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The expanding H5N1 avian influenza panzootic causes high mortality of skuas in Antarctica

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Abstract

High pathogenicity avian influenza virus H5N1 subtype (H5N1 HPAIV), clade 2.3.4.4b, is expanding its host and geographical range, and invaded Antarctica in 2023. Although mortality in Antarctic wildlife from H5N1 HPAIV has been suspected, mainly based on virological analysis of swabs collected from dead animals, it has not been unequivocally diagnosed. Here we show that H5N1 HPAIV caused high mortality in a breeding colony of skuas at one of ten sites in Antarctica we visited in March 2024. By combined virological, bacteriological and pathological analyses, we found that H5N1 HPAIV caused multi-organ necrosis and rapid death in skuas, but not in other species examined. Taken together with recent data, skuas in Antarctica are at risk of continued mortality from H5N1 HPAIV infection, threatening their already small populations. Conversely, because of their wide distribution and

ecological relevance, skuas may play a substantial role in the spread of the virus across Antarctica. Transdisciplinary surveillance is needed in coming years to monitor the impact of this poultry-origin disease on Antarctica's unique wildlife.

Introduction

The incursion of A/goose/Guangdong/1/96 (Gs/Gd) lineage high pathogenicity avian influenza (HPAI) virus (HPAIV) of the subtype H5N1 clade 2.3.4.4b into Antarctica is a potential threat to the millions of wild birds and mammals living there. This is exemplified by the incursion of H5N1 HPAIV into South America in 2022-2023, where it is estimated to have killed at least 667,000 wild birds of 83 species and 53,000 wild mammals of 11 species [1,2]. There is also a real risk that the mortality of Antarctic wildlife from this virus will go unnoticed or underreported. Such documentation is critical to support the need for transformative change to protect wildlife populations worldwide from HPAI and other anthropogenic diseases originating from ever-increasing livestock populations [3,4].

The effects of H5N1 HPAIV infection on wildlife range from no clinical signs to death [5]. This means that infected wild animals can both act as vectors of the virus, possibly carrying it thousands of kilometers during long-distance migration in a staggered manner [6], and as its victims, suffering severe die-offs [7]. It also means that detection of HPAIV in a dead wild animal needs to be complemented by pathological demonstration of virus-associated lesions to confirm that HPAI virus infection was the cause of death. Additionally, these investigations are enhanced by testing apparently healthy individuals in wildlife populations.

The presence of H5N1 HPAIV of clade 2.3.4.4b in Antarctica was first recorded in January 2024 in a kelp gull (*Larus dominicanus*) on Livingston Island (South Shetland Islands), adjacent to the Antarctic Peninsula [8,9]. The virus may have spread to Antarctica directly from the southern tip of South America [8], or it may have used Subantarctic islands as stepping stones [10]. Seabirds and marine mammals are known to migrate between these locations during their annual cycle and may have carried the virus with them [11,12].

Two closely related seabird species that are known to be susceptible to H5N1 HPAIV infection in the southern hemisphere are brown skuas (*Stercorarius antarcticus*) and south polar skuas (*Stercorarius maccormicki*) [10,13]. While brown skuas, with a global population of about 7000 pairs, exhibit a circumpolar breeding and non-breeding distribution across the southern hemisphere [14–16], south polar skuas, with a global population of 5000 to 7000 pairs, breed around the coast of Antarctica [14,17], but exhibit a trans-equatorial non-breeding distribution on the North Pacific and North Atlantic Oceans [18,19]. In some locations, these species breed in sympatry, and hybridization occurs [15]. Both species were among the first to be found infected with H5N1 HPAIV in Subantarctic South Georgia in late 2023 as well as Antarctica in early 2024 [10,13,20,21]. Although H5N1 HPAIV was detected in association with morbidity and mortality in both brown and south polar skuas, no pathological analyses have been presented to confirm HPAI as the cause of death.

Besides HPAI, avian cholera (also known as pasteurellosis), repeatedly reported to cause avian mortality in this region, needs to be considered in the differential diagnosis of unusual mortality of wild birds in the Subantarctic and Antarctic regions [22–26]. *Pasteurella multocida*, the causative

agent of avian cholera, typically causes epizootics in wetlands or breeding colony sites with high densities of birds, including seabirds [27].

Little is known about the spread of H5N1 HPAIV within and among species of wild seabirds and mammals in Antarctica, about the character and severity of disease, and the levels of mortality in affected populations. Therefore, we set up the HPAI Australis Expedition to investigate the introduction and spread of H5N1 HPAIV across the Antarctic Peninsula and its impact on local wildlife [20]. Between 17 and 28 March 2024, at the end of the austral summer, when the breeding season of skuas (October-December), gentoo penguins (*Pygoscelis papua*; November-December) and Adélie penguins (*Pygoscelis adeliae*; October-February)[28] had already concluded, we conducted epidemiological surveys in wildlife at 10 locations at the South Shetland Islands, Weddell Sea and Trinity Peninsula for wildlife morbidity and mortality, performed autopsies and collected postmortem tissue samples and environmental samples for virological, bacteriological, and pathological analyses. Here, we report our findings of H5N1 HPAIV detection in skuas at three locations (Hope/Esperanza Bay, Devil Island, Beak Island) at the Trinity Peninsula and Weddell Sea region, and confirmation of HPAI as the probable cause of a mass die-off of south polar skuas at one of those locations (Beak Island). Additionally, we present results from avian cholera, environmental samples and other species from which samples were collected.

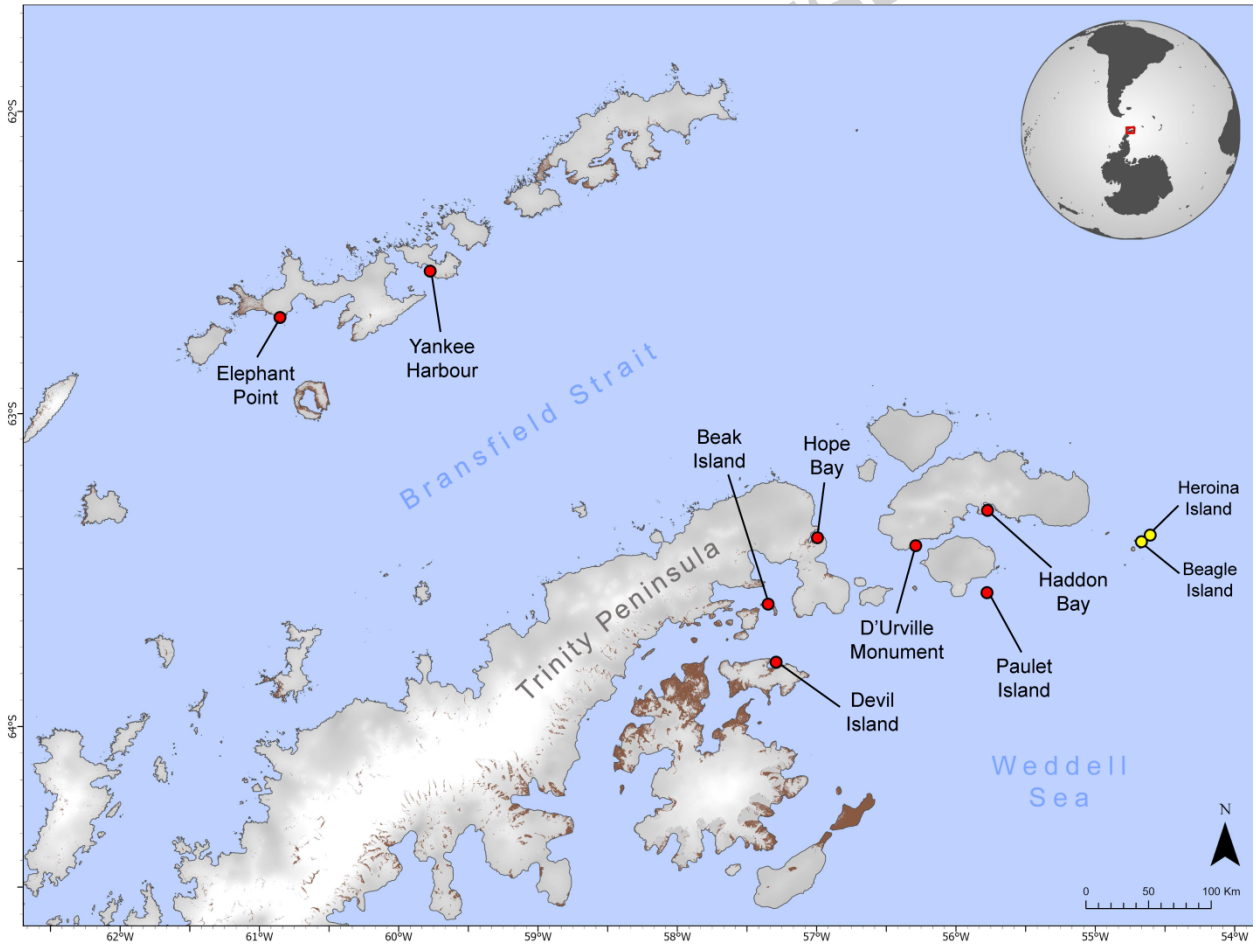

Figure 1. Map of the HPAI Australis Expedition, indicating investigated sites. Results from Heroina Island and Beagle Island (yellow) will be shown in a separate publication. The map was created using ArcGIS Pro 3.1.2 (ESRI, Redlands, California) based on shapefiles made publicly available by the British Antarctic Survey (BAS) via the UK Polar Data Centre (https://data.bas.ac.uk/)

Results

Overview of skua mortality during the HPAI Australis Expedition

During our expedition (Fig. 1), we found evidence of skua mortality at six sites (Hope Bay, Devil Island, Beak Island, Heroina Island, Beagle Island and Paulet Island), four of which are presented here (results for Heroina and Beagle Island will be shown in a separate publication) (Fig. 2, Table 1).

