

Overexpression of Head date 1 gene (*Hd1*): an adaptation of antarctic hairgrass to guano input from *Macronectes giganteus* colonies of Antarctica

Super expressão do gene Head date (Hd1): uma adaptação da grama antártica à entrada de guano das colônias de *Macronectes giganteus* da Antártica

Sobreexpresión del gen Head date 1 (Hd1): una adaptación de la hierba antártica al ingreso de guano de colonias de *Macronectes giganteus* en la Antártida

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Abstract

The Antarctic biodiversity, beyond the species composition, also comprises interactions between fauna and flora. *M. giganteus*, is one of the species that occupy the antarctic ice-free areas for reproduction. The moss *Sanionia uncinata* (Hedw.) Loeske and *Deschampsia antarctica* Desv. gress, common in Antarctica, with other species make up vast green formations and is associated with breeding areas of seabirds. These sites are large deposits of guano, because a large amount of birds those gather in colonies. Due to this large supply of guano, the soil becomes a deposit of minerals, mainly of nitrogen available in the form of ammonium and nitrate. The problem is that not all plant species tolerate high quantities of these substances so different plant species show trends in the mechanisms of tolerance to stress by ammonium, which have been proven at the molecular level. The aim of this study was to investigate the influence of breeding colonies of seabirds on plant populations in the South Shetland Islands, Antarctica, from a molecular perspective. From the analysis of the collected samples, using the RNA-Seq and qRT-PCR approach was possible to identify a single gene differential and significantly expressed in *D. antarctica*. The LOC_Os06g16380 gene among the sampled treatments (control, 1m and 10m), showed higher expression coming 1m near breeding areas of *M. giganteus*. Our results suggest that Hd1 is associated with the plants stress related to guano input since that soil analysis demonstrated a higher concentration of mineral nitrogen available near of breeding areas of seabirds.

Keywords: Abiotic stress; Ammonium; Guano; Seabirds; Giant Petrel.

Resumo

A biodiversidade antártica, além da composição de espécies, compreende também as interações entre a fauna e a flora. *Meripilus giganteus* Karst é uma das espécies que ocupa as áreas de degelo da Antártica, para sua reprodução. O

musgo *Sanionia uncinata* (Hedw.) Loeske e a grama *Deschampsia antarctica* Desv., comuns na Antártica, junto de outras espécies formam tapetes verdes, associados as áreas de reprodução de aves marinhas. Esses locais são grandes depósitos de guano, devido à grande quantidade de pássaros que se reúnem nas colônias. Com essa grande oferta de guano, o solo torna-se um depósito de minerais, principalmente de nitrogênio disponível na forma de amônio e nitrato. O problema é que nem todas as espécies de plantas toleram grandes quantidade dessas substâncias, pois diferentes espécies de plantas apresentam tendências nos mecanismos de tolerância ao estresse por amônio, que já foi comprovado em nível molecular. O Objetivo deste estudo foi investigar a influência das colônias de reprodução de aves marinhas nas populações de plantas nas ilhas Shetlands no Sul, na Antártica, sob uma perspectiva molecular. A partir da análise das amostras coletadas utilizando a abordagem RNA-Seq e qRT-PCR foi possível identificar um único gene diferencial e significativamente expresso em *D. antarctica*. O geneLOC_Os06g16380 entre os tratamentos amostrados (controle, 1m e 10m), apresentou maior expressão chegando próximo aos criadouros de *M. giganteus*. Nossos resultados sugerem que o Hd1 está associado a entrada de guano em plantas relacionadas ao estresse, uma vez que a análise do solo demonstrou uma maior concentração de nitrogênio mineral disponível próximo as áreas de reprodução de aves marinhas.

Palavras-chave: Estresse abiótico; Amônio; Guano; Aves marinhas; Petrel gigante.

