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Abscisic acid enhances cold tolerance in honeybee larvae

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The natural composition of nutrients present in food is a key factor determining the immune function and stress responses in the honeybee (*Apis mellifera*). We previously demonstrated that a supplement of abscisic acid (ABA), a natural component of nectar, pollen, and honey, increases honeybee colony survival overwinter. Here we further explored the role of ABA in *in vitro*-reared larvae exposed to low temperatures. Four-day-old larvae (L4) exposed to 25°C for 3 days showed lower survival rates and delayed development compared to individuals growing at a standard temperature (34°C). Cold-stressed larvae maintained higher levels of ABA for longer than do larvae reared at 34°C, suggesting a biological significance for ABA. Larvae fed with an ABA-supplemented diet completely prevent the low survival rate due to cold stress and accelerate adult emergence. ABA modulates the expression of genes involved in metabolic adjustments and stress responses: *Hexamerin 70b*, *Insulin Receptor Substrate*, *Vitellogenin*, and *Heat Shock Proteins 70*. AmLANCL2, the honeybee ABA receptor, is also regulated by cold stress and ABA. These results support a role for ABA increasing the tolerance of honeybee larvae to low temperatures through priming effects.

1. Introduction

Honeybees pollinate around one third of the crops cultivated worldwide, but their role in global food production has been threatened by unusually high mortality rates of managed colonies [1]. Environmental, agrochemical, parasitic, and pathogenic stressors have increased the frequency and intensity of colony failure and collapse phenomena [2,3]. It has been shown that most honeybee colony loss occurs during the winter season [4,5]. Khoury *et al.* [6] have established, through mathematical predictive models, that if colony populations become small, the colony's death is accelerated. Small colonies are unable to incubate brood or maintain nest temperature, increasing the colony's chance of dying [6]. It has also been shown that extreme cold events negatively impact on the distribution and abundance of some bee species [7]. Thus, the colony temperature is affected both by meteorological phenomena and colony size [5,6,8].

The honeybee regulates thermal homeostasis in the brood nest, keeping it between 33°C and 36°C [9]. Deviations from normal brood temperatures induce changes in the worker bee's behaviour; they crowd tightly together in clusters, reducing heat loss, and producing endothermic heat by the activation of thoracic muscles [9–11]. Honeybee broods reared at temperatures below 32°C shows delayed development, increased mortality, abnormal nervous system development, and poor behavioural performance as adult bees [12,13].

Lately, the role of naturally occurring compounds present in nectar, pollen, and honey is being revalued [14,15]. Through laboratory and field experiments, we have demonstrated that the supplementation of hives with syrup containing

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abscisic acid (ABA) enhanced the innate immune response of honeybees and the winter survival of the colony [15].

The phytohormone ABA is involved in plant responses to environmental stresses such as extreme temperature, light, and drought [16]. In animal systems, ABA is an endogenous hormone acting in inflammatory responses [17,18]. ABA is a natural constituent of pollen, nectar, and honey [19–21] and because of that, we predicted that ABA would work as a specific component of the honeybee's food, enhancing the bees' capacity to confront winter temperatures. Here, we demonstrate that honeybee larvae reared *in vitro* and fed with an ABA-supplemented diet, showed improved developmental behaviour and higher survival rates upon submitted to cold stress.

2. Material and methods

(a) *In vitro* rearing and cold stress treatment

Larvae of honeybee *Apis mellifera* (*A. mellifera ligustica*- *A. mellifera mellifera*) of 1-day-old (1st instar, L1) were collected from colonies of our experimental apiary at Santa Paula, route 226, km 10, Mar del Plata, Argentina. The larvae were transferred from brood comb to 48-well culture plates. The larval rearing plates were placed into desiccators maintained at a relative humidity of 96% (K_2SO_4 saturated) in a 34°C incubator. Daily diet volumes provided to the larvae and variation of the composition of the diet was done according to Aupinel *et al.* [22] and is detailed in electronic supplementary material, table S1. The diet was supplemented or not with 50 µM of (R,S)-abscisic acid (ABA, Sigma Aldrich). Larvae were fed from day 1 until day 6. We used ecologically relevant doses of ABA supplementation based on measurements of ABA in honey and royal jelly in our region (0.211 and 0.119 $\mu g\, g^{-1}$ of fresh weight (FW), respectively), and ABA measurements reported in honey from different origins (between 0.1 and 360 $\mu g\, g^{-1}$ of FW) [20,21]. For some experiments, diet was supplemented with 5, 50, and 500 µM of ABA (equivalent to 1.32, 13.2, and 132 $\mu g\, g^{-1}$ FW, respectively).

After 4 days growing at 34°C, a group of larvae was transferred into an incubator at 25°C for 3 days (cold-stressed larvae) and then returned to 34°C until they culminate the development as newly emerged workers (NEW). All larvae (control and cold stressed) received 160 µl as a total diet during a feeding period of 6 days (see figure 1 and electronic supplementary material, table S1).

(b) Abscisic acid extraction and quantification

For ABA quantification, individuals of 4, 6, and 8 days old were entirely used, while in adults (NEW), the midguts were extracted by pulling out from the sting and separated from the rest of the body. Each sample was frozen in liquid nitrogen and ground to a fine powder in a mortar. ABA extraction was performed following the method described by Van Norman *et al.* [23] and using the Phytodetek ABA Quantification Kit (Agdia) according to the manufacturer's instructions.

(c) Measurements of survival, fresh weight, and developmental timing

Survival was measured as the percentage of NEW from an initial number of L1 included in each experimental condition, considered as 100%. Dead individuals were counted daily and removed from the rearing plates.

Fresh weight (FW) was measured in individuals of different developmental stages: 4, 6, 8, 12 days old, and NEW, after dusting them off with filter paper. Development was measured as the time (days) between the larval stage L1 and adult emergence.

