contents (1/11) (**Fig. 17B**).

Rare, round to oval, and fluid-filled cysts, 5 mm to 1 cm in diameter, were also identified on the surface of proximal infundibulum, in the dorsal mesosalpinx (2/14).

The oviduct was well-developed and normal in size (5/14), with a diffusely congested mucosa (5/5) covered with small amounts of white caseous exudate and albuminous material (2/5), or multifocally to diffusely enlarged and distorted with abundant creamy to caseous exudate, yellow-brown in color (9/14) (**Fig. 17C-D**). In a few birds the organ was displacing the gastrointestinal tract (3/14) and/or compressing the left kidney (1/14) and large intestine, leading to megacolon (1/14) (**Fig. 17C**). In two of these cases (2/4), a large caseous mass, weighing up to 350 grams, was found within the uterus. In 3 cases, the oviductal walls had a thickened and spongy appearance, revealing variable numbers of fluid-filled mucosal cysts, 1 mm to 1.5 cm in size, admixed with rare miliary white foci (**Fig. 17E**). Multiple yellow caseous nodules, 2 to 4 cm in size, were identified in the vaginal mucosa and extending to the utero-vaginal junction, in 2 birds (**Fig. 17F**).

Small to moderate amounts of fibrinous to caseous exudate, yellow to brown in color, were visible on the surface of oviduct (9/14), gastro-intestinal tract (9/14), ovary (4/14), abdominal air sacs (3/14) and, to a lesser extent, liver (3/14), spleen (2/14), pancreas (1/14) and pericardial sac (1/14) (**Fig. 17A-B**). In two cases, creamy egg-yolk material, admixed with fibrinous flakes, was found covering the intestine, and reproductive tract.

Additional findings included moderate to marked hepatomegaly (4/14), splenomegaly (2/14), multifocal to diffuse consolidation of the left lung (left side) (2/14), multifocal star-shaped, caseous foci scattered in the hepatic parenchyma (1/14) and severe focal swelling of the caudal lobe of the right kidney (1/14).



**Figure 17. Necropsy findings in captive African houbara bustards with peritonitis/salpingo-peritonitis.**

Yellow fibrinous exudate visible on the surface of intestine (arrowhead), ovary (white asterisk) and oviduct (black asterisk) in an actively-laying bird experiencing sudden death. Note the presence of well-developed and congested ova (white asterisk) **(A)**. Single ovarian follicle exhibiting a cystic, fluid-filled appearance (arrowhead). Note the accumulation of caseous exudate, distending the infundibulum, on the right (asterisk). Other segments of the oviduct were also involved (not shown) **(B)**. Diffusely enlarged oviduct, impacted with caseous exudate (asterisk and black arrowhead) displacing the gastrointestinal tract (white arrowhead). Few caseous nodules are visible on the oviductal surface (black arrowheads). Egg binding was highly suspected **(C)**. Overview of an abnormally distended oviduct after being isolated from the coelomic cavity. Ovarian follicles are regressing, with some being flaccid and collapsed (arrowhead) **(D)**. Diffusely cystic appearance of the oviductal mucosa (shell gland). White, military foci are occasionally visible among the cysts **(E)**. Two yellow caseous nodules scattered in the vaginal mucosa. The oviduct is clearly impacted (arrowheads) **(F)**.

### **Histopathology and cytology**

Histopathological findings are summarized in **table 5**. Mild to marked peritonitis, ranging from fibrino-heterophilic to lymphohistiocytic, was identified in all birds examined (14/14). Moderate amounts of fine yolk granules were noticed admixed with fibrino-heterophilic exudate and bacterial colonies in two birds. The mesentery and serosa of small and large intestine (12/14) and oviduct (10/14) were more commonly affected, followed by ovary (6/14), gizzard (4/9), liver (3/14), spleen (2/14), proventriculus (2/14) and pancreas (1/14). Multiple granulomas, containing amorphous eosinophilic material were seen adhered to the surface of oviduct (5/14), ovary (2/14), small intestine (2/14), gizzard (1/9) and within the mesentery (4/14).

Generalized congestion and fibrino-heterophilic oophoritis were present in 3/14 birds. Paraovarian cysts, lined by ciliated columnar epithelium, were identified in the mesosalpinx, adjacent to the infundibulum (2/14). Salpingitis lesions included intraluminal accumulation of variable amounts of fibrino-heterophilic exudate, admixed with oviductal secretions and bacterial colonies (**Fig. 18A-B**). The lamina propria of the different oviductal segments examined was mildly to severely expanded by mixed inflammatory cells, including lymphoplasmic cells and heterophils (12/14). Occasional lymphoid follicles were scattered here and there (4/14). In some sections, the mucosa of magnum and shell gland appeared severely and diffusely compressed by luminal contents, and showed marked inflammatory changes, glandular hyperplasia and fibrosis (8/14) (**Fig. 18B**). Oviductal contents were eliciting a marked granulomatous response in 3/14 birds and in one case, basophilic, crystalline elements suggestive of egg shell fragments, were identified at the periphery. Multifocal to diffuse glandulo-cystic hyperplasia, characterized by the presence of fluid-filled cysts lined by ciliated,

cuboidal to columnar epithelial cells, effacing the original mucosal architecture (magnum and shell gland), ranging from mild to severe, was also present (8/14) (**Fig. 18C**). A variable number of cysts contained fibrino-heterophilic exudate, occasionally admixed with intralesional bacterial colonies (4/8), and areas of dystrophic mineralization (2/8) (**Fig. 18D**). Vaginitis was confirmed in 2/14 birds. In both cases, the mucosa was severely effaced by large caseous granulomas containing small to large numbers of bacterial colonies, extending to the tunica muscularis and invading the uterovaginal-junction, associated with extensive fibrosis in one case, further confirmed by MT stain (1/1). A single granuloma contained a large foreign body of plant origin (1/2) (**Fig. 18E**). Two birds didn't exhibit exudative and/or inflammatory changes in the oviduct, however the mucosa of the organ was diffusely congested and several bacterial colonies were readily visible within the blood vessels of the lamina propria. Extragenital lesions included mild to marked exudative to granulomatous airsacculitis (4/7), bronchopneumonia (2/12), splenitis (3/14), hepatitis (2/14), and pericarditis (1/13). Intralesional bacterial colonies comprised of Gram-negative cocco-bacilli were identified in multiple organs, including mesentery/intestinal serosae (2/2), ovary (1/1), oviductal contents and oviductal cysts (2/2), vagina (1/1), air sacs (1/1), lung (1/1), pericardium (1/1), liver (1/1) and spleen (1/1). No acid-fast bacteria or fungal elements were revealed by ZN and PAS stain, respectively, in oviduct (magnum and vagina), lung, air sacs and liver (1/1).

Renal sections showed multifocal degeneration/necrosis of the renal tubular epithelium, with areas of dystrophic mineralization, ranging from mild to moderate (6/13), intratubular urates spherules or tophi eliciting a granulomatous reaction (3/13), interstitial fibrosis and glomerulosclerosis (3/13). Moderate to marked renal lipidosis (2/13) and urolithiasis with parenchymal atrophy (1/13) were occasionally present, while a well-differentiated renal carcinoma/adenoma was identified in one bird.

Moderate, diffuse to locally extensive macrovesicular steatosis was present in 5/14 birds.

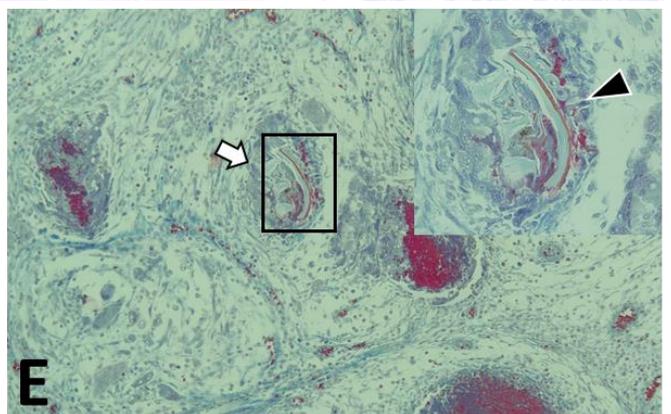
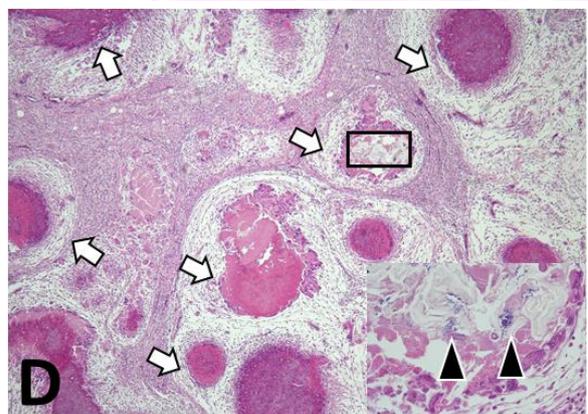
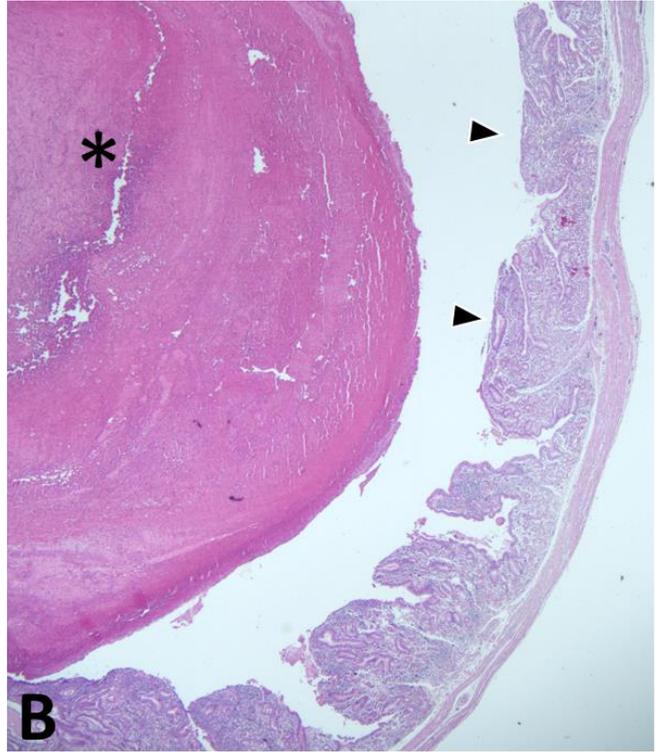
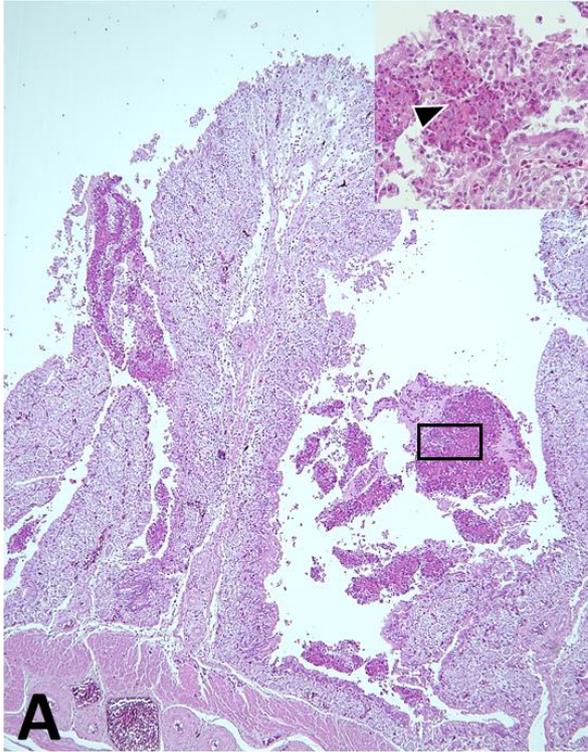
Myeloid leucosis/myelodysplasia was diagnosed in one bird, based on the identification of myeloid cells at various degrees of differentiation, including mostly myeloblasts and metamyelocytes, severely effacing the hepato-splenic parenchyma.

Brain (14/14), nerves (8/8), nasal cavity (13/13) and trachea (13/13) appeared within normal limits.

Cytological samples (liver, lung, spleen, intestine) were all negative for acid-fast bacteria (14/14)

**Table 5.** Overview of histopathological findings identified in captive African houbara bustards with gross lesions of salpingitis/salpingo-peritonitis.

<b>Histopathological lesions and changes of diagnostic significance</b>		
<b>Genital tract - oviduct</b>	<b>Frequency</b>	<b>Proposed Pathogenesis/Etiology</b>
Salpingoperitonitis, exudative, pleocellular to granulomatous	12/14 (86%)	descending infection (respiratory tract), ascending infection (cloaca), traslocation of bacteria from intestine, septicemia
Glandulo-cystic hyperplasia, multifocal to diffuse	8/14 (57%)	chronic inflammation, hormonal imbalance, senescence
Mucosal congestion, diffuse, with intravascular bacterial colonies	2/14 (14%)	septicemia
Vaginitis, granulomatous, transmural	2/14 (14%)	ascending infection (cloaca/cloacitis/cloacal prolapse), trauma-induced (including iatrogenic)
<b>Genital tract - ovary</b>	<b>Frequency</b>	<b>Proposed Pathogenesis/Etiology</b>
Regression, follicular atresia	10/14 (71%)	sepsis, chronic disease
Oophoritis, exudative	3/14 (21%)	descending infection (respiratory tract), infection extending from the oviduct, septicemia
Paraovarian cysts	2/14 (14%)	developmental (paramesonephric remnants)
<b>Coelomic cavity</b>	<b>Frequency</b>	<b>Proposed Pathogenesis/Etiology</b>
Peritonitis, with concurrent salpingitis, exudative, pleocellular to granulomatous	12/14 (86%)	infection extending from the oviduct, complicated egg yolk peritonitis, septicemia
Peritonitis, without concurrent salpingitis, exudative, yolk egg material	2/14 (14%)	descending infection from the respiratory tract, traslocation of bacteria from intestine, septicemia
<b>Urinary tract</b>	<b>Frequency</b>	<b>Proposed Pathogenesis/Etiology</b>
Nephrosis, tubular degeneration	6/13 (46%)	dehydration
Glomerulosclerosis and interstitial fibrosis of medullary tracts	3/13 (23%)	chronic renal disease
Urate nephropathy	3/13 (23%)	dehydration
Renal steatosis, multifocal	2/13 (15%)	metabolic, nutritional
Urolythiasis	1/13 (8%)	compression, dehydration
Primary renal, well-differentiated adenoma/carcinoma	1/13 (8%)	spontaneous, viral-induced
<b>Extra-urogenital organs</b>	<b>Frequency</b>	<b>Proposed Pathogenesis/Etiology</b>
Pneumoconiosis (silica dust)	11/12 (92%)	environmental, inhalation of silica dust
Silicosis (air sacs)	4/7 (57%)	environmental, inhalation of silica dust
Airsacculitis, exudative to granulomatous	4/7 (57%)	aerogenous infection, extension from the coelomic cavity/oviduct, septicemia
Intestinal flagellated protozas (cecal crypts)	6/14 (42%)	<i>Tetratrichomonas</i> spp.
Hepatic steatosis, locally extensive to diffuse	5/14 (36%)	metabolic, nutritional
Bronchopneumonia, necrotizing to granulomatous	2/12 (17%)	aerogenous infection, infection extending from the coelomic cavity/oviduct, septicemia
Hepatitis, necrotizing to granulomatous	2/14 (21%)	septicemia
Splenitis, necrotizing to exudative	3/14 (21%)	septicemia
Fibrin thrombi within hepatic sinusoids	2/14 (14%)	septicemia
Pericarditis, exudative	1/13 (8%)	septicemia
Myeloid leucosis/myelodysplasia (spleen, liver)	1/14 (7%)	spontaneous or viral-induced neoplasia / chronic inflammatory condition
Splenic accumulation of hemozoin pigment	1/14 (7%)	Haemosporidia ( <i>Haemoproteus</i> / <i>Plasmodium</i> )
Chronic enteropathy	1/14 (7%)	undetermined



**Figure 18. Significant histopathological findings identified in captive African houbara bustards with peritonitis/salpingo-peritonitis.**

Accumulation of fibrino-heterophilic exudate admixed with oviductal secretions and bacterial colonies in the lumen of the oviduct (insert and arrowhead), hematoxylin and eosin (H&E) stain (4X) (A). Severe compression of the oviductal mucosa by large amounts of inflammatory exudate admixed with oviductal secretions and bacterial colonies (H&E stain, 50X) (B). Diffuse, severe cystic glandular hyperplasia involving the mucosa of the egg-chamber. A single cyst exhibits necrotic-inflammatory changes associated with a few bacterial colonies (insert and arrowhead), H&E stain (C). Severe transmural granulomatous vaginitis. The original architecture is effaced by multiple granulomas (arrows), occasionally containing bacterial colonies comprised of cocco-bacilli (insert and arrowheads) (H&E stain, 4X and 40X) (D). Serial sections produced for special stains revealed the presence of fibrosis in addition to the granulomatous reaction and highlighted a large foreign body, of plant origin, within a single granuloma (insert, arrowheads) (Masson's Trichrome stain, 4X and 40X) (E).

**Bacteriology**

Results are summarized in **table 6**. *E. coli* was isolated in 12/14 birds. Specifically, for surgical (ante-mortem) samples, *E. coli* was recovered from the oviduct (3/3) while vaginal cultures were negative for bacteria after 48h of incubation (1/1). For post-mortem samples, *E. coli* was isolated from the oviduct (10/13), coelomic cavity (5/6), liver (3/8), ovary (2/5), air sacs (1/2), trachea (1/1), and heart (1/1). *Klebsiella pneumoniae* (1/13), *Serratia odorifera* (1/13) and *Streptococcus* spp. (1/13) were also recovered from the oviduct. No bacteria were isolated from lung (1/1), spleen (1/1) and vagina (1/1). All samples were negative for *Salmonella* spp.

