

A first report of the detection of Avipoxvirus genomic sequences in louse flies

(Diptera: Hippoboscidae)

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Abstract

The Hippoboscidae are ectoparasites of birds and mammals, which, as a group, are known to vector multiple diseases. Avipoxvirus (APV) is mechanically vectored by various arthropods and causes seasonal disease in wild birds in the United Kingdom. Signs of avian pox and the presence of louse flies (Hippoboscidae) on Dunnocks *Prunella modularis* were recorded over a 16.5-year period in a rural garden in Somerset, United Kingdom. Louse flies collected from this site and other sites in England were tested for the presence of APV DNA and RNA

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sequences. Louse flies on Dunnocks were seen to peak seasonally three weeks prior to the peak of avian pox lesions, an interval consistent with the previously estimated incubation period of APV in Dunnocks. APV DNA was detected on 13/25 louse flies, *Ornithomya avicularia* and *Ornithomya fringillina*, taken from Dunnocks, both with and without lesions consistent with avian pox, at multiple sites in England. Collectively these data support the premise that louse flies may vector APV. The detection of APV in louse flies, from apparently healthy birds, and from sites where disease has not been observed in any host species, suggests that the Hippoboscidae could provide a non-invasive and relatively cheap method of monitoring avian diseases. This could provide advanced warnings of disease, including zoonoses, before they become clinically apparent.

Keywords: Vector; Parasite; Disease detection; Methods; Avian pox; Wildlife disease

Introduction

The Diptera in the family Hippoboscidae are related to the Tsetse flies, and both sexes are obligate haematophagous ectoparasites. There are over 200 species worldwide (Dick, 2018) of which eleven species breed in the United Kingdom (UK), three on mammals (keds) and eight, louse or flat flies, on birds (Hutson, 1984; Wawman, 2024).

Some species of louse fly are proven vectors of various pathogens and others have been isolated from them without formal proof that these flies are acting as vectors (Bezerra-Santos and Otranto, 2020). *Ornithomya avicularia* and *Pseudolynchia canariensis* are proven biological vectors of *Haemoproteus* sp. (Baker, 1963; Cepeda *et al.*, 2019), and trypanosomes have been shown to develop within the midgut of *O. avicularia*, and to reach the infective stage, but transmission only occurs when birds ingest the flies (Baker, 1967). For most other pathogens, the evidence that louse flies are acting as vectors is weaker: West Nile Virus

(WNV) was detected in *Icosta americana* from WNV infected raptors (Farajollahi *et al.*, 2005); *Crataerina melbae* was proposed as a vector of trypanosomes in Alpine Swifts *Tachymarptis melba* (Cigler *et al.*, 2024); an unidentified Hippoboscid was suggested as a potential vector when found in association with Newcastle Disease Virus (NDV) infected Laughing Doves *Streptopelia senegalensis* (Obanda *et al.*, 2016). Pathogens have also been identified in louse flies taken from birds without documented signs of disease, including *Babesia* spp. from *O. avicularia* and *O. biloba* (Čisovská Bazsalovicsová *et al.*, 2023), *Rickettsia* sp. from *Crataerina pallida* (Cerutti *et al.*, 2018), and trypanosomes from *O. biloba*, *O. fringillina* and *Ornithoica turdi* (Santolíkova *et al.*, 2022).

Avipoxvirus (APV) is a genus of double-stranded DNA viruses within the Chordopoxvirinae subfamily and Poxviridae family (ICTV, 2024). The most studied member of the genus is fowlpox virus, a cause of disease in domestic poultry (Van Riper and Forrester, 2007; Yeo *et al.*, 2019). Other members of the genus cause disease in wild avian species, which is characterised by proliferative lesions on the skin, feet and legs, head and sometimes on the mucus membranes. APV is mechanically vectored by a range of arthropods, including *Aedes* and *Culex* mosquitoes (Kligler, Muckenfuss and Rivers, 1929), midges (Lee *et al.*, 2017), fleas (Smits *et al.*, 2005), the mite *Dermanyssus gallinae* (Shirinov, Ibragimova and Misirov, 1972; Huong *et al.*, 2014) and stable flies *Stomoxys calcitrans* (Alehegn, Chanie and Mengesha, 2014). APV remains viable on the proboscis of *Culex tarsalis* for up to 28 days (DaMassa, 1966). In temperate regions of the world avian pox is a seasonal disease where vectors are not active during the winter (Wawman *et al.*, unpublished results; Van Riper and Forrester, 2007; Lachish *et al.*, 2012).

The Dunnock *Prunella modularis* is a small passerine that is native across Europe. It was first reported with avian pox lesions in the UK in the 1940's (Edwards, 1955). It is affected by avian pox with a seasonal peak in August following an absence of disease in May

and June (Wawman *et al.*, unpublished results) which correlates with the seasonal peaks in vectors such as mosquitoes (Cull *et al.*, 2024). Three species of Hippoboscids have been found on UK Dunnocks, *O. avicularia*, *O. fringillina* and *O. chloropus* (Wawman, Smith and Sheldon, unpublished results; Hill, 1962) and their peaks in populations occur seasonally at the time of year which might potentially allow them to be vectors of APV in Dunnocks (Wawman, 2025). This is particularly true when the estimated incubation period, which varies from 13 to 48 days in Dunnocks (Wawman *et al.*, unpublished results), is taken into consideration.

In this study we combine knowledge of the seasonality of avian pox and Hippoboscids on Dunnocks, with molecular techniques to detect APV in association with *Ornithomya* spp.. We also consider whether louse flies and keds could be used as a non-invasive way to sample for viruses in wild populations.

Materials and methods

Samples

From February 2008 until August 2024, Dunnocks were ringed, in a rural garden, near Minehead, in Somerset, as part of the British Trust for Ornithology (BTO) Retrapping Adults for Survival Scheme (<https://www.bto.org/our-science/publications/birdtrends/2020/methods/retrapping-adults-survival-scheme>, last accessed 2024-09-09). At each capture, in addition to the usual data collected during bird ringing, records were made of signs of disease or the presence of ectoparasites. Louse flies that left Dunnocks during routine ringing activities were collected, identified according to a key (Hutson, 1984), and preserved in 70% ethanol (with distilled water and no other excipients).

