ULPGC Universidad de Las Palmas de Gran Canaria | Escuela de Doctorado

ULPGC Universidad de Las Palmas de Gran Canaria | IUSA Instituto Universitario de Sanidad Animal y Seguridad Alimentaria

TESIS DOCTORAL

Vigilancia Sanitaria en Avifauna Canaria: Detección y caracterización molecular de herpesvirus, avipoxvirus y hemoparásitos en el alcaraván (*Burhinus oedicnemus*)

ANA COLOM RIVERO

DOCTORADO EN SANIDAD ANIMAL Y SEGURIDAD ALIMENTARIA

LAS PALMAS DE GRAN CANARIA

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Tesis presentada por: **Dª. Ana Colom Rivero**

Dirigida por: **Dr. Antonio Fernández Rodríguez** y por la **Dra. Eva Sierra Pulpillo**

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(Firma)	**(Firma)**	**(Firma)**

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D. ANTONIO FERNÁNDEZ RODRÍGUEZ, COORDINADOR DEL

PROGRAMA DE DOCTORADO DE SANIDAD ANIMAL Y

SEGURIDAD ALIMENTARIA DE LA UNIVERSIDAD DE LAS PALMAS

DE GRAN CANARIA,

INFORMA,

Que la Comisión Académica del Programa de Doctorado, en su sesión de

Fecha / / tomó el acuerdo de dar el consentimiento para su

tramitación, a la tesis doctoral titulada "Vigilancia Sanitaria en Avifauna

Canaria: Detección y caracterización molecular de herpesvirus, avipoxvirus y

hemoparásitos en el alcaraván (*Burhinus oedicnemus*).

presentada por la doctoranda Dª Ana Colom Rivero y dirigida por el Catedrático

Antonio Fernández Rodríguez y la Doctora Eva Sierra Pulpillo.

Y para que así conste, y a efectos de lo previsto en el Artº 11 del Reglamento

de Estudios de Doctorado (BOULPGC 7/10/2016) de la Universidad de Las

Palmas de Gran Canaria, firmo la presente en Las Palmas de Gran Canaria,

a de del dos mil veintiséis

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A mis padres, por ayudarme, empujarme y permitirme haber llegado hasta aquí.

A todos mis amados compañeros de vida de cuatro patas que le dan sentido a mi vida: Noha, Rubi, Nano, Agustín, Veva, Duende, Elsa, Buffy, Quini, Cuba, Cloe, Patty, Cooper, Simba, Hanna, John Nieve, Noa y Kira.

“Aquí estamos, la especie más inteligente que jamás haya existido. Entonces, ¿cómo es posible que estemos destruyendo el único planeta que tenemos?”.

Jane Goodall

“…¿si no es ahora, cuándo?, ¿si no eres tú, quién?...”

Robe Iniesta

ÍNDICE

1. INTRODUCCIÓN

Introducción.

1.1. Importancia de la vigilancia epidemiológica de la fauna silvestre

La vigilancia epidemiológica de la fauna silvestre es un componente esencial para comprender y gestionar los riesgos sanitarios que afectan simultáneamente a los animales, los ecosistemas y las personas (One Health; una sola salud) (Mori et al., 2014) (WHO, 2019). Asimismo, la Organización Mundial de Sanidad Animal (OMSA/WOAH) destaca que la fauna silvestre desempeña funciones ecológicas críticas, como el control de plagas, la dispersión de semillas y la polinización, y, a la vez, está expuesta a amenazas crecientes asociadas a patógenos, contaminantes, tóxicos y presiones antrópicas (World Organisation for Animal Health & IUCN, 2024).

El planeta atraviesa actualmente un proceso de transformación ambiental acelerada, impulsado en gran medida por la actividad humana, fenómeno que diversos autores vinculan con el Antropoceno (P.J. Crutzen & Stoemer, 2000) . Este contexto de cambio global está generando alteraciones profundas en los ecosistemas, incluida la elevación de las temperaturas en medios terrestres y marinos (Illera et al., 2024). Paralelamente, se ha observado en las últimas décadas un notable aumento en la aparición de enfermedades infecciosas emergentes y reemergentes, muchas de ellas de carácter zoonótico. Estas enfermedades pueden definirse como aquellas que surgen por primera vez en una población, o bien aquellas que, aun siendo previamente conocidas, experimentan un aumento rápido en su incidencia o en su distribución geográfica (Morse, 1995). Ejemplos recientes incluyen el VIH/SIDA, el Virus del Nilo Occidental, el Dengue, la Gripe Aviar o el Sars-CoV-2, entre otras.

La emergencia de estas enfermedades infecciosas ha sido asociada a múltiples factores. Entre ellos destacan la expansión y redistribución de vectores favorecidas por el cambio climático, la intensificación de los intercambios globales, la destrucción y fragmentación de hábitats naturales y la creciente ocupación del territorio por actividades humanas. Como consecuencia, se ha producido un

incremento significativo en las interacciones entre fauna silvestre, animales domésticos y seres humanos, facilitando el intercambio y la circulación de patógenos a lo largo del continuo huésped-patógeno (Daszak et al., 2000).

En este contexto de acelerado cambio global, la vigilancia y monitorización sanitaria de la fauna silvestre adquiere una relevancia cada vez mayor, constituyendo una herramienta esencial para comprender los riesgos emergentes y anticipar posibles amenazas para la salud animal, humana y ambiental (Daszak et al., 2000).

1.2. Vigilancia como herramienta para la gestión sanitaria y la conservación

La OMSA subraya que los animales silvestres pueden experimentar enfermedad, albergar patógenos sin mostrar signos clínicos, o servir como reservorios, manteniendo ciclos de transmisión que pueden afectar a otras especies domésticas, humanas o silvestres. En este sentido, la vigilancia constituye una herramienta clave para caracterizar estos riesgos, anticipar eventos sanitarios y orientar respuestas efectivas en el marco del enfoque One Health (World Organisation for Animal Health & IUCN, 2024).

Según la guía OMSA/IUCN, la vigilancia permite:

- Detectar amenazas emergentes o reemergentes, incluyendo enfermedades nuevas o introducidas, así como variaciones en agentes conocidos.
- Monitorizar los cambios en tiempo y espacio en la presencia de patógenos, tóxicos o signos de enfermedad.
- Contribuir a la conservación de especies, ya que la enfermedad y la contaminación química figuran entre las causas reconocidas de declive poblacional (p. Ej., Lista Roja de la UICN).
- Identificar impactos directos en la salud de poblaciones silvestres, potencialmente afectando parámetros reproductivos, supervivencia o estructura demográfica.

- Reconocer eventos inusuales**,** como mortalidades inesperadas, cambios de comportamiento o patrones atípicos, que pueden indicar problemas de origen patógeno o tóxico.

La vigilancia no solo aporta información para interpretar estos fenómenos, sino que también constituye una herramienta para diseñar acciones de mitigación, planes de manejo y políticas de conservación. Además, más del 60 % de los patógenos humanos conocidos tienen origen zoonótico, muchos de los cuales están asociados a fauna silvestre (Rahman et al., 2020). Esto no implica que la fauna represente un riesgo per se, sino que la vigilancia permite comprender y gestionar adecuadamente las interfaces entre humanos, animales domésticos y vida silvestre, minimizando riesgos sin generar impactos negativos injustificados sobre los animales o sus hábitats (Sharan et al., 2023).

1.3. La vigilancia epidemiológica como sistema de alerta temprana

La OMSA señala que la vigilancia en fauna silvestre puede actuar como un sistema centinela, detectando señales tempranas de problemas ambientales, contaminantes o patógenos antes de que afecten a animales domésticos o a la salud pública. Aunque históricamente se ha enfatizado su dimensión preventiva desde una perspectiva antropocéntrica, es imprescindible destacar su valor ecológico: la salud de los ecosistemas es inseparable de la salud planetaria.

Las aves, en particular, representan indicadores ecológicos altamente sensibles a alteraciones ambientales, respondiendo con rapidez a cambios en sus hábitats y constituyendo un grupo idóneo para estudios de vigilancia sanitaria (Smits & Fernie, 2013).

1.4. La vigilancia en ecosistemas insulares: el caso de Canarias

Los ecosistemas insulares, como los de Canarias, presentan características ecológicas únicas que incrementan su vulnerabilidad (Fernández-Palacios et al., 2021a). El aislamiento geográfico, la evolución diferenciada y la limitada superficie disponible favorecen la aparición de especies altamente especializadas y endémicas (Kier et al., 2009). Aunque las islas ocupan apenas un 6,7 % de la superficie terrestre, albergan alrededor del 20 % de la biodiversidad mundial y concentran aproximadamente el 50 % de las especies amenazadas y el 75 % de las extinciones registradas (Fernández-Palacios et al., 2021b; Florencio et al., 2021; Illera et al., 2024).

Canarias, junto con otros archipiélagos atlánticos (Azores, Madeira, Cabo Verde y las Islas Salvajes), forman parte de la región biogeográfica de la Macaronesia, caracterizada por un alto grado de endemicidad. El archipiélago canario, formado por siete islas principales y varios islotes, presenta condiciones climáticas subtropicales moduladas por los vientos alisios, lo que contribuye a su amplia diversidad de hábitats (Fernández-Palacios et al., 2007).

1.5. La Red Vigía Canarias como sistema de vigilancia sanitaria de fauna silvestre

La Red Canaria de Vigilancia Sanitaria de la Fauna Silvestre (Red Vigía Canarias) es un proyecto del Gobierno de Canarias y constituye una iniciativa pionera orientada a la monitorización activa del estado de salud de la fauna silvestre del Archipiélago. Su propósito principal es identificar las causas más relevantes de mortalidad y evaluar la presencia e impacto de patologías naturales y emergentes, así como aquellas asociadas a actividades humanas. Desde 2018, la información generada se recopila de forma sistemática gracias a la participación de diversas instituciones y centros de investigación de Canarias especializados en diagnósticos anatomopatológicos y toxicológicos.

Los datos integrados en la plataforma son procesados y analizados con el objetivo de ampliar el conocimiento sobre los factores que influyen en la mortalidad y promover estrategias eficaces de conservación de la fauna canaria. En la actualidad, se aplican técnicas de minería de datos para establecer correlaciones y clasificaciones, y en el futuro se prevé incorporar herramientas de analítica predictiva que permitan detectar patrones, anticipar escenarios y facilitar la toma de decisiones orientadas a la protección de las especies. Los resultados generados por la plataforma representan un avance notable en la comprensión de las causas de mortalidad de la fauna en las Islas Canarias y aportan información esencial para impulsar acciones de conservación basadas en evidencia científica (https://www3.gobiernodecanarias.org/aplicaciones/redvigiacanarias/).

La región constituye un espacio de especial relevancia para la vigilancia sanitaria por su elevada biodiversidad, su función como corredor migratorio entre continentes y la gran sensibilidad de sus ecosistemas a las presiones antrópicas. En este marco, el Instituto Universitario de Sanidad Animal y Seguridad Alimentaria (IUSA) participa como centro colaborador, contribuyendo de manera destacada al análisis anatomopatológico de los ejemplares remitidos por la red. Dentro del conjunto de taxones evaluados, las aves representan el principal grupo monitorizado, tanto por su abundancia como por su valor como bioindicadores del estado de salud ambiental.

Canarias alberga más de 15.700 especies y subespecies, de las cuales, alrededor del 27 % de las especies y el 60 % de las subespecies son endémicas. En cuanto a la avifauna, el archipiélago cuenta con 155 especies, lo que representa aproximadamente el 0,98 % del total mundial (https://www.biodiversidadcanarias.es/biota/estadisticas?se=0&m=0).

Las aves que habitan en Canarias se clasifican en función de su distribución y su estado de conservación, considerándose las siguientes categorías:

- **Endémicas:** especies o subespecies presentes exclusivamente en las islas.
- **Nidificantes:** aquellas que crían en el archipiélago.
- **Migratorias:** especies que se desplazan entre diferentes regiones, incluyendo Canarias como área de paso, invernada o reproducción.

- **Amenazadas:** especies incluidas en el Libro Rojo de Aves de España, con subcategorías según su riesgo de extinción.
- **Introducidas:** especies incorporadas por acción humana, que pueden tener o no carácter invasor.
- **Extintas:** especies o subespecies desaparecidas históricamente en el archipiélago.

En el contexto de Canarias, la combinación de elevada biodiversidad, altos niveles de endemicidad, vulnerabilidad ecológica y presiones ambientales hace especialmente necesaria la implementación de sistemas reforzados de vigilancia. Las aves, y en particular el alcaraván, constituyen un modelo idóneo para abordar estas cuestiones desde una perspectiva molecular, epidemiológica y de conservación (Hawkes et al., 2019).

En Canarias están representados 17 órdenes de aves, de los cuales 8 cuentan con una o más especies o subespecies endémicas. Este es el caso del Orden Charadriiformes, al que pertenece el alcaraván común (*Burhinus oedicnemus*), representado en el archipiélago por dos subespecies endémicas: *B. o. distinctus* y *B. o. insularum* (Oromí, P. & A. García, 2010).

Figura 1. *Burhinus oedicnemus insularum* (Sassi, 1908). Biota (S.Scholz).

1.6. El alcaraván común (*Burhinus oedicnemus*) en Canarias

Biología y ecología del alcaraván común

Se trata de un ave limícola que presenta una notable capacidad de camuflaje en medios abiertos, esteparios y agrícolas, facilitada por su plumaje de tonalidades pardo-terrosas y por sus hábitos crepusculares y comportamiento generalmente esquivo. Morfológicamente destaca por sus grandes ojos amarillos, largas patas del mismo color y un pico amarillento rematado en una punta negra. Presenta además una franja blanca característica en la cabeza y en las alas, menos evidente en individuos juveniles y más marcada en los adultos. Estas franjas alares, delimitadas por bordes negros, son especialmente visibles en la superficie dorsal del ala durante el vuelo, mientras que la parte ventral muestra una coloración considerablemente más pálida. Durante el periodo reproductor la especie resulta más detectable gracias a la emisión de su reclamo característico; en esta etapa manifiesta un comportamiento marcadamente territorial, aunque el resto del año adopta hábitos más gregarios, frecuentando al anochecer espacios antrópicos como establos y explotaciones ganaderas, así como zonas verdes urbanas, entre ellas campos de golf. Su dieta se basa mayoritariamente en invertebrados, especialmente insectos (principalmente ortópteros, coleópteros y hormigas) (Rioja, 2001) (https://seo.org/ave/alcaravan-comun/#informacion).

La especie presenta una amplia distribución que abarca Europa, Asia occidental y el norte de África. En el archipiélago canario se encuentra presente en todas las islas, incluida La Graciosa, así como en los islotes de Alegranza y Lobos. Generalmente ocupa territorios llanos, escasamente arbolados y de carácter árido o semiárido. Dentro de este gradiente ecológico explota un espectro relativamente amplio de hábitats, que comprende tanto áreas de vegetación natural o seminatural como entornos agrícolas. En Canarias, cada una de las subespecies endémicas exhibe patrones de distribución propios, reflejo de la heterogeneidad ambiental y del aislamiento insular (Juana & Mar, 2005).

Los análisis genéticos realizados sobre el Alcaraván común, basados en marcadores de microsatélites nucleares y secuencias de ADN mitocondrial, han puesto de manifiesto una diferenciación significativa entre las poblaciones del Mediterráneo y las del archipiélago canario, lo que sugiere procesos evolutivos independientes y un grado notable de estructuración genética entre ambas regiones (Mori et al., 2014, 2017)

Distribución y ecología de *Burhinus oedicnemus distinctus* en las islas occidentales

Esta subespecie está presente en las islas de Gran Canaria, Tenerife, La Gomera, El Hierro y La Palma (Figura 2), donde mantiene poblaciones sedentarias. Aunque en Gran Canaria ha experimentado una recuperación en los últimos años, su distribución histórica era más amplia. Las estimaciones disponibles sitúan su tamaño poblacional en torno a 300-400 parejas reproductoras (Madroño et al., 2004).

En Gran Canaria, pese a su presencia relativamente extendida, el hábitat adecuado para la subespecie se halla fuertemente fragmentado. En la zona norte puede localizarse desde las proximidades de Las Palmas de Gran Canaria hasta las lomas de Gáldar-Agaete, donde la especie continúa siendo relativamente común. En la franja este y sur de la isla ocupa áreas más bajas y medias comprendidas aproximadamente entre Telde y el entorno del Castillo del Romeral (Madroño et al., 2004).

En Tenerife, la subespecie mostró históricamente una mayor representación espacial; sin embargo, en la actualidad su presencia se restringe principalmente a la vertiente meridional, desde El Porís de Abona hasta el sector de Guía de Isora (Madroño et al., 2004).

En La Gomera, la población se estima en aproximadamente 20 parejas reproductoras, concentradas principalmente en los lomos situados en los barrancos del sector sur y sureste de la isla. En El Hierro, en cambio, la subespecie presenta una población relativamente más abundante, con alrededor de 100 parejas

distribuidas en diferentes enclaves insulares. Finalmente, en La Palma, la presencia del taxón es muy reducida, con cerca de 12 parejas restringidas a áreas abiertas localizadas en los sectores occidental y noroccidental de la isla (Madroño et al., 2004).

Figura 2. Área de distribución conocida de la subespecie *B.o. distinctus* en el archipiélago canario. (https://www.biodiversidadcanarias.es/biota/especie/V00031)

La subespecie muestra preferencia por zonas bajas y xéricas del piso basal, ocupando principalmente llanos pedregosos y antiguos cultivos. No obstante, en islas como El Hierro y La Palma puede extender su presencia hacia pastizales de mayor altitud y hacia formaciones abiertas de *Pinus canariensis*. En La Gomera, su distribución se restringe a lomos con vegetación xerófita o de transición localizados en el sector meridional. En Gran Canaria y Tenerife, además de los ambientes semidesérticos característicos, la subespecie utiliza también enclaves relativamente más húmedos con presencia de pastizales. Presenta una notable fidelidad tanto a los territorios de cría como a las áreas de concentración posreproductiva. En estos enclaves se han registrado agrupaciones que oscilan entre 10 y 40 individuos en Tenerife y El Hierro, superando los 50 ejemplares en Gran Canaria. Estas concentraciones se localizan preferentemente en llanos pedregosos, antiguos terrenos agrícolas y, de manera ocasional, en campos de golf (Madroño et al., 2004).

Distribución y ecología de *Burhinus oedicnemus insularum* en las islas orientales

Esta subespecie se distribuye en las islas más orientales del archipiélago: Fuerteventura, Lanzarote, La Graciosa, Lobos y Alegranza (Figura 3). En Lanzarote y Fuerteventura, excepto en los lugares con volcanismo más reciente o en terrenos especialmente agrestes, la subespecie presenta una distribución amplia y continua. En Alegranza su presencia es más frecuente en la mitad oriental de la isla, mientras que en La Graciosa ocupa prácticamente toda la superficie que ofrece hábitats adecuados. Sin embargo, en Lobos resulta poco común, localizándose únicamente en las zonas llanas situadas en el sector central del islote. Las estimaciones disponibles sitúan la población en aproximadamente 76-548 parejas en Lanzarote y sus islotes, y entre 148-1034 parejas en Fuerteventura y Lobos (Lorenzo et al., 2004).

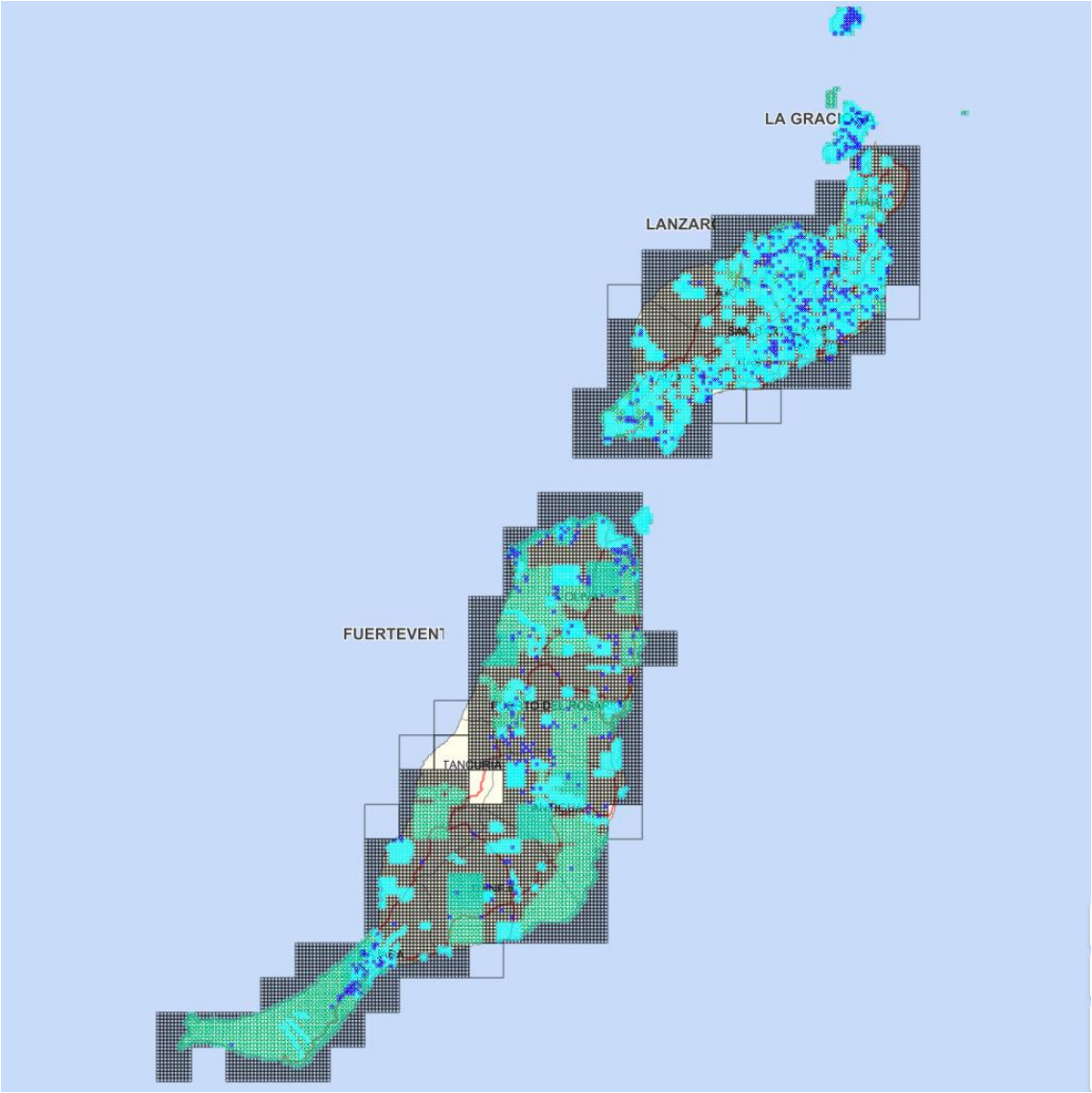


Figura 3. Área de distribución conocida de la subespecie *B.o. insularum* en el archipiélago canario. (https://www.biodiversidadcanarias.es/biota/especie/V00032)

Principales amenazas para las subespecies de alcaraván en el archipiélago canario

La destrucción, alteración y fragmentación del hábitat constituye la principal amenaza para las poblaciones de *Burhinus oedicnemus* en el archipiélago canario. En las islas occidentales, como Tenerife y Gran Canaria, la pérdida de hábitat se ha asociado inicialmente a la expansión agrícola —especialmente del cultivo del plátano— y, más recientemente, al desarrollo turístico, la urbanización y la construcción de nuevas infraestructuras viarias y edificaciones dispersas. Estas

transformaciones han provocado la desaparición de numerosos enclaves tradicionalmente ocupados por la especie. En Fuerteventura y Lanzarote, por su parte, la urbanización acelerada, la implantación de complejos turísticos, parques eólicos, explotaciones mineras y campos de golf continúan reduciendo de manera significativa la extensión de hábitats óptimos (Lorenzo et al., 2004; Madroño et al., 2004).

Las molestias de origen humano representan una amenaza transversal en todas las islas. El tránsito frecuente de personas, a menudo acompañado de perros sueltos, incide negativamente durante el periodo reproductor y puede inducir el abandono de nidos. En Fuerteventura y Lanzarote esta presión se ve acentuada por el uso de vehículos todoterreno —turísticos o vinculados a actividades ganaderas— que acceden libremente a llanos y jables utilizados para la nidificación (Lorenzo et al., 2004; Madroño et al., 2004).

La depredación por mamíferos introducidos, como gatos, ratas, erizos o la ardilla moruna, constituye otra amenaza relevante, especialmente para huevos y pollos. Aunque la magnitud real de este impacto no se conoce con precisión, la elevada abundancia de estas especies en numerosos entornos agrícolas y periurbanos indica un potencial significativo de depredación, comparable al documentado para otras aves de ambientes esteparios (Lorenzo et al., 2004; Madroño et al., 2004).

El pastoreo intensivo, particularmente el ejercido por cabras en régimen semi-extensivo en Fuerteventura, contribuye a la degradación de la vegetación natural, la pérdida de cobertura herbácea y la erosión del suelo. Todo ello favorece el procesos de desertificación y reduce la disponibilidad de invertebrados, recurso alimentario fundamental durante la reproducción (Lorenzo et al., 2004; Madroño et al., 2004).

La mortalidad por atropellos constituye otra amenaza para ambas subespecies, si bien su magnitud es difícil de estimar debido a la escasez de datos sistemáticos. A ello se suma la colisión con tendidos eléctricos, especialmente relevante en *B. o. insularum*, con numerosos ejemplares hallados muertos en Fuerteventura y Lanzarote. Se trata de un impacto potencialmente extrapolable al conjunto del archipiélago (Lorenzo et al., 2004; Madroño et al., 2004).

Finalmente, aunque en claro retroceso, la caza ilegal continúa ocasionando bajas esporádicas que pueden afectar de forma desproporcionada a poblaciones pequeñas o fragmentadas (Lorenzo et al., 2004; Madroño et al., 2004).

1.7. Procedimientos diagnósticos y caracterización de la mortalidad

Procedimientos diagnósticos aplicados a las aves ingresadas en la Red Vigía Canarias

Entre los años 2020 y 2024, y en el marco del proyecto de la Red de Vigilancia de Fauna Silvestre de Canarias, se evaluó anualmente una media de 853 aves, lo que representa aproximadamente el 80 % de los ejemplares ingresados en el centro dicho periodo. De ese total, unas 205 correspondieron a alcaravanes, con una media anual de 49 individuos (5,74 %). La única excepción la constituyó el año 2020, en el cual únicamente se evaluaron 8 ejemplares de esta especie (Figura 4.)

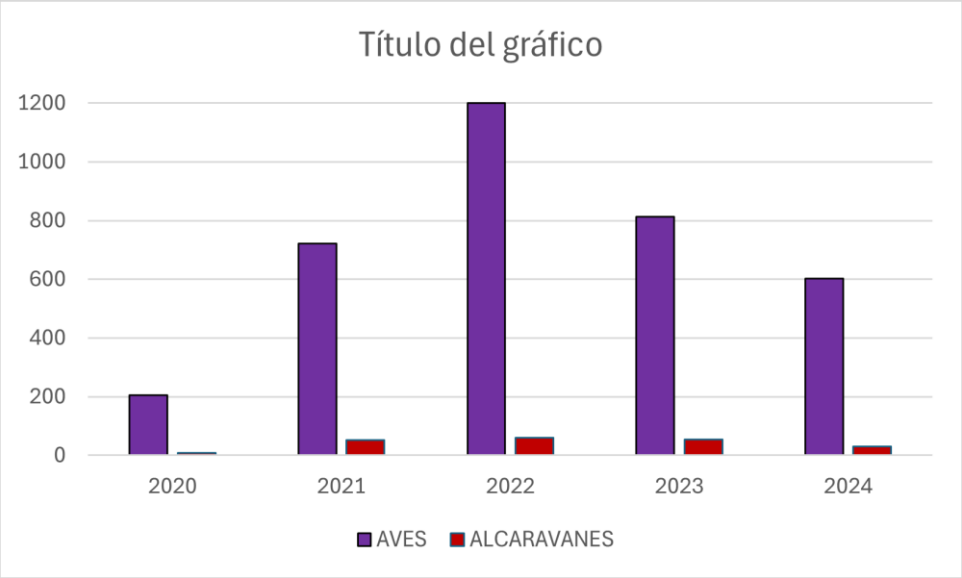


Figura 4. Representación gráfica del número de aves y alcaravanes evaluados en el marco del proyecto de la Red de Vigilancia de Fauna Silvestre de Canarias (2020 – 2024).

Con el fin de determinar las causas de muerte, los cadáveres procedentes de todas las islas son remitidos al IUSA a través de la Red Vigía. En estas instalaciones se realiza la necropsia y toma de muestras siempre que el estado del cadáver lo permite. Los ejemplares fallecidos se conservan refrigerados o congelados hasta la

realización de la necropsia, tras lo cual se clasifican según su grado de descomposición en cinco categorías: muy fresco (1), fresco (2), descomposición incipiente (3), descomposición avanzada (4) y, descomposición muy avanzada (5), siguiendo los protocolos estandarizados del IUSA (Marrero-ponce et al., 2025). Todas las necropsias se llevan a cabo mediante procedimientos homogéneos y previamente establecidos (Rae, 2003), que incluyen la revisión sistemática de todos los órganos y la documentación fotográfica de las lesiones observadas.

Durante las necropsias se obtienen muestras de los principales órganos, como hígado, pulmones, riñones, e intestinos, así como de cualquier lesión detectada. Además, se obtienen hisopos estériles e individuales, sin medio de transporte, a partir de distintas cavidades corporales (orofaringe, cloaca y cavidad celómica). Las muestras de cerebro también se recogen mediante esta técnica, siguiendo lo descrito previamente (Source et al., 2006). Todo el material biológico se archiva posteriormente en el IUSA. Las muestras frescas y sin fijar se almacenan a −80 °C hasta su procesamiento para los análisis moleculares, mientras que los tejidos paralelos se fijan en formalina tamponada al 4 % para su estudio histológico. Estas últimas se procesan siguiendo los protocolos rutinarios: inclusión en parafina, corte a 3 μm y tinción con Hematoxilina y Eosina (H&E) para su examen histopatológico.

La determinación de la edad se basa en la evaluación de características morfológicas externas, determinados rasgos esqueléticos y el grado de desarrollo gonadal, permitiendo clasificar a los individuos como pollos, juveniles o adultos, en ocasiones, determinar la edad con certeza no es posible. El estado nutricional se valora mediante la inspección visual y la palpación de la masa muscular pectoral (keel scoring), así como mediante la observación de las reservas de grasa. Para ello, se emplea una escala numérica de cinco niveles, donde 1 corresponde a caquexia, 2 a bajo peso, 3 a condición normal, 4 a sobrepeso y 5 a obesidad. Este sistema constituye una modificación del método de siete categorías propuesto por Burton et al., (2014). En nuestra adaptación, se añade la categoría de caquexia (puntuación 1) para identificar a los individuos con una atrofia muscular pectoral marcada, diferenciando así la pérdida muscular patológica del bajo peso general. Las categorías delgado y flaco descritas por Burton et al. se fusionan en un único nivel de bajo peso (puntuación 2), que incluye a aves sin atrofia muscular evidente. La

categoría ideal se corresponde con nuestra condición normal (puntuación 3), mientras que el sobrepeso moderado pasa a denominarse sobrepeso (puntuación 4). Finalmente, las categorías de sobrepeso severo y sobrepeso mórbido se integran en una sola categoría (obeso, puntuación 5), ya que en esta población de aves silvestres no representan diferencias clínicamente relevantes; ambas reflejan un exceso de grasa que altera los contornos corporales y enmascara la definición muscular.

