Mol Cell Toxicol (2019) 15:163-172
DOI 10.1007/s13273-019-0020-1

ORIGINAL PAPER

Skin transcriptome profiling reveals the distinctive molecular effects of temperature changes on Antarctic bullhead notothen

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Received: 25 June 2018 / Accepted: 10 September 2018

Abstract

Backgrounds: Temperature is an important abiotic factor that directly influences the physiology of marine fish. The Antarctic bullhead notothen *Notothenia coriiceps* inhabits water with temperatures ranging from −1.9 to 2°C at circumpolar regions. Thus, *N. coriiceps* is useful as a model animal for understanding the effects of temperature stress.
Methods: To assess the transcriptional response of skin tissue to temperature changes, Antarctic bullhead notothen were exposed to two temperature stresses, 4°C and −2°C, following acclimatization at 2°C. Twenty-four hours after the temperature change, skin transcriptomes were sequenced using the Illumina Hiseq 2000 platform and analyzed using a series of bioinformatics tools. Functional gene annotations through pathway analyses of the Gene Ontology and Kyoto Encyclopedia of Genes and Genomes databases revealed commonly or distinctively modulated transcriptional changes in skin.
Results: Both temperature stressors significantly upregulated the actin cytoskeleton regulation pathway and the skin's water barrier function, while the stressors downregulated the metabolism involved in muscle contraction, choline receptor regulation, collagen regulation, and immunity. Cold stress caused significant downregulation of the mRNA expression of genes involved in vasopressin-regulated water reabsorption. Neither the heat- nor cold-stressed skin transcriptomes exhibited significant heat shock protein expression.
Conclusion: Our results suggest that, as a first barrier for fish, the skin has complex metabolisms with high transcriptional sensitivity against environmental temperature stress. These results will be useful for understanding the skin-specific molecular mechanisms that Antarctic fish use to adapt to temperature fluctuations.

Keywords: Antarctic bullhead notothen, *Notothenia coriiceps*, Temperature, Skin, Transcriptome

Introduction

The low temperature of the sea water in Antarctica acts as a highly selective pressure for fish evolution. Water temperature is important because it affects the concentration of oxygen dissolved in sea water. The Antarctic Southern Ocean has high oxygen solubility due to its low temperature. Antarctic fish are known to be vulnerable to temperature fluctuations because they are isolated and have adapted to the cold and stable temperatures via physiological and genomic evolution[1,2]. Antarctic notothenioids have evolved in the extreme environmental conditions of the low temperature, icy, and oxygen-rich waters of the Southern Ocean. Their unique physiological and biochemical adaptations, such as the acquisition of antifreeze glycoproteins, which inhibit the growth of ice crystals, possession of

Electronic supplementary material The online version of this article (doi: 10.1007/s13273-019-0020-1) contains supplementary material, which is available to authorized users.

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cold-adapted proteins with specific activities at low temperatures, loss of the inducible heat shock response (HSR), and lack of hemoglobin expression in species of the Channichthyidae family have long been of interest to scientists[3-5]. Researchers have continuously proposed theories regarding how temperature fluctuations affect Antarctic notothenioids at the molecular and biochemical level, and their subsequent response. The responses of antioxidant defense metabolisms to temperature changes and corresponding modulation of the internal metabolic rate are known to be species specific[6-9]. Because global warming is having an obvious effect on the Southern Ocean and its endemic fauna, it is vital that we study how Antarctic notothenioids will respond to and endure the rising water temperature and its consequences, including dramatic alterations to key environmental factors such as pH, salinity, and water chemistry.

Notothenia coriiceps is an endemic Antarctic stenothermic fish species with a circumpolar distribution. Under natural environmental conditions, they can only survive at temperatures between −2.5°C to 6.0°C[1]. The thermal limits of *N. coriiceps* have been reported to be approximately 17°C following acclimatization at 4°C for 7-14 days[10,11]. We have previously developed a genomics resource for *N. coriiceps*, highlighting the loss of the phosphorylation-dependent sumoylation motif in heat shock factor 1 and the induction of HSR in the blood[12], although most notothenioids lack the capacity for inducible HSR and other chaperones[3,4]. In this study, we performed a basic bioinformatics-based analysis of the transcriptional response of *N. coriiceps* skin tissue to exposure to 4°C and −2°C following acclimatization at 2°C. Fish skin is known to act as an effective barrier to pathogenic microorganisms and ice crystal propagation[13,14]. Better knowledge of the molecular response to elevated temperatures will inspire new perspectives from which to study the adaptation mechanisms of notothenioids.

