Ekologija virusa influence u riječnim galebovima (Chroicocephalus ridibundus) u Hrvatskoj

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Doctoral thesis / Disertacija

2014

Degree Grantor / Ustanova koja je dodijelila akademski / stručni stupanj: **University of Zagreb, Faculty of Science / Sveučilište u Zagrebu, Prirodoslovno-matematički fakultet**

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:217:745243

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Download date / Datum preuzimanja: **2024-03-28**

Repository / Repozitorij:

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Sveučilište u Zagrebu

PRIRODOSLOVNO-MATEMATIČKI FAKULTET
BIOLOŠKI ODSJEK

Luka Jurinović

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DOKTORSKI RAD

Zagreb, 2014.

Sveučilište u Zagrebu

FACULTY OF SCIENCE
DIVISION OF BIOLOGY

Luka Jurinović

Ecology of influenza viruses in Black-headed Gulls (*Chroicocephalus ridibundus*) in Croatia

DOCTORAL THESIS

Zagreb, 2014

Ovaj je doktorski rad izrađen u Centru za peradarstvo, Hrvatskog veterinarskog instituta u Zagrebu, pod vodstvom dr. sc. Vladimira Savića u sklopu Sveučilišnog poslijediplomskog doktorskog studija Biologije pri Biološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu. Većina istraživanja je financirana iz sredstava projekta Ministarstva znanosti, obrazovanja i sporta Republike Hrvatske pod brojem 048-0481153-1136 „Genska karakterizacija virusa influence ptica i newcastleske bolesti u Hrvatskoj“, a dio je financiran od strane ZGOS-a.

Zahvale

Zahvaljujem Vladi na ukazanoj prilici, na vođenju i svemu naučenom.

Zahvaljujem Maji, Mirti i Željki na svesrdnoj pomoći pri laboratorijskom dijelu posla.

Zahvaljujem Duji, Kreši, Vedranu, Ivanu, Zecu, Basreku, Maši, Višnji, Borisu, Toniju, Svenu, Jeleni, Maji, Ivi, Birušu, Loreni, Tei, Milošu i ostalima bez čije pomoći terenski dio ne bi bio ni izvediv ni zabavan.

Zahvaljujem Zrinki za ljubav prema pticama, zahvaljujem Jeleni za znanost o pticama.

Zahvaljujem Toniju na tisućama odgovora.

Zahvaljujem Mladenu i Macieju na ustupljenim fotografijama.

Zahvaljujem svojoj obitelji na strpljivosti i potpori kroz cijeli moj (poduži) obrazovni period.

Zahvaljujem Maši za ljubav i Grgi za sreću.

Ovaj rad posvećujem bakama.

TEMELJNA DOKUMENTACIJSKA KARTICA

Sveučilište u Zagrebu
Prirodoslovno-matematički fakultet
Biološki odsjek

Doktorski rad

Ekologija virusa influence u riječnim galebovima (*Chroicocephalus ridibundus*) u Hrvatskoj

Luka Jurinović

Centar za peradarstvo, Hrvatski veterinarski institut, Heinzelova 55, 10000 Zagreb, Hrvatska

Ciljevi ove disertacije bili su utvrditi ulogu riječnih galebova u širenju influence ptica te odrediti o čemu ovisi pojavnost i brojnost riječnih galebova na zagrebačkom području. Riječni galebovi su gnjezdarice u Hrvatskoj, međutim najveću brojnost postižu tijekom zimskih mjeseci. Dolazak jata od preko tisuću riječnih galebova na zagrebačko područje značajno je koreliran s NAO indeksom, dok odlazak na gnijezdilišta nije koreliran ni s jednim od ispitanih klimatskih faktora. Tijekom istraživanja utvrđena je ukupna prevalencija od 0,2 % influence među riječnim galebovima na zagrebačkom području, a većinu izolata činili su virusi podtipa H13 i H16. Ipak početkom 2006. godine izdvojen je visokopatogeni virus influence podtipa H5N1 iz naizgled zdravih riječnih galebova. Ovo je prvo dokumentirano izdvajanje ovog podtipa virusa iz naizgled zdravih riječnih galebova te ukazuje da riječni galebovi mogu bez ikakvih kliničkih znakova rasprostranjivati virus na velike udaljenosti.

(61 stranica, 2 slike, 1 tablica, 61 literaturnih navoda, jezik izvornika hrvatski)

Rad je pohranjen u središnjoj biološkoj knjižnici Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu, Marulićev trg 20/II, 10000 Zagreb

Ključne riječi: divlje ptice, VPIP, H5N1, NPIP, NAO indeks.

Mentor: Dr.sc. Vladimir Savić, znanstveni savjetnik

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Prof. dr. sc. Maja Popović
Dr. sc. Jelena Kralj, znanstveni suradnik

Rad prihvaćen:

BASIC DOCUMENTATION CARD

University of Zagreb
Faculty of Science
Division of Biology

Doctoral thesis

Ecology of influenza viruses in Black-headed Gulls (*Chroicocephalus ridibundus*) in Croatia

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The aims of this dissertation were to determine the role of black-headed gulls in spreading the avian influenza virus and to determine the climatic factors affecting dynamics of black-headed gulls in Zagreb area. Black-headed gulls breed in Croatia, but they are much more numerous during winter months. The arrival of first flocks bigger than 1000 birds is correlated with the NAO index while departure to the breeding grounds is not correlated to any of the climatic factors tested. During the study, the overall prevalence of avian influenza in black-headed gulls in Zagreb area was 0.2 % and the most common viruses isolated were of the H13 and H16 subtypes. Still, in the beginning of 2006 the highly pathogenic H5N1 virus was isolated from apparently healthy black-headed gulls on Pantana marsh. It is the very first documented isolation of this virus from apparently healthy black-headed gulls and it indicates that black-headed gulls can carry the virus to great distances without showing any clinical signs.

(61 pages, 2 figures, 1 table, 61 references, original in Croatian)

Thesis deposited in the Central Biological Library, Division of Biology, Faculty of Science, University of Zagreb, Marulićev trg 20/II, 10000 Zagreb

Keywords: wild birds, HPAI, H5N1, LPAI, NAO index

Supervisor: Dr. Vladimir Savić, DVM

Reviewers: Dr. Dijana Škorić, Associate Prof.
Dr. Maja Popović, Prof.
Dr. Jelena Kralj, Research associate

Thesis accepted:

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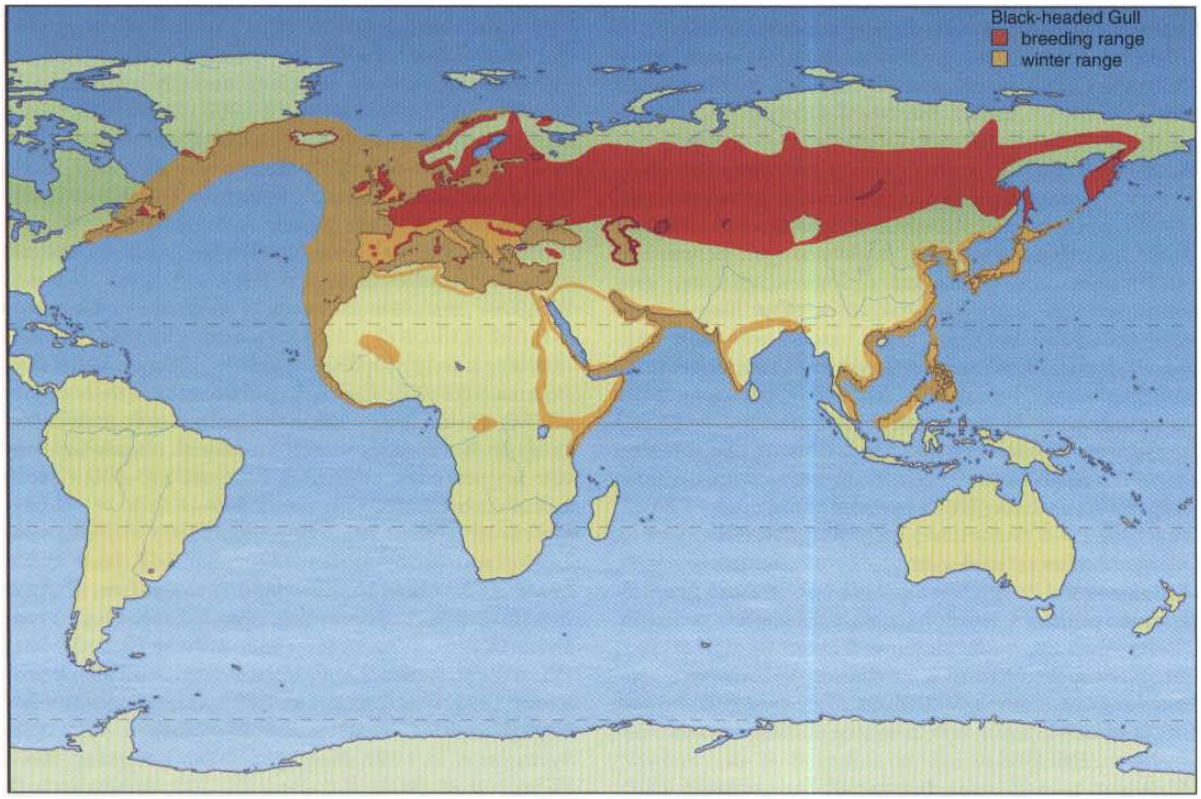
1. UVOD

1.1. Riječni galeb

Riječni galeb, *Chroicocephalus ridibundus,* (slika 1.) je jedna od najčešćih vrsta galebova u Europi i Aziji. To je mali galeb (dužine 34 - 39 cm, promjera krila 100 - 110 cm) kojeg, u gnijezdećoj sezoni, karakterizira čokoladno smeđa glava. Mužjaci su neznatno veći od ženki. Nastanjuje cijeli zapadni palearktik i istočnu Aziju (slika 2.). Gnijezdi na srednjim geografskim širinama na čitavom nizu različitih staništa, od mediteranske zone, oceanskih obala i otoka, preko unutrašnjosti kontinenta pa sve do borealne zone pa čak i subarktika. Iako nastanjuje raznolika staništa nikad ne gnijezdi na područjima snijega i leda te u pustinjama. Uvijek gnijezdi u blizini plitke, slatke, bočate ili slane vode. Najčešće se gnijezdi u gusto naseljenim kolonijma koje mogu biti velike i do nekoliko tisuća parova. Osim tijekom sezone gniježđenja, druževni su tijekom cijele godine i za migracija i za zimovanja.

Slika 1. Riječni galeb prstenovan na odlagalištu Jakuševec, a fotografiran na jezeru Blato (Foto: Mladen Ban).

Izvan sezone gniježđenja riječni galeb je pretežno migratoran ili disperzan. Područje na kojem zimuje u Europi se proteže od juga Islanda i Farskih otoka preko južne Norveške, zapadnog Baltika i Balkana te južno od juga Rusije. U velikom broju zimuje na Iberijskom poluotoku te oko Sredozemlja. Određene populacije slijede obalu Atlantskog oceana do sjeverozapada Afrike pa čak i do obala Nigerije.



Slika 2. Karta rasprostranjensti riječnog gleba (crveno-područje gniježđenja, žuto-područje zimovanja, preuzeto iz Olsen i Larsson, 2004.).

S obzirom da se već godinama prstenuju, postoji veliki broj nalaza koji ukazuju na neke pravilnosti u kretanjima pojedinih populacija ovog galeba. Ptice iz Ujedinjenog Kraljevstva se pretežno disperziraju u blizini gnjezdilišta te samo povremeno zimuju izvan otoka. Prema nalazima, ptice s Farskog otočja i Islanda zimi prate obalu Atlantika, makar postoji nekoliko nalaza na Grenlandu, Newfoundlandu i Novoj Scotiji. Iako neke ptice koje pripadaju fenoskandijskim i baltičkim populacijama ostaju zimi na jugu Skandinavije i zapadu Baltičkog mora, mnoge se kreću prema zapadu, do obala Atlantika te britanskog otočja, dok

neke prelaze cijeli europski kontinent i zimuju na Balkanu i oko Sredozemlja. Riječni galebovi koji se gnijezde u Nizozemskoj i Belgiji pretežno zimuju na britanskom otočju i atlantskoj obali. Gnjezdarice Švicarske i zapadne Njemačke su parcijalno migratorne i neke ptice dosegnu i zapad Sredozemlja i sjeverozapad Afrike te se u manjem broju kreću prema Atlantiku. Ptice iz sjeverne Njemačke, Češke, Slovačke zimi odlaze prema jugu i jugo-zapadu u smjeru Jadrana i zapadnog Sredozemlja. Nalazi ptica iz Rusije upućuju na to da zimuju u cijeloj Europi s tim da većina zimskih nalaza potječe s Balkana i iz Italije (Cramp i Simmons, 1983.; Olsen i Larrson, 2004.).

