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RNA replicon vaccination confers long-lasting protection against H5N1 avian influenza in 23 zoo bird species

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Highly pathogenic avian influenza (HPAI) H5N1 viruses (clade 2.3.4.4b) cause mass mortality in birds and have infected over 50 mammalian species, including humans. Approved avian vaccines remain limited. We report the use of a propagation-defective vesicular stomatitis virus (VSV) replicon vaccine, VSVΔG($H5_{mb}$), encoding a modified H5 hemagglutinin from a 2022 Swiss HPAI isolate. Specific pathogen-free chickens immunized twice intramuscularly develop strong neutralizing antibody responses and are fully protected from lethal H5N1 challenge, without viral shedding. The vaccine is also administered to 317 captive birds across 23 species in Bern Animal Park and Basel Zoo. No side effects are observed. Birds without prior exposure to H5Nx develop strong antibody responses after two doses; previously exposed birds respond after one vaccination. After one year, 98.7% retained significant neutralizing antibody levels, indicating durable protection. These findings suggest that VSVΔG($H5_{mb}$) is a safe, effective vaccine candidate for broad protection against H5N1 across diverse avian species.

Influenza A viruses belong to the family *Orthomyxoviridae* and are characterized by a single-stranded, negative-sense RNA genome consisting of eight distinct segments[1]. These viruses are further classified by the two major antigens of the viral envelope, hemagglutinin (HA) and neuraminidase (NA), with 19 HA and 12 NA subtypes identified[1–4]. Most subtypes circulate in wild birds, particularly waterfowl, which are considered the natural reservoir for avian influenza A viruses (AIV)[5]. These viruses usually cause no clinical disease in their natural hosts and are therefore referred to as low-pathogenic avian influenza (LPAI) viruses. LPAI viruses predominantly replicate in the gastrointestinal tract and are shed with feces in large amounts into the environment. Sporadically, spillovers of LPAI viruses from wild birds to domestic poultry or mammalian species occur.

AIV pathogenicity is largely determined by the sequence motif at the HA proteolytic cleavage site[6]. Most LPAI viruses have a monobasic cleavage site that is recognized by trypsin-like proteases restricted to specific tissues, thereby limiting viral spread and pathogenicity. However, mutations can introduce a polybasic cleavage site, enabling cleavage by ubiquitously expressed proteases like furin[6,7]. This mutation allows systemic viral spread and results in high pathogenicity avian influenza (HPAI), particularly in poultry, often with up to 100% mortality. To date, only H5 and H7 subtypes have acquired this high pathogenicity in nature.

HPAI virus of subtype H5N1 was first isolated in chickens in Scotland in 1959, and again in turkeys in England in 1991[8]. A more concerning H5N1 strain emerged in 1996 on goose farms in Guangdong, China, and has since evolved into distinct clades and subclades[9]. This lineage spread globally and became endemic in parts of Asia and Africa[10]. Human infections with various H5N1 clades have been reported, with 954 confirmed cases and 464 deaths across 24 countries between 2003 and 2024[11]. A novel H5N1 clade (2.3.4.4b) emerged in the Middle East in 2020 and became dominant in Asia, Europe, and Africa

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by 2021[12]. It spread to North America via migratory birds in late 2021[13], and subsequently reached Central and South America as well as Antarctica[14]. As of early 2024, it has spread to all continents except Oceania. This ongoing panzootic has caused mass mortality among wild birds, threatening biodiversity and conservation efforts[14–17]. Additionally, outbreaks in poultry farms have led to the death or culling of millions of birds.

Notably, clade 2.3.4.4b H5Nx viruses have crossed species barriers to infect a wide range of mammals[18], including foxes[19], minks[20], polar bears[21], cats[22,23], marine mammals[14,17], rodents[24], and recently, dairy cows[22,25]. Genetic analyses revealed mutations that were indicative of adaptation to mammals[26]. Human infections, primarily among poultry cullers and dairy farm workers, have also been reported[26–28], though most resulted in mild symptoms such as conjunctivitis[29]. So far, there is no evidence of human-to-human transmission of H5N1, but continued adaptation in mammals increases the risk of a pandemic[27].

Zoological institutions are particularly susceptible to the occurrence of H5N1 HPAI due to the proximity of diverse avian and mammalian species and the attraction of wild birds to open habitats. Multiple zoos have reported outbreaks during the ongoing panzootic. However, due to longstanding non-vaccination policies, vaccines for H5N1 are not approved in most countries of Europe and North America. In places where vaccines are approved, their use is typically restricted to domestic poultry. This creates challenges, as restrictions such as mandatory indoor housing, particularly over extended periods of time, can conflict with animal welfare.

Experimental vaccination of zoo birds with inactivated H5N2, H5N3 or H5N9 viruses has shown mixed results, with variable immune responses among species[30–32]. Despite the use of mismatched NA subtypes, the differentiation of infected from vaccinated animals (DIVA) remains difficult with these inactivated influenza vaccines, which poses a problem for HPAI surveillance and international movement of vaccinated animals[33]. There is therefore a need for safe, broadly protective, and DIVA-compatible vaccines for use in avian as well as in mammalian species. Such vaccines may not only protect animals but could also reduce the risk of zoonotic transmission.

Previous research has shown that hemagglutinin (HA)-recombinant, single-cycle vesicular stomatitis virus (VSV) replicon particles (VRPs) can protect chickens against lethal HPAI virus infection[34,35]. These VRP vaccines elicited broadly reactive antibodies, prevented virus shedding, and supported DIVA strategies[35]. In the present study, we evaluated a VSV-based replicon vaccine encoding the HA of a 2022 H5N1 HPAI (clade 2.3.4.4b) virus isolate in various bird species in two Swiss zoos. We assessed the magnitude and duration of the humoral immune response across species over a 400-day period.

Results

Generation of a propagation-defective RNA replicon particle vaccine

In February 2022, H5N1 HPAI virus infected two wild grey herons (*Ardea cinerea*) and one captive Dalmatian pelican (*Pelecanus crispus*) in Bern Animal Park, Switzerland. Infectious virus was isolated from the infected pelican and designated A/Dalmatian Pelican/Bern/1/2022 (H5N1). The viral RNA segments 4 (encoding HA) and 6 (encoding NA) of this virus were completely deciphered and submitted to the GenBank data bank (accession numbers PX149233 and PX149234, respectively). Sequence analysis classified this virus as belonging to phylogenetic clade 2.3.4.4b.

Previously, we demonstrated that chickens immunized intramuscularly with VSVΔG(HA) replicon particles, encoding the HA gene of an older H5N1 strain (clade 2.5), were protected against lethal challenge with a clade 2.2 H5N1 virus[35]. Sequence comparisons revealed that the HA_1 subunit of the new A/Dalmatian Pelican/Bern/1/2022 (H5N1) isolate differs from earlier clades in several amino acid positions of the HA_1 subunits of these older H5 clades, particularly in the globular head region containing the receptor binding domain (Supplementary Fig. 1).

To create a clade-matched vaccine, we engineered VSVΔG(H5) replicon particles encoding either the unmodified HA of A/Dalmatian Pelican/Bern/1/2022 (H5N1), containing a polybasic (pb) cleavage site, or a version with a monobasic (mb) site, mimicking low-pathogenic strains (Fig. 1a). Replicon particles were produced in helper cells expressing VSV G protein in an inducible manner[36], yielding on average 1.08 ×108.mL^{-1} infectious particles within 20 h (Fig. 1b). Concentration by ultracentrifugation produced vaccine stocks with titers of 6.19 ×108 f.f.u.mL^{-1}, a 5.7-fold increase (Fig. 1b).

SDS-PAGE of purified VSVΔG($H5_{mb}$) particles and staining with colloidal Coomassie revealed major VSV proteins (N, G, M) along with an 80-kDa band (Fig. 1c, left panel), which reacted in Western blots with a H5N1-specific polyclonal chicken serum (Fig. 1c, right panel), and likely represents the HA_0 precursor. This precursor was absent in purified VSVΔG($H5_{pb}$) particles, which contained the HA_1 and HA_2 cleavage products, consistent with intracellular cleavage by the host protease furin. These findings confirm that HA is incorporated into the VSV envelope, though at lower levels than the native VSV G protein[37]. Indirect immunofluorescence of VSVΔG($H5_{mb}$)-infected MDCK cells showed that the recombinant HA antigen is expressed on the cell surface (Fig. 1d), where budding of VSV takes place.

We next evaluated the replication competence of the replicon particles in Madin-Darby canine kidney (MDCK) cells using a low multiplicity of infection (m.o.i. = 0.0001 f.f.u./cell). VSVΔG($H5_{mb}$), with its monobasic cleavage site, did not generate infectious progeny (Fig. 1e) and remained limited to single infected cells (Fig. 1f). VSVΔG($H5_{pb}$), with the polybasic cleavage site, exhibited limited spread (Fig. 1f), and some virus release (Fig. 1e). In contrast, a construct encoding both the wild-type HA and NA from A/Dalmatian Pelican/Bern/1/2022 (H5N1), VSVΔG($H5_{pb}$:N1:GFP), replicated efficiently, reaching approximately 10^7 f.f.u. mL^{-1} by 48 h post infection (p.i.) (Fig. 1e).

These observations align with prior findings indicating that NA is critical for the efficient release of infectious viral particles[38]. Due to the absence of infectious progeny of VSVΔG($H5_{mb}$) (Fig. 1e, f), this construct was selected for further animal testing as a propagation-defective vaccine candidate. Its inability to replicate beyond initially infected cells enhances safety, while still expressing the immunogenic HA antigen to stimulate an immune response.

Chickens vaccinated with VSVΔG($H5_{mb}$) are fully protected from lethal challenge with highly pathogenic H5N1 (clade 2.3.4.4b)

The VSVΔG($H5_{mb}$) vaccine was evaluated in 5-week-old specific pathogen-free (SPF) White Leghorn chickens. The chickens were divided into four groups each consisting of eight animals of both sexes. Group A received a control vaccine, VSV*ΔG, encoding GFP but no influenza virus antigen, via the intramuscular (i.m.) route (Fig. 2a). Groups B and C were immunized i.m. with VSVΔG($H5_{mb}$), while group D received the same vaccine via the ocular route. On day 28, groups A and B were boosted i.m. with VSV*ΔG and VSVΔG($H5_{mb}$), respectively. Groups C and D received booster vaccinations via eye drop. On day 56, all groups were challenged intranasally with 10^6 $TCID_{50}$ of A/Dalmatian Pelican/Bern/1/2022 (H5N1).

