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Is low pathogenic avian influenza virus virulent for wild waterbirds?

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Although low pathogenic avian influenza virus (LPAIV) is traditionally considered to have adapted to its wild waterbird host to become avirulent, recent studies have suggested that LPAIV infection might after all have clinical effects. Therefore, I reviewed the literature on LPAIV infections in wild waterbirds. The virulence of LPAIV was assessed in 17 studies on experimental infections and nine studies on natural infections. Reported evidence for virulence were reductions in return rate, feeding rate, body weight, long-range movement and reproductive success, as well as pathological changes in infected organs. However, major caveats in studies of experimental infections were unnatural route of LPAIV inoculation, animal husbandry not simulating natural stressors and low sensitivity of clinical assessment. Major caveats in studies of natural infections were incomplete measurement of LPAIV infection burden, quasi-experimental design and potential misclassification of birds. After taking these caveats into account, the only remaining evidence for virulence was that presence and intensity of LPAIV infection were negatively correlated with body weight. Based on this correlation, together with the demonstrated LPAIV tropism for the intestinal tract, I hypothesize that LPAIV reduces digestive tract function, and suggest how future studies could be directed to test this hypothesis.

1. Introduction

Wild waterbirds are the original reservoir for 16 haemagglutinin subtypes (H1–H16) and nine neuraminidase subtypes (N1–N9) of influenza A virus. The main wild bird species involved belong to the orders Anseriformes (mainly ducks, geese and swans) and Charadriiformes (mainly gulls and waders; [1]). From this reservoir, whole viruses or gene segments thereof may cross the species barrier to new hosts, including humans and domestic animals [2]. In doing so, the virus needs to adapt to its new host, and—according to the trade-off model—part of this adaptation consists of reaching a new level of virulence that confers optimal fitness to the virus [3]. Much research has been carried out on the virulence of influenza A virus for humans and domestic animals [4–7]; by contrast, little research has been done on its virulence for wild waterbirds, and the results are ambivalent.

Influenza A viruses circulating in birds—avian influenza viruses—may be divided into the categories 'low pathogenic' and 'high pathogenic', based on their virulence for chickens [8]. When low pathogenic avian influenza viruses (LPAIVs) were first isolated from wild waterbirds in the 1970s [9], the absence or rarity of clinical signs in both naturally and experimentally infected birds led to the conventional wisdom that LPAIV in wild waterbirds is avirulent, possibly owing to the adaptation of the virus to its host over many centuries [10,11]. However, this conclusion has been questioned in recent years. For example, van Gils *et al*. [12, p. 1] suggested that 'LPAIV infections in wild migratory birds may have higher clinical and ecological impacts than previously recognized'. To try to clarify this matter, it seemed appropriate to review the literature on the subject. I did this by reviewing published articles for evidence of virulence in natural and experimental LPAIV infections of anseriform and charadriiform species.

Knowing the level of virulence of LPAIV for wild birds is important for several reasons. It may help us to understand how the virulence of a high pathogenic avian influenza virus would need to change so that it can be maintained in wild bird populations. For example, the current high pathogenic avian influenza virus of the subtype

H5N1 circulating in poultry in several countries regularly spills over into wild birds and there is concern that it may adapt and become endemic in these species, which would make eradication of this virus virtually impossible [13]. Furthermore, knowledge of the level of virulence of LPAIV for wild birds is necessary to understand its epidemiology, to evaluate the effect of LPAIV infection on the health of wild birds at the level of the individual and of the population, to understand the co-evolution of LPAIV and its wild bird hosts, and to understand the selective pressures that maximize the fitness of LPAIV in its wild bird hosts.

2. Methodology

I retrieved articles published before 1 January 2013 from PubMed and Web of Knowledge, as well as any relevant citations in the retrieved articles, on experimental and natural influenza virus infections in wild waterbirds. I limited myself to infections in which the virus was a LPAIV from an anseriform or charadriiform species, either in its free-living or domesticated form (e.g. the Pekin duck as a domesticated form of the mallard (*Anas platyrhynchos*)), and the host was an anseriform or charadriiform species. Specifically, I did not include studies using high pathogenic avian influenza viruses, of which the interaction with the host is very different from that of LPAIV [14,15].

I defined virulence (synonym: pathogenicity) as 'the ability to cause disease'. This was based on standard dictionary definitions. Dorland's medical dictionary [16, p. 741] defines virulence as 'the degree of pathogenicity of an organism' and pathogenicity as 'the quality of producing or the ability to produce pathologic changes or disease'. Webster's English dictionary [17, p. 2662] defines disease as 'an impairment of the normal state of the living animal or plant body or of any of its components that interrupts or modifies the performance of the vital functions' and pathologic as 'altered by disease'. It should be noted that other definitions of virulence are in use in the scientific literature on host–pathogen interactions. For example, Anderson & May [18] define virulence as lethality, that is, the ability of a pathogen to cause death. This definition does not account for sublethal effects. Schall [19] defines virulence as 'any consequence of infection that reduces the host's lifetime reproductive success'. It is often impossible to know whether a given consequence will reduce the host's lifetime reproductive success, and, therefore, whether this particular consequence fits the definition. Furthermore, it excludes any diseases that do not impact reproductive fitness.