(d) Quantification of transcript level by real time PCR (qPCR)

The total RNA from individuals was extracted, reverse transcribed, and cDNA used as a template for qPCR analysis. The protocol is detailed in the supplementary material and the primer sequences listed in electronic supplementary material, table S2. The expression levels of each gene were normalized to *Actin*. LinRegPCR program was employed for the analysis of qPCR data [24]. The transcript relative quantification results were determined from the ratio between the starting concentration value of the analysed mRNAs and the reference *Actin* mRNA in each sample. The mean and standard error was calculated from values of the transcript quantification obtained in each biological replicate.

(e) Statistical analysis

Statistical analyses were conducted with SigmaPlot v. 11.0 software (Systat Software Inc., CA, USA). ABA content, survival, and developmental time data were analysed using Student's *t*-test. A Mann–Whitney test was performed when the assumptions of normality and/or homogeneity of variances were violated. Fresh weight and transcript quantification data were analysed using one-way ANOVA or two-way ANOVA, with *post hoc* comparisons by the Holm-Sidak multiple comparison test. Kruskal–Wallis test (with *post hoc* comparisons by the Dunn's multiple comparison test) was used when the assumptions of normality and/or homogeneity of variances were violated.

3. Results

(a) Description of the experimental design

Figure 1 shows the experimental approach designed to investigate the role of ABA in cold stress tolerance in honeybee larvae. We decided to study the effects of cold stress on honeybee larvae at 25°C, mainly due to two reasons: (i) it has been established that brood reared at temperatures lower than 34°C increases pupal mortality, negatively influencing the emergence of adults, and affecting the synaptic organization of the adult honeybee brain [12,13,25] and (ii) preliminary results obtained in our laboratory demonstrated that larvae reared *in vitro* at temperatures below 23°C were completely unstable and eventually died.

(b) Cold-stressed honeybee larvae maintain high levels of abscisic acid

Figure 2 shows that at the control growth temperature of 34°C, ABA content in larvae decreased during development from 4-day-old larvae to 8-day-old individuals (Mann–Whitney test, $U = 0$, $p = 0.001$), while an increase was observed in NEW compared to 8-day-old individuals (Mann–Whitney test, $U = 0$, $p < 0.001$). Figure 2 also shows that the ABA content decreased at a lower rate in 4- to 8-day-old cold-stressed individuals compared with the control conditions (Mann–Whitney test, $U = 0$, $p = 0.006$). This result suggests that the higher ABA content in cold-stressed honeybee larvae might play a role in cold stress responses. Thereby, the effect of ABA supplementation in the diet of *in vitro*-reared larvae was studied. We predicted an enhanced tolerance to cold stress in ABA-supplemented honeybee larvae. Electronic

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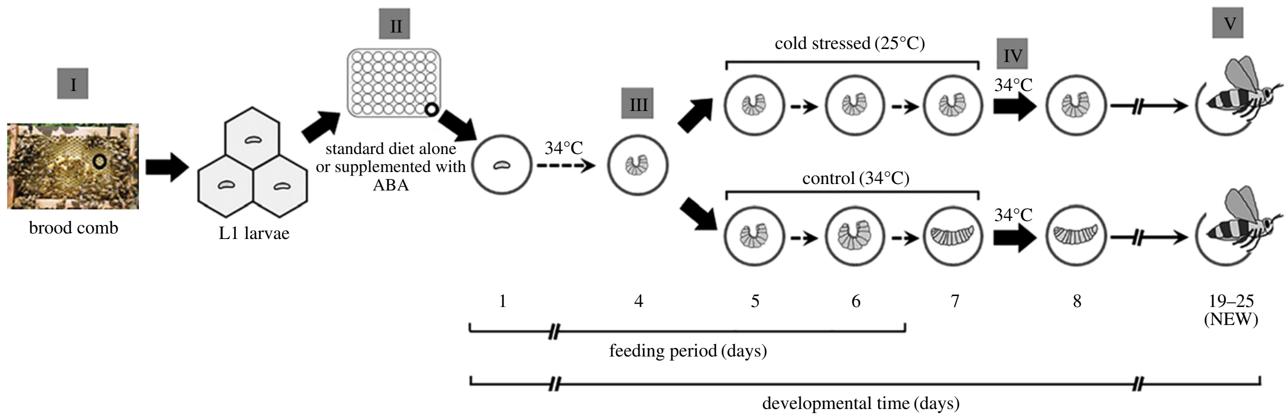

Figure 1. Experimental design for the manipulation of *A. mellifera* during the development in standardized *in vitro* growth conditions. (I) First instar larvae were collected from brood combs and transferred to 48-well culture plates. (II) *In vitro* rearing was carried out according to Aupinel *et al.* [22]. The standard diet during the larval feeding period was supplemented or not with 0, 5, 50, or 500 µM abscisic acid (ABA). (III) Four-day-old larvae were treated as follows: a group of individuals was continuously grown at the control temperature (34°C) while another group was cold stressed (25°C). (IV) After 3 days the group kept at 25°C was returned to the control temperature (34°C) until adult emergence. (V) The number of newly emerged workers (NEW) and the developmental timing was registered for each growing condition. (Online version in colour.)

supplementary material, figure S1*a* shows that, between 5 and 500 µM, ABA does not alter the FW of honeybees at any developmental stage (Kruskal–Wallis test, 4 days old: $H = 4.894$, d.f. $= 3$, $p = 0.18$; 6 days old: $H = 4.522$, d.f. $= 3$, $p = 0.21$; 8 days old: $H = 5.78$, d.f. $= 3$, $p = 0.09$; NEW: $H = 6.295$, d.f. $= 3$, $p = 0.098$). We also determined that ABA content was 1.29 ± 0.43 and 0.082 ± 0.017 µg g^{-1} FW (mean ± s.e.) in 4-day-old larvae and NEW, respectively, when the diet was supplemented with 50 µM ABA. This represents a significant increase of 35 and 9 times in 4-day-old larvae and NEW, respectively, compared to larvae fed without ABA (Student's *t*-test, 4-day-old: $t = -2.921$, d.f. $= 10$, $p = 0.015$; NEW: $t = -4.569$, d.f. $= 11$, $p < 0.001$). High concentrations of ABA do not cause any adverse consequences to honeybee health.

