Marine Biology (2021) 168:75
https://doi.org/10.1007/s00227-021-03876-1

ORIGINAL PAPER

Phenotypic plasticity in *Pygoscelis adeliae* physiology and immunity under anthropogenic pressure: a proteomic and biochemical scenario

Andrés E. Ibañez[1,2] · Carla Di Fonzo[3] · Diego Torres[1] · Martín Ansaldo[3] · Julieta Fernández[4] · Diego Montalti[1,2,3]

Received: 18 December 2020 / Accepted: 9 April 2021 / Published online: 24 April 2021

Abstract

The capacity of seabirds to shape their physiological and immune phenotypes may often be constrained by the ecological context. While phenotypic plasticity in physiological traits has been previously studied, the molecular mechanisms underlying phenotype plasticity in response to environmental stress have been little explored. This prompted us to enquire about how the nutritional and immune status are involved in physiological adaptations in breeding seabirds under anthropogenic pressure. At Esperanza (Hope) Bay, Antarctic Peninsula exists one of the biggest breeding penguin colony of *Pygoscelis adeliae* (Adélie). At this location, penguins nest nearby the Argentinian Esperanza Station and, therefore, are exposed to high levels of disturbance, whereas there is a low disturbed area, where penguins also breed far away from the Station. In both areas, the nutritional and immune status in breeding individuals was addressed and serum protein expression level was analyzed using a proteomic approach. Body mass, proteins, albumin, and triacylglycerol were higher in penguins from the low disturbance area, whereas uric acid increased in individuals from the disturbed area, indicating a poorer body condition of penguins under anthropogenic pressure. Immune responses were elevated in penguins from the disturbed area (IgY, γ-globulins and hemagglutinating activity). Finally, individuals breeding under anthropogenic pressure overexpressed proteins with immune, antioxidant, and metabolic functions. The poor nutritional status of penguins under disturbance may be the consequence of the reallocation of resources to the immune system. Altogether, this would constitute a potential strategy to preserve an adequate immune phenotype under stressed environments.

Introduction

Organisms can confront environmental changes through physiological plasticity (i.e., the expression of different phenotypes by the same genotype in different environments), which allow them to conduct different annual processes successfully (Gremillet and Charmantier 2010; Wingfield et al. 2017). Seabirds display great sensitivity to environmental changes and, therefore, provide an appropriate model to study variations in phenotypes (Burger and Gochfeld 2004). Given the changing degree of anthropogenic pressure, it is expected that seabirds display distinct physiological and immune phenotypes (Levy et al. 2020). In addition, if seabirds are to be used as long-term environmental bioindicators, the strength of the phenotypic plasticity in a population should be sustained across time (Gremillet and Charmantier 2010). This approach requires an understanding of the relationships of seabird physiological parameters with other ecosystem components; however, this information still remains scarce. In our study, we sought to test the hypothesis that anthropogenic activity level can drive distinct patterns of

Responsible editor: V. Paiva.

Reviewers: M.C. Gatt and undisclosedexperts.

Julieta Fernández and Diego Montalti equally contributed.

✉ Andrés E. Ibañez
aeibanez@fcnym.unlp.edu.ar

[1] Sección Ornitología, Div. Zool. Vert. (FCNyM-UNLP), Paseo del Bosque s/n, (B1900FWA), La Plata, Buenos Aires, Argentina

[2] Centro Científico Tecnológico del Consejo Nacional de Investigaciones Científicas y Técnicas (CCT-CONICET-La Plata), La Plata, Argentina

[3] Laboratorio de Ecofisiología y Ecotoxicología, Instituto Antártico Argentino, 25 de Mayo 1143, (B1650HMK) San Martín, Buenos Aires, Argentina

[4] Instituto de Biotecnología y Biología Molecular (IBBM)-CCT-CONICET-La Plata, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina

physiological and immune phenotypes, using *Pygoscelis adeliae* as a model species.

Seabirds are one of the most threatened groups of birds (Días et al. 2019). Several drivers of these threats occur at the colony due to human activity, whereas others occur at sea (e.g., incidental mortality (bycatch) in fisheries, and overfishing affecting resource availability). In addition, in sites where seabirds conduct most of their life cycle processes, several environmental factors that may induce changes in components of their fitness occur (Studds and Marra 2005; Cockrem 2007). Among them, we can mention the anthropogenic activity, the density of the colonies and the quality of resources availability (Mallory et al. 2010).

Immune function is increasingly being studied in the context of life-history processes of seabirds (Norris and Evans 2000; Evans et al. 2015; Kulascewicz et al. 2017; Parker et al. 2017; Palacios et al. 2018; Gatt et. al. 2021). The immune system is constituted by two arms: innate and adaptive defenses (Mauck et al. 2005). The development of adaptive immune defenses (Immunoglobulin Y, IgY) is a time-dependent exposure-driven process, during which the individual interacts with pathogens and develops an appropriate immune response. In contrast, innate immunity (complement factors, natural antibodies, acute phase proteins (APPs), and antibacterial proteins) relies on pattern recognition receptors against highly conserved elements expressed by pathogens (McDade et al. 2016). Seabirds are challenged by pathogens in their surrounding environments; however, this pressure can vary with the location (Levy et al. 2020). The ability to shape distinct immune phenotypes may affect the type of response against pathogens, individual survival and reproductive success (Råberg and Stjernman 2003; Mauck et al. 2005). Under anthropogenic pressure, the presence of different stress factors (pathogens and pollutants) is known to shape the immunological phenotype (Barbosa et al. 2013; Khan et al. 2020). Therefore, in this context, the issue of optimizing immune responses gains relevance because of the trade-offs between investment in annual cycle events, individual survival and immune system (Norris and Evans 2000; Parker et al. 2017).

Colonial seabirds display great site fidelity and, therefore, are vulnerable to human activity at the colony (Nisbet 2000). Anthropogenic pressure may induce behavioral and physiological changes in seabirds (Ropert-Courdet et al. 2019). Among behavioral changes, variations in the activity time, locomotion, and agonistic behavior were described under the effects of disturbance factors. Ultimately, these changes affected physiological traits, foraging ecology, and the energy expenditure and success of reproduction (Rodgers and Smith 1995; Tarr et al. 2010; Weismerkirch et al. 2018; Ropert-Courdet et al. 2019). Another threat for breeding seabirds in coastal areas, are the human discharges which contain potentially pathogenic bacteria and other parasites that contribute to the local microbial load (Barbosa and Palacios 2009; Barbosa et al. 2013; Khan et al. 2019). Penguins are known to carry human associated microbial pathogens as *Salmonella* spp., *Campylobacter* spp., *Pasteurella multocida*, *Chlamydia* spp., *Escherichia coli* and *Borrelia burgdorferi* (Barbosa and Palacios 2009; Barbosa et al. 2013; Mykhaylenko et al. 2020). However, there is scarce information about the impact of human pathogens on penguin's health and their prevalence in Antarctic regions (Grimaldi et al. 2015; Ropert-Courdet et al. 2019; Mykhaylenko et al. 2020).

Adélie Penguins breed around the entire Antarctic continent during the austral summer on ice-free areas. They display a long breeding season and high site fidelity (Tella 2002). Usually, breeding areas have been also used for the establishment of research stations and the development of touristic activities (Patterson et al. 2003; Bender et al. 2016); therefore, penguins and humans often occur together, raising concerns about the effects of anthropogenic activities on penguin populations. The Adélie Penguin colony at Esperanza (Hope) Bay, Antarctic Peninsula, is one of the largest in Antarctica, gathering 104,000 breeding pairs (Santos et al. 2018). At this location, some breeding groups are distributed close to an Argentinian research station (Esperanza Station) and thus, exposed to a high level of human disturbance, such as direct contact with people, the release of heavy metals and organic pollutants derived from waste disposal sites, materials for construction, chemical compounds used for effluent treatment, noise, and introduced pathogens derived from human discharges (Leotta et al. 2002; Barbosa et al. 2013). Other groups in the colony breed far away from the research station and are exposed to a lower level of disturbance. In this scenario, penguins that breed close to humans have to deal with anthropogenic factors; therefore, it is expected that penguins have shaped their physiological and immune phenotype to compensate for the greater exposure to pollutants and the risk of infection by a broader diversity of pathogens.

Among eco-immunologists, there is increasing interest to understand the underlying mechanisms involved in the phenotypic plasticity in organisms' physiology (Hegemann et al. 2012; Levy et al. 2020). As most of the biological processes are regulated by proteins, the characterization of protein patterns in tissues and body fluids has become important for providing information on biological processes that are still poorly understood (Bertile et al. 2016). For this, in recent years, new methodologies have been developed within physiological research, such as proteomic studies (Grabek et al. 2015). Few works have applied these approaches in physiological studies on wild organisms (Bertile et al. 2009, 2016; Epperson et al. 2010; Grabek et al. 2015; Phillips et al. 2020). To further understand about the molecular components and mechanisms involved in the physiological and immune plasticity, in the present work, we determined the

immune and nutritional status, combined with a proteomic approach, in Adélie penguins exposed to contrasting levels of anthropogenic pressure. We expected to find differences in the expression pattern of proteins involved in the regulation of immune responses and with energy metabolism functions. Altogether, this study provides insights into how differences in physiological and immunological traits mediate phenotypic plasticity.

