Impact of soil warming on the plant metabolome of Icelandic grasslands

Master in Industrial Chemistry and Introduction to Chemical Research

Master Thesis

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Index

List of acronyms

LC-MS	**L**iquid **C**hromatography–**M**ass **S**pectrometry
GC-MS	**G**as **C**hromatography–**M**ass **S**pectrometry
HPLC	**H**igh **P**erformance **L**iquid **C**hromatography
FTMS	**F**ourier **T**ransform **M**ass **S**pectrometry
S/N	**S**ignal-to-**N**oise ratio
m/z	**M**ass-to-**C**harge ratio
ESI	**E**lectro**S**pray **I**onization
HESI	**H**eated **E**lectro**S**pray **I**onization
RT	**R**etention **T**ime
PCA	**P**rincipal **C**omponents **A**nalysis
PC	**P**rincipal **C**omponent
PLS-DA	**P**artial **L**east **S**quares **D**iscriminant **A**nalysis
ANOVA	**AN**alysis **O**f **VA**riance
PERMANOVAs	**PER**mutational ANOVAs
RNA	**R**ibo**N**ucleic **A**cid
DNA	**D**eoxyribo**N**ucleic **A**cid
MEOH	**M**ethanol
ROS	**R**eactive **O**xygen **S**pecies

Abstract

Under the projected climate change scenario, plants will be subjected to a combination of abiotic stresses such as warming and drought. The temperature seems that will rise in higher proportion at high than at temperate and tropical latitudes.

The natural geothermal conditions in southern Iceland provide an opportunity to study the impact of warming on plants because of hot springs and fumaroles that induce stable temperature gradients at soil level. In one of the studied valleys, hot springs have been present for centuries while in another valley hot springs started in 2008 after suffering a shallow crustal earthquake.

We aim to study the impact of soil warming on the metabolomic profile of two typical species of Arctic latitudes *Agrostis capillaris L.* and *Ranunculus acris L.* and discern whether their shifts in metabolome became stronger in the long-time warming grassland than in the short-time warming grassland.

Agrostis capillaris L. and *Ranunculus acris L.* showed a different response to warming depending on the evolutionary time they were exposed. While the grass species presented a shift in metabolome on the long-time warming site, the herb species did not show a clear shift. The different responses of both species could be related to their different reproductive strategies; the grass species reproduces mainly by vegetative spread whereas the herb reproduces mainly by seedlings. This enable the grass species a faster adaptation capacity at plot level.

The temperature threshold where we observed an abrupt shift in overall metabolome profile of *Agrostis capillaris L.* and *Ranunculus acris L.* species was located between 5-10 ºC above the control temperature. Current climate models projected by the scenario of 2100 increases of annual average temperature between 3-5 ºC for the Arctic region depending on the areas. The results thus suggest that some current dominant plants in the boreal would not trigger the metabolism pathways related to warming stress.

In the long-time grassland the main compounds that we detected to be up-regulated at highest temperatures were sugars and amino acids both related to heat shock metabolic pathways and some secondary metabolites such as phenols and terpenes also associated with a wide array of stresses.

1 Introduction

The effect of human activities on environment has exponentially increased since pre-industrial era leading to higher atmospheric concentrations of carbon dioxide, methane and nitrous oxide. These atmospheric gases seem to have been the dominant cause of the observed warming since the mid-20th century (IPCC, 2013). The impact of climate change is difficult to predict because it depends on many direct and indirect effects[2–4]. Overall, the increase of global mean surface temperature at the end of the century (2081-2100) is likely to increase between 2.6 ºC to 4.8 ºC[5]. The effect of the temperature is intrinsic to all species since it alters their life cycle and each species has a range of living temperatures depending on its distribution. Often the exposure to higher temperatures implies generally a faster development but does not mean a higher production because climate change also will imply a shorter life cycle and reproductive phase reducing the yield potential[6]. The capacity of organisms to increase their activity by warming could be limited by several variables such as water and nutrients availability[7–9]. These alterations of climatic conditions usually affect living organisms shifting their seasonal activities and migration patterns[10,11].

Temperature rises across the globe and especially in the High North is melting snow and ice further altering the capacity of the hydrological system at regional but also at planetary scale. The Arctic region is one of the most studied areas in the context of climate change impacts because of it is crucial importance related to the climate change of all the planet. Over the next 100 years, climate change is expected to accelerate and to be also accompanied by several other processes such as impacts on river runoff, on atmospheric circulation and modulation of atmospheric CO_2 and CH_4 concentrations at world scale[12]. Boreal areas are receiving an extra energy input in its surface compared with other world areas, as example in the tropic areas where a greater fraction of the new warming energy is consumed into evaporation. However, in Arctic and Antarctic regions the melting of snow and ice reduces the albedo effect causing increase of heat absorption by ocean surface and land. As warming reduces the extent of sea ice, solar heat absorbed by the oceans in the summer is more easily transferred to the atmosphere during winter, and because of this absorbed energy is transported by the atmosphere and oceans affecting other regions. Under this scenario, the temperatures are projected to rise the Arctic between 3-5ºC over the land and above 7ºC in the oceans[13].

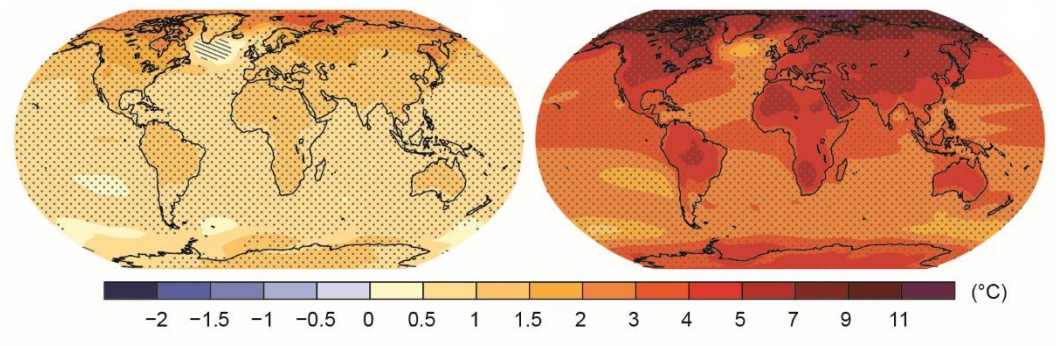

Figure 1. Maps of CMIP5 multi-model mean results for the scenarios RCP2.6 (left) and RCP8.5 (right) of change in average surface temperature (1986-2005 to 2081-2100). Source: IPCC 2013.

By this high capacity to absorb light energy in arctic, warming advances more rapidly than in the other parts of the world. In the Arctic, growing season is short and cool and thus imposing major constraints on the biota because the growing season is so compressed[14]. Therefore, faster changes as for instance the lengthening of the summer period can have a great impact on the artic ecosystems.

Plants are closely tuned to the abiotic and biotic factors and they have evolved biochemical, molecular and genetic mechanisms to avoid stress[15,16]. When there is an alteration of the normal climatic conditions, plants suffer stress and respond to avoid injury. Plants exposed in any stress conditions are able to perceive the first stress signals and trigger stress responses mechanisms. Moreover, there is an overlap among the different stress responses explains the cross-tolerance phenomenon. Several studies provide evidence that physiology of the plants are directly controlled by abiotic conditions such as: salinity[17–20], water availability[21], nutrients availability[22–24] temperature[20]. Those abiotic factors must be in a balanced ratio to reach the optimum abiotic conditions for live developing. There are many investigations providing information about the mechanisms underlying the stress response and the identification of the specific genes and metabolites that are responsible for tolerance in determined phenotypes to different abiotic stresses. One of the most important and studied stress is that due to temperature change.

Plants are subjected to continuous diurnal and seasonal temperature fluctuations and some species are adapted to extreme climates, they have acquired thermotolerance, where plants have increased the freezing or heat tolerance[25]. These high complex mechanisms of defense are reached by reprogramming the transcriptome, proteome and finally the metabolome of plants. Predictions of climate change suppose that plants will respond to different abiotic stress factors such as warming, drought, cold, salinity and/or

anthropogenic activities with the final consequence of the alteration of their metabolome as the end of the cascade of responses to adapt to new temperature situation. These changes are associated with metabolic pathways up- and down-regulation to increase resistance to stress, and are thus the consequence of the final molecular responses associated with physiological and morphological shifts such as growth, production, photosynthesis-respiration, reproductive output, defense responses and finally to the proportional allocation of resources to all these different functions and also to different plant organs.

The heat stress induces metabolic changes that frequently are associated with heat shock protein accumulations, that are responsible of this stress resistance in heat-exposed conditions[26,27]. Extreme high temperatures during the reproductive stage affect fertilization, pollen viability and even fruit formation[28,29]. The fast climate change and extreme climatic events are expected to have a large impact on plants and ecosystems, to the point of surpassing the thresholds of resistance of ecosystems[30–36]. Improving tolerance to these fast climate change and extreme events will be a target for ongoing and future agricultural and nature-conservation programmes. Species of fundamental importance in nature conservation in current boreal areas, such as the grass *Agrostis capillaris L*. and one herbaceous dicot *Ranunculus acris L*., can be affected by these extreme events. These species are thus interesting subjects for studying the impacts of extreme periods of warming on the metabolomic shifts in plants and soil.

