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Silent Travelers: The Potential of Migratory Birds and their Ticks to Introduce Emerging Viruses into Switzerland

Inaugural-Dissertation

to be awarded the Doctoral Degree of the
Vetsuisse Faculty University of Bern

submitted by

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2026

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Zusammenfassung

Vetsuisse Faculty University of Bern 2026

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Silent Travelers: The Potential of Migratory Birds and their Ticks to Introduce Emerging Viruses into Switzerland

Die Schweiz beheimatet über 400 Vogelarten, von denen viele interkontinental migrieren. Diese Vögel können zoonotische Viren wie das West-Nil-Virus (WNV) oder aviäre Influenza-Virus (AIV) einschleppen und infizierte Zecken verbreiten, was das Risiko für die Einführung neuartiger Krankheiten erhöht. Um dieses Risiko zu bewerten, haben wir 2023 im Naturschutzgebiet Bolle di Magadino im Tessin (Schweiz), einem wichtigen Rastplatz für Zugvögel, 42 Vogelarten (39 Zugvogelarten, 3 Standvögel) beprobt. Wir sammelten 496 Abstriche, 164 Seren und 22 Zecken. Während die RT-qPCR keine aktiven WNV- oder AIV-Infektionen nachwies, identifizierte der ELISA Orthoflavivirus-Antikörper in 15 von 164 Seren. Die metagenomische Sequenzierung enthüllte eine Virenlandschaft aus 30 Familien (28 in Vögeln). Innerhalb dieses Viroms dominierten DNA-Viren (*Circoviridae*, *Genomoviridae*, *Parvoviridae*) in ihrer Abundanz bei geringer Diversität. Das hochdiverse RNA-Virom spiegelt hingegen die Ernährung der Vögel und das lokale Habitat des Naturschutzgebietes wider. Bemerkenswerterweise gelang der Erstdnachweis des Bole tick virus 4 (BLTV4) in der Schweiz, identifiziert in Zecken (*Rhipicephalus turanicus*) von Langstreckenziehern. Diese Studie stellt die erste aktive Überwachung des Wildvogel-Viroms in der Schweiz dar. Sie etabliert einen Rahmen für die Früherkennung von Pathogenen und unterstreichen die Rolle von Zugvögeln als Ausbreitungsvektoren für Zecken und neuartige, durch Zecken übertragene Viren.

Avian virome, Tick virome, Migratory strategy, Metatranscriptomics, Next-generation sequencing

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Abstract in thesis language

Vetsuisse Faculty University of Bern 2026

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Silent Travelers: The Potential of Migratory Birds and their Ticks to Introduce Emerging Viruses into Switzerland

Switzerland hosts over 400 bird species, many of which migrate intercontinentally. These birds can introduce zoonotic viruses like West Nile (WNV) or avian influenza (AIV) and disperse infected ticks, risking the introduction of novel tick-borne diseases. To assess this, we sampled 42 bird species (39 migratory, 3 resident) in 2023 in the nature reserve Bole di Magadino (Ticino, Switzerland), a critical stopover site. We collected 496 swabs, 164 sera, and 22 associated ticks. While RT-qPCR detected no active WNV or AIV infection, ELISA identified Orthoflavivirus antibodies in 15/164 sera, indicating prior exposure. Metagenomic sequencing revealed a viral landscape spanning 30 families (28 identified in birds). Within this virome, DNA viruses, predominantly Circoviridae, Genomoviridae, and Parvoviridae, dominated in abundance but exhibited low diversity. In contrast, the highly diverse RNA virome reflected birds dietary habits and the local habitat at the nature reserve. Notably, we report the first detection of Bole tick virus 4 (BLTV4) in Switzerland, identified in several ticks (*Rhipicephalus turanicus*) associated with long-distance migrants. This study provides the first active surveillance of the wild bird virome in Switzerland. Our findings establish a framework for early pathogen detection and underscore the role of migratory birds as dispersal vectors for ticks and novel tick-borne viruses.

Avian virome, Tick virome, Migratory strategy, Metatranscriptomics, Next-generation sequencing

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1. Introduction

Wild birds, with over 11,000 different species globally, represent the most diverse tetrapod clade [1–3]. In Switzerland alone, more than 400 species have been recorded [4]. A key ecological feature is their migratory behaviour, with the African-Eurasian flyway acting as a corridor for over two billion birds during their biannual migration, marking it as one of the most critical migratory routes globally [5,6]. Migration distances vary widely among species, from hundreds to several thousand kilometers, with flight durations lasting from one week to a month [7–10]. During migration, birds cross numerous countries and frequently stop at selected stopover sites to rest and feed, where high bird density favours horizontal transmission of microorganisms within and across species [5,11–13].

Birds are flying endothermic vertebrates with a unique adaptive immune system, characterized by a compact genetic repertoire and mechanisms like gene conversion [14–16]. During migration, they must constantly adapt to new environments and pathogens [6,15–18]. Due to the high energetic demands of long-distance flight, a response to novel antigens may be reduced during active migration [19]. This allows for prolonged asymptomatic virus excretion and facilitates host-virus coevolution [13,14]. This phenomenon has been observed in wild waterfowl, the primary reservoir of avian influenza virus (AIV). In Europe, AIV exhibits winter seasonality as aquatic birds congregate in wetlands as viral particles maintain their infectivity for extended periods in cold aquatic environments [13,20–22]. Furthermore, the introduction of West Nile virus (WNV) Lineage 2 from endemic regions of sub-Saharan Africa into Hungary in the mid-2000s supports the role of birds as a broader viral reservoir. This virus circulates between birds and mosquitoes, which acquire WNV from viraemic birds and transmit it to other birds or incidental hosts such as humans and horses, causing local cases and outbreaks [13,14,23].

Beyond recognized zoonotic pathogens, a high diversity of unidentified viruses has been detected by metagenomic studies, including non-zoonotic or of unclear clinical relevance [24–26]. Viruses from families such as *Astroviridae*, *Picornaviridae*, *Adenoviridae*, *Circoviridae*, and *Parvoviridae* are frequently sequenced from various bird species [27,28]. Additionally, many sequences likely originate from viruses associated with insects, plants, fungi, or bacteria, reflecting dietary intake or environmental exposure rather than active infection [29]. Migratory birds also passively transport hematophagous ectoparasites, particularly ixodid ticks. As a result, African ticks such as *Hyalomma rufipes* are frequently introduced into Europe and may be infected by zoonotic pathogens, most notably the Crimean-Congo haemorrhagic fever virus (CCHFV) [13,30–33].

Despite the increase in research, metagenomic exploration of avian viruses has predominantly targeted commercial livestock, primarily poultry-related diseases such as *Salmonella* and enteric disorders like runting-stunting syndrome (RSS) or has been restricted to passive monitoring during mass mortality events [15,26,34–48]. While waterfowl are extensively monitored as the main natural reservoir for AIV [22], this narrow focus creates a gap in global health surveillance regarding other avian taxa. Consequently, research is heavily skewed towards certain species and pathogens, leaving most avian viromes, particularly passerines (songbirds such as sparrows, finches, or swallows), which represent 60% of avian diversity, largely unexplored [2]. While recent studies in China and Australia have begun to fill this gap, no metagenomic analysis has yet investigated migratory birds in Switzerland [2,24]. This represents a vulnerability in One Health preparedness, as ubiquitous birds frequently interface with urban and agricultural environments, increasing the potential for undetected zoonotic spillovers to humans and domestic animals. The limitations of such studies are largely attributable to practical challenges, ranging from complex legal frameworks and small sample sizes to the substantial resources required for viral

bioinformatics [49,50]. However, establishing a comprehensive viral landscape is essential to provide the baseline necessary for proactive risk assessment.

To address this gap, we performed next-generation sequencing (NGS) to characterize viral diversity in passerines and their associated ectoparasites at the Bolle di Magadino nature reserve (Ticino, Switzerland). This region, as a wetland of international importance and a major stopover site for migratory birds, is an excellent location for sampling and bird monitoring activities [23,51]. Its strategic position in Ticino, in close proximity to WNV-endemic areas in Northern Italy (such as the Po Valley), makes it a vital surveillance point to assess whether migratory birds facilitate the introduction of emerging viruses into Switzerland as they travel along intercontinental flyways [23]. We also investigated whether a bird's geographic origin and migratory strategy are key determinants of virome composition. In contrast to established diagnostic tools like RT-qPCR and ELISA, which are designed to detect specific pathogens such as AIV or WNV, the NGS-based approach facilitates an exploratory and unbiased detection of the viral landscape that may otherwise remain undetected[52].

2. Materials and methods

2.1 Sampling sites and collection periods

Sampling was conducted in Piano di Magadino (Ticino, Switzerland), a rural area with suitable habitat for multiple bird species located near sites where WNV-positive FTA cards and *Culex pipiens* mosquitoes were detected in 2022 [23]. The Ramsar site of Bolle di Magadino, a unique wetland and marshy habitat, serves as an important resting place for migratory breeders. The sampling season was divided into 3 periods. The spring period (April to mid-May), with mist nets (1 km total length) primarily at the Ramsar site. The reproduction period (mid-May to the end of June), with nets on private property in Cadenazzo. To minimize disturbance during this sensitive nesting period, the sampling was restricted to 10- 14-day intervals from 5 a.m. to noon. Lastly, the post-reproductive period (end of June to end of September) involved nets on private property in Cadenazzo and at a farm in Sant'Antonino. Additionally, field rangers provided samples from birds, particularly hooded crows, captured in various locations across Ticino for population containment plans. Capture and handling followed standard ornithological procedures [53,54].

2.2 Sample collection

Bird captures and sampling procedures were conducted under the authorization and supervision of the Cantonal Veterinary Office of Ticino (Permit No. N-35671-TI35/2023/2023) and adhered to Swiss animal welfare legislation. Once captured, birds were identified by species, sexed, aged (if possible), and marked with metal rings before sampling and released. To facilitate viral preservation, 496 oropharyngeal and cloacal swabs were obtained from birds using sterile swabs (DRYSWAB™ ENT MW151, Medical Wire & Equipment, Corsham, Wiltshire, England) and transferred into 1 ml UTM™ tube (Universal Transport Medium, Copan, Brescia, Italy) to preserve viruses. Swabs were stored at 4°C for 24-48 hours, then at -80°C. Blood sampling, not exceeding 1% of the birds' body weight, was performed from the brachial vein of each individual bird using a 25G 5/8 needle (0.5x16 mm, Becton Dickinson, Franklin Lakes, NJ, USA). The blood was collected into a non-heparinized calibrated capillary micropipette 100 µl (Cat. No 2-000-100, DRUMMOND, Broomall, PA, USA) and subsequently transferred to the 1.5 ml collection tube using a 15" aspirator tube (Cat. No 2-000-000, DRUMMOND, Broomall, PA, USA). A total of 164 blood samples were kept at 4 °C for 24-48 hours to allow clotting and afterwards centrifuged at 2000 x g, for 10 min at 4 °C to separate serum from clot. Sera were stored at -20 °C while clotted blood at -80 °C, until further analysis. Additionally, a total of 22 ticks found were collected, placed in Eppendorf tubes, and kept in a cooling bag until storage at -80°C (Tab. 1).

