Comprehensive Serological Analysis of Two Successive Heterologous Vaccines against H5N1 Avian Influenza Virus in Exotic Birds in Zoos

Júlia Vergara-Alert,1,†* Hugo Fernández-Bellon,2† Núria Busquets,1 Gabriel Alcántara,2 María Delclaux,2 Bienvenido Pizarro,2 Celia Sánchez,3 Azucena Sánchez,4 Natàlia Majó,1,5 and Ayub Darji1

Centre de Recerca en Sanitat Animal (CRESA), UAB-IRTA, Campus de la Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain1; Grupo Veterinario, Asociación Ibérica de Zoos y Acruarios, Secretariat, Parc Zoológico de Barcelona, Parc de la Ciutadella s/n, Barcelona, Spain2; Subdirección General de Sanidad de la Producción Primaria, Ministerio de Medio Ambiente y Medio Rural y Marino, 28071 Madrid, Spain3; Departamento de Enfermedades Emergentes, Laboratorio Central de Veterinaria, Ctra. Algete, km 8, 28110 Algete, Madrid, Spain4; and Departament de Sanitat i Anatomia Animals, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain5

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In 2005, European Commission directive 2005/744/EC allowed controlled vaccination against avian influenza (AI) virus of valuable avian species housed in zoos. In 2006, 15 Spanish zoos and wildlife centers began a vaccination program with a commercial inactivated H5N9 vaccine. Between November 2007 and May 2008, birds from 10 of these centers were vaccinated again with a commercial inactivated H5N3 vaccine. During these campaigns, pre- and postvaccination samples from different bird orders were taken to study the response against AI virus H5 vaccines. Sera prior to vaccinations with both vaccines were examined for the presence of total antibodies against influenza A nucleoprotein (NP) by a commercial competitive enzyme-linked immunosorbent assay (cELISA). Humoral responses to vaccination were evaluated using a hemagglutination inhibition (HI) assay. In some taxonomic orders, both vaccines elicited comparatively high titers of HI antibodies against H5. Interestingly, some orders, such as Psittaciformes, which did not develop HI antibodies to either vaccine formulation when used alone, triggered notable HI antibody production, albeit in low HI titers, when primed with H5N9 and during subsequent boosting with the H5N3 vaccine. Vaccination with successive heterologous vaccines may represent the best alternative to widely protect valuable and/or endangered bird species against highly pathogenic AI virus infection.

Avian influenza (AI) is an infectious disease caused by type A influenza viruses of the Orthomyxoviridae family. AI virus subtypes are classified according to their surface glycoproteins: hemagglutinin (H1 to H16) and neuraminidase (N1 to N9) (9). To date, highly pathogenic avian influenza (HPAI) viruses are restricted mainly to infections with H5 and H7 subtype viruses, which have caused unprecedented morbidity and mortality in birds within the last few years (2). Aquatic wild birds, including Anatidae (ducks, geese, and swans) and Charadriidae (shorebirds), are widely considered to be the natural reservoir of AI virus (13). Although wild birds were not known to be implicated in the initial HPAI outbreaks, in 2002, an outbreak of H5N1 HPAI virus in Hong Kong caused mortality in a wide range of avian species, including migratory birds and resident waterfowls (6). Since then, the H5N1 subtype of HPAI virus has spread throughout Asia and into Europe and Africa, affecting a large number of species. In 2005, an outbreak killed over 6,000 water birds (mainly bar-headed geese [Anser indicus], great cormorants [Phalacrocorax carbo], Pallas’s gulls [Larus ichthyaetus], brown-headed gulls [Larus brunnicephalus], and ruddy shelducks [Tadorna ferruginea]) at the Qinghai Lake National Nature Reserve in northwest China (3). Furthermore, several reports indicate direct bird-to-human transmission in some Asian countries (11, 18). These zoonotic consequences and the ecologic value of protecting avian species have emphasized the need for effective control measures.

Due to unprecedented morbidity and mortality caused by H5N1 HPAI virus and given the value of birds kept in zoos, in 2005 the European Commission directive 2005/744/EC allowed vaccination against AI virus in such birds in zoos, under strict surveillance (7). In the following years, different European countries established preventive vaccination campaigns in zoological institutions. In 2006, 15 Spanish zoos and wildlife centers underwent a vaccination program with a commercial inactivated H5N9 vaccine. Between November 2007 and May 2008, birds from 10 of these centers were vaccinated again with a commercial inactivated H5N3 vaccine, as decided by the Spanish government. The decision of changing the vaccine used in the first AI vaccination program (VP1) was based on experimental results showing that the H5N3 vaccine, a reverse genetics monovalent vaccine, was shown to elicit a strong immune response and protected chickens (10) and ducks (12) from experimental H5N1 infection, with no detection of viral shedding.

The goal of the present study was to compare the seropro-
tection elicited by inactivated H5N9 and H5N3 vaccines and evaluate the boost effect of H5N3 vaccine in inducing immune responses after priming a wide selection of avian species with H5N9 in Spanish zoos.

MATERIALS AND METHODS

Vaccination. An inactivated, commercial, water-in-oil adjuvanted H5N9 (A/CK/Italy/22A/H5N9/1998) vaccine (Poulvac i-AI H5N9-, Fort Dodge Animal Health, Weesp, Netherlands), containing at least 128 hemagglutination units (HAU) according to potency test, was used in zoos during the first AI vaccination program (VP1) in Spain. Vaccination against AI virus in some of the zoos began in March 2006, with the remaining zoos vaccinating up to September 2006. More than 2,600 birds were vaccinated in the 15 zoos participating in this study. The birds were vaccinated twice within a 3-week interval via the subcutaneous route. Eighteen months later, between November 2007 and May 2008, a second vaccination program (VP2) was carried out. At that time, an inactivated, commercial, water-in-oil adjuvanted H5N9 (strain rg-Ack/VN/CS804) vaccine (Poulvac i-AI H5N3-, Fort Dodge Animal Health, Weesp, Netherlands), containing at least 256 HAU, was used. Ten out of the 15 zoos took part in the second vaccination program. More than 450 birds were vaccinated either once (if they had been previously vaccinated with the H5N9 vaccine) or twice (those being vaccinated for the first time). Most of the animals receiving the vaccine for the first time were born after VP1.

