SURVEILLANCE OF WILD WATERBIRDS FOR AVIAN INFLUENZA VIRUSES IN LITHUANIA

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Abstract. A total of 3720 samples of 24 waterbird species belonging to 5 orders were collected in Lithuania between 2007 and 2011 for the analysis of avian influenza viruses. One hundred eigthy seven samples were positive for influenza A viruses or antibodies. All influenza A positive samples were found in the mallard (*Anas platyrhynchos*). The recorded prevalence of influenza A virus in mallards was 7.5%. Among mallards sampled in Lithuania low-pathogenic H7N2, AIVH7 and AIVH5 subtype's viruses prevailed. Other low pathogenic avian influenza subtypes (H1-H4, H6, H8, H9, H10N4 and H12) were also found in mallards, collected in various regions of Lithuania. Therefore in the future active surveillance for avian influenza virus in Lithuania (sampling of apparently healthy wild birds) should give a high priority to the mallard. Following recommendations of the European Commission (EC Directive 2005/94/EC, 2006), certain other species of wildfowl at the higher risk, such as the common pochard (*Aythya ferina*), tufted duck (*Aythya fuligula*) and mute swan (*Cygnus olor*) should be also included into the national monitoring schemes for avian influenza in the countries of the European Union. Passive surveillance (sampling of hunted birds or birds found dead) should pay special attention to the mallard, as well as to the common pochard and tufted duck. Such surveillance scheme should provide an early warning about potential presence of HPAIV virus in the country.
Keywords: waterbirds, avian influenza, bird migration, Lithuania.

LIETUVOS VANDENS PAUKŠČIŲ UŽSIKRĖTIMO PAUKŠČIŲ GRIPO VIRUSAIS STEBĖSENA

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Santrauka. 2006–2011 metais paukščių gripui nustatyti Lietuvoje buvo atliekama laukinių vandens paukščių stebėsena. Surinkti ir ištirti 24 paukščių rūšių mėginiai iš 3 720 vandens paukščių. Išaiškinti 187 individai, užsikrėtę įvairiais mažai patogeniškas paukščių gripo potipiais. Paukščių gripo virusai nustatyti tik didžiosiose antyse (*Anas platyrhynchos*), tarp kurių užsikrėtusios sudarė 7,5 proc. Dažniausiai didžiosios antys buvo užsikrėtusios paukščių gripo H7N2, AIVH7 ir AIVH5 virusų potipiais. Nustatyti ir kiti mažai patogeniški H1-H4, H6, H8, H9, H10N4 ir H12 virusų potipiai. Paukščių gripo prevencijai ir kontrolei Lietuvoje būtina tęsti laukinių vandens paukščių užsikrėtimo paukščių gripo virusu stebėseną, ypatingą dėmesį skiriant didžiosioms antims ir kitoms didelės rizikos grupei priskiriamoms vandens paukščių rūšims – rudagalvėms antims (*Aythya ferina*), kuoduotosioms antims (*Aythya fuligula*) ir gulbėms nebylėms (*Cygnus olor*).

Raktažodžiai: vandens paukščiai, paukščių gripas, paukščių migracijos, Lietuva.

Introduction. Avian influenza (AI) is a highly contagious viral infection which can affect different species of birds. Highly Pathogenic Avian Influenza (HPAI) viruses spread rapidly causing a serious disease with high mortality in almost all bird species and it has so far been restricted to H5 and H7 subtypes (Munster et al., 2007; Lvov et al., 2010). The bird-to-human transmission of highly pathogenic AIV (HPAIV) subtype H5N1 was recorded in 1997. Low Pathogenic Avian Influenza (LPAI) viruses belonging to H1-H16 subtypes usually cause a mild disease in poultry (Capua, Alexander, 2009). LPAI strains of the H5 and H7 subtypes have the potential to mutate into HPAI. Among the different subtypes of avian influenza viruses, H5 and H7 are of major interest because of the serious consequences for the poultry industry and the increasing frequency of direct transmission of these viruses to humans. Although previously HPAIV infections were rarely observed in wild birds and if so only in connection with poultry outbreaks, since the continuing outbreaks of H5N1 HPAI in Asia in 2003/2004 (Gilbert et al, 2008) wild birds have been thought to be implicated in the long distance spread of that virus. There are considerable evidences from North America and Eurasia indicating that wild birds such as greater white-fronted goose or common pochard could play a significant role in the spread of avian influenza (Irza, 2006; Peterson & Williams, 2008). Surveillance of wild birds for avian influenza viruses has been compulsory in the European Union (EU) since 2005, primarily as a means of detecting HPAI H5N1 virus and of monitoring the circulation of LPAI virus H5 and H7 strains (EC Directive 2005/94/EC, 2006).