Resumen

La biodiversidad antártica, además de la composición de especies, también comprende las interacciones entre la fauna y la flora. *Meripilus giganteus* Karst es una de las especies que ocupa las áreas libres de hielo de la Antártida para su reproducción. El musgo *Sanionia uncinata* (Hedw.) Loeske y la hierba *Deschampsia antarctica* Desv., Comunes en la Antártida con otras especies, forman vastas formaciones verdes y están asociados con áreas de reproducción de aves marinas. Estos lugares son grandes depósitos de guano, debido a la gran cantidad de aves que se reúnen en las colonias. Con este gran aporte de guano, el suelo se convierte en un depósito de minerales, principalmente nitrógeno disponible en forma de amonio y nitrato. El problema es que no todas las especies vegetales toleran grandes cantidades de estas sustancias, ya que diferentes especies vegetales muestran tendencias en los mecanismos de tolerancia al estrés amónico, lo que ya ha sido probado a nivel molecular. El objetivo de este estudio fue investigar la influencia de las colonias reproductoras de aves marinas en las poblaciones de plantas en las islas Shetland del sur de la Antártida desde una perspectiva molecular. A partir del análisis de las muestras recolectadas utilizando el enfoque RNA-Seq y qRT-PCR, fue posible identificar un único gen diferencial y expresado significativamente en *D. antarctica*. El genLOC_Os06g16380 entre los tratamientos muestreados (control, 1 m 10 m), mostró mayor expresión, acercándose a los sitios de reproducción de *M. giganteus*. Nuestros resultados sugieren que Hd1 está asociado con la entrada de guano en las plantas relacionadas con el estrés, ya que el análisis del suelo demostró una mayor concentración de nitrógeno mineral disponible cerca de las áreas de reproducción de aves marinas.

Palabras clave: Estrés abiótico; Amonio; Guano; Aves marinas; Petrel gigante.

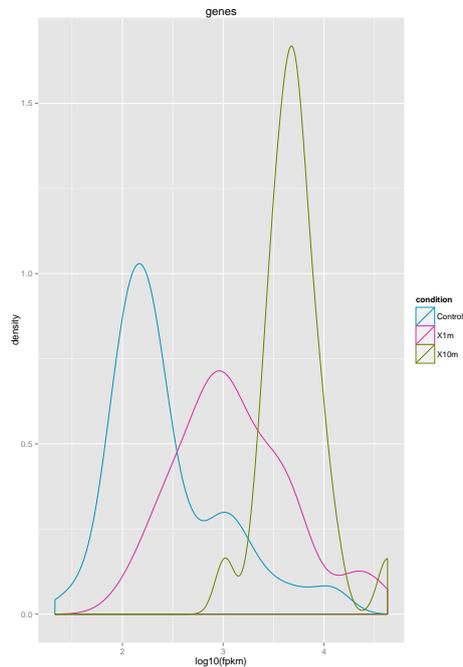
1. Introduction

The Antarctic terrestrial ecosystems are characterized by extreme abiotic conditions when compared with other continents. Around 86% of Antarctic is covered by ice, the air temperatures are low (average between the -10°C to -20°C in coastal areas) and high winds and snowstorms cover the region which also presents a short summer season (about two months). Altogether those features hinder the establishment of terrestrial biodiversity. Besides the environmental constraints the animals influence during the short austral summer, increase the accumulation of large quantities of minerals in the soil (mainly Nitrogen) during the breeding season (Barcikowski *et al.*, 2001; Alberdi *et al.*, 2002; Lee *et al.*, 2008).

Deschampsia antarctica Desv. (Figure 1a) is one of the two native flowering plant species found in Antarctica and is the only hairgrass inhabiting the region. This species is abundant mainly in the South Shetlands Islands occupying almost the entire coastal area of the Maritime Antarctic, often occurring in the vicinity of bird colonies (Edwards and Lewis-Smith *et al.*, 1988; Parnikoza *et al.*, 2011).