During 2023, both at the main avian pox study site near Minehead in Somerset, and at a second site on the Wirral Peninsula near Liverpool where lesions consistent with avian pox had been observed, flies were preserved in RNAlater for DNA and RNA analyses

Additional flies were collected from all bird species from 2020 to 2023, as part of the “Mapping the UK’s Flat Flies Project” (Wawman, 2025) and stored in 70% ethanol for later analyses.

Next Generation Sequencing (NGS)

One fly, taken from a Dunnock with avian pox at the second site, and the only one from an infected Dunnock that was preserved in RNAlater, was chosen for DNA and RNA extraction, using shotgun Next Generation Illumina Sequencing (NGS). The fly was prepared by washing in phosphate buffered saline (PBS) to remove the RNAlater, then in 5% sodium hypochlorite solution to remove contaminants, and finally twice more in PBS to remove the sodium hypochlorite.

DNA and RNA were isolated separately from the fly using the QIAgen AllPrep DNA/RNA MiniKit (Qiagen, Manchester, United Kingdom). Briefly, the fly was homogenized in 300µl RLT buffer using a single 5mm steel bead in a TissueLyserII (Qiagen) for 5 minutes at 30Hz. After this, manufacturer’s instructions were followed and DNA was eluted into 60µl buffer EB and RNA was eluted into 50µl RNase-free water. Sequencing libraries were prepared using Nexetra XT kits (Illumina, Cambridge, UK) and sequencing using a Nextseq sequencer (Illumina, Cambridge, UK). Illumina) to generate 2 x 150 base paired-end reads.

The raw data were filtered to remove adaptors and low quality reads using the programs fastp version 0.23.4 (Chen *et al.*, 2018) and multiqc v 1.19 (Ewels *et al.*, 2016). The sequences were aligned and the Dipteran host genomes removed in the program Bowtie2

(Langmead and Salzberg, 2012). Sequences were assembled using MEGAHIT (D. Li *et al.*, 2015). Taxonomic classification was carried out using the program Kracken2 (Wood, Lu and Langmead, 2019), with the program Bracken (Lu *et al.*, 2017) to estimate species abundance and facilitate removal of poor quality and low abundance sequences. Viral sequence detection was performed using ViralVerify (<https://github.com/ablab/viralVerify/tags>, last accessed 2024-09-16) and ViralComplete (<https://github.com/ablab/viralComplete> last accessed 2024-09-16) with sequences obtained from the NCBI viral database (<https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/> last accessed 2024-09-16). Avian Pox Virus sequences identified using these methods were isolated and the identity was confirmed using BLAST+ (Camacho *et al.*, 2009).

PCR screening of samples

Twenty-four of the flies previously preserved in 70% ethanol were selected from a range of sites across England, across an area where avian pox is likely to occur in Dunnocks, from sites where avian pox had not been observed on any bird species by bird ringers, as well as sites from which it had been reported from Dunnocks or other species. Twenty-three flies were from Dunnocks and one from a Great Spotted Woodpecker *Dendrocopos major*.

These flies were not washed, to avoid removing evidence that they might be mechanical vectors. Flies were homogenised in liquid nitrogen prior to DNA extraction, using the DNeasy Blood and Tissue kit (QIAGEN) according to the manufacturer's instructions. Screening for APV was carried out through amplification of a 578-bp PCR product of the Avipoxvirus 4b core protein gene (*fpv167*) as previously described, using primer sequences 5'-CAGCAGGTGCTAAACAACAA-3' and 5'-CGGTAGCTTAACGCCGAATA-3' (Lawson *et al.*, 2012). Amplification was achieved using the high-fidelity Q5 polymerase (NEB) under the following cycling conditions: 98 °C (5 min); 40×[98 °C (1 min); 64 °C (20 sec); 72 °C

(20 sec)]; 72 °C (7 min). Following confirmation of a product of the correct size on an agarose gel against a positive control of a DNA sequence from a Dunnock pox lesion at the main site (GenBank Accession number PP756527), APV was confirmed through bidirectional Sanger sequencing (Source BioScience) using the same primers as for amplification.

Computational analysis

Other analyses were performed and the phenology plotted in R version 4.2.1 (R Development Core Team, 2022) using packages dplyr (Wickham *et al.*, 2023) and lubridate (Grolemund and Wickham, 2011) to process the data. The map was plotted using the packages maps (Becker *et al.*, 2022), mapdata (Brownrigg, 2022) and scales (Wickham and Seidel, 2022).

Results

In order to investigate the association between avipoxvirus infection, small Passerine bird species and louse flies, Dunnocks were sampled from sites in England. Two species of louse flies were identified from Dunnocks, *Ornithomya avicularia* and *O. fringillina*.

Figure 1 shows the relationship between Dunnocks with evidence of avian pox infection and presence of flat flies (both species) and Dunnocks based on cumulative data over the study period. Avian pox infection were reported during the first 16 weeks of the year and then a later surge in infections during weeks 30 to 36, reaching a peak of before of 15 cases during week 34, then declining for the remaining weeks of the year. Flat flies were only detected on Dunnocks from week 24 and peaked on week 29 with another peak on week 31. This preceded the peak of avian pox cases by 2-3 weeks as shown when the louse fly frequency plot is moved to three weeks later (Figure 2).

NGS sequencing and classification with Kracken2 identified five non-overlapping sequences of DNA as APV covering a total of 598 base pairs (Table 1) from an *Ornithomya avicularia* taken from a Dunnock with avian pox at the second site (Table 2). However, the length of sequence detected (<600 base pairs) represents less than 0.2% of the Avipoxvirus genome (comparison with Magpiepox virus 2, 298,392bp GenBank Acc. No. MW485973). It is not clear if this represents residual presence of virus on the external surface of the louse fly (e.g. contamination of mouthparts following feeding on the dunnock), despite surface disinfection, or presence within the fly (gut, oral cavity, salivary glands, or other internal organs) but at very low levels. No APV RNA was detected.

To more comprehensively test for the presence of avian pox 24 further samples of louse flies were subjected to PCR-based analysis for avian pox DNA. Eleven of 23 flies tested from Dunnocks and the one from the Great Spotted Woodpecker (Table 2), were PCR positive for APV. These included one *Ornithomya fringillina* from a Dunnock with avian pox at the site on Wirral Peninsula near Liverpool. All PCR-derived sequences were identical across the 512bp where they aligned, and a 100% match to in this high-quality central region of the sequence obtained from an avian pox lesion on a Dunnock at the main site. These positive flies were from the main study site (Somerset: n=4), two sites (Corby and Stanford Reservoir in Northamptonshire) where avian pox was occasionally observed in Great Tits *Parus major*, but not in Dunnocks, either during the study or in the previous year (n=3), and three sites (Rutland Water in Rutland, near Sandy in Bedfordshire, and West Down in Devon) where avian pox had not been observed in any bird species (n=4). The locations of these sites, and those at which no flies tested positive (n=4), are shown in the map (Figure 3).