**Table 6.** Bacteriology results of a total of 14 African houbara bustard female breeders

Ring ID	ORGANS	TIMING	BACTERIOLOGY
U06M02276	oviduct	pm	<i>E. coli</i>
	ovary	pm	<i>E. coli</i>
U09M00136	oviduct	pm	<i>E. coli</i> ; <i>Streptococcus</i> spp.
	ovary	pm	no growth after 48hrs
	liver	pm	no growth after 48hrs
U09M02867	oviduct	pm	<i>E. coli</i> , <i>Klebsiella pneumoniae</i>
	oviduct	pm	<i>E. coli</i>
U09M01837	ovary	pm	no growth after 48hrs
	coelomic cavity	pm	<i>E. coli</i>
	oviduct	pm	<i>E. coli</i>
U13M01559	liver	pm	no growth after 48hrs
	lung	pm	no growth after 48hrs
	vagina	am	no growth after 48hrs
U18M07731	liver	pm	no growth after 48hrs

	spleen	pm	no growth after 48hrs
	oviduct	pm	<i>Serratia odorifera</i>
U12M07480	coelomic cavity	pm	no growth after 48hrs
	liver	pm	no growth after 48hrs
	oviduct	am	<i>E. coli</i>
U12M13784	oviduct	pm	<i>E. coli</i>
	coelomic cavity	pm	<i>E. coli</i>
	oviduct	pm	<i>E. coli</i>
U09M02501	liver	pm	<i>E. coli</i>
	heart	pm	<i>E. coli</i>
	oviduct	pm	<i>E. coli</i>
U13M02746	coelomic cavity	pm	<i>E. coli</i>
	air sacs	pm	<i>E. coli</i>
	liver	pm	<i>E. coli</i>
	oviduct	pm	<i>E. coli</i>
U05M01376	coelomic cavity	pm	<i>E. coli</i>
	liver	pm	<i>E. coli</i>
	oviduct	pm	<i>E. coli</i>
U12M16283	ovary	pm	<i>E. coli</i>
	trachea	pm	<i>E. coli</i>
	oviduct	am	<i>E. coli</i>
U10M06893	oviduct	pm	no growth after 48hrs
	ovary	pm	no growth after 48hrs
	air sacs	pm	no growth after 48hrs
	oviduct	am	<i>E. coli</i>
U12M11377	oviduct	pm	no growth after 48hrs
	coelomic cavity	pm	<i>E. coli</i>
	liver	pm	no growth after 48hrs

am = ante-mortem (surgery); pm = post-mortem (necropsy exam)

### Molecular biology

All samples tested negative for IAV, AOaV-1, IBV, MG, MS and *Chlamydia* spp. (14/14). Between 4 and 11 APEC virulence genes were detected in selected *E. coli* isolates (**Table 7**). Specifically, *IucD* and *iss* were consistently identified (100%, 9/9), while *astA* was the least represented (33%, 3/9). Positive detection of the other virulence genes was 55% (5/9) for *tsh*,

66% (6/9) for *cav*, *papC* and *hlyF*, 77% (7/9) for *ompT* and *irp-2*, and 88% (8/9) for *iroN* and *vat*. Three isolates (ID 4,7 and 8) exhibited the same profile: *tsh*, *iucD*, *iroN*, *vat*, *ompT*, *irp-2*, *cva*, *papC*, *hlyF* and *iss*

**Table 7.** Prevalence of APEC virulence-associated genes (n=11) in selected *E. coli* isolates (n=9) recovered from captive African houbaras with lesions of peritonitis, salpingo-peritonitis and vaginitis.

Isolate ID	Pathology	Location	APEC virulence-associated genes*										
			<i>tsh</i>	<i>iucD</i>	<i>astA</i>	<i>iroN</i>	<i>vat</i>	<i>ompT</i>	<i>irp-2</i>	<i>cva</i>	<i>papC</i>	<i>hlyF</i>	<i>iss</i>
U13M02746	ap	E	-	+	-	+	+	+	-	-	-	-	+
U12M13784	csp	E	-	+	-	+	+	-	-	-	-	-	+
U12M16283	ap	E	+	+	+	-	+	+	+	+	+	+	+
U12M11377	csp	E	+	+	-	+	+	+	+	+	+	+	+
U09M02501	asp	E	-	+	-	+	-	-	+	-	-	-	+
U09M02867	csp-v	M	-	+	+	+	+	+	+	+	+	+	+
U09M01837	csp	M	+	+	-	+	+	+	+	+	+	+	+
U06M02276	csp	M	+	+	-	+	+	+	+	+	+	+	+
U05M01376	asp	M	+	+	+	+	+	+	+	+	+	+	+

\* Presence (+) or absence (-) of each gene;

\*\*Isolates obtained at surgery (ante-mortem)

ap= acute peritonitis/septicemia; asp = acute salpingo-peritonitis/septicemia, csp = chronic salpingo-peritonitis; csp-v = chronic salpingo-peritonitis and vaginitis

E= Enjil; M = Missouri

## Discussion

We presented the first comprehensive description of a series of cases of peritonitis and salpingo-peritonitis involving African houbara bustard breeders maintained under captive conditions as part of an *ex-situ* conservation project. The clinico-pathological findings identified were consistent with those previously described in poultry (176,181,201). Bustards dying spontaneously (4/14) had signs of acute septicemia, together with peritonitis (2/4), and salpingo-peritonitis (2/4), and the majority was actively laying between 1 and 10 days prior to the *exitus* (3/4). Euthanized birds presented signs consistent with chronic salpingo-peritonitis and oviductal impaction characterized by extensive inflammatory changes, further confirmed at histopathology, occasionally extending to extragenital organs. (2/10). Egg binding was highly suspected at necropsy (2/10) or confirmed at surgery (1/10). In addition, histopathology revealed a marked granulomatous reaction centered around the oviductal contents (3/10), including some egg-shell fragments (1/3). Chronic forms originated from both breeding centers, while the majority of acute cases (3/4) occurred in Enjil. *Escherichia coli* was isolated from the reproductive tract of the majority of birds and molecular characterization of selected isolates revealed the presence of several virulence factors associated with avian pathogenic *E. coli* (APEC). In summary, a minimum of four virulence genes were identified. The iron uptake-related genes (*iucD*, *ironN*), the serum survival gene (*iss*) and the vacuolating autotransporter toxin gene (*vac*) (88%) were the most represented. These findings are consistent with previous studies conducted on *E. coli* isolates originating from salpingo-peritonitis cases in commercial poultry flocks (182,201–203). On the other end *astA* was present only in 33% of isolates. Three isolates, obtained from euthanized birds with chronic salpingo-peritonitis belonging to both breeding centers, shared the same profile (*tsh*, *iucD*, *ironN*, *vat*, *ompT*, *irp-2*, *cva*, *papC*, *hlyF* and *iss*). However, besides these three isolates, specific pathotypes couldn't be identified, as the same pathological presentation (acute peritonitis/septicemia, acute salpingo-peritonitis/septicemia, chronic salpingo-peritonitis) was present with different virulence profiles. Overall, the variability of profiles identified suggests the involvement of multiple strains, supporting a secondary opportunistic *E. coli* infection. From this point of view it's important to emphasize that, in addition to the presence of specific virulence genes, management factors, promoting bacterial exposure and colonization of the genital tract are important determinants of the pathogenic potential of *E.coli* (181). Host factors, such as immune status and individual susceptibility, should also be considered (201,203).

In our study, an ascending infection from the cloaca was suspected to be the most prevalent mechanism in birds exhibiting oviductal impaction and chronic salpingo-peritonitis,

while birds dying spontaneously presented septicemic changes. Vaginitis may occur as a result of cloacitis or cloacal trauma, including improper insemination practices (50,196). Iatrogenic trauma related with artificial insemination was suspected in one bird, while cloacal prolapse, occurring a year before euthanasia, was mentioned in the history of the second bustard. Interestingly, in this last bird, histopathology revealed the presence of an intralesional foreign body comprised of a large plant debris, which likely resulted from environmental contamination of mucosal lesions originating from the prolapse.

Considering the chronicity of the lesions observed in several cases, the involvement of other pathogens couldn't be completely excluded, despite a negative molecular testing. For example, IAV, AOaV-1, IBV, and *Mycoplasma* (MG and MS) can replicate in the trachea and the reproductive tract of poultry, damaging the mucosal epithelium, predisposing to colibacillosis and negatively impacting egg production (183,204–208). However, egg-shell quality issues, including misshapen, soft-shelled or discolored eggs, and respiratory signs (191,192,194) were not reported in studied bustards. Similarly, besides airsacculitis (4/14) and bronchopneumonia (2/14) associated with intralesional bacteria in a few cases, there were no microscopic lesions suggestive of a viral or mycoplasma infection were observed in the sections of trachea and the upper respiratory tract examined. Serology was not attempted, first because all birds received multiple vaccinations against IAV and AOaV-1 over the course of their life, complicating the differentiation of vaccinated birds from naturally-exposed ones and, secondly, due to the lack of reliable, bustard-specific serological testing for significant avian pathogens, with the exception of competitive enzyme-like immunosorbent assay (ELISA) and inhibition of hemagglutination (HI) targeting IAV and AOaV-1. Routine serological screenings of non-vaccinated sentinels over the years were negative for IAV. On the other end, circulation of AOaV-1 in the area was supported by the occasional serological detection in sentinel breeders, in the absence of clinical signs. Sporadic cases of mortality with a positive PCR for AOaV-1 were registered in wild doves at the beginning of 2021 (Reneco, unpublished data).

In terms of specific histopathological findings, 57% (8/14) of the bustards examined revealed microscopic lesions consistent with oviductal glandulo-cystic hyperplasia. A condition known as cystic endometrial hyperplasia (CEH), characterized by similar morphological changes and potentially predisposing to secondary bacterial infections (pyometra) have been described in several mammalian species (209–218). The proposed etiology includes excessive estrogenic stimulation, endogenous or exogenous. Subclinical infections, leading to prolonged exposure to bacterial toxins promoting endometrial proliferation, are also considered to play a role (209,219). Hyperestrogenism may originate from gonadal tumors, ovarian follicular cysts,

ingestion of certain plants (211,217) and mycotoxins, such as zearalenone (220) or hormonal treatment (209,221). CEH has also been considered an age-related condition, being reported more frequently in middle-aged and old dogs and representing a common finding in aging mice and rats (222,223). Unfortunately, available literature on avian species is more limited. Cystic oviductal hyperplasia, possibly resulting from developmental disorders and/or hormonal imbalances, has been described in psittacines, such as budgerigars, canaries and poultry (189,224). In our study, the only hormonal treatment provided was prostaglandin F<sub>2</sub>α, which was administered to a total of 3 birds (1-2 daily injections) due to a suspect retained egg. No neoplastic lesions were identified microscopically in the sections of the reproductive tract examined. On the other end, an ovarian cyst was noticed in one bird, at necropsy, while parafollicular cysts, likely of developmental origin, were present in two birds. Ovarian cysts may result from endocrine imbalances, anatomic abnormalities, as well as chronic inflammation and may also represent a risk factor for the development of carcinomas (225–227).

Selected birds ranged from 4 to 17 years of age (average 11 years). The maximum longevity of captive houbara bustards is considered to be 32 years, compared to 15 years into the wild (24,228,229), the majority of captive females tend to lay eggs between 2 and 16 years of age (average 10 years). Some birds may be productive up to 24 years or longer, with the oldest breeding bird ever registered in the Moroccan centers being 27 years old (Reneco, unpublished data). In poultry, older birds tend to lay larger eggs (230), and present a reduced oviductal peristalsis and transportation efficiency due to aging, which could predispose to egg binding (185). Senility was also considered a potential factor in the development of reproductive disorders, particularly oviductal obstruction, in non-domesticated avian species (189). In addition to age, stress, nutritional deficiencies (ex. hypocalcemia, hypovitaminosis A), inadequate body frame development and weight can negatively impact ovulation and oviductal activity (185), and should be considered among potential risk factors for the development of peritonitis and salpingo-peritonitis. For example, obese chickens, in addition to also laying larger eggs (186,231), exhibit a higher incidence of erratic ovulation, potentially leading to internal laying and yolk peritonitis, egg binding and oviductal/cloacal prolapse due to the presence of excessive coelomic fat (232).

In order to limit the occurrence of *E. coli* genital infections in captive bustard breeders, bacterial exposure should be limited ensuring excellent hygiene levels and husbandry conditions. Specific critical control points to be considered include: water and feed quality, proper application of cleaning and disinfection protocols (drinking and feeding systems, AI tools) and litter management, training and continuous education of personnel involved in

artificial insemination procedures and manipulation of birds, and enhanced biosecurity to avoid the introduction of harmful pathogens such as AIV, AOaV-1 and *Mycoplasma* spp. within the breeder flocks. Stress and immunosuppressive factors should also be limited. Furthermore, additional studies are needed to define the role played by host-related factors (age, weight, egg size) in the development of peritonitis and salpingo-peritonitis in the African houbara bustard.

### 3. Conclusions and future perspectives

Chronic salpingo-peritonitis and oviductal impaction, impairing the reproductive potential of affected birds, was the most common pathological presentation identified in our bustards, followed by acute septicemic forms.

*E. coli*, exhibiting several APEC virulence genes, based on molecular analysis of selected isolates, was recovered from the majority of affected birds. Several “virulence” profiles were identified, suggesting the involvement of multiple strains. These findings were further supported by antimicrobial sensitivity testing (Kirby-Bauer disk diffusion method) conducted on a total of 24 isolates recovered from both genital and extragenital organs (**Table 8**). For this purpose, nine medically-important antibiotics were used, including amoxicillin (AMX), amoxicillin/clavulanic acid (AMC), sulfamethazole/trimethoprim (SXT), neomycin (NEO), enrofloxacin (ENR), doxycycline (DOX), chloramphenicol (CHL) and cephalexin (CEX). Several antimicrobial resistance patterns were identified. In summary, forty-two percent (10/24) of isolates were resistant to AMX, 38% (9/24) to DOX, 33% (8/24) to CEX and 25% (6/24) to AMC. However, 100% (24/24) and 96% (23/24) of isolates were sensitive to ENR and SXT, respectively. Twenty-nine percent (7/24) of the isolates was resistant to 3 or 4 molecules.

**Table 8.**Antimicrobial sensitivity results conducted on *E. coli* isolates (n=24) recovered from different organs of captive African houbara bustards with gross lesions of peritonitis/salpingo-peritonitis

Bird	Organ	Antimicrobial sensitivity test - <i>E.coli</i> isolates							
		AMX	AMC	SXT	NEO	ENR	DOX	CHL	CEX
U06M02276	ovary	R	I	S	S	S	I	S	I
U09M00136	oviduct	R	I	S	I	S	I	S	R
U09M02867	oviduct	R	I	S	I	S	I	S	R
U09M01837	oviduct	R	R	S	I	S	I	I	R
	coelom	R	R	S	I	S	I	I	I
U13M01559	oviduct	R	I	S	I	S	R	S	R
U12M13784	oviduct	I	I	S	S	S	I	I	I
	coelom	I	I	S	S	S	I	I	I
U09M02501	oviduct	I	I	S	R	S	R	I	I
	heart	I	I	S	R	S	R	I	I
	liver	I	I	S	R	S	R	I	I
U13M02746	oviduct	I	I	S	I	S	R	S	I
	coelom	I	I	S	I	S	R	S	I
	liver	I	I	S	I	S	R	S	I
	air sacs	I	I	S	I	S	R	S	I
U05M01376	oviduct	I	I	S	I	S	I	I	I
	coelom	I	I	S	I	S	I	I	I
	liver	I	I	S	I	S	I	I	I
U12M16283	oviduct	R	R	S	R	S	I	S	R
	ovary	R	R	I	R	S	I	S	R
	trachea	R	R	S	R	S	I	S	R
U10M06893	oviduct	I	I	S	S	S	R	I	I
U12M11377	oviduct	R	R	S	I	S	I	S	R
	coelom	I	I	S	I	S	I	I	I

S = sensitive, I = intermediate, R = resistant

Characterization of additional *E.coli* isolates obtained from previous and new cases, combining serotyping, molecular methods and proteomics (PCR-based APEC virulence profiling, multilocus sequence typing (MLST), MALDI-TOF MS) (201,233) is recommended. This approach would allow to better assess and understand the involvement of polyclonal or clonal bacterial populations and their spatio-temporal distribution. This would also help to identify potential introduction events or emergence of specific strains within the flocks and to assess the feasibility of specific control strategies in the future, such as autovaccines. Overall, amelioration of hygiene levels, management and breeding practices over the years, was accompanied by a progressive reduction of cases. Specific corrective actions and points of improvement included:

- ❖ Retraining and continuing education of personnel involved in artificial insemination activities.
- ❖ implementation of a reverse osmosis and UV light system for water treatment.
- ❖ flushing of the water lines at the entrance of the breeding centers with hydrogen peroxide, at specific time points and in the event of water contaminations based on routine bacteriological analysis.
- ❖ inclusion of mycotoxin adsorbents in the feed.

The role played by captive conditions as well as host-related factors on the occurrence of peritonitis and salpingo-peritonitis in African Houbaras requires additional studies. This semi-desertic avian species is less selected and domesticated, compared to poultry. Breeders are kept for an extended period of time, under different husbandry conditions and present a complex diet (22,234). Captive rearing and artificial insemination practices are pivotal for the genetic management of Houbaras, however they may lead to stressors related with sustained egg production and frequent contacts with humans and handling. Compared to commercial poultry flocks, which are managed by a relatively limited number of people, Houbara breeders are followed individually, which is more labor intensive and requires the recruitment and specific training of more personnel. Furthermore, the remote location of the Houbara conservation projects poses additional challenges, in terms of resources availability and accessibility. The goal is to find a balance between “industrialization”, promoting the production of healthy individuals to be released into the wild for repopulation purposes, and “conservation”, maintaining the genetic traits of wild populations. The expansion of captive breeding operations

should be accompanied by a proportionate amelioration of management practices and by a reinforced biosecurity.