Larvae FW significantly diminished in 6- and 8-day-old individuals exposed to cold stress, compared to individuals grown at 34°C (one-way ANOVA, 6 days old: $F_{3,170} = 190.761$, $p < 0.001$; Kruskal–Wallis test, 8 days old: $H = 63.727$, d.f. $= 3$, $p < 0.001$; electronic supplementary material, figure S1*b*). However, individuals exposed to cold stress for 3 days that emerged as adults (NEW) showed a similar FW to those that were grown continuously at 34°C (one-way ANOVA, $F_{3,96} = 0.933$, $p = 0.428$; electronic supplementary material, figure S1*b*), indicating that after returning to the standard temperature, the larvae were able to recover. We observed that 4-day-old larvae transferred to 25°C did not eat or did so slowly, which correlates with the lower gain of weight detected in 6- and 8-day-old larvae (electronic supplementary material, figure S1*b*). This was also confirmed by the accumulation of food in the wells of the plate of cold-stressed larvae. The survival rate and developmental timing was then studied to understand the consequences of the altered weight gain of larvae under cold stress.

(c) Abscisic acid prevents the decrease in the survival rate and recovers partially the delayed development of cold-stressed larvae

Survival rate is an indicator broadly used to study the impact of cold stress in insects [26]. Figure 3*a* shows that the exposure of honeybee larvae to 25°C for 3 days diminished the survival rate, compared to individuals reared at the standard temperature (34°C) (Student's *t*-test, $t = 2.967$, d.f. $= 6$, $p = 0.025$). Figure 3*a* also shows that the survival rate is 60.6% at 34°C, while cold stress decreases it to 35.7%. However, when the diet was supplemented with 50 µM ABA, the survival rate of cold-stressed individuals reaches a value of 57.4%, which is close to the rate of non-stressed individuals (Student's *t*-test, $t = -3.96$, d.f. $= 4$, $p = 0.017$). In addition, ABA supplementation in the diet has no effect on the survival rate of non-stressed individuals (Student's *t*-test, $t = -1.15$, d.f. $= 8$, $p = 0.283$, figure 3*a*).

Figure 3*b* demonstrates that while individuals growing at 34°C reach adult emergence after 21.5 ± 1.105 days (mean ± s.e.), cold-stressed individuals emerged after 24.8 ± 0.422 days (mean ± s.e.), showing a significant developmental delay (Mann–Whitney test: $U = 1$, $p < 0.001$). Supplementation with ABA accelerated the emergence of cold-stressed individuals, which achieved this phase after 23.7 ± 0.92 days (mean ± s.e.) (Mann–Whitney test: $U = 32$, $p = 0.004$; figure 3*b*). Again, the supplementation with ABA had no significant effect on the development of individuals growing in standard conditions (Mann–Whitney test: $U = 522.5$, $p = 0.262$; figure 3*b*). Figure 3*c* shows representative pictures of individuals at different developmental times growing either in standard or cold stress conditions. Cold-stressed 6-day-old larvae are smaller than non-stressed ones and this observation correlates with their lower FW (electronic supplementary material, figure S1*b*). In addition, 11-day-old individuals grown at standard conditions were found at the pupae developmental stage while cold-stressed individuals remained at the pre-pupae stage (figure 3*c*). Individuals reared at standard temperatures started emergence as adults after 20 days, while cold-stressed individuals were found still at pupae stages (figure 3*c*). Figure 3*d* shows that cold-stressed 17-day-old individuals displayed pink eyes and non-pigmented body while the ABA-supplemented individuals presented purple eyes and light brown pigmented body, indicating that ABA accelerates development.

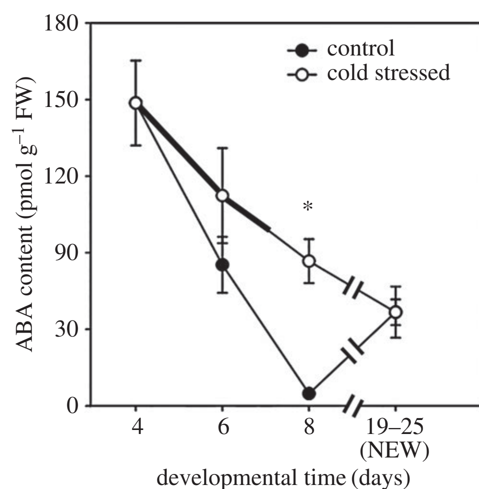

Figure 2. Cold-stressed honeybee larvae reduces ABA content at a slow rate. Four-day-old larvae reared *in vitro* were continuously grown at control conditions (34°C) or exposed to cold stress (25°C) for 3 days and then returned to standard conditions (see figure 1). The wide black line indicates the period of cold stress exposure. A competitive ELISA was employed for the quantitative determination of ABA (Phytodetek ABA Test Kit, AGDIA). *Significant difference for a $p = 0.006$ (Mann–Whitney test). FW: Fresh weight. NEW: Newly emerged workers.