1.8. Clasificación y distribución de las causas de muerte en alcaravanes evaluados (2020–2024)

Las causas de la muerte se clasificaron en tres categorías: "muerte natural", "muerte antropogénica" y "no determinada", esta última asignada cuando no fue posible alcanzar una conclusión diagnóstica. Desde un punto de vista cuantitativo, en 112 casos (55 %) no se logró determinar la causa de la muerte, principalmente debido al avanzado estado de descomposición o deterioro del cadáver en el momento de su recepción. En 56 casos (27 %) la muerte se atribuyó a causas antropogénicas, mayoritariamente vinculadas a colisiones o atropello con vehículos motorizados. Finalmente, 37 casos (18 %) se clasificaron como muerte natural. Dentro de este último grupo, el 40 % de (15/37) presentó indicios de un proceso infeccioso y el 19 % (7/37) evidenció la presencia de un proceso parasitario, atendiendo a la clasificación del diagnóstico etiológico (Figura 5).

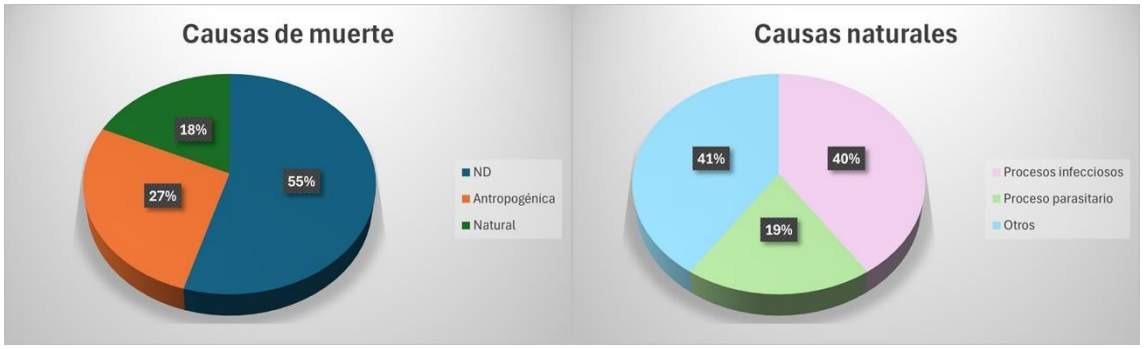


Figura 5. Representación gráfica de causas de muerte de los alcaravanes evaluados en el marco del proyecto de la Red de Vigilancia de Fauna Silvestre de Canarias 2020 y 2024.

En relación con el diagnóstico etiológico, esta tesis se centra específicamente en las enfermedades infecciosas y parasitarias identificadas en los ejemplares analizados. Dentro de las enfermedades infecciosas, se abordarán de forma prioritaria aquellas

de etiología vírica, con especial atención a los herpesvirus y el avipoxvirus, dada su relevancia epidemiológica y su impacto potencial sobre la salud y conservación de las poblaciones de aves. Por su parte, el estudio de las enfermedades parasitarias se focaliza en la detección y caracterización de hemoparásitos, cuyo análisis complementa la comprensión integral del estado sanitario de las especies evaluadas.

Diagnóstico etiológico y enfoque metodológico aplicado

Aunque las guías de la OMSA recomiendan integrar metodologías diagnósticas complementarias, como técnicas moleculares, análisis histopatológicos, serovigilancia y métodos de muestreo no invasivo, en esta tesis se han aplicado específicamente aquellas técnicas compatibles con el tipo de muestras disponibles, ya que el trabajo se ha desarrollado íntegramente sobre animales muertos remitidos al sistema de vigilancia. En este contexto, las metodologías empleadas, el diagnóstico molecular, mediante reacción en cadena de la polimerasa (PCR) y secuenciación, y el diagnóstico anatomopatológico, constituyen las herramientas más adecuadas y eficaces para la identificación etiológica, la detección de infecciones subclínicas, la caracterización de patrones lesionales y la comprensión de la epidemiología de los patógenos presentes en la fauna silvestre.

La vigilancia epidemiológica de la fauna, enmarcada en el enfoque One Health promovido por la OMSA, es esencial para la protección de la salud animal y humana y para la conservación de especies y ecosistemas. Sobre esta base metodológica y conceptual, la presente tesis profundiza en el estudio de herpesvirus, avipoxvirus y hemoparásitos en el alcaraván de Canarias, así como en su relevancia biológica, ecológica y sanitaria.

1.9. Patógenos objeto de estudio

Herpesvirus aviares

Los herpesvirus (HVs), pertenecientes al orden *Herpesvirales*, son virus ADN bicatenarios, lineales y envueltos; los viriones constan de un núcleo, una cápside, un tegumento y una envoltura (Figura 6), capaces de infectar una amplia diversidad de hospedadores, tanto vertebrados como invertebrados (Davison et al., 2009).

Dentro de este orden, la familia *Orthoherpesviridae* se organiza en tres subfamilias: *Alphaherpesvirinae*, *Betaherpesvirinae* y *Gammaherpesvirinae* (Gatherer et al., 2021).

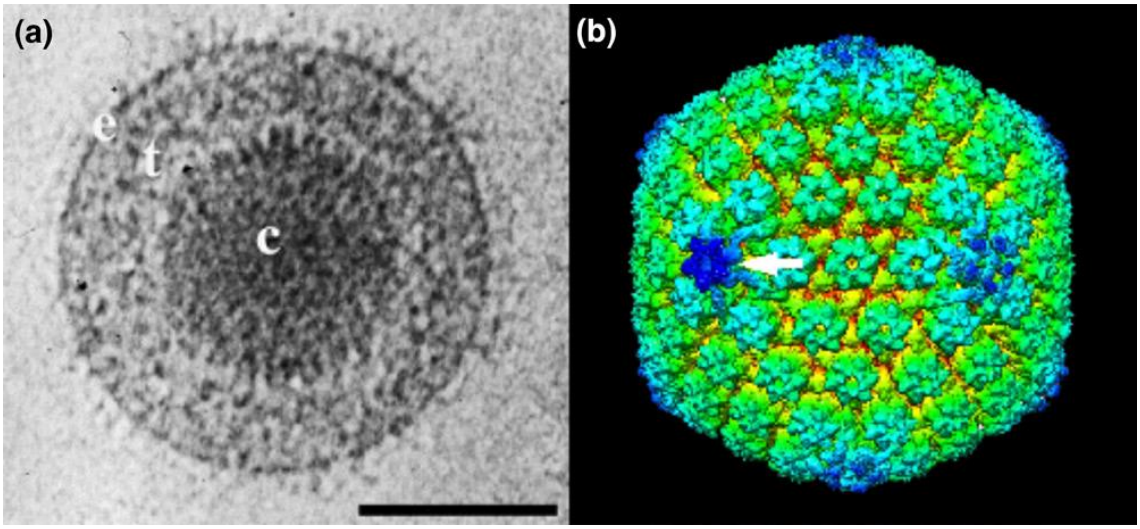


Figura 6. Estructura del virión y la cápside del virus del herpes simple tipo 1. (a) Imagen criomicroscópica electrónica de un virión que muestra la cápside (c), el tegumento (t) y la envoltura (e). Escala de 100 nm. (b) Reconstrucción tridimensional de una cápside que muestra hexones, pentones y el portal (flecha). Imagen perteneciente al artículo ICTV Virus Taxonomy Profile: *Herpesviridae* 2021 (Gatherer et al., 2021; McElwee et al., 2018; McGeoch et al., 2006).

Los HVs representan un notable éxito evolutivo, ya que se han descrito a lo largo de prácticamente todo el espectro de vertebrados y, al menos, en un invertebrado. En condiciones naturales, cada especie viral suele estar asociada estrechamente a un hospedador específico, y en muchas especies ampliamente estudiadas coexisten varios HVs distintos. Por ello, es probable que las aproximadamente 120 especies reconocidas que actualmente componen la familia *Herpesviridae* (Erhard F. Kaleta & Docherty, 2008) representen solo una fracción mínima de la diversidad real. La especificidad de hospedador que caracteriza a muchos HVs sugiere un largo proceso de coevolución, durante el cual estos virus han alcanzado un elevado grado de adaptación a sus hospedadores. Esta hipótesis se ve reforzada por estudios filogenéticos y por la escasa patogenicidad que suelen mostrar en condiciones naturales. Por el contrario, los casos de elevada virulencia observados en humanos

o animales domésticos suelen ser consecuencia de situaciones de desequilibrio ecológico generadas por actividades humanas (Davison, 2002).

Habitualmente, la infección primaria sistémica se establece mediante una viremia asociada a células, seguida de una fase de latencia en la que el virus puede reactivarse esporádicamente. Para persistir, los HVs emplean múltiples estrategias destinadas a modular la respuesta inmune del hospedador (Gatherer et al., 2021).

En las aves, los herpesvirus aviares (AHVs) se incluyen exclusivamente en la subfamilia *Alphaherpesvirinae* ,la cual comprende cinco géneros: *Simplexvirus*, *Varicellovirus*, *Mardivirus*, *Iltovirus* y *Scutavirus* (Gatherer et al., 2021). A pesar de que se han descrito numerosos AHVs, principalmente pertenecientes a *Mardivirus* e *Iltovirus*, muchos de los detectados en aves silvestres continúan pobremente caracterizados a nivel genómico y taxonómico (Erhard F. Kaleta & Docherty, 2008).

Los AHVs pueden causar diferentes cuadros clínicos y los individuos que superan la infección suelen establecer estados de latencia prolongados. En numerosos casos, la presencia simultánea de otros agentes infecciosos, factores ambientales adversos o estrés social o reproductivo favorece la aparición de formas clínicas manifiestas y, ocasionalmente, epizootias que pueden generar mortalidades notables (Niemeyer et al., 2017). No obstante, las enfermedades inducidas por AHVs rara vez comprometen la supervivencia de poblaciones bien establecidas (Davison, 2002). Más frecuentemente, las pérdidas afectan a la descendencia de determinadas parejas o provocan la eliminación de un número variable de individuos de distintas edades dentro de una población (Erhard F. Kaleta & Docherty, 2008)

Existe una gran variedad de AHVs capaces de producir distintas manifestaciones patológicas en aves de todo el mundo (E. F. Kaleta & Brinkmann, 1993; Pinkerton et al., 2008). La migración natural, el comercio internacional y la participación en exposiciones contribuyen significativamente a su dispersión (George et al., 2020). Además, las infecciones simultáneas con bacterias oportunistas, micoplasmas, clamidias, hongos o protozoos (flagelados, coccidios) pueden enmascarar los efectos primarios de los AHVs sobre sus hospedadores (Erhard F. Kaleta & Docherty, 2008). La obtención de información detallada sobre los AHVs de cualquier especie aviar es fundamental por tres razones principales: (a) algunos

AHVs pueden causar enfermedades graves y, en ocasiones, mortalidades elevadas; (b) el conocimiento del rango de hospedadores de nuevos aislados permite establecer medidas de precaución para evitar su diseminación; y (c) la susceptibilidad a la infección por un AHV determinado puede emplearse como herramienta complementaria para inferir relaciones filogenéticas entre especies de aves (E. F. Kaleta, 1990; Mettenleiter et al., 2019; Sacristán et al., 2024; Sibalin et al., 1974; Žlabravec et al., 2021).

Dado que la mayoría de los AHVs presentan un rango de hospedadores relativamente estrecho en condiciones naturales, su distribución geográfica tiende a reflejar la de sus especies hospedadoras. En aves migratorias, esta distribución puede alcanzar dimensiones continentales, desde Alaska hasta Sudamérica o desde el norte de Europa y Asia hasta África o el sur asiático. Tanto la migración natural como el transporte internacional de aves exóticas favorecen la diseminación global de estos virus, lo que dificulta establecer patrones regionales claros sobre su presencia (Erhard F. Kaleta & Docherty, 2008).

Finalmente, aún se desconoce por completo el rango natural de hospedadores de muchos AHVs. Mientras algunos muestran una especificidad muy estrecha, otros pueden infectar numerosas especies pertenecientes a familias u órdenes distintos. En este contexto, es probable que las aves mantenidas en cautividad y procedentes de distintos continentes estén expuestas a AHVs que anteriormente eran endémicos de regiones geográficas concretas (Erhard F. Kaleta & Docherty, 2008).

Avipoxvirus y viruela aviar

Los poxvirus se encuentran entre los virus animales de mayor tamaño y complejidad. La familia Poxviridae agrupa virus envueltos, generalmente de morfología ovalada o con forma de ladrillo que pueden alcanzar hasta 400 nm de diámetro, dimensiones solo ligeramente inferiores a las de muchas bacterias comunes, cuyo genoma consiste en una molécula lineal única de ADN (Figura 7) (McInnes et al., 2023). A diferencia de la mayoría de los virus ADN, cuya replicación ocurre en el núcleo, los poxvirus se replican en el citoplasma de las células infectadas (Agustina, 2019).

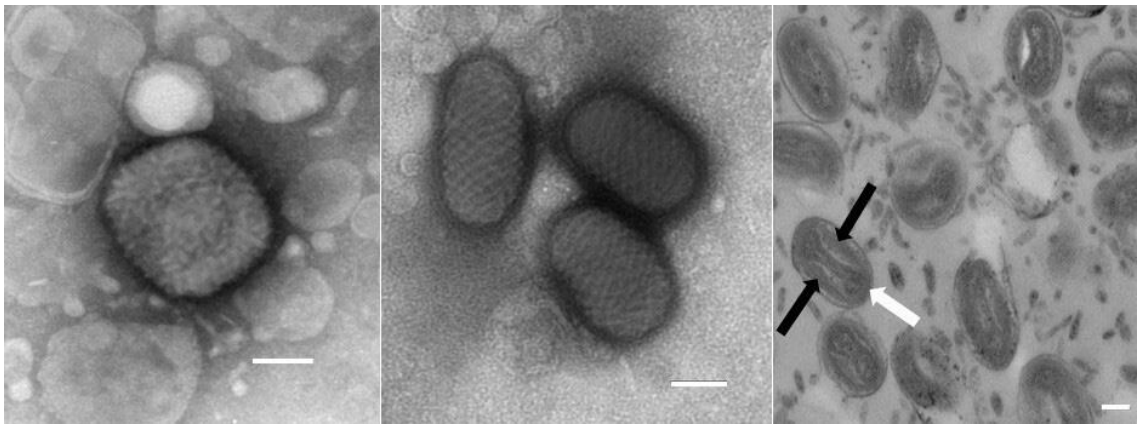


Figura 7. Morfología del virión. Micrografías electrónicas de preparaciones con tinción negativa de un virión maduro de ortopoxvirus (izquierda); viriones maduros de parapoxvirus (centro); y viriones de avipoxvirus que muestran dos cuerpos laterales (flechas negras) a ambos lados del núcleo en forma de mancuerna (flecha blanca) (derecha). Barras, 100 nm. Imágenes cortesía de David J. Everest, Agencia de Sanidad Animal y Vegetal (APHA), Surrey, Reino Unido pertenecientes al artículo ICTV Virus Taxonomy Profile: *Poxviridae* 2023 (McInnes et al., 2023).

Esta familia se divide en dos subfamilias: Chordopoxvirinae, que incluye virus que infectan vertebrados, y Entomopoxvirinae, compuesta por virus que afectan a insectos. Dentro de Chordopoxvirinae se encuentran patógenos relevantes para humanos, ganado y fauna silvestre, incluidas especies acuáticas. Actualmente, siete géneros de esta subfamilia infectan vertebrados, entre ellos el género Avipoxvirus, responsable de las infecciones en aves (Agustina, 2019; Le Loc'h et al., 2015).

El Comité Internacional de Taxonomía de Virus (ICTV) reconoce actualmente 12 especies dentro de este género, y se han propuesto dos más recientemente basadas en análisis filogenéticos y rangos de hospedador (McInnes et al., 2023). Los estudios filogenéticos basados en regiones genómicas seleccionadas han permitido clasificar los avipoxvirus en al menos tres clados principales: **f**owlpox-like, canarypox-like y psittacinepox-like (Boyle, 2007; Jarmin et al., 2006; McInnes et al., 2023). No obstante, los límites entre especies permanecen poco definidos. Un estudio molecular reciente identificó 152 secuencias virales únicas, reflejando una notable diversidad genética dentro del género (Williams et al., 2021). Tradicionalmente, esta diversidad se ha investigado mediante un fragmento conservado de 578 pb del gen de la proteína central 4b (Lüschow et al., 2004),

aunque actualmente se emplean con mayor frecuencia aproximaciones genómicas más amplias.

Los Avipoxvirus son la causa de la viruela aviar, una enfermedad infecciosa de distribución mundial (Smits et al., 2005). Dentro este género existe múltiples especies y cepas que varían en su patogenicidad y especificidad de hospedador (Atkinson, 2007). Las infecciones por avipoxvirus se han documentado en unas 230 especies de aves silvestres y domésticas en todo el mundo, tanto en ambientes terrestres como marinos. Esta amplia distribución plantea interrogantes sobre las vías de transmisión y los mecanismos de dispersión global del virus (Gyuranecz et al., 2013).

Debido a su extrema durabilidad, los avipoxvirus pueden sobrevivir fuera del hospedador durante el tiempo suficiente para que su transmisión pueda ocurrir de varias formas, ya sea por contacto directo entre aves, contacto indirecto con superficies, objetos o insectos contaminados. Sin embargo, no pueden penetrar a través de la piel intacta y deben ingresar al cuerpo a través de abrasiones en la piel o las membranas mucosas o por insectos hematófagos, principalmente mosquitos. Estos últimos sirven como principales vectores en la mayoría de las zonas. Debido a su importancia en el transporte del virus de una ave a otra, esta enfermedad es más común en primavera y otoño, debido al aumento de densidad de los mosquitos durante los periodos de lluvias (Agustina, 2019).

La viruela aviar es una enfermedad de evolución relativamente lenta, caracterizada por lesiones proliferativas y bien delimitadas en la piel de los dedos, patas o cabeza (Figura 8), así como en las mucosas de la cavidad oral y el tracto respiratorio superior. En algunos casos pueden producirse infecciones sistémicas (Tripathy & Reed, 2013). Las células infectadas suelen presentar grandes inclusiones intracitoplásmicas acidófilas, conocidas como cuerpos de Bollinger (C. van Riper & Forrester, 2007).

Figura 8. Lesión típica de avipoxvirus en cabeza y rostro en una tórtola (*Streptopelia turtur*). Cedida por la Dra. Soraya Déniz.

La gravedad clínica depende de diversos factores, como la susceptibilidad del hospedador, la virulencia de la cepa viral, la localización y el número de lesiones, y la presencia de infecciones secundarias o condiciones ambientales adversas (Tripathy & Reed, 2013; Van Riper et al., 2002). Aunque la viruela aviar suele ser autolimitante y se asocia a tasas de mortalidad bajas (P. S. da Silva et al., 2009; Singh et al., 2003), pueden ocurrir cuadros graves, especialmente cuando se desarrollan infecciones bacterianas o fúngicas secundarias (Reza et al., 2013; R. A. F. Silva et al., 2023). Estas coinfecciones pueden agravar la evolución clínica y conducir a enfermedad sistémica (Echenique et al., 2016).

Existen reportes de casos de infecciones por avipoxvirus en alcaravanes silvestres, un ejemplo es el caso de un alcaraván de Cerdeña con confirmación patológica y molecular por PCR (Lecis et al., 2017) o el de la detección molecular en dos ejemplares recogidos en las Islas Baleares en 1980 (Pérez-Tris et al., 2011) y en Marruecos en 2013 (Le Loc'h et al., 2015), respectivamente, aunque sin descripción patológica en ambos casos. Por último, existen descripciones clínicas y patológicas, con análisis moleculares en individuos cautivos (Lierz et al., 2007).

Hemoparásitos aviares

Los hemosporidios (Sporozoa: Haemosporida) son un grupo de parásitos que habitan en anfibios, reptiles, aves y mamíferos y utilizan insectos dípteros hematófagos (Insecta: Diptera) como vectores. Debido a que incluyen los agentes de la malaria, que sigue siendo una de las enfermedades humanas más comunes en países tropicales, cuyos casos importados se conocen en todo el mundo, son uno de los grupos de parásitos protistas más estudiados y conocidos. Sin embargo, el nivel de conocimiento entre las distintas familias de hemosporidios es notablemente desigual. La gran mayoría de los trabajos se han dedicado a estudiar los agentes de la malaria humana y a un pequeño número de especies pertenecientes a la familia Plasmodiidae, que han servido de modelos en la investigación de esta enfermedad. El resto de grupos de hemosporidios, principalmente Haemoproteidae, Leucocytozoidae y Garniidae, están menos estudiados.(Valkiunas, 2004). Por tanto, podemos decir que se ha subestimado la importancia de estos hemoparásitos en las aves. Estos parásitos sanguíneos son transmitidos por dípteros hematófagos (Haemoproteus por moscas piojo o mosquitos picadores, Leucocytozoon por moscas negras o mosquitos picadores, y Plasmodium por mosquitos) (Figura 9) (Carter T. Atkinson et al., 2009; Fecchio et al., 2019; Ilgūnas et al., 2022; Valkiunas, 2004; Valkiūnas & Iezhova, 2023). Es importante destacar que estos parásitos causan enfermedades en las aves domésticas, disminución en la productividad o incluso la muerte, así, también las aves que viven en zoológicos o aviarios enferman y mueren por hemosporidiosis de etiología tanto conocida, como no conocida (Agbemelo-Tsomafo et al., 2023; Alvarado-Piqueras et al., 2025; C. T. Atkinson & Van Riper, 1991; Bennett et al., 1993). Ejemplos de estos agentes son *Leucocytozoon caulleryi, l. simondi, l.smithi*, *Haemoproteus mansoni, Plasmodium durae,* y otros muchos (Valkiunas, 2004)

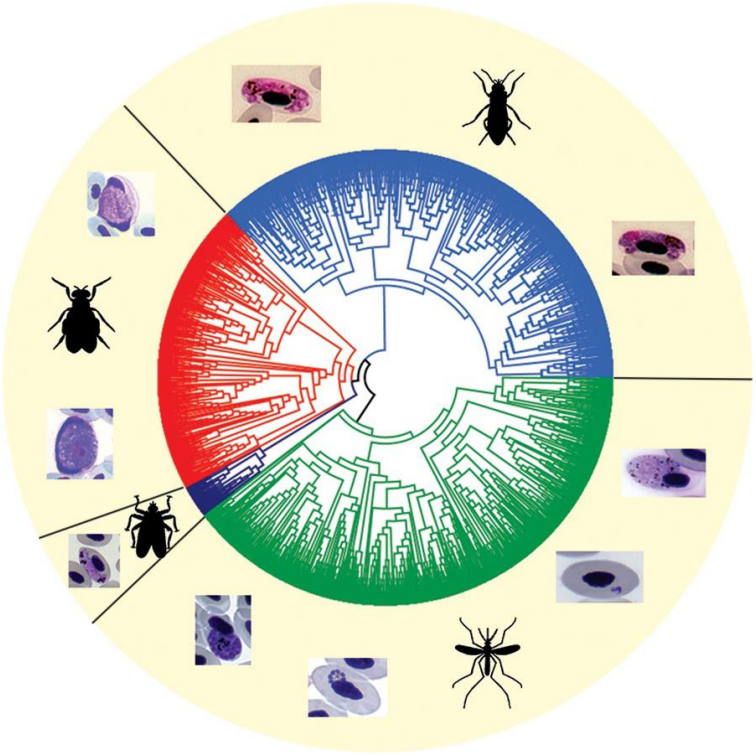


Figura 9. Filogenia de inferencia bayesiana de los linajes genéticos *citocromo b* de los tres géneros principales *Plasmodium*, *Haemoproteus* y *Leucocytozoon* de parásitos hemosporidios aviares. Las ramas coloreadas en azul, rojo y verde representan los linajes Haemoproteus, Leucocytozoon y Plasmodium, respectivamente. Imagen obtenida del artículo "Evolutionary ecology, taxonomy, and systematics of avian malaria and related parasites" (Fecchio et al., 2020).

Las especies aviares pertenecientes a los géneros *Plasmodium, Haemoproteus* y *Leucocytozoon* comparten características morfológicas y de desarrollo similares, sin embargo, una de sus diferencias fundamentales se observa en su ciclo reproductivo, donde el género *Plasmodium* realiza su reproducción asexual o merogonia en el interior de los eritrocitos circulantes mientras que *Haemoproteus* y *Leucocytozoon* lo hacen en los tejidos(Carter T. Atkinson et al., 2009).

Los Haemoproteus son parásitos intraeritrocíticos que afectan a las aves, siendo los más comunes y extendidos de las aves silvestres, sin embargo, su papel como agentes patógenos aún no está bien estudiado (Carter T. Atkinson et al., 2009).

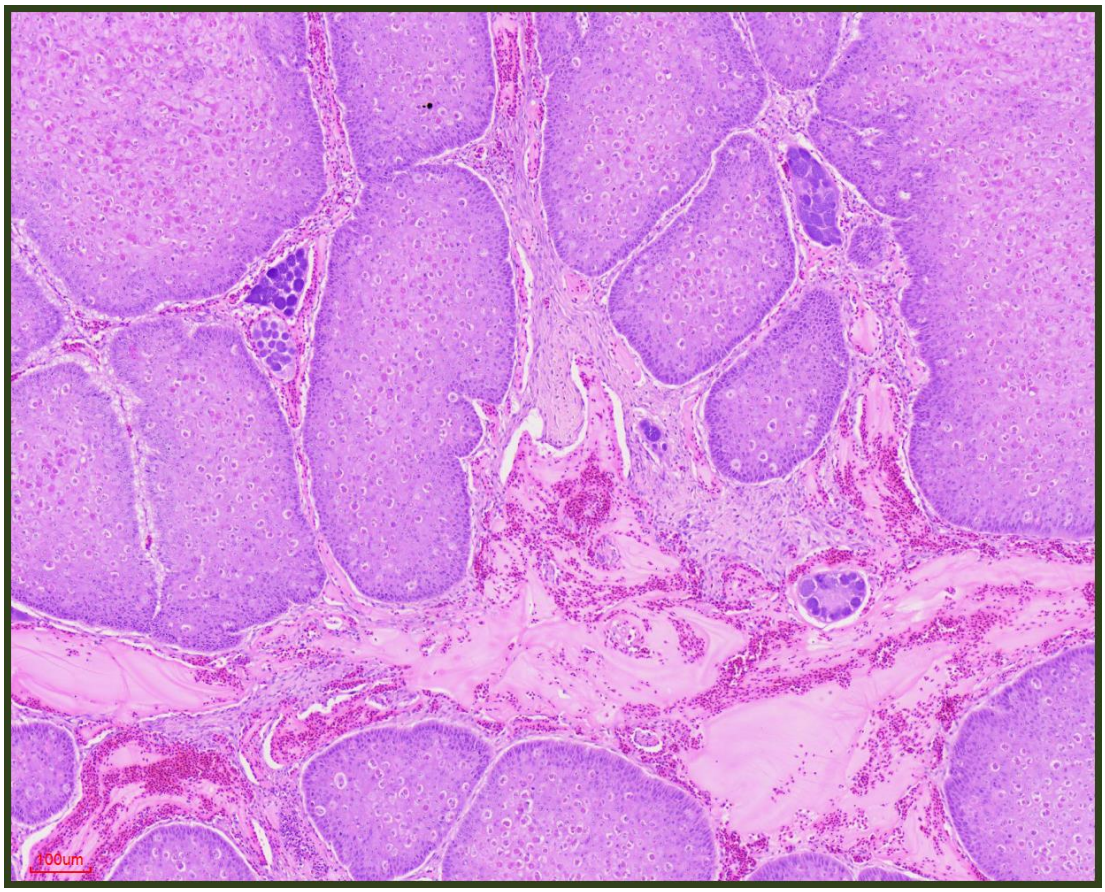
Por otro lado, la malaria aviar es una enfermedad transmitida por mosquitos causada por protozoos del género *Plasmodium*. Más de 40 especies pueden infectar aves,

mostrando una amplia variabilidad en su rango de hospedadores, distribución geográfica, vectores implicados y patogenicidad (Carter T. Atkinson et al., 2009).

La leucocitozoonosis es una enfermedad protozoaria transmitida por vectores y causada por diversas especies del género *Leucocytozoon*. Aunque existen numerosas especies, solo unas pocas se consideran patógenas. Los grupos de aves más susceptibles incluyen anátidas, colúmbidos, galliformes, rapaces y avestruces. Algunas especies producen mortalidades significativas en aves domésticas y *Leucocytozoon simondi* puede originar brotes localizados en anátidas silvestres. Otras especies provocan impactos menores y aún están poco estudiadas, por lo que es probable que se identifiquen nuevos efectos patógenos a medida que se conozcan mejor sus ciclos biológicos (Carter T. Atkinson et al., 2009).

Los *Leucocytozoon* presentan una marcada especificidad de hospedador, generalmente a nivel de orden, aunque algunas especies son específicas de familia o incluso de una sola especie. Están estrechamente relacionados con *Plasmodium* y *Haemoproteus*, con los que comparten características del ciclo vital, pero se diferencian por su transmisión, que depende principalmente de simúlidos (Simuliidae), excepto *L. caulleryi*, transmitido por ceratopogónidos (Carter T. Atkinson et al., 2009).

2. OBJETIVOS



Objetivos de la tesis

2.1. Objetivo general

Evaluar la utilidad de un enfoque diagnóstico integrador, basado en la combinación de técnicas moleculares e histopatológicas, para la detección, caracterización y comprensión del impacto de agentes infecciosos y sus manifestaciones patológicas en el alcaraván común (*Burhinus oedicnemus*), con el fin de aportar información relevante para la vigilancia sanitaria y la conservación de sus poblaciones, incluidas las subespecies endémicas del archipiélago canario.

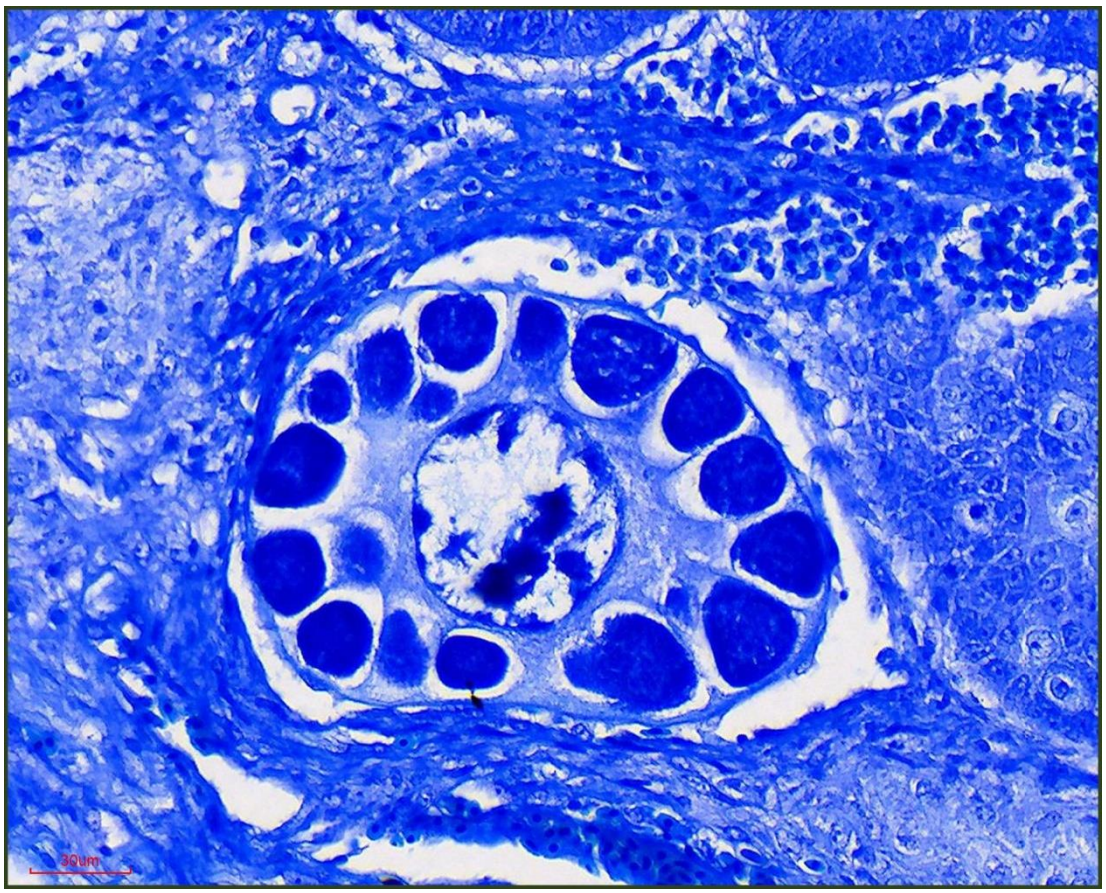
2.2. Objetivos específicos

1. Identificar y caracterizar los herpesvirus presentes en ejemplares de alcaraván común del archipiélago canario mediante técnicas moleculares y análisis filogenéticos, así como evaluar su distribución tisular y la presencia de posibles efectos citopáticos o lesiones asociadas mediante el estudio histopatológico de los ejemplares positivos.

