Wild birds have been implicated in the expansion of highly pathogenic avian influenza virus (H5N1) outbreaks across Asia, the Middle East, Europe, and Africa (in addition to traditional transmission by infected poultry, contaminated equipment, and people). Such a role would require wild birds to excrete virus in the absence of debilitating disease. By experimentally infecting wild ducks, we found that tufted ducks, Eurasian pochards, and mallards excreted significantly more virus than common teals, Eurasian wigeons, and gadwalls; yet only tufted ducks and, to a lesser degree, pochards became ill or died. These findings suggest that some wild duck species, particularly mallards, can potentially be long-distance vectors of highly pathogenic avian influenza virus (H5N1) and that others, particularly tufted ducks, are more likely to act as sentinels.

The currently ongoing outbreaks caused by highly pathogenic avian influenza virus (HPAIV) of the subtype H5N1 are of concern not only to the poultry industry but also to public health (1,2). This virus, which causes a high fatality rate among infected patients, may adapt to efficient human-to-human transmission and thus initiate a new human influenza pandemic (3). Since 1996, when the ancestor virus was identified in domestic geese from China (4), outbreaks have spread and now encompass countries in Asia, the Middle East, Europe, and Africa (5). This spread of HPAIV among poultry flocks is traditionally thought to occur by transport of infected poultry, contaminated equipment, and persons associated with the poultry industry (6). HPAIV has occasionally been detected in wild birds near affected poultry flocks, but these birds have had limited or no role in virus dissemination (7). In the current outbreaks, however, wild birds are suspected of playing a major role as long-distance virus vectors.

During the expansion of HPAI (H5N1) outbreaks from Asia to Europe, 2 events implicated wild birds, particularly waterbirds, as long-distance virus vectors (8). First, virus outbreaks in 2005 rapidly spread westward from Russia and Kazakhstan in July and August to Turkey, Romania, and Ukraine in October. Wild waterbirds were suggested as a vector because the virus spread through areas that had no record of any virus presence and coincided with the fall migration of wild waterbirds between these areas. Second, at the beginning of 2006, HPAIV (H5N1) was detected in many wild waterbirds in western Europe, often in areas where no outbreaks had been detected among intensively surveyed poultry (9–12); this event overlapped with unusual waterbird movements associated with cold weather in the Black Sea area. Quantitative analysis of the global spread of HPAIV (H5N1) also supports the potential role of migratory wild birds in virus spread (13). In June and July of 2007, Germany, France, and the Czech Republic again reported HPAIV (H5N1) in wild waterbirds (14), which illustrates their ongoing involvement in the epidemiology of this viral infection.

The main argument against the view that wild waterbirds are long-distance vectors of HPAIV (H5N1) is that most wild waterbirds in which this virus was identified were either sick or dead, which suggests that they were too severely affected to spread the virus over any substantial distance (15). This argument is supported by experimental evidence that over time HPAIV (H5N1) has become more pathogenic for domestic ducks. Although domestic ducks did not show clinical disease or death from HPAIV (H5N1) isolates from 2001 or before, experimental infection of domestic ducks with an HPAIV (H5N1) isolate from 2002 caused neurologic disease and death (16). This high pathogenicity has been shown to be associated with molecular changes in the polymerase genes PA and PB1.
A. penelope

Asian wigeon (mallard)

4 species of dabbling ducks belonging to the genus *Aythya*.

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The harvested allantoic fluid had a titer (19) of $1.3 \times 10^6$ median tissue culture infectious dose (TCID$_{50}$/mL) and was diluted with phosphate-buffered saline (PBS) to obtain a final titer of $3.3 \times 10^3$ TCID$_{50}$/mL. All experiments with HPAIV (H5N1) were performed under Biosafety Level 3+ conditions.

Virus Preparation

A virus stock of influenza virus A/turkey/Turkey/1/2005 (H5N1) was prepared by 2 passages in 10-day-old embryo-transport medium (VT). Allantoic fluid had a titer (19) of $1.3 \times 10^6$ median tissue culture infectious dose (TCID$_{50}$/mL) and was diluted with phosphate-buffered saline (PBS) to obtain a final titer of $3.3 \times 10^3$ TCID$_{50}$/mL. All experiments with HPAIV (H5N1) were performed under Biosafety Level 3+ conditions.