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Table 1: Bird species, their migratory behaviour, nutrition and numbers of samples collected in this study

Migratory behavior	Bird species	Swab	Serum	Tick	Nutrition
Resident birds	<i>Corvus corone cornix</i>	10	1		Omnivores
	<i>Dendrocopos major</i>	1	1		Insects, seeds
	<i>Picus viridis</i>	2	1		Insects
Partial migrants	<i>Carduelis chloris</i>	2	1		Seeds
	<i>Cettia cetti</i>	1			Insects, spiders, worms
	<i>Falco tinnunculus</i>	1			Insects, small mammals, birds
	<i>Fringilla coelebs</i>	3	2		Insects, Seeds
	<i>Garrulus glandarius</i>	1			Omnivores
	<i>Parus major</i>	3	1		Insects, seeds
	<i>Passer italiae</i>	2	1		Insects, seeds
	<i>Passer montanus</i>	3	2		Insects, seeds
	<i>Sylvia melanocephala</i>	1			Insects, berries
	<i>Turdus merula</i>	7	6	1	Fruits, insects, worms
	<i>Turdus philomelos</i>	2	1		Insects, berries, snails, worms
Short-distance migrants	<i>Emberiza schoeniclus</i>	4	2		Insects, seeds
	<i>Erithacus rubecula</i>	53	4	2	Insects, berries, spiders
	<i>Luscinia svecica cyanecula</i>	1			Insects, spiders
	<i>Phylloscopus collybita</i>	21			Insects, spiders
	<i>Remiz pendulinus</i>	1			Insects, seeds
	<i>Serinus serinus</i>	4			Seeds
	<i>Sylvia atricapilla</i>	154 ^a 143 ^b	76		Insects, berries, spiders
	<i>Turdus iliacus</i>	1		1	Insects, berries, worms
	<i>Turdus philomelos</i>	2	1		Insects, berries, snails, worms
	<i>Lymnocyptes minimus</i>	1			Insects, seeds
Long-distance migrants	<i>Acrocephalus arundinaceus</i>	25 ^a 24 ^b	15	2	Insects
	<i>Acrocephalus schoenobaenus</i>	7 ^a 6 ^b	1		Insects
	<i>Acrocephalus scirpaceus</i>	34 ^a 32 ^b		4	Insects
	<i>Curruca communis</i>	30 ^a 27 ^b	7	2	Insects
	<i>Curruca curruca</i>	5 ^a 4 ^b	3		Insects
	<i>Ficedula hypoleuca</i>	5 ^a 4 ^b			Insects
	<i>Hirundo rustica</i>	14	11		Insects, spiders
	<i>Jynx torquilla</i>	6	5		Insects
	<i>Lanius collurio</i>	13	4		Insects, small mammals, reptiles
	<i>Locustella naevia</i>	2			Insects
	<i>Luscinia megarhynchos</i>	4			Insects, spiders
	<i>Luscinia svecica</i>	2	1		Insects, spiders
	<i>Muscicapa striata</i>	1			Insects, spiders
	<i>Otus scops</i>	1			Insects
	<i>Phoenicurus phoenicurus</i>	20	8	1	Insects, spiders
	<i>Phylloscopus trochilus</i>	23			Insects
	<i>Saxicola rubetra</i>	12	3	9	Insects, spiders
	<i>Sylvia borin</i>	12	6		Insects
	<i>Ixobrychus minutus</i>	1	1		Insects, fish, snails, amphibians
	Total	496^a	164	22	
		476^b			

^a Number of sampled birds ^b Number of samples available for NGS.

2.3 Molecular and serological analysis

Automated viral RNA extraction from oropharyngeal and cloacal swabs was performed on the Maxwell® RSC platform (Promega Corporation, Madison, WI, USA) using 200 µl of UTM™ as starting material. Purification was carried out using the Maxwell® RSC Viral Total Nucleic Acid Purification Kit AS1330 (Promega Corporation, Madison, WI, USA), following the manufacturer's instructions, with an elution volume of 50 µl of nuclease-free water. Viral RNA from blood clots was extracted manually using the RNeasy Plus Universal kit (Qiagen, Hilde, Germany) and eluted in 60 µl of RNase-free water. The presence of WNV in extracted RNA was analyzed by RT-qPCR using primers and probes, as previously described [23]. Mengo virus strain vMC0 was added to each sample as an internal control of the extraction process and subsequently analyzed by specific RT-qPCR [55]. All RT-qPCR assays were performed on a 7500 Real-Time PCR System (Applied Biosystems™, Waltham, MA, USA). Sera were tested for neutralizing antibodies against WNV using the INGEZIM West Nile COMPAC kit (Gold Standard Diagnostics, Madrid, Spain) following the manufacturer's instructions. Optical density was measured at 450 nm with Infinite® F50 plate reader and Magellan™ software (Tecan Group Ltd., Männedorf, Switzerland). The inhibition percentage (IP) was calculated in accordance with the protocol. Samples with IP ≥40% were classified as positive, ≤30% as negative and 30-40% as doubtful. To account for the high level of serological cross-reactivity within the *Orthoflavivirus* genus, samples yielding positive or doubtful results were subjected to confirmatory serum neutralization testing (SNT) [56]. The test was performed in vitro with Vero cells (2x10⁴ cells/0.1mL), GMEM medium, sodium bicarbonate 7.5% solution, antibiotics (penicillin and streptomycin), antifungal (Amphotericin B) (all Gibco, ThermoFisher Scientific, Waltham, Massachusetts, USA), West Nile virus (isolate France 2018/7025, titer 10e1.9) and crystal violet staining. To determine the titer of the neutralizing antibodies, a logarithmic dilution of the sera samples was prepared. However, due to the low volume of the starting serum material (<10µL), to be able to perform the assay in duplicates, samples were diluted 1:32, 1:64, 1:128, 1:256. (Supp. Tab. S2). Consequently, there was insufficient material to perform neutralization assays for other potentially cross-reactive *Orthoflaviviruses*.

2.4 Birds metagenomic sample processing

2.4.1 Swab Pooling

All 496 oropharyngeal and cloacal swab samples collected from 42 bird species were screened by RT-qPCR for AIV and WNV. However, 20 samples were excluded from further analysis due to material limitations, like insufficient sample volume after primary screening. Consequently, 476 samples were available for next-generation sequencing (NGS). First, instead of processing each sample individually, samples were pooled predominantly by species (42), with each pool containing a single species, however, four pools contained two species belonging to the same taxonomic family. Overall, 154 pools were prepared, including pools with four birds (n = 96), three (n = 11), two (n = 12), and only one bird (n = 35). Individual samples were analyzed for birds from which ticks had been removed and from those with a positive WNV antibody ELISA result (INGEZIM West Nile COMPAC kit, Gold Standard Diagnostics, Madrid, Spain). Finally, 200 µl of medium from each sample was transferred to a 1.5-ml Eppendorf tube. The total volume, ranging from 200 to 800 µl, varied depending on the number of birds (one to four). The Mengovirus strain vMC0 (Mengovirus Extraction Control Kit, bioMérieux, Geneva, Switzerland) served as an exogenous internal control at a ratio of 0.5 µl per 100 µl of medium. This control ensured consistent nucleic acid recovery and monitored potential inhibitors [57]. The tube was shaken for 15 seconds (Vortex Genie 2 Digital) and centrifuged at 14,000 rpm for 10 minutes (Eppendorf Centrifuge 5427 R).

2.4.2 Filtration

For the filtration process, the supernatant was collected using a 20G needle (0.90 x 40 mm, B. Braun Sterican 100, Melsungen, Germany) and a 1-ml syringe (B. BRAUN Omnifix-F, Melsungen, Germany). The supernatant was then pressed through a 0.45-µm syringe filter (Puradisc, 13 mm, Whatman GE Healthcare, Chicago, Illinois, USA) into a 2-ml Eppendorf tube. This step has been introduced to remove bacteria and host cells bigger than the filter size [58,59].

2.4.3 Nucleic acid isolation and SISPA amplification

To achieve a relative enrichment of viral particles, host and environmental nucleic acids not protected by a viral capsid were removed via nuclease treatment, followed by nucleic acid isolation as described previously [59]. To maximize the recovery of the avian virome, total nucleic acids were isolated using the QIAamp Viral RNA Mini Kit (Qiagen, Hombrechtikon, Switzerland) with modifications such as the omission of the RNA carrier to prevent host-derived background interference and addition of β-mercaptoethanol to a final concentration of 1% in the AVL buffer (Gibco, 50 mM, Thermo Fisher, Waltham, Massachusetts, USA) to eliminate any residual nuclease activity from previous nuclease treatment. To elute DNA and RNA viruses, 30 µl of RNase-free water and 30 µl of Tris-EDTA (TE) buffer (1x molecular biology grade, Thermo Fisher, Waltham, Massachusetts, USA) were used. Reverse transcription and second-strand synthesis were also performed as previously described using the SISPA (sequence-independent single-primer amplification) method [58]. Random amplification of dsDNA was performed using HotStarTaq polymerase (5U/µl; Qiagen, Hombrechtikon, Switzerland) and a 20-cycle protocol to increase the probability of detecting low-frequency viral sequences in samples from passerine birds. After amplification, the samples were purified using the QIAquick PCR Purification Kit (Qiagen, Hombrechtikon, Switzerland) following the manufacturer's instructions. Finally, the concentration of dsDNA was measured using the Qubit Fluorometer 4 (Invitrogen, Carlsbad, California, USA) [58,59].

2.5 Ticks metagenomic sample processing

2.5.1 Tick collection and identification

Twenty-two ticks (n=19 engorged, n=3 non-engorged) in adult or nymph stages were collected from 14 birds belonging to 10 species in the nature reserve Bolle di Magadino and Piano di Magadino, canton Ticino (TI, Switzerland, Tab. 2). Ticks were stored at -80°C until further processing. Morphological classification to the species level was achieved via stereomicroscope, following two identification guides provided by Estrada Peña [60,61].

Table 2: Overview of sampled ticks, their species, feeding status and collection timepoint, the host bird species with corresponding migratory behaviour, and Bole tick virus 4 detection.

Number of ticks	Tick species	Engorgement status	Bird species	Migration behaviour	Collection month in 2023	BLTV4 detection ^c
1	<i>Ixodes frontalis</i>	engorged	<i>Turdus iliacus</i>	short-distance	April	-
2 ^b	<i>Ixodes frontalis</i>	non-engorged	<i>Erithacus rubecula</i>	short-distance	April October	-
1	<i>Ixodes frontalis</i>	engorged	<i>Acrocephalus scirpaceus</i> ^a	long-distance	May	-
2	<i>Ixodes frontalis</i>	engorged	<i>Curruca communis</i>	long-distance	May	-
1	<i>Ixodes frontalis</i>	engorged	<i>Phoenicurus phoenicurus</i>	long-distance	May	-
1	<i>Ixodes frontalis</i>	engorged	<i>Arcocephalus arundinaceus</i> ^a	long-distance	May	-

1	<i>Rhipicephalus turanicus</i>	engorged	<i>Arcocephalus arundinaceus</i> ^a	long-distance	May	-
9 ^b	<i>Rhipicephalus turanicus</i>	engorged	<i>Saxicola rubetra</i>	long-distance	April	tick (1x)
3 ^b	<i>Rhipicephalus turanicus</i>	engorged	<i>Acrocephalus scirpaceus</i> ^a	long-distance	April May	tick (2x)
1	<i>Rhipicephalus turanicus</i>	non-engorged	<i>Turdus merula</i>	partial migrant	June	-
22						

^a Different tick species were collected from different individuals of the same bird species., ^b ticks collected from two different birds of the same species, ^c NGS Results

2.5.2 Sample processing

After the morphological identification, individual ticks were prepared for NGS. For surface decontamination, each tick was first washed twice with 80% Ethanol, dried with a paper towel and then homogenized in 650 µl of Dulbecco's phosphate-buffered saline (DPBS, St. Louis, Missouri, USA) with a stainless-steel bead (5mm, QIAGEN, Hilden, Germany) using a TissueLyzer II (Qiagen, Hilden, Germany) at 30 Hz for 5 minutes. Afterwards, the resulting homogenate was centrifuged at 16'060 x g for 6 minutes [62].

2.5.3 Nucleic acid isolation and SISPA amplification

The 230 µl supernatant was first enriched for virus particles as described for the bird samples. Total nucleic acids (RNA and DNA) were extracted using the QIAamp Viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany) with the same modification as previously. Furthermore, tick samples were processed following the same procedures as those applied to the avian samples. The final dsDNA concentration was quantified using the Qubit Fluorometer 4 (Invitrogen, Carlsbad, California, USA) [58,59,62].