The impact of climate change on grasslands on high latitudes is higher than in grasslands of mid- and low-latitudes due to a rapid increased of climate change in these cold-climate regions. This makes the boreal ecosystems especially helpful to understand the sensitivity of plant communities in terms of community change. Northern ecosystems are characterized by stress-tolerant species, adapted to nutrient-poor conditions and having low leaf phosphorus and mainly nitrogen concentrations[37,38]. Several records provides the evidence of relatively fast vegetation changes in response to past climate change in Iceland. During the last years a metaanalysis of phenological, growth and reproductive responses of individual plants on moderate warming sites shows that species respond in different ways and to different degrees depending on growth form and initial environmental conditions[39,40].

Previous studies showed that warming may cause a shift in abundance of species due to a change on nitrogen availability and decomposition of soil organic carbon[41]. On the other hand, nitrogen is an important nutrient present in proteins and in essential enzymes and the limitation of this element could have crucial effects on plant

productivity. Higher temperature also means higher decomposition rate, increasing the carbon released to the atmosphere and thus a positive feedback on global warming. Some experiments on tundra vegetation evidence an increase in nitrogen availability and nutrient accumulation in response to warming, suggesting that could be an important indirect effect of warming[42]. An increase on nitrogen availability can favour more competitive species against more stress-tolerant.

Metabolomics is a powerful tool for improving our understanding of the changes in metabolism and biochemical composition of organisms, i.e. the ultimate phenotypic response to environmental changes[43–47]. It is increasingly applied to ecological studies in what has been called ecometabolomics[45,46,48]. Ecometabolomics can explore the effects of the ecological organism-environment interaction by detecting the final phenotypic response of the organism and by detecting the metabolic pathways that are up- and down-regulated in response to environmental changes. Within the context of metabolomics, a metabolite is defined as an organic molecule, weighting less than 1kDa. Metabolites are the final products of the activity of different cellular processes and, hence, define the phenotype in the sense of the final response of the genotype to environmental changes. Metabolites can be divided in primary metabolites that are synthesized by the cell because of their indispensability for their growth, development and reproduction, such as organic acids or nucleotides, and secondary metabolites, which are those that are produced by an organism not for primary metabolic processes but are important for ecological functions such as terpenes or phenols. The principal goal of studying metabolomics is to monitor metabolic changes during stress responses, to identify metabolites belonging to the groups that are responsible for stress tolerance and the shift in metabolic pathways and functions of the plants in response to warming stress.

The concept of top-down omics approaches allows to begin at the highest conceptual level and work down to the details. In this way, metabolomics provides a better analysis of the different response capacities conferred by the phenotypic plasticity of each species, allowing for elucidation of metabolic pathways that might be involved in a specific phenotypic response. Metabolomics can be also used as a preliminary screening study of the metabolome response. This does not exclude the simultaneous or subsequent use of targeted chemical analyses.

Previous metabolomic studies of warming stress on plants have observed increases in the concentrations of saturated fatty acids[49,50] in the thylakoid[51] and plasma membranas[52]. Metabolomic studies have allowed to observe that under combined drought and warming conditions plants respond differently at shoot than at root levels[53].

Under high temperature stress, the level of saturation of membrane lipids extracted from the leaves of creeping bent grass increased, whereas no change in membrane lipids was observed in root tissues[54]. These metabolomic changes usually drive warming to increase biomass production in several ecosystems[41]. Warming can thus have a positive effect on growth and biological activity but only when water or nutrients are not limited warming can also have a negative effect on plant growth and primary productivity in other ecosystems, often because warming reduces water availability and/ or nutrient availability or up-take capacity[55,56].

Ecometabolomics has recently been used to monitor the phenotypic changes of a particular genotype in response to temperature shifts[48,57–64]. The effects of warming on leaf metabolomes have been widely studied[46,48,58–60,62,65]. Nevertheless, undo less is known about the combined effect of warming on plant-soil system by metabolomic analyses of both compartments at once, and little is known on the tipping point of increased warming.

In this master thesis two grassland ecosystem, submitted to natural temperature gradients of warming are studied in Iceland to consider plant and soil simultaneously to search for tipping points in the responses to progressive warming, and to discern the role of evolutionary adaptation, the long-term responses in comparison with immediate short-term responses to warming. Iceland is an island formed in the zone between the American and Eurasian continental plates[66]. The area around Hveragerði is extremely geothermally active, because it is situated in the direct surroundings of the Hengill volcano system[67]. This geothermal activity manifests itself as hot springs and fumaroles. These hot spots warm the surrounding soil layers, creating a soil temperature gradient. We studied two valleys that have housed fumaroles and hot rivers for centuries. The hot spots are present in the first grassland from at least the 1980ties and is assumed that this valley can be considered as a study of long time exposure and the plant species communities can be considered to be near to equilibrium. The second valley suffered a shallow crustal earthquake with a strength of 6.3 on the Richter magnitude scale in 2008. This earthquake changed the

Figure 2. Natural soil warming in natural grassland in Iceland.

course of underwater systems and as a result new fumaroles appeared and the soil began to head up. Thus, this valley can be considered as a proxy to study the short-medium time exposure and the plant species communities can be considered to be far to equilibrium.

We investigated the impact of warming on the metabolomic structure of aboveground organs (leaves) of the grass *Agrostis capillaris L*. and the herbaceous dicot *Ranunculus acris L*., in both sites. We tested the hypothesis that plants respond differently depending on the time that their population have been exposed to warming. The interest of this study is that the temperature gradient of soil in these valleys of southern Iceland provides the possibility to study the impact of the predicted temperature rise at short- and at long-time and to study the likely abrupt shifts in the progressive warming by comparing plots in a gradient from control to +15 ºC. The temperature gradient in the experimental sites has a lot of advantages in comparison with the human induced warming experiments such as lack of experimental artefacts in addition to the zero cost of warming. Moreover, as in the old valley was likely warmed during centuries or at the very least by more than 30 years, and the newest valley is warmed only since 2008 they offer an excellent chance to study the impact of temperature on many ecosystems process at different time scenarios.

2 Objectives

The objectives of this Master Thesis were the following ones:

1. To study the impact on soil warming on the metabolic profile in two plant species, the grass *Agrostis capillaris L* and the herb *Ranunculus acris L*. a grass and herb, i.e. two plants species of very different morphological and physiological traits.

2. To discern whether the growth shifts in the metabolome in response to warmer soils are stronger in the grassland submitted to long-time warming than in the grassland submitted to short-medium time warming.

3. To find out which metabolites are in higher or lower concentration in relation with the different degrees of warming, and therefore which metabolic pathways are up-regulated and which ones are down-regulated.

4. To search for tipping points, i.e. for thresholds, in the response of the metabolomes to increasing warming.

3 Results

Both studied species, *Agrostis capillaris L.* and *Ranunculus acris L.*, had different overall metabolome profiles (PERMANOVA pseudo-*F* = 243; *P* < 0.001). The metabolome profile was also affected by site (pseudo-*F* = 13.2; *P* < 0.001), and marginally significantly by warming (pseudo-*F* = 2.02; *P* < 0. 1). There was a significant interaction between species and site (pseudo-*F* = 6.29; *P* < 0.001) and between site and warming (pseudo-*F* = 2.03; *P* < 0.05), but not between species and warming (pseudo-*F* = 1.26; *P* > 0.05). The temperature threshold where we observed an abrupt shift in overall metabolome profile of both species was located between 5 and 10 ºC above the control temperature (Table S1).

Soil pH and C and N concentrations were different between sites (pH; *F* = 5.42; *P* < 0. 05; %C: *F* = 10.5; *P* < 0. 001; %N: *F* = 155; *P* < 0. 001) and among warming levels (pH; *F* = 6.03; *P* < 0. 001; %C: *F* = 7.92; *P* < 0. 001; %N: *F* = 4.18; *P* < 0. 005). No significant differences were found for soil RNA/DNA neither with site nor with warming (pseudo-*F* = 0.56; *P* > 0.05) (Table S2).

When all data were analysed together in a Principal Component Analysis (PCA), PC1 accounted for the differences between species, whereas PC2 separated samples of both sites (Figure 3) consistent with the results of PERMANOVA analysis. PCs 1 and 2 explained 31 and 7% of variance respectively in the PCA conducted with the leaf samples (including species, site and warming). Species is thus the primary factor and site the secondary factor explaining leaf metabolomic profile variance. Concentrations of amino acids, some amino acid and sugar related compounds (RCAAS), some nitrogen bases, some phenols and most organic acids were higher in *Agrostis capillaris L.* species (Figure 3). The concentrations of some sugars such as ribose, lyxose, sorbose and trehalose, some organic acids such as malic acid, and some phenols were higher in *Ranunculus acris L.* species (Figure 3).