At Hope Bay, 16 non-breeding brown skuas and 3 non-breeding south polar skuas were previously found dead between February 5th and March 9th, 2024, through routine surveillance conducted by the Argentine National Antarctic Program, and stored under rocks to prevent scavenging. At the time of our visit, we could retrieve 6 of these dead adult skuas (1 south polar, 2 brown, 3 unidentified). Additionally, about 60 live skuas (apparently healthy) were present at the time of our visit. We autopsied two skuas and diagnosed avian cholera as the probable cause of death (SK01, SK06), although in one (SK01) a fungal infection probably contributed to death. We detected moderate to high levels of *P. multocida* in the brains of four additional dead skuas (SK02 to SK05). Two of these also had low (SK03) to moderate (SK02) levels of H5N1 HPAIV RNA in the brains (Table 2), so the confidence level for the avian cholera diagnosis was scored as "medium" (Supplementary table 10).

At Devil Island, we found a dead juvenile skua (unidentified species; SK07), and diagnosed HPAI as its probable cause of death. An additional 15 apparently healthy live skuas were present at the time of our visit.

We visited Beak Island because we considered its topography to provide a likely breeding habitat for skuas. There, we found 46 dead skuas (18 south polar, 28 unidentified species; 26 adult, 19 juvenile, 1 undetermined age class). Additionally, about 100 apparently healthy live skuas were also present at the time of our visit; these were occasionally seen inspecting or scavenging on skua carcasses, in addition to agonistic interactions with other live skuas and kelp gulls. We autopsied five of the dead skuas (SK08 to SK12) and diagnosed HPAI as the most probable cause of death. We detected moderate (SK15) or high levels (SK13, SK14, SK16, SK17) of H5 HPAIV in the brains of five additional dead skuas, supporting HPAI as cause of death.

At Paulet Island, we found three dead skuas (unidentified species; SK29 to SK31) and we determined that the possible causes of death were avian cholera in two of them (SK29, SK31), and either fungal disease or avian cholera in the third (SK30). About 30 apparently healthy live skuas were present at the time of our visit.

Table 1. Overview of diagnostic criteria for high pathogenicity avian influenza (HPAI), avian cholera, and fungal infection as proposed causes of death of 20 skuas found dead. The confidence level of the diagnosis applies to all diagnoses within the same animal.

Location	Individual	Species	Age class	State of autolysis	Nutritional condition	HPAI		Avian cholera		Fungal disease	Diagnosis	
						Brain H5N1 HPAIV RNA	Viral histo	Brain *P.m.* DNA	*P.m.* histo	Fungus histo	Diseases at death in order of severity	Confidence level
Hope Bay	**SK01**	BRSK	Ad	Moderate	M	-	-	+++	+	+	1. Fungal disease 2. Avian cholera	High
	SK02	BRSK	Ad	Advanced	P	++	na	++	na	na	1. Avian cholera 2. HPAI	Medium
	SK03	SPSK	Ad	Advanced	VP	+	na	+++	na	na	1. Avian cholera 2. HPAI 3. Starvation	Medium
	SK04	UNSK	Ad	Advanced	VP	-	na	++	na	na	1. Avian cholera 2. Starvation	Medium
	SK05	UNSK	Ad	Advanced	P	-	na	++	na	na	1. Avian cholera	Medium
	SK06	UNSK	Ad	Moderate	M	-	-	+++	+	-	1. Avian cholera	High
Devil Is.	**SK07**	UNSK	Juv	Minimal	G	+++	+	-	-	-	1. HPAI	High
Beak Is.	**SK08**	SPSK	Ad	Moderate	VP	+++	+	-	-	+	1. HPAI 2. Fungal disease 3. Starvation	High
	SK09	UNSK	Juv	Mild	VP	+++	+	-	-	-	1. HPAI 2. Starvation	High
	SK10	UNSK	Juv	Mild	G	+++	+	-	-	-	1. HPAI	High
	SK11	UNSK	Juv	Mild	G	+++	+	-	-	-	1. HPAI	High
	SK12	UNSK	Juv	Mild	G	+++	+	-	-	-	1. HPAI	High
	SK13	UNSK	nd	nd	nd	+++	na	-	na	na	1. HPAI	Medium
	SK14	UNSK	nd	nd	nd	+++	na	-	na	na	1. HPAI	Medium
	SK15	UNSK	nd	nd	nd	++	na	-	na	na	1. HPAI	Medium
	SK16	UNSK	nd	nd	nd	+++	na	-	na	na	1. HPAI	Medium
	SK17	UNSK	nd	nd	nd	+++	na	-	na	na	1. HPAI	Medium
Paulet Is.	**SK29**	UNSK	Ad	Moderate	nd	-	-	+++	+	-	1. Avian cholera	High
	SK30	UNSK	Ad	Moderate	P	(+)	-	++	+*	+	1. Fungal disease 2. Avian cholera	High
	SK31	UNSK	Ad	Moderate	na	-	-	+++	na†	-	1. Avian cholera	Medium

Species: BRSK: brown skua, SPSK: south polar skua, UNSK: unidentified skua.
Age class: Ad: adult, Juv: juvenile
Nutritional condition: VP, very poor; P, poor; M, moderate; G, good
nd: not determined
na: not applicable (not sampled)
Brain H5N1 HPAIV RNA: Result of HPAI virus M1 RT-qPCR expressed as 40 – Cq, with following categories: -, not detected; +, 1 to 10; ++, 11 to 20; +++, 21 to 30, (+) not detected in this study, however low levels of HPAIV RNA had been detected in a brain swab in earlier testing [20,29], and no avian influenza-associated disease was detected.
Viral histo: HPAI virus NP antigen expression and associated lesions present (+) or absent (-) in tissues.
Brain *P.m.* DNA: Result of *Pasteurella multocida kmt1* qPCR expressed as 40 – Cq, with following categories: -, no *P. multocida* DNA detected; +, 1 to 7; ++, 8 to 14; +++, 15 to 21.
P.m. histo: Aggregates of *P. multocida* and associated lesions characteristic for avian cholera present (+) or absent (-) in tissues.
Fungus histo: Fungal hyphae and associated lesions characteristic for fungal disease present (+) or absent (-) in tissues.
* As liver was not sampled for this skua, it is not possible to detect if *P. multocida* is associated with lesions there. Abundant bacteria with a morphology that is consistent with *Pasteurella* spp are present within blood vessels in the lungs.

† As liver was not sampled for this skua, it is not possible to detect if *P. multocida* is associated with lesions there. The lungs were in an advanced state of autolysis and showed a mixture of bacterial growth and therefore it was not possible to determine if *P. multocida* was present.

Macroscopic findings in skuas found dead

Some of the skuas that were found dead had abnormal postures, possibly related to HPAI: opisthotonos (backward arching of head, neck, and spine; 15/37), torticollis (twisting of the neck; 8/37) and/or wings spread (32/37; Supplementary Table 1, Supplementary Fig. 1). Out of 6 dead skuas (1 from Devil Island, 5 from Beak Island) diagnosed with HPAI as a probable or possible cause of death, one had opisthotonos (SK07), one had torticollis (SK08), and four had wings spread (SK08 to SK11). However, out of two dead skuas from Paulet Island (SK29 and SK30) diagnosed with avian cholera as a probable or possible cause of death, SK30 had opisthotonos and both had wings spread, indicating that these abnormal postures are not restricted to death from HPAI. The postures of the dead skuas from Hope Bay were not recorded as the carcasses had been previously manipulated to avoid scavenging by healthy skuas.