In total, 13 out of 25 louse flies tested positive for APV, 6/9 *Ornithomya fringillina* and 7/16 *O. avicularia*. Six of the flies were recorded as being female, four male and in three the sex was not recorded (Table 2).

None of the Dunnocks without active disease at the time of capture, from which the flies were taken, were later seen to develop avian pox, although most (7/10) were not recaptured. However, most (7/10) were juveniles at the time of capture and would have been expected to leave their natal areas (the ringing sites) during their postnatal dispersal.

Discussion

APV DNA was detected in 13 of 25 louse flies, of two species, *Ornithomya avicularia* and *O. fringillina*, that were obtained from sites across England. All the 512 base pair sequences obtained from the louse flies by PCR were identical to that obtained from an avian pox lesion on a Dunnock at the main site, and matched reference sequences from NCBI Genbank. The APV positive flies included one *O. avicularia* and one *O. fringillina* that had been taken from Dunnocks with avian pox lesions, the rest having been taken from Dunnocks with no signs of disease, and a single fly from an apparently healthy Great Spotted Woodpecker. Field studies showed that peaks in the presence of louse flies on Dunnocks were followed three weeks later by peaks in the prevalence of avian pox.

The three week interval falls within the estimate of the incubation period of avian pox in Dunnocks of 13-48 days (Wawman *et al.*, unpublished data). However, phenological comparisons may not be a reliable means of identifying a vector because other vectors' emergence may be triggered by the same climatic conditions as that of the louse flies. Arthropod vectors are known to down-regulate their hosts' immune systems which may promote transmission of avian pox (Wikel, 1999). Moreover, reproduction-associated stress or hormonal induced immunosuppression may also make Dunnocks more susceptible to avian pox at particular times of the year.

Although conclusive evidence that APV is vectored by Hippoboscidae may be challenging to obtain, the detection of avian pox DNA associated with louse flies is strong

evidence of the potential for a role in transmission. If, as expected from knowledge of its mode of transmission by other vectors, APV is mechanically vectored, evidence of viral replication in the vector, such as Bollinger Bodies (Bollinger, 1873) or the presence of viral mRNA, would not be expected, and indeed RNA was not detected in this study, although only a single specimen taken from a Dunnock with avian pox was tested, and further testing is needed to confirm this result. Using sequences to compare viral DNA in avian hosts and parasites might confirm that the same strains are circulating in both (Yeo *et al.*, 2019), but would not prove beyond doubt that Hippoboscids are responsible for transmission. In this study, all of the 512 base pair sequences obtained by PCR were identical, and matched that taken from an avian pox lesion on a Dunnock at the main site, but longer sequences may be necessary to fully establish that they are the same strain of the virus.

Isolating APV DNA from a Hippoboscid taken from a Dunnock that later developed avian pox, might more strongly implicate Hippoboscids as vectors. However, finding APV DNA in 52% of the louse flies tested, including flies from sites where no avian pox had been observed in any species of bird, indicates that APV may be circulating in the wild bird population at a far higher rate than expected purely from the observed presence of clinical signs. Lesions were only observed in 8.3% of Dunnocks (Wawman *et al.*, unpublished results) and 7.2% of House Finches *Haemorrhous mexicanus* (McGraw *et al.*, 2022), but PCR detected APV in the spleens of 43% of wild birds in Italy (Bertelloni *et al.*, 2022), and 69.2% of a sample of avian species introduced into New Zealand were seropositive (Ha *et al.*, 2013). It would be necessary to use other methods such as invasive sampling techniques. to detect the timing of seroconversion in birds in relation to the presence of flies carrying APV, which would be difficult in a wild host population.

Detecting APV in louse flies at sites where no avian pox has been observed, and at sites where avian pox has only been observed in species other than Dunnocks, such as at

Stanford Reservoir and Corby, where it was only seen occasionally in Great Tits, might suggest that avian pox is of low virulence in Dunnocks. It is also likely that it exists in an enzootic cycle between different wild bird species and louse flies and other arthropod vectors. The two species of louse flies found to be positive for avian pox in this study, *Ornithomya avicularia* and *O. fringillina* are found on a wide range of bird species (Wawman, Smith and Sheldon, unpublished results) and could be vectoring APV between Dunnocks, Great Tits and other affected species. This hypothesis is supported by the presence of an APV-positive louse fly on a Great Spotted Woodpecker.

Louse flies may have potential as a means of monitoring the potential for vector-transferred disease in wild bird populations. NGS sequencing could be used to determine which pathogens of potential interest are present in louse flies, potentially by pooling specimens to minimise the cost. Pathogen targeted PCR could be used in cases where focal pathogens have already been identified as a potential cause for concern and sequences compared to track different strains of concern. Individual based analyses can be used to quantify the numbers of infected individuals, including those in the prodromal phase of disease and those in which infections remain subclinical, and to determine the geographical areas in which diseases are present. Expanding this approach to keds, could also allow diseases to be tracked in the wild deer population, and in livestock such as horses, sheep and goats in regions where they are frequently infected.

Other parasites have been used to take samples from wild birds, for example, blood sucking bugs, *Dipetalogaster maximus*, contained within dummy eggs have been used avoid stress when taking blood samples from adult nesting birds including Common Terns *Sterna hirundo* for cortisol assays (Arnold *et al.*, 2008), and Eurasian Kestrel for counting blood parasites, leucocyte profiles and microsatellite analysis for paternity tests and genetic sexing (Sumasgutnet, Rubin and Gamauf, 2014), and have been shown to be a reliable method in

Common Swift *Apus apus* (Bauch *et al.*, 2013). Bat flies (Diptera: Nycteribiidae) which are closely related to the louse flies, and sometimes included as part of the Hippoboscidae, have been used to investigate the presence of *Bartonella* spp. *Polychromophilus* spp. and *Trypanosoma* spp. in bats (Szentiványi *et al.*, 2020).