To evaluate immune responses, virus-neutralizing antibody titers were measured using a surrogate chimeric virus, VSVΔG($H5_P$:$N1_P$:GFP), encoding both HA and NA of A/Dalmatian Pelican/Bern/1/2022 (H5N1) and GFP as a reporter (Supplementary Fig. 2a). This system allowed us to perform virus-neutralisation tests at biosafety level 2 and provided a clear assay readout via GFP expression. The neutralization titers (ND_{50}) obtained with this surrogate system were consistent with those obtained with A/Dalmatian Pelican/Bern/1/2022 (H5N1), with a correlation coefficient of $r = 0.9692$ (Supplementary Fig. 2b). No cross-neutralization was observed with VSV*ΔG(EBOV-GP), a surrogate virus

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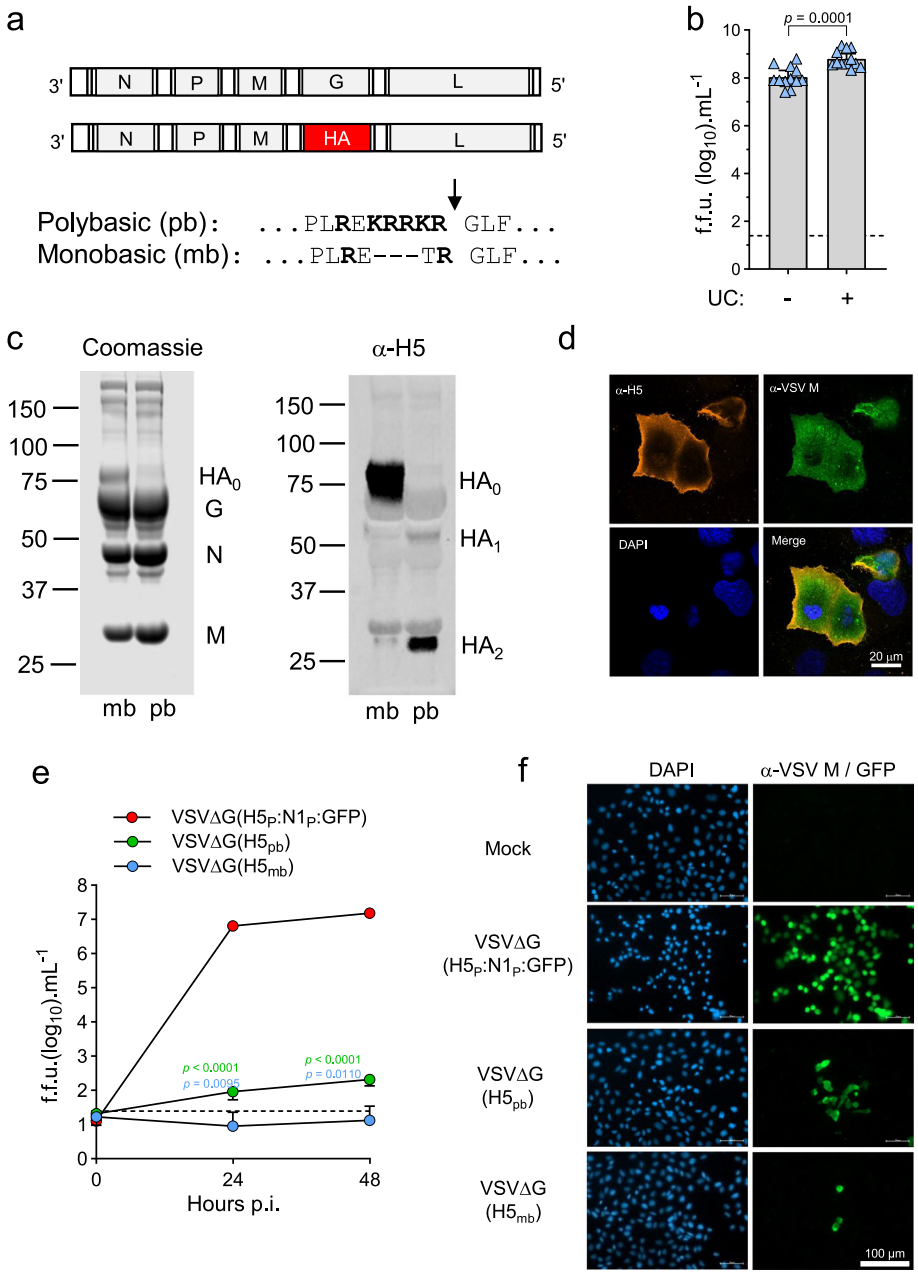

Fig. 1 | Generation and characterization of VSVΔG(H5) replicon particles. **a** Genome maps of VSV and VSVΔG(H5) vector. VSV contains five genes (N, P, M, G, and L). In VSVΔG(H5), the G gene is replaced by the HA gene of A/Dalmatian Pelican/Bern/1/2022 (H5N1) encoding either a polybasic (pb) or monobasic (mb) cleavage site. **b** Infectious virus yield on BHK-G43 helper cells infected with VSVΔG($H5_{mb}$) at m.o.i. 0.05 f.f.u./cell. At 20 h p.i., virus particles were concentrated from 200 mL of cell culture supernatant by ultracentrifugation (UC) and resuspended in 20 mL PBS. Infectious titers before and after concentration were determined on BHK-21 cells. The infectious titers of n = 12 vaccine batches are shown (blue triangles). The height of the bars indicates the geometric mean value. The error bars show the 95% confidence interval. Statistical significance was tested by the unpaired, two-sided Student's *t* test (df = 22; t = 4.732). **c** Western blot of VSVΔG($H5_{pb}$) and VSVΔG($H5_{mb}$) particles after ultracentrifugation. Viral proteins were separated by SDS-PAGE under reducing conditions, stained with colloidal Coomassie (left panel), or immunostained with chicken polyclonal anti-H5 serum. The relative molecular mass (in kDa) according to the migration of molecular weight markers is indicated on left hand side. A representative experiment out of two performed is shown. **d** Surface H5 antigen was detected with bovine α-H5 serum. VSV matrix (M) protein was stained in permeabilized cells using mAb 23H12 (α-VSV M). VSV*ΔG-infected cells visualized by GFP. Nuclei stained with DAPI. Scale bar = 20 µm. A representative experiment out of two performed is shown. **e** Multicycle virus replication on MDCK cells. Cells were infected with the indicated viruses (m.o.i. = 0.0001) and cell culture supernatant sampled at 1, 24, and 48 h p.i. Infectious titers were determined on BHK-21 cells (mean ± SD of 3 infection experiments). The detection limit (25 f.f.u. mL^{-1}) is indicated (dashed line). The two-way ANOVA with Tukey's multiple comparison test compared VSVΔG($H5_P$:$N1_P$:GFP) to VSVΔG($H5_{pb}$) and VSVΔG($H5_{mb}$). *P* values are indicated in the graph. **f** Immunofluorescence analysis of MDCK cells at 20 h p.i. with the indicated viruses (m.o.i. = 0.02). Cells infected with VSVΔG($H5_P$:$N1_P$:GFP) were detected by GFP fluorescence. Cells infected with either VSVΔG($H5_{mb}$) or VSVΔG($H5_{pb}$) were detected by indirect immunofluorescence using a monoclonal antibody directed to the VSV M protein. Cell nuclei were stained with DAPI. The bar is equivalent to 100 µm. A representative experiment out of three performed is shown. Source data are provided as a Source Data file.

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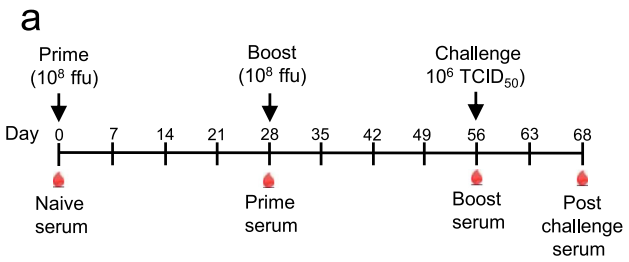
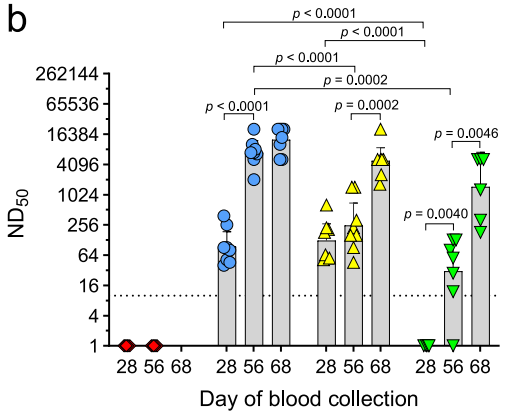
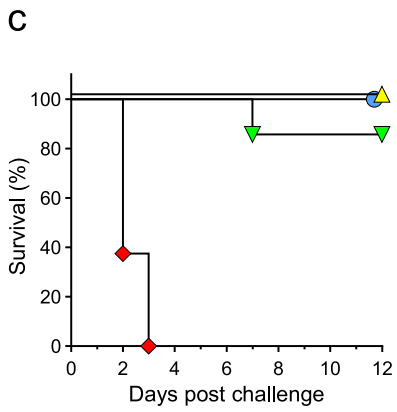
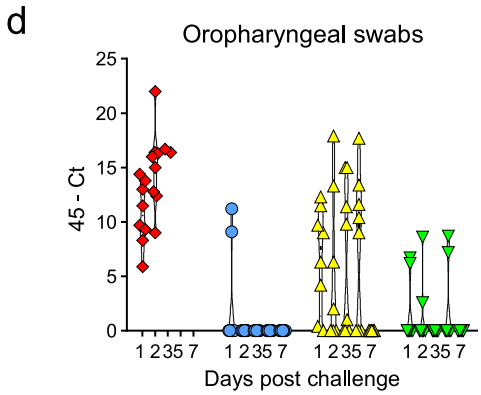


Group	Code	Vaccine	Dose (f.f.u.)/ animal	Administration route	
				Prime	Boost
A	◆	VSV*ΔG	10^8	i.m.	i.m.
B	●	VSVΔG($H5_{mb}$)	10^8	i.m.	i.m.
C	▲	VSVΔG($H5_{mb}$)	10^8	i.m.	ocular
D	▼	VSVΔG($H5_{mb}$)	10^8	ocular	ocular







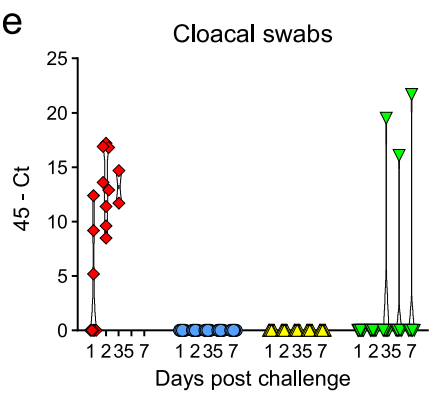


Fig. 2 | Evaluation of the VSVΔG($H5_{mb}$) vaccine in specific pathogen-free chickens. a Schematic representation of the experimental setup. Red drops below the timeline indicate time points of blood sampling. Four vaccine groups (n = 8 each group) with the corresponding symbol/color code, vaccine name, dose, and administration route are depicted on the right-hand side. Group A: Red rhombs; group B: Blue circles; group C: Yellow triangles; group D: Green inverted triangles. **b** Determination of the virus neutralization dose 50% (ND_{50}) in serum of immunized animals (n = 8 for each group). Data are presented as scatter dot plots. The height of the bars indicates the geometric mean value. The error bars show the 95% confidence interval. The two-way ANOVA test with Tukey's multiple comparisons was used to identify significant ND_{50} values between prime, boost and post-challenge sera. *P* values are indicated in the graph. **c** Survival rate of SPF chickens following intranasal infection with 10^6 $TCID_{50}$ of A/Dalmatian Pelican/Bern/1/2022 (H5N1). Determination of virus load by RT-qPCR in oropharyngeal (**d**) and cloacal (**e**) swab samples collected at the indicated days p.i. (n = 8 for each group). Source data are provided as a Source Data file.

encoding the Ebola virus glycoprotein[39], and immune sera from animals vaccinated with the Ebola glycoprotein vector did not neutralize the H5N1 surrogate virus (Supplementary Fig. 2c), confirming antigen specificity.

