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## EMBRYONIC AND NEONATAL MORTALITY FROM SALMONELLOSIS IN CAPTIVE BRED RAPTORS

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**ABSTRACT:** In a captive breeding center near Rome (Italy), cases of embryonic and neonatal death were recorded during the breeding seasons in the European eagle owl (*Bubo bubo*), peregrine falcon (*Falco peregrinus*), buzzard (*Buteo buteo*), and lanner falcon (*Falco biarmicus*). *Salmonella havana* and *S. virchow* were isolated. Three pulli, clinically infected with *S. havana*, were successfully treated with enrofloxacin. From two groups of healthy 3- to 4-wk-old eagle owls, *Salmonella* sp. group 61 (61:r:-) and *S. havana* were collected. A strain of *S. paratyphi* B was detected in a pharyngeal swab and a fecal sample from an adult female goshawk (*Accipiter gentilis*), affected with pharyngeal trichomoniasis. A *S. hadar* strain was collected from a healthy 1-yr-old female eagle owl and *S. livingstone* was isolated from a 1-mo-old female peregrine, dead of an acute respiratory syndrome. Lesions of fibrinous polyserositis and multivisceral congestion were observed. From frozen 1-day-old chicks, on which adult and young raptors were fed, *S. havana* and *S. livingstone* isolates with similar biochemical and drug susceptibility patterns to those isolated from raptors were identified. A surveillance program on infectious diseases reduced embryonic and neonatal death rates in the following breeding seasons.

**Key words:** Captive breeding, embryonic death, neonatal death, raptors, *Salmonella* spp.

### INTRODUCTION

In 1993, a surveillance program on infectious and parasitic diseases was started in the Center for Conservation and Captive Breeding in Campagnano di Roma (Rome, Italy), where several species of raptors (Accipitriformes, Falconiformes and Strigiformes) and some species of wild carnivores (Felidae, Mustelidae) are kept. Detailed clinical examinations and periodic sampling of different materials (blood, feces, cloacal swabs, occasional corpses, non-hatched eggs) from the center are conducted. The samples are submitted for hematological, hematochemical, serological, microbiological, parasitological and gross anatomical and histopathological examinations. Necropsy materials, swabs and non-hatched eggs are screened for viruses, *Mycoplasma* spp., *Chlamydia* spp., bacteria and fungi. The program was started when cases of embryonic and neonatal deaths had occurred in some species of raptors during the 1993 breeding season. The observations made during a 3-yr-period (1993 to 1995) are reported herein. This study was initiated to determine if a particular etiological agent was associated

with these cases and whether a control program could improve hatchability and survival rate of these birds.

### MATERIALS AND METHODS

During April and May 1993, cases of embryonic and neonatal deaths occurred in some breeding pairs of hawks, falcons and owls. Six eagle owl (*Bubo bubo*), four peregrine falcon (*Falco peregrinus*), two golden eagle (*Aquila chrysaetos*), and two buzzard (*Buteo buteo*) pulli ( $n = 14$  birds) were necropsied and submitted for microbiological examinations, along with four peregrine falcons, one eagle owl and one lanner falcon (*Falco biarmicus*) dead embryos. Cultures also were made from fecal samples and cloacal swabs from other pulli with or without symptoms and from all the pairs with unsuccessful broods. This procedure then was extended to all the birds bred in the center; it was repeated monthly until the end of 1993, then performed every three months. Cultures also were made from frozen poultry meat and 1-day-old chicks (DOC) used in the diet of adult and young individuals.

Bacteriological and mycological examinations were conducted on cloacal, tracheal and pharyngeal swabs and on necropsy specimens (brain, heart, lung, liver, intestine). Each specimen was streaked on a plate of blood agar base n. 2 (Unipath Ltd., Hampshire, United Kingdom) containing 5% sheep defibrinated blood,

