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# Does fluctuating thermal regime trigger free amino acid production in the parasitic wasp *Aphidius colemani* (Hymenoptera: Aphidiinae)?

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**Abstract**

When stressful cold-exposure is interrupted by short warm intervals, physiological recovery is possible, and this improves markedly the survival of insects. Fluctuating thermal regime (FTR) may act as a cue triggering the initiation of a metabolic response involving synthesis of cryoprotective compounds, such as free amino acids (FAA). Since specific changes in FAA levels can provide a good indication of the overall response of an organism to stressful conditions, we investigated temporal changes in FAA body contents of the parasitoid *Aphidius colemani* Viereck during exposure to FTR (4 °C: 20 °C for 22 h: 2 h per day) versus constant low temperature (4 °C). Physiological response during cold-exposure was clearly dissimilar between thermal treatments. Under constant cold-exposure FAA pool increased, whereas it decreased with cold-exposure duration in FTR. No single FAA accumulation could explain the higher survival under FTR. We propose that instead of considering FAA as a part of cryoprotective arsenal, FAA accumulation should rather be regarded as a symptom of a cold-induced physiological response. This is much less manifest under FTR, as the warm intervals likely allow a periodic reactivation of normal metabolic activities and a recovery of developmental processes.


*Keywords:* Chill injury; Amino acids; Low temperature; Recovery; Parasitoid

## 1. Introduction

Temperature has profound effects on ectotherms and is undoubtedly one of the most important abiotic factors governing insect life. It simultaneously affects numerous physiological processes and biophysical structures and influences metabolic activities, developmental rates and growth (e.g., Sinclair et al., 2003). Frequent exposure of insects to temperature variations has led to the evolution of protective biochemical and physiological mechanisms. Due to seasonal cycles, many insects species are frequently exposed to sub-optimal low temperatures in their natural environments (Hance et al., 2007). When the "dose" of cold-exposure (i.e., a combination of exposure time and temperature) exceeds a specific threshold, chill injuries accumulate, become progressively irreversible and eventually lethal (Bale, 1996; Kostal et al., 2006). In many insect species, lethal cumulative injuries occur even at temperatures above 0 °C, but the main causes of death are still not well understood (Renault et al., 2002; Kostal et al., 2004, 2006). Chill injuries probably result from various physiological dysfunctions including a loss of membrane potential, a reduction in protein synthesis resulting in the leakage of cytoplasmic solutes (Slachta et al., 2002), a reduction or unbalance of metabolites transfer leading to the accumulation of potentially toxic metabolic waste substances (Nedved et al., 1998), neuromuscular injuries (Kelty et al., 1996), thermoelastic stress (Lee and Denlinger, 1991), ion homeostasis perturbation (Kostal et al., 2004, 2006) and production of free radicals (Rojas and Leopold, 1996).

Several studies have shown that exposing insects to fluctuating thermal regimes (FTRs) (i.e., prolonged exposures at low temperatures combined with periodic short pulses at

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warm temperatures), in contrast to constant low temperatures, increases survival in most species tested to date (Chen and Denlinger, 1992; Nedved et al., 1998; Renault et al., 2004; Colinet et al., 2006a). FTRs reduce the level of accumulated injuries and thus mortality, either because less chill injuries accumulate at FTR, as the insects are exposed to low temperature for a shorter time, or because the effect of chilling is compensated by the exposure to warmer temperatures (Hanč and Nedvěd, 1999; Renault et al., 2004). In some cases, it has been demonstrated that chill injuries were completely repaired, resulting in a highly reduced mortality (Renault et al., 2004).

Few studies have investigated the metabolic responses of insects experiencing thermally variable environments. Hanč and Nedvěd (1999) hypothesized that higher temperature may allow a physiological processes of cold-hardening that is cued by the low temperature, but requires a stay at higher temperatures for effective expression. Pio and Baust (1988) reported periodic variation in glycerol and sorbitol concentrations with thermal fluctuations (e.g., intermittent bouts of chilling and warming). In the beet armyworm *Spodoptera exigua*, haemolymph osmolality and glycerol content did not differ significantly between cyclic and constant temperature regimes (Kim and Song, 2000). The authors speculated that, instead of glycerol, other polyols or free amino acids (FAA) may be involved. Recently, Wang et al. (2006) also attempted to investigate why FTR enhance cold-hardiness in *Locusta migratoria* eggs. They found that FTR improved survival and induced the accumulation of heat shock proteins (*Hsps*), myo-inositol, trehalose, mannitol, and sorbitol. However, there was a discrepancy between survival rate and these accumulations, as the highest survival did not correspond to the highest levels of cryoprotectants and *Hsps*. The authors speculated that some other factors that affect cold hardiness may probably be involved.

