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Laboratory findings on the health status of the endemic rock-partridge (*Alectoris graeca whitakeri*) population during a two-year conservation programme in Sicily

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SUMMARY

Sicily (Italy) hosts an 'endangered', endemic population of *Alectoris graeca whitakeri*, commonly known as Sicilian Rock Partridge. An EU-funded Life Natura 2000 project has been founded, involving Istituto Zooprofilattico Sperimentale of Sicily for veterinary aspects: a total of 15 Sicilian Rock Partridge found dead were collected, identified and processed by postmortem examination and laboratory investigations. The evidence of internal parasites was the most relevant finding, showing different types of infections by Nematoda, Cestoda and Coccidia. 60 per cent of these cases were infected with more than one parasite. In one single case, a pathogenic strain of *Escherichia coli* related to granulocytic lesions in liver was found and another cause of death was due to respiratory disease caused by *Aspergillus fumigatus*. The study represents the first veterinary report on this rare species and underlines the importance to monitor the health status of wild species in the Italian environment in order to preserve local biodiversity.

BACKGROUND

The genus *Alectoris* includes seven recognised species with a distribution from Southern Europe and Northern Africa to much of Asia and the Arabian Peninsula[1–3] (figure 1). Sicily (Italy) hosts an 'endangered', endemic population classified as *Alectoris graeca whitakeri*,[4] commonly known as the Sicilian Rock Partridge.[5] The Sicilian Rock Partridge is the smallest and lightest subspecies among the *Alectoris* genus. The Sicilian Rock Partridge was first described more than 170 years ago with several clusters associated with Sicilian mountainous regions including Mount Etna and the Madonie mountains.[6] *Alectoris graeca* is considered 'near threatened' by the International Union for Conservation of Nature (IUCN), but the Sicilian subspecies is rather more endangered with an IUCN Red List rating of endangered.[7] The only numerical indication of the Sicilian Rock Partridge population dates from the early 1990s, with an estimate of about 1500 pairs.[8] Another study conducted in the eastern part of Sicily reported a low density in 2013 (0.67 pairs/km^2) compared with 1989 (3.3 pairs/km^2).[9] *A g whitakeri* has been included in Annex I of the 'Birds Directive' (79/409/EEC) as well as Annex III of the Berne Convention, and, in recent years, all subspecies of *Alectoris graeca* have been included in Annex I of the 'Birds Directive' (2009/147/EEC). The major reasons for decline of the Sicilian Rock Partridge are unclear, but likely relate to environmental factors including new intensive agricultural management, progressive urban expansion, periodic fires within its territories and particularly its nesting grounds, massive illegal hunting[10] and more recently wild boars. To date, there have been no published veterinary reports of diseases affecting Sicilian endemic *A g whitakeri*; the few available concern other European and Asiatic rock-partridges as wild cases in *Alectoris rufa*,[11–14] captive/domestic chukars[15–18] and wild chukars,[19 20] with one study on reproductive disturbance in captive *Alectoris graeca saxatilis* associated with nematode infection.[21]

Data on the likely cause of mortality of *A g whitakeri* and the most prevalent pathologies are important because of the limited population size and may inform on the future risk of extinction caused by both non-infectious (eg, trauma/poisoning) and infectious risks. Data collected during this study will be important for future strategic conservation and restocking actions.

CASE PRESENTATION

Fifteen carcases of Sicilian Rock Partridge found dead were collected from the protected area classified as S.P.A. ITA010029 during a Life project on the conservation of *A g whitakeri* in Sicily (Life09 NAT/IT/000099–Sicalecons). All birds were identified for their phenotypic compliance[1] to the endemic subspecies and age was estimated. Sex was determined by the presence/absence of spurs and confirmed by observation of the gonads during postmortem examination. Initially, birds were screened by external examination in order to detect superficial lesions and/or the presence of infestations Oropharyngeal, cloacal and conjunctival swabs were also taken when death was considered to have been recent (ie, within 24–48 hours, according to the season). Swabs were then inserted in Amies transport medium with and without charcoal (Thermo Fisher), transported to the laboratory at 4°C and processed within 24 hours. After postmortem examination, in case of suggestive pathology, sections/