Both vaccines are effective against the virus type in circulation and support the DIVA (differentiating infected from vaccinated animals) principle, as the N antigen differs from N1, which makes it possible to distinguish vaccinated birds from H5N1-infected birds while maintaining acceptable efficacy. Further details may be obtained from the manufacturer. In the two campaigns, the vaccine dose administrated was adapted to body weight. Thus, birds with a body weight of <2 kg were given 0.2 ml, those 2 to 10 kg were given 0.5 ml, and those >10 kg were given 1 ml. Published mean body weights of the different species were used instead of using individual weights (4).

Sampling. Blood was collected from the right jugular, brachial, or ulnar vein (left or right). In VP1, samples were obtained on the day of both first (n = 2,672 samples from 17 taxonomic orders) and second (n = 947 samples from 17 taxonomic orders) vaccinations, as well as 9 (n = 933 samples from 17 taxonomic orders) and 18 (n = 542 samples from 16 taxonomic orders) weeks following the first vaccination dose. In VP2, blood was collected on the day of vaccination (n = 469 samples from 16 taxonomic orders) and 6 (n = 398 samples from 14 taxonomic orders) and 12 (n = 376 samples from 15 taxonomic orders) weeks after the first vaccination. In VP2, birds receiving an AI vaccine for the first time (107 out of 469) were revaccinated after 6 weeks (Fig. 1).

The official sampling protocol also included collecting cloacal swabs to detect the presence of AI virus by reverse transcription-PCR (RT-PCR), as described previously (13).

Serology. Sera prior to vaccinations with H5N9 (A/CK/Italy/22A/H5N9/1998) and H5N3 (rg-Ack/VN/CS804) were examined for the absence of total antibodies against influenza A nucleoprotein (NP) by a commercial competitive enzyme-linked immunosorbent assay (cELISA) kit (ID VET, Montpellier, France). The cELISA is based on recombinant AI virus NP as the antigen and a conjugated antibody directed against the NP of AI virus. The assay was performed according to manufacturer instructions.

To evaluate the humoral immune response induced after both vaccinations, homologous H5-specific antibody titers were determined by an HI test by following standard procedures (14). Briefly, chicken erythrocytes and 4 HAU of an H5 antigen (GD-Animal Health Service Deventer, Netherlands) were used for the test. Sera from some bird species may cause agglutination of the chicken erythrocytes used in the HI test, which may mask low levels of HI activity. For that reason, before doing the test, sera from all animals were pretreated with a 50% suspension of chicken erythrocytes for 1 h. Fifty microliters of pretreated serum was diluted by 2-fold serial dilution (1:2 to 1:1,024) in phosphate-buffered saline (PBS) solution in U-bottomed microwell plastic plates (Nunc, Copenhagen, Denmark), and 4 HAU of virus was added to each well. Following incubation at room temperature for 30 min, 50 μl of 0.6 to 0.75% chicken red blood cells (RBC) was added to each well, and the plates were incubated at room temperature for 30 to 45 min to allow RBC to settle. The HI titer was determined as the value of the highest dilution of serum causing complete inhibition of the 4 HAU. Vaccine-induced titers of ≥32 were considered to be a measure of vaccine efficacy, and titers <16 were considered negative according to 92/40/EEC guidelines (8). In poultry, HI titers of >16 were shown to indicate protection against infection when animals were challenged with HPAI H7N7 virus after vaccination with inactivated H7 AI vaccines (17). Since performing challenge experiments in valuable zoo species is not possible and in accordance with the European Food Safety Authority (EFSA), we chose an HI titer of 32 as a threshold of protective vaccine efficacy, as vaccine manufacturers do (5).

To evaluate the specific immune response against an HPAI H5N1 virus strain and to test the breadth of antibody response, postvaccination serum was tested against A/Mallard/It/3401/05 (H5N1) and A/Tky/Eng/647/77 (H7N7).

No adverse reactions to vaccination were reported in any of the participating centers.

Statistical analysis. For each species and for each order, the geometric mean titer (GMT) and the percentage of animals with titers higher than 32 were calculated. Differences of GMT values between orders were tested with the Mann-Whitney test. Statistical analyses were performed using SPSS for Windows, version 17.0.

RESULTS

Humoral response against H5N9 vaccination (VP1). Detailed data concerning humoral immune response against an inactivated H5N9 vaccine from each order and species studied is provided in Table 1. Before receiving the vaccine, only 33 birds out of 2,672 (1.2%) showed antibodies against AI virus NP when tested by cELISA. Similarly, less than 1% of the animals were seropositive for H5 AI virus by an HI test using the homologous antigen. These 25 birds, presenting HI titers of 32 or higher, belonged to four orders (Phoenicopteriformes [n = 19 birds], Anseriformes [n = 3 birds], Ciconiiformes [n = 2 birds], and Pelecaniformes [n = 1 bird]).