and on a plate of Sabouraud dextrose agar (Unipath Ltd.) and inoculated in nutrient broth n. 2 (Unipath Ltd.). The blood agar plates and nutrient broths were incubated at 37 C for 24 hr, then screened for bacterial growth. Plates of Sabouraud dextrose agar were incubated at 30 C and screened daily for 2 wk for fungal growth. *Mycoplasma* spp. isolations were attempted using a modified Frey's broth and agar media (Kleven et al., 1991). Swabs from trachea, lungs, air sacs and yolk sacs were inoculated in broth medium, incubated at 37 C, observed daily, then transferred on agar medium after 1 wk. Agar plates were incubated at 37 C and inspected after 3 and 7 days for the presence of *Mycoplasma* spp. colonies with a light microscope. For virus isolations, tissue samples (brain, lung, liver, spleen) were triturated in a tissue grinder and diluted 1:10 in phosphate buffer isotonic saline (PBS), pH 7.2 containing antibiotics (penicillin 2,000 units/ml, streptomycin 2 mg/ml, gentamycin 50 µg/ml, mycostatin 1,000 units/ml). Choanal and cloacal swabs were placed in the same PBS medium and mixed thoroughly. Antibiotic concentration was 5 times higher for cloacal swabs. Samples were centrifuged at 1,000 × g at 4 C for 10 min. The supernatant was transferred, mixed with an equal volume of medium and centrifuged as described. The resulting supernatant was stored at -70 C until attempted isolation. Each sample was thawed at room temperature and inoculated into the allantoic sac (0.2 ml) of 5 specific pathogen-free (SPF) embryonated chicken eggs (SPAFAS Inc., Norwich, Connecticut, USA) of 9 days incubation and onto confluent primary chicken embryo fibroblasts (CEF) monolayers in wells of a 6 well space-saver (Becton Dickinson, Franklin Lakes, New Jersey, USA). Embryonated chicken eggs were incubated at 37 C, and inspected daily for 7 days to observe any dead or dying embryos. After 7 days, embryos were chilled at 4 C and embryos and chorioallantoic membranes were screened for lesions. Amnio-allantoic fluids (AAF) were harvested and tested for hemagglutination activity (Alexander, 1991). Three serial blind passages were made before a sample was considered negative. Chicken embryo fibroblasts were trypsinized and suspended at  $8 \times 10^5$  cells/ml in Eagle minimal essential medium (EMEM) (Gibco Laboratories, Life Technologies, Inc., Grand Island, New York, USA) supplemented with 10% fetal calf serum (Gibco Laboratories) and 5% tryptose phosphate broth. Wells were incubated at 37 C, 5% CO<sub>2</sub> and examined daily for cytopathic effect with an inverted light microscope. When no cytopathic effect appeared within 6 days, the cells and fluids were harvested and centrifuged at

1,000 × g for 10 min. Each supernatant was subinoculated onto CEF monolayer for a second passage. Similarly, three serial passages were made before a sample was considered negative.

Slide impressions of tissues, yolk sacs of dead embryos and inoculated SPF chicken embryos, smears of fecal samples and cloacal swabs were examined for *Chlamydia* spp. with the direct immunofluorescence method (*Chlamydia* direct I.F. Biomerieux, Montalieu Vercieu, France), as previously described (Office International des Epizooties, 1992).

For *Salmonella* spp. isolation, the samples (brain, heart, lung, liver, intestine) were inoculated in buffered peptone water (BPW, Unipath Ltd.) and incubated at 37 C for 24 hr. For enrichment, 1 ml of BPW was placed in Rappaport-Vassiliadis-soy-peptone broth (Unipath Ltd.), and incubated at 42 C for 24 hr. Selective isolation was attempted subculturing 0.1 ml of the enrichment broths on SS-agar (Unipath Ltd.) and MacConkey-agar (Unipath Ltd.), incubated at 37 C for 24 hr. Suspect colonies were then subcultured in triple sugar iron agar (Unipath Ltd.), and finally identified using biochemical (API 20E, Biomerieux) and serological (sera from Difco Laboratories, Detroit, Michigan, USA) tests.

Twelve *Salmonella* spp. isolates were tested with the Kirby-Bauer procedure (Chengappa, 1990) for susceptibility to 12 antimicrobial drugs commonly used in raptor therapy (Table 1).

For histopathology, tissues collected at necropsy were fixed in 10% neutral buffered formalin. These were subsequently embedded in paraffin, cut into 5 µm thick sections and finally stained with hematoxylin and eosin (H&E) for light microscopic examination.

Data from births and deaths were statistically analyzed by means of the chi square test (BMDP Statistical Software Inc., Los Angeles, California, USA).

