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Serological evidence of antibodies to *Flaviviridae* in wild birds in Portugal

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Abstract

Emerging infectious diseases are a major threat to biodiversity and an important public health issue. Flaviviruses are the cause of several emerging vector-borne zoonotic arboviruses whose distribution is currently increasing in Europe. The evidence that West Nile virus (WNV) circulates in resident and migratory species has implications for both animal and public health and should therefore be studied in depth. USUTU (USUV), Bagaza (BAGV) and tick-borne encephalitis virus (TBEV) are other viruses that are beginning to spread more widely. An integrated surveillance program, namely in birds, is essential for reducing the risk of infection in human populations within the One Health principles. In the present study, wild birds admitted to wildlife rehabilitation centers in Portugal were sampled. Two hundred eight blood samples were assayed serologically for antibodies to flaviviruses by using a commercial ELISA kit. An overall seroprevalence of 19.6% (95% confidence interval [CI]: 13.7–26.7%) was observed. Antibodies against flaviviruses were detected in 13 (35.1%) different species of wild birds. Accipitriformes (26.7%; 95% CI: 18.5–36.2%) and Strigiformes (26.7%; 95% CI: 14.6–42.0%) were the orders with the highest seroprevalence rates recorded. There were no statistically significant differences ($p = 0.725$) between the geographical regions (NUTS II) studied, but a statistically significant difference ($p = 0.017$) was found between sex (male: 34.4%; female: 4.8%). A higher seroprevalence was detected in adults (32.1%) than in juvenile birds (9.3%) ($p = 0.014$), and age was considered a risk factor for flavivirus infection in wild birds (odds ratio 1.4; 95% CI: 0.5–4.0). More epidemiological studies are needed in Portugal since the actual spread of the genus Flavivirus throughout the country is unknown.

Keywords ELISA, Flavivirus, One Health, Seroprevalence, West Nile virus, Zoonosis

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Introduction

There are already some endemic flaviviruses in Europe, and climate change, the evolution of viruses and social factors could lead to the emergence and expansion of other flaviviruses from tropical regions of the world. Controlling the spread of flaviviruses is very difficult, given the flavivirus cycle between arthropod vectors and animal reservoir hosts; differentiating distinct flavivirus infection is also very challenging owing to nonspecific clinical signs, cross-reactivity and low periods of viremia (Kaaijk and Luytjes 2018). Vector-borne diseases account for approximately 17% of all infectious diseases worldwide, and the World Health Organization (WHO) estimates a considerable risk for the global human population of contracting one or more vector-borne diseases (WHO 2020); thus, addressing flaviviruses is highly important.

West Nile virus (WNV) is a mosquito-borne flavivirus with a zoonotic transmission cycle based on mosquitoes and avian species and is spread almost worldwide (Mackenzie et al. 2004; Weissenböck et al. 2010). WNV infection is now a disease of public health concern in Europe (Beck et al. 2013; Parkash et al. 2019). The high genetic and phenotypic diversification of the virus (Habarugira et al. 2020) and its endemic circulation in many different countries require an intensification of integrated and transdisciplinary research and surveillance efforts (Rizzoli et al. 2015).

Wild migratory birds represent important reservoir hosts and vectors of endemic or reemerging zoonotic pathogens, contributing to their wide geographic distribution and being part of the corresponding transmission cycles (Hubálek 2004). Simultaneously, climate change has occurred in recent years, and globalization continues to favor the dispersal of mosquito species to new regions and the long-distance movement of infectious hosts around the world, resulting in an increase in the number of WNV outbreaks recently reported (Farooq et al. 2023; Giesen et al. 2023). Other antigenically related flavivirus are present in Europe, all of which are associated with neurologic disease in both animals and humans and have zoonotic potential, such as USUV (Angeloni et al. 2023) and BAGV (Queirós et al. 2022). Although several studies have focused on the prevalence of WNV in Spain (Bravo-Barriga et al. 2021; García-Bocanegra et al. 2022; Marzal et al. 2022) and other western European countries (Scaramozzino et al. 2021; Ziegler et al. 2022), information in Portugal is limited. A previous article reported the results of a serological and virologic survey of birds (and horses) (Barros et al. 2011), and more recently, another study reported a serological surveillance study (Costa 2021); however, despite being a notifiable disease in Portugal, WNV surveillance remains passive. Despite evidence of WNV circulation in Portugal since 1969 (Filipe and Pinto 1969), there has not yet been a WNV human epidemic in the country (Geraldes et al. 2023). It is still not clear whether WNV is endemic in Portugal, but the climate conditions are now definitely suitable for the transmission of the virus. The southern region of the country has been identified as the main region affected thus far (Lourenço et al. 2022). Tick-borne encephalitis virus is transmitted mainly by infected ticks (genus *Ixodes*). The main USUV vectors are the same mosquitoes that transmit WNV and BAGV. To date, no data have been reported concerning USUV or TBEV in Portugal (ECDC, 2022; Angeloni et al. 2023), and a BAGV outbreak was reported in wild birds in Portugal in 2021 (Queirós et al. 2022).

