Published in final edited form as:
Avian Pathol. 2013 February ; 42(1): 60–71. doi:10.1080/03079457.2012.759176.

Expression and distribution of sialic acid influenza virus receptors in wild birds

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Abstract

Avian influenza (AI) viruses have been detected in more than 105 wild bird species from 12 different orders but species-related differences in susceptibility to AI viruses exist. Expression of α2,3-linked (avian-type) and α2,6linked (human type) sialic acid (SA) influenza virus receptors in tissues is considered to be one of the determinants of the host range and tissue tropism of influenza viruses. We investigated the expression of these SA receptors in 37 wild bird species from 11 different orders by lectin histochemistry. Two isoforms of *Maackia amurensis* (MAA) lectin, MAA1 and MAA2, were used to detect α2,3-linked SA and *Sambucus nigra* (SNA) lectin was used to detect α2,6-linked SA. All species evaluated expressed α2,3-linked and α2,6-linked SA receptors in endothelial cells and renal tubular epithelial cells. Both α2,3-linked and α-2,6-linked SA receptors were expressed in respiratory and intestinal tract tissues of aquatic and terrestrial wild bird species from different taxa, but differences in SA expression and in the predominant isoform of MAA lectin bound were observed. With a few possible exceptions, these observed differences were not generally predictive of reported species susceptibility to AI viruses based on published experimental and field data.

Introduction

Avian influenza (AI) viruses belonging to all known 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes have been isolated from wild birds (Stallknecht & Brown, 2008). Infection with low pathogenic (AI) viruses is usually asymptomatic in wild birds and these viruses are occasionally transmitted to domestic poultry. Birds within the orders Anseriformes (ducks, geese and swans) and Charadriiformes (gulls, terns and shorebirds) are reservoirs of avian influenza (AI) viruses (Stallknecht & Brown, 2008). Most AI virus isolations are reported from dabbling ducks, especially mallards (*Anas platyrhynchos*) (Stallknecht & Shane, 1988), gulls (Olsen *et al*., 2006), and shorebirds at Delaware Bay, USA (Krauss *et al*., 2010). Avian influenza viruses are only occasionally isolated from aquatic bird species belonging to other orders including Ciconiiformes, Gaviiformes, Gruiformes, Pelecaniformes, Podicipediformes and Procellariiformes and from terrestrial bird species in the orders Columbiformes, Falconiformes, Passeriformes, Piciformes and Piscittaciformes (Stallknecht & Brown, 2008).

The expression and distribution of sialic acid (SA) receptors in tissues may in part contribute to the host range and species barrier of AI virus infections (Suarez, 2008; Shinya *et. al*., 2006). The basic composition of the SA receptors recognized by the HA receptor binding site of influenza A viruses is SAα2,3(6)Galβ1,3(4)GlcNacβ1 (Suzuki, 2005). Avian

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influenza viruses usually have tropism to N-acetylneuraminic acid linked to galactose in an α2,3 configuration, while human influenza A viruses preferentially recognize N-acetylneuraminic acid linked to galactose in an α2,6 configuration (Matrosovich *et al.*, 1997; Rogers *et al.*, 1983). Detection of these receptors in tissues has been performed by lectin histochemistry using the lectins *Sambucus nigra* and *Maackia amurensis* that detect α2,6 and α2,3-linked SA receptors, respectively. The fine receptor specificity of AI viruses to SA receptors also depends on the linkage between the SAα2,3Gal disaccharide and the penultimate N-acetylhexosamine residue of the carbohydrate chain. It was previously reported that duck AI viruses preferentially bind to SAα2,3Galβ1,3GalNacβ1 while some gull isolates have preferred tropism to SAα2,3Galβ1,4GlcNacβ1 (Gambaryan *et al.*, 2005). Lectin histochemistry has shown that bird species that are highly susceptible to AI virus infections, such as chickens and Pekin ducks, strongly express α2,3-linked SA receptors in the respiratory and intestinal tracts. (Kuchipudi *et al.*, 2009; Pillai & Lee, 2010; Wan & Perez, 2006) while more resistant species, such as pigeons, had weak expression of α2,3-linked SA in most of the tissues examined (Liu *et al.*, 2009). Studies on the expression and distribution of SA influenza receptors in tissues of wild birds are lacking. To evaluate the potential role of SA receptors in determining differences in susceptibility to infection to AI viruses, we analyzed the expression of two avian-type α2,3-linked SA receptors and the human-type α-2,6-linked SA receptor in tissues of wild bird species from 11 different orders by lectin histochemistry.

Materials and Methods

Lectin histochemistry

Thirty-seven aquatic and terrestrial wild bird species (Tables 1 and 2) from 11 different orders including Anseriformes, Charadriiformes, Ciconiiformes, Gaviiformes, Gruiformes, Pelecaniformes, Passeriformes, Psittaciformes, Columbiformes, Falconiformes and Accipitriformes were analyzed for the presence of SA influenza virus receptors by lectin histochemistry. Formalin-fixed tissues or paraffin-embedded tissue sections were provided by the Southeastern Cooperative Wildlife Disease Study, Southeastern Poultry Research Laboratory, USGS National Wildlife Health Center and Miami Metrozoo. Tissues evaluated for SA receptor expression included nasal turbinates, trachea, lungs, duodenum, jejunum, ileum, caeca, heart, liver, kidney and brain. Formalin-fixed tissues were routinely processed for histology and paraffin-embedded tissue sections were deparaffinized in xylene and hydrated in decreasing alcohol solutions. The SA retrieval was performed in citrate buffer at pH 6.0 for 45 minutes using a steamer. Lectin histochemistry was performed similarly to a previously described method with some modifications (Kuchipudi *et al.*, 2009). The tissues were stained with *Maackia amurensis* (MAA) lectins for detection of avian-type α2,3-linked SA receptors or *Sambucus nigra* (SNA) lectin for detection of α2,6-linked SA.

To detect α2,3-linked SA receptors, the tissue sections were double-labeled with two isoforms of MAA (also known as MAL), MAA1 and MAA2. MAA1 and MAA2 lectins (Vector Laboratories, Burlingame, CA) detect SAα2,3Galβ1,4GlcNac and SAα2,3Galβ1,3GalNac, respectively (Gambaryan *et al.*, 2005). Sections were blocked with Carbo-Free blocking solution (Vector Laboratories, Burlingame, CA) for 15 minutes prior to lectin staining with MAA. After washing with PBS, tissue sections were incubated with a 1:100 dilution of fluorescein isothiocyanate-labeled (FITC) MAA1 lectin for 1 hour at room temperature in the dark. After 3 washes with PBS, sections were incubated with a 1:50 dilution of biotinylated MAA2 for 1 hour at room temperature and in the dark. This was followed with 3 washes with PBS and incubation with a 1:100 dilution of Alexa fluor 546 streptavidin conjugate (Molecular Probes Inc., Eugene, OR) for 2 hours at room temperature in the dark. For detection of α-2,6-linked SA receptors, tissue sections were incubated with a 1:100 dilution of FITC-labeled SNA lectin (Vector Laboratories, Burlingame, CA) for 1

hour at room temperature in the dark. After washing the stained slides with PBS, a drop of ProLong Gold antifade reagent (Molecular Probes Inc., Eugene, OR) was added and the tissue sections were sealed with a coverslip. The slides were observed in an Olympus microscope and in a Zeiss confocal microscope with FITC and tetramethylrhodamine-isothiocyanate (TRITC) filters.

Large intestine sections of mallard were used as positive controls for MAA lectin binding and sections of pig trachea were used as positive controls for SNA binding. In addition, large intestine sections of mallard, small and large intestine sections of ring-billed gull and pig trachea were treated with 250mU/ml to 1 U/ml concentration of Sialidase A (N-acetylneuraminate glycohydrolase; Prozyme, San Leandro, CA) at 37 ° C for 18 to 48 hours prior to lectin staining to ensure that the sialic acids were specifically targeted. Sialidase A cleaves all the non-reducing terminal sialic acid residues from complex carbohydrates and glycoproteins in the order α(2,6)> α(2,3)> α(2,8)> α(2,9). Mallard large intestine and pig trachea incubated with PBS were also used as omission controls.

