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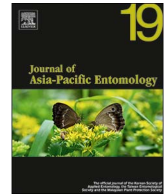

Supercooling capacity along with up-regulation of glycerol content in an overwintering butterfly, *Parnassius bremeri*

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ABSTRACT

Eggs of an endangered butterfly, *Parnassius bremeri*, hatch during December and young larvae grow during winter and early spring. Cold tolerance is required for survival and growth of the young larvae. Supercooling points (SCPs) were below − 20 °C for the young larvae compared to − 9.8 °C for the last fifth instar (L5) larvae, which developed during May. To investigate the depression of SCPs in young third instar (L3) larvae, polyols and free amino acids in hemolymph were quantified using a BioLC. Compared to L5 larvae, L3 larvae had higher polyols (glycerol and mannitol). However, L5 larvae had much higher levels of trehalose than L3 larvae in hemolymph. Increase of glycerol in L3 larvae was explained by enhanced expression of genes associated with glycerol metabolism. RNA-Seq analysis indicated that expression level of *glycerol-3-phosphate dehydrogenase* in L3 larvae was higher than that of L5 larvae and more number of *glycerol kinase* contigs was detected in L3 larvae. In contrast, trehalose-6-phosphate dehydrogenase was highly expressed in L5 larvae by more than 3.2 folds compared to L3 larvae. These results suggest that deep supercooling along with glycerol and other small cryoprotectants is associated with cold tolerance of young larvae of *P. bremeri*.

Introduction

Non-migratory insects in temperate zone overwinter to continue generations, in which they cope with harsh environments, such as lack of food and low temperatures. Lack of food can be overcome by depressing overall metabolic rate to minimize unnecessary energy usage (Storey and Storey, 1990). However, low temperature may cause serious damages against the poikilothermic animals. Damages induced by low temperatures during winter are divided into freezing injury and nonfreezing (= cold shock) injury (Kelly et al., 1996). Freezing injury includes a physical damage induced during ice growth from its nucleator (Salt, 1961). Also, freezing prevents water availability, and leads to osmotic shock and denaturation of biological materials. Nonfreezing injury includes disturbance in fluidity of phospholipid bilayers in response to low temperature and denaturation of proteins, which result in malfunctioning of biological membrane (Salt, 1961; Michaud and Denlinger, 2004).

To overcome these low temperature injuries, insects have developed at least two strategies (Baust and Rojas, 1985; Lee, 1989). Freeze-tolerant insects restrict ice formation only in extracellular space, which allows an igloo effect on intracellular components. To stabilize biological membrane under freezing desiccation status, polyols are produced and localized in intracellular space (Storey and Storey, 2012). In contrast, freezing-susceptible insects prevent internal ice formation by depressing body freezing point measured by supercooling point (SCP). Low SCPs are achieved by accumulating cryoprotectants within the body. Small polyol molecules, such as glycerol and sorbitol, are well known to supercool the body fluid because the freezing point depression depends on the number of solutes and their alcohol groups behave like water molecules to stabilize biological membrane.

The red-spotted Apollo butterfly, *Parnassius bremeri* Bremer, is a high altitude butterfly which is found in Far Eastern Asia such as Russia, Korea and China. It has been known as a member of the snow Apollo genus *Parnassius* of the Swallowtail (Papilionidae) family (Omoto et al., 2004; Choi and Kim, 2012). The butterfly in genus *Parnassius* has been designated as the most endangered insect species around the world and in this regard Korean government also designated *P. bremeri* as an endangered species in 1989 (www.korearedlist.go.kr). Furthermore, genus *Parnassius* was classified as vulnerable taxon to be extinct by the International Union for Conservation of Nature and Natural Resources (IUCN) Red List (www.iucnredlist.org), and listed on Appendix II of on the Convention on International Trade in Endangered Species (CITES)

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(www.cites.org).

One of the factors threatening the butterfly populations might be a reduction of host habitats. Kim et al. (2011) analyzed metapopulations of *P. bremeri* by mark-release-recapture method and showed its distance-dependent migration. An anthropogenic or natural removal of local habitat patches may prevent its seasonal migration according to host plants, which may result in frequent collapses of local populations (Ko et al., 2004). In addition, a global warming of climate change may accelerate the reduction of host habitats for *P. bremeri*. A perennial herb, *Sedum kamtschaticum*, which is drought-resistant and sprouts during winter, is usually consumed by young larvae of *P. bremeri* that hatch during winter in Korea (Kim et al., 1999). This suggests that *P. bremeri* should be tolerant to low temperatures to be protected from cold and freezing damages.

This study investigated supercooling capacity of young larvae of *P. bremeri* because they develop during low temperature seasons. To explain the low SCPs of young larvae, cryoprotectants in hemolymph were identified and quantified using a BioLC. Finally, RNA-Seq was performed to compare transcript levels of genes associated with polyol biosynthesis between young and old larvae to explain the significant difference in SCPs.

