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Does cold tolerance plasticity correlate with the thermal environment and metabolic profiles of a parasitoid wasp?

Vincent Foray [a,d,*], Emmanuel Desouhant [a], Yann Voituron [b], Vanessa Larvor [c], David Renault [c], Hervé Colinet [c,d], Patricia Gibert [a]

[a] Université de Lyon, F-69000, Lyon, Université Lyon 1, CNRS, UMR 5558, Laboratoire de Biométrie et Biologie Evolutive, F-69622, Villeurbanne, France
[b] Laboratoire d'Ecologie des Hydrosystèmes Naturels et Anthropisés, UMR CNRS 5023, Université Claude Bernard Lyon 1, Université de Lyon, 69622 Villeurbanne cedex, France
[c] Université Rennes 1, UMR CNRS 6553 Ecobio, 263 Avenue du Gal Leclerc, CS 74205, 35042 Rennes Cedex, France
[d] Earth and Life Institute, Biodiversity Research Centre, Catholic University of Louvain, Croix du Sud 4–5, B-1348 Louvain-la-Neuve, Belgium

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ABSTRACT

Tolerance of ectotherm species to cold stress is highly plastic according to thermal conditions experienced prior to cold stress. In this study, we investigated how cold tolerance varies with developmental temperature (at 17, 25 and 30 °C) and whether developmental temperature induces different metabolic profiles. Experiments were conducted on the two populations of the parasitoid wasp, *Venturia canescens*, undergoing contrasting thermal regimes in their respective preferential habitat (thermally variable *vs.* buffered). We predicted the following: i) development at low temperatures improves the cold tolerance of parasitoid wasps, ii) the shape of the cold tolerance reaction norm differs between the two populations, and iii) these phenotypic variations are correlated with their metabolic profiles. Our results showed that habitat origin and developmental acclimation interact to determine cold tolerance and metabolic profiles of the parasitoid wasps. Cold tolerance was promoted when developmental temperatures declined and population originating from variable habitat presented a higher cold tolerance. Cold tolerance increases through the accumulation of metabolites with an assumed cryoprotective function and the depression of metabolites involved in energy metabolism. Our data provide an original example of how intraspecific cold acclimation variations correlate with metabolic response to developmental temperature.

1. Introduction

Many ectotherms including insects have to cope with cold periods during their lifetime, and this may have strong consequences on their fitness, which in turn can contribute to determine their geographical distribution (Bale, 2002; Chown and Terblanche, 2007). Given the importance of winter periods on population dynamics, cold tolerance in insects usually exhibits a high degree of phenotypic plasticity (*e.g.* Ayrinhac et al., 2004). The ability of such organisms to sustain a cold stress first depends on the basal thermal tolerance and second on their capacity to respond to thermal variations *via* plasticity (Nyamukondiwa et al., 2011). Cold tolerance can be enhanced by pre-exposure to low temperatures during larval or adult stages (Colinet and Hoffmann, 2012), the so-called adaptive thermal acclimation (Rako and Hoffmann, 2006). The amplitude of thermal acclimation that a species can reach is expected to be linked to the environmental variability and predictability of the thermal conditions of their habitats, *i.e.* species growing in highly variable habitats are expected to exhibit a higher capacity for cold acclimation compared to their relatives from more buffered environments (van Tienderen, 1991; Gabriel and Lynch, 1992; DeWitt et al., 1998; Angilletta, 2009). Although, differences in the level of cold acclimation have been reported among species from distinct geographical origins in several arthropod species (*e.g.* Bahrndorff et al., 2009; Overgaard et al., 2011; Boher et al., 2012), the possible relationship between the level of cold acclimation and the thermal characteristics of habitats has been less examined at the intra-specific level (but see Klok and Chown, 2003; Ayrinhac et al., 2004; Cooper et al., 2012; Sinclair et al., 2012).