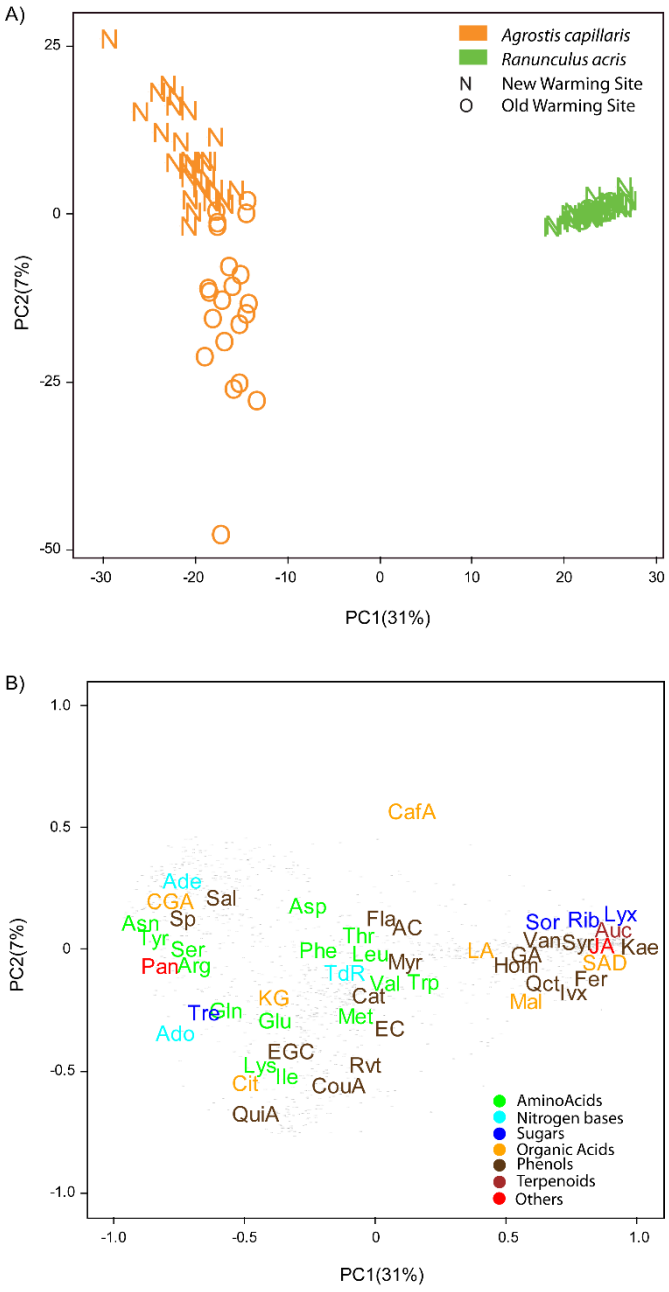

Figure 3. Plots of cases and variables in the PCA conducted with the physic-chemical, elemental composition, biological and metabolomic variables in *Ranunculus acris L. and Agrostis capillaris L.* using PC1 versus PC2. (A) The cases are categorized by site and specie. Species are indicated by different colours (green, *Ranunculus acris L.*; orange, *Agrostis capillaris L*. The two sites are indicated by symbols N, new warming site; O, old warming site). (B) Loadings of the metabolomic variables in PC1 and PC2. The various metabolomic families are represented by colours: dark blue, sugars; green, amino acids; orange, related compounds to the metabolism of amino acids and sugars; cyan, nucleotides; brown, phenolics; dark red, terpenes and red others. Metabolites: Arginine (Arg), asparagine (Asn), aspartic acid (Asp), glutamic acid (Glu), glutamine (Gln), isoleucine (Ile), lysine (Lys), leucine (Leu), methionine (Met), phenylalanine (Phe), serine (Ser), tryptophan (Trp), threonine (Thr), tyrosine (Tyr), valine (Val), adenine (Ade), adenosine (Ado), thymidine (TdR), chlorogenic acid (CGA), trans-caffeic acid (CafA), α-ketoglutaric acid (KG), citric acid (Cit), L-malic acid (Mal), lactic acid (LA), succinic acid (SAD), pantothenic acid hemicalcium salt (Pan), jasmonic acid (JA), 5,7-dihydroxy-3,4,5-trimethoxyflavone (Fla), acacetin (AC), epicatechin (EC), epigallocatechin (EGC), homoorientin (Hom), isovitexin (Ivx), kaempferol (Kae), myricetin (Myr), quercetin (Qct), resveratrol (Rvt), saponarin (Sp), catechin hydrate (Cat), 3-coumaric acid (CouA), gallic acid (GA), quinic acid (QuiA), Sodium salicylate (Sal), syringic acid (Syr), trans-ferulic acid (Fer), vanillic acid (Van), 2-deoxy-D-ribose (Rib), D-(-)-lyxose (Lyx), D-(+)-Sorbose (Sor), D-(+)-Trehalose dehydrate (Tre), Aucubin (Auc). Unassigned metabolites are represented by small grey points.

3.1 Effects of site and warming on the metabolome of *Agrostis capillaris L.*

PCs 1 and 2 explained 13 and 8% of variance respectively in the PCA conducted with the leaf samples (including site and warming) of *A. capillaris* plants (Figure 4). PC1 accounted for the differences between sites. RNA/DNA ratios are higher in the old site (Figure 4).

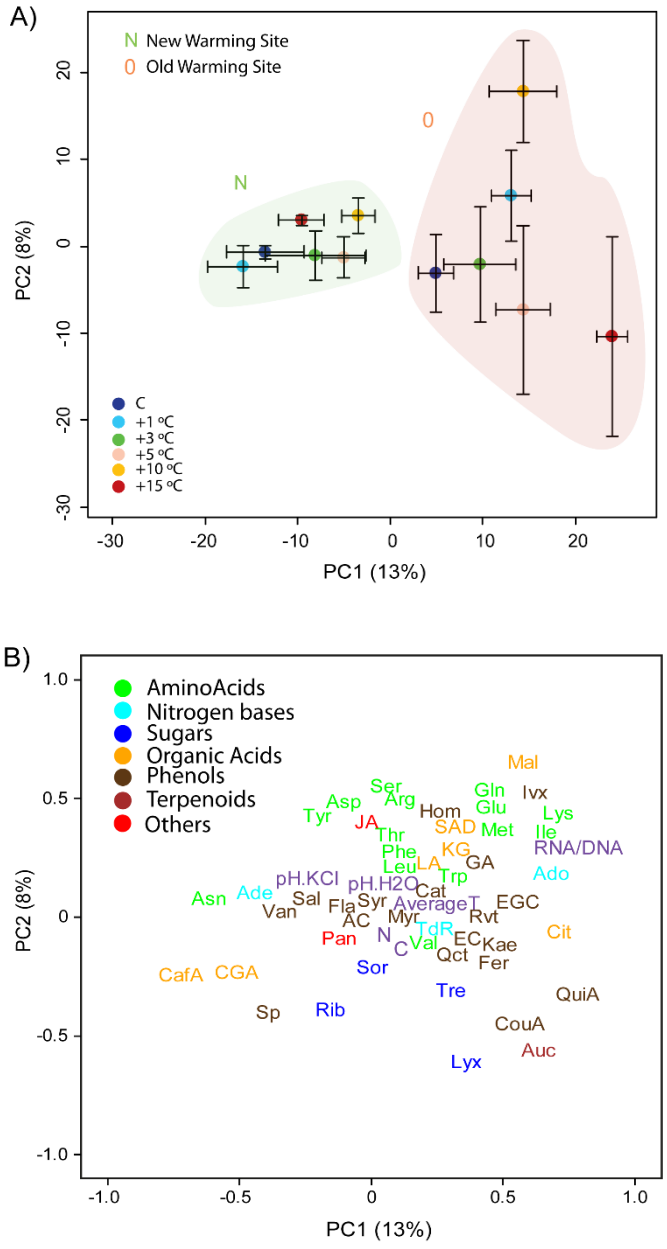

Figure 4. Plots of cases and variables in the PCA conducted with the physic-chemical, elemental composition, biological and metabolomic variables in *Agrostis capillaris L.* using PC1 versus PC2. (A) Samples categorised scores (mean ± S.E.) in both sites (new warming site, N and old warming site, O). (B) Loadings of the various physic-chemical, biological and metabolomic variables in PC1 and PC2. Physic-chemical variables, C, N and RNA/DNA concentrations are shown in purple. The various metabolomic families are represented by colours: dark blue, sugars; green, amino acids; orange, related compounds to the metabolism of amino acids and sugars; cyan, nucleotides; brown, phenolics; dark red, terpenes and red others. Metabolites as in Figure 3. Unassigned metabolites as in Figure 3 are not depicted in this figure.

The PCA showed differences among the different warming levels and this result is backed up with the PERMANOVA results showing that *A. capillaris* plants growing at higher temperatures had clearly different metabolome structure than those growing at lower temperatures in the old warming site. Differently, in the new warming site there were not differences in metabolome structure among plants growing at different soil temperatures (Figure 4 and table 1 and 2).

The main differences observed in *A. capillaris* plants growing in old warming site at different temperatures were the increase of the concentrations of the most determined phenols and terpenes under extreme warming conditions (+15ºC). However, plants growing in soil at +10ºC had higher the concentrations of some amino acids and had the highest concentration of malic acid (Figure 4 and 5).