From a pathological perspective, combining necropsy and histopathological findings we were able to provide the first description of cystic oviductal hyperplasia (COH) or cystic salpingitis, in the African Houbara. Additional studies are needed to assess the prevalence of COH in captive breeders, define predisposing and etiological factors and clarify its role in the development of salpingitis/salpingo-peritonitis. CEH has been considered an important risk factor for ascending uterine infections (pyometra) in dogs, facilitating bacterial colonization and overgrowth (209). However, recent evidences support that CEH doesn't necessarily correlates with pyometra and that not all CEH progress to pyometra (234). In terms of practical necropsy recommendations, the reproductive tract should be carefully examined in its entirety, assessing the status of the ovary and the morphology of ovarian follicles (developed, regressed, cystic). After isolating the oviduct from the coelomic cavity, the organ should be longitudinally sectioned to evaluate its contents and the mucosal appearance of the different segments. Proper lighting and a magnifying glass would help to identify small lesions, including cysts. The presence of an impacted oviduct with inflammatory exudate may complicate interpretation, due to mucosal compression/adhesions. However, histopathological assessment would help the identification of cystic changes in similar cases.

## C. STUDY 4

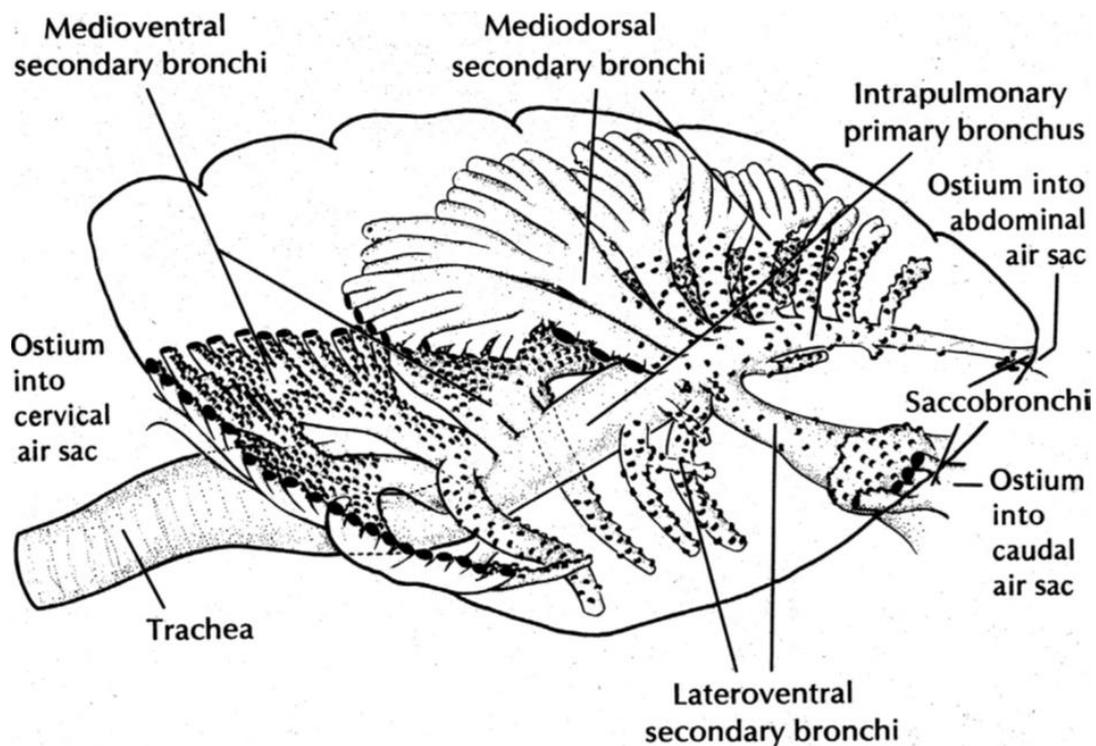
### 1. Introduction

Respiratory syndromes represent one of the most significant diagnostic challenges in veterinary medicine. A remarkable example in domestic mammals is provided by the **canine infectious respiratory disease complex (CIRDC)** also known as « kennel cough », an endemic respiratory form, commonly observed in overcrowded conditions (235,236). The list of infectious agents involved has been expanded over the years, thanks to significant advancements in the field of molecular diagnostics. Contributing pathogens include bacteria, such as *Bordetella bronchiseptica*, *Streptococcus equi* subsp. *zooepidemicus*, *Mycoplasma cynos* and viral agents like *canine mastadenovirus A*, *canine morbillivirus* (canine distemper), *canine alphaherpesvirus-1*, canine influenza virus type A H3N2 or H3N8, canine parainfluenza virus type 2, canine respiratory coronavirus and canine pneumovirus (236,237). As regards livestock species, several syndromes have been described and investigated, particularly in cows and pigs. The **bovine respiratory disease complex (BRDC)** or « shipping fever » is

characterized by the involvement of a wide range of bacterial (*Histophilus somni*, *Mannheimia haemolytica*, *Pasteurella multocida* and *Mycoplasma bovis*) and viral pathogens (bovine viral diarrhoea virus, bovine respiratory syncytial virus, bovine adenovirus-1,-3,-7,-10, bovine alpha herpes virus 1, bovine coronavirus, bovine parainfluenza 3 virus and influenza D virus) combined with stress (dietary and environmental changes, overcrowding conditions, transportation) and host-related factors (immune status, age, genetics) (128,238–240). The **porcine respiratory disease complex (PRDC)** has been reported in post-weaning and weaning-to-finishing pigs, exhibiting signs of pneumonia and poor weight gain. Both infectious (viruses and bacteria) and non-infectious factors (environmental conditions and management practices) are involved and the overall etiology may vary significantly between farms, different units belonging to the same farm and even, over time, in the same complex. Commonly identified pathogens include *Betaarterivirus suis 1* (porcine reproductive and respiratory syndrome virus), swine influenza A virus, *Suid Alphaherpesvirus 1* (Aujeszky's disease), porcine circovirus type 2 (PCV-2), *Mycoplasma hyopneumoniae*, *Pasteurella multocida*, *Bordetella bronchiseptica*, *Glaessrella (Haemophilus) parasuis*, *Streptococcus suis* and (list not exhaustive) (128,241–243).

The **avian respiratory disease complex (ARDC)** is a multifactorial condition of poultry caused by several viral (avian influenza virus, avian orthoavirulavirus-1, infectious bronchitis virus, infectious laryngotracheitis virus, avian metapneumovirus) bacterial (*Mycoplasma gallisepticum*, *Mycoplasma synoviae*, *Chlamydia psittaci*, *Escherichia coli*, *Ornithobacterium rhinotracheale*, *Pasteurella multocida*, *Reimerella anatipestifer*, *Avibacterium paragallinarum*, *Gallibacterium anatis biovar haemolytica*, *Staphylococcus spp.*) and fungal (*Aspergillus spp.*) agents, in a variety of combinations (129,244,245).

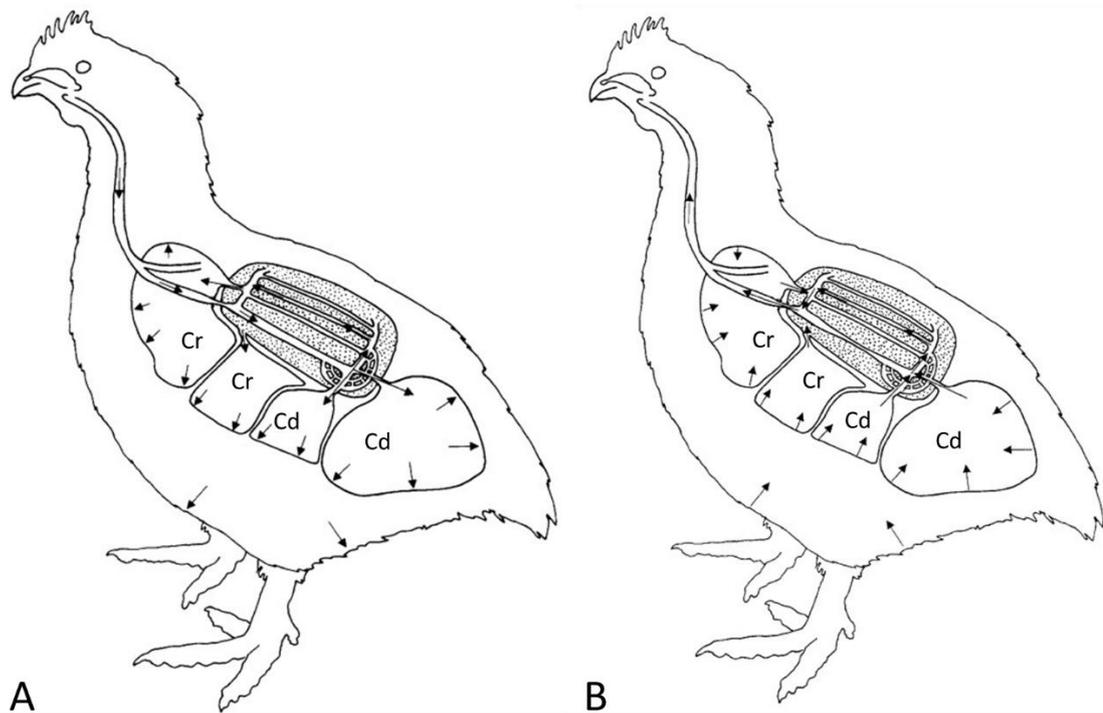
Besides pathogens exposure, susceptibility to respiratory infections in avian species can be significantly affected by adverse **environmental and husbandry conditions and management factors**. Poor ventilation, prolonged heat stress (246,247), excessive dust levels, exposure to toxic gases such as ammonia (248) as well as malnutrition (hypovitaminosis A) and immunosuppression (stress-related or viral-induced) are important predisposing and contributing factors (249,250). **Anatomical and physiological** traits should also be considered. The avian respiratory system has a unique organization. It includes the nares, with the nasal cavity, larynx, trachea, syrinx, lungs and air sacs (179,251). The trachea is comprised of complete cartilage rings and bifurcates at the syrinx, the site of vocalization, giving rise to primary, and secondary bronchi (conducting airways) and tertiary bronchi (the functional units, forming hexagonal lobules involved into the gas exchange) (**Figure 19**).



**Figure 19.** Organization of the conducting airways in the left lung of the mute swan (*Cygnus olor*). From Duncker (1971) (252).

Primary bronchi, divided into extrapulmonary and intrapulmonary bronchi, and secondary bronchi, organized in three functional groups (medioventral, mediodorsal and lateroventral).

Lungs are relatively small and rigid, located dorsally into the thoraco-abdominal cavity. They are firmly adhered to ribs and vertebrae and do not expand during the respiration cycle (251,253). Air sacs mechanically ventilate the lungs, acting as bellows and they are not involved in the gas exchange (**Figure 20**). They are poorly vascularized, and their number, relative size and location vary according with the different species. Two air sac groups are recognized: the cranial group (cervical, clavicular and craniothoracic air sacs) and a the caudal group (caudothoracic and abdominal air sacs) (253). The blood-air barrier in the avian lung is 56-67% thinner, compared to that of a mammal of the same body mass, while the respiratory surface area is greater than 15% (128,251,253). Unfortunately, such an efficient gas exchange system is also responsible of the well-known avian susceptibility to aerogenous toxins (179). An historical example of this trait, is provided by the use of canaries as sentinel birds inside coal mines to detect dangerous gas leaks in a timely manner.



**Figure 20.** Pathways of air flow in the respiratory system of a chicken (*Gallus gallus*) during inspiration (A) and expiration (B), mediated by the change of volume of air sacs.

Cr = cranial air sacs (cervical, craniothoracic), Cd = caudal air sacs (caudothoracic, abdominal).  
Modified from Fedde et al 1998 (251).

Inspirited air is filtered in the nasal chambers and in trachea and conducting airways, thanks to the presence of a mucosa comprised of ciliated epithelial cells, mucous glands and goblet cells. These elements form the **mucociliary escalator**, a primary innate defense mechanism, essential for entrapping and eliminating particulate material and pathogens from the respiratory tract. Any damage to this system, can predispose to respiratory disease (179). Foreign particles reaching the lungs may also be phagocytosed and eliminated by the epithelial cells lining the atria and infundibula in the respiratory lobules. The air arriving into cranial air sacs group is filtered, passing through the lungs, while this step is not present for the caudal group, making this area more vulnerable to infections. From the air sacs, infection can rapidly spread to the lungs. Similarly, the lack of diaphragm and the contacts of air sacs with coelomic organs, facilitate the spread of infection to adjacent areas (179,253).

Respiratory infections have been rarely documented in captive Houbaras (53,59,60,254). Since 2012, a respiratory syndrome with a complex and poorly understood etiology, characterized by chronic forms and recurrent relapses, has been affecting Asian Houbaras part of two captive breeding operations in the UAE: the National Avian Research Center (NARC) and the Sheikh Khalifa Houbara Breeding Center (SKHBC-AD) (41). While

an in-depth epidemiological study is currently in progress, a seasonal pattern seemed to be present, with cases occurring more commonly in spring and summer. In order to better understand the clinico-pathological features of this syndrome, and identify potential etiological and contributing factors involved, a series of cases registered between February and August 2022 were selected for characterization and diagnostic investigation.

## 2. Article 4

The present article, under preparation, will be submitted to the journal *Veterinary Microbiology* with the following reference:

### **Insights on the bustard respiratory complex: a respiratory syndrome affecting captive Asian houbara bustards (*Chlamydotis macqueenii*) in the United Arab Emirates.**

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## Introduction

The Asian houbara bustard is a steppic bird residing in Asia and the Middle East. Since 2014 is included in the Red List of the International Union for the Conservation of Nature (IUCN) following the drastic decline of wild populations due to progressive habitat loss and overhunting. As a result, several captive breeding projects have been established over the years (41).

Maintaining a healthy breeder flock is essential for a successful ex-situ conservation strategy. Respiratory infections represent one of the most significant diagnostic challenges in veterinary medicine, affecting a variety of species. They can result in significant losses in terms of morbidity/mortality, poor performances and treatments costs, and negatively impact animal welfare. They are characterized by a complex etiology, combining a variety of primary and opportunistic pathogens, combined with environmental, management and host-related factors (128,255). A few remarkable examples include canine infectious respiratory disease complex (CIRDC) or « kennel cough » (235,236), the bovine respiratory disease complex (BRDC) or « shipping fever » in cows (128,238–240), the porcine respiratory disease complex (PRDC) in pigs (128,241–243) and the avian respiratory disease complex (ARDC) in poultry (128,129,244,245). The latter is a major issue for the poultry industry worldwide, resulting in significant economic losses due to morbidity and mortality and increased costs related with vaccinations and treatment. In terms of viral agents, avian Orthoaviularius-1 (AOaV-1), infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), fowlpox virus, avian metapneumovirus (aMPV), *Alphainfluenzavirus influenzae* (IAV) can significantly damage the respiratory tract of poultry (104,146,245,256–258). *Mycoplasma*, particularly *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS), bacteria belonging to the *Pasteurellaceae* family, *Escherichia coli*, *Bordetella avium*, *Bordetella hinzii*, *Staphylococcus* spp., *Chlamydia psittaci*, *Aspergillus* spp. and *Cryptosporidium* spp. can also result in respiratory forms, alone or in a combination with other pathogens (245,259–264). Exposure to adverse environmental conditions, such as extreme temperatures, excessive ammonia and dust levels (246–248), and nutritional deficiencies (hypovitaminosis A) (249) can compromise the activity integrity of the respiratory epithelium, predisposing to secondary infections. Immunosuppression, stress-related or viral induced can also play a role (265).

Respiratory forms have been rarely documented in houbara bustards. A dual infection involving AOaV-1 and avipox virus resulted in the rapid death of 3-month-old birds, in Saudi Arabia (254). Nasal discharge, tracheitis, airsacculitis and occasional pulmonary abscesses

were observed during a chlamydiosis outbreak registered in quarantined birds originating from Pakistan (59). Avian Metavulavirus-2 (formerly avian paramyxovirus-2) was isolated from a single bustard that died of an unidentified respiratory disease (60), while aspergillosis was commonly observed in confiscated birds, heavily stressed and in poor body conditions (53). More recently, a study was conducted on captive African houbara bustards (*Chlamydotis undulata*) diagnosed with respiratory disease in Morocco, between 2020 and 2023 (162). The majority of cases were registered between May and August and presented with fibrino-heterophilic and lymphocytic tracheitis, airsacculitis and bronchopneumonia. Low pathogenicity avian influenza (LPAI) H9N2 and, occasionally, avian coronavirus were detected by molecular screening, while a variety of bacteria, including *E. coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterococcus*, as well as *Aspergillus* spp. were isolated from the respiratory tract. All birds tested negative for AOaV-1, ILTV, aMPV, MG and *Chlamydia* spp. (162).

Since 2012, a respiratory syndrome with a complex and poorly understood etiology, characterized by chronic forms and recurrent relapses, has been observed in outdoor, captive Asian houbara bustards (*Chlamydotis maqueenii*) residing in the United Arab Emirates (UAE). While an in-depth epidemiological study is on-going, a seasonal pattern seemed to be present, with cases occurring more commonly in spring and summer. In order to better understand the clinico-pathological features of this syndrome, and identify potential etiological and contributing factors involved, a series of cases were selected for characterization and diagnostic investigation.

## **Materials and methods**

### **Captive breeding**

Selected birds were destined to be released into the wild or for falconry training. In order to develop strong survival skills, they were housed in outdoor tunnels, 12x4x2m in size and close to sand dunes, in groups of 12-24 individuals. The vaccination protocol includes a polyvalent inactivated vaccine against low pathogenic avian influenza (LPAI) subtype H9N2 and avian orthoavulavirus-1 (AOaV-1) Ulster strain (Gallimune® 208 Merial), provided at 10 and 40 days with a booster every 6 months and an inactivated subunit vaccine against H5 HPAI and AOaV-1 la Sota strain (VOLVAC® B.E.S.T, AI + NDV) inoculated at 30 and 60 days of age, followed by annual boosters. A live attenuated canary pox vaccine (KP1 strain, Poulvac® Zoetis) is also

administered at 30 and 60 days of age, followed by annual boosters. Deworming treatments are provided at 30 days of age and once a year.