The presence of Flaviviruses can be detected via serology tests, neutralization assays, viral detection via reverse transcription polymerase chain reaction (RT–PCR), and virus isolation via cell culture (Lustig et al. 2018). RT–PCR has limited value for the routine primary diagnosis of WNV and other flaviviruses because of the low level and short-term viremia they induce. Specific serological tests are currently the most commonly used approach for WNV diagnosis, with immunoglobulin M (IgM) being the first to be detected after infection and immunoglobulin G (IgG) appearing later. However, since antibodies to WNV can persist for long periods of time in circulation and cross-reactivity with other flaviviruses can occur, commercial enzyme-linked immunosorbent assay (ELISA) kits cannot be used to diagnose acute infections caused by WNV by themselves (Busch et al. 2008). A confirmation test should be carried out (Cvjetković et al. 2023).

Collaborative efforts on flavivirus surveillance and control must be implemented and serve as an example to follow for a One Health approach toward zoonotic diseases. Prevention and control efforts depend substantially on effective surveillance of infection in birds, vectors, animals, and humans (Lustig et al. 2018). Vaccines for WNV are currently available for horses (Cavalleri et al. 2022) and are currently being tested in birds (Bergmann et al. 2023) but are not yet available for the avian class or people (Bergmann et al. 2023; ECDC 2024).

The aim of the present study was to contribute updated information on the seroprevalence of flavivirus infection in wild birds admitted to distinct rehabilitation centers in Continental Portugal.

Main results

Antibodies against flavivirus were detected in 42 out of 208 birds, with an overall seroprevalence of 19.6% (95% confidence interval [CI]: 13.7–26.7%). Four birds had

Table 1 Seroprevalence of West Nile virus and possibly other flaviviruses by species of wild birds in Portugal