HI antibody titers 3 weeks after the first vaccination (at the time of the second vaccination) (n = 947 birds) and 9 (n = 933...
Table 1. Humoral immune response of avian species in zoos, vaccinated twice (within a 3-week interval) with an inactivated H5N9 vaccine (VP1).

| Order            | Species                        | No. of birds | GMT | % of birds with HI titers of ≥32 |
|------------------|--------------------------------|--------------|-----|----------------------------------|
| Anseriformes     | Total                          | 179          | 61  | 67.2                             |
| Mandarin duck    | Aix galericulata               | 1            | 4   | 0                                |
| Egyptian goose   | Anas acrytus                   | 6            | 13  | 33.3                             |
| Northern pintail | Anas acyptea                   | 1            | 256 | 100                              |
| Northern shoveler| Anas clypeata                  | 1            | 256 | 100                              |
| Baikal teal      | Anas formosa                   | 6            | 228 | 100                              |
| Eurasian wigeon  | Anas platyrhynchos             | 2            | 181 | 100                              |
| Mallard          | Anas platyrhynchos             | 8            | 19  | 37.5                             |
| Chiloe wigeon    | Anas sibilatrix                | 9            | 4   | 0                                |
| Greylag goose    | Anser anser                    | 4            | 24  | 57.1                             |
| Swan goose       | Anser cygnoides                | 17           | 234 | 94.1                             |
| Bar-headed goose | Anser indicus                  | 1            | 32  | 100                              |
| Magpie goose     | Anseranas semipalmata          | 1            | 32  | 100                              |
| Canada goose     | Branta canadensis              | 1            | 2,048 | 100                     |
| Barnacle goose   | Branta leucopsis               | 2            | 256 | 100                              |
| Red-breasted goose| Branta ruficollis             | 9            | 299 | 100                              |
| Hawaiian goose   | Branta sandvicensis             | 1            | 512 | 100                              |
| Muscovy duck     | Cariatina moschata             | 16           | 18  | 50                               |
| Mute swan        | Cygnus olor                    | 11           | 53  | 54.5                             |
| Fulvous whistling-duck | Dendrocygna bicolor    | 1            | 1,024 | 100                     |
| Marbled duck     | Marmaronetta angustirostris    | 1            | 12  | 100                              |
| Rosbyll          | Netta peposaca                 | 9            | 299 | 88.9                             |
| Red-crested pochard | Netta rufina                 | 8            | 91  | 62.5                             |
| Knob-billed duck | Sarkidiornis melanotos         | 3            | 51  | 66.7                             |
| Ruddy shelduck   | Tadorna ferruginea             | 6            | 7   | 16.7                             |
| Raja shelduck    | Tadorna radjah                 | 1            | 1,024 | 100                     |
| Common shelduck  | Tadorna tadorna                | 2            | 362 | 100                              |
| Charadriiformes  | Total                          | 17           | 40  | 47.1                             |
| Eurasian oystercatcher | Haematopus ostralegus    | 4            | 23  | 50                               |
| Audouin’s gull   | Larus audouini                 | 1            | 4   | 0                                |
| Caspian gull     | Larus cachinnans               | 5            | 7   | 20                               |
| Pied Avocet      | Recurvirostra avosetta         | 5            | 42  | 60                               |
| Masked lapwing   | Vanellus miles                 | 2            | 64  | 100                              |
| Ciconiiformes    | Total                          | 82           | 14  | 33.7                             |
| Abdim’s stork    | Ciconia abdimii                | 1            | 256 | 100                              |
| White stork      | Ciconia ciconia                | 20           | 13  | 30                               |
| Ibis stork       | Ciconia ibis                   | 3            | 51  | 100                              |
| Scarlet ibis     | Eudocimus ruber                | 18           | 5   | 5.6                              |
| Northern bald ibis| Geronticus eremita            | 4            | 64  | 100                              |
| Marabou stork    | Leptoptilos crumeniferus       | 9            | 13  | 22.2                             |
| Yellow-billed stork| Mycteria ibis                | 1            | 4   | 0                                |
| Roseate spoonbill| Platea alba                    | 3            | 128 | 66.7                             |
| African spoonbill| Platea alba                    | 3            | 128 | 66.7                             |
| African sacred ibis| Threskiornis aethiopicus     | 19           | 15  | 37                               |
| Straw-necked ibis| Threskiornis spinicollis       | 1            | 8   | 0                                |
| Columbiformes    | Total                          | 79           | 6   | 12.5                             |
| Nicobar pigeon   | Caloenas nicobarica            | 6            | 20  | 66.7                             |
| Speckled pigeon  | Columba guinea                 | 7            | 4   | 0                                |
| Rock pigeon      | Columbina livia                | 56           | 5   | 7.1                              |
| Common wood pigeon| Columba palumbus               | 1            | 4   | 0                                |
| Victoria crowned pigeon | Goura victoria          | 2            | 4   | 0                                |
| Barbury dove     | Streptopelia risoria           | 7            | 10  | 28.6                             |
| Coraciiformes    | Total                          | 27           | 5   | 7.4                              |
| Knobbed hornbill | Aceros cassidix                | 2            | 4   | 0                                |

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| Common name                                | Scientific name       | GMT | % of birds with HI titers of ≥32 |
|-------------------------------------------|-----------------------|-----|----------------------------------|
| Mindanao wrinkled hornbill                | Aceros leucocephalus  | 2   | 0                                |
| Black hornbill                            | Anthracoceros malayanus | 2   | 0                                |
| White-crowned hornbill                    | Berenicornis comatus   | 2   | 0                                |
| Great hornbill                            | Buceros bicornis       | 1   | 0                                |
| Rhinoceros hornbill                       | Buceros rhinoceros     | 1   | 0                                |
| Abyssinian ground hornbill                | Bucorvus abyssinicus   | 1   | 0                                |
| Southern ground hornbill                  | Bucorvus leadbeateri   | 8   | 12.5                             |
| Silvery-cheeked hornbill                  | Bycanistes brevis      | 1   | 0                                |
| Trumpeter hornbill                        | Bycanistes buccinator  | 1   | 0                                |
| Gray-cheeked hornbill                     | Bycanistes subcylindricus | 2   | 0                                |
| Laughing kookaburra                       | Dacelo novaeguineae    | 4   | 7                                |
| **Falconiformes**                         |                       |     |                                  |
| **Total**                                 |                       | 75  | 42                               |