Materials & Methods

Ethics statement

This study, including fish sample collection and the experimental research conducted on animals, was conducted according to the regulations on activities and environmental protection in the Antarctic and approved by the Minister of Foreign Affairs and Trade of the Republic of Korea.

Fish sample preparation in response to temperature stress

The *N. coriiceps* (35 cm long) specimens were collected from Marian Cove, near King Sejong Station on the northern Antarctic Peninsula (62°14′S, 58°47′W), at depths of 20 to 30 m using the hook-and-line method, and the water temperatures were monitored and measured to be 1.6±0.8°C. The fish were transported in insulated containers of aerated sea water to the King Sejong Station, and acclimatized in large tanks of circulating fresh sea water at 2.0±0.2°C for at least 14 days prior to the experiments. We prepared three other large tanks at 4°C, 2°C, and −2°C for the heat stress, control, and cold stress experiments, respectively. Following acclimatization, three groups of nine specimens each of *N. coriiceps* were kept in the cold, normal, and heated tanks of aerated sea water. Three groups of three specimens of *N. coriiceps* each were sacrificed after 24 h of temperature stress. Their skin tissues were dissected, lysed, and immersed in RNAlater, and stored at −70°C for future experiments.

Illumina sequencing

For the RNA-sequencing (RNA-Seq) experiments, we prepared mRNA from blood samples from three skin specimens from each individual sample at each temperature condition. The total RNA was extracted using the RNeasy® Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted RNA was stored in RNAstable (Biometrica, San Diego, CA, USA) to prevent RNA degradation. The RNA was qualified and quantified using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). High-quality mRNA (2μg) was used to generate a double-stranded cDNA library using poly A selection then sequenced using an Illumina Hiseq 2000. The generated reads, which were approximately 75 bases in length and with approximate qualities of 20, were trimmed using Sickle (ver. 1.2) and the index sequencing primer and adaptor sequences were trimmed using Trimmomatic[15]. Low-quality reads were removed using the FASTX tool kit[16] with the parameters -t 20, -l 70, and -Q 33.

Gene expression under temperature stress

The *N. corriceps* specimens were transported to the King Sejong Station in insulated containers of aerated sea water and acclimatized in large tanks of circulating fresh sea water at 2.0±0.2°C for at least 3 days prior to the experiments. We prepared two other large tanks, for a total of three tanks at −2°C, 2°C, and 4°C for the cold stress, control, and heat stress experiments, respectively. Following acclimatization, three groups of nine specimens each of *N. coriiceps* were kept in cold, normal, and heated tanks of aerated sea water. Three groups of three specimens of *N. coriiceps* each

Table 1. Summary statistics of the RNA sequencing.

Sample ID	Run format	Assembled into genome	Trimmed reads	Total reads
Control	2×101	58,220,882	70,816,384	86,889,450
Cold	2×101	53,107,540	63,966,766	77,110,504
Heat	2×101	49,051,404	63,548,692	77,090,498

were sacrificed 0, 24 and 48 h after stress. We then collected blood samples and dissected each tissue of *N. coriiceps*. The dissected tissues were lysed, immersed in RNAlater, and stored at −70°C for future experiments.

For the RNA-Seq experiments, we prepared mRNA from skin samples from three specimens kept at each temperature condition. An Illumina Hiseq 2000 was used to sequence the mRNA, and the generated reads were trimmed using Sickle (ver. 1.2) with lengths of ~101 bases and base qualities of ~20 (Table 1). The trimmed reads were mapped to the annotated scaffold of the *N. coriiceps* genome[12] using TopHat[17], and the differentially expressed genes were assessed using Cuffdiff[18], which compares fragments per kilobase of exon per million fragments mapped values between each sample and calculates fold changes in expression for each gene based on statistical significance (cutoff, $P \leq 0.05$).

Data deposition

The obtained raw RNA-Seq data were deposited in the NCBI Sequence Read Archive (accession numbers SRX382171 and SRX382172) under bioproject number PRJNA208772.