Order	Common name (*Scientific name*)	Number (%) tested	Number (%) of seropositive	95% CI
Accipitriformes	Northern goshawk (*Accipiter gentilis*)	13 (6.3)	0 (0.0)	0.0–21.0
	Sparrowhawk (*Accipiter nisus*)	10 (4.8)	1 (10.0)	0.0–44.5
	Cinereous vulture (*Aegypius monachus*)	5 (2.4)	1 (20.0)	0.0–71.6
	Spanish imperial eagle (*Aquila adalberti*)	1 (0.5)	1 (100)	0.0–100
	Bonelli's eagle (*Aquila fasciata*)	1 (0.5)	0 (0.0)	0.0–97.5
	Common buzzard (*Buteo buteo*)	31 (14.9)	15 (48.4)	30.2–67.0
	Short-toed snake-eagle (*Circaetus gallicus*)	1 (0.5)	1 (100)	0.0–100
	Montagu's harrier (*Circus pygargus*)	2 (1.0)	0 (0.0)	0.0–84.2
	Griffon vulture (*Gyps fulvus*)	18 (8.7)	1 (5.6)	0.0–27.3
	Booted eagle (*Hieraaetus pennatus*)	10 (4.8)	7 (70.0)	34.8–93.3
	Black kite (*Milvus migrans*)	6 (2.9)	0 (0.0)	0.0–45.9
	Red kite (*Milvus milvus*)	7 (3.4)	1 (14.3)	0.0–57.9
Apodiformes	Common swift (*Apus apus*)	1 (0.5)	0 (0.0)	0.0–97.5
	Pallid swift (*Apus pallidus*)	1 (0.5)	0 (0.0)	0.0–97.5
Caprimulgiformes	European nightjar (*Caprimulgus europaeus*)	3 (1.4)	0 (0.0)	0.0–70.7
Charadriiformes	Yellow-legged gull (*Larus michahellis*)	3 (1.4)	0 (0.0)	0.0–70.7
Ciconiiformes	Gray heron (*Ardea cinerea*)	4 (1.9)	0 (0.0)	0.0–60.2
	White stork (*Ciconia ciconia*)	17 (8.2)	2 (11.8)	0.0–36.4
Columbiformes	Rock pigeon (*Columba livia*)	3 (1.4)	0 (0.0)	0.0–70.7
	Common wood-pigeon (*Columba palumbus*)	1 (0.5)	0 (0.0)	0.0–97.5
	Collared dove (*Streptopelia decaocto*)	1 (0.5)	0 (0.0)	0.0–97.5
Coraciiformes	Kingfisher (*Alcedo atthis*)	1 (0.5)	0 (0.0)	0.0–97.5
Falconiformes	Peregrine falcon (*Falco peregrinus*)	6 (2.9)	0 (0.0)	0.0–45.9
	Common kestrel (*Falco tinnunculus*)	4 (1.9)	0 (0.0)	0.0–60.2
Passeriformes	European greenfinch (*Chloris chloris*)	2 (1.0)	0 (0.0)	0.0–84.2
	Common raven (*Corvus corax*)	1 (0.5)	0 (0.0)	0.0–97.5
	Carrion crow (*Corvus corone*)	5 (2.4)	0 (0.0)	0.0–52.2
	Western house-martin (*Delichon urbicum*)	2 (1.0)	0 (0.0)	0.0 (84.2)
	Eurasian jay (*Garrulus glandarius*)	1 (0.5)	0 (0.0)	0.0–97.5
	Eurasian magpie (*Pica pica*)	1 (0.5)	0 (0.0)	0.0–97.5
Piciformes	Eurasian green-woodpecker (*Picus viridis*)	1 (0.5)	0 (0.0)	0.0–97.5
Strigiformes	Short-eared owl (*Asio flammeus*)	1 (0.5)	0 (0.0)	0.0–97.5
	Long-eared owl (*Asio otus*)	2 (1.0)	1 (50.0)	0.0–98.7
	Little owl (*Athene noctua*)	4 (1.9)	1 (25.0)	0.0–80.6
	Eurasian eagle-owl (*Bubo bubo*)	3 (1.4)	0 (0.0)	0.0–70.7
	Tawny owl (*Strix aluco*)	22 (10.6)	8 (36.4)	17.2–59.3
	Barn owl (*Tyto alba*)	13 (6.3)	2 (15.4)	0.0–45.5
Total		**208 (100)**	**42 (20.2)**	**15.0–26.3**

The information is presented in alphabetical order, both for the orders and scientific names of the birds

CI confidence interval

doubtful results. Antibodies were detected in 13 (35.1%) of the 37 species under study (Table 1).

Table 2 presents the seroprevalence of WNV and flaviviruses from the same serocomplex infection in wild birds admitted to the WRC across Portugal, according to the variables studied. By the WRC, 19.2% (95% CI: 13.8--25.7%) of the birds were seropositive at CRAS-HVUTAD, and 33.3% (95% CI: 14.6--57.0%) were seropositive at CERAS. No seropositive results were found at CERVAS, CIARA or RIAS. However, no statistically significant differences ($p = 0.362$) were found between centers.

In terms of taxonomic order, the highest seroprevalence was found in Accipitriformes (26.7%; 95% CI: 18.5–36.2%) and Strigiformes (26.7%; 95% CI: 14.6–42.0%).

