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Morphological and biochemical responses of a neotropical pest insect to low temperatures

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ABSTRACT

As traditionally cold areas become warmer due to climate change, temperature could no longer be a barrier to the establishment of non-native insects. This is particularly relevant for pest insects from warm and tropical areas, mainly those with some tolerance to moderately low temperatures, which could expand their range into these new locations. From this perspective, in this work we studied the morphological and biochemical responses of the Neotropical pest *Paysandisia archon* to low temperatures, as part of a possible strategy to colonize new areas. To that end, wild larvae were exposed for 7 days to either low (1 and 5 °C) or ambient (23 °C) temperatures. We then quantified the inner and outer morphological changes, by X-Ray Computer Tomography and Digital Holographic Microscopy, as well as the accumulation of metabolites acting as potential endogenous cryoprotectants, by Spectrophotometry. We found that *Paysandisia archon* developed a cold-induced response based on different aspects. On the one hand, morphological changes occurred with a significant reduction both in fluids susceptible to freezing and fat body, together with the thickening, hardening and increased roughness of the integument. On the other hand, we found an increase in the hemolymph concentration of cryoprotective substances such as glucose (6-fold) and glycerol (2-fold), while trehalose remained unchanged. Surprisingly, this species did not show any evidence of cold-induced response unless the environmental temperature was remarkably low (1 °C). These results could be useful to improve models predicting the possible spread of such a pest, which should incorporate parameters related to its resistance to low temperatures.

1. Introduction

Thermal stress has a major impact on insect distribution (Andrew et al., 2013). Indeed, low environmental temperatures disrupt the homeostasis of insects (Harrison et al., 2013), threatening their survival and acting as a barrier that limits their spread. Global warming is another relevant factor also driving the redistribution of insects (Kellermann and Van Heerwaarden, 2019; Daly et al., 2023). However, the effects of warming on cold areas have been poorly studied from the perspective of colonization by new insects. As traditionally cold areas become warmer due to climate change (Rind, 1986), temperature could no longer be a barrier to the invasion of these new places by some insects. Such a consequence of climate change could have economic and environmental effects that are especially relevant in the case of pest insects. In this context, global warming could lead pest insects native to warm and tropical areas, particularly those with a certain tolerance to periods of moderately low temperatures, to expand their range toward these new locations.

The Neotropical palm borer *Paysandisia archon* (Lepidoptera: Castniidae) was introduced into Europe and several areas of Asia through international trade of infested palm trees in the 1990s (Muñoz-Adalia and Colinas, 2020), and soon became one of the worst palm pest insects in these non-native areas. Its detection is difficult because it remains inside the palm tree during most of its life cycle, in larval stages that usually cover 9 instars (occasionally 8 or 7 instars). Larvae are endophagous and feed inside the stem of numerous species of palm trees,

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causing severe damage by the galleries they bore, as well as by secondary infections (Sarto i Monteys and Aguilar, 2005). Some places of great economic and cultural interest are currently threatened by this pest. This is the case, among others, of the natural population of dwarf palm (*Chamaerops humilis*), the only native palm tree in the western Mediterranean region (Guzmán et al., 2017), or the palm grove of Elche (Spain), a UNESCO World Heritage Site.

Several aspects on the physiology of *Paysandisia archon* remain to be studied, probably due in part to its long life cycle, which can be annual or biennial depending on the moment the laying is carried out. In particular, little is known about its physiological responses to low temperatures, as part of a possible strategy to colonize new areas. In this regard, insects have developed many successful and varied strategies to survive during adverse cold periods, including behavioral, bioenergetics, morphological or biochemical adaptations (Lencioni, 2004; Colinet et al., 2017; Noer et al., 2022). Each species develops its own combination of survival strategies and, therefore, requires individual study (Denlinger and Lee, 2010). Possible morphological and physiological changes induced by low temperatures include reduction in body size, decreased water content, melanism, loss or reduction of wings in adults, or even an almost complete arrest of development (Block, 1990). Regarding the biochemical response to cold, one of the main mechanisms that allow insects to survive at low temperatures is the accumulation of low molecular weight substances (Storey and Storey, 1988; Toxopeus and Sinclair, 2018), mainly polyols (e.g., glycerol), sugars (e.g., trehalose and glucose) and free amino acids (e.g., proline). These substances have several cryoprotective functions: they stabilize proteins and cell membrane structure, act as water replacement molecules, and lower the freezing point of fluids to reduce potential damage from ice crystal formation (Worland et al., 2010; Sømme, 2011; Toxopeus and Sinclair, 2018).

