Capillary Electrophoresis Assessment of Plasma Protein Changes in an African Penguin (*Spheniscus demersus*) With Aspergillosis

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**ABSTRACT:** A decrease of avian biodiversity in the African continent has been the result of anthropogenic pressure in the region. This has resulted in the African penguin (*Spheniscus demersus*) being placed on the endangered species list and requires conservation efforts to maintain its free-ranging population and placement under managed care. In the latter environment, infection by *Aspergillus fumigatus* can be common. The diagnosis and treatment of this fungal disease in birds has presented with many difficulties, largely due to the diversity and limited knowledge that exists about this species. In this study, we implement a high-resolution capillary electrophoresis system for the fractionation of African penguin plasma, followed by mass spectrometry analysis for the identification of proteins associated with aspergillosis. Several protein differences were revealed, including changes in acute phase proteins and lipid metabolism. In addition, our results demonstrated that fibrinogen β chain is a protein largely present during the inflammatory process in an African penguin infected with *A. fumigatus*. These findings present a new avenue for the measurement of plasma proteins as a potential method for identifying important biomarkers to aid in monitoring African penguin health.

1. INTRODUCTION

Biodiversity in the African continent has been experiencing tremendous pressure during the last century, which has been largely propagated by an increase in anthropogenic pressure. From the seabird family, the most threatened members are the albatross (*Diomedeidae*) and the African penguin (*Spheniscus demersus*), which has experienced a 70% decrease in population from 2001 to 2013.²⁻⁴ Although conservation efforts have been relatively successful in retaining populations under managed care, other factors have emerged to threaten this endangered species. These include reproduction difficulties and infectious diseases among animals under human care in zoological institutions and aquaria of which aspergillosis is one of the most common diseases afflicting African penguins.³ Although previous efforts to monitor and diagnose this disease have met with difficulties, since birds are often present with nonspecific signs and the tests available do not provide certainty.⁴ Furthermore, treatment for aspergillosis has also met with many difficulties since there is limited knowledge about the efficacy and pharmacokinetics in different bird species.⁵

Despite these limitations, our group has documented an electrophoretogram skewed shift observed in the plasma of an African penguin with aspergillosis.⁶ However, the composition of this shift remains largely unknown. This study aims to bridge this gap in the literature by applying the high-resolution capacity of a capillary electrophoresis (CE) system, coupled with protein mass spectrometry analysis for the identification of the constituents contributing to this shift.

2. RESULTS AND DISCUSSION

Avian aspergillosis is a fungal disease caused by infection of the respiratory tract primarily mediated by *Aspergillus fumigatus*. Aspergillosis is among the most common disease afflicting birds in captivity and is one of the leading causes of mortality in this group.⁷ A previous report has documented the electrophoretic shift observed in African penguin plasma, but the protein composition of this shift remains under investigation.⁸

2.1. Capillary Electrophoresis Analysis. To address this gap in the literature, we implemented CE as our primary mode of separation, due to the high separation efficiency provided by CE when compared to other separation techniques. In addition, the high resolution of CE provides a direct approach for fractionating complex mixtures. CE was able to replicate the shift previously reported, which constituted a left skewed (normal) to right skewed shift (abnormal) (Figure 1A). The CE results demonstrated that this shift is primarily due to a change in the charge-to-frictional coefficient ratio, which can be interpreted as an increase in charge and a decrease in the

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mass of analytes. Such changes are driven by the fractionation of proteins, which reduce the molecular size and provide a larger surface area for ionization. However, it is important to recognize the limitations of this interpretation as proteins with a higher molecular mass can still have a low retention time when their charge state significantly overcomes the drag force provided by a higher molecular mass.

2.2. Mass Spectrometry Proteomic Analysis. To further analyze the composition of the analytes that contributed to this shift, this region was collected for the mass spectrometry analysis (shown in brackets, Figure 1A). Following mass spectrometry analysis using both the UniProt G. gallus database (Table 1) and the S. demersus database (Table 2), the comparison revealed a small number of high confidence proteins found in the CE fraction (Figure 1B). This small number of proteins provided a specific representation of the proteins found in the CE fraction; however, due to the limitations of the S. demersus database, the majority of the proteins founds using this database were not peer-reviewed and curated. To corroborate the results found in the UniProt database, the same analysis was done using the NCBI database. This analysis revealed more potential in the proteins found in the CE fraction; however, due to the limitations of the database, the same analysis was done using the NCBI database.