2.6 Library preparation, quality control and sequencing

Downstream processing was conducted at the Next Generation Sequencing Platform (Bern, Switzerland). Genomic libraries were constructed using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, Massachusetts, USA). PhiX174 (PhiX Control v3 Library) was used as a run control according to the manufacturer's manual (Illumina, San Diego, California, USA). Sequencing was performed on the NovaSeq 6000 system using a high-output flow cell, generating 2 x 150 nucleotide paired-end reads [63].

2.7 Data analysis

Raw reads were mapped to an inhouse database containing 73,697 full length viral genomes from the NCBI (downloaded in April 2025) using the metagenomic pipeline of SeqMan NGen v. 18 and SeqMan Pro v. 18 software (both DNASTar, Lasergene, Madison, Wisconsin, USA) [64,65]. Low-quality hits were discarded, and only viral species with more than 10 mapped reads per reference genome were considered for downstream analyses. For taxonomic annotation, the generated consensus sequences were compared against the NCBI nucleotide database [66] using BLASTN (version 2.17.0) [67,68] with default parameters. Resulting hits were ranked by bit score, and only those with an e-value $\leq 1 \times 10^{-5}$ and bit score ≥ 100 were retained for taxonomic annotation. Following manual inspection of the alignments, the naïve best-hit method was applied to assign specific taxa to each contig. The resulting viral sequences were subsequently aligned using MAFFT (v7.526) under default settings (FFT-NS-2 algorithm) [69,70], followed by phylogenetic analysis in MEGA (v12.1) on both nucleotide and amino acid level [71]. Trees were constructed

using the maximum likelihood method. The best-fit substitution model for each dataset was automatically determined, and node support was assessed with 1,000 bootstrap replicates. All other statistical analyses were conducted in R version 4.5.2 [72]. Specifically, the total viral relative abundance (RPM) between migratory groups was compared using the approximative two-sample Fisher-Pitman permutation test from the coin package (v1.4-2). For all tests, a p-value < 0.05 was considered statistically significant.

2.8 Accession numbers

The raw sequence reads generated in this study are available in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1459659. Nucleotide sequences of selected viruses identified in this study have been deposited in GenBank under the accession numbers: PX613657 to PX613659 and PX695504 to PX695485.

3. Results

A total of 496 birds from 42 species were sampled. Of these, 486 individuals were captured using a catch-and-release method. An additional 10 birds (9 *Corvus corone cornix* and 1 *Falco tinnunculus*) were obtained from various locations across Ticino through population containment plans. From the total cohort, 496 oropharyngeal and cloacal swabs, 164 serum samples, and 22 ticks directly removed from the birds were collected. Due to material limitation a subset of 476 samples was included in the comparative migration analysis (Tab. 1). They belonged to 16 families and five orders, with the majority belonging to the order *Passeriformes* (90.5%, n=38) [1].

3.1 Avian species diversity and migratory pattern

The captured bird species exhibited diverse migratory behaviour and were initially divided into five categories: resident birds (n=3), partial migrants (n=9), short-distance migrants (n=10), intermediate migrants (n=1), and long-distance migrants (n=19). While capturing the birds, temporal variation in species composition was observed. In the initial phase (April), captures were dominated by short-distance migrants, whereas long-distance migrants were caught in the second phase (end of April to May). Resident species were consistently represented throughout the sampling period. To ensure statistically robust comparisons of migration strategies, only the 19 bird species exhibiting strictly long-distance behaviour were retained in this category. The remaining categories were grouped into resident and short-distance migrants. This resulted in two categories: residents and short-distance migrants (23 species, n=268 samples analyzed, see Fig. 1A), and long-distance migrants (19 species, n=208 samples analyzed, see Fig. 1B). Sample numbers ranged widely among species, with *Sylvia atricapilla* (short-distance, n=143) and *Erithacus rubecula* (short-distance, n=53) representing the most frequently sampled species.

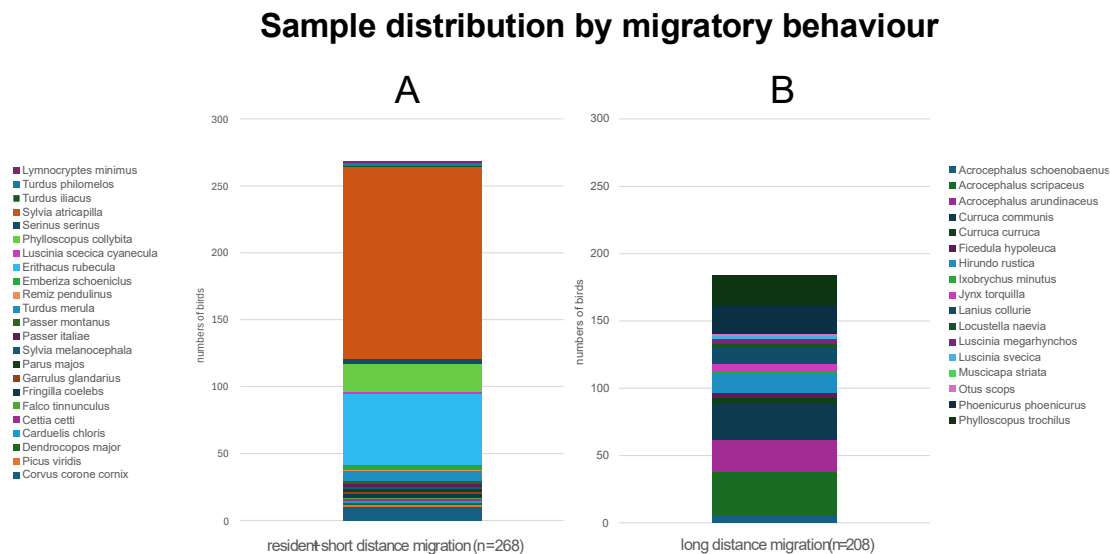


Fig. 1: Overview of swab samples distribution across bird migratory strategies. **A.** Stacked column chart shows the number of swab samples collected from bird species with short-distance migratory strategies, including resident species and *Lymnocyrtus minimus*, a species that may also exhibit long-distance migration. **B.** Stacked column chart shows the number of swab samples collected from long-distance migratory species.

3.2 Targeted molecular surveillance of AIV and WNV

First, oropharyngeal-cloacal swabs had been screened using RT-qPCR for two zoonotic pathogens, i.e., Avian Influenza virus (AIV) and West Nile virus (WNV), all of which yielded negative results. Additionally, 164 sera samples were tested for WNV antibodies using ELISA, yielding 12 doubtful and 15 positive results. To confirm these findings, a WNV serum neutralization test (SNT) was performed, starting at 1:32 serum dilution, due to the limited amount of available material. However, no neutralizing antibodies were detected, Supp. Tab. S2). Further specific SNTs for other flaviviruses were not possible due to the limited sample quantity.

3.3 Distribution of the avian virome

Metagenomic sequencing of 476 oropharyngeal and cloacal swab samples generated 3.56×10^9 reads, with individual pools yielding between 7×10^6 and 4×10^7 reads. Bioinformatics analysis identified 6.22×10^5 viral reads. After filtering for quality (E-value $\leq 10^{-5}$, bit score ≥ 100) and robustness (> 10 mapped reads per reference), 4.32×10^5 classified viral reads remained for analysis. These were assigned to 16 RNA (57.1%) and 12 DNA families (42.9%), encompassing a diverse virome of both single-stranded (ss) and double-stranded (ds) genomes. RNA viruses had higher family level diversity but were less abundant per individual, whereas DNA viruses were less diverse yet more prevalent in read counts and across bird species. Overall, viral reads represented only a minor fraction of the total sequencing output. Statistical comparison of the $\log_{10}$ -transformed viral relative abundance ($\log_{10}$ -RPM) revealed no significant difference between short-distance and long-distance migrants (approximative two-sample Fisher-Pitman permutation test, $p=0.44$, Fig. 2) [73]. To account for unequal numbers of species and samples across categories, data were normalized to RPM to ensure comparability across pools with different sequencing depths [57,74]. Furthermore, the use of a permutation-based test allowed for robust comparison of groups with different sizes without assuming a normal distribution of the viral load [75].

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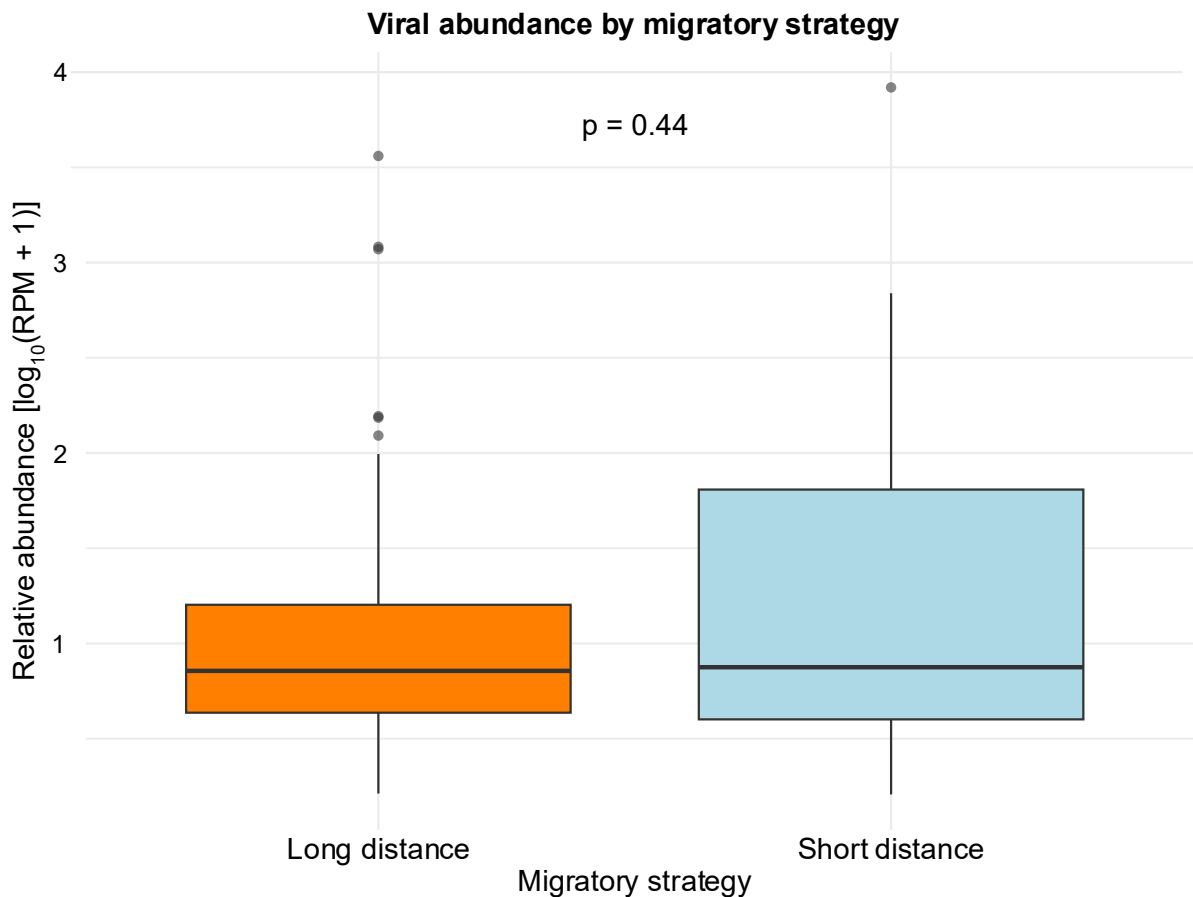
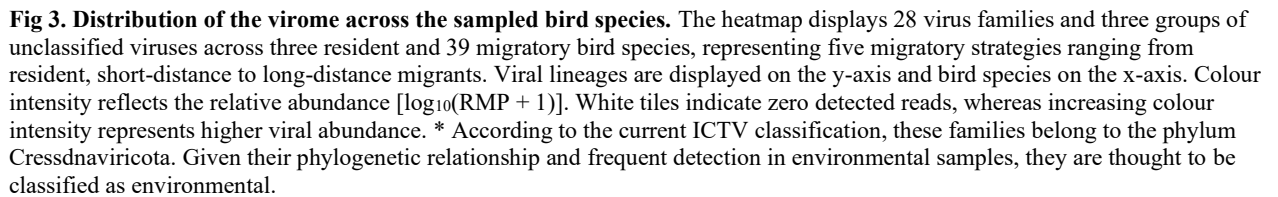


Fig. 2. Statistical overview of viral relative abundance by migratory strategy. Boxplots illustrate the viral relative abundance ($\log_{10}(\text{RPM}+1)$) for short-distance (blue) and long-distance (orange) migratory birds at the pool level. The central horizontal line indicates the median, with the box spans the interquartile range (25th–75th percentile). Whiskers extend to the 5th and 95th percentiles. Values outside this range are displayed as individual points (outliers), each presenting a single pool. No significant difference in viral abundance was observed between the two groups (approximative two-sample Fisher-Pitman permutation test, $Z=-0.78$, $p=0.44$, $n_{\text{resample}}=9999$; coin package).