Moreover, mosses are important representatives of the Antarctic terrestrial ecosystems, often composing the main landscape of coastal areas and forming large Green carpets of vegetation. *Sanionia uncinata* (Hedw.) Loeske (Figure 1b) is one of the most abundant species of mosses in Polar Regions and contributes to the accumulation of organic matter in the Antarctic soils (Mendonça *et al.*, 2011; Neufeld *et al.*, 2015). This moss species occurs mainly in environments with constant water supply, like those close to the drain lines coming from defrosted water (Lud *et al.*, 2002; Tojo *et al.*, 2012).

Figure 1. Selected plant species for the study. (a) Antarctic Hairgrass *Deschampsia Antarctica* Desv. (Photo: Clarissa Kappel Pereira). (b) *Sanionia uncinata* (Hedw.) Loeske, the most common moss carpet for antarctic ice-free areas.



Source: Photo: Filipe de Carvalho Victoria.

Considering that the spatial distribution of vegetation in Antarctica is closely linked to marine animals, understanding how changes in the seabird communities may affect terrestrial communities becomes necessary to determine the degree of interaction between plants and seabirds (Barcikowski, 2001). It is expected that one of the key elements is the input of nitrogenous compounds originating from the excrement of seabirds that may influence both positively and negatively the plant communities. Increases in ammonium input in studies of soil composition influenced by seabirds were reported in several analysis of soil composition in areas under the influence of marine animals, such as Santos *et al.* (2006), Park *et al.* (2007), Sun *et al.* (2002), Theobald *et al.* (2013).

In the nitrogen cycle, ammonium is replaced by an important role in living organisms that receive the nitrogen supply to its basic functions, and seabirds are responsible for large amounts of this issue (Zhu *et al.*, 2011). This especially occurs in the Antarctic coastal regions and sub Antarctic places where the ocean has great productivity resulting from large numbers of seabirds breeding over Antarctica every year (Riddick *et al.*, 2012).

The main nitrogen source come from the energy flow from the fish- and crustaceans-based diet of penguins, petrels and gulls (Copello *et al.*, 2008; Hebert *et al.*, 2009; Petry *et al.*, 2008, 2010). Although the average NH_4^+ concentrations of soils are often 10–1000 times lower than those of NO_3^- (Marchner, 1995), the difference in soil concentrations does not necessarily reflect the uptake ratio of each N source. Indeed, the role of NH_4^+ in plant nutrition has probably been under estimated, because most plants preferentially take up NH_4^+ when both forms are present. Ammonium requires less energy for uptake and assimilation than nitrate, mainly because NO_3^- has to be reduced prior to assimilation (Bloom *et al.*, 1992). Optimal plant growth is however usually achieved when N is supplied in both forms (Bloom *et al.*, 1999). On the other hand, the excess of ammonium in soils, may adversely affect the growth, productivity, tolerance to drought or frost and resistance to diseases and insects, leading to long-term changes in species composition. The excess of ammonium also causes soil acidification and eutrophication (Fangmeier *et al.*, 1994; Wilson *et al.*, 2004), changing the atmospheric composition and land nutrient supply (Blackall *et al.*, 2007).

Abiotic stress caused by cold, drought and increased salinity (e.g. excess of ammonium), generated a selective pressure for plants to develop mechanisms that would enable their development in environments where these factors reach extremes. This adaptive process resulted in a gene pool facing to a successful survival strategy for climate change, especially those of unexpected and extreme level (Lee et al., 2013). Lee et al. (2008), using the Expressed Sequences Tag (EST) approach, generated by large scale single-pass sequencing of cDNA clones, found novel genes of *D. antarctica*, related to the differential response of the species abiotic stress in Antarctic environment, demonstrating the strong selective pressure under the Antarctic plants, but the action of these genes have not experimentally clarified.

Thus, the biological question involving the breeding influence under the adaptation of plants to the Antarctic environment is which genes (if so) are differentially expressed when those plants are exposed to the higher nitrogen compounds input, as guano. Within this work we attempted to answer this question by using the transcriptome-based analysis of two species of Antarctic plants, *D. antarctica* and *S. uncinata*, both common species in the nesting areas of Southern Giant Petrel (*M. giganteus*) and comparing the transcriptome of those plants with plants from the same species in areas without influence of guano.