In studies of both experimental and natural infections, the infection status—LPAIV-positive or LPAIV-negative—was determined by detection of influenza A viral RNA by reverse transcriptase-polymerase chain reaction, by detection of infectious influenza A virus by virus culture, or both. For live birds, these tests were performed on cloacal swabs, on pharyngeal swabs or both. Additionally, for dead birds, these tests were performed on internal organs, in some studies supplemented by detection of influenza A viral antigen detection by immunohistochemistry. In two studies [12], the presence of antibody against influenza A virus in the serum was determined either by enzyme-linked immunosorbent assay and by a haemagglutination inhibition assay against all known haemagglutinins [12], or by a haemagglutination assay against subtype H13 [20]. In another study [21], the threshold cycle value of the real-time reverse transcriptase-polymerase chain reaction was used as a measure of the shedding intensity of LPAIV.

3. Evidence for virulence from experimental infections

By literature review, there were a total of 17 studies that reported on virulence of experimental infections of wild or domesticated anseriform or charadriiform species with LPAIV strains originating from wild anseriform or charadriiform species (table 1). All studies were performed on captive birds in the laboratory, except for one study [35], which was performed on free-living birds in the field. All studies used mallards (10 studies) or domestic ducks (six studies), except one study that used Bewick's swans (*Cygnus bewickii*; [35]) and another study that used redheads (*Aythya americana*), wood ducks (*Aix sponsa*) and laughing gulls (*Leucophaeus atricilla*) in addition to mallards [34]. Most studies used H5 or H7 subtypes alone (nine studies) or in combination with other subtypes (five studies). The interest in these subtypes is based on their ability to change from a low to a high pathogenic form in poultry. Four studies [24,35–37] used only subtypes other than H5 or H7. Most studies inoculated via the upper digestive tract (oral cavity, pharynx and oesophagus; five studies), via the upper respiratory tract (nasal cavity, choanae and trachea; six studies) or a combination of the two (five studies). Other routes of inoculation were intravenous (two studies), aerogenic (one study), supra-ocular (one study, in combination with nasal cavity and pharynx) and intrarectal (one study). The dose of the virus inoculum per bird ranged from 1×10^5 to $1 \times 10^{8.7}$ median egg infectious dose or comparable unit of measurement.

(a) Mortality

No mortality was recorded in any of the 17 studies on experimental infections (table 2). This includes one study on Bewick's swans, in which no significant difference in mortality (based on reduced resighting rate at the wintering area in the winter after banding) was observed between 13 Bewick's swans inoculated intratracheally and intra-orally with LPAIV and a control group of 16 swans sham-inoculated with phosphate-buffered saline solution (PBS; [35]).

(b) Body weight

The body weights of 16 mallards increased significantly less in the first week after combined intratracheal and intra-oesophageal inoculation with a mallard-origin LPAIV—which replicated abundantly in the digestive tract—than those of mallards after inoculation with a ringed-bill gull (*Larus delawarensis*)-origin LPAIV—which replicated poorly in the digestive tract ([36]; table 2). This resulted in a difference of 20.2 g in average body weight gain between groups at 5 days post inoculation (dpi; [36]). By contrast, there was no significant difference in body weight at 7 and 15 dpi between a group of 27 mallards inoculated intravenously with LPAIV and a control group inoculated with sterile saline solution [28]; there was no significant difference in body weight—based on visually scored abdominal profile index—between 13 Bewick's swans inoculated intratracheally and intra-orally with LPAIV and a control group of 16 swans sham-inoculated with PBS [35]; and there was no difference in body weight gain between a group of five mallards inoculated intrachoanally with LPAIV and two sham-inoculated control birds [37]. This change was not analysed statistically. Body weights were not reported in the other 13 studies.

(c) Body temperature

A body temperature increase of about 0.5°C, starting at the time of virus shedding and lasting about 2 days, was

Table 1. Experimental and natural low pathogenic avian influenza virus infections of wild waterbirds. (AER, aerosol; CHO, intrachoanal; IV, intravenous; NAS, intranasal; OCU, supra-ocular; OES, intra-oesophageal; ORA, intra-oral; PHA, intrapharyngeal; REC, intrarectal; TRA, intratracheal. EID_{50}, median egg infectious dose; $TCID_{50}$, median tissue culture infectious dose; PFU, plaque-forming units. n.a., not appropriate.)