None of the 11 skuas examined had significant macroscopic lesions, with three exceptions. One skua diagnosed with HPAI as probable cause of death (SK07) had diffusely dark red and wet lungs (pulmonary edema) (Supplementary Fig. 1C). Two skuas diagnosed with fungal disease as probable or possible cause of death (SK01, SK08) had multiple characteristic white, dull, firm, well demarcated foci (3 to 30 mm in diameter) on tracheal mucosa, lungs and heart. The nutritional conditions of the autopsied skuas ranged from very poor to good, and were not correlated with the diagnoses of HPAI, avian cholera, or fungal disease as causes of death (Table 1). All the autopsied skuas had empty stomachs, suggesting they had not fed recently before death or had regurgitated their last meal.

Nine dead skuas were immediately tested on-site for the presence of influenza A virus by use of a rapid antigen test on swabs from different tissues (Supplementary Table 1). Out of 5 skuas that were subsequently H5 HPAIV-negative by RT-qPCR (Table 1; SK01, SK06, SK29 to SK31), 4 tested negative by rapid antigen test (SK01, SK06, SK29 and SK30) and for 1 the test result was uncertain (SK31). Out of 4 skuas that were subsequently H5N1 HPAIV-positive by RT-qPCR (SK02, SK07, SK09 and SK10; Table 1), 1 tested positive by rapid antigen test (SK07), 1 tested negative (SK02) and for 2 the test results were uncertain (SK09 and SK10).

Figure 2. Dead skuas on Beak Island being examined by two expedition members. The animals were moved from their original positions and lined up, as shown in the photo, for external examination and triage for autopsy. The people depicted in the photograph are co-authors of this manuscript and have consented to including their images.

Virological and bacteriological analyses of swabs and tissues and serological analyses from skuas found dead

Influenza A virus RNA was detected by RT-qPCR from swabs and tissues of sampled skuas. H5N1 HPAIV RNA was detected in swabs and tissues of 13 of 20 dead skuas tested, based on the combination of positive M1, H5, and HPAI RT-qPCRs for each tissue or swab. N1 RT-qPCR was additionally performed on a subset of samples to confirm the subtype (Table 2, Supplementary Table 2). RNA loads (40 – Cq) of the GAPDH housekeeping gene ranged between 11.7 and 25.0 (19.6 ± 3.3 on average), indicating an overall good RNA preservation in the carcass samples (Supplementary Table 9). To confirm the presence of the multi-basic cleavage site (MBCS), a region of the hemagglutinin gene was sequenced from at least one sample from each H5N1 HPAIV-positive skua (Supplementary Table 2). Samples from all positive skuas were confirmed to have the same MBCS (PLREKRRKR/GLF). For each of the 13 skuas that tested positive, all sampled tissues and swabs tested positive by the four RT-qPCRs - indicating systemic H5N1 HPAIV infection - except for the tracheal swab of one dead skua (SK08). The highest viral RNA loads were consistently measured in the brain, except for one skua (SK12), in which the liver had the highest viral RNA load. Average viral RNA loads (expressed as 40 – Cq) were several units higher in the brain (21.4 ± 5.7) than in tracheal swabs (16.3 ± 2.9) and oropharyngeal swabs (11.1

± 6.3). The dead skuas sampled at Hope Bay had viral RNA loads in the brain (5.6 and 18.6) that were substantially lower than the dead skuas sampled at Devil Island and Beak Island (23.1 ± 3.3 on average). Overall, the highest viral loads in skuas that were diagnosed with HPAI as a possible cause of death (SK07 to SK12) were found in the brain (23.4 ± 2.5 on average) (Fig. 3).

No antibodies against H5 influenza A viruses or nucleoprotein (NP) were detected by competitive ELISA in body fluid samples collected from two skuas (SK07 and SK12), both of which were diagnosed with HPAI as probable cause of death.

To determine whether or not avian cholera contributed to mortality, all the skuas were tested for the presence of *P. multocida* DNA (*kmt1* gene) by qPCR. *P. multocida* DNA was detected in swabs and tissues from all dead skuas in Hope Bay and Paulet Island (Table 2). The qPCR amplicon sequencing revealed up to 100% identity with *P. multocida* strains via BLASTn search. The *P. multocida* bacterial DNA load (40 – Cq) differed substantially between skuas, ranging from 8.2 to 18.5 in the brain. In contrast, none of the tested organs or swabs from any of the skuas from Beak Island or Devil Island tested positive for *P. multocida* DNA by qPCR (Table 2).

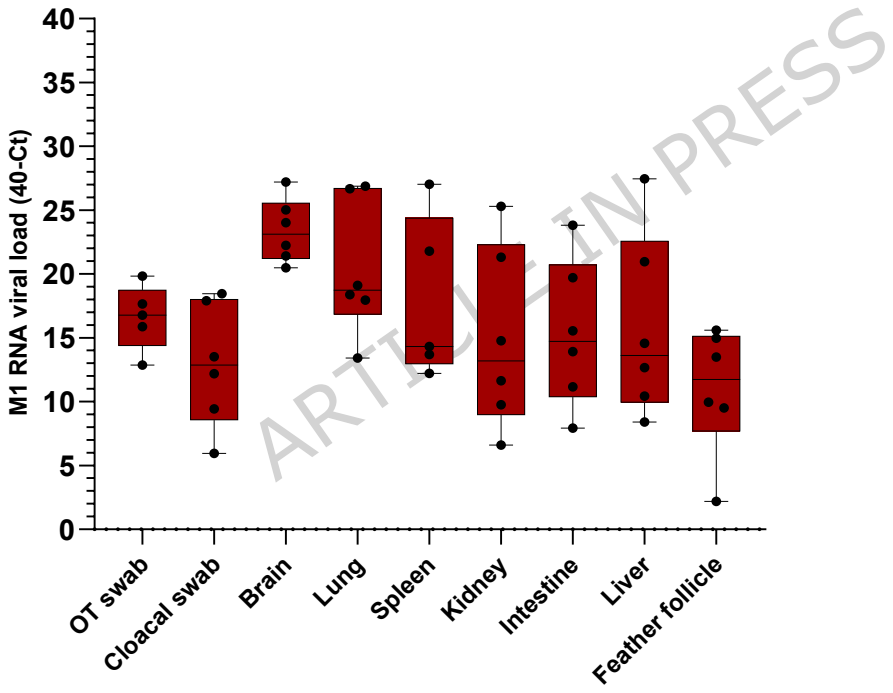

Figure 3. Influenza A RNA viral loads in different organs of skuas. Boxplot of the relative expression of M1 viral RNA loads (40 – Cq) in different organs and tissue swabs (OT swab: oropharyngeal or tracheal swab) of skuas diagnosed with HPAI as cause of death and tested positive for M1, H5, N1 and HPAI RT-qPCRs (SK07-12). Each black dot represents a single detection.

Table 2. H5N1 HPAIV viral loads (40-Cq, *M1* gene) and *P. multocida* (PM) bacterial loads (40-Cq, *kmt1* gene) in swabs and tissues of skuas found dead. Spleen, kidney and feather follicle were not tested for the presence of *P. multocida* DNA. The IDs from onboard testing are indicated to compare with previous results [20,29].

Location	Individual	ID onboard test (*40*)	Tracheal swab		Oropharyngeal swab		Cloacal swab		Brain		Lung		Intestine		Liver		Spleen	Kidney	Feather follicle
			HPAIV	PM	HPAIV	PM	HPAIV	PM	HPAIV	PM	HPAIV	PM	HPAIV	PM	HPAIV	PM	HPAIV	HPAIV	HPAIV
Hope Bay	**SK01**	Skua_1_Hope	na	na	nd	17.2	nd	16.8	nd	15.8	nd	18.5	nd	18.9	nd	4.1	nd	nd	nd
	SK02	Skua_2_Hope	na	na	10.6	4.1	7.4	9.9	18.6	9.7	na	na	na	na	na	na	na	na	na
	SK03	Skua_3_Hope	na	na	5.2	13.2	0.2	15.4	5.6	16.0	na	na	na	na	na	na	na	na	na
	SK04	Skua_4_Hope	na	na	nd	8.5	nd	4.9	nd	8.2	na	na	na	na	na	na	na	na	na
	SK05	Skua_5_Hope	na	na	nd	14.0	nd	14.2	nd	12.4	na	na	na	na	na	na	na	na	na
	SK06	Skua_6_Hope	na	na	nd	14.5	nd	16.1	nd	18.0	nd	19.9	nd	18.7	nd	22.3	nd	nd	nd
Devil Is.	**SK07**	Skua_1_Devil	na	na	17.7	nd	17.9	nd	27.2	nd	26.7	nd	19.7	nd	21.0	nd	21.8	21.3	13.5
Beak Is.	**SK08**	Skua_1_Beak	nd	nd	na	na	6.0	nd	20.5	nd	13.4	nd	7.9	nd	8.4	nd	na	6.6	2.2
	SK09	Skua_2_Beak	15.9	nd	na	na	12.2	nd	22.2	nd	18.4	nd	11.2	nd	14.6	nd	14.3	9.8	15.0
	SK10	Skua_3_Beak	16.8	nd	na	na	13.5	nd	21.4	nd	19.1	nd	15.5	nd	12.7	nd	13.7	14.8	9.5
	SK11	Skua_4_Beak	12.9	nd	na	na	9.4	nd	24.0	nd	18.0	nd	13.9	nd	10.4	nd	12.2	11.6	10.0
	SK12	Skua_5_Beak	19.8	nd	na	na	18.5	nd	25.0	nd	26.9	nd	23.8	nd	27.5	nd	27.0	25.3	15.6
	SK13	Skua_6_Beak	na	na	na	na	na	nd	28.0	nd	na	nd	na	nd	na	nd	na	na	na
	SK14	Skua_7_Beak	na	na	na	na	na	nd	21.7	nd	na	nd	na	nd	na	nd	na	na	na
	SK15	Skua_8_Beak	na	na	na	na	na	nd	16.3	nd	na	nd	na	nd	na	nd	na	na	na
	SK16	Skua_9_Beak	na	na	na	na	na	nd	22.2	nd	na	nd	na	nd	na	nd	na	na	na
	SK17	Skua_10_Beak	na	na	na	na	na	nd	25.0	nd	na	nd	na	nd	na	nd	na	na	na