Group A chickens, immunized with the control vector, developed no neutralizing antibodies (Fig. 2b), and had to be euthanized or succumbed to H5N1 infection by day 3 p.i. (Fig. 2c), with high levels of viral RNA (vRNA) detected in both oropharyngeal and cloacal swabs starting one day after infection (Fig. 2d). In contrast, all chickens in groups B and C developed protective immunity. Four weeks after the first immunization with VSVΔG($H5_{mb}$) group B had geometric mean ND_{50} titers of 94 while group C reached 125. Following a second intramuscular immunization in group B, neutralizing antibody titers rose 75-fold to an average ND_{50} of 7020. Group C, boosted via the ocular route, did not show further increase of ND_{50} titers. Nonetheless, all animals in both groups survived the challenge with HPAI A/Dalmatian Pelican/Bern/1/2022 (H5N1) (Fig. 2c), without showing symptoms of disease (Supplementary Table 2). Minimal shedding of vRNA was detected by RT-qPCR analysis of oropharyngeal and cloacal swabs of vaccinated chickens. In group B, shedding was restricted to a single day in two birds and only from the oropharyngeal site (Fig. 2d). Group C showed oropharyngeal but not cloacal shedding. Post-challenge sera from group B revealed only a small, non-significant titer increase (Fig. 2b), while group C showed a significant increase of ND_{50} titer, indicating some replication of the challenge virus had occurred and enhanced the immune response.

Group D chickens, immunized ocularly, did not develop significant virus-neutralizing antibody levels after the first dose (Fig. 2b). However, following a second ocular dose, they developed significant titers (mean ND_{50} of 31). Except for one non-responder, all animals of this group survived the challenge (Fig. 2c), without showing symptoms of disease (Supplementary Table 2). Nevertheless, some animals shed

vRNA from the oropharynx and/or cloaca (Fig. 2d, e). As with group C, the challenge infection boosted antibody titers significantly.

All chicken groups contained animals of both sexes, but immune response and immune protection of the animals were not differentiated by gender. The immune response of the mixed-sex animal group as a whole was analysed.

A single vaccine dose protects chickens against H5N1 HPAI but does not prevent virus excretion

To determine the minimal ND_{50} titer needed for protection against HPAI, a follow-up study was conducted using 5-week-old SPF chickens (Supplementary Fig. 3a). Group A (n = 4) received the VSV*ΔG control vaccine while groups B to F (n = 8 each) were immunized i.m. with decreasing doses of VSVΔG($H5_{mb}$): 10^8, 5×10^7, 2×10^7, 10^7, and 5×10^6 f.f.u., respectively. On day 21 post-vaccination, sera were collected to assess ND_{50} titers (Supplementary Fig. 3b). Control group A produced no neutralizing antibodies. Groups B to F developed geometric mean ND_{50} titers of 79, 87, 49, 40, and 16, respectively. Only the ND_{50} titers of group F were significantly lower than those in groups B and C. On day 22, all animals were challenged intranasally with 10^6 $TCID_{50}$ of A/Dalmatian Pelican/Bern/1/2022 (H5N1). All vaccinated animals in groups B to F survived without symptoms, except one non-responder in group F ($ND_{50} < 10$) that succumbed to infection (Supplementary Fig. 3c). Remarkably, even animals with ND_{50} titers as low as 10 or 20 remained healthy. However, high levels of H5N1 vRNA were detected in oropharyngeal swabs from all vaccinated groups route on days 1 - 5 p.i., with significant reduction only by day 7 (Supplementary Fig. 3d). Some cloacal shedding was also observed (Supplementary Fig. 3e). While no correlation was observed between the neutralizing serum antibody titer and oropharyngeal shedding, the ND_{50} titer correlated inversely with cloacal virus shedding (Supplementary Fig. 3f). These results suggest that a single intramuscular dose of VSVΔG($H5_{mb}$) is sufficient to protect chickens from clinical disease, even at low antibody titers, but it does not fully prevent viral shedding, especially from the upper respiratory tract.

Since the two-dose vaccination of chickens via the intramuscular route revealed superior results in terms of neutralizing antibody levels and reduced H5N1 shedding, this vaccination strategy was used in the following immunization experiments in zoo birds.

Immunization of captive birds in Bern Animal Park and Basel Zoo

Following successful trials in SPF chickens, the VSVΔG($H5_{mb}$) vaccine was tested in zoo birds at Basel Zoo and Bern Animal Park. In total 23 bird species from eight families participated (see Table 1), including wild and domestic birds. The domesticated species were chickens (*Gallus gallus domesticus*) and geese (*Anser anser domesticus*), specifically Orpington chickens (n = 3), Appenzeller Spitzhaube chickens (n = 5), Silky chickens (n = 34) and Diepholzer geese (n = 2), respectively. Most species were housed in small groups, though larger populations were present, such as 32 Silky chickens, 45 African penguins (*Spheniscus demersus*), 19 Eastern white pelicans (*Pelecanus onocrotalus*), 8 Dalmatian pelicans (*Pelecanus crispus*), and 101 Greater flamingos (*Phoenicopterus roseus*) in Basel. Bern Animal Park also housed 10 Dalmatian pelicans and 66 Greater flamingos. The species, number of animals, sex, and date of birth of all zoo birds included in the vaccination trial are listed in Supplementary Data 1. All groups containing at least two animals represented both sexes.

To assess pre-existing immunity, serum samples from all animals were tested by ELISA for antibodies against influenza nucleoprotein (NP), a conserved antigen across influenza A subtypes. Most species were negative for NP-specific antibodies (Table 1). However, all Greater flamingos from both zoos tested positive, indicating prior infection with AIV of unknown subtype or pathotype. Additional NP antibody-positive species included Eastern white pelicans (*Pelecanus onocrotalus*), common Eider ducks (*Sommateria mollissima*), Barnacle geese (*Branta leucopsis*), Nene geese (*Branta sandvicensis*), Bar-headed geese (*Anser indicus*), Black swans (*Cygnus atratus*), and Coscoroba swans (*Coscoroba coscoroba*). Notably, these birds shared access to pond systems frequented by wild ducks, grey herons, and other free-ranging species, which may have been sources of exposure. Sera positive for NP antibodies were further tested for H5-specific antibodies (Table 1). All NP-positive Greater flamingos (*Phoenicopterus roseus*) in Bern, and many of the other NP-positive birds, were also positive for H5, suggesting previous infection with H5 subtype AIV. However, ELISA could not distinguish whether the infecting H5Nx viruses were low- or highly pathogenic.

Vaccinated zoo birds produce high levels of long-lasting H5N1-neutralizing antibodies

A total of 317 birds from various species were vaccinated intramuscularly with a high dose (10^8 f.f.u.) of adjuvant-free VSVΔG($H5_{mb}$) (Fig. 3a). The birds received a second dose 35 days after the first and a third dose one year later. The vaccine was well tolerated across species, with no adverse effects observed. Blood samples were collected at days 1, 35, 70, 365, and 400. Sera were analysed for virus-neutralizing activity using the VSVΔG($H5_P$:$N1_P$:GFP) reporter virus. Pre-vaccination sera from Greater flamingos and Black swans showed a baseline virus-neutralizing activity, while most other species had low or undetectable levels (Fig. 4). Nearly all birds developed protective levels of neutralizing antibodies ($ND_{50} \geq 10$) after the first dose (Figs. 3 and 4), with immune responses varying with species. Birds with pre-existing H5 immunity, such as Greater flamingos, Red-breasted geese (*Branta ruficollis*), and Black swans (*Cygnus atratus*) had strong responses after the first dose but showed only modest titer increases after the second. In contrast, species without prior H5 immunity, like Silky chickens, showed significantly increased titers after the booster, rising from ND_{50} of 41 to 907 (Fig. 3b). Titers remained stable over the year, with only slight decline, and increased again after the third dose (Fig. 3b). NP-ELISA confirmed that no AIV infections occurred in this time (Table 1). African penguins (*Spheniscus demersus*) maintained protective titers, with ND_{50} values declining from 628 (day 70) to 207 (day 365) and rising again to 942 after the third immunisation (Figs. 3c and 4).

Greater flamingos at Bern and Basel showed pre-existing H5-specific antibodies by ELISA (see Table 1). The serum antibodies also had H5-specific neutralizing activity with a geometric mean ND_{50} titer of 19 in Bern (Fig. 3d, d1), and of 63 in Basel (see Fig. 3e, d1). These titers increased to over 470 post-prime (Fig. 3d, e, d35) and surpassed 750 after the second dose (Fig. 3d, e d70). One year later, titers had declined but remained within the protective range (ND_{50} = 124 and 383, respectively) (Fig. 3d, e, d365). ND_{50} titers rose again after the third dose (Fig. 3d, e, d400).

Dalmatian pelicans from Bern (n = 10) and Basel (n = 6) showed no pre-existing H5-specific antibodies but reached a geometric mean ND_{50} of 55 after the first immunisation, which did not significantly increase following the second dose (ND_{50} = 76) (Fig. 3f). Titers declined over the year (ND_{50} of 24), with one animal's titer falling below the detection limit. Vaccination on day 365 restored ND_{50} titers to 180. In comparison, Eastern white pelicans (n = 19) responded more robustly, with higher initial and sustained ND_{50} titers (mean ND_{50} = 100 at day 365) (Fig. 3f). Eight of these birds had low but detectable pre-vaccination titers.