| Common name                                | Scientific name       | GMT | % of birds with HI titers of ≥32 |
|-------------------------------------------|-----------------------|-----|----------------------------------|
| Cinereous vulture                         | Aegypius monachus      | 3   | 8                                |
| Steppe eagle                              | Aquila nipalensis      | 3   | 51                               |
| Verreaux’s eagle                          | Aquila verreauxii      | 1   | 128                              |
| Red-tailed hawk                           | Buteo jamaicensis      | 1   | 16                               |
| Variable hawk                             | Buteo poecilochrous    | 1   | 128                              |
| Royal hawk                                | Buteo regalis          | 4   | 32                               |
| Turkey vulture                            | Cathartes aura         | 4   | 11                               |
| Short-toed eagle                          | Circaetus gallicus     | 3   | 20                               |
| Black vulture                             | Coragyps atratus       | 1   | 4                                |
| Lanner falcon                             | Falco biarmicus        | 1   | 512                              |
| Lesser kestrel                            | Falco naumanni         | 3   | 203                              |
| Black-chested buzzard eagle               | Geranoaetus melanoleucus | 2  | 4                                |
| Palm-nut vulture                          | Gypohierax angolensis  | 1   | 8                                |
| White-backed vulture                      | Gyps africanus         | 1   | 4                                |
| Griffon vulture                           | Gyps fulvus            | 3   | 102                              |
| Himalayan vulture                         | Gyps himalayensis      | 1   | 256                              |
| White-tailed eagle                        | Haliaeetus albicilla    | 2   | 32                               |
| Bald eagle                                | Haliaeetus leucocephalus | 4  | 54                               |
| African fish eagle                        | Haliaeetus vocifer      | 4   | 38                               |
| Black kite                                | Milvus migrans         | 3   | 64                               |
| Red kite                                  | Milvus milvus          | 5   | 194                              |
| Hooded vulture                            | Necrosyrtes monachus    | 6   | 81                               |
| Egyptian vulture                          | Neophron percnopterus  | 2   | 362                              |
| Osprey                                    | Pandion haliaetus      | 3   | 40                               |
| Harris’s hawk                             | Parabuteo unicinctus   | 2   | 256                              |
| Honey buzzard                             | Pernis apivorus        | 3   | 4                                |
| Southern caracara                         | Polyborus plancus      | 4   | 54                               |
| King vulture                              | Sarcoramphus papa      | 2   | 64                               |
| White-headed vulture                      | Trigonops occipitalis  | 1   | 4                                |
| Andean condor                             | Vultur gryphus         | 3   | 4                                |
| **Galliformes**                           |                       |     |                                  |
| **Total**                                 |                       | 69  | 30                               |

| Common name                                | Scientific name       | GMT | % of birds with HI titers of ≥32 |
|-------------------------------------------|-----------------------|-----|----------------------------------|
| Vulturine guineafowl                       | Acryllium vulturinum  | 3   | 25                               |
| Lady Amherst’s pheasant                    | Chrysophosphus amherstiae | 3  | 8                                |
| Golden pheasant                           | Chrysophosphus pictus | 1   | 4                                |
| Great curassaw                            | Crax rubra            | 1   | 32                               |
| Red junglefowl                            | Gallus gallus         | 26  | 55                               |
| Silver pheasant                           | Lophura nycthemera    | 2   | 4                                |
| Indian peafowl                            | Pavo cristatus        | 31  | 29                               |
| Common pheasant                           | Phasianus colchicus   | 2   | 4                                |
| **Gruiformes**                            |                       |     |                                  |
| **Total**                                 |                       | 31  | 10                               |

| Common name                                | Scientific name       | GMT | % of birds with HI titers of ≥32 |
|-------------------------------------------|-----------------------|-----|----------------------------------|
| Blue crane                                | Anthropoides paradisea | 3   | 4                                |
| Demoiselle crane                          | Anthropoides virgo    | 10  | 8                                |
| Black crowned crane                       | Balearica pavonina    | 1   | 4                                |
| Gray crowned crane                        | Balearica regulorum   | 11  | 18                               |
| Seriema                                   | Cariama cristata      | 3   | 6                                |
| Common crane                              | Gruus grus            | 3   | 13                               |
| **Passeriformes**                         |                       |     |                                  |
| **Total**                                 |                       | 9   | 8                                |

| Common name                                | Scientific name       | GMT | % of birds with HI titers of ≥32 |
|-------------------------------------------|-----------------------|-----|----------------------------------|
| Pied crow                                 | Corvus albus          | 3   | 4                                |
| Carrion crow                              | Corvus corone         | 1   | 4                                |
| Corn bunting                              | Emberiza calandra     | 1   | 16                               |