Materials and methods

Study area and sample collection

At Esperanza (Hope) Bay, Antarctic Peninsula (63°24'S, 57°01'W), the Research Station Esperanza has been established more than 60 years ago, showing human activity during the entire year. During the Antarctic summer, when most birds are breeding, the human population reaches a maximum number of approximately 100 people. In this area, it was reported that several anthropogenic pollutants contribute to local impacts such as oil spills and paint, heavy metals coming from waste disposal sites, materials used in constructions, chemical compounds used for effluent treatment, and human discharges to the coastal area (Acero et al. 1996; Corsolini et al. 2008; Cebuhar et al. 2017). It is possible to find oil slicks in the surroundings of the station derived from the fuel used to provide electric service. In addition, aircraft and helicopters used for logistics and transportation fly over the penguin colony during the breeding season contribute to another type of disturbance (Carlini et al. 2007).

The penguin colony at Esperanza (Hope) Bay is one of the largest in Antarctica (Santos et al. 2018). At this site, Adélie Penguin breeds in a colony nearby to the Research Station and exposed to high levels of human disturbance, indicating that penguins have been habituated to human presence for at least 60 years. On the other hand, few kilometers away, there is a non (or low)-disturbed area, geographically protected and with no nearby stations, where penguins breed during these months (Carlini et al. 2007). Penguins were sampled in zones differentially disturbed: high disturbance (HDZ) and low disturbance (LDZ) (Fig. 1).

During the Antarctic summer, from December to February of seasons 2014–2015 and 2015–2016, samples were

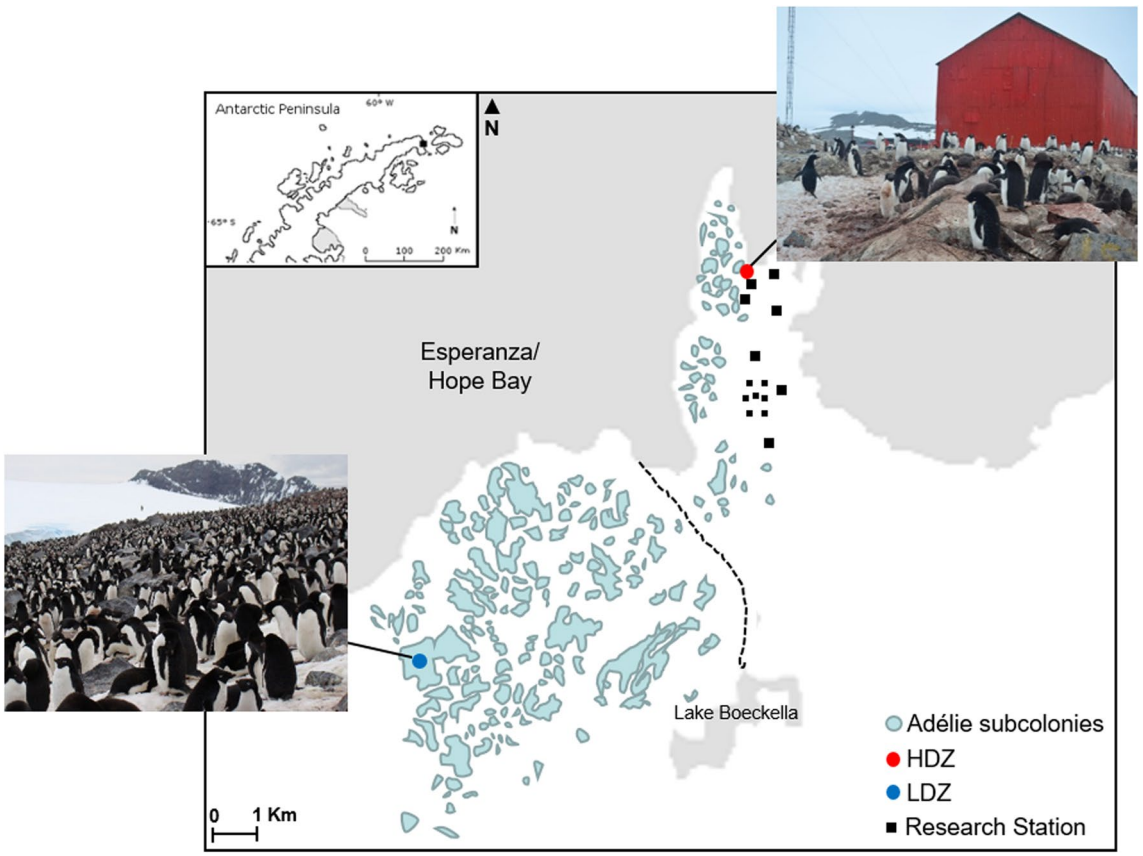

Fig. 1 Map of the study area at Esperanza/Hope Bay, Antarctic Peninsula (63°24'S, 57°01'W). Red and blue points represent the sampling point in the colony in "High disturbance zone" HDZ (63°23'S, 56°59'W) and "Low disturbance zone" LDZ (63°24'S, 57°00'W), respectively. The dotted line represents a geographic point, where the Adélie Penguin (*P. adeliae*) colony is divided in the HDZ and LDZ

extracted from adult breeding individuals 6–10 days after egg hatching (hatching dates span approximately 3 weeks), assuring that when removing the adult from the nest both chicks can thermoregulate (Olmastroni et al. 2004). Breeding individuals were randomly selected in each zone, and only nests with two hatched chicks were considered at the moment of sampling to avoid differences associated with parental effort. Of note, in both seasons individuals were sampled in the same sampling point from each area (HDZ = 63°23'S, 56°59'W; LDZ = 63°24'S, 57°00'W). Penguins ($n = 60$; $n_{HDZ} = 30$ and $n_{LDZ} = 30$) were extracted manually from the nest, and chicks were protected from predators. To take blood, penguins were held with the flippers restrained and the head placed under the arm of the handler preventing movement, to minimize stress during handling. A second handler took up to 3 ml blood from the metatarsal vein using a 22-G needle and 5 ml syringe; finally, the area was cleaned with an alcohol swab. During each season, $n = 30$ individuals were sampled (15 penguins from each zone). To avoid variation in blood parameters due to handling, blood was extracted within 3–5 min after capture. Finally, the individuals were weighed. After that, half of the blood was incubated for 4 h at 4 °C and the other half was introduced in a tube with heparin kept at 4 °C. Then, samples were centrifuged 10 min at $400 \times g$ to obtain serum and plasma, respectively. The sex of the individuals was not determined; therefore, this variable was not considered for the analysis. In some cases, the volume of sample was not enough, consequently it was not possible to measure the complete set of hematological parameters in each individual.

Hematological determinations

To assess the nutritional status of penguins from each zone ($n = 30$ individuals from each zone), circulating plasmatic concentration of total proteins, albumin, uric acid, and triacylglycerol were determined in each individual using colorimetric commercial kits (Architect, Abbott). Serum protein fractions (α-, β- and γ-globulins) were analyzed by electrophoresis. Briefly, agarose gel electrophoresis was performed using semi-automated equipment (Hydrasis, Sebia Electrophoresis, France) (Ibañez et al. 2015). After that, gels were stained with 0.2% Amido Schwartz solution. The electrophoretic profile of each sample was obtained and analyzed by densitometry using PHORESIS software. Finally, the percentage of each protein fraction (Albumin, α-, β- and γ-globulins) was calculated by considering the area under the curve for each peak (corresponding to a particular fraction) and the total area (the sum of the area of each peak) (Fig. 3a).

Circulating levels of total Immunoglobulin Y (IgY) were determined in serum by direct ELISA using peroxidase-conjugated anti-chicken IgY antibodies (Sigma, St Louis, MO, USA, A-9046). Briefly, 96-well microtiter plates (Nunc PolySorp; Nunc, Roskilde, Denmark) were coated (1 h, 37 °C) with serum samples diluted in 0.1 M carbonate-bicarbonate buffer (pH = 9.6), then washed (×3 with PBS-Tween-20; phosphate-buffered saline, pH = 5 supplemented with 0.05% Tween-20), and incubated with 1% non-fat milk (Nestlé coffee-mate) in PBS-Tween-20 (1 h, 37 °C, blocking of free binding sites). After new washing, the wells were incubated with peroxidase-conjugated anti-chicken IgY. The linear range of the sigmoidal curve for this antibody-antigen response, as well as the optimal serum dilution (1/30,000), were determined as was previously described by Martínez et al. (2003). Finally, the wells were washed, ABTS [2,2-azino-di (3-ethylbenzthiazoline sulfonate)] was added as substrate, and after 30 min (at room temperature) color development was stopped with oxalic acid 2% and then read as optical density (OD) at 405 nm. The positive control sample was made of two randomly chosen plasma samples that were combined and defined as the positive (100%) control sample. Positive control samples and blank wells with only carbonate–bicarbonate buffer (negative control) were included on each plate. Controls and samples were run in duplicate wells. If readings differed by more than 5% between duplicate wells, the sample was reassayed. The inter- and intra-coefficient of variation were 3.12% and 1.8%, respectively.