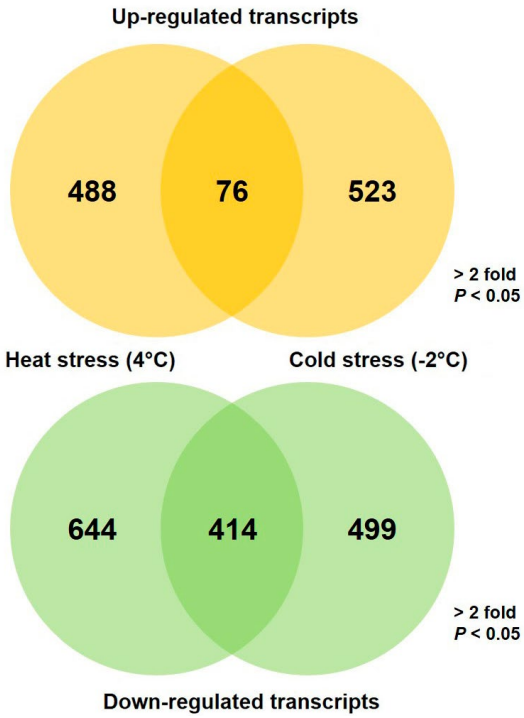

Figure 1. Number of statistically significant transcripts (i.e., over ±2 fold; $P<0.05$) in the skin tissue of *Notothenia coriiceps*, with the number of uniquely or commonly up- or down-regulated transcripts following exposure to heat (4°C) or cold (−2°C) stress.

Results & Discussion

We analyzed three libraries for each temperature (i.e., 4°C and −2°C) and three libraries for the control group (2°C) using the Illumina RNA-Seq platform. The sequenced skin cDNA libraries contained a large number of raw reads, with 49 to 58 million reads per library. After the trimming process, we retained 63-70 million reads from each library (Table 1). We aligned each library from the three groups (i.e. 4°C, 2°C, and −2°C) individually to the assembled reference transcriptome so that we could compare differentially expressed transcripts.

A total of 1,622 transcripts exhibited significant differences between the mRNA expression levels of the control and heat-stressed *N. coriiceps* with the criteria > ±2-fold change and $P<0.05$ (564 transcripts upregulated; 1,058 transcripts downregulated), while 1,512 transcripts exhibited significant differences between the cold-stressed and control *N. coriiceps* (599 transcripts upregulated; 913 transcripts downregulated) (Figure 1). Of the upregulated transcripts, 488 heat- and 523 cold-stressed *N. coriiceps* mRNAs were preferentially expressed depending on temperature, whereas 76 mRNAs were commonly upregulated in both temperatures. In the case of downregulated transcripts, 414 mRNAs were common to both temperatures, while 644 and 499 mRNAs were unique to the hot and cold conditions, respectively.

We compared the global functionalities of the heat- and cold-stressed skin transcriptomes to the functionality of the control group. We assessed the distinct sensitivities of the skin transcriptome profiling of the heat- and cold-stressed samples by conducting func-