Antibody responses in smaller animal groups are shown in Fig. 4. Responses varied even among closely related species. For example, Barnacle geese (n = 2) showed low post-boost titers (ND_{50} = 20 and 28) (Fig. 4k), while Red-breasted geese (n = 2) developed high titers (ND_{50} = 1600 and 3200) (Fig. 4l). However, small group sizes limit statistical analysis of interspecies differences.

Overall, 228 of the 317 vaccinated birds were tested serologically one-year post-vaccination. Of these, 225 (98.7%) had protective ND_{50}

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Table 1 | Serological analysis of avian species included in the vaccination program[a]

Species name[b]	Family[b]	Common name	Site	Animal number	NP$^+$ (d1)	H5$^+$ (d1)	NP$^+$ (d365)
Gallus gallus dom.	*Phasianidae*	Orpington chicken	Basel	3	0/3	ND[c]	0/2
Gallus gallus dom.	*Phasianidae*	Silky chicken	Basel	32	0/32	ND	0/27
Gallus gallus dom.	*Phasianidae*	Appenzeller Spitzhaube chicken	Bern	5	0/4	ND	0/5
Tetrao urogallus	*Phasianidae*	Western capercaillie	Bern	7	0/7	ND	0/3
Alectoris graeca	*Phasianidae*	Rock partridge	Bern	8	0/8	ND	0/5
Pyrrhocorax pyrrhocorax	*Corvidae*	Chough	Bern	2	0/2	ND	0/2
Ciconia nigra	*Ciconiidae*	Black stork	Bern	2	0/2	ND	0/2
Bubo bubo	*Strigidae*	Eurasian eagle owl	Bern	2	0/2	ND	0/2
Aegolius funureus	*Strigidae*	Boreal owl	Bern	2	0/2	ND	0/1
Athene noctua	*Strigidae*	Little owl	Bern	2	0/2	ND	0/1
Athene noctua	*Strigidae*	Little owl	Basel	2	0/2	ND	0/1
Nyctea scandica	*Strigidae*	Snowy owl	Bern	2	0/2	ND	0/2
Phoenicopterus roseus	*Phoenicopteridae*	Greater flamingo	Bern	66[d]	33/33	33/33	33/33
Phoenicopterus roseus	*Phoenicopteridae*	Greater flamingo	Basel	101[d]	54/54	54/54	52/52
Pelecanus crispus	*Pelecanidae*	Dalmatian pelican	Bern	10	1/10	1/1	1/10
Pelecanus crispus	*Pelecanidae*	Dalmatian pelican	Basel	8	0/8	ND	0/7
Pelecanus onocrotalus	*Pelecanidae*	Eastern white pelican	Basel	19	10/19	7/10	11/19
Spheniscus demersus	*Spheniscidae*	African penguin	Basel	45	0/45	ND	1/45
Aythya nyroca	*Anatidae*	Ferrruginous duck	Bern	3	0/3	ND	0/3
Bucephala clangula	*Anatidae*	Common goldeneye	Bern	1	0/1	ND	ND
Sommateria mollissima	*Anatidae*	Common Eider duck	Bern	4	3/4	1/3	3/4
Branta ruficollis	*Anatidae*	Red-breasted goose	Basel	4	3/4	ND	2/2
Branta leucopsis	*Anatidae*	Barnacle goose	Basel	2	1/2	0/1	0/2
Branta sandvicensis	*Anatidae*	Nene goose	Basel	4	4/4	0/4	1/4
Anser indicus	*Anatidae*	Bar-headed goose	Basel	2	1/2	0/1	0/2
Anser anser	*Anatidae*	Greylag goose	Bern	4	0/4	ND	3/3
Anser anser dom.	*Anatidae*	Diepholzer goose	Bern	2	0/2	ND	0/2
Cygnus atratus	*Anatidae*	Black swan	Basel	2	2/2	2/2	2/2
Coscoroba coscoroba	*Anatidae*	Coscoroba swan	Basel	2	1/2	1/1	2/2

[a]Source data are provided as a Source Data file.
[b]Species and family names are given in italics.
[c]ND = Not determined.
[d]Serological examination was performed on half of the vaccinated birds.

titers ($ND_{50} \geq 10$), demonstrating that the VSVΔG($H5_{mb}$) vaccine induced long-lasting and broadly effective immunity across diverse bird species. While all animal groups with at least two animals represented both sexes (Supplementary Data 1), the immune response of the animals to vaccination was not disaggregated according to sex.

VSVΔG($H5_{mb}$) induces significant levels of VSV-neutralizing antibodies

To test whether neutralizing antibodies against VSV G protein could interfere with repeated vaccination, we tested sera from vaccinated Dalmatian pelicans and Silky chickens for VSV-neutralizing activity (Supplementary Fig. 4). No significant VSV-neutralizing antibodies were detected in pre-immune sera nor in non-vaccinated pelicans. By day 35, some vaccinated pelicans developed VSV-neutralizing antibodies and all showed significant levels by day 70 (Supplementary Fig. 4a). These titers declined by day 365, in some cases below the detection limit ($ND_{50} = 10$), but rose again after the booster at day 365, showing similar kinetics to H5-specific antibodies (see Fig. 2f). In Silky chickens, the kinetics of VSV-specific (Supplementary Fig. 4b) and H5-specific neutralizing antibodies (Fig. 3b) were also comparable. These results indicate that the G protein on the surface of the replicon particles triggers a VSV-specific antibody response. However, after initial immunization (prime) VSV-specific antibodies are too low to impair the success of the second application (booster).

VSVΔG($H5_{mb}$) particles elicit antibodies with broadly H5-specific neutralizing activity

We identified several amino acid differences in the globular head domain of clade 2.3.4.4b H5 hemagglutinin compared to older H5 clades (see Supplementary Fig. 1). To reflect these antigenic changes, the VRP vaccine used in this study was adapted to match the currently circulating panzootic H5N1 HPAI. To evaluate cross-clade protection, day 70 sera from vaccinated Silky chickens and African penguins were tested for neutralizing activity against VSVΔG(H5:N1:GFP) surrogate viruses encoding the HA antigen of older clades (1.1, 2.5, 2.3.2.1, and 2.3.4), using the clade 2.3.4.4b surrogate virus as reference (Fig. 5). ND_{50} titers against older H5 clades were significantly lower than those against the homotypic clade 2.3.4.4b virus, though most animals still had titers considered protective (Fig. 5a). However, some penguins had no detectable inhibitory activity against older clades (Fig. 5b). These findings suggest that the antigenic drift in clade 2.3.4.4b significantly reduced cross-neutralization, supporting the need to update the vaccine to better match the currently circulating H5N1 viruses.

Discussion

For decades, many countries worldwide have prohibited the general prophylactic vaccination of poultry against H5 and H7 HPAI. This policy stems from concerns about existing vaccine types. Live-attenuated influenza vaccines (LAIV) based on low-pathogenic H5 or

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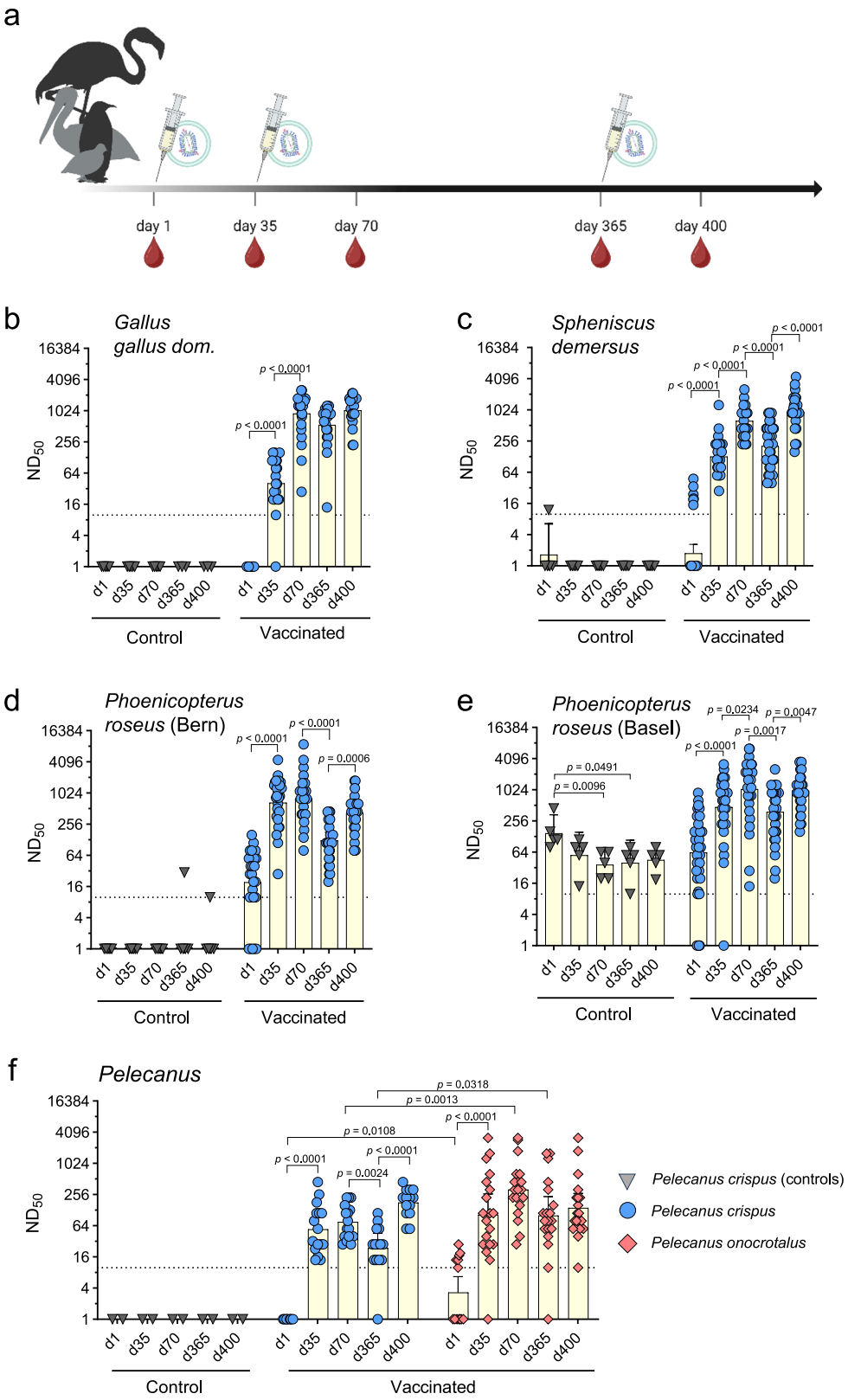

H7 viruses may mutate and become highly pathogenic. Inactivated vaccines, though safer, raise concerns that vaccinated birds could silently carry and shed HPAI viruses, allowing undetected transmission. Additionally, conventional vaccines typically do not support the DIVA principle, which is crucial for surveillance and trade[40]. Thus, non-vaccination strategies focused on biosecurity, surveillance, stamping out, and quarantine have prevailed, especially while HPAI outbreaks remained sporadic[41]. However, the ongoing global spread of clade 2.3.4.4b H5N1 HPAI has highlighted the urgent need for modern, effective vaccines.