To determine the hematocrit value, a heparinized capillary was filled with 100 µl of blood from the Eppendorf tube that contained heparinized blood once in the laboratory. To calculate hematocrit ($n_{HDZ} = 10$ and $n_{LDZ} = 10$), blood was centrifuged at 5000 rpm for 15 min (Ibañez et al. 2015). Then, using a digital caliper the total volume of blood in the capillary was measured and it was considered as 100%. After centrifugation, the red blood cells fraction was measured and the% was calculated as a proportion of the total volume of blood.

Finally, the determination of the hemagglutinating and lytic activity of plasma ($n_{HDZ} = 9$ and $n_{LDZ} = 11$) was conducted according to Matson et al. 2005. This is a highly repeatable qualitative scoring assay for characterizing natural antibodies (Nabs) mediated complement activation and RBC agglutination titers and useful for analyzing and compare innate humoral immunity. In particular, lysis reflects the interaction of complement and NAbs, whereas agglutination results from NAbs only (Matson et al. 2005).

Serum proteomic assay

Serum proteins from Adélie Penguins ($n = 4$ individuals from each zone) were separated by 1 Dimension (1-D) SDS/acrylamide electrophoresis (12.5%). For this, 10 µg of proteins from each individual were electrophoresed for 20 min at 80 V and 100 min at 100 V. Proteins were stained and

visualized by a colloidal Coomassie blue method (G 250, Fluka, Buchs, Switzerland). Stained gels were then washed three times with deionized water. Then, for each analyzed sample, the gel was excised in three sections and proteins were extracted for their identification by mass spectrometry (MS) (Fig. 5). First, proteins were digested with trypsin (Promega V5111) and desalted with ZipTip C18 columns (Millipore). Generated peptides were analyzed by nanoHPLC (Thermo Scientific, EASY-nLC 1000) coupled to a mass spectrometer (Thermo Scientific, Q-Exactive model) with an Orbitrap analyzer for their identification. A reverse-phase column was used for peptide separation (Easy-Spray ColumnPepMap RSLC) (P/N ES801) at 35 °C. Peptide ionization was performed by electrospray at 2.5 kV (Thermo Scientific, EASY-SPRAY). Proteins were identified and quantified using the software Proteome Discoverer 2.1 (Thermo Scientific). This software is a search tool in which the database and the details linked to the sample preparation can be selected. Based on this information and the data obtained from the mass spectrometer, the software provides a report indicating which proteins were identified and the peptides used for the identification. The analysis was conducted using the following criteria: Database: Chordata (Uniprot), Enzyme: Trypsin, Miscleavage: 2, Dynamic modifications: oxidation, Static modifications: Carbamidamethylation. The peptide and fragment mass tolerance were set to 10 ppm and 0.05 Da, respectively. A significance threshold value of $P < 0.05$ and a peptide cut-off score of 20 were also applied. The exponentially modified protein abundance index was measured for the identified proteins, based on the number of peptides found for the protein versus the number of total peptides. After protein identification in each individual from each zone, to conduct the analysis we selected those proteins that individuals had in common, and that showed significant differences in their abundance index. Finally, for these proteins the Ratio of expression (HDZ^{LDZ}) was calculated and the increase or decrease expression level was inferred as the Ratio of Expression *fold* (the number of times a protein was under- or over-expressed). Protein digestion and Orbitrap data acquisition were performed by the Center of Chemical and Biological Studies by Mass Spectrometry [Centro de Estudios Quımicos y Biológicos por Espectrometría de Masa (CEQUIBIEM), National University of Buenos Aires].

Once the proteins that displayed a differential expression pattern between the individuals from each zone were identified, the functional relationship between them was evaluated. To identify protein–protein interaction networks, the Search-Tool for the Retrieval of Interacting Proteins (STRING) analysis (https://string-db.org/) was used (Szklarczyk et al. 2021). Because the lack of species-specific proteins in the STRING database, protein-interaction network analysis was based on human protein identifiers. Protein networks were assessed using the function of "search multiple proteins" in STRING and using default settings and medium confidence. Color lines between nodes indicate evidence-based interactions for network edges as follows: known interactions, predicted interactions or via text mining, co-expression or protein homology. Colored nodes in the analysis represent query proteins and the first shell of interactors; white nodes represent the second shell of interactors. The PPI enrichment *P* value for the protein network was reported. Such an enrichment value indicates that the identified network of proteins has significantly more interactions than expected for a random set of proteins (Szklarczyk et al. 2021).

Statistical analysis

Statistical analysis and plotting were performed using GraphPad Software, Inc. (2007) and the software R (R Core Team 2014). Normality and homogeneity of variance were tested using Kolmogorov- Smirnov (K–S) and Levene tests. As all compared data were normally distributed (K-S $P > 0.01$), a GLM was conducted to analyze each hematological parameter considering the zone (HDZ and LDZ) and the season (2014–2015/2015–2016) as fix factors, and sampling date (6,7,8,9, and 10 days after egg hatching) as covariate. To test differences between individuals from each zone in the proteomic approach, data were analyzed using an unpaired *t* test. Results are presented as mean ± SD and in the estimate (est), *t* and *P* values are shown and the significance level was taken $P \leq 0.05$.

Results

Adélie Penguins showed significant differences in body condition between zones, though, this variation was not related to the year and the sampling day (Supplementary Table 1). Body mass (est = 202.74, t = 2.526, P = 0.0144, $n_{HDZ} = 30$, $n_{LDZ} = 30$), total proteins (est = 0.309, $t = 3.007$, $P = 0.0039$, $n_{HDZ} = 30$, $n_{LDZ} = 30$), albumin (est = 0.0533, $t = 5.044$, P = 0.004, $n_{HDZ} = 30$, $n_{LDZ} = 30$) and triacylglycerol (est = 0.3204, $t = 3.116$, $P = 0.0028$, $n_{HDZ} = 30$, $n_{LDZ} = 30$) were higher in the LDZ (Fig. 2). Conversely, uric acid concentration was higher in HDZ penguins (LDZ: est = −0.098, $t = -2.353$, $P = 0.0221$, $n_{HDZ} = 30$, $n_{LDZ} = 30$) (Fig. 2).

Serum protein fractions of Adélie Penguins were scattered in five peaks corresponding to albumin, α_1, α_2, β- and γ-globulins (Fig. 3a). Differences were observed in the γ-globulins fraction, whereas no differences for α_1-, α_2-, β-globulins values were addressed between zones (Fig. 3b). The abundance of γ-globulins was higher in penguins breeding in HDZ (LDZ: est = −2.296, $t = -4.999$, $P = 0.002$, $n_{HDZ} = 30$, $n_{LDZ} = 30$). Moreover, in individuals belonging to the HDZ the level of serum IgY was higher than in those from the LDZ (LDZ: est = −0.050, $t = -3.255$, $P = 0.0021$,

Table 1 List of differentially expressed serum proteins from Adélie Penguins (*P. adeliae*) exposed to contrasting anthropogenic activity level ($n_{HDZ}=4$ and $n_{LDZ}=4$)

Accession number	(% coverage)	Protein name	Function	Ratio HDZ/LDZ	*Ratio Expr. Fold*
A0A093NPW0	(42.95)	Beta-2-glycoprotein	Immune	2.29	> 1
A0A093PLD6	(48.17)	Alpha-1-antiproteinase 2	Immune	12.42	> 10
A0A093NV14	(30.87)	Complement Factor H	Immune	10.40	> 10
A0A093NMA3	(38.3)	Ig lambda chain	Immune	27.02	> 10
A0A093NJ67	(21.90)	Complement component C8 gamma chain	Immune	51.51	> 10
A0A093NJC7	(25.59)	Complement C4	Immune	8.76	> 1
P81605	(22.80)	Dermcidin	Immune	12.63	> 10
A0A093NSD6	(43.47)	Complement C3	Immune	2.41	> 1
A0A093NHQ3	(22.55)	Pantetheinase	Antioxidant + Immune	4.21	> 1
A0A1D5PVW1	(59.74)	Retinol Binding Protein 4	Antioxidant + Immune + Metab	9.75	> 10
A0A093R7V4	(35.79)	Vitamin D binding protein	Transport + Immune + Metab	0.89	< 1