In Lithuania, the monitoring of hunted wild birds or birds found dead for the detection of avian influenza viruses (according to the National Avian Influenza Action Plan) was implemented by the National Food and Veterinary Risk Assessment Institute between 2006 and 2011. The monitoring of live migratory and wintering waterbirds for detection of HPAI and LPAI virus strains was performed in Lithuania as part of the international programme on avian influenza coordinated by Wetlands International and OMPO (the European Institute for the Management of Wild Birds and their Habitats) in 2009 – 2011. The results of surveillance of wild waterbirds for avian influenza in Lithuania are presented in this study.

Material and methods. Samples of dead waterbirds (hunted birds and individuals found dead) were collected in all regions of Lithuania between 2007 and 2009. Most samples were collected during the open hunting season on wildfowl in Lithuania (August–November). Laboratory tests in the National Food and Veterinary Risk Assessment Institute were conducted in accordance with the Diagnostic Manual for Avian Influenza of the European Union (EC Directive 2005/94/EC, 2006). Type A influenza viruses were classified on the basis of two of these proteins fixed on the surface of virus particles: the hemagglutinin (HA) and neuraminidase (NA) glycoproteins (OIE). The RNA was isolated from cloacal and oropharyngeal swabs/or tissuess by means of viral RNA kits (QIAGEN, Hilden, Germany) and analyzed by real-time RT-PCR for influenza virus matrix (M) gene fragments. In positive A type influenza samples, H5- and H7 - specific real-time RT-PCRs were used to identify or exclude respective subtypes (Slomka et al., 2007). H5 and H7 isolates were pathotyped following the European Union Directive 2005/94/EC approved in 2006. Direct hemagglutinin (HA) typing or sequencing of positive samples was carried out following standard methods (Hoffmann et al., 2001; Phipps et al., 2004). Molecular pathogenicity of H5 and H7 subtype–positive samples was determined by sequencing the hemagglutinin gene segment (using BigDye Terminator v3.1 cycle sequencing kit, Applied Biosystems, Foster City, CA, USA).

Live and apparently healthy migratory and wintering waterbirds were captured and sampled in the main areas of their concentration in Lithuania, designated during earlier investigations (Švažas et. al. 1998, 1999; Švažas, Raudonikis, 2009). The majority of samples of migratory waterbirds were collected in the Nemunas River delta (55°15´N, 21°20´E) with adjacent wetlands in September–November and March–May during 2009–2011. Samples of the wintering waterbirds were collected mainly in coastal wetlands and in the permanently ice-free stretch of the Nemunas River at the Kaunas Hydro Power Station. Most waterbirds were captured with cannon-nets of different size provided by OMPO. All captured waterbirds were measured and ringed. Oral-pharyngeal/cloacal samples were collected using sterile cotton cloacal and tracheal swabs. The samples were stored in transport medium (Hank's balanced salt solution containing 0.5% lactalbumin, 10% glycerol, 200 U/ml penicillin, 200 µg/ml streptomycin, 100 U/ml polymyxin B sulphate, 250 µg/ml gentamycin, and 50 U/ml nystatin) and further at -80°C in the nitrogen freezer. The collected samples (stored in dry ice) were shipped for the avian influenza analysis to the designated AI Reference Laboratories. The analysis of samples collected in 2009 for AI was performed at the Norwegian Veterinary Institute and at the Italian Zooprophylactic Institute as part of the European Union "New FluBird" program. The analysis of samples collected in 2010–2011 for AI was performed at the Erasmus Medical Centre, the Netherlands. RNA was isolated using a MagnaPure LC Total nucleic acid isolation kit and influenza A viruses were detected using a real-time RT-PCR specific for the highly conserved matrix gene of influenza A viruses (Munster et al., 2009). Matrix RRT-PCR positive samples (cycle threshold (Ct) value, <40) were subsequently used for detecting H5 and H7 influenza A viruses using a RRT-PCR targeting the H5 gene as described previously (Munster et al. 2009). Oligonucleotides 5'-GGC-AAC-AGG-AAT-GAA-GAA-TGT-TCC-3' and 5'-AAT-CAG-ACC-TTC-CCA-TCC-ATT-TTC-3' and the double labeled probe 5'-6-FAM-AGA-GGC-CTA-TTT-GGT-GCT-ATA-GCG-GGT-