2. Methodology

Plant materials. Plant samples were collected in Copacabana, near the US Refuge in King George Island (62° 23' 0" S, 58° 27' 0" W) and, in Stinker Point, Elephant Island (61° 13' 20" S, 55° 21' 35" W), during the Antarctic austral summer of 2014/2015. *D. antarctica* and *S. uncinata* were sampled at pre-defined locations according to the breeding areas of *M. giganteus* (Southern Giant Petrel). Plants were collected from approximately 1m, 5m and 10m from the center of the breeding area, with three replicates per sample, totalizing 9 samples per plant. The aerial part, and roots in the case of *D. antarctica*, were removed from soil and placed in zip-loc bags. Four different individuals were taken along each sampling distance. The same procedure was applied to collect plant samples in an area without influence of bird colonies. Subsequently the samples were storage at -80°C until RNA extraction.

RNA Extraction. Specimens collected in the field were washed with autoclaved ultrapure water, and homogenized with liquid nitrogen, crucible and pestle. The homogenate obtained for each sample were used for total RNA extraction. RNA extraction was performed for each treatment, three replicates (n = 18) using the connector kit of pure RNA Mini kit ® (Ambion Life Technologies, Carlsbad, California, USA) according to the manufacturer's instructions. The quantity and quality of total RNA was measured by spectrophotometry using a Spectrophotometer NanoVue™ Plus (GE Healthcare, Little Chalfont, United Kingdom).

mRNA Enrichment. Total RNA were subjected to enzymatic digestion of DNA using the Kit™ TURBO DNA-free (Ambion Life Technologies, Carlsbad, California, USA) according to the manufacturer's instructions. The cytoplasmic depletion (5S, 5.8S, 18S, and 28S) and mitochondrial (12S and 16S) ribosomal RNA (rRNA) was performed with the Eukaryote v2 RiboMinus™ System (Ambion Life Technologies, Carlsbad, California, USA) following the manufacturer's instructions, and mRNA was quantified by fluorometry using a Qubit - RNA Assay Kit (Invitrogen, Carlsbad, California, USA).

Library preparation and sequencing. Eighteen libraries (one for each treatment for both species) were generated using Ion Total RNA-Seq Kit v2 (Ambion Life Technologies, Carlsbad, California, USA). Ion OneTouch™ 2 System and Ion PGM™ Template OT2 400 Template Kit were used to prepare RNA library, sequencing was performed using the Ion PGM™ Sequencing 400 in Ion system PGM™ using three Ion 318™ Chip v2 (six loaded samples per chip).

Assembly and mapping transcripts. The quality filtering of RNA-seq reads for each library were independently mapped using TopHat2 against the *Physcomitrella patens* v3.0 and *Oryza sativa* v3.0 as reference genomes for *S. uncinata* e

D. antarctica, respectively. The levels of gene expression and isoforms were first normalized using a variation of the FPKM method (Fragments Per Kilo-base of mRNA length per Million mapped reads) performing in the Cufflinks tools (Cufflinks2, CuffMerge and Cuffdiff2) on the alignments of TopHat, and *P. patens* genome annotation v3.1 and v3.1 *O. sativa*. All analysis was performed in the Galaxy, the Galaxy Rättsch Lab platform (galaxy.cbio.mskcc.org/). The genomes of *P. patens* and *O. sativa* annotation of genes were downloaded from Phytozome V10.1 (phytozome.jgi.doe.gov). Statistics and graphical analysis of the differential expression on the transcripts detected were performed in the program R (version 3.1.1) with CummeRbund extension (Goff et al., 2012).