Data indicates the accession number, the sequence coverage (%) which represents the percentage of protein sequence covered by the identified peptides, the ratio of expression (arbitrary units) between zones (HDZ^{LDZ}) and the expression ratio *fold*

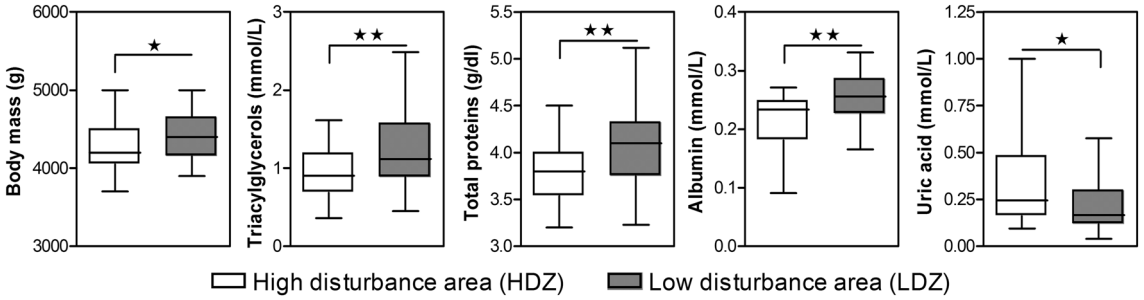

Fig. 2 Nutritional status of Adélie Penguins (*P. adeliae*) from HDZ and LDZ. Data ($n_{HDZ}=30$ and $n_{LDZ}=30$) are represented as Mean ± SE of each parameter: Body mass (g), triacylglycerols (mmol l^{-1}), total proteins (g dl^{-1}), albumin (mmol l^{-1}) and uric acid (mmol l^{-1}). Statistical results correspond to the *P* values calculated by GLM considering the zone (HDZ and LDZ) (*$P<0.05$ and **$P<0.01$)

$n_{HDZ}=30$, $n_{LDZ}=30$) (Fig. 4a). The hemagglutinating activity was greater in Adélie Penguins from the HDZ compared with those from LDZ (LDZ: est = −2.907, $t=-6.877$, $P=3.61e\text{-}03$, $n_{HDZ}=9$, $n_{LDZ}=11$), whereas no differences were observed in the lytic activity (Fig. 4b). Hematocrit (%) value was higher in penguins from LDZ than in those from the HDZ (est = 7.680, $t=5.054$, $P=0.008$, $n_{HDZ}=10$, $n_{LDZ}=10$) (Fig. 4c).

By proteomic analysis of serum samples ($n=4$ individuals from each zone), a total of 109 (Supplementary Table 2) proteins were identified after comparison of generated peptides against the Chordata protein database (Uniprot). Analysis of each individual revealed that, among the total proteins identified, 11 common proteins between all the individuals showed a differential expression level. Then, the abundance of these common proteins were compared between the two zones (HDZ and LDZ). Identified proteins are summarized in Table 1 indicating the accession number, the number of peptides and sequence coverage (%), the ratio of expression (as arbitrary units) between zones (DZ^{LDZ}) and the Ratio of expression *fold*. Innate immunity proteins such as complement factors (C8 and C3) ($t=2.558$, $P=0.0430$; $t=2.554$, $P=0.0432$, respectively; $n_{HDZ}=4$, $n_{LDZ}=4$), factor H ($t=4.219$, $P=0.0056$, $n_{HDZ}=4$, $n_{LDZ}=4$) and complement factor C4 ($t=2.374$, $P=0.0500$, $n_{HDZ}=4$, $n_{LDZ}=4$), acute phase proteins (APPs) α_1-antiproteinase ($t=2.889$, $P=0.0277$, $n_{HDZ}=4$, $n_{LDZ}=4$), β2-glycoprotein ($t=3.168$, $P=0.0194$) and dermcidin ($t=2.622$, $P=0.0470$, $n_{HDZ}=4$, $n_{LDZ}=4$) and, Ig lambda V-I chain conforming antibodies from the adaptive arm of the immune system ($t=2.960$, $P=0.0416$, $n_{HDZ}=4$, $n_{LDZ}=4$) were overexpressed in penguins from the HDZ. Retinol binding protein (RBP) was overexpressed in individuals from DZ ($t=4.327$, $P=0.0025$, $n_{HDZ}=4$, $n_{LDZ}=4$), whereas vitamin D binding protein (VDBP) was underexpressed ($t=3.498$, $P=0.0250$, $n_{HDZ}=4$, $n_{LDZ}=4$). Among proteins with both antioxidant and immune functions, pantetheinase was overexpressed in penguins from HDZ ($t=2.878$, $P=0.0347$, $n_{HDZ}=4$, $n_{LDZ}=4$) (Fig. 5).

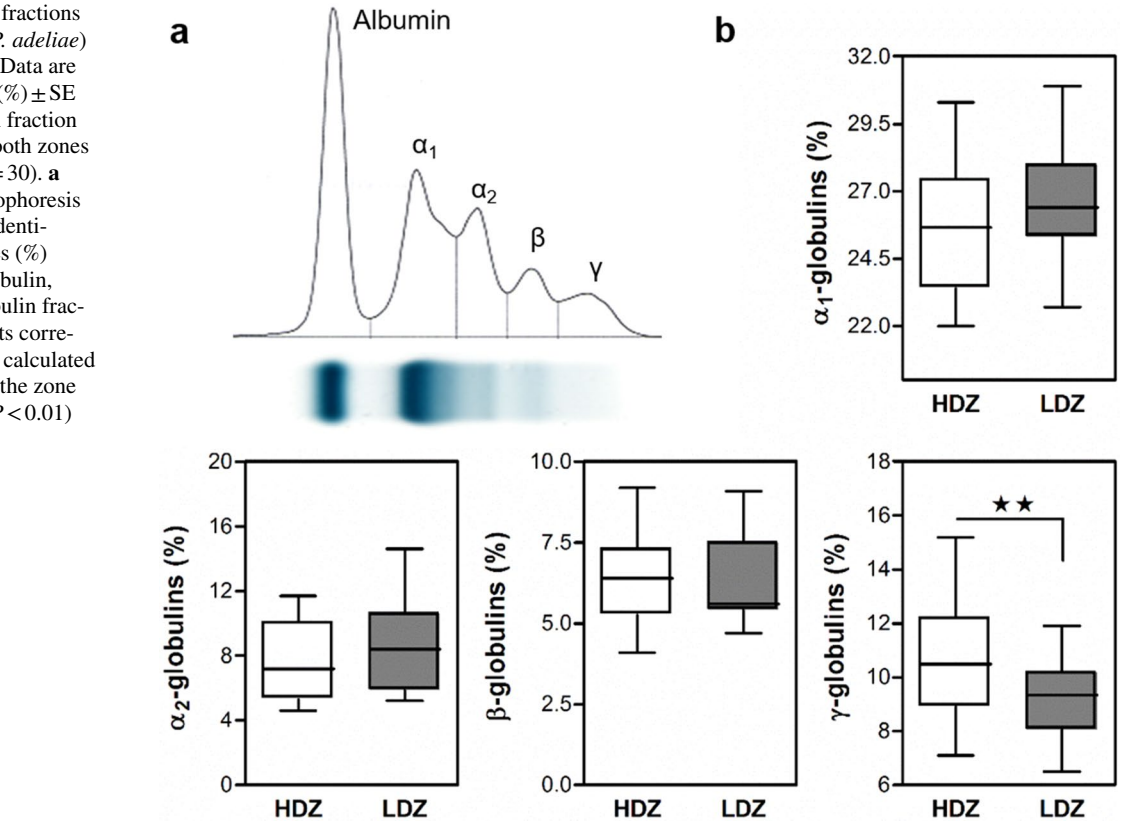

Fig. 3 Serum protein fractions of Adélie penguins (*P. adeliae*) from HDZ and LDZ. Data are represented as Mean (%) ± SE of each serum protein fraction for individuals from both zones ($n_{HDZ}=30$ and $n_{LDZ}=30$). **a** Representative electrophoresis and protein fraction identification. **b** Percentages (%) of α_1-globulin, α_2-globulin, β-globulin and γ-globulin fractions. Statistical results correspond to the *P* values calculated by GLM considering the zone (HDZ and LDZ) (**$P<0.01$)