2.3. Statistical Analysis. To determine the variability between samples, a multivariable dimension-reduction analysis was implemented. To compute total (common and unique) variance and fit each component variance in a linear model, a principal component analysis (PCA) was done using proteomic results from the G. gallus analysis (Figure 2A, left panel). From this analysis, two features became prominent: (1) the cluster of individual samples represents the variability within that dataset (abnormal plasma contains a higher variability when compared to normal) and (2) the 95% confidence region between the normal and abnormal samples does not overlap, indicating that these samples are compositionally distinct. To validate a categorical distinction (normal vs abnormal), a bilinear factor model analysis was implemented. The partial least squares discriminant analysis (PLS-DA) demonstrated that there is a distinct categorical separation between the two datasets, as no overlap is observed between the normal and abnormal samples (Figure 2A, right panel).

2.4. Plasma Inflammatory Markers. Several proteins with significant changes in the abnormal plasma sample were identified using the chicken and African penguin databases (UniProt and NCBI). These changes included a significant increase in the fibrinogen β chain (FGB: F1NUL9, Q02020) in the abnormal plasma (Figure 2B). Although not statistically significant, fibrinogen α chain was also elevated in the abnormal plasma.

Additional changes included a significant decrease in albumin. As an important negative acute phase protein, decreases have been previously observed in birds with inflammatory processes and reported in African penguins with aspergillosis. Decreased ceruloplasmin was also noted; as it is a positive acute phase protein (APP) in the chicken, the low levels here may be related to the severity of the chronic infection in this penguin. This is contrasted by the increased levels of the positive APP haptoglobin. Glutathione peroxidase 3 levels were significantly decreased. In humans, this protein was found to be significantly decreased with severe inflammatory response syndrome. Changes in keratin levels are likely consistent with liver injury related to the disease process. Decreased levels vitellogenin and apolipoprotein may signify changes in lipid metabolic processes related to the disease process and/or inflammatory processes. Increased isocitrate dehydrogenase levels may also be associated with lipogenesis. The increase in prostaglandin E2 receptor EP4 also requires further investigation. Prostaglandin E2 is known to increase with inflammation. The EP4 receptor can be expressed in many tissues including platelets where it helps with controlling homeostasis. The presence of the EP4 receptor in the plasma may reflect tissue or cellular damage related to the aspergillosis disease process. A decrease in kininogen was observed; this protein has a role in coagulation and inflammation. Additional studies should be undertaken to examine repeated measures during infection to better understand the kinetics of these potential inflammatory biomarkers.

To understand the potential pathways over-represented in the abnormal sample, the PANTHER classification system was utilized using both the UniProt and NCBI G. gallus databases. These revealed that the abnormal sample has an increased activation of the blood coagulation pathway (raw $p$-value $= 3.75 \times 10^{-3}$ and FDR $= 6.04 \times 10^{-3}$), suggesting the potential role of clotting proteins in contributing to the shift observed in the CE electropherogram (Figures 1A and 2D).

2.5. Aspergillus Protein Analysis. Examination of the expression of protein relative to the Aspergillus protein
database revealed several uncharacterized proteins and many be involved in cellular processes (Figure 2C, right panel). Of possible note is a decrease in isocitrate lyase. This enzyme has been reported to aid the persistence of Mycobacterium infection and other infectious agents that persist in macrophages. Of note is a decrease in isocitrate lyase. This enzyme has been reported to aid the persistence of Mycobacterium infection and other infectious agents that persist in macrophages.15 In addition, increases were also observed in macrophages containing A. fumigatus conidia.16 A more detailed examination of the other proteins would be best studied in in vivo models.

Table 1. Capillary Electrophoresis Fraction Comparison between Normal and Abnormal Penguin Plasma Using G. gallus UniProt Protein Database