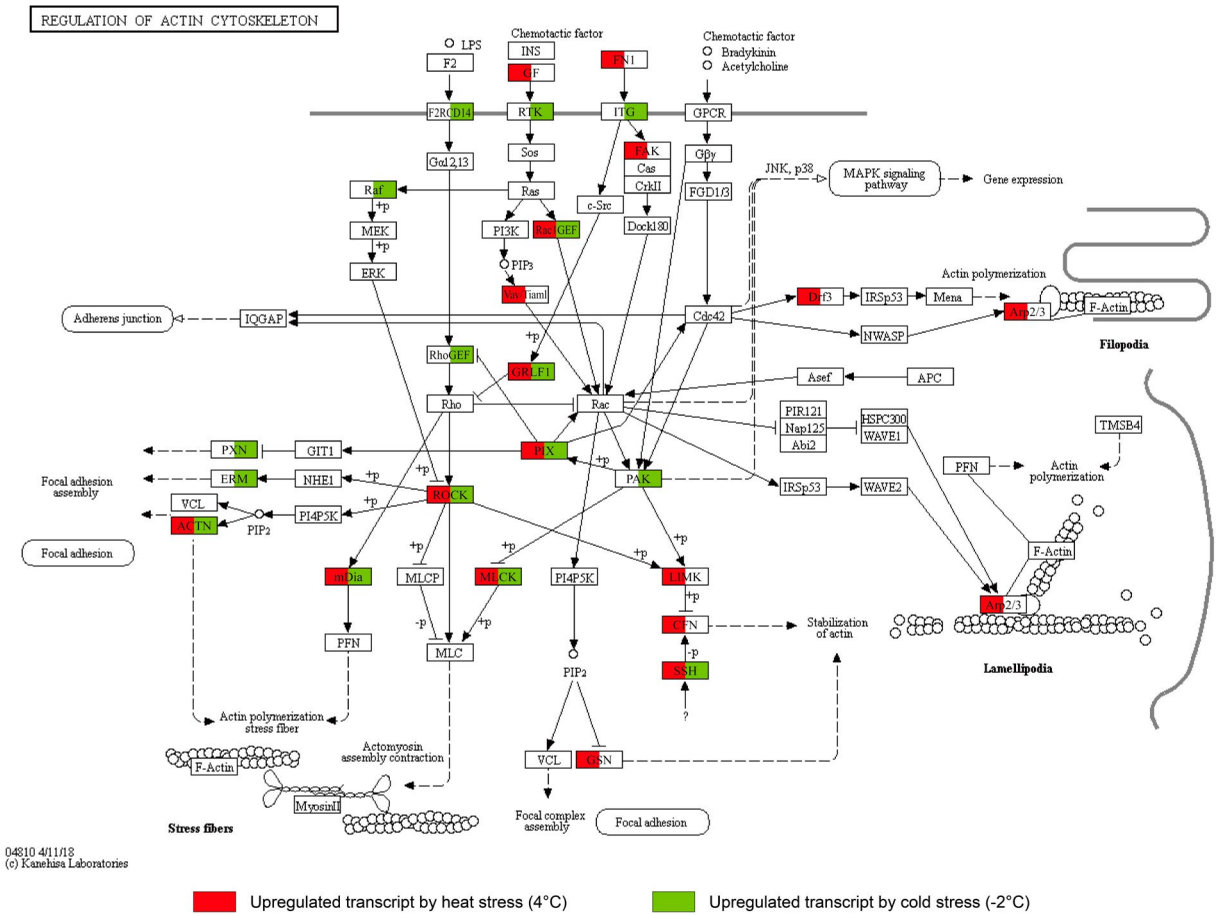

Figure 2. Comparison of mapped upregulated transcripts (i.e., over 2 fold; $P<0.05$) by both heat (4°C) and cold (−2°C) stresses in the *regulation of actin cytoskeleton* KEGG pathway. The transcript mapped from the heat-stressed *N. coriiceps* skin transcriptome is shown in red and the transcript mapped from the cold-stressed *N. coriiceps* skin transcriptome is shown in green.

tional classification via both Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway mapping. The results of the overall comparison between highly matched transcripts indicated that the transcriptional expression of genes associated with the muscle cytoskeleton in skin tissue is modulated at both temperatures. Many of the transcripts from both heat- and cold-stressed skin transcriptomes satisfying the criteria of $>\pm 2$-fold change and $P<0.05$ that aligned directly to KEGG pathways were matched with upregulation in the *regulation of actin cytoskeleton* pathway (Figure 2 and Supplementary Table 1 and 2). Many of the significantly downregulated transcripts were aligned to the *vascular smooth muscle contraction* pathway (Figure 3A and Supplementary Table 3 and 4). The results of the GO network analysis also supported the conclusion that a series of GO terms related to muscle contraction metabolism were downregulated by both heat and cold stresses (Figure 3B). The skin is the first barrier that protects fish against pathogens and numerous environmental factors, and protection by the underlying epithelium against natural, chemical, and mechanical damage means that its integrity is important for mucus production. Thus, skin strength should be maintained to lower the potential for exogenous damage and infections. Previous reports have suggested that fluctuations in environmental factors can affect skin morphology by modulating mucus cells in the fish epidermis[19-23]. Although there is limited information available on the effects of temperature on the skin tissues of stenothermic fish, modulations of the muscle cytoskeleton and its motility would be detrimental because they increase the susceptibility of *N. coriiceps* to physical disturbances, skin lesions, or infections.

Although certain genes associated with similar pathways were commonly up- or downregulated by heat or cold stress, we observed notable distinctive clear