Using louse flies to screen for the presence of disease in a population would have several advantages compared to using serology or other samples from birds. Firstly, serology and other blood tests require invasive sampling techniques, performed by veterinary surgeons or individuals who have undergone additional training and licensing, which limits the sample size and geographical area over which a study can take place, and adds costs to any project: louse flies can be collected by any bird ringer, or wildlife rehabilitator or member of the public who comes across a sick, injured or dead bird hosting louse flies. Citizen science projects, such as the Mapping the UK's Flat Flies Project (Wawman, 2025) could allow widespread coverage across a region. Additionally, serology only indicates that a bird has produced an immune response at some point during its life, with no way of determining the timing of the infection, whereas, because of the short life span of Hippoboscids, assuming that transovarial and transstadial transmission can be ruled out, any infection within them is likely to have been acquired in the same season. Adult louse flies have a life span of around three to six months (Hutson, 1984), so APV virions would have to be acquired in the same year. Using PCR-sequencing approaches to detect viral DNA is specific for the disease under investigation, whereas relying on observations of sick birds in the field, or even in the hand by trained bird ringers, risks both mis-diagnoses and a significant underestimate of prevalence of infection.

A major disadvantage of using wild louse flies is that the sampling is likely to be somewhat random in nature, as only birds with ectoparasites that can be caught can be sampled, and there may be differences in the health of birds with and without parasites,

especially if co-infections are present. Quantitative results, such as an estimate of disease prevalence will be difficult to obtain. Sick birds may be easier to catch if flying weakly, or be so sick that they remain hidden and are not caught. Ectoparasites may be affected by the presence of avian endoparasites: the louse fly *Olfersia spinifera*, was found to be less likely to switch hosts when infected with *Haemoproteus iwa* (Levin and Parker, 2014) and ectoparasites may aggregate on certain hosts with co-infection. The prevalence of micro-organisms may not be consistent between vectors and their hosts – a study of bats and bat flies showed twice the prevalence of *Trypanosoma* spp. and *Polychromophilus* spp. in hosts compared to their ectoparasites, but a similar prevalence of *Bartonella* spp. in both groups (Szentiványi *et al.*, 2020).

Vertical transmission leading to infection of louse flies might also be a potential issue for some diseases in which it occurs and might explain the higher rate of detection of APV in the louse flies compared to their hosts. *Bartonella schoenbuchensis* is vertically transmitted in the Deer Ked *Lipoptena cervi* (de Bruin *et al.*, 2015), and *Anaplasma ovis* in the Sheep Ked *Melophagus ovinus* (Zhao *et al.*, 2018), but vertical transmission of viral pathogens has not been reported in louse flies. However, vertical transmission of endosymbionts occurs (Duron *et al.*, 2014) and some arthropod viruses are vertically transmitted via both ova and spermatozoa (Longdon and Jiggins, 2012). Some sigmaviruses, a group of RNA viruses found naturally in Diptera, are known from Hippoboscids, such as the Wuhan Louse Fly sigmaviruses (C.-X. Li *et al.*, 2015) and Aksy-Durag Melophagus sigmavirus (Litov *et al.*, 2021). It is possible that compared to the transovarial route, reproduction by adenotrophic viviparity in the Hippoboscidae, could favour vertical transmission, as there is prolonged maternal contact between the female and her offspring, during which time it is fed from a specialised milk gland.

Despite these issues, useful results might be obtained similar to those used in the surveillance used in monitoring other vector borne diseases such as the European network for medical and veterinary entomology (VectorNet) (<https://www.ecdc.europa.eu/en/about-us/partnerships-and-networks/disease-and-laboratory-networks/vector-net>, last accessed 22nd November 2024), which monitors the ranges mosquitos, ticks, biting sandflies, and midges across Europe, or other studies such as VB-RADAR in the United Kingdom, which monitors the flaviviruses, WNV and Usutu Virus in ticks (<https://www.vb-radar.com>, last accessed 22nd November 2024). Using louse flies may have advantages over using other arthropod vectors to monitor disease, because, although louse flies switch hosts (Corbet, 1956), those collected by ringers are taken from a host with a ring bearing a unique identifying code, which allows the bird to be followed throughout its life, potentially giving additional information about the progress of diseases that would not be obtained otherwise.

From the phenological and molecular evidence presented in this paper, it is likely that the *Ornithomya* spp. present in the UK, especially *Ornithomya avicularia* and *Ornithomya fringillina*, are vectors of APV in Dunnocks. Further research will be required to determine the exact role of louse flies in vectoring APV in Dunnocks, and in other avian species, and how this occurs. Although mechanical transmission would be the expected route, the possibility of vertical transmission facilitating autochthonous disease transmission should be considered.

Louse flies may provide a relatively cheap and non-invasive method of monitoring disease outbreaks (or assessing potential at risk populations) in wild birds, when combined with targeted DNA sequencing for specific pathogens.

Data availability. Details of the flies sequenced can be found in Table 1 together with the accession numbers for the APV sequences.

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Ethical standards. All birds handled in this study were ringed under licence: BTO ringing permits S5420 issued to DCW, and S3144 issued to JET for the two main sites, and the permits of other trained ringers taking part in the Mapping the UK’s flat flies project elsewhere. All those mentioned in the acknowledgements have given informed written consent for their names to be published.

References

- Alehegn, E., Chanie, M. and Mengesha, D. (2014) 'A Systematic Review of Serological and Clinicopathological Features and Associated Risk Factors of Avian Pox', *British Journal of Poultry Sciences*, 3(3), pp. 78–87. Available at: <https://doi.org/10.5829/idosi.bjps.2014.3.3.8553>.
- Arnold, J.M. *et al.* (2008) 'Taking the stress out of blood collection: comparison of field blood-sampling techniques for analysis of baseline corticosterone', *Journal of Avian Biology*, 39(5), pp. 588–592. Available at: <https://doi.org/10.1111/j.0908-8857.2008.04265.x>.
- Baker, J.R. (1963) 'Transmission of Haemoproteus sp. of English Wood-pigeons by Ornithomyia avicularia', *the Journal of Protozoology*, 10(4), pp. 461–465.
- Baker, J.R. (1967) 'A Review of the Role Played by the Hippoboscidae (Diptera) as Vectors of Endoparasites', *Journal of Parasitology*, 53(2), pp. 412–418. Available at: [/www.jstor.org/stable/3276603](http://www.jstor.org/stable/3276603).
- Bauch, C. *et al.* (2013) "'Bug-eggs" for Common Swifts and other small birds: minimally-invasive and stress-free blood sampling during incubation', *Journal of Ornithology*, 154(2), pp. 581–585. Available at: <https://doi.org/10.1007/s10336-013-0931-x>.
- Becker, R.A. *et al.* (2022) 'maps: Draw Geographical Maps. R package version 3.4.1'. Available at: <https://cran.r-project.org/package=maps>.
- Bertelloni, F. *et al.* (2022) 'Molecular Detection of Avipoxvirus in Wild Birds in Central Italy', *Animals*, 12(3), pp. 1–7. Available at: <https://doi.org/10.3390/ani12030338>.
- Bezerra-Santos, M.A. and Otranto, D. (2020) 'Keds, the enigmatic flies and their role as vectors of pathogens', *Acta Tropica*, 209, p. 105521. Available at: <https://doi.org/10.1016/j.actatropica.2020.105521>.