In insects, the enhancement of the cold tolerance level involves physiological adjustments particularly on membrane composition, enzyme activity and concentration of compatible solutes (Sinclair et al., 2003; Chown and Terblanche, 2007; Clarke and Worland, 2008). This physiological remodelling prevents the accumulation of lethal chill injuries and allows a faster recovery when the environmental conditions are permissive again for the insect species. During acclimation, the progressive alteration of the concentrations of several compounds related to energetic metabolism provides useful biochemical fingerprints allowing a reliable monitoring of the acclimatory response of cold exposed insects (Bundy et al., 2009; Colinet et al., 2012a). In addition, the thermal-induced accumulation of compatible solutes

* Corresponding author at: Earth and Life Institute, Biodiversity Research Centre, Catholic University of Louvain, Croix du Sud 4–5, B-1348 Louvain-la-Neuve, Belgium. Tel.: +32 10 47 34 96; fax: +32 10 47 34 90.
E-mail addresses: vincent.foray@uclouvain.be (V. Foray), emmanuel.desouhant@univ-lyon1.fr (E. Desouhant), vanessa.larvor@univ-rennes1.fr (V. Larvor), david.renault@univ-rennes1.fr (D. Renault), herve.colinet@uclouvain.be (H. Colinet), patricia.gibert@univ-lyon1.fr (P. Gibert).

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such as polyols, sugars and free amino acids represents an ubiquitous physiological response in cold acclimated insects (Storey and Storey, 2005; Michaud and Denlinger, 2007; Koštál et al., 2011). Metabolic fingerprinting approaches have been used to depict the cold acclimation responses of adult insects (Lalouette et al., 2007; Michaud and Denlinger, 2007; Overgaard et al., 2007; Michaud et al., 2008), however metabolomic analyses of developmental acclimation of cold tolerance have not been studied to the same extent (Koštál et al., 2011; Colinet et al., 2012a). So far, no study has simultaneously investigated amplitude of cold acclimation according to thermal habitat characteristics and physiological adjustments among insect populations using metabolic fingerprints.

The parasitoid *Venturia canescens* Gravenhorst (Hymenoptera: Ichneumonidae) is characterized by a high level of intra-specific variation, which manifests itself in distinct reproduction modes among populations that grow and develop into distinct habitats (Beukeboom et al., 1999). The parthenogenetic arrhenotokous populations (sexual strain) live exclusively in natural environments, where they are subjected to seasonal and daily thermal fluctuations. Conversely, the parthenogenetic thelytokous populations (asexual strain) thrive in anthropogenic environments (*i.e.*, granaries and mills) that confer buffered thermal conditions (Amat et al., 2006). Thelytokous wasps are unlikely to survive in natural habitats during the cold season (Amat, 2004), and anthropogenic habitats serve as a refuge during winter periods.

In this study, we used targeted gas chromatography and mass-spectrometry (GC/MS) to examine changes in metabolic profiles between thelytokous and arrhenotokous populations of *V. canescens* that were reared under controlled conditions at different temperatures. We assumed that development at low temperature would promote cold tolerance, and hypothesize that this may be associated with metabolic changes. Because the arrhenotokous population thrives in thermally variable habitats, we expected that they may be characterized by a higher capacity for cold acclimation than thelytokous individuals, and that the two populations will display specific metabolic profiles according to the developmental temperature.

2. Materials and methods

2.1. Biological material and cultures

V. canescens is a Mediterranean endoparasitoid of lepidopteran larvae, mainly of the family Pyralidae (Salt, 1976). We conducted our experiments on thelytokous and arrhenotokous populations established from a large sample of wild individuals collected in orchards and near anthropogenic habitats near Valence, France (North: 44°58′34″, East: 4°55′66″, Gotheron INRA station). Thelytokous parthenogenesis is not induced by endosymbiotic bacteria (Mateo-Leach et al., 2009; Foray et al., in press). The thelytokous and arrhenotokous wasps that we used for cold tolerance assays were collected in the summer of 2006 and 2007, respectively, and both strains were maintained under controlled conditions (25 ± 1 °C, 70 ± 5% RH and 12:12 (L:D) for 20 and 8 generations, respectively). Parasitoids used for metabolic profiling were collected during summer 2010 and maintained under controlled conditions for 8 generations. Such a short-duration stay prevents laboratory adaptation in *V. canescens* (Foray et al., 2011). *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) larvae were used as hosts for the development of the parasitoids. They were reared with wheat semolina as a substrate.