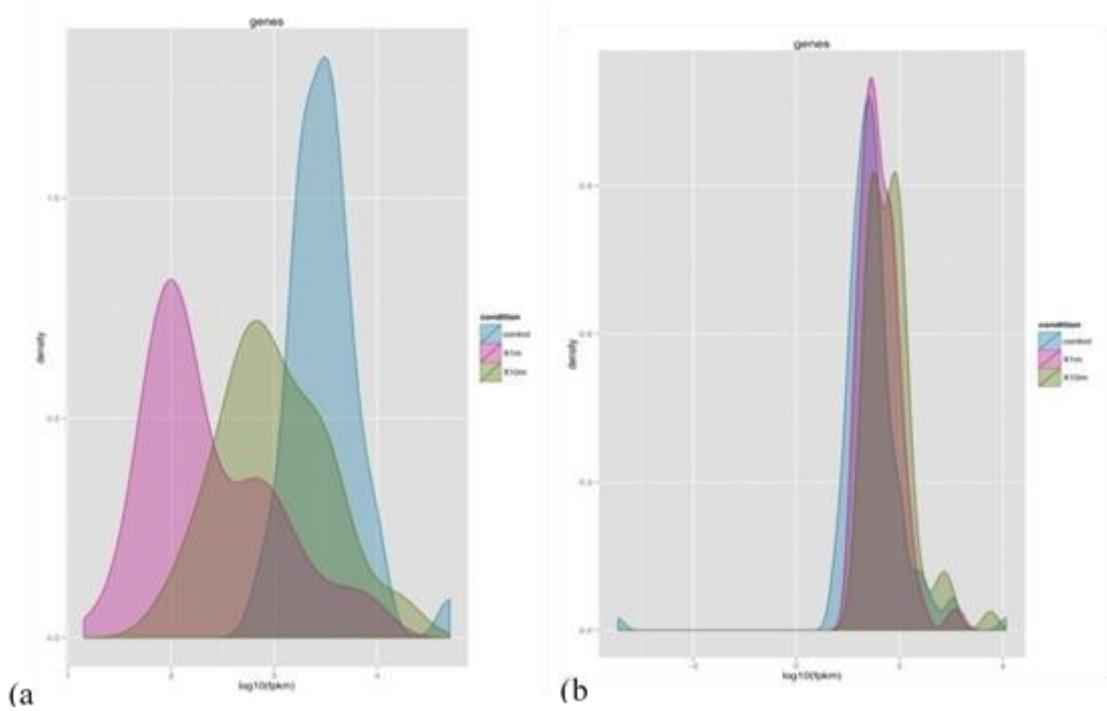
qRT-PCR analysis. The candidate gene selected from the RNA Sequence analysis had the expression patterns analyzed by qRT-PCR using SYBR® Green detection system (Applied Biosystems®, California, USA). The quantitative variation between different samples was evaluated using the comparative CT method ($\Delta\Delta CT$), and the data of target gene expression, normalized to the level of expression of TIP41-like genes, used as standard reference in internal control (endogenous) (Caldana, et al., 2007, Jain et al., 2006). The qRT-PCR reactions were performed in triplicate techniques from: 2.0 ul 10x buffer; 1.2 uL of 50 mM MgCl₂; 0.4 uL of 5 mM dNTPs; 1 ul of each oligonucleotide (10 mM); 0.05 uL Taq Platinum - DNA polymerase (5 U / uL); 2 uL of Syber Green (1x); 0.4 uL ROX, the first tape 1 ul cDNA (diluted 1: 5) and water to make a final volume of 20 uL. The cycling conditions used for amplification were 50 °C for 2 minutes, 95 °C for 10 minutes, and 40 cycles of 95 °C for 30 seconds, 60 °C for 1 minute and 72 °C for 1 minute, occurring reading fluorescence in this last step. Finally, a cycle of 72 °C for 5 minutes.

Soil mineral nitrogen analysis. In each GHG sampling event, sSoil samples were taken for analysis of mineral nitrogen (nitrate – NO₃ and ammonium – NH₄ by Kjeldahl distillation), determined according to Tedesco et al. (1995).