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| Order                  | Species                      | No. of birds | GMT | % of birds with HI titers of ≥32 |
|-----------------------|------------------------------|--------------|-----|----------------------------------|
|                       | Rosy starling                | 1            | 128 | 100                              |
|                       | Red-billed chough            | 11           | 6   | 0                                |
|                       | Common blackbird             | 24           | 0   |                                   |
|                       | Great white pelican          | 20           | 32  | 60                               |
|                       | Pink-backed pelican          | 8            | 4   | 0                                |
|                       | Great cormorant              | 3            | 4   | 0                                |
| Pelecaniformes        | Total                        | 31           | 15  | 38.7                             |
|                       | Pelicaniformes               | Total        | 93  | 122 86                          |
|                       | Lesser flamingo              | 19           | 143 | 89                               |
|                       | Chilean flamingo             | 5            | 256 | 80                               |
|                       | American flamingo            | 69           | 111 | 85.5                             |
|                       | Piciformes                   | Total        | 3   | 13 33.3                         |
|                       | Toco toucan                  | 1            | 4   | 0                                |
|                       | Keel-billed toucan           | 1            | 128 | 100                              |
|                       | Black-mandibled toucan       | 1            | 4   | 0                                |
| Psittaciformes        | Total                        | 177          | 15  | 42.9                             |
|                       | Blue-fronted amazon          | 3            | 8   | 33.3                             |
|                       | Orange-winged amazon         | 2            | 16  | 50                               |
|                       | Yellow-shouldered amazon     | 9            | 30  | 56                               |
|                       | Festive amazon               | 5            | 21  | 40                               |
|                       | Yellow-crowned amazon        | 3            | 32  | 66.7                             |
|                       | Red-spectacled amazon        | 3            | 10  | 0                                |
|                       | Vinaceous amazon             | 3            | 10  | 0                                |
|                       | Hyacinth macaw               | 1            | 128 | 100                              |
|                       | Great green macaw            | 3            | 51  | 100                              |
|                       | Blue-and-yellow macaw        | 27           | 16  | 66.7                             |
|                       | Red-and-green macaw          | 17           | 9   | 23.5                             |
|                       | Scarlet macaw                | 15           | 37  | 86.7                             |
|                       | Military macaw               | 13           | 19  | 38.5                             |
|                       | Red-fronted macaw            | 13           | 16  | 53.8                             |
|                       | Chestnut-fronted macaw       | 4            | 8   | 25                               |
|                       | Blue-crowned parakeet        | 1            | 4   | 0                                |
|                       | Finch’s parakeet             | 1            | 4   | 0                                |
|                       | White cockatoo               | 8            | 9   | 25                               |
|                       | Sulfur-crested cockatoo      | 5            | 11  | 40                               |
|                       | Goffins cockatoo             | 1            | 8   | 0                                |
|                       | Salmon-crested cockatoo      | 1            | 32  | 100                              |
|                       | Western corella              | 8            | 4   | 0                                |
|                       | Yellow-crested cockatoo      | 1            | 16  | 100                              |
|                       | Eclectus parrot              | 7            | 16  | 57.1                             |
|                       | Golden parakeet              | 6            | 11  | 16.7                             |
|                       | Scaly-headed parrot          | 1            | 4   | 0                                |
|                       | Pesquet’s parrot             | 1            | 4   | 0                                |
|                       | African gray parrot          | 15           | 13  | 40                               |
| Sphenisciformes       | Total                        | 16           | 9   | 18.8                             |
|                       | Humboldt penguin             | 5            | 21  | 60                               |
|                       | African penguin              | 11           | 6   | 0                                |
| Strigiformes          | Total                        | 12           | 7   | 16.7                             |
|                       | Little owl                   | 2            | 11  | 50                               |
|                       | Eurasian eagle owl           | 7            | 7   | 14.3                             |
|                       | Snowy owl                    | 2            | 4   | 0                                |
|                       | Barn owl                     | 1            | 4   | 0                                |
| Struthioniformes      | Total                        | 33           | 11  | 30.3                             |
|                       | Emu                          | 9            | 7   | 22.2                             |
|                       | Greater rhea                 | 19           | 9   | 21.1                             |
|                       | Ostrich                      | 5            | 37  | 80                               |
| All                  |                             | 933          | 103 | 48.2                             |

*The geometric mean titers (GMT) and the percentages of birds with a postvaccination serum hemagglutination inhibition (HI) titer of ≥32 shown were measured 6 weeks after the second vaccination.*
titer (GMT) was 81, and 31.8% of birds reached a serum antibody titer of ≥32 against the H5 antigen. On average, after the booster vaccination, the GMT reached 103, and 51.4% had titers ≥32 against the H5 antigen. To evaluate long-lasting immunity, titers 15 weeks after the second vaccination were studied. More than 45% of the birds were considered positive, and the overall GMT was 59. Of the 7 taxonomic orders for which more than 45 individuals were subjected to serological follow-up, 6 reached mean titers greater than 32 (Fig. 2). Falconiformes, Pelecaniformes, Phoenicopteriformes, and Struthioniformes presented HI titers over 120. In contrast, Anseriformes reached a serum antibody titer of ≥32 against the H5 antigen. The other birds that had a titer of ≥32 against the H5 antigen. The Falconiformes order showed a weaker response, with a GMT of 50. The other birds that had not been vaccinated previously had a GMT of less than 32. Among animals vaccinated in VP1, Galliformes showed a very high response (GMT = 437) 12 weeks after receiving the H5N3 vaccine. The Psittaciformes and Struthioniformes orders reached seropositivity with a GMT of 58 and 128, respectively (Fig. 4).

After H5N3 vaccination, 338 birds were evaluated for the presence of serum antibody titers against an HPAI H5N1 strain circulating in Europe (A/Mallard/It/3401/05) and for the presence of A/Tky/Eng/647/77 (H7N7)-specific antibodies. The response obtained against H5N1 was compared to those elicited against the H5N3 vaccine component. Moreover, two groups were differentiated between those being H5N9 and H5N3 vaccinated and those receiving only the H5N3 vaccine. The frequencies of birds reaching a seroprotective titer (≥32) are similar when testing antibody titers against H5N1 as well as for the vaccine compound in both the studied groups (Fig. 5). No immune response against the H7N7 strain was detected in any of the studied animals.

**Virus detection.** No AIV antigen was detected in collected cloacal swabs in VP1. Prior to VP2, two animals that were RT-PCR positive were probably exposed to AI virus during this time interval. Both animals were from the Phoenicopteriformes order.

**Humoral response against H5N3 vaccination (VP2).** Detailed data concerning humoral immune response against an inactivated H5N9 vaccine from each order and species studied are provided in Table 2. Of 469 birds tested prior to VP2, 190 tested positive by the cELISA (40%). Most of the seropositive birds were from the following orders: Phoenicopteriformes \((n = 74)\), Anseriformes \((n = 51)\), Psittaciformes \((n = 16)\), and Ciconiiformes \((n = 15)\). However, only 26 out of 190 animals were not vaccinated in the previous vaccination program (VP1). By HI test, 279 out of 469 (60%) birds were seronegative for H5 AIV.