| UniProt Acc # | protein description | #PSMs | score MS Amanda |
|--------------|---------------------|-------|-----------------|
| unique to normal | A0A3Q2U743 | A2M_recep domain-containing protein | 79 | 6757.63 |
| | E1BQC2 | Ovotransferrin | 35 | 975.9 |
| | E1C7T1 | SERPIN domain-containing protein | 29 | 0 |
| | Q197X2 | Apolipoprotein B | 32 | 266.76 |
| | A0A1DSPWR4 | uncharacterized protein | 24 | 0 |
| | O93601 | Apolipoprotein AIV | 7 | 471.12 |
| | A0A146F0A0 | Keratin, type II cytoskeletal coechal | 13 | 788.66 |
| | F1NJP8 | uncharacterized protein | 7 | 471.12 |
| | A0A3Q2UKP2 | MG2 domain-containing protein | 19 | 418.74 |
| | E1BV78 | Fibrinogen C-terminal domain-containing protein | 6 | 206.84 |
| | A0A3Q2U324 | A2M domain-containing protein | 12 | 1093.8 |
| | F1NP5N | SERPIN domain-containing protein | 2 | 0 |
| | P51890 | Lumican | 6 | 0 |
| | Q90633 | Complement C3 | 19 | 13.51 |
| | A0A1DSPX29 | Uncharacterized protein | 13 | 38.63 |
| | A0A3Q2URG6 | Ig-like domain-containing protein | 26 | 1648.05 |
| | F1NX74 | uncharacterized protein | 11 | 0 |
| | Q6BCB8 | Vitellogenin (fragment) | 49 | 2596.24 |
| | F1NAK4 | TOG domain-containing protein | 15 | 358.21 |
| | A0A2U8UYC6 | BG protein | 11 | 0 |
| | A0A146F031 | Type II α-keratin IIB | 4 | 23.84 |
| | A0A11RHN7 | IF rod-domain-containing protein | 7 | 0 |
| | A0A11RRQ8 | SERPIN domain-containing protein | 5 | 0 |
| | A0A1DSPRL3 | uncharacterized protein | 14 | 121.24 |
| | F19121 | Serum albumin | 217 | 1751.1 |
| | P08250 | Apolipoprotein A-1 | 208 | 15973.42 |
| | F1NDN6 | Keratin 12 | 17 | 1395.89 |
| | A0A1DSPMQ5 | IF rod domain-containing protein | 39 | 3118.05 |
| | A0A11RJW5 | IF rod domain-containing protein | 18 | 2643.58 |
| | A0A11RWG9 | Keratin, type I cytoskeletal 19 | 23 | 1688.5 |
| | A0A11TPQ422 | Transferrin | 17 | 738.56 |
| | F1NUL9 | Fibrinogen β chain | 6 | 422.5 |
| | A0A11RRK4 | IF rod-domain-containing protein | 20 | 1743.7 |
| | F1P4V1 | Fibrinogen α chain | 5 | 302.83 |
| | E1BWR7 | J-domain-containing protein | 30 | 1552.64 |
| | A0A1DSPCD2 | A2M_recep domain-containing protein | 37 | 3310.88 |
| | A0A3Q3AU25 | TED_complement domain-containing protein | 23 | 2056.6 |
| | A0A146F047 | Type II α-keratin IIC | 30 | 1207.63 |
| | F1NPQ2 | Isocitrate dehydrogenase [NADP] | 6 | 547.32 |
| | Q5ZLC5 | ATP synthase subunit β, mitochondrial | 9 | 335.35 |
| | A0A1DSPQ9 | uncharacterized protein | 13 | 495.72 |
| | F1NDN5 | IF rod domain-containing protein | 14 | 1102.05 |
| | Q01406 | Src substrate protein p85 | 9 | 388.04 |
| | A0A3Q2UHJ9 | RMS-binding protein 2 | 11 | 126.64 |
| | A0A1DSPAG3 | FHA domain-containing protein | 10 | 509.57 |
| | F1NKX8 | uncharacterized protein | 14 | 702.97 |
| | A0A11RZ04 | IF rod-domain-containing protein | 22 | 608.59 |

“Proteins identified using the G. gallus protein database (Downloaded from UniProt on January 2020) were filtered for high confidence peptides. Comparison was made on Proteome Discoverer 2.0. Black Bold = proteins significantly identified in the volcano plot analysis.