Table 2 Seroprevalence of WNV infection and possibly other flavivirus in wild birds admitted to the WRC across Portugal

Variable	Number (%) tested	Number (%) of seropositive	95% CI
Rehabilitation center			
CERAS	21 (10.1)	7 (33.3)	14.6–57.0
CERVAS	1 (0.5)	0 (0.0)	0.0–97.5
CIARA	3 (1.4)	0 (0.0)	0.0–70.7
CRAS-HVUTAD	182 (87.5)	35 (19.2)	13.8–25.7
RIAS	1 (0.5)	0 (0.0)	0.0–97.5
		$p=0.362$	
Order			
Accipitriformes	105 (50.5)	28 (26.7)	18.5–36.2
Ciconiiformes	21 (10.1)	2 (9.5)	0.0–30.4
Falconiformes	10 (4.8)	0 (0.0)	0.0–30.9
Passeriformes	12 (5.8)	0 (0.0)	0.0–26.5
Strigiformes	45 (21.6)	12 (26.7)	14.6–42.0
Other[a]			
		$p<0.001$	
Age			
Juvenile	54 (26.0)	5 (9.3)	0.0–20.3
Adult	28 (13.5)	9 (32.1)	15.9–52.4
Undetermined[b]	126 (60.6)	28 (22.2)	15.3–30.5
		$p=0.014$	
Geographical region			
North	133 (63.9)	26 (19.5)	13.2–27.3
Centre	21 (10.1)	6 (28.6)	11.3–52.2
Lisbon Metropolitan Area	1 (0.5)	0 (0.0)	0.0–97.5
Alentejo	9 (4.3)	2 (22.2)	0.0–60.0
Unknown[b]	44 (21.2)	8 (18.2)	0.0–32.7
		$p=0.725$	
Sex			
Female	21 (10.1)	1 (4.8)	0.0–23.8
Male	32 (15.4)	11 (34.4)	18.6–53.2
Undetermined[b]	155 (74.5)	30 (19.4)	13.5–26.5
		$p=0.017$	
Migratory behavior			
Resident	158 (76.0)	31 (19.6)	13.7–26.7
Migratory	27 (13.0)	8 (29.6)	13.8–50.2
Mixed	23 (11.1)	3 (13.0)	0.0–33.6
		$p=0.334$	
TOTAL	**208 (100)**	**42 (20.2)**	**15.0–26.3**

CI confidence interval, *CERAS* Wildlife Study and Rehabilitation Centre, *CERVAS* Centre for Ecology, Recovery and Surveillance of Wild Animals, *CIARA* Environmental Interpretation and Animal Recovery Centre, *CRAS-HVUTAD* Wildlife Rehabilitation Centre of the Veterinary Teaching Hospital of UTAD, *RIAS* Wildlife Rehabilitation and Research Centre of Ria Formosa

[a] Other: Apodiformes, Caprimulgiformes, Charadriiformes, Columbiformes, Coraciiformes, Piciformes

[b] Not included in the statistical analysis

Ciconiiformes had a seroprevalence of 9.5% (95% CI: 0.0–30.4%). No seropositive results were found in the orders Falconiformes, Passeriformes or any other order included in the study. The differences were statistically significant ($p<0.001$) across all the tested orders.

No significant differences ($p=0.725$) were detected for geographical regions, with a seroprevalence of 19.5% (95% CI: 13.2--27.3%) in the North, 28.6% (95% CI: 11.3--52.2%) in the Centre, 0.0% (95% CI: 0.0--97.5%) in Lisbon (metropolitan area), 22.2% (95% CI: 0.0--60.0%) in Alentejo, or 18.2% (0.0--32.7%) in birds of unknown origin. Antibodies against flaviviruses were found in rescued birds in the following districts: Braga, Bragança, Castelo Branco, Évora, Guarda, Portalegre, Porto, Viana do Castelo, Vila Real and Viseu (Fig. 1).

The comparison of the variable sex ($n=53$) revealed significantly different seroprevalence rates between female (4.8%; 95% CI: 0.0--23.8%) and male (34.4%; 95% CI: 18.6--53.2%) ($p=0.017$) wild birds. With respect to the variable migratory behavior, the seroprevalence was 29.6% (95% CI: 13.8--50.2%) in migratory birds, 19.6% (95% CI: 13.7--26.7%) in resident birds, and 13.0% (95% CI: 0.0--33.6%) in mixed migratory behavior birds. A comparison of the variable age ($n=82$) revealed significantly different seroprevalence rates between juvenile (9.3%; 95% CI: 0.0--20.3%) and adult (32.1%; 95% CI:

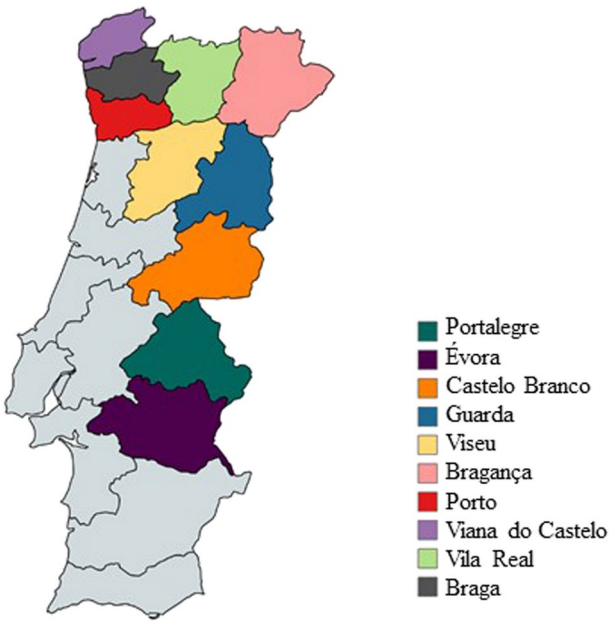

Fig. 1 Districts of Portugal where the birds tested positive for flavivirus antibodies: Braga (B), Bragança (Br), Castelo Branco (CB), Évora (E), Guarda (G), Portalegre (Po), Porto (P), Viana do Castelo (VC), Vila Real (VR) and Viseu (V)

15.9--52.4%) ($p=0.014$) wild birds. Age was the only suggested risk factor. Compared with the reference category (juvenile; arbitrary OR = 1), adult birds had an odds ratio (OR) of 1.4 (95% CI: 0.5–4.0; $p=0.019$).

The present results reveal the existence of antibodies to flaviviruses (from the Japanese encephalitis [JE] serocomplex) in wild birds from different parts of Portugal. The positive birds were from districts in the North, Centre and South of the country, mostly from interior areas. The absence of positive results in three of the WRCs is likely related to the low number of birds tested. To the best of our knowledge, cases of WNV infection in Portugal reported thus far have been restricted to South Portugal (Lourenço et al. 2022), so it is important to highlight the spread of seropositive animals to other geographical areas.

In the present study, four birds had doubtful results, and these were not considered for the statistical analysis. These results require repeated ELISA or confirmatory testing.

We found an overall seroprevalence of antibodies against flavivirus of 19.6%. It is relevant to establish a comparison with previous studies in the Iberian Peninsula. In a previous study in Spain, IgG antibodies against flaviviruses were found in 32.7% of the wild birds tested (56/171; 95% CI: 26.8–38.6) by blocking ELISA (bELISA), and the individual WNV seroprevalence was 19.3% (95% CI: 14.3–24.3) after the virus neutralization test (VNT) (García-Bocanegra et al. 2022). Even though the results are somewhat similar, the types of ELISA used differ. In the present study, we used competitive ELISA (cELISA), a screening tool that requires confirmation, which we have not yet performed. In addition, a seroepidemiological study in wild ungulates in Spain revealed seroprevalence values ranging from 20% to 24.9% (95% CI: 23.2–26.7%) (Casades-Martí et al. 2023). In other European countries, there is a wide range of flavivirus seroprevalence values in wild birds. It spans from 14.8% to 16.2% in East Germany (Ziegler et al. 2022) and from 1.3% in Cyprus (Pallari et al. 2021). There is a notable lack of recent studies in many countries, where outbreaks have even occurred.