The viral reads were assigned to 28 distinct virus families and a group of unclassified viruses, which was further divided into three subgroups, i.e. *Cressdnaviricota*, other DNA viruses, and RNA viruses not specifically classified in the NCBI Taxonomy Browser [76] (Fig. 3). Among the 42 bird species, *S. atricapilla* ($n = 143$) had both the highest number of collected samples and the highest viral diversity, with 17 families. Interestingly, viral richness did not strictly correlate with sample size, *E. rubecula* ($n = 53$) hosted only nine virus families despite its large representation. By contrast, *Hirundo rustica* (long-distance, $n=14$) hosted 11 families, while *Turdus merula* (partial migrant, $n=7$) and *Jynx torquilla* (long-distance, $n=6$) each harboured ten families. The lowest viral richness was observed in *Muscicapa striata* (long-distance, $n=1$), with viral reads assembled only to the family *Circoviridae*. For further analysis, virus families were categorized based on their primary hosts: (i) vertebrates, (ii) invertebrates, (iii) environment, (iv) bacteriophages, and (v) unclassified and newly established families.



Seven virus families were identified based on reads assembled to viral genomes known to infect vertebrates, mainly mammals and birds: *Adenoviridae*, *Circoviridae*, *Gandrviridae*, *Papillomaviridae*, *Parvoviridae*, *Picornaviridae*, and *Spinareoviridae*, alongside unclassified viruses of the phylum *Cressdnaviricota*. This category had the highest viral abundance in the study, with 2.05×10^5 reads. Notably, *Circoviridae* dominated with 1.85×10^5 reads, predominantly, in *H. rustica* (9.96×10^4 reads) and *Acrocephalus arundinaceus* (long-distance, 2.73×10^4 reads). Overall, *Circoviridae* and the unclassified *Cressdnaviricota* group (1.05×10^4 reads) were detected in nearly all species (37/42). *Parvoviridae* (9.56×10^3 reads) was detected across 21 bird species, while *Picornaviridae* (11 reads) was the least abundant, detected only in *S. atricapilla*.

Reflecting the diet of most investigated species (40/42), five RNA viral families primarily infecting insects were identified: *Dicistroviridae*, *Iflaviridae*, *Orthototiviridae*, *Permutotetraviridae*, and *Polycipiviridae*. The overall read count was 1.31×10^4 , with high concentrations in specific species, particularly in *S. atricapilla* (2.9×10^3 reads for *Dicistroviridae*) and *J. torquilla* (6.96×10^3 reads for *Polycipiviridae*). In contrast, *Permutotetraviridae* (60 reads) showed the lowest prevalence, detected only in *Phylloscopus collybita* (short-distance) and *Sylvia borin* (long-distance). Additionally, some *Parvoviridae* reads likely correspond to the subfamily *Densovirinae*, which specifically infect arthropod hosts, were also detected.

3.3.3 Environment-associated

The category with the highest family richness comprised 12 families predominantly associated with plant and fungal hosts: *Alphaflexviridae*, *Botourmiaviridae*, *Bromoviridae*, *Genomoviridae*, *Orthototiviridae*, *Partitiviridae*, *Secoviridae*, *Solemoviridae*, *Tombusviridae*, *Tymoviridae*, *Usaviridae*, and *Virgaviridae*. Although this group represented 42.9% of the total family richness (12 out of 28 identified families), its cumulative abundance was relatively low, accounting for only 6.4% of the total classified viral reads (2.78×10^4 reads). Within this category, five families were identified in only a single bird species. However, *Genomoviridae* (1.81×10^4) was identified in more than half of the species (23/42), with its highest viral load in *S. atricapilla* (1.37×10^4 reads) and *Curruca communis* (long-distance, 1.7×10^3 reads).

3.3.4 Bacteriophages

Although this study targeted eukaryotic viruses and included nuclease treatment to reduce bacterial and host nucleic acids, sequences from one bacteriophage family were detected. The family *Microviridae* (515 reads) was identified in 14 of the 42 bird species.

3.3.5 Newly established and unclassified families

This last category included recently established taxa within the phylum *Cressdnaviricota* such as *Cruciviridae* (11 reads, 1/42 species), *Mahapunaviridae* (100 reads, 1/42 species), *Kanorauviridae* (212 reads, 12/42 species), and *Geplanaviridae* (26 reads, 1/42 species) [77,78]. The unclassified DNA viruses represented the highest overall sequence abundance (3.71×10^5 reads, 36/42 species), dominated by *E. rubecula* with 3.59×10^5 reads, and *Corvus corone cornix* (resident, 1.04×10^4 reads). In contrast, unclassified RNA viruses (4.97×10^3 reads) were less prevalent, detected in only six species.

3.4 Diversity and phylogeny of avian DNA viruses

To further investigate the evolutionary relationships of the identified DNA virome, representative sequences from the most prevalent families were analyzed. In parallel, a statistical comparison was performed to determine if migratory strategy influenced the viral load. Consistent with the overall family prevalence data, no significant difference in the log₁₀-transformed DNA viral relative abundance was observed between short-distance and long-distance migrants (approximative two-sample Fisher-Pitman permutation test, $p=0.15$, Fig.4) [73,75]. To account for the technical bias introduced by varying sequence depths and unequal numbers of species and samples, data were again normalized to RPM to ensure comparability [57,74]. Notably, this statistical pattern was not fully reflected in the mapped consensus sequences, while group-wide abundance showed no significant difference, five out of the six DNA sequences with sufficient coverage for phylogenetic analysis were derived from the mapping data of short-distance migrants (Fig 5 and 6).

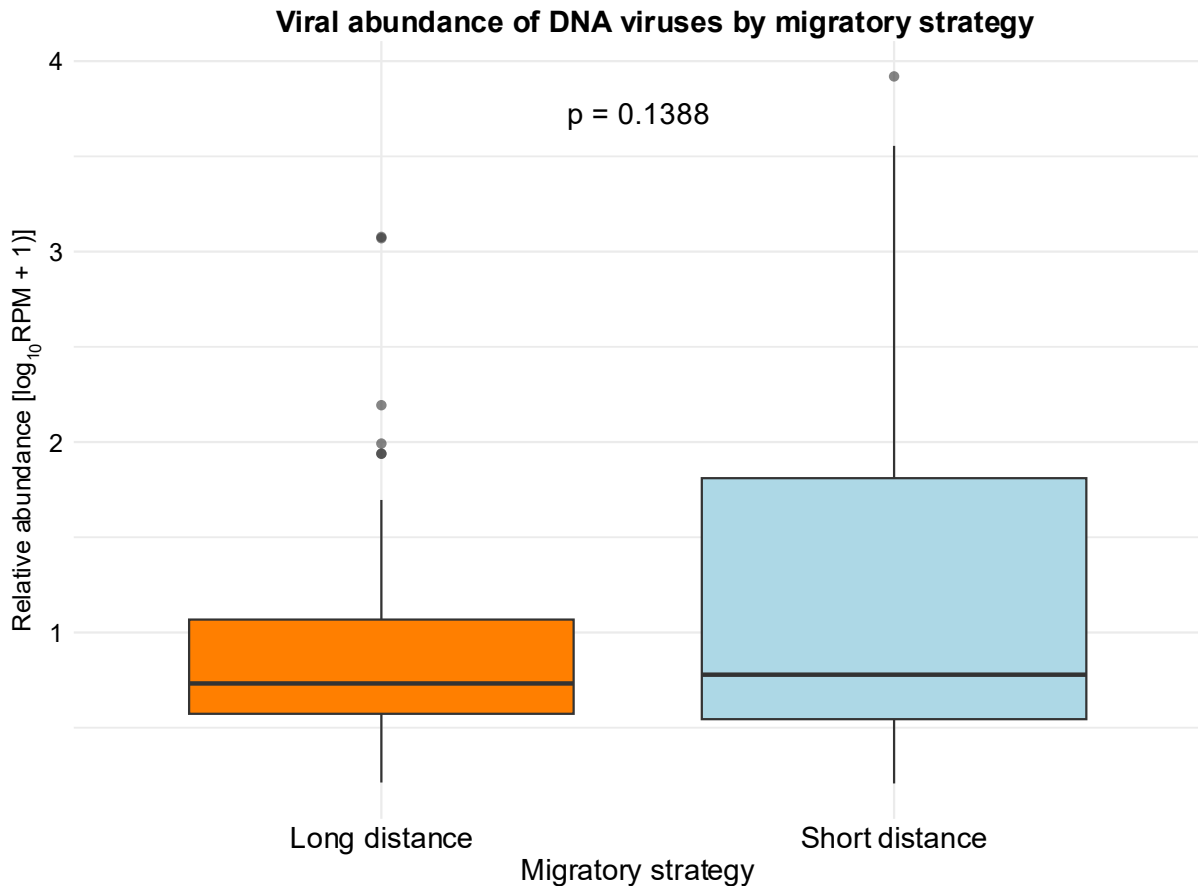


Fig. 4. Statistical overview of viral reads derived from DNA genomes. Boxplots show abundance of the DNA-specific viral relative abundance ($\log_{10}(\text{RPM}+1)$) for short-distance (blue) and long-distance (orange) migratory birds. The central horizontal line indicates the median with the box spans the interquartile range (25th–75th percentile). Whiskers extend to the 5th and 95th percentiles. Values outside this range are shown individually (outliers). Each point corresponds to a specific viral family detection within a pool. No significant difference in DNA viral abundance was observed between the two groups (approximative two-sample Fisher-Pitman permutation test, $Z=-1.45$, $p=0.15$, $n_{\text{resample}}=9999$, coin package).

3.4.1 Circoviridae

Viruses belonging to the family *Circoviridae* (phylum *Cressdnaviricota*) represented a major component of the study's findings, being detected in 38 of 42 bird species. Most identified consensus sequences were closely related to the tick-associated circular virus 2. Notably, a complete genome sequence ([PX695504](#); 2,123 nt) recovered from *S. atricapilla* showed 99.7% identity to a previously identified tick-associated circular virus 2 strain ([ON456885](#)) and has the typical two ORF structure encoding the capsid and Rep proteins. Additionally, a partial sequence ([PX695499](#); 795 nt) was identified in *A. arundinaceus* with 97.5% identity to an Arizlama virus ([NC_137451](#)) and contained a single open reading frame (ORF) encoding replication-associated (Rep) protein.

3.4.2 Genomoviridae

Within the same phylum as *Circoviridae*, members of the family *Genomoviridae* were identified in several short- & long-distance migratory bird species. Here, a partial consensus sequence ([PX695500](#); 1,222 nt) had 98% identity to a previously detected *Gemycircularvirus* ([MH378453](#)). This sequence contained two partial ORFs encoding capsid (Cap) and Rep protein.