type of infection	host species	host age	source of virus	virus subtype	route of inoculation	dose of inoculum	reference
experimental	Pekin duck	eight to 20-week-old	free-living mallard	H5N3	AER	not specified[a]	[22]
	Pekin duck	one to four-month-old	feral teal	H5N2	NAS or ORA	1×10^8 EID_{50}	[23]
	Pekin duck	four to eight-week-old	mallard	H2N2	ORA or REC	1×10^7 EID_{50}	[24]
	Pekin duck	eight-week-old	35 isolates, shorebirds and gulls	several	ORA and TRA	1×10^7 EID_{50}	[25]
	mallard	adult	free-living duck	H5N2	ORA and TRA	1×10^7 EID_{50}	[26]
	mallard	two-week-old	seven isolates, bl.-w. teal (one) and mallard (six)	several	IV	1.1×10^7 to 7.1×10^8 EID_{50}	[27]
	mallard	seven-month-old	hunter-killed mallard	H5N1	IV	1.25×10^5 EID_{50}	[28]
	mallard	two-week-old	hunter-killed mallard	H5N1	IV	1.1×10^6 EID_{50}	[28]
	comm. layer type duck	three-week-old	goose	H5N2	NAS	$1 \times 10^{7.2}$ EID_{50}	[29]
	Cherry Valley dom. duck	five-week-old	migratory ducks	H5N1	NAS	1×10^8 EID_{50}	[30]
	mallard	13-week-old	mallard and duck	H4N6 and H5N2	OCU, NAS and PHA	1×10^6 EID_{50}	[31]
	mallard	three-month-old	mallard	H7N7	OES	$1 \times 10^{8.7}$ EID_{50}	[32]
	mallard	three-month-old	mallard	H5N2	OES	$1 \times 10^{8.7}$ EID_{50}	[32]
	mallard	juvenile	northern pintail	H5N9	OES and PHA	1.5×10^6 PFU	[33]
	mallard	10–16-week-old	several, all from wild mallards	several	CHO	1×10^6 EID_{50}	[34]
	redhead	10–16-week-old	several, all from wild mallards	several	CHO	1×10^6 EID_{50}	[34]
	wood duck	10–16-week-old	several, all from wild mallards	several	CHO	1×10^6 EID_{50}	[34]
	laughing gull	12-week-old	several, all from wild mallards	several	CHO	1×10^6 EID_{50}	[34]
	Bewick's swan	adult	mallard	H4N6	ORA and NAS	1×10^8 $TCID_{50}$	[35]
	mallard	22-week-old	mallard and gull	H2N3 and H13N6	OES and TRA	1×10^8 EID_{50}	[36]
	mallard	four-week-old	mallard	H3N8	CHO	$1 \times 10^{6.5}$ EID_{50}	[37]
	mallard	four-week-old	mallard	H3N8 and H5N2	NAS	1×10^5 to 1×10^6 EID_{50}	[38]

(*Continued.*)

Table 1. (*Continued.*)

type of infection	host species	host age	source of virus	virus subtype	route of inoculation	dose of inoculum	reference
natural	Bewick's swan	adult and yearling	n.a.	H6N8 and H6N2	n.a.	n.a.	[12]
	several shorebirds	various ages	n.a.	several	n.a.	n.a.	[39]
	mallard	various ages	n.a.	not specified	n.a.	n.a.	[21]
	greater white-fronted goose	various ages	n.a.	several	n.a.	n.a.	[40]
	ring-billed gull	three- and five-week-old	n.a.	H13N6	n.a.	n.a.	[20]
	mallard	two-month-old	n.a.	several	n.a.	n.a.	[41]
	Bewick's swan	various ages	n.a.	not specified	n.a.	n.a.	[35]
	black-headed gull	juvenile	n.a.	H13N8, H16N3	n.a.	n.a.	[42]
	ruddy turnstone	various ages	n.a.	not specified	n.a.	n.a.	[43]

[a]Birds were exposed to virus aerosols ranging from 3.3×10^3 to 1.6×10^5 EID_{50} per litre of air.

measured in four of six intra-oesophageally inoculated mallards that had a subcutaneous electronic thermometer ([32]; table 2). This change was not analysed statistically. In three mallards with an intracoelomic transponder recording body temperature, there was a non-significant trend on day 1 after combined intratracheal and intra-oesophageal inoculation with LPAIV, whereby the average body temperature was 0.4°C higher than during the 6 days prior to inoculation [36]. Body temperatures were not reported in the other 15 studies.

(d) Other clinical signs

Clinical signs other than reduced body weight and increased body temperature were reported present in one of 15 studies [28]: a group of 50 mallard hens inoculated intravenously with LPAIV had significantly lower egg production (207 eggs) in the first week after inoculation than a control group inoculated with PBS (242 eggs). No clinical signs were reported in the other 14 studies (table 2). This included one study in which intra-oesophageal inoculation of LPAIV into six mallards was not associated with any change in activity level, based on the number of movements per minute measured by use of a surgically implanted subcutaneous transponder [32]; and one study of Bewick's swans, in which there was no significant difference between 13 birds inoculated intratracheally and intra-orally with LPAIV and a control group of 16 swans sham-inoculated with PBS in bite rate or bites per produced faecal dropping [35].

(e) Gross lesions

Multifocal pulmonary consolidation was observed at 2, 3 and 4 days after combined intra-oral and intratracheal inoculation of LPAIV into mallards ([26]; table 2). Similarly, multifocal pulmonary congestion and consolidation were observed at 1, 3 and 6 days after combined intra-oesophageal and intratracheal inoculation of LPAIV into mallards [36]. Additionally, opacity of the air sacs was observed at 1 dpi, and accumulation of mucus in the pharyngeal cavity and choanae was observed at 6 dpi. These lesions were absent in sham-inoculated birds [36]. Other studies reported absence of gross lesions at 1–6 [38], 3–4 [23], 7 [32] and 21 dpi [34]. Gross lesions were not recorded in the remaining 11 studies.