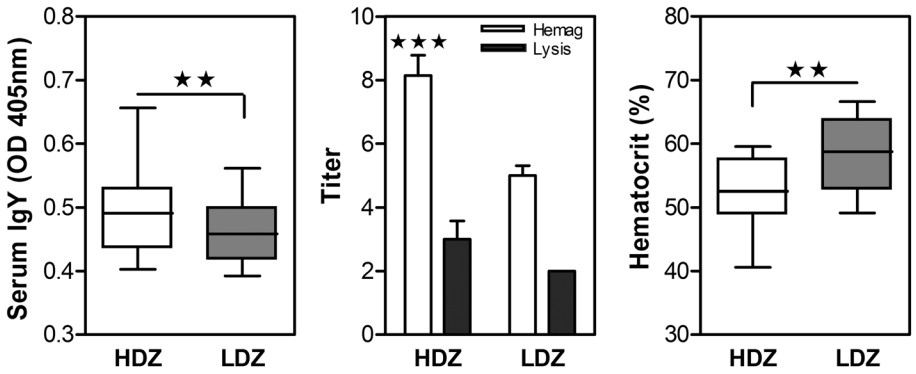

Fig. 4 Immune status of Adélie penguins (*P. adeliae*) from HDZ and LDZ. Data are represented as Mean ± SE for each immune parameter from individuals exposed to contrasting levels of anthropogenic activity. **a** Level of IgY (OD at 450 nm) ($n_{HDZ}=30$ and $n_{LDZ}=30$), **b** Hemagglutinating and lytic activity ($n_{HDZ}=9$ and $n_{LDZ}=11$) and, **c** hematocrit value (%) ($n_{HDZ}=10$ and $n_{LDZ}=10$). Statistical results correspond to the *P* values calculated by GLM considering the zone (HDZ and LDZ) (**$P<0.01$, ***$P<0.001$)

Based on the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) analysis, the PPI enrichment *P* value for the proteins identified in Adélie Penguins was < 1.0e-16. Such, enrichment indicates that the proteins are at least partially biologically connected, as a group. Human protein homologues were used for the assessment of the protein–protein interaction networks. Some biological GO (Gene Ontology) pathways were identified (Fig. 6), which included complement activation, humoral and innate immune responses, immune effector processes and response to stress, regulation of acute inflammatory responses, to external stimulus and, metabolic processes and vitamin metabolism. In addition, response wound healing, stress and immune system processes including complement, coagulation cascades and bacterial infection defense pathways (*Pertussis* and *Staphylococcus aureus*) and, pantothenate and CoA metabolism were identified.

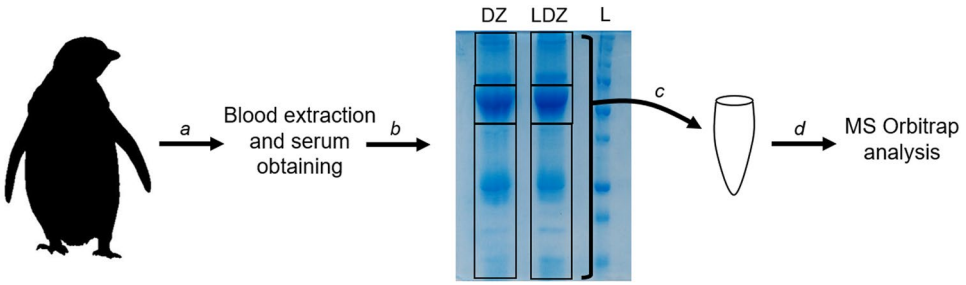

Fig. 5 Proteomic analysis of serum samples. Representation of the processing of samples. Image shows one sample from each zone (HDZ and LDZ). This procedure was conducted for all individuals assayed ($n = 4$/zone). **a** Blood was extracted from individuals from each zone and serum was obtained for each individual. **b** Total proteins were quantified and 10 µg of proteins were separated by 1-D SDS/acrylamide Gel electrophoresis for each individual. Gel was stained with colloidal Coomassie Blue. Then each lane on the gel was cut into three sections for each sample. **c** Proteins were extracted from each section of the gel and trypsin treatment was conducted. **d** Protein fragments were separated by HPLC chromatography and generated peptides were analyzed in an Oritrap mass spectrometer

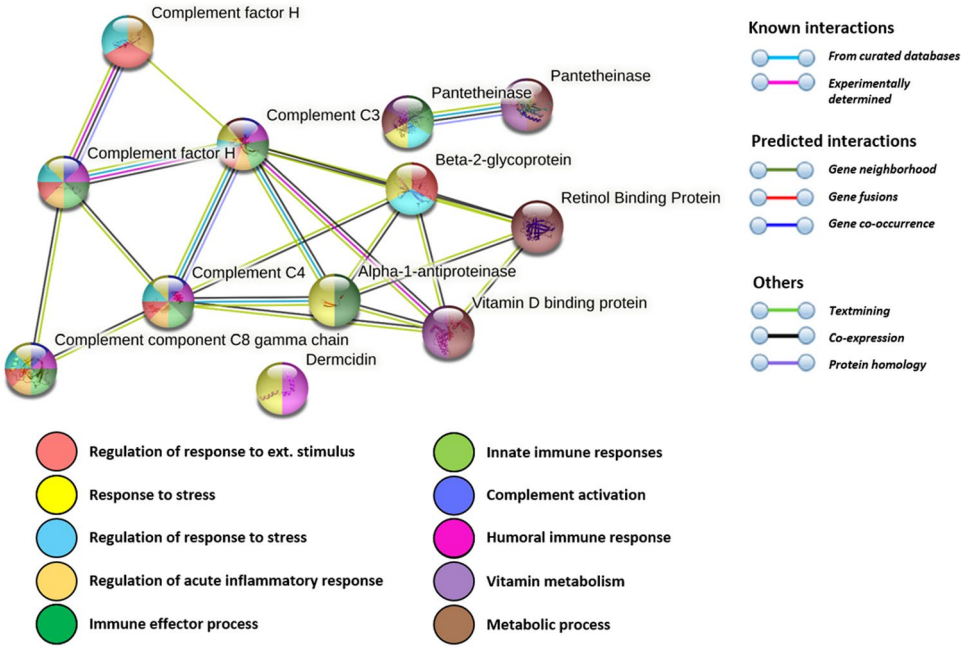

Fig. 6 Protein–protein interaction networks of serum proteins differentially expressed Adélie Penguins (*P. adeliae*) from both areas (HDZ and LDZ). Protein–protein interactions plot based on known and predicted interactions of human homologue proteins to proteins identified in Adélie Penguins using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) analysis. Biological Gene Ontology (GO) pathways relating to identified proteins are highlighted showing regulation of response to external stimulus; response to stress; regulation of response to stress; regulation of acute inflammatory response; immune effector process; innate immune responses; complement activation; humoral immune response; vitamin metabolism and metabolic process. Colored nodes represent query proteins and first shell of interactors. Colored lines indicate whether protein interactions are identified via known interactions (curated databases, experimentally determined), predicted interactions (gene neighborhood, gene fusion, gene co-occurrence) or via text mining, co-expression or protein homology

Discussion

Understanding the dynamics of physiological and immune functions in wild organisms has become a topic of great interest in ecological studies (Levy et al. 2020). Because humans are changing environments, the introduction of new pathogens (Leotta et al. 2002) and pollutants, and the chronic exposure of organisms to them may lead to variation in physiological phenotypes (Gremillet and Charmantier 2010; Dehnhard et al. 2015). In this scenario, the ability to resist infections is an important component of the individual's overall fitness. Therefore, shaping immune responses, while avoiding damaging processes

(e.g., oxidative damage) are thought to underlie phenotype's plasticity in changing environments (Isaksson and Sheldon 2011; Marri and Ritcher 2014).

To our knowledge, only a few studies have conducted a proteomic approach on wild seabirds to address physiological traits (Bertile et al. 2016; Phillips et al. 2020). The present study describes the nutritional and immune status in breeding Adélie Penguins exposed to different levels of anthropogenic activity. In addition, using a proteomic approach, we identified differentially expressed proteins involved in the regulation of immune responses and stress, antioxidant processes, metabolism and vitamin transport. Considering the differences observed in penguins' physiological status between areas, these proteins constitute potential molecular biomarkers involved in the modulation of phenotype plasticity in physiological traits in changing environmental conditions.

Physiological changes, because anthropogenic activity demonstrates severe implications in seabirds' survival or reproduction (Viblanc et al. 2012). Previous studies have reported the negative effects of human impact in penguins' physiology, considering areas with different level of anthropogenic pressure (Barbosa et al. 2007a,2013; Cebuhar et al. 2017). We report that penguins from the HDZ showed a reduced body condition compared to those from the LDZ. Body mass, hematocrit and plasma metabolites (triacylglycerols, total proteins and albumin) were lower in individuals from HDZ indicating a poor body condition (Cherel et al 1988; Graña Grilli et al. 2018; Ibañez et al. 2018). A similar corporal and hematological pattern was addressed in seabirds exposed to polluted environments (Bustnes et al. 2008; Siebert et al. 2012; Blevin et al. 2014). In addition, previous studies conducted at this location on Adélie Penguins addressed oxidative damage in penguins exposed to anthropogenic pressure. An increase in Glutathione S-transferase activity and plasmatic uric acid concentration was observed in penguins from HDZ compared to those from LDZ (Cebuhar et al. 2017). Complementing this study, we also report a higher uric acid concentration in penguins from HDZ and differences in body condition according to the level of human activity. Uric acid has antioxidant properties and often reflects the onset of a disease (Costantini 2011). Moreover, under sustained energy-demanding processes or immune responses (which often induce oxidative damage) protein catabolism occurs for energy obtaining, ultimately inducing an increase in uric acid (Cherel et al. 1988; Cebuhar et al. 2017). Therefore, the higher uric acid concentration in penguins from HDZ is in agreement with this statement.