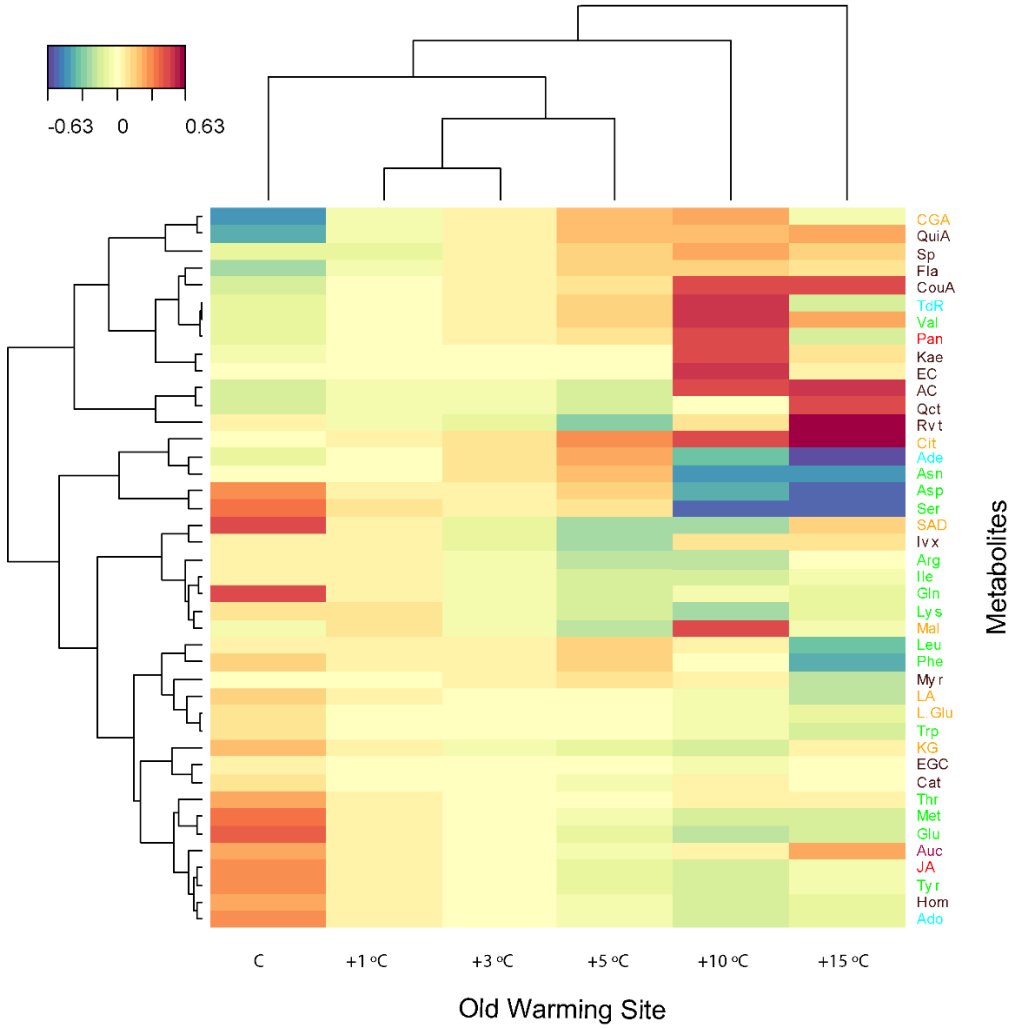

Figure 5. Clustered image maps of the metabolites in the old warming site of *Agrostis capillaris L.* based on the data of the PLS analysis. The red and blue colours indicate positive and negative correlations respectively.

The physic-chemical variables such as soil pH and soil C and N concentration did not show differences between sites. Soil pH and temperature were linked to metabolome shifts in both sites, while foliar RNA/DNA ratio was linked to metabolome shifts only in the new grassland.

Table 1. PERMANOVA results of *Agrostis capillaris L*. Bold type indicates significant effects (P < 0.05). Italics type indicates marginally significant effects (P < 0.1).

	Df	F.Model	*P-value*
Site	1	13.3136	**0.0005**
Temperature	5	1.6354	**0.0210**
SitexTemp	5	1.4068	*0.0815*

Table 2. Post-hoc Turkey HSD tests from the one-way ANOVA. The table shows the results of t-test statistics for the comparisons of different warming levels of *Agrostis capillaris L. (A) in* the old warming site, and (B) the new warming site regarding the PCA scores. Bold type indicates significant effects (*P* < 0.05) and italic type indicated marginal effect (*P* < 0. 1).

A)

Agrostis capillaris L. Old warming site					
	B	C	D	E	F
A	0.92	1.000	1.00	**0.01**	**0.047**
B		0.936	0.92	*0.08*	**0.004**
C			1.00	**0.04**	**0.01**
D				*0.05*	**0.05**
E					**0.03**

B)

Agrostis capillaris L. New warming site					
	B	C	D	E	F
A	1.00	0.70	0.23	**0.02**	*0.08*
B		0.93	0.49	**0.04**	*0.07*
C			0.98	*0.07*	*0.07*
D				1.00	*0.09*
E					0.82

3.2 Effects of site and warming on the metabolome of *Ranunculus acris L.*

PC2 accounted for the differences between sites in the metabolomes of *Ranunculus acris L.* (Figure 6). PCs 1 and 2 explained 9 and 7% of variance respectively in the PCA conducted with the leaf samples (including site and warming). The metabolomes were more different among plants submitted at different soil temperatures in the new warming site than in the old warming site (Figure 6).

The metabolome was different in the sets of plants growing in the two sites, and in the different levels of warming. In the new warming site, the highest warming levels

(+15ºC, +10ºC) had different metabolome profile than the plants growing under other soil temperatures (Table 3).

The physic-chemical variables such as soil pH and stoichiometry were different between sites. In *R. acris,* soil pH and temperature were linked to metabolome differences in the new warming site while the foliar RNA/DNA and soil C and N were linked to metabolome shifts in the old warming site.

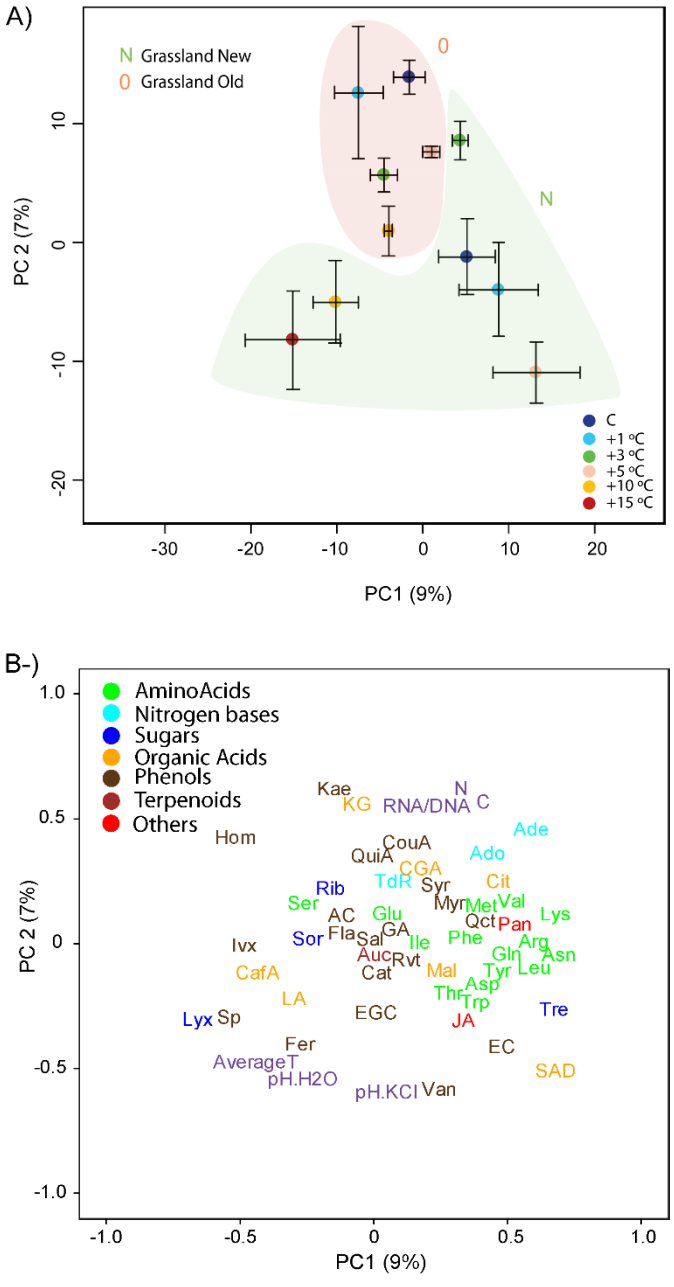

Figure 6. Plots of cases and variables in the PCA conducted with the physic-chemical, elemental composition, biological and metabolomic variables in *Ranunculus acris L.* using PC1 versus PC2. (A) Samples categorised scores (mean ± S.E.) in both sites (new warming site, N and old warming site, O). (B) Loadings of the various physic-chemical, biological and metabolomic variables in PC1 and PC2. Physic-chemical variables, C, N and RNA/DNA concentrations are shown in purple. The various metabolomic families are represented by colours: dark blue, sugars; green, amino acids; orange,

related compounds to the metabolism of amino acids and sugars; cyan, nucleotides; brown, phenolics; dark red, terpenes and red others. Metabolites as in Figure 3. Unassigned metabolites as in Figure 3 are not depicted in this figure.