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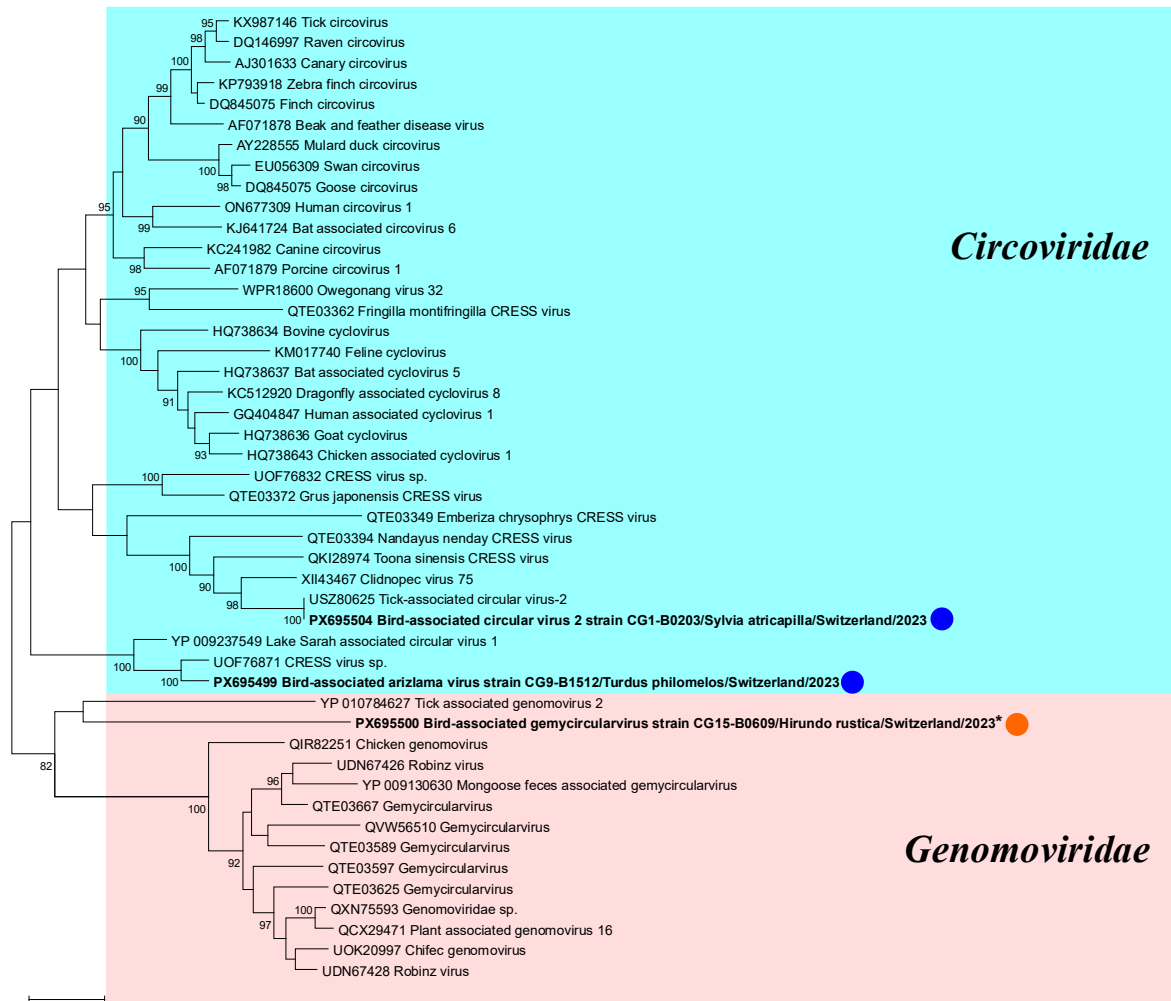


Fig. 5. Phylogenetic analysis of the phylum *Cressdnaviricota*. The Maximum Likelihood (ML) tree was constructed in MEGA (v12.1) based on an alignment of amino acid (aa) sequences of the replication-associated protein (Rep) performed with MAFFT (v7.526). Representative members of the families *Circoviridae* (including the genera *Circovirus* and *Cyclovirus*) and *Genomoviridae* were included for taxonomic context. Numbers at the internal nodes represent bootstrap support values derived from 1,000 replicates. For clarity, only bootstrap values $\geq 80\%$ are displayed. Sequences identified in this study are shown in bold and annotated with a blue (short-distance migrant) or orange (long-distance migrant) dot. Asterisks (*) indicate partial Rep sequences.

3.4.3 Parvoviridae

Within the subfamily *Densovirinae*, a nearly complete consensus genome sequence ([PX695503](#); 3,980 nt) identified in *S. serinus* and a partial sequence ([PX695497](#); 1,649 nt) detected in *P. italiae* showed 99.8% identity to *Acheta domestica* densovirus (KF015278), a known insect pathogen [79]. The first sequence contains two ORFs encoding NS1 and NS2, along with partial ORFs for NS3, VP1, and VP2 (structural proteins). The second contains only ORFs for NS2 and a partial NS1. A third sequence ([PX695494](#); 865 nt) shared 99% identity with a previously identified *Grus japonensis* parvoviridae sp. ([PQ438377](#)) and contained one partial ORF encoding for NS1 protein.

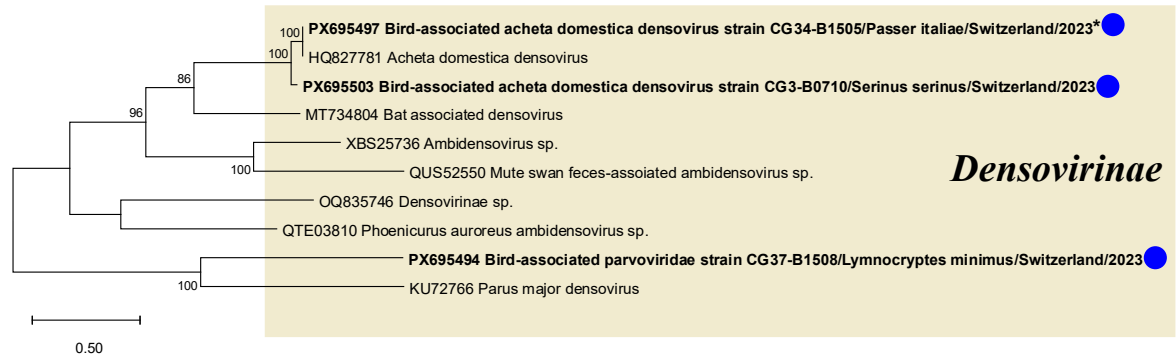


Fig. 6. Phylogenetic analysis of *Parvoviridae*. The Maximum Likelihood (ML) tree was constructed in MEGA (v12.1) based on an alignment of amino acid (aa) sequences of the non-structural protein (NS1) performed with MAFFT (v7.526). Representative members of the *Densovirinae* belonging to the family *Parvoviridae* were included for taxonomic context. Numbers at the internal nodes represent bootstrap support values derived from 1,000 replicates. For clarity, only bootstrap values $\geq 80\%$ are displayed. Sequences identified in this study are shown in bold and annotated with a blue (short-distance migrant) or orange (long-distance migrant) dot. Asterisks (*) indicate partial Rep sequences.

3.5 Diversity and phylogeny of avian RNA viruses

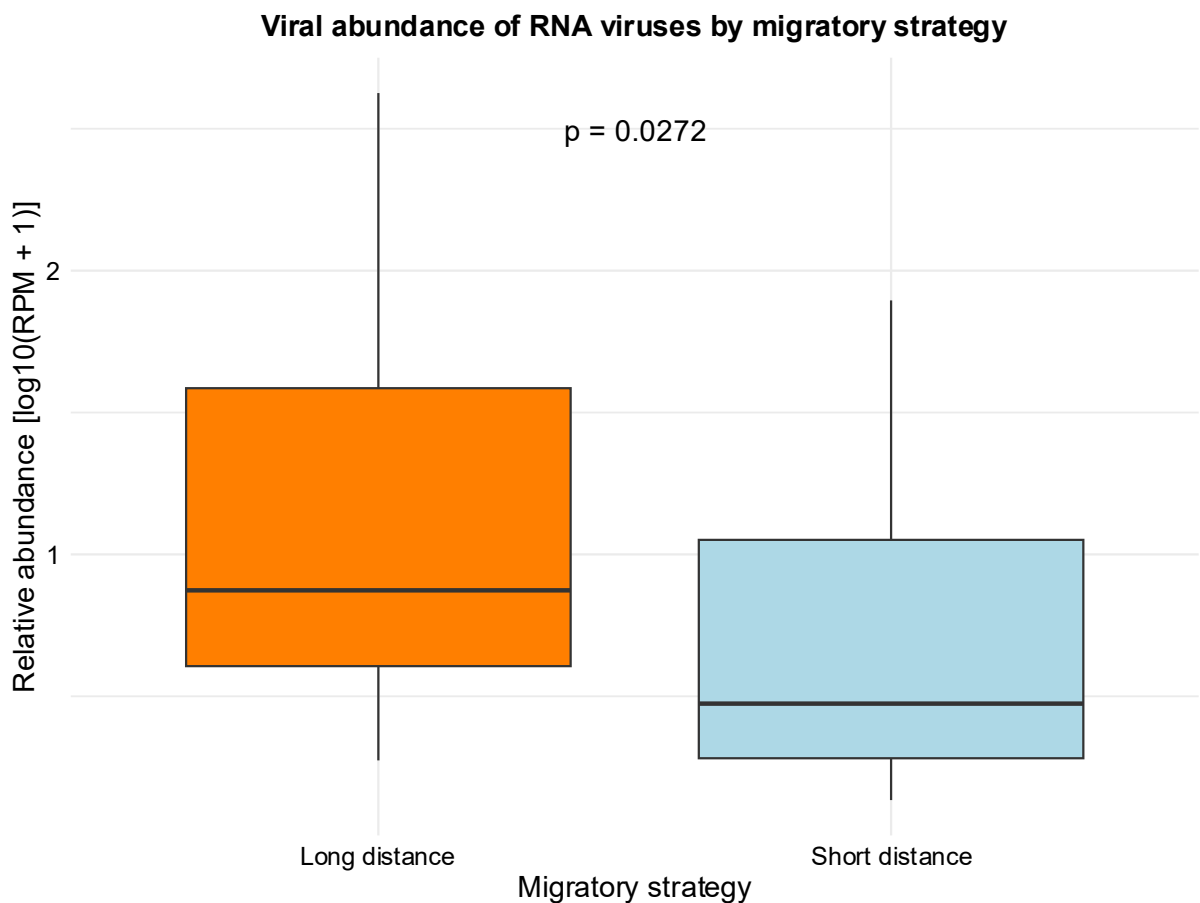


Fig. 7. Statistical overview of viral reads derived from RNA genomes. Boxplots show abundance of the RNA-specific viral relative abundance ($\log_{10}(\text{RPM}+1)$) for short-distance (blue) and long-distance (orange) migratory birds. The central horizontal line indicates the median with the box spans the interquartile range (25th–75th percentile). Whiskers extend to the 5th and 95th percentiles. A significant higher RNA viral abundance is observed in the long-distance migrants (approximative two-sample Fisher-Pitman permutation test, $Z=2.19$, $p=0.0281$, $n_{\text{resample}}=9999$, coin package).

3.5.1 Invertebrate-associated

A total of seven consensus sequences were assembled and classified into four viral families: *Dicistroviridae*, *Orthototiviridae*, *Iflaviridae*, and *Polycipiviridae* (Fig. 8), with five detected in long-distance migrants. Within the family *Dicistroviridae*, three sequences encoding structural and non-structural proteins were identified. The first identified in *S. atricapilla* (PX695490; 2,209 nt) showed 95% identity to a *Formica exsecta* virus (NC_023021). The second from *S. atricapilla* (PX695492; 2,144 nt) exhibited 99.66% identity to a *Rhopalosiphum padi* virus (MN535735). Finally, a third sequence detected in *J. torquilla* (PX695491; 2,193 nt) had 96% identity to a *Cripavirus* (PP872551). In *Orthototiviridae*, two sequences containing partial RdRp ORFs were detected. One identified from *H. rustica* (PX695486; 2,334 nt) showed 96% identity to a known *Broome virga-like virus* (MT498833), and another identified from *J. torquilla* (PX695488; 1,102 nt) exhibited 89% identity to a *Sogatella furcifera* toti-like virus 2 (ON402806). A nearly complete *Polycipiviridae* genome sequence (PX695494; 11,622 nt) from *J. torquilla* showed 96% identity to a *Myrmica scabrinodis* virus (NC_035457), with ORFs encoding capsid, hypothetical, and RdRp proteins. The final invertebrate host virus was detected in *A. arundinaceus* (PX695493; 1,812nt) and assigned to *Iflaviridae* with 99% identity to *Vespa velutina* Moku virus (MF346349), covering part of a polyprotein ORF.