U Hrvatskoj se gnijezdi na ribnjacima i močvarnim staništima uz Savu i Dravu (Kralj, 1997.). Izvan sezone gniježđenja skupljaju se u velika jata. Takva jata se mogu vidjeti u priobalju Hrvatske od listopada do travnja (Kralj, 1997.). U unutrašnjosti se zimi drži gradova te se najčešće hrani na odlagalištima otpada. Na zagrebačkom odlagalištu otpada Jakuševec su prisutni od listopada do travnja, a maksimalnu brojnost postižu između siječnja i ožujka. Najveći broj zabilježen na Jakuševcu je 13500 ptica i to u siječnju 2003. godine (Jurinović, 2006.). Nalazi ptica prstenovanih na Jakuševcu i ptica prstenovanih drugdje, a nađenih na Jakuševcu tijekom zimskih mjeseci upućuju da se ondje hrane ptice iz cijele Europe od Engleske do Rusije te od Finske do Tunisa (neobjavljeni podaci, Jurinović). Nalazi riječnih galebova prstenovanih tijekom zime na odlagalištu Jakuševec, a nađenih tijekom sezone gniježđenja te nalazi ptica prstenovanih u gnijezdu, a nađenih tijekom zime na Jakuševcu upućuju na to da se većina ptica koja se zimi hrani na Jakuševcu gnijezdi u istočnoj Europi. Većina nalaza potječe iz Poljske, Mađarske i Litve te nešto iz Slovačke, Njemačke, Italije, Latvije, Finske i Rusije (slika 1., znanstveni rad 2).

1.2. Influenca ptica

Virusi influence ptica pripadaju rodu *Influenzavirus A* i porodici *Orthomyxoviridae*. Razvrstani su u podtipove temeljem posjedovanja jednog od 16 različitih hemaglutininskih antigena (H1 do H16) i jednog od devet neuraminidaznih antigena (N1 do N9). Iz ptica su izdvojeni virusi naizgled svih mogućih kombinacija, a genski rezervoar za kruženje virusa influence ptica (IP) u prirodi su uglavnom vodene ptice (Capua i Alexander, 2004.; Fouchier i sur., 2005.). Prvenstveno su to divlje ptice koje pripadaju redovima guščarica *Anseriformes* (patke, guske i labudovi) i šljukarica *Charadriformes* (galebovi, čigre i ćurlini), (Webster i sur., 1992.). Sama virusna čestica je sferičnog oblika veličine 80-120 nm te ima segmentiranu

Tablica 1. Popis segmenata RNA virusa influence te uloga proteina koje kodiraju.

Segment	Veličina	Kodirani protein	Opis uloge proteina
1	2341	PB2	RNA-polimeraza, "cap snatching" – uzima 5'-metilgvanozinsku „kapu“ od mRNA domaćina koja se koristi za početnicu pri sintezi pozitivne jednolančaneRNA.
2	2341	PB1	RNA-polimeraza
		PB1-F2	inducira apoptozu, nije prisutan u svim virionima
3	2233	PA	RNA-polimeraza
4	1778	HA	hemaglutinin, vezuje se za stanične receptore
5	1565	NP	protein koji se vezuje uz segmente RNA-genoma
6	1413	NA	neuraminidaza, enzim koji cijepa sijalinsku kiselinu, potreban za izlazak sintetiziranih viriona iz stanice domaćina
7	1027	M1	protein matriksa
		M2	strukturni protein i ionski kanal
8	890	NS1	nestrukturni protein, uloga u sprečavanju imunosnog odgovora stanice domaćina i u sintezi virusne RNA
		NEP (NS2)	sudjeluju u prijenosu rinbonukleoproteina iz jezgre u citosol

negativno orjentiranu jednolančanu ribonukleinsku kiselinu i virusnu ovojnicu. Morfološki se na samoj površini ističe oko 500 izdanaka. To su antigenski proteini hemaglutinina i

neuraminidaze. Sama RNA se sastoji od 8 segmenata koji kodiraju 11 proteina, a uloga pojedinih gena dana je u tablici 1. (prema Bouvier i Palese, 2008.).

Prvi opis bolesti koja uzrokuje veliki pomor peradi opisao je Perroncito 1878. godine i nazvao je kuga peradi. Za uzročnika ove bolesti je tek 1901. dokazano da je ultrafiltrabilna čestica, „virus“, a tek je 1955. godine potpuno odvojen od ostalih virusa (npr. virusa newcastleske bolesti) koji uzrokuju slične ili blaže simptome kod peradi (prema Lupiani i Reddy, 2009.). Prvi slučaj izdvajanja virusa influence iz divlje ptice je visokopatogeni virus podtipa H5N3 koji je 1961. godine izazvao veliki pomor crvenokljunih čigri, *Sterna hirundo*, u južnoj Africi (Becker, 1966.). Sustavna istraživanja influence u divljim pticama su počela tek sredinom 1970-ih godina i otkrila veliko značenje divljih ptica kao rezervoara različitih virusa. Do sada su niskopatogeni virusi influence izdvojeni iz barem 105 vrsta divljih ptica iz 26 porodica (Olsen i sur., 2006.).

Virusi influence A su, s obzirom na bolest koju uzrokuju u domaće peradi, podijeljeni na viruse niske i visoke patogenosti. Virusi visoke patogenosti uzrokuju visokopatogenu influencu ptica (VPIP) koja može rezultirati uginućem i do 100 % jedinki. Ovakvi virusi su do sada ograničeni isključivo na podtipove H5 i H7, iako VPIP ne uzrokuju svi virusi iz ove dvije skupine. Treba naglasiti da niskopatogeni virusi podtipova H5 i H7 u određenim okolnostima mogu mutirati u visokopatogene viruse te su stoga i niskopatogeni virusi ova dva podtipa također vrlo značajni za domaću perad (Capua i Alexander, 2004.). Hemaglutinin se sastoji od dvije podjedinice: HA1 i HA2. Do infektivnosti virusa dolazi tek nakon cijepanja hemaglutinina u te dvije podjedinice pomoću enzima domaćina. Patogenost virusa IP za perad ovisi ponajprije o višestrukoj zastupljenosti bazičnih aminokiselina na mjestu cijepanja proteina hemaglutinina u procesu infekcije stanice domaćina. Ukoliko na mjestu cijepanja nisu višestruko zastupljene bazične aminokiseline poput arginina ili lizina, hemaglutinin se može cijepati jedino pomoću enzima tripsina i njemu sličnih enzima kojeg nalazimo samo u određenim tkivima, poput dišnog i probavnog sustava kokoši i pura. Ukoliko pak jesu, za cijepanje je dovoljan i enzim poput furina koji je zastupljen u većini stanica. Na taj način virus se može umnožavati u većini stanici i tako uzrokovati fatalnu bolest (Stienke-Grober i sur., 1992.; Rott, 1992.).

Kod divljih ptica virusi influence se najčešće rasprostranjuju orofekalnim putem te stoga ptice koje su životom vezane uz vodene površine, naročito one koje se okupljaju u velika jata, tijekom seobe ili zimovanja, imaju veću prevalenciju virusa. Način prehrane

također uvelike doprinosi širenju influence pa tako patke plivarice, *Anatidae*, koje se hrane filtriranjem površine vode, npr. divlja patka, *Anas plathyrhynchos*, imaju veću prevalenciju od ostalih pataka i gusaka. Kako se virus influence rasprostranjuje fecesom vrlo su bitni vanjski čimbenici koji utječu na preživljavanje virusa izvan tijela domaćina. Među njima se ističe temperatura, a virusi influence lakše preživljavaju na nižim temperaturama te su stoga i na višim geografskim širinama veće prevalencije ovih virusa (Munster i sur., 2007.; Stallknecht i Brown, 2008.; Munster i Fouchier, 2009.).

1.3. Dijagnostika virusa influence

Najprikladniji uzorci za dijagnostiku influencu su sadržaj crijeva i organi mrtvih ptica, primarno dišni sustav, slezena, bubrezi, jetra mozak i srce. Od živih ptica najbolje je uzeti obrisak ždrijela i obrisak kloake. Nakon uzimanja, uzorke treba staviti u izotoničnu otopinu antibiotika. Sastav ove otopine može varirati u zavisnosti o lokalnim uvijetima, ali bi trebao sadržati penicilin (2000 IU/ ml), streptomicin (2 mg/ml), gentamicin (50 µg/ml) i mikostatin (1000 IU/ml) za tkiva i obriske ždrijela. Za feces i obriske kloake ova koncentracija se može i upeterostručiti. Najčešći način umnažanja virusa influence je inokulacija embrioniranih kokošjih embrija. Supernatant suspenzije organa ili obrisaka se, nakon centrifugiranja, inokulira u alantoisnu šupljinu 9 do 11 dana starih embrija. Embrionirana jaja se inkubiraju na 37°C 2-7 dana. Nakon tog, ili prije ukoliko embriji uginu, alantoisna tekućina se pomoću 10% suspenzije eritrocita pijetla testira na hemaglutinacijsku aktivnost. U slučaju pozitivnog ishoda, virusni izolat se provjerava testovima specifičnim za podtipove i to inhibicijom hemaglutinacije (HI) te inhibicijom neuraminidaze (NI). Pomoću ovih testova se potvrđuje da je izolat upravo virus influence te se određuje kojem podtipu izolat pripada (Anonymous, 2009.).

Već se neko vrijeme uz klasične metode uvelike primjenjuju i molekularne metode u dijagnostici influence. Kao polazišni materijal za izolaciju ribonukleinske kiseline (RNA) može se koristiti supernatat suspenzije organa ili obrisaka, ali i virusni izolat. Nakon izolacije ukupne RNA, uzorak se može testirati metodom lančane reakcije polimerazom u stvarnom vremenu uz prethodnu reverznu transkripciju (RT-qPCR) specifičnom za gen M virusa influence ptica (Spackman i sur., 2002.) kako bi se potvrdila nazočnost ovog virusa. Pozitivne uzorke se može testirati RT-qPCR specifičnom za gen H5 (Slomka i sur.,2007.) i gen H7 (Slomka i sur., 2009.) kako bi se potvrdila ili isključila moguća nazočnost ovih dvaju podtipova influence. Ukoliko se dokaže nazočnost virusa influence podtipa H5 i H7 svakako treba odrediti njihovu patogenost, a to je najlakše učiniti određivanjem slijeda aminokiselina na mjestu cijepanja hemaglutinina. Ukoliko se prilikom dijagnostike koristi virusni izolat, količina RNA u njemu je puno veća te su signali prilikom pretraga puno jači, a sekvence jasnije.