2.6. Fibrinogen as an Inflammatory Marker. Despite the various potential markers identified using the NCBI database, the UniProt databased identified fibrinogen as the only statistically significant marker, which highlights the advantage of using the UniProt database as fibrinogen was manually curated and annotated in the database. The presence of fibrinogen also corroborates the PANTHER pathway findings as both the NCBI and UniProt databases provided the same results.
Fibrinogen is known to migrate in the β globulin fraction of agarose gel electrophoresis and may be, in part, responsible for the electrophoretic shift seen in penguins with aspergillosis. It is notable that fibrinogen β chain along with fibrinogen α chain is involved in the elevation of the blood coagulation pathway. In humans with infection by A. fumigatus, tissue necrosis and subsequent thrombosis (blood coagulation) have been reported. Coagulation is the process in which a series of molecular events leads to the formation of a blood clot, whose primary role is to maintain homeostasis, prevent excessive hemorrhage, and allow tissue repair. This process requires an intricate network of interactions between different molecular components and is separated into three major pathways: (1) intrinsic, (2) extrinsic, and (3) common. The activation of the intrinsic and extrinsic pathways intersects by the activation of prothrombin to thrombin, which in turn cleaves fibrinogen into fibrin monomers (Figure 2D). Mediation of the coagulation pathway is important in maintaining homeostasis but upon disruption pathological manifestations occur, which often results in disease.

There are many cellular and molecular components within this pathway but during fungal infection platelet activation can be induced by secreted fungal factors, which in turn stimulates platelet’s antifungal properties. These include increased sensitivity to foreign particles, inhibit fungal growth, and increase host’s immune response. Despite the benefits provided by platelet activation, some harmful effects can be considered, which include excessive inflammation and the production of thrombotic factors. Such factors include fibrinogen, a soluble glycoprotein synthesized in the liver composed of three separate polypeptide chains (Aα, Bβ, and γ). Fibrinogen plays a crucial role in the regulation and propagation of pathological conditions, given that different biological functions have been attributed to this protein. This diversity in biological function has been attributed to unique epitopes within fibrinogen that are associated with unique interactions and molecular cascades. This highlights fibrinogen as a pivotal regulator in pathological conditions making fibrinogen a potential target for therapeutic intervention.

Fibrinogen has previously been described in experimental models in chickens and identified as a positive acute phase protein in this species. This study is inclusive of increases with turpentine injection and in response to infection with Escherichia coli, Eimeria tenella, and Streptococcus. However, no increases were observed with infectious bursal disease virus. Despite the association of fibrinogen with inflammatory lesions, due to the lack of accurate fibrinogen detection methods, increases in fibrinogen in Gentoo penguins with an inflammatory lesion named bumblefoot were undetected. In addition, no increases were observed in recently captured Humboldt penguins and in Magellanic penguins, which were in rehabilitation facilities. This calls for the development of epitope-specific detection methods, such as the generation of antibodies that accurately recognize fibrinogen across multiple species (Figure 3). The area highlighted in red indicates a potential region that is conserved between multiple species and can be targeted for the development of antibody detection methods. By focusing on antibodies that recognize this region, an early detection method can be beneficial for monitoring the health of endangered species.
that there is a very distinct protein profile that requires further study. 

Hosts and Aspergillus proteins were also identified that also replicate the shift previously documented and was also able to be collected from penguin plasma. The CE system was able to accurately collect the constituents comprising this shift. A shotgun proteomics approach was able to identify fibrinogen as the constituent most likely responsible for this shift; however, this shotgun proteomics approach comes with its limitations. These include a general protein profile that only takes into consideration major protein changes, which does not explain minor changes that can lead to the overall effect. To elucidate a more detailed proteomic profile, the isobaric tags for relative and absolute quantitation (iTRAQ) protein mass spectrometry is required for more specific and confident results. This method was implemented in a previous study comparing African penguins with aspergillosis at different disease time points. However, fibrinogen was not able to be detected, which can be due to the variation in disease severity between samples.

### Table 3. Capillary Electrophoresis Fraction Comparison between Normal and Abnormal Penguin Plasma Using G. gallus NCBI Protein Database

| A/N | NCBI Acc # | protein description | #PSMs | score MS Amanda |
|-----|------------|---------------------|-------|-----------------|
| unique to normal | BAE16337.1 | ovotransferrin BB type | 47 | 3170.43 |
| (-) | NP_001264422.1 | α-1-antitrypsin precursor | 37 | 1895.87 |
| (-) | ABF70173.1 | apolipoprotein B | 43 | 1726.98 |
| (-) | XP_418162.6 | keratin, type I cytoskeletal 19 | 29 | 2282.34 |
| (-) | CAA76273.1 | apolipoprotein AIV | 13 | 1011.79 |
| (+) | ITPF | α Chain A, transthyretin | 35 | 0 |
| (-) | NP_001156704.1 | glutathione peroxidase 3 precursor | 10 | 1244.25 |
| (-) | NP_001263286.1 | lumican precursor | 11 | 698.47 |
| (-) | XP_015143259.1 | α-1-antitrypsinase isoform X1 | 4 | 399.12 |
| (-) | NP_990320.2 | fibrinogen γ chain precursor | 9 | 452.51 |
| (-) | BAD32701.1 | vitellogenin, partial | 5 | 252.01 |
| (+) | AAA64694.1 | complement C3 precursor | 25 | 786.7 |
| (+) | XP_004937173.1 | dnah homolog subfamily B member 5 isoform X1 | 68 | 4311.68 |
| (+) | XP_004935636.2 | utrophin isoform X1 | 20 | 1278.33 |
| (+) | AAA92202.1 | type 1 α-keratin 14 | 14 | 1155.4 |
| (+) | XP_025006915.1 | α-1-antitrypsin isoform X1 | 10 | 611.66 |
| (+) | XP_015130883.1 | eIF-2-α kinase activator GCN1 isoform X1 | 16 | 420.96 |
| (+) | AAA48630.1 | B-G | 12 | 50.99 |