Although the importance of FAA during cold-exposure has been much less investigated than other low molecular weight compounds (Renault et al., 2006), some correlations between cold-hardiness and levels of some FAA have been found in arthropods (Hanzal and Jegorov, 1991; Goto et al., 1997; Storey, 1997; Fields et al., 1998; Issartel et al., 2005). A relationship between increased proline levels and cold-tolerance has been suggested in different insect species (e.g., Fields et al., 1998; Ramlov, 1999), and other amino acids, such as glycine, alanine and leucine have also been suspected to play a role during cold-acclimation. Apart from their potential role in cold-tolerance, amino acids can be used as an effective monitoring agent for physiological conditions in many invertebrates. Indeed, most specific stress phenomena initiate specific metabolic responses in the FAA pools (Powell et al., 1982). Since metabolites, such as FAA, are downstream of both gene transcripts and proteins, specific changes in metabolite levels can provide a good indication of the overall response of an organism to stressful conditions (Malmendal et al., 2006).

Although cold-resistance has been studied in many insect species, little is known about low temperature effects on insect parasitoids (Rivers et al., 2000). Since insect parasitoids are commonly used in biological control, the possibility of using cold storage as an aid to mass-release was examined intensively over the last 70 years (Archer et al., 1973; Hofsvang and Hagvar, 1977; Jarry and Tremblay, 1989; Levie et al., 2005; Colinet et al., 2006b). The small parasitic wasp *Aphidius colemani* Viereck (Hymenoptera: Aphidiinae), is commercially produced and distributed as an aphid biocontrol agent, targeting primarily *Myzus persicae* Sulzer (Homoptera: Aphididae) in glasshouses of several European countries. This parasitoid stops feeding at the end of the third larval stage (Muratori et al., 2004), spins its cocoon inside the empty cuticle of the aphid, forms a mummy and pupates (Hagvar and Hofsvang, 1991). From this moment onwards, it no longer feeds and all metabolic processes, including metamorphosis, make use of energetic reserves accumulated during the larval stages.

A recent study demonstrated that the cold-survival of *A. colemani* was substantially increased under FTRs (Colinet et al., 2006a). FTRs may act as a cue triggering the initiation of a metabolic response involving synthesis of cryoprotective compounds such as FAA, thus resulting in increased survival. However, the physiological roles of most FAA are still not fully understood (Yi and Adams, 2000), no study has investigated the effects of different thermal treatments on FAA pool in parasitoids. The main objectives of this study were to test if any FAA accumulation may explain the increased survival under FTRs, to use FAA metabolic responses and/or trajectories to compare specific cold conditions, and to verify if cold stress disrupts normal FAA levels. As model, we use the freezing-and chill-intolerant parasitoid *A. colemani* Viereck (Hymenoptera: Aphidiinae).

## 2. Materials and methods

### 2.1. Rearing aphids and parasitoids

The green peach aphid, *M. persicae*, was used as a host in parasitoid rearing and laboratory cultures were established from individuals collected in agricultural fields around Louvain-la-Neuve, Belgium (50.3 °N Latitude) in 2000. Aphids were reared in 0.3 $m^3$ cages on sweet pepper (*Capsicum annuum* L.) under 18±1 °C, ±60% RH and LD 16:8 h. *A. colemani*, originally provided by Biobest Co. (Belgium), was subsequently reared in the laboratory under the same conditions.

To obtain standard mummies, batches of 50 standardized three-day-old aphids were offered to a mated female parasitoid for 4 h. Aphids were all synchronized at the same age in order to avoid host-age effects on parasitoid development (Colinet et al., 2005). Parasitoid females were less than 48 h old, naïve, and mated. The resulting parasitized aphids were then reared under controlled conditions (18±1 °C, ±60% RH and LD 16:8 h) until mummification. Newly formed mummies were left to develop for one day, under the same rearing conditions, before cold-exposure. One-day-old mummies were used in the experiment because young mummies are known to be more cold-tolerant (Hofsvang and Hagvar, 1977; Levie et al., 2005).

### 2.2. Thermal treatment and survival

For aphid parasitoids, temperatures used for cold-storage usually range between 0 and 7 °C (Archer et al., 1973; Singh

and Srivastava, 1988; Rigaux et al., 2000). In the present study, the parasitoids were exposed to 4 °C, a temperature close to the thermal threshold of *A. colemani* (2.8 °C) (Elliott et al., 1995). Batches of one-day-old mummies were placed in small plastic Petri dishes. Mummies were then exposed to low temperature inside thermo-regulated LMS® incubators, with saturated relative humidity and in complete darkness. Batches of mummies were assigned randomly to either constant or FTRs:

— Treatment C: constant temperature: 4 °C for the entire duration of the experiment
— Treatment F: fluctuating thermal regime: the 4 °C exposure was interrupted daily (every 22 h) by a transfer to 20 °C for 2 h.

To investigate the effects of the cold-treatments on parasitoid survival, three batches of 50 mummies were removed at weekly intervals for each experimental condition and kept at 20 °C. The survival after one, two and three weeks of cold-exposure, expressed as the emergence rate, was assessed as the number of adults that successfully emerged from the mummies when replaced at 20 °C. A non-cold-exposed control (i.e., three batches of 50 mummies) was maintained at 20 °C.