Materials and Methods

Virus Preparation

A virus stock of influenza virus A/turkey/Turkey/1/2005 (H5N1) was prepared by 2 passages in 10-day-old embryo-transport medium (VT). Allantoic fluid had a titer (19) of $1.3 \times 10^6$ median tissue culture infectious dose (TCID$_{50}$/mL) and was diluted with phosphate-buffered saline (PBS) to obtain a final titer of $3.3 \times 10^3$ TCID$_{50}$/mL. All experiments with HPAIV (H5N1) were performed under Biosafety Level 3+ conditions.

Experimental Design

For each species, 8 birds were housed together in negatively pressurized isolator units. Each bird was inoculated with $1 \times 10^4$ TCID$_{50}$ HPAIV (H5N1), 1.5 mL intratracheally and 1.5 mL intrasophageal. We used this low dose to increase the chance of inducing a subclinical infection and to simulate field circumstances. In addition, 4 birds per duck species, which served as negative controls, were sham inoculated in the same manner with PBS-diluted sterile allantoic fluid. Each day, a qualified veterinarian scored clinical signs of disease in all birds according to a standardized list. Cloacal and pharyngeal swabs were collected in 1 mL transport medium (21) daily for the first 14 days and every 2 days thereafter.

<table>
<thead>
<tr>
<th>Common name (taxonomic name), n = 8 each</th>
<th>No. ducks with clinical signs</th>
<th>No. ducks that excreted virus from*</th>
<th>Pharynx</th>
<th>Cloaca</th>
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</thead>
<tbody>
<tr>
<td><strong>Tufted duck (Aythya fuligula)</strong>†</td>
<td>Mild 4 Severe 3</td>
<td>Virus isolation 6 RT-PCR 7</td>
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<tr>
<td><strong>Eurasian pochard (Aythya ferina)</strong>†</td>
<td>3 1</td>
<td>7 7</td>
<td>2 5</td>
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<td>0 0</td>
<td>8 8</td>
<td>0 5</td>
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<td>0 0</td>
<td>3 7</td>
<td>1 4</td>
<td></td>
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<tr>
<td><strong>Eurasian wigeon (Anas penelope)</strong></td>
<td>0 0</td>
<td>4 7</td>
<td>0 0</td>
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<tr>
<td><strong>Gadwall (Anas strepera)</strong></td>
<td>0 0</td>
<td>7 8</td>
<td>0 8</td>
<td></td>
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<tr>
<td><strong>Total</strong></td>
<td>7 4</td>
<td>35 44</td>
<td>3 27</td>
<td></td>
</tr>
</tbody>
</table>

*Positive result from any swab collected during the experiment. RT-PCR, reverse transcription–PCR.
†One bird removed after inoculation because of concurrent disease.
We randomly divided each group of 8 birds into 2
groups of 4. One group was euthanized by exsanguination
under isoflurane anesthesia for pathologic examination
at 4 dpi; the other group was monitored for virus excre-
tion until 18–21 dpi. Two ducks were removed after in-
oculation: 1 tufted duck because of concurrent aspergillo-
sis and 1 pochard because of concurrent staphylococcosis.
Also, 1 pochard and 3 tufted ducks were chosen for
pathologic examination at 4 dpi because they were dead
or moribund. Although this was not random sampling, it
does reflect the field situation because dead ducks no longer
actively excrete virus. As expected, by 2 dpi 100% of the
positive-control chickens were sick or dead, whereas the
negative-control ducks showed no clinical signs and were
euthanized at 4 dpi. Animal studies were approved by an
independent animal ethics committee and performed under
Biosafety Level 3+ conditions.