TTC-AT-TAMRA-3' were used to detect the H5 and H7 gene. Pools of 4 - 5 samples were prepared and analysed in parallel with influenza A virus positive and negative controls. Next RNA isolation and RRT-PCR detection of influenza A viruses was repeated for individual samples of RRT-PCR positive pools. Individual influenza A virus RRT-PCR positive samples were used for virus isolation. 200 µl of the original material was inoculated in the allantoic cavity of 11-day old embryonated chicken eggs. After 2 days, the allantoic fluid was harvested and tested for AIV presence in a hemagglutination assay with turkey erythrocytes. The subtype of the viral isolates was determined using a hemagglutination inhibition (HI) assay for the characterization of the subtype of the HA and a RT-PCR to determine the subtype of the NA (Munster et al., 2009).

The data received from the Lithuanian Bird Ringing Centre on long-term bird ringing of the mallard (*Anas platyrhynchos*) and mute swan *(Cygnus olor)* were analysed in this study.

Results. A total of 3720 samples of 24 waterbird species belonging to 5 orders were collected in Lithuania between 2007 and 2011, and analysed for avian influenza viruses. Out of 2291 samples collected from dead birds (mostly from hunted ducks submitted by hunters) 181 samples (7.9%) were found positive for A type influenza virus (Table 1).

Table 1. **Results of the analysis of hunted or found dead waterbirds studied for avian influenza viruses in Lithuania in 2007–2011**

Order	Family	Species	No. of samples	No. of positive samples	Prevalence (%)
Pelecaniformes	Phalacrocoracidae	Great Cormorant *Phalacrocorax carbo*	2	0	0
Anseriformes	Anatidae	Mute Swan *Cygnus olor*	114	0	0
		Whooper Swan *Cygnus cygnus*	3	0	0
		Bean Goose *Anser fabalis*	1	0	0
		Common Teal *Anas crecca*	16	0	0
		Northern Shoveler *Anas clypeata*	2	0	0
		Mallard *Anas platyrhynchos*	2085	181	8,6
		Garganey *Anas querquedula*	2	0	0
		Eurasian Wigeon *Anas penelope*	1	0	0
		Gadwall *Anas strepera*	1	0	0
		Common Pochard *Aythya ferina*	6	0	0
		Tufted Duck *Aythya fuligula*	10	0	0
Gruiformes	Rallidae	Common Coot *Fulica atra*	44	0	0
		Water Rail *Rallus aquaticus*	1	0	0
Charadriiformes	Laridae	Black-headed Gull *Larus ridibundus*	2	0	0
		Common Gull *Larus canus*	1	0	0

Table 2. **Results of the analysis of waterbirds captured alive in Lithuania and studied for avian influenza viruses** (study of swabs has been carried out in EU Reference Laboratories in 2009–2011)