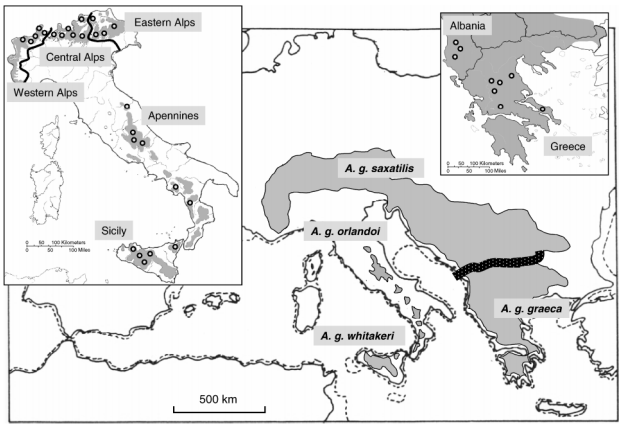

Figure 1 Eurasian distribution of *Alectoris* genus.

fragments of tissues and/or swabs were taken for parasitology, microbiology and/or histological testing following the procedures described below. The intestine was always screened for the presence of internal parasites.

INVESTIGATIONS

External parasites were screened by simple observation, with the help of a brush and a white sheet. Once collected, all samples were fixed in 70 per cent (v/v) ethanol for further morphological identification. All external parasites were identified by morphological keys comparison according to the standards.[22]

Postmortem examination was performed with a Y incision from both shoulders down to the sternum, separating the skin from the rib cage and abdomen and opening the carcase for examination of the coelomic cavity and evaluation of internal organs.[23]

Parasitological investigations

Internal parasites were investigated using a direct mount technique through preparation of smears obtained from faecal samples (collected from the cloaca) and the contents from three sections of the intestine (mid-points of the duodenum, jejunum and caecum). Faecal material collected from the intestine was routinely processed by a saturated sugar (density 1.27 g/mL) flotation technique,[24] observing the upper supernatant phase by direct light microscopy at 100–400× magnification in order to identify eggs in faecal material. Internal parasites were fixed in 70 per cent (v/v) ethanol in order to evaluate characteristic features and morphology using the classification approaches for Nematoda and Cestoda.[25–27] Determination of the prevalences and intensities of each parasite species was calculated for every sample. To collect oocysts directly from the intestines, in case of suspected lesions, scrapings of the mucosa were made and the material rinsed into a beaker with 2.5 per cent (w/v) potassium dichromate solution to release the unsporulated oocysts.[28] The tissue suspension was filtered through cheesecloth into a beaker using a spatula to agitate the suspension. The sticky proteinaceous material present in the caeca was broken down by suspending the homogenate in 10–20 per cent (v/v) sodium hypochlorite solution in an ice-bath for 10–15 minutes.[29] This procedure was useful if clean suspensions of oocysts were required, but the sporulation of oocysts may sometimes be abnormal. Alternatively, homogenised pieces of intestine were treated for 3 hours in 1N sodium hydroxide solution,[30] after which a fairly clean suspension of oocysts was obtained. Oocysts belonging to the *Eimeria* genus were classified for respective species according to their morphology[31] and according to the method proposed for coccidia of poultry,[32] based on both morphological and biological features including intestinal location of endogenous stages, gross lesion appearance, size and shape of the parasite. Semi-quantitative determination of the intensity of coccidiosis infection used an arbitrary score of infection density, designating: 1+ (≤10 oocysts per microscopic field 400×) for dead birds with no evidence of diarrhoea around the cloaca and where small numbers of oocysts were detectable; 2+ (11 to ≤50 oocysts per field) for dead animals with moderate infection and slight diarrhoea and 3+ (more than 50 oocysts per field) for dead birds with acute infection and diarrhoea (visible around the cloacae) and carcase emaciation.