2.2. Developmental acclimation

To produce individuals for the assays, we randomly chose 40 thelytokous females and 40 arrhenotokous couples and placed them in boxes containing approximately 500 *E. kuehniella* third-instar larvae with access to food (50% water-diluted honey on a piece of cotton wool). Wasps were free to mate and to parasitize hosts during a 96-h period. This procedure was performed under controlled conditions (25 ± 1 °C, 70 ± 5% RH and 12:12 L:D). The parasitized hosts were then randomly distributed among three identical MLR-352 H incubators (SANYO Electric Biomedical Co., Ltd., Osaka, Japan) set at 17, 25 and 30 °C (± 1 °C) to continue development until adulthood. These temperatures are within the lower and the upper thermal thresholds for development of *V. canescens* (Eliopoulos and Stathas, 2003). The hosts were inspected twice a day at the onset of emergence of *V. canescens* (between 8:00 and 12:00 p.m.) to collect newly emerged wasps for assays.

2.3. Cold tolerance assay

The newly emerged wasps were placed individually, without anaesthesia, into plastic vials (Ø: 30 mm, h: 70 mm) with a piece of cotton wool soaked with 2 ml of water under controlled conditions (25 ± 1 °C, 70 ± 5% RH, 12:12 L:D). The cold tolerance of 1-day-old adult females was assessed by measuring their recovery time from chill coma following an exposure to cold stress. Cold exposure consisted in placing females individually into a dry 2 ml Eppendorf vials immersed in a glycol solution cooled to −7 °C for 7 h. Preliminary tests revealed that such conditions are non-lethal and do not induce freezing of female parasitoids (data not shown). Chill coma recovery time (CCRT) was measured by monitoring the necessary time for the females to stand on their legs after being transferred to room temperature (25 ± 1 °C). This index has been linked to adaptive patterns that match expectations based on climatic conditions (Ayrinhac et al., 2004; Hoffmann et al., 2005). The maximum observation time was 2 h; beyond this period, CCRT was considered censored.

2.4. Metabolic fingerprinting

Metabolic profiling was made using whole body extracts of thelytokous and arrhenotokous females developed at 17, 25 and 30 °C and frost at −80 °C at their emergence until analysis. The wasps were weighed (wet mass) using a microbalance accurate to within 0.01 mg (Mettler microbalance). For each modality, an analysis was performed using 8 true biological replicates ($n = 8$), each consisting of a pool of 3 wasps, except at 17 °C, where 7 and 10 replicates were used for arrhenotokous and thelytokous wasps, respectively. We used a GC-MS platform to measure metabolites from the whole insect body as described in details by Colinet et al. (2012b). Briefly, metabolites were extracted using methanol-chloroform (2:1) and then derivatized with methoxyamine HCl hydrochloride in pyridine followed by MSTFA. We completely randomized the injection order of the samples. All samples were run under the SIM mode. We therefore only screened for the 60 pure reference compounds included in our custom-made spectral database. Quantification was based on calibration curves obtained from pure reference compounds. The system consisted of a CTC CombiPal autosampler (GERSTEL GmbH & Co.KG, Germany), a Trace GC Ultra chromatograph and a Trace DSQII quadrupole MS (Thermo Fischer Inc., USA). Chromatograms were deconvoluted using XCalibur v2.0.7.

2.5. Statistical analyses

To compare the CCRT, we used parametric survival analysis assuming a non-constant hazard function following a Weibull distribution. This model allowed for the incorporation of censored data. The significance of the explanatory variables was assessed using z statistics. The instantaneous probability of wasp recovery over time was analysed by testing whether the scale parameter differed from unity (Crawley, 2007).

Metabolite profiles were analysed using a between principal component analysis (Between PCA, Dolédec and Chessel, 1991) to test a clustering effect according to the experimental modalities, *i.e.*, the population and the developmental temperature. Between PCA finds linear combinations of variables maximising the between-modalities. The inertia calculated in a Between PCA represents the part of the total

variance that is due to the differences between modalities (Dolédec and Chessel, 1991). The data were normalised to prevent the effects of the metabolite concentration means and ranges of variability on the correlations with the principal components. A Monte-Carlo test (number of iterations = 999) was used to determine whether the samples were randomly distributed in variable space according to their experimental modality. All data analyses and graphics were performed using R 2.12.1 (R Development Core Team, 2010) with "ade4" (Chessel et al., 2004) and "survival" packages.