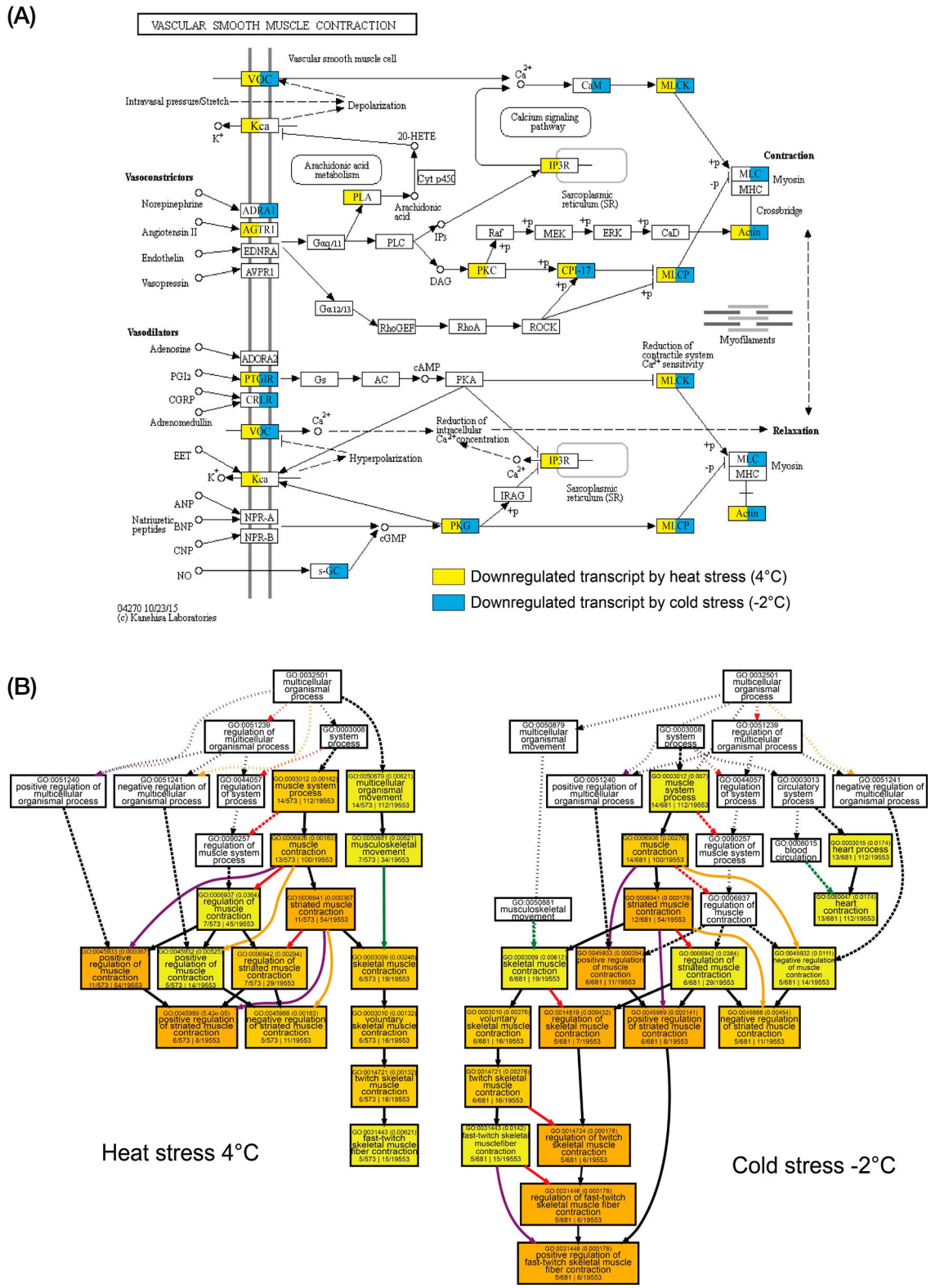

Figure 3. (A) Comparison between the mapped transcripts downregulated (i.e., over −2 fold; $P<0.05$) by both heat (4°C) and cold (−2°C) stresses in the *vascular smooth muscle contraction* KEGG pathway. The transcripts mapped from the heat and cold-stressed *N. coriiceps* skin transcriptomes are shown in yellow and cyan, respectively. (B) GO network analysis of the transcripts exhibiting significant downregulation in response to both heat and cold stress.

mRNA expression patterns in several isoform groups. Of those commonly upregulated by both temperatures, six epidermis-type lipoxygenase 3 (*aloxe3*) isoforms were detected in the heat- or cold-stressed skin transcriptome (Figure 4A). The *Aloxe3* gene is a member of the lipoxygenase gene family and mediates skin differentiation via hydroperoxide isomerase[24]. *Aloxe3* plays an important role in creating and maintaining the skin's ability to act as a water barrier, and is strongly associated with ichthyosiform disease, which is characterized by skin barrier dysfunction and transepidermal water loss[25]. To the best of our knowledge, there are no previous reports on the putative function of *aloxe3*. We assumed that the mRNA expression of its isoforms would be significantly upregulated to exert the observed control over water balance in skin tissue in the event of possible disturbances in muscle integrity. The transcriptional expression of several collagen isoforms (e.g., *col1, col6*) was significantly downregulated at both temperatures (Figure 4A). Collagen is the main structural protein in connective skin tissue and is important for maintaining the characteristics of fibrous tissues, such as rigidity or compliance. Although the potential functions of *col* isoforms in relation to temperature changes have rarely been studied in fish, the downregulation of *col* isoforms is strongly associated with muscle integrity. Interestingly, numerous transcripts coding for the nicotinic acetylcholine receptor (*ChRN*) gene in the skin transcriptome were downregulated significantly at both temperatures (Figure 4B). In fact, temperature changes significantly affected ChRN function[26]. The temperature dependencies of neuromuscular ChRN gating and agonist binding have also been investigated[27,28]. The cholinergic system is important for fish physiology and movement, meaning that the downregulation of *ChRN* transcripts affects the cognition and/or locomotion of *N. coriiceps*[29].