Microbiological investigations

Bacterial cultures were carried out from mucosal swabs (transport swabs with Amies transport medium with and without charcoal, Thermo Fisher) taken from oropharyngeal and conjunctival tracts and from cloaca. Samples were cultured by standard procedures on Columbia agar containing 5 per cent sheep blood (Oxoid Limited, Hampshire, UK), MacConkey's agar (Oxoid, Hampshire, UK) and incubated aerobically at 37°C for up to 48 hours. Contents of the large intestine and faecal samples were also incubated in Buffered Peptone Water (Oxoid, UK) at 37°C±1°C for 16–20 hours, then plated on modified semisolid Rappaport-Vassiliadis agar (Oxoid, UK) for 24 hours and on xylose-lysine-desoxycholate agar (Oxoid, UK) for biochemical identification, according to international standard procedures for Salmonella isolation (ISO 6579). For all other bacteria, including fastidious species, samples of trachea, lung, duodenum and caecum were inoculated directly into tryptone soya agar medium, modified brilliant green medium and Columbia III with 5 per cent SB medium (BD Diagnostic, Diagnostic Systems, Sparks, Maryland, USA) and incubated at 37°C for 72 hours. For isolation of *Mycoplasma* species, samples of trachea and lung were inoculated on to *Mycoplasma* broth base (PPLO, BD Diagnostics, Diagnostic Systems) supplemented with phenol red, glucose, horse serum, nicotinammide adenina dinucleotide, cysteine, thallium acetate and penicillin and incubated at 37°C in an atmosphere containing 10 per cent CO_2 for 7 days according to Ref. 33. *Mycoplasma* broth cultures were then streaked on to *Mycoplasma* agar prepared as above but with 1.3 per cent agar (BD Diagnostics, Diagnostic Systems), maintained under identical conditions and examined daily for 25 days. Lesions were subcultured on to Sabouraud dextrose sugar, which is a medium specific for fungi, and incubated at 25°C and 37°C for 4–10 days. Additionally, portions of the same damaged organs were homogenised and plated on blood agar to screen for possible bacterial coinfection.

Viral diseases were also routinely investigated. Specifically, selected portions of the following tissues were processed by RT-PCR in order to monitor avian influenza from lung and spleen;[34] flavivirus DNA (West Nile Disease) from spleen[35] and coronavirus from lung and oropharyngeal swab.[36]

Histological investigations

All suspected lesions were sampled and fixed in 10 per cent buffered formalin, then embedded in paraffin-wax following standard methods.[37] Four-micrometre-thick sections were obtained using a microtome, then mounted on slides and stained with H&E. In order to detect fungal infection, suspected sections were also stained by PAS (periodic acid-Schiff) and PASM

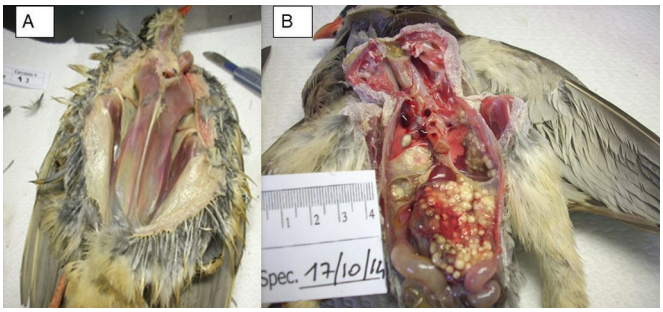

Figure 2 (A) One of the carcases of rock partridges enrolled in the study showing weak body condition. (B) Carcase showing granulomatous lesions on surface of internal organs.

(periodic Schiff-methenamine) to investigate the presence of hyphae infiltrating the tissues.