The main effects of warming were higher in concentrations of phenols such as coumaric acid, quinic acid, saponarin, resveratrol and some terpenes, and some sugars detected at high concentrations in plants growing under the extreme warming conditions. In contrast, the concentrations of some amino acids and organic acids such as malic acid were higher in *R. acris* plants growing under lower warming treatments (Figure 6 and 7).

In the new warming site, the metabolome profile of *R. acris* plants growing at higher temperatures were clearly separated from the rest; while in the old warming site *R. acris* plants growing at different soil temperatures presented less difference in metabolome structure (Figure 6). The PCA showed differences among the different warming levels in both sites and this result is backed up with the PERMANOVA results (Table 4).

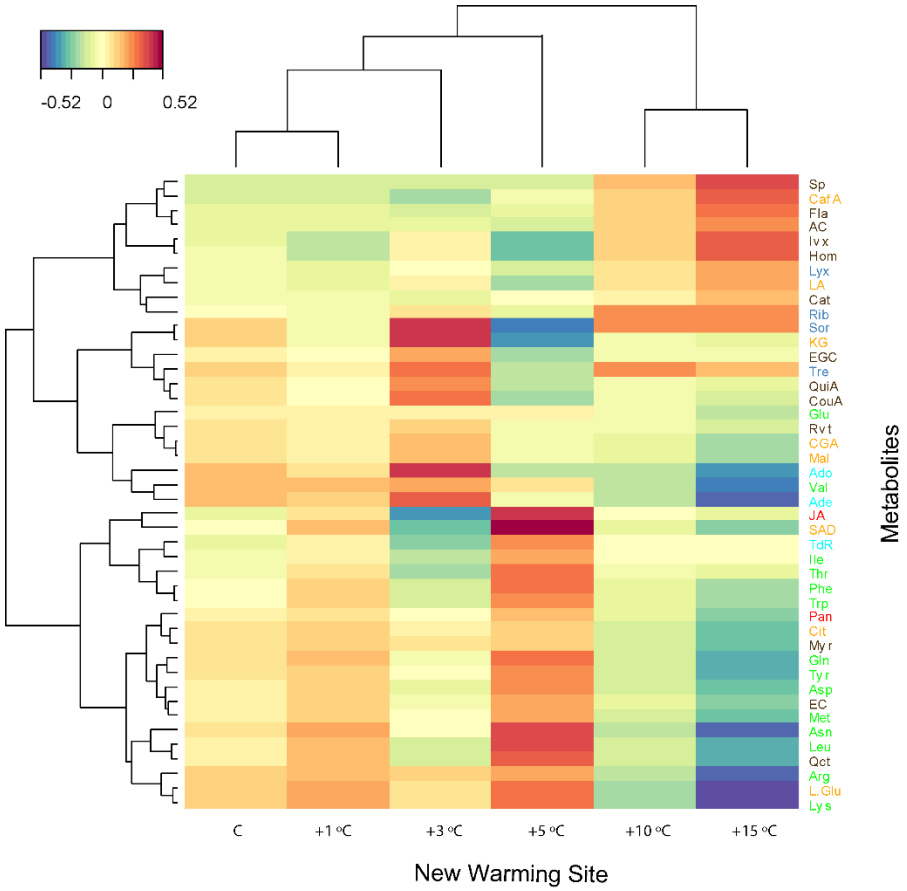

Figure 7. Clustered image maps of the metabolites in the new warming site for *Ranunculus acris L.* based on the data of the PLS analysis. The red and blue colours indicate positive and negative correlations respectively.

Soil total carbon and nitrogen concentrations were negatively related with metabolome shifts associated with increasing temperature in *R. acris*, and foliar RNA/DNA of *R. acris* was negatively related with metabolome shifts associated with increasing temperature.

Table 3. Post-hoc Turkey HSD tests from the one-way ANOVA. The table shows the results of t-test statistics for the comparisons of different warming levels of *Ranunculus acris L. (A)* in the old warming site and (B) the new warming site regarding the PCA scores. Bold type indicates significant effects ($P < 0.05$) and italic type indicated marginal effect ($P < 0.1$).

A)

Ranunculus acris L. Old warming site				
	B	C	D	E
A	***0.05***	0.14	0.99	0.40
B		0.92	***0.05***	0.53
C			0.12	0.91
D				0.31

B)

Ranunculus acris L. New warming site					
	B	C	D	E	F
A	0.99	1.00	0.74	**0.02**	**0.02**
B		0.96	0.97	**0.007**	**0.006**
C			0.62	**0.03**	**0.03**
D				**0.01**	**0.001**
E					0.89

Table 4. PERMANOVA results of *Ranunculus acris L*. Bold type indicates significant effects ($P < 0.05$). Italics type indicates marginally significant effects ($P < 0.1$).

	Df	F.Model	*P-value*
Site	1	2.9063	**0.0005**
Temperature	5	1.9687	**0.0005**
SitexTemp	4	1.0586	0.2334

4 Discussion

The effect of warming in metabolome of both species was abrupt in temperatures higher than 5-10ºC respect to control; the metabolome suffered significant changes (table S1) between the two sets of plants growing at these temperatures. Under climate change scenario, temperatures are projected to rise between 3-5ºC in boreal areas[13] (Figure 1). According to our results, both species would adapt to this increase in temperature without significantly shifting their metabolome.

The variability of the metabolome was lower in the *Ranunculus acris L.* samples than in the *Agrostis capillaris L.* samples (Figure 3). The shift in the metabolome of *Ranunculus acris L.* individuals growing at different soil temperatures was much less significant and smaller than those *Agrostis capillaris L*. The coefficient of variation of the PC2 scores was 15% for *Ranunculus acris L.* and 57% for *Agrostis capillaris L*. The metabolomic analyses results strongly suggested that the metabolism of *Ranunculus acris L.* appears to be much more conservative and homeostatic than that of *A. capillaris*. These results are consistent with previous studies reporting that heath herbs showed higher metabolomic homeostasis and less metabolomic flexibility in response to changes in environmental conditions than grass species[68].

The grass species did not shift its metabolome significantly with soil warming at short-term but it did at long-term warming. A possible reason could be that *Agrostis capillaris L.* propagates vegetatively by rhizomes and stolons but also could be by way of seeds. The large proportion of *A. capillaris* clones and low proportion of seedlings in populations suggests that much of its reproduction is vegetative. Contrarily, the heath herb species shifted its metabolomic profile much less than the grass, but the little change occur at short time but not at long-term warming. These results thus showed contrasting different time-response when comparing both species. The seeds of *R. acris*, while having no obvious dispersal mechanism and tending to fall close to the parent plant[70], can be carried long distances on the pelts or hooves of stock and in the gut of grazing animals[71–74]. Sarukhan (1974) found that a large proportion of *R. acris* seeds fed to cows was still viable after being voided in faeces and Dore and Raymond (1942) calculated that one cow could disperse approximately 9400 tall buttercup seeds. The different strategies to spread the new individual plants can be underlying the different results observed between both species. *A. capillaris* have mainly vegetative reproduction and thus the new individuals grow near their parental cohort. This should facility the local evolution and a progressive adaptation to the temperature gradient at short distances.

Thus a progressive selection at short distances explained why we observed greater differences among plots under different temperatures with the pass of time. In contrast, the great dispersion of the new individuals of *R. acris* with respect the parent cohort will prevent this effect.

The metabolome profile of *A. capillaris* among different individual plants submitted to different soil temperatures in the old warming site is more different depending on soil temperature (the highest warming plot are clearly different than the others that indicate the specialization through time) in the old than the in new field. The metabolome did not significantly change with warming in the new warming site. The ratio of RNA/DNA is higher in the old grassland in the highest warmed plot of *A. capillaris* plants, also suggesting that *A. capillaris* plants growing in old warming site have higher transcriptome activity or a higher activity of gene expression and protein synthesis in the long-term warming exposed population than in short-time warming exposed population. A selection towards more metabolically active individuals by long-time warming is thus suggested in this species. Warmed grassland plants had more active metabolism pathways related with sugars and amino acids but also with some important secondary metabolite groups such as phenols and terpenes. This up-regulation of primary metabolic pathways coincided with a higher RNA/DNA ratio in plants indicating a higher capacity of the system to increase its DNA consistent with a rise in plant activity.

Agrostis capillaris L. plants in the old warming site presented an increase of amino acids and their derivates (citric acid, threonine, glutamine, glutamic acid, and malate) phenols and terpenes with warming; whereas in the new warmed grassland the organic acids, sugars and phenols concentrations were present in higher concentrations (Figure 5). These results are consistent with previous ones that found similar metabolomic shifts under drought or warming conditions observing a metabolomic shift towards a pronounced mobilization of sugars in soluble form and by a synthesis of soluble amino acids. All of them contribute to an increase of cell osmotic potential and prevents water losses[76–78].