For each condition, 25 mummies were dissected just after the insects were removed from the cold incubator. Moreover the mummies that did not emerge after 7 days at 20 °C (i.e., dead) were also dissected in order to identify the developmental stage reached, as described in Colinet et al. (2006b). This procedure was performed to ensure that insects were still alive at the end of the cold-exposure (and thus at the moment of FAA dosage), as indicated by any progress in the development between the two moments of dissection.

### 2.3. Free amino acids

To investigate the impact of the cold-exposure, the FAA pool was measured under three different conditions:

1 Constant temperature — Treatment C: FAA measured in mummies kept at a constant temperature of 4 °C.
2 Fluctuating thermal regimes — Treatments F: FAA measured in mummies kept under FTR — (4 °C exposure interrupted every 22 h by a transfer to 20 °C for 2 h). The treatment F was divided in 2 sub-treatments:
   — Treatment Fc: FAA measured in mummies removed from the incubator at the end of the cold period, just before the temperature rose to 20 °C for 2 h.
   — Treatment Fw: FAA measured in mummies removed from the incubator at the end of the warm period, just before the temperature dropped to 4 °C for 22 h.

For each experimental condition, nine samples containing 25 mummies were removed from the incubator after one, two and three weeks of cold-exposure. Mummies were immediately weighed (fresh mass) using a Mettler® micro-balance (accurate to 0.01 mg) and frozen in liquid nitrogen. They were then stored at −80 °C until the assay of FAA.

FAA were also analysed in control mummies (exposed at 20 °C, $n=9$).

### 2.4. Sample preparation

FAA were extracted from fresh material. The mummies were homogenized in 1 mL of 70% ethanol and Fontainebleau sand, before adding 1 mL of 40% ethanol and 1 mL of ultra pure water. The homogenate was then centrifuged for 10 min at 4500 *g* and 4 °C, and the supernatant collected. The first pellet was re-suspended in 1 mL of ultrapure water and centrifuged for 10 min at 4500 *g* and 4 °C, and the supernatant collected. The combined supernatants ($n=2$) were pooled in a balloon flask and dried by evaporation using a rotary-evaporator. The insoluble residue was re-suspended in 800 μL of ultra pure water. Samples were stored at −80 °C.

### 2.5. Analytical procedure

FAA were assayed as described by Bouchereau et al. (1999). Amino acids were characterized and quantified by HPLC after pre-column derivatization with 6-aminoquinolyl-*N*-hydroxy-succinimidylcarbamate (AQC) (using a Waters Accq-Tag amino acid analysis system, Waters Corporation, Milford, USA) and reversed-phase liquid chromatographic separation (see Bouchereau et al., 1999 for a full description of the method). Fifteen microliter aliquots of the crude aqueous extracts were assayed using the procedure optimised by Cohen and Michaud (1993) and Issartel et al. (2005).

### 2.6. Statistical analysis

Arcsine square root transformation was required to normalize the distribution of the emergence rate (Hardy, 2002). The emergence rate was analysed using a two-way ANOVA (Proc GLM, SAS Institute, Cary, NC, USA, 1990) with thermal treatment and duration of cold-exposure as fixed factors.

The log-transformed FAA concentrations were analysed using a two-way ANOVAs (Proc GLM, SAS Institute, Cary, NC, USA, 1990) with thermal treatment and duration of cold-exposure (from one to three weeks) as fixed factors. Multiple comparisons were then performed on each factor using Tukey's test to describe differences between groups. Dunnett's pairwise multiple comparisons *t*-tests were used to compare the mean values to the control means. A significance level of $\alpha=0.05$ was used for all tests. Data presented in the figures are untransformed.

## 3. Results

### 3.1. Survival

As expected, emergence rate (Fig. 1) was significantly affected by thermal treatment ($F=62.77$, $P<0.001$) and by the duration of cold-exposure ($F=46.52$, $P<0.001$). Under FTRs (treatment F), the emergence rate remained high, with 75% emergence after three weeks of cold-exposure. The number of

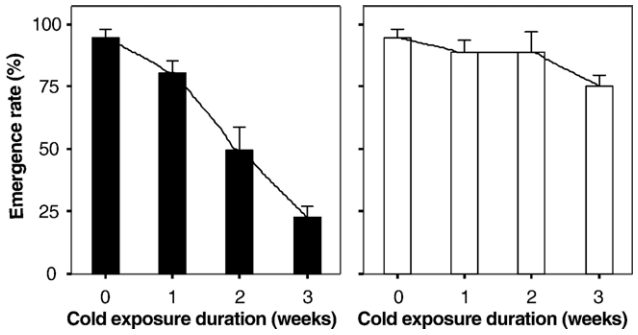


Fig. 1. Percentage of emerging adults (mean±SE) as a function of the duration of cold exposure for each thermal treatment: constant 4 °C (treatment C, black bars) and fluctuating thermal regime with daily warming (treatment F, white bars).

emerging adults in treatment C (constant 4 °C) was highly significantly reduced. It only reached 50 and 23% after two and three weeks, respectively (Fig. 1). The significant interaction between thermal treatment and duration ($F=14.20$, $P<0.001$) indicates that the temporal reduction in emergence was treatment-specific. These results confirm the beneficial impact of fluctuating temperatures on survival of the cold-exposed mummies.