**Pathologic Examination and
Immunohistochemical Testing**

Necropsies and tissue sampling were performed ac-
cording to a standard protocol. After fixation in 10% neut-
ral-buffered formalin and embedding in paraffin, tissue
sections were examined by 1 of 2 methods: hematoxylin
and eosin staining for histologic evaluation or an immuno-
histologic method that used a monoclonal antibody against
nucleoprotein of influenza A virus as a primary antibody
for detection of influenza viral antigen (22). The positive
control was lung tissue of an HPAIV (H5N1)–infected
domestic cat; negative controls were omission of primary
antibody, substitution of primary antibody by an irrelevant
monoclonal antibody of the same isotype, and testing of
tissues from sham-inoculated ducks The following tissues
were examined: brain (cerebrum, cerebellum, brainstem),
trachea, bronchus, lung, caudothoracic or abdominal air
sac, esophagus, proventriculus, duodenum, pancreas, liver,
jejunum, ileum, cecum, colon, bursa of Fabricius, spleen,
kidney, gonad (testis or ovary), heart, pectoral muscle, and
adrenal gland.

**RT-PCR and Virus Titration**

Tissue samples were weighed and homogenized in 3
mL of transport medium by use of a homogenizer (Kine-
matica Polytron, Lucerne, Switzerland). RNA isolation and
RT-PCR were performed as described (23). Briefly, RNA
from swabs and tissue suspensions was isolated by using a
MagNaPure LC system with the MagNaPure LC Total Nu-
cleic Acid Isolation Kit (Roche Diagnostics, Almere, the
Netherlands). Real-time RT-PCR assays were performed on
an ABI Prism 7000 Sequence Detection System machine
(Applied Biosystems, Foster City, CA, USA) by using the
TaqMan EZ RT-PCR Core Reagents Kit (Applied Biosys-
tems, Nieuwerkerk a/d IJssel, the Netherlands) according
to the manufacturer’s instructions. The test used a hybrid-
ization probe (5′-6-FAM-TTT-ATT-CAA-CAG-TGG-
CGA-GTT-CCC-TAG-CAC-T-TAMRA-3′) and specified
primers (forward: 5′-GAG-AGG-AAA-GTG-GAG-
TAA-AAT-TGG-A-3′ and reverse: 5′-AGA-ATA-GAC-
CAG-CTA-CCA-TGA-TTG-C-3′) to detect the hemagglu-
tinin gene of HPAIV (H5N1). For each run the samples
were prepared and processed in parallel with several nega-
tive and positive control samples. Virus titers were deter-
mined by serial 10-fold dilution of the homogenized tissue
samples and swabs on MDCK cells, as described (19). Vi-
rus titrations were performed in quadruplicate.

**Results**

Despite the low doses of virus used to inoculate the
ducks, rates of productive infection in the 6 species were
high: 76% according to virus isolation and 93% accord-
ting to RT-PCR (Table). HPAIV (H5N1) infection caused
clinical signs of disease in only tufted ducks and pochards,
both of which are diving ducks in the genus *Aythya* (Table).
In contrast, the remaining 4 species—all dabbling ducks
belonging to the genus *Anas*—were clinically unaffected.
Clinical signs, which were more severe in tufted ducks
than in pochards, developed at 3 to 4 dpi and consisted of
labored breathing, increased recumbency, and neurologic
signs (torticollis [Figure 1, panel A], circling, loss of bal-
ance, and head tremors). Severely affected birds died or
were euthanized in a moribund state at 4 dpi. Mildly af-
fected birds recovered by 7 or 8 dpi.

Severe neurologic signs in tufted ducks and pochards
were associated with multifocal viral encephalitis. Al-
though no gross brain lesions were noted (online Appendix
Table 1, available from www.cdc.gov/EID/content/14/4/
600-appT1.htm), according to laboratory analysis these
birds had multiple foci of influenza virus antigen expres-
sion (Figure 1, panel B; online Appendix Table 2, available
from www.cdc.gov/EID/content/14/4/600-appT2.htm) as-
associated with severe necrosis and inflammation (Figure 1,
panel C) and high virus titers (10^{1.5} to 10^{3.2} TCID_{50} per g tis-
sue) in the brain (online Appendix Table 3, available from
www.cdc.gov/EID/content/14/4/600-appT3.htm). The only
other ducks with evidence of HPAIV (H5N1) infection of
the brain were gadwalls, none of which showed neurolog-
ic signs. Gadwalls had only focal influenza virus antigen
expression (Figure 1, panel D; online Appendix Table 2),
mild encephalitis (Figure 1, panel E), and low virus titers
(10^{1.5} TCID_{50} per g tissue) in the brain (online Appendix
Table 3). No other species had evidence of HPAIV (H5N1)
infection in the brain according to immunohistochemical
testing, histologic examination, or virus isolation (Figure 1,
panels F and G; online Appendix Tables 2 and 3), although
individual animals did have positive RT-PCR results for the brain (online Appendix Table 4, available from www.cdc.gov/EID/content/14/4/600-appT4.htm).