Paulet Is.	**SK29**	Skua_1_Paulet	na	na	na	na	nd	na	nd	18.5	nd	21.2	na	na	na	na	na	na	na
	SK30	Skua_2_Paulet	na	na	na	na	nd	na	nd	8.9	nd	18.6	na	na	na	na	na	na	na
	SK31	Skua_3_Paulet	na	na	na	na	nd	na	nd	15.1	nd	15.7	na	na	na	na	na	na	na

nd: not detected
na: not applicable (not sampled)

Histopathology of tissues from skuas found dead

Virus antigen expression in H5 HPAIV RNA-positive skua carcasses

Influenza A virus NP antigen was detected by immunohistochemistry (IHC) in all (n=6) H5N1 HPAIV RNA-positive skua carcasses (Supplementary Table 3). Brain was the organ that most consistently expressed virus antigen (5/6 skuas). Other tissues in which virus antigen expression was detected in parenchymal cells were lung, pancreas, thyroid, adrenal, preen gland and gonad (testicle) (Fig. 4, Supplementary Fig. 2). One bird (SK12) had abundant virus antigen expression in endothelial cells in every tissue examined. Virus antigen was rarely detected in parenchymal cells of kidneys (1/5), and not in those of livers (0/6), or in hearts (0/6) (Supplementary Table 3).

In the brain, virus antigen was detected in cerebrum, cerebellum and brainstem in a pattern that was seemingly random and differed per bird. Virus antigen also was detected in peripheral ganglia of one bird (Supplementary Fig. 2). The cells that expressed virus antigen in the nervous tissues were both neurons and glial cells, based on morphology and their location relative to other cells. In one bird (SK07), a large stretch of ependymal cells and the underlying neuropil also expressed virus antigen, suggesting entry via the cerebrospinal fluid (Supplementary Fig. 2). By *in situ* hybridization (ISH), we detected influenza A virus RNA in the brain section of a skua (SK11) that was virus antigen negative by IHC (Supplementary Text 1, Supplementary Fig. 4).

In the lung (2/6), virus antigen was detected in air capillaries. Because of their close alignment, it was not possible to determine whether endothelial cells and/or epithelial cells of the air capillaries expressed virus antigen. Virus antigen distribution was multifocal to coalescing and involved 90 to 100% of the air capillaries per lung section.

In other tissues, the cell types expressing virus antigen were exocrine glandular epithelial cells in pancreas (Fig. 4) and epithelial cells of the preen gland, sperm producing cells (spermatogonia) of inactive testis, epithelial cells in thyroid gland (Supplementary Fig. 2) and in adrenal gland. In most tissues, virus antigen was mostly expressed by clusters of tens of cells.

All five skuas that tested negative for H5N1 HPAIV RNA by RT-qPCR (Table 2) and of which tissues were sampled for histopathology (Hope Bay – SK01 and SK06; Paulet Island – SK29 to 31) were also negative for influenza A virus antigen expression.

Co-localization of virus antigen expression and histological lesions

Histological lesions were attributed to H5N1 HPAIV infection based on their co-localization with virus antigen expression (co-localization was assessed on a serial H&E slide; Fig. 4, Supplementary Fig. 2, Supplementary Table 4). Virus antigen expression in the lungs co-localized with diffuse distension of blood vessels with erythrocytes (SK07 and SK12; Supplementary Fig. 3) and flooding of approximately 10 to 30% of the lumina of all parabronchi and infundibula with eosinophilic homogenous material (edema) and fibrillary eosinophilic material (fibrin) (SK07 only). Virus antigen expression in the brain co-localized with neuronal necrosis and presence of a few heterophils around larger foci of necrosis (Fig. 4). Virus antigen expression in the pancreas co-localized with necrosis of exocrine glandular epithelial cell necrosis with mild heterophil infiltration around larger foci. In both testicle and thyroid gland, it was co-localized with few degenerate and necrotic cells (Supplementary Fig. 2).

Microscopic findings associated with avian cholera

All five skuas that tested negative for influenza virus RNA by RT-qPCR, tested positive for *P. multocida* DNA by qPCR (Table 2). Three (SK01, SK06, SK29) of the five skuas had histological

lesions in the liver suggestive of *P. multocida* infection (Supplementary Fig. 5, Supplementary Table 4); liver was not sampled in the other two. These lesions were characterized by the presence of randomly distributed, large aggregates of uniformly shaped coccoid bacteria in the liver, in some aggregates co-localized with heterophils and necrotic hepatocytes (SK06 and SK29). In addition, large aggregates of coccoid bacteria, consistent with *P. multocida*, and confirmed by ISH (Supplementary Text 2), were also present in the blood vessels of other organs sampled in all five birds that tested positive by qPCR (Supplementary Fig. 5).

Microscopic findings associated with fungal infection

Histological evidence of fungal infection was present in three skuas (SK01, SK08, SK30) in variable combinations of tissues: trachea, lung, air sac, heart (table S4). Fungal infection was characterized by abundant presence of fungal hyphae (3-6 µm thick, septate, with regular dichotomous branching, consistent with *Aspergillus fumigatus*)[30,31] (Supplementary Fig. 6). The presence of fungal hyphae was particularly extensive in the lung. Fungal hyphae were co-localized with necrosis and infiltration with heterophils, macrophages and admixed with fibrin and extravasated erythrocytes.

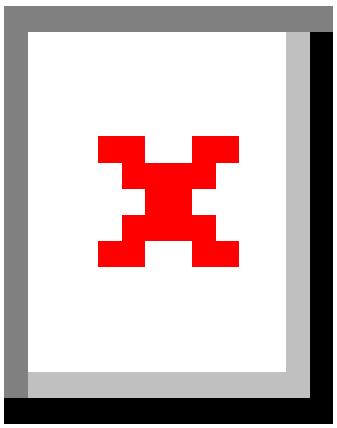

Figure 4. Serial sections of hematoxylin and eosin staining (H&E) and influenza IHC in tissues of skuas found dead. Brain and pancreas belong to SK07, air sac belongs to SK09. Cells positive for virus antigen by immunohistochemistry (IHC) stain red in the nucleus and cytoplasm. Brain and pancreas: the center of the H&E picture contains necrotic debris (loss of architecture, hypereosinophilia, karyorrhexis and pyknosis) close to neuronal cells and exocrine pancreatic cells that express virus antigen (visible in IHC). Air sac: virus-antigen-positive cells largely have their normal morphology, with no associated lesions.

Virological and bacteriological analyses of environmental fecal swabs from live skuas and bacteriological analysis of environmental matrix samples

None of the environmental fecal swabs collected from live skuas at the sites where dead skuas were found tested positive for H5N1 HPAIV RNA: Beak Island (0/42 fecal swabs), Hope Bay (0/20), Devil Island (0/11), Paulet Island (0/11). For Beak Island, considering that there were approximately 100 live skuas at the time of our visit, it is possible to state that our sample size was sufficient to conclude with 90% confidence that the prevalence of HPAIV in the feces of the live skuas was lower than 5% (assuming test sensitivity of 90% and random sampling) (Supplementary Table 5). None of the 12 environmental fecal swabs collected from skuas at any of the other sites (Elephant Point, D'Urville Monument, Haddon Bay, Yankee Harbour) tested positive for influenza A virus; however, the sample size of environmental fecal swabs from skuas at these locations was lower and does not allow for a statement on the prevalence of the virus (Supplementary Table 5).