In VP2, antibody titers at 6 \((n = 398\) samples) and 12 \((n = 376\) samples) weeks postvaccination were studied. In both cases, the number of seropositive animals was around 40%, and the overall GMTs were different between those animals vaccinated in the previous vaccination program (VP1 with H5N9) and those vaccinated for the first time with H5N3 (Fig. 3 and 4). Six weeks after the second dose of the H5N3 vaccine, Galliformes and Pelecaniformes orders (that were included in the VP2 with only the H5N3 vaccine) manifested a GMT higher than 150 (Fig. 3). The Falconiformes order showed a weaker response, with a GMT of 50. The other birds that had not been vaccinated previously had a GMT of less than 32. Among animals vaccinated in VP1, Galliformes showed a very high response (GMT = 437) 12 weeks after receiving the H5N3 vaccine. The Psittaciformes and Struthioniformes orders reached seropositivity with a GMT of 58 and 128, respectively (Fig. 4).

After H5N3 vaccination, 338 birds were evaluated for the presence of serum antibody titers against an HPAI H5N1 strain circulating in Europe (A/Mallard/It/3401/05) and for the presence of A/Tky/Eng/647/77 (H7N7)-specific antibodies. The response obtained against H5N1 was compared to those elicited against the H5N3 vaccine component. Moreover, two groups were differentiated between those being H5N9 and H5N3 vaccinated and those receiving only the H5N3 vaccine. The frequencies of birds reaching a seroprotective titer (≥32) are similar when testing antibody titers against H5N1 as well as for the vaccine compound in both the studied groups (Fig. 5). No immune response against the H7N7 strain was detected in any of the studied animals.

**DISCUSSION**

In the present work, we demonstrate that carrying out two vaccination programs with successive heterologous vaccines in wild animals from Spanish zoos can be the key to widely protect species from taxonomic orders which did not develop HI antibody to a unique vaccine. In 2005, when the European Commission directive 2005/744/EC allowed vaccination against avian influenza (AI) in zoos (7), other European countries also embarked on the mass vaccination program in zoo birds. Lately, results from some of the zoos, judging the efficacy of different vaccine formulations used, have been reported (1, 15, 16). Comparison of different vaccine formulations in eliciting a strong humoral response could be instrumental to decide future vaccination programs against AI virus.

In 2006, both Spain (data from present study, VP1) and Denmark (1) used inactivated H5N9 vaccines from different manufacturers in their vaccination programs in zoo birds. We observed that 51.4% of the H5N9-vaccinated birds in Spanish zoos had an HI titer of ≥32 after booster vaccination, with an overall GMT of 103. The present data were comparatively lower than those previously reported by Bertelsen et al. (1), also using the H5N9 vaccine, where 76% of the zoo birds developed a titer of 32 with a GMT of 137. The differences in seroprotection efficacy between our results and those reported by Bertelsen et al. (1) may be due to different amounts of antigen or adjuvants used in the vaccine preparation, since the inactivated H5N9 vaccine studied by the Danish group was derived from a different manufacturer. Moreover, it should be noted that the present work is comprised of a large number of exotic birds \((n = 933\) after booster vaccination) from various orders, which may influence the amount of the overall GMT. This fact may also explain the heterogeneity in the antibody
TABLE 2. Humoral immune response of avian species in zoos vaccinated twice (within a 6-week interval) with an inactivated H5N3 vaccine (VP2)