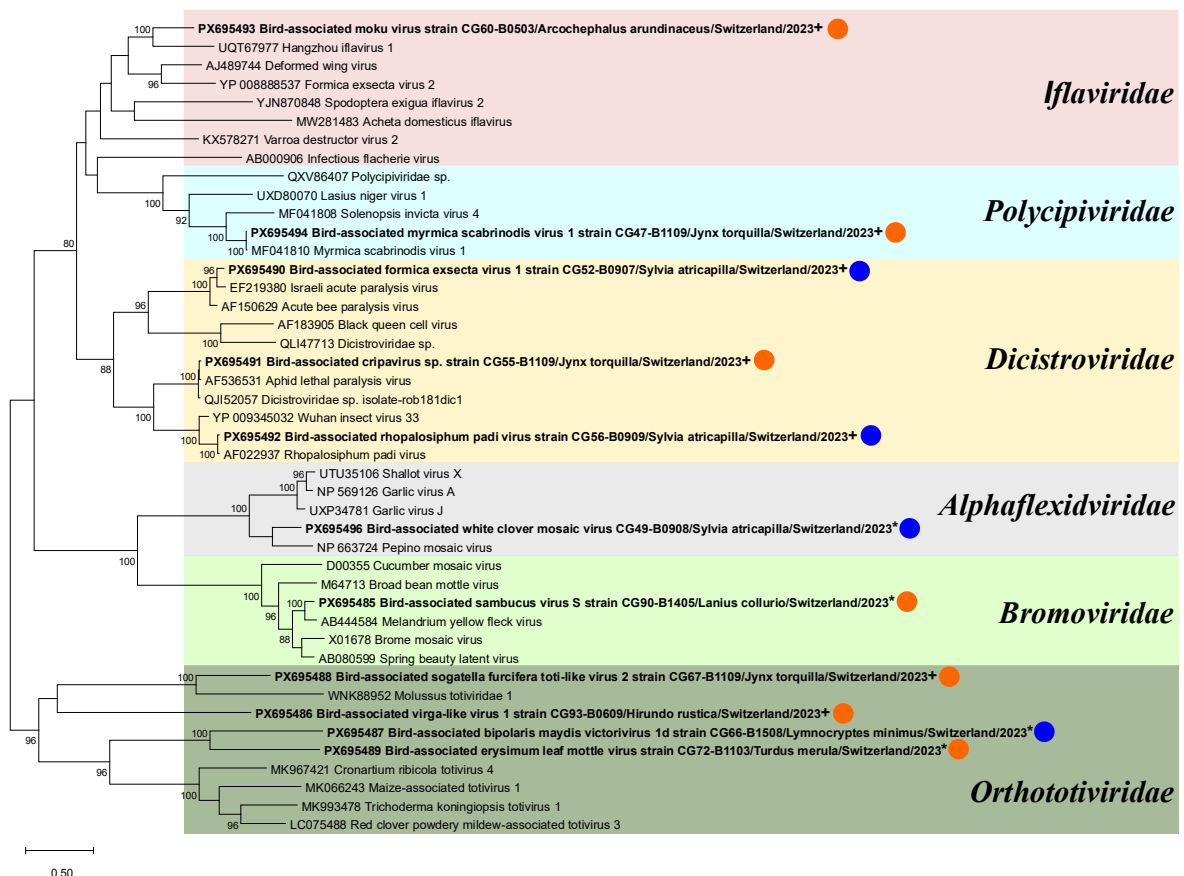


Fig. 8. Phylogenetic analysis of RNA genomes. The Maximum Likelihood (ML) tree was constructed in MEGA (v12.1) based on an alignment of amino acid (aa) sequences of the RNA-dependent RNA polymerase (RdRp) performed with MAFFT (v7.526). Representative members of different RNA virus families were included for taxonomic context. Numbers at the internal nodes represent bootstrap support values derived from 1,000 replicates. For clarity, only bootstrap values $\geq 80\%$ are displayed. Sequences obtained in this study are indicated in bold and annotated with a blue (short-distance migrant) or orange (long-distance migrant) dot. * represents the environment infecting viruses, while + depicts invertebrates infecting viruses.

3.5.2 Environment-associated

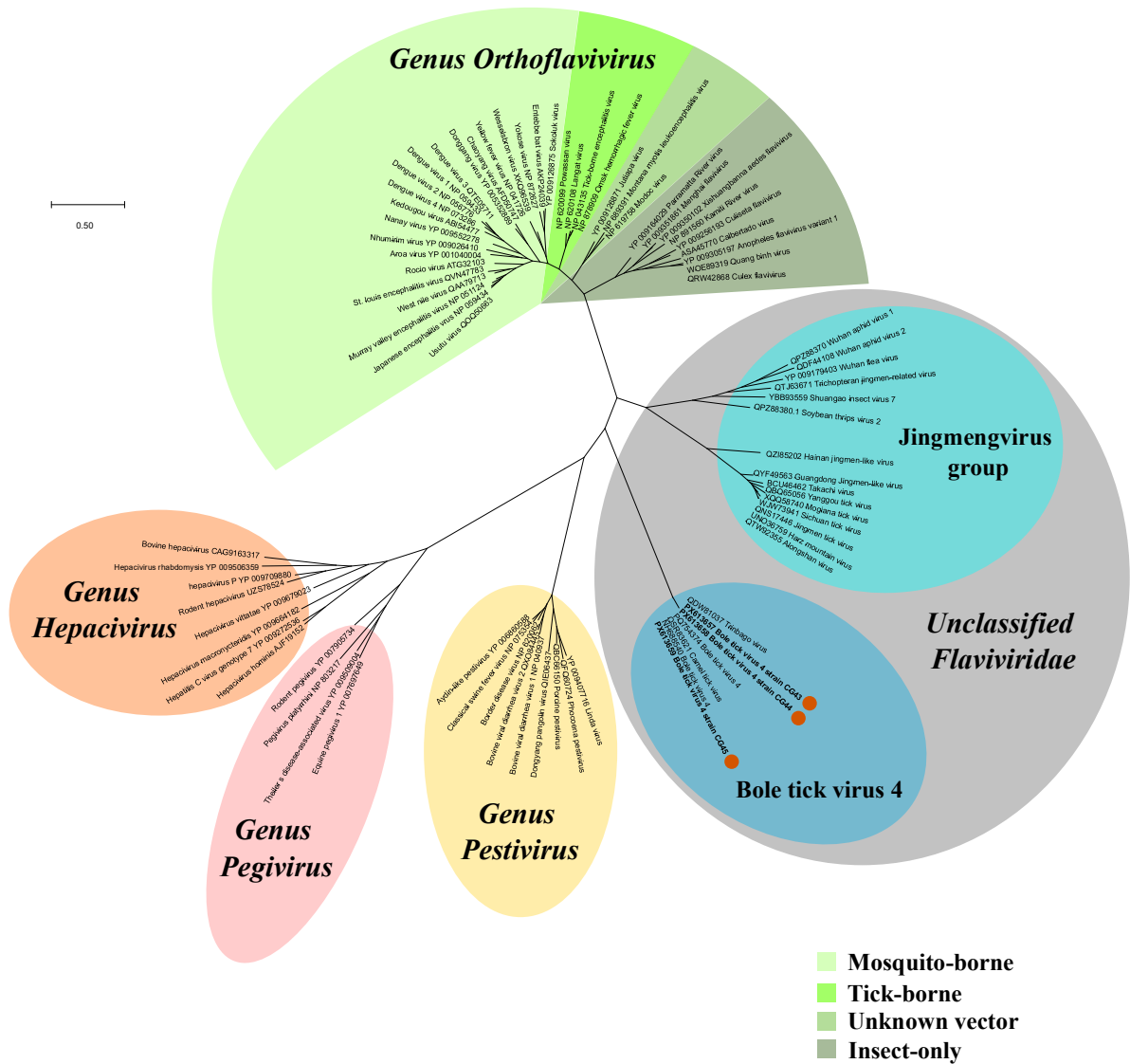
A total of four consensus sequences were mapped, which were detected in both short-distance and long-distance migrants. Within the family *Alphaflexiviridae*, a sequence ([PX695496](#); 4006 nt) was identified in *S. atricapilla* and showed 95% identity to White clover mosaic virus ([PQ162444](#)). This sequence contained partial ORFs for replication and coat proteins, as well as triple gene block (tgb1, tgb2, tgb3). In *Bromoviridae*, a sequence ([PX695485](#); 831 nt) was assembled from *Lanius collurio* (long-distance), sharing 96% identity with Sambucus virus 2 ([NC_040389](#)) and containing a partial ORF for protein 2a. Additionally, two sequences showed strong associations with the recently established family *Orthototiviridae*. In *Turdus merula*, a sequence ([PX695489](#); 1,892 nt) shared 91% identity to *Erysimum* leaf mottle virus ([OL420671](#)). Notably, while this virus is taxonomically assigned to the *Tymoviridae*, in our phylogenetic analysis it clustered within the *Orthototiviridae* clade. This sequence encoded partial ORFs for replication and movement protein. Further, in *Lymnocyrtus minimus* (intermediate migrant), a sequence ([PX695487](#); 1,082 nt) was found that shared 92% identity with a *Bipolaris maydis* victorivirus ([MN935505](#)), including a partial RdRp ORF.

3.6 Phylogenetic investigation of *Flaviviridae*

To investigate the potential role of birds as dispersal vectors for tick-borne pathogens, we analyzed 22 ticks (19 engorged) removed from captured birds. In total, 14 birds from ten species were found to be tick-infested (Tab. 2). Seven of these species exhibited long-distance migratory behaviour, wintering in central or southern Africa, while the three remaining species only migrate to the Mediterranean region and North Africa [33]. Nearly all ticks (21/22) were collected between April and June, coinciding with the arrival of birds from Africa. Shotgun sequencing of the ticks generated 5.03×10^8 reads, of which 7.52×10^4 viral reads, mapped to four families: *Circoviridae*, *Flaviviridae*, *Nairoviridae* and *Partitiviridae*. Notably, no major tick-borne human pathogens sequences including Tick-borne encephalitis virus (TBEV) were detected. However, 99.73% of the viral reads (7.5×10^4) were classified into the family *Flaviviridae*. These reads were detected in three engorged *Rhipicephalus turanicus* ticks. A complete consensus genome sequence ([PX613658](#); 16,230 nt) and a nearly complete sequence ([PX613659](#); 16,181 nt), both sharing 95% nt identity with a Bole tick virus 4 (BLTV4) strain from China ([MW561976](#)), were assembled. These genomes encode a single long ORF for a polyprotein. Additionally, a third partial sequence ([PX613657](#); 1,653 nt) showed 94% nt identity to another strain from China ([PP945237](#)).