3. Results and Discussion

Differential expression analysis. As expected the gene expression analysis distribution across treatments were distinct for each species tested. The moss species presented lower differential expression detected within treatments (Figure 2a). Furthermore Kernel distribution of FPKM scores for overall genes detected across the Antarctic hairgrass *D. antarctica* indicated a similar distribution of transcripts within the treatments (Figure 2b), meaning that only this plant was affected by the guano from the Southern Giant Petrel. Following the kernel analysis Cufflink tool was applied in order to determine which genes were differentially expressed in *S. uncinata* and *D. antarctica* were identified a single significant gene expressed and only for the grass species. The LOC_Os06g16380 gene had its expression in *D. antarctica*, and among the three treatments (control, 1m and 10m), with higher expression close to the *M. giganteus* colonies (1m treatment). The moss species does not show a significant gene expressed for both treatments.

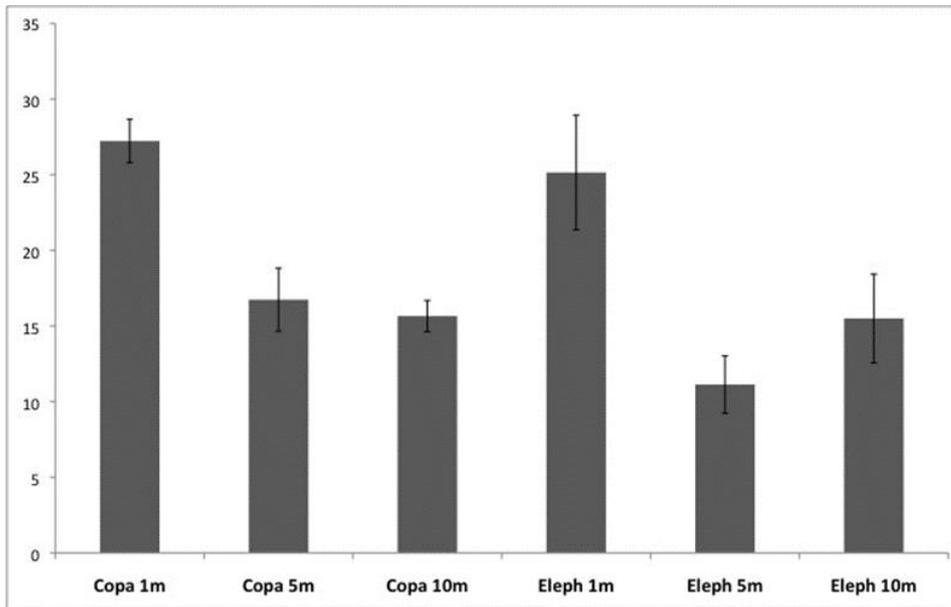
Figure 2. Kernel distribution of FPKM scores for overall genes detected across samples in the Control, 1m and 10m of distance from birds colonies in both plant species analyzed. (a) Density plot of *Sanionia uncinata* show as lower distribution on genes detected across distances, otherwise displayed for *Deschampsia antarctica* (b), suggesting the influence of the distances from birds colonies only for the Antarctic hairgrass species.



Source: Photo: Filipe de Carvalho Victoria.

Confirmation of differentially expressed genes by qRT-PCR analysis. Having found the LOC_Os06g16380 gene differentially expressed in *D. Antarctica* by RNA-Seq, the next step was to perform a qRT-PCR on total mRNA to confirm the expression patterns. A higher and significant concentration of the target gene fragments was observed in *D. Antarctica* at 1m of distance from *M. giganteus* colonies in both sampling places (Copacabana and Stinker Point) (Figure 3). At 5m and 10m of distance from the breeding colonies, the expression patterns were similar to each other but smaller than at 1m of distance (Figure 3). A higher amount of ammonium in the soil near the breeding colonies (e.g. 1m) in both sites (Copacabana - 1.8 mg/dm³ and Stinker Point - 1.5 mg/dm³; Figure 4) was detected, suggesting the influence of nitrogen in the differential expression of LOC_Os06g16380 gene.