Lectin binding distribution and intensity were scored semiquantitatively. Intensity of lectin binding was graded as 0 (negative), 1 (mild), 2 (moderate) and 3 (strong). Distribution scores were classified as 0 (negative), 1 (staining >0–25% of the cells), 2 (staining >25–50% of the cells), 3 (staining >50–75% of the cells) and 4 (>75% of the cells with positive staining). A final lectin binding score was obtained by multiplying the intensity and distribution scores and classified as mild (score 0–3), moderate (score 4–6) and strong (score 8–12).

Results

Differences in SA expression between wild bird species were only observed in the respiratory and intestinal tracts. The sialidase treatment prior to lectin histochemistry abolished the binding of MAA2 and SNA lectins in mallard large intestine and pig trachea, respectively (data not shown). The sialidase treatment decreased MAA1 binding in the large intestine of mallard and abolished the binding of this lectin in intestine sections of ring-billed gull (data not shown). Omission controls did not have background fluorescence. Similar SA receptor expression was observed between individual birds of most species evaluated (Tables 1 and 2). Ruddy turnstone was the only species that had birds of different age groups evaluated and SA expression was similar between birds (Tables 1a and 1b).

Expression of α2,3-linked SA receptors in respiratory and intestinal tracts of aquatic bird species

The α2,3-linked SA receptor expression (MAA) in the respiratory and intestinal tract of aquatic bird species is shown in Table 1a. The predominant isoforms of MAA lectin bound and type of cell with α2,3-linked SA can be found in Table 1b. Twenty-two aquatic bird species from 6 different orders were examined. Most species in the order Anseriformes had abundant expression of α2,3-linked SA in the respiratory and intestinal tracts and staining was similar between species of the same genus. Except for the red head duck, all birds in the order Anseriformes either had abundant binding of both isoforms of MAA or strongly bound only MAA2 in ciliated and goblet cells of the respiratory tract (Fig. 1a). The red head was the only species of Anseriformes examined that had predominant binding of MAA1 lectin in the respiratory tract (Fig. 1b).

All species of Anseriformes expressed α2,3-linked SA receptors in the intestinal tract. Abundant expression of α2,3-linked SA, mostly in goblet cells, was observed in the duodenum and jejunum of Anseriformes species, except for the red head duck, black swan and northern pintail which had limited expression. All species of Anseriformes strongly

expressed α2,3-linked SA in enterocytes and goblet cells of the ileum and caeca with strong binding of both isoforms of MAA (Fig. 1e) or predominant binding of MAA2.

The α2,3-linked SA receptor expression in tissues was often similar between species of Charadriiformes of the same genus. All birds in the order Charadriiformes strongly expressed α2,3-linked SA in epithelial cells of the respiratory tract. In the respiratory tract of shorebird species of the genera *Calidris* and *Arenaria*, there was either strong binding of MAA1 and MAA2 or predominant binding of MAA1 in epithelial cells (Fig. 1c). In gulls of the genus *Larus*, the epithelial cells had strong binding of both isoforms of MAA or predominant binding of MAA2. Except for the red knot (Fig. 1g), all other species of Charadriiformes expressed α2,3-linked SA receptors in the intestinal tract (Fig. 1f).

Aquatic wild bird species in the orders Ciconiiformes, Gaviiformes, Gruiformes and Pelecaniformes also abundantly expressed α2,3-linked SA receptors in the respiratory and intestinal tracts. Most of these other waterfowl species bound both isoforms of MAA or predominantly bound MAA2 in the respiratory tract (Fig. 1d). Red-throated loon was the only species that predominantly bound MAA1 lectin in the respiratory tract. All examined birds in these orders strongly bound MAA1 and MAA2 lectins in the intestines (Fig. 1h).

Expression of α2,3-linked SA receptors in respiratory and intestinal tracts of terrestrial bird species

The α2,3-linked SA receptor expression (MAA) in the respiratory and intestinal tract of terrestrial bird species is shown in Table 2a. The predominant isoforms of MAA lectin bound and type of cell with α2,3-linked SA are detailed in Table 2b. Fifteen terrestrial bird species from 5 different orders were examined. All species of Passeriformes examined expressed α2,3-linked SA in epithelial cells of the respiratory tract. Within the order Passeriformes, the European starling had predominant binding of MAA1 in the respiratory tract (Fig. 2b) and the lowest α2,3-linked SA expression in the trachea. The other species of Passeriformes had strong binding of both isoforms of MAA (Fig. 2a) or predominant binding of MAA2 lectin in the respiratory tract.

All species of Passeriformes expressed α2,3-linked SA receptors in the intestines. House finch (Fig. 2e), zebra finch, house sparrow and kinglet were the species with the strongest α2,3-linked SA expression in the intestinal tract with binding of both isoforms of MAA lectin in enterocytes and goblet cells. The European starling was the species with the weakest expression of α2,3-linked SA in the intestines and expression of this receptor type was not detected in the small intestine of this species (Fig. 2f).

The respiratory tract and the intestine of rock pigeons showed abundant α2,3-linked SA receptor expression with predominant binding of MAA1 lectin (Fig. 2c, 2g). Mourning dove had weak α2,3-linked SA expression in the respiratory tract and also had predominant binding of MAA1 lectin in the intestine.

Budgerigar was the only species of Psitacifformes examined. This species had weak α2,3-linked SA receptor expression in the upper respiratory tract, but strong binding of both isoforms of MAA in the lung and small intestine.

The two hawk species examined strongly expressed α2,3-linked SA receptors in the respiratory tract (Fig. 2d) and had similar abundant binding of both isoforms of MAA in enterocytes and goblet cells (Fig. 2h). The American kestrel and bald eagle had abundant expression of α2,3-linked SA in the respiratory tract, but these species mildly expressed this receptor type in the intestinal tract.

Expression of α-2,6-linked SA receptors in respiratory and intestinal tracts of aquatic bird species

The α2,6-linked SA receptor expression (SNA) in the respiratory and intestinal tract of aquatic bird species is shown in Table 1a. Table 1b shows the type of cell with α2,6-linked SA. Except for the wood duck, all the other species of Anseriformes strongly expressed α2,6-linked SA receptors in epithelial cells of the trachea and lung. Similar α2,6-linked SA expression in the respiratory tract was observed between species of ducks of the genus *Anas* (Fig. 3a) and swans of the genus *Cygnus*. All species of Anseriformes examined had abundant expression of α2,6-linked SA in enterocytes of the ileum and caeca (Fig. 3d) and varying expression of this receptor in duodenum and jejunum.

Expression of α2,6-linked SA receptors was variable in shorebird species. All gull species had strong expression of α2,6-linked SA in the respiratory tract (Fig. 3b). Intestinal expression of α2,6-linked SA was negative to mild in most species of Charadriiformes examined (Fig. 3e).

All other waterfowl species in the orders Ciconiiformes, Gaviiformes, Gruiformes and Pelecaniformes examined also expressed α2,6-linked SA receptors in respiratory tract tissues; none of these species expressed this receptor type in the ileum and caeca.

Expression of α2,6-linked SA receptors in respiratory and intestinal tracts of terrestrial bird species

The α2,6-linked receptor expression (SNA) in the respiratory and intestinal tract of terrestrial bird species is shown in Table 2a. Table 2b shows the type of cell with α2,6-linked SA. Expression of α2,6-linked SA was observed in the respiratory tract of all species of Passeriformes examined (Fig. 3c). American crow, European starling and house sparrow had the strongest expression of this receptor type in the intestinal tract. House finch, kinglet and Eastern meadow lark did not express α2,6-linked SA in the intestines.

Budgerigars did not express α2,6-linked SA receptors in the respiratory tract, but strongly expressed this receptor type in the small intestine (Fig. 3f).

Moderate to strong expression of α2,6-linked SA receptors was observed in the respiratory tract of rock pigeon and mourning dove. Rock pigeon did not express this receptor type in the intestinal tract.

The α2,6-linked SA receptor expression was variable in raptor species. The red-tailed hawk and the bald eagle were the species that had the most abundant expression of α2,6-linked SA in the respiratory tract. All raptor species examined did not express α2,6-linked SA in the duodenum and jejunum. The red-tailed hawk was the only raptor species that expressed this receptor type in the ileum and caeca.