3. Results

3.1. Chill coma recovery time (CCRT)

The survival analysis revealed that the CCRT significantly varied with the developmental temperature, the population and the interaction of these two factors (Fig. 1). The CCRT increased with increasing developmental temperature ($|z| = 12.05$, $P<0.001$). After development at 17 °C, the median CCRTs for this temperature were 5.0 and 5.5 min for arrhenotokous and thelytokous populations, respectively. By contrast, after developmental acclimation at 30 °C, 58% of arrhenotokous wasps recovered, while no thelytokous individuals did. The arrhenotokous population recovered faster than the thelytokous population ($|z| = 2.55$, $P = 0.01$), regardless of their developmental temperature. The difference between the two populations decreased along with decreased developmental temperatures, resulting in a significant interaction ($|z| = 4.18$, $P<0.001$). In addition, the instantaneous probability to recover tended to decrease over time because the scale parameter is significantly less than 1 (scale = 0.614, $|z| = 5.54$, $P<0.001$).

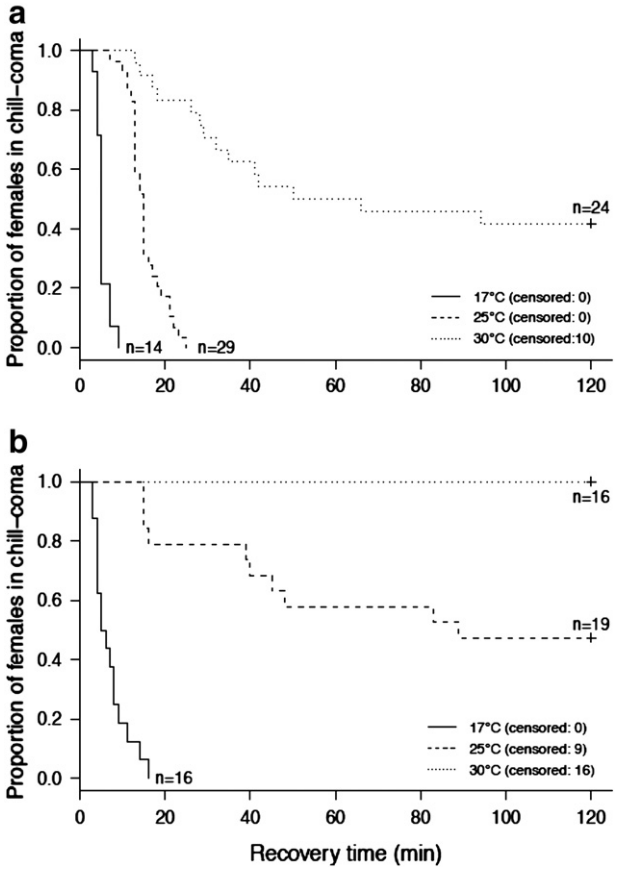

Fig. 1. Kaplan–Meier representations of the chill coma recovery time (CCRT) of arrhenotokous (a) and thelytokous (b) wasps after a cold shock at −7 °C during 7 h according to their developmental temperature (17, 25 and 30 °C). The sample size is denoted by "n"; this sample identifies censored data by "censored".

3.2. Metabolic profiles

The GC/MS analysis allowed the identification and quantification of 31 metabolites in female *V. canescens* (Table 1: 11 were amino acids, 10 sugars or polyols, 6 metabolic intermediates and 4 diverse metabolites). Proline, trehalose, glucose, glutamate and glucose-6-phosphate (G6P) were among the most abundant metabolites (Table 1).

The first two principal components of the PCA were used to analyse the distribution of the samples (Fig. 2A). A clustering effect of developmental temperature and population was found (Monte-Carlo test, $P<0.001$). The first principal component (PC1) accounted for 60.5% of the inter-modality variability and separated the samples according to the developmental temperature. More than half of the metabolites were significantly correlated with PC1, either negatively or positively (Fig. 2B) (Spearman correlation tests, $P \leq 0.01$), and therefore varied with respect to the developmental temperature. The metabolites that were negatively correlated with PC1 (G6P, putrescine, glucose, leucine, mannose, isoleucine, alanine, glycine, cadaverine, glycerate and fructose) increased with decreasing developmental temperature (see Online Resource 1 for details). Conversely, the metabolites that were positively correlated with PC1 (namely inositol, trehalose, fumarate, glutamate, phenylalanine, serine, malate, citrate and sucrose) increased with increasing developmental temperature (see Online Resource 1 for details).

The second principal component (PC2) explained 18.8% of the inter-modality variability and was characterised by a clear opposition between the two populations (Fig. 2A). Maltose, succinate, sucrose and glycerol were negatively correlated with PC2, while phenylalanine, threonine and serine were positively correlated with PC2 (Fig. 2C) (Spearman correlation tests, $P \leq 0.01$). Among these metabolites, those that were positively correlated with PC2 were more abundant in the arrhenotokous, while those that were negatively correlated with PC2 were more abundant in the thelytokous population (see Online Resource 1 for details).