The transcriptional expression of numerous genes involved in immunity, including *complement and coagulation cascade, leukocyte transendothelial migration* and *phagosome* pathways, was significantly decreased at both temperatures (Figure 5 and Supplementary Table 3 and 4). In fact, we expected the temperature fluctuations to cause a decrease in the transcriptome because the immunosuppressive effects of changes in the water temperature on the systemic immunity of fish have been described in detail[30]. However, it is notable that the skin tissue of *N. coriiceps* has conserved complex immunity and transcriptional sensitivity against changes in temperature. The immune system is considered to be one of the most vulnerable systems to temperature changes, which can alter both innate and adaptive immunity. Thus, the transcriptional downregulation of immune-related pathways is involved in the dysregulation of

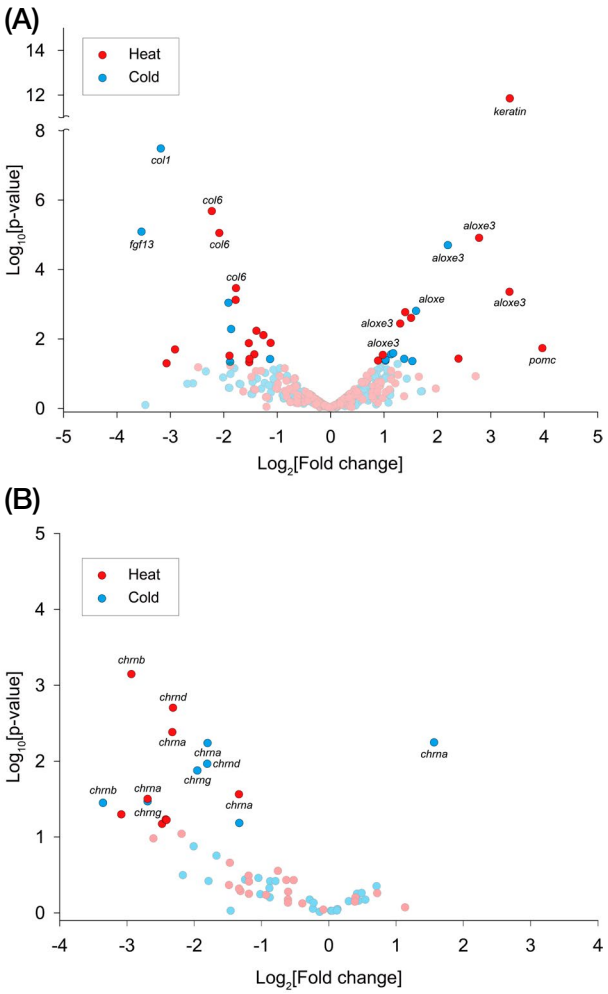

Figure 4. Volcano plots of distinctive clear mRNA expression patterns by both heat (4°C, red) and cold (−2°C, cyan) stresses observed in several isoform groups: (A) epidermis-type lipoxygenase 3 (*aloxe3*) and collagen, and (B) nicotinic acetylcholine receptor (*ChRN*). The normalized values were transformed by the $\log_2$ fold change and $-\log_{10} P$ value and plotted as the fold change (X-axis) against the P value (Y-axis). The shaded colors indicate insignificant P values ($P > 0.05$) with $< \pm 2$ fold expression.

immunity and homeostasis because the skin, which is the physical and chemical barrier of the innate immune system, controls the risk of exposure to numerous waterborne pathogens in fish.

Heat shock proteins (Hsps), which are a promising biomarker for temperature fluctuations, play a crucial role in maintaining protein homeostasis under sub-lethal stress conditions (e.g., heat, salinity, and aquatic pollutants) in fish[31]. Although all members of the Hsp family (e.g., *Hsp20, Hsp40, Hsp60, Hsp70* and *Hsp90*) are conserved in Antarctic fish, notothenioids are known to lack an inducible HSR under temperature