1.4. Dosadašnja istraživanja influence na galebovima

U povijesti postoji jako malo istraživanja influence ptica koja su bila rađena isključivo na galebovima. Kod većih i opsežnijih istraživanja su galebovi činili jedan, obično manji dio uzorka. Nakon zime 2005/06. godine i pojavljivanja visokopatogenog virusa influence podtipa H5N1 u većini Europe mnoge su zemlje provodile, osim pasivnog, i aktivni monitoring. Na taj način je obrađeno puno više uzoraka divljih ptica što je dalo i puno bolji uvid u ulogu divljih ptica, pa tako i galebova, u širenju influence ptica. U Italiji je tako u periodu od 2005. do 2007. godine pretraženo ukupno 1435 uzoraka fecesa i brisova kloake divljih ptica, od čega su galebovi činili 4,6 % (66 uzoraka). Svi uzorci iz galebova dali su negativan rezultat (Perez-Ramırez i sur., 2010.). U Češkoj su u prvoj polovici 2006. godine pretražili lešine 2101 ptice od kojih su galebovi činili samo 2,95 % odnosno 61 uzorak koji su svi bili negativni (Nagy i sur., 2007.). U periodu od 2004. do 2006. godine u sjevernoj Italiji su pretražili 4083 od čega su galebovi činili 3,3 % (135 uzoraka). Svi osim jednog uzorka crnoglavog galeba su bili negativni (Terregino i sur., 2007.). Tijekom 2005. i 2006. godine u Bosni i Hercegovini je pretraženo ukupno 394 lešina divljih ptica. Od tog broja galebovi su činili 0,5 % (2 lešine) i oba su bila negativna (Goletić i sur., 2010.). U Sloveniji su tijekom perioda od 2006. do 2010. godine pretražili 2547 ptica, od čega je šljukaricama pripadalo svega 81 uzorak odnosno 3,2 %. Od toga su tri galeba klaukavca bila pozitivna na virus influence i to jedan virus podtipa H13N6 i dva netipizirana virusa influence (Slavec i sur., 2012.). Prema udjelu galebova u ispitanom uzorku ističu se istraživanja iz Norveške tijekom 2006. i 2007. godine (Germundsson i sur., 2010.) i Gruzije u periodu od 2009. do 2011. godine (Lewis i sur., 2013.). Od ukupno 2742 pretražene ptice u Norveškoj galebovi su činili 44,2 % odnosno 1213 ptica. Virus influence nađen je u 74 galeba, a najčešći podtipovi su H13 i H16 , dok su povremeno izdvajani i podtipovi H1, H4, H5 i H6. U Gruziji je od ukupno 5220 pretraženih ptica bilo je 2545 galebova ili 48,8 %. Od ukupnog broja galebova 53 su ptice bile pozitivne na virus influence, a izdvojeni su virusi podtipova H9, H11 i H13. Većina virusa nađena je u riječnih galebova i to njih 36. Osim europskih i mnoge ostale zemlje su provodile monitoring nakon zime 2005./06. I tamo su galebovi u manjini ukupnog uzorka. U Iranu je u razdoblju od 2003. do 2007. godine od ukupno 1146 ptica pregledano svega 25 galebova (2,2 %) i svi su uzorci bili negativni (Fereidouni i sur., 2010.). U sjevernoj Americi je u periodu od 2006. do 2008. godine pretraženo ukupno 145055 ptica od čega je galebova, čigri i njorki ukupno bilo 4 %, ni jedan virus influence nije nađen u galebovima (Pedersen i

sur., 2010.). Tijekom 2006. i 2007. godine u Argentini je pretraženo 2895 ptica od čega 8,6 % galebova (250 ptica). Jedan od galebova bio je pozitivan i iz njega je izdvojen virus podtipa H13N9 (Pereda i sur., 2008.).

Izolati influence iz galebova najčešće pripadaju podtipovima H13 i H16. Ova dva podtipa čine zasebnu skupinu izolata koji su gotovo isključivo nađeni samo u galebovima (Munster i sur., 2007.). Podtip H16 je i opisan upravo na riječnom galebu 2005. godine (Fouchier i sur., 2005.) dok je H13 opisan na američkim galebovima (Hinshaw i sur., 1982.).

Verhagen i suradnici su u periodu od 2006. do 2010. godine proučavali influencu na riječnim galebovima u Nizozemskoj. I u tom istraživanju su kao najčešći izolati se pojavljivali podtipovi H13 i H16. Epizootija influence među riječnim galebovima u Nizozemskoj traje svake godine od lipnja do srpnja i to isključivo među pticama u prvoj kalendarskoj godini (poletarcima i svježe opernaćenim pticama). Zanimljivo da ni jedan izolat H13 niti H16 nije nađen u pticama izvan sezone gniježđenja te autorima ostaje nepoznanica gdje virusi perzistiraju (Verhagen i sur., 2014.). Lvov i suradnici su u periodu od 1975. do 1999. godine izolirali ukupno 264 izolata influence iz galebova koji su gnijezdili na području delte rijeke Volge. Većina izolata (190) potječe od mladunaca velikog crnoglavog galeba, *Ichtyaetus ichtyaetus*, i to su gotovo isključivo virusi koji pripadaju podtipu H13 (Lvov i sur., 2001.). U Rusiji su 1978. izdvojeni virusi influence iz potrkušaca riječnih galebova te 1979. iz embrija u istoj koloniji (Roslaia i sur., 1984.). Tijekom istraživanjau Gruziji, Lewis i suradnici nisu uspjeli detektirati virus influence ni kod mladih niti kod odraslih ptica u koloniji armenskih galebova, *Larus armanicus*, dok su ih redovito izdvajalii kod više vrsta galebova (uključujući i riječne galebove) izvan sezone gniježđenja (Lewis i sur., 2013.).

1.5. Ciljevi istraživanja

Dva su osnovna cilja ovog istraživanja:

1) odrediti o čemu ovisi pojavnost i brojnost riječnih galebova na zagrebačkom području,
2) otkriti utjecaj riječnih galebova na rasprostranjivanje virusa influence.

Naročito je bitno otkriti kolika je prevalencija virusa podtipova H5 i H7 koji mogu potencijalno biti visokopatogeni i kao takvi opasni za perad. Virus VPIP podtipa H5N1 je, osim za perad, posebno opasan i za ljude. Također planiram odrediti prevalenciju i protutijela na virus influence kao pokazatelj dodira galebova s virusima influence.

2. ZNANSTVENI RADOVI

Rad 1

Savić V, Labrović A, Amšel Zelenika T, Balenović M, Šeparović S, **Jurinović L** (2010) Multiple introduction of Asian H5N1 avian influenza virus in Croatia by wild birds during 2005-2006 and isolation of the virus from apparently healthy black headed gulls (*Larus ridibundus*). Vector-borne and zoonotic diseases 10(9): 915-920.

Rad 2

Jurinović L, Kralj J (2013) Effect of weather conditions on dynamics of the black-headed Gull, Chroicocephalus ridibundus, wintering on Jakuševec, Zagreb city rubbish dump. Pakistan J. Zool. 45(6): 1773-1776.

Rad 3

Jurinović L, Savić V, Balenović M, Lisičić D, Lucić V (2014) Virological and serological investigation of avian influenza in black-headed gulls captured on a rubbish dump in Croatia. Vet arhiv *in press*.

Znanstveni rad 1

Multiple introduction of Asian H5N1 avian influenza virus in Croatia by wild birds during 2005-2006 and isolation of the virus from apparently healthy black-headed gulls (*Larus ridibundus*)

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Key Words: Birds, Epidemiology, Influenza

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Abstract

This study describes the introduction and spread of avian influenza A (H5N1) subtype in Croatia. Seventeen isolates were identified during the period from October 2005 to March 2006, all originating from wild birds. The full-length nucleotide sequence analysis of the hemagglutinin (HA) gene of seven representative isolates revealed that three distinct genetic strains involved in the outbreaks, implicating at least three independent introductions of the virus into Croatia during a relatively short period of time. All three genetic strains belonged to clade 2.2 (Qinghai-like viruses) and each strain displayed significant similarity to concurrent H5N1 viruses from other European countries. The dominant strain of the virus was present in all four affected areas and in all three bird species (mute swan, mallard, and black-headed gull), indicating cross-species transmission of the virus. Two other genetic strains were found, together with the dominant strain, only in a marsh at the Adriatic coast during late February and early March 2006, which could be associated with frozen water surfaces in the continental part of Croatia as well as in Eastern Europe in early 2006 and the movement of birds toward warmer areas. This is also the first isolation of highly pathogenic avian influenza virus of H5N1 subtype from apparently healthy black-headed gulls.

Introduction

Highly pathogenic avian influenza (HPAI) causes flock mortality in susceptible poultry species as high as 100% (Alexander and Capua 2008). Some HPAI viruses (HPAIV), particularly HPAIV of subtype H5N1 of Asian origin, also infect humans (Claas et al. 1998, Subbarao et al. 1998) and the infection is often fatal. The virus possibly has pandemic potential (Hien et al. 2004).

Table 1. Primers used for amplification of the full-length hemagglutinin gene of H5N1 Qinghai-like viruses

Primer	Sequence (5'–3') or reference nucleotid	Nucleotide position[a]	Size (bp)
Bm-HA-1	Hoffmann et al. (2001)	(14+)[b] 1-14	657
Q-H5R-643	CCTTGTCTGCTCTGCCGCATCATTTG	618-643	
Q-H5F-587	GATCTTTTGGTACTGTGGGGGATTC	587-611	619
Q-H5R-1205	CATCTATTGCCTTTTGAGTGGATTC	1181-1205	
Q-H5F-1142	CATAGCAACGAGCAGGGGAGTGG	1142-1164	653
Bm-NS-890R	Hoffmann et al. (2001)	1760-1779 (+15)[b]	

[a]Based on GenBank accession number EF205157 (A=goose=Krasnoozerskoe=627=05 [H5N1]).
[b]Number of noncomplementary nucleotides preceding or exceeding the hemagglutinin gene complementary sequences.

Asian HPAIV of H5N1 subtype apparently emerged in domestic poultry in China already in 1996 (Xu et al. 1999) and circulated locally with sporadic spread to neighboring Hong Kong (Sims et al. 2005). The situation changed dramatically between 12 December, 2003 and 27 January, 2004 when eight countries in South-East Asia first reported the presence of the virus in poultry (Capua and Alexander 2004). The widespread presence of HPAI H5N1 viruses in poultry in the Far East inevitably resulted in spill-over into wild bird populations in May 2005 (Alexander and Capua 2008). Probably both wild bird migrations and the movement of poultry were responsible for further spread of the virus westward (Alexander and Capua 2008), and there is strong evidence that wild birds could spread the virus long distances (Kilpatrick et al. 2006, Normile 2006).

Early isolates from wild mute swans (*Cygnus olor*) were obtained in Croatia already in October 2005 (OIE, 2009). These infected swans were a forerunner of the apparent importance of these birds in the spread of the HPAIV H5N1, and from January to April 2006, wild mute swans and other wild birds were shown to be infected in numerous countries in Europe, Caucasus and Middle East (Alexander and Capua 2008). Genome analysis of HPAI H5N1 viruses from wild and domestic birds and humans in Europe and Africa show their relationship with the isolates from wild birds at the Qinghai Lake in China. This Euro-African lineage, which was the cause of several fatal human infections in Middle East and Africa, has been introduced at least 3 times into the European-African region and has split into 3 distinct, independently evolving sublineages (Salzberg et al. 2007).

Apart from the initial introduction and finding of the HPAI H5N1 virus in wild swans in October 2005, the virus has been found in several wild bird species in four major areas in Croatia until late March 2006. In this study we described details of the HPAI H5N1 virus infection in wild birds in Croatia from October 2005 until March 2006 as well as genetically analyzed and compared representative virus isolates in order to better understand the spread and epidemiology of this infection. This information may be useful for prevention and control of HPAIV H5N1 infection elsewhere.

Materials and methods

Samples

A total of 6142 wild bird and 844 poultry samples were collected from September 2005 until June 2006. Most of the wild bird samples were examined for AIV within the frame

of passive surveillance across Croatia. Remaining samples from wild birds and poultry were taken within active surveillance in the areas where HPAIV subtype H5N1 had been previously confirmed. Mainly organs (brain, trachea, lungs, liver, spleen, kidneys and intestines) and sporadically cloacal and tracheal swabs were tested. Live birds and poultry were tested exclusively by tracheal and cloacal or in some cases only by cloacal swabbing.

Virus isolation

Virus isolation in chicken embryos and virus isolate subtyping by hemagglutination inhibition and neuraminidaseinhibition tests were carried out according to the standard procedure (OIE 2004). A panel of monospecific antisera covering all 16 H subtypes and 9 N subtypes of avian influenza virus (AIV) (Istituto Zooprofilattico Sperimentale delle Venezie, Padova, Italy) and monospecific sera against avian paramyxoviruses: APMV-1, -2, -3, -4, -6, -7, -8 and -9 (Veterinary Laboratories Agency, Weybridge, UK) were used.

Molecular methods

Viral RNA for selected H5 isolates was extracted from allantoic fluids using High Pure Viral Nucleic Acid Kit (Roche Applied Science, Germany). Two-step reverse transcription and polymerase chain reaction (PCR) were carried out using and GeneAmp® Gold RNA PCR Core Kit (Applied Biosystems, USA). cDNAs were synthesized using random hexamers. Three partially overlapping fragments spanning the whole HA gene were amplified for each isolate by PCR using three primer pairs (Table 1), each in a final concentration of 1 mM, and the thermocycling conditions consisted of initial denaturation at 95°C for 5 min, followed by 40 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 3 min, and a final extension at 72°C for 10 min. All PCR products were electrophoresed in 2% agarose gel with addition of ethidium bromide and visualized on UV transilluminator. Specific bands were excised and purified with Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI). Sequencing of all PCR amplicons was performed in both directions with Big Dye Terminator v3.1 Sequencing Standard Kit (Applied Biosystems) in a 3730XL DNA sequencer (Applied Biosystems) and with same primers used for PCR. After sequencing, the primer sequences were trimmed off. The trimmed sequences for each virus isolate were assembled with ALIGN Plus 2.0 software. Phylogenetic analysis was performed with MEGA software version 4 (Tamura et al. 2007) by using Neighbor Joining algorithm with 1000 bootstrap replicates.