(d) Abscisic acid regulates the expression of genes associated with metabolic adjustments and stress responses

Since ABA supplementation has an impact on the survival rate and the development of cold-stressed honeybees, we studied the effect of ABA on the expression of genes involved in metabolic, signalling and stress protection processes. The effect of cold stress and ABA supplementation was studied during and after the period of stress. Two time points were chosen to analyse changes in gene expression: 2 days of cold stress (6-day-old larvae) and 1 day after the stress (8-day-old individuals) (see figure 1). First, we determined the transcript accumulation of genes associated with metabolic regulations: *Hexamerin 70b* (*Hex70b*), *Cytochrome C* (*CytC*), *Insulin Receptor Substrate* (*IRS*), and *Target of Rapamycin* (*TOR*) [27–29]. Figure 4*a* shows that *Hex70b* transcript levels significantly increased in cold-stressed 8-day-old individuals when compared to control individuals, and that this increase was higher in ABA-supplemented larvae (two-way ANOVA, $N_{34°C,Control} = 11$, $N_{34°C,ABA} = 9$, $N_{25°C,Control} = 6$, $N_{25°C,ABA} = 6$, temperature: $F_{1,20} = 144.19$, $p < 0.001$; treatment: $F_{1,20} = 21.756$, $p < 0.001$; temperature x treatment: $F_{1,20} = 21.877$, $p < 0.001$). At the standard temperature (34°C), the highest *Hex70b* transcript level was found in larval stages (electronic supplementary material, figure S2). In contrast, the levels of *CytC* transcript were low in larvae and pupae and notably higher in NEW (electronic supplementary material, figure S2). Figure 4*b* shows that *CytC* transcripts significantly increased in 6- and 8-day-old cold-stressed individuals, compared to individuals reared at a standard temperature (two-way ANOVA, 6 days old: $N_{34°C,Control} = 12$, $N_{25°C,Control} = 6$, $F_{1,32} = 27.295$, $p < 0.001$; 8 days old: $N_{34°C,Control} = 12$, $N_{25°C,Control} = 6$, $F_{1,\ 31} = 92.194$, $p < 0.001$). ABA supplementation itself had no effect on *CytC* expression (two-way ANOVA, 6 days old: $N_{34°C,ABA} = 12$, $N_{25°C,ABA} = 6$, $F_{1,32} = 0.891$, $p = 0.352$; 8 days old: $N_{34°C,ABA} = 11$, $N_{25°C,ABA} = 6$, $F_{1,31} = 0.492$, $p = 0.488$).

Figure 4*c*,*d* show that *IRS* and *TOR* transcripts were higher in 6-day-old cold-stressed individuals compared to control ones (two-way ANOVA, IRS: $N_{34°C,Control} = 8$, $N_{25°C,Control} = 9$, $F_{1,34} = 14.356$, $p < 0.001$; TOR: $N_{34°C,Control} = 12$, $N_{25°C,Control} = 6$, $F_{1,35} = 28.496$, $p < 0.001$), suggesting a role for these genes in the response to low temperatures. At this time point, ABA supplementation slightly reduced the effect of the stress on the expression of *IRS* transcript (Kruskal–Wallis test, $N_{34°C,Control} = 8$, $N_{34°C,ABA} = 9$, $N_{25°C,Control} = 9$, $N_{25°C,ABA} = 9$, $H = 18.003$, d.f. $= 3$, $p < 0.001$; figure 4*c*).

Secondly, we studied the expression of a gene set associated with stress responses: *Vitellogenin* (*Vg*), *Mn-Superoxide Dismutase* (*MnSOD*), *Heat-shock 70* (*HSP70*), and *Heat-shock 90* (*HSP90*) [26,30,31]. Figure 5*a* shows that *Vg* transcript levels significantly increased in 6- and 8-day-old individuals exposed to cold stress, compared with individuals grown at the control temperature (two-way ANOVA, 6 days old: $N_{34°C,Control} = 9$, $N_{25°C,Control} = 9$, $F_{1,26} = 6.433$, $p = 0.018$; 8 days old: $N_{34°C,Control} = 6$, $N_{25°C,Control} = 6$, $F_{1,22} = 6.183$, $p = 0.022$). Interestingly, ABA supplementation induced the expression of *Vg* in 6-day-old individuals, growing at either control or low temperatures (Kruskal–Wallis test, $N_{34°C,Control} = 9$, $N_{34°C,ABA} = 9$, $N_{25°C,Control} = 9$, $N_{25°C,ABA} = 9$, $H = 11.23$, d.f. $= 3$, $p = 0.011$; figure 5*a*). Figure 5*a* also indicates that *Vg* expression was maintained high in 8-day-old individuals that returned to standard temperature, independently of ABA supplementation (two-way ANOVA, $N_{34°C,Control} = 6$, $N_{34°C,ABA} = 6$, $N_{25°C,Control} = 6$, $N_{25°C,ABA} = 6$, temperature: $F_{1,22} = 6.183$, $p = 0.022$; treatment: $F_{1,22} = 0.365$, $p = 0.561$). The levels of *Vg* transcript are particularly low during developmental stages previous to adult emergence (electronic supplementary material, figure S2).

The expression of *MnSOD* and *HSP90* was also high in 8-day-old individuals exposed to cold stress that were returned to the control temperature (two-way ANOVA, *MnSOD*: $N_{34°C,Control} = 6$, $N_{25°C,Control} = 6$, $F_{1,20} = 13.358$, $p = 0.002$; figure 5*b*, and *HSP90*: $N_{34°C,Control} = 6$, $N_{25°C,Control} = 6$, $F_{1,20} = 8.037$, $p = 0.01$; figure 5*d*). In contrast, the level of *HSP70* transcripts increased in 6-day-old cold-stressed individuals and those growing at 34°C that were ABA-supplemented (Kruskal–Wallis test, $N_{34°C,Control} = 6$, $N_{34°C,ABA} = 6$, $N_{25°C,Control} = 6$, $N_{25°C,ABA} = 6$, $H = 10$, d.f. $= 3$, $p = 0.019$; figure 5*c*). In 8-day-old individuals that were either growing at 34°C or submitted to cold stress, ABA supplementation induced a decrease of *HSP70* transcript levels, suggesting that ABA-supplemented individuals might be sensing a less severe stress (two-way ANOVA, $N_{34°C,Control} = 6$, $N_{34°C,ABA} = 6$, $N_{25°C,Control} = 6$, $N_{25°C,ABA} = 6$, temperature: $F_{1,\ 20} = 1.016$, $p = 0.326$; treatment: $F_{1,\ 20} = 4.906$, $p = 0.039$; figure 5*c*).