OUTCOME AND FOLLOW-UP

According to the phenotypic features of all bird carcases, nine females (three adults and six young) and six males (two adults and four young) were confirmed to belong to the endemic subspecies *A g whitakeri*.

At postmortem examination, 7 out of 15 birds showed poor nutritional status, emaciation, ruffled feathers (figure 2A).

One of these birds was affected by severe granulomatous lesions ranging in size from 1 mm to 1 cm in the liver and lungs with presence of blood-serum liquid (figure 2B).

Histologically, granulomas were characterised by a central necrotic area surrounded by histiocytes, lymphocytes and multinucleated giant cells. Avian tuberculosis was excluded by the absence of acid fast bacilli in smears stained with Ziehl-Neelsen and after 4 weeks culture.

Another carcase showed a total loss of breast musculature and a severe pneumonia with large yellowish nodules, ranging in size from 20 to 40 mm, greyish in colour, firm with irregular margins (figure 3A). A dark red area was also observed in

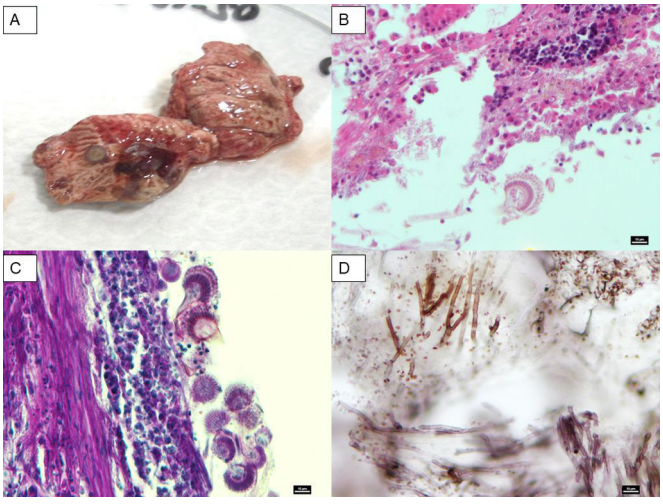

Figure 3 (A) Lungs affected by multinodular lesions. (B) Histological section of the lung's lesion showing septate fungal hyphae infiltrating the respiratory tissue (400×). (C) PAS staining which shows hemispherical vesicles flattened at the top (400×). (D) PASM staining which shows dichotomous, branching filamentous structure of the fungus (400×). PAS, periodic acid-Schiff; PASM, periodic acid-Schiff-methenamine.

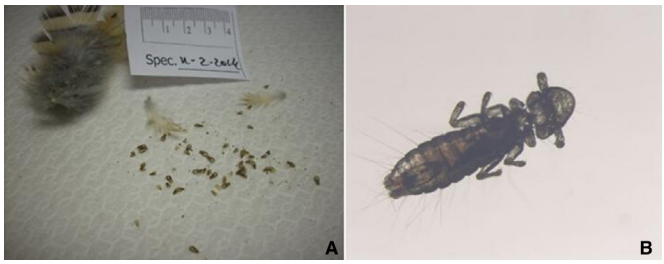

Figure 4 (A) Infestations were picked up using brush against the feather of birds. (B) *Goniodes colchici* at 100× magnification.

the middle lobe of the right lung. Additionally, the serosa of the small intestine appeared moderately hyperemic. Histologically, the nodules presented fungal hyphae spreading from the centre of necrotic lesion, surrounded by lymphocytes and macrophages (figure 3B). Morphology of the fungus[38] was compatible with the genus Aspergillus (ie, presence of a head in long column, long conidiophores, hemispherical vesicles flattened at the top, a single row of phialides, tight and regular globular conidia, septates and dichotomous hyphae). PAS, PASM staining (figure 3B–D) and culture test confirmed infection by *Aspergillus fumigatus*.[39] The histological findings confirmed the lung and related serosae were the primary site of infection.