- Bollinger, O. (1873) 'Ueber Epithelioma contagiosum beim Haushuhn und die sogenannten Pocken des Geflügels', *Archiv für Pathologische Anatomie und Physiologie und für Klinische Medizin*, 58(3–4), pp. 349–361. Available at: <https://doi.org/10.1007/BF02105254>.
- Brownrigg, R. (2022) 'mapdata: Extra Map Databases. R package version 2.3.1', pp. 1–7. Available at: <https://cran.r-project.org/web/packages/mapdata/mapdata.pdf>.
- de Bruin, A. *et al.* (2015) 'Vertical transmission of Bartonella schoenbuchensis in Lipoptena cervi', *Parasites & Vectors*, 8(1), p. 176. Available at: <https://doi.org/10.1186/s13071-015-0764-y>.
- Camacho, C. *et al.* (2009) 'BLAST+: architecture and applications', *BMC Bioinformatics*, 10(1), p. 421. Available at: <https://doi.org/10.1186/1471-2105-10-421>.
- Cepeda, A.S. *et al.* (2019) 'Experimental characterization of the complete life cycle of Haemoproteus columbae, with a description of a natural host-parasite system used to study this infection', *International Journal for Parasitology*, 49(12), pp. 975–984. Available at: <https://doi.org/10.1016/j.ijpara.2019.07.003>.
- Cerutti, F. *et al.* (2018) 'The microbiota of hematophagous ectoparasites collected from migratory birds', *PLOS ONE*. Edited by B. Stevenson, 13(8), p. e0202270. Available at: <https://doi.org/10.1371/journal.pone.0202270>.
- Chen, S. *et al.* (2018) 'fastp: an ultra-fast all-in-one FASTQ preprocessor', *Bioinformatics*, 34(17), pp. i884–i890. Available at: <https://doi.org/10.1093/bioinformatics/bty560>.
- Cigler, P. *et al.* (2024) 'Trypanosomiasis: An emerging disease in Alpine swift (Tachymarptis melba) nestlings in Switzerland?', *International Journal for Parasitology: Parasites and Wildlife*, 23(December 2023), p. 100895. Available at: <https://doi.org/10.1016/j.ijppaw.2023.100895>.
- Čisovská Bazsalovicsová, E. *et al.* (2023) 'Bird Louse Flies Ornithomya spp. (Diptera:

- Hippoboscidae) as Potential Vectors of Mammalian Babesia and Other Pathogens', *Vector-Borne and Zoonotic Diseases*, 0(0), pp. 1–9. Available at: <https://doi.org/10.1089/vbz.2022.0088>.
- Corbet, G.B. (1956) 'The Life-History and Host-Relations of a Hippoboscid Fly *Ornithomyia fringillina* Curtis', *The Journal of Animal Ecology*, 25(2), p. 403. Available at: <https://doi.org/10.2307/1934>.
- Cull, B. *et al.* (2024) 'iNaturalist community observations provide valuable data on human-mosquito encounters', *Journal of Vector Ecology*, 49(2). Available at: <https://doi.org/10.52707/1081-1710-49.2.r12>.
- DaMassa, A.J. (1966) 'The Role of *Culex tarsalis* in the Transmission of Fowl Pox Virus', *Avian Diseases*, 10(1), p. 57. Available at: <https://doi.org/10.2307/1588207>.
- Dick, C.W. (2018) 'Checklist of the world Hippoboscidae (Diptera: Hippoboscoidea)', (January). Available at: https://www.researchgate.net/publication/322579074_CHECKLIST_OF_WORLD_NYC_TERIBIIDAE_DIPTERA_HIPPOBOSCOIDEA.
- Duron, O. *et al.* (2014) 'Origin, acquisition and diversification of heritable bacterial endosymbionts in louse flies and bat flies', *Molecular Ecology*, 23(8), pp. 2105–2117. Available at: <https://doi.org/10.1111/mec.12704>.
- Edwards, G.R. (1955) 'Excrescences about the eyes and on the legs and feet of dunnocks', *British Birds*, 48(4), pp. 186–187. Available at: <https://britishbirds.co.uk/content/notes-200>.
- Ewels, P. *et al.* (2016) 'MultiQC: summarize analysis results for multiple tools and samples in a single report', *Bioinformatics*, 32(19), pp. 3047–3048. Available at: <https://doi.org/10.1093/bioinformatics/btw354>.
- Farajollahi, A. *et al.* (2005) 'Detection of West Nile virus RNA from the louse fly *Icosta*

- americana (Diptera: Hippoboscidae)', *Journal of the American Mosquito Control Association*, 21(4), pp. 474–476. Available at: [https://doi.org/10.2987/8756-971X\(2006\)21\[474:DOWNVR\]2.0.CO;2](https://doi.org/10.2987/8756-971X(2006)21[474:DOWNVR]2.0.CO;2).
- Grolemund, G. and Wickham, H. (2011) 'Dates and Times Made Easy with lubridate.', *Journal of Statistical Software*, 40(3), pp. 1–25. Available at: <https://www.jstatsoft.org/v40/i03/>.
- Ha, H.J. *et al.* (2013) 'The seroprevalence of avipoxvirus and its association with avian malaria (*Plasmodium* spp.) infection in introduced passerine birds in the southern regions of the North Island of New Zealand', *Avian Diseases*, 57(1), pp. 109–115. Available at: <https://doi.org/10.1637/10285-061912-ResNote.1>.
- Hill, D.S. (1962) 'A STUDY OF THE DISTRIBUTION AND HOST PREFERENCES OF THREE SPECIES OF ORNITHOMYIA (DIPTERA: HIPPOBOSCIDAE) IN THE BRITISH ISLES', 37(4–6), pp. 37–48. Available at: <https://doi.org/10.1111/j.1365-3032.1962.tb00286.x>.
- Huong, C.T.T. *et al.* (2014) 'Molecular Detection of Avian Pathogens in Poultry Red Mite (*Dermanyssus gallinae*) Collected in Chicken Farms', *Journal of Veterinary Medical Science*, 76(12), pp. 1583–1587. Available at: <https://doi.org/10.1292/jvms.14-0253>.
- Hutson, A.M. (1984) 'Keds, Flat-flies and Bat-Flies: Diptera, Hippoboscidae and Nycteribiidae', *Handbooks for the Identification of British Insects*, 10(7), p. 40. Available at: https://www.royensoc.co.uk/sites/default/files/Vol10_Part07_Hutson.pdf.
- ICTV (2024) 'ICTV Master Species List 2023MSL39.v3'. International Committee on Taxonomy of Viruses. Available at: <https://ictv.global/msl>.
- Kligler, I.J., Muckenfuss, R.S. and Rivers, T.M. (1929) 'TRANSMISSION OF FOWL-POX BY MOSQUITOES', *The Journal of Experimental Medicine*, 49(4), pp. 649–660. Available at: <https://doi.org/10.1084/jem.49.4.649>.