None of the environmental matrix samples – air (n=4), water (n=10) or soil (n=4) - from Hope Bay, Beak Island or Paulet Island tested positive for *P. multocida* DNA by qPCR, despite the presence of *P. multocida*-positive dead skuas at Hope Bay and Paulet Island.

Overview of mortality and diagnostic findings in species beyond skuas

During our expedition, we also found and autopsied carcasses of gentoo penguins, Adélie penguins and Antarctic fur seals (*Arctocephalus gazella*) (Table 3). Although we detected low levels of H5N1 HPAIV RNA in some of the tissue samples from penguins, our investigations supported other possible causes of death than HPAI (Table 3). Additionally, we collected brain samples from one snowy sheathbill (*Chionis albus*) and five southern elephant seals (*Mirounga leonina*), but we did not find any evidence of HPAIV infection (Table 3). These carcasses were in variable states of autolysis and decomposition (also based on GAPDH RT-qPCR data; Supplementary Table 9) ranging from freshly deceased carcasses to skeletal remains with dried-out fragments of brain tissue.

H5N1 HPAIV RNA was detected by RT-qPCR in 5 of 25 Adélie penguins from Devil Island and 1 of 7 gentoo penguins from D'Urville Monument (Supplementary Table 6). 4 of the 5 positive Adélie penguins were confirmed to have a MBCS (PLREKRRKR/GLF). Overall, viral RNA loads in these penguins were lower than in skuas and not detected in all the tissues and swabs. On average, the highest viral RNA load in H5 HPAIV-positive Adélie penguins was detected in the oropharyngeal swabs (10.1 ± 2.1), but comparable results were also found in the brain (9.4 ± 3.2). The positive gentoo penguin showed the highest viral RNA load in the brain (11.8) (Supplementary Table 6). We also detected influenza A viral RNA in tissues and swabs of additional Adélie (n=1, Paulet Island) and gentoo penguins (n=4, D'Urville Monument), but these samples were negative in the H5 and/or HPAI RT-qPCRs (Supplementary Table 7). No antibodies against H5 influenza A viruses or NP were detected by competitive ELISA in a body fluid sample collected from one gentoo penguin from D'Urville Monument (GEPE09).

Virus antigen expression was not detected in any of the penguin carcasses that were positive for H5 HPAIV RNA. Rare cells in the brain tissues of two Adélie penguins (AP01 and AP02) were positive by ISH (Supplementary Table 8). By histopathological examination, no other clear cause of death was detected for any of these six penguins, which fits with their probable cause of death

based on macroscopic examination being either starvation (3/6), egg retention (1/6), or trauma (1/6) (Table 3). For 1/6 penguins (AP06), neither macroscopic assessment was conducted nor histological samples collected.

Nearly all 370 environmental fecal swabs collected from species other than skuas tested negative for influenza A virus RNA (Supplementary Table 5). The exceptions were two environmental fecal swabs collected from apparently healthy individuals: one, which tested positive for H5N1 HPAIV RNA, from a southern giant petrel (*Macronectes giganteus*) at Yankee Harbour, and the other one, which could not be subtyped or pathotyped due to its low viral load, from a gentoo penguin at D'Urville Monument.

Table 3. Overview of diagnoses in gentoo penguins (GP), Adélie penguins (AP), snowy sheathbills (SB), southern elephant seals (ES) and Antarctic fur seals (FS) found dead. All animals tested negative for *P. multocida* DNA by qPCR in all organs.

Location	Individual	Age class	State of autolysis	Sex	Nutritional condition	HPAI		Observations		Diagnosis	
						Brain viral RNA	Viral histo	Macroscopic	Microscopic	Diseases at death in order of severity	Confidence level
Elephant Point	**GP01-03**	Juv, Ad	Minimal*	nd	P	-	na	Starvation	na	Unknown	Inconclusive
	GP04	Juv	Advanced	nd	nd	-	na	Unknown	na	Unknown	Inconclusive
	GP06	Ad	Minimal*	nd	G	-	na	Unknown	na	Unknown	Inconclusive
	ES01-05	Juv	Advanced	nd	nd	-	na	Unknown	na	Unknown	Inconclusive
	FS01	Ad	Advanced	nd	nd	-	na	Unknown	na	Unknown	Inconclusive
Devil Is.	**AP01-02**	Ch, Juv	Moderate/Advanced	M	VP	+	-†	Starvation	nad	1. Starvation 2. HPAI	Medium
	AP03	Juv	Advanced	nd	VP	-‡	-	Starvation	nad	1. Starvation 2. HPAI	Medium
	AP04	Ad	Advanced	F	G	++	-	Egg retention	nad	1. Egg retention 2. HPAI	Medium
	AP05	Ch	Moderate	M	VP	-	na	Starvation	nad	Starvation	Medium
	AP06	nd	nd	nd	nd	+	na	Unknown	na	Unknown	Inconclusive
	AP07-25	nd	nd	nd	nd	-	na	Unknown	na	Unknown	Inconclusive
D'Urville Monument	**GP08-09**	Ch, Juv	Minimal	F	VP	-	na	Starvation	nad	Starvation	High
	GP10	Ad	Minimal	M	G	++	-	Predation, HPAI	Mild histiocytic thyroiditis; nad	1. Predation 2. HPAI	High
	GP11	Juv	Minimal*	M	VP	-	na	Starvation	nad	Starvation	High
	GP12	nd	nd	nd	nd	-	na	nd	na	Unknown	Inconclusive
	GP14	Ch	Minimal	nd	nd	-	na	Unknown	nad	Unknown	Inconclusive
	GP15, AP26	Juv	Minimal/Moderate	nd	VP	-	na	Starvation	nad	Starvation	High
	SB01	nd	Advanced	nd	nd	-	na	nd	na	Unknown	Inconclusive
Haddon Bay	**FS02**	nd	Advanced	nd	nd	-	na	nd	na	Unknown	Inconclusive
Paulet Is.	**AP60-61, AP63-64, AP67-68**	Ch	Advanced	nd	nd	-	na	nd	na	Unknown	Inconclusive
	AP62	Juv	Moderate	nd	VP	-	na	Starvation	nad	Starvation	Medium
	AP65	Juv	Advanced	nd	nd	-	na	nd	na	Unknown	Inconclusive
	AP66	Juv	Moderate	nd	VP	-	na	Starvation	nad	Starvation	Medium
Yankee Harbour	**GP16-19, GP21-22**	Ad	Minimal*	nd	nd	-	na	Predation	nad	Predation	Medium
	GP20	Ad	Advanced*	nd	nd	-	na	Unknown	na	Unknown	Inconclusive
	FS06	Juv	Minimal*	M	Mo	-	na	Pneumonia¶	Bacterial pneumonia#	Bacterial infection	High

Age class: Ch: chick, Juv: juvenile, Ad: adult
Sex: M: male, F: female
Decomposition state: VP: very poor, P: poor, Mo: moderate, G: good
nd: not determined
na: not applicable (not sampled)
nad: no abnormalities detected
* Scavenged or predated
† Tissues were negative for virus antigen by IHC, and lesions were not detectable. For these cases, ISH was performed on the brain tissue, and few cells were positive.
‡ Animal positive for HPAI viral RNA but not in the brain.
¶ Severe chronic embolic pyogranulomatous pneumonia.
# Severe chronic embolic pyogranulomatous pneumonia with intralesional bacteria

Discussion

In our survey of sites at the South Shetland Islands and Trinity Peninsula in March 2024 [20], we diagnosed HPAI as the probable cause of an unusual mortality event involving at least 10 of 46 skuas found dead at the time of our visit at Beak Island. Evidence supporting this diagnosis included relevant macroscopic observations, high loads of H5N1 HPAIV RNA, virus antigen expression and typical lesions in the brains and other organs of skuas found dead, combined with lack of evidence of other potential causes of death. These results establish HPAI as a significant cause of mortality of skuas in the Antarctic Peninsula and Weddell Sea region of Antarctica.

Skuas in Antarctica may be both victims and potential vectors of H5 HPAIV, as indicated previously for wild birds in Europe [32,33] and North America [34].

As victims, the 46 skua deaths at Beak Island attributed to HPAI are undoubtedly far fewer than the actual number of skua deaths from HPAI there during the 2023-2024 breeding season, because of the dead skuas we missed, those that already had been scavenged by the time of our survey or died outside the colony site [35,36]. In addition, the skua deaths that may have occurred at other unsurveyed sites of the Antarctic Peninsula were missed.