Lanthionine synthetase C-like protein (LANCL2) has been established as a G protein-coupled peripheral membrane protein and a key component of the ABA signal transduction pathway in mammalian cells [32,33]. Sequence alignment of human and *A. mellifera* LANCL2 shows 41.3% amino acid identity and 71% similarity (electronic supplementary material, figure S3). Electronic supplementary material, figure S4*a*, shows a specific and saturated binding of $[^3H]$ABA to recombinant AmLANCL2, with an affinity similar to that observed with human LANCL2. Protein purity was

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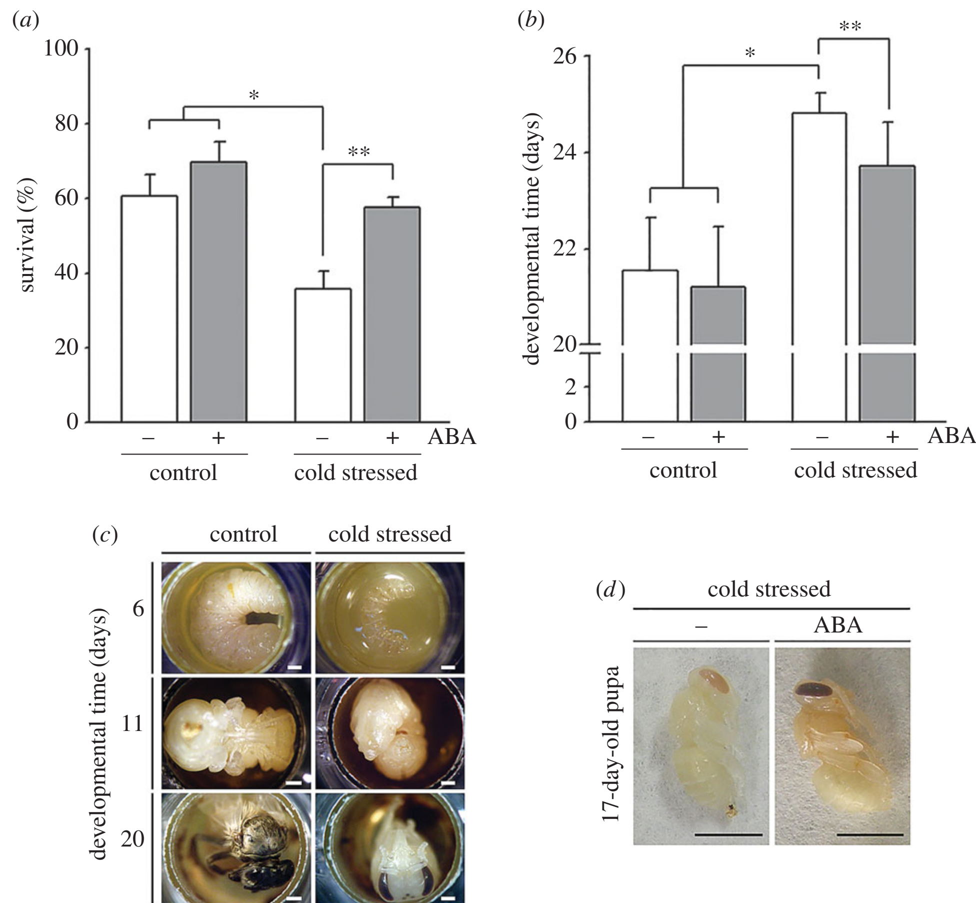

Figure 3. ABA increases the survival rate and accelerates the developmental timing of honeybee larvae exposed to cold stress. Four-day-old larvae *in vitro* reared were grown at control conditions (34°C) or exposed to cold stress (25°C) for 3 days. Larvae were fed with a standard diet supplemented or not with 50 μM ABA (see figure 1). (*a*) Survival rates were estimated as the percentage of newly emerged workers (NEW) that survived from an initial number of L1 larvae included in each experimental condition. *Significant differences for a $p = 0.025$ (Student's *t*-test). **Significant differences for a $p = 0.017$ (Student's *t*-test). (*b*) Developmental time (days) required to reach adult emergence (NEW). *Significant differences for a $p < 0.001$ (Mann–Whitney test). **Significant differences for a $p = 0.004$ (Mann–Whitney test). (*c*) Representative images of honeybee individuals grown at control conditions (34°C) or exposed to cold stress (25°C). Images were taken at 6, 11, and 20 days of development. White bar: 0.1 cm. (*d*) Representative images of 17-day-old pupae exposed to cold stress and supplemented or not with 50 μM ABA. Scale bar, 0.5 cm.

determined by SDS-PAGE analysis (electronic supplementary material, figure S4*b*). The expression of *AmLANCL2* presents slight changes between larvae to pupae stages with a significant increase in NEW (electronic supplementary material, figure S2). Figure 6 shows that *AmLANCL2* transcript levels significantly increased in 6-day-old cold-stressed individuals (one-way ANOVA, $N_{34^{\circ}\mathrm{C,Control}} = 12$, $N_{34^{\circ}\mathrm{C,ABA}} = 12$, $N_{25^{\circ}\mathrm{C,Control}} = 6$, $N_{25^{\circ}\mathrm{C,ABA}} = 6$, $F_{3,32} = 18.881$, $p < 0.001$), while ABA supplementation slightly reduced the transcript level of *AmLANCL2*. Actin gene expression used to normalize the expression of the studied genes is not altered by treatments (electronic supplementary material, figure S5).