2. Identificar y caracterizar, desde un punto de vista molecular y patológico, las infecciones por *Avipoxvirus* en alcaravanes comunes del archipiélago canario.

3. Evaluar la presencia de hemoparásitos aviares (*Haemoproteus*, *Plasmodium* y *Leucocytozoon* spp.) en ejemplares de alcaraván común del archipiélago canario mediante técnicas moleculares e histopatológicas.

3. RESUMEN DE LOS ARTÍCULOS QUE INTEGRAN LA TESIS



Resumen de los artículos que integran la tesis

3.1. Primer artículo: Detección molecular de un nuevo herpesvirus en el alcaraván (*Burhinus oedicnemus*) de las Islas Canarias

Los herpesvirus (HVs) son virus ADN de doble cadena pertenecientes al orden *Herpesvirales*, con capacidad para infectar una amplia variedad de hospedadores vertebrados e invertebrados. En aves, los HVs se clasifican exclusivamente dentro de la subfamilia *Alphaherpesvirinae*, que comprende cinco géneros. Aunque numerosos HVs aviares han sido descritos, especialmente en especies domésticas, aquellos detectados en aves silvestres continúan estando pobremente caracterizados genómica y taxonómicamente. Más de un centenar de especies de aves silvestres han sido identificadas como hospedadoras de distintos HVs; sin embargo, su rango real de hospedadores, dinámica de infección y factores asociados a la latencia y reactivación viral permanecen escasamente estudiados.

El alcaraván común (*Burhinus oedicnemus*) es un ave limícola de hábitos preferentemente nocturnos que se distribuye por Europa, el norte de África y Asia, que ocupa principalmente ambientes áridos, incluyendo zonas ocupadas por el ser humano. En el archipiélago canario están reconocidas dos subespecies endémicas (*B. o. insularum* y *B. o. distinctus*) de especial interés para la conservación. A pesar de la limitada información disponible acerca de su ecología y estado sanitario, se han descrito diversas patologías de origen natural en esta especie, lo que pone de manifiesto la necesidad de continuar los estudios sobre las amenazas sanitarias que afectan a sus poblaciones.

En el presente estudio se analizaron 50 alcaravanes comunes encontrados muertos entre 2020 y 2023 en el marco del programa de vigilancia de fauna silvestre de Canarias (Red Vigía Canarias). Durante las necropsias se evaluaron parámetros biológicos básicos, como el estado de conservación, la condición corporal, la edad y el sexo, y se recogieron. muestras de tejidos (hígado, pulmón, riñón, intestino y

encéfalo), así como hisopos orofaríngeos, cloacales y celómicos. Un total de 181 muestras fueron procesadas para la detección molecular de HVs mediante una PCR anidada modificada (semicuantitativa), dirigida al gen UL30 de la ADN polimerasa viral. Las muestras positivas fueron secuenciadas mediante el método de Sanger y analizadas filogenéticamente utilizando métodos de Máxima Verosimilitud y Neighbor-Joining. Adicionalmente, se llevó a cabo una extracción de ARN con digestión DNasa, seguida de retrotranscripción y PCR (RT-PCR), con el objetivo de detectar ARN mensajero del virus y determinar su estado replicativo.

El ADN de HV se detectó en 4 de los 50 individuos analizados (8 %), correspondientes a 7 de las 181 muestras estudiadas (4 %), procedentes de encéfalo, riñón, pulmón, cavidad celómica y orofaringe. Mediante secuenciación se identificó un fragmento de 163 pb en todas las muestras positivas y, en dos individuos, un amplicón adicional de 385 pb, depositado en GenBank (PQ332998). En todas las muestras positivas para ADN de HVs, no se detectó ARN mensajero del virus, lo que sugiere ausencia de replicación activa de éste al momento de la muerte de las aves.

El estudio histopatológico no mostró lesiones específicas compatibles con una infección por HVs. Los análisis filogenéticos situaron las secuencias obtenidas dentro de la subfamilia *Alphaherpesviridae*, con identidades nucleotídicas del 67 % al 75 % respecto a otros HVs aviares, lo que sugiere la detección de una nueva variante viral no descrita previamente.

3.2. Segundo artículo: Caracterización patológica y molecular de la infección por Avipoxvirus en *Burhinus oedicnemus* en Canarias.

Este estudio pretende analizar de forma integral la presencia, caracterización molecular y manifestaciones patológicas de infecciones por *Avipoxvirus* en ejemplares de alcaraván común (*Burhinus oedicnemus*) procedentes del archipiélago canario recogidos entre los años 2021 y 2024. Los *Avipoxvirus* son

patógenos de ADN ampliamente distribuidos y con una elevada diversidad genética, descritos en más de 374 especies de aves, con un rango de especificidad variable de hospedador. Causantes de la viruela aviar con una presentación clínica más frecuentemente en la forma cutánea, que se caracteriza por lesiones nodulares proliferativas o ulcerativas que pueden favorecer infecciones secundarias.

Se evaluaron ocho alcaravanes con lesiones compatibles con viruela aviar: tres animales vivos atendidos en un centro de recuperación y cinco procedentes de necropsias. Se registraron datos biológicos, estado nutricional, distribución anatómica de lesiones, y evolución clínica. Se realizaron estudios histopatológicos y análisis moleculares para la detección de ADN viral mediante PCR convencional y semicuantitativa dirigida al gen P4b. Seis casos fueron analizados mediante técnicas moleculares, seis mediante histopatología y, de ellos, cuatro mediante ambos enfoques.

Las lesiones macroscópicas observadas consistían en nódulos proliferativos y/o ulcerados, principalmente en patas y dedos, con un grado de severidad variable, desde formas leves hasta lesiones graves que, por su extensa afectación de las falanges, pudieran suponer un compromiso funcional para el animal. El examen histopatológico reveló hiperplasia en la epidermis, degeneración balonizante, necrosis y presencia de cuerpos de inclusión intracitoplasmáticos (cuerpos de Bollinger), confirmando la infección por *Avipoxvirus*. En un individuo se identificó además una hiperplasia compatible con carcinoma de células escamosas, algo infrecuente pero descrito previamente en asociación con infecciones crónicas por *Avipoxvirus*.

Se detectó ADN viral mediante técnicas moleculares en todas las muestras evaluadas. La secuenciación del gen P4b reveló la presencia de tres variantes del virus distintas en el grupo estudiado, agrupadas en los clados A2, B1 y B2, lo que pone de manifiesto la diversidad genética de *Avipoxvirus* en alcaravanes del archipiélago. Este hallazgo coincide con estudios previamente publicados que describen la elevada variabilidad genética del género y su capacidad para infectar múltiples hospedadores.

La presencia de infecciones secundarias como bacterias intralesionales y, especialmente, estructuras fúngicas compatibles con *Aspergillus* spp., lo cual se confirmó en seis de los ocho casos mediante PCR, identificándolo como *Aspergillus fumigatus*, sugiere que estas coinfecciones podrían agravar la evolución clínica y aumentar el riesgo de mortalidad de estos animales.

Este trabajo representa la primera caracterización sistemática de viruela aviar en alcaravanes silvestres del archipiélago canario y pone de manifiesto la presencia de diversas variantes virales en dos subespecies endémicas de Canarias. Los resultados ponen en valor la vigilancia sanitaria en especies insulares vulnerables y sustentan la necesidad de estandarizar protocolos de diagnóstico que permitan mejorar la comparabilidad entre estudios y apoyar estrategias de conservación basadas en evidencia científica.

3.3. Tercer artículo: Estudio de parásitos hemosporidios en alcaravanes silvestres (*Burhinus oedicnemus*) en Canarias: Primera evidencia molecular e histopatológica de infección por *Leucocytozoon* sp.

Este estudio aporta la primera descripción molecular e histopatológica de infección por *Leucocytozoon* sp. en el alcaraván común (*Burhinus oedicnemus*) del archipiélago canario. Entre 2020 y 2024 se analizaron 47 ejemplares silvestres mediante un enfoque diagnóstico combinado que incluyó la PCR anidada dirigida al gen mitocondrial *del citochromo b* y el examen histopatológico de múltiples tejidos. Las aves procedían de distintas islas del archipiélago y representaban las dos subespecies endémicas reconocidas (*B. o. insularum* y *B. o. distinctus*), siendo la mayoría de los individuos hallados muertos en el marco del programa de vigilancia sanitaria de fauna silvestre del Gobierno de Canarias.

La detección molecular identificó un único individuo positivo (prevalencia del 2 %), correspondiente a una hembra adulta procedente de Gran Canaria, que presentaba coinfección por *Avipoxvirus* y *Aspergillus fumigatus*. La secuenciación mostró una identidad del 100 % con el linaje CIAE02 de *Leucocytozoon*, previamente descrito en numerosas especies de aves y regiones geográficas,

especialmente en rapaces, lo que sugiere un linaje altamente generalista o un complejo de linajes crípticos no resolubles con el marcador utilizado. El análisis filogenético confirmó su agrupamiento dentro de este linaje con alto soporte estadístico.

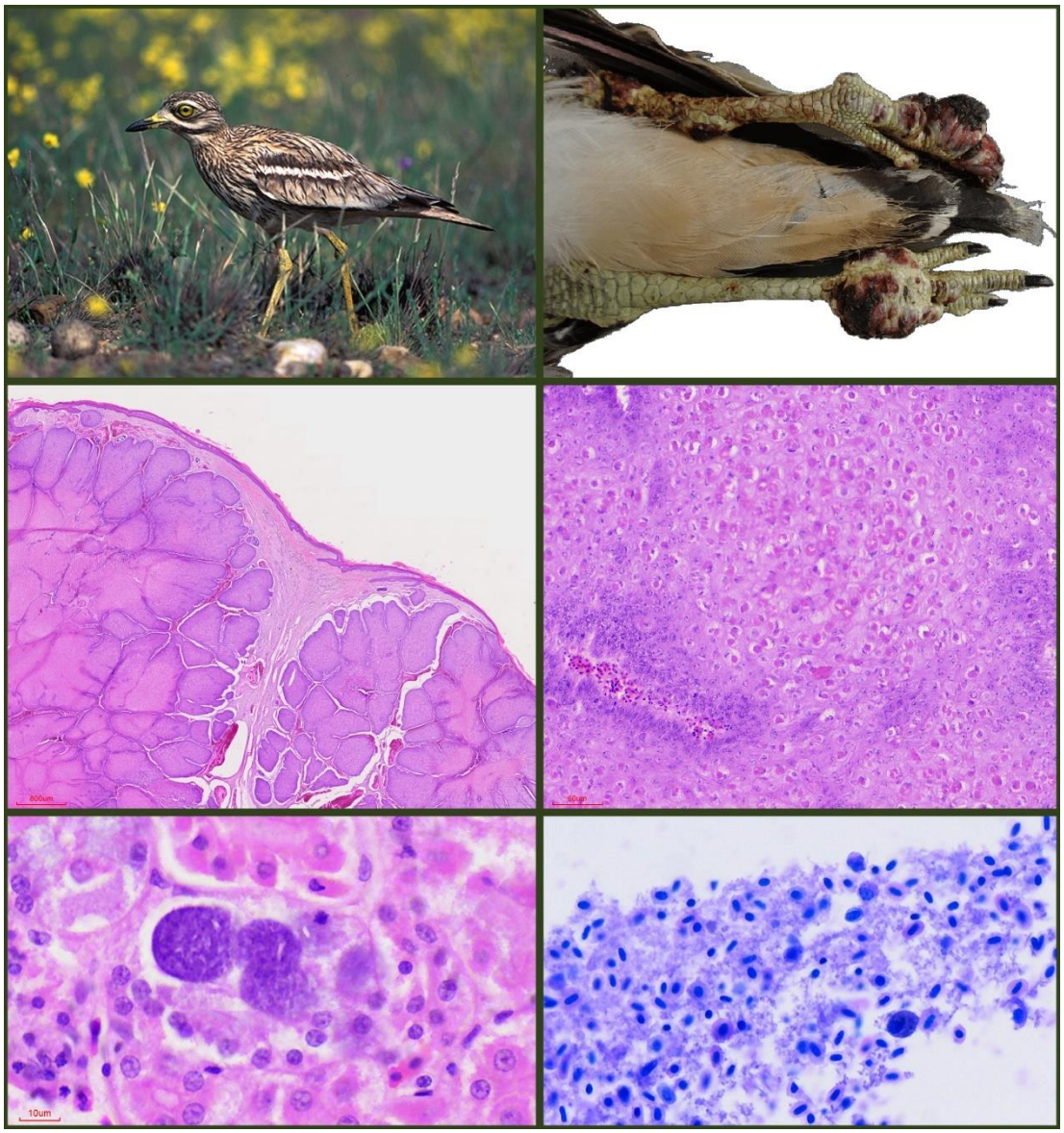
El estudio histopatológico reveló un patrón de infección sistémica caracterizado por la presencia de merontes y megalomerontes en hígado, riñón y piel. Las estructuras parasitarias, localizadas principalmente en células epiteliales y capilares dérmicos, mostraron características morfológicas compatibles con el desarrollo exoeritrocítico típico de *Leucocytozoon*. En la piel se identificaron numerosos megalomerontes de gran tamaño, un hallazgo poco frecuente en esta localización. A pesar de la triple coinfección, la respuesta inflamatoria fue escasa en la mayoría de los tejidos, lo que sugiere una infección subaguda, crónica o potencialmente abortiva en un hospedador no completamente adaptado. De forma ocasional, se observaron estructuras compatibles con gametocitos intraeritrocíticos, indicativas de un desarrollo parasitario incompleto o de una parasitemia muy baja.

Este trabajo se engloba dentro del conocimiento actual sobre hemoparásitos aviares, destacando la limitada información disponible en Charadriiformes y, en particular, en el género *Burhinus*, en el que previamente solo se había documentado *Haemoproteus burhinus*. La detección del linaje CIAE02 de *Leucocytozoon* en el alcaraván común amplía su rango de hospedadores y refuerza la necesidad de estudios integrativos que combinen estudios moleculares, morfológicos y ecológicos para identificar linajes y comprender las dinámicas de transmisión, especialmente en sistemas insulares.

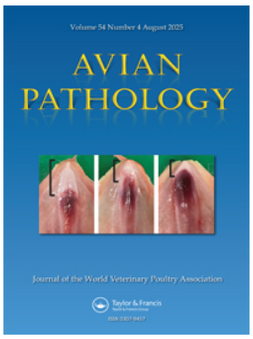
Desde una perspectiva epidemiológica y de conservación, la presencia de vectores competentes, principalmente simúlidos, en las islas, junto con el carácter vulnerable de las subespecies endémicas y el reducido tamaño de sus poblaciones, hace necesario incluir estos hemoparásitos en los programas de vigilancia sanitaria. El estudio demuestra además la utilidad del análisis post mortem de tejidos como herramienta complementaria a los muestreos sanguíneos, capaz de revelar infecciones latentes o restringidas a órganos específicos que podrían pasar desapercibidas mediante métodos convencionales.

En conjunto, el trabajo amplía el conocimiento sobre la diversidad y biología de los haemosporidios en aves no paseriformes y pone de relieve la importancia de aplicar enfoques diagnósticos combinados para detectar infecciones crípticas en especies de especial interés conservacionista en ecosistemas insulares.

4. PUBLICACIONES CIENTÍFICAS



4.1. ARTÍCULO 1: **Molecular detection of a novel herpesvirus in the stone-curlew (*Burhinus oedicnemus*) from the Canary Islands.**



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ORIGINAL ARTICLE

Molecular detection of a novel herpesvirus in the stone-curlew (*Burhinus oedicnemus*) from the Canary Islands

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ABSTRACT

Avian herpesviruses (AHVs) are widely distributed and associated with a variety of diseases affecting bird populations globally. Despite the increasing detection of AHVs in recent years, there remains a significant gap in knowledge regarding their classification and host range. This study aimed to detect herpesvirus (HV) in two vulnerable, endemic subspecies of stone-curlew (*Burhinus oedicnemus*) in the Canary Islands. Forty-six pooled tissue swabs (liver, kidney, and lung) and 135 individual swabs (brain, cloaca, and oropharyngeal cavity) were collected from 50 stone-curlews recorded as deceased wildlife specimens between 2020 and 2023. DNA from a novel alpha-HV was successfully amplified from seven out of the 181 tissue samples (4%) and from four out of 50 birds analysed (8%) using a semi-nested polymerase chain reaction (PCR) approach with degenerate primers. Positive samples were distributed across various tissue types: brain ($n = 1$), kidney ($n = 1$), lung ($n = 2$), coelomic cavity ($n = 1$), and oropharyngeal swab ($n = 2$). Some individuals tested positive in multiple tissue types, although no histopathological features indicative of HV infection were observed in any of the birds. Sequencing of all positive samples revealed identical HV nucleotide sequences across all specimens. The longest PCR amplicon, obtained with the TGV and KG1 primer combination, yielded identical sequences in two of the seven positive samples. Based on these findings, we propose the designation of this novel HV as *Burhinus oedicnemus alphaherpesvirus*.

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KEYWORDS
Avian; novel herpesviruses; wildlife; Canary Islands; stone-curlew; polymerase chain reaction; sequence analysis; phylogenetic analysis

Introduction

Herpesviruses (HVs), classified in the order *Herpesvirales*, are linear, enveloped, double-stranded DNA viruses infecting a broad range of hosts, including vertebrate and invertebrate species (Davison *et al*., 2009). Within this order, the family *Orthoherpesviridae* is divided into three subfamilies: *Alpha-, Beta-,* and *Gammaherpesvirinae*. Avian herpesviruses (AHVs) are exclusively classified under the subfamily *Alphaherpesvirinae* (Gatherer *et al*., 2021), which encompasses five genera: *Simplexvirus, Varicellovirus, Mardivirus, Iltovirus and Scutavirus* (Gatherer *et al*., 2021). Despite the identification of numerous AHVs, most of which belong to the genera *Mardivirus* and *Iltovirus*, several viruses detected in avian species, particularly wild birds, remain poorly characterized at the genomic and taxonomic levels (Kaleta & Docherty, 2007).

Over 100 different free-living bird species have been identified as hosts for various HV strains, but the full spectrum of host range and infectivity remains largely unexplored. While naturally infectious HV are often assumed to exhibit low genetic diversity, certain HV strains have demonstrated the capacity to infect multiple bird species across different families and even orders, highlighting their potential for broad host adaptation (Niemeyer *et al*., 2017). Herpesviruses can persist in a latent state for extended periods in many avian species without causing clinical signs. However, under certain specific conditions, such as co-infections or stress from reproductive activity, social interactions, or environmental changes, latent HV may reactivate, leading to symptomatic disease or increased mortality (Niemeyer *et al*., 2017).

The stone-curlew, *Burhinus oedicnemus*, is a medium-sized wader in the *family Burhinidae*, characterized by its cryptic plumage, large yellow eyes, and nocturnal habits (Lorenzo *et al*., 2004). Despite its name, it is not closely related to true curlews but instead belongs to the order Charadriiformes. This species is distributed across Europe, North Africa, and parts of Asia, primarily inhabiting open, arid environments with sparse vegetation. However, it exhibits notable adaptability to human-modified landscapes. The species also demonstrates marked behavioural flexibility; while typically territorial during the breeding season, it becomes more social outside this period. It often congregates in groups at night, using areas like farmland, golf courses, and stables as

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roosting sites, particularly as evening approaches (Lorenzo *et al.*, 2004). Conservation concerns have arisen for the stone-curlew across its range, as populations face threats from habitat loss and disturbance. However, significant gaps remain in understanding its ecology and behaviour, with only limited studies available (Giunchi *et al.*, 2015).

In the Canary Islands, two endemic subspecies of the stone-curlew are recognized: *Burhinus oedicnemus insularum* (Sassi, 1908) and *Burhinus oedicnemus distinctus* (Bannerman, 1914). *B. o. insularum* is found in Alegranza, La Graciosa, Lanzarote, Lobos and Fuerteventura, while *B. o. distinctus* inhabits Gran Canaria and the Western Islands (Martín, 1987). Both subspecies are listed in the Canary Islands Catalogue of Protected Species and are considered important for regional biodiversity conservation (Martín, 1987).

Although research on the factors influencing their populations is limited, the primary reported causes of mortality are anthropogenic, with power line collisions being particularly significant (Rioja, 2001). In addition, natural pathologies, including bacterial, viral, and parasitic infections and diseases, have been occasionally reported in this species (Georgiev *et al.*, 1996; Kent *et al.*, 2006; Lierz *et al.*, 2007; Zylan *et al.*, 2008; Schmidt *et al.*, 2009; Calabuig *et al.*, 2011; Lecis *et al.*, 2017; Suárez-Santana *et al.*, 2022). This highlights the need for further investigation into the threats facing these endemic subspecies to inform effective conservation strategies. This study expands our understanding of HV infection in stone-curlews in the Canary Islands, as assessing this pathogen prevalence is essential for evaluating potential health risks to these vulnerable populations.

Materials and methods

Birds and tissue sampling

This study included 50 deceased stone-curlews collected between 2020 and 2023 as part of a Canarian Government survey on wildlife mortality causes, known as *Red Vigía Canarias* (Order No. 134/2020, May 26, 2020). The dead birds submitted for necropsy were categorized based on their preservation status (decomposition state: 1 = very fresh, 2 = fresh, 3 = incipient decomposition), by the veterinary pathology protocol established at the Institute of Animal Health and Food Safety (IUSA) at the University Las Palmas de Gran Canaria (data provided in Table 1). The nutritional condition of the birds was evaluated by examining the pectoral muscle mass (keel score) and assessing fat reserves. The condition was rated on a scale from 1–5, where 1 corresponds to cachexia, 2 to thin, 3 to normal, 4 to overweight, and 5 to obesity, based on a modified version of the scoring system proposed by Burton *et al.* (2014). Age estimation was performed by assessing body morphology, skeletal structure, and gonadal development, and individuals were categorized as chicks, juveniles, or adults. All necropsies were performed using standardized and systematic procedures (Rae, 2003).

During necropsy, tissue samples were collected from key organs, including the liver, lungs, kidneys, and intestines (Table 1). Additionally, swab samples were obtained from the oropharyngeal, cloacal, and coelomic cavities, as well as the brain, using sterile, individually wrapped swabs without transport medium. Given the brain's fragility, meticulous care was taken during necropsy to preserve its integrity for histopathological analysis. Virology samples were obtained by carefully removing the dorsal calvarium, incising the meninges, and gently swabbing the brain surface. Tissue swabbing has been shown to yield PCR results equivalent to tissue dissection, ensuring reliable diagnostics while minimizing damage (Errington *et al.*, 2014; Helm *et al.*, 2021). Fresh, unfixed tissues and swab-collected samples were stored at −80°C until processing for molecular virology analysis. Simultaneously, the same organ samples were fixed in 10% buffered formalin for subsequent histopathological examination.

Of the 50 stone-curlews, 21 were identified as female (21/50; 42%), 18 as male (18/50; 36%), and the sex of 11 individuals (11/50; 22%) could not be determined due to the condition of the carcases. In terms of age distribution, 28 birds were adults (28/50; 56%), 18 were juveniles (18/50; 36%) and two were chicks (2/50; 4%) with the age of two birds undetermined (2/50; 4%). Body condition assessments revealed that 15/50 (30%) were cachectic (very thin), 20/50 (40%) were thin, and 13/50 (26%) were in a good body condition. In two cases, the body condition could not be evaluated due to the advanced decomposition of the carcases (Table 1).

Molecular study: DNA extraction and PCR

During sample processing, small tissue sections from each available organ were collected for each animal, pooling lung, liver, and kidney tissues into a single sample using sterile swabs (Vircell S.L., Granada, Spain). These pooled tissue samples were then placed into tubes containing transport medium (Transport Medium for the Collection and Preservation of Viruses, Chlamydia, and Mycoplasma, Vircell S.L.), formulated with HEPES, gelatine, bovine serum albumin, sucrose, and compatible antibiotics to ensure the stability and viability of the pathogens during transport and storage. Likewise, swab samples obtained during necropsy were also

Table 1. Data from 50 stone-curlews (*Burhinus oedicnemus*) included in the present study (2020–2023), comprising biological information (age and sex), location and condition (dead or alive) at the time of discovery, state of decomposition at necropsy, body condition, and samples tested for HV DNA detection.

								Tested samples
ID	Age	Sex	FL	FD	S	DC	BC	Swabs
SA447/21	J	U	L	24/05/2021	D	3	2	Pool*
SA255/21	J	M	F	20/06/2018	D	3	3	Pool*
SA359/21	A	U	T	29/04/2021	D	3	3	Pool*, brain
SA485/21	A	F	L	08/04/2021	D	2	3	Pool*, brain, cloaca
SA518/21	J	M	L	06/04/2021	D	2	2	Pool*, brain, intestine
SA597/21	J	M	L	21/12/2018	D	2	2	Pool*, brain, cloaca
SA598/21	A	F	L	25/05/2019	D	3	1	Pool*, brain, cloaca
SA849/21	J	U	GC	27/08/2021	D	3	3	Pool*, brain
SA870/21	J	M	GC	U	D	2	1	Brain, oropharyngeal cavity, liver, kidney, lung
SA875/21	J	F	L	07/06/2021	D	2	U	Pool*, brain
SA324/20	A	U	L	30/07/2020	D	3	1	Pool*
SA131/21	J	F	L	08/08/2020	D	2	1	Pool*
SA132/21	A	F	L	03/09/2020	D	3	1	Pool*
SA133/21	A	F	L	24/11/2020	D	3	1	Pool*
SA134/21	J	M	L	04/12/2020	D	2	3	Pool*
SA341/21	*P*	M	GC	29/04/2021	D	3	2	Pool*
SA1133/21	A	M	T	22/0/2021	D	2	2	Pool*, oropharyngeal cavity
SA1156/21	A	F	F	25/10/2021	D	3	2	Pool*, brain, oropharyngeal cavity
SA876/21	A	M	L	08/07/2021	D	2	2	Pool*, brain, cloaca, oropharyngeal cavity
SA877/21	J	M	L	29/06/2021	D	3	3	Pool*, brain, cloaca, oropharyngeal cavity
SA994/21	J	M	L	31/08/2021	D	3	3	Pool*, brain, conjunctiva, cloaca, oropharyngeal cavity
SA1262/21	A	F	T	04/11/2021	D	2	3	Pool*, brain, intestine, cloaca, oropharyngeal cavity
SA1295/21	J	F	GC	U	D	3	2	Pool*, brain, intestine, cloaca, oropharyngeal cavity
SA1296/21	J	F	GC	18/09/2021	D	2	1	Pool*, brain, intestine, cloaca, oropharyngeal cavity, fur
SA1297/21	A	F	GC	28/09/2021	D	2	3	Pool*, brain, intestine, cloaca, oropharyngeal cavity
SA1298/21	J	F	GC	U	D	1	3	Pool*, brain, cloaca, oropharyngeal cavity
SA1349/21	A	U	L	18/09/2021	D	3	3	Pool*, brain, intestine, oropharyngeal cavity
SA1411/21	A	F	T	30/11/2021	D	3	2	Pool*, brain, intestine, oropharyngeal cavity
SA1417/21	A	F	T	07/09/2021	D	2	3	Pool*, brain, intestine, oropharyngeal cavity
SA447/21	J	U	L	24/05/2021	D	3	1	Pool*, brain, cloaca, oropharyngeal cavity
SA1438/21	A	F	GC	12/11/2021	D	2	2	Pool*, brain, intestine, oropharyngeal cavity
SA1439/21	A	M	GC	U	D	2	2	Pool*, brain, intestine, oropharyngeal cavity, joint
SA1505/21	A	U	L	26/11/2021	D	2	1	Pool*, brain, intestine, oropharyngeal cavity
SA1506/21	A	F	L	10/11/2021	D	3	1	Pool*, brain, cloaca, oropharyngeal cavity, coelomic cavity
SA061/22	A	M	L	10/12/2021	D	3	2	Lung, brain, oropharyngeal cavity
SA301/22	A	F	L	27/02/2022	D	3	2	Pool*, brain, intestine, oropharyngeal cavity
SA341/22	U	U	F	22/01/2022	D	2	1	Brain
SA1267/22	A	F	GC	31/05/2022	A	3	2	Pool*, brain, cloaca, oropharyngeal cavity, coelomic cavity
SA1277/22	U	M	GC	U	D		2	Pool*, brain, lung, liver, kidney, cloaca, oropharyngeal cavity
SA1246/22	*P*	U	GC	05/05/2022	D	3	3	Pool, brain, oropharyngeal cavity, cloaca, coelomic cavity
SA1269/22	J	U	GC	08/08/2022	A	2	U	Pool, brain, cloaca, oropharyngeal cavity
SA1283/22	J	M	GC	26/06/2022	A	2	2	Pool, brain, intestine, cloaca, oropharyngeal cavity
SA1642/22	A	M	L	03/10/2022	D	2	1	Pool*, brain, intestine, cloaca, oropharyngeal cavity, coelomic cavity
SA334/21	A	M	GC	22/04/2021	D	2	2	Pool*
SA1365/22	A	F	H	U	D	3	2	Pool*, brain, intestine, oropharyngeal cavity, coelomic cavity, cloaca, spleen
SA1508/22	A	M	GC	28/09/2022	D	3	1	Pool*, brain, intestine, cloaca, oropharyngeal cavity, coelomic cavity
FS0229/23	J	U	L	12/02/2023	D	2	1	liver, intestine, brain, cloaca
FS0453/23	A	M	L	08/04/2023	D	2	2	Pool*, brain, intestine, cloaca, oropharyngeal cavity, coelomic cavity
FS0546/23	A	F	GC	U	D	2	2	Pool*, brain, cloaca, oropharyngeal cavity, coelomic cavity
FS415/23	A	F	GC	05/05/2023	A	1	1	Pool*, brain, cloaca, oropharyngeal cavity, coelomic cavity

Notes: *Pool = Liver, kidney, lung; FD (finding date); FL (finding location: GC = Gran Canaria; T = Tenerife; F = Fuerteventura; L = Lanzarote); S (status: A = alive; D = dead); DC (decomposition code); DC (1 = very fresh, 2 = fresh, 3 = incipient decomposition); BC (body condition); BC (1 = cachexia, 2 = thin (slim), 3 = good body condition, U = undetermined); Sex (F = female, M = male, U = undetermined), Age (A = adult, J = juvenile, U = undetermined).

immersed in the same transport medium in individual tubes.