Dissections revealed that none of the mummies that were removed weekly from the incubator had reached the adult stage, whereas most of the mummies that did not emerge after the cold-exposure contained fully-formed adults (92, 82 and 91%, respectively for one, two and three weeks of cold-exposure in treatment F, and 89, 93 and 85%, respectively for one, two and three weeks in treatment C). This high proportion of imagos in non-emerged mummies confirms that metamorphosis indeed took place once mummies were returned to 20 °C.

### 3.2. FAA in control group

Eighteen different FAAs were detected in the whole body extracts of the parasitic wasp *A. colemani* (Figs. 2 and 3). Due to the difficulty of accurately distinguishing Asn from Ser, and Arg from Thr, the results were presented as combined amounts of Asn/Ser and Arg/Thr. In the control group, Gln, Tyr, Glu, Ala, Arg/Thr, Pro and Trp were the major components comprising about 80% of the total FAA pool, with Gln, Tyr and Glu being the most abundant amino acids (representing roughly half of the total FAA pool).

### 3.3. FAA pool of cold-exposed mummies compared to the control

A one-week exposure to a constant 4 °C induced a significant increase in several FAA (Glu, Ala, Arg/Thr, Pro, Asn/Ser, Gly, Leu, Lys, Ile), whereas the Trp level significantly decreased (Figs. 2 and 3). Under constant cold-exposure conditions, the level of most FAA remained significantly higher than in the control after two and three weeks of cold exposure (Figs. 2 and 3). The total FAA pool (Fig. 4) was 76.0±3.37 nmol $mg^{-1}$ fresh mass in control mummies (20 °C), and reached 95.98±6.83 nmol $mg^{-1}$ fresh mass after one week of cold-exposure in treatment C (25% of increase). This total FAA pool remained high even after two and three weeks of cold-exposure.

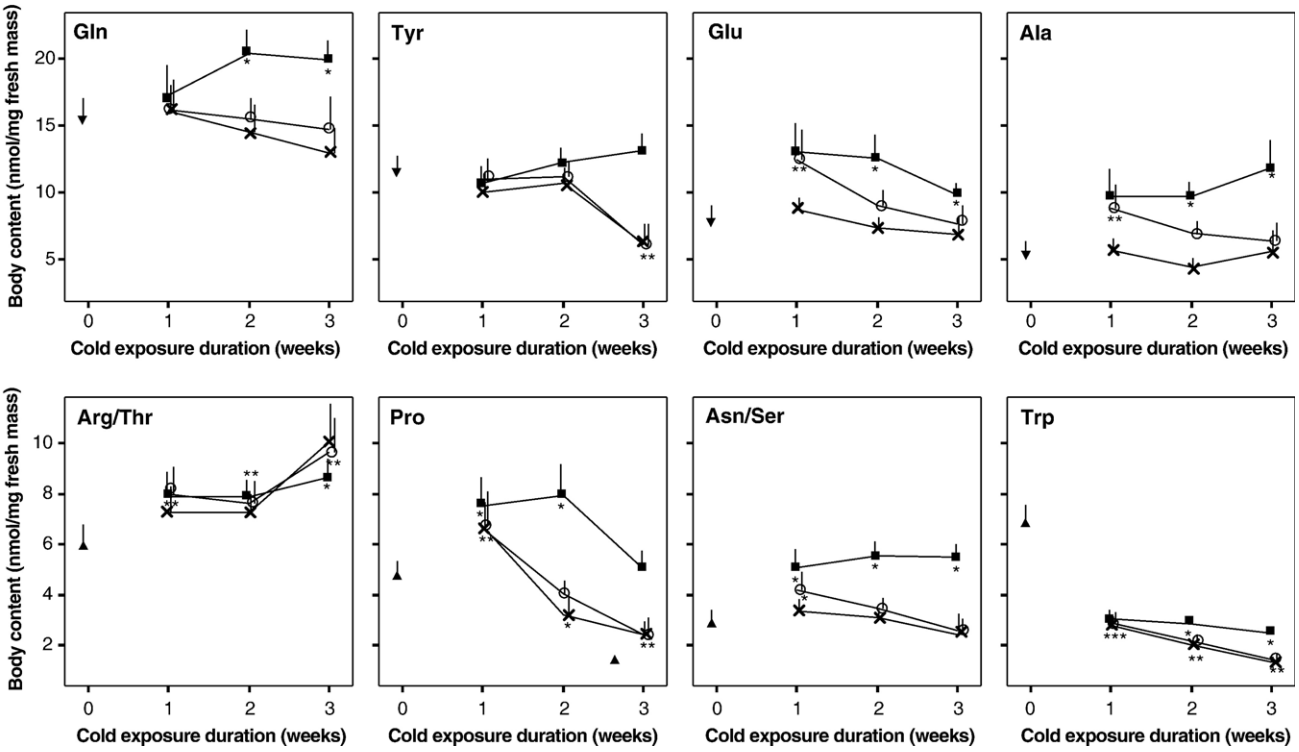


Fig. 2. Changes in the contents of some FAA (mean+SE, $n=9$) as a function of cold exposure duration for each thermal treatment, (■) for treatment C, (○) for treatment Fc, (x) for treatment Fw. The symbol (∗) indicates a significant difference from control value (Dunnett *t*-test). Graphs are ranked with decreasing concentrations.