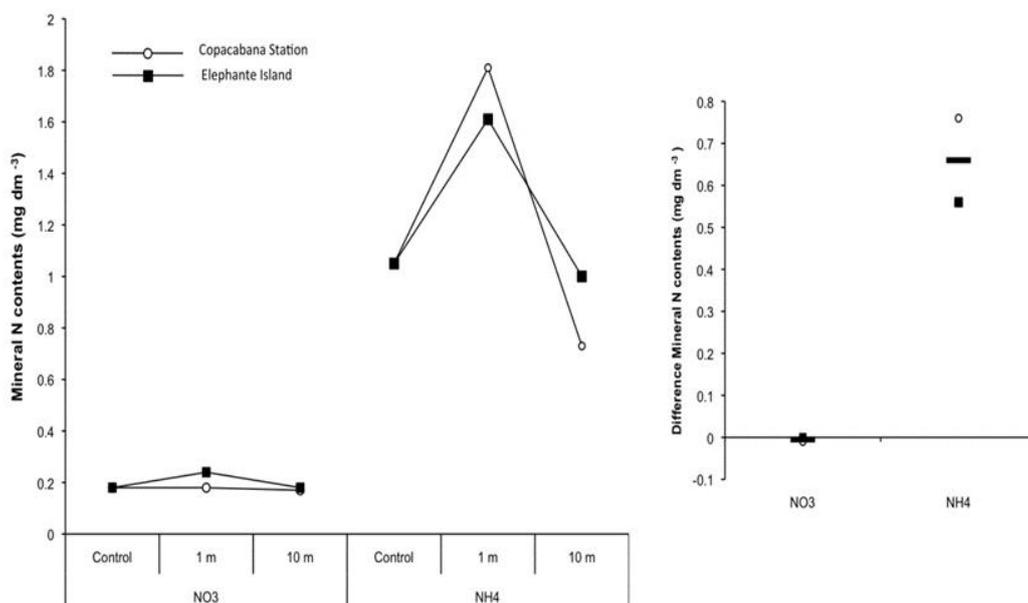
Figure 3. Fragments quantities of LOC_Os06g16380 gene in the three distances (1m, 5m and 10m), obtained from of three specimens sampled in Copacabana, at King George Island (Copa) e Stinker Point at Elephant Island (Eleph) each. The bar represent the average plus/minus SD values of three replicates runs of qRT-PCR analyses.



Source: Photo: Filipe de Carvalho Victoria.

Soil mineral nitrogen analysis. Through the Kjeldahl distillation of mineral nitrogen in the soil, there was obtained values nitrogen as ammonia and nitrate. There was a higher amount of ammonium in the soil near 1m colonies breeding of birds in both sites: Copacabana (1.8 mg / dm³) and Stinker Point (1.5 mg / dm³). Nitrogen in nitrate form does not significantly varied between the three samples at both locations (Figure 4).

Figure 4. Mineral Nitrogen concentrations in control treatments, 1m and 10m in the two sampling sites, Copacabana (King George Island) and Stinker Point (Elephant Island).



Source: Photo: Filipe de Carvalho Victoria.

The LOC_Os06g16380 gene was previously found and described by Zhang et al. (2012) as belonging to a region related with the Heading date gene I (*Hdl*) found in rice. This gene is an orthologous gene of *CONSTANS* gene identified in *Arabidopsis* model species (Takakashi & Shimamoto, 2011), and regulates the expression of florigen gene *Hd3*, responsible for controlling the mechanism involved with the transition from the vegetative to the reproductive phase in flowering plants (Kojima et al., 2002; Sonoda et al., 2003; Park et al., 2008). In rice, the *Hdl* gene is reported as the major quantitative trait locus (QTL) controlling response to photoperiod (Yano et al., 2000) that determines the regional and seasonal adaptation of rice crops (Zhang et al., 2012). This trait conferring short or long vegetative phase, susceptible to use in breeding program to increase the yield in distinct latitudes (Takakashi & Shimamoto, 2011; Zhang et al., 2015). However, the pleiotropic effect from *Hdl* expression on the productivity/yield and growth in rice was already observed (Zhang et al., 2012), although this gene did not affect these characteristics, and thus expression was not detected in roots. Our results in *D. antarctica* suggests that the higher input of NH_4^+ close to the seabirds colonies induced an increase in the LOC_Os06g16380 expression and that this region can be related with the capacity of this grass species to respond to high contents of ammonium in soil and even be related to the transport of these mineral in grass roots. These findings corroborate the theory of guano input from sea mammals and birds enables in which nutrients can change the chemical and organic characteristics of the soil and in turn can determine the spatial distribution of *D. antarctica* (Smykla et al., 2007; Park et al., 2012).