Expression of α2,3 and α2,6-linked SA receptors in other tissues

The expression of SA influenza receptors in the heart, liver, kidney and brain was similar between different species. The endocardium and endothelial cells from all examined species expressed α2,6-linked SA receptor (data not shown) and the α2,3-linked SA receptor type recognized by MAA2 lectin (Fig. 4). In the kidney, all examined species similarly expressed abundant amounts of α2,6 (data not shown) and α2,3-linked (Fig. 5) SA receptors in distal convoluted tubules and tubules in the medullary cone. There was no SA receptor expression in cardiac myocytes, neurons or hepatocytes in all examined species.

Discussion

We performed the most extensive study ever undertaken on the distribution of SA influenza virus receptors in tissues of wild birds. In this study, we evaluated the tissue distribution, cell type and intensity of expression of α2,3 (avian-type) and α2,6 (human-type)-linked SA receptors in 37 wild bird species representing 11 different taxonomic orders. We used two isoforms of MAA lectin, MAA1 and MAA2, in order to determine the diversity of α2,3-linked SA receptors present in tissues of wild birds. Although MAA1 lectin is not specific for α2,3-linked SA receptors, this lectin should be used in conjunction with MAA2 lectin, since some influenza viruses were previously reported to predominantly bind SAα2,3Galβ1,4GlcNac (Gambaryan *et al.*, 2005; Nicholls *et al.*, 2007). Also, some duck isolates of AI virus were reported to have preferred binding to SAα2,3Galβ1,3GalNac polymeric conjugates of sialooligosaccharides, while some gull isolates preferred SAα2,3Galβ1,4GlcNac (Gambaryan *et al.*, 2005). In our study, sialidase treatment abolished MAA1 lectin staining in intestine of ring-billed gull, but only decreased the intensity of MAA1 lectin staining in mallard large intestine, which suggests that the large intestine of ducks also contains non-sialic acid α2,3 glycans detected by MAA1 lectin. Few species including red head, dunlin, red-throated loon, American crow, European starling and budgerigar had predominant or exclusive binding of MAA1 in tissues (Tables 1b and 2b). Since MAA1 lectin is non-specific, some of these results may also represent binding to non-sialic acid glycans.

We observed that ducks, geese and swans have abundant expression of α2,3-linked SA receptors in the respiratory and intestinal tracts, which correlates with the role of Anseriformes as natural reservoirs of AI viruses and their high susceptibility to infection to these viruses. The strong binding of MAA2 lectin in tissues of various species of Anseriformes may explain why AI viruses isolated from ducks mainly bind to SAα2,3Galβ1,3GalNac polymeric conjugates of sialooligosaccharides (Gambaryan *et al.*, 2005). Although abundant expression of α2,3-linked SA receptors was observed in tissues of Anseriformes, it is well known that LPAI virus prevalence and susceptibility to H5N1highly pathogenic AI (HPAI) viruses vary in birds of this order. Most LPAI virus isolations in this order are from dabbling ducks and mallard is the species with most frequent AI virus isolations (Stallknecht & Brown, 2008). Variations in AI virus shedding patterns and susceptibility to H5N1 HPAI viruses have been reported in various species of ducks including mallard, red head, blue-winged teal, wood duck and northern pintail (Brown *et al.*, 2006). We observed that the red head duck differed from the other duck species with predominant binding of MAA1lectin in the respiratory tract, which may in part explain the decreased susceptibility of this species to H5N1 HPAI virus infection in that study. However, in the case of swans and geese that in contrast to ducks are highly susceptible to H5N1 HPAI virus infection (Brown *et al.*, 2008), avian-type α2,3-linked SA receptor expression in the respiratory tract did not significantly differ from most of the other Anseriformes species evaluated in this study.Most LPAI virus isolations in the order Charadriiformes have been from ruddy turnstones in Delaware Bay during the spring (Hanson *et al.*, 2008). Other shorebird species including dunlin, sanderling and red knot have been detected with LPAI viruses in cloacal swabs, but with much lower prevalence (Hanson *et al.*, 2008). Strong expression of α2,3-linked SA was observed in the respiratory and intestinal tract tissues of shorebird species, including ruddy turnstone, dunlin and sanderling, which support the susceptibility of these species to AI viruses. Interestingly, red knots did not express α2,3-linked SA in the intestines, which may partially explain the lower isolation rate of low pathogenic AI (LPAI) viruses from cloacal swabs in this species. On the other hand, other factors such as feeding behavior, habitat utilization and immunity are also important to determine species-related variation in susceptibility to LPAI in shorebird species (Hanson *et al.*, 2008).

Strong expression of α2,3-linked SA receptors recognized by both isoforms of MAA in ring-billed gulls and laughing gulls is consistent with experimental and surveillance data on the susceptibility of these birds to a wide diversity of LPAI virus subtypes (Brown *et al.*, 2011). We observed that expression of α2,3-linked SA in the respiratory and intestinal tracts of herring gull is lower than in the other gull species evaluated. However, α2,3-linked SA receptor expression does not totally explain susceptibility as herring gull and laughing gull were found to be similarly susceptible to clinical disease caused by experimental infection with Mongolia/05 HPAI H5N1 after intranasal inoculation (Brown *et al.*, 2006; Brown *et al.*, 2008).

The role of aquatic bird species in the orders Ciconiiformes, Gaviiformes, Gruiformes and Pelecaniformes in the ecology of AI viruses is still unclear and isolation of AI viruses from these species have rarely been reported (Stallknecht & Shane, 1988; Lebarbenchon *et al.,* 2010). Binding of both isoforms of MAA lectin in tissues of great blue heron, red-throated loon, Stanley crane, American coot and American white pelican suggests that these species would be susceptible to a wide range of AI viruses.

Terrestrial wild birds are rarely infected with AI viruses under natural conditions (Stallknecht & Brown, 2008) but are susceptible to some HPAI viruses such as the Eurasian H5N1 HPAI viruses (Boon *et al.*, 2007). In one study, the susceptibility of house sparrow, house finch, zebra finch, European starling and budgerigar to H5N1 HPAI viruses was evaluated. Zebra finch was the most susceptible species with high morbidity and mortality followed by the house finch and budgerigar (Perkins & Swayne, 2003). The abundant α2,3-linked SA expression observed in tissues of finches in our study may explain the increased susceptibility of these species to H5N1 HPAI. The authors also reported that the European starling was the least susceptible species with no morbidity and mortality, lack of gross and microscopic lesions, and absence of AI virus antigen in tissues (Perkins & Swayne, 2003). In another study, it was observed that European starlings infected with a H3N8 LPAI virus had rare cloacal shedding (Nemeth *et al.,* 2010). The lower α2,3-linked SA expression observed in tissues of European starling may explain the increased resistance of this species to H5N1 HPAI and the lower cloacal shedding after infections with AI viruses (Nemeth *et al.*, 2010; Perkins & Swayne, 2003). Expression of α2,3-linked SA recognized by both isoforms of MAA was also observed in the respiratory and intestinal tracts of various other species of Passeriformes examined in our study. This suggests that these species may be susceptible to a wide diversity of AI viruses, and therefore the low prevalence of infection associated with these species apparently relates to factors other than receptor expression.

The low expression of α2,3-linked SA receptors in the respiratory and intestinal tracts of pigeons was previously suggested as a possible explanation for their increased resistance to H5N1 HPAI (Liu *et al.*, 2009). However, the previous study only evaluated the expression of SAα2,3Galβ1,3GalNac by using the MAA2 lectin. Since rock pigeon predominantly bound MAA1 lectin, this species may be more susceptible to AI viruses that have tropism for the SA receptor SAα2,3Galβ1,4GlcNac, which is detected by MAA1 lectin. The mourning dove had mild expression of α2,3-linked SA in the respiratory tract, which suggests that this species may also be more resistant to AI virus infections; however, there is no experimental evidence to support increased resistance in this species.

Raptor species, such as birds in the orders Falconiformes and Accipitriformes, may potentially be infected with AI viruses by ingestion of infected carcasses and environmental exposure. Some species are long distant migrants and may potentially spread AI to different regions and countries. We observed that different raptor species express α2,3-linked SA receptors in the respiratory and intestinal tracts, suggesting that they may be susceptible to infection. Indeed, the American kestrel was previously reported to be highly susceptible to

H5N1 HPAI after intranasal inoculation with high morbidity and mortality (Hall *et al.*, 2009). Like passerines, LPAI virus isolations have only rarely been reported from raptors (Goyal *et al.*, 2010), but strong α2,3-linked SA expression in the respiratory and intestinal tract suggests that they are susceptible to infection.