| shared between normal and abnormal |
|-------------------------------------|
| (-) | NP_990592.2 | albumin precursor | 355 | 35948.54 |
| (-) | AAA48592.1 | apolipoprotein A-I precursor | 265 | 30932.8 |
| (+) | XP_024998571.1 | α-2-macroglobulin isoform X1 | 156 | 13563.35 |
| (+) | XP_015128103.1 | keratin, type II cytoskeletal 8 | 15 | 549.28 |
| (-) | NP_990736.3 | complement C3 | 36 | 2155.21 |
| (+) | BAU68261.1 | keratin, type II cytoskeletal cochleal | 13 | 549.28 |
| (+) | NP_001161155.1 | fibrinogen β chain precursor | 12 | 869.42 |
| (+) | NP_990340.2 | keratin, type I cytoskeletal 19 | 15 | 1163.59 |
| (+) | XP_015158591.1 | keratin, type II cytoskeletal 6B-like | 17 | 1400.32 |
| (+) | NP_001258801.1 | fibrinogen α chain isoform 1 precursor | 14 | 639.03 |
| (+) | XP_004998050.1 | α-2-macroglobulin-like | 58 | 4909.92 |
| (+) | XP_00123972.1 | keratin, type I cytoskeletal 19 | 40 | 3118.05 |
| (+) | NP_0001026652.2 | ATP synthase subunit β, mitochondrial precursor | 9 | 335.35 |
| (+) | XP_025000170.1 | isocitrate dehydrogenase [NADP] cytoplasmic | 7 | 552.07 |
| (+) | JAS03214.1 | GTPase activator GCN1 isoform X1 | 7 | 491.9 |
| (+) | NP_000103112.2 | mitochondrial | 14 | 1102.05 |
| (+) | NP_001268427.1 | keratin, type I cytoskeletal 15 | 17 | 738.56 |
| (+) | Q01406.1 | transthyretin | 8 | 388.04 |
| (+) | XP_015131556.2 | Srrc substrate protein p85, Cortactin, P80 | 11 | 702.97 |
| (+) | XP_015139455.1 | arf-GAP with SH3 domain, H+ transporting, part I | 10 | 509.57 |

Proteins identified using the G. gallus protein database (Downloaded from NCBI on September 2020) were filtered for high confidence peptides. Comparison was made on Proteome Discoverer 2.0. A/N = relative abundance ratio between abnormal and normal plasma, + = increase in abnormal, and − = decrease in abnormal.

### 3. CONCLUSIONS

Our findings support the premise that monitoring thrombotic factors such as fibrinogen and in particular fibrinogen β chain is of important clinical relevance in monitoring the health status of African penguins in captivity. Currently, there are no standardized methods of fibrinogen measurement in avian species although an imprecise method used in older veterinary research studies is a manual heat precipitation method, which presents with many inaccuracies. Importantly, several other hosts and Aspergillus proteins were also identified that also require further study.