## RESULTS

During the 1993 breeding season, salmonellae were isolated from 10 dead pulli of the 14 examined (71%) and from two dead embryos of the six examined (33%). Particularly, *S. havana* (13,23:f,g,[s]:-) was isolated from three eagle owl, two peregrine falcon, and two buzzard dead pulli and from two dead embryos (one eagle owl and one lanner falcon), while *S. virchow* (6,7:r:1,2) was isolated from three eagle owl dead pulli. *Salmonella havana*

TABLE 1. *In vitro* susceptibility of 12 *Salmonella* spp. isolates from the Center in Campagnano di Roma against 12 antimicrobial agents.

Antimicrobial agent	Disk potency	<i>S. havana</i> (5 isolates)			<i>S. virchow</i> (2 isolates)			<i>Salmonella</i> sp. 61:r:-			<i>S. ittingstone</i> (2 isolates)			<i>S. hadar</i>			<i>S. paratyphi</i> B			Total % on 12 isolates			
		S <sup>a</sup>	I <sup>b</sup>	R <sup>c</sup>	S <sup>a</sup>	I <sup>b</sup>	R <sup>c</sup>	S <sup>a</sup>	I <sup>b</sup>	R <sup>c</sup>	S <sup>a</sup>	I <sup>b</sup>	R <sup>c</sup>	S <sup>a</sup>	I <sup>b</sup>	R <sup>c</sup>	S <sup>a</sup>	I <sup>b</sup>	R <sup>c</sup>	S <sup>a</sup>	I <sup>b</sup>	R <sup>c</sup>	
Amikacin	30 µg	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	—
Amoxicillin	30 µg	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	—
Ampicillin	100 µg	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	—
Carbenicillin	100 µg	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	—
Chloramphenicol	30 µg	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	—
Doxycycline	30 µg	100	—	—	50	50	—	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	—
Enrofloxacin	5 µg	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	—
Flumequine	30 µg	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	—
Gentamicin	10 µg	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	—
Oxytetracycline	30 µg	—	100	—	—	100	—	—	—	100	—	—	—	—	100	—	—	—	—	—	—	—	—
Spectinomycin	100 µg	40	20	40	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Trimethoprim-sulfamethoxazole	100 µg	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	100	—	—	—

a % Susceptible.

b % Intermediate.

c % Resistant.

also was detected in cloacal swabs and feces of three pulli with clinical symptoms. Sudden death characterized all the 14 lethal cases. The three diseased birds showed lethargy and refused food. *Salmonella* sp. group 61:r:- was isolated also from two fecal pools of three, collected in aviaries where symptomless 3- to 4-wk-old eagle owls were kept. Isolates were not detected from feces or cloacal swabs collected from the breeding pairs. Other pathogens were not collected from necropsy materials or from other samples.

At necropsy, all pulli infected with *S. havana* showed intense multivisceral congestion. Histologically, a severe vasculitis was observed in various tissues (brain, liver, lungs). The birds infected with *S. virchow* had fibrinous polyserositis, duodenal abscessation (one eagle owl pullus) and gelatinous edema in the cervical region (one eagle owl pullus). No relevant lesions were observed in the other dead pulli. Cloacal swabs and fecal cultures from the three birds presenting symptoms (two peregrine falcons, one eagle owl) were positive for *S. havana*. They were treated successfully with enrofloxacin (Baytril® 2,5%, Bayer AG Leverkusen, Germany; 0.4 ml/Kg, s.c. for 1 wk). Another strain of *S. havana* with identical biochemical and similar drug susceptibility patterns (Table 1) was isolated from frozen 1-day-old chicks (DOC), used in the diet of adult and young raptors beginning 2 mo before the onset of reproductive disorders.

In July 1993, a mixed infection of *S. paratyphi* B (4,12:b:-) and *Trichomonas* spp. was observed in an adult female goshawk (*Accipiter gentilis*) affected with a severe pharyngitis. The bacterium was isolated from both pharyngeal lesions and feces.

A strain of *S. hadar* (6,8: z<sub>10</sub>:enx) was the only isolate in 1994. It was obtained from a healthy 1-yr-old female eagle owl. During the year, periodical assays of cloacal swabs and feces, as well as seven dead pulli (five eagle owls, two buzzards), were