### **Epidemiology, selection of cases and gross lesions.**

A total of 34 individual cases of chronic respiratory infections, originating from the Sheikh Khalifa Houbara Breeding Center (SKHBC-AD) were selected for this study. Selected bustards included 19 females and 15 males, ranging between 4.5 months and 8.5 years of age (average 1.3 year). They started exhibiting clinical signs between February and August 2022 and at the time of sampling they have been hospitalized for a minimum of 12 days and a maximum of 80 days (average 39 days). Spontaneous death occurred in 2 birds, while 32 individuals were humanely euthanized by means of intravenous injection of T61 (200) due to poor response to treatment, recurrent clinical relapses and severe degradation of general conditions. A complete necropsy was performed within 2 to 24 hours after death and lesions were recorded.

### **Bacteriology.**

Amie's swabs were aseptically collected from nasal cavity (n=6), trachea (n=3), air sacs (n=9) lung (n=15) and liver (n=9) at necropsy. Samples were plated on general and differential media (nutrient agar, eosin methylene blue agar, hektoen enteric agar and chapman agar). All plates were incubated aerobically and under microaerophilic conditions at 37°C and examined at 24 and 48hrs post-incubation. Bacterial isolates were identified based on colony's morphology, biochemical tests and Analytical Profile Index (API) test strips (bioMerieux, Inc).

### **Molecular biology.**

Oropharyngeal, tracheal and cloacal swabs and sections of lung and cecal tonsils (n=34) were applied on Flinders Technology Associates (FTA®) cards. After suspension of FTA cards' sections in TE-buffer (198), nucleic acids were extracted using Mag Fast Extraction Kit (MAGFAST384) and IDEAL-96HP extraction robot (Innovative diagnostics, France) and placed at -80°C until analysis (cecal tonsils and cloacal samples were pooled for extraction and mentioned throughout the text as ceca).

For viral pathogens, reverse transcription-quantitative polymerase chain reaction (RT-qPCR), targeting *Alphainfluenzavirus influenzae* (matrix gene) (IAV), avian orthoaviularivirus-1 (AOaV-1), infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), avian metapneumovirus A, B and C (aMPV A, B, C) (Innovative diagnostics, France) were performed. Reverse RT-nested PCR targeting the fusion gene (F gene) of AOaV-1 (266) was conducted on selected positive samples for AOaV-1 by RT-qPCR, exhibiting the lowest Ct

value (lung n=3, trachea n = 2, ceca n=2; oropharynx n= 1). A total of 17 birds, presenting hyperplastic tracheal and bronchial changes at histopathology, with no apparent viral inclusions, was also tested for avipoxvirus (locus P4b or 4b core protein gene) by conventional PCR (267).

For bacterial agents, quantitative polymerase chain reaction (qPCR) targeting *Chlamydia spp.*, *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) (Innovative diagnostics, France), *Mollicutes* 16S ribosomal RNA (rRNA) gene (268) and *Bordetella avium* (95) were conducted. In addition, samples collected from 17 birds with gross and/or microscopic changes of rhinitis/rhinosinusitis were tested for *Avibacterium paragallinarum* by qPCR (92)

Samples with a Ct value higher than 35 were considered negative.

All positive samples for F gene AOaV-1 and positive samples for *Mollicutes* 16S rRNA with the lowest Ct value (trachea n= 10, lung n = 4, ceca n =3 and oropharynx n = 1) were sent to sequencing analysis (Sanger method).

### **Histopathology.**

Sections of brain (n=33), eyelid (n=30), nasal cavity (n=30), trachea (n=34), lung (n=34), air sacs (n=23), liver (n=34), pancreas (n=33), esophagus (n=34), proventriculus (n=34), gizzard (n=34), small and large intestine (n=34), kidney (n=31), spleen (n=34), thymus (n=25) and bursa of Fabricius (n=17) were collected at necropsy and placed in 10% buffered formalin. Tissues were routinely processed, paraffin-embedded, sectioned at 3 µm thickness, stained with hematoxylin and eosin (H&E) and examined by light microscopy. Gram, Periodic acid-Schiff (PAS), Congo red (CR) and Ziehl-Neelsen (ZN) special stains were performed on selected lung, air sacs, liver and spleen sections.

### **Transmission Electron Microscopy.**

Ultrastructural analysis was performed on tracheal (n=3) and lung tissue sections (n=2), obtained from three clinically-affected birds humanely euthanized, with a positive *Mollicutes* PCR and exhibiting severe lymphoplasmacytic tracheitis and broncho-interstitial pneumonia at histopathology. Tracheal and lung (including primary bronchi) sections collected from a healthy bird, dying of acute trauma and not included in the 34 selected bustards, were used as negative controls. Tissues were stored in 10% buffered formalin. Tissues were sectioned (10 transversal sections in total), immersed in 2% glutaraldehyde in Sorensen's phosphate buffer (0.1 mol/L, pH = 7.4) for 1 h, and washed with Sorensen's phosphate buffer for 12 h. Subsequently, samples were incubated with 1% OsO<sub>4</sub> in Sorensen's phosphate buffer (0.05 mol/L, glucose 0.25 mol/L, OsO<sub>4</sub> 1%) for 1 h, dehydrated in an ascending ethanol series until

ethanol 100% and then with propylene oxide, and embedded with epoxy resin (EMBed 812). After 48h of polymerization at 60°C, serial ultrathin sections (70 nm) were mounted on 100 mesh collodion-coated copper grids and post-stained with 3% uranyl acetate in 50% ethanol and with 8.5% lead citrate before being examined on a HT 7700 Hitachi electron microscope at an accelerating voltage 80 KV.

## Results

### Clinico-pathological findings.

The overall clinical picture was characterized by mild to marked gurgling, oculo-nasal discharge with clear mucous/fibrinous exudate (**Fig. 21A-B**), soiled/conglutinated periocular and crown feathers (**Fig. 21A-B**) gasping for air and changes or loss of voice (**Fig. 21C**). Reduced activity, progressive weight loss and dehydration with/without concurrent cloacal impaction, following prolonged decubitus, were also reported.

At necropsy, affected birds were mildly to markedly emaciated (23/34) and dehydrated (20/34). Small amounts of white to dry brown exudate were noticed around the nostrils (12/34), and at the edges of the eyelids (4/34). The tracheal mucosa was moderately congested and variable amounts of mucus, with a foamy appearance, were found within the lumen (33/34) (**Fig. 21D**). Dense mucus (22/34), occasionally admixed with few fibrinous flakes (2/34), was noticed within the nasal cavity. Abdominal air sacs were covered with small to moderate amounts of white, fibrinous exudate (12/34) or yellow, mucoid exudate (3/34). In one case, they were severely impacted with a large collection of yellow caseous material. Bronchial caseous casts and diffusely consolidated lungs were rarely present (1/34). Lungs revealed multifocal, pale pink to grey foci, and areas of consolidation, usually with a symmetric distribution (**Fig. 21E**). Upon incision, the intrapulmonary bronchi appeared diffusely thickened, with a reduced lumen, and contained variable amounts of grey, mucoid exudate extending to the adjacent parenchyma (25/34) (**Fig. 21E-F**). Additional findings included mild to moderate increased amounts of urates within the ureters (11/34) and a deformed beak (rhinotheca) (1/34).



**Figure 21.** Clinical signs and post-mortem findings in captive Asian houbara bustards with chronic respiratory disease.

Ocular discharge (arrowheads and insert) and soiled periorbital feathers (A). Accumulation of small amounts of white fibrinous exudate around the nostrils (arrowhead). Periorbital and crown feather appear conglutinated and soiled (B). Respiratory distress, gasping (arrowhead). Gurgling was also present (C). Markedly congested tracheal mucosa and increased amount of mucous, with a foamy appearance, within the lumen (arrowhead) (D). Multifocal, bilateral pale foci visible on the lungs (arrowheads) corresponding to areas of consolidation. Note also the poor body conditions, including generalized pallor, atrophied liver, and complete absence of coelomic fat (E). Intrapulmonary bronchus exhibiting markedly thickened walls (mural thickening) and distended lumen with grey mucoid exudate, occasionally extending to the adjacent parenchyma (arrowheads) (F).

## **Histopathology.**

Histopathological changes identified in the respiratory tract are summarized in **Table 9**.

In the vast majority of birds (33/34), the tracheal mucosa was diffusely expanded by lymphoplasma cells and occasional heterophils. Mucous glands were either depleted or markedly hyperplastic (**Fig. 22A**). The respiratory epithelium exhibited various degrees of deciliation, blebbing, and superficial heterophilic infiltration or was replaced by hyperplastic and/or metaplastic cells with a squamous appearance (**Fig. 22B**). The tracheal cartilage presented marked degenerative, necrotic changes in 3 cases (**Fig. 22C**).

Broncho-interstitial pneumonia (28/34), with/without concurrent exudative (heterophilic) broncho-pneumonia (23/34) and markedly hyperplastic bronchus-associated lymphoid tissue (BALT) (25/34) were prominent findings (**Fig. 22D-E**). Lymphoid follicular aggregates were also noticed in air sacs (10/23) and, to a lesser extent, trachea (7/34), conjunctiva (hyperplastic conjunctival associated-lymphoid tissue - CALT) (5/30) and nasal cavity/intraorbital sinuses (3/30) (**Fig. 22F**). The bronchial mucosa (primary/secondary bronchi) was diffusely and severely expanded by hyperplastic/hypertrophic mucous glands and lymphoplasma cells. The lumen was often distended (bronchiectasis) (27/34) and contained variable amounts of sloughed mucosal epithelium admixed with mucus and mixed inflammatory cells. Multifocal hyperplasia/hypertrophy of the lining of tertiary bronchi (proliferative response), atria and air capillaries, with areas of smooth muscle hypertrophy and fibrosis was also present.

Rare heterophilic granulomas, occasionally containing bacterial colonies, comprised on Gram-negative cocco-bacilli, were identified in a few sections of lung (2/34) and air sacs (3/23). Mild to marked pneumoconiosis, associated with birefringent crystals, consistent with silica dust (silicosis), with or without concurrent lymphocytic infiltration, was a common finding (28/34) (**Fig. 22G**). Dust-laden macrophages, forming nodular aggregates, were also present in the air sacs (21/23) (**Fig. 22H**).

Occasional findings included severe necrotizing bronchopneumonia with intralesional bacterial colonies (1/34) and angioinvasive fungal elements (*Aspergillus* spp.) (1/34) and severe granulomatous stomatitis and osteomyelitis (1/30), involving the nasal cavity and associated with PAS-positive, filamentous elements (hyphae, pseudohyphae) admixed with yeast forms (*Candida* spp.). No acid-fast bacilli were identified in lung and air sacs sections by ZN stain (2/2).

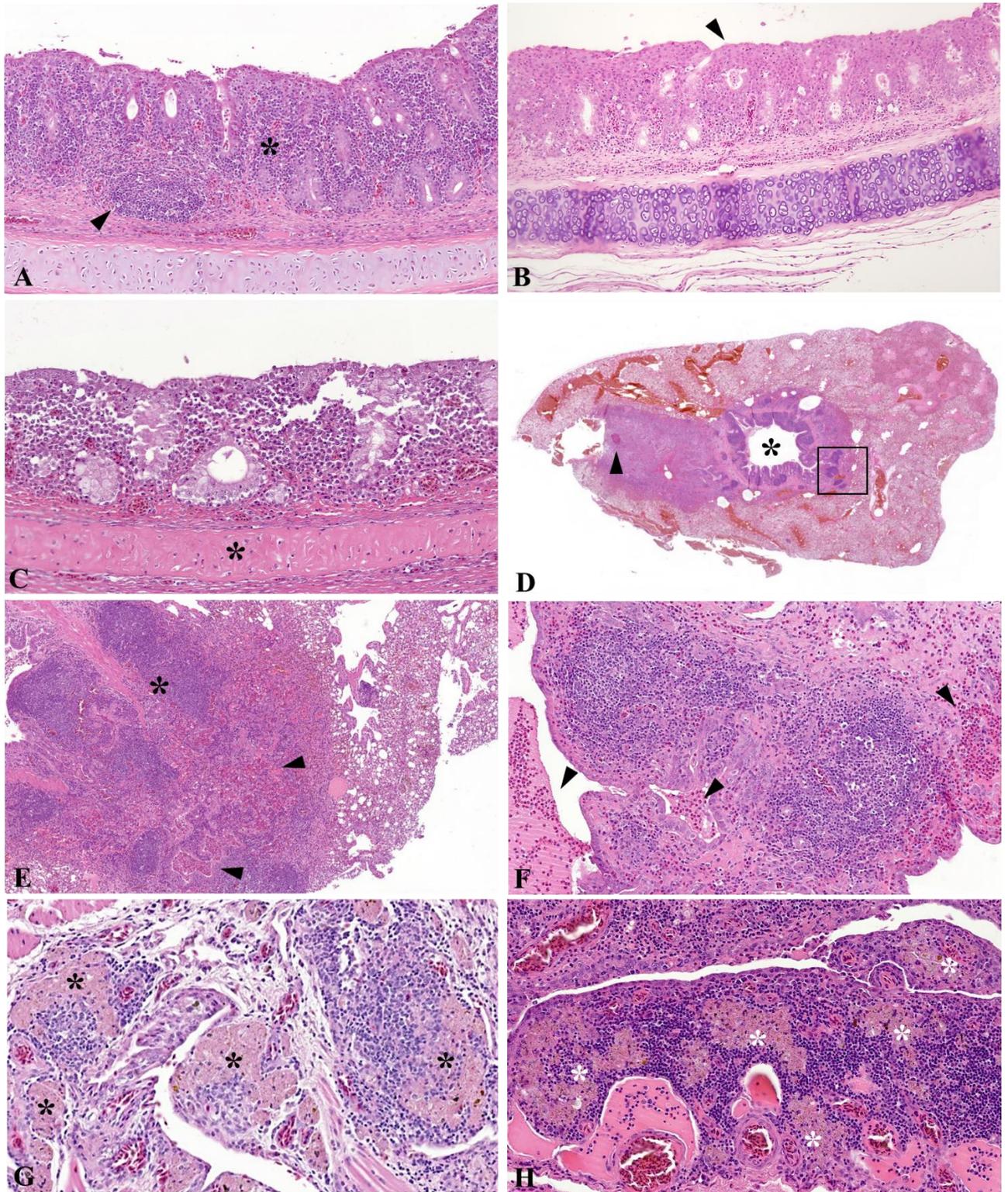
As regards extra-respiratory changes, the digestive tract showed moderate to severe cestodiasis localized to the small intestine (duodenum and proximal jejunum) (9/34), moderate depletion of zymogen granules in pancreatic acinar cells (10/33), mild to moderate lymphocytic

pancreatitis (4/33) with concurrent interstitial fibrosis and ductular hyperplasia (3/4) and moderate, locally-extensive necrotic-hemorrhagic proventriculitis (1/34).

Additional findings included mild to marked bursal atrophy (17/17), moderate to marked thymic atrophy (22/25), marked hepato-splenic amyloidosis (1/34) further confirmed by CR stain, and accumulation of moderate amounts of hemozoin pigment (Perls's negative) within hepatic Kupffer's cells and splenic phagocytic cells (8/34). Renal sections showed mild to moderate nephrosis (7/31) and tubular mineralization (12/31). Rare lipogranulomas, were present in the subcutis of the neck region, adjacent to the thymus or esophagus (8/34). Brain (33/33), gizzard (34/34) and large intestine (34/34) appeared within normal limits.

**Table 9.** Overview of histopathological findings identified in the upper and lower respiratory tract of captive Asian Houbara bustards with chronic respiratory disease.

Organ	Histopathological changes	Frequency	%
<b>Trachea</b>			
	Tracheitis, lympho-plasmacytic, with mucosal hyperplasia/metaplasia, diffuse	15/34	44%
	Tracheitis, lympho-plasmacytic, heterophilic, with mucosal hyperplasia/metaplasia, diffuse	14/34	41%
	Tracheal squamous metaplasia and plasma cell rich infiltration, diffuse	2/34	6%
	Cartilage necrosis, multifocal	1/34	3%
	Transmural tracheitis, lympho-plasmacytic, with squamous metaplasia and cartilage necrosis, multifocal to diffuse	2/34	6%
	Tracheitis, lymphofollicular infiltration, multifocal	7/34	21%
	Within normal limits	1/34	3%
<b>Lung</b>			
	Bronchitis lympho-plasmacytic, heterophilic, with mucosal hyperplasia/metaplasia, diffuse	3/34	9%
	Bronchitis, exudative, intraluminal casts	1/34	3%
	Pneumonia, broncho-interstitial, lympho-plasmacytic with mucosal hyperplasia, diffuse	1/34	3%
	Pneumonia, broncho-interstitial, lympho-plasmacytic, heterophilic, with mucosal hyperplasia, diffuse	2/34	6%
	Pneumonia, broncho-interstitial, lymphoplasmacytic, with mucosal and BALT hyperplasia, diffuse	25/34	74%
	Bronchiectasis	27/34	79%
	Broncho-pneumonia, exudative, multifocal	23/34	68%
	Broncho-pneumonia, granulomatous, with or without intralesional bacteria, focal to multifocal	2/34	6%
	Broncho-pneumonia, granulomatous, with intralesional foreign bodies, focal	1/34	3%
	Broncho-pneumonia, fibrino-necrotizing with intralesional fungi, multifocal	1/34	3%
	Broncho-pneumonia, fibrino-necrotizing with intralesional bacteria, multifocal	1/34	3%
	Pneumoconiosis (silicosis), multifocal	28/34	82%
	Within normal limits	1/34	3%
<b>Air sacs</b>			
	Aisaccultitis, lympho-plasmacytic/histiocytic and heterophilic, with epithelial hyperplasia, diffuse	16/23	69%
	Airsaccultitis, lympho-follicular infiltration, multifocal	10/23	43%
	Airsaccultitis, fibrinoid/fibrino-heterophilic exudate	10/23	43%
	Airsaccultitis, granulomatous with/without intralesional bacteria, focal to multifocal	3/23	13%
	Silicosis, multifocal	21/23	91%
	Fibrosis, diffuse	10/23	43%
<b>Nasal cavity</b>			
	Rhinitis, lympho-plasmacytic, with mucosal hyperplasia, diffuse	6/30	26%
	Rhinitis, lympho-plasmacytic, heterophilic, with mucosal hyperplasia, diffuse	8/30	35%
	Rhinitis, lymphofollicular infiltration, multifocal	1/30	3%
	Rhinitis, heterophilic infiltration, multifocal	2/30	7%
	Rhinitis, exudative, intraluminal casts	3/30	10%
	Rhinitis, necrotizing, fibrino-heterophilic with intralesional bacteria	2/30	7%
	Sinusitis, lympho-plasmacytic and heterophilic, diffuse	6/30	20%
	Sinusitis, lympho-follicular infiltration, multifocal	2/30	7%
	Stomatitis, osteomyelitis and chondritis, granulomatous, with intralesional fungi, multifocal	1/30	3%
	Nasal gland adenitis, lymphoplasmacytic, diffuse	1/30	3%
	Within normal limits	10/30	43%
	Uninterpretable (trimming artefacts)	3/30	10%
<b>Eyelid</b>			
	Conjunctivitis, lympho-plasmacytic, heterophilic, with mucosal hyperplasia, multifocal to diffuse	14/30	47%
	Conjunctivitis, lympho-plasmacytic, with mucosal hyperplasia, multifocal to diffuse	3/30	10%
	Conjunctivitis, lympho-plasmacytic, with mucosal and CALT hyperplasia, multifocal to diffuse	5/30	17%
	Conjunctivitis, heterophilic, multifocal	5/30	17%
	Within normal limits	5/30	17%



**Figure 22.** Photomicrograph of the respiratory tract of captive Asian Houbara bustards with chronic respiratory disease.