A

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B

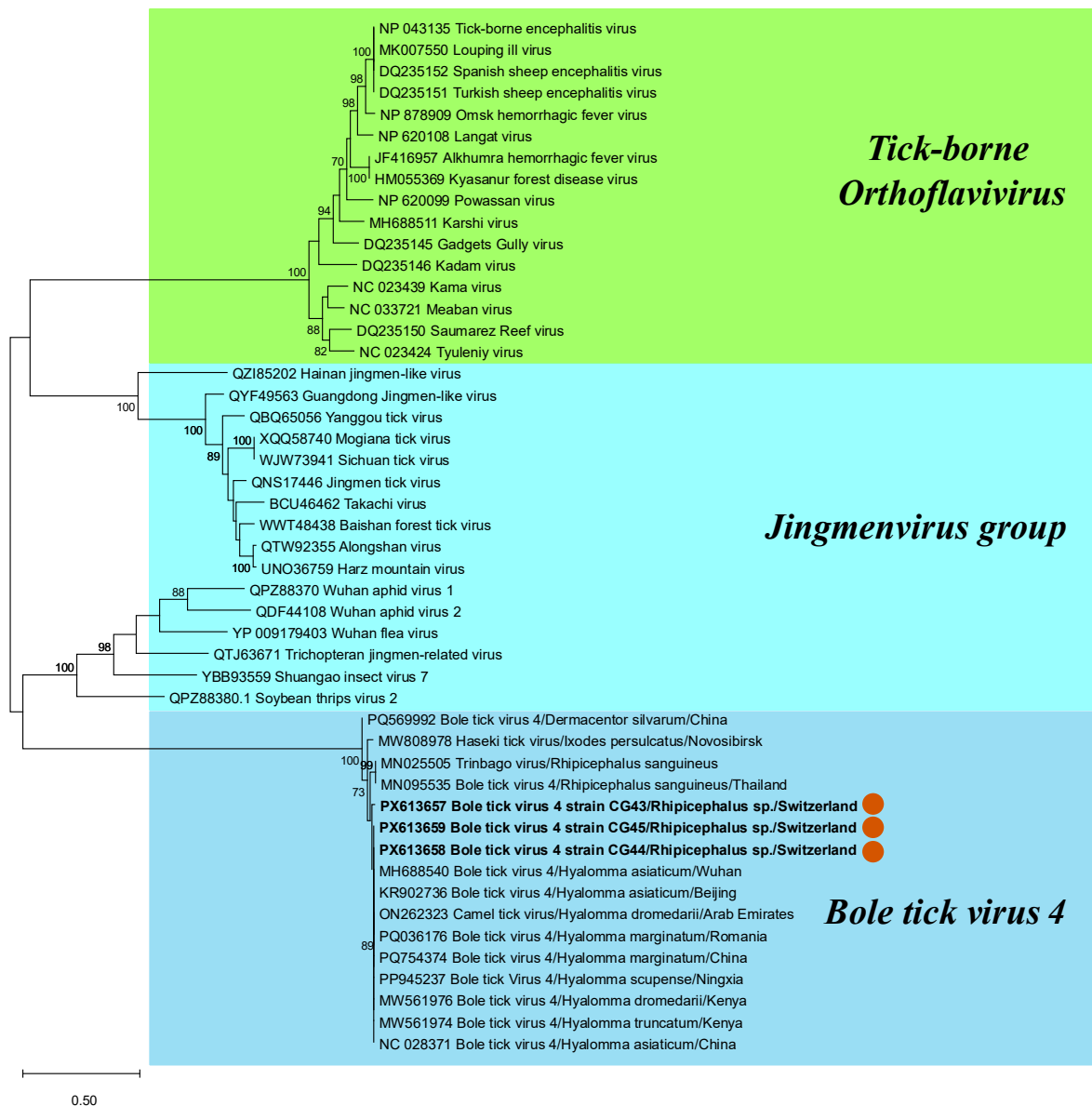
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Figure 9. Phylogenetic analysis of *Flaviviridae*. The Maximum Likelihood (ML) tree was constructed in MEGA (v12.1) based on an alignment of amino acid (aa) sequences of the RNA-dependent RNA polymerase (RdRp) performed in MAFFT (v7.526). Numbers at the internal nodes represent bootstrap support values derived from 1,000 replicates. For clarity, only bootstrap values $\geq 80\%$ are displayed. **A.** Phylogenetic analysis of representative members of the genera *Orthoflavivirus*, *Pestivirus*, *Pegivirus* and *Hepacivirus*, as well as unclassified *Flaviviridae*, including the Jingmenvirus group and Bole tick virus 4. The sequences obtained in this study are indicated in bold and marked with an orange dot (long distance migration host bird species). **B.** Detailed phylogenetic analysis of representative members of known tick-borne *Flaviviridae* and unclassified *Flaviviridae* (including the Jingmenvirus group and Bole tick virus 4). The sequences obtained in this study are indicated in bold and marked with an orange (long distance migration host bird species) dot.

Phylogenetic analysis based on conserved aa domains of the RdRp (Fig. 9A) revealed that our sequences form a robust monophyletic cluster with other previously identified BLTV4 strains. This clade is phylogenetically distinct from the four main *Flaviviridae* genera: *Orthoflavivirus* (formerly *Flavivirus*), *Hepacivirus*, *Pegivirus* and *Pestivirus*. Furthermore, no close relationship was observed to the Jingmenvirus group, which consists of segmented tick-borne flavi-like viruses such as Alongshan virus (ALSV). Detailed analysis of tick-borne *Flaviviridae* reinforces the global

clustering of Swiss BLTV4 consensus sequences with BLTV4 strains from China, Russia, and Africa. While the group forms a robust monophyletic cluster (bootstrap value 100), the short internal branch lengths and moderate bootstrap value of 73 indicate a high degree of sequence conservation across these distant geographic regions. This suggests a stable evolutionary lineage that is phylogenetically distinct from other known tick-borne *Flaviviridae* (Fig. 9B).

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4. Discussion

Over a seven-month period, 496 wild birds were sampled, representing three resident and 39 migratory species, ranging from short- to long-distance migrants. Oropharyngeal-cloacal swabs from all birds were initially screened via RT-qPCR for specific viruses, with 476 samples and 22 associated ticks subsequently undergoing metagenomic shotgun NGS. To complement these findings, 164 sera were analyzed by virus-specific ELISA, with all positive or borderline results validated through serum neutralization tests (SNT).

4.1 Host Ecology, Migration, and Seasonal Dynamics

The diversity of bird species identified ($n = 42$), and the distribution of sampled individuals ($n = 496$) are closely linked to the ecological function of the sampling site Bolle di Magadino and the sampling strategy. Reflecting seasonal shifts in avian population, the data sets comprised 268 resident and short-distance migrants and 208 long-distance migrants (Fig. 1A and 1B). This distribution is primarily driven by high sampling intensity during the spring migration peak in April and May, when numerous species fly through this area, including long-distance migrants arriving from South-East Africa via the Po Valley, as well as individuals following South-West routes. During the breeding season (mid-May to end of June), avian movement becomes more localized. Accordingly, the sampled species were primarily territorial residents or breeding migrants and may reflect a relatively stable, so called “local virome” of the Ticino avian population. The post-breeding season is defined by the dispersal of juvenile birds and the arrival of migrants from northeastern routes. The dominance of *Sylvia atricapilla* ($n = 143$) and *Erithacus rubecula* ($n = 52$) in the datasets reflects their status among the most frequent and abundant bird species in Switzerland [80,81]. In a broader context, this representation has particular significance, according to previous studies *S. atricapilla* has been identified as one of just 16 species contributing to approx. 73% of the billions of birds migrating annually along the Africa-Eurasian flyway [6]. Furthermore, it underscores the importance of the Magadino delta as a major stopover point for rest before continuing migration across the Alps into Central Europe [6,33,51]. From an epidemiological perspective, the site facilitates close intermingling among bird species that are otherwise geographically separated, thereby increasing the potential for interspecies pathogen exchange and dissemination across various migratory flyways [82].

4.2 Molecular and Serological Results for Zoonotic Viruses

Zoonotic viruses such as AIV and WNV are of particular concern due to their significant impact on both public health and animal population [82–84]. In our samples, *Orthomyxoviridae* were detected neither in RT-qPCR nor NGS. The absence of AIV is most plausibly explained by the host species composition, in which 90.5% of the sampled individuals belonged to the order *Passeriformes*, while key reservoir hosts for AIV, particularly *Anseriformes* (waterfowl) and domestic poultry were not included [45,85,86]. Although some passerines can be experimentally infected with AIV, there is currently insufficient evidence to support their role as natural reservoirs or key transmitters under field conditions [21]. However, sporadic detections in wild passerines, such as the identification of highly pathogenic avian influenza (H5N1) in a dead crow in Kerala (India) in 2026, illustrate that these species can become infected in wildlife [87,88]. Within the *Flaviviridae* family, WNV was a primary target due to its relevance for vector-borne monitoring in Ticino, where the first detection of WNV Lineage 2 in mosquitoes was reported in 2022 [23]. The detection of WNV in mosquitoes represented an early signal of viral circulation and preceded the first confirmed autochthonous human case in Switzerland in 2025 [89]. Birds, particularly *Passeriformes*, are considered highly competent amplifying hosts, frequently developing high viremia levels sufficient to infect mosquitoes, despite this, no WNV RNA has been detected in

PCR or NGS [82,90,91]. However, positive results were yielded in ELISA testing, indicating prior exposure to WNV in the absence of detectable active viremia. This discrepancy between serological and molecular findings is consistent with the observations from Germany (2014–2016), where seropositivity in birds preceded the first autochthonous cases in 2018 despite an initial lack of detectable viral RNA [92]. The absence of viral reads in our unbiased NGS survey, consistent with the negative RT-qPCR results, is likely explained by the biological kinetics of avian WNV infections, where systemic viremia usually lasts only one to eight days. Following the acute phase, viral loads outside the bloodstream decline rapidly, substantially reducing the detection from swabs, even in recently exposed birds [48,93,94]. The inability to confirm the ELISA positive results using the serum neutralization test (SNT) may be attributed first to antibody titers falling below the assay detection limit, potentially compounded by limited serum volume available for testing. Secondly, serological cross-reactivity within *Flaviviridae*, specifically among members of the genus *Orthoflavivirus*, remains a well-recognized limitation. In particular, WNV antibodies exhibit extensive cross-reactivity with other antigenically related viruses, such as Usutu virus (USUV), Japanese encephalitis virus (JEV), and Zika virus (ZIKV), primarily due to highly conserved epitopes within the fusion loop domain of the envelope (E) protein [56,92,95]. In conclusion, while our serological results suggest prior exposure to *Orthoflaviviruses*, the absence of genomic reads via NGS and the negative RT-qPCR results suggest that these findings are more likely explained by survivors infected in other regions who reached Bolle di Magadino after their viremic phase. However, additional spillover events can't be excluded, including transmission via infected mosquitoes within Ticino or via birds arriving while still viremic [23,93,94,96].

4.3 Virome Composition of the birds

The quantitative dominance of small DNA viruses is a recurrent feature of vertebrates viromes, frequently representing the vast majority of identified viral sequences in fecal and swab samples. Consistent with prior metagenomic studies, our detected DNA viruses, dominated by the families *Circoviridae* and *Parvoviridae*, showed lower diversity but accounted for the most viral reads [20,28,37,47,59,97–99], with no significant abundance difference across migratory strategies (Fig. 4). These suggests that the core viromic composition remains stable across migratory strategies, indicating that while migration facilitates pathogen dispersal, the overall swab virome is not primarily shaped by migration. Conversely, the RNA virome showed a significantly higher viral load in long-distance migrants (Fig. 7), a finding that aligns with the observation that RNA viruses exhibited higher diversity but lower overall abundance throughout our study. This supports previous studies showing long-distance migrants accumulate more diverse RNA viral loads as they cross multiple ecological zones [26,29,44]. Furthermore, this accumulation is likely enhanced by flight-associated stress, which can cause immunosuppression and increased viral shedding, thereby enhancing virus detectability in NGS or in molecular assays [13,82]. A substantial proportion of the viral families identified in our study is associated with either environmental hosts (e.g., plants and fungi) or to invertebrate-infecting viruses (e.g., insects), with most being positive-stranded ssRNA viruses [29,40,100]. The high genetic diversity of these viral groups likely facilitates their widespread distribution across various environmental reservoirs [34,101], aligning with previous research, which consistently detected similar viral composition regardless of the specific avian order analyzed, spanning *Passeriformes*, *Anseriformes*, and *Charadriiformes* [24,44,46,102]. Overall, the viral composition is strongly influenced by sample type (oropharyngeal-cloacal swabs) and the dietary habits of the host, which likely reflect a mixture of local dietary possibilities and habitat at the sampling site, rather than host species or migratory strategy alone [2]. Consequently, our findings refute the initial hypothesis that the geographic origin of the birds significantly influences the overall virome composition. Instead, the high similarity in environmental and dietary viral signatures across groups indicates that shared exposure at the

Bolle di Magadino is the primary determinant of the swab virome. Consequently, [24,59] many detected viruses may be transient, acquired through ingestion of food, water or through contact with arthropods, rather than indicating active infection in the host.