Pharyngeal excretion of HPAIV (H5N1) varied significantly among the 6 duck species (1-way analysis of variance of area under pharyngeal excretion curve up to 4 dpi, p<0.001), by virus isolation (Figure 2, panel A) and by RT-PCR (Figure 2, panel C). The ducks could be divided into a high-excretion group consisting of tufted ducks, pochards, and mallards, and a low-excretion group consisting of teals, wigeons, and gadwalls (Figure 2, panels B and D). Pharyngeal excretion also varied substantially among individuals within species (online Appendix Figures 2 and 3, available from www.cdc.gov/EID/content/14/4/600-appG2.htm and www.cdc.gov/EID/content/14/4/600-appG3.htm, respectively.). This finding was most extreme in tufted ducks and pochards, the species in which the individuals with the highest excretion level were also those showing severe clinical signs (Figure 2, panels B and D).

Pharyngeally excreted HPAIV (H5N1) likely originated from lung, air sac, or both, because these were the only tissues in the respiratory tract that had immunohistochemical evidence of virus replication (Figure 2, panels E and F; online Appendix Table 2) and because virus was frequently detected in these tissues by virus isolation (online Appendix Table 3) and RT-PCR (online Appendix Table 4). The histologic lesions corresponding to influenza virus antigen expression in these tissues were bronchointerstitial pneumonia (Figure 2, panel F) and lymphocytic airsacculitis (Figure 2, panel H). Despite frequent isolation of HPAIV (H5N1) from trachea and extrapulmonary bronchus (online Appendix Table 3), these tissues had neither histopathologic nor immunohistochemical evidence of HPAIV (H5N1) replication (online Appendix Table 2), which suggests that virus isolated from these sites at 4 dpi originated from elsewhere in the respiratory tract.

Cloacal excretion of HPAIV (H5N1) was uncommon; virus was detected in cloacal swabs of only 7% of ducks by virus isolation and 59% by RT-PCR (Table). Cloacal excretion was exceeded by pharyngeal excretion in all 6 duck species, according to virus isolation (Figure 3, panels A and B; online Appendix Figure 2) and RT-PCR (Figure 3, panels C and D; online Appendix Figure 3).

Cloacally excreted virus likely originated from pancreas, liver, or both, on the basis of the significant association between virus antigen expression in these tissues (online Appendix Table 2) and virus detection in cloacal swabs by RT-PCR (online Appendix Table 4); 7 of 8 birds with virus antigen expression in liver, pancreas, or both had PCR-positive cloacal swabs between 1 and 4 dpi, in contrast to 0 of 15 birds without virus antigen expression in these tissues (Fisher exact test, p<0.00001). In the pancreas, tufted ducks and pochards had multifocal necrosis (Figure 3, panel E), which was the most prominent gross lesion associated with HPAIV (H5N1) infection in this study (online Appendix Table 1). Virus antigen expression (Figure 3, panel F) occurred at the transition between normal parenchyma and these necrotic foci (Figure 3, panel G) and
corresponded with high virus titers (10^{1.5} to 10^{6.2} TCID_{50} per g tissue) in the pancreas (online Appendix Table 3). In the liver, widespread virus antigen expression (Figure 3, panel H) was associated with necrotizing hepatitis (Figure 3, panel I) and variable virus titers (no virus isolated to 10^{6.2} TCID_{50} per g tissue) in the liver (online Appendix Table 3). Virus produced in pancreas and liver could potentially have reached the intestinal lumen through pancreatic and bile ducts, respectively. Although virus antigen expression was detected in several other tissues, virus originating from these sites likely did not contribute to virus excretion.