In positive pooled samples, individual tissue samples were then tested.

For molecular analysis, a 100µl aliquot of each sample dilution was mixed with an equal volume of DNA shield. This preparation was used for simultaneous DNA/RNA extraction via the magnetic bead method on a robotic platform, following the manufacturer's protocol for the ZYMO DNA/RNA extraction kit (ZYMO Research, Freiburg, Germany). To ensure the accuracy and reliability of the extraction process, both a negative control (nuclease-free water) and a positive control (a known herpesvirus-positive sample, previously identified in our laboratory) were included in the protocol.

The presence of herpesvirus DNA in 181 samples (46 pooled tissue swabs and 135 individual swaps) was evaluated using a semi-quantitative (sq) modification of the conventional semi-nested polymerase chain reaction (PCR) method, originally described by Vandevanter *et al.* (1996) and adapted in recent studies (Sierra *et al.*, 2022; Segura-Göthlin *et al.*, 2023). This method employs degenerate primers designed to amplify a conserved region of the DNA polymerase gene (UL30). In the first external PCR, three primers were employed: DFA (5′-

GAYTTYGCNAGYYTNTAYCC-3′), ILK (5′-TCCTG GACAAGCAGCARNYSGCNMTNAA-3′) and KG1 (5′-GTCTTGCTCACCAGNTCNACNC-CYTT-3′). This was followed by a second internal PCR using the primers TGV (5′ TGTAACTCGGTG-TAYG GNTTYACNGGNGT-3′) and IYG (5′ -CACAGAGTCCGTRTC NCCRTADAT-3′), with both PCRs conducted under identical thermal cycling conditions. To enable longer sequence amplification from positive samples, additional conventional semi-nested PCRs were performed using various combinations of external and internal primers derived from the initial external PCR: DFA (external forward) with IYG (internal reverse) and TGV (internal forward) paired with KG1 (internal reverse) (Vandevanter *et al.*, 1996; Sacristán *et al.*, 2019). Diethylpyrocarbonate (DPEC)-treated water was used as the negative control for all PCRs, while a commercial herpesvirus DNA control, Amplirun® herpes simplex 1 DNA (Vircell, S.L.), served as the positive control to ensure the accuracy and reliability of the assay. All birds were tested at least three times, except for SA1508/22, which underwent a single test and sequencing.

Detection and sequencing of PCR products and phylogenetic analysis

PCR products from positive samples were purified using a Real Clean spin kit (REAL, Valencia, Spain) for bidirectional sequencing using the Sanger method. The obtained sequences were compared among themselves and against previously published sequences in Gen Bank using BLAST (Basic Local Alignment Search Tool) search via the BLASTN algorithm). Multiple sequence alignments of HV sequences were performed using ClustalW.

Phylogenetic and molecular evolutionary analyses were conducted with MEGA version 11 (Tamura K, Stecher G, and Kumar S 2021), incorporating 45 nucleotide (nt) sequences from α- and γ-HVs from various species, including birds, mammals, and reptiles, retrieved from GenBank. Gammaherpesvirus sequences were used as the outgroup for phylogenetic reconstruction.

The Neighbor-Joining (NJ) and BioNJ algorithms were applied for tree generation, utilizing pairwise distances estimated by the Maximum Composite Likelihood (MCL) method with the General Time Reversible (GTR) model (Nei & Kumar, 2000). Evolutionary rate differences among sites were modelled using a discrete Gamma distribution with five rate categories (+G, parameter = 2.4878). The robustness of the tree topology was assessed using a bootstrap consensus tree based on 1000 replicates, with branches reproduced in fewer than 50% of bootstrap replicates collapsed. The percentage of bootstrap replicates supporting each clade (out of 1000) was indicated next to the branches (Tamura *et al.*, 2021).

For further analysis, nt sequences from positive samples were translated into deduced amino acid (aa) sequences. Additionally, 45 sequences (42 alphaherpesvirus and three gammaherpesvirus) were retrieved from GenBank. The phylogenetic tree based on the deduced aa sequences was constructed using the Maximum Likelihood (ML) method and the Le_Gascuel_2008 model (Le & Gascuel, 2008). A bootstrap consensus tree, also based on 1000 replicates (Felsenstein 1985), was generated to infer the evolutionary relationships among taxa. Branches with less than 50% bootstrap support were collapsed, and the initial tree(s) were constructed using the NJ and BioNJ algorithms based on pairwise distances estimated using the JTT model. The final tree topology was selected based on the highest log-likelihood value, and evolutionary rate differences among sites were modelled with a discrete Gamma distribution (+G, parameter = 1.1679).

Herpesvirus RNA detection to assess active replication

To detect active HV replication, viral messenger RNA (mRNA) was analysed in the HV-positive tissue and swab samples. RNA extraction was performed using the RNeasy kit (Qiagen, Hilden, Germany), which included DNase digestion to remove contaminating DNA, following the manufacturer's "Purification of Total RNA from Animal Tissues" protocol. The absence of DNA contamination ensures that any detected signal in the subsequent HV PCR analysis will originate solely from viral mRNA, providing evidence of active viral replication. To ensure the quality and success of RNA extraction, a normalizing control sq reverse transcription (RT)-PCR was carried out using glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primers: GAPDH F (5′-GTCTA-CATGTTCCAGTATGACTCC-3) and GAPDH R (5′-GCCTTCTCCATGGTGGTGAAGAC-3′) (Suzuki *et al.*, 2000).

To detect HV mRNA, we employed the same HV PCR protocol incorporating an initial RT step to synthesize complementary DNA from the viral mRNA (Vargas-Castro *et al.*, 2021). Subsequently, a HV sqPCR was conducted to confirm the complete elimination of DNA during the RNA extraction process with DNase treatment.

Results

PCR and sequencing

Herpesvirus DNA was detected in four out of 50 animals analysed (8%), across five different tissue samples or swabs with seven positive samples out of 181 analysed (4%). The positive samples were distributed as follows: brain ($n = 1$), kidney ($n = 1$), lung ($n = 2$), coelome ($n = 1$) and oropharyngeal swab (n

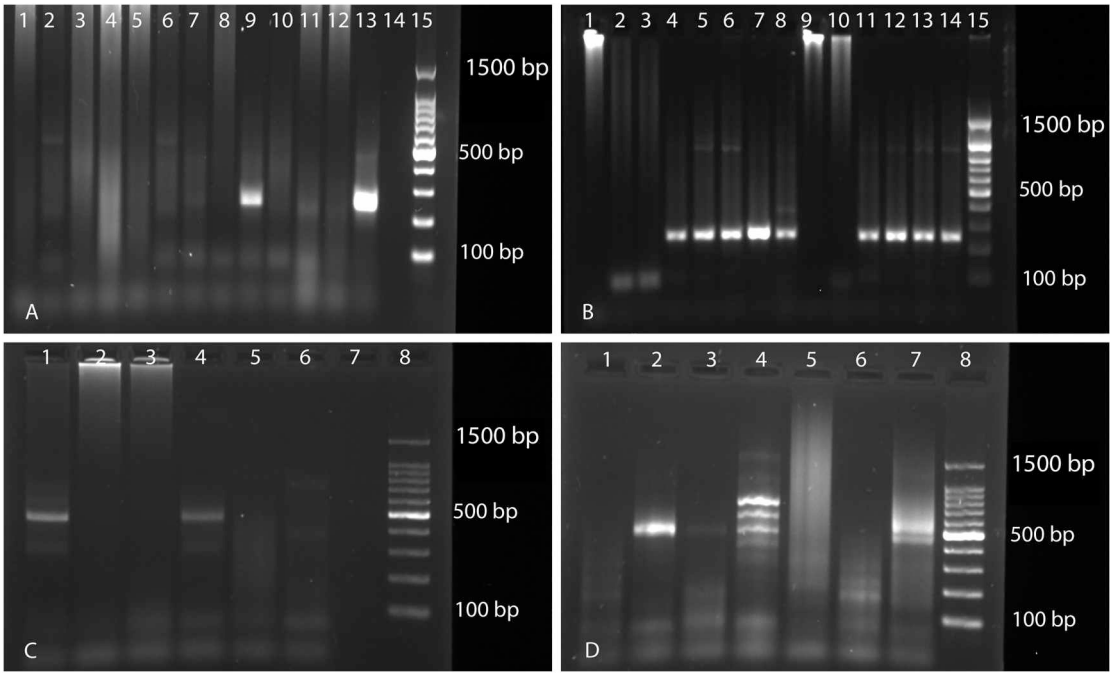

Figure 1. Agarose gel (2%) electrophoresis of SYBR Green real-time PCR products (A–B) and conventional PCR products (C-D). (A). Specific HV amplified products (~250 bp) are detected in lane 9 (SA870/21 brain) and lane 13 (PCR positive control). Lane 15 contains a 100-bp DNA ladder. (B). Expected ~250 bp amplicons of the HV DNA polymerase gene are observed in four and three replicates of sample SA1277/22 lung (Lanes 4, 5, 11, and 13) and kidney (lanes 6, 12, and 14), respectively, as well as in the extraction positive control (lane 7) and PCR positive control (lane 8). Lane 15 contains a 100-bp DNA ladder. (C). A target-specific band of ~500 bp is detected in lane 1 (SA870/21 oral cavity) and lane 4 (PCR positive control). Lane 8 contains a 100 bp DNA ladder. (D). Specific HV amplicons (~500 bp) are observed in lane 2 (SA870/21 oral cavity), lane 4 (SA12277/22 Pool), and lane 7 (PCR positive control). Lane 8 contains a 100 bp DNA ladder.

= 2). Some birds tested positive in multiple tissue samples. The HV-positive samples were analysed based on their Ct values, with samples amplified beyond a Ct value of 35 not considered positive. PCR amplicons resulting from the semi-nested sq-PCR were analysed on a 2% agarose gel, revealing a band of the expected size (approximately 250 bp) (Figure 1(A–B)). Sequencing was performed using the product of the second internal PCR from the semi-nested PCR with primers TGV and IYG, resulting in an identical 163 bp consensus fragment after primer removal, indicating a consistent viral strain. Only two of the positive samples generated the longest PCR amplicons using the TGV and KG1 primer combination, producing a band of approximately 500 bp on the agarose gel (Figure 1(C–D)). Sequencing of these amplicons yielded an identical 385-bp consensus sequence (excluding primers), which encompassed the 163 bp fragment obtained with the primers TGV and IYG, further validating the results. This sequence has been deposited in GenBank under the accession number PQ332998. A summary of this information is provided in Table 2.

Histopathological findings

Histopathological analysis of the HV-positive birds revealed various lesions unrelated to HV infection. These findings included multiorgan congestion and oedema, as well as varying degrees of multiorgan chronic microscopic lesions associated with or without a wasting body condition. Additionally, suspected unidentified bacterial infections were frequently observed, manifesting as pododermatitis (Figure 2(A)), osteomyelitis, liver abscesses (Figure 2(B)), and enteritis, along with parasitic infestations (Figure 2(C)) and unspecified traumatic lesions (Figure 2(C)). In one case, emaciation and myofibre necrosis of unknown aetiology were the only pathological findings (Figure 2(D)). Notably, no histological evidence of HV-associated cytopathic effects, such as nuclear

Table 2. Characteristics of the HV-positive PCR samples.

Bird sample	PCR type	Ct	Sequence size (bp)
SA870/21 B	Semi-nested sq-PCR	23.75	163
SA870/21 O.C.	Semi-nested sq-PCR	2.47	163
SA870/21 I	Semi-nested sq-PCR	33.02	163
SA1349/21 C.C.	Semi-nested sq-PCR	25.50	163
SA1277/22 POOL	Semi-nested sq-PCR	25.49	163
SA1277/22 I	Semi-nested sq-PCR	21.46	NA
SA1277/22 K	Semi-nested sq-PCR	18.67	163
SA1508/21 C.C.	Semi-nested sq-PCR	3.23	163
SA870/21 O.C	Conventional semi-nested PCR	NA	385
SA1277/22 POOL	Conventional semi-nested PCR	NA	385

Notes: Bird samples (B = brain; O.C. = oropharyngeal cavity; L = lung; K = kidney; C.C: = coelomic cavity; POOL = liver, kidney, lung). Ct = cycle threshold; NA = not applicable, not available.

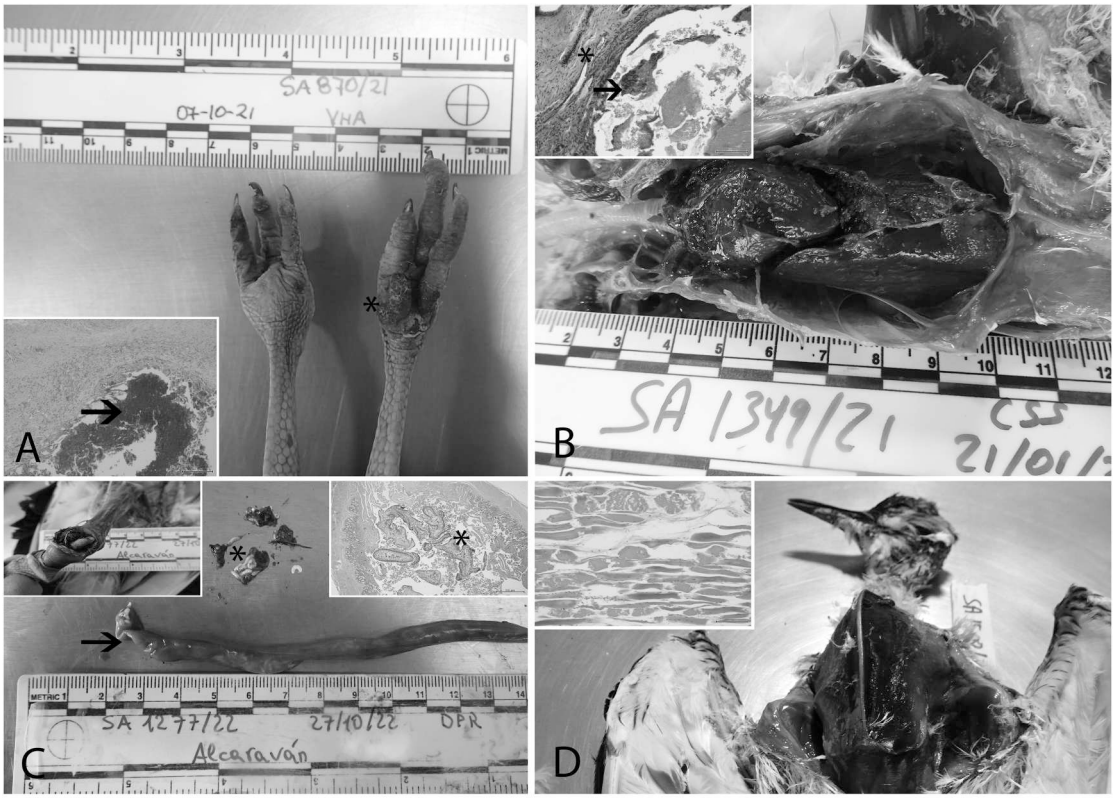

Figure 2. Gross and microscopic lesions in the HV-positive PCR stone curlews. (A). SA870/21. Pododermatitis. The right hindlimb at the feet shows an elevated, flushed, and swollen circular tissue measuring 5 × 6 mm (asterisk). Inset: the dermis is markedly thickened diffusely due to abundant connective tissue (fibrosis) and is focally infiltrated by numerous degenerate heterophils and macrophages (extensive focal chronic pyogranulomas) (arrow). H&E, bar = 100 µm. (B). SA1349/21. No gross lesions were observed in the liver during the macroscopic examination. Inset: large heterophilic granuloma with central necrosis and multinucleated giant cells (arrows), expanding the adjacent hepatic parenchyma (asterisk). H&E, bar = 100 µm. (C). SA1277/22. Large helminths (asterisk) (0.5–1 cm in length) were found throughout the entire intestine (arrow). Upper left inset: oblique open fracture in the diaphysis of the left tibiotarsus (distal third). Upper right inset: intestine. Abundant intraluminal presence of cestodes (asterisk). H&E, bar = 100 µm. (D). SA1508/22. Bilateral atrophy of the pectoral muscles. Inset: moderate multifocal segmental myofibre necrosis. H&E, bar = 100 µm.

inclusion bodies typically linked to HV infection, was observed in these PCR-positive animals (Table 3).

Phylogenetic analysis

The commonly obtained nucleotide sequences of the partial DNA polymerase UL30 gene from the seven HV-positive samples were analysed to assess their phylogenetic relationships. Phylogenetic comparison of these sequences, derived from wild birds on the Canary Islands, with other avian, reptilian and mammalian alpha- and gamma-HVs, revealed significant phylogenetic diversity among the alpha-HVs (Figure 3). The nt sequences obtained in this study (specifically the longer sequences) clustered within the alpha-HV group, demonstrating nt identity ranging from 67.28% with 98% query coverage to 75.00% with 53% query coverage.

Furthermore, a comparative analysis of the derived partial protein sequence was conducted against DNA

Table 3. Summary of HV PCR-positive birds categorized by age, sex, histopathologic findings unrelated to HV, and aetiological diagnoses proposed as potential causes of death.

ID	Age	Sex	HV-positive samples	Body condition	HV-nonrelated histopathologic findings aetiological diagnosis
SA870/21	J	M	Brain, lung, oropharyngeal swab	Cachectic	Moderate acute multifocal pulmonary oedema and moderate multifocal congestion. Malnutrition. Pododermatitis and infectious osteomyelitis.
SA1349/21	A	U	Coelomic swab	Good	Moderate multifocal pulmonary oedema. Malnutrition. Moderate-severe multifocal, coalescent liver abscesses.
SA1277/22	U	M	Lung, kidney	Thin	Marked multifocal emphysema and moderate multifocal pulmonary oedema and congestion of large vessels. Parasitic enteritis. Malnutrition. Trauma.
SA1508/22	A	M	Oropharyngeal swab	Cachectic	Severe diffuse congestive oedema. Abundant presence of coccoid bacteria. Associated with oedema and haemorrhage. Intravascular coagulation. Possible diphtheroid enteritis. Malnutrition. Compatible with infectious process.

Notes: Sex (F = female, M = male, U = undetermined), Age (A = adult, J = juvenile, u = Undetermined).

polymerase UL30 gene protein sequences from avian, reptilian and mammalian species, revealing substantial phylogenetic diversity. This analysis identified distinct clustering patterns, showcasing variations in the position of sequences from our study within the phylogenetic tree (Figure 4). The consensus herpesvirus protein sequence analysed in this study clustered within the alpha-HV group, exhibiting amino acid (aa) identity ranging from 62.50% with 81% query coverage to 74.02% with 99% query coverage.

Herpesvirus RNA detection to assess active replication

GAPDH RNA was not detected in any of the seven HV-positive samples that contained herpesvirus

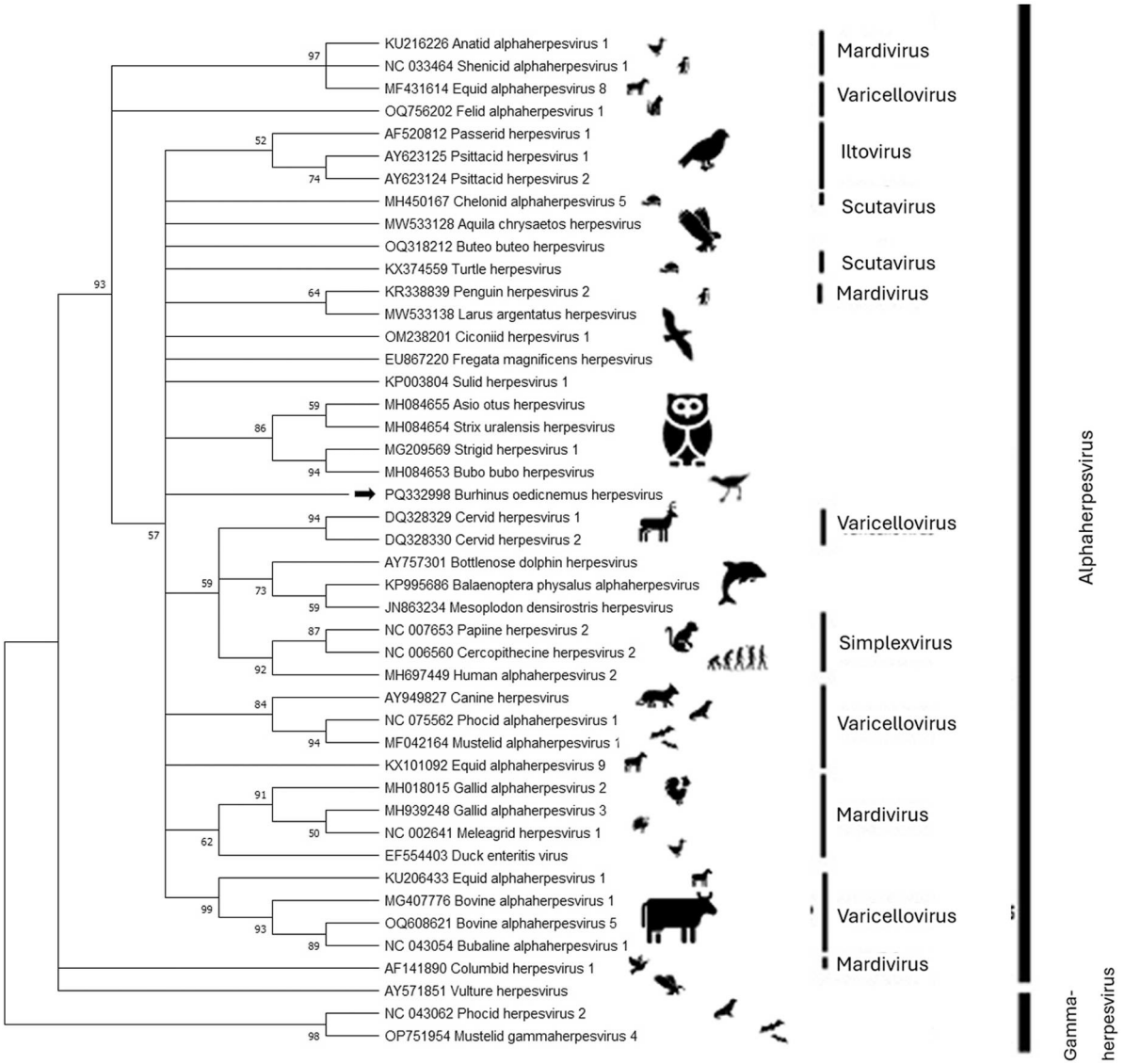

Figure 3. Phylogenetic analysis of the novel *Burhinus oedicnemus* HV (GenBank accession number: PQ332998; 385 bp: GGCAACGGTCTGCTGCCATGCCTGCCCGTGGCGGCTACGGTGACCACAGTAGGCAGAGACATGCTTTTGGCAACTAGGGATTACATA-CACTCGCGGTGGGCCACGATAGAAATGCTGAAGGCCGCATTTGGAGACTCGGCGGAAAACGCTGGGGACGGGAGTTCGTACGAGGT-GAACGTTATTTACGGAGACACGGATTCGGTGTTCTGTAGGTTCAGGGGCGTAGCGACCGAGTGGCTAGTGAAAATGGGGGACGCCATGGC-GAAACGCATCACCGGAGACCTTTTCAGGGCACCGATCAAGTTGGAGTGCGAGAAGACGTTCACAAAGCTGCTGCTGATAACTAAGAAAAAG-TATATAGGAACCATCTGCGGGGGGAAGATGC), obtained from sample SA870/21, based on partial sequences of the DNA polymerase gene from different host species, with sequencing coverage ranging from 98% to 53%. The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible model (Nei & Kumar, 2000). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates are shown next to the branches (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories (+*G*, parameter = 2.7892)). This analysis involved 50 nucleotide sequences. There was a total of 554 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura, Stecher, & Kumar, 2021).

Figure 4. Phylogenetic analysis of the novel *Burhinus oedicnemus* HV (GenBank accession number: XHS94912; 128aa: GNGLLPCLPVAATVTTVGRDMLLATRDYIHSRWATIEMLKAAFGDSAENAGDGSSYEVNVIYGDTDSVFCRFRGVATEWLVKMGDAMAK-RITGDLFRAPIKLECEKTFTKLLLITKKKYIGTICGGKM), obtained from sample SA870/21, based on partial sequences of the protein polymerase gene from different host species, with sequencing coverage ranging from 81% to 99%. The evolutionary history was inferred by using the Maximum Likelihood method and Le_Gascuel_2008 model (Le & Gascuel, 2008). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates are shown next to the branches (*Felsenstein J. (1985). Confidence Limits on Phylogenies: An Approach Using the Bootstrap. Evolution 39:783-791.*, n.d.). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories (+*G*, parameter = 1.1679)). This analysis involved 46 amino acid sequences. There was a total of 141 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura, Stecher, & Kumar, 2021).

DNA. Moreover, both herpesvirus RNA and DNA were undetectable following the RNA extraction process.

Discussion

In this study, we report the detection of a novel α-HV in stone-curlew samples, with an observed prevalence rate of 8%, as four out of 50 birds tested positive. Previous research on HV prevalence in wild birds has yielded varying results across different species. Studies focusing on single species, like the present one, have reported HV prevalence rates of 9.41% (16/170) and 14.5% (8/55) in owls (Žlabravec *et al.*, 2018; Žlabravec, Vrezec, *et al.*, 2021). Research on species within the same order as stone-curlews has documented prevalence ranging from 3.8% (4/104) to 5.6% (14/250) in seabirds (Niemeyer *et al.*, 2017; Verdugo *et al.*, 2019) and 0.8% (4/525) in passerines (Žlabravec, Trilar, *et al.*, 2021). Additionally, studies spanning multiple avian orders have reported HV prevalence of 7.6% (34/447) (Žlabravec, Slavec, Vrezec, Kuhar, & Rojs, 2022) and up to 20.4% (18/88) in wild birds in Poland (Woźniakowski *et al.*, 2013). The lowest prevalence, 0.7%, was recorded in Australia in a study of various genera of wild birds (Amery-Gale *et al.*, 2018). The variability in HV prevalence observed across different species may stem from several factors, including the origin of the samples analysed, and the use of different PCR assay methods (Woźniakowski *et al.*, 2013). Additionally, HV detection in these studies generally results from

opportunistic screening for multiple viruses as part of health surveillance in wild birds.

In terms of organ-specific incidence, HV DNA was detected molecularly in stone-curlew samples from brain, lung, kidney, oropharyngeal, and coelomic cavities. Although the low number of positive cases in our study limits definitive conclusions, larger studies targeting single species, such as that on owls (Žlabravec, Vrezec, *et al.*, 2021), have identified the virus exclusively in oropharyngeal swabs. In studies encompassing a broader range of species and sampling methods, no consistent pattern has emerged, suggesting a higher incidence in specific organs (Niemeyer *et al.*, 2017; Amery-Gale *et al.*, 2018; Woźniakowski *et al.*, 2013; Žlabravec, Trilar, *et al.*, 2021).

Histopathological examination of HV-positive tissues from stone-curlews in our study did not reveal general characteristic HV-associated lesions, such as necrosis, intranuclear inclusion bodies, or inflammation (Arbelo *et al.*, 2010; Suárez-Santana *et al.*, 2022). This finding aligns with molecular phylogenetic studies and the generally mild pathogenicity of HV in their natural hosts (Davison, 2002), supporting the hypothesis that *Burhinus oedicnemus* may act as a natural reservoir for this novel HV. While the absence of HV-associated lesions may suggest limited viral replication (Dake & Squire, 1993), RNA extraction in our study was unsuccessful; thus, although HV DNA was detected, no evidence of active viral replication was present at the time of analysis. The absence of detectable RNA likely reflects degradation prior to extraction, potentially due to factors such as inadequate sample handling, extended storage, or suboptimal extraction conditions (Fleige & Pfaffl, 2006). This RNA degradation emphasizes the need for stringent sample preservation and handling protocols to ensure the integrity of nucleic acids in future studies.

The identification of a novel HV in stone-curlews, for which the authors propose the formal species designation *Burhinus oedicnemus herpesvirus* (GenBank accession number: PQ332998), represents the first global report of HV in this species, to the authors' knowledge. This finding offers valuable insights into the genetic diversity and evolutionary dynamics of HVs within the order *Charadriiformes*. While a HV strain from the same order, specifically from *Larus argentatus* (MW533138.1) (Žlabravec, Slavec, Vrezec, Kuhar, Zorman Rojs, *et al.*, 2022), has been previously reported, comparative sequence analysis using the NCBI BLAST tool revealed less than 85% nt identity between the two sequences. This significant sequence divergence suggests that the novel strain identified in stone-curlews represents a distinct HV, emphasizing the high degree of genetic variability that can exist among HVs infecting avian hosts, possibly shaped by ecological, geographical, and host-specific factors (McGeoch *et al.*, 2000). The geographic isolation of the Canary Islands, coupled with the migratory behaviour of stone-curlews, may have contributed to the unique evolutionary trajectory of this HV (Madroño *et al.*, 2004; Altizer *et al.*, 2011).

Of the four HV-positive stone-curlews, three were from Gran Canaria and one from Lanzarote. Notably, all obtained sequences were identical, and no previous records of them exist in public databases, underscoring the novelty of the virus. The geographic distribution of positive birds across two islands of the Canarian archipelago raises interesting questions about the ecology and transmission dynamics of this novel HV. The identical nature of the sequences obtained from individuals on different islands could point to recent or ongoing transmission events, suggesting that this HV may be circulating within stone-curlew populations in the Canary Islands. Alternatively, the uniformity of the sequences could point to a shared evolutionary origin, suggesting that the novel HV may have descended from a common ancestral strain and is now widely distributed among different subspecies of stone-curlews. This scenario is consistent with the idea of long-term host-virus coevolution, where the virus persists across geographically separated populations while maintaining genetic similarity (McGeoch *et al.*, 2000; Davison, 2002). Further genetic and ecological studies are necessary to determine whether the virus is actively spreading or reflects a long-standing association with its host species.

Herpesviruses represent a remarkable evolutionary success, exhibiting a close association with specific host species (Davison, 2002). In nature, each HV is typically linked to a single host, with some animal species also infected by several distinct HVs (Esperón *et al.*, 2008; Davison *et al.*, 2009; Bellière *et al.*, 2010; Žlabravec, Slavec, Vrezec, Kuhar, & Rojs, 2022). Herpesvirus infections in their natural hosts are generally mild and lead to latent infection. However, cross-species transmission can trigger severe and often fatal outcomes, as observed in avian populations (Kaleta & Docherty, 2008; Woźniakowski & Samorek-Salamonowicz, 2015). For example, *Columbid herpesvirus* 1 (CoHV-1) has been transmitted to other avian species, primarily through direct contact or the consumption of infected pigeon meat by birds of prey (Gailbreath & Oaks, 2008). Although the absence of closely related sequences in public databases indicates that this novel HV may have evolved independently in stone-curlews, without significant cross-species transmission, the potential for HV to affect predators of stone-curlews or other bird species remains an open question. Further studies are essential to more comprehensively characterize this novel herpesvirus detected in stone curlews from the Canary Islands. These studies should include the sequencing

of additional viral genes or, ideally, full genomic sequencing to gain a deeper understanding of the virus structure, and genetic diversity, and its potential for host adaptation. Moreover, additional research is necessary to determine the extent and impact of HV transmission across different avian species. In light of this, conducting screenings for this AHV in other avian species could significantly enhance our understanding of the ecological dynamics surrounding the stone-curlew and its interactions within the broader avian community (Madroño *et al*., 2004). Such an approach may also yield valuable insights into the potential impacts of HV transmission on wildlife health, contributing to more informed conservation strategies. Identifying transmission pathways and understanding the role of HVs in avian populations could be crucial for assessing potential risks to both predator species and overall biodiversity.