Severe lymphoplasmacytic tracheitis with concurrent heterophilic infiltration of the respiratory epithelium. The lamina propria is expanded by lymphoplasma cells (asterisk), including occasional lymphoid aggregates (arrowhead). Hematoxylin and eosin (H&E) stain (16X) (A). Diffuse squamous metaplasia of the tracheal respiratory epithelium. H&E stain (10X) (B). Severe chondronecrosis (asterisk) involving the tracheal cartilage, with concurrent lymphoplasmacytic tracheitis. H&E stain

(26X) (C). Severe interstitial bronchopneumonia. Note the diffuse thickening of the walls of an intrapulmonary bronchus, bronchiectasis and BALT hyperplasia. Foci of heterophilic infiltration (arrowheads) are also present. H&E stain (1.4X and 13.7X) (D-E). Severe airsacculitis, lymphofollicular and heterophilic (arrowheads). H&E stain (20X) (F). Pneumoconiosis and silicosis characterized by the presence of nodular clusters of dust-laden macrophages within the walls of tertiary bronchi (G) and scattered in the air sacs (H), associated with lymphocytic reaction. H&E stain (20X and 62X).

### **Bacteriology**

*Escherichia coli* was isolated from the nasal cavity (3/6), together with *Pseudomonas* spp. and *Staphylococcus* spp. (2/6), followed by air sacs (2/9), lung (2/15) and liver (1/9). *Pseudomonas* spp. was isolated from the trachea (2/3), while *Klebsiella* spp. was recovered from air sacs (1/9), lung (1/15) and liver (1/9). The rest of the samples was negative for bacterial growth after 48hrs of incubation.

### **Molecular biology**

In terms of viral agents, all samples were negative for IAV, IBV, ILT, aMPV A, B and C and avipoxvirus. **AOaV-1** was detected in oropharynx (5/34), lung (6/34), trachea (4/34) and ceca (5/34) (Ct values between 16.87 and 33.99, average 27.72). RT nested PCR targeting the F gene was positive in trachea, lung and ceca belonging to a single bird and partial sequencing of the F gene, revealed a cleavage site with an aminoacidic motif consistent with a **virulent AOaV-1** strain (G-R-R-Q-K-R↓F-I) (257,269,270).

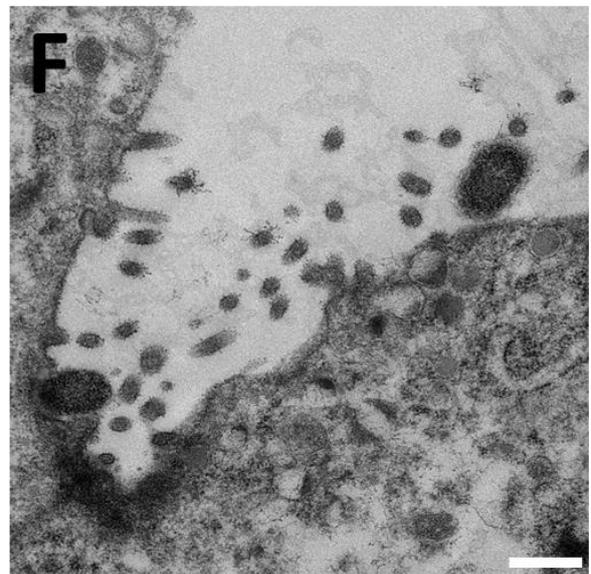
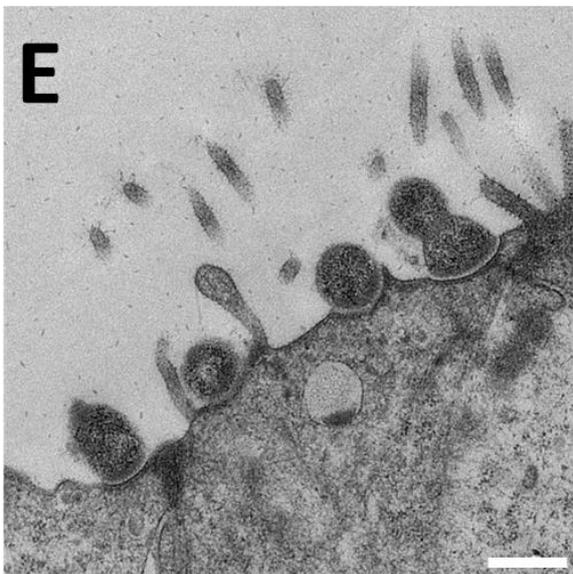
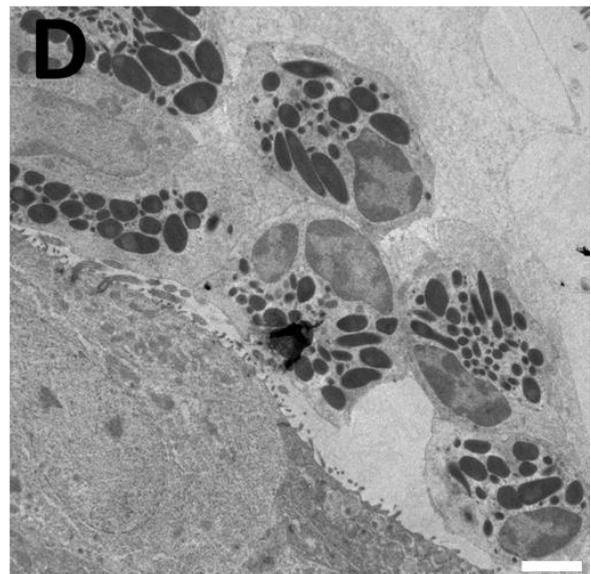
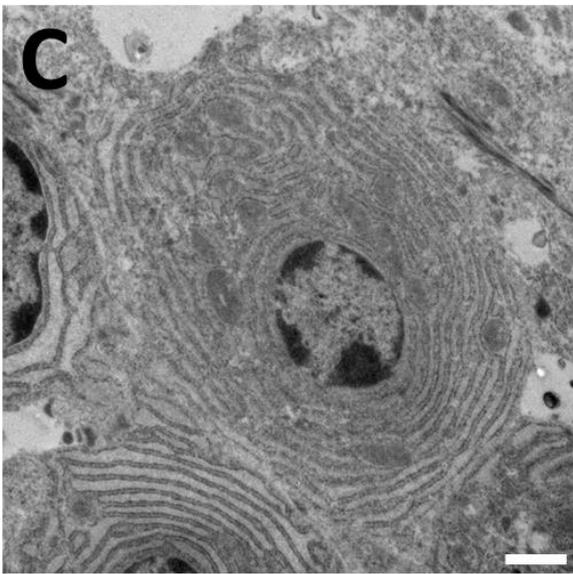
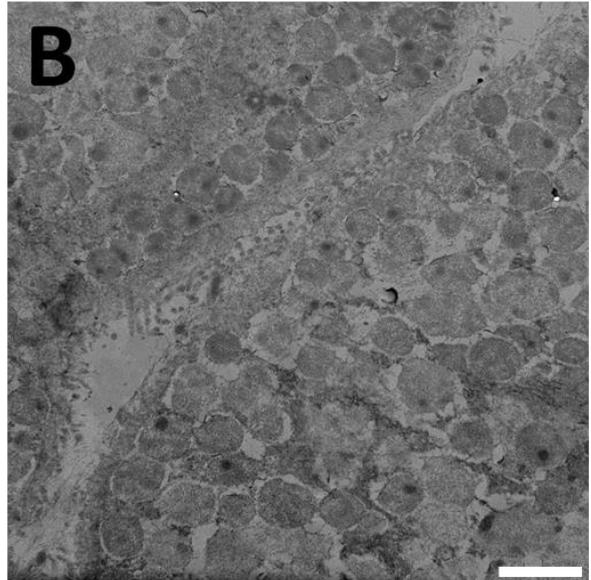
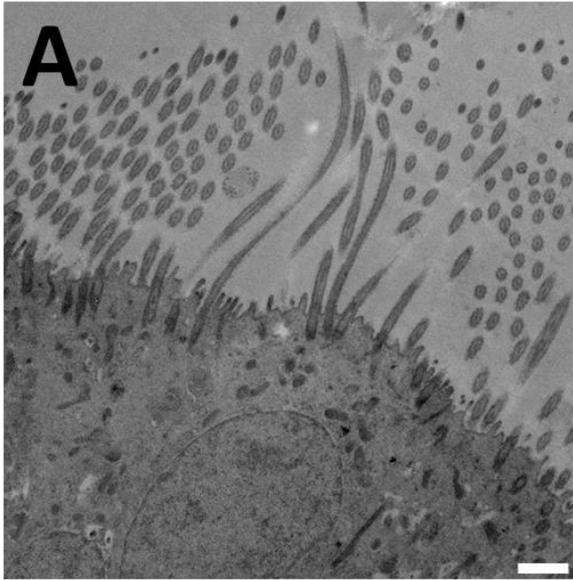
For bacterial pathogens, all samples were negative for *Av. paragallinarum*, MG and *Chlamydia* spp. **Mollicutes 16S rRNA** was positive in trachea (26/34), ceca (23/34), lung (15/34) and oropharynx (3/34) (Ct values between 23.5 and 34.57, average 29.83). Sequencing analysis (280 base pair) conducted on selected positive samples, revealed the presence of a *Mycoplasma* spp. exhibiting a homology of 91.40% to *Mycoplasma iowae* (gene bank accession CP129194.1) in trachea (7/10), lung (3/4) and oropharynx (1/1). In a few trachea (3/10), ceca (3/3) and lung (1/4) samples sequencing was inconclusive due to the presence of a mixed signal. **MS** was detected in trachea (13/34), lung (1/34) and ceca (1/34) (Ct values between 24.26 and 33.44, average 29.20). All positive samples were also positive for *Mollicutes* 16S rRNA. In addition, among positive *Mollicutes* 16S rRNA samples in which *Mycoplasma iowae*-like was detected, trachea (1/10) and lung (1/3) were also positive for MS.

A single trachea was positive for *B. avium* (1/34) (Ct value 30.94).

### **Transmission Electron Microscopy**

Tracheal and bronchial sections obtained from the control bird showed a mucosal epithelium comprised of abundant ciliated epithelial cells and sparse goblet cells. The underlying lamina

propria appeared within normal limits (**Fig. 23A**). In affected birds, trachea (3/3) and bronchi (2/2) exhibited marked mucosal changes including diffuse attenuation and/or loss of cilia and goblet cell hyperplasia (**Fig. 23B**). The underlying lamina propria was expanded with mononuclear cells, predominantly plasma cells (**Fig. 23C**). Heterophils were present in the tracheal lumen, admixed with mucous (**Fig. 23D**). In one bird, electron-dense ovoid-shaped and anucleate organisms, with a granular appearance and approximately 0.5µm wide, were seen closely associated with the cytoplasmic membrane and microvilli of the tracheal epithelium (**Fig. 23E-F**).



**Figure 23.** Ultrastructural changes identified in captive Asian houbara bustards with chronic respiratory disease by transmission electron microscopy (TEM).

Normal tracheal mucosa, comprised of ciliated epithelial cells (**A**) (Bar = 1  $\mu\text{m}$ ). Diffuse attenuation and/or loss of cilia and goblet cell hyperplasia, with marked reduction of the lumen of a bronchus (**B**) (Bar = 2  $\mu\text{m}$ ). Lymphoplasmacells are expanding the lamina propria (**C**) (Bar = 1  $\mu\text{m}$ ), while heterophils are present in the tracheal lumen while (**D**) (Bar = 2  $\mu\text{m}$ ). Electron-dense ovoid-shaped and anucleate organisms, with a granular appearance and approximately 0.5  $\mu\text{m}$  wide, are seen closely associated with the cytoplasmic membrane and microvilli of tracheal epithelial cells (**E-F**) (Bar = 0.5  $\mu\text{m}$ ).

## Discussion

Respiratory infections represent a major issue in animal breeding and a significant diagnostic challenge. They can affect a variety of species, and are usually characterized by a complex multifactorial etiology (237,240,241,245). Here we provided the first comprehensive description of a respiratory form affecting captive Asian houbara bustards, including clinico-pathological and diagnostic findings. Both juvenile (less than 12 months) and adult (older than 12 months) birds, appeared to be affected. The most prevalent agents detected included AOaV-1, *Mycoplasma synoviae* and a *Mycoplasma iowae*-like, sharing a 91.4% homology with *Mycoplasma iowae*, and potentially representing a novel species. Gram negative bacteria, such as *E. coli*, *Pseudomonas* spp. and *Klebsiella* spp., were occasionally isolated from the upper and lower respiratory tract and likely represented secondary invaders.

Several histopathological patterns were identified, often in the same bird, suggesting a variegated etiology consistent with the involvement of a combination of viral and bacterial pathogens. Among the most consistent findings, broncho-interstitial pneumonia and lymphoplasmocytic tracheitis have been observed with several viral agents, including AOaV-1, while BALT hyperplasia and lymphofollicular infiltration have been commonly reported with mycoplasmosis, although not exclusively (179,255).

Based on sequencing analysis, a virulent strain of AOaV-1 was identified in the lung of a bird sampled in September 2022. This finding is consistent with previous studies, showing that captive houbara bustards residing in the UAE are particularly at risk of being exposed to a variety of class II AOaV-1, all sharing an aminoacidic motif consistent with highly pathogenic strains and carried by peridomestic wild birds, visiting the captive breeding sites seeking food, water and shelter (“oasis effect”) (271). The breeding center object of this study is surrounded by artificial forests watered by an irrigation system, inhabited by a variety of wild birds. In spring and summer direct/indirect contacts with wild birds, potentially carrying harmful pathogens (271) may increase, due to the occurrence of the breeding season in several species, and the aforementioned oasis-effect. In addition, several commercial poultry farms, including

at least meat-type and egg-type chickens, and a few processing plants are present within a 15 km radius. The sanitary status and management practices of these poultry flocks is largely unknown.

*Bordetella avium*, a Gram-negative cocco-bacillus responsible of a highly contagious respiratory form in young turkeys, known as turkey coryza, and an opportunistic pathogen in chickens (261) was detected in the trachea of a single bustard by molecular analysis. In addition to poultry, *B. avium* has been detected in asymptomatic wild birds either directly, or indirectly (261,272–274) but it has never been reported in houbara bustards before. The clinico-pathological findings of *B. avium* infection are related with the production of five different toxins: heat stable endotoxin, tracheal cytotoxin, heat-labile toxin, dermonecrotic toxin and osteotoxin (261). The binding and subsequent damage to the respiratory epithelium predisposes to secondary infections. Polymicrobial forms, including also AOaV-1, *Mycoplasma* spp. and *E.coli*, are commonly reported (261). Additional findings suggesting the involvement of *Bordetella* spp. in our study included the presence of cartilage necrosis in the trachea of three birds and the identification of electron-dense elements, consistent with prokaryote organisms, adhered to the tracheal respiratory epithelium by ultrastructural analysis (TEM). In poultry, besides bordetellosis, tracheal degenerative changes have been described with aspergillosis (*Aspergillus flavus*) (275), and following the inhalation of cotton defoliant combined with polymicrobial infections (276). Tracheal trauma, including iatrogenic, may also play a role (277). In our cases, intralesional fungal elements were not identified, while there was no indication of exposure to toxic irritants or occurrence of a traumatic event. The limited PCR detection observed in our cases could have resulted from the chronic course of the disease and the fact that selected birds received antibiotic treatment for 7 to 22 days (average 17 days), provided orally, via nebulization and/or intratracheal administration. In addition, since we relied on PCR primers specifically designed for *B. avium* (95), the involvement of other *Bordetella* spp., such as *B. hinzii* and *B. bronchiseptica*, couldn't be excluded.

The exact role played by the infectious agents detected and their contribution to the respiratory syndrome described, including the possibility of a synergistic effect and the occurrence of a carrier status, requires additional studies. For example, dual infections involving *M. imitans*, a minimally pathogenic mycoplasma species closely related to MG, and IBV can result in extensive deciliation and prolonged inflammation of the respiratory tract of chickens (183,278). Combined infections with MS and *M. meleagridis* lead to sinusitis in turkeys, while airsacculitis lesions are exacerbated by concurrent involvement of AOaV-1 and

IBV, including live attenuated vaccines, adverse environmental conditions and immunosuppression (260).