The orders with the most different species analyzed were Accipitriformes (12 species), Passeriformes (6 species) and Strigiformes (6 species). Previously, in the Iberian Peninsula, wild birds belonging to the order Accipitriformes also presented the highest frequency of seropositivity for WNV (46.3%; 19/41), followed by Strigiformes (16.1%; 9/56), which were in third place (García-Bocanegra et al. 2022). Among the 13 species in which antibodies to WNV have been detected, most have already been reported as susceptible to infection in Europe, such as the barn owl (*Tyto alba*) (García-Bocanegra et al. 2022), booted eagle (*Hieraaetus pennatus*), common buzzard (*Buteo buteo*) (Csank et al. 2018), griffon vulture (*Gyps fulvus*), long-eared owl (*Asio otus*) (Jurado-Tarifa et al. 2016), short-toed snake eagle (*Circaetus gallicus*) (Alba et al. 2014; Jurado-Tarifa et al. 2016), Spanish imperial eagle (Aquila *adalberti*) (Höfle et al. 2008), sparrowhawk (*Accipiter nisus*) (Erdélyi et al. 2007), tawny owl (*Strix aluco*) (Michel et al. 2018) and white stork (*Ciconia ciconia*) (Linke et al. 2007; Alba et al. 2014). The sample size was small for the orders that yielded negative results. (Passeriformes, $n=12$; Falconiformes, $n=10$).

The main tools used to diagnose WNV include serological (or indirect) tests that aim to detect antibodies to WNV, such as ELISA, hemagglutination-inhibition tests (HAITs) or immunofluorescence assays (IFATs). ELISAs are the most commonly used diagnostic assays because they are relatively simple, quick and inexpensive and allow many samples to be screened at once. Competitive ELISA (cELISA) is the most sensitive of all the developed serological technologies but is mostly used for screening purposes, a circumstance derived from its lower level of specificity (Beck et al. 2013). Cross-reactions between related and co-circulating flaviviruses are common. Infection with a virus from the group has been shown to induce antibodies that generate positive results in cELISA and other rapid serological diagnostic tests, but which virus cannot be distinguished (Beck et al. 2017; Llorente et al. 2019). The aim of this study was to show evidence of the presence of antibodies to WNV in wild birds present in Portugal, and a commercial cELISA kit was used for this purpose. There are still not many studies on the prevalence of flavivirus infections in Portugal. A very recent study reflects on the concern of the emerging threats of BAGV, USUV and WNV in the country, and attempts to shed light on the epidemiological dynamics of their infections. It has proved the endemic circulation of WNV and sporadic circulation of BAGV and USUV in Portugal, although only one species of bird has been tested (Fontoura-Gonçalves et al. 2024). This proof of co-circulation is definitely a conditioning factor when evaluating ELISA results, as several types of antibodies may be detected simultaneously. The kit that was used was proven to detect antibodies to a wide range of flaviviruses in multiple species. No confirmatory test was carried out for these results. Therefore, seropositivity must be interpreted with care because cross-reactions among flaviviruses are frequently observed (Calisher et al. 1989; Beck et al. 2013). Flaviviruses are antigenically related, and diagnostics should be based on tests that prove to be specific enough to avoid cross-reactivity between related flaviviruses that may cocirculate in the same geographical area. WNV and USUV, for example, belong to the JE serocomplex and share common

distribution areas in Europe (Scaramozzino et al. 2021; Simonin 2024), which means that cross-neutralization can occur, leading to misinterpretation of results due to false positives. Although other serological assays could be used as confirmatory methods (Kuno 2003; Weingartl et al. 2003; Mackenzie et al. 2004), these methods also have drawbacks. According to the manufacturer, the commercial ELISA used here exhibited greater sensitivity than the hemagglutination inhibition assay did, detecting seroconversion concurrently or prior to the VNT.

Serosurveys of free-ranging birds should be carefully interpreted. The role of migratory birds in the maintenance and dissemination of zoonotic pathogens, such as flaviviruses, could be extremely relevant in assessing public health risks (Malik et al. 2021). Some species are long-distance migrants, choosing Portugal for nesting during the Spring–Summer seasons. These birds can be previously infected with WNV and other flaviviruses elsewhere during migration and wintering (Lopez et al. 2008). However, while they are in Portugal, they act as hosts for potential vectors to be infected during their blood meals. Moreover, sedentary bird species may also be exposed to a wide variety of flaviviruses (Aguero et al. 2011; Zannoli and Sambri 2019).