4. Discussion

In the present study the metabolic changes according to developmental temperature were examined in the two populations of *V. canescens* undergoing contrasted thermal regimes in their respective habitats. Both cold tolerance and metabolic profiles depended on the developmental temperature, the population origin and their interaction; even if the effect of the population origin had a smaller magnitude than the temperature one. Yet, our study highlights a case of intraspecific variation in thermal acclimation correlated with metabolic changes.

4.1. Thermal acclimation and associated metabolomic changes

The CCRT, measured as a proxy of the cold tolerance of *V. canescens* populations, was highly improved when larval development occurred at a low temperature (17 °C) and decreased gradually with increasing developmental temperature. This pattern of reaction norm is common among insects (Ayrinhac et al., 2004; Terblanche et al., 2005; Zeilstra and Fischer, 2005) and is consistent with the definition of adaptive thermal acclimation (Lagerspetz, 2006). Studies conducted on *Drosophila* species highlighted the complexity of the cold adaptation process (Rako and Hoffmann, 2006; Cooper et al., 2010; Boher et al., 2012), and the diversity of methods used to assess it (see discussion in Terblanche et al., 2011). The CCRT is a sensitive index of cold adaptation that is capable of detecting plasticity in cold tolerance in a wide range of insect species and, in some cases, is correlated to cold stress survival (David et al., 1998; Gibert et al., 2001; Anderson et al., 2005; Lachenicht et al., 2010; MacMillan and Sinclair, 2011; Weldon et al., 2011; Dierks et al., 2012).

Table 1
List of the metabolites identified in female parasitoid wasps of *Venturia canescens* by GC/MS. Metabolites were classified according to four categories: amino acids, sugars and polyols, metabolic intermediates and other metabolites. The range of the concentrations ($nmol \cdot mg^{-1}$) found for each metabolite is indicated in brackets [min–max].

Amino acids	Sugars and polyols	Metabolic intermediates	Other metabolites
Valine [0.388–1.170]	Ribose [0.006–0.022]	Citrate [0.686–1.675]	Putrescine [1.120–3.490]
Leucine [0.343–0.988]	Fructose [0.029–0.232]	Succinate [0.570–2.025]	Cadaverine [0.006–0.023]
Isoleucine [0.193–0.647]	Mannose [0.010–0.972]	Fumarate [0.150–0.429]	Pipecolate [0.137–0.766]
Proline [4.746–29.179]	Glucose [0.897–17.523]	Malate [0.590–1.576]	Gluconate lactone
Glycine [0.602–2.047]	Sucrose [0.005–0.056]	Glycerate [0.003–0.012]	[0.129–1.281]
Serine [0.518–1.497]	Maltose [0.002–0.076]	Glucose-6-phosphate (G6P)	
Threonine [0.283–0.881]	Trehalose [3.235–21.330]	[0.083–4.090]	
Alanine [0.417–1.942]	Glycerol [0.220–0.873]		
Phenylalanine [0.110–0.447]	Adonitol [0.008–0.045]		
Ornithine [0.007–0.077]	Inositol [0.063–0.349]		
Glutamate [3.576–10.633]			

As predicted, developmental acclimation resulted in different metabolic profiles in *V. canescens*, and these differences might account for differences in cold tolerance. Following developmental acclimation at 17 °C, in particular, *V. canescens* wasps from both populations accumulated several compounds with assumed cryoprotective properties. Specifically, glucose, fructose, alanine and glycine are usually upregulated in response to cold treatments (Colinet et al., 2007; Lalouette et al., 2007; Overgaard et al., 2007) and during diapause in various insect species (Michaud and Denlinger, 2007; Li et al., 2001). Polyamines, like putrescine and cadaverine, should also be conserved metabolic fingerprints of cold acclimation response in insect. They accumulate at low developmental temperatures during induction of diapause or cold acclimation (Koštál et al., 2011; Colinet et al., 2012a). Polyamines are involved in the stress tolerance and the regulation of heat shock protein (HSPs) synthesis of plants (Gill and Tuteja, 2010; Koenigshofer and Lechner, 2002). The accumulation of glucose, alanine and glucose-6-phosphate, concomitantly with the decrease of glutamate, may denote a higher allocation of energy to the nervous system by activation of the glucose-alanine cycle (Tsacopoulos et al., 1994). This hypothesis requires further experimental work to test whether it might explain the plasticity of CCRT; coma induced by cold shock and the progressive recovery of coordinated mobility suggest that cold temperatures alter characteristics of the nervous system (David et al., 1998; MacMillan and Sinclair, 2011).