To address the limitations of traditional vaccines, we previously developed a propagation-defective RNA replicon particle vaccine encoding the HA antigen of a 2004 H5N1 virus (clade 2.5)[42]. This vaccine induced strong neutralizing antibodies and protected chickens

Fig. 3 | Evaluation of the VSVΔG($H5_{mb}$) vaccine in zoo birds. a Study design: 317 captive birds from Bern Animal Park and Basel Zoo, representing 23 different species, were vaccinated intramuscularly with VSVΔG($H5_{mb}$) containing 2×10^8 f.f.u. mL^{-1}. Small birds (< 1.5 kg) received 250 µL and large birds (≥ 1.5 kg) 500 µL of VSVΔG($H5_{mb}$) suspension. Boosters were given on days 35 and 365 using the same dose and administration route. Blood samples were collected on days 1, 35, 365 and 400, for virus-neutralization tests and ELISA. Created in BioRender. Stettler, M. (2025) https://BioRender.com/vhzijz7. **b**–**f** H5-specific virus-neutralizing antibody titers in sera from vaccinated and control birds at the indicated time points. **b** Silky chickens (*Gallus gallus domesticus*; n = 27, + 5 controls). **c** African penguins (*Spheniscus demersus*; n = 40, +5 controls). **d**, **e** Greater flamingos (*Phoenicopterus roseus*) living in Bern Animal Park (**d**, n = 28, +5 controls) and Basel Zoo (**e**, n = 48, + 5 controls). One flamingo with an exceptionally high ND_{50} values was excluded (d1: 44,668, d35: 64,000, d70: 44,668, d365: 64,000, d400: 89,125). **f** Dalmatian pelicans (*Pelecanus crispus*, n = 16, + 2 controls) from both locations and Eastern White pelicans (*Pelecanus onocrotalus*, n = 19) from Basel. Data are presented as scatter dot plots (**b**–**f**). Non-vaccinated animals are represented by grey inverted triangles. ND_{50} titers of vaccinated animals are represented by either blue circles (**b**–**e**) or blue circles (for *P. crispus*) and red rhombs (for *P. onocrotalus*) (**f**). The height of the bars indicates the geometric mean value. The error bars show the 95% confidence interval. The detection limit $ND_{50} = 10$ is indicated. Statistically significance was evaluated by the one-way ANOVA test with Tukey's multiple comparisons (**b**–**f**). For pelican interspecies comparison, the two-way ANOVA test with Sidak's multiple comparisons was used. *P* values are indicated in the graphs. Reported F and DF values are: **b**, F = 257.5, DF = 123; **c**, F = 300, DF = 187; **d**, F = 52.53, DF = 135; **e**, F = 3.364, DF = 20 (controls); F = 37.32, DF = 227 (vaccinated); **f**, F = 91.84, DF = 71 (*P. crispus*, vaccinated); FD = 23.86, DF = 90 (*P. onocrotalus*). Source data are provided as a Source Data file.

from lethal HPAI[35]. In this study, we updated the vaccine to match the currently circulating H5N1 viruses by using the HA of A/Dalmatian Pelican/Bern/1/2022 (H5N1), a clade 2.3.4.4b virus isolated from a diseased Dalmatian pelican at Bern Animal Park.

The updated VRP vaccine does not replicate autonomously but can be propagated to high titers in helper cells expressing the VSV G protein[36]. When administered intramuscularly to SPF chickens, a single dose generated virus-neutralizing serum antibodies and protected against lethal HPAI challenge. Notably, chickens with antibody titers of $ND_{50} \geq 10$ survived infection, suggesting this ND_{50} titer as a threshold for protective immunity. However, these birds continued to shed viral RNA for several days post infection, indicating that HA-specific serum antibodies were not secreted in sufficient quantities into the mucosal surfaces of the respiratory and gastrointestinal tract. It was only after the second immunisation that the antibody level increased sufficiently to prevent viral shedding. This result is consistent with an earlier finding that chickens vaccinated twice via the intramuscular route with RNA replicon particles did not pass H5N1 HPAI challenge virus on to unvaccinated sentinel chickens in the same cage[35]. Our results are also in line with the observation that serum antibodies have access to the mucosal surfaces of chickens[43]. However, it seems that high levels of antibodies must be present in the blood to achieve concentrations in mucosal tissues that can reduce the shedding of avian influenza viruses effectively[44,45]. In our experiments, a single intramuscular dose of VSVΔG($H5_{mb}$) induced serum antibodies with a geometric mean ND_{50} titer of 74 (Supplementary Fig. 3b), while two intramuscular applications resulted in an ND_{50} titer of 7019 (Fig. 2b). This suggests that the cut-off titer required to prevent viral RNA shedding must be somewhere between these two values. Further studies are certainly needed to determine whether the serum antibody level is the only parameter affecting the secretion of the antibodies into mucosal surfaces, or whether other factors such as antibody affinity maturation are also involved.

In contrast to prior trials with inactivated vaccines that yielded variable immune responses across bird species[31,46,47], our study showed that the majority of the 23 avian species responded well to vaccination with adjuvant-free VSVΔG($H5_{mb}$) replicon particles, producing high levels of neutralizing antibodies. This broad efficacy may be due to the VSV G protein used during vaccine production, which facilitates viral entry across diverse cell types via the low-density lipoprotein (LDL) receptor family[48,49]. Most birds responded robustly although avian LDL receptor homologs are divergent[50], possibly limiting vaccine uptake in certain species like the Ferruginous duck *(Aythya nyroca*), the Common goldeneye (*Bucephala clangula*), and the Barnacle goose (*Branta leucopsis*).

Unlike vector vaccines based on either Newcastle disease virus[51], avian herpesviruses[52,53], or fowlpox virus[54], VSV is not a known avian pathogen. Thus, birds lack pre-existing VSV immunity that could impair vaccine effectiveness. However, virus-neutralizing antibodies directed against VSV G protein were detected following immunization with VSVΔG($H5_{mb}$), particularly in Dalmatian pelicans. These birds responded poorly to the second immunization but showed a stronger immune response one year later, once VSV-specific antibodies had waned. Interestingly, some zoo birds, such as Greater flamingos, showed pre-existing H5-specific immunity, likely from prior exposure to H5Nx viruses. Yet, these birds responded well to the vaccine, indicating that pre-existing H5-specific virus-neutralizing antibodies do not interfere with VSVΔG($H5_{mb}$) efficacy.

The vaccine also fulfills DIVA criteria. Using a commercial ELISA, vaccinated and infected birds could be distinguished. This is significant because it meets two critical requirements for modern HPAI vaccines: (1) robust protection that halts virus shedding (after prime/boost vaccination) and (2) compatibility with surveillance and trade standards.

Furthermore, VSVΔG($H5_{mb}$) induced cross-clade protection. Birds vaccinated with the clade 2.3.4.4b HA antigen produced antibodies capable of neutralizing older H5N1 strains, suggesting a level of cross-reactivity that could protect against future antigenic drift.

Another major benefit observed was the long-lasting immune response that was induced by the VSVΔG($H5_{mb}$) vaccine. One year after the second immunization, 98.7% of vaccinated zoo birds maintained protective antibody titers ($ND_{50} \geq 10$). For example, Silky chickens, which had not encountered H5Nx viruses after vaccination, still displayed high ND_{50} titers at day 365. While species like Greater flamingos and African penguins showed moderate titer declines, the overall durability of the immune response indicates that booster vaccinations may only be required at extended intervals. This long-term protection reduces the need for restrictive indoor housing of captive birds, improving animal welfare in zoos and conservation centers.

Given its efficacy in captive wild birds, the VSVΔG($H5_{mb}$) vaccine may also protect highly endangered free-living birds such as California condors (*Gymnogyps californianus*) and African penguins[55,56]. However, as repeated administration of the vaccine to wild birds is logistically challenging, this approach is probably only realistic for critically endangered species with small populations.

Our previous and current studies show that VSV-based RNA replicon particles are generally effective in protecting chickens against HPAI[34,35]. The compatibility of the vaccine with the DIVA principle also makes it suitable for HPAI control in poultry. Importantly, while a single intramuscular dose provides protection, only the prime-boost regimen efficiently inhibits viral shedding. This is critical, as infected but asymptomatic birds can silently spread HPAI viruses. However, mass vaccination of poultry using intramuscular delivery is regarded impractical. Encouragingly, this study showed that ocular immunization with VSVΔG($H5_{mb}$) was nearly as effective, suggesting mucosal delivery routes could be optimized for large-scale use. Vaccination of poultry could dramatically reduce economic losses from HPAI outbreaks but also lower the risk of zoonotic transmission. This has precedent - China's mass vaccination