Our study showed that various wild bird species in different taxonomic groups express the α2,6-linked (human-type) SA receptor in the respiratory and intestinal tracts and many species express α2,3 and α2,6-linked SA in the same tissue and type of cell (Tables 1b and 2b). It was previously suggested that poultry species that co-express both avian and human-type SA receptors in cells may have the potential to act as "mixing vessels" and change the receptor tropism of AI viruses to human-type receptors (Kuchipudi *et al.*, 2009; Wan & Perez, 2006). Although we detected α2,6-linked SA receptors in many wild bird species, there is no evidence to date that human influenza viruses can replicate in tissues of wild birds. Virus histochemistry in trachea and colon of some wild bird species showed strong attachment of a H3N2 seasonal human influenza virus in the trachea of herring gull and rock pigeon, while no binding was observed in the colon of these species (Jourdain *et al.*, 2011). This correlates with our finding of abundant expression of α2,6-linked SA receptors in the trachea and no expression of this receptor type in the lower intestine of the herring gull and rock pigeon.

We observed that endothelial cells in all examined species bound MAA2 lectin, which is specific for SAα2,3Galβ1,3GalNac receptors. Tropism of AI viruses for endothelial cells has been demonstrated in some wild bird species (Brown *et al.* 2008; Perkins & Swayne, 2003). Expression of α2,3-linked SA receptors recognized by both MAA isoforms was observed in renal tubules of all examined bird species. This correlates with the ability of AI viruses to replicate in kidneys causing lesions after natural and experimental infections and the isolation of these viruses from kidneys of infected birds (Swayne & Pantin-Jackwood, 2008; Swayne & Slemons, 1995). Replication of LPAI viruses in the kidney and in chicken kidney cells is also supported by the presence of trypsin-like enzymes in renal tubular epithelial cells (Suarez, 2008; Swayne & Pantin-Jackwood, 2008).

In summary, wild bird species reported to be susceptible to AI viruses have abundant expression of α2,3-linked SA receptors in the respiratory and intestinal tracts, but observed variations in receptor specificity patterns across taxon do not provide a clear indicator of species-related differences in susceptibility to either LPAI or H5N1 HPAI viruses. While presence of specific SA receptors may partially influence both susceptibility and shedding patterns, other host and viral factors are also important in determining differences in AI virus prevalence, viral shedding and the outcome of disease caused by HPAI viruses.

Acknowledgments

We would like to acknowledge Southeast Poultry Research Laboratory (SEPRL), USGS National Wildlife Health Center, Miami Metrozoo and the Drs. Justin Brown, Taiana Costa, Sonia Hernandez, Kevin Keel, Steven Kubiski, David Perpinan, Jeffrey Hall and Page Luttrell for providing the tissues used in this study. We also would like to thank the histology laboratory staff at the University of Georgia for making the tissue blocks and unstained slides, and also Deb Carter and Jamie Barber for their technical assistance.

This work was funded by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH), Department of Health and Human Services, under Contract No. HHSN266200700007C. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH.

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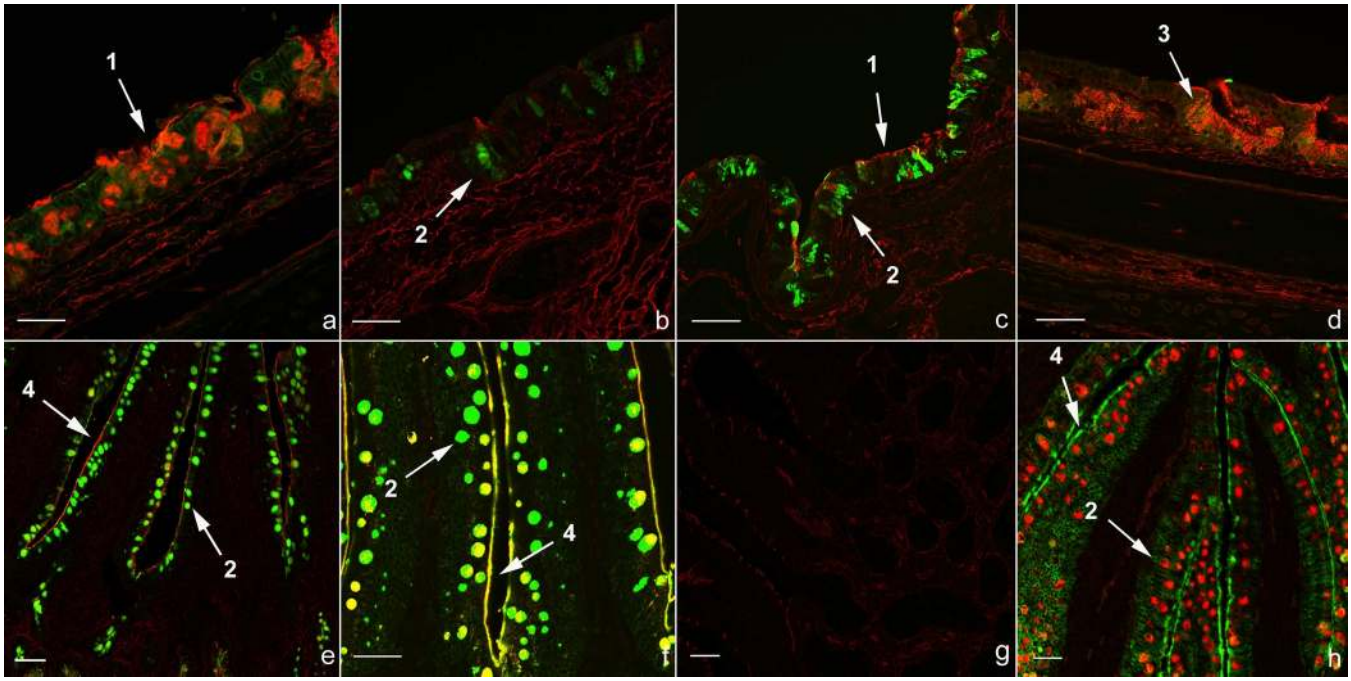

Figure 1.
Lectin binding of *Maackia amurensis* 1 (MAA1) and *Maackia amurensis* 2 (MAA2) for α2,3-linked SA receptors in the respiratory and intestinal tract of aquatic bird species. MAA1 binding in green, MAA2 binding in red and binding of both lectins in yellow. Respiratory tract: a - wood duck (trachea), b – red head (trachea), c – ruddy turnstone (bronchus), d – American coot (trachea). Intestinal tract: e – wood duck (ileum), f – dunlin (small intestine), g – red knot (small intestine), h – American Coot (small intestine). 1 – ciliated cells; 2 – goblet cells; 3 – mucous glands; 4 – enterocytes. Bar: 50 μm.