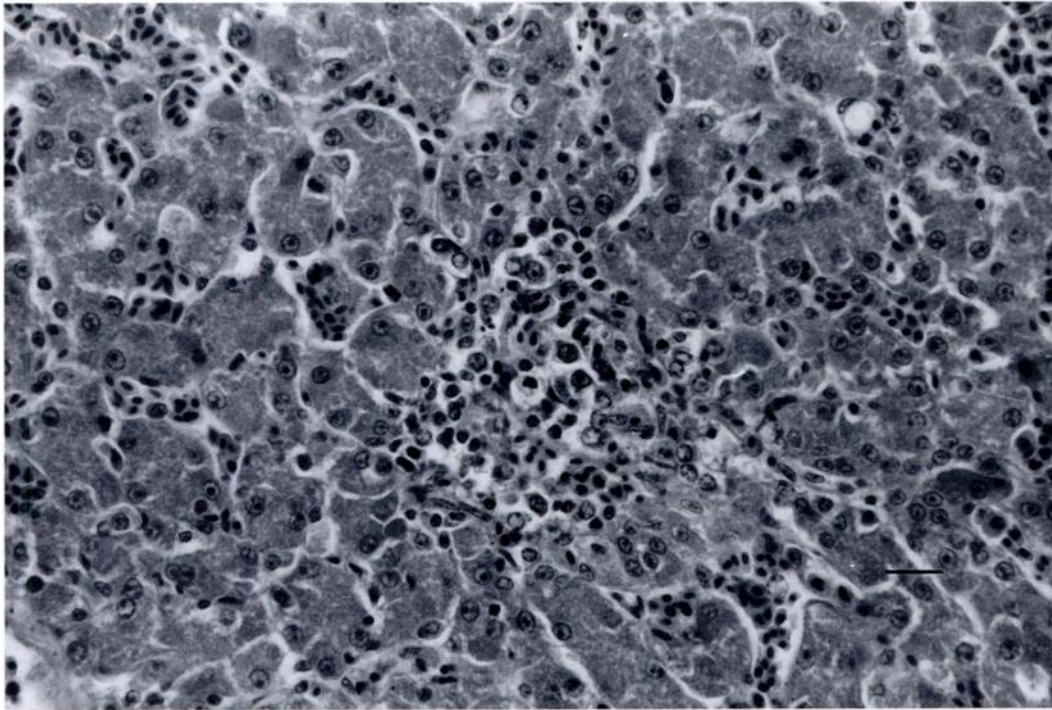


FIGURE 1. A microgranulomatous lesion within the lobular parenchyma of the liver of a peregrine falcon (*Falco peregrinus*). H&E. Bar = 8  $\mu$ m.

negative for *Salmonella* spp. and other pathogens.

In May 1995, a *S. livingstone* (6,7:d:l,w) strain was isolated from necropsy specimens (brain, heart, liver, lungs, intestine) from a 1-mo-old female peregrine falcon. The bird suffered from an acute respiratory syndrome and before the exitus showed torticollis.

In this case, post-mortem findings consisted of a fibrinous polyserositis, also involving the liver and the pericardium. Histologically, the inflammatory exudate was characterized by a marked presence of heterophils. A granulomatous, nonpurulent, multifocal hepatitis was observed (Fig. 1). This was accompanied by a marked congestion of the sinusoids, along with severe thrombosis affecting portal triad vessels (Fig. 2). Bacterial colonies were often seen within thrombi. More chronic lesions evolved through organization of thrombi, followed by vascular lumen obliteration, endothelial proliferative changes,

and thickening of vessel walls. Congestion, associated with less evident thrombotic lesions (Fig. 3), also was found in the lungs. Valvular thromboendocarditis and small foci of purulent myocarditis, along with edema and dissociation of myofibers, was seen in the heart.

Another strain of *S. livingstone* with identical biochemical and drug susceptibility patterns (Table 1) was isolated from DOC which were used as food without being previously screened for *Salmonella* spp. Plasmid typing technique is being carried out on the two isolates referred in the present paper and on another *S. livingstone* isolate from the hatchery where the DOC had been purchased. Other deaths did not occur and other pathogens were not isolated from sampled materials through 1995.

The results of susceptibility tests to 12 chemotherapeutic agents, performed on 12 isolates among those cultured in a 3-yr control program, are presented in Table 1.