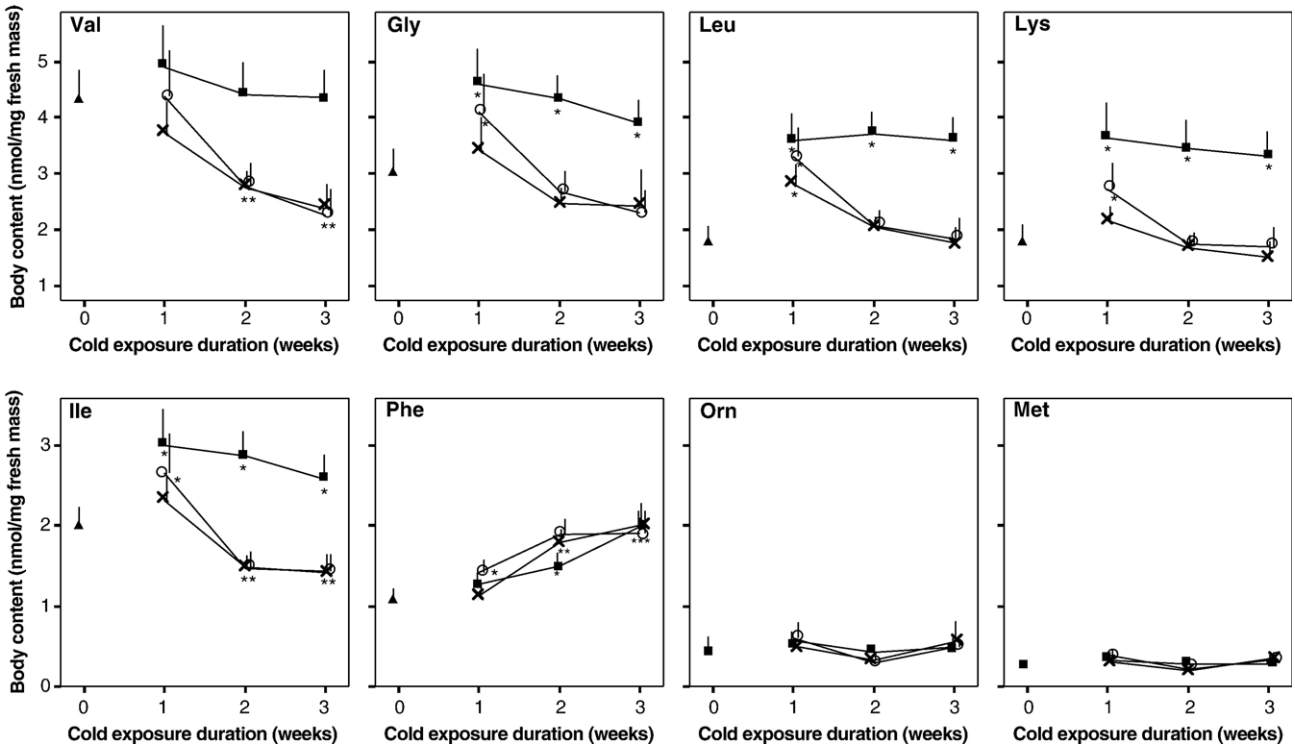


Fig. 3. Changes in the contents of some FAA (mean+SE, $n=9$) as a function of cold exposure duration for each thermal treatment: (■) for treatment C, (○) for treatment Fc, (x) for treatment Fw. The symbols (∗) indicate significant differences with the control value (Dunnett $t$-test). Graphs are ranked with decreasing concentrations.

After one week of FTR, FAA levels measured in the mummies at the end of the warm interval (treatment Fw) were similar from the control values. Pro and Leu were the only amino acids that significantly increased, whereas Trp decreased (Figs. 2 and 3). After two and three weeks of FTR, the concentrations of FAA in treatment Fw were similar to those found in control mummies, but some FAA were significantly less abundant (Tyr, Pro, Trp, Val, Ile) (Figs. 2 and 3). After one week of FTR, the total FAA content in treatment Fw (76.79± 4.90 nmol $mg^{-1}$ fresh mass) (Fig. 4) was the same as in the control, whereas it was significantly lower after three weeks, as a result of cumulated reductions in several amino acids levels.