Parasitological findings: Four out of 15 carcases showed considerable infestation with a small infestation which lacked wings (figure 4a and b), later classified as *Goniodes colchici* (Denny), a species of louse. All carcases showed notable evidence of internal parasite infection (table 1).

Coinfection with multiple parasites was detected in 9/15 (60 per cent) of the carcases, including between two and four parasite species. Coccidial parasites were detected in all birds with varied levels of infection. No significant differences were detected between *Eimeria* species occurrence or level of infection in male and female birds. Intestinal protozoa were the most prevalent parasites detected in *A g whitakeri*, with four different species identified: *Eimeria kofoidi, E. caucasica, E. legionensis* and an as yet unidentified *Isospora* species. All coccidia were detected in the caeca. Parasitological and pathological findings suggest the existence of an association between the intensity of infection and severity of symptoms in the host species, especially when associated with other concurrent parasites. A single Cestode species was observed in four birds: *Raillietina tetragona* (Molin 1858), (figure 5a and b). All birds infected by this taenid exhibited

Table 1 Parasites detected in Sicilian Rock Partridges (*Alectoris graeca whitakeri*), including the site(s) of localisation, the respective rates of prevalence and intensity and the average number of each sex of the parasites found (where relevant)

Parasite	Site of infection	Incidence (per cent)	Parasite burden (male)	Parasite burden (female)
Ascaridia compar	Duodenum	1/15 (6)	2	7
Raillietina tetragona	Small intestine	4/15 (26)	9	14
Goniodes colchici	Body skin	4/15 (26)	43	36
Eimeria caucasica	Caecum	3/15 (20)	Average infection score +1	
Eimeria kofoidi	Caecum	12/15 (80)	+3	
Eimeria legionensis	Caecum	8/15 (53)	+2	
Isospora species	Caecum	5/15 (33)	+2	

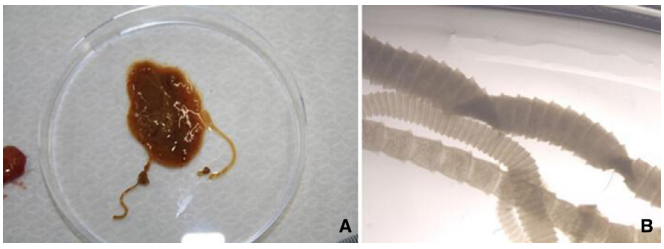

Figure 5 (A) *Raillietina* species removed from the intestine with haemorrhagic and catarrhal intestinal content. (B) Morphological characteristics of *Raillietina tetragona*.

catarrhal enteritis, with different degrees of severity (depending on the size of the infection). The area surrounding the site of the scolex attachment was defined by a yellowish mucous exudate, with the mucosa of the small intestine appearing thickened and showing nodular lesions. One nematode species was detected in a single bird, *Ascaridia compar* (Shrank 1790). Nine worms were found in the duodenum, where the lumen contents presented as a liquid-blood serum. A slight flattening of the duodenal mucosa was observed, likely a consequence of mechanical compression due to the presence of the parasites (figure 6a and b). A large egg with a thin shell was seen to have been retained in the oviduct of the same bird.

Bacteriological findings: All oral-pharyngeal and ocular swabs were negative for *Mycoplasma* and other pathogenic bacteria. For 14 of 15 birds' culture of cloacal swabs did not result in the identification of any pathogenic enteric bacteria. Cultures showed colonies of Gram-positive and Gram-negative microorganisms such as *Proteus*, *Faecal Coliform*, *Eubacterium*, *Lactobacillus*, *Streptococcus*, *Propionibacterium* and *Bacteroides*. *Escherichia coli* was isolated from the only one carcase showing systemic granulomatous lesions: bacterial culture in Tryptic soy broth (TSB) selective media and on Sorbitol MacConkey's agar from tissue fragments and swabs from the lesions confirmed the anatomo-pathological diagnosis of classic coligranulomatosis due to an aggressive strain of *E coli*, later confirmed by standard biochemical procedures.[40] In the bird, Sabourad Dextrose Agar (SDA) culture showed clear growth in 4 days, with the presence of flat, dusty and initially green colonies, which became greyish after a few days, confirming the growth of *Aspergillus* species.