Nucleotide sequences were also translated into amino acid sequences in order to predict pathogenicity of each analyzed isolate according to the HA0 cleavage site.

Results

During the investigation period, 17 AIV subtype H5N1 were isolated from wild birds on 6 sites in four major areas. No AIV was isolated from Croatian poultry. Details about location, date, bird species, bird health status and type of surveillance regarding virus isolation are shown in Table 2. Results of phylogenetic analysis of selected isolates from all four areas and all affected birds species are shown in Figures 1 and 2. All analyzed AIV subtype H5N1 isolates from Croatia during 2005-2006 possessed multiple basic amino acids at the HA0 protein cleavage site (PQGERRRKKR*GLF) indicating high pathogenicity of the avian influenza isolates (Rott 1992).

In addition to AIV subtype H5N1, a low pathogenic AIV subtype H5N3 with HA protein cleavage site motif PQRETR*GLF and AIV subtype H10N7 were isolated from mallards (*Anas platyrhinchos*) whereas one PMV-1 was isolated from a mute swan.

Discussion

HPAIV H5N1 was confirmed in dead mute swans in Croatia on fishponds "Grudnjak" and "Ribnjak 1905" in Slavonia in October 2005, on the island of Čiovo and nearby marsh of Pantana in Dalmatia in February 2006, as well as in Baranja and Zagreb in March 2006. HPAIV H5N1 virus was also confirmed in apparently healthy black headed gulls (*Larus ridibundus*) in the marsh of Pantana at the end of February and early March 2006 as well as in a dead mallard from the same location in early March 2006. As anticipated, all seven analyzed Croatian isolates are H5N1 Qinghai-like viruses, that is, they belong to clade 2.2 (WHO/OIE/FAO H5N1 Evolution Working Group 2008). Most of these isolates belong to subclade 2.2.2. and are closely grouped with the index case isolate in Croatia (A/Cygnus olor/Croatia/1/05) from dead swans found on the fishpond "Grudnjak" (Fig. 1). Other members of this group are isolates from dead swans from Baranja and Zagreb, and the isolate from apparently healthy gulls from the marsh of Pantana. Interestingly, five out of six H5N1 isolates from the reemerged outbreak in wild birds at the Qinghai Lake in China in May 2006 (QH06) were adjacent to A/Cygnus olor/Croatia/1/05 whereas one was related to the strain A/duck/Novosibirsk/02/05 from Russia (Wang et al. 2008). Finding that A/Cygnus olor/Croatia/1/05-like viruses were dominant H5N1 viruses circulating in Croatia during 2006 corroborates implication that the QH06 viruses of the Qinghai Lake may travel back via migratory birds (Wang et al. 2008). Other three analyzed isolates from Croatia belong to 2.2.1 subclade but, unlike Croatian isolates from subclade 2.2.2, which circulated in Croatia

Table 2. Isolates of highly pathogenic avian influenza virus of H5N1 subtype from Croatia during 2005 and 2006.

Location	**Date of sampling**	**Bird species**	**Sample**	**Health status**[4]	**Surveillance**	**Isolate designation**
Fishpond "Grudnjak" - Zdenci *(45°37'N 18°03'E)* **Slavonia**	19.10.2005.	Mute swans (*Cygnus olor*)	Organs of 6 birds[2]	Died with symptoms	Passive	A/Cygnus olor/Croatia/1/2005[5]
	29.10.2005.	Mute swan (*Cygnus olor*)	Organs	Carcass	Active	A/Cygnus olor/Croatia/616-1/2005
		Mute swan (*Cygnus olor*)	Organs	Carcass	Active	A/Cygnus olor/Croatia/616-2/2005
	19.11.2005.	Mute swan (*Cygnus olor*)	Organs and pharingeal and cloacal swabs[2]	Dead with symptoms	Active	A/Cygnus olor/Croatia/1089/2005
Fishpond "Ribnjak 1905" – Našice *(45°32'N 18°08'E)* **Slavonia**	21.10.2005.	Mute swan (*Cygnus olor*)	Cloacal swab[3]	Carcass	Passive	A/Cygnus olor/Croatia/168/2005
	24.10.2005.	Mute swans (*Cygnus olor*)	Cloacal swabs of 4 birds[2]	Carcasses	Active	A/Cygnus olor/Croatia/181/2005
	28.10.2005.	Mute swan (*Cygnus olor*)	Organs	Carcass	Active	A/Cygnus olor/Croatia/613/2005
Slatina - Čiovo *(43°30'N 16°16'E)* **Dalmatia**	15.2.2006.	Mute swan (*Cygnus olor*)	Organs	Carcass	Passive	A/Cygnus olor/Croatia/229/2006[5]
Pantana marsh– Trogir[1] *(43°31'N 16°16'E)* **Dalmatia**	21.2.2006.	Mute swan (*Cygnus olor*)	Organs	Carcass	Active	A/Cygnus olor/Croatia/315/2006[5]
	28.2.-3.3.2006.	Black headed gulls (*Larus ridibundus*)	Cloacal swabs of 5 birds[2]	Healthy	Active	A/Larus ridibundus/Croatia/680-1-5/2006
		Black headed gulls (*Larus ridibundus*)	Cloacal swabs of 5 birds[2]	Healthy	Active	A/Larus ridibundus/Croatia/680-6-10/2006[5]

		Black headed gulls (*Larus ridibundus*)	Cloacal swabs of 5 birds [2]	Healthy	Active	A/Larus ridibundus/Croatia/680-11-15/2006
		Black headed gulls (*Larus ridibundus*)	Cloacal swabs of 5 birds [2]	Healthy	Active	A/Larus ridibundus/Croatia/680-16-20/2006
		Black headed gulls (*Larus ridibundus*)	Cloacal swabs of 5 birds [2]	Healthy	Active	A/Larus ridibundus/Croatia/680-21-25/2006
	3.3.2006.	Mallard (*Anas platyrhynchos*)	Organs	Carcass	Active	A/Anas platyrhynchos /Croatia/676/2006[5]
Draž *(45°50'N 18°47'E)* **Baranja**	3.3.2006.	Mute swan (*Cygnus olor*)	Organs	Carcass	Passive	A/Cygnus olor/Croatia/635/2006[5]
Zagreb *(45°46'N 15°53'E)*	28.3.2006.	Mute swan (*Cygnus olor*)	Organs	Carcass	Passive	A/Cygnus olor/Croatia/1422/2006[5]

[1]The samples from the Pantana marsh were taken within active surveillance after positive finding in mute swan in nearby village of Slatina.

[2]Individual samples were processed as a pool.

[3]Pharingeal swab was also taken, but gave negative result.

[4]*Died with symptoms:* sick bird was noted and tested after it died; *Carcass*: dead bird tested without known health history; *Healthy*: tested bird did not show any disease symptoms at the moment of sampling.

[5]Isolates of highly pathogenic avian influenza virus that were genetically analyzed.

FIG. 1. Phylogenetic tree of the hemagglutinin (HA) gene (full-length sequence) of highly pathogenic avian influenza virus (HPAIV) (H5N1) clade 2.2 (WHO/OIE/FAO H5N1 Evolution Working Group 2008). The tree was constructed by using Neighbor Joining algorithm with 1,000 bootstrap trials. Length of the horizontal lines is proportional to the genetic distance among isolates. Scale bar indicates substitutions per site. Isolates from Croatia are in bold and index case isolate is underlined. GenBank accession numbers for index case isolate and isolates from other countries are given in parentheses. The tree is rooted with A/Goose/Guangdong/1/96 (H5N1) with Genbank accession number AF144305 (the isolate is not shown in the figure).

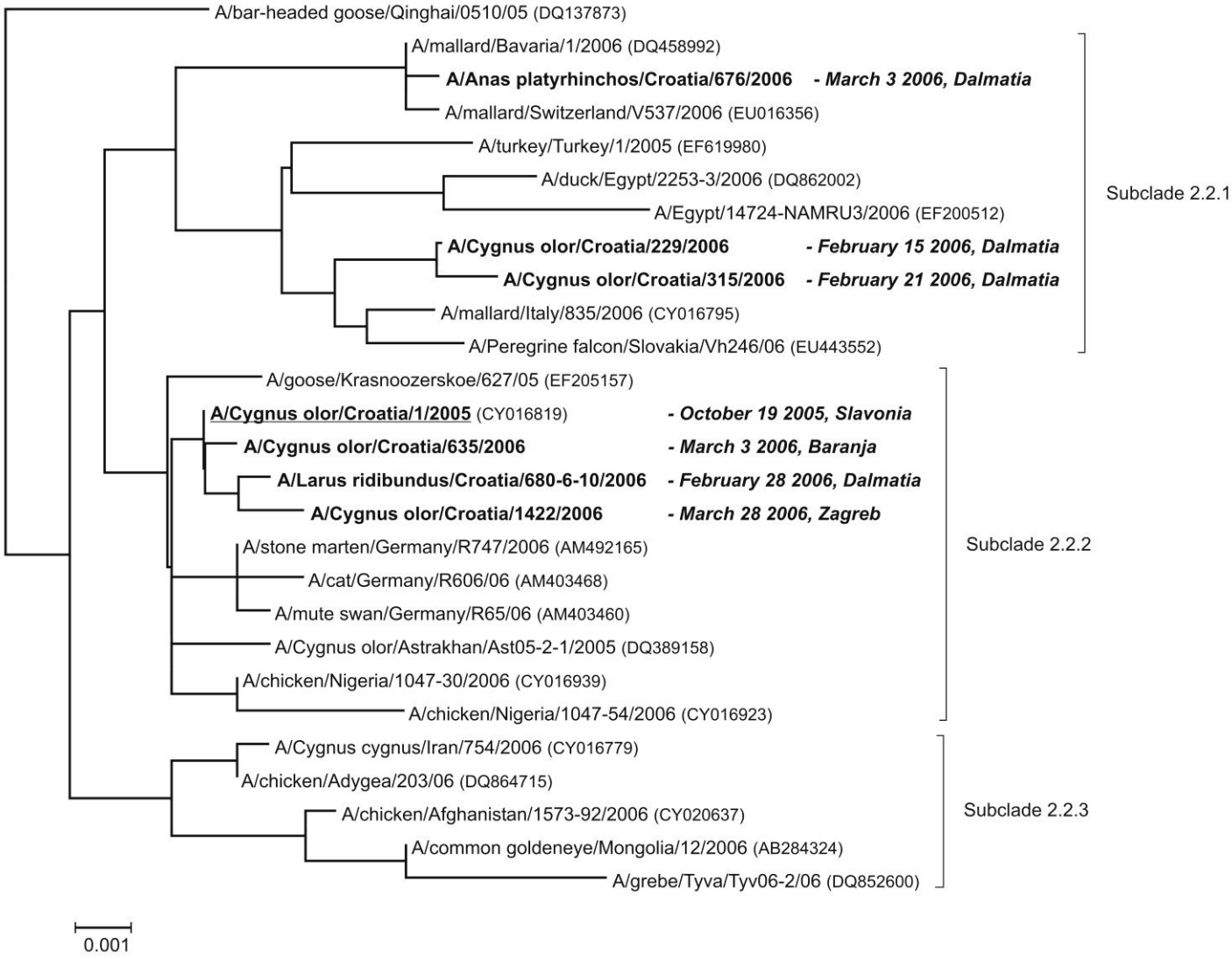

FIG. 2. Map of Croatia with locations and years of outbreaks, affected bird species and genetic subclades of analyzed highly pathogenic avian influenza virus (HPAIV) (H5N1) isolates. Squares, circle and triangle represent mute swans (*Cygnus olor*), mallard (*Anas platyrhynchos*) and black headed gulls (*Larus ridibundus*), respectively. White figures represent 2.2.1 subclade and black figures 2.2.2. subclade (WHO/OIE/FAO H5N1 Evolution Working Group 2008).

apparently for 6 months and remained genetically close, 2.2.1 subclade isolates show greater diversity and indicate there are two distinct genetic strains (Fig. 1). This diversity of concurrent isolates is a result of independent evolution of the viruses that most likely took place before introduction into Croatia. Therefore, phylogenetic analysis of Croatian isolates from October 2005 until March 2006 implicates at least three independent introductions of H5N1 viruses by wild birds. Similarly, multiple introductions of H5N1 Qinghai-like viruses

occurred in Germany (Starick et al. 2008) and in France (Gall-Recule´ et al. 2008) in early 2006, but with poultry infections involved in the outbreaks.