- Lachish, S. *et al.* (2012) 'Epidemiology of the Emergent Disease Paridae pox in an Intensively Studied Wild Bird Population', *PLoS ONE*, 7(11), p. e38316. Available at: <https://doi.org/10.1371/journal.pone.0038316>.
- Langmead, B. and Salzberg, S.L. (2012) 'Fast gapped-read alignment with Bowtie 2', *Nature Methods*, 9(4), pp. 357–359. Available at: <https://doi.org/10.1038/nmeth.1923>.
- Lawson, B. *et al.* (2012) 'Emergence of a Novel Avian Pox Disease in British Tit Species', *PLoS ONE*. Edited by R.A.M. Fouchier, 7(11), p. e40176. Available at: <https://doi.org/10.1371/journal.pone.0040176>.
- Lee, H.R. *et al.* (2017) 'MOLECULAR EPIDEMIOLOGY OF AVIAN POXVIRUS IN THE ORIENTAL TURTLE DOVE (*STREPTOPELIA ORIENTALIS*) AND THE BITING MIDGE (*CULICOIDES ARAKAWAE*) IN THE REPUBLIC OF KOREA', *Journal of Wildlife Diseases*, 53(4), p. 749. Available at: <https://doi.org/10.7589/2016-10-230>.
- Levin, I.I. and Parker, P.G. (2014) 'Infection with *Haemoproteus iwa* affects vector movement in a hippoboscid fly-frigatebird system', *Molecular Ecology*, 23(4), pp. 947–953. Available at: <https://doi.org/10.1111/mec.12587>.
- Li, C.-X. *et al.* (2015) 'Unprecedented genomic diversity of RNA viruses in arthropods reveals the ancestry of negative-sense RNA viruses', *eLife*, 4(4), pp. 1–26. Available at: <https://doi.org/10.7554/eLife.05378>.
- Li, D. *et al.* (2015) 'MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph', *Bioinformatics*, 31(10), pp. 1674–1676. Available at: <https://doi.org/10.1093/bioinformatics/btv033>.
- Litov, A.G. *et al.* (2021) 'Possible Arbovirus Found in Virome of *Melophagus ovinus*', *Viruses*, 13(12), p. 2375. Available at: <https://doi.org/10.3390/v13122375>.
- Longdon, B. and Jiggins, F.M. (2012) 'Vertically transmitted viral endosymbionts of insects: Do sigma viruses walk alone?', *Proceedings of the Royal Society B: Biological Sciences*,

- 279(1744), pp. 3889–3898. Available at: <https://doi.org/10.1098/rspb.2012.1208>.
- Lu, J. *et al.* (2017) ‘Bracken: estimating species abundance in metagenomics data’, *PeerJ Computer Science*, 3(1), p. e104. Available at: <https://doi.org/10.7717/peerj-cs.104>.
- McGraw, K.J. *et al.* (2022) ‘Poxvirus infection in house finches (*Haemorhous mexicanus*): Genome sequence analysis and patterns of infection in wild birds’, *Transboundary and Emerging Diseases*, 69(5), pp. e2318–e2328. Available at: <https://doi.org/10.1111/tbed.14575>.
- Obanda, V. *et al.* (2016) ‘COMPLETE GENOMIC SEQUENCE OF VIRULENT PIGEON PARAMYXOVIRUS IN LAUGHING DOVES (*STREPTOPELIA SENEGALENSIS*) IN KENYA’, *Journal of Wildlife Diseases*, 52(3), pp. 599–608. Available at: <https://doi.org/10.7589/2015-07-199>.
- R Development Core Team (2022) ‘R: A language and environment for statistical computing.’ R Foundation for Statistical Computing, Vienna, Austria. Available at: <https://www.r-project.org/>.
- Van Riper, C. and Forrester, D.J. (2007) ‘Avian Pox’, in *Infectious Diseases of Wild Birds*. Ames, Iowa, USA: Blackwell Publishing Professional, pp. 131–176. Available at: <https://doi.org/10.1002/9780470344668.ch6>.
- Santolíkuvá, A. *et al.* (2022) ‘Avian Louse Flies and Their Trypanosomes: New Vectors, New Lineages and Host–Parasite Associations’, *Microorganisms*, 10(3), p. 584. Available at: <https://doi.org/10.3390/microorganisms10030584>.
- Shirinov, F.B.; Ibragimova, A.I.; and Misirov, Z.G. (1972) ‘[Spread of fowl pox virus by the mite *Dermanyssus gallinae*.]’, *Veterinarnyi Institut, USSR.*, 4, pp. 48–49. Available at: <https://www.cabdirect.org/cabdirect/abstract/19722200639>.
- Smits, J.E. *et al.* (2005) ‘An Epizootic of Avian Pox in Endemic Short-toed Larks (*Calandrella rufescens*) and Berthelot’s Pipits (*Anthus berthelotti*) in the Canary Islands,