(f) Histological lesions

In the digestive tract, disorientation and absence of columnar epithelial cells lining the cavity of bursa of Fabricius were observed at 3 days after intrarectal inoculation with LPAIV into Pekin ducks. Serial sections of the same tissue showed influenza virus antigen in columnar epithelium by immunofluorescence ([24]; table 2). By contrast, no histological lesions were observed in the intestine or bursa of Fabricius at 1, 3 or 6 days after combined intratracheal and intra-oesophageal inoculation with LPAIV into mallards [36], or at 1 to 4 days after intranasal inoculation with LPAIV into mallards [38], despite abundant influenza virus antigen expression in epithelial cells of both intestine and bursa by immunohistochemistry (figure 1).

In the respiratory tract, tracheitis, pneumonia and air-sacculitis were observed at 2, 3 and 4 days after combined intra-oral and intratracheal inoculation of LPAIV into mallards [26]. Tracheitis was characterized by infiltration of mainly lymphocytes and macrophages in the tracheal lamina propria and epithelium. Pneumonia was characterized by infiltration of

Table 2. Evidence of virulence in wild waterbirds experimentally or naturally infected with low pathogenic avian influenza virus. (1, unnatural route of inoculation; 2, animal husbandry not simulating natural stressors; 3, low sensitivity of clinical assessment; 4, incomplete measure of LPAIV infection burden; 5, quasi-experimental design; 6, potential misclassification of birds. Dashes denote not stated.)

			clinical signs			lesions					
type of infection	host species	mortality[a]	lower body weight	higher body temperature	other	gross	histological	reduced long-range movement	reduced reproductive success	caveats to consider	reference
experimental	Pekin duck	no	—	—	no	—	—	—	—	1,2,3	[22]
	Pekin duck	no	—	—	no	no	—	—	—	2,3	[23]
	Pekin duck	—	—	—	—	—	yes	—	—	1,2,3	[24]
	Pekin duck	no	—	—	no	—	—	—	—	1,2,3	[25]
	mallard	no	—	—	no	yes	yes	—	—	1,2,3	[26]
	mallard	—	—	—	—	—	no	—	—	1,2,3	[27]
	mallard	no	no	—	yes	—	—	—	—	1,2,3	[28]
	comm. layer type duck	no	—	—	no	—	—	—	—	2,3	[29]
	Cherry Valley dom.duck	no	—	—	no	—	no	—	—	2,3	[30]
	mallard	no	—	—	no	—	—	—	—	2,3	[31]
	mallard	no	—	yes	no	no	no	—	—	2,3	[32]
	mallard	—	—	—	no	—	—	—	—	2,3	[33]
	several species[b]	no	—	—	no	no	no	—	—	2,3	[34]
	Bewick's swan	no	no	—	no	—	—	no	no	3,4,6	[35]
	mallard	no	no	—	no	—	—	—	—	2,3	[37]
	mallard	no	yes	yes	no	yes	yes	—	—	1,2,3	[36]
	mallard	no	—	—	no	no	yes	—	—	2,3	[38]
natural	Bewick's swan	no	yes[c]	—	yes	—	—	yes	—	4,5,6	[12]
	several shorebirds	—	—	—	—	—	—	—	—		[39]
	mallard	—	yes	—	—	—	—	yes	—	4,5,6	[21]
	gr. white-fronted goose	no	yes	—	—	—	—	no	—	5	[40]
	ring-billed gull	—	—	—	no	—	no	—	—		[20]
	mallard	—	—	—	—	no	no	—	—		[41]
	Bewick's swan	yes	no	—	no	—	—	no	yes	4,5,6	[35]
	black-headed gull	—	—	—	—	—	no	—	—		[42]
	ruddy turnstone	no	—	—	—	—	—	—	—	4,5,6	[43]

[a]In studies of natural infection, probability of resighting of banded birds was used as a proxy for mortality.
[b]Mallard, redhead, wood duck and laughing gull.
[c]Based on abdominal profile index.