Agrostis capillaris L. plants metabolome in the old warming site growing under higher temperature showed an increase of sugars that revealed a regulatory effect on the heat stress effect[79], phenolic compounds, some organic acids and terpenes such as aucubin concentrations, all them involved in anti-stress mechanisms[80] (Figure S1). *Agrostis capillaris L.* plants in the new warming site at higher temperature had the lowest concentration of the free amino acids such as methionine, lysine, and isoleucine (Figure S1). This decrease in free amino acids could be associated to the incorporation of amino

acids in heat stress protective proteins. However, there was an increase in asparagine, a metabolite that plays the role in nitrogen storage and transport and contributes to the maintenance of osmotic pressure[81]. An extreme increase in soil temperature can lead plant to water deficit producing more soluble sugars and amino acids that could act as osmolytes that contribute to turgor maintainance by osmotic adjustment[78,82,83]. The metabolome in leaves of *Agrostis capillaris L.* in the new warming site in higher temperature, showed an increase of jasmonic acid that acts as a regulator of plant growth and development process. Jasmonic acid is converted to a variety of derivatives including their esters that may also be conjugated to amino acids.

Ranunculus acris L. had higher concentrations of free amino acids, most phenols and organic acid and sugars in the new warming site than in the old warming site (Figure 7 and S2). However, in the old warming site we detected higher concentration of nitrogen bases, flavonols, and higher foliar RNA/DNA ratio. This heath herb, with long-time generation time and probably with general traits closer to a stress-tolerant species has less metabolomic flexibility and variability among different individuals and the differences among individual plants growing at different temperatures tend to be small with time.

When metabolomic profiles of *R. acris* plants of both sites were analysed together, we observed higher concentration of free amino acids and some nucleotide bases in the lowest temperature plots in the old and the new warming sites. The production of amino acids indicates activation of primary metabolism suggesting that under control temperatures plants had more up regulated the primary metabolism and less the secondary metabolism than under heat stress. In the new warming site *Ranunculus acris L.* had higher concentration of organic acids such as lactic acid, trans-caffeic acid, sugars such as lyxose, ribose, sorbose and trehalose, and secondary metabolites such as phenols than in the old warming sites (Figure 7 and S2). These patterns are in accordance to previous studies reporting that during stress period plants can accumulate non-reducing disaccharides such as lyxose[84]. Plants in the warming treatments had higher concentrations of metabolites related to the heat shock response, such as sucrose and glucose and normally were associated with higher concentrations of amino acids. *R. acris* did not have an increase in the pool sizes of amino acids (asparagine, leucine, isoleucine, threonine, alanine and valine), and derivatives such as oxaloacetate and pyruvate[78]. The extreme increase in soil temperature can lead plant to water deficit producing more soluble sugars and amino acids that could act as osmolytes that contribute to turgor maintainance by osmotic adjustment[78,82,83]. Tyrosine was found in a high concentration due to its important role in photosynthesis because acts as an electron donor in the reduction of oxidized chlorophyll and it has been shown to have

relation in the levels of stress hormones[85]. Phenylalanine, which is in higher concentration in the highest temperature, is the substrate of phenylalanine ammonia lyase (PAL) which is the key enzyme in the phenolic biosynthesis pathway. This is in agreement with the increase of concentration of phenolics compounds the highest temperature. These phenols seem to protect the plants against abiotic and biotic stresses[86].

Ranunculus acris L. growing in the old warming site under moderate warming (control to +5ºC), presented metabolome with higher concentration of polyphenolics such as quinic acid and organic acids that are metabolites associated to the Krebs cycle such as malic acid, chlorogenic acid, quercetin and α-Ketoglutaric acid (Figure S2). α-Ketoglutaric acid is an intermediary of the Krebs cycle and a precursor to the amino acid biosynthesis of glutamine and glutamate. These compounds have been frequently associated with antioxidant function. Quinic acid is a precursor in the shikimic acid pathway, a common metabolic pathway in the biosynthesis of aromatic amino acids such as tyrosine, tryptophan, and phenylalanine[87]. These kind of amino acids are precursors of a large variety of secondary metabolites such as lignins, flavonoids, alkaloids, and phytodexins[88]. *Ranunculus acris L.* growing in the old warming site under higher temperatures (+5 to +10ºC) had also higher concentration of phenols, terpenes and flavonols such as kaempferol and quercetin, that could have a protective effect against biotic stressors[89,90].

Warming does usually affects the stability of proteins, the efficiency of enzymatic reactions and the nucleic acids causing severe metabolic imbalance. The most important primary metabolites are sugar and amino acids which concentration in plant tissues are affected by stress, usually as result of an increase of impairment in the CO_2 assimilation process[75]. The high concentration of secondary metabolites cannot be explained as a result from variation in their primary metabolite precursors and usually is result of a complex regulatory process. The heat stress frequently affects the plasma membrane of cells, the stability of proteins, the efficiency of enzymatic reactions and the nucleic acids, and all together causes severe metabolic imbalance and also changes on the impairment in electrons transport chains and production of ROS.

Changes in temperature generated the production of different metabolites and thus a different composition of available C[91]. Under moderate warming, favourable conditions for growth, the assimilated C is allocated to growth and energy supply (more primary metabolism) whereas under less favourable conditions of strong warming the assimilated

C is allocated increasingly to anti-stress compounds such as phenols and terpenes (more secondary metabolism).

5 Conclusions

Plant species responded differently to soil warming depending on the time they were exposed. The shifts in metabolome composition were stronger in the long-time warming site than in the short-time warming site in the case of the grass species but this was not so clear in the heath herb species.

The temperature threshold where we observed an abrupt shift in overall metabolome profile of *Agrostis capillaris L.* and *Ranunculus acris L.* species was located between 5-10 ºC more than control temperature. Above this threshold, both species tended to up-regulate the metabolic pathways related to warming stresses.

The variability of the metabolome was lower in the *Ranunculus acris L.* samples than in the *Agrostis capillaris L* samples. The shift in the metabolome of *Ranunculus acris L.* individuals growing at different soil temperatures was much less significant and smaller than the shift in *Agrostis capillaris L*.

In the long-time warming site, plants contained higher concentration in sugars and amino acids that are related to heat shock metabolism pathways. In addition, in this long-time warming site, plants had higher concentration of some important secondary metabolite groups such as phenols and terpenes that could have a protective effect against biotic stressors. This up-regulation of primary metabolic pathways coincided with a higher foliar RNA/DNA ratio.

Agrostis capillaris L. plants in the old warming site presented an increase of amino acids and their derivates (citric acid, methionine, glutamine, glutamic acid, and malate) phenols and terpenes with warming; whereas in the new warming site the organic acids, sugars and phenols concentrations were present in higher concentrations with warming. The ratio of RNA/DNA has higher in the old grassland in the highest temperatures plots.

The metabolome of *Ranunculus acris L.* plants of both sites, showed higher concentration of free amino acids and some nucleotide bases in the lowest temperature plots. In the new warming site *Ranunculus acris L.* had more organic acids such as lactic acid and trans-caffeic acid, sugars such as lyxose, ribose, sorbose and trehalose, and secondary metabolites such as phenols than the old warming sites. However, in the old warming site we detected higher concentration of nitrogen bases, flavonols, and higher foliar RNA/DNA ratio.

In the long-time warming site the main compounds that we detected to be up-regulated at highest temperatures were sugars and amino acids both related to heat shock metabolic pathways and some secondary metabolites such as phenols and terpenes also associated with a wide array of stresses.

6 Material and Methods

6.1 Description of the study area

6.1.1 Situation

Iceland is a volcanic island situated on the conjuncture of the Atlantic and Arctic ocean close to the Arctic Circle between the latitudes 62°23'N and 66°32'N and longitudes 13°30'W and 24°32'W. Because of its high latitude, the solar angle is never high and there is a large difference in day length between summer and winter. The climate of South West Iceland is maritime with cool summers and mild winters influenced by the cold East Greenland Current and the warm North Atlantic Current[93]. The island itself is a dome uplift of the Mid-Atlantic ridge with mountain peaks of up to more than 2000 m. Glaciers cover about 11.5% of Iceland's total area[67]. Its position on the Mid-Atlantic ridge makes it a very active volcanic area that played a major role in shaping the island together with the erosive power of the past and present glacial cover[94].

6.1.2 Study sites

The two grasslands studied in this thesis are located in the South West part of Iceland, near the town of Hveragerði. The mean annual temperature is 4.1°C, with a mean of -1.1°C in the coldest month (January) and 10.8°C in the warmest (July), and an annual precipitation of 1372mm (Icelandic Met Office, 2014). The soil type is a brown andosol with a volcanic origin. This is a freely drained soil type, rich in allophane clay minerals and ferrihydrates, evolved from eolian and tephra materials originating from neighbouring active volcanos[95,96]. The geothermal activity occurring in this area, mainly in the form of hot springs and fumaroles, originates from the Hengill volcanic system. This system is situated on the intersect of three volcanic zones, the Hengill system, the Hrómundartindur system and the Hveragerdi system[67,97].

The old warming site (O) ecosystem is situated in a valley several kilometres north of Hveragerði, known for its geothermal activity for centuries[98]. Transects in this area will be studied as a proxy for long-term effects of soil warming. The new warming site, (N), only has been warmed since an earthquake in May 2008 modified the underground hot water systems[99]. Transects of this grassland are situated near the university campus of Hveragerði and will be examined as an indicator for short-term effects of soil warming on vegetation.

The selected plants included one grass species (*Agrostis capillaris* L.) and one herbaceous dicot (*Ranunculus acris* L.).

