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

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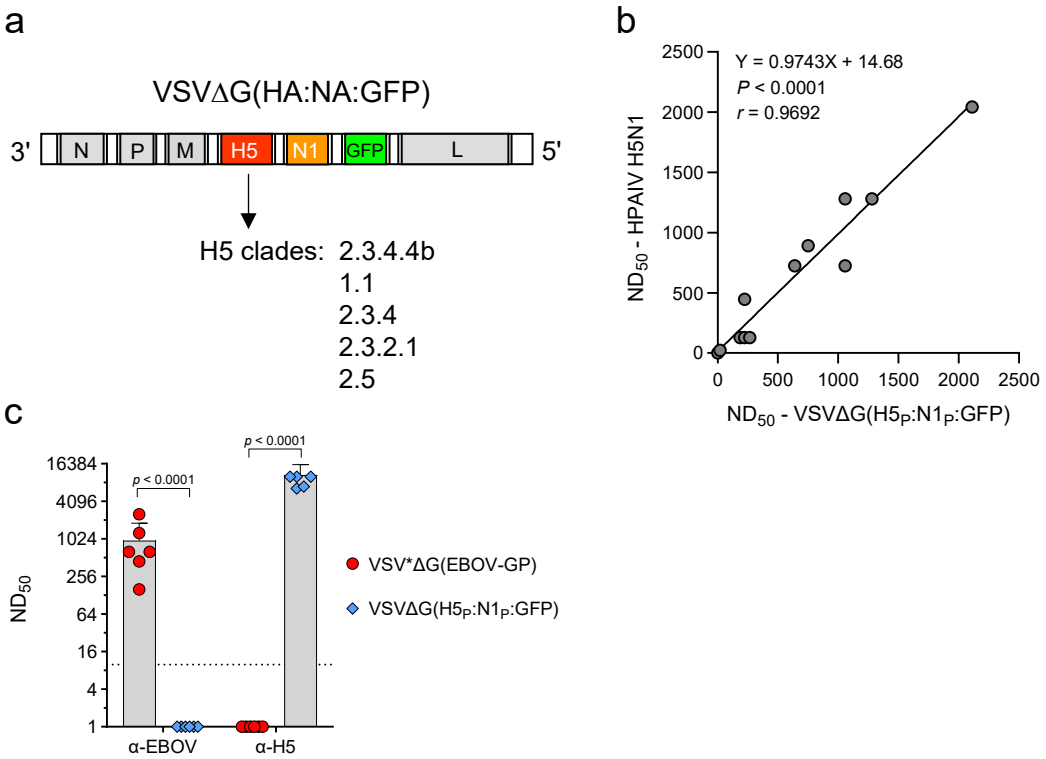
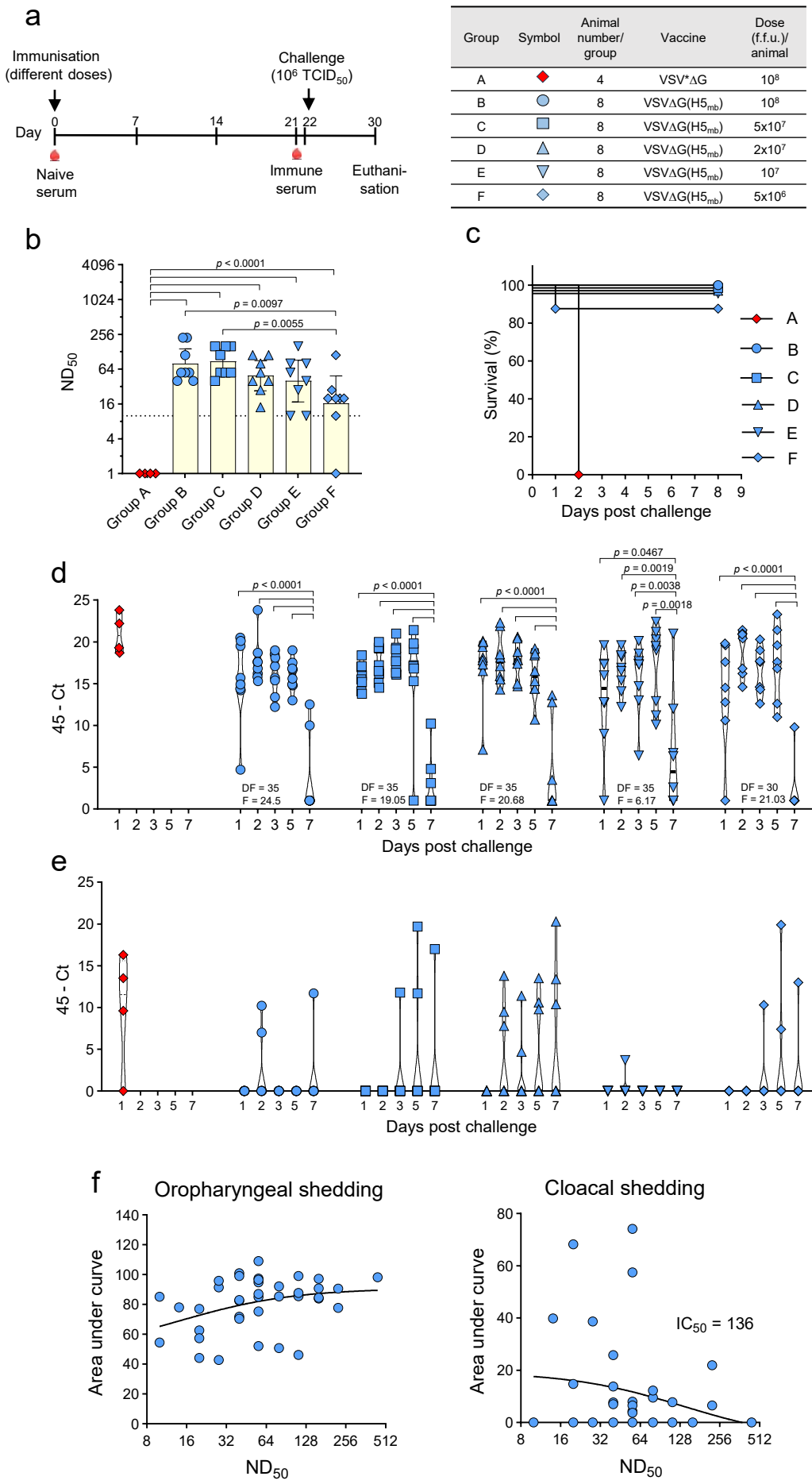
Supplementary Information