Order	Family	Species	No. of samples	No. of positive	Prevalence (%)
Pelecaniformes	Phalacrocoracidae	Great Cormorant *Phalacrocorax carbo*	20	0	0
Ciconiiformes	Ciconidae	White Stork *Ciconia ciconia*	8	0	0
	Ardeidae	Great White Egret *Ardea alba*	1	0	0
Anseriformes	Anatidae	Mute Swan *Cygnus olor*	331	0	0
		Bean Goose *Anser fabalis*	11	0	0
		Greater White-fronted Goose *Anser albifrons*	86	0	0
		Barnacle Goose *Branta leucopsis*	22	0	0
		Mallard *Anas platyrhynchos*	390	6	1,5
		Eurasian Wigeon *Anas penelope*	1	0	0
Gruiformes	Rallidae	Common Coot *Fulica atra*	18	0	0
Charadriiformes	Charadriidae	Northern Lapwing *Vanellus vanellus*	53	0	0
		Ruff *Philomachus pugnax*	9	0	0
	Laridae	Black-headed Gull *Larus ridibundus*	79	0	0
		Common Gull *Larus canus*	15	0	0
		Herring Gull *Larus argentatus*	383	0	0
		Great Black-headed Gull *Larus marinus*	2	0	0

Avian influenza viruses positive samples were identified exclusively from mallards, hunted in various regions of the country. Among mallards the detected prevalence was 8.6%. Among the analysed "A" type influenza positive samples from mallards there was one sample positive for low-pathogenic AIV H5 subtype and two samples of low-pathogenic AIVH7 subtype. Out of 79 other A type influenza positive samples, 61 were successfully sequenced using a set of primers targeting hemagglutinin gene. Using a set of bioinformatics tools, hemagglutinin subtype was identified as H1 in 5 analysed samples, H2 – in 8, H3 – in 11, H4 – in 10, H6 – in 10, H8 – in 2, H9 – in 11 and H12 – in 4.

Six (0.4 %) out of 1429 samples collected from live waterbirds were identified as A type influenza positive during the analysis performed at the Erasmus Medical Centre (Table 2).

All influenza positive samples were identified only in the mallard. Among the mallard, the recorded infection rate accounted for 1.5%. Four viruses found in mallards were cultured and isolated from eggs. Two of them were characterized as low pathogenic H7N2 viruses and two – as H10N4 viruses. There were two other H7 positive samples identified by PCR on the original material, but these viruses could not be cultured. The pathogenicity of H7 subtype-positive samples was determined by sequencing the basic cleavage site of the hemagglutinin gene.

Mallards sampled in Lithuania were infected by low-pathogenicity H7N2, AIVH7 and AIVH5 subtypes viruses. The subtype H7 was much more prevalent (found in 6 samples) than subtype H5 (1 sample). The presence of LPAI H5 and H7 viruses in Mallards can potentially raise the risk of generating new HPAI viruses, particularly in sites where these ducks occasionally mix with poultry. A variety of other LPAI subtypes (H1-H4, H6, H8, H9, H10N4 and H12) were also found in mallards, sampled in various regions of Lithuania.

Discussion. Mallards sampled in Lithuania clearly dominated among birds identified as A type influenza positive. The recorded prevalence of influenza A virus in mallards (7.5%) was very similar to the prevalence (7.3%) earlier identified in this species in other countries of Europe (Munster et al., 2007). Many factors including the year, season, location, species and age of birds influence the prevalence of avian influenza virus, as was recorded in wild birds in Europe, America and Africa (Munster et al., 2007; Latorre-Margalef et al., 2009; Gaidet et al., 2010). By experimentally infecting wild ducks, recently it has been found that mallards can potentially be the main long-distance vectors of HPAI H5N1 (Keawcharoen et al., 2008). The mallard was the only species to show abundant virus excretion without clinical or pathologic evidence of debilitating disease and this can explain almost total absence of dead mallards in wild bird die-offs from HPAIV (H5N1) in Europe and Asia in 2005–2007 (Keawcharoen et al., 2008).