A review of works that investigate the effect of seabirds on continental plant communities in tropical and subtropical regions carried out by Ellis (2005), analyzed fifty seven studies, which indicate evidence that seabird nests affect the biomass of seabirds. Aboveground plants and significantly alters population richness and plant community composition. These data are confirmed for the plant communities of ice-free areas, in which a significant number of species, mainly mosses and lichens, are classified as ornithocrophiles or ornithocrophobes (Victoria et al., 2013; Schmitz et al., 2018).

Studies on impacts of seabird colonies on plant communities in ice-free areas of Antarctica are rare, for example Zwolicki (2015), which does not assess stress but the occurrence of plant populations grouped in six zones that were classified based on the occurrence of *Prasiola crispa* (Lightfoot) Menegh, *Deschampsia antarctica* Desv., *Colobanthus quitensis* (Kunth.) Bart. and a lichen identified as *Usnea* sp.

The higher and significant concentration of the target gene fragments was observed in *D. Antarctica* at 1m of distance from *M. giganteus* colonies in both sampling places (Copacabana and Stinker Point) (Figure 3). This can be explained by the fact that the passage of nitrate (NO_3^-) and ammonium (NH_4^+) through the cell wall of leaf epidermis cells and after its entry into the cell, nitrate can be reduced to nitrite (NO_2^-), in the cytosol, through the enzyme nitrate reductase (RN) and, soon after, converted to ammonium (NH_4^+) in the plastid, through the enzyme nitrite reductase (RNi). Ammonium is then incorporated into amino acids by the enzymes glutamine synthetase (GS) and glutamate synthase (GOGAT), forming glutamine (GLN), glutamate (GLU) and other amino acids and their metabolites. Therefore, the greater proximity of the nests, the concentration of guano is greater mainly due to the fact that the strong winds that occur in Antarctica take the guano that is released on the plant populations. (Crawford, 1995; Bredemeier et al., 2000; Victoria et al., 2013; Schmitz et al., 2018).

Regarding *S. uncinata*, no significant results relating to differential gene expression in the three treatments suggest that this is not an ideal moss to this type of analysis. This is probably due to their morphological plasticity associated with its great ecological amplitude (Gimingham & Smith, 1971; Hebel et al., 2012.), being the mechanisms that control these adaptations remains unknown up to date. The ecological amplitude of *S. uncinata* is because this species does not suffer a significant effect from the presence of seabird colonies, occurring near or far from them. Among many other species of mosses, such as *Polytrichastrum alpinum* Hedw. are considered ornithocrophiles because they occur in areas that have guano input, whereas *Polytrichum juniperinum* Hedw. It is classified as ornithocrophobic because it does not occur in areas that have guano input (Victoria et al., 2013, Schmitz et al., 2018).

Another ecological characteristic that explains the non-expression of the studied gene is the fact that the rhizoids of *S. uncinata* do not have contact with the soil. This species grows mainly on rock fragments forming mats, which is very characteristic in the plant communities of ice-free areas. Its use in this research is because it is the species with the highest Index of Ecological Importance (IES), and with large areas of occurrence (Raven & Edwards, 2001; Victor & Dolan, 2012).

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