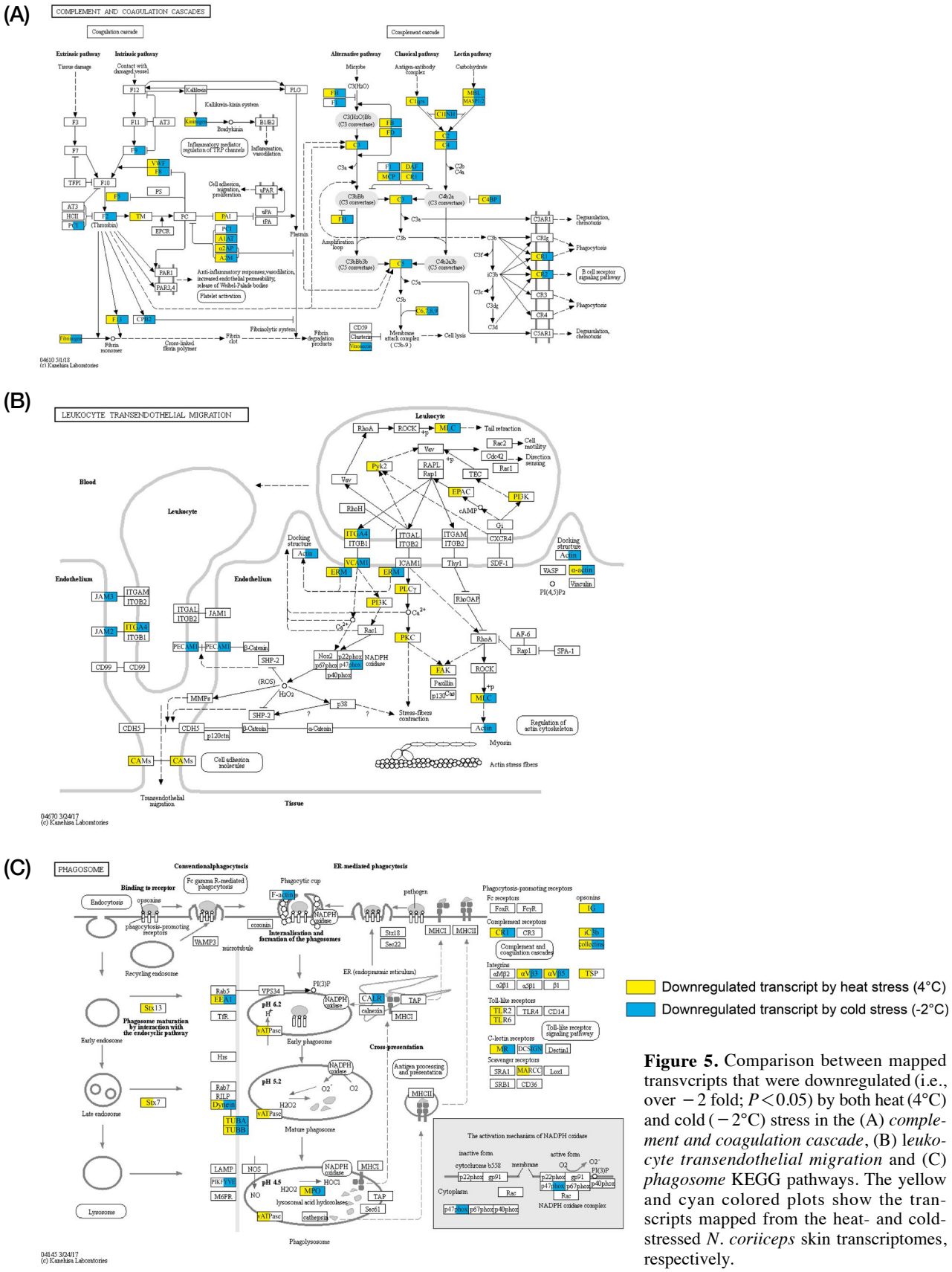

Figure 5. Comparison between mapped transvcripts that were downregulated (i.e., over −2 fold; $P<0.05$) by both heat (4°C) and cold (−2°C) stress in the (A) *complement and coagulation cascade*, (B) *leukocyte transendothelial migration* and (C) *phagosome* KEGG pathways. The yellow and cyan colored plots show the transcripts mapped from the heat- and cold-stressed *N. coriiceps* skin transcriptomes, respectively.