The FAA levels in mummies exposed to FTR and measured at the end of the cold interval (treatment Fc) showed an intermediate response between treatment C and Fw. As in the treatment C, levels of several amino acids increased compared to the control (Glu, Ala, Arg/Thr, Pro, Asn/Ser, Gly, Leu, Lys, Ile) after one week of exposure (Figs. 2 and 3), resulting in an increase in the total amount of FAA (89.54±7.17 nmol $mg^{-1}$ fresh mass) (Fig. 4). However, as in the Fw mummies, the total amount of FAA was significantly lower than in the control after three weeks (Figs. 2 and 3).

The influence of the cold-exposure on the metabolic response was detectable in all the treatments. However, it seemed to be more manifest in treatment C. Indeed, among all exposure durations, there were 33, 25 and 15 situations (out of 48), in treatment C, Fc and Fw, respectively, in which FAA contents were significantly different from the control values (Dunnett's pairwise $t$-test, $P<0.05$) (Figs. 2 and 3).

### *3.4. Effect of thermal treatments and duration of exposure on FAA*

The most abundant amino acids were Gln, Tyr, Glu and Ala representing more than 50% of the FAA pool in treatment C, and Gln, Tyr, Glu and Arg/Thr, representing more than 50%, in treatments Fw and Fc. Cold-exposure (from one to three weeks) affected the level of almost all FAA (Table 1, Figs. 2 and 3). Gln was the only amino acid for which the concentration remained relatively constant during the cold-exposure. Except for Arg/Thr, Ala and Phe, which were characterized by increased

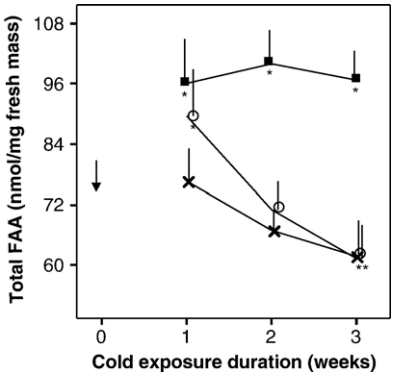


Fig. 4. Changes in the total FAA content (mean+SE, n=9) as a function of cold exposure duration for each thermal treatment: (■) for treatment C, (○) for treatment Fc, (x) for treatment Fw. The symbols (∗) indicate significant differences with the control value (Dunnett $t$-test).

Table 1
Two-way analysis of variance on FAA contents with thermal treatment (C, Fc and Fw) and duration of cold exposure (from 1 to 3 weeks) as factors

| | Duration | | | Comparison Tukey's test | | | treatment | | | Comparison Tukey's test | | | Interaction duration * treatment | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *df* | *F* | *P* | 1 week | 2 weeks | 3 weeks | *df* | *F* | *P* | C | Fc | Fw | *df* | *F* | *P* |
| Gln | 2 | 1.19 | 0.31 | a | a | a | 2 | 18.27 | <0.001 | a | b | b | 4 | 2.52 | 0.049 |
| Tyr | 2 | 24.20 | <0.001 | a | a | b | 2 | 18.46 | <0.001 | a | b | b | 4 | 11.58 | <0.001 |
| Glu | 2 | 16.66 | <0.001 | a | b | c | 2 | 27.65 | <0.001 | a | b | c | 4 | 1.29 | 0.283 |
| Ala | 2 | 3.03 | 0.04 | ab | b | a | 2 | 47.18 | <0.001 | a | b | c | 4 | 2.48 | 0.050 |
| Arg+Thr | 2 | 12.32 | <0.001 | b | b | a | 2 | 0.27 | 0.765 | a | a | a | 4 | 1.20 | 0.320 |
| Pro | 2 | 59.30 | <0.001 | a | b | c | 2 | 37.27 | <0.001 | a | b | b | 4 | 5.78 | <0.001 |
| Asp+Ser | 2 | 8.97 | <0.001 | a | a | b | 2 | 44.92 | <0.001 | a | b | b | 4 | 3.55 | 0.011 |
| Trp | 2 | 63.00 | <0.001 | a | b | c | 2 | 30.13 | <0.001 | a | b | b | 4 | 6.02 | <0.001 |
| Val | 2 | 23.25 | <0.001 | a | b | b | 2 | 34.22 | <0.001 | a | b | b | 4 | 3.54 | 0.011 |
| Gly | 2 | 17.40 | <0.001 | a | b | b | 2 | 29.31 | <0.001 | a | b | b | 4 | 1.99 | 0.106 |
| Leu | 2 | 22.41 | <0.001 | a | b | b | 2 | 52.95 | <0.001 | a | b | b | 4 | 6.32 | <0.001 |
| Lys | 2 | 13.53 | <0.001 | a | b | b | 2 | 65.63 | <0.001 | a | b | b | 4 | 2.18 | 0.081 |
| Ile | 2 | 33.71 | <0.001 | a | b | b | 2 | 53.88 | <0.001 | a | b | b | 4 | 5.08 | 0.001 |
| Phe | 2 | 45.38 | <0.001 | c | b | a | 2 | 2.33 | 0.105 | a | a | a | 4 | 3.08 | 0.022 |
| Orn | 2 | 12.37 | <0.001 | a | b | a | 2 | 0.93 | 0.398 | a | a | a | 4 | 0.83 | 0.513 |
| Met | 2 | 15.43 | <0.001 | a | b | a | 2 | 0.63 | 0.535 | a | a | a | 4 | 3.38 | 0.014 |
| Total FAA | 2 | 7.04 | 0.002 | a | ab | b | 2 | 26.43 | <0.001 | a | b | b | 4 | 2.43 | 0.056 |