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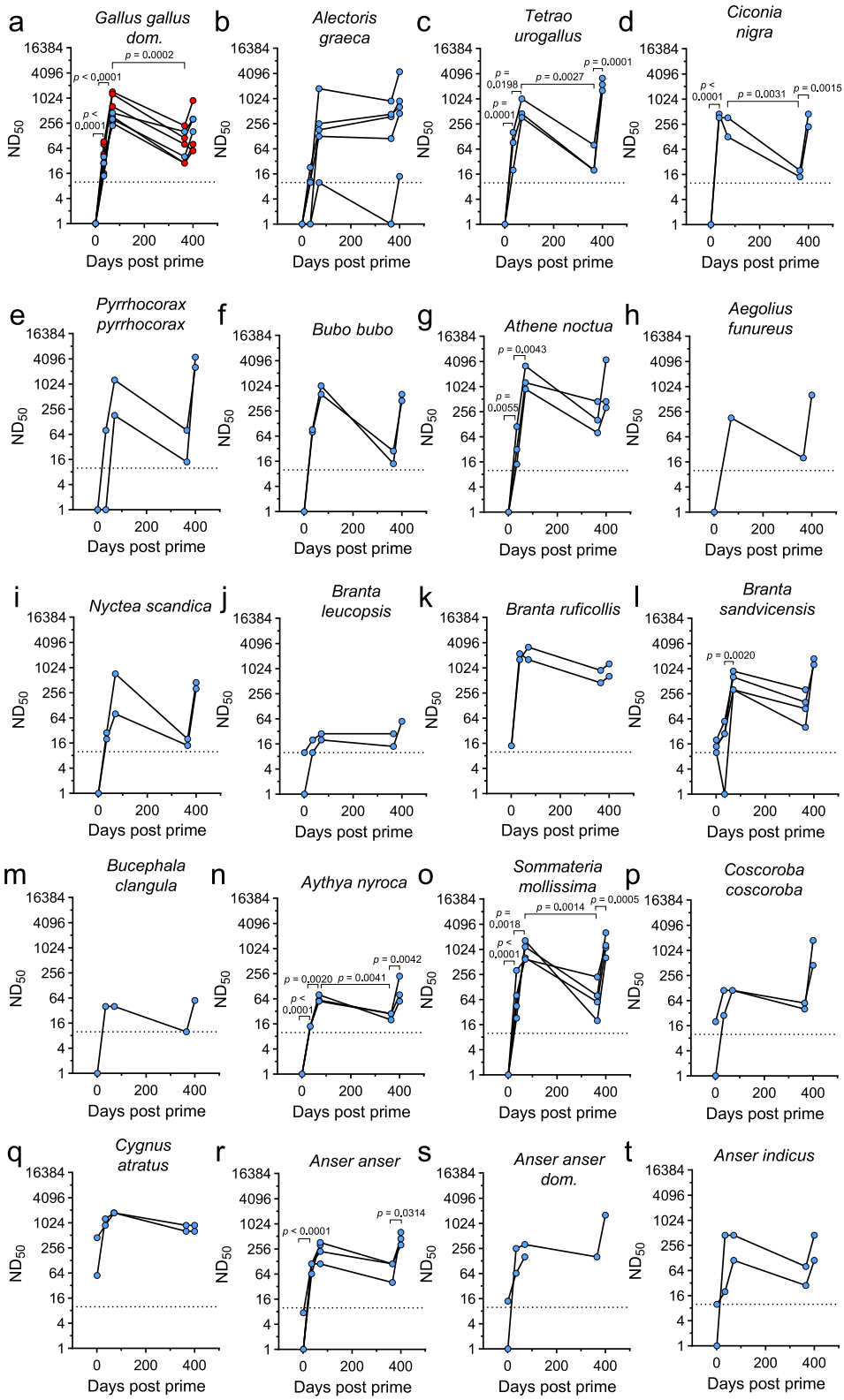

campaign against H7N9 significantly reduced human cases[57]. A similar strategy using the VSVΔG($H5_{mb}$) vaccine could mitigate current and future H5N1 outbreaks.

Beyond birds, VSV-based influenza vaccines have also proven effective in mammals[58–60]. The current panzootic has affected a wide range of mammalian hosts, including dairy cows[26], minks[20], and cats[23]. The VSVΔG($H5_{mb}$) vaccine could be adapted for use in these species, offering cross-species protection and helping to contain the expanding host range of H5N1 HPAI.

In conclusion, we have developed a safe, efficacious, and DIVA-compatible vaccine based on RNA replicon particles encoding the HA of currently circulating H5N1 HPAI (clade 2.3.4.4b). Used in a prime/boost immunization protocol, the vaccine provides strong protection against mortality and viral shedding in chickens and diverse wild bird

Fig. 4 | Virus-neutralizing antibody titers in serum of vaccinated zoo birds. **a**–**t** H5-specific neutralizing antibody titers in serum samples prepared from the indicated avian species at day 1, 35, 70, 365, and 400 of the vaccination trial. **a** Domestic Orpington chickens, (*Gallus gallus dom*., Basel, n = 3, blue circles) and Appenzeller Spitzhaube chickens (*Gallus gallus dom*. Bern, n = 5, red circles); **b** Rock partridge (*Alectoris graeca*, Bern, n = 5); **c** Western capercaillie (*Tetrao urogallus*, Bern, n = 3); **d** Black stork (*Ciconia nigra*, Bern, n = 2). **e** Chough (*Pyrrhocorax pyrrhocorax*, Bern, n = 2); **f** Eurasian eagle owl (*Bubo bubo*, Bern, n = 2); **g** Little owl (*Athene noctua*, Bern and Basel, n = 3); Boreal owl (*Aegolius funureus*, Bern, n = 1); **i** Snowy owl (*Nyctea scandica*, Bern, n = 2); **j** Barnacle goose (*Branta leucopsis*, Basel, n = 2); **k** Red-breasted goose (*Branta ruficollis*, Basel, n = 2); **l** Nene goose (*Branta sandvicensis*, Basel, n = 4); **m** Common goldeneye (*Bucephala clangula*, Bern, n = 1); **n** Ferruginous duck (*Aythya nyroca*, Bern, n = 3); **o** Common Eider duck (*Sommateria mollissima*, Bern, n = 4); **p** Coscoroba swan (*Coscoroba coscoroba*, Basel, n = 2); **q** Black swan (*Cygnus atratus*, Basel, n = 2); **r** Greylag goose (*Anser anser*, Bern, n = 4); **s** Domestic Diepholzer goose (*Anser anser dom*., n = 2); **t** Bar-headed goose (*Anser indicus*, Basel, n = 2). The detection limit ND_{50} = 10 is indicated by a dotted line. Statistical significance was computed for groups with n ≥ 3 animals using the one-way ANOVA test and Tukey's multiple comparisons. *P* values are indicated in the graphs. **a** F = 82, DF = 33; **b** F = 9.474, DF = 20; **c** F = 59.06, DF = 10; **d** F = 116.3, DF = 6; **g** F = 29.94, DF = 10; **l** F = 13.53, DF = 13; **n** F = 82.99 DF = 10; **o** F = 58.01, DF = 15; **r** F = 46.53, DF = 13. Source data are provided as a Source Data file.

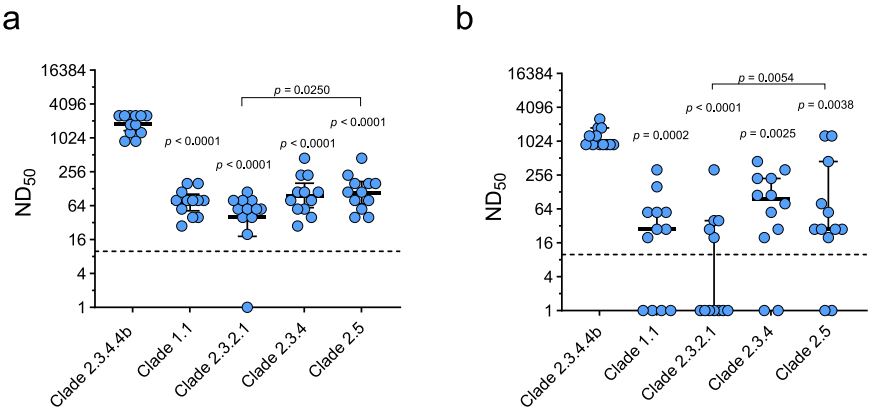

Fig. 5 | Potency of vaccine-induced neutralizing antibodies against older H5 clades. Immune sera were prepared from Silky chickens (*Gallus gallus domesticus*, n = 12) (**a**), and African penguins (*Spheniscus demersus*, n = 12) (**b**) 5 weeks after the second immunization (day 70) and analyzed for H5N1-neutralizing activity against VSVΔG(H5:N1:GFP) surrogate viruses encoding the HA antigens of indicated H5 clades. Data are presented as scatter dot plots (blue circles) (**a**, **b**). Geometric mean values are indicated by big horizontal bars. Error bars below and above the respective geometric mean bar indicate the 95% confidence interval. The detection limit (ND_{50} = 10) is indicated by an interrupted line. ND_{50} titers significantly different compared to clade 2.3.4.4b were computed by the one-way ANOVA test with Tukey's multiple comparisons (**a**, F = 41.47, DF = 55; **b**, F = 19.49, DF = 11). *P* values are indicated in the graphs. Source data are provided as a Source Data file.

species, induces long-lasting immunity, and shows potential for cross-clade protection. Its compatibility with the DIVA principle and potential for mass application position it as a valuable tool for managing the ongoing H5N1 HPAI panzootic. With further optimization for mass application, this vaccine could transform HPAI control strategies, protect wildlife and livestock, and reduce the public health risk posed by zoonotic H5N1 transmission.

Methods

Cells

Madin-Darby canine kidney (MDCK, type II) cells were kindly provided by Georg Herrler (University of Veterinary Medicine, Hannover, Germany) and grown in Earle's Minimal Essential Medium (MEM, Life Technologies, Zug, Switzerland) supplemented with 5% fetal bovine serum (FBS, Pan Biotech, Aidenbach, Germany). BHK-21 cells were purchased from the German cell culture collection (DSZM, Braunschweig, Germany; cat. no. ACC-61) and grown in Glasgow's Minimum Essential Medium (GMEM, Life Technologies). BHK-G43 cells, a transgenic baby hamster kidney (BHK-21) cell clone producing the VSV G protein in a regulated manner[36], were cultured with GMEM supplemented with 5% FBS. Vero E6 cells (American Type Culture Collection, ATCC, Manassas, Virginia, USA; cat. no. CRL-1586) were kindly provided by Christian Drosten and Marcel Müller (Charite, Berlin, Germany) and maintained in Dulbecco's minimal essential medium (DMEM; Life Technologies) supplemented with 10% FBS and non-essential amino acids (Life Technologies).

Viruses

A/Dalmatian Pelican/Bern/1/2022 (H5N1) was isolated from biopsy material prepared from a diseased Dalmatian Pelican in Bern Animal Park in February 2022. Briefly, brain and kidney tissues were homogenized and subjected to centrifugation (5 min, 14,000 × *g*, 4 °C). The clear supernatant was sterile-filtrated (Minisart NML cellulose acetate standard syringe filter with 0.2 μm pore size). The material that passed through the filter was inoculated onto confluent MDCK monolayers for 2 days at 37 °C. The supernatant was collected, cell debris removed by centrifugation, and the clear supernatant supplemented with 10% FBS, and stored in aliquots at -70 °C. Infectious virus titers were determined on MDCK cells by limiting dilution. The infected cells were washed once with PBS and fixed with 10% formalin containing 1% (w/v) of crystal violet. The tissue infectious dose 50% ($TCID_{50}$) was calculated according to the Spearman-Kärber formula[61].