4. Discussion

Phytochemicals present in the natural bee diet impact on the physiology, behaviour, and health of honeybees. In this work, we report that ABA is present in honeybees and its concentration is kept high in cold-stressed individuals. Moreover, endogenous ABA concentration varies along the different developmental stages of the honeybee. From figure 2 and electronic supplementary material, figure S1*b*, the concentration of ABA is calculated as 0.59 and 3.81 *p*moles ABA individual^{-1} at 8 days old and NEW, respectively. Because individuals are not fed between these two developmental stages, it is postulated that the sevenfold increase of ABA content observed in NEW is a consequence of genuine endogenous ABA synthesis, as was already discussed in Negri *et al.* [15]. More interestingly, the endogenous ABA concentrations found in honeybees are in the nanomolar range, the same concentration found in human granulocytes and macrophages, where ABA levels also increase under stress situations [18,34].

The harmful effects of cold stress in insects, and particularly in honeybees, change with developmental stages, temperature, and time of exposure [12,13,25,35]. Under chronic or severe cold exposure insects experience loss of neuromuscular function, caused by decreased membrane potential and reduced excitability of the neuromuscular system, decreased membrane fluidity, disruption of ion and water homeostasis across membranes, impairment of cellular metabolism, depletion of cellular ATP, protein denaturation and ROS, among others. Several molecular and physiological mechanisms have been identified to confront chill injuries [35,36]. Here, we describe that an ABA dietary supplement augments the survival rate of cold-stressed larvae (25°C for 3 days) at levels of individuals reared at standard

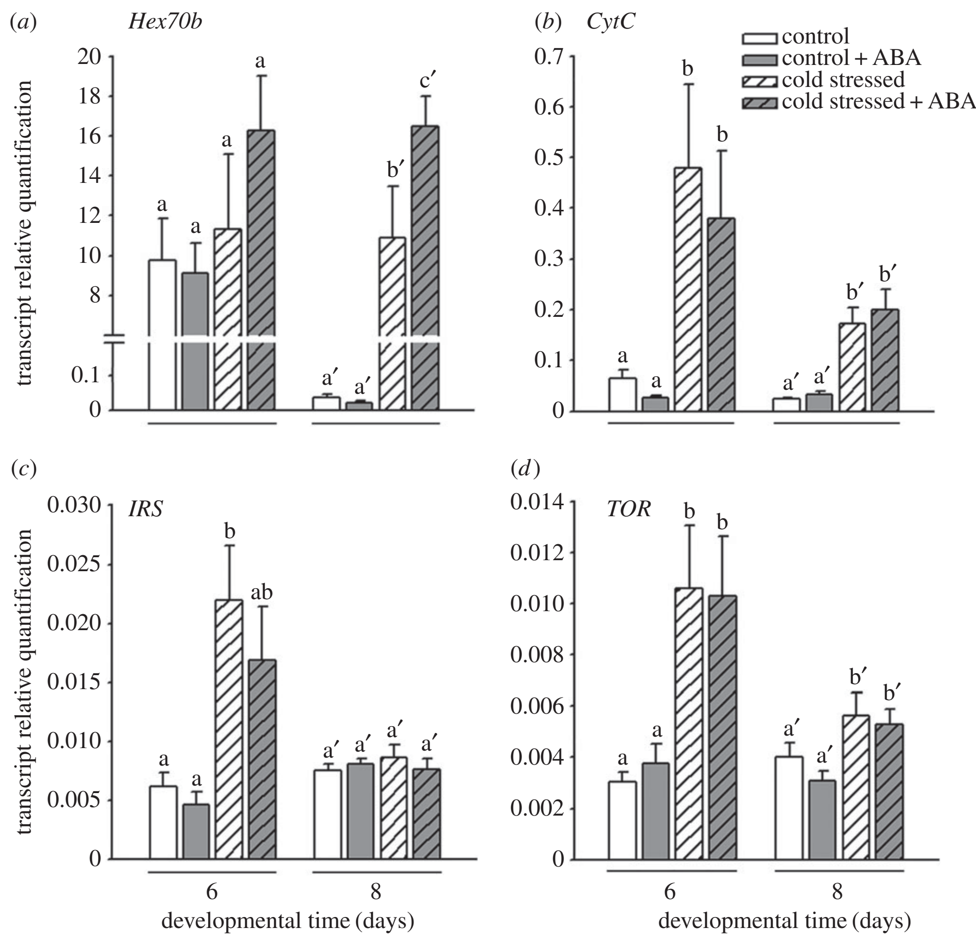

Figure 4. Cold stress and ABA modulate the transcript level of genes associated with metabolic and signalling pathways in honeybees. Four-day-old larvae *in vitro* reared were grown at control conditions (34°C) or exposed to cold stress (25°C) for 3 days. Larvae were fed with a standard diet supplemented or not with 50 μM ABA (see figure 1). qPCR analyses were used for quantifying the expression of: (*a*) *Hexamerin 70b* (*Hex70b*), (*b*) *Cytochrome C* (*CytC*), (*c*) *Insulin receptor substrate* (*IRS*), and (*d*) *Target of rapamycin* (*TOR*). The qPCR analysis was performed in 6-day-old larvae (2 days of cold stress) and in 8-day-old individuals (1 day after the cold stress). Expression of *Actin* was used for normalization. Statistical analysis for Hex70b, CytC, and TOR: two-way ANOVA with *post hoc* comparisons by the Holm-Sidak multiple comparison test; IRS: Kruskal–Wallis test with *post hoc* comparisons by the Dunn's multiple comparison test. Different letters indicate transcript levels significantly different for a $p < 0.05$.

temperature conditions (34°C) (figure 2*a*). ABA also accelerates the development of individuals exposed to low temperatures, allowing them to complete metamorphosis in less time (figure 3*b* and electronic supplementary material, figure S1*b*).

In animals, compensatory growth is related to the rapid recovery when organisms accelerate their growth to catch up after a period of low growth [37,38]. In this work, we show that L4 larvae exposed to cold stress emerged as adults with the same FW as individuals grown at 34°C, but they required 3.5 more days to reach emergence. In contrast, cold-stressed individuals that were ABA-supplemented showed an accelerated growth compared to non ABA-supplemented, as they attained the same FW in a shorter time (figure 3*b*). This may imply a putative function for ABA in activating mechanisms related to compensatory growth in honeybee.