For each factor, multiple comparisons were performed using Tukey's test to describe differences between groups, different letters indicate significant differences (significance level of $\alpha = 0.05$).

amounts with cold-exposure duration, the general response was a reduction in FAA concentrations, with maximum values observed after one week of cold-exposure, before reaching a plateau after two weeks of cold-exposure (Table 1). Among all treatments, the total FAA pool (Fig. 4) illustrates the general pattern with higher, intermediate and lower concentrations observed after one, two and three weeks of cold-exposure, respectively (Table 1).

The metabolic response to cold-exposure was clearly affected by the thermal treatment. Except for Arg/Thr, Phe, Orn and Met, significant treatment effects were observed in all FAA (Table 1). In most cases, FAA levels were significantly higher in treatment C than in treatment Fc and Fw, which were not significantly different. The significant interactions between duration and thermal treatment (Table 1) indicate that the temporal changes in FAA pools were treatment-specific in many cases.

## 4. Discussion

As shown in a previous study (Colinet et al., 2006a), the survival of immature parasitoids is markedly improved when the exposure to 4 °C is interrupted by daily transfers to 20 °C. It has been suggested that the normal physiological state of the cold-exposed insects may be re-activated during the short pulses at warm temperature. Transferring the mummies to 20 °C may reduce the speed and the amount of accumulated injuries. Additionally, warm temperatures may counteract the negative effects of the cold-exposure by allowing a repair of the cumulative damages due to cold (Nedved et al., 1998; Renault et al., 2004; Colinet et al., 2006a). The strong differences in survival between constant and FTRs indicate that the experimental conditions should have a strong impact on physiological and metabolic responses of the parasitoids.

None of the mummies dissected just after incubator removal had reached the adult stage, whereas fully-formed adults were found in most of the mummies that did not emerge when replaced at 20 °C after the cold-exposure. The high proportion of imagos in dead mummies after the cold-exposure indicates that metamorphosis indeed took place once mummies were replaced at 20 °C, as observed by Levie et al. (2005) and Colinet et al. (2006b).

Compared with other animals, FAA are generally highly concentrated in the haemolymph of insects (Mansingh, 1967; Chen, 1985). Our data are consistent with that of Mullins (1985), which showed that endopterygotes are generally characterized by low levels of Asn, Phe, Leu and Ile and high amounts of Glu, Gln and Pro. In *A. colemani*, Gln, Tyr and Glu are the main FAA composing roughly half of the total FAA pool. Like many other koinobiont parasitoids, *A. colemani* feeds on host haemolymph during larval development, and consumes all host tissues in the final part of its larval development before mummy formation and pupation (Muratori et al., 2004). Many organisms are supposed to contain approximately the same FAA pool as their food sources, i.e., the host in the case of a parasitoid (Quicke and Shaw, 2004). Therefore, the high level of Tyr in mummies probably results from the hypertyrosinaemic syndrome triggered by parasitism in aphids, as observed with *Aphidius ervi* (Rahbé et al., 2002).

A sharp decrease in Trp, compared to control conditions, was observed in all treatments. In insects, Trp is particularly known to serve as a precursor for the synthesis of eye pigment (ommochromes) (Hooper et al., 1999). Hence, the Trp decrease may be related to a developmental process; indeed a sharp fall in Trp concentration has been observed in *Carausius morosus* during larval development and was linked to ecdysis (Stratakis, 1980). The onset of the pupal stage in mummies may explain the reduction in Trp levels, especially since the formation and pigmentation of eyes occurs during the pupal stage (Starý, 1970).

Although the present study provides information about FAA levels rather than the pathways involved, it can provide useful information about physiological mechanisms occurring during cold-exposure. If any FAA had a specific role in cold-tolerance, it would imply the occurrence of very high concentration of this amino acid. At the start of the cold-exposure, initial increases were observed in most FAA, particularly in treatment C, suggesting that these initial accumulations were not related to cold-tolerance, since most insects died in treatment C. Except for Arg/Thr, Ala and Phe for which the concentrations continued to increase, the general temporal pattern among all treatments, was a reduction in the FAA pool with duration, from maximum values observed after one week of cold-exposure. The increases observed in Arg/Thr and Phe were not different between the three thermal treatments, suggesting that these accumulations were not the reasons behind the treatment-related differences in survival. Arg plays an important role in metabolism, and its accumulation may result from an alteration of metabolic pathways, induced by low temperature (Fields et al., 1998). Ala content increased in treatment C, whereas it remained relatively stable under fluctuating regimes. An increase in Pro, Ala and Gly seem to be a common feature accompanying acclimation of insects at low temperatures (Morgan and Chippendale, 1983; Storey, 1983; Hanzal and Jegorov, 1991; Fields et al., 1998; Ramlov, 1999; Yi and Adams, 2000; Li et al., 2001). The relationship between high Ala concentration following acclimation and cold-hardiness, and the factors affecting the Ala levels are still not well understood. Goto et al. (2001a) found that the levels of Ala were always high under anaerobic conditions, and also under aerobic conditions. They suggested that an increase in Ala content at low temperatures and aerobic conditions may be due to a decrease in $O_2$ uptake caused by lower temperatures. This may explain why Ala accumulates under constant cold-exposure, since temperature was constantly low under treatment C.