- Spain', *Veterinary Pathology*, 42(1), pp. 59–65. Available at: <https://doi.org/10.1354/vp.42-1-59>.
- Sumasgutnet, P., Rubin, I. and Gamauf, A. (2014) 'Collecting blood samples in Eurasian Kestrels (*Falco tinnunculus*) (Aves : Falconidae) via blood-sucking bugs (Insecta : Hemiptera : Reduviidae) and their use in genetics and leucocyte profiles Author (s): P . Sumasgutner , I . Rubin and A . Gamauf', *Annalen des Naturhistorischen Museums in Wien. Serie B für Botanik und Zoologie*, pp. 247–257.
- Szentiványi, T. *et al.* (2020) 'Host conservation through their parasites : molecular surveillance of vector-borne microorganisms in bats using ectoparasitic bat flies', *Parasite*, 27(72), pp. 1–10. Available at: <https://doi.org/10.1051/parasite/2020069>.
- Wawman, D.C. (2024) 'Ornithomya biloba , Pseudolynchia garzettae and Pseudolynchia canariensis (Diptera : Hippoboscidae): three new United Kingdom colonists and potential disease vectors', *Medical and Veterinary Entomology*, 38(2), pp. 160–171. Available at: <https://doi.org/10.1111/mve.12703>.
- Wawman, D.C. (2025) 'Citizen scientists mapping the United Kingdom's and Republic of Ireland's flat flies (louse flies) (Diptera: Hippoboscidae) reveal a vector's range shift.', *Medical and Veterinary Entomology*, pp. 1–17. Available at: <https://doi.org/10.1111/mve.12795>.
- Wickham, H. *et al.* (2023) 'Package 'dplyr' : A Grammar of Data Manipulation'. Available at: <https://cran.r-project.org/package=dplyr>.
- Wickham, H. and Seidel, D. (2022) 'scales: Scale Functions for Visualization'. Available at: <https://cran.r-project.org/package=scales>.
- Wikel, S.K. (1999) 'Modulation of the Host Immune System by Ectoparasitic Arthropods', *BioScience*, 49(4), pp. 311–320. Available at: <https://doi.org/10.2307/1313614>.
- Wood, D.E., Lu, J. and Langmead, B. (2019) 'Improved metagenomic analysis with Kraken

2', *Genome Biology*, 20(1), p. 257. Available at: <https://doi.org/10.1186/s13059-019-1891-0>.

Yeo, G. *et al.* (2019) 'Characterization of Fowlpox virus in chickens and bird-biting mosquitoes: A molecular approach to investigating avipoxvirus transmission', *Journal of General Virology*, 100(5), pp. 838–850. Available at: <https://doi.org/10.1099/jgv.0.001209>.

Zhao, L. *et al.* (2018) 'First report of *Anaplasma ovis* in pupal and adult *Melophagus ovinus* (sheep ked) collected in South Xinjiang, China', *Parasites & Vectors*, 11(1), p. 258. Available at: <https://doi.org/10.1186/s13071-018-2788-6>.

Table 1. Avian pox contig BLAST results from NGS sequencing. This table shows the results for the top blast hit of each of the contigs identified as being avian pox, from the *Ornithomya avicularia* taken from a Dunnock at the second site on the Wirral Peninsula near Liverpool, England.

DNA sequence	Top BLAST hit	Identity (%)	Genbank accession number	E-value	Query coverage (%)	alignment length (bp)
1	Crowpox virus	100	ON408417.1	7.00E-25	100	67
2	Canarypox virus	98.9	AY318871.1	2.00E-36	100	91
3	Canarypox virus	99.21	AY318871.1	4.00E-56	100	127
4	Canarypox virus	93.62	AY318871.1	2.00E-50	100	141
5	Canarypox virus	100	AY318871.1	4.00E-66	100	142

Table 2. Louse flies *Ornithomya* spp. tested for APV. Results of sequencing with details of bird host and site and GenBank accession numbers, for the Avipoxvirus 4b core protein gene sequences.

Date collected	Species	APV detected method	Fly sex	Bird age	Bird with pox?	Species with avian pox at site	Site	Latitude	Longitude
Dunnock <i>Prunella modularis</i>									
1 Jul 2021	<i>O. avicularia</i>	no		juvenile	no	Dunnocks	Wirral Peninsula, Cheshire	53.31	-3.20
5 Jul 2022	<i>O. fringillina</i>	no		juvenile	no	Dunnocks	Minehead, Somerset	51.20	-3.51
1 Jul 2020	<i>O. avicularia</i>	no		juvenile	no	Dunnocks	Minehead, Somerset	52.20	-3.51
1 Aug 2020	<i>O. fringillina</i>	no		juvenile	no	Dunnocks	Minehead, Somerset	53.20	-3.51
1 Aug 2022	<i>O. avicularia</i>	no	f	juvenile	no	Dunnocks	Minehead, Somerset	55.20	-3.49
5 Jul 2021	<i>O. avicularia</i>	no		juvenile	no	none seen	West Down, Devon	51.14	-4.29
1 Jul 2021	<i>O. avicularia</i>	no	f	juvenile	no	none seen	North Hill, Minehead, Somerset	54.21	-3.51
5 Jun 2022	<i>O. avicularia</i>	no	f	juvenile	no	none seen	Brandon, Suffolk	52.39	0.50
4 Jun 2022	<i>O. avicularia</i>	no	f	fledgling	no	none seen	Cameley, North Somerset	51.25	-2.57
1 Sep 2022	<i>O. fringillina</i>	no	m	juvenile	no	none seen	Cameley, North Somerset	51.25	-2.57
2 Jul 2022	<i>O. avicularia</i>	no	m	adult	no	none seen	Little Snoring, Norfolk	52.83	0.82
5 Jul 2023	<i>O. avicularia</i>	no	f	juvenile	no	none seen	Bridford, Devon	50.61	-3.70
4 Jul 2023	<i>O. avicularia</i>	NGS	f	juvenile	yes	Dunnocks	Wirral Peninsula, Cheshire	53.31	-3.20
10 Nov 2021	<i>O. fringillina</i>	PCR		juvenile	yes	Dunnocks	Wirral Peninsula, Cheshire	53.31	-3.20
2 Aug 2021	<i>O. fringillina</i>	PCR	m	juvenile	no	Dunnocks	Minehead, Somerset	51.20	-3.51
3 Aug 2022	<i>O. fringillina</i>	PCR	m	juvenile	no	Dunnocks	Minehead, Somerset	51.20	-3.51
10 Jul 2022	<i>O. avicularia</i>	PCR	f	juvenile	no	Dunnocks	Minehead, Somerset	51.20	-3.51
7 Aug 2013	<i>O. fringillina</i>	PCR		adult	no	Dunnocks	Minehead, Somerset	51.20	-3.51
5 Jul 2022	<i>O. fringillina</i>	PCR	f	juvenile	no	Great Tits	Stanford Reservoir, Northamptonshire	52.41	-1.12
1 Jul 2022	<i>O. avicularia</i>	PCR	m	juvenile	no	Great Tits	Corby, Northamptonshire	52.41	-0.83
1 Aug 2022	<i>O. avicularia</i>	PCR	f	adult	no	Great Tits	Corby, Northamptonshire	52.41	-0.83
10 Jul 2021	<i>O. avicularia</i>	PCR	f	adult	no	none seen	Near Sandy, Bedfordshire	52.41	-0.38
2 Jul 2022	<i>O. avicularia</i>	PCR		juvenile	no	none seen	Rutland Water, Rutland	52.59	-0.82