The reduction of several Krebs cycle intermediates, such as fumarate, citrate and malate in cold acclimated wasps suggest an alteration of the energetic metabolism. Metabolic alteration after a cold acclimation is also observed in *D. melanogaster* and beetles and seems to induce cold hardiness when it is associated to the accumulation of low molecular weight sugars (Evans, 1981; Koštál et al., 2011; Colinet et al., 2012a). This response might be related to the metabolic depression observed in diapausing insects and hypothesized as an adaptive energy-saving strategy (Lee, 1980; Zeng et al., 2008). The reduction of metabolic intermediates should also be interpreted as a consequence of their more rapid utilisation (*i.e.* turnover) that is consistent with higher mitochondrial density and efficiency; this has also been reported in other insect species after cold treatments (Joanisse and Storey, 1995; McMullen and Storey, 2008). In both cases, the Krebs cycle represents an interesting biomarker candidate of physiological adjustments in response to low temperatures, as pointed out earlier in other insect species (Michaud et al., 2008).

4.2. Cold tolerance and metabolomic differences between populations

The arrhenotokous population that likely experiences lower temperatures in their natural habitats is more cold tolerant than the thelytokous population, irrespective of development temperature. This difference was the largest in the group grown at 30 °C, from which none of the thelytokous wasp recovered from cold shock. The interaction between acclimation and population effects resulted in a smaller difference in the level of cold tolerance between thelytokous and arrhenotokous populations after development at 25 and 17 °C. Such variations in thermal acclimation between the two populations, originally thriving in distinct thermal habitats, are consistent with the predictions of the available models that explored the evolution of phenotypic plasticity (van Tienderen, 1991; Gabriel and Lynch, 1992). Experiments on *Drosophila* species reported these variations at the interspecific level (Hoffmann and Watson, 1993) and not at the intraspecific level (Cooper et al., 2012). Our study highlights intraspecific variation in developmental acclimation of cold tolerance. However, in contrast to our expectations, arrhenotokous wasps displayed a lower degree of plasticity for cold tolerance than thelytokous wasps. This result could be explained if basal cold tolerance is traded off against cold tolerance plasticity, as reported across *Drosophila* species (Nyamukondiwa et al., 2011). The adaptive value of thermal tolerance ideally requires demonstration under field conditions to incorporate all of the factors affecting the overall balance between costs and benefits (Kristensen et al., 2008; Overgaard et al., 2010) and trade-off with life history traits (Marshall and Sinclair, 2010; Basson et al., 2011). In *V. canescens*, cold tolerance might be at the cost of fecundity, as arrhenotokous wasps have a lower egg production than thelytokous ones (Pelosse et al., 2007; Foray et al., 2011).

Overall, the thelytokous and arrhenotokous populations exhibited distinct metabolic profiles; however, we found no evidence of massive accumulation of cryoprotective substances in the coldest tolerant population. The thelytokous population had higher concentrations of succinate, maltose and sucrose than arrhenotokous one. These variations may relate to differential energetic needs and/or allocation to reproduction. Further, arrhenotokous wasps have a higher level of some free amino acids, phenylalanine, threonine and serine. These compounds have no known cryoprotective functions. Their accumulation likely suggests particular development, protein synthesis/catabolism and metabolic pathways (Fields et al., 1998; Colinet et al., 2007), unrelated to cold tolerance. In spite of this, we cannot rule out that the cold tolerance variation between both populations involves other physiological adaptations, such as membrane remodelling (Overgaard et al., 2008) or the production of HSPs (Hoffmann et al., 2003; Sørensen et al., 2003; Chown and Nicolson, 2004). As adaptation to thermal variations can also imply thermoregulatory compensation (Huey and Pascual, 2009), future experiments should account for the thermoregulation capacity of thelytokous and arrhenotokous wasps.