Sex identification in birds by phenotype and external morphology cannot be performed for approximately half of the species that occur in Europe. This situation is even more difficult in young juvenile birds. Sexing can be performed surgically, cytologically, or molecularly (Griffiths 2000), but any of these options would be extremely expensive and not feasible for WRC. This is why so many of the individuals sampled were classified as "undetermined" in terms of sex. To date, sex has not been identified as a risk 264 factor associated with positive Flaviviruses cases (Verbeek and Caffrey, 2002; Ludwig et 265 al. 2010). Aging is also not an easy task. A high level of knowledge about the molting patterns of different species is needed, and the people who receive birds at the WRC do not always have that experience. Migratory behavior, climatic variation, nutritional status and other intraspecific factors also influence molt duration and progression, making this process very complex (Newton 2009; Zuberogoitia et al. 2018). This is why we also have a high number of samples classified as "undetermined" age. In this study, age was the only confirmed risk factor. Compared with juveniles, adult birds presented a 1.4-fold greater risk of being infected. Adult birds have a longer life span and therefore a longer period of exposure, so this result is expected (García-Bocanegra et al. 274 2022). Age influences social behaviour, which in turn can influence the risk of 275 transmission (high population densities, the greater the risk) (Ludwig et al. 2010). Age over 1 year was also previously described as one of the main risk factors for WNV seropositivity. Species group (raptors) and size (large) were other risk factors described (García-Bocanegra et al. 2022). Nevertheless, if it had been possible to determine the exact age of more animals, the results would have been more accurate.

The transmission cycle of WNV is complex, and proper control requires targeted preventative measures and actions. WNV outbreaks are responsible for dramatic bird mortality events, causing severe declines in some bird species (LaDeau et al. 2007; Ip et al. 2014; CDC 2024). For conservation purposes, it is highly important to assess the status of virus dispersal in our wild populations since the Iberian Peninsula is home to some critically endangered bird species (Birdlife International 2015).

On the basis of previous experience from countries where the disease is endemic (Paternoster et al. 2017; Todoric et al. 2022), the surveillance plans to be implemented should be based on a holistic vision and multidisciplinary teams between regional institutions involved in every health sector: public, animal, and environmental. A One Health approach is the best way to achieve good results in terms of human case prevention, as it is based on early detection of the viral circulation in the main vertebrate hosts (birds and horses) and the integration of data. In addition to WNV, other flaviviruses are an expanding threat in Europe and are associated with important human diseases. Although no WNV confirmation test has been carried out, the high detection of antibodies to flaviviruses in this study is highly relevant (Daep et al. 2014; Pierson and Diamond 2020).

Considering the epidemiology of WNV in Europe over the last three decades, the establishment of appropriate surveillance systems is fundamental, as the risk for bird populations and horse and human health is a reality (Chevalier et al. 2011). Likewise, the analysis of WNV cases in horses can help identify risk areas for humans (García-Carrasco et al. 2023), and the assessment of circulation in birds, especially resident species, is also useful. This kind of investigation is essential for designing prevention and control measures properly under One Health strategies.

Conclusions

This study demonstrated the circulation of flaviviruses in various species of wild, migratory and resident birds in Portugal.

The circulation of zoonotic flaviviruses in Europe is a reality that should not be ignored, as these viruses represent serious emerging threats for animal and public health. The symptoms they can cause in humans are very serious and even fatal, and in wildlife, severe outbreaks can have a very significant impact on the dynamics of populations.

The real epidemiological status of flaviviruses in Portugal remains uncertain. This study revealed that there is circulation of anti-flavivirus antibodies in birds that reside in or pass through Portugal, which means that there should be a wake-up call to the possible focus of transmission and amplification of the disease. WNV is the main agent studied and the most likely cause because it was previously detected in the area, but the reality is that other flaviviruses are arriving, and they are no less serious or worrying. We should consider the results obtained as "undetermined flaviviruses", due to the limitations of the laboratory method related to cross-reactions. However, in spite of no specific antibodies to a specific virus were detected, the infection is most likely caused by WNV, BAGV or USUV (whose presence is known in the country). True knowledge of the spread of these viruses, their expression and their affected hosts is important for establishing prophylactic measures. The local epidemic status of other flaviviruses is still unknown, and climate and environmental changes are expected to affect the epidemiology of these viruses. Knowledge about the expression of *Flaviviridae* in Portugal is still very limited, so basic seroprevalence studies such as this one are very important and necessary to reliably update the distribution of these viruses.