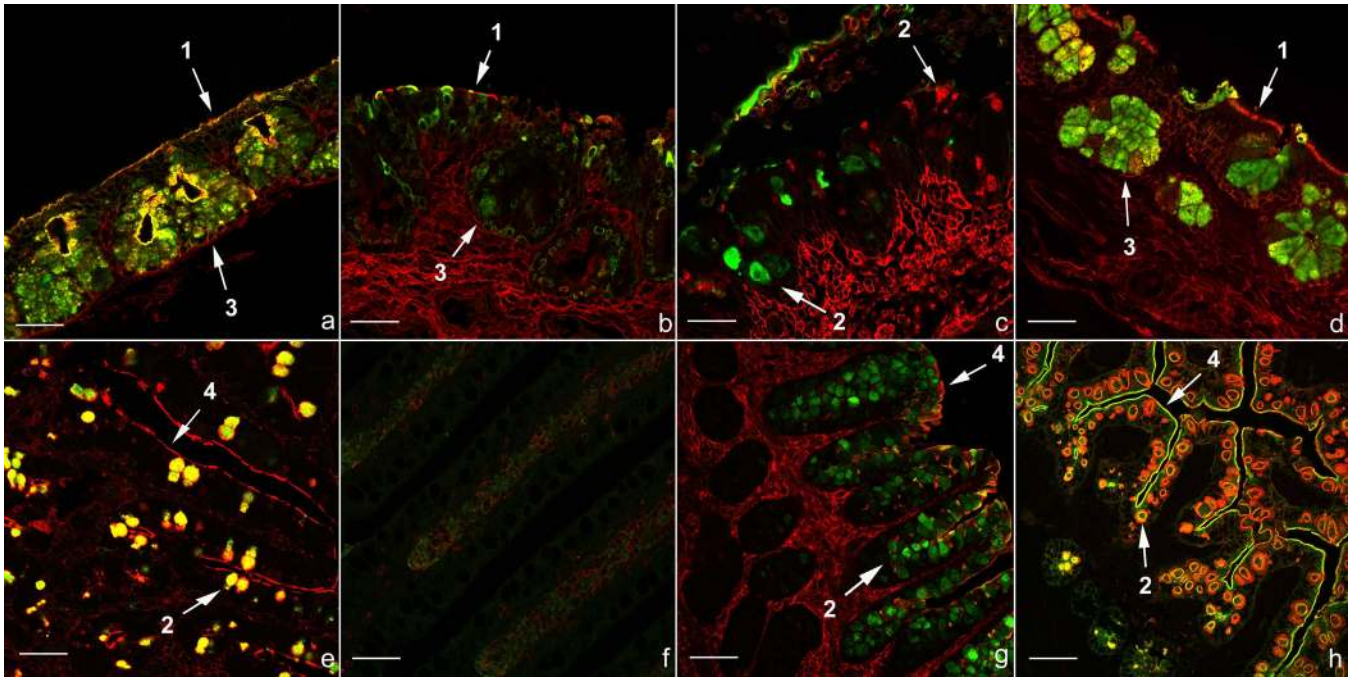

Figure 2.
Lectin binding of *Maackia amurensis* 1 (MAA1) and *Maackia amurensis* 2 (MAA2) for α2,3-linked SA receptors in the respiratory and intestinal tract of terrestrial bird species. MAA1 binding in green, MAA2 binding in red and binding of both lectins in yellow. Respiratory tract: a – house finch (trachea), b – European starling (nasal turbinates), c – rock pigeon (bronchus), d – Cooper's hawk (trachea). Intestinal tract: e – house finch (small intestine), f – European starling (small intestine), g – rock pigeon (large intestine), h – Cooper's hawk (small intestine). 1 – ciliated cells; 2 – goblet cells; 3 – mucous glands; 4 – enterocytes. Bar: 50 μm.

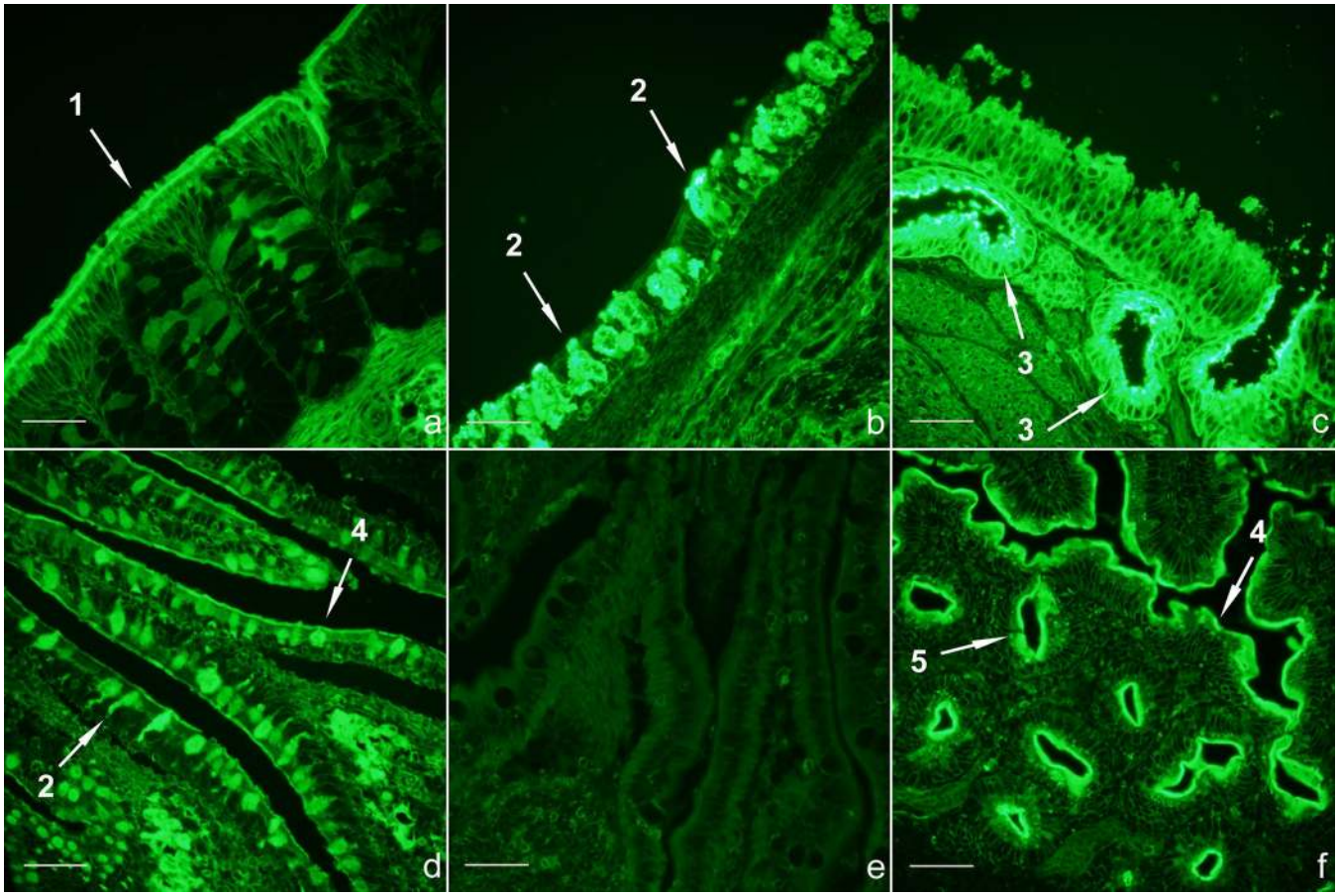

Figure 3.
Lectin binding of *Sambucus nigra* (SNA) for α2,6-linked SA receptors in the respiratory and intestinal tract of aquatic and terrestrial bird species. Respiratory tract: a – northern pintail (nasal turbinates), b – ring-billed gull (trachea), c – European starling (nasal turbinates). Intestinal tract: d – wood duck (ileum), e – laughing gull (small intestine), f – budgerigar (small intestine). Arrows: 1 – ciliated cells; 2 – goblet cells; 3 – mucous glands; 4 – enterocytes; 5 – crypts. Bar: 50 μm.