Virological investigation resulted negative for the presence of intercurrent infections.

DISCUSSION

Data collected in this study represent the first report on infections and lesions in the rare *A g whitakeri*. General consideration of the causes of mortality prevalent underlines the importance of environmental condition as a major risk for specie conservation.

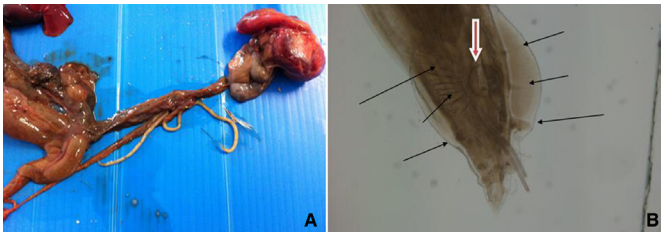

Figure 6 (A) *Ascaridia compar* coming out from partridges' duodenum. (B) *Ascaridia compar*: caudal papillae (black arrows), pericloacal sucker (red arrow) 400×.

Learning points

- The study represents the first veterinary report on *Alectoris graeca whitakeri*.
- All carcases showed notable evidence of internal parasite infection.
- Coinfection with multiple parasites was detected in 60 per cent of the carcases.
- The present work sought to highlight the importance of health status monitoring for wild species in the Italian environment and the collection of information suitable to preserve local biodiversity.

Poor body condition due to multiple parasitosis is probably most important since coligranulomatosis and aspergillosis were represented by one case only.

Parasitic pathogens: It cannot be confirmed whether mortality in any of the Rock Partridges sampled was caused by parasite infection, but it is likely that parasite colonisation compromised effective utilisation of sufficient food, resulting in an aggravated wasting process. The evidence of heavy parasitic infection in carcases of *A g whitakeri* mirror previous observations in other species within the *Alectoris* genus.[12] Moreover, this study reports the first finding of *Ascaridia compar* in Sicilian *Alectoris*, underlining a possible role for *Ascaridia compar* in compromising bird fertility (even in low-level infections, as suggested in Ref. 21, and destabilising the host-parasite system. The low-level occurrence of bacterial and fungal infection in wild *Alectoris* precludes significant conclusion, although they are common among poultry when flock management is poor. The cases detected here may have been exacerbated by stress-related immunosuppression, caused by suboptimal ecological conditions impacting on a species strongly linked to its territory and ethological habits. Microbiological/bacterial findings: *Alectoris rufa*, genetically similar to *A g whitakeri*, is known to be highly susceptible to *colibacillosis*.[41 42] Stressors, such as starvation, thermal and/or migratory stress, toxicosis, adverse environmental conditions or trauma, can cause immunosuppression and promote colonisation of tissues by opportunistic pathogens[44,45].

The present work sought to highlight the importance of health status monitoring for wild species in the Italian environment and the collection of information suitable to preserve local biodiversity. In this modern context, Public Veterinary Health can play a central role in investigating and surveil environmental, toxic, infectious and genetic risks. Advocacy for the One Health agenda has driven development of new approaches for the management of wild species, recognising the importance of parallel environmental, veterinary and human health, as in the case of avian influenza. Several examples of this new 'global health vision' have been reported in regions such as the EU, USA and Australia[44], where the role for the veterinarian has become especially significant in so called 'pathosurveillance', developing from a simple scientific database to an important tool of Public Health. Regional reports concerning morbidity and mortality of wild species should be introduced into a new epidemiological approach to investigate and explain/understand these phenomena, supporting effective risk assessment and planning emergency/contingency actions.

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