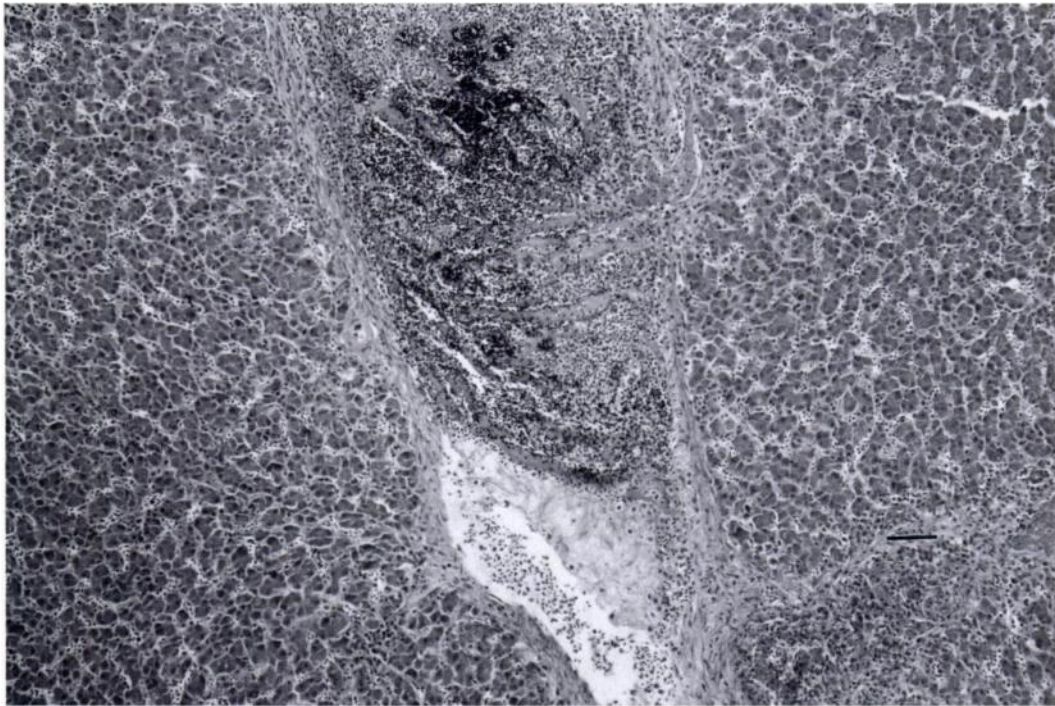


FIGURE 2. Thrombotic lesions partially occluding the lumen of periportal vessels and severe vasculitis are seen in the liver of a peregrine falcon. H&E. Bar = 30  $\mu$ m.

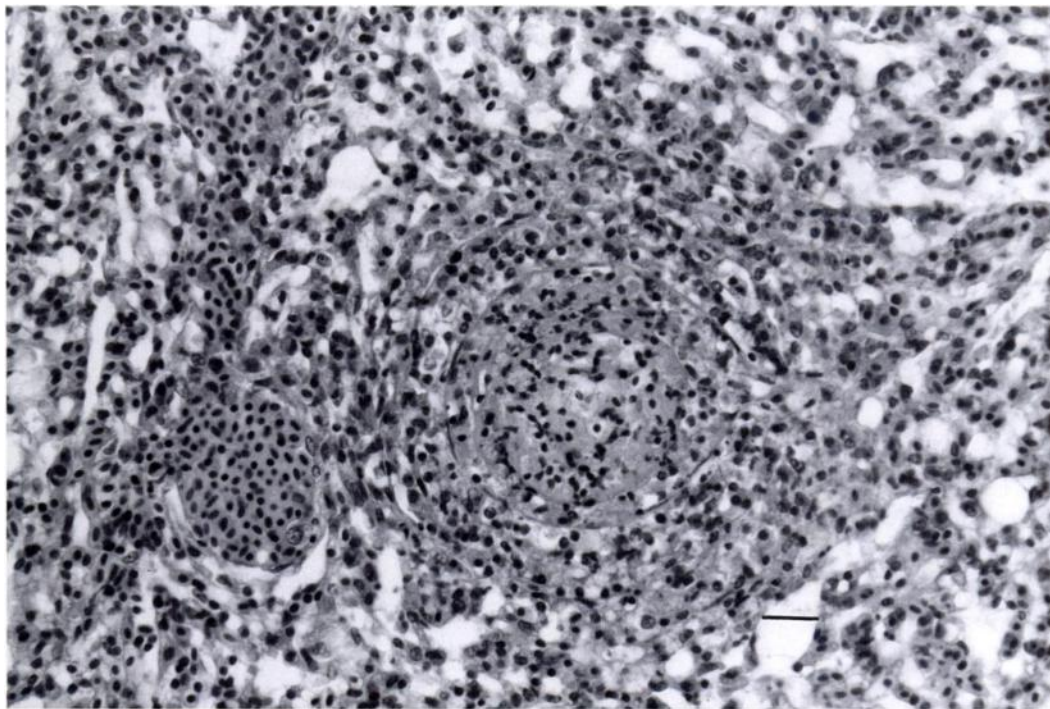


FIGURE 3. Microthrombus formation occurring within blood vessels in the lung tissue of a captive peregrine falcon. H&E. Bar = 8  $\mu$ m.