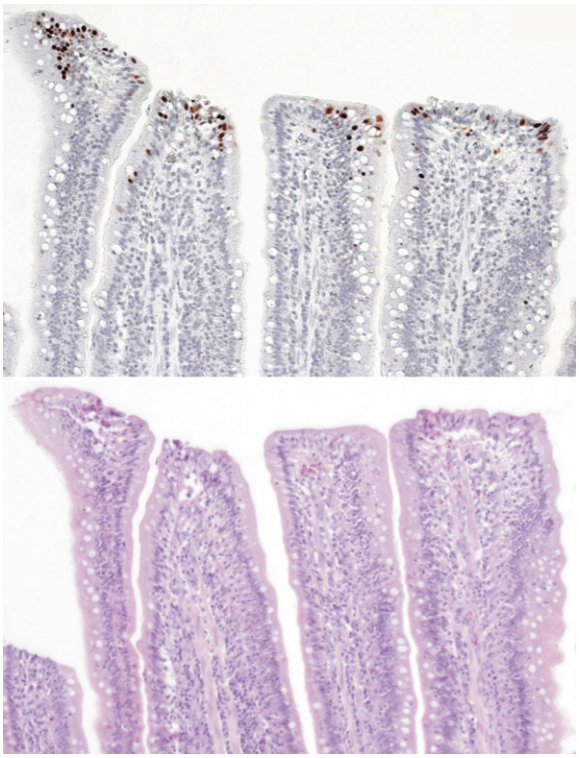

Figure 1. Microscopic view of the small intestine of a mallard, 3 days after intratracheal and intra-oesophageal inoculation with low pathogenic avian influenza virus, subtype H2N3. Many epithelial cells lining the mucosal villi express influenza virus antigen, visible as brown staining (*a*). Immunohistochemistry stain with mouse anti-influenza A virus nucleoprotein as primary antibody, and haematoxylin counterstain. However, in a serial section (*b*) of the same tissue as in (*a*), there is no evidence of necrosis or inflammation. Haematoxylin and eosin stain. Reprinted from Daoust *et al*. [36].

mainly lymphocytes and macrophages in between pulmonary lobules and around airways and blood vessels, by infiltration of lymphocytes and macrophages mixed with oedema fluid and fibrin in parabronchial and atrial lumina and by both desquamation and hyperplasia of parabronchial and atrial epithelium. Airsacculitis was characterized by infiltration of lymphocytes and macrophages, mixed with oedema fluid, into airsac walls. There was intermittent influenza virus antigen expression in epithelial cells of the airways by immunohistochemistry, but not in the lung tissue, including consolidated areas. Sham-inoculated birds had much milder infiltration of inflammatory cells in lungs and trachea.

A locally extensive interstitial, exudative and proliferative pneumonia was observed at 1, 2 and 3 days after combined intratracheal and intra-oesophageal inoculation of both mallard-origin and ring-billed gull-origin LPAIV into mallards, associated with virus antigen expression in epithelium of air capillaries, air sacs, parabronchi and bronchi, including in consolidated lung tissue [36]. However, there was no clear evidence of tracheitis or airsacculitis associated with LPAIV infection. Sham-inoculated control birds either had no or much milder pulmonary lesions.

A mild to moderate lymphocytic tracheitis and laryngitis were observed at 1 and 2 days after intranasal inoculation of LPAIV into mallards [38]. This was associated with virus antigen expression in a few tracheal and laryngeal epithelial cells.

Four other studies reported the absence of histological lesions at 3 and 14 [30], 3 and 5 [27], 7 [32] and 21 dpi [34]. Histological lesions were not recorded in the remaining nine studies.

(g) Reduction in long-range movements or reproductive success

No significant difference between 13 Bewick's swans inoculated intratracheally and intra-orally with LPAIV and a control group of 16 swans sham-inoculated with PBS was seen in mean dispersal distance at four weeks after capture or in the reproductive success 1 year after capture [35]. Long-range movements and reproductive success were not studied in the 16 other studies.

4. Evidence for virulence from natural infections

By literature review, there were nine studies that reported on virulence of natural LPAIV infections of wild anseriform or charadriiform species (table 1). Six of these [12,21,35,39,40,43] were mark–recapture studies that included observations on body weight, behaviour, long-range movements and reproductive success. The three remaining studies [20,41,42] were pathology studies that examined the association between LPAIV infection and the presence of lesions.

(a) Mortality

There was evidence of mortality (based on reduced resighting rate at the wintering area 1 year after banding) in one study on Bewick's swans: 19 birds infected with LPAIV at the beginning of winter had significantly lower probability of being seen the following winter than 63 uninfected birds (infected: 42%, uninfected: 68%; Fisher's exact test $p = 0.038$). This was most apparent in juvenile swans, which were less than half as likely to return when they had been infected compared with those that had not been infected (infected: 27%, uninfected: 63%; Fisher's exact test $p = 0.079$; [35]; table 2). There was no evidence for mortality in three studies: 1 year after catching Bewick's swans, three out of the four banded seropositive birds (75%) and five out of eight banded seronegative birds (63%) had been seen back at their wintering grounds, suggesting that the survival rate was not affected [12]; LPAIV-positive ($n = 67$) and LPAIV-negative greater white-fronted geese (*Anser albifrons*; $n = 1722$) wintering in The Netherlands showed no significant differences in resighting after capture [40]; and resighting rates of ruddy turnstones (*Arenaria interpres*) in the year following capture were not significantly different between LPAIV-negative birds (494 of 1070, 46%) and LPAIV-positive birds (73 of 163, 45%; [44]). Mortality was not recorded in the remaining five studies.