| Group Order | Species                                                                 | No. of birds | GMT | % of birds with HI titers of ≥32 |
|-------------|------------------------------------------------------------------------|--------------|-----|----------------------------------|
| Anseriformes | Total                                                                   | 44           | 10  | 11                               |
| Anseriformes | Egyptian goose                                                          | 4            | 4   | 0                                |
| Anseriformes | Mallard                                                                 | 12           | 10  | 0                                |
| Anseriformes | Greylag goose                                                           | 2            | 4   | 0                                |
| Anseriformes | Bar-headed goose                                                        | 2            | 32  | 100                              |
| Anseriformes | Magpie goose                                                            | 1            | 4   | 0                                |
| Anseriformes | Hawaiian goose                                                          | 5            | 16  | 20                               |
| Anseriformes | Cape Barren goose                                                       | 1            | 4   | 0                                |
| Anseriformes | Andean goose                                                            | 4            | 11  | 0                                |
| Anseriformes | Black swan                                                              | 6            | 16  | 0                                |
| Anseriformes | Mute swan                                                               | 1            | 16  | 0                                |
| Anseriformes | Fulvous whistling-duck                                                  | 1            | 64  | 100                              |
| Anseriformes | Rosybill                                                               | 5            | 7   | 20                               |
| Columbiformes | Total                                                                   | 5            | 16  | 40                               |
| Columbiformes | Common wood pigeon                                                     | 1            | 4   | 0                                |
| Columbiformes | Diamond dove                                                            | 2            | 64  | 100                              |
| Columbiformes | Barbary dove                                                            | 2            | 8   | 0                                |
| Coraciiformes | Total                                                                   | 2            | 4   | 0                                |
| Coraciiformes | White-crowned hornbill                                                  | 2            | 4   | 0                                |
| Falconiformes | Total                                                                   | 4            | 49  | 75                               |
| Falconiformes | Common buzzard                                                          | 2            | 108 | 100                              |
| Falconiformes | Griffon vulture                                                        | 1            | 4   | 0                                |
| Falconiformes | Black kite                                                             | 1            | 128 | 100                              |
| Galliformes | Total                                                                   | 11           | 187 | 100                              |
| Galliformes | Red junglefowl                                                          | 5            | 56  | 100                              |
| Galliformes | Indian peafowl                                                          | 6            | 512 | 100                              |
| Gruiformes | Total                                                                   | 1            | 4   | 0                                |
| Gruiformes | Demoiselle crane                                                       | 1            | 4   | 0                                |
| Pelecaniformes | Total                                                                   | 4            | 152 | 75                               |
| Pelecaniformes | Great white pelican                                                    | 3            | 512 | 100                              |
| Pelecaniformes | Great cormorant                                                        | 1            | 4   | 0                                |
| Phoenicopteriformes | Total                                                                    | 4            | 8   | 0                                |
| Phoenicopteriformes | American flamingo                                                      | 4            | 8   | 0                                |
| Strigiformes | Total                                                                   | 2            | 11  | 50                               |
| Strigiformes | Barn owl                                                                | 1            | 4   | 0                                |
| Strigiformes | Spectacled owl                                                         | 1            | 32  | 100                              |
| Anseriformes | Total                                                                   | 91           | 20  | 42                               |
| Anseriformes | White-checked pintail                                                   | 1            | 4   | 0                                |
| Anseriformes | Chestnut teal                                                          | 2            | 4   | 0                                |
| Anseriformes | Mallard                                                                 | 11           | 4   | 0                                |
| Anseriformes | Greylag goose                                                           | 3            | 40  | 33.3                             |
| Anseriformes | Emperor geese                                                           | 5            | 4   | 0                                |
| Anseriformes | Swan goose                                                              | 5            | 9   | 20                               |
| Anseriformes | Barnacle goose                                                          | 1            | 4   | 0                                |
| Anseriformes | Red-breasted goose                                                     | 3            | 16  | 33.3                             |
| Anseriformes | Hawaiian goose                                                          | 1            | 16  | 0                                |
| Anseriformes | Cape Barren goose                                                       | 1            | 11  | 0                                |
| Anseriformes | Andean goose                                                            | 6            | 4   | 0                                |
| Anseriformes | Ashy-headed goose                                                       | 2            | 4   | 0                                |
| Anseriformes | Ruddy-headed goose                                                     | 7            | 4   | 0                                |
| Anseriformes | Black swan                                                              | 6            | 102 | 83.3                             |
| Anseriformes | Black-necked swan                                                       | 3            | 6   | 0                                |
| Anseriformes | Rosybill                                                               | 6            | 323 | 100                              |
| Anseriformes | Red-crested pochard                                                     | 2            | 181 | 100                              |
| Anseriformes | Ruddyl shedduck                                                         | 14           | 61  | 78.6                             |
| Anseriformes | Common shelduck                                                         | 12           | 85  | 83.3                             |
| Charadriiformes | Total                                                                   | 4            | 13  | 50.0                             |
| Charadriiformes | Caspian gull                                                           | 4            | 13  | 50.0                             |
| Ciconiiformes | Total                                                                   | 25           | 16  | 44                               |
| Ciconiiformes | White stork                                                             | 3            | 4   | 0                                |
| Ciconiiformes | Glossy ibis                                                             | 17           | 31  | 64.7                             |
| Ciconiiformes | African sacred ibis                                                     | 5            | 4   | 0                                |
| Columbiformes | Total                                                                   | 9            | 4   | 0                                |
| Columbiformes | Common wood pigeon                                                     | 9            | 4   | 0                                |
| Coraciiformes | Total                                                                   | 6            | 9   | 33.3                             |
| Coraciiformes | Knobbed hornbill                                                        | 2            | 4   | 0                                |
| Coraciiformes | Mindanao wrinkled hornbill                                              | 2            | 4   | 0                                |
| Coraciiformes | Black hornbill                                                          | 2            | 45  | 100                              |
| Falconiformes | Total                                                                   | 7            | 9   | 28.6                             |
| Falconiformes | Turkey vulture                                                          | 2            | 64  | 100                              |
| Falconiformes | Himalayan vulture                                                       | 1            | 4   | 0                                |
| Falconiformes | Bald eagle                                                              | 1            | 4   | 0                                |
| Falconiformes | Hooded vulture                                                          | 1            | 4   | 0                                |
| Falconiformes | Harris’s hawk                                                           | 2            | 4   | 0                                |
| Galliformes | Total                                                                   | 22           | 437 | 95.5                             |
| Galliformes | Indian peafowl                                                          | 22           | 437 | 95.5                             |

Continued on following page
responses that we observed in serological analysis in vaccinated birds, which varied greatly, not only between taxonomic orders but also between species of a single order and even within species. Similar observations with an inactivated H7N1 vaccine were published by Philippa et al. (15), who described a high seroprotection rate of 81.5% and an overall GMT of 190, with variations in HI titers among different bird orders examined. In general, based on the serological analysis from a huge number of H5N9-vaccinated Spanish zoo birds, we observed that more than 75% of birds from Phoenicopteriformes manifested a GMT of $\frac{1}{32}$, and from the other 15 orders studied after booster vaccination, 12 had a protection rate less than 50%.

| Group          | Order          | Species                             | No. of birds | GMT | % of birds with HI titers of $\geq 32$ |
|----------------|----------------|-------------------------------------|--------------|-----|---------------------------------------|
| Gruiformes     | Total          | Anthropoides paradisea              | 6            | 9   | 33.3                                  |
| Gruiformes     | Blue crane     | Anthropoides virgo                  | 3            | 4   | 4                                     |
| Gruiformes     | Demoiselle crane | Anthropoides virgo                 | 2            | 45  | 100                                   |
| Gruiformes     | Gray crowned crane | Balearica regulorum           | 1            | 4   | 0                                     |
| Passeriformes  | Total          | Carduelis chloris                  | 1            | 4   | 0                                     |
| Pelecaniformes | Total          | Phoeniconaias minor                | 8            | 4   | 0                                     |
| Pelecaniformes | Pink-backed pelican | Pelecanus rufescens             | 8            | 4   | 0                                     |
| Phoenicopteriformes | Total | Phoenicopterus ruber              | 91           | 18  | 29.7                                  |
| Phoenicopteriformes | Chilean flamingo | Phoenicopterus chilensis       | 9            | 276 | 100                                   |
| Phoenicopteriformes | American flamingo | Phoenicopterus ruber           | 51           | 27  | 35.3                                  |
| Psittaciformes | Total          | Articulated species                | 7            | 58  | 100                                   |
| Psittaciformes | Red-and-green macaw | Ara chloroptera               | 1            | 32  | 100                                   |
| Psittaciformes | Military macaw | Ara militaris                      | 3            | 32  | 100                                   |
| Psittaciformes | Eclectus parrot | Eclectus roratus                  | 3            | 128 | 100                                   |
| Sphenisciformes | Total          | Spheniscus demersus                | 16           | 10  | 0                                     |
| Sphenisciformes | African penguin | Spheniscus humboldti              | 4            | 4   | 0                                     |
| Sphenisciformes | Humboldt penguin | Spheniscus humboldti            | 12           | 14  | 0                                     |
| Strigiformes   | Total          | Bubo bubo                          | 3            | 4   | 0                                     |
| Strigiformes   | Eurasian eagle owl | Nyctea scandiaca               | 2            | 4   | 0                                     |
| Strigiformes   | Snowy owl      | Nyctea scandiaca                  | 1            | 4   | 0                                     |
| Struthioniformes | Total         | Dromaius novaehollandiae          | 3            | 128 | 33.3                                  |
| Struthioniformes | Emu            | Rhea americana                    | 1            | 16  | 0                                     |
| Struthioniformes | Greater rhea   | Rhea americana                    | 2            | 362 | 100                                   |