All the isolates were fully susceptible in vitro to 7 of the 12 (58%) antimicrobial drugs tested. On five *S. havana* isolates tested against spectinomycin, two were susceptible (one from peregrine falcon, one from buzzard), two were resistant (one from lanner falcon, one from eagle owl embryonated eggs), and one was intermediate (from DOC). The lowest susceptibility was observed for tetracyclines (oxytetracycline, doxycycline).

#### DISCUSSION

The cases reported here confirm the pathogenicity of *Salmonella* spp. to raptors (Cooper, 1993; Halliwell and Graham, 1986; Keymer, 1972; Locke and Newman, 1970; Schroeder, 1981; Wilson and McDonald, 1967). *Salmonella havana*, *S. virchow* and *S. livingstone* were isolated from diseased organs (brain, heart, liver, lung, intestine) presenting lesions similar to those described previously (Halliwell and Graham, 1986; Locke and Newman, 1970). Moreover, the recovery of the three *S. havana*-infected pulli after chemotherapeutic treatment with enrofloxacin suggests clinical response to paratyphoid infection. Pathogenicity of these serotypes of *Salmonella* spp. is well documented both in human and in veterinary medicine (Ashdown and Ryan, 1990; Ghosh et al., 1989; Lax et al., 1995; Makarem, 1982; Mehra-bian et al., 1988; Nathwani et al., 1991; Old et al., 1994; Poernomo et al., 1983).

Apparently, ours are the first recorded cases of salmonellosis caused by these serotypes in raptors. Moreover, among different *Salmonella* spp. serotypes cultured from raptors bred in the Center, some were associated with disease, while other serotypes were isolated from healthy birds (*Salmonella* group 61:r:- and *S. hadar*). In the cases occurring in 1993, we observed symptoms and deaths only in pulli <2-wk-old (ranging from 1- to 11-day-old). Furthermore, the only other case of death due to salmonellosis (*S. livingstone*) occurred in 1995 in a 1-mo-old female peregrine falcon. A greater virulence of such sero-

types in young individuals could be hypothesized. Breeding pairs did not show any symptoms and were *Salmonella* spp. negative.

Transmission of *Salmonella* spp. infection in embryos and young birds in the Center can be attributed to shell contamination, direct ovarian transmission or direct infection of chicks with contaminated food. The most important source of egg infection, at least in domestic birds, appears to be shell contamination after fecal contact. Motile salmonellae are able to penetrate the shell and multiply within the egg (Padron, 1990; Nagaraja et al., 1991; Garcia Espinosa et al., 1996). Another important source of embryonic infection is shell contamination from contaminated food carried into nests with eggs, which is a common practice in owls.

Direct ovarian transmission does not seem to be as common as shell penetration, at least in domestic birds (Nagaraja et al., 1991), although isolations of motile strains from the ovary and/or the peritoneal cavity have been reported frequently (Yamamoto et al., 1961; Snoeyenbos et al., 1969). It seems that at least some non-host-adapted serotypes of *Salmonella* spp. cause local infections of the ovary and peritoneum of laying birds, producing egg contamination prior to shell formation. Moreover, strain differences in producing direct egg transmission were observed among strains of the same serotype (Nagaraja et al., 1991).

We isolated strains of *S. havana* in 1993 and *S. livingstone* in 1995 from food (DOC) on which adult and young were fed. Strains of the same serotype, with similar biochemical and drug susceptibility patterns, were previously cultured from dead or diseased pulli. In the first outbreak, the stock of DOC had been used for 2 mo before the occurrence of deaths and hatching failure, therefore we cannot distinguish between embryonic infection and birds infected after the ingestion of contaminated food. The first hypothesis seems to be more likely for the 1- and 2-

day-old dead pulli (50%; 5/10) found positive, as well as for the three of the same age with symptoms. Otherwise, peracute infections should be postulated in these birds to explain sudden deaths or the onset of symptoms after being infected by their first meals. Rodents, birds, reptiles and insects, which frequently are carriers or vectors of *Salmonella* spp., could have been involved in those cases where we could not find the source of infection. In such cases, undetected infections in parents could be retained as the source of infection for their chicks. Carnivores bred in the Center were not fed DOC and their fecal cultures were always negative for *Salmonella* spp.