VSV* is a propagation-competent VSV encoding the green fluorescent protein (GFP) reporter protein. VSV*ΔG is a propagation-defective VSV encoding the GFP reporter protein in place of the G protein gene[62]. Both viruses were titrated on either BHK-21 or Vero cells[62]. VSV*ΔG(EBOV-GP), a chimeric virus encoding the Ebola virus envelope glycoprotein in place of the VSV G gene, was propagated and titrated on Vero cells[39].

The propagation-competent chimeric viruses VSVΔG (HA_Y:NA_Y:GFP) encoding the HA and NA proteins of A/chicken/Yamaguchi/7/2004 (H5N1), clade 2.5, VSVΔG(HA_Y:NA_Y:GFP) encoding the HA protein of A/Muscovy duck/Vietnam/OIE-559/2011 (H5N1), clade 1.1, and VSVΔG(HA_{HK}:NA_Y:GFP) encoding the HA protein of A/peregrine falcon/Hong Kong/810/2009 (H5N1), clade 2.3.4, and VSVΔG(HA_{Ho}:NA_Y:GFP) encoding the HA protein of A/whooper swan/Hokkaido/4/2011 (H5N1), clade 2.3.2.1, have been described in a previous report[38]. These chimeric reporter viruses were propagated and titrated on MDCK cells.

Plasmids

Viral RNA was isolated with TRIzol reagent (Life Technologies, cat. no. 15596026) from 200 µL of A/Dalmatian Pelican/Bern/1/2022 (H5N1) stock (passage 2 on MDCK cells) according to the manufacturer's instructions. The RNA was reverse-transcribed by SuperScript III Reverse Transcriptase (Life Technologies, cat. no. 18080093) using the Uni12 primer that is complementary to the conserved 12 nucleotides of the 3′-end of the viral RNA[63]. Segment 4 and segment 6 cDNAs were amplified with Phusion™ High-Fidelity DNA Polymerase (Life Technologies, cat. no F530L) using segment-specific sense and antisense primers[64]. The PCR products were gel-purified, cloned into the pJET1.2 plasmid (Life Technologies, cat. no. K1231), and sequenced according to Sanger. The cDNA sequences of segment 4 and segment 6 have been deposited at the GenBank databank (accession nos. PX149233 and PX149234, respectively).

The open reading frame of A/Dalmatian Pelican/Bern/1/2022 (H5N1) HA was amplified with Phusion high fidelity DNA polymerase using the oligonucleotide primers IF-H5(P)-S(MluI) and IF-H5(P)-AS(NheI) (Supplementary Table 1) and cloned into the *Mlu*I and *Nhe*I sites of the pVSV*ΔG(HA) plasmid[34], resulting in plasmid pVSVΔG($H5_{pb}$). The HA polybasic (pb) proteolytic cleavage site REKRRKR was changed into a monobasic (mb) sequence motif (RETR) by generating two overlapping cDNA fragments[65]. Fragment 1 was amplified by PCR using the primers IF-H5(P)-S(MluI) and H5(P) mb-AS, and fragment 2 was produced using the primers H5(P)mb-S and IF-H5(P)mb-AS(NheI) (Supplementary Table 1). The two cDNA fragments were separated by agarose gel chromatography, excised from the gel, and purified. The purified cDNA fragments were mixed at equimolar ratio, denatured by heating at 95 °C, annealed to each other at 60 °C, and incubated with Phusion DNA polymerase at 72 °C to obtain a complete double-stranded chimeric cDNA. Finally, the modified H5 cDNA was amplified using the IF-H5(P)-S(MluI) and IF-H5(P)-AS(NheI) primers and cloned into the pVSV*ΔG(HA) plasmid resulting in pVSVΔG($H5_{mb}$). To generate a propagation-competent chimeric VSV reporter virus, the HA and NA genes of A/Dalmatian Pelican/Bern/1/2022 (H5N1) were amplified using the IF-H5(P)-S(MluI)/IF-H5(P)-AS(MluI) and IF-N1(P)-S(XhoI)/IF-N1(P)-AS(XhoI) primer pairs (Supplementary Table 1) and inserted into the *Mlu*I and *Xho*I sites of pVSVΔG(HA_R:NA_R:GFP)[66] by In-Fusion cloning (In-Fusion Snap Assembly Master Mix, Takara, Saint-Germain-en-Laye, France, cat. no. 638948), resulting in the plasmid pVSVΔG(HA_P:NA_P:GFP).

Generation of recombinant RNA replicon particles

Virus replicon particles (VRPs) have been produced according to a published procedure[35]. Briefly, BHK-G43 cells were inoculated for 1 h with modified vaccinia virus Ankara encoding the T7 phage RNA polymerase (MVA-T7)[67], using a multiplicity of infection (m.o.i.) of 3 focus-forming units (f.f.u.) per cell. Subsequently, the cell culture medium was replaced with GMEM containing 5% FBS and 10^{-9} M mifepristone (Merck KGaA, Darmstadt, Germany). The cells were transfected with Lipofectamine 2000 reagent (Life Technologies) and a mixture of four plasmids: pVSVΔG($H5_{mb}$:N1:GFP (or pVSVΔG($H5_{pb}$:NA:GFP) and plasmids encoding the VSV N, P, and L genes, respectively, all under the control of the T7 promoter (Kerafast, Boston, USA; cat. no. EH1012). One day post transfection, the cells were dissociated with trypsin/EDTA (Life Technologies) and co-cultured for 24 h with an equal number of non-transfected BHK-G43 cells in the presence of 10^{-9} M mifepristone. The cell culture supernatant was collected and cell debris removed by centrifugation (1200 x g, 10 min, 4 °C). The clarified cell culture supernatant was passed through a 0.2 µm pore size filter for depletion of MVA-T7. The replicon particles VSVΔG($H5_{mb}$) and VSVΔG($H5_{pb}$) were propagated on mifepristone-treated BHK-G43 cells and stored in aliquots at -70 °C in the presence of 10% FBS.

Titration of RNA replicon particles

Infectious virus titers were determined on BHK-21 cells grown in 96-well microtiter plates. The cells were inoculated in duplicate with 40 µL per well of serial 10-fold virus dilutions for 1 h at 37 °C. Thereafter, 60 µL of GMEM were added to each well and incubated for 24 h at 37 °C. The cells were fixed for 30 min with 3.7% formalin in PBS, permeabilized with 0.25% (v/v) of Triton X-100, and incubated for 60 min with a monoclonal antibody directed to the VSV matrix protein (mAb 23H12, diluted 1:25 with PBS, KeraFast, Boston, MA, cat. no. EB0011) and subsequently for 60 min with goat anti-mouse IgG conjugated with Alexa Fluor-488 (diluted 1:500 in PBS; Life Technologies, cat. no. A28175). Infected cells were detected by fluorescence microscopy (Observer.Z1 microscope, Zeiss, Feldbach, Switzerland), and infectious virus titers were calculated and expressed as focus-forming units (f.f.u.)/mL.

Preparation of the RNA replicon vaccine

For a typical vaccine batch preparation, BHK-G43 cells were seeded into five T150 flasks and maintained in 40 mL/flask of GMEM medium with 5% FBS. When confluency was reached, the medium was aspirated and the cells maintained at 37 °C for 6 h with 40 mL/flask of GMEM containing 10^{-9} M of mifepristone. VSVΔG($H5_{mb}$) was added (m.o.i of 0.05 f.f.u./cell), and incubated with the cells for 20 h at 37 °C. The cell culture supernatant was transferred to 50-mL Falcon tubes and cell debris removed by centrifugation (1000 x g, 15 min, 4 °C). The replicon particles were pelleted from the cleared supernatant by ultracentrifugation (105,000 x g, 60 min, 4 °C), and resuspended in 20 mL of PBS. The VSVΔG($H5_{mb}$) replicon vaccine was stored in 5-mL aliquots at -70 °C.

Western blot analysis

Confluent monolayers of BHK-G43 cells grown in T75 flasks were treated for 6 h with mifepristone (10^{-9} M) and subsequently infected with either VSVΔG($H5_{pb}$) or VSVΔG($H5_{mb}$) using an m.o.i. of 0.1 f.f.u./cell. At 24 h p.i., the cell culture supernatant of the infected cells was collected and cell debris removed by centrifugation (1200 x g, 15 min, 4 °C). Subsequently, virus particles were pelleted from the clarified cell culture supernatant by ultracentrifugation and solubilized by adding preheated (95 °C) sodium dodecyl sulfate (SDS) sample buffer containing 0.1 M of dithiothreitol (DTT) to the pellets. The solubilized proteins were separated by SDS polyacrylamide gel electrophoresis (PAGE) using two 4–12% gradient gels (SurePAGE™; Genscript, Leiden, The Netherlands). The separated proteins were visualized by incubating the first gel overnight with colloidal Coomassie (GelCode Blue Stain Reagent, Life Technologies, cat. no. 24590). The separated proteins of the second gel were transferred to a nitrocellulose membrane by semi-dry blotting. The nitrocellulose membrane was blocked overnight at 4 °C with Odyssey Blocking Reagent (Li-COR Biosciences, Lincoln, NE) and subsequently incubated with a polyclonal chicken immune serum which was directed against the low-pathogenic A/duck/Hokkaido/Vac-1/2004 (H5N1)[35]. The membrane was washed four times with PBS containing 0.1% Tween-20 and incubated with the secondary antibodies IRDye 800CW donkey anti-chicken IgY (LI-COR Biosciences, Cat. no. 926-32218) diluted 1:5000 in PBS. Following several washing steps with PBS/0.1% Tween 20, the blots were scanned with the Odyssey Infrared Imaging system (LI-COR Biosciences, Bad Homburg, Germany).

Indirect immunofluorescence analysis

MDCK cells grown on 12-mm glass coverslips were inoculated for 90 min with either VSV*ΔG, VSVΔG($H5_{pb}$), VSVΔG($H5_{mb}$) or VSVΔG($H5_P$:$N1_P$:GFP) using an m.o.i. of either 0.02 or 0.1 f.f.u./cell. The cells were washed once with MEM, further incubated for 16 h at 37 °C, and fixed for 30 min with PBS containing 3.7% formalin. The cells were incubated for 1 h with immune serum (1:100 in PBS) prepared from dairy cows 21 days post infection with A/cattle/Texas/063224-24-1/2024

(H5N1)[68]. The cells were washed with PBS, permeabilized for 5 min with 0.25% (vol/vol) of Triton X-100 in PBS and then incubated for 1 h with a monoclonal antibody directed to VSV matrix (M) protein (mAb 23H12, KeraFast, Boston, MA, cat. no. EB0011), diluted 1:25 in PBS. Thereafter, the cells were washed with PBS and then incubated for 1 h with goat anti-mouse IgG conjugated to Alexa Fluor-488 (1:500; Life Technologies, cat. no. A11001) and rabbit anti-bovine IgG conjugated to Rhodamine Red-X (1:250; Jackson ImmunoResearch Laboratories, West Grove, PA, USA, cat no. 301-295-003). Cells infected with VSV* or VSVΔG($H5_P$:$N1_P$:GFP) were not labelled with the VSV M-specific antibody but visualized taking advantage of the GFP reporter protein. Finally, the cells were stained for 5 min with 4′,6-diamidino-2-phenylindole (DAPI, 0.1 μg/mL; Merck KGaA, Darmstadt, Germany, cat. no. 9542), embedded in Mowiol 4–88, and analyzed by fluorescence microscopy.