It is unlikely that cloacally excreted virus originated from the intestinal, urinary, or genital tracts, although all 3 tracts empty into the cloaca. In the intestine, no virus antigen expression was found in the intestinal epithelium of any of the 23 ducks examined. Virus antigen expression was found in neurons and satellite cells in the peripheral nervous system (submucosal and myenteric plexi, mesenteric ganglia) of the small intestine (online Appendix Figure 4, panel A, available from www.cdc.gov/EID/content/14/4/600-appG4.htm), in association with necrotizing ganglionitis (online Appendix Figure 4, panel B), and in myocytes in the lamina muscularis mucosae of the colon, without associated histologic lesions. However, these tissues do not empty into the intestinal lumen. No virus antigen expression was found in tissues of urinary tract (kidney) or genital tract (testis or ovary) (online Appendix Table 2). The occasional isolation of HPAIV (H5N1) from kidney samples (online Appendix Table 3) may be explained by inadvertent sampling of overlying air sac wall, which did express influenza virus antigen.

Evidence of HPAIV (H5N1) replication was found sporadically in tissues other than those of the respiratory, digestive, and nervous systems (online Appendix Table 2). Virus antigen expression was detected in multiple foci of medullary and cortical cells of the adrenal gland (online Appendix Figure 4, panel C) and was associated with necrotizing adrenalitis (online Appendix Figure 4, panel D). Virus antigen expression was also detected in multiple foci of myocytes of the heart and was associated with myocardial necrosis.

**Discussion**

Our study shows that of the 6 wild duck species studied, the mallard is the prime candidate for being a long-distance vector of HPAIV (H5N1) because it was the only species to show abundant virus excretion without clinical or pathologic evidence of debilitating disease (Table; Figure 2, panels B and D). These findings fit with the absence of dead mallards in wild bird die-offs from HPAIV (H5N1) in Europe and Asia in 2005 and 2006 (14, 24, 25), although HPAIV (H5N1) was detected in 1 dead mallard during the recent 2007 HPAI (H5N1) outbreak in wild birds in Germany (26). Other characteristics of the mallard support its potential role as a vector (27): online Appendix Figure 1): it is the most abundant anatid species in Western Eurasia (>9 million birds); part of the population migrates long distances northeast to southwest between breeding and winter-
ing areas; and it is found on nearly every type of wetland and is very tolerant of human presence, thus forming a potential link between wild waterfowl, domestic animals, and humans.

Pochards and tufted ducks are less likely candidates as long-distance virus vectors because those individuals that excreted the most virus also developed severe neurologic disease (Figure 2, panels B and D) and therefore would not have been able to fly far before succumbing. Instead, they are more likely to act as sentinels for HPAIV (H5N1) in wild bird populations, as do mute swans (Cygnus olor) (9). However, pochards cannot be ruled out as potential vectors because some birds excreted abundant virus in absence of severe clinical signs (Figure 2, panels B and D). Our results correspond with field observations of pochards and tufted ducks involved in wild bird die-offs from HPAIV (H5N1) infection in France, Germany, and Sweden early in 2006 (14). Some of these birds showed clinical signs of neurologic disease, e.g., compulsively swimming around in circles (28). Therefore, close surveillance of these 2 Aythya species for unusual illness, particularly neurologic disease, or death should provide early warning for HPAIV (H5N1) infection in an area. Redheads (Aythya americana), which are diving ducks indigenous to North America, experimentally infected with a 2005 isolate of HPAIV (H5N1) neither showed clinical signs nor died (29). Of the 6 species tested, the 3 remaining Anas species—gadwall, teal, and wigeon—are the least likely candidates as long-distance virus vectors because they had limited virus excretion (Figure 2, panels B and D).