The geometric mean titers (GMT) and the percentages of birds with a postvaccination serum hemagglutination inhibition (HI) titer of $\geq 32$ shown were measured 6 weeks after the second vaccination. Animals are grouped into two groups: the nonvaccinated in VP1 and the ones that were vaccinated in VP1.

FIG. 3. Humoral immune response following vaccination with an inactivated H5N9 vaccine (VP1). An inactivated H5N9 vaccine was used and administered twice within a 3-week interval. Bars represent the geometric mean titers (GMT) with standard errors (SE) of different taxonomic orders. The statistical significance of the difference (Mann-Whitney test) between taxonomic orders for each time point is indicated with a letter ($P < 0.05$).

FIG. 4. Humoral immune response in birds vaccinated with an inactivated H5N3 vaccine (VP2) and vaccinated previously with an inactivated H5N9 vaccine in VP1. An inactivated H5N3 vaccine was used and administered once. Bars represent the geometric mean titers (GMT) with standard errors (SE) of different taxonomic orders. The statistical significance of the difference (Mann-Whitney test) between taxonomic orders for each time point is indicated with a letter ($P < 0.05$).
For the second vaccination program (VP2), the Spanish Ministry replaced the H5N9 vaccine with an H5N3 recombinant vaccine. The decision was based on the results given by the manufacturer, showing that H5N3 (a reverse genetics vaccine), besides protecting chickens (10) and ducks (12) from experimental AI infection, also prevented viral shedding. Masking disease signs while the bird continues to shed viruses may be a serious problem both for valuable exotic birds and humans. Thus, limiting virus shedding and further transmission is of extreme importance.

Vaccination with inactivated recombinant H5N3 vaccine was equally effective as VP1 in eliciting high titers of HI antibodies against H5 among most of the bird orders studied, except for birds belonging to Psittaciformes, which did not develop HI antibodies to either vaccination protocol. Interestingly, however, priming with H5N9 and subsequently boosting with the H5N3 vaccine induced a significant antibody response in Psittaciformes birds, albeit at lower titers than the others. Similarly, Galliformes and Struthioniformes birds responded to the H5N3 vaccine with much higher HI titers after booster vaccination. This strategy (prime-boost) could be used in some of the orders or species which do not respond to a unique vaccine. However, we also have to carefully pay attention to the antibody titer length. As shown in Fig. 2, GMT after 18 months decreased drastically. Thus, some of the orders receiving H5N3 vaccine only once, because they were previously vaccinated with H5N9 (Fig. 4), did not show high titers. Philippa et al. (16), based on previous reports, have pointed to the need of a revaccination between 6 to 10 months after vaccination to maintain seroprotective titers among different wild species in zoos. This was similar to the results we obtained in VP1 18 months after the single vaccination, where seroprotection titers started to decrease. The effect of a booster vaccination is seen clearly in VP2, in those animals nonvaccinated previously in VP1 (Fig. 3), especially for the orders of Galliformes and Pelecaniformes, where GMT increased four times. These results are similar to those obtained by Philippa et al. (16), after booster vaccination increased the GMT by 30% (from 50.5% after single vaccination to 80.5% after booster vaccination) (16).

To design future vaccination strategies in exotic wild birds, it is important to evaluate both the response against the vaccine and the durability of HI antibodies. Sera 80 weeks after a single H5N9 dose were analyzed. On average, the birds had titers less than 20, meaning that 1.5 years after vaccination, we cannot detect HI titers in serum samples.

Antibody titers against HPAI H5N1 showed a similar trend as those against the homologous strain, with 34.1% of birds developing a titer of ≥32 (animals vaccinated with successive vaccines, H5N9 and H5N3) and 20.3% of the animals receiving only the H5N3 vaccine showing seroprotective titers. However, both groups showed lower titers than the results reported by Philippa et al. (16), where 61.2% of the birds had a titer of ≥40 against the HPAI strain tested, and more than 80% had a seroprotective titer against the homologous strain.

Taking into account that inactivated H5N3 vaccine induces strong immune responses and, more importantly, limits viral shedding (sterile immunity), a prime (H5N9)-boost (H5N3) vaccine strategy in future vaccination programs within exotic valuable zoo birds and in particular in the Psittaciformes, Galliformes, and Struthioniformes orders would be more adequate and advisable. Together with increased biosecurity measures and monitoring, vaccination may represent the best alternative to protect valuable and/or endangered bird species against HPAI virus infection. However, variations in elicited antibody responses among different bird orders and species must be carefully scrutinized in designing future vaccination programs. This will not only protect vaccinated birds from infection but also restrict further dissemination of otherwise devastating HPAI virus.

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