The methodology implemented in this study demonstrates that there is a very distinct protein profile that contributes to the electropherogram shift between normal and abnormal penguin plasma. The CE system was able to accurately replicate the shift previously documented and was also able to collect the constituents comprising this shift. A shotgun proteomics approach was able to identify fibrinogen as the constituent most likely responsible for this shift; however, this shotgun proteomics approach comes with its limitations. These include a general protein profile that only takes into consideration major protein changes, which does not explain minor changes that can lead to the overall effect. To elucidate a more detailed proteomic profile, the isobaric tags for relative and absolute quantitation (iTRAQ) protein mass spectrometry is required for more specific and confident results. This method was implemented in a previous study comparing African penguins with aspergillosis at different disease time points. However, fibrinogen was not able to be detected, which can be due to the variation in disease severity between samples.
Table 4. Capillary Electrophoresis Fraction Comparison between Normal and Abnormal Penguin Plasma Using *S. demersus* NCBI Protein Database

| A/N | NCBI Acc # | protein description | #PSMs | score MS Amanda |
|-----|------------|---------------------|-------|-----------------|
| unique to normal | | | |
| (-) | KAF1443202.1 | hypothetical protein FQV21_0001894, partial | 66 | 3243.8 |
| (-) | KAF1460030.1 | Apolipoprotein A-IV, partial | 41 | 2831.5 |
| (-) | KAF1440412.1 | Vitamin α-binding protein, partial | 35 | 2665.58 |
| (-) | KAF1437930.1 | Glutathione peroxidase 3, partial | 48 | 4027.82 |
| (-) | KAF1451635.1 | α-1-antiprotease 2, partial | 29 | 2240.39 |
| (-) | KAF1462588.1 | Complement C4, partial | 12 | 704.55 |
| (-) | KAF1462353.1 | Antithrombin-III, partial | 17 | 749.18 |
| (-) | KAF1448111.1 | IgGFc-binding protein, partial | 7 | 419.08 |
| (-) | KAF1445884.1 | Lumican, partial | 12 | 705.35 |
| (-) | KAF1448559.1 | Kininogen-1, partial | 9 | 607.52 |
| (-) | KAF1440420.1 | Immunoglobulin J chain, partial | 6 | 404.59 |
| (-) | KAF1436065.1 | α-1-antiproteinase 2, partial | 5 | 446.56 |
| (-) | KAF1442536.1 | α-2-antiplasmin, partial | 7 | 418.29 |
| (-) | KAF1461885.1 | Complement C4, partial | 12 | 704.55 |
| (-) | KAF1462355.1 | Antithrombin-III, partial | 17 | 749.18 |
| (-) | KAF1448583.1 | α-2-HS-glycoprotein, partial | 2 | 155.5 |
| (-) | KAF1433771.1 | α-2-macroglobulin, partial | 6 | 215.12 |
| (-) | KAF1450162.1 | Ceruloplasmin, partial | 16 | 1011.78 |
| (-) | KAF1448853.1 | α-2-macroglobulin, partial | 16 | 298.93 |
| (-) | KAF1461872.1 | Vitellogenin-2, partial | 15 | 250.62 |
| (-) | KAF1433539.1 | Fibrinogen α chain, partial | 7 | 419.08 |
| (-) | KAF1437685.1 | Vitronectin, partial | 3 | 0 |
| (-) | KAF1450162.1 | Ceruloplasmin, partial | 16 | 1011.78 |
| (-) | KAF1448583.1 | α-2-HS-glycoprotein, partial | 2 | 155.5 |
| (-) | KAF1460517.1 | Keratin, type II cytoskeletal cochleal, partial | 11 | 562.27 |
| (-) | KAF1440393.1 | Serum albumin, partial | 882 | 90551.6 |
| (-) | KAF1461539.1 | Ovotransferrin, partial | 428 | 29264.01 |
| (-) | KAF1455946.1 | Apolipoprotein A-I, partial | 478 | 49003.21 |
| (-) | KAF1433561.1 | α-2-macroglubulin, partial | 117 | 12637.97 |
| (-) | KAF1451546.1 | α-1-antiproteinase 2, partial | 129 | 11830.1 |
| (-) | KAF1464563.1 | α-2-macroglobulin, partial | 41 | 5923.86 |
| (-) | KAF1464562.1 | Pregnancy zone protein, partial | 48 | 4907.35 |
| (-) | KAF1433885.1 | α-2-macroglobulin, partial | 75 | 6403.21 |
| (-) | KAF1446481.1 | Transthyretin, partial | 31 | 847.92 |
| (-) | KAF1432482.1 | hypothetical protein FQV21_0005409 | 22 | 2007.11 |
| (+) | KAF1460518.1 | Keratin, type II cytoskeletal 75, partial | 23 | 1688.01 |
| (+) | KAF1460516.1 | Keratin, type II cytoskeletal 75, partial | 24 | 1637.88 |
| (+) | KAF1463905.1 | Vascular non-inflammatory molecule 2, partial | 6 | 529.87 |
| (+) | KAF1442749.1 | Haptoglobin, partial | 79 | 6603.76 |
| (+) | KAF1460527.1 | Keratin, type II cytoskeletal 6A, partial | 40 | 3009.32 |
| (+) | KAF1440571.1 | Isocitrate dehydrogenase [NADP] cytoplasmic, partial | 6 | 547.32 |
| (+) | KAF1435572.1 | Transthyretin, partial | 20 | 1251.63 |
| (+) | KAF1443465.1 | Fibrinogen γ chain, partial | 7 | 491.9 |
| (+) | KAF1455495.1 | ATP synthase subunit α, mitochondrial, partial | 7 | 1482.1 |
| (+) | KAF1457308.1 | hypothetical protein FQV21_0013721, partial | 13 | 404.11 |
| (+) | KAF1450900.1 | Src substrate protein p85, partial | 11 | 292.36 |
| (+) | KAF1437491.1 | ATP synthase subunit β, mitochondrial, partial | 8 | 491.06 |
| (+) | KAF1437567.1 | hypothetical protein FQV21_0002520, partial | 59 | 2059.02 |
| (+) | KAF1440713.1 | Protein bassoon, partial | 13 | 633.47 |
| (+) | KAF1462098.1 | Titin, partial | 30 | 669.19 |
| (+) | KAF1440406.1 | Ras GTPase-activating protein nGAP, partial | 10 | 248.84 |
| (+) | KAF1445851.1 | Ankyrin repeat domain-containing protein 17, partial | 15 | 511 |
| (+) | KAF1446351.1 | TBC1 domain family member 8B, partial | 11 | 19.3 |