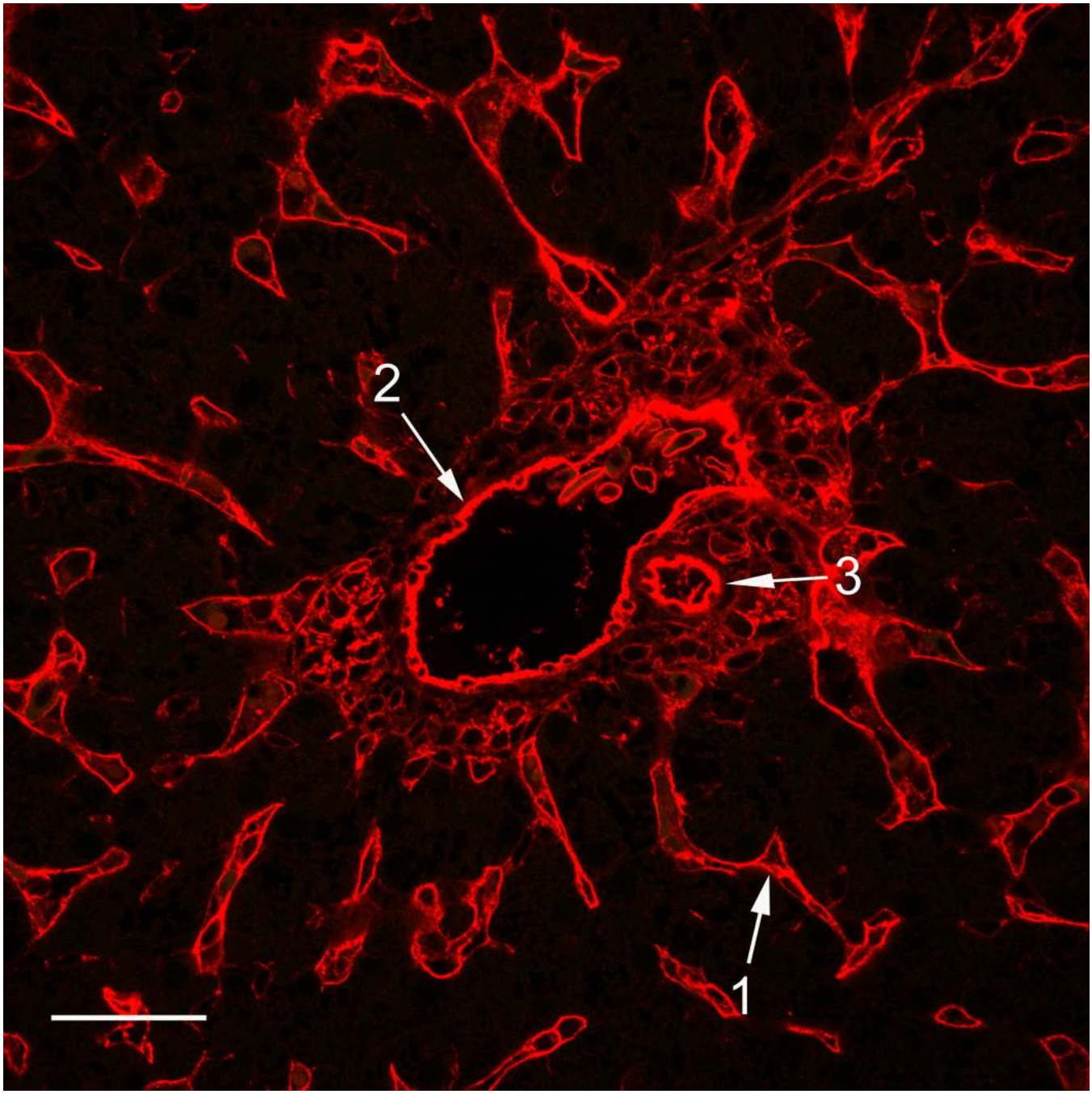

Figure 4.
Mallard liver. Lectin binding of *Maackia amurensis* 2 (MAA2) for SAα2,3Galβ1,3GalNac receptors in endothelial cells of hepatic sinusoids (arrow 1), portal vein (arrow 2) and portal arteriole (arrow 3). Bar: 50 μm.

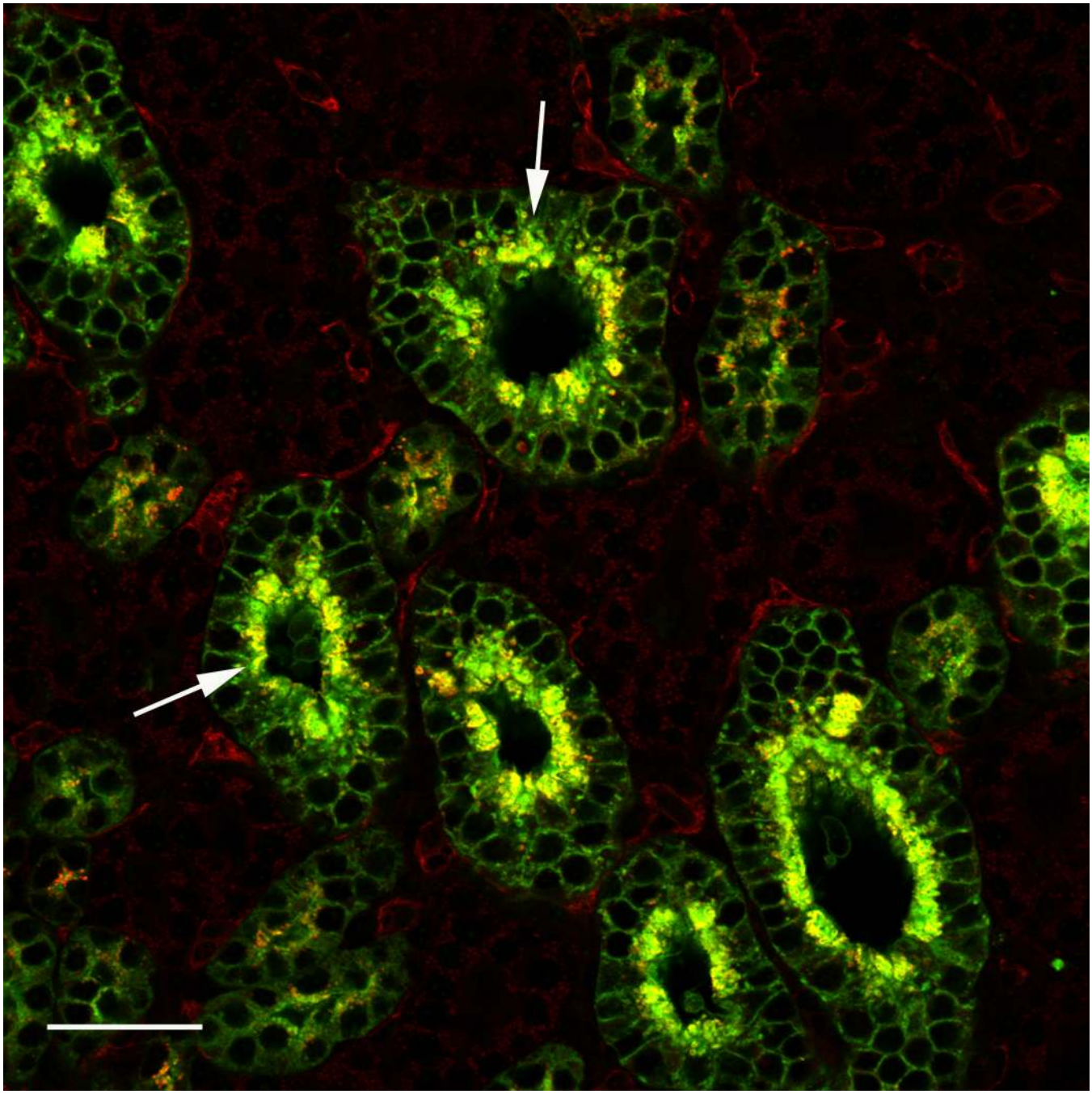

Figure 5.
Ring-billed gull kidney. Lectin binding of *Maackia amurensis* 1 (MAA1) and *Maackia amurensis* 2 (MAA2) in tubular epithelial cells (arrows). Bar: 50 μm.

Table 1

a. Lectin binding in respiratory and intestinal tracts of waterfowl species using *Maackia amurensis* (MAA) and *Sambucus nigra* (SNA) lectins.

Order/Species	Age[a]	Lectin binding[b]									
		Nasal turbinates		Trachea		Lung (bronchi)		Duodenum/ jejunum		Ileum/caeca	
		MAA	SNA	MAA	SNA	MAA	SNA	MAA	SNA	MAA	SNA
Anseriformes											
Mallard *Anas platyrhynchos* (n=3)	4 w	++/ +++	−	+++	+++	+++	+++	+++	−	+++	+++
Blue-winged teal *Anas discors* (n=3)	10 w	+++	−	+++	+++	+++	+++	+++	+++	+++	+++
Cinnamon teal *Anas cyanoptera* (n=1)	8 w	+++	−	+++	+++	+++	+++	+++	+++	+++	+++
Northern Pintail *Anas acuta* (n=3)	12 w	+++	+++	+++	+++	+++	+++	−/+	−	+++	+++
Red Head *Aythya Americana* (n=2)	16 w	+++	+++	+++	+++	+++	+++	+	−	+++	+++
Wood duck *Aix sponsa* (n=2)	12 w	+++	+	+++	+	+++	−	+++	++	+++	+++
Black Swan *Cygnus atratus* (n=1)	4 w	+++	−	+++	+++	+++	+++	+	+++	+++	+++
Mute Swan *Cygnus olor* (n=1)	5–6 w	+++	−	+++	+++	+++	+++	+++	−	+++	+++
Bar-headed goose *Anser indicus* (n=2)	8 w	+++	+	+++	+++	+++	−	+++	−	+++	+++
Cackling goose *Branta hutchinsii* (n=2)	8 w	+++	+++	+++	+++	+++	+++	+++	−	+++	+++
Charadriiformes											
Dunlin *Calidris alpina* (n=2)	Juv[c]	ND[e]	ND	+++	+++	+++	+++	+++	−	+++	−
Sanderling *Calidris alba* (n=2)	Ad[d]	ND	ND	+++	−	+++	−	+++	+++	ND	ND
Red Knot *Calidris canutus* (n=1)	Ad	+++	−	+++	−	+++	++	−	−	−	−
Ruddy turnstone *Arenaria interpres* (n=2)	Juv/Ad	ND	ND	ND	ND	+++	+++	+++	−	+++	−
Herring gull *Larus smithsonianus* (n=3)	12 w	−/+++	+++	+++	+++	++	+++	+	−	++	−