(b) Lower body weight, higher body temperature and other clinical signs

The body weight of mallards ($n = 3389$) was significantly lower for LPAIV-positive birds than for LPAIV-negative birds ([21]; table 2). Also, the body weight of mallards ($n = 260$) was lower for juvenile mallards shedding more LPAIV than for juvenile mallards shedding less LPAIV [21]. In one of four study years, 2008–2009 ($n = 1510$), greater white-fronted geese with a lower body weight had a higher probability of LPAIV infection [40]. Hanson *et al*. [39] did not perform structural comparison of LPAIV infection and body weight, but mentioned that a ruddy turnstone found positive for LPAIV on 16 May 2002 had gained 45 g when recaptured on 3 June 2002 and weighed 9 g more than the average of 30 other

ruddy turnstones captured on 3 June 2002. In two Bewick's swans infected with LPAIV at spring migration, the body weight increase—based on rate of change in visually scored abdominal profile index—was lower than in four uninfected birds, whereas body weight at capture and abdominal profile index upon departure did not differ [12]. However, in a follow-up study with a larger number of Bewick's swans ($n = 82$, of which 19 were infected at capture), there was no significant difference between infected and uninfected birds in body weight at capture, abdominal profile index at departure, or rate of change in abdominal profile index [35]. Body weights were not reported in the other four studies. Body temperatures were not reported in any of the nine studies.

Other than loss in body weight, the only clinical signs reported were in two LPAIV-infected Bewick's swans, which had lower food intake than four uninfected birds, based on a lower bite rate and fewer bites per produced faecal dropping [12]. However, in a follow-up study with a larger number of Bewick's swans ($n = 82$, of which 19 were infected at capture), there was no significant difference between infected and uninfected birds in bite rate or bites per produced faecal dropping [35]. Other clinical signs were reported absent in one other study [20] and were not recorded in the remaining four studies.

(c) Gross and histological lesions

Gross lesions were absent in mallards infected with LPAIV ([41]; table 2). Gross lesions were not reported in the other two pathology studies. No histological lesions were present at the sites of LPAIV infection—based on expression of influenza viral antigen—in the epithelium of cloacal bursa and intestine of nine mallards [41] and 15 black-headed gulls (*Chroicocephalus ridibundus*; [42]) infected with LPAIV. No influenza virus antigen expression was detected in other tissues tested: respiratory tract (mallards and black-headed gulls) and other major organs (mallards only). These findings suggest that natural LPAIV infection in these two species is limited to the intestinal tract and cloacal bursa and causes no or minimal histological lesions. In 240 ring-billed gulls, seropositivity against influenza virus was significantly associated with a higher probability of myocarditis, interstitial nephritis, pancreatic infiltration, periportal infiltration and/or necrosis in the liver [20]. However, none of these histological lesions co-localized with the expression of influenza viral antigen by immunohistochemistry; therefore, LPAIV infection could not be confirmed as their cause. Histological lesions were not reported in the remaining six studies.

(d) Reduction in long-range movements or reproductive success

Juvenile mallards in September ($n = 9$) shedding more LPAIV had longer staging periods in Ottenby, Sweden ([21]; table 2). In two Bewick's swans infected with LPAIV at spring migration, the departure date from the study site in The Netherlands was later and the next stopover site was nearer in eight to ten uninfected birds [12]. In a follow-up study with a larger number of Bewick's swans ($n = 82$, of which 19 were infected at capture), LPAIV-positive birds had a tendency to remain closer to the catch site than birds that were LPAIV-negative at capture. Four weeks after capture [35], infected birds had displaced roughly half the distance of uninfected birds, although these differences were not significant at the $p = 0.05$ level after accounting for the effect of the day in December on which they were caught. LPAIV-positive and LPAIV-negative greater white-fronted geese wintering in The Netherlands showed no differences in mean dispersal distance in the first 12 days after testing [40]. Long-range movements were not studied in the five other studies.

Reduced reproductive success was observed in Bewick's swans infected with LPAIV: 28 per cent (9 out of 32) of the uninfected birds of known breeding status were seen to return with young in the winter after capture, compared with none (0 out of 5) of the infected birds. This difference, while based on a small sample size, was significant: breeding status of adults in the year after capture differed on the basis of infection status in the winter of capture ($\chi^2 = 2.87$, $p = 0.09$; ordinal logistic regression including the effect of prior breeding status ($\chi^2 = 5.43$, $p = 0.020$; [35]). Similarly, neither of the infected swans from the 2005/2006 winter, studied by van Gils *et al.* [12] returned with offspring the following winter, while two of eight uninfected birds from that season returned with offspring [35].

5. Discussion

The results of this literature review need to take into account that virulence of LPAIV infection, if present, may be dependent on host species and only a small proportion of the species comprising the Anseriformes and Charadriiformes are represented in these studies. Furthermore, the caveats involved in studies of both experimental and natural LPAIV infections need to be taken into account. To quote Gary Wobeser, a long-time researcher of wildlife diseases [44, p. 150]: 'It is inappropriate to conclude that an agent has no significant effect on a wild species based on tests done in artificial situations, in which the test animals have abundant food and are protected from other stressors. It may be equally misleading to judge the significance of a disease by comparing naturally infected and naturally uninfected animals'.