Disclosure statement

No potential conflict of interest was reported by the authors.

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4.2. ARTÍCULO 2: **Pathological and Molecular Characterization of *Avipoxvirus* Infection in *Burhinus oedicnemus* in the Canary Islands**

veterinary sciences

MDPI

Article

Pathological and Molecular Characterization of *Avipoxvirus* Infection in *Burhinus oedicnemus* in the Canary Islands

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Simple Summary

Wild birds are vulnerable to diseases that can affect their health and survival. In this study, we examined eight Stone-curlews from the Canary Islands with signs of avian pox, a viral skin disease. Some birds had mild lesions and recovered with treatment, while others developed severe foot injuries that could hinder movement, feeding, and predator avoidance. Genetic analysis revealed different viral strains, showing high diversity within this host species. In addition to the virus, we detected skin fungal co-infections that may worsen the disease. Moreover, one bird presented an unusual tumor-like lesion, which has previously been described in association with avian pox in other species, expanding the range of known disease manifestations. These findings improve our understanding of health threats in wild birds and stress the need to consider multiple infections in wildlife disease research.

Abstract

Avian poxvirus was diagnosed in eight wild Stone-curlews (*Burhinus oedicnemus*) from the Canary Islands, based on a combination of pathological and molecular analysis. Affected birds exhibited lesions consistent with poxvirus infection; three of four with mild lesions ($\leq$2 on pelvic limbs, excluding phalanges) were successfully rehabilitated and released, while four with moderate ($\leq$2 on phalanges) to severe lesions ($\geq$3 on phalanges) potentially faced impaired mobility, increased predation risk, and reduced foraging efficiency. Histopathology of six individuals revealed Bollinger bodies, characteristic of *Avipoxvirus* infection, and molecular analysis confirmed the presence of viral DNA in six cases. Three genetically distinct viral variants were identified, each associated with different phylogenetic clades and subclades, suggesting substantial viral diversity within this host species. Co-infection with *Aspergillus fumigatus* was also detected in six of the eight birds, confirmed by molecular analysis in either skin lesions or lung tissue. To our knowledge, this represents the first report of *A. fumigatus* co-infection in Stone-curlews with *Avipoxvirus*. Additionally, one individual presented a tumor-like lesion, expanding the known pathological manifestations of the disease. These findings provide new insights into avian pox and highlight the importance of considering fungal co-infections in the differential diagnosis, given their potential to exacerbate disease severity.

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Keywords: avian pox; Stone-curlew; *Aspergillus fumigatus*; poxvirus; squamous cell carcinoma

1. Introduction

Avian pox is a globally distributed infectious disease affecting birds caused by viruses of the genus *Avipoxvirus* (family *Poxviridae*) [1]. The International Committee on Taxonomy of Viruses (ICTV) currently recognizes 12 species within this genus, with two additional species recently proposed based on phylogenetic divergence and host range patterns [2]. Phylogenetic analyses of selected genomic regions have grouped *Avipoxvirus* strains into at least three major clades: fowlpox-like, canarypox-like, and psittacinepox-like [2–4]. However, despite this clade-level structure, species boundaries remain unresolved. A recent molecular survey identified 152 unique viral sequences, highlighting substantial genetic diversity within the genus [5], which has traditionally been characterized using a conserved 578 bp fragment of the 4b core protein gene [6], although broader genomic approaches are now increasingly applied.

Avian poxviruses infect a wide range of both domestic and wild avian species, with infections documented in more than 374 bird species spanning 23 avian orders, highlighting their broad host range [5]. However, while many viral lineages appear capable of infecting a broad range of host species across diverse avian orders, the extent of host specificity remains debated. Some strains show evidence of generalist behavior, potentially driven by anthropogenic factors, whereas others may display a more restricted, taxon-specific host range [3,7].

Clinically, avian pox manifests in three primary forms: cutaneous, diphtheritic, or mixed, with the cutaneous form being most prevalent [8]. This form typically involves nodular lesions on the comb, wattles, eyelids, and legs [9]. Disease severity varies depending on host susceptibility, viral strain virulence, location and number of injuries, and the presence of secondary infections or environmental stressors [9,10]. Although avian pox is typically self-limiting and associated with low mortality rates [11,12], severe outcomes can occur, particularly when secondary bacterial or fungal infections develop [13–16]. Such co-infections can exacerbate disease progression and may result in systemic illness [13,17].

The impact of Avipoxviruses on island bird populations is particularly concerning, with the virus implicated in population declines and potential local extinctions [18,19]. The Stone-curlew (*Burhinus oedicnemus*) is a ground-nesting bird of conservation concern in Europe. Genetic studies have revealed significant differentiation between mainland and island populations, with two endemic subspecies recognized in the Canary Islands: *B. o. insularum* (Fuerteventura and Lanzarote, including La Graciosa and Alegranza), and *B. o. distinctus* (Gran Canaria, Tenerife, La Gomera, El Hierro and La Palma) [20–22]. Despite its ecological and conservation significance, the susceptibility of this species to *Avipoxvirus* infection remains poorly understood [23–25].

This study seeks to explore the occurrence and genetic variability of *Avipoxvirus* in Stone-curlew populations, with special attention to the endemic subspecies of the Canary Islands. Through an integrated approach involving clinical examination, anatomopathological analysis, and molecular techniques, we aim to characterize the viral strains, determine their phylogenetic relationships, and enhance the understanding of avian pox ecology in wild bird species of conservation concern.

2. Materials and Methods

2.1. Animals and Tissue Sampling

This study includes eight Stone-curlews with cutaneous avian pox-like lesions, comprising three live cases and five necropsied individuals, conducted within the framework of the Canarian Wildlife Health Surveillance Network, a monitoring program on causes of mortality in Canarian wildlife established by the Canarian Government (Red Vigía Canarias; Order No. 134/2020, 26 May 2020). Of the eight individuals included in the present study, seven originated from Gran Canaria and one from Lanzarote (Table 1).

Table 1. Case information for Stone-curlews (*Burhinus oedicnemus*) included in this study, summarizing case number, internal identification code, status at discovery in the field, finding location and date, admission to the WRCT (when applicable), rehabilitation history, clinical outcome, and sample origin.

Case Code	ID Ref.	Status		FL	FD	Admission at WRCT		Days at WRCT	Clinical Outcome at WRCT			Sample Origin	
		Dead	Alive			Yes	No		Died	Euthanized	Released	Necropsy	Biopsy
1	SA518/21	X	-	L	06/04/21	-	X	NA	NA	NA	NA	X	-
2	123/23	-	X	GC	08/03/23	X	-	74	-	-	X	-	X
3	237/23	-	X	GC	14/04/23	X	-	45	-	-	X	-	X
4	FS415/23	-	X	GC	04/05/23	X	-	<1 (few hours)	-	X	-	X	-
5	FS81/24	-	X	GC	29/07/23	X	-	11	X	-	-	X	-
6	FS499/24	-	X	GC	03/09/24	X	-	1	X	-	-	X	-
7	FS601/24	-	X	GC	25/06/24	X	-	4	-	X	-	X	-
8	757/24	-	X	GC	18/08/24	X	-	44	-	-	X	-	X
Total	8	1	7	-	-	7	1	-	2	2	3	5	3

Notes: FL (finding location: GC = Gran Canaria; L = Lanzarote); FD (finding date); WRCT = Wildlife Rehabilitation Center of the Cabildo of Gran Canaria; NA = not applicable.

The prospective component included three Stone-curlews admitted to the Wildlife Rehabilitation Center of Tafira (WRCT) (Cabildo of Gran Canaria) between 2023 and 2024 with cutaneous lesions (on the pelvic limbs and/or beak) compatible with *Avipoxvirus* infection. Upon admission, all individuals underwent clinical examination, and when available for each specific case, skin biopsies and/or swabs were collected for further diagnostic investigation. These birds were monitored during the rehabilitation process and, following improvement in clinical symptoms and visible healing of cutaneous lesions, were subsequently released back into the wild (Table 1; Cases 2, 3, and 8).

The retrospective component involved the review of 68 Stone-curlews necropsied between 2021 and 2024 at the University Institute of Animal Health and Food Safety (Instituto Universitario de Sanidad Animal y Seguridad Alimentaria, IUSA), using standardized necropsy reports, routine histopathological evaluations, and macroscopic image analyses. Among these, five birds exhibited macroscopic cutaneous lesions suggestive of avian poxvirus infection, particularly affecting featherless areas of the pelvic limbs (legs and feet). These comprised one bird found dead in the field (Table 1; Case 1) and four admitted to the WRCT for veterinary care and potential release, of which two died naturally and two were humanely euthanized due to poor prognosis (Table 1; Cases 4–7).

Of the 8 Stone-curlews, 2 were identified as female and 3 as male; the sex of the remaining 3 could not be determined due to the condition of the carcasses or, in the case of live animals, the absence of external sexual dimorphism characteristic of this species. Age classification was based on external morphology, skeletal features, and gonadal development, and individuals were assigned as juvenile (n = 2) or adult (n = 4), while the age of 3 birds could not be determined. Nutritional status was assessed through

visual and manual evaluation of the pectoral muscle mass (keel scoring) and the presence of fat reserves and classified on a five-point numerical scale, with 1 indicating cachexia, 2 underweight, 3 normal, 4 overweight, and 5 obese. This system represents a modification of the seven-category body condition scoring method described by Burton et al. (2014) [26]. In our approach, the category *cachexia* (score 1) was introduced to denote individuals with marked pectoral muscle atrophy, thereby distinguishing severe pathological muscle wasting from general underweight status. The categories *thin* and *lean* in Burton et al. were combined into a single *underweight* category (score 2), representing individuals without grossly appreciable muscle atrophy. *Ideal* corresponds to our *normal* (score 3), and *moderately overweight* corresponds to *overweight* (score 4). Finally, *severely overweight* and *morbidly overweight* were merged into a single category (*obese*, score 5), as these two categories did not reflect biologically or clinically meaningful differences in our study population of wild birds; both were characterized by excessive fat deposition that obscured body contours and masked pectoral muscle definition. Body condition assessments revealed that 3 were cachectic (grade 1) and 5 were underweight (grade 2). This case-specific information (including body weight) is summarized in Table 2.

Table 2. Summary data from eight Stone-curlews (*Burhinus oedicnemus*) included in the present study (2021–2024), comprising biological information (age and sex), carcass preservation status at necropsy and decomposition state at necropsy (when applicable), body weight, body condition, and samples analyzed for *Avipoxvirus* by histopathology and DNA detection.

Case Code	MP	DC	W	BC	AGE	SEX	Cutaneous Lesion Tested	Histopathology	Molecular Testing
1	F	2	145.9	2	J	M	NA	Yes	No
2	NA	NA	295	2	U	U	Beak Skin 1 Skin 2	Yes	Yes
3	NA	NA	320	2	A	U	Skin 1 Skin 2 Skin 3	No	Yes
4	R	1	213.9	1	A	F	Skin	Yes	Yes
5	F	3	174.5	2	J	F	NA	Yes	No
6	R	2	275.6	1	A	M	Skin	Yes	Yes
7	F	2	212.8	1	A	M	Skin	Yes	Yes
8	NA	NA	310	2	U	U	Skin	No	Yes

Notes: DC = decomposition code: (1 = very fresh, 2 = fresh, 3 = incipient decomposition); MP = method of carcass preservation (F = freezing; R = refrigeration); W = weight; BC = body condition: (1 = cachexia, 2 = thin (slim); AGE (A = adult, J = juvenile, U = undetermined); SEX (F = female, M = male, U = undetermined); NA = not available or not applicable.

Deceased Stone-curlews were stored under refrigeration (Cases 4 and 6) or frozen (Cases 1, 5, and 7) until necropsy (Table 2). Carcasses were subsequently classified according to their preservation status based on the degree of decomposition (1 = very fresh, 2 = fresh, and 3 = early decomposition), following standardized protocols established at the IUSA (Table 2).

All necropsies were performed in accordance with consistent and standardized procedures [27] and involved the systematic examination and photographic documentation of all organs and any observed lesions. During necropsy, tissue samples were collected from major organs, including the liver, lungs, kidneys, intestines, and any observed skin lesions (Table 2). Additionally, sterile, individually packaged swabs (Vircell S.L., Granada, Spain)—without transport medium—were used to collect samples from various body cavities, including the oropharyngeal cavity, cloaca, and coelomic cavity. Brain samples

were also collected via this method as previously described [28,29]. All collected biological material was archived at the IUSA.

Frozen tissue samples from six individuals were processed for molecular analysis, and histopathological examination was performed on six cases (with partial overlap) from the same cohort of eight individuals (Table 2). All fresh, unfixed tissue and swab samples were stored at −80 °C until molecular virological analyses were conducted. Parallel tissue specimens were fixed in 4% buffered formalin for histopathological processing.

2.2. Gross and Histopathological Examination

During external evaluation, the number and anatomical distribution of visible hyperplastic, pox-like lesions were documented for each affected bird. Formalin-fixed tissue samples from each collected lesion were routinely processed, paraffin-embedded, sectioned at 3 µm, and stained with Hematoxylin and Eosin (H&E) for histopathological examination. The diagnosis of avian pox was based on the presence of characteristic lesions, including large, solid or ring-shaped eosinophilic intracytoplasmic inclusions (Bollinger bodies) within affected epithelial cells [9]. In cases with suspected secondary fungal infections, additional histochemical stain techniques, such as Grocott's Methenamine Silver Nitrate (GMS) and/or Periodic acid Schiff (PAS) staining, were applied to assess the morphological characteristics of potential pathogens.

Lesion severity was evaluated based on anatomical location, lesion count, and the presence of secondary infections [1,12,16]. Given the terrestrial habits of Stone-curlews and the functional importance of their pelvic limbs in locomotion, lesions were classified as mild when limited to the pelvic limbs (excluding the phalanges) and numbering no more than two. Lesions were considered moderate if involving the phalanges but limited to two or fewer, and severe if affecting the phalanges with three or more lesions present.

2.3. Molecular Analysis

Cutaneous lesion samples, obtained through necropsies and biopsies, were processed by mechanical maceration of 0.025 g of tissue in 400 µL of DNA/RNA Shield™ (Zymo Research) using 2 mL ceramic bead Precellys® tubes, followed by centrifugation. Two hundred microliters of each skin lesion macerate were used for simultaneous DNA and RNA extraction employing a magnetic bead-based method on an automated robotic platform, following the manufacturer's instructions for the ZYMO DNA/RNA extraction kit (ZYMO Research, Freiburg, Germany). To ensure the accuracy and reliability of the extraction process, both a negative control (nuclease-free water) and a positive control (a herpesvirus-positive sample previously confirmed in our laboratory) were included in each extraction batch [29].

The presence of the *Avipoxvirus* (AVP)-DNA was assessed in 10 samples corresponding to 9 skin lesions and 1 beak lesion. For the detection of viral DNA, a real-time semiquantitative PCR (sq-PCR) assay targeting the P4b gen, which encodes the conserved structural 4b core protein, was performed, as previously described [30]. The primer pair vAAPV-124f (5′-ACGTCAACTCATGACTGGCAAT-3′) and vAAPV-246r (5′-TCTCATAACTCGAATAAGATCTTGTATCG-3′) was used along with an internal hydrolysis probe vAAPV-159p-(5′-FAM-AGACGCAGACGCTATA-MGB-3′) labelled with a 5′ reporter dye (FAM) and a 3′ quencher (Non-Fluorescent Quencher Minor Grove Binding, NFQMGB). Subsequently, all samples were subjected to a conventional PCR protocol to amplify a longer 578 bp of the same gene, as previously described [31]. The primers used were P1 (5′-CAGCAGGTGCTAAACAACAA-3′) and P2 (5′-CGGTAGCTTAACGCCGAATA-3′), enabling downstream sequence analysis for species identification. PCR products (5 µL) were analyzed by horizontal electrophoresis on a 2% agarose gel containing GelRed® (Bi-

otium, Inc., Fremont, CA, USA). Diethylpyrocarbonate (DPEC)-treated water was used as the negative control for both PCRs, while an AVP-positive sample previously confirmed in our laboratory served as the positive control.

In this study, the definitive molecular status of cutaneous lesion samples for *Avipoxvirus* (positive or negative) was determined by the combined results of all molecular assays performed, including both conventional and real-time PCR techniques. A sample was classified as positive if at least one of the PCR methods yielded a positive result. These molecular findings were further interpreted in conjunction with the histopathological evaluation of the corresponding formalin-fixed paraffin-embedded tissue samples.

In one case involving suspected secondary fungal infection, lung, liver, and kidney tissues were directly pooled into a composite sample using a sterile swab. The swab was then transferred into a tube containing viral transport medium (VTM) (Transport Medium for the Collection and Preservation of Viruses, Chlamydia, and Mycoplasma, Vircell S.L.), which contains HEPES buffer, gelatine, bovine serum albumin, sucrose, and compatible antibiotics to ensure pathogen stability and viability during both transport and storage. The sample was thoroughly mixed, and 100 µL of VTM was combined with an equal volume of DNA/RNA Shield™ (Zymo Research, Freiburg, Germany) prior to nucleic acid extraction, using the same magnetic bead-based automated method described above.

To confirm the presence of fungal infection, specifically *Aspergillus fumigatus*, a sq-PCR assay was performed using the primer pair Af25S-F (5′-GGGTCGAACGGTCAAGT-3′) and Af25S-R (5′-GAGAGTCCATGGAGGTGGAG –3′), which amplifies a 202 bp fragment of the 25S rRNA gene based on the GenBank sequence U15421. The sq-PCR was conducted using SYBR Green chemistry (Bio-Rad), following the manufacturer's recommended protocols.

2.4. Detection, Sequencing of PCR Products, and Phylogenetic Analysis

Conventional PCR amplicons from AVP-DNA-positive samples were purified using the Real Clean spin kit (REAL, Valencia, Spain) and subsequently subjected to bidirectional Sanger sequencing. The resulting nucleotide sequences were analyzed and compared both internally and with publicly available sequences in GenBank through the BLAST 2.15.0 (Basic Local Alignment Search Tool, (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 22 October 2023) algorithm. Multiple sequence alignments of AVP sequences were carried out using ClustalW. The resulting nucleotide sequences were deposited in GenBank (accession numbers: PV976817, PV976818, PV976819, PV976820 and PV976821).

Phylogenetic and molecular evolutionary analyses were performed using MEGA version 12 [32], incorporating 41 AVP nucleotide sequences retrieved from GenBank. Phylogenetic trees were constructed using the Neighbor-Joining (NJ) and BioNJ algorithms based on pairwise genetic distances estimated by the Maximum Composite Likelihood (MCL) method under the Tamura 3 parameter (1985) substitution model Nei & Kumar, 2000. Rate variations among sites were modelled using a discrete Gamma distribution with 5 categories (+G, shape parameter = 0.3839). The analysis included 45 nucleotide sequences, with 1050 aligned positions in the final dataset. The topology robustness was assessed using a bootstrap consensus tree based on 1000 replicates. Branches supported by fewer than 50% of bootstrap replicates were collapsed, and bootstrap values (expressed as percentages) were displayed next to the corresponding branches [33].

3. Results

3.1. Gross and Histopathological Findings

Gross examination of the eight animals from our study revealed multiple raised, wart-like cutaneous vesicles or nodules, varying in color, typically pink, red (Figure 1), or yellow, as observed in Case 1 (Figure 2A). These lesions were predominantly located on the pelvic

limbs, including the area of the tibiotarsus, the tarsometatarsus, and phalanges. Lesion numbers varied among individuals, ranging from two to more than three per animal, and displayed diverse morphologies and surface characteristics, from a small, smooth, yellowish nodule, to multiple ulcerated, coalescing, nodules (Table 3) (Figures 1 and 2A).

Figure 1. Gross cutaneous manifestations of *Avipoxvirus* infection in the Stone-curlews included in this study, presented in increasing severity from mild to severe. (**A**). Case 3. Mild. Small vesicular lesion on the tarsometatarsus (arrow). (**B**). Case 2. Mild. Multiple pink ulcerative and vesicular lesions on the tarsometatarsus, treated with iodine at the WRCT (arrows). (**C**) Case 6. Moderate. One vesicular lesion on the phalanges (arrow). (**D**). Case 5. Severe. Multiple red nodular lesions on the leg tarsometatarsus and phalanges (arrows). (**E**). Case 4. Severe. Multiple red eroded nodules on the tarsometatarsus and phalanges (arrows). (**F**). Case 7. Severe. A large, ulcerated nodule occupying nearly the entire phalangeal surface (arrow).

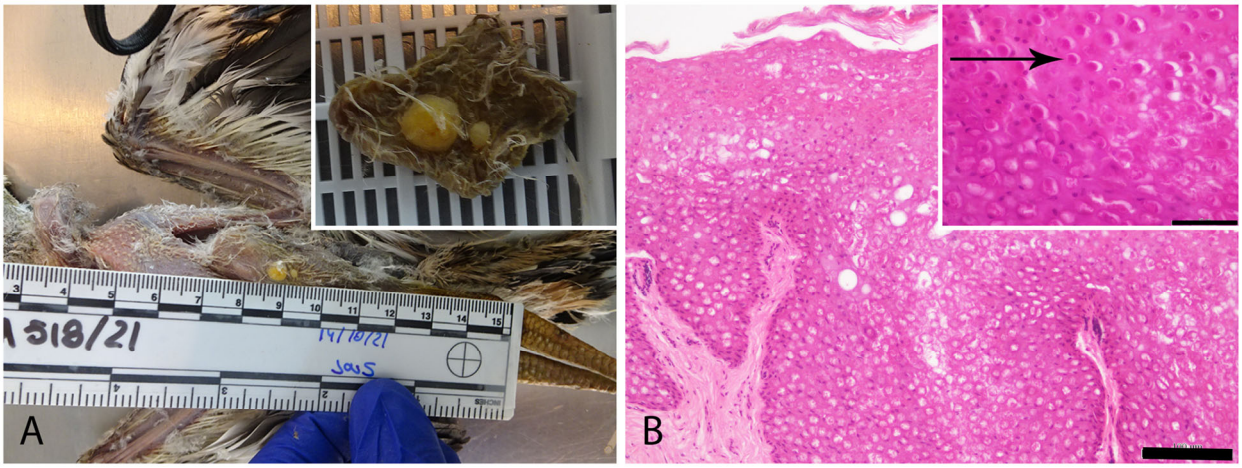

Figure 2. Characteristics of the pox-like lesion in Case 1 (**A**). Gross presentation of the cutaneous yellow form of *Avipoxvirus* infection, characterized by two yellowish contiguous nodular lesions on the skin. (**B**). Histopathological section showing characteristic epidermal hyperplasia, ballooning degeneration of keratinocytes, and the presence of solid intracytoplasmic inclusion bodies indicative of *Avipoxvirus* infection (Bollinger bodies: BB) (Bar = 100 µm). Inset: Higher magnification of the BB (arrow) (Bar = 50 µm).

Table 3. Summary of gross and histopathological characteristics of cutaneous lesions observed in the eight animals included in this study.

Case Code	Gross Findings				Histopathology											
	L	NI	S	TS	BB	HP	HQ	BD	II	N	E	U	H	KP	F	B
1	T	2	Mild	Skin	+	+	-	+	+	+	-	-	-	-	+	-
2	TM	2	Mild	Skin	+	+	+	+	+	+	+	+	+	+	+	+
	Beak	1		Skin	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
3	TM	2	Mild	Skin	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
4	TM, PH	+3	Severe	Skin	+	+	+	+	+	+	+	+	+	+	+	+
5	TM, PH	+3	Severe	Skin	+	+	-	+	+	+	+	+	+	-	-	+
6	PH	2	Moderate	Skin	+	+	+	-	+	+	-	-	-	-	+	-
7	TM, PH	+3	Severe	Skin	+	+	-	+	+	+	+	+	+	-	+	+
8	TM	2	Mild	Skin	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Note: Gross findings [L = Location (T = Tibiotarsus; TM = Tarsometatarsus; PH = Phalanges); NI = Number of lesions; S = Severity of lesions]; Histopathology [(TS = Tissue Sample; BB = Bollinger bodies; HP = hyperplasia of the epithelium; HQ = hyperkeratosis; BD = ballooning degeneration of keratinocytes; II = inflammatory infiltrates; N = necrosis; E = erosion; U = ulceration; H = hemorrhage; KP = keratin pearls; F = Fungus; B = Bacteria); (+ = presence; - = absence; NA = Not available, not applicable)].

Histopathological examination of six of the eight cases from our study showed focal to multifocal epidermal thickening due to keratinocyte hyperplasia, particularly within the stratum spinosum, the presence of characteristic eosinophilic intracytoplasmic inclusion bodies (Bollinger bodies) in the infected keratinocytes (solid or ring-shaped), and variable degrees of epidermal necrosis (Figures 2B and 3). Additional histopathological findings included heterophilic inflammatory infiltrates in five of six cases (83.3%), ballooning degeneration in five of six (83.3%), hemorrhage in four of six (66.7%), erosion in four of six (66.7%), and ulceration in four of six (66.7%) (Figure 3). Keratin pearls were identified exclusively in two cases (Case 2 and Case 4) (Figure 3). Histopathological evaluation could not be performed for Cases 3 and 8 due to the unavailability of biopsy material.

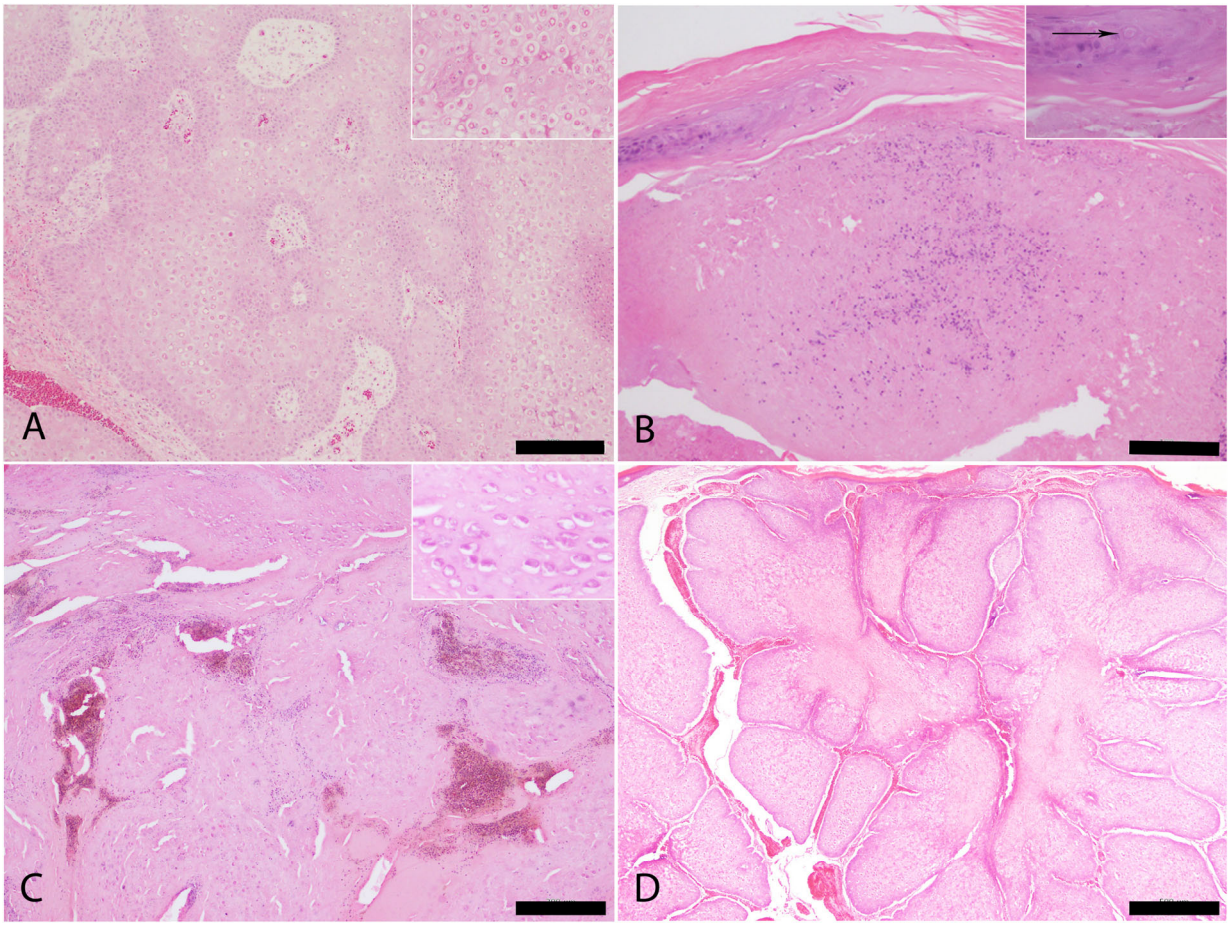

Figure 3. Histopathological findings related to *Avipoxvirus* infection. Hematoxylin and Eosin stain. (**A**). Case 2. Severe epidermal hyperplasia with ring-like eosinophilic intracytoplasmic inclusion bodies (Bollinger bodies) (Bar = 200 µm). Inset: higher magnification of the BB. (**B**). Case 6. Extensive areas of necrosis and heterophilic inflammation, accompanied by mild epidermal hyperplasia and scattered solid eosinophilic intracytoplasmic inclusion body-like inclusions (Bar = 200 µm). Inset: higher magnification of the intracytoplasmic inclusion body-like inclusions (arrow). (**C**). Case 7. Marked epidermal hyperplasia with the presence of solid eosinophilic intracytoplasmic inclusion bodies (BB) in keratinocytes (Bar = 200 µm). Inset: higher magnification of the BB. (**D**). Case 4. Pronounced epidermal hyperplasia featuring characteristic eosinophilic intracytoplasmic inclusion bodies (BB) and central areas of necrosis (Bar = 500 µm).

Tumor-like proliferative lesions associated with cutaneous *Avipoxvirus* infection were identified in Case 7 (Figure 4A). Histologically, adjacent to areas of marked epithelial hyperplasia with Bollinger bodies (Figure 3C), an invasive proliferation was observed. It was composed of irregular infiltrative cords of proliferating keratinocytes and nests of atypical epithelial cells with prominent squamous differentiation. Other histological features included keratinization, intercellular bridges, and nuclear pleomorphism, consistent with a diagnosis of locally invasive squamous cell carcinoma (Figure 4B).

Regarding co-infections, superficial and/or intralesional bacteria were observed in four of the six animals evaluated histologically. Additionally, 3.5 to 4.5 µm, tubular, septate hyphae, with parallel walls, and dichotomous branching, morphologically consistent with *Aspergillus* spp. were identified in cutaneous lesions from five cases (Cases 1, 2, 4, 6, and 7), as well as in the lung tissue of Case 7 (Figure 5) (Table 3).