Differences in body condition and physiological traits may be attributed to individual quality, sex, age and breeding chronology (Dehnhard et al. 2015; Colominas-Ciuró et al. 2017; Flo et al. 2019). At Hope Bay, Adélie Penguin breeding chronology (egg laying and hatching dates) between zones was similar in both seasons. In addition, slight differences were addressed in breeding success between HDZ and LDZ zones, being higher in HDZ during the season 2014–2015, whereas during 2015–2016, the success was similar (Silvestro et al. unpublished results). Altogether, these indicates that breeding chronology may not be associated to the differences observed in penguin's physiology. On the other hand, individual quality, sex, and age were not determined in this study; thus, it represents a limitation in our conclusions as we cannot exclude them from driving differences in phenotypes.

Anthropogenic activity influence seabirds' immunocompetence through the release of pollutants (heavy metals, metalloids and organic) or by the spread of novel pathogens (Ropert-Coudert et al. 2019). Moreover, the fact that penguins nest in dense aggregations could increase the probability of disease transmission. Thus, the introduction of pathogens that could give rise to emergent infectious diseases is a serious concern for penguin conservation. Serum proteins constitute a relevant group of molecules which provide an accurate assessment of the immune function and health status (Gruys et al. 2005). We addressed five protein fractions corresponding to albumin, α_1, α_2, β- and γ-globulins as was previously described (Ibañez et al. 2015). No differences related to anthropogenic activity were observed in α- and β-fractions. Our results contrast to the previous descriptions by Grassman et al. 2000 in which α- and β-globulins were positively correlated with the presence of organic pollutants. On the other hand, lower and higher values of albumin and γ-globulins, respectively, were observed in penguins from the HDZ compared to those from LDZ. Supporting these observations, it was reported that albumin decreases during inflammation (Cray et al. 2009) and, γ-globulins increase in response to immune-mediated diseases (Ivey 2000; Cray et al. 2005, 2007). Moreover, penguins from HDZ showed an increased immune function with higher levels of IgY antibody (adaptive humoral defenses) and hemagglutinating activity (constitutive innate antibody defenses). These results agree with previous studies which addressed the effect of human activity on immune system (Staszewski et al. 2007; Barbosa et al. 2007a; 2013). Altogether, our observations indicate that in the study area human-derived pathogens may mediate effects on both innate and adaptive humoral immune system.

Organisms response to environmental changes depends on their intrinsic sensitivity and plasticity, and hence their capacity to buffer against poor conditions by adapting their physiology and immune responses to cope with change (Ropert-Courdet et al. 2019). Assuming the costs of mounting an immune response and the biochemical substrates required (i.e., amino acids and lipids) (Evans et al. 2015; McDade et al. 2016), the decline of body condition observed in penguins from HDZ may be associated with

the reallocation of resources to the immune system. Altogether, this scenario may shape the reproductive process in terms of energy costs (Brock et al. 2013; Kulaszewicz et al. 2017; Tieleman 2018). However, the knowledge about the molecular mechanisms that mediate such variation is scarce (Hasselquist and Nilsson 2012). Studies on breeding seabirds have described that immune function decreases because of a trade-off with reproduction events (incubation and chick-rearing) (Evans et al. 2015; Kulaszewicz et al. 2017; Ibañez et al. 2018). Whereas, in sites where exposition to human activity occurs immune responses are often increased (Barbosa 2007a,2013). In agreement with this, breeding Adélie Penguins exposed to anthropogenic activity increased their immune responses, which represents an extra-energy cost during breeding. To gain further insight into the molecular basis underlying physiological adaptation to changing environments, we determined the serum protein expression pattern. For this, four individuals from each zone were randomly selected, and applying a proteomic approach differences in the protein expression pattern were observed. Proteins involved in antioxidant processes, immune functions, and vitamin transport and metabolism and, responses to stress were overexpressed in penguins from HDZ.

Regarding immune proteins, α1-antiproteinase (or α1-antitrypsin), β2-glycoprotein, dermcidin, components of the complement complex (C8 and C3), factor H, complement component C4 and, Ig lambda chain are involved in humoral innate and adaptive immune defenses. The α1-antiproteinase and β2-glycoprotein are APPs overexpressed during inflammatory processes, possess immunomodulatory functions and prevents tissue damage (Gruys et al. 2005; Stockley et al. 2015; McDonell et al. 2020). Dermcidin acts as an antimicrobial anionic peptide capable of killing bacteria (Ageitos et al. 2017). In addition, among innate immune proteins, complement factors constitute the main protection barrier of the host against infectious agents (Gruys et al. 2005). During inflammation, complement factor C3 is overexpressed and is involved in the opsonization and trapping of microorganisms and their products, in the complement activation, and modulating the host's immune response (Gruys et al. 2005). Factor H is an abundant glycoprotein expressed by the liver and, can be also expressed locally by a variety of cell types. This factor acts in the regulation of the alternative complement pathway activation by interacting with C3b component (Ferreira et al. 2010). Complement component C4 is a central protein in the classical and lectin pathways within the complement system. During activation of complement, its major fragment C4b becomes covalently attached to the surface of pathogens and alter self-tissue (Mortensen et al. 2015). On the other hand, the Ig lambda chain is representative of the light chain of immunoglobulins and is involved in the activation of the complement complex by interacting with the component C3 during infection. Altogether, in agreement with the differences in the immune function under environmental stress penguins may increase the expression of both innate and adaptive immune components probably promoting a strong protection against human-introduced pathogens.

Proteins with antioxidant and immune function as Pantetheinase, RBP and DBP showed differences in their expression level. Pantetheinase and RBP were overexpressed in penguins from HDZ, while DBP was slightly higher in penguins from LDZ. Pantetheinase is involved in tissue tolerance to oxidative stress and also, in the balance of damage induced by pathogens. Previous reports using two pathogens as infectious agents, schistosomas parasites and rickettsias bacteria, revealed that the absence of tissue pantetheinase expression had little impact on pathogen burden, whereas tolerance to the disease was considerably affected (Naquet et al. 2014). Indeed, the overexpression of pantetheinase in penguins from HDZ may be involved in tissue protection against oxidative damage induced by the elevated immune responses. On the other hand, RBP is a transporter of retinol (vitamin A) from the liver to the target cells and tissues. The increased expression of this transporter in individuals from HDZ would be associated in part with an increase of retinol transport to lymphoid tissues, where they act as immunomodulatory/antioxidant agents (Walrand et al. 2005; Hõrak et al. 2006). DBP is involved in the transport of vitamin D metabolites and modulation of immune and inflammatory responses (Gomme and Bertolini 2004). The expression level of DBP was lower in penguins from the HDZ. This is consistent with previous studies in which during restricted energy or high energy demand processes there is a reduction in circulating levels of DBP (Laing and Fraser 2002; Bertile et al. 2009).

Based on information obtained from literature and the protein–protein network analysis by the STRING tool, it was possible to infer that the identified proteins (Table 1) are partly connected in terms of their biological function. The PPI enrichment *P* value for the group of proteins analyzed indicates that the identified network of proteins may contribute to the regulation of a broad range of physiological functions including immunity, defense mechanisms and metabolic pathways.

The overexpression of proteins with antioxidant, metabolic and immune functions may have a critical role in shaping physiology in penguins exposed to anthropogenic activity. This group of proteins may be considered as potential biomarkers involved in physiological plasticity to changing environments. Altogether, these results provide relevant for future studies on ecophysiology or ecotoxicology on different seabird species under changing environments.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00227-021-03876-1.

Acknowledgements This work was made possible thanks to Instituto Antártico Argentino (IAA), which provided logistical support and permission to carry out the fieldwork at Esperanza (Hope) Bay. Special thanks to Dr. Marcela Pari and Dr. Amalia Figueroa (Hospital Interzonal General de Agudos (HIGA) Luisa C. Gandulfo) for their help in plasma metabolite determination, and to Dr. Facundo Xavier Palacio for the help with statistical analysis. We thank the Editor's and reviewer's criticism and comments, which help to improve the presentation of our research.

Author's contribution AEI conducted field-work, sample, experiments and data analysis and wrote the manuscript. DM and MA contributed to manuscript redaction and sample analysis. JF contributed to the proteomic analysis and manuscript preparation. CDF and DT contributed to the field-work with sample obtaining, experimental design and data analysis.