The dominant A/Cygnus olor/Croatia/1/05-type strain of H5N1 virus in Croatia was found in all four affected areas (Slavonia, Baranja, Dalmatia and Zagreb) and was the only detected strain in Slavonia, Baranja and Zagreb. However, all three types were found in three different bird species near the town of Trogir in Dalmatia (Fig. 2). The first finding of H5N1 in Dalmatia was on 15 February 2006 in a swan carcass on the seashore near Slatina village on the island of Čiovo. The carcass was obviously washed by sea stream from the neighboring marsh of Pantana about a kilometer away. A week later another swan carcass was found in the marsh of Pantana and the virus was phylogenetically very similar to the virus isolated the previous week, indicating that both isolates were epidemiologically linked. Nevertheless, both isolates were significantly different from other H5N1 viruses isolated in Croatia. Subsequent active surveillance in the marsh of Pantana resulted in isolation of H5N1 viruses from apparently healthy black-headed gulls and from a dead mallard. The gulls were captured by mist nets, and were released immediately after sampling and ringing (ring numbers available on request). Isolates from gulls were of A/Cygnus olor/Croatia/1/05-type strain whereas the isolate from the mallard differed from all other H5N1 isolates from Croatia and was close to certain isolates from Western Europe. Finding of three different genetic strains of H5N1 virus at the same time in the marsh of Pantana which is located on the Adriatic coast and has moderate winters could be associated with frozen water surfaces in the continental part of Croatia as well as in Eastern Europe in early 2006 and the movement of birds toward warmer areas. Assumption about unusual migration pattern of wild birds from the Black Sea basin in other parts of Europe due to harsh winter conditions 2005-2006 was also mentioned by Kilpatric et al. (2006) and Alexander and Capua (2008).

During 2005 and 2006, three genetic strains of H5N1 virus were found in three different wild bird species, however, no particular relationship between the virus strain and the bird species was found, indicating cross species transmission of the virus. Isolation of H5N1 virus from black headed gulls is not common, especially from apparently healthy wild birds. Most of H5N1 virus detections were from dead or severely ill birds including documented isolations of H5N1 virus from black headed gulls (Ellis et al. 2004). Therefore, this is the first documented isolation of Asian HPAIV of H5N1 subtype from apparently healthy black headed gulls. It is possible that the gulls were captured in the incubation phase while they shed the virus before exhibiting clinical signs of the infection. It is not known whether the gulls would have shown clinical sign or died later since they were released

immediately after sampling and were not found afterwards. If black-headed gulls do not exhibit clinical signs after infection with H5N1 virus and if they shed the virus, they are a possible link for virus transmission among wild birds. Pathogenicity of Asian HPAIV of H5N1 subtype for certain gull species apparently increased through viral evolution between 1997 and 2005 from asymptomatic or mild infection to fatal disease (Perkins and Swayne 2003, Brown et al. 2008a, 2008b). This implicates an assumption that the black-headed gulls captured in the marsh of Pantana were in the incubation phase and died afterward. Experimental infection of black-headed gulls with appropriate virus could give a reliable answer to this question.

Acknowledgements

All activities regarding sample collection, avian influenza virus detection and field epidemiological investigation were funded by the Ministry of Agriculture, Fisheries and Rural Development of the Republic of Croatia. Genetic analysis of the avian influenza viruses was funded by the Ministry of Sciences, Education and Sports of the Republic of Croatia within the research grant "Genetic characterization of AI and ND viruses isolated in Croatia" (048-0481153-1136).

Author Disclosure Statement

No competing financial interests exist.

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Znanstveni rad 2

Effect of Weather Conditions on Dynamics of the Black-headed Gull, *Chroicocephalus ridibundus*, Wintering on Jakuševec, Zagreb City Rubbish Dump

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Abstract.- Temperature and precipitation are known to have an effect on the onset of bird migration, but studies were mostly taken on Passeriformes. Between 2001 and 2006 we studied the wintering numbers and dynamics of migration of a short-distance migrant, black-headed gull, Chroicocephalus ridibundus, on Zagreb city rubbish dump, Jakuševec. Mean monthly numbers showed significant negative correlation with the local winter temperature, with higher numbers in the coldest months. Arrival of black-headed gulls correlated with North Atlantic Oscillation (NAO) index for the period from September to November. Precipitation didn't have an effect on the timing of migration or maximum numbers. Our results indicate that arrival time for black-headed gull depended on the conditions in breeding areas, while departure time did not respond to NAO or local temperature.

Key words: Black-headed gull, NAO index; onset of migration.

The effect of climate on bird migration has been intensively studied during the last few decades. Temperature and precipitation are known to have an effect on the onset of bird migration (Hüppop and Hüppop, 2003; Hubalek, 2004). The North Atlantic Oscillation (NAO) index is defined as the difference of normalized atmospheric pressure at sea level between Island and Azores. Negative winter NAO index indicates colder and dryer winters in

Europe, while positive indicates warmer winter with more precipitation. It is frequently used as the weather indicator for western and central Europe and near-coastal areas (Hüppop and Hüppop, 2003; Zalakevicius et al., 2006). It has been shown that changes in the timing of arrival are greater among short-distance migrants than long-distance migrants (Hubalek, 2004; Rainio et al., 2006). However, not all authors observed such differences, especially in eastern Europe where the NAO index has a weaker impact (Zalakevicius et al., 2006). Data from many more localities are needed, especially for non-passerine birds that are less represented in studies of bird–climate relationship (Tryjanowski et al., 2002; Zalakevicius et al., 2006; Kralj and Dolenec, 2008), before a general conclusion about the timing of migration over wider areas can be reached.

Phenological studies in Croatia were up to now restricted to the spring arrival of breeding passerines (Dolenec, 2003; Kralj and Dolenec, 2008; Dolenec et al., 2009). We chose a short-distance migrant wintering in Croatia: black-headed gull to see whether the weather conditions had an influence on their wintering numbers and dynamics of migration.

Materials and methods

Black-headed gull is one of the most common gull species in the Western Palaearctic (Cramp and Simmons, 1983). The Croatian breeding population is around 1500 pairs, however the population is greatly increased during migration and in winter (Kralj, 1997). Black-headed Gulls that breed more northerly migrate and winter more southerly than those breeding in more southern areas. (Cramp and Simmons, 1983; Olsen and Larrson, 2004).

Zagreb, the largest city in the country, is situated in the north-western part of Croatia. Having around one million inhabitants it has the biggest rubbish tip and is the place with the highest concentration of wintering gulls in the country. During the winter months more than 15,000 gulls feed at the Zagreb city rubbish dump, Jakuševec (Jurinović and Kralj, 2012).

Recoveries of black-headed Gulls ringed in winter in Zagreb and observed during breeding season as well as the recoveries of black-headed gulls ringed as chicks and found wintering in Zagreb showed that they mostly originated from eastern Europe (Fig. 1).

Gulls were counted once a week, two hours after sunrise, during the whole period from January 4th 2001 till December 26th 2006. We used a method of estimation counts to

assess the number of birds present at the rubbish tip (Bibby et al., 1992). Mean monthly numbers were calculated for each month.

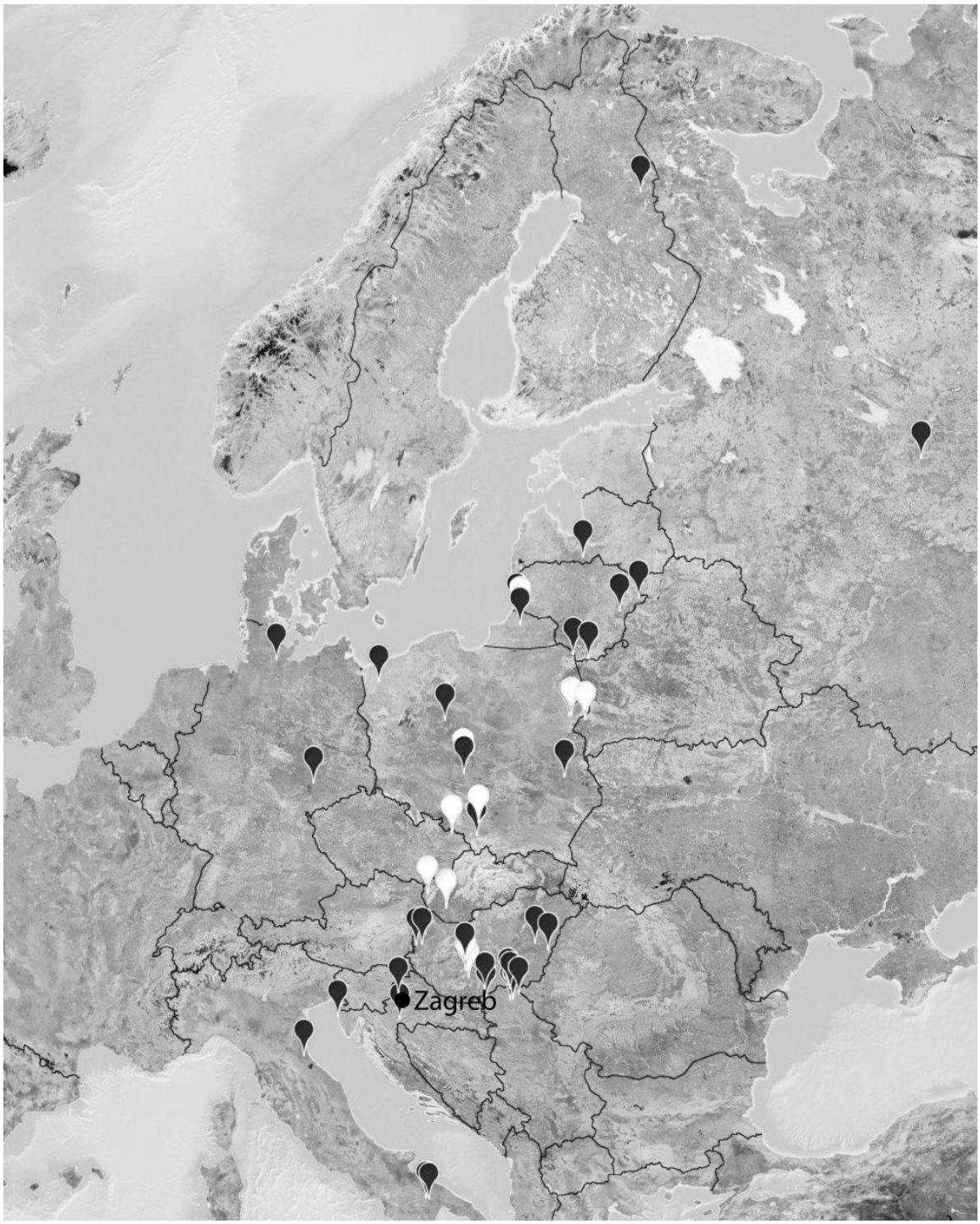

Fig. 1. The origin of the black-headed gulls wintering on Jakuševec, Zagreb. White markers: ringing sites for chicks recorded on Jakuševec during winter. Black markers: observation sites between April and June for black-headed Gulls ringed in winter on Jakuševec.

We compared the arrival and departure dates with climate conditions on both breeding and wintering grounds. Small flocks of black-headed gulls or individual birds can however be seen throughout the year. Therefore, instead of analysing the first arrival dates and departure dates, we analysed the first or last occurrence of flocks over 1000 black-headed gulls. We used NAO index (NAO index data for investigated period was downloaded from URL http://www.cru.uea.ac.uk/cru/data/nao.htm on 9th March 2013) to represent weather conditions at the breeding grounds. The mean NAO winter indices for September-November were calculated, according to Hüppop and Hüppop (2003) and Marra et al. (2005). To describe the weather at the wintering grounds, the mean monthly temperature and monthly precipitation in Zagreb for six year period (provided by the Meteorological Office in Zagreb) were used. Calendar days of the arrival and departure of flocks were converted into Julian dates (1 for 1st July) with correction for leap-years. Julian dates were converted back to conventional format when reporting results. Pearson correlation ($P < 0.05$) was used to examine the relationships between the gull data (mean monthly numbers and first and last occurrence of flocks over 1000 black-headed gulls) and average temperature and precipitation in Zagreb, NAO index and Julian dates.

Results

Black-headed Gull is the most numerous gull species on Jakuševec with daily maximum of up to 13,500 birds recorded. They are present at Jakuševec from October till April (Fig. 2). In the rest of the year they are sporadic and present in small numbers.

Mean monthly numbers showed significant negative correlation with the local winter temperature (November to March), showing higher numbers in the coldest months ($r = -0.724$, $P < 0.001$). Arrival of black-headed gulls (first occurrence of flocks over 1000 birds) was correlated with NAO index for the period from September to November ($r = 0.927$, $P < 0.05$), while the departure was not correlated with NAO index or with the local weather. We did not find any significant correlation between local precipitation and gull numbers or timing of their movements (Table I).