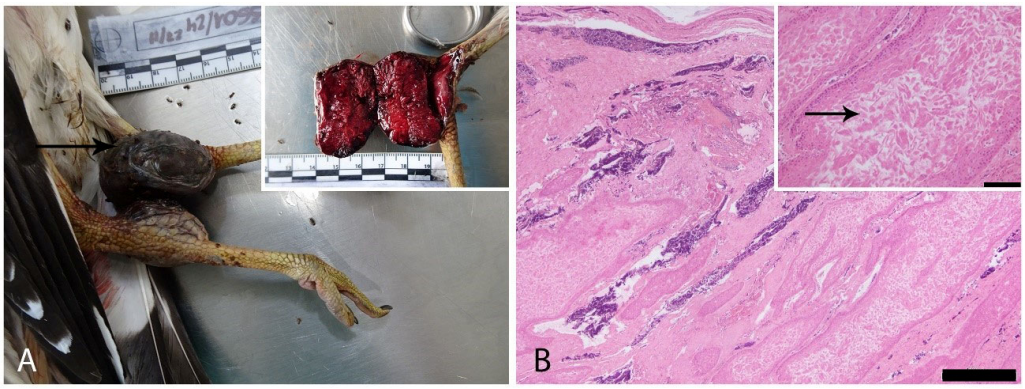

Figure 4. Tumor-like lesions compatible with a squamous cell carcinoma associated to *Avipoxvirus* infection in Case 7. (**A**). Gross finding. Tumor-like lesions identified in Case 7. (**B**). Histopathological changes consistent with irregular infiltrative cords of keratinocytes (Bar = 200 μm). Inset: squamous differentiation of proliferative and invasive keratinocytes (arrow), compatible with a squamous cell carcinoma (Bar = 100 μm).

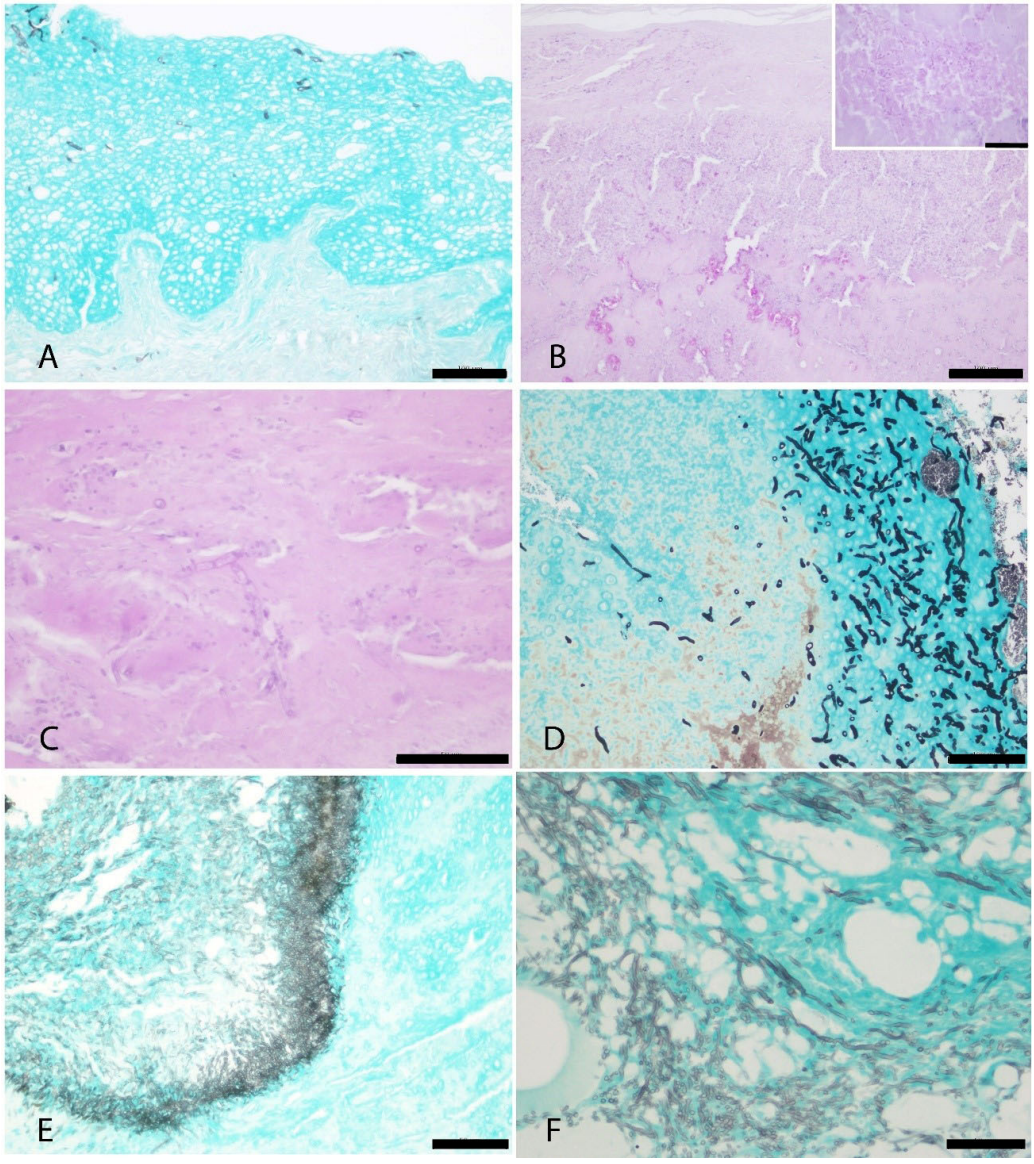

Figure 5. Histopathological findings of secondary fungal co-infections. (**A**). Case 1. Fungal hyphae were observed in the superficial stratum of the epidermis, without associated histopathological alterations. GMS stain (Bar = 100 μm). (**B**). Case 6. Fungal hyphae are present in middle layers of the

hyperplastic epidermis, accompanied by necrosis and inflammation. PAS stain (Bar = 100 µm). Inset: higher magnification of the intralesional hyphae. PAS stain (Bar = 50 µm). (**C**). Case 4. Branched, parallel, dichotomous hyphae, consistent with *Aspergillus fumigatus*, are embedded within a necrotic and inflamed area of the hyperplastic epidermis. PAS stain (Bar = 50 µm). (**D**). Case 2. Infiltrating and invasive fungal hyphae were observed within the proliferative epithelium. GMS stain (Bar = 50 µm). (**E**). Case 7, skin. Short, thin septate fungal hyphae stained black, with angular and dichotomous branching, suggestive of *Aspergillus* sp. (Bar = 50 µm). (**F**). Case 7, lung. Similar fungal hyphae observed, consistent with *Aspergillus* infection, confirmed by PCR as *A. fumigatus* (Bar = 50 µm).

3.2. PCR and Sequence Analyses

Avipoxvirus P4b DNA was detected in skin lesion samples from all six birds analyzed by molecular methods. Of the ten samples tested, eight (exclusively obtained from the pelvic limbs) were positive (Table 4). All positive samples yielded amplification with both conventional and sq-PCR assays, except in two cases (Table 3), in which amplification was detected using only one of the two assays. The AVP-DNA positive samples were further evaluated based on their cycle threshold (Ct) values, with amplification beyond a Ct of 35 considered negative by the sq-PCR criteria. In Case 6, only the sq-PCR assay produced a positive result, while the conventional PCR failed to generate an amplicon of the expected size. In contrast, in Case 7, no amplification was observed by sq-PCR; however, a distinct band of 578 bp, corresponding to the expected product of the conventional PCR, was visualized on the 2% agarose gel (Figure 6) (Table 4).

Table 4. Results of the PCR methods for the detection of AVP-DNA and *Aspergillus fumigatus*.

Case Code	Source for DNA Extraction	Sq-RT-PCR (123 bp)	Ct	Conventional PCR (587 bp)	Sq	Sq-RT-PCR *Aspergillus fumigatus* Results/Ct
1	Skin	NA	NA	NA	NA	NA
	Beak	-	NA	-	NA	-
2	Skin 1	+	30.03	+	Yes	-
	Skin 2	+	20.90	+	Yes	- 30.80
3	Skin 1	-	NA	-	NA	-
	Skin 2	+	28.25	+	Yes	+ 31.13
	Skin 3	+	24.98	+	Yes	+ 31.73
4	Skin	+	30.32	+	Yes	+ 36.44
5	NA	NA	NA	NA	NA	NA
6	Skin	+	29.53	-	NA	+ 32.06
7	Skin	-	NA	+	Yes	-
	Pool *	NA	NA	NA	NA	+ 33.28
8	Skin	+	23.48	+	Yes	-

Notes: Ct = cycle threshold; NA = not applicable, not available; Sq = sequencing.

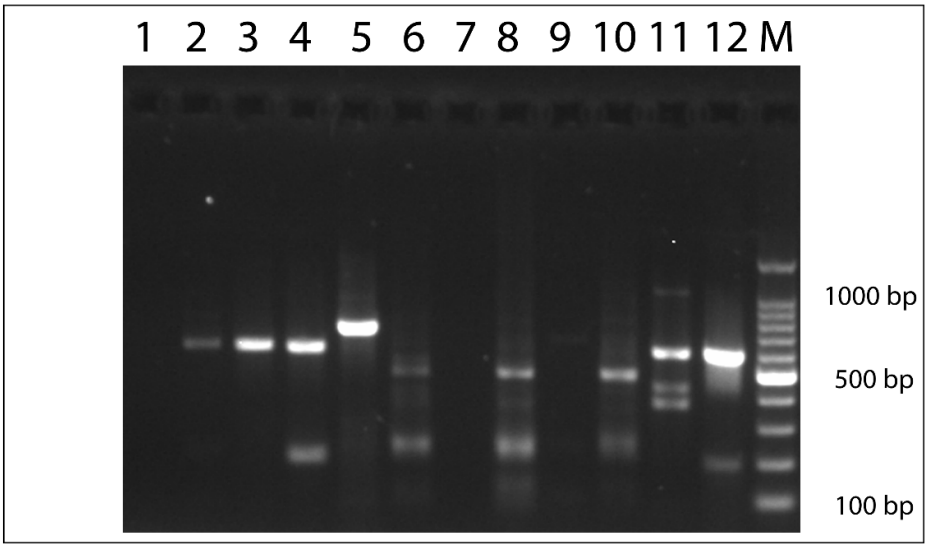

Figure 6. Agarose gel (2%) electrophoresis of conventional PCR products. (A). Specific AVP-DNA amplified products (~578 bp) are detected in lane 2 (Case 2), lane 3 (Case 3), lane 4 (Case 7), lane 11 (Case 8), and lane 12 (Case 4 and our PCR positive control). Last lane (M) contains a 100 bp DNA ladder.

Fresh frozen tissue samples were not available for Case 1 and Case 5, precluding confirmation of viral presence through PCR-based methods. Amplicons obtained from conventional PCR (Cases 2, 3, 4, 7, and 8) and the sq-PCR product from Case 6 were successfully sequenced. BLAST analysis of the conventional PCR amplicons revealed varying degrees of homology between the P4b gene sequences from each bird and previously described AVP sequences. These sequences, with 100% coverage, showed identities ranging from 100% to 97.05%, depending on the AVP species.

DNA of *Aspergillus fumigatus* was detected in five of the eight birds analyzed, supporting the histopathological findings. An exception was noted in the skin sample from Case 7, which showed fungal elements upon microscopic examination (Figure 5) but yielded a negative PCR result. However, the corresponding pooled sample, which included lung tissue, tested positive.

3.3. Phylogenetic Analysis

The P4b gene nucleotide sequences obtained from six AVP-positive animals were analyzed to evaluate their phylogenetic relationships. A fragment of approximately 578 bp from each P4b sequence was used to construct a phylogenetic tree, incorporating 41 reference AVP sequences representing the three major clades. Phylogenetic inference, performed using the T92 + G substitution model (Figure 7), placed the newly generated sequences within the established AVP clades, as previously described by Gyuranecz et al. (2013), Jarmin et al. (2006), and McInnes et al. (2023) [2,3,7]. The tree showed strong bootstrap support for the branching pattern. The AVP sequences from this study were distributed among distinct clades: three cases grouped within clade A2 (100%), clustering with two previously identified AVP sequences from Stone-curlews (HM627224 [34], KU551306 [24]); Case 4 was assigned to clade B2 (87%); and Case 5 was grouped within clade B1 (84%), together with another AVP-DNA sequence previously reported in a Stone-curlew (AY530310 [6]) (Figure 7).

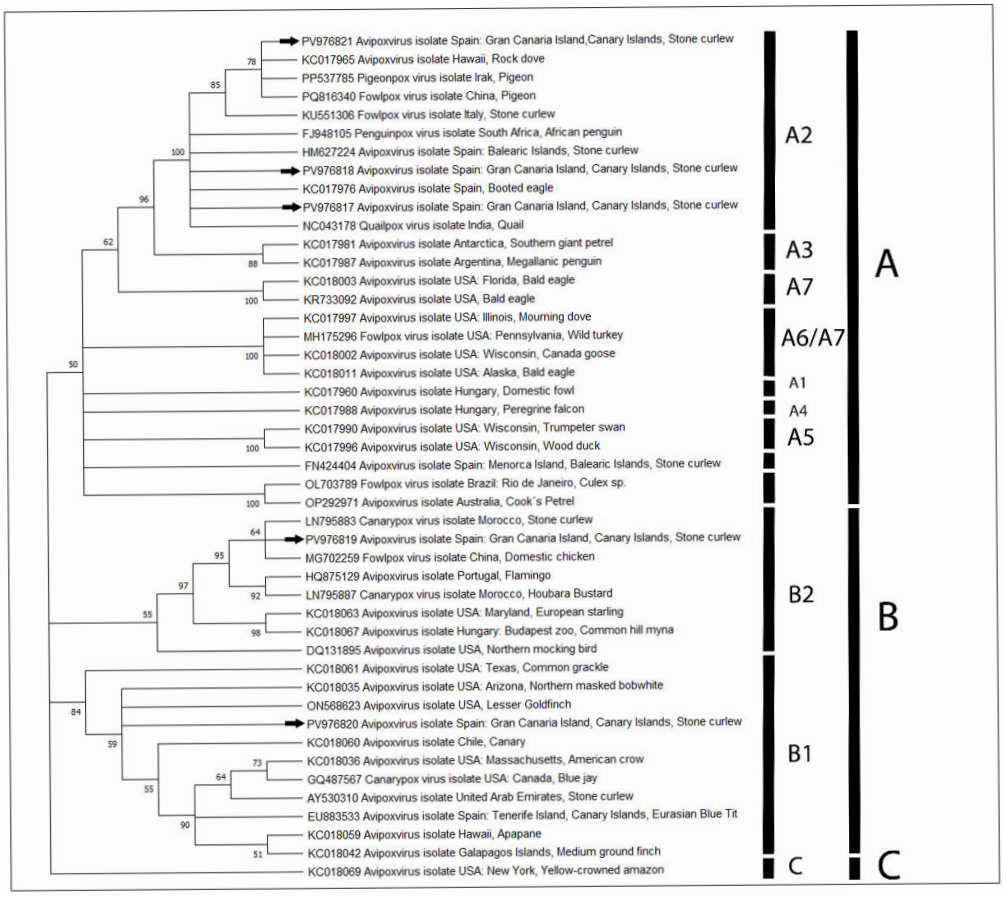

Figure 7. Phylogenetic analysis of cases 2, 3, 4, 7, and 8 AVP-positive (arrow) (GenBank accession numbers: PV976817, PV976818, PV976819, PV976820, and PV976821), obtained from skin samples, based on partial sequences of the P4b gene from different host species. Clades A, B, and C (and their subclades, see Gyuranecz et al., 2013 [7]) are labelled. The evolutionary history was inferred by using the Maximum Likelihood method [33]. The bootstrap consensus tree inferred from 1000 replicates [35] is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates are shown next to the branches [35]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining [36] and BioNJ [37] algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) [33] approach and then selecting the topology with superior log likelihood value. A discrete Gamma distribution across 5 categories (+G, parameter = 0.3839). The analytical procedure encompassed 46 nucleotide sequences with 1.050 positions in the final dataset. Evolutionary analyses were conducted in MEGA12 [32].

4. Discussion

The Stone-curlew is a migratory species of conservation concern, included in Annex I of the EU Birds Directive. *Avipoxvirus* infections have been reported in both wild and captive populations of this species across various regions, with a notably high prevalence in island environments like the Canary Islands, Balearic Islands, and Sardinia. Consistent with our findings, these infections frequently manifest as cutaneous lesions on the legs [6,23–25,34].

Avian pox was diagnosed in 8 out of 71 (11.3%) Stone-curlews examined, which included 68 deceased individuals and three that were rehabilitated and subsequently released into the wild following a period of recovery at a wildlife rehabilitation center (WRCT). Of the eight individuals exhibiting gross lesions suggestive of poxvirus infection, six underwent histopathological examination, all of which showed features consistent with *Avipoxvirus* infection. Molecular confirmation (detection of AVP-DNA) was achieved in

four of these six cases. Additionally, AVP-DNA was detected in two lesion samples from individuals that were released and therefore not subjected to histopathological evaluation, as biopsies were only collected for molecular confirmation of the infection.

Our study represents the first systematic investigation of avian pox in wild Stone-curlews, integrating gross, histopathological, and molecular analyses from a collection of affected individuals. Prior reports of *Avipoxvirus* infection in this species have been largely limited to anecdotal or isolated cases. For instance, in the Canary Islands, five wild Stone-curlews admitted to a wildlife rehabilitation center (WRCT) showed gross lesions, with electron microscopy confirming the diagnosis in two cases but lacking molecular analysis [25]. Other documented cases include a wild Stone-curlew from Sardinia with both pathological and molecular confirmation of APV infection by PCR [24], and molecular detection from two specimens collected in the Balearic Islands in 1980 [34] and in Morocco in 2013 [38], respectively, although pathological descriptions were not provided for these latter cases. Clinical descriptions, together with pathological and molecular analysis, have also been reported in captive, purchased individuals, which likely became infected via cross-contamination with a wild passerine bird introduced into the same farm [23]. In contrast, the Stone-curlews in our study became infected in their natural habitat, representing the first comprehensive characterization of naturally occurring avian pox in a cohort of wild individuals.

Macroscopic and microscopic examination of eight *Avipoxvirus*-infected Stone-curlews in this study revealed no evidence of systemic involvement, supporting their classification as localized, non-generalized cases of the dry cutaneous form of avian pox. Lesions predominantly affected featherless areas of the pelvic limbs (legs and toes) and were either proliferative and/or erosive-ulcerative. However, in one case, two lesions were also observed on a feathered region of the thigh, presenting as prominent, smooth, yellow nodules, a manifestation that although occasionally reported in avian pox infections, is not considered typical [9]. Based on lesion count and anatomical distribution, the majority of cases were classified as minimal (50%), whereas moderate (12.5%) and severe (37.5%) lesions together comprised the same proportion (50%). Mild infections typically present as small lesions (1–5 mm) and are generally non-debilitating [34]. Notably, three of the four cases classified as minimal involved animals that were rehabilitated and subsequently released into the wild without long-term field follow-up, thereby limiting our understanding of the risk of disease recurrence or potential long-term impacts on the local population. This observation is consistent with the self-limiting nature of the infection in immunocompetent individuals, although veterinary intervention may have contributed to lesion resolution and the overall favorable clinical outcome observed in these individuals. Conversely, larger lesions, as observed in three individuals and primarily affecting the phalangeal region, covering more than 10% of the surface area, were classified as severe in our study due to their potential to significantly impair locomotor function [5,39]. In ground-nesting, steppe-associated species such as the Stone-curlew, reduced mobility due to extensive cutaneous lesions may heighten susceptibility to predation and hinder foraging efficiency, thereby indirectly contributing to increased mortality risk [1,20,21,25].

Moreover, although the cutaneous form of avian pox is rarely a direct cause of death, it can negatively impact both survival and reproductive success by predisposing affected individuals to secondary infections [5]. Opportunistic bacterial and fungal pathogens are among the most frequently reported complications associated with the cutaneous form of avian pox, contributing significantly to disease severity and clinical outcome [40]. In the present study, co-infections with fungal pathogens were detected in nearly all examined individuals, and bacterial co-infections were also observed in several cases, highlighting the role of opportunistic infections in disease progression and clinical outcome. Although

bacterial agents could not be identified at the genus or species level, their detection in skin tissue samples indicates a possible contribution to the observed pathology. Nevertheless, the absence of taxonomic resolution hampers our understanding of their ecological role and their interactions with the primary infection. Further investigation is required to elucidate these relationships. In contrast, fungal pathogens were more clearly characterized, with *Aspergillus fumigatus* molecularly confirmed in skin lesions or lung tissue in six out of eight Stone-curlews affected by avian pox. To our knowledge, this is the first documentation of this co-infection in Stone-curlews, although similar dual infections have been reported in other avian species [14,17]. The cutaneous lesions associated with avian pox may have facilitated secondary fungal invasion, as previously suggested for other fungal species like *Aspergillus* spp. and *Candida* spp. [16]. *A. fumigatus* is a widespread airborne fungus that causes aspergillosis, a common respiratory disease in birds, as it was observed in one Stone-curlew from our study, particularly when immunity is compromised [41]. Our findings highlight the importance of considering fungal co-infections, particularly with *A. fumigatus*, in the evaluation of avian pox cases, especially when respiratory or systemic signs are present.

Additionally, tumor-like lesions were observed in one individual infected with *Avipoxvirus*. Histopathological examination revealed features resembling squamous cell carcinoma, a neoplastic condition that has been associated with avian poxvirus infections in previous studies [42,43]. Some authors have suggested that chronic *Avipoxvirus*-induced epithelial proliferation and inflammation may contribute to neoplastic transformation, although the causal relationship remains unclear [9,44].

This study reveals notable molecular variability among *Avipoxvirus* strains infecting two Stone-curlew subspecies in the Canary Islands, while prior research had only confirmed *Avipoxvirus* infection in the subspecies *B. o. distinctus* via electron microscopy [25]. Our analysis identified three distinct viral variants in just six APV-DNA-positive individuals. These variants were associated with different phylogenetic clades and subclades, indicating potentially high genetic diversity within APV strains affecting this host species. This diversity likely reflects a combination of ecological and epidemiological factors, including vector diversity, interspecies transmission, and anthropogenic movement of birds [9]. Different insect vectors can carry distinct viral strains [8,45,46], while sympatric bird species and human-mediated translocations may facilitate cross-species spillover and local mixing of variants [7,45]. Such mechanisms are consistent with previous observations of high *Avipoxvirus* diversity within single host species across localized populations [47]. Nonetheless, the presence of several low-support branches (collapsed at the 50% bootstrap threshold) in our analysis suggests that alternative phylogenetic topologies cannot be ruled out, and the inferred relationships among these variants should therefore be interpreted cautiously. Within this context of limited confidence, the observed clustering remains broadly consistent with previous molecular studies that revealed a remarkable genomic diversity among Avipoxviruses, surpassing the number of species currently recognized by the ICTV, and highlighted the capacity of several strains to infect multiple avian hosts [5]. Recent phylogenetic analyses have demonstrated that the divergence between APV clades is sometimes comparable to, or even exceeds, that observed among mammalian poxvirus genera. This has led to calls for a taxonomic reassessment of the genus *Avipoxvirus* [2].

The detection of *Avipoxvirus* in endemic Stone-curlew subspecies populations raises important conservation concerns, not only for this species but also for other vulnerable and range-restricted avifauna in the Canary Islands. Historical epizootic events in island ecosystems, including those in Hawaii, the Galápagos, and the Canary Islands, have demonstrated the highly invasive and pathogenic nature of Avipoxviruses in immunologically naive bird populations, with infection prevalence reaching up to 88% and contributing to population

declines in several endemic taxa, including finches, pipits, larks, and albatrosses [19,25,48]. In this context, our study provides a detailed pathological and genetic characterization of avian poxviruses infecting wild Stone-curlews in the Canary Islands during a 4-year period, offering a comprehensive overview and a more complete understanding of the pathology, distribution, and diagnostic features of avian pox in this island-endemic and endangered population. We systematically documented lesions, confirmed infection through histopathological features consistent with poxvirus, and detected AVP-DNA using both conventional and semi-quantitative PCR. By integrating gross, histopathological, and molecular analyses across multiple natural cases of infection, our findings contribute novel and systematic information that was previously lacking in this species, including the detection of *A. fumigatus* co-infection and the presence of a tumor-like lesion associated with avian pox. However, a limitation of this study was the absence of molecular confirmation in two cases and the inability to perform histopathological assessment in another two. These constraints emphasize the need for standardized diagnostic approaches, as consistent use of molecular and histopathological techniques would enhance comparability, strengthen analyses, and improve result interpretation, especially in rare species or small-sample studies.

This infectious disease may represent a potential threat to the conservation of endemic Stone-curlew populations within the archipelago. Although infectious diseases are less frequently listed as primary drivers of extinction relative to habitat loss or overexploitation, their role in population declines should not be underestimated. According to the IUCN Red List, infectious diseases have been implicated in approximately 3.7% of documented vertebrate extinctions over the past five centuries [18], underscoring the importance of integrating disease surveillance and management into conservation planning for susceptible species such as the Stone-curlew.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ICTV	International Committee on Taxonomy of Viruses
WRCT	Wildlife Rehabilitation Center of Tafira (Cabildo of Gran Canaria)
AVP	Avipoxvirus

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4.3. ARTÍCULO 3: **Survey of Haemosporidian Parasites in Wild Stone-Curlews (*Burhinus oedicnemus*) in the Canary Islands: First Molecular and Histopathological Evidence of Leucocytozoon sp. Infection**

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Survey of Haemosporidian Parasites in Wild Stone-Curlews (*Burhinus oedicnemus*) in the Canary Islands: First Molecular and Histopathological Evidence of *Leucocytozoon* sp. Infection

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Simple Summary

Haemosporidian parasites are vector-borne protozoans infecting a wide range of birds and occasionally causing disease in susceptible species. However, information on these parasites in the Stone-curlew (*Burhinus oedicnemus*), especially in the Canary Islands, is lacking. In this study, 47 wild Stone-curlews were examined using molecular and histopathological methods to detect these parasites. We report the first molecular and histopathological confirmation of *Leucocytozoon* sp. infection in this species. Parasite DNA was found in several organs, and characteristic tissue stages were identified in the liver, kidney, and skin. Phylogenetic analysis revealed that the detected lineage (CIAE02) has previously been described in raptors and other birds. These findings expand the known host range of *Leucocytozoon* CIAE02 and emphasize the importance of the combined use of molecular and histopathological tools to uncover hidden parasite diversity in wild avifauna.

Abstract

Avian haemosporidians are globally distributed protozoan parasites transmitted by hematophagous vectors, yet information on their occurrence in the Stone-curlew (*Burhinus oedicnemus*), particularly from the Canary Islands, is scarce. Between 2020 and 2024, 47 Stone-curlews were examined for *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* spp. using nested PCR targeting the *cytochrome b* gene, followed by sequencing and phylogenetic analysis. Histopathological examination was performed on formalin-fixed tissues. *Leucocytozoon* sp. DNA was detected in one individual (case FS415/23), with identical sequences amplified from multiple organs. Phylogenetic analysis placed this isolate within lineage CIAE02, previously reported in raptors and other avian taxa. Microscopic evaluation revealed megalomeronts in the liver, kidney, and skin, consistent with *Leucocytozoon* infection. Despite concurrent infection with *Avipoxvirus* and *Aspergillus fumigatus*, no prominent inflammatory reaction surrounded the haemosporidian tissue states. The only prior haemosporidian reported in *Burhinus* is *Haemoproteus burhinus*, described from *B. oedicnemus saharae* in Iraq, and no *Leucocytozoon* infections have previously been recorded in this genus. Therefore, this represents the first evidence of *Leucocytozoon* infection in the Stone-curlew, extending the known host range of lineage CIAE02. These findings highlight the relevance of integrative diagnostic approaches for detecting latent or cryptic haemosporidian infections in non-passerine avian hosts.

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1. Introduction

Hemoparasites are vector-borne protozoans with a cosmopolitan distribution that infect a broad range of vertebrate hosts. Among birds, the most frequently reported are avian haemosporidian (order Haemosporida, phylum Apicomplexa), with more than 250 species classified into the three most common avian blood parasite genera: *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* [1,2]. These blood parasites are transmitted by hematophagous dipterans (*Haemoproteus* by louse flies or biting midges, *Leucocytozoon* by black flies or biting midges, and *Plasmodium* by mosquitoes) [3–7].

The life cycle of avian haemosporidians is complex, involving both vertebrate and invertebrate hosts. It includes three principal stages: (i) an exo-erythrocytic phase (merogony or schizogony) phase, during which asexual replication occurs within the tissues of the intermediate vertebrate host; (ii) an erythrocytic stage, where gametocytes develop within blood cells and become infectious to hematophagous insect vectors; and (iii) sexual and sporogonic phases within the dipteran definitive host vector, where fertilization and sporozoite formation occur. Mature sporozoites are subsequently transmitted to new avian hosts during vector blood meals [8,9].

Molecular approaches have markedly enhanced the accuracy of diagnosing avian haemosporidians. In particular, mitochondrial DNA primers targeting the *cytochrome b* gene have enabled the reliable detection and identification of *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* species across diverse avian hosts and regions. These molecular tools have overcome many of the limitations of traditional microscopic methods, particularly in cases of low parasitemia or mixed infections [10].

However, recent research has revealed a greater diversity of avian haemosporidians than historically recognized, highlighting the need for further investigations to resolve their phylogenetic relationships [1,11–16]. Sequencing remains essential to achieve fine-scale taxonomic resolution. Only through the analysis of sequence data can parasite lineages be accurately identified, cryptic diversity detected, and morphologically similar but genetically divergent species distinguished [3,17].

The genetic diversity within the order Haemosporida is substantial, with over 5312 different lineages currently listed in the MalAvi database [18] is no longer accessible through its former public URL, we used an updated version of the database provided directly by one of the authors (Javier Pérez-Tris, pers. comm.) (accessed on 3 July 2025). Nevertheless, the existence of distinct genetic lineages does not necessarily imply that all represent valid species. Establishing species boundaries requires integrative analysis employing multiple genetic markers and morphological characterization [18]. Addressing this taxonomic uncertainty remains challenging, largely due to the difficulty of sampling diverse avian hosts across extensive geographic ranges [5].

Beyond their taxonomic and genetic diversity, avian haemosporidians are of considerable pathological importance. Infection outcomes vary widely, ranging from subclinical infections to severe disease, depending on the parasite species, host susceptibility, and infection intensity [3,9]. Common pathological manifestations include anemia, hepatosplenomegaly, vascular damage, and multifocal necrosis in parenchymatous organs. Exo-erythrocytic stages, particularly the large megalomeronts of *Leucocytozoon* spp., can cause extensive tissue destruction, inflammation, and hemorrhage, occasionally leading to mortality in susceptible hosts [3,9].

In addition, abortive development of haemosporidian parasites is increasingly recognized as a relevant source of pathology. In non-compatible or accidental hosts, parasites may initiate development but fail to complete their life cycle, resulting in the formation of atypical exo-erythrocytic stages [3,9,19]. Although such infections do not produce circulating gametocytes, they may induce severe tissue damage and are often underdiagnosed when relying solely on blood smears.