HPAIV (H5N1) infection in these wild ducks contrasts in pattern of excretion with that of low-pathogenicity avian influenza virus infection in wild ducks and contrasts in pattern of disease with that of HPAIV infection in chickens. Both contrasts can be explained by the specific tissue tropism of HPAIV (H5N1) in wild ducks. With regard to pattern of excretion, low cloacal excretion was associated with lack of evidence for HPAIV (H5N1) replication in intestinal epithelium of any of the 23 ducks examined (online Appendix Table 2), in contrast to most low-pathogenicity avian influenza viruses for which intestine is the main replication site (30). Instead, HPAIV (H5N1) replicated preferentially in the respiratory tract (online Appendix Tables 2 and 4), which corresponds with high pharyngeal excretion. How this preferential pharyngeal excretion of HPAIV (H5N1) affects its spread and persistence in a wild duck population remains to be determined.

Severe clinical disease in the HPAIV (H5N1)–infected tufted ducks and pochards manifested itself mainly as neurologic signs at about 4 dpi, although pathologic examination also showed virus-induced lesions in organs other than the brain. These findings differ substantially from those of HPAIV (H5N1)–infected chickens, which are characterized mainly by widespread hemorrhage and edema and death by about 2 dpi (31). Again, this contrast can be explained by differences in tissue tropism. Whereas the cardiovascular lesions in poultry are associated with widespread replication of HPAIV (H5N1) in endothelium lining the blood vessels (31), no such endotheliotropism was detected in any of 23 ducks examined.

The knowledge gained from this study has several implications for surveillance in wild ducks. Active surveillance (sampling of apparently healthy wild birds) should give priority to mallards and, to a lesser degree, pochards. Sampling should not be limited to cloacal swabs, as is the custom in surveillance for low-pathogenicity avian influenza virus, but should include pharyngeal swabs. Passive surveillance (sampling of diseased or dead birds), should pay extra attention to tufted ducks and pochards, particu-
larly those exhibiting neurologic disease. Sampling of wild duck carcasses should not be limited to cloacal, pharyngeal, and tracheal swabs and should include internal organs such as brain, trachea, lung, pancreas, liver, kidney, and spleen (online Appendix Tables 3 and 4).

Acknowledgments

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Dr Keawcharoen is a veterinarian at the Erasmus Medical Center in Rotterdam. Her research interests include the role of animal reservoirs in emerging zoonoses and the molecular biology of influenza virus.

References


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## Gross lesions in wild ducks at 4 days postinoculation with highly pathogenic avian influenza virus (H5N1)

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*Severity of lesion: white, no visible lesion; yellow, mild; orange, moderate; red, marked.
†No gross lesions were observed in other organs.
‡Airsacculitis, characterized by mild, multifocal to diffuse opacity and thickening of the air sacs.
§Multifocal pulmonary consolidation.
¶Multifocal pancreatic necrosis, consisting of red or gray foci of 1–4 mm in diameter affecting up to 25% of the pancreas.
#Diffuse hepatic necrosis.
Appendix Table 2. Expression of antigen in organs of wild ducks at 4 days postinoculation with highly pathogenic avian influenza virus (H5N1)

<table>
<thead>
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<th>System</th>
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*White, no cells positive; yellow, rare cells positive; orange, moderate number of cells positive; red, many cells positive.
†No virus antigen expression was observed in other organs or tissues.
‡Presence of histologic lesions consistent with infection but no viral antigen expression.
### Appendix Table 3. Isolation of highly pathogenic avian influenza virus (H5N1) from organs of wild ducks at 4 days after inoculation and from swabs at 1–4 days postinoculation

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*TCID<sub>50</sub>, median tissue culture infectious dose; white, no virus isolated; yellow, virus titer 0.8–1.9; orange, 2.0–3.9; red, 4.0–5.9; dark red, 6.0–6.2; dpi, days postinoculation.
Appendix Table 4. Detection of highly pathogenic avian influenza virus (H5N1) by reverse transcription–PCR from organs of wild ducks at 4 days postinoculation and from swabs at 1–4 days postinoculation

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*White, no virus detected; yellow, 30–39 Ct; orange, 20–29 Ct; red, 10–19 Ct; dpi, days postinoculation.