It is important to define the distribution and migratory routes of mallards breeding in Lithuania and of those of birds of Northern Europe origin migrating through Lithuania, as this information is essential to understanding of the dynamics of virus transmission. Several biogeographic populations of mallard defined on the basis of their main wintering regions have been recently recognized in Europe. Birds breeding in the Baltic States, northwest Russia and Finland, and wintering mainly in West and Northwest Europe were ascribed to Northwest Europe population, estimated at about 4.5 million individuals (Delany, Scott 2006). The analysis of recoveries of mallards ringed in Lithuania in 1930–2011 indicates that main wintering and staging sites of these birds are located in Western and Central Europe, though birds of Lithuanian origin were also recovered in southern and northern Russia, in Siberia, in the Mediterranean and Caspian Sea Regions (Fig. 1).

Recoveries of mallards ringed as day-old ducklings in Latvia during their post-nesting dispersal and first autumn migration were also obtained mainly in Western and Central Europe, though a large-scale mixture occurs between individuals breeding, staging and wintering in different regions of Europe (Viksne et al., 2010). All mallards of the Eastern Baltic origin were strictly migratory until the 1970s, while at present most local breeders are partially migratory (Švažas et al. 2001a, 2001b). During migration periods, staging mallards were recorded in numerous pools and ponds used for production of domestic ducks and geese in various regions of Lithuania, particularly in coastal floodplains.

Mallards are the most abundant wildfowl species in Eastern Europe, with about 2 million breeding pairs estimated (Viksne et al., 2010). These ducks are very tolerant of human presence and are common in urban and agricultural habitats, thus forming a potential link between wild waterfowl, poultry and humans (Keawcharoen et al., 2008). Lithuania is located on the crossroad of different flyways of mallards, with local ringed birds being recovered in all regions of Europe. Therefore the surveillance of this species for the avian influenza viruses in the country is of particular importance.

Among other duck species the common pochard (*Aythya ferina*) and tufted duck (*Aythya fuligula*) are more likely to act as sentinels for HPAIV H5N1 in wild bird populations and close surveillance of these species for unusual illness or death should provide an early warning about HPAIV H5N1 infection in an area (Keawcharoen et al., 2008). It is important to continue the surveillance of both species for the avian influenza virus in Lithuania.

The mute swan has also been ascribed to the group of species at the highest risk related to avian influenza (European Commission, 2006). Mute swans dominated among waterbird species particularly affected by the H5N1 virus during the last major outbreak of avian influenza in the Baltic Sea region in February – May 2006 (71% of all reported H5N1 infections were detected in mute swans), with infected individuals being found at the southeastern Baltic coast close to Lithuania (European Commission, 2006). The mute swan has been identified as primarily a sentinel species for HPAIV H5N1 **(**Keawcharoen et al. 2008).