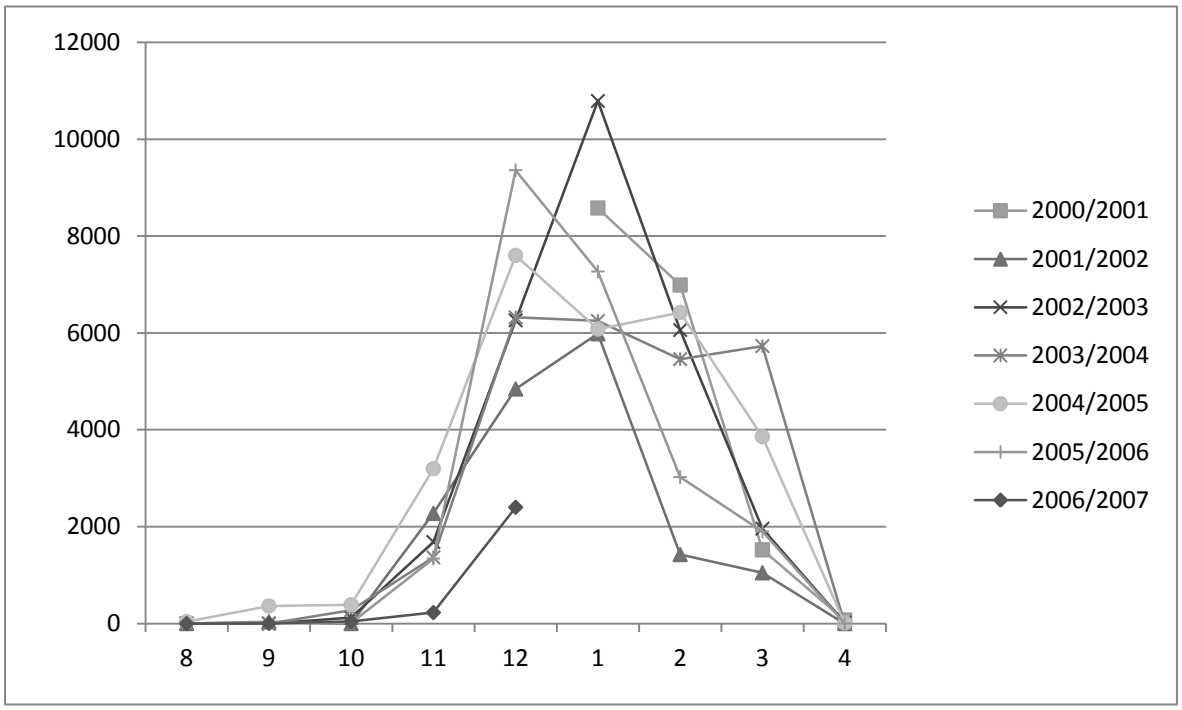

Fig. 2. Mean monthly numbers of black-headed gulls found on Jakuševec in months of the year starting with August.

Individual black-headed gulls are present on Jakuševec for almost the whole year, their numbers increase in November. First dates with numbers higher than 100 varied between years (30th August to 3rd November), but first dates with numbers over 1000 showed a much more uniform pattern (from 8th November to 8th December) which was shown to be positively correlated with autumn (September to November) NAO index.

Discussion

During the last 15 years many studies have shown the migratory movements correlated with weather conditions. These studies were mostly done on migratory passerine birds (Tryjanowski et al., 2002; Zalakevicius et al., 2006, Kralj and Dolenec, 2008). Our study showed that black-headed gulls wintering in Croatia harmonize the autumn migration (measured through the timing of their arrival to wintering grounds) according to the weather conditions on breeding grounds. NAO index did not have any effect on the departure from wintering ground in Croatia (situated far away from the northern coasts), which confirmed its decreasing effect in the eastern Europe away from the northern sea coast (Zalakevicius et al.,

2006). Precipitation did not show an effect on the timing of migration or maximum numbers. Precipitation has been proven to have an effect on migratory birds, but mostly through food availability (Studds and Marra, 2011) that has the great impact on the selection of wintering grounds (Chamberlain et al., 2007). Due to the daily disposal of garbage on the landfill sites, the availability of food usually did not change with time or local weather conditions. Even the high snow cover does not have any effect on the food availability.

Lack of correlation between the local climate factor or NAO index and departure date of the black-headed gulls indicates that the departure time in this population is either intrinsic or triggered by some other extrinsic factor (such as length of the day). This finding is contrary to Hubalek's (2004), who found that during a period of more than 100 years black-headed Gull showed negative correlation with NAO index for their spring arrival in Czech Republic. Rainio et al. (2006) also showed that the spring migration of Finnish black-headed gulls breeding in the boreal and arctic zone responded to NAO index. However, study of the spring migration phenology is usually restricted to arrival dates, while birds are known to adjust their migration en route (Tøttrup et al., 2008), hence the departure from wintering grounds and arrival to breeding sites, must not necessarily be correlated with the same factors. Such adjustments are known for long-distance migrants, but might also be present, to a lesser extent, in short-distance migrants.

Acknowledgements

We thank the Meteorological Office in Zagreb for providing climate data. This study was partly financed by ZGOS.

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(Received 3 July 2013, revised 25 October 2013)

Table I.-The first and the last occurrence of flocks over 1000 black-headed gulls at Zagreb city rubbish dump and NAO and climate data for the study period.

Winter	**Arrival date**	**Departure date**	**NAO index (Sept-Nov)**	**Temperature (ºC)**						**Precipitation (mm)**					
				Sept	**Oct**	**Nov**	**Dec**	**Jan**	**Feb**	**Sept**	**Oct**	**Nov**	**Dec**	**Jan**	**Feb**
2001/02	10.11.	17.3.	-0,98	14.4	14.0	3.1	-2.4	-0.1	5.9	211.6	7.2	114.7	36.7	91.4	18.2
2002/03	8.11.	9.3.	-1,78	15.2	11.2	9.7	1.9	-1.8	-2.7	98.6	99.1	83.0	79.7	19.7	53.7
2003/04	13.11.	7.3.	-1,01	15.5	9.2	7.8	1.4	-0.6	2.5	114.9	118.1	57.8	30.7	76.2	37.5
2004/05	15.11.	17.3.	-0,07	16.0	13.0	6.6	1.6	-0.7	-2.2	79.1	138.6	46.0	54.2	68.6	69.0
2005/06	15.11.	17.3.	-0,32	16.7	11.6	4.9	1.0	-1.7	1.2	87.3	30.7	73.5	135.2	27.7	73.9
2006/07	8.12.	19.3.	-0,43	17.5	12.9	8.4	3.6	5.6	6.6	64.9	4.8	60.2	35.9	53.8	41.2
Median	**14.11.**	**17.3.**													

Znanstveni rad 3

Virological and serological investigation of avian influenza in black-headed gulls captured on a rubbish dump in Croatia

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Abstract

A total of 142 black-headed gulls (BHGs), *Chroicocephalus ridibundus*, were captured during February and March 2009 at Zagreb city rubbish dump (45.45 N 16.01 E) in order to collect cloacal swabs and serum samples. Cloacal swabs were tested by virus isolation in embryonated chicken eggs which resulted in isolation of one avian influenza virus (AIV) that was of H16 subtype. Collected sera were tested by blocking ELISA for avian influenza antibodies resulting in 28.2% positive samples which were retested by haemagglutination inhibition (HI) using H5 and H7 subtype antigens. Only one serum sample was positive for H5 and none for H7 antibodies. Statistically, no significant difference was found between ages of AIV seropositive birds ($\chi 2$= 2.08, df= 1, P=0.15). In contrast, seroprevalence in different months of capturing where higher proportion of positive gulls during March than

during February was found ($\chi 2$= 4.53, df= 1, P=0.03), especially in younger birds ($\chi 2$= 7.67, df= 1, P=0.006). Such finding suggests that BHGs might contract AIV infection during their aggregations in large numbers at rubbish dumps or similar feeding sites during winters. Although only one of 142 tested birds was positive for H5 subtype antibodies, this finding cannot be neglected knowing that apparently healthy BHGs can carry highly pathogenic AIV of H5N1 subtype. Nevertheless, the results of our study have shown that BHGs are more often infected with other AIV subtypes and therefore are most likely not the primary carriers of H5 AIV. To our knowledge this is the first detection of H16 AIV in Croatia.

Key words

avian influenza, H5 subtype, gulls, *Chroicocephalus ridnibundus*

Introduction

Highly pathogenic avian influenza (HPAI) is a viral disease that can cause high mortality of both poultry and wild birds. Main natural reservoir of avian influenza (AI) are birds belonging to orders *Anseriformes* (ducks, geese and swans) and *Charadriformes* (gulls, terns and waders) (WEBSTER et al., 1992). AI of H13 subtype is the most frequent one found in gulls (OLSEN et al., 2006). It is closely related to H16 subtype and they are both genetically distant from influenza viruses found in hosts other than gulls. During the study in Norway in 2006/07 it was found that H13 and H16 are the most frequently occurring AI subtypes found in gulls, but the authors also isolated H1, H4, H5 and H6 AI subtypes from these birds (GERMUNDSSON et al., 2010).

In May of 2005 HPAI H5N1 virus killed more than 6,000 wild birds at Lake Qinghai. Two out of six bird species that were affected were gulls: great black-headed gull *(Ichthyaetus ichthyaetus*) and brown-headed gull (*Chroicocephalus brunnicephalus*) (ZHOU et al., 2006).

During the outbreak of HPAI H5N1 virus of Asian lineage in Croatia in winter 2005/06 seventeen H5N1 viruses were isolated from wild birds. Five of them were from black-headed

gulls (BHGs), *Chroicocephalus ridibundus.* All of the gulls were apparently healthy in contrast to infected mute swans (*Cygnus olor*) and a mallard (*Anas plathyrynchos*) which have shown clinical sign or were found dead (SAVIĆ et al., 2010).

This virus can be also fatal to humans. In period from 2003 until submitting of this paper there were 637 confirmed cases of people infected with HPAI H5N1 virus, 378 of which had fatal outcome (ANONYMOUS, 2013).

Zagreb city rubbish dump Jakuševec is one of places with highest aggregation of BHGs in Croatia. During winter months up to 13,500 BHGs feed on Jakuševec (JURINOVIĆ and KRALJ, 2012). That concentration of gulls creates a great opportunity for spread of AI viruses (AIVs) among these birds (OLSEN et al., 2006).

The aim of this study is to determine whether the AI viruses of H5 and H7 subtypes are circulating in gulls feeding on Zagreb city rubbish dump.

Material and methods

Sampling

During February and March 2009 gulls were captured at Zagreb city rubbish dump (45.45 N 16.01 E) with clap net. Captured gulls were divided in three age groups according to their plumage; unknown age, second calendar year and third calendar year or older (OLSEN and LARSSON, 2004). Cloacal swabs and serum samples were collected from all the gulls.

Serology

All of the sera were tested by ELISA for presence of antibodies against avian influenza virus using FlockCheck AI MultiS-Screen Ab Test (IDEXX, Maine, USA) according to the manufacturer's instructions.

Positive and suspicious samples were tested by haemaglutination inhibition (HI) using H5 and H7 antigens (Istituto Zooprofilattico Sperimentale delle Venezie, Padova, Italy). For the first test H5N1 and H7N1 antigens were used respectively. Samples that were positive for H5 antibodies were retested with H5N2 antigen in order to eliminate neuraminidase cross reactivity.

Virus isolation

AIV isolation was carried out in embryonated chicken eggs according to the standard procedures (ANONYMOUS, 2009). A battery of monospecific antisera covering all 16 H subtypes of AIV and monospecific serum against avian paramyxoviruse type 1 (Istituto Zooprofilattico Sperimentale delle Venezie, Padova, Italy) were used for typing of haemagglutinating isolates.

Molecular methods

Viral RNA was extracted from all haemaglutinating allantoic fluids using High Pure Viral Nucleic Acid Kit (Roche Applied Science, Mannheim, Germany).

Extracted RNA samples were tested by RT-qPCR specific for type A influenza viruses (SPACKMAN et al., 2002) and positive samples were then tested by RT-q PCR specific for H5 (SLOMKA et al., 2007) and H7 subtype (SLOMKA et al., 2009). All RT-qPCR tests were carried out in LightCycler 1.5 (Roche Applied Science, Mannheim, Germany) with slight modifications of the original protocols due to the different platform (available upon request).

Statistical analysis

Data were analyzed using Statistica software package (StatSoft, Inc. 8.0, Tulsa, USA). Possible differences between months of capturing or age classes were tested using $\chi 2$ tests. Differences at $P<0.05$ were regarded statistically significant.