6.1.3 Experimental design

Five replicate transects were established along the temperature gradients in the two valleys, the old-warming site and the new-warming site. Each transect consisted out six different soil temperature levels. The orientation of the slopes was chosen as similar as possible for all transects in both warming sites.

The temperature levels were established in by vegetation plots of 0.2x0.5m in which the average temperature corresponds with one of the six temperature levels. Control, +1 ºC, +3 ºC +5 ºC +10 ºC and +15 ºC.

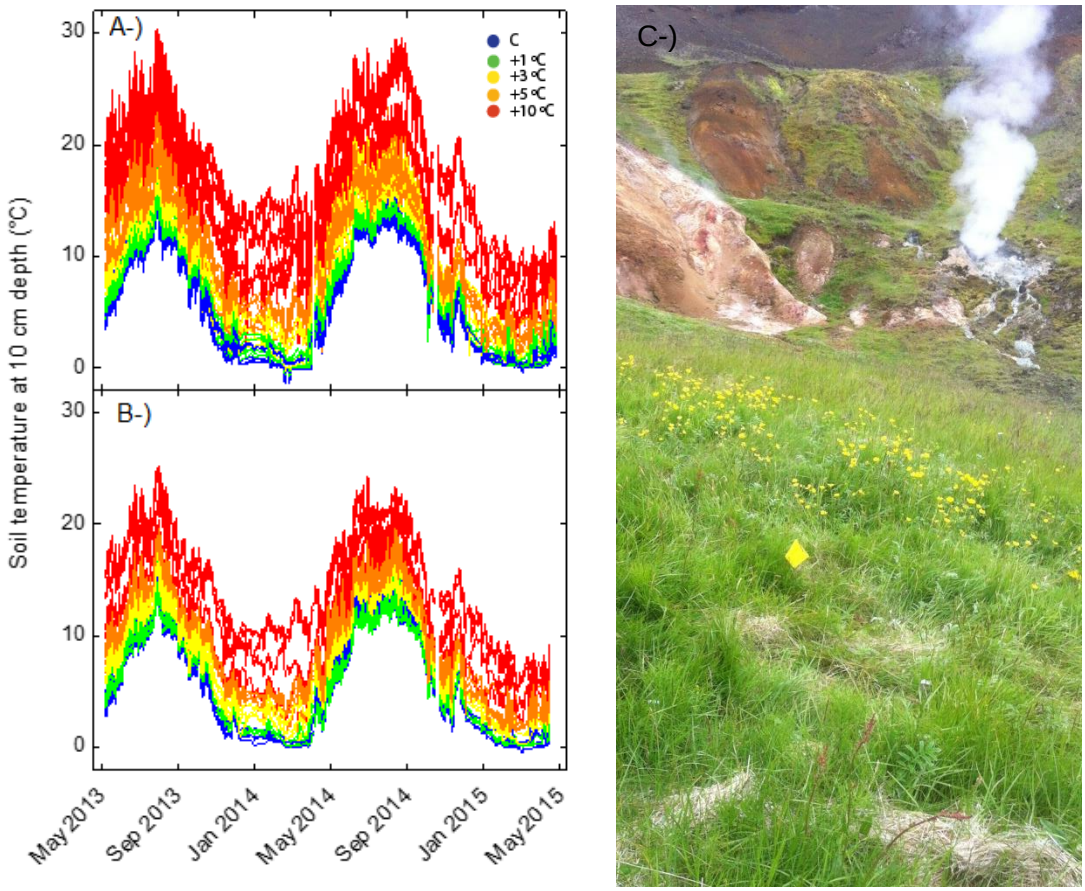

Figure 8. Soil temperatures at 10 cm depth from May 2013 to May 2015 in every measurement plot in (A) the short-term warming site and (B) the long-term warming site. Colours show targeted soil temperature elevations: blue for unwarmed soils, green for +1°C, yellow for +3°C, orange for +5°C and red for +10°C. (C) Natural soil warming in natural grassland in Iceland.

6.2 Collection and preparation of tissue samples

Samples were collected at the end-growing season in July. Leaves 113 tissue samples were collected (2 species × 2 sites (N and O) × 6 warming treatment × 5 transects (plots). The procedure for sample preparation is described in detail by Rivas-Ubach *et al*. (2013). Briefly, the frozen samples were lyophilized and stored in plastic cans at -80 °C. The samples were ground with a ball mill (Mikrodismembrator-U, B. Braun Biotech International, Melsungen, Germany) at 1700 rpm for 2 min, producing a fine powder that was stored at -80 °C until the extraction of the metabolites. See the supplementary material of Gargallo-Garriga *et al.* (2014) for details.

6.3 Extraction of metabolites for liquid chromatography–mass spectrometry (LC-MS) analysis

The extraction of metabolites followed the protocol of t'Kindt et al. (2008) with minor modifications. The Eppendorf tubes received 150 mg of sample powder from each sample, and then 1ml of MeOH/H_2O (80: 20) was added to each tube. The tubes were vortexed for 10 min, sonicated for 5 min at room temperature and then centrifuged at 23 000 g for 5 min. After centrifugation, 0.7 ml of the supernatant from each tube was collected using crystal syringes, filtered through 0.22-lm pore microfilters and transferred to a labelled set of high-performance liquid chromatography (HPLC) vials. The vials were stored at -80°C until the LC-MS analysis. This procedure was repeated for two extractions of the same sample.

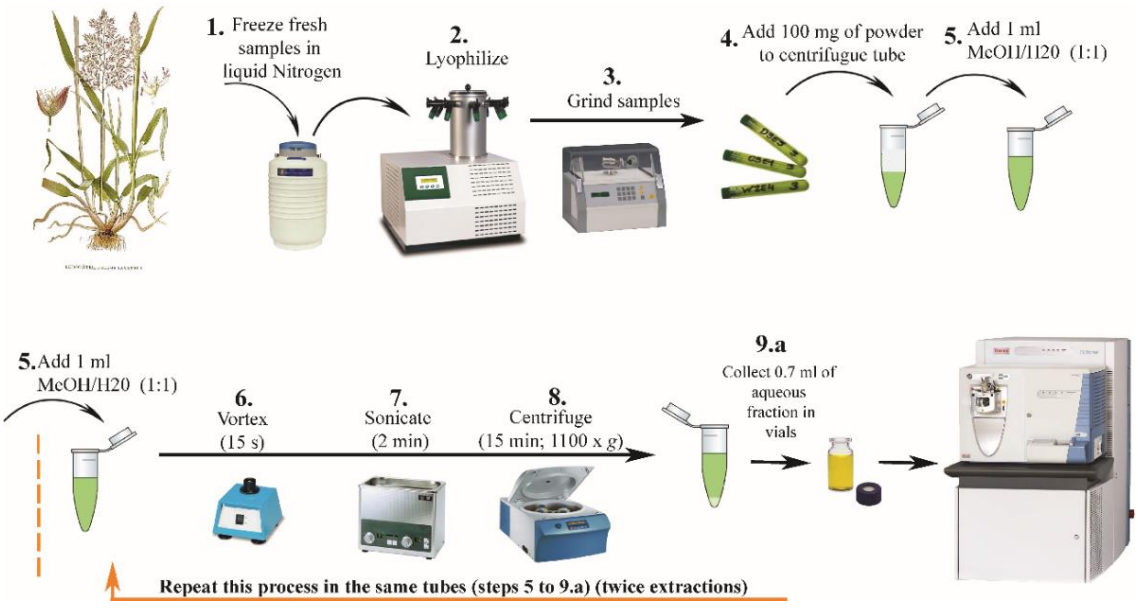

Figure 9. Experimental procedure for the preparation of extracts of plant tissue for its posterior LC-MS analysis.

6.4 LC-MS analysis

LC-MS chromatograms were obtained with a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific/Dionex RSLC, Dionex, Waltham USA) coupled to an LTQ Orbitrap XL high-resolution mass spectrometer (Thermo Fisher Scientific, Waltham, USA) equipped with an HESI II (heated electrospray ionisation) source. Chromatography was performed on a reversed-phase C18 Hypersil gold column (150 × 2.1 mm, 3-µ particle size; Thermo Scientific, Waltham, USA) at 30 °C. The mobile phases consisted of acetonitrile (A) and water (0.1% acetic acid) (B). Both mobile phases were filtered and degassed for 10 min in an ultrasonic bath prior to use. The elution gradient, at a flow rate of 0.3 mL per minute, began at 10% A (90% B) and was maintained for 5 min, then to 10% B (90% A) for the next 20 min. The initial proportions (10% A and 90% B) were gradually recovered over the next 5 min, and the column was then washed and stabilised for 5 min before the next sample was injected. The injection volume of the samples was 5 µL. HESI was used for MS detection. All samples were injected twice, once with the ESI operating in negative ionisation mode (-H) and once in positive ionisation mode (+H). The Orbitrap mass spectrometer was operated in FTMS (Fourier Transform Mass Spectrometry) full-scan mode with a mass range of 50-1000 m/z and high-mass resolution (60000). The resolution and sensitivity of the spectrometer were monitored by injecting a standard of caffeine after every 10 samples, and the resolution was further monitored with lock masses (phthalates). Blank samples were also analysed during the sequence. The assignment of the metabolites was based on the standards, with the retention time and mass of the assigned metabolites in both positive and negative ionisation modes.