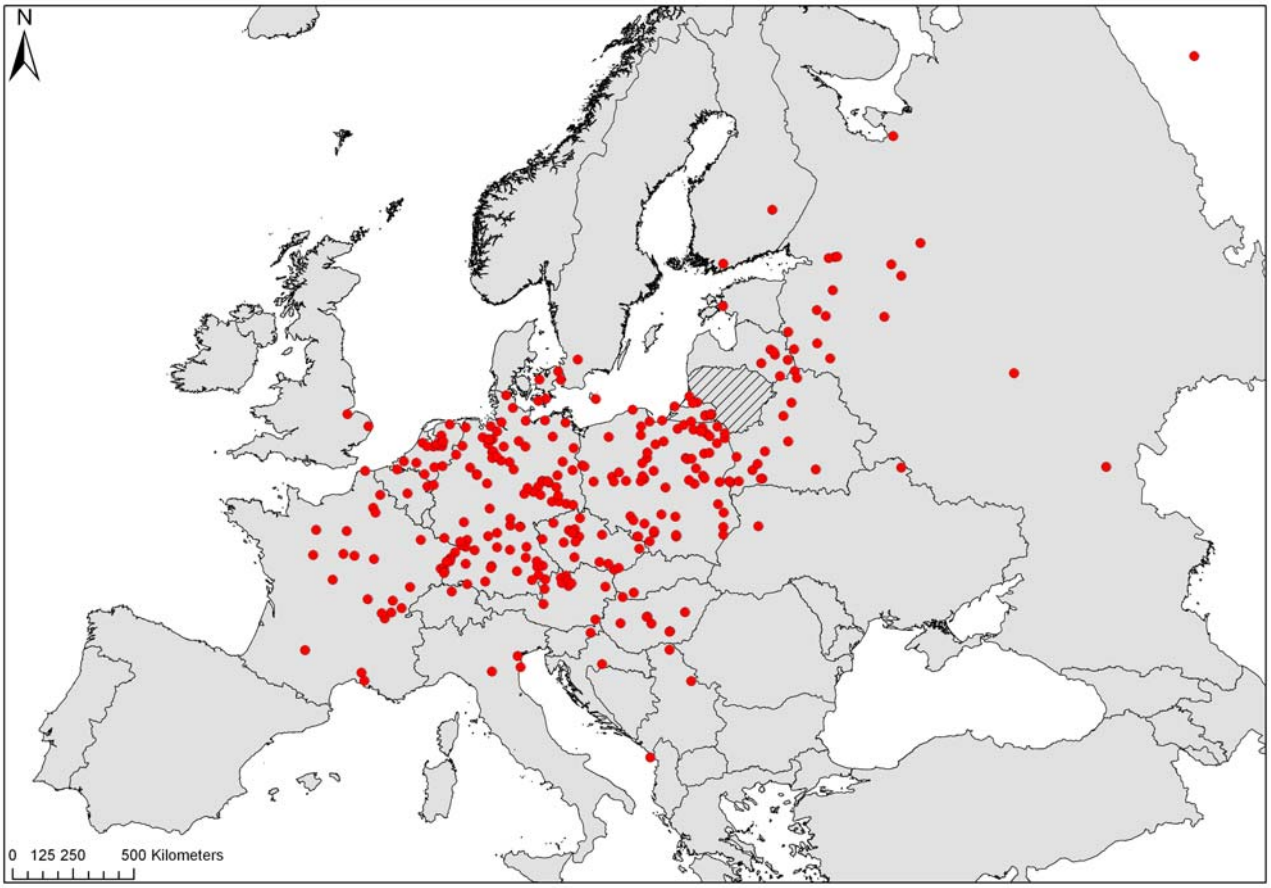

Fig. 1. **Recoveries of mallards** (*Anas platyrhynchos*) **ringed in Lithuania in 1930–2011**

Birds breeding in the Baltic States and other countries of Central and Northwest Europe are attributed to the Northwest/Central Europe population, estimated at about 250000 individuals (Delany, Scott 2006). The recoveries of mute swans ringed in Lithuania in 1930–2011 indicate that main wintering and staging sites of these birds are located in Central and Northwestern Europe, particularly along the coasts of the Baltic and North Seas (Fig. 2).

The analysis of long-term ringing data indicates that the main wintering sites of mute swans of eastern Baltic origin during the last 20 years have shifted 450 km eastwards, with most local birds wintering along the coasts of the Baltic Sea close to their breeding grounds (Švažas et al., 2001a, 2001b). However, recently recoveries of mute swans breeding in Lithuania have been also obtained from the Black Sea region and Hungary.

Mute swans are very tolerant towards human presence and are common in urban habitats. Therefore it is necessary to continue their surveillance for the avian influenza virus in Lithuania.

In the future active surveillance for avian influenza virus in Lithuania (sampling of apparently healthy wild birds) should give a clear priority to mallards and (to a lesser degree) to other species of wildfowl at the highest risk, like common pochards, tufted ducks and mute swans. The surveillance for avian influenza should be focused particularly in Lithuanian coastal wetlands, holding internationally important concentrations of migratory wildfowl (Švažas et al., 1998, 1999). Sampling should not be limited to cloacal swabs, but following EU recommendations (EC Directive 2005/94/EC, 2006) should include pharyngeal swabs. Passive surveillance (sampling of hunted birds or birds found dead) should pay special attention to mallards (annually hunted ducks in different regions of Lithuania), as well as to common pochards and tufted ducks. Such surveillance scheme should provide early warning about potential presence of the HPAIV H5N1 virus in the country. The main value of surveillance in wild birds for the poultry industry is the early detection of infection with H5 and H7 avian influenza strains, which could lead to outbreaks of highly pathogenic avian influenza in poultry after mutation in the poultry host (Lukauskas et al., 2006).