(a) Important caveats of experimental infection studies

Important caveats involved in studies of experimental infections are related to route of inoculation, animal husbandry and clinical examination. If we consider free-living waterbirds to be infected with LPAIV by faecal–oral transmission [11], then inoculation in the nasal cavity or on the eye (exposed by dabbling behaviour), in the oral cavity, choanae, pharynx or oesophagus (exposed by ingestion of contaminated water) may be considered as good approximations of the natural route of infection. By contrast, inoculation in the trachea, in the rectum, in the vein, or by aerosol may be considered as poor approximations of this route and may cause different organs to be infected and diseased than observed in natural LPAIV infection. Intratracheal inoculation of LPAIV into mallards was associated with pneumonia [26,36]. By contrast, pneumonia from LPAIV infection was detected neither in naturally infected birds [20,41,42] nor in birds inoculated experimentally via another route than the trachea [23,32,34,38]. Intravenous inoculation of LPAIV into mallards was associated with reduced egg production [28]. Intrarectal inoculation of LPAIV into Pekin ducks was associated with histological damage to the cloacal bursa [24], but this lesion was not detected in naturally infected mallards [41]. Together, these findings suggest that pneumonia, reduced egg production and damage

to the cloacal bursa are artefacts of an artificial route of inoculation, and are not relevant in free-living wild waterbirds.

A second major caveat of experimental infection studies is that the husbandry of captive birds in the laboratory results in very different circumstances from those of free-living birds in the field. In the field, a bird undergoes stress from adverse weather, competition for food, migration, egg production and moulting [44, p. 16]. Within the free-living bird's limited energy budget, there is competition between the energy required to deal with these stressors and the energy required to mount an immune response against LPAIV infection [45]. By contrast, a captive bird in the laboratory is kept at room temperature, is provided with food and water ad libitum, and generally does not need to migrate, produce eggs or moult. Because it can allocate more energy to combat infection with LPAIV, the observed virulence may be lower. Therefore, failure to observe virulence of LPAIV under laboratory circumstances, as reported in the majority of studies on experimental infections [22,23,25,27, 29–31,33,34,37] does not mean that LPAIV is not virulent for wild waterbirds under field circumstances.

A third major caveat of experimental infection studies is the lack of sensitivity to detect clinical signs, other than changes in body weight or body temperature. Most studies [22,23,25–30,32,33,38] actually did not state the method of clinical examination. Other studies [31,34,36,37] did state the method, but limited the description to the statement that birds were examined once [31,36] or twice [34,37] daily for clinical signs [31,34,36,37] or behavioural changes [34,37]. While this method will allow detection of mortality and obvious clinical signs, such as abnormal movements, panting and ruffled feathers, it will miss more subtle clinical signs, such as reduced motility, changes in allocation of time to different activities and diarrhoea. Interestingly, some of the studies of natural LPAIV infections in free-living wild waterbirds do include detailed observation of foraging behaviour [12,35], even though such observation is much more difficult to perform in the field than in the laboratory. Together, this suggests that the lack of reported clinical signs of LPAIV infection in the above studies does not rule out that clinical signs occurred; they may not have been observed because the method of clinical examination was not sensitive enough.

(b) Important caveats of natural infection studies

Important caveats involved in studies of natural infections are related to the practical difficulty of adequately measuring the burden of LPAIV infection in free-living waterbirds, to the quasi-experimental design of most of these studies and to potential misclassification of birds. Adequately measuring the burden of LPAIV infection in free-living waterbirds is difficult because of the natures of both the virus and the host. Unlike pathogens such as avian poxvirus [46] and *Mycoplasma gallisepticum* [47], which infect external organs and cause clinical signs of disease that can be diagnosed by observation of birds from a distance, LPAIV infects internal organs and requires capture and sampling of birds for diagnosis. Furthermore, the epidemiology of LPAIV in waterbirds is characterized by infection lasting one to two weeks [21,48], the possibility of serial infections during one season [21,49] and a high cumulative incidence of infection [11,50]. This means that over a period of time—for example, breeding season or migratory season—the burden of LPAIV infection, based on frequency, duration and intensity of infection, can vary substantially among individual birds. Estimation of this burden of infection would require repeated sampling at frequent intervals (e.g. twice a week) in order not to miss any infections, and identification and quantification of any LPAIV present in the samples. However, the wariness, ability to fly and migratory behaviour of free-living waterbirds makes it difficult to capture and sample them for LPAIV infection more than one time in their lifetime in most circumstances.

It is valid to use such a one-time measurement to study the association between LPAIV infection and other variables determined at the same time, for example, body weight. However, such a one-time measurement is inadequate as a measure of the LPAIV infection burden needed to study the association with variables determined weeks (time of departure from staging or stopover site), months (distance travelled) or even a year (survival rate, reproductive success) after the time of capture. For example, in a study correlating burden of LPAIV infection with survival rate, two birds may test positive for LPAIV at the time of banding. However, between the time of banding and the time of observation for survival rate (based on resighting banded birds 1 year later), one bird may have had several more LPAIV infections, while the other did not. Therefore, the cumulative burden of LPAIV infection during the study period would differ substantially between the two birds, despite having been placed in the same category 'infected'.