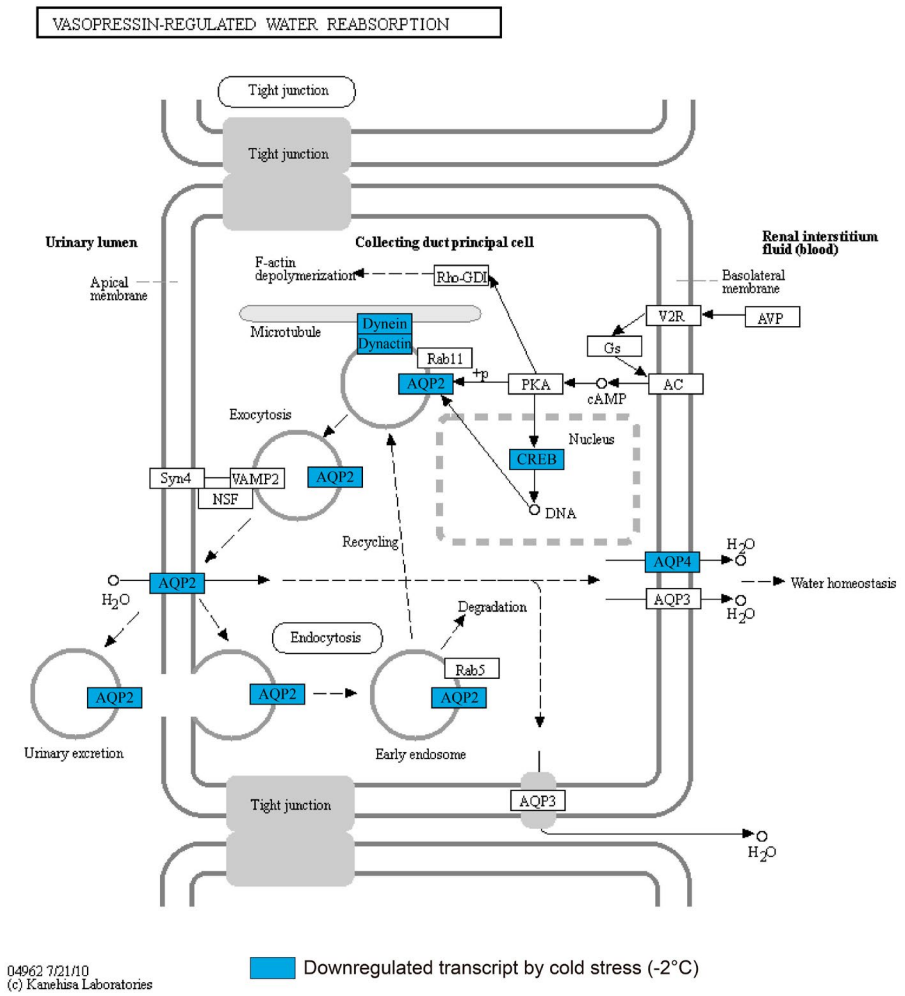

Figure 6. Analysis of the mapped transcripts downregulated (i.e., over −2 fold; $P<0.05$) by cold (−2°C) stress in the *vasopressin-regulated water reabsorption* KEGG pathway. The transcript mapped from the cold-stressed *N. coriiceps* skin transcriptome is shown in cyan.

stress[32-37]. Similarly, we did not observe any significant increase or decrease in the *Hsp* transcripts of heat- or cold-stressed *N. coriiceps* skin transcriptomes (data not shown), though HSR induction has previously been observed in the blood transcriptome of *N. coriiceps*[12]. Further studies are warranted to explore tissue-specific HSR in Antarctic notothenioids, with confirmation of their expression at the protein level.

We did not find any novel heat stress- or cold stress-specific pathways because we only analyzed the GO terms and KEGG pathways using significant transcripts (> ±2-fold change and $P<0.05$). In the case of the cold-stressed skin transcriptome, transcripts such as aquaporin-2 (*AQP2*) and *AQP4*, which are involved in *vasopressin-regulated water reabsorption* metabolism, were significantly downregulated (Figure 6 and Supplementary Table 4). Water permeability is controlled by vasopressin through the AQP2 water channel protein, which is known to play an important role in controlling the water content through phosphorylation and translocation from intracellular storage vesicles to the apical plasma membrane[38]. Particularly in marine fish, water excretion should be highly controlled to regulate the osmotic water permeability with respect to the salt content. The transcriptional decrease of this pathway is presumably associated with controlling the water balance of skin tissue, as observed via modulation of the *aloxe3* isoforms, which controls the skin's functionality as a water barrier.

Conclusion

Taken together, we have presented the entire spectrum

of transcriptional responses involved in common induction and inhibition mechanisms found in the heat- and/or cold-stressed skin transcriptome in *N. coriiceps*. Our results show that the application of transcriptome profiling is a promising approach to the study of the effects of temperature fluctuations, as each transcriptome can respond to temperature stress via both specific and common response mechanisms. Further research on the effects of temperature fluctuations will enable us to gain a more comprehensive understanding of the underlying metabolism governing how Antarctic fish respond and adapt to changing temperatures.

Acknowledgements This work was supported by the Polar Genome 101 Project (PE19080) grant, funded by the Polar Research Institute of South Korea.

Conflict of Interest Bo-Mi Kim, Do-Hwan Ahn, Seunghyun Kang, Jihye Jeong, Euna Jo, Bum-Keun Kim, Jin-Hyoung Kim & Hyun Park declares that they have no conflict of interest.

Human and animal rights The experimental procedures followed the actual law of animal protection that was approved by the Animal Welfare Ethical Committee and the Animal Experimental Ethics Committee of the Korea Polar Research Institute (Incheon, South Korea).

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