In addition to their putative role as cryoprotectants, FAA are involved in many metabolic processes including development, protein synthesis/catabolism and energetic pathways (Fields et al., 1998; Renault et al., 2006). During winter diapause, low-temperature exposure and cold-acclimation, the total FAA pool often increases in insects (Mansingh, 1967; Rains and Dimock, 1978; Morgan and Chippendale, 1983; Hanzal and Jegorov, 1991; Fields et al., 1998; Renault et al., 2006). Our results confirm these observations since one-week constant cold-exposure induced a significant increase in nine FAAs, resulting in a global increase in the total FAA pool, compared to the control condition. This initial increase was smaller in treatment Fc and insignificant in treatment Fw, emphasizing the importance of the 2 h-warm intervals for recovering from a thermal stress. Recently, Malmendal et al. (2006) analysed the metabolic profile of *Drosophila melanogaster* following a heat stress. They reported variations in many major metabolite concentrations, including FAAs, with an initial increase in some FAAs, followed by a recovery phase where homeostasis was rapidly reached (after 4 h). In our study, the repeated 2 h-warm intervals may allow a return to normal physiological conditions, which is not the case under a continuous cold-exposure regime. In treatment C, FAA concentrations were generally significantly higher compared to the control, even after two and three weeks. Increased levels of FAA in the mummies during the first days of cold-exposure might result from both protein degradation and/or reduction in protein synthesis. Temperature (high and low) may act as a stress resulting in protein degradation (Renault et al., 2006; Malmendal et al., 2006). Protein damage (unfolding) is commonly observed in cells exposed to stress, and many terminally damaged proteins are removed by proteolytic degradation (Kültz, 2005).

Hanč and Nedvěd (1999) suggested that the physiological processes of cold-hardening, which is cued by the low temperature, may require a stay at higher temperatures for effective expression. In several insects species, low temperature exposures induce synthesis of *Hsps* during recovery periods at higher temperature (Nunamaker et al., 1996; Joplin et al., 1990; Yocum et al., 1991). It was recently shown that thermoperiod cycles stimulate the expression of *Hsps*, particularly with repeated cycles (Wang et al., 2006). Under FTRs, *Hsps* may be synthesised during the pulses at high temperature, consuming the FAA pool, this assumption being supported by our recent proteomic study where *Hsps* show significant up-regulations under FTR (Colinet et al., unpublished data). Chen and Denlinger (1992) suggested that energy supplies depleted after long cold-exposure, as observed in *A. colemani* (Colinet et al., 2006b), may be regenerated during the pulse of high temperature. This would involve reactivation or synthesis of FAA-consuming proteins involved in energetic pathways. Our recent proteomic data also corroborate this assumption, since several proteins involved in energy production/conversion are up-regulated under FTR (Colinet et al., unpublished data). The cold-induced initial increase of the FAA pool suggests that replenishment of the pool, probably from protein breakdown and/or reduction in protein synthesis, exceeds the amino acid utilization for development, protein synthesis and energetic pathways. This is particularly manifest in treatment C, where development and metabolic activity are permanently slowed down, whereas under FTRs, warming periods allow a more rapid utilization and incorporation of amino acids into the developmental, repair and energetic processes.

Since most stress phenomena initiate specific metabolic responses in the FAA pool in invertebrates (Powell et al., 1982), the amino acid pool may be used as a monitoring agent for physiological response of parasitoids exposed to stressful cold-conditions. We hypothesized that temperature fluctuations may act as a cue triggering the initiation of a metabolic response involving synthesis of cryoprotective compounds, such as FAA. The present study clearly demonstrates that the physiological responses exhibited by *A. colemani* during cold-exposure were dependent on thermal treatments, but contrary to predictions, the fluctuating temperature induced a much less accumulation of FAA than constant cold-exposure. This likely signifies that an increase in the FAA pool is a cold-induced symptom probably resulting from protein breakdown, disruption of metabolic pathways and reduction of protein synthesis. This would be much less manifest under fluctuating temperature, since warm intervals allow the reactivation of developmental processes and

normal metabolic activities. Some FAA have been suspected to play a cryoprotective role in insects, but no clear relationship between their evident cold-induced accumulation and the survival has yet been established (e.g., Goto et al., 2001a,b). Therefore, instead of considering FAA as a part of the cryoprotective arsenal, FAA accumulation should rather be regarded as a symptom of cold-induced multiple physiological perturbations.

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