Regarding antimicrobial susceptibility, we found that none of the 12 strains tested were sensitive to oxytetracycline, and only one (8%) to doxycycline, probably because of the extensive use of tetracyclines in animal therapy in the past decades. Spectinomycin, although recommended in salmonellosis (Weaver and Cade, 1991), was fully effective against only seven isolates (58%). One isolate (*Salmonella* spp. 61:r:-) resulted resistant to gentamicin and one (*S. hadar*) to flumequine, antimicrobial drugs introduced more recently in veterinary therapy.

During 1994 and 1995, we observed a reduction of embryonic and neonatal deaths, in comparison with the 1993 breeding season. Moreover, neither *Salmonella* spp. nor other pathogens were isolated from dead embryos and pulli. The embryonic death rate (EDR) decreased from 22% (10/46) in 1993 to 13% (7/54) in 1994, and in 1995 (7/56). Using the chi square test this decrease was not significant, probably because the sample size was too small. However, there was a significant difference between the neonatal death rate (NDR) <14-day-old, observed in 1993 (39%; 14/36) and the NDR in 1994 (15%; 7/47) ( $\chi^2 = 5.006$ ;  $P = 0.0025$ ). The NDR again decreased in 1995 to 12% (6/49). Both the EDR and the NDR were calculated from the data referring to the different species bred in the Center, all of

them belonging to families of raptors in which death cases caused by *Salmonella* spp. previously have been reported (Keymer, 1972; Schroeder, 1981). The Center for conservation and captive breeding in Campagnano di Roma is a private organization, supported by voluntary assistance. The first objective of this facility is to breed in captivity certain endangered raptor species like golden eagle, goshawk, sparrowhawk (*Accipiter nisus*), peregrine falcon, lanner falcon, and eagle owl for conservation purposes and to produce young birds for reintroduction programs. Up to 1996, the Center has released successfully to the wild 26 pairs of eagle owls and 6 pairs of peregrine falcons. Youngsters to be released are usually put out at hack when they are 42-days-old. By this time, feces, cloacal swabs and choanal swabs are collected and screened for viruses, *Chlamydia* spp., *Mycoplasma* spp., and *Salmonella* spp. at days 7 and 28. If no pathogens are collected and there is no clinical sign of disease, the birds can leave the Center. If any of these pathogens are collected, birds are put in separate aviaries and treated with appropriate antimicrobial drugs. Particularly, if any *Salmonella* spp. isolate is collected even in symptomless birds, they are treated with an antimicrobial agent for 1 wk. Although the isolation of *Salmonella* spp. from healthy raptors may represent just the transitory passage of these bacteria in their intestinal tract and treatment may be inadvisable for some authors (Cooper, 1993), we prefer to adopt this protocol to avoid any risk of introducing pathogens in wild populations. The birds are screened again for the pathogen 7 and 14 days later. If negative and not too old for introduction purposes (the Center does not hack birds more than 8-wk-old) the birds are utilized. If any major viral infection is diagnosed, the birds must be isolated and are considered no longer suitable for release. When a new bird is received, it is kept in a quarantine facility for 30 days and clinically and microbiologically screened (including fecal



smears and cultures for *Mycobacterium* spp.). Our experience indicates that the application of a control program against major infectious diseases can reduce losses in captive breeding.

Salmonellosis seems to be a relatively rare disease in wild raptors and the prevalence of *Salmonella* spp. appears to be low also in captive birds of prey (Cooper, 1985), although both the clinical form as well as the carrier state have been described (Keymer, 1972; Halliwell et al., 1986; Cooper, 1993). In our routine examinations we never observed cases of salmonellosis and we never cultured any *Salmonella* spp. from more than 100 wild birds of prey collected from Central Italy. Since some serotypes can cause mortality in raptor species, this should engender concern about the release of captive bred individuals into the wild without the application of a control program in the breeding facilities. Our approach consists also in the microbiological screening of the stocks of frozen DOC and poultry meat, which are considered a major source of *Salmonella* spp. The aim of this monitoring program is to avoid the release of birds with acute or latent infections which could affect the wild population, or the introduction of birds otherwise suffering from disorders of the reproductive system.

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