Results

A total of 142 BHGs were captured (Table 1).

Virus isolation yielded two haemaglutinating agents and RNA of only one was positive for avian influenza type A virus, but negative for H5 and H7 subtypes. Serotyping of the isolate has shown it to be of H16 subtype. The other isolate was serotyped as avian paramyxovirus type 1. The bird harbouring the AIV was in its third calendar year or older.

Results of ELISA are given in Table 1. Briefly, 28.2% sera were positive for avian influenza antibodies while only one of them was positive for H5 antibodies with titre of 4 log2 with both H5N1 and H5N2 antigens, and none of them was positive for H7 antibodies.

When testing for variations in proportions of gulls positive versus negative for avian influenza antibodies, no significant statistical difference were found between ages ($\chi 2$= 2.08, df= 1, P=0.15). Significant statistical difference was found when testing all gulls in different months of capturing ($\chi 2$= 7.67, df= 1, P=0.006). Consequent analysis has shown no significant difference between months of capturing for gulls that were in their 3rd calendar year or older ($\chi 2$= 1.96, df= 1, P=0.16), but revealed significantly higher proportion of positive gulls during March than during February for gulls in their 2nd calendar year ($\chi 2$= 4.53, df= 1, P=0.03).

Table 1. Results of ELISA test for presence of antibodies against avian influenza in Black-headed Gulls sera.

	Tested birds by months		Positive birds by months		Total tested birds	Total positive birds
	February	March	February	March		
Unknown age	1	0	0 (0%)	0 (0)	1	0 (0%)
2nd calendar year	64	13	**12 (18.75%)**[a]	**6 (46.16%)**[a]	77	18 (23.38%)
3rd calendar year or older	31	33	8 (25.80%)	14(42.42%)	64	22 (34.38%)
Total for all ages	96	46	**20(20.83%)**[b]	**20(43.48%)**[b]	142	40 (28.17%)

[a] Statistically significant differences among months of sampling for BHGs in their second year (P=0.03).

[b] Statistically significant differences among months of sampling for all BHGs sampled (P=0.006).

Discussion

BHGs are one of the most common gull species in Europe. In cities with large water areas, such as lakes or rivers, they interact very closely with humans and can serve as vectors for many diseases. Prevalence of influenza virus in BHGs varies greatly between different ages of birds, population and time of year when birds were sampled. Percentages can be very misleading in small samples such as 60% prevalence in juvenile birds caught in August 1999 in Sweden (FOUCHIER et al., 2005) or 21% in Norway in 2006 (GERMUNDSSON et al., 2010) with samples of 10 and 21 birds respectively. Other authors (GLOBIG, 2006, PEREZ-RAMIREZ et al., 2010) failed to detect AI viruses in small samples. In bigger samples like 1,583 birds from Northern Europe and Netherlands (MUNSTER et al., 2007) or 4,303 birds from the European Union (HESTERBERG et al., 2009) prevalence was less than 1%, 0.9% and 0.5% respectively and is probably closer to the real prevalence. In all mentioned studies RT-qPCR was used except in one study (GLOBIG, 2006) where virus isolation in embryonated chickens egg was used. Virus isolation success from RT-q PCR positive samples varies from 83.3% (FOUCHIER et al., 2005) to 33.5% (MUNSTER et al., 2007). The results of our study are in accordance with bigger samples and represent 0.7% (1/142) of birds positive for AI virus.

No AIV of H5 subtype was isolated during this study. Most of the documented cases of isolation of HPAI H5N1 virus from BHGs was from dead birds (SHARASHOV et al., 2010, ELLIS et al., 2004) but SAVIĆ et al. (2010) described isolation from apparently healthy BHGs. However, AIV of H16 subtype was isolated from a BHG and to our knowledge this is the first detection of this AIV subtype in Croatia.

Overall AIV seroprevalence in BHGs on Jakuševec in February and March 2009 was 28.2%. It was higher in older birds (34.4%) than in birds in their second calendar year (23.4%), but this difference does not show statistical significance. The probable cause of this can be found in fact that older birds have had more time to get infected (GRAVES 1992, DE MARCO 2004).

The percentage of seropositive birds was significantly higher in March (overall 43.48%) than in February (overall 20.83%) both for birds in their second calendar year and older. In period 2001 – 2005 there was big difference between February and March in average number of

BHGs in a day. It was 5302.00 for February and 2742.38 for March (JURINOVIĆ, 2006). This change in number and subsequently exchange of flocks feeding in Jakuševec is the probable reason for such change in seroprevalence.

Although only one of 40 seropositive birds was positive for H5 antibodies, this finding cannot be neglected. The bird was in its second calendar year. Nevertheless, the results of our study indicate that BHGs are not the primary carriers of H5 AIV. Most probably they contracted infection from other water birds during massive feeding or roosting on water surfaces because waterfowls are very efficient in influenza viruses transmission via fecal material in the water supply (WEBSTER et al., 1992). Gulls are not typical migratory birds with regular routes of their movements. They are, especially before reaching sexual maturity, capable of crossing very long distances in small periods of time, and are therefore capable of transmitting the disease over a vast area before showing symptoms.

Thus there is a need of systematic monitoring of gulls on massive feeding or roosting sites (such as rubbish tips or big water areas) in order to timely prevent possible spread of the virus to domestic poultry and eliminate subsequent potential major economic losses.

Acknowledgement

We acknowledge the Ministry of Science, Education and Sports of the Republic of Croatia - project 048-0481153-1136 that supported this work.

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JURINOVIĆ, L., V. SAVIĆ, M. BALENOVIĆ, D. LISIČIĆ, V. LUCIĆ**: Virološko i serološko istraživanje influence ptica u riječnih galebova uhvaćenih na gradskom odlagalištu otpada u Hrvatskoj**

SAŽETAK

Tijekom veljače i ožujka 2009. na gradskom odlagalištu otpada u Zagrebu (45.45 N 16.01 E) uhvaćena su 142 riječna galeba (*Chroicocephalus ridibundus*) u cilju prikupljanja brisova nečisnica i uzoraka krvi. Brisovi nečisnica su pretraženi izdvajanjem virusa u embrioniranim kokošjim jajima što je rezultiralo izdvajanjem virusa influence ptica koji je bio podtipa H16. Prikupljeni uzorci seruma su pretraženi imunoenzimnim testom za protutijela virusa influence ptica od kojih je 28,2% bilo pozitivno. Pozitivni uzorci su dodatno pretraženi inhibicijom hemaglutinacije s antigenima podtipa H5 i H7 među kojima je samo jedan bio pozitivan na H5 i nijedan na H7 protutijela. Statistički nije nađena značajna razlika među različitoj dobi seropozitivnih ptica ($\chi2$= 2.08, df= 1, P=0.15). Nasuprot tome, seroprevalencija u različitim mjesecima lova je imala veći udio tijekom ožujka nego tijekom veljače ($\chi2$= 4.53, df= 1, P=0.03), naročito u mlađih ptica ($\chi2$= 7.67, df= 1, P=0.006). Ovakav nalaz ukazuje na moguću infekciju riječnih galebova tijekom njihovog brojnog okupljanja na odlagalištima otpada ili sličnim mjestima za hranjenje tijekom zime. Iako je samo jedna od 142 ptice bila pozitivna na protutijela za H5 podtip, nalaz se ne smije zanemariti znajući da naizgled zdravi riječni galebovi mogu nositi virus jako patogene influence podtipa H5N1. Unatoč tome, rezultati našeg istraživanja pokazuju da riječni galebovi češće bivaju inficirani drugim

podtipovima virusa influence ptica te stoga najvjerojatnije nisu primarni nosioci virusa influence podtipa H5. Sukladno našim spoznajama, ovo je prvi dokaz virusa influence podtipa H16 u Hrvatskoj.

3. RASPRAVA

Tijekom jeseni i zime 2005./2006. godine visokopatogeni virus influence podtipa H5N1 došao je i do Europe, pojavljujući se u peradi u Turskoj, Rusiji, Ukrajini i Rumunjskoj (Savić, 2006.). U listopadu 2005. godine virus je izdvojen iz mrtvih crvenokljunih labudova, *Cygnus olor*, nađenih na ribnjacima „Grudnjak“ i „Ribnjak 1905“ u Slavoniji. U veljači 2006. godine isti je virus izdvojen i iz mrtvih crvenokljunih labudova s otoka Čiova, obližnje močvare Pantane, iz Zagreba te iz Baranje. U ožujku 2006. godine virus je izdvojen iz naizgled zdravih riječnih galebova te mrtve divlje patke, *Anas plathyrhynchos,* ponovo s močvare Pantane. Sveukupno je izdvojeno 17 virusa influence A podtipa H5N1. Pomoću univerzalnih početnica za H5 gen (Slomka i sur., 2007.) napravljen je RT-PCR te sekvenciran PCR proizvod veličine oko 300 parova baza svih 17 virusnih izolata oko područja koje kodira mjesto cjepanja hemaglutinina. Iz tih, relativno kratkih, sekvenci napravljeno je više filogenetskih analiza koristeći razne supstitucijske modele. Na temelju toga izabrano je 7 reprezentativnih izolata iz kojih je ponovo izolirana ukupna RNA te je napravljen PCR za cijeli gen hemaglutinina. Za filogenetsku analizu je primijenjen Maximum Composite Likelyhood supstitucijski model. Očekivano, svi su izolati bili slični virusu izdvojenom na jezeru Quinghai, odnosno liniji 2.2 (WHO/OIE/FAO H5N1 Evolution Working Group, 2008.). Većina ovih izolata je pripadala podliniji 2.2.2. i usko su grupirani s prvim izolatom iz Hrvatske (A/Cygnus olor/Croatia/1/05) izdvojenim iz mrtvog crvenokljunog labuda nađenog na ribnjaku “Grudnjak” (Slika 1, Znanstveni rad 1). Drugi pripadnici ove skupine su izolati iz mrtvih crvenokljunih labudova iz Baranje i Zagreba te izolati iz naizgled zdravih riječnih galebova s Pantane. Zanimljivo, pet od šest H5N1 izolata iz ponovnog izbijanja bolesti u divljih ptica na jezeru Qinghai u Kini u svibnju 2006. (QH06) su pridruženi izolatu A/Cygnus olor/Croatia/1/05 dok je jedan srodan izolatu A/duck/Novosibirsk/02/05 iz Rusije (Wang i sur., 2008.). Ovo saznanje potkrjepljuje implikaciju da se QH06 virusi sa Quinghai jezera mogu vratiti putem ptica selica (Wang i sur., 2008.).

Ostala 3 izolata iz Hrvatske pripadaju podliniji 2.2.1. ali, za razliku od hrvatskih izolata iz podlinije 2.2.2. koji su očito kružili po Hrvatskoj šest mjeseci i ostali genetički bliski, izolati iz podlinije 2.2.1. pokazuju veliku raznovrsnost i upućuju na postojanje dvije odvojene genetičke linije (slika 1., znanstveni rad 1). Ova raznolikost konkurentnih izolata je rezultat odvojene evolucije virusa koja se najvjerojatnije odvijala prije introdukcije u

Hrvatsku. Stoga, filogenetska analiza hrvatskih izolata implicira barem tri odvojene introdukcije H5N1 virusa uz pomoć divljih ptica.

Nakon prvog nalaza H5N1 u lešinama crvenokljunih labudova na močvari Pantana u blizini Trogira, u okviru aktivnog monitoring izdvojili smo H5N1 iz naizgled zdravih riječnih galebova te iz mrtve divlje patke. Galebove smo uhvatili pomoću vertikalnih ornitoloških mreža te smo ih odmah nakon prstenovanja i uzorkovanja pustili. Izolati iz mrtvih labudova su filogenetički prilično srodni, što ukazuje da su epidemiološki povezani, ali značajno različiti od ostalih izolata iz Hrvatske. Izolati iz galebova su slični A/Cygnus olor/Croatia/1/05 izolatu dok je izolat iz patke različit od svih izolata iz Hrvatske i sličan određenim izolatima iz zapadne Europe. Nalaz tri genetički odvojena soja virusa H5N1 u isto vrijeme na močvari Pantana koja se nalazi na jadranskoj obali te ima umjerene zime, može se povezati sa smrznutim vodenim površinama u kontinentalnom dijelu Hrvatske, kao i u cijeloj istočnoj Europi početkom 2006. godine i pomicanjem ptica prema toplijim područjima. Pretpostavke o neuobičajenim obrascima pri seobi divljih ptica s područja oko Crnog mora prema toplijim dijelovima Europe zbog iznimno jake zime 2005./2006. godine su spomenuli Kilpatric i suradnici (2008.) te Alexander i Capua (2008.).