a. Lectin binding in respiratory and intestinal tracts of waterfowl species using *Maackia amurensis* (MAA) and *Sambucus nigra* (SNA) lectins.

Order/Species	Age[a]	Lectin binding[b]									
		Nasal turbinates		Trachea		Lung (bronchi)		Duodenum/ jejunum		Ileum/caeca	
		MAA	SNA	MAA	SNA	MAA	SNA	MAA	SNA	MAA	SNA
Laughing gull *Larus atricilla* (n=2)	2–3 w	+++	+++	+++	+++	+++	+++	+++	+	+++	+
Ring-billed gull *Larus delawarensis* (n=2)	6 w	+++	+++	+++	+++	+++	+++	+++	–	+++	+
Ciconiiformes											
Great Blue Heron *Ardea herodias* (n=1)	Ad	+	+++	+++	+++	+	+++	+++	+	+++	–
Gaviiformes											
Red-throated Loon *Gavia stellata* (n=1)	Ad	ND	ND	+++	+++	+++	+++	+++	–	+++	–
Gruiformes											
American Coot *Fulica americana* (n=1)	Ad	+++	–	+++	+++	+++	–	+++	–	+++	–
Stanley Crane *Anthropoides paradiseus* (n=1)	4 w	+++	+++	+++	+++	+++	+++	+++	+++	+++	–
Pelecaniformes											
American White Pelican *Pelecanus onocrotalus* (n=1)	Ad	ND	ND	ND	ND	+++	+++	+++	–	+++	–

b. Cell type distribution of MAA and SNA lectins and types of MAA isoforms observed in respiratory and intestinal tracts of aquatic bird species.

Order/species	Cell type distribution									
	Nasal turbinates		Trachea		Lung (bronchi)		Duodenum/ jejunum		Ileum/caeca	
	MAA	SNA	MAA	SNA	MAA	SNA	MAA	SNA	MAA	SNA
Anseriformes										
Mallard *Anas platyrhynchos* (n=3)	** Cil[ab]	–	*MAA2 Cil[a]	Cil[a]	*MAA2 Cil[a]	Cil	MAA2 Gc	–	*MAA2 Ent[a]	Ent[a]
Blue-winged teal *Anas discors* (n=3)	*MAA2 Cil[ab]	–	** Cil[a]	Cil[a]	*MAA2 Cil	Cil	** Ent[a]	Ent	** Ent[a]	Ent[a]
Cinnamon teal *Anas cyanoptera* (n=1)	** MuG	–	** Cil[a]	Cil	** Cil	Cil	** Ent[a]	Ent[a]	** Ent[a]	Ent[a]

b. Cell type distribution of MAA and SNA lectins and types of MAA isoforms observed in respiratory and intestinal tracts of aquatic bird species.

Order/species	Cell type distribution									
	Nasal turbinates		Trachea		Lung (bronchi)		Duodenum/ jejunum		Ileum/caeca	
	MAA	SNA	MAA	SNA	MAA	SNA	MAA	SNA	MAA	SNA
Northern Pintail *Anas acuta* (n=3)	** Cil[ab]	Cil	*MAA2 Cil[a]	Cil[a]	*MAA2 Cil	Cil	** Gc	–	*MAA2 Ent[a]	Ent[a]
Red Head *Aythya Americana* (n=2)	** MuG	Cil	*MAA1 Cil[a]	Cil[a]	*MAA1 Gc	Cil[a]	MAA2 Ent	–	*MAA2 Ent[a]	Ent
Wood duck *Aix sponsa* (n=2)	*MAA2 MuG[a]	Gc	*MAA2 Cil[a]	Gc	*MAA2 Cil[a]	–	MAA1 Ent	Ent	** Ent[a]	Ent[a]
Black Swan *Cygnus atratus* (n=1)	** MuG	–	** Cil[a]	Gc	** Cil[a]	Gc	MAA1 Ent[a]	Gc	** Ent[a]	Ent[a]
Mute Swan *Cygnus olor* (n=1)	*MAA1 MuG	–	** Cil[a]	Gc	** Cil[a]	Gc	MAA1 Gc	–	** Ent[a]	Gc
Bar-headed goose *Anser indicus* (n=2)	** MuG	MuG	** Cil[a]	Gc	MAA2 Cil	–	*MAA2 Gc	–	** Ent[a]	Ent[a]
Cackling goose *Branta hutchinsii* (n=2)	** MuG	MuG	** Cil[a]	Gc	** Cil[a]	Cil	*MAA2 Gc	–	** Ent[a]	Ent[a]
Charadriiformes										
Dunlin *Calidris alpina* (n=2)	ND	ND	MAA1 Gc	Cil	MAA1 Gc	Cil	** Ent[a]	–	** Ent[a]	–
Sanderling *Calidris alba* (n=2)	ND	ND	** Cil	–	** Gc	–	** Gc	Ent[a]	ND	ND
Red Knot *Calidris canutus* (n=1)	** MuG[a]	–	** Gc	–	*MAA1 Gc	Cil	–	–	–	–
Ruddy turnstone *Arenaria interpres* (n=2)	ND	ND	ND	ND	** Cil[a]	Gc	** Gc	–	** Gc	–
Herring gull *Larus smithsonianus* (n=3)	MAA2 MuG	Cil[b]	MAA2 Gc	Cil[a]	*MAA2 Cil[a]	Gc	MAA2 Gc	–	*MAA2 Gc	–
Laughing gull *Larus atricilla* (n=2)	*MAA1 MuG	Cil[ab]	** Cil[a]	Cil[a]	** Cil[a]	Cil[a]	** Ent[a]	Gc	** Ent[a]	Gc
Ring-billed gull *Larus delawarensis* (n=2)	*MAA2 Cil[a]	Cil[ab]	MAA2 Gc	Gc	** Gc	Gc	** Gc	–	** Gc	Gc
Ciconiiformes										

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b. Cell type distribution of MAA and SNA lectins and types of MAA isoforms observed in respiratory and intestinal tracts of aquatic bird species.

Order/species	Cell type distribution									
	Nasal turbinates		Trachea		Lung (bronchi)		Duodenum/ jejunum		Ileum/caeca	
	MAA	SNA	MAA	SNA	MAA	SNA	MAA	SNA	MAA	SNA
Great Blue Heron *Ardea herodias* (n=1)	MAA2 MuG	MuG	MAA2 Cil[a]	Gc	** Gc	Gc	*MAA1 Gc	Gc	** Gc	–
Gaviiformes										
Red-throated Loon *Gavia stellata* (n=1)	ND	ND	MAA1 Cil	Cil	MAA1 Cil	Cil	** Gc	–	** Gc	–
Gruiformes										
American Coot *Fulica americana* (n=1)	MAA1 MuG	–	*MAA2 MuG	Gc	MAA2 Gc	–	** Ent[a]	–	** Ent[a]	–
Stanley Crane *Anthropoides paradiseus* (n=1)	** Cil[ab]	MuG	*MAA2 Cil[a]	Gc	*MAA2 Cil[a]	Gc	** Gc	Gc	** Gc	–
Pelecaniformes										
American White Pelican *Pelecanus onocrotalus* (n=1)	ND	ND	ND	ND	** Cil	Cil	** Gc	–	** Gc	–

[a] Age, weeks.