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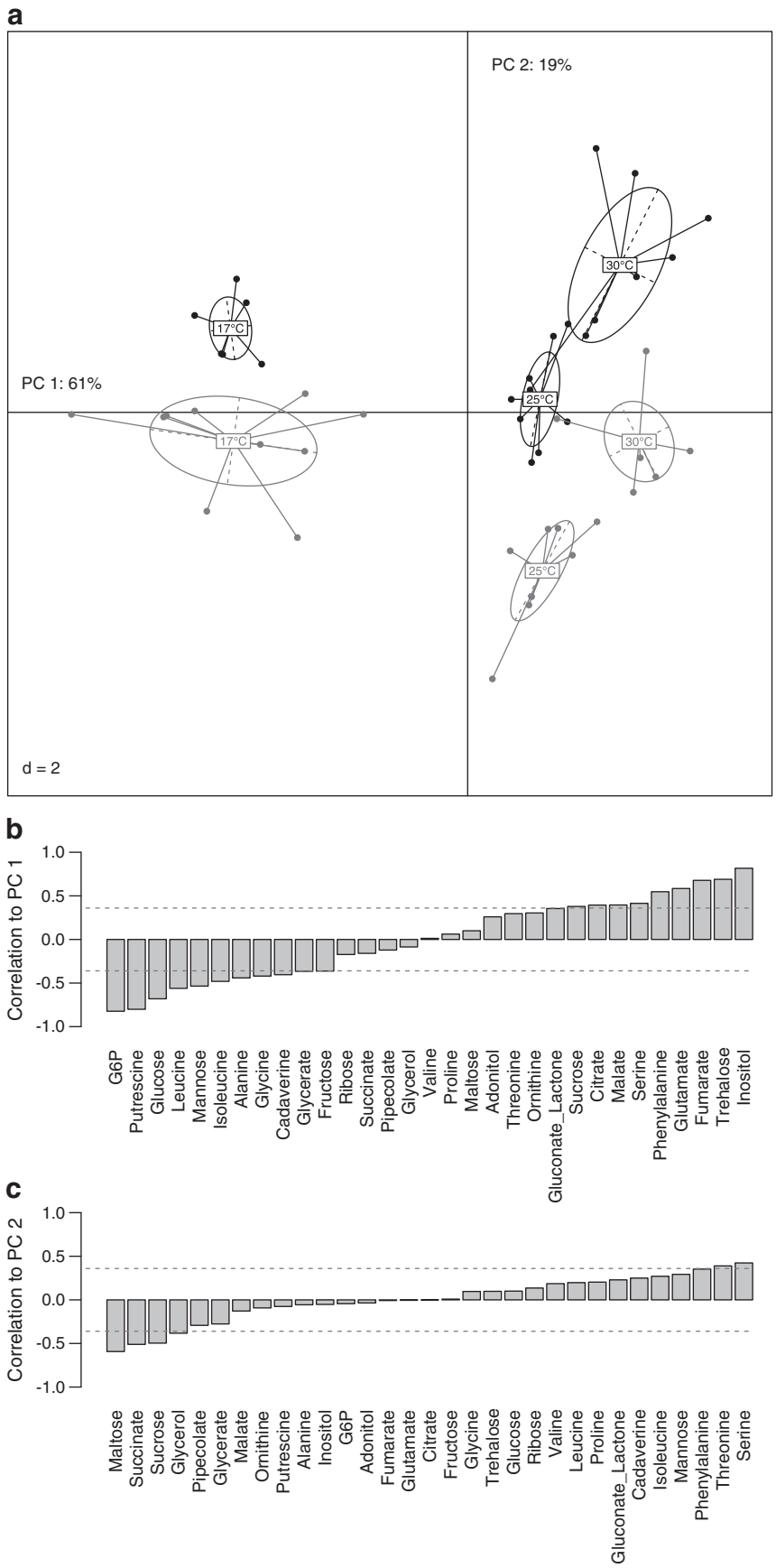

Fig. 2. (a) Between principal component analysis of metabolomic samples from arrhenotokous (*black*) and thelytokous (*grey*) wasps developed at 17, 25 and 30 °C. The ellipsoids of inertia are encompassing 65% of the samples of each modality, and the centres of the ellipsoids represent the mean values for each modality. The first two principal components (PCs) comprise 70% of the inter-modality variability: 61% and 19% for PC 1 and 2, respectively. "d" corresponds to the numbers of axes kept in the analysis. (b) and (c) correlations of the different metabolite concentrations to the principal components PC1 and PC2 in the between principal component analysis. Dotted lines indicate significant correlations at $P \leq 0.01$.

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