Serology was not attempted, partially because affected birds received multiple vaccinations against AIV, AOaV-1 and canary pox, and due to the lack of reliable, bustard-adapted, serological tests, except for competitive ELISA and inhibition of hemagglutination (HI) for IAV and AOaV-1. However, over the years, IAV (H9) and AOaV-1 have been detected by serological screening of non-vaccinated sentinel birds and molecular analysis of symptomatic and asymptomatic birds.

The lesions identified in our bustards were indicative of prolonged and/or repeated damaged to the respiratory epithelium, compromising the mucociliary escalator and the structural integrity of bronchial walls (bronchiectasis), predisposing to opportunistic and reoccurring infections. From this point of view, environmental conditions should also be considered among potential contributing/exacerbating factors and investigated further. Based on available epidemiological data (data not shown), respiratory cases occurred more frequently in spring and summer, particularly during the warmer months of the year, and progressively increased with the increase of flock density over the course of the breeding season. Birds destined to be released into the wild and falconry bustards are moved to outdoor tunnels as young as 30 days of age, to become familiar with wild conditions. This is a delicate step, that could result in significant stress, with detrimental effects on the immune system. In summer, environmental temperatures in UAE may reach 49°C (Reneco, weather broadcast 2022). In addition, since avian species lack sudoriferous glands, to cope with elevated environmental temperatures they increase their respiratory rates and promote evaporative cooling through open-mouth breathing (panting) and gular flutter. Panting may predispose to respiratory infections, because the natural filter provided by the nasal cavity is bypassed (246). The Asian houbara bustard is a partial migratory species, wintering in Pakistan, Iran, Iraq and the Arabian Peninsula. Some resident populations can be found in several parts of Central Asia, Middle East and Arabian Peninsula. Birds with a migratory genetic may not be adapted to summer conditions in UAE. Captive-bred birds released in UAE were able to survive and establish resident populations. However, these birds were released in reserves with plantations, providing significant resources in terms of food and shelter (16,279,280).

Morbidity rates were also suspected to increase after sand storms. Exposure to sand and dust, supported by the high prevalence of pneumoconiosis and silicosis in our birds, may have played a role exacerbating clinical signs, following the irritation of an already compromised respiratory epithelium, and contributing to the dissemination of pathogens (281–284). Houbara bustards

are likely to come in contact with dust and sand particles into the wild, considering their natural geographical distribution. However, in the event of a dust storm, captive birds are actually confined inside tunnels and, despite significant efforts made to provide the best environmental enrichments, respecting the natural ecology of the species, tunnel birds may not be able to seek sufficient shield and protection. Additional studies are needed to properly define the role played by environmental factors in the respiratory forms observed.

In conclusion, we were able to show that the investigation of chronic respiratory forms affecting captive Houbara using a multidisciplinary approach, despite some limitations, allowed us to characterize a complex, multifactorial syndrome, raising some questions in terms of etiological and contributing factors involved and defining the direction of future studies.

## **B. Conclusions and future perspectives**

This work was able to shed some light on a complex respiratory syndrome affecting captive Asian Houbaras in UAE. We chose to focus on chronic forms because chronicity and re-infections were considered a significant component of the problem. We also wanted to have an idea of the most commonly and severely affected segments of the respiratory tract, allowing the disease to express its “full pathogenic potential”. Finally, we wanted to prove the feasibility of relying on a multidisciplinary approach in challenging, long-lasting forms.

Our clinico-pathological assessment highlighted significant lesions (lymphoplasmacytic tracheitis, squamous metaplasia of the respiratory epithelium, broncho-interstitial pneumonia with BALT hyperplasia and bronchiectasis, exudative bronchopneumonia) explaining poor response to treatment and clinical relapses. While squamous metaplasia of the respiratory epithelium represents a reversible change, upon removal of the insulting agent/s, bronchiectasis is in fact irreversible. Despite the lack of pathognomonic changes, highlighting a single etiology, several “diagnostic leads” were provided, defining the basis for additional studies.

We detected a potentially novel *Mycoplasma* species exhibiting an homology of 93.19% to *Mycoplasma penetrans* (gene bank accession JN935872.1) implicated in urogenital and respiratory infections in human immunodeficiency virus-infected patients (285–288). The closest avian *Mycoplasma* species, sharing a homology of 91.4%, was *Mycoplasma iowae* (gene bank accession, which is known for inducing reduced hatchability, embryo mortality, leg abnormalities and airsacculitis in turkeys (260). The next step would be to properly identify and characterized this agent, through full genome sequencing and phylogenetic analysis, including the identification of potential virulence genes. Subsequently, its exact role in the respiratory syndrome observed (primary/opportunistic pathogen) would need to be defined, assessing its

prevalence within captive bustard flocks (healthy vs clinically-affected birds), association with the respiratory lesions observed, and tissue distribution, combining, specifically-designed molecular and in situ-detection methods.

Proper interpretation of bacteriological findings in Houbaras requires a good understanding of their normal microflora. A single study assessed the composition of the microflora of the upper respiratory tract (nasal passages) and conjunctiva of healthy bustards maintained in captivity (289). Overall, Gram-positive organisms (*Micrococcus* spp., *Aerococcus* spp., coagulase negative *Staphylococcus* spp., *Bacillus* spp.) predominated in both anatomical sites, while Gram negative bacteria (*Providentia rettgeri*) were occasionally recovered from the nasal cavity, while *Mycoplasma* cultures were all negative. Unfortunately, tracheal samples were not part of the investigation.

To evaluate the role played by bacterial pathogens, other than *Mycoplasma*, bacteriology was performed in a limited number of cases. This choice was based on the chronic stage of infection, combined with a history of prolonged antibiotic treatment. While the majority of samples was negative for bacterial growth after 48hrs of incubation, Gram negative bacteria, including *E. coli*, *Pseudomonas* spp. and *Klebsiella* spp. were occasionally recovered from the respiratory tract and likely represented secondary invaders.

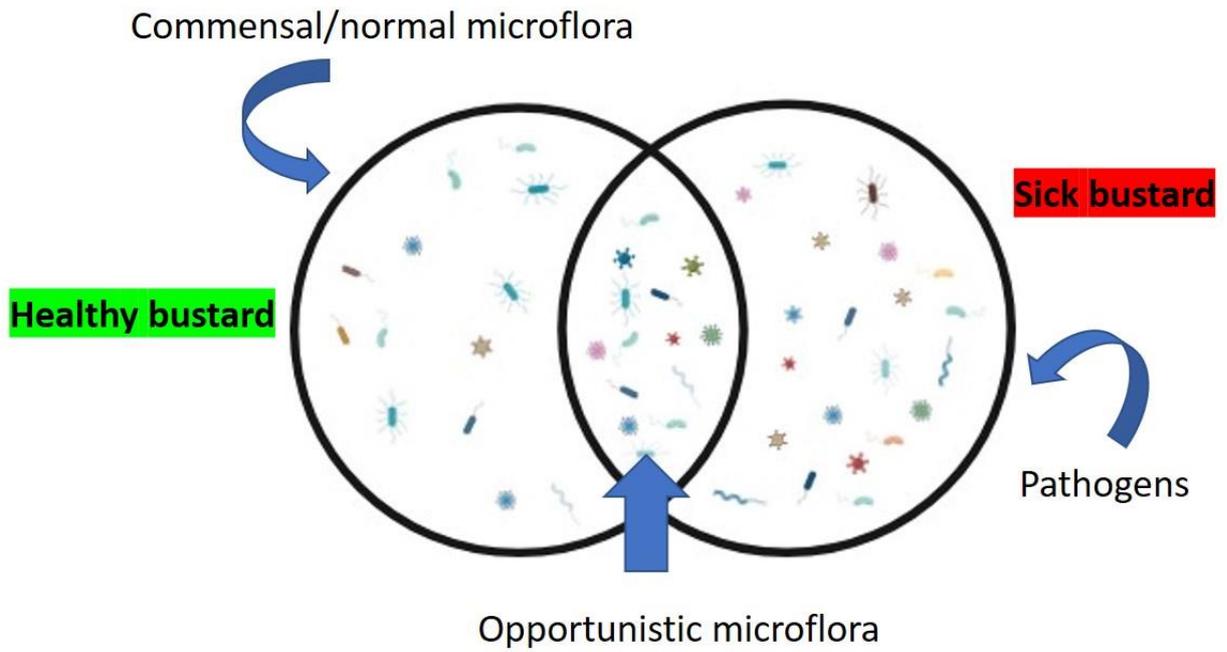
We relied on molecular analysis to assess the involvement of slow growing, fastidious organisms, including *Mycoplasma* spp. The presence of gross and/or microscopic lesions of rhino-sinusitis in some bustards and the proximity to several poultry farms, questioned the involvement of *Av. paragallinarum*, a Gram-negative bacterium belonging to the *Pasteurellaceae* family, responsible of infectious coryza in chickens, pheasants, peacocks and Temmick's tragopans (262). However, no positive birds were detected by PCR (92). On the other end, *Bordetella avium*, was detected from the trachea of a single bird with tracheitis (95) and its involvement was further suggested by histopathological and TEM findings. Based on these results, diagnostic protocols and bacteriology operative procedures were adapted, including the re-introduction of blood agar media, previously discontinued starting from 2018. The following year (2023) tracheal swabs were collected from birds with respiratory disease and plated on blood agar. Subsequently, bacterial colonies consistent with *Bordetella* were isolated and three different species, *B. avium*, *B. hinzii* and *B. bronchiseptica* were confirmed by 16S rRNA sequencing analysis. Interestingly, tracheal samples collected from bustards dying of non-respiratory causes, such as trauma, were also positive for *Bordetella* spp. by aerobic cultures. In chickens, *B. avium* is commonly recovered from the respiratory tract of healthy individuals and is considered an opportunistic agent. In turkeys, both *B. avium* and

*B.hinzii* can result in primary respiratory forms (261). *Bordetella bronchiseptica* is a well-known respiratory pathogen in mammals, with a zoonotic potential (290–293). It is one of the etiological agents of kennel cough, in dogs (237), the PRDC and atrophic rhinitis in pigs (294,295), suppurative bronchopneumonia in cats (296) and respiratory infections in laboratory animals (297). It has also been detected in wildlife, including avian species, with no apparent disease (298,299). Additional studies are needed to properly define the role of *Bordetella* spp. in the respiratory tract of Houbaras (primary or opportunistic agents), the occurrence of a carrier status and potential sources of exposure (poultry, wild birds). The first step would be to implement molecular screening based on species-specific PCR (93,95) targeting both healthy and symptomatic (respiratory) Houbaras as well as peridomestic wild birds visiting tunnels for food and shelter. This approach would allow to evaluate a large number of birds, with a shorter turnaround time for the results, compared to cultures. *Bordetella* spp. are slow growing bacteria, producing small, non-hemolytic colonies after 48 hours of incubation that could be easily shadowed by the overgrowth of (261). In addition, with this strategy, potential issues related with the overgrowth of fast-growing bacteria (for example *E. coli* or *Pseudomonas aeruginosa*), or post-mortem contaminants (*Proteus*) would be overcome.

In terms of viral agents, in addition to properly characterize the AOV-1 detected by full genome sequencing and phylogenetic analysis and to have an overview of the viral strains circulating in the bustard flocks, over time, as part of the on-going epidemiological study, it would be interesting to expand the panel of diagnostic targets, particularly in regards to avian coronaviruses (aCoVs). Based on the frequent and severe involvement of trachea and primary bronchi and the proximity to poultry flocks, infectious bronchitis virus (IBV) was considered among our list of differential diagnoses. IBV is an enveloped, single-stranded RNA virus belonging to the genus *Gammacoronavirus*. It is responsible of significant economic losses to the poultry industry worldwide, resulting in respiratory forms, egg production issues and nephritis (183,256). Other clinically-significant *Gammacoronaviruses* in birds include turkey coronavirus (TCoV), the etiological agent of an enteric form in young turkeys (poults) known as « mud fever » (300), guineafowl coronavirus (gfCoV) including fulminating enteritis and pancreatic degeneration in guineafowl (301) and pheasant coronavirus (PhCoV) associated with respiratory and renal disease in pheasants (256). *Gammacoronaviruses*, including IBV live vaccines, have been detected in a variety of wild and captive birds and the number of positive avian species is continuously increasing (256,302–304). However, except for Galliformes, detection of viral RNA was not associated with disease (256). An undefined avian coronavirus (*Gammacoronavirus*) was detected in African houbara bustards exhibiting respiratory signs,

alone or in combination with LPAI H9N2 but further characterization was not attempted (162). Viruses belonging to a recently described genus, *Deltacoronavirus*, have been detected in several wild birds in different countries, mostly belonging to the order Passeriformes, as well as an Asian houbara bustard in UAE (Dubai). However, no clinical signs were reported in sampled birds (305–307). The only exception is represented by the isolation of a deltacoronavirus from a green-cheeked Amazon parrot (*Amazona viridigenalis*) presenting anorexia, regurgitation and maldigestion, at the clinical exam, and revealing a thin-walled and dilated proventriculus and gizzard, at necropsy (308). From a practical point of view, the use of broad range or « Pan » Coronavirus PCR (one-step RT-PCR) would expand our diagnostic capacity (126,309). However, a positive detection in clinically-affected birds should be followed by the application of *in situ* detection tools, such as IHC or RNAscope-ISH, using custom-designed antibodies or probes, to assess the presence of the viral target in lesional tissues.

Considering the multifactorial nature of the respiratory syndrome described in Houbaras, an important development would be to define the « pathobiome » of affected birds and to compare it with the microbiome of healthy individuals, using a metagenomic approach (**Figure 24**). This strategy could allow us to explore the complexity of microbial interactions in the respiratory tract of Houbaras, assessing the occurrence of perturbations or displacements of the normal microflora in diseased birds (128,310). An epidemiological study would further support this analysis, identifying potential contributing and exacerbating factors (ex. outdoor vs indoor housing, exposure to sand storms, extreme environmental temperature.) and, as a result, critical control points. Comparing and « crossing » the microbiota composition of different groups, according to the impact factors identified could provide useful insights to unveil the pathogenesis of the **bustard chronic respiratory syndrome (BCRS)** and identify the most effective management and control strategies.



**Figure 24.** Investigation of the microflora of the respiratory tract of Houbaras (created in BioRender.com).

Assessing the composition of the microflora of the respiratory tract in both healthy and sick birds would help to identify pathogens, opportunistic agents and commensal microbes, and to study microbial interactions.

## CHAPTER 3: FINAL DISCUSSION

The present work highlights the complexity of diagnostics when dealing with spontaneous field cases and the need of relying on a multidisciplinary approach, sequentially combining different techniques based on the hypothetico-deductive model, to increase diagnostic sensibility and accuracy. This strategy is particularly important for endangered species maintained in captivity, for which diseases susceptibility and clinico-pathological presentation are only partially characterized and documented.

### A. Limitations and points of strength of the study

#### 1. Limitations

In terms limitations, the **chronic stage** of disease, combined with antibiotic treatment in some of the birds object of this study, may have prevented the detection of primary etiological agents. Moreover, **local regulations and restrictions** related with the import of biological products and the shipping of bacterial isolates prevented serotyping and further characterization of *E. coli* isolates originating from Morocco.

In the present study, we relied solely on direct diagnostic methods. However, **serology** is also an important part of the diagnostic investigation and is essential for disease surveillance, and to assess the efficacy of vaccination programs. Unfortunately, the only reliable tools currently implemented in captive Houbaras, monitoring non-vaccinated sentinels, include competitive ELISA and inhibition of hemagglutination (HI) for IAV and AOaV-1. Proper assessment of the serological response of Houbaras against other relevant pathogens is currently hampered by the lack of species-specific reagents. There is a need for additional studies focusing on the validation of serological assays in the Houbara.

Finally, a complete analytical **epidemiological study**, although on-going, was not fully available. Solid epidemiological data should be considered an important pre-requisite of any diagnostic investigation. They help selecting the most representative individuals, defining the list of differential diagnosis and identifying potential risk and contributing factors.

#### 2. Strengths

A major point of strength of our work is its methodology. Through the sequential combining of classical and novel diagnostic tools we were able to improve the etiological diagnosis of emerging and re-emerging conditions in the Houbara, defining the direction of future research efforts. An important aspect of this approach is the fact that it allows to

approximate Koch's postulates, limiting the need of conducting experimental infections, representing an ethically questionable option for endangered species.

This work significantly expanded the overall knowledge of the pathology of an endangered avian species. Specifically:

- We presented the first description of **H5N8 HPAI natural infection** in captive Houbaras. This is important because HPAIV are evolutive pathogens and can result in significant changes in terms of pathobiological traits and lesional patterns. Prompt identification of cases is crucial to ensure the implementation of the most effective control strategies. For this reason, the present work helps understanding clinical presentation, tissue tropism, and common lesions of H5N8 HPAI in Houbaras, ameliorating diagnostic procedures and surveillance activities.
- We detailed macroscopic and microscopic lesions associated with **peritonitis and salpingo-peritonitis** in captive Houbara breeders. In addition to the pathological characterization of these conditions, we could propose presumptive pathogenesis (ascending infection) associated with the detection of multiple *E. coli* strains, supporting the involvement of management factors. We also identified changes consistent with cystic oviductal hyperplasia, discussing potential risk factors involved (age, hormonal imbalance, chronic inflammation) and questioning its role in the development of genital infections.
- We provided an overview of a **chronic respiratory form** in outdoor captive Houbaras, identifying predominant pathological patterns, as well as potential etiological and contributing factors. The identification of severe, chronic irreversible changes, explained poor response to treatment and clinical relapses. Compared to acute forms, the number of studies on chronic respiratory infections in avian species is more limited (311,312), except for the chronic respiratory disease associated with *Mycoplasma* infections in poultry (260,313,314). As regards non-avian species, a few studies described histopathological lesions in cattle and sheep with chronic pneumonia (315–317) or documented the evolution of pulmonary lesions in experimentally-infected ruminants (315–317). Romero et al. compared chronic pulmonary lesions observed in cattle with those reported in humans with chronic pulmonary disease (318). Our data encourage to pursue and complete the syndromic surveillance to identify specific time-frame and clarify the role played by the infectious agents detected (primary or secondary pathogens) and other contributing factors (environmental conditions and dust exposure).