Funding The study was supported by National Geographic Waitt Grant Program (#W401-15) and Agencia Nacional de Promoción Científica y Tecnológica (PICT-2014–3323) (to AEI), Proyecto de Investigación Plurianual (PIP- CONICET n°:0158) and (PICTA-2010–0080) (to DM). Field-work activities and logistic were supported by the Dirección Nacional del Antártico (DNA) and Instituto Antártico Argentino (IAA).

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval All applicable international, national, and institutional guidelines for sampling, care and experimental use of animals for the study were followed as established by the Article III, Annex II of the Madrid Protocol, Law 24.216 (Taking, Harmful Intrusion and Introduction of Species) within the framework of the projects evaluated and approved by the Environment Office of the IAA and Dirección Nacional del Antártico (DNA).

References

Acero JM, Agraz JL, Aguirre CA (1996) Revisión ambiental de las actividades antárticas en Bahía Esperanza. Instituto Antártico Argentino, pp 1–35

Ageitos JM, Sánchez-Pérez A, Calo-Matac P, Villa TG (2017) Antimicrobial peptides (AMPs): Ancient compounds that represent novel weapons in the fight against bacteria. Biochem Pharmacol 133:117–138

Barbosa A, Palacios MJ (2009) Health of Antarctic birds: a review of their parasites, pathogens and diseases. Polar Biol 32:1095–1115

Barbosa A, Merino S, Benzal J, Martinez J, García-Fraile S (2007) Geographic variation in the immunoglobulin levels in pygoscelid penguins. Polar Biol 30:219–225

Barbosa A et al (2013) Pollution and physiological variability in gentoo penguins at two rookeries with different levels of human visitation. Ant Sci 25(2):329–338

Bender NA, Crosbie K, Lynch HJ (2016) Patterns of tourism in the Antarctic Peninsula region: a 20-year analysis. Antarct Sci 28(3):194–203

Bertile F, Schaeffer C, Le Maho Y, Raclot T, Van Dorsselaer A (2009) A proteomic approach to identify differentially expressed plasma proteins between the fed and prolonged fasted states. Proteomics 9:148–158

Bertile F, Fouillen L, Wasselin T, Maes P, Le Maho Y, Van Dorsselaer A, Raclot T (2016) The safety limits of an extended fast: lessons from a non-model organism. Sci Rep 6:39008

BirdLife International (2018) State of the world's birds: taking the pulse of the planet. BirdLife International

Blévin P, Tartu S, Angelier F, Leclaire S, Bustnes JO, Moe B, Herzke D, Gabrielsen GW, Chastel O (2014) Integument colouration in relation to persistent organic pollutants and body condition in arctic breeding black-legged kittiwakes (*Rissa tridactyla*). Sci Total Environ 1(470–471):248–254

Bonnedahl J, Broman T, Waldenstrom J, Palmgren H, Niskanen T, Olsen B (2005) In search of human-associated bacterial pathogens in Antarctic wildlife: report from six penguin colonies regularly visited by tourists. Ambio 34:430–432

Brock PM, Hall AJ, Goodman SJ, Cruz M, Acevedo-Whitehouse K (2013) Immune activity, body condition and human-associated environmental impacts in a wild marine mammal. PLoS ONE 8(6):e67132

Brommer JE (2004) Immunocompetence and its costsduring development: an experimental study in blue tit nest-lings. Proc R Soc Lond B 271:S110–S113

Burger J, Gochfeld M (2004) Marine birds as sentinels of environmental pollution. EcoHealth 1:263–274

Bustnes JO, Fauchald P, Tveraa T, Helberg M, Skaare JU (2008) The potential impact of environmental variation on the concentrations and ecological effects of pollutants in a marine avian top predator. Environ Int 34(2):193–201

Carlini AR, Coria RN, Santos MM, Libertelli MM, Donini G (2007) Breeding success and population trends in Adélie penguins in areas with low and high levels of human disturbance. Polar Biol 30:917–924

Cebuhar JD, Di Fonzo CI, Dopchiz LP, Ansaldo M (2017) Anthropogenic impact modifies the blood antioxidant status in *Pygoscelis adeliae* and *Pygoscelis papua* at Hope Bay Antarctic Peninsula. Ecotoxicol Latin Am 4:105–118

Cherel Y, Robin JP, Walch O, Karmann H, Netchitailo P, Le Maho Y (1988) Fasting in the king penguin i: hormonal and metabolic changes during breeding. Am J Physiol 254:R170–R177

Cockrem JF (2007) Stress, corticosterone responses and avian personalities. J Ornithol 148:S169–S178

Colominas-Ciuró R, Santos M, Coria M, Barbosa A (2017) Reproductive effort affects oxidative status and stress in an Antarctic penguin species: an experimental study. PLoS ONE 12(5):e0177124

Core Team R (2015) R: A language and environment for statistical computing. R foundation for statistical computing, Viena, Autria. URL: http://www.R-project.org/

Corsolini S (2008) Industrial contamination in Antarctic Biota. J Chromatogr A 1216:598–612

Costantini D (2011) On the measurement of circulating antioxidant capacity and the nightmare of uric acid. Meth Ecol Evol 2:321–325

Cray C, Zielezienski-Roberts K, Bonda M, Stevenson R, Ness R, Clubb S, Marsh A (2005) Serologic diagnosis of Sarcocystosis in *Psittacine* birds: 16 cases. J Avian Med Surg 19:208–215

Cray C, Rodriguez M, Zaias J (2007) Protein electrophoresis of *psittacine* plasma. Vet Clin Pathol 36:64–72

Cray C, Zaias J, Altman N (2009) Acute phase response in animals: a review. Comp Med 59:517–526

de Villiers M (2008) Review of recent research into the effects of human disturbance on wildlife in the Antarctic and sub-Antarctic region. Working Paper XXXI Antarctic Treaty Consultative Meeting, Ukraine

Dehnhard N, Eens M, Demongin L, Quillfeldt P, Poisbleau M (2015) Individual consistency and phenotypic plasticity in rockhopper penguins: female but not male body mass links environmental conditions to reproductive investment. PLoS ONE 10(6):e0128776