The Stone-curlew (*Burhinus oedicnemus*) (order Charadriiformes, family Burhinidae) is a nocturnal wader of conservation concern, widely distributed across Europe, North Africa, and parts of Asia, and represented in the Canary Islands by two endemic subspecies: *B. o. insularum* (eastern islands) and *B. o. distinctus* (western islands) [20–26]. As a species adapted to steppe environments, the Stone-curlew is particularly sensitive to global environmental change and is regarded as both a flagship and an umbrella species, since conservation efforts directed toward its protection also benefit other fauna associated with steppe habitats and contribute to the maintenance of these ecosystems [26,27]. In the Canary Islands, the species inhabits open, arid environments such as coastal plains, scrublands, and cultivated fields, typically nesting directly on the ground among sparse vegetation. It primarily feeds on insects, other invertebrates, and small vertebrates, exhibiting crepuscular and nocturnal activity patterns that reduce competition and predation risk. The Stone-curlew is largely sedentary within the archipelago, although some local movements occur in response to food availability and climatic conditions [23]. Both Canarian subspecies are legally protected due to their limited distribution and ecological vulnerability [20–22]; however, their populations continue to face threats, primarily from anthropogenic factors, such as collisions with power lines, as well as from natural causes, including infectious and parasitic diseases [28–33]. Given their restricted range and conservation concern [34], a thorough understanding of infectious agents, including blood parasites, viruses, and fungi, is essential. The present study aims to determine the presence of avian hemosporidia in the Canary Islands Stone-curlew and discuss the potential implications of these findings for the conservation of the endemic subspecies.

2. Materials and Methods

2.1. Study Area and Climate

The Canary Islands are a volcanic archipelago located off the northwestern coast of Africa (27°37′–29°25′ N, 13°20′ and 18°10′ W) (Figure 1), composed of seven main islands that vary in topography and climate. The region has a predominantly subtropical climate characterized by mild temperatures throughout the year, low annual rainfall, and the influence of the northeast trade winds. Rainfall is irregular and mainly concentrated between November and March, while the summer months are typically dry. The eastern islands (Lanzarote and Fuerteventura) are more arid and dominated by steppe and semi-desert habitats, whereas the western islands (Tenerife, La Palma, La Gomera, and El Hierro) are more humid, with higher altitudes and greater vegetation cover [https://www3.gobiernodecanarias.org/ (accessed on 10 November 2025)] [https://www.gevic.net/index.php (accessed on 10 November 2025)].

Figure 1 shows the locations where the specimens included in this study were found. Geographical coordinates are provided in Table A1 in some cases, the coordinates are approximate depending on the precision of the location site.

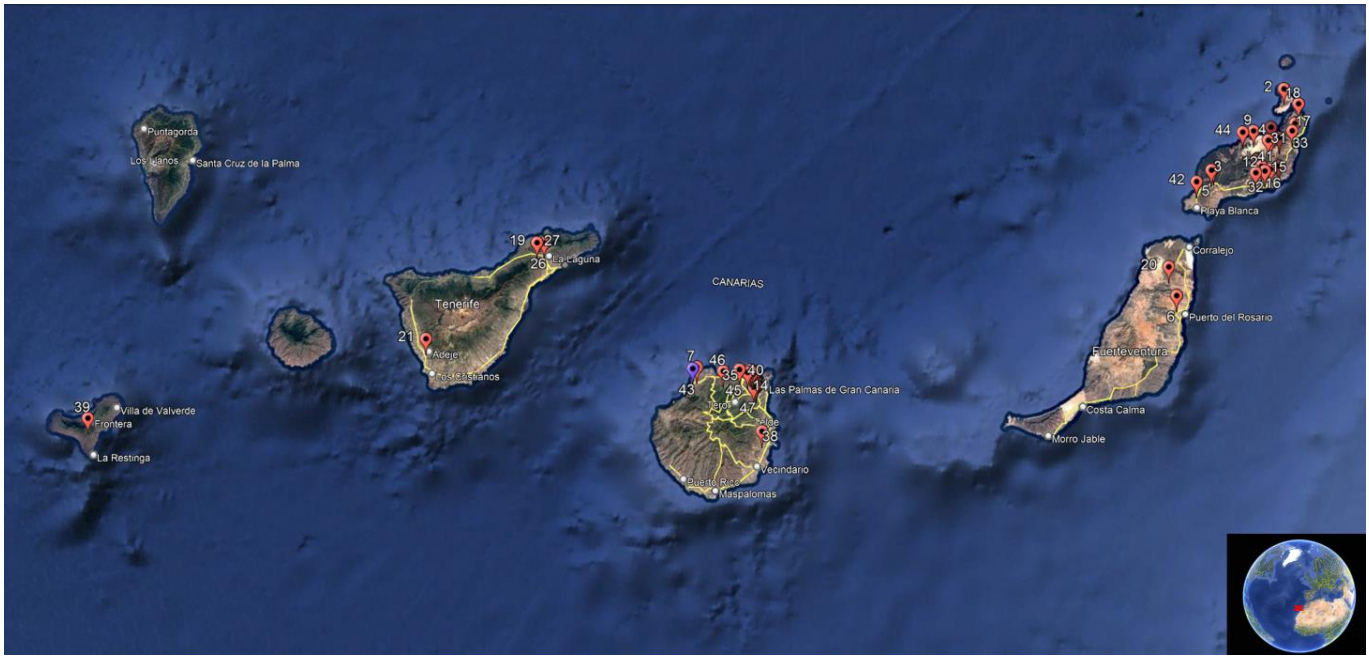

Figure 1. Map of the Canary Islands showing the locations where the birds included in the study were found. The positive *Leucocytozoon* case is indicated. (📍 Positive case).

2.2. Birds and Tissue Sampling

The stone-curlew is distributed across the Canary Islands with an estimated 1000–2500 resident pairs. Population densities are highest in the eastern islands, with 485–3972 individuals in Lanzarote and 278–1351 in Fuerteventura, and smaller populations in La Graciosa (7–59 individuals) and Alegranza (~10 pairs). In contrast, the western islands host fewer breeding pairs: approximately 250 in Gran Canaria, 50–60 in Tenerife, 100 in El Hierro, and around 20 and 12 pairs in La Gomera and La Palma, respectively [35].

A total of 47 deceased Stone-curlews were collected between 2020 and 2024 through the Canarian Wildlife Health Surveillance Network, a governmental program established to investigate wildlife mortality in the Canary Islands (Red Vigía Canarias; Order No. 134/2020, 26 May 2020). Carcasses were submitted for necropsy at the University Institute of Animal Health and Food Safety (IUSA), University of Las Palmas de Gran Canaria (ULPGC), and selected for this study based on their decomposition status following the veterinary pathology protocol of the IUSA; only specimens categorized as state: 1 (very fresh), 2 (fresh), or 3 (incipient decomposition) were included, while those in advanced (state 4) or very advanced decomposition (state 5) were excluded. Biological information, including age, sex, date of carcass discovery, and body condition (assessed following previously established protocols [29,36,37]), is provided in Table A1.

All necropsies were performed using standardized and consistent procedures [38], which include a thorough macroscopic assessment of each carcass and photographic documentation of all organs and any detected lesions. Tissue samples were obtained from major organs, such as the liver, lungs, kidneys, and intestines, as well as from any skin areas exhibiting lesions (Table A1). Sterile, individually wrapped swabs without transport medium (Vircell S.L., Granada, Spain) were used to collect samples from the oropharyngeal cavity, cloaca, coelomic cavity, and brain, as previously described [28,39,40]. All biological material was archived at the IUSA. Fresh, unfixed tissue was stored at −80 °C for subsequent molecular analyses, whereas additional samples were fixed in 10% buffered formalin for histopathological examination.

In cases where sex, age, or body condition could not be determined, this limitation was primarily attributed to the absence of suitable tissue resulting from the cause of death, such as trauma from vehicle collisions, or to the lack of macroscopically identifiable organs, such as the gonads, which prevented sample collection for histopathological examination (Table A1).

2.3. Molecular Analysis of Avian Hemosporidia

For each animal, small tissue samples from the lung, liver, and kidney were pooled into a single composite sample using a sterile swab, except in one case where only a lung tissue sample was available. These samples were placed in tubes containing viral transport medium with HEPES buffer, gelatine, bovine serum albumin, sucrose, and compatible antibiotics to preserve pathogen stability and viability during transport and storage (Transport Medium for the Collection and Preservation of Viruses, Chlamydia, and Mycoplasma, Vircell S.L., Granada, Spain). Skin samples were mechanically homogenized in DNA/RNA Shield. For molecular extraction, a 100 µL aliquot of each diluted pooled sample was combined with an equal volume of DNA/RNA Shield, while 200 µL of the macerated skin sample was used after centrifugation.

A simultaneous DNA/RNA extraction was conducted using a magnetic bead-based method on an automated robotic platform, following the manufacturer's protocol for the ZYMO DNA/RNA extraction kit (ZYMO Research, Freiburg, Germany). To validate the extraction process, both a negative control (nuclease-free water) and a positive control (an *Avipoxvirus* positive sample previously confirmed in our laboratory) were included [29].

A total of forty-six pooled samples were analyzed, comprising tissue swabs from liver, kidney, and lung. In one bird, only a lung sample was available. In cases where pooled samples tested positive, individual tissue samples were subsequently analyzed, including those originally included in the pool as well as additional organs that showed structures compatible with haemosporidian infection in the histopathological study (e.g., skin) (Table A1). To detect avian haemosporidian parasites (*Haemoproteus*, *Plasmodium*, and *Leucocytozoon*), conventional polymerase chain reaction (PCR) was used following a nested PCR protocol previously described by Hellgren et al. (2004) [17], which targets the *cytochrome b* gene of the mitochondrial genome. Specifically, DNA templates (2 µL) were amplified in a 12.5 µL reaction mixture containing 1× PCR buffer with $MgCl_2$ (2.5 mM), 0.4 µM of each primer, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 0.05 U/µL of Taq DNA polymerase (Roche Applied Science, Vienna, Austria), and diethylpyrocarbonate (DEPC)-treated water. Primary PCRs were conducted with an initial denaturation at 94 °C for 3 min, followed by 20 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 45 s, with a final extension at 72 °C for 10 min. Secondary PCRs were performed under the same conditions, except that 35 cycles were applied instead of 20. The external PCR employed the primers HaemNFI (5′-CATATATTAAGAGAAITATGGAG-3′; where "I" denotes the universal base, inosine) and HaemNR3 (5′-ATAGAAAGATAAGAAATACCATTC-3′). For the internal PCR, the primers HaemF and HaemR2 were used to amplify *Plasmodium* spp. and *Haemoproteus* spp., while HaemFL (59-ATGGTGTTTTAGATACTTACATT-39) and HaemR2L (5′-CATTATCTGGATGAGATAATGGIGC-3′) were used for *Leucocytozoon* spp.

PCR products (5 µL) were analyzed by horizontal electrophoresis on a 2% agarose gel stained with GelRed® (Biotium, Inc., Fremont, CA, USA). Diethylpyrocarbonate (DPEC)-treated water was employed as the negative control in both PCR assays, while a commercially available purified DNA of *Plasmodium falciparum* (Vircell S.L. Ref.: MBC148-R) was used as the positive control. Successful amplification of *Leucocytozoon* spp was indicated by PCR products of 478 base pairs in length (bp), excluding primer sequences, and 480 bp for *Haemoproteus* spp. and *Plasmodium* spp.

2.4. Detection and Sequencing of PCR Products and Phylogenetic Analysis

As the PCR assay amplifies fragments of nearly identical size for *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* spp., the specific genus could not be determined based solely on the electrophoretic band pattern. Therefore, amplicons obtained from conventional PCR [17] of positive samples were purified using the Real Clean spin kit (REAL, Valencia, Spain) and subsequently subjected to bidirectional Sanger sequencing. Sequencing was performed using the product of the second internal PCR from the semi-nested PCR, employing primers HaemF, HaemR2, for *Plasmodium* spp. and *Haemoproteus* spp. and HaemFL, HaemR3L for Leucocytozoon spp. The resulting nucleotide sequences were analyzed both internally and by comparison with publicly available sequences in GenBank through BLASTN (Basic Local Alignment Search Tool) (+ 2.17.0 version) analysis on 16 September 2025 and MalAvi database lineage name [18] consultation on 3 July 2025. Multiple sequence alignments of Plasmodium spp., *Haemoproteus* spp. and *Leucocytozoon* spp. sequences were carried out using ClustalW.

Phylogenetic and molecular evolutionary analyses were performed using MEGA version 12 [41], incorporating 21 nucleotide sequences retrieved from GenBank, 19 of *Leucocytozoon* spp., and two *Plasmodium* spp. sequences as the outgroup. Phylogenetic trees were constructed using the Neighbor-Joining (NJ) and Maximum Parsimony (MP) algorithms based on pairwise genetic distances estimated by the Maximum Composite Likelihood (MCL) method according to the Tamura (1992) [42] model. The rate model allowed for 59.10% of sites to be evolutionarily invariable (I). The analysis included 22 nucleotide sequences, with 526 aligned positions in the final dataset. The topology robustness was assessed using a bootstrap consensus tree based on 1000 replicates. Branches supported by fewer than 50% of bootstrap replicates collapsed, and bootstrap values (expressed as percentages) were displayed next to the corresponding branches [42].

2.5. Histopathological Examination

Formalin-fixed tissue samples were routinely processed for histopathological evaluation. Samples were dehydrated through graded alcohols, cleared in xylene, and embedded in paraffin wax. Three µm-thick sections were stained with hematoxylin and eosin (H&E) following standard protocols and examined under a light microscope (Olympus BX51, Olympus Corp., Tokyo, Japan). Although all available tissues were processed, histopathological results are presented only for the individual that tested PCR-positive for haemosporidian infection. The evaluation focused on identifying tissue alterations associated with haemosporidian infection, including the presence, localization, and morphology of exo-erythrocytic stages (meronts and megalomeronts), inflammatory responses, necrosis, and vascular lesions. Special attention was given to the detection of abortive developmental stages in non-erythrocytic tissues, as well as the presence of intra-erythrocytic gametocytes. Representative parasitic stages were photodocumented using a digital microscope camera (DP21) and a 0.5X (U-TV0.5XC-3) adapter (Olympus Corp., Tokyo, Japan).

3. Results

3.1. Birds and Tissue Sampling

The sample included representatives of both Stone-curlew subspecies present in the Canary Islands. Of these, 22 individuals (47%) belonged to *B. o. insularum* (20 from Lanzarote and 2 from Fuerteventura), while the remaining 25 birds (53%) corresponded to *B. o. distinctus* (19 from Gran Canaria, 5 from Tenerife, and 1 from El Hierro). Of the 47 Stone-curlews examined, 20 were identified as female (20/47), 19 as male (19/47), and the sex of 8 individuals (8/47) could not be determined. In terms of age distribution, 27 birds were adults (27/47), 18 were juveniles (18/47), and 1 was a chick (1/47), with

the age of 1 bird being undetermined (1/47). Body condition assessment revealed that 14 (14/47) individuals were cachectic (grade 1), 18 (18/47) were underweight (grade 2), and 13 were in an ideal stage (grade 3). In 2 cases, the body condition could not be evaluated. Full biological information for each specimen is provided in Table A1.

3.2. Detection and Sequencing of PCR Products

A fragment of the mitochondrial *cytochrome b* gene consistent with Haemosporida parasites was initially detected in the pooled tissue sample (liver, kidney, and lung) and later confirmed in the corresponding individual tissues, as well as the skin of the same bird (case FS415/23). The positive Stone-curlew, an adult female, was found alive in an urban area in northern Gran Canaria (Table A1). Due to the severe lesions on its pelvic limbs consistent with advanced avian poxvirus infection, combined with the overall poor clinical condition of the bird, euthanasia was performed shortly after admission to the Wildlife Recovery Center of Tafira. The avian poxvirus infection was later confirmed by PCR and histopathological analysis [29]. In addition, an *Aspergillus fumigatus* infection was detected and confirmed by PCR [29].

Amplicons obtained from the semi-nested PCR revealed a band of the expected size (approximately 522 bp) (Figure 2). Further sequencing of the positive samples produced a uniform consensus sequence of approximately 476 bp (primers excluded). This sequence clustered within the genus *Leucocytozoon*, showing 100% nucleotide identity and query coverage with multiple *Leucocytozoon* spp. sequences from diverse avian hosts across different geographic regions, and has been deposited in GenBank under accession number PX377274. The same skin sample had previously tested positive for *Avipoxvirus* (AVP) and *Aspergillus fumigatus* DNA by PCR, as reported in an earlier study [29].

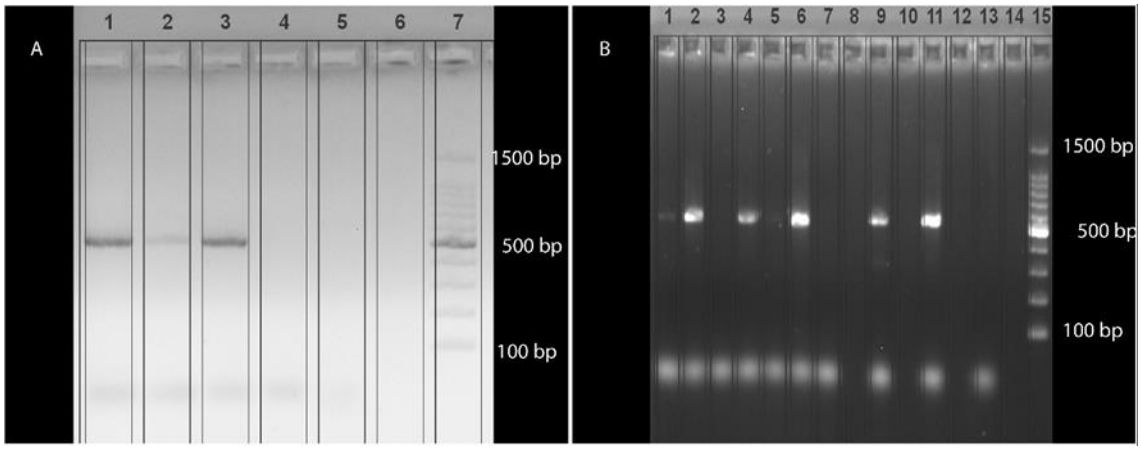

Figure 2. Agarose gel (2%) electrophoresis of conventional semi-nested PCR products targeting the Haemosporidian *cytochrome b* gene. (**A**). Amplified products of the expected size (≈522 bp) are detected in lanes 1 and 2, corresponding to two replicates of the pooled tissue sample of case FS415/23. Lane 3 represents the commercial positive control (*Plasmodium falciparum*), lane 4 the negative control, lanes 5 and 6 are empty, and lane 7 contains the 100 bp DNA ladder. (**B**). Amplicons of the expected size (≈522 bp) are observed in liver (lane 2), lung (lane 4), kidney (lane 6), and skin (lane 9) samples, as well as in the commercial positive control (lane 11). Lane 13 corresponds to the negative control, and lane 15 contains the 100-bp DNA ladder.

3.3. Phylogenetic Analysis

The mitochondrial *cytochrome b* gene sequence obtained from the Haemosporidia-positive Stone-curlew was analyzed to assess its phylogenetic relationships. This sequence (GenBank accession ID: PX377274) was included in a phylogenetic analysis with 21 other haemosporidian sequences retrieved from GenBank, comprising 19 *Leucocyto-*

zoon spp. and two *Plasmodium* spp. sequences used as outgroups. The phylogenetic tree was constructed using the T92 + I substitution model (Figure 3). Our sequence clustered closely with the CIAE02 linage reported from several countries, including Turkey [KP000840, KC962151, KC962152, KP000841*(BUTBUT01 linage)], Spain (GQ371174, HF543631, MK330160), the Philippines (JX418201), Mongolia (KJ577832), and Germany (EF607287), showing 100% nucleotide identity in BLASTn (BLAST+ 2.17.0) searches and strong phylogenetic support in maximum likelihood analyses (bootstrap = 100%) (Figure 3). Lineage assignment was determined by comparison with publicly available sequences in the GenBank and MalAvi databases (mbio-serv2.mbioekol.lu.se/Malavi) [18].

Figure 3. Phylogenetic analysis of positive case sequence (arrow) (GenBank accession number: PX377274), obtained from liver, kidney, and skin samples, based on partial mitochondrial cytochrome b sequences from various host species (indicated in brackets next to the *Leucocytozoon* and *Plasmodium* species names). Lineages from previous studies are labeled in parentheses. Evolutionary history was inferred using the Maximum Likelihood method [42]. The bootstrap consensus tree, inferred from 1000 replicates [42], represents the evolutionary relationship of the taxa analyzed, with branches supported in less than 50% of replicates collapsed. The percentage of replicate trees in which associated taxa clustered together is shown next to the branches [42]. The initial tree for the heuristic search was selected as the one with the superior log-likelihood between a Neighbor-Joining (NJ) tree [43] and a Maximum Parsimony (MP) tree. The NJ tree was generated using a matrix of pairwise distances computed with the p-distance [44]. The rate model allowed for 59.10% of sites to be evolutionarily invariable (I). The analysis included 22 coding nucleotide sequences, incorporating 1st, 2nd, 3rd, and non-coding positions, with 526 positions in the final dataset. Evolutionary analyses were conducted in MEGA12 [41] utilizing up to 3 parallel computing threads.

3.4. Histopathological Findings

In histological sections, exo-erythrocytic stages consistent with Haemosporidia infection were identified in three of the four PCR-positive tissues. Meront-like structures at various maturation stages were observed within hepatocytes (Figure 4a) and within renal epithelial cells (Figure 4b–f). These parasitic stages were characterized by rounded (10–20 µm in diameter) to oval (35–70 µm in length) structures containing multiple basophilic nuclei. No inflammatory infiltrate, necrosis, or other tissue reaction was evident in any of the examined tissues.

In the skin, mature meronts (Figure 5) and numerous megalomeronts (Figure 6) exhibiting considerable morphological variability were observed within the dermis. These exo-erythrocytic stages were mostly located inside capillaries. The megalomeronts appeared as large, round to oval structures—often exceeding 50 µm in diameter—with a thick eosinophilic wall that frequently enclosed an enlarged host cell nucleus. The internal content consisted of numerous cytomeres arranged within a granular to finely vacuolated basophilic cytoplasm, containing multiple nuclei at various stages of development. The

affected skin also exhibited marked epidermal hyperplasia, ballooning degeneration, and intracytoplasmic eosinophilic inclusion bodies (Bollinger bodies) consistent with *Avipoxvirus* infection, as well as fungal hyphae morphologically compatible with *Aspergillus* spp. in the necrotic crusts and superficial dermis. Despite the presence of these concurrent infections, no prominent inflammatory reaction was observed surrounding the haemosporidian stages. On rare occasions, small, round, basophilic structures consistent with free merozoites were observed within the vascular plasma, apparently representing stages preceding erythrocytic invasion. These were surrounded by a localized inflammatory response composed predominantly of macrophages and heterophils (Figure 5c).

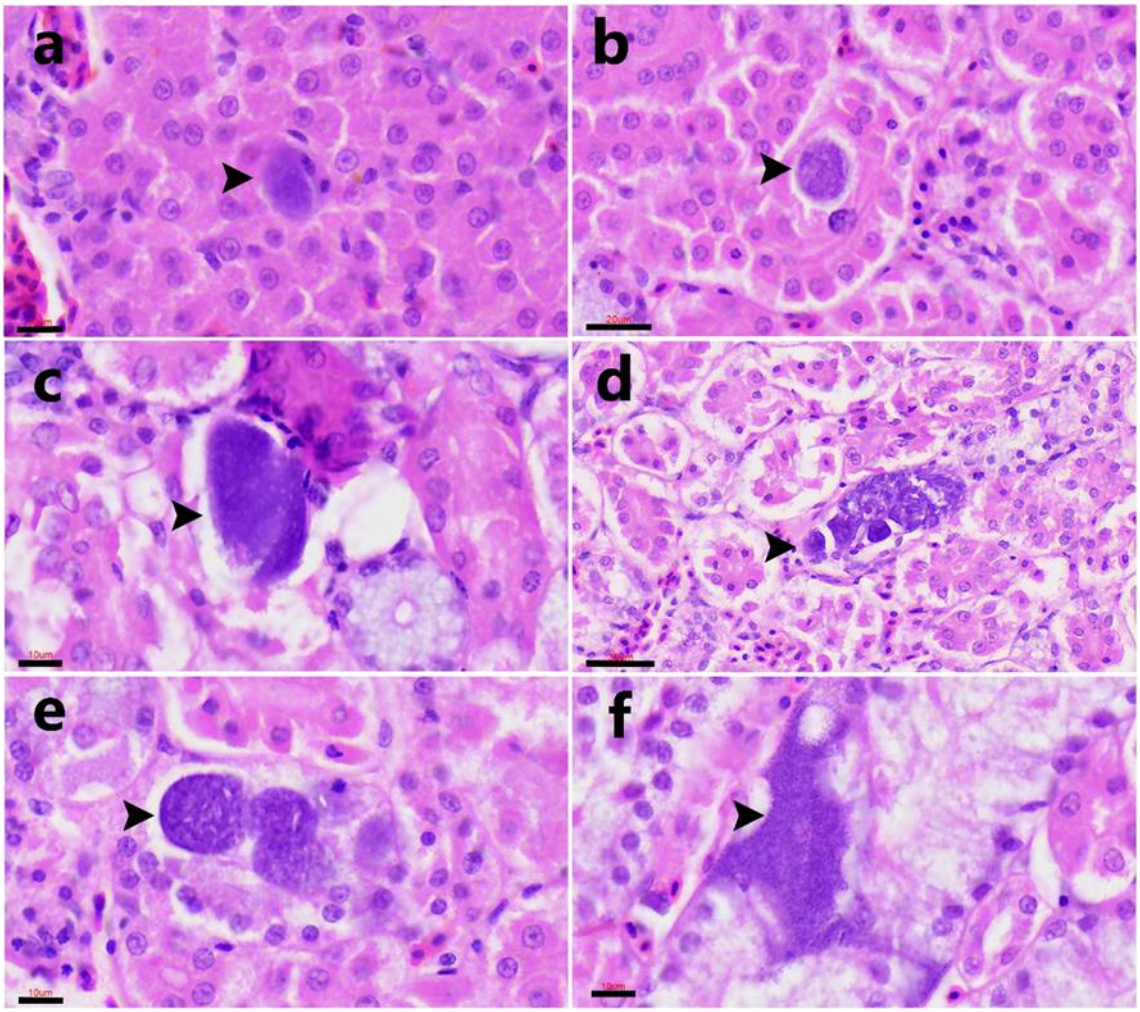

Figure 4. Case FS415/23. Exo-erythrocytic tissue stages of *Leucocytozoon* sp. parasites (arrowheads) in hematoxylin-eosin (H&E) stained sections. (**a**) Early hepatic meront (scale bars =10 μm). (**b**–**f**) Meronts of irregular shape and size were detected within the epithelial cells of renal tubules. (**b**). A small meront in a renal epithelial cell (scale bars = 20 μm). (**c**). A more mature meront packed with merozoites, occupying the entire intracellular space and causing protrusion into the tubular lumen (scale bar = 30 μm). (**d**,**e**) Developing megalomeronts in the kidney ((**d**). Scale bar = 30 μm). (**f**). Mature meront within a renal epithelial cell. Scale bars =10 μm, unless otherwise indicated.

Occasionally, structures compatible with intra-erythrocytic stages of *Leucocytozoon* sp. were observed within the blood vessels of the liver (Figure 7) and kidney. The infected circulating cells appeared enlarged and distorted, containing eccentrically placed basophilic nuclei and cytoplasmic inclusions consistent with developing gametocytes.

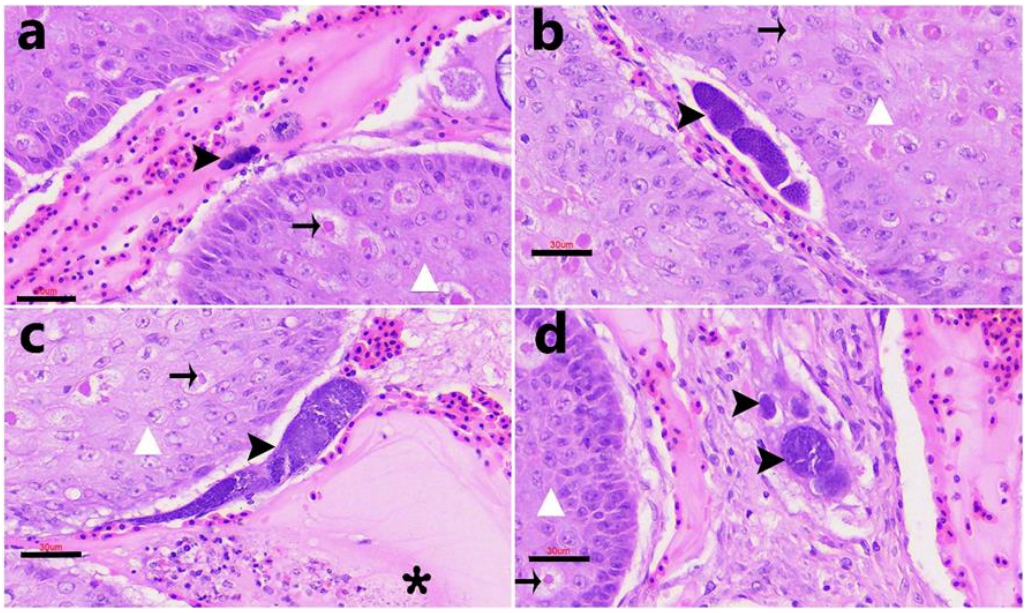

Figure 5. Case FS415/23. Exo-erythrocytic stages of *Leucocytozoon* sp. in hematoxylin-eosin (H&E)-stained skin sections. The skin shows severe epidermal hyperplasia (white triangle) with characteristic *Avipoxvirus* eosinophilic intracytoplasmic inclusion bodies (Bollinger bodies) (arrow). (**a**). Intravascular meront (arrowhead) (**b**). Incipient megalomeront (arrowhead) within a dermal vessel. (**c**). Incipient megalomeront (arrowhead) within a dermal vessel, with multiple free merozoites surrounded by inflammatory cells (asterisk). (**d**). Incipient megalomeronts within the dermis. Scale bars = 30 µm.

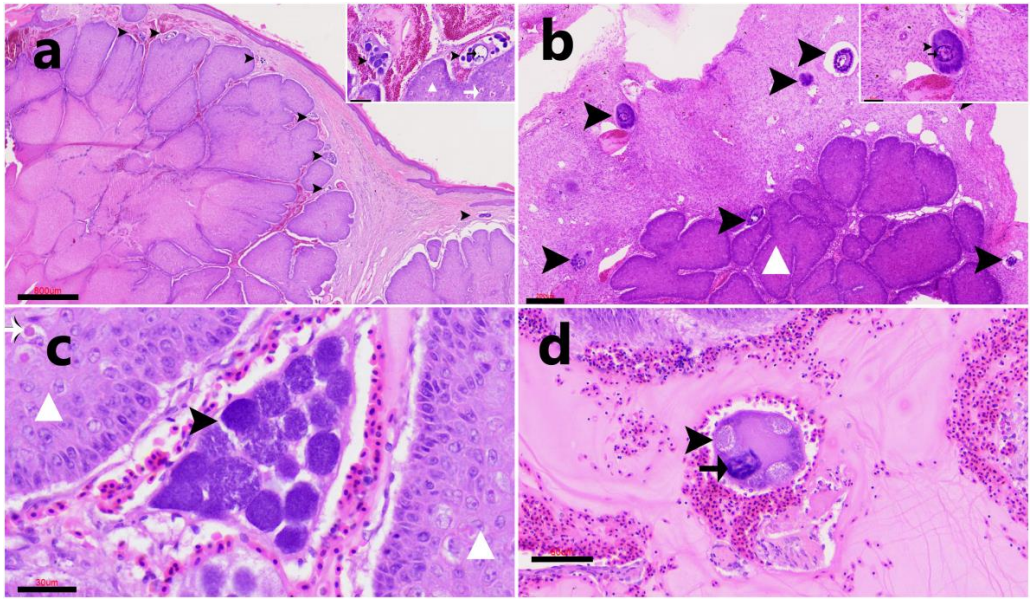

Figure 6. Case FS415/23. Histological sections (Hematoxylin & Eosin stain) of avian skin showing exo-erythrocytic stages of *Leucocytozoon* sp. and concurrent *Avipoxvirus* infection characterized by epidermal hyperplasia (white triangle); Bollinger bodies (white arrow). (**a**). Overview of the dermis and epidermis showing multiple large parasitic stages (arrowheads) consistent with *Leucocytozoon* megalomeronts beneath marked epidermal hyperplasia. Scale bar = 800 µm. Inset: higher magnification of a blood vessel containing developing megalomeronts (arrowhead) with multiple internal nuclei (merozoites) and hypertrophied host cell nuclei (arrow). Scale bar = 70 µm. (**b**). Multiple incipient megalomeronts within or adjacent to dermal vessels (arrowheads). Scale bar = 200 µm. Inset: developing megalomeront (arrowheads) with internal nuclei (merozoites) and hypertrophied host cell nuclei (arrow). Scale bar = 60 µm. (**c**). Mature meront (arrowhead) in a dermal vessel. Scale bar = 30 µm. (**d**). Vascular-associated megalomeront (arrowheads) with internal merozoites and a hypertrophied host cell nucleus (arrow). Scale bar = 60 µm.