We enriched the **diagnostic tool box** of the Houbara:

- **Development of RNAscope ISH.** The possibility of relying on a sensitive and customizable tool, less affected by autolytic changes, compared to conventional IHC, is extremely relevant. The extreme temperatures that characterized the environment of captive Houbaras in summer can accelerate autolytic changes, with a detrimental effect on tissue morphology and immunoreactivity. RNAscope ISH could support the etiological role of infectious agents identified by culture and/or molecular analysis.
- **Storage and shipping of FFPE tissues.** Based on our results, FFPE blocks could become the diagnostic sample of the future for the Houbara. Compared to fresh or formalin-fixed samples, requiring specific packaging and permits related with infectious/toxic risks, they are easily shipped and can be stored at room temperature. They can be used for standard histopathology, special stains, in situ detection methods, as well as molecular analysis, overcoming issues related with the isolation of pathogens.
- **FFPE-based and molecular pathology.** FFPE tissues allow target sampling, visualizing representative areas exhibiting specific lesions and/or intralesional agents and they can be used for defining the microbiome and pathobiome of selected individuals and tissues by metagenomic analysis. Since metagenomic analysis is time consuming and costly, its use in a routine diagnostic setting is more limited. However, sequencing data could be used for defining PCR assays, including multiplex assays, to be implemented under field conditions for diagnostic and surveillance purposes and in situ detection methods to assess the presence of an infectious agents in lesional tissues. A significant example of this application is provided by the characterization and etiological investigation of lymphoproliferative neoplasms in the Houbara, currently in progress. In this study, classical pathology, histopathology and immunophenotyping by IHC of neoplastic tissues are integrated by metagenomic analysis conducted on FFPE tissues (**Annex**).

#### **A. General diagnostic guidelines**

Thanks to our study, we can provide some practical guidelines for investigating pathological entities in the Houbara. The first step is to obtain a complete clinical and epidemiological overview of the problem, to select the most representative individuals and formulate diagnostic hypothesis. Sudden and unexplained deaths should always be investigated for AI. A complete necropsy should be performed, as soon as possible to limit the occurrence of autolytic changes, refrigerating or freezing carcasses in the event of prolonged delay. The post-mortem exam

should be conducted systematically, starting with an external examination of the carcass after moistening the feathers with soapy water. Birds with suspect chlamydiosis and avian influenza should always be examined under a laminar flow hood or, wearing specific personal protective equipment. An external exam, combined with palpation of neck, keel, wings and limbs, is essential to assess the nutritional status of the animal and the presence of traumatic lesions. Dry skin, firmly adhered to underlying muscles is another indication of dehydration. Soiled periorbital and crown feathers, accumulation of dry exudate around the eyelids, nostrils and swollen sinuses are indicative of a respiratory problem. Pasted vent with feces and excessive amounts of urates can be seen in the event of diarrhea and dehydration. Specific recommendations, based on the system/organs of intestine are detailed in **table 10**.

**Table 10.** Specific necropsy and sampling recommendations

<b>Organ/system</b>	<b>Specific recommendations</b>
<b>Brain</b>	Remove the organ from the skull. In the absence of visible lesions, a section of the cerebro-cortex should be saved for molecular analysis, reserving the rest of the organ for histopathology.
<b>Digestive tract</b>	There is no crop
<b>Nasal cavity</b>	Cross-sectioned it at its base, as close as possible to the eyes, and assess the status of nasal turbinates and infraorbital sinuses.
<b>Trachea</b>	Perform a longitudinal section starting from the laryngeal inlet, to evaluate the mucosa and the presence of intraluminal exudate. Culture the middle tract, avoid collecting swabbed portions for histopathology
<b>Air sacs</b>	Assess their status at the opening the coelomic cavity and before removing the digestive tract. Culture at the very beginning.
<b>Lungs</b>	Section the parenchyma, following the course of primary bronchi, to evaluate the presence of mural thickening, bronchiectasis, exudate or consolidated areas
<b>Genital tract</b>	Assess the status of the ovary (well developed, small, collapsed, consolidated, cystic follicles); Assess the status of the oviduct in situ, then isolate it from the coelomic cavity. Open it longitudinally, throughout its length, to assess the mucosa and luminal contents
<b>Bursa of Fabricius</b>	It lies dorsally to the cloaca (urodeum), like in poultry. It is round, dark grey to pink-gray in color and has a granular appearance
<b>Cecal tonsils</b>	Multiple mucosal nodules (islands) located at the base of the ceca

Mottled spleen, liver and pancreas and fibrinous peritonitis should arise a suspicion of HPAI. Bacteriological sampling should be performed at the beginning of the necropsy, avoiding excessive manipulations to limit contaminations. For the same reason, the digestive tract should

be opened at the end. For *Bordetella* spp. isolation, respiratory swabs (nasal cavity, trachea, bronchi/lung) should be plated on blood agar and, depending on availability, Macconkey agar and incubated aerobically at 37°C. Cultures should be examined for up to 72hrs.

All organs exhibiting gross changes should be recorded, describing and documenting lesions following a photography plan (**Table 11**). A magnifying glass and a supplementary light source may be required for optimal assessment. All organs exhibiting gross changes, as well as apparently unremarkable organs involved in the clinical signs observed or reported (for example, the brain in a neurological bird) should be sampled for bacteriology. A representative section should also be collected for histopathology and molecular analysis (stored at -80°C).

**Table 11.** Minimum requirements for documenting necropsy lesions

<b>Photography plan</b>
Overview of the carcass (dorsal decubitus)
In situ overview of affected organ/s
Isolated organ/s*
Cut sections of organ/s or specific lesion/s to show the extension of changes*
Pictures with and without flash
Always include animal identification number and scale

\* place organs on a solid background (suitable colors include black, dark blue, grey)

Systematic sampling should include: oropharyngeal, tracheal and cloacal swabs (molecular analysis) brain, trachea, lung (including primary bronchi), heart, liver, spleen, kidney, duodenum with pancreas tissue sections (histopathology and molecular analysis). In addition to the aforementioned swabs, specific set of organs, defined according the pathological conditions investigated in this study include: **(i) HPAI:** brain, trachea, heart, lung, liver, spleen, duodenum with pancreas, jejunum, ileum with ceca, cecal tonsils, kidney and gonad. **(ii) Peritonitis/salpingo-peritonitis:** trachea, lung, air sacs, heart, liver, spleen, ovary, oviduct (affected segments, minimum magnum and uterus), abnormal oviductal contents, duodenum and other intestinal segments visibly affected, pancreas, kidney. **(iii) Respiratory forms:** brain, eyelid, nasal cavity, trachea, lung (including primary bronchi), air sacs, duodenum with pancreas, kidney, bursa of Fabricius, thymus and cecal tonsils.

Molecular analysis should be performed based on clinico-pathological and microbiological findings as well as epidemiological data, relying on species-specific or Pan PCR assays

(oriented approach). Unbiased next-generation sequencing (NGS) and metagenomics approach could help identify novel infectious agents in clinical samples as well as FFPE tissues blocks (319–321). In situ detection methods should be implemented to assess the presence of pathogens of interest in lesional tissues, selecting samples based on histopathological and molecular findings.

### **B. Diagnostic challenges in the modern era of captive breeding**

The Houbara is facing new dynamic challenges. On one end, the expansion of captive breeding activities, supported by continuous technological advancements, should be accompanied by a proportionate increase of management resources to maximize breeding performances, while ensuring elevated biosecurity and sanitary standards. On the other end, artificialization and human development of previously depopulated areas could result in the emergence of novel infectious hazards. For example, the breeding centers in the UAE were strategically created in a desertic area, located 70 km from the nearest city. However, over the last years, several commercial poultry farms, including both meat-type and egg-type chickens and a few processing plants, were established within a 15 km radius. The sanitary status and management practices of these poultry flocks have been largely unknown. The movement of poultry wastes/litter and live birds at the end of the production cycle, from the poultry house to the processing plant, can pose significant treats for diseases transmission (322–325). In addition to the proximity to poultry flocks, the occurrence of direct and indirect contacts with wild and peridomestic avian species, supported by the « oasis-effect » produced by the captive breeding facilities in desertic areas, provide a fertile ground for pathogens transmission, evolution and adaptation (271,326,327) and call for enhanced biosecurity and sensitive and reliable diagnostic tools for screening purposes. Environmental and meteorological factors should also be considered as part of this dynamic context. Adverse environmental conditions, including poor air quality, exposure to toxic gas and dust as well as extreme temperatures can promote respiratory infections in poultry (247,248). Exposure to airborne dust, carried by winds, and high temperatures are considered risk factors for the development of invasive bacterial disease, such as *Neisseria meningitidis*, in people residing in sub-Saharan Africa (281,284). Climate changes can significantly impact the evolution of infectious diseases. They can affect, directly or indirectly, vectors distribution, birds' migration, in terms of migratory timing and latitude, ecology and physiology, allergens and pollution patterns, as well as commercial practices. These factors can lead to new infectious disease patterns, transmission dynamics and evolution of pathogens (326,328–333).

In conclusion we showed that combining classical and novel diagnostic tools we were able to significantly improve the etiological diagnosis of emerging and re-emerging conditions in the Houbara. This approach should be promoted to study sanitary issues in other endangered species, characterized by a limited availability of samples.

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## ANNEX

### **Metagenomic analysis as a diagnostic game changer in a multidisciplinary context: the example of lymphoproliferative neoplasia in captive Houbaras.**

#### **Introduction**

Lymphoproliferative disorders have been extensively studied and characterized in poultry and a viral etiology has been recognized. Spontaneous forms have also been reported in adult chickens, although rarely (102,334). Among non-poultry species, passerines and psittacines appear to be commonly affected by lymphoid neoplasia, but a viral etiology has yet to be established (82–84). Similarly, the role played by infectious agents in the development of lymphoid tumors in birds of prey has not been defined (84,85).

As regard Houbaras, lymphoproliferative forms have been sporadically reported. The first reports mentioning lymphoid leucosis were solely based on post-mortem findings identified in imported or captive individuals in UAE (60,196). Between 2000 and 2004, seven cases of lymphoproliferative disease were identified in a flock of captive Asian Houbaras residing in Saudi Arabia, based on histopathological assessment (335). However, characterization of the neoplastic lesions observed by immunostaining was not attempted. In 2011, a retrospective study conducted on 1082 Asian Houbaras over a 10 years period, between 1999 and 2009, identified a total of 19 cases of lymphoid leucosis (prevalence 0.95%) (336). Histopathology and immunostaining for T (CD3) and B lymphocytes (CD79) revealed neoplastic lymphoid cells, mostly CD3 positive, severely effacing the original architecture of several tissues. Viral isolation on frozen organs obtained from affected birds was inconclusive and screening of a captive breeder flock in UAE, using an antigen ELISA test (Flockcheck ALV-Ag, IDEEX, UK) on cloacal swabs, didn't reveal positive birds (60).

The diagnosis of lymphoproliferative disorders in poultry typically relies on a stepwise approach combining standard (epidemiology, clinical history, gross pathology and histopathology) and advanced criteria (immunophenotyping of neoplastic cells, molecular analysis and viral isolation) (102). Proper diagnosis is essential to ensure the application of the most effective control measures. Marek's disease has been controlled by widespread vaccination, as early 18 days of incubation (*in ovo*), combined with enhanced biosecurity and selection of genetically-resistant individuals. On the other end, due to the lack of vaccines, lymphoid leucosis has been controlled through eradication, eliminating positive birds from breeding flocks and ensuring strict sanitation practices. A similar approach is applied to reticuloendotheliosis, despite the low prevalence of the disease (82,102).

## **Object of the study**

In order to better understand the clinico-pathological presentation and etiology of lymphoid neoplasia in captive Houbaras and secure conservation efforts, we characterized a series of cases, combining gross pathology, histopathology, immunohistochemistry and metagenomic analysis on FFPE blocks.

## **Materials and methods**

We worked on a total of 18 cases, registered between February and October 2022, including 10 Asian Houbaras and 8 African Houbaras originating from UAE and Morocco, respectively.

A complete necropsy was performed between 24 and 48 hrs. after death. Lesions were recorded and documented. Sections of brain (n=5), peripheral nerves (n=3), skin (n=6), larynx (n=4), lung (n=16), heart (n=8), liver (n=18), pancreas (n=7), spleen (n=17), thymus (n=4), kidney (n=8), pharynx (n=1), esophagus (n=5), proventriculus (n=6), intestine (n=14), ovary (n=), testis (n=6), adrenal and thyroid gland (n=4) were collected at necropsy and fixed in 10% neutral buffered formalin, paraffin-embedded and sectioned at 3 µm. Slides were routinely stained with hematoxylin and eosin (H&E) and examined under light microscopy.

For the detection of T and B lymphocytes, immunohistochemistry (IHC) targeting CD3 (cluster of differentiation 3, part of the T-cell receptor) and CD268/BAFF-R (B-cell activating factor receptor) were performed on selected cases, based on histopathological findings and degree of autolytic changes (337). For CD3, the immunohistochemical protocol included a heat-induced epitope retrieval step (HIER, 30 minutes at 95°C in EDTA buffer, pH 9) (PT link, Agilent Technologies), followed by a peroxidase blocking step (5 minutes at room temperature - RT) (S2023, Dako) and by the saturation of non-specific binding sites with normal goat serum (X0907, Dako) (25 minutes at RT). Subsequently, slides were incubated with a mouse monoclonal antibody raised against human CD3 (clone F7.2.38, Dako) applied at a 1:50 dilution, for 50 min at RT. Immunostaining was visualized using the EnVision FLEX HRP detection system (Dako Omnis) with 3,3'-diaminobenzidine (DAB) as a chromogen (Dako).

For CD268/BAFF-R, a similar protocol was used except for the HIER (30 minutes at 95°C, citrate buffer, pH 6) (PT link, Agilent Technologies). Slides were incubated with a mouse monoclonal antibody raised against chicken CD268/BAFF-R (clone 2C4, Bio-Rad) applied at a 1:1000 dilution, for 50 min at RT.

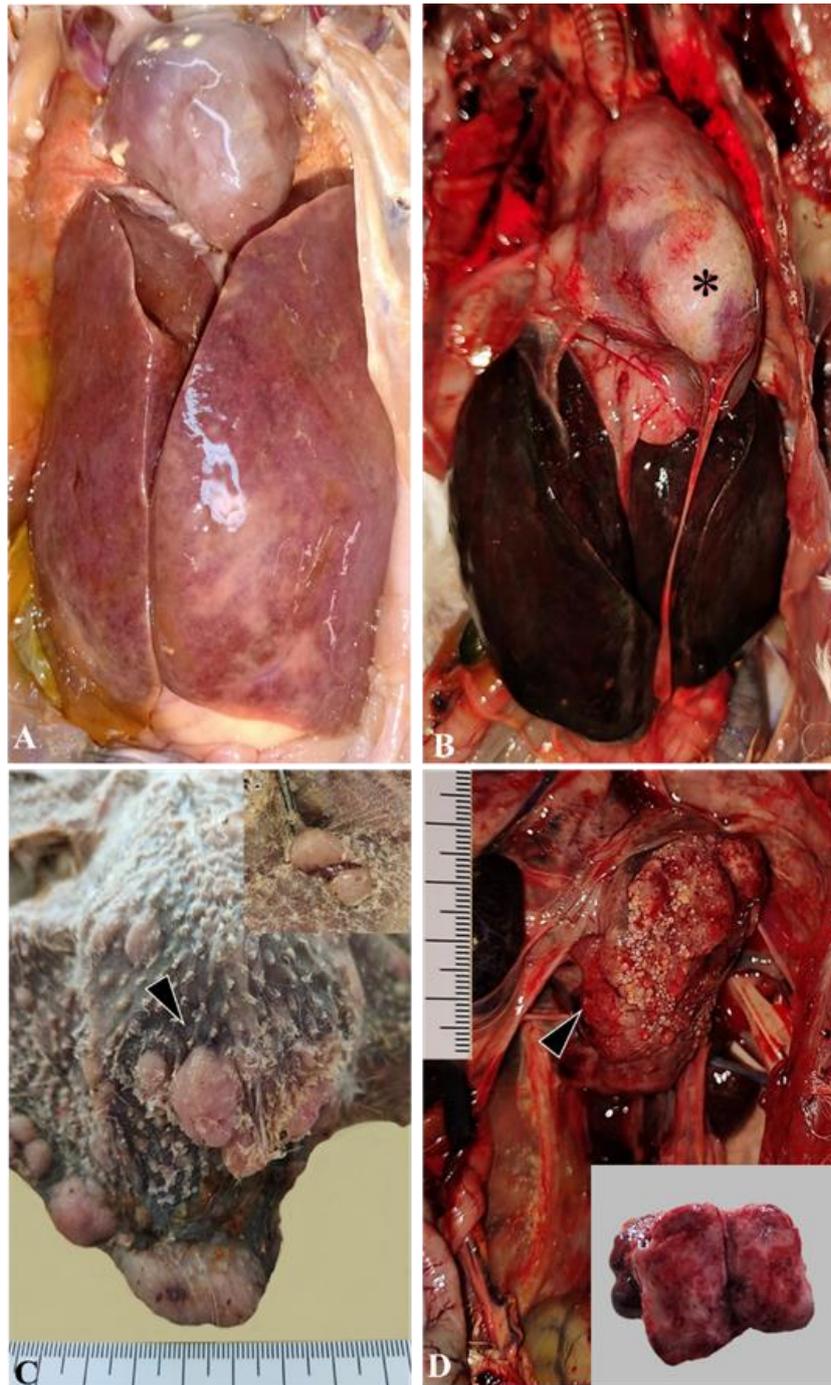
Cases were classified as B or T cell lymphoma based on the dominant immunoreactivity of neoplastic cells (> 70%). Non-B/Non-T cell neoplasia was mentioned in case of lack of immunoreactivity. Inconclusive cases resulted from the occurrence of non-specific staining, preventing proper assessment, while in the presence of extensive autolytic changes, immunostaining was not attempted.