4.4 Viral families of interest in the avian virome

4.4.1 DNA virome

The metagenomic analysis revealed a quantitative dominance of DNA viruses, particularly from the phylum *Cressdnaviricota* (Fig. 5), a pattern frequently observed in vertebrate metaviromics [24,59]. This phylum comprises small circular ssDNA viruses, including the *Circoviridae* and *Genomoviridae*, belonging to the group known as CRESS-DNA viruses (circular Rep-encoding single-stranded DNA viruses), which are characterized by genomes ranging from 1 to 25 kb that encode a Rep protein to initiate replication via a rolling-circle mechanism [103–105]. While birds are recognized as important hosts for these viruses, the full extent of their circular ssDNA diversity remains poorly characterized. Notably, our three CRESS-DNA consensus sequences did not cluster with known avian pathogens, a finding consistent with large-scale research from China, which has shown that *Passeriformes* serve as an important natural reservoir for highly diverse CRESS-DNA lineages, often forming divergent clades related to environmental or vector-associated viruses [24]. Furthermore, although CRESS viruses have been detected in human clinical samples, their pathogenic relevance remains unconfirmed [106]. This DNA dominance is further reflected in the family *Parvoviridae*, which was among our most abundant and diverse groups. The viruses of this family are frequently present in the avian intestinal tract, often found in healthy and diseased birds [25,107–109]. Interestingly, the three *Parvoviridae* contigs identified in this study were all classified within the subfamily *Densovirinae* (Fig. 6). Given that densoviruses primarily infect arthropods, their presence likely reflects dietary acquisition of insects rather than an active vertebrate infection. The host composition of our study is directly reflected in the low abundance or absence of certain viral families that are otherwise ubiquitous in avian metaviromics. While *Adenoviridae* and *Caliciviridae* are considered global residents in wild birds, they were present at low abundance or absent in our samples [26,100,102,110–113]. This distinct viral profile may be attributed to the lack of aquatic birds (*Anseriformes* and *Charadriiformes*) that typically serve as major reservoirs for many of these absent families [2]. Finally, members of the *Microviridae* were identified despite nuclease treatment, representing a highly diverse group of circular ssDNA bacteriophages previously described in the gut virome of mammals and birds, particularly within the order *Passeriformes* [114,115].

4.4.2 RNA virome

Avian gastrointestinal viromes are generally characterized by a core group of viral RNA families, typically dominated by the *Picornaviridae*, which often account for approximately 30% of viral reads in avian faecal and gut samples [20,107,116]. Our data, however showed a low prevalence, suggesting that the local virome at the Bolle di Magadino is primarily shaped by site-specific biodiversity and diet rather than a stable core of vertebrate viruses. This ecological signature is further evidenced by the presence of invertebrate-infecting virus families (Fig. 8), such as *Dicistroviridae*, *Iflaviridae*, and *Polycipiviridae*, which likely represent transient “passengers” acquired through arthropod ingestion rather than active vertebrate infection [26,114–116]. While our findings for *Dicistroviridae* align with prevalences reported in migratory populations across China, the contrastingly lower levels of *Iflaviridae* likely reflect the specific local composition and prey availability at the Bolle di Magadino site [26,29,102]. Beyond their role as dietary markers, these findings highlight the potential role of migratory birds as incidental mechanical vectors of agriculturally relevant pathogens across different ecological zones [13,120]. The birds' close

interaction with the local ecosystem is further exemplified by viruses with broader environmental associations. This includes the detection of *Orthototiviridae*, which encompassed contigs linked to both insect and plant hosts [66]. Together with the presence of plant-infecting families such as *Alphaflexiviridae* and *Bromoviridae*, these findings reflect an exposure profile resulting from direct consumption of infected material or insects and contact with the surrounding vegetation at the Bolle di Magadino site. [102,121,122].

u^b

4.5 Detection of Bole tick virus 4 (BLTV4) in Ticks

Within the family *Flaviviridae*, we identified Bole tick Virus 4 (BLTV4) in ticks of the species *Rhipicephalus turanicus*, marking its first detection in Switzerland. Initially described in 2015 through metagenomic sequencing in China, BLTV4 has subsequently been reported in multiple tick genera, predominantly *Hyalomma* and *Rhipicephalus*, across Asia, Europe, Africa and the Caribbean. Members of the *Flaviviridae*, including the genera *Orthoflavivirus* (formerly *Flavivirus*), *Pestivirus*, *Hepacivirus*, and *Pegivirus*, are typically characterised by non-segmented positive-strand ssRNA genomes ranging from 9 to 13 kb [83,131]. BLTV4 distinguishes itself from these classical members by possessing an enlarged, non-segmented, positive-stranded ssRNA genome of approx. 16.3 kb, encoding a single large polyprotein [125,127,128]. While BLTV4 shares conserved non-structural protein (NS3 and NS5) with the Jingmenvirus (JMV) group e.g., Alongshan virus and Jingmen tick virus, it lacks their characteristic genomic segmentation [132,133]. Instead, BLTV4 represents an evolutionary lineage that occupies an intermediate phylogenetic position relative to classical flaviviral genera, still forming a separated clade in phylogenetic analysis (Fig. 9A + B). While the replication mechanisms of BLTV4 and its zoonotic potential remain incompletely understood, sequences of this virus and its close relatives e.g. Haseki tick virus have been detected in human serum samples from febrile patients [125]. Furthermore, metagenomic analysis of the skin infectome at the tick bite site has identified sequences related to BLTV4, often associated with localized clinical signs such as papule at the site of attachment [134]. The detection of BLTV4 in ticks collected from *A. arundinaceus* at the Bolle di Magadino highlights the potential role of migratory birds, particularly long-distance migrants, in the dispersal of ectoparasites and their associated pathogens. These ticks may have acquired in Mediterranean or sub-Saharan regions prior to flight into Switzerland. In our dataset 22 ticks were collected from 496 birds, representing only a small fraction relative to the scale of avian migration across the Palearctic-Afrotropical flyways, where more than two billion birds migrate annually between Europe and Africa [6]. Migratory birds can therefore act as mechanical dispersal host, transporting attached ticks across continental borders and ecological barriers within days [135]. Consequently, the detection of BLTV4 highlights the need for continued surveillance of avian-associated ectoparasites to better assess the introduction and circulation of emerging or novel tick-borne viruses in Switzerland.

5. Conclusion

This study represents the first active monitoring of wild bird virome in Switzerland, providing a comprehensive investigation of oropharyngeal-cloacal samples from 496 birds (42 species) and 22 associated ticks. We identified 30 viral families, expanding the understanding of avian viral diversity. While no emerging or exotic viruses were identified within the avian viromes, our results support the hypothesis that migratory birds may increase the risk of introducing novel pathogens into Switzerland by transporting infected ectoparasites, as demonstrated by the first detection of Bole tick virus 4 in ticks collected from long-distance migrants. Overall, the composition of the avian virome primarily reflects the local ecological interaction, habitat, and dietary behaviour. The majority of detected viruses appear to be transient, reflecting environmental exposure rather than active infection, and no evidence supporting zoonotic potential in the avian virome was identified here [26,102]. However, the positive WNV ELISA results indicate that some birds had previous exposure to *Orthoflaviviruses*, representing survivors of an earlier infection phase. While these seropositive birds may have reached the Bolle di Magadino after their acute viremia, it is noteworthy that the viremic phase in passerines typically lasts between five and seven days [96]. This duration is sufficient for individuals to reach subsequent stopover sites while still infectious. Once introduced at a new location closer to Switzerland, the virus can establish a local transmission cycle, thereby contributing to the step-by-step dispersal of virus across ecological zones [12]. However, it should be noted that while swab-based metagenomics is highly effective for revealing diversity, provide limited sensitivity for detecting systematic infections at low viral loads. In this context, although limited and more challenging, blood-based virome may be necessary to capture more comprehensively active infections [136]. Taken together, our results highlight the importance of continued monitoring of not only migratory birds but also bird-associated ectoparasites, particularly ticks to improve early detection and monitoring emerging tick-borne threats.

6. Supplementary

S2 Table: Overview of serological results.

Species	Migration behaviour	ELISA ^{ab} results (PI %)	ELISA ^{ab} Interpretation	WNV SNT	Collection month in 2023
<i>Corvus corone cornix</i>	resident	32.20	doubtful	negative ^c	June 2023
<i>Sylvia atricapilla</i>	short-distance	65.59	positive	negative ^c	April
		60.25	positive	negative ^c	April
		49.85	positive	negative ^c	April
		47.42	positive	negative ^c	April
		47.16	positive	- ^d	April
		36.62	doubtful	negative ^c	April
		35.26	doubtful	negative ^c	April
		35.07	doubtful	negative ^c	April
		32.97	doubtful	negative ^c	April
		32.35	doubtful	negative ^c	April
		31.31	doubtful	negative ^c	April
		31.25	doubtful	negative ^c	April
		30.61	doubtful	negative ^c	April
<i>Arcocephalus arundinaceus</i>	long-distance	63.74	positive	negative ^c	April
<i>Curruca curruca</i>	long-distance	72.06	positive	- ^d	April
<i>Curruca communis</i>	long-distance	78.08	positive	negative ^c	April
		45.82	positive	- ^d	April
		39.04	doubtful	negative ^c	May
		31.72	doubtful	negative ^c	May
<i>Hirundo rustica</i>	long-distance	86.62	positive	negative ^c	July
		32.20	doubtful	- ^d	July
<i>Ixobrychus minutus</i>	long-distance	93.78	positive	- ^d	May
<i>Jynx torquilla</i>	long-distance	54.02	positive	negative ^c	April
<i>Phoenicurus phoenicurus</i>	long-distance	83.69	positive	negative ^c	April
		87.41	positive	negative ^c	April
		41.37	positive	negative ^c	May
<i>Saxicola rubetra</i>	long-distance	34.91	doubtful	negative ^c	May

^bINGEZIM West Nile COMPAC kit, Gold Standard Diagnostics, Madrid, Spain, ^b ELISA Compac Ingenasa Positivity Index (PI) evaluation guidelines: $\leq 30\%$ (negative), $31\% - 39\%$ (doubtful) and $\geq 40\%$ (positive), ^cnegative result in a 1:32 dilution, ^d the entire sample was used for ELISA analysis.

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Acknowledgements

First and foremost, I would like to sincerely thank the Department of Diagnostics and Development at the Institute of Virology and Immunology, especially Claudia Bachofen and Jakub Kubacki. Without their guidance and support, this work would not have been possible. I am also deeply grateful to Valeria Guidi and Giulia Zezza from the Institute of Microbiology at the University of Applied Sciences and Arts of Southern Switzerland (SUPSI) for their excellent collaboration. Furthermore, I thank the Next Generation Sequencing Platform of the University of Bern for performing the high-throughput sequencing experiments, as well as Marie-Anne Berazategui and Kin Morandi for their invaluable contribution to the collection of bird samples. I am also grateful to the collaborators at the Fondazione Bolle di Magadino and Ficedula, in particular Nicola Patocchi, Roberto Lardelli, and Chiara Scandolara, for their dedicated support during the bird sampling activities. Lastly, I would like to thank the Institute of Parasitology at the University of Bern, specifically Walter Basso and Maryna Galat, for their expert contribution to the morphological characterization of the ticks.

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Declaration of Originality

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I hereby declare that I have written this work independently and that I have not used any sources other than those indicated. All passages, whose wording or meaning was taken from any sources, are all cited and distinctly noted as such.

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