Supplementary Fig. 1 | Comparison of the HA_1 subunit amino acid sequences of different H5 clades. The HA_1 primary sequence of A/Dalmatian Pelican/Bern/1/2022 (H5N1), clade 2.3.4.4b (GenBank acc. no. PX149233), was compared with the HA_1 amino acid sequences of A/Peregrine falcon/Hong Kong/810/2009 (H5N1), clade 2.3.4 (GenBank acc. no. BAI39636), A/Whooper swan/Hokkaido/4/2001 (H5N1), clade 2.3.2.1 (GenBank acc no. BAJ71684), A/chicken/Yamaguchi/7/2004 (H5N1), clade 2.5, (GenBank acc. no. AB166864), A/Muscovy duck/Vietnam/OIE-559/2011 (H5N1), clade 1.1 (GenBank acc. no. BAK39622) using CLUSTAL W. The globular head region is indicated by a dashed line on top of the sequences. The 130-loop, 190-helix and 220-loop that are part of the receptor-binding pocket are indicated by grey background. Amino acid positions differing from the 2.3.4.4b reference sequence are highlighted by yellow background. Amino acid residues that are unique to the clade 2.3.4.4b HA_1 primary sequence are marked by bold red letters.

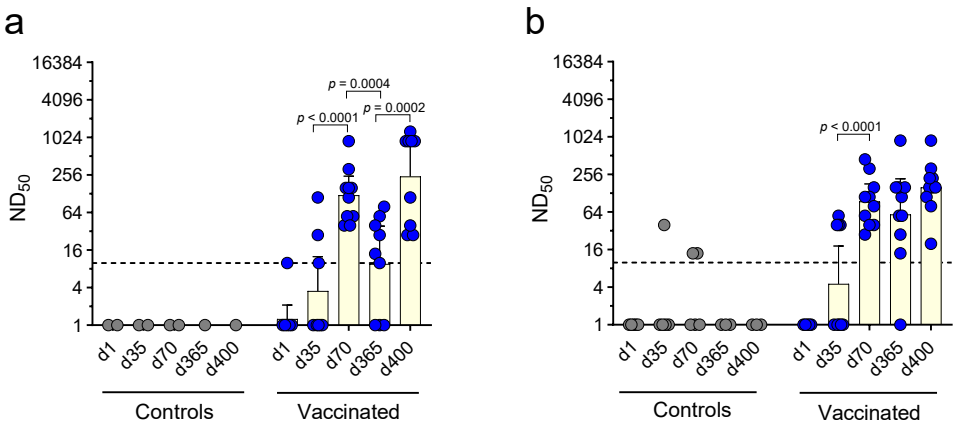


Supplementary Fig. 2 | Virus neutralization test using chimeric VSV reporter virus. **a** Genome map of the propagation-competent VSVΔG(H5:N1:GFP) vector encoding the HA and NA envelope glycoproteins of H5N1 HPAI viruses and a GFP reporter. Surrogate viruses encoding the HA antigen derived from either of the indicated H5 clades were used in this study. **b** Linear regression analysis of surrogate and traditional virus neutralization tests. Twelve pre-immune and immune sera prepared from the blood of VSVΔG($H5_{mb}$)-immunized chickens were analyzed by virus neutralization test using either A/Dalmatian Pelican/Bern/1/2022 (H5N1) or the VSVΔG($H5_P$:$N1_P$:GFP) surrogate virus. ND_{50} titers obtained with either of the two assays were analyzed by linear regression. **c** Specificity of the neutralizing antibody response. Virus-neutralization tests were performed with either VSV*ΔG(EBOV-GP) (red circles) or VSVΔG($H5_P$:$N1_P$:GFP) (blue rhombs) using immune sera (α-EBOV) from guinea pigs (n = 6) immunized with VSV*ΔG(EBOV-GP) and immune sera (α-H5) from chickens (n = 6) immunized with VSVΔG($H5_{mb}$). The unpaired, two-tailed Student`s *t* test was used to assess significant differences between the antibody-mediated neutralization of VSV*ΔG(EBOV-GP) (t = 16, df = 10) and VSVΔG($H5_P$:$N1_P$:GFP) (t = 56, df = 10). Source data are provided as a Source Data file.



Group	Symbol	Animal number/ group	Vaccine	Dose (f.f.u.)/ animal
A	◆	4	VSV*ΔG	10^8
B	●	8	VSVΔG($H5_{mb}$)	10^8
C	■	8	VSVΔG($H5_{mb}$)	$5x10^7$
D	▲	8	VSVΔG($H5_{mb}$)	$2x10^7$
E	▼	8	VSVΔG($H5_{mb}$)	10^7
F	◆	8	VSVΔG($H5_{mb}$)	$5x10^6$

Supplementary Fig. 3 | A single vaccine dose is protective but does not prevent virus excretion. **a** Schematic representation of the experimental design. Red drops below the timeline indicate time points of blood sampling. The vaccine groups with the identifying colors/symbols, number of animals, vaccine name, and vaccine dose are depicted on the right-hand side. **b** Determination of the virus neutralization dose 50% (ND_{50}) in serum of animals 3 weeks after immunization. Data are presented as scatter dot plots. The height of the bars indicates the geometric mean. The error bars show the 95% confidence interval. The one-way ANOVA test with Tukey`s multiple comparisons was used to identify significant ND_{50} values between groups (F = 18.04; DF = 38). (**c**) Survival rate of SPF chickens following nasal infection with 10^6 $TCID_{50}$ of A/Dalmatian Pelican/Bern/1/2022 (H5N1). **d, e** Determination of virus load by RT-qPCR in oropharyngeal (**d**) and cloacal (**e**) swab samples collected at the indicated days p.i.. The one-way ANOVA test with Tukey`s multiple comparisons was used to identify significant levels of virus load between days post challenge. F and DF values are indicated. **f** Non-linear regression analysis (curve fitting) of virus shedding (viral RNA load) versus neutralizing serum antibody titers (ND_{50}). For each animal, the viral RNA load (45 – Ct value) in oropharyngeal and cloacal swab samples was plotted as a function of time (days 1–7 after infection). The area under the curve (AUC) was calculated and then plotted against the ND_{50} titer of the corresponding animals. Source data are provided as a Source Data file.