Comparison of the virus distribution, viral loads, and associated pathological changes in the tissues of these south polar skuas and brown skuas and those in the tissue of great skuas (*Stercorarius skua*) with HPAI are informative. In skuas from both hemispheres, neurological clinical signs were associated with HPAIV infections, resulting in (per)acute death of affected individuals [10,37]. Despite a limited sample size for postmortem examination, we confirmed systemic HPAIV infections, including a particular tropism for the brain, which suggests that sampling this organ is the most efficient way to detect HPAIV in this species, but also the lungs, based on high viral RNA loads and virus antigen expression. Thus, neurological clinical signs could have occurred before the onset of death, although we did not observe any apparently sick birds alive during our expedition. In this context, body posture appeared to be a limited indicator of HPAI, as in one case (Paulet Island) the skua was more likely to have died of avian cholera. Virus distribution and associated pathological changes in tissues appeared to be more severe and widespread in great skuas [37], suggesting south polar and brown skuas may have an even faster fatal outcome from the disease than great skuas.

Local transmission of HPAIV among skuas at Beak Island was likely due to contact with other infected skuas. Our results of virological analysis of skua carcasses support different routes of transmission. Local transmission among skuas may have occurred via intra-species scavenging or cannibalism (based on multiple virus-positive organs that are typically eaten, as well as our observations of skuas scavenging skua carcasses on Beak Island), via contact with contaminated feces (based on viral RNA-positive cloacal swabs) or via contact with contaminated respiratory secretions (based on viral RNA-positive oropharyngeal or tracheal swabs). The freshwater lakes on Beak Island may have been a fomite for HPAIV transmission of skuas bathing together. In a HPAI-associated unusual mortality event of great skuas on Foula (Shetland Isles), the rapid spread of HPAIV among great skuas was considered to have been facilitated by their habit to bathe and socialize at freshwater lochs and pools, where close conspecific interactions occurred [38]. Alternatively, the higher density of carcasses near freshwater lakes on Beak Island could be related to agonal processes rather than transmission dynamics. Transmission of HPAIV may have been enhanced because of the low ambient temperatures, which ranged between –15 and +5°C at the sampling locations during our expedition. It is known that persistence of virus infectivity in infected carcasses [39] and contaminated water [40,41] is higher at lower temperatures.

As potential vectors, the results of our study do not provide strong evidence either for or against skuas as long-distance vectors of H5N1 HPAIV. Whereas some skuas seem to die acutely upon infection, we did not follow these infected individuals over time. Therefore, we cannot estimate whether, for example, they can act as vectors of HPAIV in the early stages of infection. Also, we do not know whether other skuas may be infected without showing clinical signs, as occurs in some duck species [42]. The unusual mortality event of skuas from HPAI in the Weddell Sea area may have started with introduction of H5N1 HPAIV from elsewhere by an infected skua. This fits with the facts that HPAIV had been detected in skuas on the South Shetland Islands and in other parts of the Antarctic Peninsula in February 2024 [13], and we did not observe mortality of other species at Beak Island. Additionally, this is consistent with the more recent detections of H5N1 HPAIV in skuas on both the east and west coasts of the Antarctic Peninsula, South Georgia, the South Shetland, Falkland, Marion, Crozet and Kerguelen Islands (https://scar.org/library-data/avian-flu). At Hope Bay, breeding brown skuas arrived earlier in the 2023-2024 season than the non-breeding population, which displayed a peak between mid-January and early February, and which was the only population affected by HPAI (Instituto Antártico Argentino, unpublished data). This would also suggest a possible introduction of H5N1 HPAIV in the Antarctic Peninsula by non-breeding skuas migrating from locations where the virus was already established (e.g. South Georgia and South Shetland Islands). The preferential brown skuas migration for the southwest Atlantic sector of the Southern Ocean and the Patagonian shelf [16] also supports this hypothesis. Alternatively, the unusual mortality event may have started with the introduction by an (undetected) infected bird of another species or marine mammal, with subsequent spillover into skuas [8,10].

The results of virological analysis of environmental fecal samples suggested lack of HPAIV circulation in apparently healthy birds at the times of our visits except for a single environmental fecal swab of a southern giant petrel (out of 13 environmental fecal swabs collected from this species). The failure to detect HPAIV RNA in environmental fecal swabs of apparently healthy birds contrasts with successful detection in samples of birds found dead, particularly in skuas on Beak Island. This is in agreement with previous studies showing that the usefulness of avian fecal samples for HPAI monitoring programs in wild bird populations is limited [43–45]. In this case, elaborate and adapted protocols are required and recommended for subsequent analyses to allow identification of nucleic acids in feces [43,46,47], e.g. including additional clean-up steps to remove possibly high levels of inhibitors that potentially interfere with downstream analyses or carrying an internal control to track likely inhibited samples. Alternatively, and in agreement with the low viral loads from cloacal swabs, these results suggest low HPAIV shedding into feces, which would influence transmission, spread and environmental persistence of the virus. Thus, avian influenza surveillance by fecal sampling, although easy to perform and reliable for LPAIVs detection [45,48], clearly needs careful consideration for HPAIV in the Antarctic context, considering its apparently low reliability to detect HPAIV circulation.

We diagnosed avian cholera rather than HPAI as possible cause of death of skuas at Hope Bay. Evidence supporting this diagnosis includes high loads of *P. multocida* in multiple tissues (indicating systemic bacterial spread), multifocal coccoid bacterial aggregates in the liver colocalized with necrosis and inflammation (indicating severe acute disease from *P. multocida* infection), and no detectable H5N1 HPAIV RNA in tissues or swabs. Previously, avian cholera had been diagnosed by bacteriological and pathological analyses in brown and south polar skuas found dead in Hope Bay in the breeding seasons of 1999-2000 and 2000-2001 [23]. These results underline the importance of

considering multiple possible causes when investigating unusual mortality events in skuas or other wildlife species, even in the presence of HPAI outbreaks.

Although we found evidence of H5N1 HPAIV infection in both dead Adélie penguins at Devil Island and a dead gentoo penguin at D'Urville Monument, we diagnosed other probable causes of death, mainly starvation. HPAI was ruled out as cause of death based on the low viral RNA levels by RT-qPCR in tissues and lack of virus antigen expression by IHC and associated lesions by histopathology in the same tissues. There also were two dead skuas at Hope Bay in which tissues low to medium levels of H5N1 HPAIV were detected by RT-qPCR, but they possibly or likely died from avian cholera. These results fit with the broad range of disease outcomes in multiple wild bird species infected with HPAIV, from subclinical infection to acute death [5,42,49]. Therefore, the detection of H5N1 HPAIV RNA by itself in the avian brain does not necessarily mean that HPAIV infection was the cause of death. However, the lack of clinical signs would represent a concern for viral persistence in animal populations and potential spread to susceptible species.

Whereas not investigated in this study, speculation on how the virus is transmitted between skuas and other species is relevant for the epidemiology of H5N1 HPAIV in this setting. Both brown and south polar skuas are opportunistic scavengers and predators of other birds. However, considering their dietary and behavioral differences [15,17,50,51], brown skuas are expected to have more and closer contacts with penguins, and might be more likely to be involved in virus transmission to and from penguins than south polar skuas. However, longitudinal studies throughout the breeding season and phylogenetic analyses of the viral isolates would be needed to assess this.

We conclude from the detection of H5N1 HPAIV in five species (brown skua, south polar skua, gentoo penguin, Adélie penguin, southern giant petrel) at four sites at a geographical distance of up to 50 km from each other in our study, together with the detection in other parts of the Antarctic Peninsula by us and others [8,13,20], that H5N1 HPAIV already was widespread in the northern part of the Antarctic Peninsula in March 2024, just two months after its first detection in Antarctica [8]. We also conclude that H5N1 HPAIV has the potential to cause marked mortality in south polar and brown skuas of the Antarctic Peninsula, up to levels that can potentially cause declines in these small populations. In 1984, it was estimated that there were only 650 breeding pairs of south polar skuas and 150 breeding pairs of brown skuas in the Antarctic Peninsula, out of global populations of 5000 to 8000 breeding pairs of south polar skuas and 7000 breeding pairs of brown skuas [52]. We are not aware of more recent estimates for the investigated sites, with the exception of Hope Bay. At Hope Bay, the brown skua breeding and non-breeding populations were overall increased between 2014 and 2023, and remained stable between the breeding seasons 2022-2023 and 2024-2025 (39-43 nests and 75-90 individuals, respectively; Ibañez et al., unpublished data); hence, the mortality at Hope Bay (primarily due to avian cholera) would represent roughly 20% of the local non-breeding population.