Animal experiments

Vaccination and infection experiments with chickens were approved by the cantonal veterinary authority in Bern, Switzerland, under the authorization numbers BE26/2023 (see also the Ethics statement). Specific pathogen-free (SPF) White Leghorn chickens (5 to 6 weeks old, mixed sex) were obtained from the IVI breeding unit. A first experiment evaluated the protective efficacy of VSVΔG($H5_{mb}$) using a prime-boost regimen (Fig. 2). Chickens of groups A, B, and C (n = 8, each) were immunized intramuscularly (i.m.) by injecting 250 µL of either VSV*ΔG (group A) or VSVΔG($H5_{mb}$) (groups B and C) into the left and the right *musculus pectoralis major* (500 µL in total, corresponding to 10^8 f.f.u./animal). Animals of group D were immunized with VSVΔG($H5_{mb}$) by dropping 100 µL of VRP suspension into each eye (corresponding to 10^8 f.f.u./animal). The animals were kept for 4 weeks employing deep litter management and *ad libitum* access to feed and water. At day 28, chickens of groups A and B were boosted (i.m.) with 10^8 f.f.u. of VSV*ΔG and VSVΔG($H5_{mb}$), respectively, while animals of groups C and D were immunized via eye drop using 10^8 f.f.u./animal of VSVΔG($H5_{mb}$). At day 58, the chickens were infected via the nasal route with 50 µL of MDCK cell-grown (passage 2) A/Dalmatian Pelican/Bern/1/2022 (H5N1) containing 10^6 $TCID_{50}$. Following infection, the animals were surveyed daily for clinical signs of disease including general behavior of the animal, body shape, appearance of the feathers and skin, appearance of the eyes, respiratory, neurological, and intestinal/renal symptoms. A clinical scoring system was used to assess the severity of the disease and to define the humane endpoint: 0, no symptoms; 1, mild symptoms; 2, moderate symptoms, 3, severe symptoms. Animals were immediately euthanized if they reached a total clinical score of 5 or more, or if a clinical score of 2 was reached at two consecutive days, or if a score of 3 was reached for one or more of the clinical parameters at any time.

Swab samples were collected on days 1, 2, 3, 5, and 7 p.i. and tested for viral RNA by RT-qPCR (see below). At day 68, all surviving animals were euthanized. Blood was also collected on day 1 (pre-immune), day 28 (prime), day 56 (boost), and day 68 (*post mortem*). Serum was prepared by centrifugation of coagulated blood and stored at -20 °C before analysis by ELISA or virus neutralization test (see below). A second experiment evaluated the minimal protective dose after a single immunization of doses of 10^8 to $5x10^6$ f.f.u./animal under similar conditions as the first experiment, applying the challenge infection 22 days after vaccination (Supplementary Fig. 3).

Vaccination experiments in zoo birds were approved by the cantonal veterinary authority in Bern, Switzerland, under the authorization numbers BE24/2023 (see also the Ethics statement). For vaccination of captive birds, 348 birds belonging to 23 different species were selected in Basel Zoo and Bern Animal Park. Preference was given to birds living in outdoor enclosures with potential exposure to avian influenza. Prior to immunization, all birds were subject to clinical examination including assessment of general health, body condition, and body weight. Only birds deemed clinically healthy were included in the study. The species, number of animals, sex, and date of birth of all zoo birds included in the vaccination trial are listed in Supplementary Data 1. The zoo birds were vaccinated on six different dates between August 23, 2023, and September 13, 2023. Blood samples were collected at days 1, 35, 70, 365, and 400 using either the *vena jugularis*, *vena ulnaris* or the *vena metatarsalis medialis* as sampling sites. Approximately 1.0 mL of blood was collected per bird using 22 G to 27 G disposable hypodermic needles (100 Sterican, B. Braun Melsungen AG, Germany), depending on the size of the bird. A total of 317 birds were vaccinated three times (at days 1, 35, and 365) using 10^8 f.f.u./mL of VSVΔG($H5_{mb}$). The vaccine was usually administered by injection into the *musculus pectoralis major*. However, in the case of pelicans, which have subcutaneous air sacs with large extensions, *musculus gastrocnemius* was injected. Birds with body weights of less than 1.5 kg received 0.25 mL of the vaccine (corresponding to $5x10^7$ f.f.u./animal.), while birds with more than 1.5 kg of body weight received 0.5 mL of the VRP suspension. Following immunization, zoo birds were closely monitored for seven days by the attending veterinarians and animal caretakers to detect potential adverse reactions or changes in animal behavior in response to the vaccination.

RT-qPCR

To determine virus loads in oropharyngeal and cloacal swab samples, swab tips were placed into 0.5 mL of RA1 lysis buffer (Macherey-Nagel, Düren, Germany; cat. no. 740961) containing 1% (v/v) β-mercaptoethanol. After a short centrifugation step, RNA was extracted from 200 µL lysate using the NucleoMag Vet kit (Macherey Nagel, cat. no. 744200) according to the manufacturer's protocol. Reverse transcription from RNA to cDNA and real-time quantitative PCR (qPCR) were performed on the QuantStudio 5 real-time PCR system (Thermo Fisher Scientific) using the AgPath-ID One-Step RT-PCR kit (Life Technologies, cat. no. AM1005) and segment 7-specific oligonucleotide primers and probe[69,70]. Data were acquired and analyzed using Design and Analysis Software v1.5.2 (Thermo Fisher Scientific).

Enzyme-linked Immunosorbent Assay (ELISA)

For detection of serum antibodies directed against influenza nucleoprotein (NP) and H5 hemagglutinin, the ID Screen® Influenza A Antibody Competition Multi-species ELISA (ID-Vet, Montpellier, France, cat. no. FLUACA) and the ID Screen® Influenza H5 Antibody Competition (ID-Vet, cat. no.: FLUACH5-5P) were used, respectively. ELISA tests were performed according to the manufacturer's instructions. Absorbance was read at 450 nm using a GloMax Discover GM3000 plate reader equipped with software 4.0.0, firmware 4.92.0 (Promega Madison, Wisconsin, USA).

Virus neutralization tests

Serum samples were heated for 30 min at 56 °C to inactivate factors of the complement system. Twofold serial dilutions of heat-inactivated immune sera were prepared in duplicates or quadruplicates in 96-well cell culture plates using MEM cell culture medium (50 µL/well). Sera of guinea pigs immunized with the VSV*ΔG(EBOV-GP) vaccine were used as controls[39]. To each well, 50 µL of cell culture medium containing 100 f.f.u. of either VSVΔG($H5_P$:$N1_P$:GFP), VSV*, or VSV*ΔG(EBOV-GP) were added and incubated for 30 min at 37 °C. Subsequently, the antibody/virus mixture was transferred to confluent MDCK or Vero cell monolayers in 96-well cell culture plates and incubated at 37 °C for 24 h. Infected cells were detected by fluorescence microscopy taking advantage of the virus-encoded GFP reporter. Neutralization dose 50% (ND_{50}) values were calculated according to the Spearman and Kärber method[61].

Neutralization tests with authentic A/Dalmatian Pelican/Bern/1/2022 (H5N1) were performed analogously. However, the assay was performed in quadruplicates using 100 $TCID_{50}$ of virus per well. The

antibody/virus mixtures were incubated with MDCK cells for 48 h. Thereafter, the cells were washed once with 200 µl of PBS/well and fixed for 60 min at 21 °C with 10% formalin containing 1% crystal violet. Finally, the microtiter plates were rinsed with tap water and the ND_{50} titer calculated as above.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 10, version 10.1.2 (GraphPad Software, Boston, Massachusetts, USA). Unless noted otherwise, data are presented as scatter dot plots with geometric mean values and 95% confidence intervals (CI) also indicated. Specific statistical tests such as the one-way or two-way ANOVA test were used to assess significant differences in serum antibody responses in vaccinated animals as indicated in the figure legends. P values < 0.05 were considered significant.

Biosafety

Work with HPAI A/Dalmatian Pelican/Bern/1/2022 (H5N1) has been approved by the Swiss Federal Office of Public Health (license no. A230074-01) and was performed at IVI laboratories and animal facilities complying with biosafety level 3. Immunization with the genetically modified virus replicon vaccine was approved by the Swiss Federal Office for the Environment (FOEN) (reference no. BAFU-217.2344635/2) with consent by the Federal Office of Public Health (FOPH), the Federal Food Safety and Veterinary Office (FSVO), and the Federal Office for Agriculture (FOAG).

Ethics statement

This study was conducted in compliance with Swiss animal welfare regulations (TSchG SR 455; TSchV SR 455.1; TVV SR 455.163). The committee on animal experiments of the canton of Bern, Switzerland, reviewed the experiments, and the cantonal veterinary authority (Amt für Landwirtschaft und Natur LANAT, Veterinärdienst VeD, Bern, Switzerland) approved the study under the authorization numbers BE24/2023 (for zoo birds) and BE26/2023 (for chickens).

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All data generated in this study are provided without restriction in the main text, figures and Supplementary files of this publication. The nucleotide sequences of genome segments 4 and 6 of the HPAI isolate A/Dalmatian Pelican/Bern/1/2022 (H5N1) have been deposited at the GenBank data bank under accession numbers PX149233 and PX149234, respectively. Source data are provided with this paper and are also available at Zenodo (https://doi.org/10.5281/zenodo.15753718). Source data are provided with this paper.

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Author contributions

S.H., C.W., and G.Z. designed and supervised the study. L.B., M.S., and G.Z. produced and characterized the viral vector vaccine. L.B., M.S., K.D., and G.Z. performed serological and molecular diagnostic assays. E.H., S.H., N.R., M.S., C.W., F.W., and G.Z. were involved in the organization and performance of the animal experiments. M.S. and G.Z. carried out data analysis and processing. M.S. and G.Z. wrote the manuscript draft. K.D., E.H., S.H., N.R., M.S., F.W., C.W., and G.Z. edited the manuscript. S.H. and G.Z. were involved in funding acquisition.

Competing interests

The authors declare no competing interests.

Additional information

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