Supplementary Fig. 4. Detection of VSV-neutralizing antibodies in serum of VSVΔG($H5_{mb}$)-immunized animals. a, b Dalmatian pelicans (*Pelecanus crispus*, n = 10, + 2 controls) in Bern (**a**), and Silky chickens (*Gallus gallus domesticus*, n = 10, + 5 controls) in Basel (**b**) were immunized intramuscularly with VSVΔG($H5_{mb}$) particles produced on VSV G-expressing cells. The animals were boosted with the same vaccine at days 35 and 365. Two Dalmatian pelicans and five Silky chickens were not vaccinated and served as controls. After blood was withdrawn from the animals at the indicated days, sera were prepared and tested for the presence of VSV-specific neutralizing antibodies using the propagation-competent VSV* reporter virus. Data are presented as scatter dot plots. Non-vaccinated control animals are represented by grey circles and vaccinated animals by blue circles. The height of the bars indicates the geometric mean. The error bars show the 95% confidence interval. The dashed line represents the lower detection limit (ND_{50} = 10). The one-way ANOVA test with Tukey`s multiple comparisons was used to assess significantly different ND_{50} titers between different time points of blood collection (**a**, F = 23.38, total DF = 43; **b**, F = 26.69, total DF = 45). Source data are provided as a Source Data file.

Supplementary Table 1. Oligonucleotide PCR primers used in this study.[a]

Primer	Sequence
IF-H5(P)-S(MluI)	5`-CGATCTGTTTACGCGTAAAATGGAGAACATAGTACTTCTTCTT-3`
IF-H5(P)-AS(MluI)	5`-AGGATTTGAGACGCGTCTAAATGCAAATTCTGCACTGTAACGAC-3`
IF-H5(P)-AS(NheI)	5`-GAAGAATCTGGCTAGCCTAAATGCAAATTCTGCACTGTAACGAC-3`
H5(P)mb-S	5`-TAGTCCTCTAAGAGAAACAAGAGGCCTGTTTGGGGCGAT-3`
H5(P)mb-AS	5`-TCGCCCCAAACAGGCCTCTTGTTTCTCTTAGAGGACTATTTCT-3`
IF-N1(P)-S(XhoI)	5`-AGATATCACGCTCGAGAAAATGAATCCAAATCAAAGGATAATAAC-3`
IF-N1(P)-AS(XhoI)	5`-GAAGAATCTGCTCGAGCTACTTGTCAATTGTGAATGGCAACTC-3`

[a] Oligonucleotide primers were synthesized by Microsynth AG, Balgach, Switzerland.

Supplementary Table 2. Clinical score of SPF chickens following nasal challenge infection with A/Dalmatian Pelican/Bern/1/2022 (H5N1).

Animal no.	Clinical score at the indicated day post challenge[a]											
	d1	d2	d3	d4	d5	d6	d7	d8	d9	d10	d11	d12
A1	0	1	5[b]									
A2	0	Dead										
A3	0	5[b]										
A4	0	3	5[b]									
A5	0	5[b]										
A6	1	Dead										
A7	0	5[b]										
A8	1	2	Dead									
B1	0	0	0	0	0	0	0	0	0	0	0	0
B2	0	0	0	0	0	0	0	0	0	0	0	0
B3	0	0	0	0	0	0	0	0	0	0	0	0
B4	0	0	0	0	0	0	0	0	0	0	0	0
B5	0	0	0	0	0	0	0	0	0	0	0	0
B6	0	0	0	0	0	0	0	0	0	0	0	0
B7	0	0	0	0	0	0	0	0	0	0	0	0
B8	0	0	0	0	0	0	0	0	0	0	0	0
C1	0	0	0	0	0	0	0	0	0	0	0	0
C2	0	0	0	0	0	0	0	0	0	0	0	0
C3	0	0	0	0	0	0	0	0	0	0	0	0
C4	0	0	0	0	0	0	0	0	0	0	0	0
C5	0	0	0	0	0	0	0	0	0	0	0	0
C6	0	0	0	0	0	0	0	0	0	0	0	0
C7	0	0	0	0	0	0	0	0	0	0	0	0
C8	0	0	0	0	0	0	0	0	0	0	0	0
D1[b]												
D2	0	0	0	0	0	0	0	0	0	0	0	0
D3	0	0	0	0	0	0	0	0	0	0	0	0
D4	0	0	0	0	0	0	0	0	0	0	0	0
D5	0	0	0	0	0	0	0	0	0	0	0	0
D6	0	0	0	0	0	2	6[c]					
D7	0	0	0	0	0	0	0	0	0	0	0	0
D8	0	0	0	0	0	0	0	0	0	0	0	0

[a] Clinical symptoms of disease were rated 0 (no symptoms), 1 (mild symptoms), 2 (moderate symptoms), 3 (severe symptoms). The total score corresponds to the cumulative sum of all symptoms rated and was assigned for each animal every day after challenge infection.

[b] Animal was euthanized due to severe symptoms of disease.

[c] Animal died prior to challenge infection.