6.5 Processing of LC-MS

The LC-MS raw data files were processed using MZMINE 2.10 [100] (see Table S3 of the Supporting Information for details). Before the numerical database was exported in “csv” format, the chromatograms were base-line-corrected, deconvoluted, aligned and filtered. Metabolites were assigned by comparison with the analyses of the standards (retention time and mass spectrometry). Assigned variables corresponding to the same molecular compounds were summed. The LC-MS data for the statistical analyses corresponds to the absolute peak area at each retention time (RT). The area of a peak is directly proportional to the concentration (i.e. µg/mL) of its corresponding (assigned) metabolite in the sample. Thus, a change in the area of a peak will mean a change in the concentration of its assigned metabolite.

6.6 Statistical analyses

HPLC-MS data was analysed by univariate and multivariate statistical analyses. We conducted permutational multivariate analyses of variance (PERMANOVAs)[101] using the Euclidean distance, with site (N and O), species (Ra, Ao), warming treatment (six levels of warming), and plant as fixed factors and individuals as random factors. Multivariate ordination principal component analyses (PCAs) (based on correlations) and partial least squares discriminant analyses (PLS-DAs) were also performed to detect patterns of sample ordination in the metabolomic and stoichiometric variables. The profiles of leaves from different site were additionally submitted to separate PCAs (Fig. 1). The PC scores of the cases were subjected to one-way ANOVAs to determine the statistical differences among groups with different levels of the categorical independent variables studied (species, plant and warming treatment). The PERMANOVAs, PCAs, PLS analyses, and clustered image maps were conducted by the *mixOmics* package of R software (R Development Core Team 2008). The Kolmogorov-Smirnov (KS) test was performed on each variable to test for normality. All assigned and identified metabolites were normally distributed, and any unidentified metabolomic variable that was not normally distributed was removed from the data set. Statistica v8.0 was used to perform the ANOVAs, post hoc tests, and KS tests.

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Supporting information

Table S1. Post-hoc Bonferrani tests from the one-way ANOVA. Shows the results of t-test statistics for the comparisons of different warming levels of both species, *Agrostis capillaris L.* and *Ranunculus acris L.* regarding the PCA scores. Bold type indicates significant effects ($P < 0.05$) and cursive indicated marginal effect ($P < 0.1$).

Temperature	B	C	D	E	F
A	0.79	0.13	0.96	**<0.001**	**0.001**
B		0.80	0.99	**0.01**	*0.05*
C			0.60	**0.02**	*0.05*
D				**0.005**	**0.03**
E					**0.005**

Tables S2. One-way ANOVAs of the physic-chemical and biological traits of the soils. Bold type indicates significant effects ($P < 0.05$). Italics type indicates marginally significant effects ($P < 0.1$).

	Degr. of	AverageT		pH H2O		pH KCl		RNA/DNA		%C		%N	
	Freedom	F	p	F	p	F	p	F	p	F	p	F	p
Site	1	8.70	**0.01**	5.42	**0.03**	10.8	**0.001**	0.56	0.46	10.5	**0.001**	155	**0.001**
Temp	5	127	**0.001**	6.03	**0.001**	2.14	*0.09*	0.98	0.44	7.92	**0.001**	4.18	**0.005**
Site*Temp	5	1.52	0.21	0.46	0.80	0.41	0.84	0.87	0.51	1.61	0.18	3.10	**0.02**

Table S3. Processing parameters of LC-MS chromatograms using MzMine 2.10[100]. Chromatograms presented correspond to the total ion current (TIC).

		(+H) Chromatograms		(-H) Chromatograms
	Baseline correction			
	Chromatogram type	TIC		TIC
1	MS level	1		1
	Smoothing	10E6		10E6
	Asymmetry	0.001		0.001
2	**Mass detection** (Exact Mass)			
	Noise level	4.5×10^5		4.5×10^5
	Chromatogram builder			
3	Min time span	0.05		0.05
	Min height	25000		25000
	m/z tolerance	0.002		0.002
4	**Smoothing**			
	Filter width	5		5
	Chromatogram deconvolution (Local minimum search)			
	Chromatographic threshold	70%		70%
5	Search minimum in RT range (min)	0.1		0.1
	Minimum relative height	7.0%		7.0%
	Minimum absolute height	30000		30000
	Min ratio of peak top/edge	2		2
	Peak duration range	0.0-2.0		0.0-2.0

6	**Chromatogram alignment (join alignment)**			
	m/z tolerance	0.001		0.001
	weight for m/z	80		80
	RT tolerance	0.3		0.3
	Weight for RT	20		20
7	**Gap filling (Peak Finder)**			
	Intensity tolerance	20%		20%
	m/z tolerance	0.001		0.001
	Retention time tolerance	0.1		0.1
	RT correction	marked		marked
8	**Filtering**			
	Minimum peaks in a row	25		25
	Ions excluded from database	<75 Between 0.0 and 1 min Between 28.5 and 30 min		<85 Between 0.0 and 1,1 min Between 27.0 and 30 min

Figure S1. Clustered image maps of the metabolites in the new warming site for *Agrostis capillaris L.* based on the data of the PLS analysis. The red and blue colours indicate positive and negative correlations respectively.

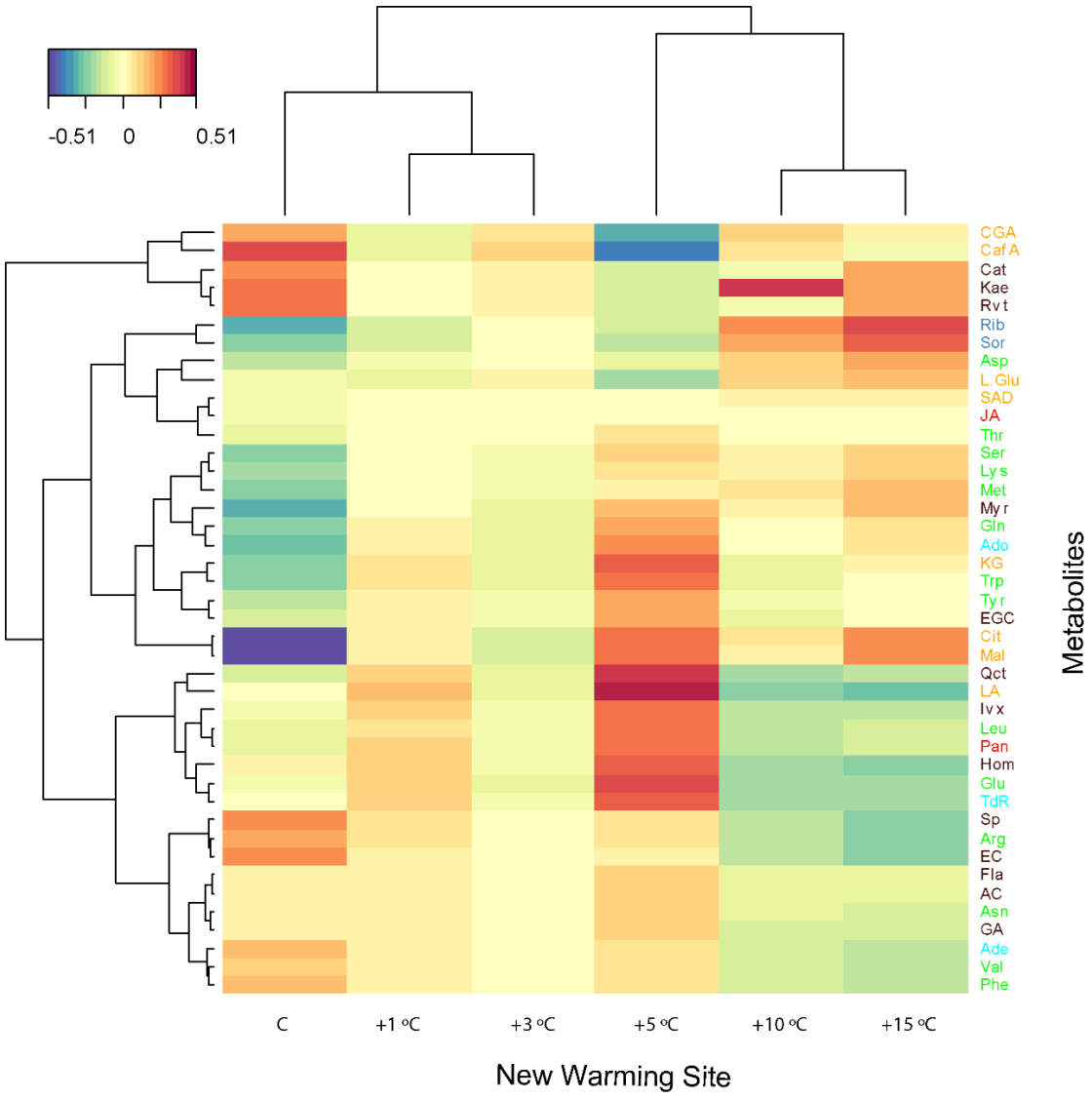

Figure S2. Clustered image maps of the metabolites in the old warming site for *Ranunculus acris L.* based on the data of the PLS analysis. The red and blue colours indicate positive and negative correlations respectively.