Whereas we previously showed that H5N1 HPAIV RNA was detected in the brain of one of three skuas from Paulet Island (SK30) [20,29], we could not confirm this result hereby. One possible explanation is that since the RNA viral load was low, and given the focal nature of HPAI lesions in the brain [53] (as we also showed in the skuas we sampled), one sample might have included a cluster of HPAIV-positive cells, while another one did not. Despite the presence or absence of viral RNA, we diagnosed this animal with a cause of death other than HPAI.

The specific implication of demonstrating that south polar and brown skuas are victims of H5N1 HPAIV infection is that these two species are probably at risk of continued high mortality in coming years, especially if the virus is circulating early in the breeding season. The presence of H5 HPAIV in Antarctica in the 2024/2025 breeding season was demonstrated by its detection in 11 dead skuas sampled at different locations of the Antarctic Peninsula [54]. This mortality will potentially result in declines of their already small populations, both on the Antarctic Peninsula and elsewhere in their ranges. For comparison, the number of apparently occupied territories of great skuas on Foula (Shetland, UK), the world's largest breeding colony of this species, was estimated to have declined by about 60-70% due to HPAI [38]. Similar estimates have also been made for the whole UK [55]. To be able to assess the effects of HPAI on their populations, it is important to closely monitor as many as possible breeding sites of these species for morbidity and mortality from HPAI. A related priority action is to perform regular population censuses of brown skuas and south polar skuas as frequently as possible in coming years.

The specific implication of evidence for south polar and brown skuas as potential vectors of HPAIV is that they may play a substantial role in further spread of the virus. Breeding colonies of brown skuas are distributed across the Subantarctic region, while those of south polar skuas are distributed along the whole coast of the Antarctic continent [14–19]. Therefore, they could play a role in spreading HPAIV widely, despite their small numbers. However, relatively little is known about movements of skuas between breeding colonies. To better assess their potential roles as vectors, it is important to learn more about their movement ecology, for example, by attaching miniature data loggers to breeding and non-breeding birds to be able to follow their movements through the environment [56–58], as well as sampling apparently healthy skuas [59] and performing phylogenetic analysis with host, subtype and region discrete trait mappings [6]. Additionally, longitudinal studies conducted throughout the breeding season could provide valuable insights into the relevant reservoirs of infection and transmission pathways in the Antarctic context.

The general implication of H5N1 HPAIV being so widespread across the Antarctic Peninsula in several species in the summer of 2023/2024 is that it has gained a foothold in Antarctica and may not only persist but also spread across the continent in coming years. Based on the history of H5N1 HPAIV in European wildlife in recent years, where the unusual mortality events occurred in different species from one year to the next[60] (EFSA reports 2020 to 2024, from: https://www.efsa.europa.eu/en/topics/topic/avian-influenza), it may be expected that additional species will undergo unusual mortality events from HPAI in Antarctica in the future. Therefore, close avian influenza surveillance by transdisciplinary teams will be needed across Antarctica to be able to record not only virus spread but also diagnose unusual mortality events from HPAI. Such surveillance should include serological screening for both HPAIVs and LPAIVs to understand the role of different species for virus persistence in the Antarctic and be performed according to agreed biosafety guidelines. To be confident that unusual mortality events are caused by HPAI and not by avian cholera or other mortality factors, adequate diagnostic expertise is required. Just as important as recording virus spread and associated mortality, the population sizes of affected species need to be counted in coming years to be able to assess the impact of the virus on wildlife population sizes. This is all the more important for Antarctic wildlife, which is threatened by multiple factors such as global warming, increased scientific activities and infrastructure, increased tourist numbers, invasion of non-native species, overfishing, and pollution [61]. These avian influenza surveillance and population impact

studies fit in a global exercise to assess the impact of this poultry-origin disease on worldwide wildlife populations, and to provide a well-documented basis to take action to prevent future spillover of anthropogenic diseases from livestock to wildlife [4].

Materials and Methods

Field work: observations and sample collection

All the samples described in this study have been collected between March 17th and March 28th, 2024, at the following sites:

- Yankee Harbour, Greenwich Island (62°31'55"S, 59°46'32"W)
- Elephant Point, Livingston Island (62°41'21"S, 60°51'58"W)
- Hope Bay, Trinity Peninsula (63°24'16"S, 56°59'49"W)
- Haddon Bay, Joinville Island (63°18'41"S, 55°46'28"W)
- D'Urville Monument, Joinville Island (63°25'36"S, 56°17'13"W)
- Paulet Island (63°34'31"S, 55°46'30"W)
- Beak Island (63°36'49"S, 57°20'50"W)
- Devil Island (63°47'50"S, 57°17'29"W)

The selection of locations we visited was based on sites with a diverse range of wildlife species, especially skua, and previously reported observations of morbidity and mortality. A scouting team was responsible for surveying the territory, recording the number of animals present and observing clinical signs of HPAI in wildlife. The resulting live animal count data do not intend to cover respective population dimensions, but convey the scenario present at the moment of our visit.

Environmental fecal swab samples were collected from apparently healthy individuals that had been directly observed to defecate, or based on their appearance and on the fact that the feces were freshly collected from areas where skuas (and no other species) were present minutes earlier, by swabbing the fresh dropping off the substrate, and placing it into 1.5 mL NucleoProtect VET buffer (Macherey-Nagel). Autopsies on a subset of dead animals were performed on site, and samples for virological analyses were collected and stored in 1 mL DNA/RNA Shield (Zymo). Samples for histopathological analyses were also collected and stored in 10% neutral buffered formalin.

Air was collected by filtration through nanofiber filters and filters introduced in 2 mL CLB as described previously [62]. Likewise, water and soil samples (environmental matrix samples) were mixed with 0.5 mL Cell Lysis Buffer (CLD; Promega).

All the samples were stored refrigerated at 4°C until the end of the expedition.

The temperature conditions at the sampling locations were variable and ranged between –15 and +5°C.

Immediate on-site testing for the detection of influenza A virus antigen with the FASTest® AIV Ag test kit (MEGACOR Veterinary Diagnostics, Germany) was conducted for a subset of avian carcasses. Single or multi-organ swabs (table S1) were utilized according to the manufacturer's instructions; nevertheless, the suboptimal environmental conditions (e.g. low ambient temperature) might have impaired the kit functionality and caused uncertain results in some cases.

All the operations described above were performed with adequate personal protective equipment (PPE), including N95 face mask, goggles/eyewear, coveralls, and gloves (https://scar.org/~documents/science-4/life-sciences/ag-daw/updated-predictions-and-recommendations-for-highly-pathogenic-avian-influenza-in-antarctica-v7?layout=default). Since all

samples were stored in infectious agent-inactivating buffers, no BSL3 regulations were required after sample collection.
The activities undertaken during this expedition were in agreement with the permit "Meagan Dewar – Avian Influenza research (2023-24 season)" granted to Meagan Dewar by the Australian Government, Department of Climate Change, Energy, the Environment and Water – Australian Antarctic Division (AAD).
We applied levels of confidence for our disease diagnoses because we investigated carcasses of free-ranging animals found dead for which we have no disease history knowledge. Additionally, not all carcasses could be investigated with the same tests, either because of lack of time, or because the state of decomposition or scavenging hampered taking a full set of samples. We distinguished three confidence levels: high, medium, inconclusive (Supplementary Table 10). The assigning of these categories was based on i) the samples available for testing, ii) the level of matching of the different test results, and iii) the result of a host housekeeping gene (GAPDH) as internal control for RNA preservation in the carcass. We assigned a category to each autopsied individual for which we made disease diagnosis. GAPDH RNA was detected in all our carcass samples and therefore negative viral and bacterial genes qPCR results were taken into account.

Laboratory analyses: carcass samples

Nucleic acid extraction

All samples were stored at –70°C. Tissue samples were homogenized prior to virological and bacteriological screenings. RNA was extracted for virological analyses using the High Pure RNA Isolation Kit (Roche) according to the manufacturer's instructions. For bacteriological analyses, 200 µL of tissue swab or environmental sample supernatant (air, soil, water) and 100 µL of tissue homogenate were mixed with 600 µL of MagNA Pure External Lysis Buffer (Roche) and PBS up to 1 mL. 20 µL of phocine distemper virus (PDV) extraction control was added to each sample [63]. Total nucleic acids were extracted using MagNA Pure 96 System (Roche).