The high ABA levels and delayed development observed in cold-stressed honeybee larvae is reminiscent of what is found in plant seeds. ABA helps seeds to overcome the stress conditions and to germinate only when the environmental parameters are conducive for germination and growth [39]. In honeybees, like in plants, ABA could act as a signal to adjust the metabolic rate until the growing conditions become favourable. To study this possibility, the expression of genes associated with metabolic adjustments and stress responses was analysed. Hexamerins (Hex) are amino acid storage proteins that are upregulated in the feeding stage of larvae, when they gain weight before entering metamorphosis. We demonstrate that 8-day-old individuals that were exposed to cold stress retained high levels of *Hex70b* transcript compared with individuals kept at the standard temperature (figure 4*a*). ABA supplementation slightly increased *Hex70b* transcript accumulation in 8-day-old individuals that were cold stressed, which could represent an increase in nutrients stored for accelerating growing processes in those individuals.

CytC is an electron carrier for mitochondrial complexes III and IV. Cold-stressed individuals present *CytC* transcript accumulation. It was reported that *CytC* expression is induced under elevated metabolic rates occurring, for example, during the queen development [28]. The cold-induced increase of *CytC* transcript level observed in this study indicates a raise in the mitochondrial metabolic rate and/or an increase of the mitochondrial number per cell in cold-stressed larvae. This might be associated with an effort to increase the metabolic heat production in cold-stressed individuals to protect themselves from temporary low temperatures.

IRS and TOR are central components of a conserved signalling pathway that regulates cell and organism growth in

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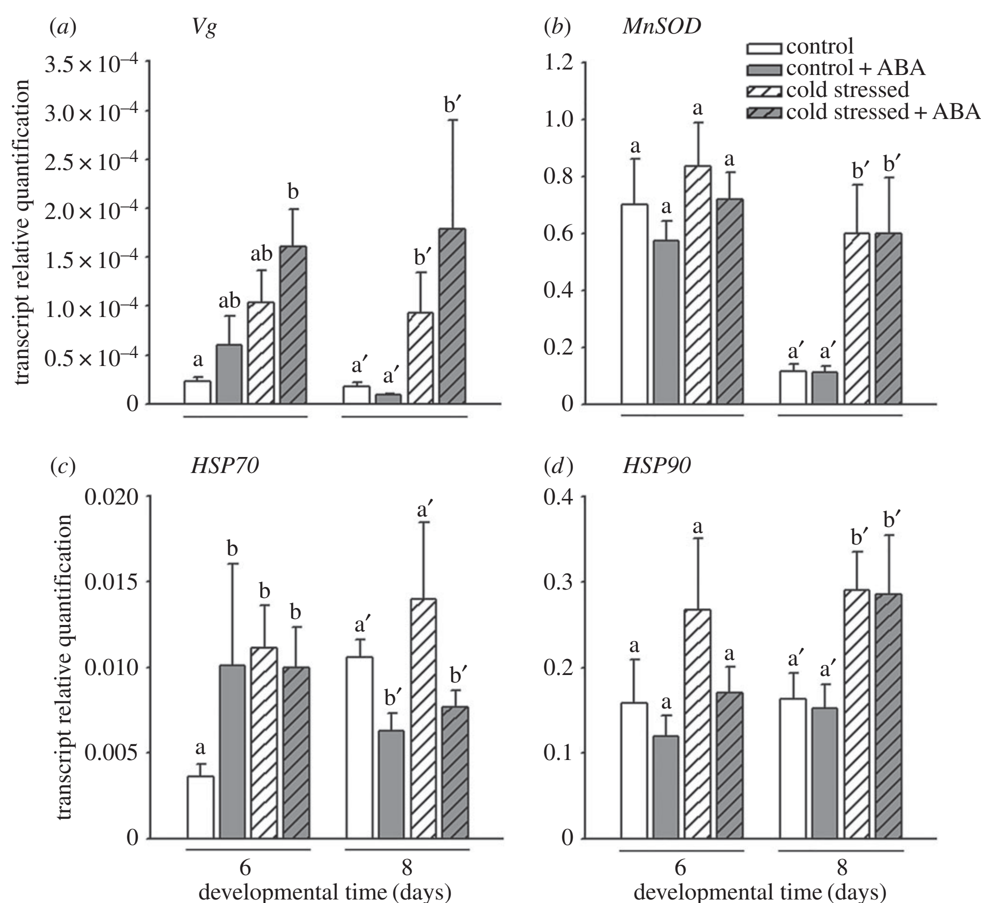

Figure 5. Cold stress and ABA modulate the expression of genes involved in stress responses in honeybees. Four-day-old larvae *in vitro* reared were grown at control conditions (34°C) or exposed to cold stress (25°C) for 3 days. Larvae were fed with a standard diet supplemented or not with 50 μM ABA (see figure 1). qPCR analyses were used for quantifying the transcript level of: (*a*) *Vitellogenin* (*Vg*), (*b*) *Mn-Superoxide dismutase* (*MnSOD*), (*c*) *Heat shock protein 70* (*HSP70*), and (*d*) *Heat shock protein 90* (*HSP90*), as described in figure 4. Statistical analysis: two-way ANOVA with *post hoc* comparisons by the Holm-Sidak multiple comparison test. Different letters indicate transcript levels significantly different for a $p < 0.05$.

response to nutrient status [29]. Given that *IRS* and *TOR* transcript accumulation is high in cold-stressed 6-day-old individuals, it is suggested their involvement in metabolic adjustments is associated with early responses to lower food intake [40]. It was reported that the ablation of the insulin-like peptide-producers cells in the brain of *Drosophila melanogaster,* leads to reduced tolerance of heat and cold [41]. The insulin–insulin like signalling (IIS) associated with the TOR branch has been linked to increased resistance to some types of stress [42]. Thus, ABA could positively influence the honeybee metabolism through the IIS pathway involving IRS.