Materials and methods

Rearing P. bremeri

Four pairs of adults were first collected in Samcheok, Korea with a permission of Wonju Local Environmental Administration in 2005. A whole rearing procedure of *P. bremeri* was performed in field conditions (Hweongsung, Korea; see inset map of Fig. 1) with intense handlings. Briefly, laid eggs were manually attached to the fallen oak tree leaves and kept in field conditions for 180 days in double net (0.1 × 0.3 mm mesh) cage to protect from any attacks of parasitoids or predators. The eggs were then transferred to young larval cage (40 × 50 × 70 cm) and the resulting newly hatching larvae were collected into a plastic petri dish (10 cm diameter × 4 cm height) with supplement of host plant, *S. kamtschaticum*. At 4th instar (L4, late April), the larvae were separated into each of 30 individuals and kept in a metal cage (71 × 51 × 88 cm covered with 1 × 1 mm metal mesh) with host plants. Developed adults were transferred to mating cages (270 × 190 × 220 or 190 × 130 × 170 cm covered with 1 × 1 mm metal mesh depending on population size) and provided with host plant and *Cirisium japonicum* for oviposition sites. Field aerial temperatures in the experimental station were measured daily for 4 years (2012–2015) with an auto-recording thermometer (DT-127, CEM, Shenzhen, China).

SCP measurement

SCPs of all developmental stages of *P. bremeri* were measured according to the method of Kim and Kim (1997). The measurement of each developmental stage was replicated three times with three different individuals.

Polyol analysis using a BioLC

Hemolymph from third (L3) and fifth (L5) instar larvae of *P. bremeri* were collected into 1.5 mL tubes containing a pinch of anticoagulant powder, phenylthiourea (PTU, Sigma-Aldrich Korea, Seoul, Korea), and diluted with the distilled and deionized water. Carbohydrate and polyol were analyzed using an ion exchange HPLC (BioLC, Dionex, Sunnyvale, CA, USA) by the method of Park et al. (2014).

Transcriptome analysis of P. bremeri larvae using RNA-Seq

Total RNAs were isolated from L3 and L5 larvae of *P. bremeri* using Trizol reagent (Invitrogen, Carlsbad, CA, USA). L3 sample used 20 larvae and L5 sample used 3 larvae. Each extracted RNA sample was resuspended in 40 μL diethyl pyrocarbonate-treated water. RNA integrity for subsequent RNA-Seq was analyzed using Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). This RNA QC was evaluated based on an RNA Integrity Number value greater than or equal to 7. Our samples recorded as 7.7–8.5.

From total RNAs, cDNA library was constructed with Triseq RNA kit (Invitrogen, Seoul, Korea) and sequenced by Illumina HiSeq 2000 (Illumina Inc., San Diego, CA, USA) by 101 bp reads. Raw reads were trimmed by Trimmomatic 0.32 program (http://www.usadellab.org/cms/?page=trimmomatic) under a criterion of 230 (phred score base quality 30% or more) and then assembled using Trinity program (http://trinityrnaseq.sourceforge.net/). The assembled contigs were used to be calculated into FPKM (fragment per kilobase of transcript per million mapped reads) with RSEM (1.2.15) program (http://deweylab.biostat.wisc.edu/rsem/) and annotated using BlastX program of NCBI GenBank. Based on the annotated contigs, three genes of *GK*, *GPDH*, and *TPS* were chosen and compared in their FPKM levels between L3 and L5 larvae. Sequences of all these genes were uploaded to GenBank with accession numbers of KY565575 for *GK*, KY565577 for *GPDH*, and KY565578 for *TPS*.

Data analysis

All bioassays were performed in three independent replicates.

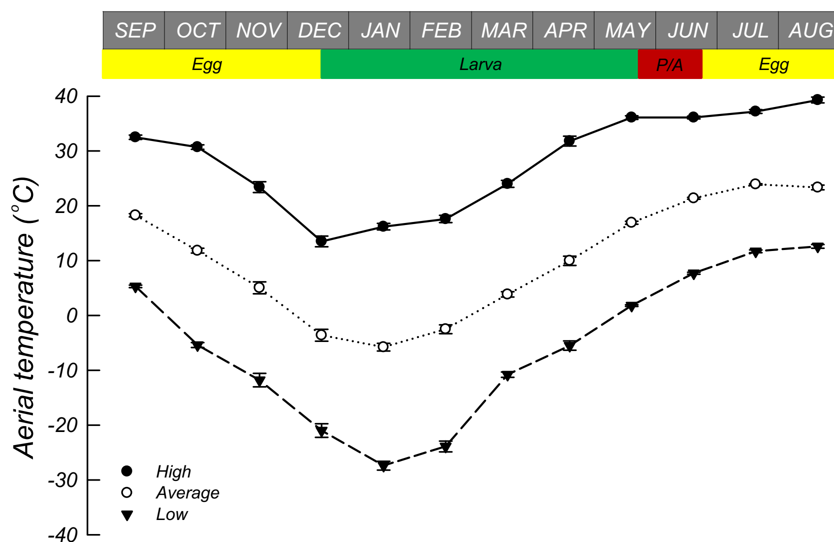

Fig. 1. A life cycle of *P. bremeri* and monthly aerial temperatures in Hweongsung, Korea (see inset figure), where it developed on host plant, *S. kamtschaticum*, in field conditions. Field ambient temperatures were measured in the experimental station for 4 years (2012–2015). Temperatures are depicted in means of high, average, low temperatures in each month. Error bars indicate standard deviations. 'P/A' indicate pupa and adult developmental stages, respectively.

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