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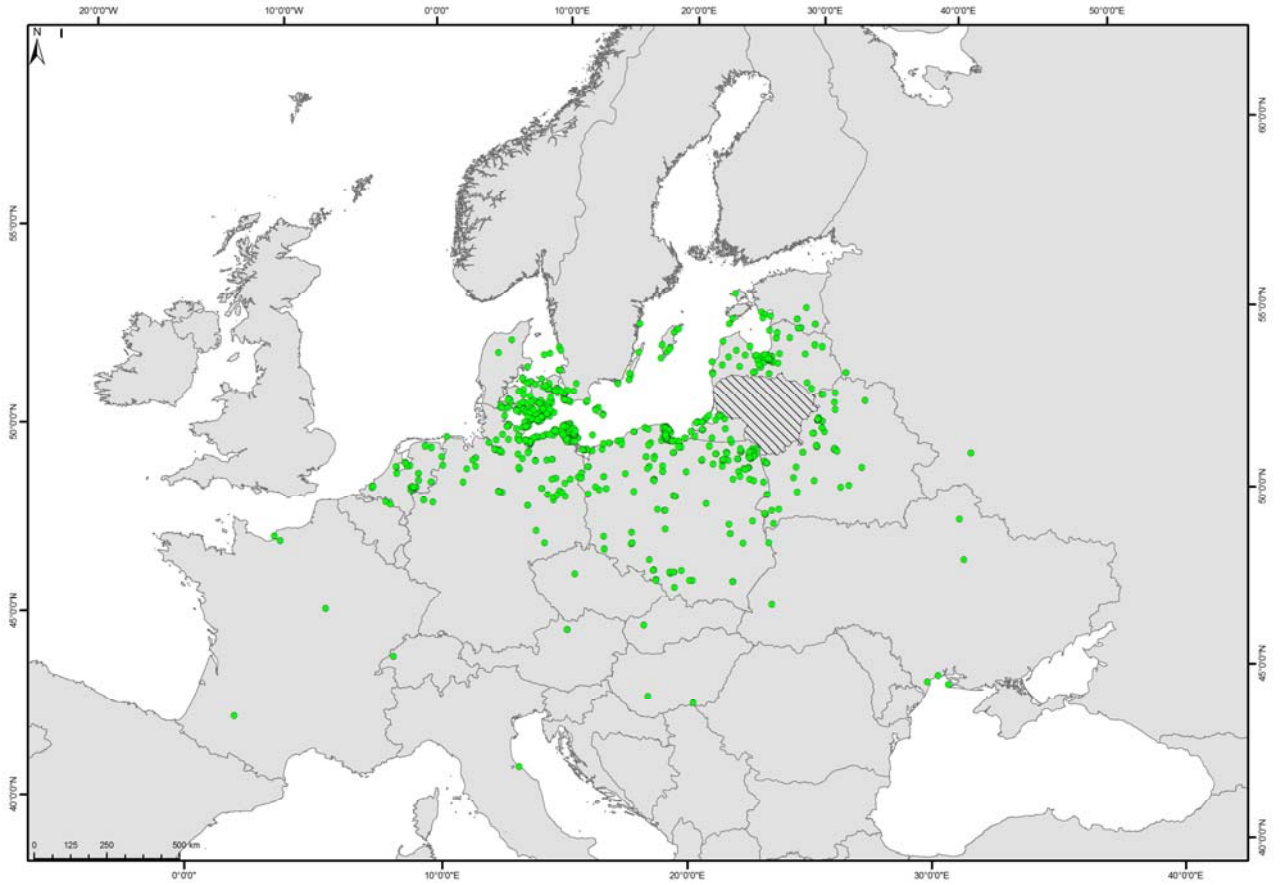

Fig 2. **Recoveries of mute swans** (*Cygnus olor*) **ringed in Lithuania in 1930–2011**

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