Tijekom 2005. i 2006. godine tri genetički različita soja virusa podtipa H5N1 su nađena u 3 različite vrste divljih ptica, međutim nije pronađena nikakva povezanost između sojeva virusa i vrsta divljih ptica koja bi upućivala na prenošenje virusa među vrstama. Izdvajanje virusa podtipa H5N1 iz riječnih galebova nije uobičajeno, a posebno ne iz naizgled zdravih ptica. Većina virusa ovog podtipa je nađena kod mrtvih ili jako bolesnih ptica, uključujući i dokumentirano izdvajanje virusa podtipa H5N1 iz riječnih galebova u Hong Kongu (Ellis i sur., 2004.). Stoga, ovo je prvo dokumentirano izdvajanje azijskog visokopatogenog virusa influence ptica podtipa H5N1 iz naizgled zdravih riječnih galebova. Moguće je da su galebovi ulovljeni u fazi inkubacije dok još nisu pokazivali kliničke znakove infekcije, a rasprostranjivali su virus. Nepoznato nam je jesu li galebovi naknadno imali ikakve kliničke simptome ili su kasnije uginuli jer nisu više nađeni nakon prstenovanja. Ako riječni galebovi ne pokazuju simptome nakon zaražavanja visokopatogenim virusom H5N1, a rasprostranjuju virus, oni su moguća poveznica za rasprostranjenost virusa među divljim pticama. Patogenost azijskog visokopatogenog virusa influence ptica podtipa H5N1 za pojedine vrste galebova se povećala kroz virusnu evoluciju između 1997. i 2005. godine od asimptomatične ili blage infekcije do fatalne bolesti (Perkins i Swayne, 2003.; Brown i sur., 2008.; Brown i sur., 2008.a). Ovo upućuje na to da su galebovi ulovljeni u fazi inkubacije te

da su nakon toga vjerojatno uginuli. Pokusna infekcija riječnih galebova prikladnim virusom dala bi odgovor na ovo pitanje.

Riječni galeb kao i divlja patka je najčešća vrste galeba, odnosno najčešća vrsta pataka u Euroaziji. Nalazi prstenovanih ptica upućuju da jedinke obje ove vrste tijekom sezone gniježđenja borave u Rusiji, a zimuju u Hrvatskoj (Jurinović i Kralj, 2013.; Kralj, 2013.). Obje vrste su jako brojne na cijelom području Euroazije te jako druževne i često dolaze u jatima koja broje i nekoliko tisuća. Takva su mjesta idealna za cirkuliranje i prijenos virusa te predstavljaju moguću poveznicu između Azije i Europe u smislu prijenosa virusa influence.

Mnoge su studije tijekom posljednjih 15 godina pokazale da su migracijska kretanja ptica povezana s vremenskim uvjetima. Većina ovih studija je provedena na migratornim pjevicama, *Passeriformes* (Tryjanowski i sur., 2002.; Zalakevicius i sur., 2006.; Kralj i Dolenec, 2008.). Naše je istraživanje utjecaja vremenskih parametara na brojnost i dinamiku riječnih galebova na zagrebačkom odlagalištu otpada Jakuševec pokazalo da je jesenska migracija riječnih galebova koji zimuju ondje uvjetovana vremenskim uvjetima na gnjezdilištima. Jesensku migraciju smo kvantificirali kroz datum dolaska ptica na mjesto zimovanja, a klimatske faktore na gnjezdilištu kroz NAO (North Atlantic Oscilation) indeks. NAO indeks je definiran kao razlika normaliziranog atmosferskog tlaka na morskoj razini između Islanda i Azora. Negativni NAO indeks ukazuje na hladnije i suše zime u Europi, dok pozitivni ukazuje na tople zime s više padalina. Često se koristi kao vremenski indikator za zapadnu i središnju Europu te područja blizu obala (Hüppop i Hüppop, 2003.; Zalakevicius i sur., 2006.). Iz naših istraživanja proizlazi da NAO indeks nije imao utjecaj na vrijeme napuštanja zimovališta što je u sukladnosti sa Zalakeviciusom i sur., 2006. koji su utvrdili da se utjecaj NAO indeksa smanjuje u istočnoj Europi te s udaljenošću od sjeverne obale mora. Također, nismo utvrdili nikakav utjecaj količine oborina niti na početak migracije niti na najveće brojnosti tijekom istraživanog perioda. Oborine dokazano imaju utjecaj na ptice selice, ali najčešće kroz dostupnost hrane (Studds i Marra, 2011.). Međutim zbog svakodnevnog odlaganja otpada na Jakuševcu, čak i po najnepovoljnijim vremenskim uvjetima organska komponenta komunalnog otpada je dostupna pticama. Čak ni visoki snježni pokrivač ne predstavlja problem pticama.

Nedostatak korelacije između lokalnih klimatskih faktora i NAO indeksa s datumom odlaska sa zimovališta ukazuje na to da je to uvjetovano nekim drugim faktorima (poput duljine dana) ili urođeno. Iako je ovo činjenično u suprotnosti sa Hubalekom (2004.) koji je

utvrdio značajnu negativnu korelaciju između NAO indeksa i datuma dolaska riječnih galebova u Češku tijekom perioda od više od 100 godina, te Rainiom i sur. (2006.) koji su utvrdili korelaciju između NAO indeksa i proljetnog dolaska finskih riječnih galebova, treba imati na umu da se fenologija dolaska galebova na gnjezdilišta mjeri datumom dolaska na mjesto gniježđenja, a da se ptice mogu prilagoditi uvjetima na migracijskom putu (Tøttrup i sur., 2008.). Stoga datum odlaska sa zimovališta i datum dolaska na gnjezdilišta ne moraju nužno biti korelirani ni međusobno ni s istim faktorima.

U gradovima s velikim vodnim područjima, kao što su jezera ili rijeke, riječni galebovi često dolaze u bliske kontakte s ljudima te mogu biti prenosioci raznih bolesti. Prevalencija virusa influence ptica kod riječnih galebova uvelike se razlikuje u ovisnosti o populaciji kojoj pripadaju, starosti ptica te dobu kad su ptice uzorkovane. Postoci mogu biti prilično varljivi u malim uzorcima kao što su 60% učestalosti u juvenilnih ptica uhvaćenih u kolovozu 1999. u Švedskoj (Fouchier i sur., 2005.) ili 21% u Norveškoj 2006. godine (Germundsson i sur., 2010.) s uzorcima od 10 odnosno 19 ptica. Drugi autori (Globig, 2006.; Peres Ramirez i sur 2010.) u malim uzorcima nisu uspjeli zabilježiti viruse influence u riječnih galebova. U većim uzorcima kao što su 1583 ptica iz sjeverne Europe i Nizozemske (Münsteru i sur., 2007.) ili 4303 ptice iz Europske unije (Hesterberg i sur., 2009.) učestalost je manja od 1 % , točnije 0,9 % i 0,5 %. Ovi su postoci vjerojatno bliži stvarnoj prevalenciji. U svim spomenutim studijama je primjenjen RT-qPCR, osim u jednoj studiji (Globig, 2006.) u kojem je korišten izdvajanje virusa u oplođenim kokošjim jajima. Uspješnost izdvajanja virusa iz pozitivnih uzoraka s RT-qPCR-a varira od 83,3 % (Fouchier i sur., 2005.) do 33,5 % (Münster i sur., 2007.) Rezultat našeg istraživanja 2009. godine je u skladu s većim uzorcima i iznosi 0,7 % (1/142) ptica pozitivnih za viruse influence.

Tijekom istraživanja nisam izolirao ni jedan virus podtipa H5. Većina dokumentiranih slučajeva izolacije visokopatogenih virusa influence podtipa H5N1 u riječnih galebova je iz mrtvih ptica (Sharashov i sur., 2010.; Ellis i sur., 2004.), ali Savić i suradnici (2010.) su 2006. izdvojili ovaj virus iz naizgled zdravih riječnih galebova. Izdvojeni virus podtipa H16 kojeg smo izdvojili 2009. godine je prvi dokaz o pojavljivanju ovog podtipa u Hrvatskoj.

Ukupna seroprevalencija influence u riječnih galebova na Jakuševcu u veljači i ožujku 2009. godine je bila 28,2 %. Nešto je viša u starijih ptica (34,4 %) nego u ptica u drugoj kalendarskoj godini (23,4 %), ali ta razlika ne pokazuje statističku značajnost. Vjerojatni

uzrok toga može se naći u činjenici da su starije ptice imali više vremena da se zaraze (Graves, 1992.; De Marco, 2004.) .

Postotak seropozitivnih ptica bio je značajno veći u ožujku (sveukupno 43,48 %) nego u veljači (sveukupno 20,83 %) za obje kategorije starosti. U razdoblju od 2001. do 2005. godine postojala je velika razlika u prosječnom broju riječnih galebova u danu na odlagalištu Jakuševec i to 5302,00 u veljači i 2742,38 u ožujku (Jurinović, 2006.). Ova promjena u brojnosti i sukladno tome i promjena jata riječnih galebova koja se hrane na Jakuševcu su vjerojatni razlog u tolikoj promjeni seroprevalencije.

Iako je samo jedna od 40 seropozitivnih ptica bila pozitivna na protutijela za H5 podtip, ovaj nalaz ne smije proći nezapaženo. Ipak rezultati naše studije pokazuju da riječni galebovi nisu primarni nosioci podtipa H5 influence nego su se vjerojatno zarazili na masovnim hranilištima i odmorištima na vodenim površinama. Influenca se vrlo efikasno prenosi među pticama vodaricama preko fekalnog materijala (Webster i sur., 1992.). Galebovi nisu tipične selice s točno određenim rutama kretanja te su, osobito prije spolne zrelosti sposobni prijeći velike razdaljine u relativno kratkom periodu te su sposobni prenijeti viruse preko ogromnog prostora prije pojave ikakvih simptoma.

Sukladno nalazima iz 2009. godine nastavili smo pretraživati riječne galebove na odlagalištu otpada Jakuševec te smo u periodu od 2010. do 2013. godine ukupno pretražili 3193 ptice. Ovaj uzorak dao nam je još bolji uvid u prevalenciju virusa influence u riječnim galebovima koji se hrane na odlagalištu otpada Jakuševec. Ukupno smo izdvojili još 6 virusa influence ptica. Od toga je bilo 5 izolata podtipa H13 i još 1 podtipa H16 (neobjavljeni vlastiti podaci).

Ovi podaci daju ukupnu prevalenciju virusa influence u riječnim galebovima od 0,2 % zimi na zagrebačkom području. Ovaj broj je u skladu s podacima koje je Graves dobio u studiji na prstenokljunim galebovima, *Larus delawarensis*, u Sjevernoj Americi (Graves, 1992.). Gravesovi rezultati govore o prevalenciji od 0,26 % kod prstenokljunih galebova u „hladnim“ mjesecima te je uzorak od 1200 riječnih galebova pretraženih izvan sezone gniježđenja (Verhagen i sur., 2014.) nedostatan da bi se sa 95 % sigurnošću utvrdila prevalencija od 0,2 % (Hoye i sur., 2010.).

4. ZAKLJUČCI

1) Filogenetskom analizom izolata je dokazano da je tijekom 2005. i 2006. godine visokopatogeni virus influence podtipa H5N1 u tri odvojena navrata introduciran u Hrvatsku.

2) Riječni galebovi mogu nositi i rasprostranjivati virus influence, a da ne pokazuju nikakve simptome bolesti te su zbog toga jedan od načina kojima se ova bolest može „neprimjetno“ širiti na velike udaljenosti

3) Termin dolaska riječnih galebova na zimovanje u Hrvatsku koreliran je s NAO indeksom za period od rujna do prosinca, odnosno vremenskim uvjetima na gnjezdilištima.

4) Termin odlaska riječnih galebova sa zimovališta (iz Hrvatske) nije povezan niti s jednim od ispitanih klimatoloških parametara (NAO indeks, prosječne oborine i temperatura u Zagrebu) te je vjerojatno uvjetovan nekim drugim faktorima (kao npr. duljina dana).

5) Ukupna prevalencija influence u riječnih galebova tijekom zimskih mjeseci na Jakuševcu tijekom istraživanja iznosi 0,2 %.

6) Odrasli riječni galebovi su u većem broju seropozitivni na virus influence, nego galebovi u prvoj godini života.

7) Seroprevalencija je značajno veća u ožujku, nego u veljači i za odrasle i za mlade riječne galebove.

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6. ŽIVOTOPIS

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