Methods

Wild birds admitted to two main different wildlife rehabilitation centers (WRCs), the Wildlife Rehabilitation Centre of the Veterinary Teaching Hospital of UTAD (CRAS-HVUTAD) (n = 182) in Vila Real and the Wildlife Study and Rehabilitation Centre (CERAS) ($n = 21$) in Castelo Branco, were sampled between 2021 and 2023. Five samples from other WRCs were also included: one from the Centre for Ecology, Recovery and Surveillance of Wild Animals (CERVAS – Gouveia), three from the Environmental Interpretation and Animal Recovery Center (CIARA – Torre de Moncorvo), and one from the Wildlife Rehabilitation and Research Centre of Ria Formosa (RIAS – Olhão). The causes of admission vary and include orphans or birds that have suffered traumatic injuries, namely, collisions, electrocution, traffic accidents or illegal shooting. A physical examination was carried out on all the birds to assess their health status and body condition, and further diagnostic procedures were performed as needed. Blood samples (approximately 0.3 mL) were collected from the ulnar vein, metatarsal vein or jugular vein, according to the anatomy of each species, and transferred into heparin-lithium tubes. The samples were centrifuged at 2000 rpm for 10 min, and the plasma was then separated and stored at -20°C until further analysis. The data gathered for analysis included species, order, location where the bird was rescued and migratory habits. Age and sex were also recorded whenever possible.

All the plasma samples were tested for antibodies to WNV via a commercial ELISA kit (ID Screen® West Nile Competition Multispecies) following the manufacturer´s instructions. Two negative and positive controls were included in each 96-well plate, as recommended. This test allows the detection of anti-pr-E antibodies in multiple species, including birds. The results were classified as positive, negative or doubtful after verifying the test validation criteria. Doubtful results were not considered in the present study for statistical analysis.

The birds were grouped by order, family and sex (females, males, and undetermined). Individuals of species that do not exhibit sexual dimorphism could not be distinguished and were therefore classified as undetermined. In terms of age, birds were, in a simplified manner, classified as juvenile, adult or undetermined because a more in-depth classification is often difficult to determine. The juvenile category includes all ages before adulthood, namely, nestlings, fledglings and juveniles themselves. For the origin, the municipality where the bird was rescued was recorded whenever possible. With respect to their migratory habits, birds are described as estival, wintering or resident (this classification may be cumulative with one of the first).

Statistical analyses were performed with BM SPSS Statistics 27 (IBM; Armonk, NY, USA). The prevalence of WNV flavivirus antibodies was calculated as the ratio of positive samples to the total number of plasma samples tested, using a 95% confidence interval (CI), determined by sample size calculators. Pearson's chi-square test or Fisher's exact test was used to compare seroprevalence values related to epidemiological variables (multivariable analysis). The outcome variable was dichotomized as positive versus not positive to identify any risk factors associated with seropositivity. Multiple logistic regression was used to model the odds ratio (OR) and its 95% CI of being seropositive in relation to the variables. Significant potential risk factors at $p < 0.05$ (two-tailed; $\alpha = 0.05$) were then assessed via stepwise regression to construct a multiple model (Wald test stepwise p-Wald value to enter $p < 0.05$). The multiple logistic model was developed via a stepwise approach. Backward elimination followed by forward selection for each variable at a time was performed via a likelihood ratio test at each step, with 0.05 (two-tailed; $\alpha = 0.05$) as the significance level for removal or entry. The adequacy of the models was assessed via the Hosmer and Lemeshow goodness-of-fit test (Hosmer and Lemeshow 2000). The model was repeated until all remaining variables presented statistically significant values ($p < 0.05$).

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Authors' contributions
FL and ACC: Conceptualization; FL, CP, LC and ACC: methodology; LC and ACC: formal analysis; FL: writing—original draft preparation; FL and ACC: data curation; LC, ACM, JRM, CP, APL, MF, RB, CL, FS, MM and ACC: writing—review and editing; ACC, ACM and MM: supervision; LC and ACC: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Declarations

Ethics approval and consent to participate
The animal study protocol was approved by the Ethics Committee of the University of Trás-os-Montes e Alto Douro.

Consent for publication
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The authors declare that they have no conflicts of interest.

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