The second major caveat of studies of natural infections is that their design is generally quasi-experimental. This means that the birds are not assigned randomly to groups, but instead are assigned on the basis of the results of their infection status at the time of capture. It is therefore not possible to control external factors from influencing the results, so that cause and effect cannot be established [51]. For example, as discussed for the study of Latorre-Margalef *et al*. [21], if one finds in such a study of natural infection that LPAIV-infected individuals have significantly lower body weight than uninfected individuals, one may not conclude that LPAIV infection causes body weight loss, because there may be an external factor, such as immunocompetence, that influences both LPAIV status and body weight [52,53]. The conclusion is that the results from such studies need to be interpreted with great caution.

The third major caveat of studies of natural infections is that birds may be misclassified [43]. In the same example of a study on survival rate as above, where the infection status at the time of banding is correlated with the survival rate based on resighting banded birds 1 year later, one might find a bird negative for LPAIV at the time of banding. However, this bird may have abrogated a LPAIV infection just before the time of banding, or may have become infected with LPAIV between the time of banding and the time of resighting 1 year later. Despite these possibities, the bird would be classified as 'uninfected'.

6. Conclusions

One or more of the above caveats apply to most of the studies reviewed here (table 2). Therefore, these caveats need to be taken into account in the interpretation of the results of these studies. With regards to the nine studies [12,21,24,26,28, 32,35,38,40] reporting positive evidence of disease associated with or owing to LPAIV infection, only three remain after

accounting for these caveats (table 2): LPAIV inoculated intranasally into mallards caused mild to moderate tracheitis and laryngitis [38]; mallards with natural LPAIV infection had significantly lower body weight than uninfected mallards [21]; level of LPAIV shedding in naturally infected juvenile mallards was negatively correlated with body weight [21] and greater white-fronted geese with a lower body weight had a higher probability of LPAIV infection in one of four study years [40]. The latter two studies are correlational, so that a cause–effect relationship cannot be inferred.

Based on these results of decreased body weight, together with the observation that the intestine is the main site of LPAIV replication, as shown by extensive infection of intestinal epithelium in naturally infected mallards [41] and black-headed gulls [42], I hypothesize that LPAIV infection of the intestine reduces digestive tract function. Although neither gross nor histological lesions have been detected in association with LPAIV infection in these species [41,42], this does not preclude reduced digestive tract function. In mammals, rotavirus [54,55], coronavirus [55] and astrovirus [56], which also have tropism for intestinal epithelium, are known to cause clinical disease, particularly diarrhoea. However, the severity of clinical signs does not always correlate with the histological damage to the intestine. The pathogenesis of diarrhoea from these three viruses is considered to be multifactorial, involving primary damage to intestinal epithelium by virus infection, hypersecretion and malabsorption. Interestingly, rotavirus has an enterotoxin, non-structural protein 4, that is able to induce diarrhoea in absence of histological damage. Diarrhoea is one of the clinical signs exhibited occasionally by poultry infected with LPAIV [8]. Diarrhoea in LPAIV-infected wild birds has not been recorded, but this may be because they were not specifically checked for this clinical sign.

7. Future directions

Future studies, both in the laboratory and in the field, could be designed to determine whether LPAIV infection of wild waterbirds decreases digestive tract function. In the laboratory, a route of inoculation should be used that emulates faecal–oral transmission as closely as possible: intra-oral, intrapharyngeal, intra-oesophageal, intrachoanal and/or intranasal inoculation to mimic infection by dabbling in water contaminated with infected faeces. Husbandry of birds in the laboratory should emulate—as much as possible—naturally occurring stressors: limited feeding rather than ad libitum feeding, ambient rather than room temperature, exercise rather than inactivity. It may be difficult to mimic some of these naturally occurring stressors because of regulatory issues on husbandry of laboratory animals. In the field, experimental inoculation would allow birds to be randomly assigned to infected and uninfected groups, and so improve the ability to prove cause–effect relationships of LPAIV infection. Both in the laboratory and in the field, clinical examination of birds should emphasize variables related to digestive tract function, and should use methods that are sensitive enough to detect small differences. Both in the laboratory and in the field, such variables include proportion of time allocated to different activities, including foraging, bite rate, rate of defaecation and abdominal profile index. Additionally, in the laboratory, variables such as change in body weight, diarrhoea and food conversion rate could be measured.

It is clear from this review that LPAIV infection does not cause direct mortality in wild waterbirds. However, even a small reduction in digestive tract function could have a substantial effect on free-living wild waterbirds, because they delicately balance energy intake and energy output. This balance is particularly difficult to maintain at the time of migration, when they need to find high-quality food at staging and stopover sites in order to satisfy the high energy demands of long-distance flying and—in spring—of subsequent breeding (reviewed in [35,57]). Reduced digestive tract function, and thus reduced food conversion, means that birds need to find more high-quality food for the same level of refuelling, delaying the timing of their migration. Delayed migration may result in reduced availability of suitable food at the next stopover site [58], and delayed arrival at the breeding ground can decrease a bird's chance to occupy a prime breeding site [59] and reduce the survival prospects of its offspring [60]. Therefore, a negative effect of LPAIV infection on the digestive tract function of migratory waterbirds could potentially reduce their survival and reproductive success. At the population level, the cost of endemic LPAIV infection in a particular wild waterbird species may be a genetic adaptation against too severe reduction in digestive function.

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