Note: Shares the NCBI Acc # with Table 3.

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33285
Additional studies should be undertaken with other repeated measures from African penguins with aspergillosis. Of note, the current sample was obtained after the start of antifungal and very close to death. Samples provided earlier in the disease process may provide information on other biomarkers. Overall, while many of these observed protein changes are not specific for aspergillosis, they do reflect a greater understanding of the pathogenesis of aspergillosis and the acute phase response in this species. Although mass spectrometry is able to elucidate protein changes in avian plasma samples, the amount of time required to process and analyze samples often comes at the cost of the lives of these endangered species. The African penguin along with other avian species that are endangered require a quick and precise approach for monitoring their health decline and conservation. Mass spectrometry is not a cost-effective method that can be used in the field, but it only provides an avenue for the development of methods that can focus on a quick and accurate approach for monitoring health decline in endangered avian species.

### 4. EXPERIMENTAL SECTION

#### 4.1. Acquisition of African Penguin (S. demersus) Samples

A 15 year old female penguin under managed care (Brookfield Zoo, Chicago, IL) was presented with weight loss over a 3 month period. A complete blood count revealed a significant leukocytosis and radiographs supported the presence of a respiratory infection. The animal received treatment with antibiotics and antifungal medication. It was noted that the appetite improved and attitude remained good. On recheck approximately 1.5 weeks later, a mild weight gain was present, the body condition was still poor, and the leukocytosis was improved but still present. Three days later,
the animal was observed to have respiratory difficulty but still ate; the following day, the animal was found dead. Necropsy confirmed the presence of systemic aspergillosis. The samples that were used in this project included plasma obtained on the recheck date and a banked plasma sample approximately one year previous when the animal was in good health.

4.2. Separation via Capillary Electrophoresis (CE).

Fractions were separated using the Agilent Capillary Electrophoresis 7100 coupled with a bare fused silica capillary tube (Agilent, G1600−63311). CE buffer consisted of 100 mM formic acid, 5 mM ammonium acetate, and 100 mM Tris at a pH of 7.3. The sample was prepared at a 1:10 ratio of raw penguin plasma to CE buffer. Parameters for the CE system included sample injection at 50 mbar for 10 s, high voltage at 30 KV, current set at 10 μA, power set at 6 W, and cassette temperature of 25 °C, coupled with a high-pressure system of 10 mbar for the duration of the separation (60 min). Signal was detected using UV absorbance at 280 nm (bandwidth 4 nm) and no reference wavelength. Fractions were collected in separate collection tubes from the region represented by brackets in the CE electropherogram (Figure 1).