M1, H5, N1 and HPAI real time RT-PCR

The presence of influenza A virus and H5 hemagglutinin (HA) RNA in the samples was determined by duplex RT-qPCR as described previously [64]. The neuraminidase 1 (N1) subtype was determined by RT-qPCR as described previously [65] on a subset of samples.
The presence of a MBCS in the HA sequence was first determined by RT-qPCR. A partial sequence from segment 4 (HA) was amplified using optimized primers and probes [66] for the detection of HPAI H5 viruses (FW: CCTTGCGACTGGGCTCAG, RV: ATCAACCATTCCCTGCCA, probe1: FAM-AGAAGAAARAGAGGGCTGTTTGGGGCT-BHQ-1, probe2: FAM-AGAAGAAARAGAGGCCTGTTTGGGGCT-BHQ-1). RT-qPCR mix included 20 µL containing 5 µL of RNA, 500 nM of each primer, 250 nM of each probe, 5 µL 4x TaqMan™ Fast Virus 1-Step Master Mix (Applied Biosystems) and nuclease-free water to a final volume of 20 µL. The amplification protocol was 5 min 50°C, 20 s 95°C, 45x (3 s 95°C, 20 s 56°C, 31 s 60°C). Fit point analysis was used to determine the Cq values. Negative and positive controls were included. Samples with Cq>40 were considered negative. The following categories were established based on the M1 viral RNA load (40 – Cq): 0, negative (-); 1 to 10, low (+); 11 to 20, moderate (++); 21 to 40, high (+++).

MBCS sequencing

To further confirm the presence of a MBCS, the segment region including the MBCS was amplified by RT-PCR and sequenced. cDNA was first synthesized using 80 nM of non-segment-specific H5N1 primer (AGCRAAAGCAGG), 2 µL dNTPs 10 mM, 20 U RNAse inhibitor and 11.5 µL RNA. This mix was incubated for 5 min at 65°C and then placed on ice for denaturation of RNA secondary structures. 20 U RNAse inhibitor, 1x Superscript IV buffer, 1 µL DTT 0.1 M, 200 U Superscript IV Reverse Transcriptase (Invitrogen) were added to the mix in a final volume of 25 µL. cDNA synthesis was performed with the following parameters: 5 min 25°C, 15 min 50°C, 10 min 80°C. A segment region of 310 bp including the MBCS was amplified using specific primers (J3 and B2a) as described previously [67]. The reaction mix consisted of 5 µL cDNA, 200 nM of each primer, 5 µL GeneAmp 10X Gold Buffer, 5 µL $MgCl_2$ 25 mM, 5 U AmpliTaq Gold DNA Polymerase (Applied Biosystems), 1 µL dNTPs 10 mM and nuclease-free water in a final volume of 50 µL. The RT-PCR amplification was performed with the following parameters: 6 min 95°C, 40x (20 s 95°C, 30 s 50°C, 1 min 72°C), 6 min 72°C. After separation on a 2% agarose gel electrophoresis, bands corresponding to the cleavage site amplicon were purified with MiniElute Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The amplicon was then prepared for Sanger sequencing. 2 µL of DNA were amplified using 600 nM of the same primers specific for the cleavage site, 0.5 µL BigDye 5X Sequencing Buffer, 0.5 µL BigDye™ Terminator v3.1 (Applied Biosystems) and water to a final volume of 10 µL. The amplification parameters were the following: 10 s 96°C, 30 s 45°C, 30x (4 min 60°C). The amplification products were then purified on a Sephadex plate PERFORMA V3 (EdgeBio). Sequencing was performed in a 3500xL genetic analyser (Applied Biosystems). Sequences were analysed using BioEdit or SnapGene. A reference sequence was submitted to GenBank (accession number: PV570239).

GAPDH and β-actin real time RT-PCR

To estimate the degree of RNA integrity in the carcass samples, ubiquitously expressed host gene RNAs were amplified by RT-qPCR. From bird samples, GAPDH RNA was amplified using specific primers and probe designed based on Adélie penguin GAPDH gene (Gene ID: 103921100) (FW: CAACCCCCAATGTCTCTGTT, RV: TATATGCCAGGATGCCCTTC, probe: FAM-AAGGCTGCTGCTGATGGGCC). From seal samples, β-actin RNA was amplified using in-house designed primers cross-reactive with seals (FW: GGCATCCATGAAACTACCTT, RV: AGCACTGTGTTGGCATAGAG, probe: FAM-ATCATGAAGTGTGACGTTGACATC). The 20 µL RT-qPCR mix included 5 µL of extracted RNA, 20 µM of each primer, 10 µM of probe, 5 µL 4x TaqMan™ Fast Virus 1-Step Master Mix (Applied Biosystems) and nuclease-free water to a final volume of 20 µL. The amplification protocol included 5 min 50°C, 20 s 95°C, 45x (3 s 95°C, 31 s 60°C). Fit point analysis was used to determine the Cq values. Samples with Cq>40 were considered negative.

Pasteurella multocida kmt1 *real time PCR*

The presence of *P. multocida* in the samples was determined by qPCR. A partial sequence of the *kmt1* gene, which is conserved among all *P. multocida* strains, was targeted using primers as previously described [68]. The qPCR mix included 5 µL of extracted DNA, 20 µM of each primer, 10 µM of probe, 5 µL 4x TaqMan™ Fast Virus 1-Step Master Mix (Applied Biosystems) and nuclease-free water to a final volume of 20 µL. The amplification protocol was 20 s 95°C, 45x (15 s 95°C, 20 s 53°C, 31 s

60°C). Fit point analysis was used to determine the Cq values. Negative and positive controls were included. Samples with Cq>40 were considered negative.
To further confirm the presence of the *P. multocida kmt1* gene, a subset of samples from different locations were used to amplify and sequence the same amplicon (211 bp) of the qPCR reaction. The PCR mix consisted of 5 µL input DNA, 200 nM of each primer, 1 µL SuperScript III RT/Platinum Taq Mix (Invitrogen), 25 µL 2X Reaction Mix and nuclease-free water to a final volume of 50 µL. The amplification protocol was 6 min 95°C, 40x (20 sec 95°C, 30 sec 53°C, 60 sec 72°C), 6 min 72°C. The PCR product isolation and sequencing steps were the same as described above. The sequences were analyzed using BLASTn against the NCBI GenBank database to identify closely related sequences. The following categories were established based on the *kmt1* DNA load (40 – Cq): 0, negative (-); 1 to 7, low (+); 8 to 14, moderate (++); 15 to 40, high (+++).

Serology

When available, fluid from the celomic cavity of carcasses selected for autopsies was collected with a disposable pipette and processed as described previously [69]. This fluid is referred to as "body fluid" throughout the text. The supernatant was tested for the detection of antibodies against influenza A viral nucleoprotein (NP) and H5 hemagglutinin (HA) using ID Screen Influenza A Antibody Competition Multi-species and ID Screen Influenza H5 Antibody Competition 3.0 Multi-species (IDvet, Innovative Diagnostics), according to the manufacturer's instructions.

Immunohistochemistry (IHC), in situ hybridization (ISH) and histopathology

IHC for the NP antigen of influenza A virus was performed as described previously [70]. ISH for the NP segment of influenza A virus was performed as described previously [71]. ISH for *Pasteurella multocida* was performed with the same protocol described previously [71], but using the RNAscope probe B-P.multocida-Pm1-dnaA (Catalog #1267471-C1; BioTechne). The UBC positive control probe worked for the species for which ISH was performed (Adélie penguin, south polar skua, brown skua). 3 µm formalin-fixed paraffin-embedded tissue sections were deparaffinized using xylene, rehydrated using graded ethanol, stained with hematoxylin (Klinipath) and eosin (QPath; H&E), and assessed by use of a light microscope by a qualified veterinary pathologist for any histopathological changes.

Laboratory analyses: environmental fecal samples

RNA extraction

All samples were stored at –70°C. 250 µL of original (first step) or 1:4 diluted (second step) supernatant was treated with Proteinase K for 20min at RT and spun down at 13,000 rpm for 1 min. A pre-clean up by incubation with TRIzol™ LS reagent (Thermo Fisher Scientific, Waltham, MA, USA) and chloroform and RNA extraction was performed as described before [72]. A heterologous RNA (IC2) was added during the extraction process in 1:10 ratio to the later on elution volume of 110 µL as internal control.

Real time RT-PCR

The extracted RNA was used as input for a duplex RT-qPCR screening, targeting IAV M1-gene and the EGFP-control gene of IC2 as described previously [33]. Positive IAV samples were tested to

665 determine subtype and pathotype. The German National Reference Laboratory for avian influenza
666 RT-qPCR approach has been used for confirmation/exclusion of NP-, H5-, N1- and HPAIV H5-
667 specific targets [73]. Samples without a clear IC2 signal (Cq>34) have been considered as inhibited and
668 thus not evaluable. Those inhibited samples underwent a second round of previously described clean-
669 up, RNA extraction and RT-qPCR screening, but with a 1:4 diluted original sample material in PBS.
670 If in this second round a sample did not reveal an IC2 signal, it remained not evaluable.

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Competing interests

Authors declare that they have no competing interests.

Data availability

A reference sequence including the multi-basic cleavage site (MBCS), generated in this study, is available in GenBank under accession number PV570239.