3 Jul 2022	<i>O. avicularia</i>	PCR	m	juvenile	no	none seen	Rutland Water, Rutland	52.59	-0.82
potted Woodpecker <i>Dendrocopos major</i>									
8 Sep 2023	<i>O. avicularia</i>	PCR	m	juvenile	no	none seen	West Down, Devon	51.14	-4.29

Figure 1. Weekly cases of avian pox in Dunnocks (green bars) and numbers of louse flies (*Ornithomya avicularia* and *O. fringillina* combined) found on Dunnocks (black stars and solid lines) for all years combined, from a 16.5 year study in Somerset, UK.

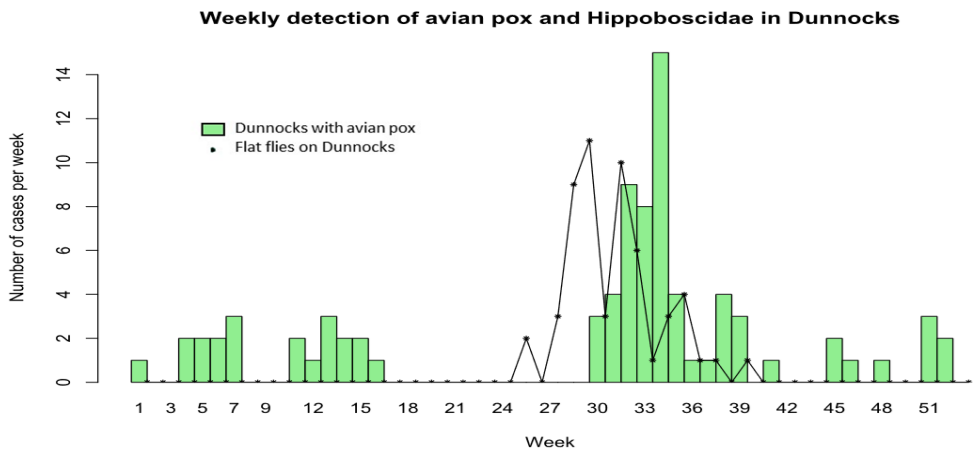


Figure 2. Weekly detection of avian pox in Dunnocks (green dots) and presence of louse flies on Dunnocks (black stars), with the louse flies plotted 3 weeks later than their actual dates, to show the similarity in peaks, otherwise separated by the approximate incubation period of avian pox in Dunnocks.

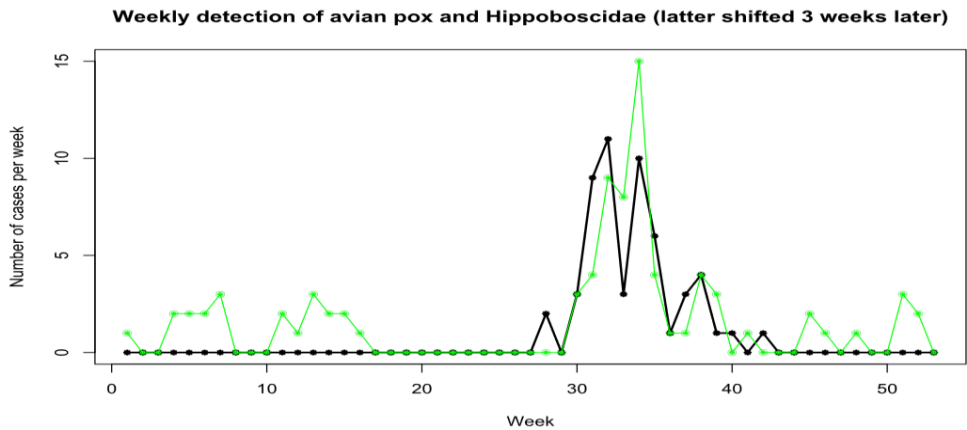
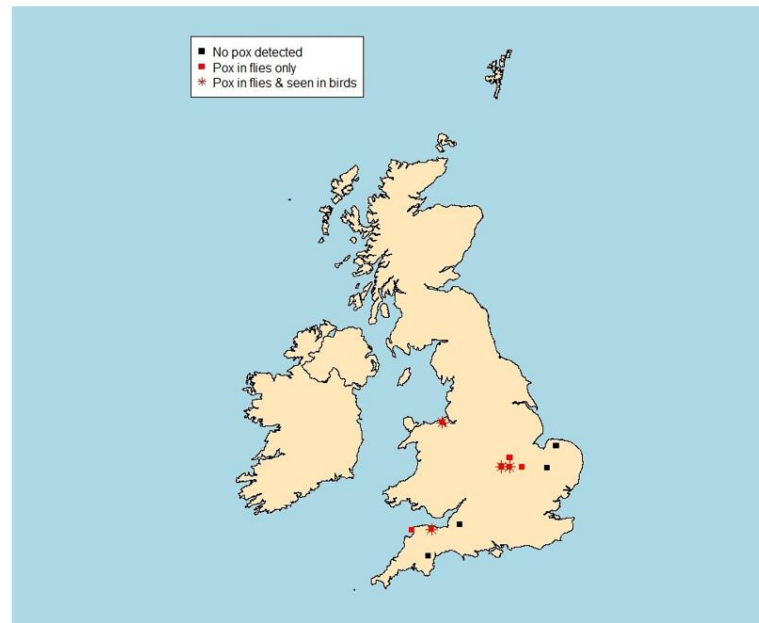


Figure 3. Map of the sites from which flies were tested: black squares - no avian pox detected, in either tested louse flies or observed in birds; red squares - avian pox detected but no birds observed with avian pox; red stars – birds observed with avian pox and flies tested positive.



Accepted