[b] The lectin binding score was graded as mild (+), moderate (++) and strong (+++). Variation in lectin binding between individual birds is separated by a slash (/).

[c] Juv, juvenile.

[d] Ad, adult.

[e] ND, not done.

Cil, ciliated cells. Gc, goblet cells. MuG, epithelial cells in mucous glands. Ent, enterocytes. ND, not done.

[a] Goblet cells also positive.

[b] Epithelial cells in mucous glands also positive. - negative;

* Predominant MAA isoform bound.

** Binding of both MAA isoforms.

Table 2

a. Lectin binding in respiratory and intestinal tracts of terrestrial bird species using *Maackia amurensis* (MAA) and *Sambucus nigra* (SNA) lectins. The lectin binding score was graded as mild (+), moderate (++) and strong (+++).Variation in lectin binding between individual birds separated by slash (/).

Order/Species	Age[a]	Lectin binding[b]									
		Nasal turbinates		Trachea		Lung (bronchi)		Duodenum/ jejunum		Ileum/caeca	
		MAA	SNA	MAA	SNA	MAA	SNA	MAA	SNA	MAA	SNA
Passeriformes											
American Crow *Corvus brachyrhynchos* (n=1)	Ad[c]	+++	+++	+++	−	ND[e]	ND	+++	+++	+++	+++
Brown-headed Cowbird *Molothrus ater* (n=1)	Ad	+++	+++	+++	+++	+++	+++	−	−	+++	+++
Eastern Meadow Lark *Sturnella magna* (n=1)	Ad	+++	+++	+++	−	ND	ND	+++	−	−	−
European Starling *Sturnus vulgaris* (n=2)	Ad	+/++	+++	+	++	++	+++	−	+++	+	+++
Kinglet *Regulus sp* (n=1)	Ad	+++	−	+++	+++	+++	+++	+++	−	+++	−
House Sparrow *Passer domesticus* (n=3)	Ad	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
House Finch *Carpodacus mexicanus* (n=2)	Ad	+++	+++	+++	+++	+++	+++	+++	−	+++	−
Zebra Finch *Taeniopugia guttata* (n=1)	Ad	+++	+++	+++	+++	−	−	+++	−	+++	+
Columbiformes											
Mourning Dove *Zenaida macroura* (n=1)	Ad	+	+++	+	+++	+	+++	−	−	++	+++
Rock Pigeon *Columba livia* (n=2)	4 w	+++	+++	+++	+++	+++	++	+++	−	+++	−
Psittaciformes											
Budgerigar *Melopsittacus undulatus* (n=2)	Ad	+	−	+	−	+++	−	+++	+++	ND	ND
Falconiformes											
American Kestrel *Falco sparverius* (n=2)	Unk[d]	ND	ND	+++	+	+++	−	+	−	−	−
Cooper's Hawk *Accipiter cooperi* (n=1)	Unk	+++	+++	+++	−	+++	−	+++	−	+++	−

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a. Lectin binding in respiratory and intestinal tracts of terrestrial bird species using *Maackia amurensis* (MAA) and *Sambucus nigra* (SNA) lectins. The lectin binding score was graded as mild (+), moderate (++) and strong (+++).Variation in lectin binding between individual birds separated by slash (/).

Order/Species	Age[a]	Lectin binding[b]									
		Nasal turbinates		Trachea		Lung (bronchi)		Duodenum/ jejunum		Ileum/caeca	
		MAA	SNA	MAA	SNA	MAA	SNA	MAA	SNA	MAA	SNA
Red-tailed Hawk *Buteo jamaicensis* (n=1)	Unk	+++	+++	++	+++	+++	+++	+++	–	+++	++
Acciipitriformes											
Bald Eagle *Haliaeetus leucocephalus* (n=1)	Ad	+++	+++	–	–	+++	+++	+	–	+	–

b. Cell type distribution of MAA and SNA lectins and types of MAA isoforms observed in respiratory and intestinal tracts of terrestrial bird species.

Order/species	Cell type distribution									
	Nasal turbinates		Trachea		Lung (bronchi)		Duodenum/ jejunum		Ileum/caeca	
	MAA	SNA	MAA	SNA	MAA	SNA	MAA	SNA	MAA	SNA
Passeriformes										
American Crow *Corvus brachyrhynchos* (n=1)	MAA1 MuG	MuG	MAA1 Gc	_[c]	ND[d]	ND	*MAA1 Gc	Ent[a]	*MAA1 Gc	Gc
Brown-headed Cowbird *Molothrus ater* (n=1)	** MuG	MuG[a]	MAA2 Gc	Gc	MAA2 Cil	Gc	–	–	*MAA1 Gc	Gc
Eastern Meadow Lark *Sturnella magna* (n=1)	** MuG	MuG[a]	*MAA2 Cil[a]	–	ND	ND	*MAA1 Gc	–	–	–
European Starling *Sturnus vulgaris* (n=2)	** Cil[ab]	MuG	MAA1 Gc	Gc	*MAA1 Gc	Gc	–	Ent[a]	** Ent	Gc
Kinglet *Regulus sp* (n=1)	** MuG	–	*MAA2 Cil	Cil	** Gc	Cil	** Ent[a]	–	** Ent[a]	–
House Sparrow *Passer domesticus* (n=3)	** Cil[ab]	MuG	*MAA2 Cil[a]	Cil	MAA1 Cil	Cil	** Ent[a]	Gc	** Ent[a]	Gc
House Finch *Carpodacus mexicanus* (n=2)	** MuG	MuG	** Cil[ab]	MuG	MAA1 Gc	Gc	** Ent[a]	–	** Ent[a]	–
Zebra Finch *Taeniopugia guttata* (n=1)	** Cil[ab]	MuG	** Cil[ab]	MuG	–	–	** Ent[a]	–	** Ent[a]	Ent
Columbiformes										

b. Cell type distribution of MAA and SNA lectins and types of MAA isoforms observed in respiratory and intestinal tracts of terrestrial bird species.

Order/species	Cell type distribution									
	Nasal turbinates		Trachea		Lung (bronchi)		Duodenum/ jejunum		Ileum/caeca	
	MAA	SNA	MAA	SNA	MAA	SNA	MAA	SNA	MAA	SNA
Mourning Dove *Zenaida macroura* (n=1)	MAA1 MuG	MuG	** Gc	MuG[a]	MAA2 Gc	Gc	–	–	MAA1 Gc	Ent
Rock Pigeon *Columba livia* (n=2)	*MAA1 Cil[ab]	Cil[b]	*MAA1 Gc[b]	Cil	*MAA1 Gc	Cil	*MAA1 Ent[a]	–	** Ent[a]	–
Psittaciformes										
Budgerigar *Melopsittacus undulatus* (n=2)	MAA1 MuG	–	MAA1 MuG	–	** Cil[a]	–	** Ent	Ent	ND	ND
Falconiformes										
American Kestrel *Falco sparverius* (n=2)	ND	ND	** Cil[a]	Gc	** Cil[a]	–	MAA1 Ent	–	–	–
Cooper's Hawk *Accipiter cooperi* (n=1)	** MuG	MuG	** Cil[a]	–	** Cil[a]	–	** Ent[a]	–	** Ent[a]	–
Red-tailed Hawk *Buteo jamaicensis* (n=1)	** Cil[ab]	MuG	MAA2 Cil	Gc	MAA2 Cil	Gc	** Ent[a]	–	** Ent[a]	Ent[a]
Acciipitriformes										
Bald Eagle *Haliaeetus leucocephalus* (n=1)	MAA2 MuG	MuG	–	–	MAA2 Gc	Gc	MAA2 Gc	–	MAA2 Gc	–

[a] Age, weeks.

[b] The lectin binding score was graded as mild (+), moderate (++) and strong (+++). Variation in lectin binding between individual birds is separated by a slash (/).

[c] Ad, adult.

[d] Unk, unknown.

[e] ND, not done.

Cil, ciliated cells. Gc, goblet cells. MuG, epithelial cells in mucous glands. Ent, enterocytes.

[a] Goblet cells also positive.

[b] Epithelial cells in mucous glands also positive.

[c] –, negative.

[d] ND, not done.

* Predominant MAA isoform bound.

** Binding of both MAA isoforms