In honeybees, Vitellogenin (Vg) is thought to play a central role in the extended lifespan of the winter bees [43]. *Vg* transcript levels are upregulated in 6- and 8-day-old individuals exposed to cold stress (figure 5*a*), suggesting that Vg could be playing a protective role or mediating the recognition of damaged cells as previously shown [30,44]. Moreover, ABA supplementation resulted in a higher induction of *Vg* expression in cold-stressed larvae (figure 5*a*), indicating enhanced Vg-mediated protection.

In insects, low temperatures induce the expression of genes of the antioxidant system and genes related with the heat shock response [26,31,45]. *MnSOD* and *HSP90* transcripts accumulation were increased in cold-stressed 8-day-old individuals, as expected (figure 5*b–d*). Recently, Kharenko *et al.* [46] reported the interaction between ABA and human HSP70 family members. In that paper, authors highlight the implications of ABA-HSP70 interactions to the intracellular protein folding activities. Given that *HSP70* expression is increased in ABA-supplemented 6-day-old larvae reared at 34°C, it would be interesting to explore whether the interaction of HSP70 and ABA is conserved in honeybees.

Vg and *HSP70* expression were induced by ABA supplementation in 6-day-old individuals growing at 34°C, reaching similar levels to cold-stressed larvae. This indicates that ABA could have a 'priming' effect in individuals that might result in a stronger and faster response to confront low temperatures. Insects are capable of cold-hardening; a brief exposure to nonlethal low temperatures significantly enhances the tolerance to a subsequent cold shock [35]. ABA treatment could act as a stimulus for cold-hardening triggering physiological changes driving cold tolerance processes. This is a very interesting point that deserves further studies.

At 8 days old, ABA decreases *HSP70* expression in both control and cold-stressed larvae, suggesting a tight control of ABA on *HSP70* gene expression. Nevertheless, the analysis of protein abundance and/or protein activity should reinforce our study. Štětina *et al.* [47] showed that different low-temperature treatments induce the accumulation of *Hsp70* mRNA in wild-type larvae of *Drosophila melanogaster*. However, the Hsp70-null mutant lacking all six copies of *Hsp70* genes only showed a detrimental effect on survival in the case of severe acute cold shock [47]. Even if ROS

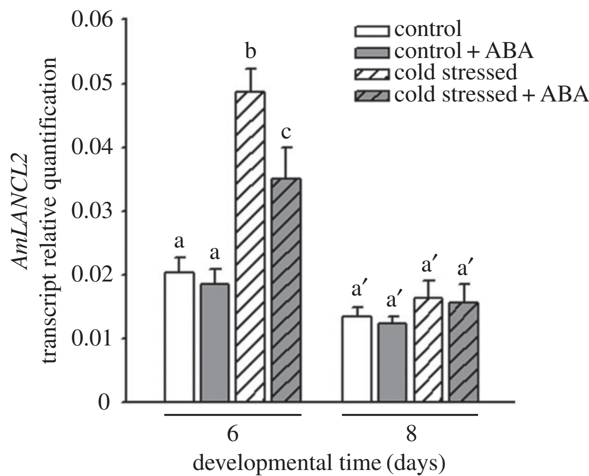

Figure 6. Cold stress induces the expression of the ABA receptor LANCL2 in honeybee larvae. Four-day-old *in vitro* reared larvae were grown at control conditions (34°C) or exposed to cold stress (25°C) for 3 days. Larvae were fed with a standard diet supplemented or not with 50 µM ABA (see figure 1). qPCR analysis was performed as described in figure 4. Different letters indicate transcript levels significantly different for a $p < 0.05$ (one-way ANOVA test with *post hoc* comparisons by the Holm-Sidak multiple comparison test).

increase is a common consequence of cold stress exposure no oxidative stress markers were found in cold-stressed *Drosophila suzukii*, suggesting that either only well protected individuals survive or that oxidative balance is not involved in the cold response in this species [48]. At the organizational level, approaches at sub-organismal scale may also help to determine the role of ABA in cold tolerance in honeybees, in a way that is not attained by whole-body measurements. Since the analyses of transcriptome, proteome, and metabolome have become a core pillar to scan the molecular changes during stress responses in insects [49], such approaches would help our study providing a whole panorama of responses activated by ABA in cold-stressed honeybee larvae.

Some components of the mechanism underlying ABA actions in mammals [32] have been identified in honeybees. We demonstrated that recombinant AmLANCL2 binds ABA and that endogenous ABA content increases in cold-stressed larvae, thereby suggesting that ABA, ABA receptor, and ABA signalling components could be regulating responses to low temperatures in honeybees. Calcium signalling has been established as an essential cold-sensing mechanism mediating rapid responses in insects [50]. Because in plants and animals it has been demonstrated that second messenger calcium takes part in ABA-mediated signal transduction [17,51], it's hypothesized that calcium could also be implicated in ABA-mediated responses to cold stress in honeybees.

Previous results have suggested that honeybees receive nutritional components from honey that are not provided by alternative food sources widely used in apiculture [52]. ABA is a natural constituent of honey and due to its beneficial effects on the individual and colony fitness, beekeepers might consider supplementing alternative foods with ABA when they harvest the honey. ABA could be acting as a multitask compound through the activation of different metabolic pathways, enhancing the immunological response and protecting against stresses like low temperatures.

Data accessibility. Data supporting the results are available in the electronic supplementary material.

Authors' contributions. Experiments were designed by L.R. and L.L. and conducted by L.R., P.N., L.S., L.G., and T.V. The manuscript was prepared and written by L.R. and L.L., and revised and discussed by all the authors. The design and direction of the project were performed by L.L.

Competing interest. We declare we have no competing interests.

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