4.3. Sample Preparation for Protein Mass Spectrometry.

Samples from CE fraction were added 4 times their volume of acetone at −20 °C and incubated overnight (about 16−18 h) at −20 °C. Samples were centrifuged at 10 000 g at 4 °C for 10 min (Beckman Microfuge 18). The supernatant was discarded and the pellet (very small) was air-dried for 10 min. The sample was dispersed in 8 μL of 0.1% (v/v) formic acid in ultrapure water, corresponding to a ratio of 5:100 (v/v) of 50% formic acid to sample volume. Samples were either stored at −20 °C or immediately processed for desalting and protein enrichment.

Desalting and protein enrichment was carried out using the Pierce Graphite Spin Columns (Thermo, 88302) using the manufacturer's recommendations. Samples were evaporated using a CentriVap concentrator system (Labconco, 7810016) coupled with a CentriVap cold trap (Labconco, 7811020) and resuspended in 30 μL of 0.1% (v/v) formic acid in ultrapure water.
formic acid, 2% (v/v) acetonitrile, in mass spectrometry grade ultrapure water.

4.4. High-Performance Liquid Chromatography-Mass Spectrometry. Mass spectrometry proteomics was carried out as described in detail in our previous publication for a total of 3 technical replicates. In brief, mass spectrometry was performed on a Q-Exactive instrument after fractionation on a coupled Easy nLC 1000 nano-liquid chromatography system (Thermo Fisher Scientific). This was equipped with the Acclaim PepMap C18 RSLC Analytical column, Nano Viper, 75 μm x 15 cm diameter and length, 2 μm particle size (Cat. # 164534, Thermo Fisher Scientific). The following gradient was set up using Thermo Scientific Xcalibur (Version 4.1.31.9, Released 2017), 20 min of 2% solvent B, 20 min of 30% solvent B, 15 min of 40% solvent B, 20 min of 70% solvent B, and 30 min of 98% solvent B, all at a constant rate flow of 350 nL/min. Data was acquired using Thermo Scientific Xcalibur software (Version 4.1.31.9, Released 2017), proteins were identified and analyzed using Proteome Discoverer 2.2 (Version 2.2.0.388, Released 2017) and MS Amanda 2.0 (Version 2.0, Released 2017), UniProt and NCBI sequence databases were used for the identification of proteins G. gallus, S. demersus, and A. fumigatus (UniProt: downloaded January 2020, NCBI: downloaded September 2020). Proteome Discoverer search parameters for trypsin-digested proteins (max missed cleavage sites: 2, min. peptide length: 6, max peptide length: 144), dynamic post-translational modifications: oxidation +15.995 Da (M), acetylation +42.011 Da (N-term), static post-translational modifications: carbamidomethylation +57.021 Da (C), max modification per peptide: 3, precursor mass tolerance: 10 ppm, fragment mass tolerance: 0.02 Da, signal/noise threshold for spectra: 1.5, false discovery rate: strict for PSMs 0.01, strict for peptides 0.01. In brief, false discovery rates are calculated as follows: first, the software ascertains whether there are q-values and PEPs available for PSMs. If so, the software uses them and assigns the PSM confidences based on Target FDRs for PSMs. Next, the software calculates q-values and PEPs for peptides engaging the Quality algorithm. Peptide confidence is then assigned based on Target FDRs for peptides. If there are no q-values and PEPs available for PSMs, the PSM confidence is set based on our defined Target FDRs for PSM employing the respective search engine scores.

4.5. Statistical Analysis. Data from Proteome Discoverer was analyzed using MetaboAnalyst 4.0. Data was normalized to a generalized logarithm transformation. The multivariate principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) displayed a 95% confidence region with specific principal component 1 on X-axis and principal component 2 on X-axis. The volcano plot analysis had a fold change threshold of 1.0, a comparison type ratio of abnormal to normal, nonparametric test, p-value threshold of 0.001 (raw data), and equal group variance. A PANTHER overrepresentation test for pathway analysis was carried out using the PANTHER classification system (PANTHER version 15, released February 2020). The G. gallus reference database was used for this analysis using Fisher's exact test, correcting using the calculated false discovery rate and setting threshold for a false discovery rate of p < 0.05.

ASSOCIATED CONTENT

Accession Codes
Raw mass spectrometry data was deposited on PRIDE Archive (Project accession: PXD018096).

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ABBREVIATIONS
CE, capillary electrophoresis; PCA, principal component analysis; PLS-DA, partial least squares discriminant analysis; PC, principal component; EPF, electrophoretic force; EOF, electroosmotic flow; APP, acute phase protein.
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