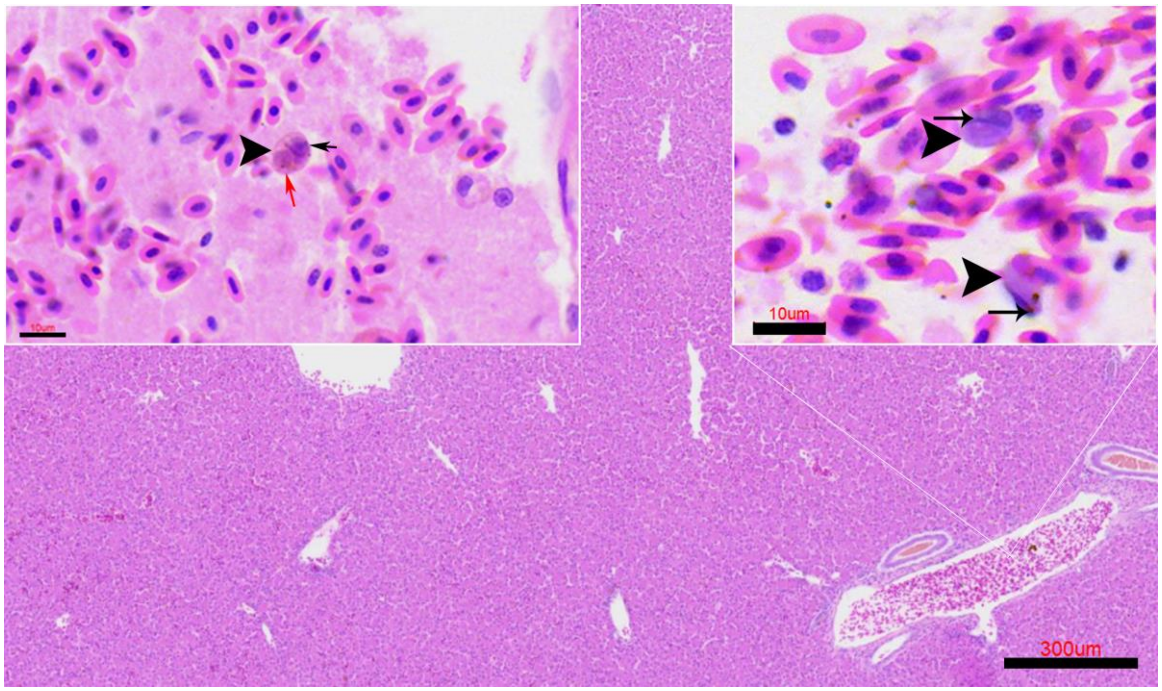

Figure 7. Case FS415/23. Intra-erythrocytic-like stage of *Leucocytozoon* sp. (arrowheads) observed in hematoxylin-eosin (H&E)- stained liver sections. Scale bar = 300 μm. Upper left inset: liver vessel showing a parasite nucleolus (red arrow). Upper right inset: blood vessel containing structures compatible with the intra-erythrocytic form of *Leucocytozoon* sp.; host cell nuclei indicated (black arrow). Inset scale bar = 10 μm.

4. Discussion

This study represents the first molecular and histopathological confirmation of *Leucocytozoon* sp. infection in the Stone-curlew. The detection of the only *Leucocytozoon*-positive Stone-curlew in Gran Canaria is consistent with the distribution and population structure of the species in the archipelago. Gran Canaria holds one of the largest and most stable populations of *Burhinus oedicnemus distinctus* in the western islands, with approximately 250 breeding pairs [35], which increases the likelihood of encountering infected individuals compared with islands where the species is less abundant. In our study, samples were geographically distributed across the main islands where the species occurs, and thus not restricted to a single locality. However, population sizes differ markedly between islands, and this must be considered when interpreting the spatial pattern of infection.

The identification of parasite DNA in several organs, including the skin, liver, kidney, and lungs, together with the observation of characteristic exo-erythrocytic stages, provides clear evidence of a systemic infection and confirms the susceptibility of this species to haemosporidian parasites. To date, information on blood parasite diversity within the genus *Burhinus*, and particularly in the Eurasian Stone-curlew, remains scarce. A notable exception is the report of *Haemoproteus burhinus* in the Saharan subspecies *Burhinus oedicnemus saharae* from Iraq, described by Mohammad (1998) [45], which was characterized by hypertrophied infected erythrocytes and lateral displacement of the host cell nucleus in microgametocytes. Taken together, these findings broaden our understanding of haemosporidian diversity and host–parasite associations within the family Burhinidae.

Moreover, the detection of *Leucocytozoon* sp. in this species demonstrates the capacity of this parasite to infect Charadriiform hosts, a group in which such infections are infrequently reported. According to the MalAvi database, more than 5300 haemosporidian lineages have been identified to date, but only 67 have been associated with Charadriiformes. Most of these belong to *Haemoproteus* (23 lineages) and *Plasmodium* (39 lineages), while *Leucocytozoon*

accounts for merely five lineages, emphasizing the rarity of such infections in this avian order [18].

Molecular characterization based on *cytochrome b* sequencing and comparison with the MalAvi database confirmed that the detected *Leucocytozoon* isolate belongs to the lineage CIAE02. According to MalAvi records, this lineage exhibits an exceptionally broad host and geographical range, having been reported from more than ten avian orders, including Falconiformes, Strigiformes, Gruiformes, and Charadriiformes, and across four continents: Europe, Asia, Africa, and North America [46–52]. It has been previously reported in raptors such as Eleonora's Falcon (*Falco eleonorae*) in Alegranza Island (Canary Islands) and Black Kite (*Milvus migrans*) in southern Spain [47,53].

Although CIAE02 is most frequently associated with raptors (Accipitridae and Falconidae), its occurrence in hosts occupying diverse ecological niches, such as rails (*Crex crex*), gulls (*Larus* spp.), woodpeckers (*Dryocopus martius*), and even parrots (*Brotogeris cyanoptera*), indicates either an unusually generalist parasite with low host specificity or a complex of closely related cryptic lineages that cannot yet be genetically distinguished. Detection of this lineage in the Stone-curlew further extends its known host spectrum to the family Burhinidae and suggests possible ecological overlap in vector exposure or occasional host switching among sympatric bird species. Further molecular and morphological investigations are required to clarify the taxonomic boundaries of CIAE02 and to better understand host–parasite associations and transmission dynamics of *Leucocytozoon* in Charadriiform birds.

The identification of exo-erythrocytic meronts and megalomeronts in the liver and kidney is consistent with the typical life cycle of *Leucocytozoon* spp., in which asexual replication occurs in multiple tissues before gametocyte formation in circulating blood cells. The histological detection of meronts and megalomerts in the liver and kidneys agrees with previous descriptions regarding their morphology and localization in other avian hosts [4,6,9,10,54–61]. However, the presence of megalomeronts in the skin represents an uncommon finding, as their development in dermal tissue has been rarely documented [9]. Their localization within dermal capillaries may indicate broader tissue tropism or atypical development, potentially influenced by host species or concurrent infections [3,9]. In this individual, *Avipoxvirus* lesions and *Aspergillus fumigatus* infection were present in the skin [28,29], potentially altering local tissue conditions and facilitating aberrant parasite development.

Although no previous reports of coinfection involving these specific pathogens have been found, several studies have documented concurrent *Plasmodium* and *Avipoxvirus* infections in island bird species, where such associations have been linked to population declines and even local extinctions [62–65]. The frequent coexistence of these pathogens is often explained by the overlap of their insect vectors [3,63,66]. However, in the present case, the vectors involved are not generally shared between both pathogens [1,67–69]. While all hemosporidian parasites are transmitted by Diptera, each genus is typically associated with a specific dipteran family [1,7,66]. Only *Leucocytozoon caulleryi* is known to be transmitted by biting midges (*Culicoides* spp.) [1], whereas all other *Leucocytozoon* species are vectored by black flies (*Simuliidae*). Their ecological distribution and microhabitat preference play a key role in shaping parasite transmission dynamics and can impose constraints on the parasite's host range [70,71]. Although many ornithophilic black fly species do not exhibit strict host specificity, they frequently show strong fidelity to particular habitat niches, such as foraging or breeding predominantly in the upper forest canopy, which in turn influences which bird species they are most likely to encounter and infect [72,73].

Although black flies generally require humid environments with continuous flowing water for larval development, the ecological conditions in the Canary Islands are compar-

atively challenging due to the limited and patchy distribution of permanent freshwater streams. Even under these constraints, five Simuliidae species (*Simulium guimari*, *S. intermedium*, *S. paraloutetense*, *S. ruficorne*, and *S. velutinum*) are currently recognized in the archipelago, with *S. guimari* consistently recorded as the most abundant and widespread. Species richness also varies among islands; notably, Gran Canaria and La Gomera each support all five taxa [74]. The persistence of these species indicates that even small or seasonal water sources, including ravines, remnants of laurel forest, and human-created water channels, provide sufficient habitat to maintain black fly populations that may interact with local avifauna and facilitate the transmission of *Leucocytozoon* parasites [53,75–77].

Common pathological alterations associated with leucocytozoonosis include tissue damage and inflammatory reactions related to the presence of megalomeronts, which represent the final stage of exo-erythrocytic development. In addition, vascular congestion and focal tissue degeneration may occur because of intravascular accumulations of intraerythrocytic stages, potentially impairing blood flow and oxygen delivery, thereby contributing to local hypoxia and organ dysfunction [3,9]. Notably, no marked inflammatory response was detected around most parasitic stages, despite concurrent *Avipoxvirus* and *Aspergillus fumigatus* infections, suggesting a subacute or chronic infection phase, or possibly a degree of host adaptation. In contrast, focal inflammatory responses were observed, characterized by leukocytosis associated with free merozoites within dermal blood vessels, indicating that rupture of parasitic stages and antigen release can locally activate the host immune system. Structures resembling intra-erythrocytic stages were rarely observed in the liver, kidneys, and lungs, indicating a predominance of exo-erythrocytic development and suggesting a chronic or subclinical infection. These findings align with the low parasitaemia typically reported in *Leucocytozoon* infections [3,7,9,10].

The low infection prevalence detected in our study (2%) contrasts with previous surveys in the archipelago based on blood smears. For instance, Bodawatta et al. (2020) [78] reported a prevalence of 15% in passerine species, while the survey conducted in Tenerife documented 13.4% infection, primarily in Passeriformes but including one positive *Accipiter nisus* carrying the same lineage identified in our material (CIAE02) [75]. Spurgin et al. (2021) [65] recorded a prevalence of 9.6% in Berthelot's pipit (*Anthus berthelotii*) in 2006, which declined to 0% in 2009. Additionally, a prevalence of 0.5% was reported for Eleonora's falcon, also infected with the same lineage found in our study [53]. These differences highlight the influence of sampling method, species composition, and temporal variation on observed prevalence patterns and underscore the need for integrating both blood-based and tissue-based approaches in future haemosporidian surveys.

Most studies on avian haemosporidians rely on blood samples from live birds, which enable the detection of circulating gametocytes and estimation of parasitemia levels. However, recent research has demonstrated that these parasites can persist in internal organs and may not always be detectable in peripheral blood [5,79]. More recently, Heaver et al. (2025) [80] detected *Plasmodium* and *Haemoproteus* DNA in 13.5% of over 850 wild birds examined postmortem in Great Britain and confirmed exoerythrocytic parasite stages and lesions consistent with avian malaria in several cases. Collectively, these studies highlight that postmortem tissue screening can reveal latent or tissue-restricted infections and provide histopathological context that is unavailable from blood samples alone. Nevertheless, factors such as postmortem degradation, uneven parasite distribution, and differences in tissue tropism may influence detection sensitivity. Consequently, infection data derived from tissues should be interpreted with caution and not directly compared with blood-based prevalence estimates. Instead, both approaches are best viewed as complementary, together offering a more comprehensive understanding of haemosporidian diversity, distribution, and host–parasite dynamics in wild bird populations.

In the present study, blood smears were not available, and parasite detection relied exclusively on molecular screening of tissues and histopathological examination. Although the lack of blood films limits the morphological characterization of blood stages and precludes quantification of parasitemia, the combination of molecular and histological evidence still provides important insight into the infection process. The detection of parasite DNA in multiple organs, together with the presence of exo-erythrocytic stages and rare gametocyte-like forms, suggests that parasite development was initiated but possibly not completed. The limited inflammatory reaction observed in association with these lesions further supports the hypothesis of an early or abortive infection rather than a fully established systemic parasitemia.

In non-adapted hosts, *Leucocytozoon* spp. may undergo incomplete development, resulting in the formation of tissue stages that fail to mature into gametocytes, while still inducing marked histopathological alterations [3,9,62]. This phenomenon reflects a mismatch between parasite developmental requirements and host cellular or immunological conditions. Accordingly, the dermal megalomeronts observed in this Stone-curlew likely represent abortive development in a non-evolutionarily adapted host, a scenario previously reported in *Leucocytozoon simondi* infections in non-adapted waterfowl [9].

From an epidemiological perspective, the occurrence of *Leucocytozoon* in a Stone-curlew from the Canary Islands raises questions about the distribution of competent vectors and the potential impact on local bird populations. The presence of suitable vectors in the archipelago, combined with the insular isolation and small population size of the endemic subspecies (*B. o. insularum* and *B. o. distinctus*), may pose a potential conservation concern, as even low-prevalence infections could contribute to morbidity or reduced fitness in vulnerable populations. Previous studies in island birds have demonstrated that limited genetic diversity and restricted dispersal can amplify the effects of infectious diseases on population dynamics [3,5,81].

5. Conclusions

The present findings expand the known host range of *Leucocytozoon* and emphasize the value of combining molecular screening with histopathology to detect low-parasitemia or cryptic infections, particularly in non-passerine hosts. Integrative taxonomic approaches that incorporate morphological, molecular, and vector data are essential to determine whether this lineage represents a distinct species or an intraspecific variant. The histological evidence of atypical tissue tropism and possible abortive development observed in this Stone-curlew suggests that this host–parasite relationship may represent a recent or accidental host shift rather than a coevolved association.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

IUSA University Institute of Animal Health and Food Safety
ULPGC University of Las Palmas de Gran Canaria

Appendix A

Table A1. Data from 47 stone-curlews (*Burhinus oedicnemus*) included in the present study (2020–2024), comprising biological information (age and sex), island of origin and recorded coordinates (latitude and longitude), condition (dead or alive) at the time of discovery, state of decomposition at necropsy, body condition, and the samples tested for Haemosporidian DNA detection.

ID	Age	Sex	FL	GC	FD	SS	DC	BC	Sample Tested	Haemosporidian PCR Results
SA324/20	A	U	L	29°6′35.86″ N, 13°33′21.72″ W	30/07/2020	D	3	1	Pool*	-
SA131/21	J	F	L	29°14′51.89″ N, 13°30′44.34″ W *	08/08/2020	D	2	1	Pool*	-
SA132/21	A	F	L	28°56′57.90″ N, 13°46′16.37″ W *	03/09/2020	D	3	1	Pool*	-
SA133/21	A	F	L	29°3′49.73″ N, 13°33′55.21″ W *	24/11/2020	D	3	1	Pool*	-
SA134/21	J	M	L	28°56′37.94″ N, 13°46′47.15″ W *	04/12/2020	D	2	3	Pool*	-
SA255/21	J	M	F	28°30′21.84″ N, 13°53′2.39″ W	20/06/2018	D	3	3	Pool*	-
SA334/21	A	M	GC	28°6′13.79″ N, 15°41′58.93″ W *	22/04/2021	D	2	2	Pool*	-
SA359/21	A	U	T	29°4′34.42″ N, 13°31′19.56″ W	29/04/2021	D	3	3	Pool*	-
SA447/21	J	U	L	29°5′31.98″ N, 13°37′24.62″ W	24/05/2021	D	3	1	Pool*	-
SA485/21	A	F	L	28°59′35.28″ N, 13°36′18.00″ W	08/04/2021	D	2	3	Pool*	-
SA518/21	J	M	L	28°59′35.28″ N, 13°36′18.00″ W	06/04/2021	D	2	2	Pool*	-
SA597/21	J	M	L	28°56′55.55″ N, 13°36′22.06″ W	21/12/2018	D	2	2	Pool*	-
SA849/21	J	U	GC	28°5′1.74″ N, 15°27′18.20″ W	27/08/2021	D	3	3	Pool*	-
SA870/21	J	M	GC	28°6′38.85″ N, 15°28′21.07″ W *	U	D	2	1	Pool*	-
SA875/21	J	F	L	28°57′55.56″ N, 13°32′52.69″ W *	07/06/2021	D	2	U	Pool*	-
SA876/21	A	M	L	28°57′27.70″ N, 13°34′14.15″ W	08/07/2021	D	2	2	Pool*	-
SA877/21	J	M	L	29°7′52.43″ N, 13°27′45.56″ W	29/06/2021	D	3	3	Pool*	-
SA994/21	J	M	L	29°12′0.67″ N, 13°27′9.54″ W	31/08/2021	D	3	3	Pool*	-
SA1133/21	A	M	T	28°28′29.84″ N, 16°20′53.60″ W *	22/0/2021	D	2	2	Pool*	-

Table A1. *Cont.*

ID	Age	Sex	FL	GC	FD	SS	DC	BC	Sample Tested	Haemosporidian PCR Results
SA1156/21	A	F	F	28°36′2.63″ N, 13°55′19.53″ W *	25/10/2021	D	3	2	Pool*	-
SA1262/21	A	F	T	28°6′37.96″ N, 16°43′51.44″ W *	04/11/2021	D	2	3	Pool*	-
SA1295/21	J	F	GC	28°4′8.43″ N, 15°27′13.40″ W	U	D	3	2	Pool*	-
SA1296/21	J	F	GC	28°4′8.43″ N, 15°27′13.40″ W	18/09/2021	D	2	1	Pool*	-
SA1297/21	A	F	GC	28°4′8.43″ N, 15°27′13.40″ W	28/09/2021	D	2	3	Pool*	-
SA1298/21	J	F	GC	28°4′8.43″ N, 15°27′13.40″ W	U	D	1	3	Pool*	-
SA1411/21	A	F	T	28°28′37.20″ N, 16°19′15.71″ W	30/11/2021	D	3	2	Pool*	-
SA1417/21	A	F	T	28°28′37.20″ N, 16°19′15.71″ W	07/09/2021	D	2	3	Pool*	-
SA1438/21	A	F	GC	28°4′8.43″ N, 15°27′13.40″ W	12/11/2021	D	2	2	Pool*	-
SA1439/21	A	M	GC	28°4′8.43″ N, 15°27′13.40″ W	U	D	2	2	Pool*	-
SA1505/21	A	U	L	28°58′44.49″ N, 13°31′4.29″ W	26/11/2021	D	2	1	Pool*	-
SA1506/21	A	F	L	29°3′50.86″ N, 13°33′20.79″ W *	10/11/2021	D	3	1	Pool*	-
SA061/22	A	M	L	28°58′22.42″ N, 13°35′7.82″ W	10/12/2021	D	3	2	Lung	-
SA301/22	A	F	L	29°6′15.60″ N, 13°28′21.07″ W	27/02/2022	D	3	2	Pool*	-
SA1246/22	C	U	GC	28°4′8.43″ N, 15°27′13.40″ W	05/05/2022	D	3	3	Pool*	-
SA1267/22	A	F	GC	28°7′12.30″ N, 15°28′56.82″ W *	31/05/2022	A	3	2	Pool*	-
SA1269/22	J	U	GC	28°6′32.11″ N, 15°27′51.01″ W *	08/08/2022	A	2	U	Pool*	-
SA1277/22	U	M	GC	28°7′11.14″ N, 15°28′37.43″ W *	U	D		2	Pool*	-
SA1283/22	J	M	GC	27°55′14.36″ N, 15°24′50.89″ W	26/06/2022	A	2	2	Pool*	-
SA1365/22	A	F	H	27°42′36.42″ N, 17°59′55.14″ W *	U	D	3	2	Pool*	-
SA1508/22	J	M	GC	28°6′41.54″ N, 15°29′26.38″ W *	28/09/2022	D	2	3	Pool*	-
SA1642/22	A	M	L	28°59′51.72″ N, 13°36′13.03″ W *	03/10/2022	D	2	1	Pool*	-
FS229/23	J	U	L	28°54′0.03″ N, 13°50′1.64″ W	12/02/2023	D	2	1	Pool*	-
FS415/23	A	F	GC	28°6′10.56″ N, 15°41′59.18″ W *	05/05/2023	A	1	1	Pool*, Liver, kidney, lung, skin	*Leucocytozoon*. CIAE02 linage.
FS0453/23	A	M	L	29°5′4.78″ N, 13°39′55.37″ W	08/04/2023	D	2	2	Pool*	-
FS0546/23	A	F	GC	28°7′6.68″ N, 15°31′21.48″ W *	U	D	2	2	Pool*	-
FS499/24	A	M	GC	28°6′27.51″ N, 15°35′0.19″ W *	03/09/24	A	2	1	Pool*	-
FS601/24	A	M	GC	28°3′53.96″ N, 15°27′44.07″ W	25/06/24	A	2	1	Pool*	-

Notes: Pool* = Liver, kidney, lung; FD (finding date); FL (finding location: GC = Gran Canaria; T = Tenerife; F = Fuerteventura; L = Lanzarote); GC (Geographical coordinates, Latitude/Longitude (* Approximate coordinates by locality); S (status: A = alive; D = dead); DC (decomposition code); DC (1 = very fresh, 2 = fresh, 3 = incipient decomposition); BC (body condition); BC (1 = cachexia, 2 = thin (slim), 3 = good body condition, U = undetermined); Sex (F = female, M = male, U = undetermined), Age (A = adult, J = juvenile, C = chick, U = undetermined); Underlined = Positive individual.

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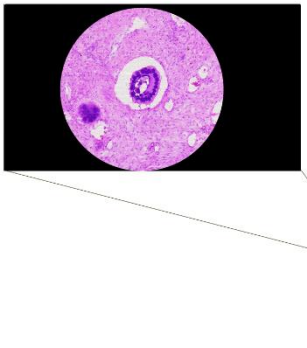
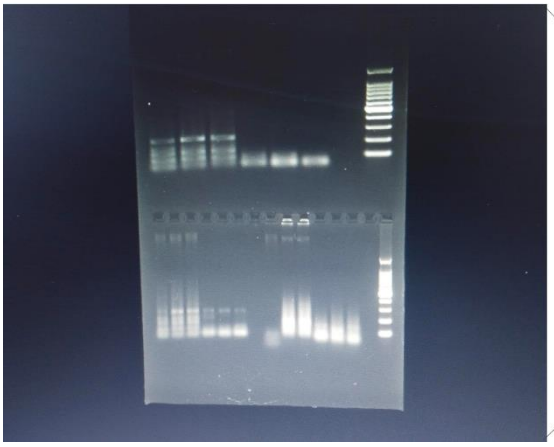
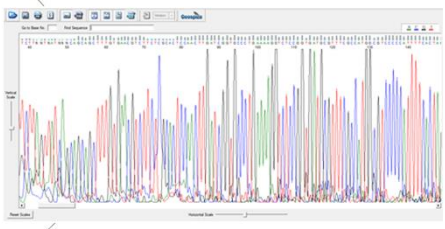
5. CONCLUSIONES







Conclusiones

1. La aplicación de técnicas moleculares en tejidos de alcaraván común obtenidos durante necropsias realizadas en el marco del programa Red Vigía durante el periodo 2020–2024 permitió detectar ADN de herpesvirus en el 8 % de los individuos analizados. Aunque esta prevalencia puede considerarse baja (<10 %), los resultados evidencian que la especie es susceptible a la infección por herpesvirus.
2. La detección de ADN de herpesvirus en una amplia variedad de muestras tisulares y de hisopos (hígado, riñón, pulmón, encéfalo, cloaca y orofaringe) sugiere que el virus no presenta un tropismo marcado por un tejido específico o que es capaz de diseminarse a múltiples órganos durante el curso de la infección.
3. La evaluación histopatológica no reveló lesiones compatibles con infección por herpesvirus en los individuos positivos, lo que sugiere una baja patogenicidad del virus detectado y/o un alto grado de adaptación del hospedador a la infección.
4. En todos los individuos positivos se obtuvo una secuencia idéntica que mostró una similitud nucleotídica inferior al 75 % con las secuencias de *Alphaherpesvirus* previamente descritas, lo que indica la detección de un herpesvirus novel en el alcaraván común.
5. La aplicación combinada de técnicas moleculares y el estudio histopatológico de lesiones cutáneas permitió identificar *Avipoxvirus* como agente causal en el 11,3 % de los ejemplares de alcaraván común analizados en el marco del programa Red Vigía de Canarias durante el periodo 2021–2024, una prevalencia compatible

con la descrita (10–20 %) en estudios focalizados o en especies susceptibles.

6. La infección por *Avipoxvirus* mostró un espectro de lesiones de severidad variable, con afectación preferente de las patas, lo que podría comprometer la movilidad en una especie de hábitos fundamentalmente esteparios.
7. La coinfección con *Aspergillus fumigatus* se confirmó en el 75 % de los ejemplares infectados por *Avipoxvirus*, lo que indica una elevada frecuencia de esta asociación y sugiere que puede constituir un factor agravante de la enfermedad, tanto a nivel local como sistémico, representando además la primera descripción de esta coinfección en el alcaraván común.
8. El análisis de secuencias del gen P4b, un marcador conservado del genoma de *Avipoxvirus*, permitió identificar la circulación de múltiples variantes genéticas infectando a los alcaravanes comunes, lo que pone de manifiesto la complejidad epidemiológica de la viruela aviar en una especie insular de interés para la conservación.
9. La aplicación combinada de técnicas moleculares y el estudio histopatológico en tejidos de 47 alcaravanes comunes analizados en el marco del programa Red Vigía durante el periodo 2020–2024 permitió detectar la presencia de *Leucocytozoon* sp. en el 2,1 % de los individuos, lo que indica una baja prevalencia de este hemoparásito en la muestra estudiada, especialmente en el contexto de hemoparásitos aviares en aves no paseriformes (<5 %).
10. La detección del linaje CIAE02 de *Leucocytozoon* en el alcaraván común amplía el rango de hospedadores conocido para este hemoparásito y constituye la primera evidencia de infección por *Leucocytozoon* en el género *Burhinus*.
11. La ausencia de una respuesta inflamatoria marcada asociada a las estructuras tisulares de *Leucocytozoon*, incluso en presencia de coinfecciones concomitantes (*Avipoxvirus* y *Aspergillus fumigatus*), sugiere un curso de infección subclínico, crónico o abortivo, lo que

pone de manifiesto la capacidad de estos hemoparásitos para pasar desapercibidos sin un enfoque diagnóstico integrador.

12. En conjunto, los estudios incluidos en esta tesis revelan una frecuencia global de detección de agentes infecciosos del 16,9 % en el alcaraván común del archipiélago canario, con presencia de coinfecciones, lo que sugiere una presión sanitaria moderada y una circulación persistente de distintos patógenos.
13. Estos resultados ponen de manifiesto la importancia de una vigilancia sanitaria basada en un enfoque diagnóstico integrador para la conservación de la especie y de sus subespecies endémicas, al permitir detectar infecciones crípticas, caracterizar la diversidad de los agentes implicados y reconocer manifestaciones patológicas poco frecuentes que podrían pasar desapercibidas mediante métodos convencionales.

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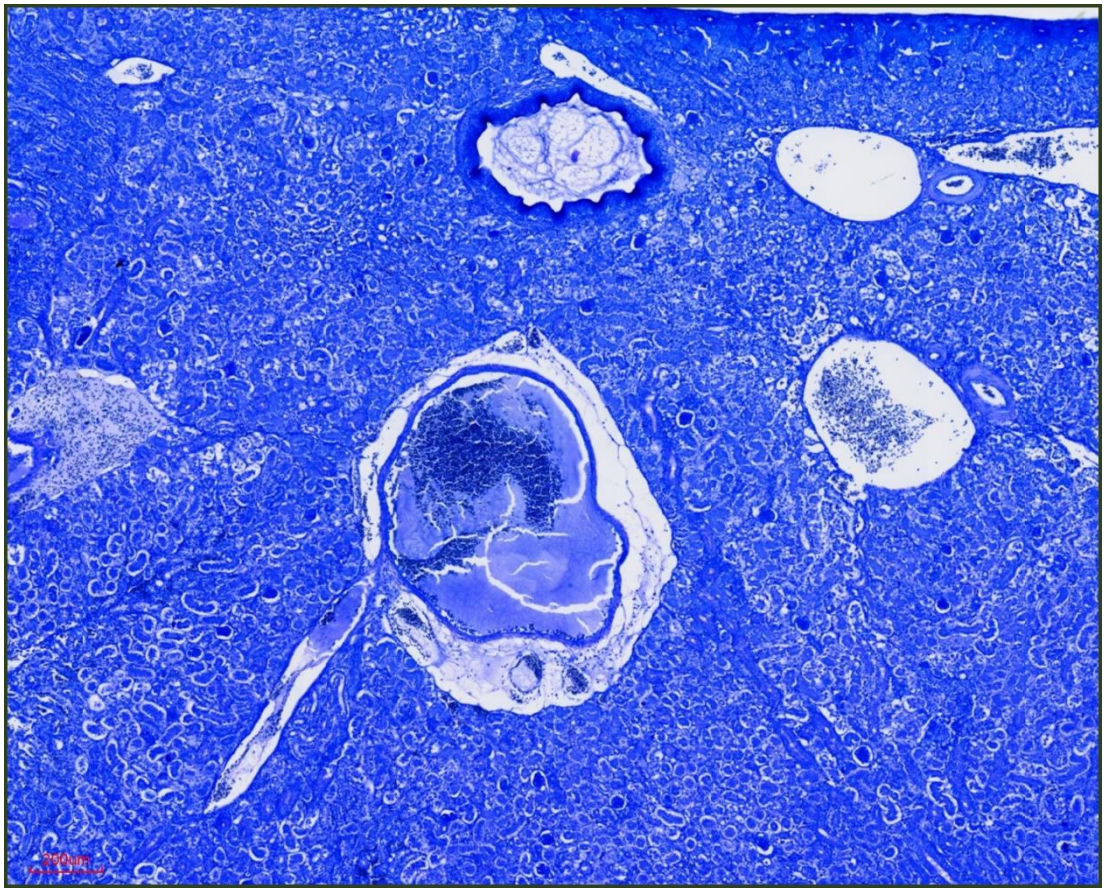

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