Dias M et al (2019) Threats to seabirds: a global assessment. Biol Cons 237:25–537
Epperson LE, Rose J, Carey HV, Martin SL (2010) Seasonal proteomic changes reveal molecular adaptations to preserve and replenish liver proteins during ground squirrel hibernation. Am J Physiol Regul Integr Comp Physiol 298:R329–R340
Evans JK, Dann P, Frankel T (2015) Variation in innate immune function during incubation, chick-rearing and moult in Little Penguins (*Eudyptula minor*). Emu 115:63–71
Ferreira VP, Pangburn MK, Cortés C (2010) Complement control protein factor H: the good, the bad, and the inadequate. Mol Immunol 47(13):2187–2197
Flach EJ, Stevenson MF, Henderson GM (1990) Aspergillosis in Gentoo penguins (*Pygoscelis papua*) at Edinburgh Zoo, 1964 to 1988. Vet Rec 126(4):81–85
Flo J, Løseth ME, Sonne C, Jaspers VLB, Brun-Hansen H (2019) Plasma protein fractions in free-living white-tailed eagle (*Haliaeetus albicilla*) nestlings from Norway. BMC Vet Res 15:290
Gatt MC et al (2021) Costs of reproduction and migration paid in later return to the colony, not in physical condition, in a long-lived seabird. Oecologia 195:287–297
Gomme PT, Bertolini J (2004) Therapeutic potential of vitamin D-binding protein. Trends Biotechnol 22:340–345
Grabek KR, Martin SL, Hindle AG (2015) Proteomics approaches shed new light on hibernation physiology. J Comp Physiol B 185:607–627
Graña Grilli M, Pari M, Ibañez AE (2018) Poor body conditions during the breeding period of a low breeding success seabird population. Mar Biol 165(9):142
Grasman KA (2002) Assessing immunological function in toxicological studies of avian wildlife. Integr Comp Biol 42:34–42
Grasman KA, Armstrong M, Hammersley DL, Scanlon PF, Fox GA (2000) Geographic variation in blood plasma protein concentrationsof young herring gulls (*Larus argentatus*) and Caspian terns (*Sterna caspia*) from the Great Lakes and Lake Winnipeg. Comp Biochem Physiol C 125:365–375
Gremillet D, Charmantier A (2010) Shifts in phenotypic plasticity constrain the value of seabirds as ecological indicators of marine ecosystems. Ecol App 20(6):1498–1503
Grimaldi WW, Seddon PJ, Lyver POB, Nakagawa S, Tompkins DM (2015) Infectious diseases of Antarctic penguins: current status and future threats. Polar Biol 38(5):591–606
Gruys E, Toussaint MJ, Niewold TA, Koopmans SJ (2005) Acute phase reaction and acute phase proteins. J Zhejiang Univ Sci B 6(11):1045–1056
Hasselquist D, Nilsson J (2012) Physiological mechanisms mediating costs of immune responses: what can we learn from studies of birds? Ani Behav 83(6):1303–1312
Hegemann A, Matson KD, Both C, Tieleman BI (2012) Immune function in a free-living bird varies over the annual cycle, but seasonal patterns differ between years. Oecologia 170(3):605–618
Hõrak P, Zilmer M, Saks L, Ots I, Karu U, Zilmer K (2006) Antioxidant protection, carotenoids and the costs of immune challenge in greenfinches. J Exp Biol 209:4329–4338
Ibañez A, Najle R, Larsen K, Figueroa A, Pari M, Montalti D (2015) Hematological values of three Antarctic penguins: Gentoo (*Pygoscelis papua*), Adélie (*P. adeliae*) and Chinstrap (*P. antarcticus*). Polar Res 34:1
Ibañez AE, Graña Grilli M, Figueroa A, Pari M, Montalti D (2018) Declining health status of Brown Skua (*Stercorarius antarcticus lonnbergi*) parents and their offspring during chick development. Polar Biol 41:193–200
Isaksson C, Sheldon BC (2011) The Challenges of integrating oxidative stress into life-history biology. Bioscience 61:194–202
Ivey ES (2000) Serologic and plasma protein electrophoretic findings in 7 psittacine birds with aspergillosis. J Avian Med Surg 14:103–106
Khan JS, Provencher JF, Forbes MR, Mallory ML, Lebarbenchon C, McCoy KD (2019) Chapter One - Parasites of seabirds: A survey of effects and ecological implications. Adv Marine Biol Acad Press 82:1–50
Kulaszewicz I, Wojczulanis-Jakubas K, Jakubas D (2017) Trade-offs between reproduction and self-maintenance (immune function and body mass) in a small seabird, the little auk. J Avian Biol 48:371–379
Laing CJ, Fraser DR (2002) Changes with malnutrition in the concentration of plasma vitamin D binding protein in growing rats. Br J Nutr 88:133–139
Leotta GA, Paré J, Sigler L, Montalti D, Vigo G, Petruccelli M, Reinoso EH (2002) Thelebolus microsporus mycelial mats in the trachea of wild brown *skua Catharacta antarctica lonnbergi* and South Polar Skua *C. maccormicki* carcasses. J Wild Dis 38(2):443–447
Levy H et al (2020) Evidence of pathogen-induced immunogenetic selection across the large geographic range of a wild seabird. Mol Biol Evol 37(6):1708–1726
Mallory ML, Robinson SA, Hebert CE, Forbes MR (2010) Seabirds as indicators of aquatic ecosystem conditions: a case for gathering multiple proxies of seabird health. Mar Pollut Bull 60:7–12
Marri V, Richner H (2014) Immune response, oxidative stress and dietary antioxidants in great tit nestlings. Comp Biochem Physiol A 179C:192–196
Martin LB II, Scheuerlein A, Wikelski M (2003) Immune activity elevates energy expenditure of house sparrows: a link between direct andindirect costs? Proc R Soc Lond B 270:153–158
Martínez J, Tomás G, Merino S, Arriero E, Moreno J (2003) Detection of serum immunoglobulins in wild birds by direct ELISA: a methodological study to validate the technique in different species using antichicken antibodies. Funct Ecol 17:700–706
Matson KD, Ricklefs RE, Klasing KC (2005) A hemolysishemagglutination assay for characterizing constitutive innate humoral immunity in wild and domestic birds. Dev Comp Immunol 29:275–286
Mauck RA, Matson KD, Philipsborn J, Ricklefs RE (2005) Increase in the constitutive innate humoral immune system in Leach's Storm Petrel (*Oceanodroma leucorhoa*) chicks is negatively correlated with growth rate. Funct Ecol 19:1001–1007
McDade TW, Georgiev AV, Kuzawa CW (2016) Trade-offs between acquired and innate immune defenses in humans. Evol Med Public Health 1:1–16
McDonell T, Wincupa C, Buchholz I, Pericleous C, Gilesa I, Ripolla V, Cohen H, Delcea M, Rahman A (2020) The role of beta-2-glycoprotein I in health and disease associating structure with function: More than just APS. Blood Rev 39:100610
Mortensen S et al (2015) Structural basis for the function of complement component C4 within the classical and lectin pathways of complement. J Immunol 194(11):5488–5496
Mykhailenko A et al (2020) First record of *Serratia marcescens* from Adelie and gentoo penguin faeces collected in the *Wilhelm Archipelago*, Graham Land, West Antarctica. Polar Biol 43:903–910
Naquet P, Pitari G, Dupre S, Galland F (2014) Role of the Vnn1 pantetheinase in tissue tolerance to stress. Biochem Soc Trans 42:4
Nisbet IC (2000) Disturbance, habituation, and management of waterbird colonies. Waterbirds 23:312–332
Norris K, Evans MR (2000) Ecological immunology: life history tradeoffs and immune defense in birds. Behav Ecol 11:19–26
Olmastroni S, Pezzo F, Volpi V, Focardi S (2004) Effects of weather and sea ice on the reproductive performance of the Adélie penguin at Edmonson point, Ross Sea. CCAMLR Sci 11:99–109

Palacios MJ et al (2018) Cellular and humoral immunity in two highly demanding energetic life stages: reproduction and moulting in Chinstrap Penguin. J Ornithol 159:283–290

Parker BJ, Barribeau SM, Laughton AM, Griffin LH, Gerardo NM (2017) Life-history strategy determines constraints on immune function. J Animal Ecol 86:473–483

Patterson DL, Easter-Pilcher AL, Fraser WR (2003) The effects of human activity and environmental variability on longterm changes in Adélie Penguin populations at Palmer Station, Antarctica. In: Huiskes AHL, Gieskes WWC, Rozema J, Schorno RML, van der Vies SM, WolV WJ (eds) Antarctic biology in a global context. Proceedings of VIIIth SCAR international biology symposium, Backhuys Publishers, Leiden, pp 301–307

Phillips RA, Kraev I, Lange S (2020) Protein deimination and extracellular vesicle profiles in antarctic seabirds. Biology 9(1):15

Råberg L, Stjernman M (2003) Natural selection onimmune responsiveness in blue tits Parus caeruleus. Evolution 57:1670–1678

Rodgers JA, Smith HT (1995) Set-back distances to protect nesting bird colonies from human disturbance in Florida. Conserv Biol 9:89–99

Ropert-Coudert Y et al (2019) Happy feet in a hostile world? The future of penguins depends on proactive management of current and expected threats. Front Mar Sci 6:248

Santos MM, Hinke JT, Coria NR, Fusaro B, Silvestro A, Juáres MA (2018) Abundance estimation of Adélie penguins at the Esperanza/Hope Bay mega colony. Polar Biol 41:2337–2342

Siebert U, Schwemmer P, Gue N, Harder T, Garthe S, Prenger-Berninghoff E, Wholsein P (2012) Health status of seabirds and coastal birds found at the German North Sea coast. Acta Vet Scand 54(1):43

Staszewski V, McCoy KD, Tveraa T, Boulinier T (2007) Interannual dynamics of antibody levels in naturally infected long-lived colonial birds. Ecology 88(12):3183–3191

Stockey RA (2015) The multiple facets of alpha-1-antitrypsin. Ann Transl Med 3(10):130

Studds E, Marra PP (2005) Nonbreeding habitat occupancy and population processes: an upgrade experiment with a migratory bird. Ecology 86:2380–2385

Szklarczyk D et al (2021) The STRING database in 2021: customizable protein–protein networks, and functional characterization of user-uploaded gene/measurement sets. Nuc Ac Res 49(D1):605–612

Tarr NM, Simons TR, Pollock KH (2010) An experimental assessment of vehicle disturbance effects on migratory shorebirds. J Wild Man 74:1776–1783

Tella JL (2002) The evolutionary transition to coloniality promotes higher blood parasitism in birds. J Evol Biol 1(5):32–41

Tieleman BI (2018) Understanding immune function as a pace of life trait requires environmental context. Behav Ecol Soc 72:55

Tin T et al (2009) Impacts of local human activities on the Antarctic environment. Ant Sci 21:3–33

Viblanc VA et al (2012) Coping with continuous human disturbance in the wild: insights from penguin heart rate response to various stressors. BMC Ecol 12:10

Walrand S, Farges MC, Dehaese O, Cardinault N, Minet-Quinard R, Grolier P, Bouteloup-Demange C, Ribalta J, Winklhofer-Roob BM, Rock E (2005) In vivo and in vitro evidences that carotenoids could modulate the neutrophil respiratory burst during dietary manipulation. Eur J Nutr 44:114–120

Weimerskirch H, Prudor A, Schull Q (2018) Flights of drones over sub-Antarctic seabirds show species and status-specific behavioural and physiological responses. Polar Biol 41:259–266

Wingfield JC, Pérez JH, Krause JS, Word KR, González-Gómez PL, Lisovski S, Chmura HE (2017) How birds cope physiologically and behaviourally with extreme climatic events. Philos Trans R Soc Lond B Biol Sci 372(1723):20160140