Four FFPE liver samples, including three neoplastic birds diagnosed with B-cell lymphoma, T-cell lymphoma and Non-B/Non-T neoplasia, respectively, and a non-neoplastic bird that died due to acute trauma, used as a negative control, were selected for metagenomics analysis (RNA and DNA fraction).

## Results

Affected bustards ranged between 1 and 16 years of age (average of 7 years). The overall clinical picture included progressive weight loss, reduced activity or sudden death with no clinical signs prior to the *exitus*. Chronic dermatitis with feather loss (1/18) and cutaneous pox (1/18) were occasionally mentioned in the clinical history.

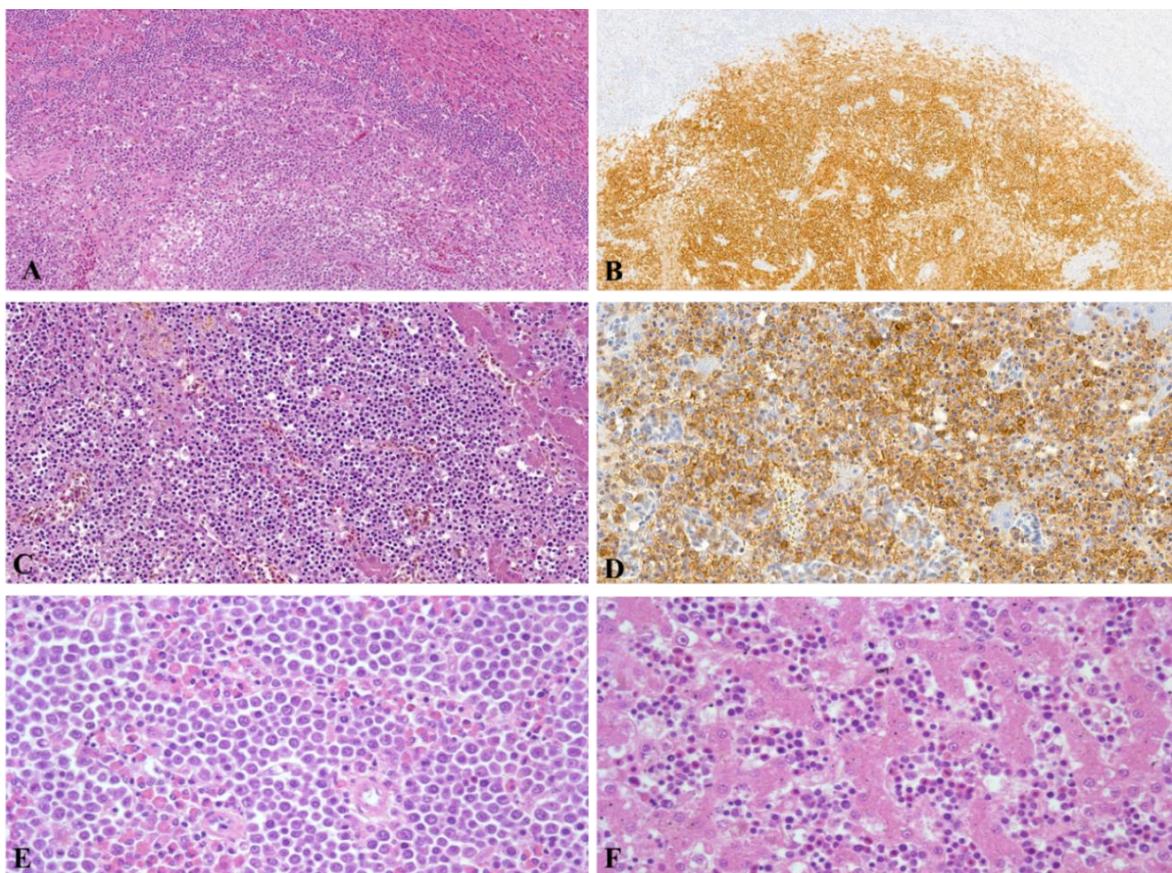
At necropsy, the majority of birds (11/18) were emaciated, while severe coelomic distension with ascites (1/18) and hydropericardium (1/18) were rarely present. Hepato-splenomegaly (10/18) with a diffusely pale or marbled parenchyma were commonly observed. Multiple nodules, pink-white in color and with a lardaceous appearance, were noticed in liver (7/18), lung (5/18), spleen (3/18), kidney (2/18), heart (2/18) and gastro-intestinal tract (4/18) (**Fig. 25A-B**). Similar nodules were seen infiltrating the feather follicles of neck, dorsum and tail region (1/18), the subcutis of the neck region (2/18) and extending to thymus and thyroid glands (1/18) (**Fig. 25C**). In one case the bone marrow appeared diffusely pale brown in color and had a firm, rubbery consistency. The ovary and adrenal gland were replaced by a large polygonal pink mass, relatively soft and exhibiting necrotic-hemorrhagic areas at the surface of cut (1/18) (**Fig. 25D**). Additional lesions included hemorrhagic spleen (1/18), dark and firm liver (1/18), mottled liver (2/8) and spleen (1/18) and multiple hemorrhages scattered in the intestinal walls (1/18).



**Figure 25. Necropsy findings identified in captive Houbaras diagnosed with a lymphoproliferative disorder.**

Diffusely enlarged liver with a mottled appearance (A). Multifocal to coalescent white nodules scattered within the heart (asterisk) (B). Several pale pink nodules, with a lardaceous, glistening appearance at the surface of cut (insert), are infiltrating the feather tracts of the dorsum and tail region, upon feather removal. The melanic skin is a physiological trait of houbara bustards (C). The ovary and possibly the adrenal gland are replaced by a voluminous lardaceous mass, exhibiting necrotic-hemorrhagic foci at the surface of cut (insert). Regressed pin-point ovarian follicles are still recognizable on the surface (arrowhead) (D).

Microscopically, neoplastic changes were identified in all cases examined (18/18). Among sampled organs, spleen (16/17), liver (17/18), intestine (10/14) and lung (8/16) were most commonly affected. Autolytic changes prevented detailed assessment of cellular morphology in 8/18 cases. In the rest of the cases (10/18), sheets of round to polygonal cells, relatively monomorphic (6/10) or pleomorphic (4/10), were seen forming nodules or diffusely infiltrating multiple organs, obliterating the original architecture. Neoplastic cells appeared medium-large in size, had a round basophilic nucleus with finely stippled to clumped, chromatin, one to two nucleoli, variable amounts of basophilic to eosinophilic cytoplasm with no visible granules (lymphoblast morphology) (**Fig. 26**).



**Figure 26. Histopathological and immunohistochemical findings identified in Houbaras. diagnosed with a lymphoproliferative disorder.**

Locally extensive infiltration of the hepatic parenchyma by neoplastic lymphoid cells with loss of the original architecture obtained from two different birds. Hematoxylin and eosin (H&E) stain (**A**, **C**). The vast majority of infiltrating cells exhibit a strong membranous positivity for CD268 (**B**) or CD3 (**D**). The bone marrow is extensively infiltrated by neoplastic cells. Islands of extra-sinusoidal granulopoiesis are still visible (**E**). Neoplastic cells are also seen expanding the hepatic sinusoids (leukemic form) (**F**) and extending to the adjacent parenchyma (not shown).

Immunohistochemistry allowed the identification of four different groups, including round cell neoplasia, B cell lymphoma (**Fig 26B**), T cell lymphoma (**Fig 26D**) and Non-B, non-T cell neoplasia with a lymphoblast morphology (**Table 11**). The larger group (8/18, 44%) was the round cell neoplasia, which included cases exhibiting non-specific immunohistochemical staining (4/8) and cases for which IHC was not attempted due to marked autolytic changes (4/8). B-cell lymphomas accounted for 27% of cases (5/18), followed by Non-B/non-T cell neoplasia with a lymphoblast morphology (16% 3/18) and T cell lymphomas (11%, 2/18).

### **Preliminary discussion**

In this study, autolytic changes prevented proper morphological and immunohistochemical assessment of neoplastic lesions in 44% of cases. In the rest of the birds examined, T cell lymphomas accounted for 11% of cases, while B cell lymphomas and Non-B/non-T neoplasia with a lymphoblast morphology were identified in 27% and 16% of cases, respectively. As regards the Non-B/non-T neoplasia group, lack of immunoreactivity may have resulted from prolonged fixation or advanced autolytic changes. However, this hypothesis was discarded by the presence of internal positive controls. Other possible explanations include the loss of conventional B and T cell markers in neoplastic lymphoid cells, or the occurrence of myeloid leukosis. Myeloblasts, are relatively uniform in size, lack eosinophilic cytoplasmic granules and may be difficult to differentiate from lymphoblasts.

In this study, CD268/BAFF was used as a B cell marker. Other markers, such as CD78 and CD20 tend to have variable and inconsistent results in avian species (83). PAX5 (pair box protein 5) was considered an acceptable option but, compared to CD268/BAFF, but provided inferior results. In the future, it would be interesting to assess the suitability of other B cell markers, such as MUM-1 (multiple myeloma 1 protein).

Overall, our findings depicted a complex pathological picture, more variegated compared to the one provided by available literature (60,196,335,336). Altogether, gross pathology, histopathology and immunohistochemistry were not sufficient to provide strong etiological insights.

**Metagenomic analysis on selected liver tissue sections is currently in progress and may help to provide additional information.**

**Table 11.** Overview of immunohistochemical findings identified in captive Asian and African houbara bustards diagnosed with a lymphoproliferative disorder

Histopathological and Immunohistochemical findings																												
Bird ID	Species	Gender	Age (years)	Histopathological Diagnosis	CD3	CD268	Spleen	Liver	Intestine	Lung	Kidney	Pancreas	Esophagus	Proventriculus	Skin	Heart	Bone marrow	Testis	Ovary	Adrenal	Thymus	Thyroid	Oviduct	Larynx	Pharynx	CNS	PNS	Comments
							1	1	1	1	0	NA	NA	NA	0	0	NA	0	0	NA	0	/	NA	NA	NA	/	NA	
1	<i>C. macqueenii</i>	M	12	B cell lymphoma	20%	80%	1	1	1	1	0	NA	NA	NA	0	0	NA	0	/	NA	NA	NA	/	NA	NA	NA	0	
2	<i>C. macqueenii</i>	M	11	B cell lymphoma	20%	80%	1	1	1	0	1	1	NA	NA	NA	NA	NA	1	/	NA	NA	NA	/	NA	NA	NA	NA	
3	<i>C. macqueenii</i>	NA	4	B cell lymphoma	30%	70%	1	1	1	0	NA	0	NA	NA	NA	0	NA	NA	NA	NA	NA	NA	NA	0	NA	NA	NA	
4	<i>C. macqueenii</i>	NA	11	T cell lymphoma	90%	10%	NA	1	NA	1	NA	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	
5	<i>C. macqueenii</i>	NA	9	Non-B/non-T cell neoplasia	-	-	1	1	NA	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	
6	<i>C. macqueenii</i>	NA	4	Non-B/non-T cell neoplasia	-	-	1	0	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	
7	<i>C. macqueenii</i>	NA	8	round cell neoplasia	IN	IN	1	1	1	0	NA	NA	1	1	NA	NA	NA	NA	NA	1	NA	NA	NA	NA	NA	NA	0	
8	<i>C. macqueenii</i>	M	1	round cell neoplasia	NT	NT	1	1	0	1	1	NA	NA	0	0	0	NA	0	/	NA	0	0	0	0	NA	0	NA	
9	<i>C. macqueenii</i>	NA	12	round cell neoplasia	IN	IN	1	1	NA	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	
10	<i>C. macqueenii</i>	NA	7	round cell neoplasia	NT	NT	1	1	0	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	
11	<i>C. undulata</i>	M	3	B cell lymphoma	30%	70%	0	1	0	1	1	NA	0	0	0	0	NA	1		NA	NA	NA	0	NA	NA	NA		
12	<i>C. undulata</i>	F	5	B cell lymphoma	20%	80%	1	1	1	1	NA	NA	1	1	NA	1	1	/	1	NA	NA	NA	1	1	NA	NA	dermis, subcutis and feather follicles	
13	<i>C. undulata</i>	F	13	T cell lymphoma	70%	30%	1	1	1	1	1	1	1	1	1	1	1	/	1	NA	0	0	0	NA	NA	0	0	subcutis
14	<i>C. undulata</i>	M	8	Non-B/non-T cell neoplasia	-	-	1	1	1	1	1	1	NA	NA	1	1	1	/	1	1	1	/	NA	NA	1	NA	severe intravascular, leukemic form	
15	<i>C. undulata</i>	NA	2	round cell neoplasia	NT	NT	1	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA		
16	<i>C. undulata</i>	NA	16	round cell neoplasia	IN	IN	1	1	0	0	1	NA	NA	NA	NA	NA	0	NA	NA	NA	NA	NA	NA	NA	NA	NA		
17	<i>C. undulata</i>	NA	2	round cell neoplasia	NT	NT	1	1	1	0	NA	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA		
18	<i>C. undulata</i>	M	1	round cell neoplasia	IN	IN	1	1	1	1	0	1	1	1	0	NA	0	/	0	1	1	/	NA	1	0	0		

1= affected, 0=not affected, NA = not available

NT = not tested by IHC; IN = inconclusive IHC



**Titre :** Utilisation d'une approche multidisciplinaire pour déterminer l'étiologie de syndromes cliniques chez une espèce aviaire menacée : l'exemple de l'Outarde Houbara

**Mots clés :** Outardes, Houbara, Conservation, Diagnostique, Multidisciplinaire, Pathologie

**Résumé :** Dans le travail présenté ici, nous avons évalué l'intérêt d'une approche diagnostique multidisciplinaire pour l'investigation d'entités pathologiques mal caractérisées en utilisant l'Outarde Houbara comme espèce modèle. Cette espèce fait l'objet de plusieurs projets d'élevages conservatoires en Afrique du Nord, au Moyen Orient et en Asie centrale. Nous nous sommes intéressés à trois entités pathologiques ayant toutes le potentiel de mettre en péril les efforts de conservation : l'influenza aviaire hautement pathogène (IAHP), les infections génitales chez les oiseaux inséminés artificiellement et un syndrome respiratoire affectant les oiseaux élevés à l'extérieur et devant être renforcé pour les populations sauvages. Pour la première entité, nous avons validé l'utilisation de l'hybridation *in situ* par RNAscope pour détecter le gène de matrice des virus influenza aviaires dans des tissus fixés au formol et inclus en paraffine. Cela nous a permis de décrire de manière exhaustive et pour la première fois un épisode infectieux à virus IAHP H5N8 chez l'Outarde Houbara, qui s'est manifestée par des formes cliniques suraigues à aigues de la maladie, associées à un pantropisme tissulaire, avec endothéliotropisme et neurotropisme viral. Pour la seconde entité pathologique, nous avons caractérisé une série de cas de péritonite et salpingo-péritonite associés à des infections par *Escherichia coli*. La diversité des profils de virulence de la bactérie, identifiée par biologie moléculaire, suggère l'implication de nombreuses souches. Les formes chroniques étaient prédominantes et une infection ascendante a été suspectée. De plus, l'examen histologique a permis l'identification de modifications tissulaires compatibles avec une hyperplasie kystique endométriale, pouvant intervenir comme facteur de risque dans le développement des infections génitales chez l'Houbara. Enfin, pour la troisième entité pathologique, nous nous sommes concentrés sur un syndrome respiratoire multifactoriel d'évolution essentiellement chronique. Divers agents pathogènes ont été détectés chez les oiseaux affectés, y compris une espèce potentiellement nouvelle de Mycoplasme. Des facteurs environnementaux, comme le stress thermique et l'exposition à des tempêtes de sable sont considérés comme des facteurs contributifs importants. Nous avons montré dans ce travail que nous améliorions considérablement le diagnostic étiologique des affections émergentes et réémergentes chez l'Outarde Houbara par une approche multidisciplinaire. Cette approche devrait être encouragée pour étudier la santé d'autres espèces menacées, notamment lors d'une disponibilité limitée en échantillons.

**Title:** The use of a multidisciplinary approach to investigate the etiology of clinical forms in an endangered avian species: the example of the Houbara Bustard

**Key words:** Bustards, Houbara, Conservation, Diagnostic, Multidisciplinary, Pathology

**Abstract:** In the present work, we assessed the use of a multidisciplinary diagnostic approach to successfully investigate poorly characterized pathological entities using an endangered avian species as a model: the Houbara Bustard. This species is the object of several captive breeding operations located in North Africa, the United Arab Emirates and Central Asia. We focused our attention on three conditions that could potentially jeopardize conservation efforts: high pathogenicity avian influenza (HPAI), genital infections in artificially-inseminated breeders and a respiratory syndrome affecting outdoor birds destined to be released. For the first condition, we successfully validated an RNA scope *in situ* hybridization (ISH) assay for the detection of the avian influenza A virus matrix gene in formalin-fixed and paraffin-embedded (FFPE) tissues. We then provided the first comprehensive description of HPAI H5N8 natural infection in the Houbara, resulting in hyperacute and acute forms exhibiting marked tissue pantropism, endotheliotropism and neurotropism. For the second condition we characterized a series of cases of peritonitis and salpingo-peritonitis. Chronic forms predominated and an ascending infection was highly suspected. Most of the cases were associated with the isolation of *Escherichia coli*. The identification of a variety of virulence profiles by molecular analysis of selected bacterial isolates suggested the involvement of multiple strains. Furthermore, histopathology allowed the identification of changes consistent with cystic oviductal hyperplasia, expanding the list of potential risk factors involved in the development of genital infections in the Houbara Bustard.

For the third condition, we were able to shed some light on a multifactorial respiratory syndrome, focusing on long-lasting, chronic forms. A variety of viral and bacterial pathogens were detected, including potentially a novel *Mycoplasma* species. Environmental conditions, such as heat stress and exposure to dust storms, were considered significant contributing factors. We showed that combining classical and novel diagnostic tools we were able to significantly improve the etiological diagnosis of emerging and re-emerging conditions in the Houbara. This approach should be promoted to study sanitary issues in other endangered species, characterized by a limited availability of samples.