In this work, we studied the physiological response of the Neotropical lepidopteran pest *Paysandisia archon* to sudden low temperatures, from both a morphological and a biochemical approach. To that end, we measured morphological changes through a combination of two modern imaging techniques. The inner morphology was studied by means of X-Ray Computer Tomography (X-Ray CT), while the outer features was analyzed through Digital Holographic Microscopy (DHM). Until recently, research on the inner morphology of insects required dissection and other destructive procedures. Now, X-ray CT allows non-destructive morphological studies of a whole animal. In addition, it provides an accurate measurement of the volume of different tissues and components, including fluids like hemolymph (Smith et al., 2016; León-Quinto et al., 2020). On the other hand, DHM allows the precise reconstruction of the 3D structure of the cuticle, a relevant structure that protects insects from adverse environmental conditions and infections of microorganism (Liao et al., 2018). Indeed, as it is the outermost layer of the integumentary system, it is directly exposed to the environment and therefore its structure could be affected by environmental changes. An approach to measure possible cold-induced changes on the cuticle is by quantifying its surface roughness through DHM (Kim, 2011; León-Quinto et al., 2020). Finally, we studied the biochemical response to cold by quantifying the levels in hemolymph of glycerol, trehalose and glucose. These substances have previously been identified in the literature as potential endogenous cryoprotectants in tropical insects (Chowanski et al., 2015, 2017; León-Quinto and Serna, 2022) and Lepidoptera (Zeng et al., 2008; Vrba et al., 2017; Kelleher et al., 1987; Goto et al., 2001; Hou et al., 2009; Vatanparast and Park, 2022).

Knowledge of the physiological mechanisms underlying the response of *Paysandisia archon* to low temperatures could be relevant to implement more effective control programs for this pest. In particular, in a context of climate change, this information could be useful to improve distribution models and prevent its spread towards new regions that are becoming less adverse due to global warming.

2. Materials and methods

2.1. Insect groups

Wild larvae were collected during three years (2021–2023) in the palm grove of Elche, Spain, by the staff of the public company TRAGSA, which deals with the maintenance of this site. The collection of larvae was carried out in spring and autumn, seasons where the average temperature for the periphery of the palm trees in this area of Spain is about 23 °C (Dembilio and Jacas, 2011). For the present study, we selected only larvae in the latest instar, the most exposed in wild conditions to environmental temperature. Indeed, early-instar larvae remain thermally protected inside the palm trunk, while those in the latest one approach the periphery to build the cocoon and then become vulnerable to external temperature conditions (Muñoz-Adalia and Colinas, 2020). Since each infested palm tree generally had at most one or two last-instar larvae, the animals used in our study basically come from different palm trees. Nevertheless, since all the larvae came from the same palm grove and from the same tree species (*Phoenix dactylifera*), their rearing conditions were very homogeneous.

The selected animals were haphazardly distributed into three groups, with the only requirement that all groups had the same number of larvae for each season. A group of animals was placed for seven days in a climatic chamber under conditions of moderately low temperature, 5.0 ± 0.3 °C, while a second group was exposed for the same time to a temperature of 1.0 ± 0.3 °C, remarkably low for a Neotropical insect. The remaining larvae were used as a control group. They were placed for seven days in a room with a controlled temperature of 23 ± 1 °C, the average value mentioned above for the periphery of palm trees in spring and autumn. The relative humidity was the same for the three groups, 65 % ± 5 %. Since cold-treated larvae stop feeding, we did not provide food to the control insects. In this way, temperature was the main environmental difference between the three groups of animals.

In a first phase (autumn 2021-spring 2022), larvae were used for studies with X-Ray CT. Since this technique is non-invasive, these same animals were fed and monitored during the week following the end of the cold treatment, in order to estimate their survival rates. In a second phase (autumn 2022-spring 2023), new larvae were used to extract hemolymph for biochemical studies. The larvae for DHM studies were all collected in spring 2023 (some of them were also used for biochemical studies along with new ones). Note that the effect of the collection year is already included when analyzing the collection season, as autumn and spring correspond to different years for each type of study.

2.2. X-Ray Computer Tomography

To investigate possible cold-induced effects on the inner morphology, we scanned 45 larvae (15 per temperature group) with X-Ray CT, a non-invasive imaging tool based on X-ray absorption. Scanning was carried out with the Albira Si system (Bruker Corporation, Karksruhe, Germany) using its high resolution setting (45 keV and 0.4 mA), which provides for each animal 1000 slices of 70 × 70 mm^2 and an effective spatial resolution of 85 μm. The X-Ray CT technique and the characteristics of the Albira Si system were previously described in the literature (e.g., Sánchez et al., 2013).

In order to visualize and select a volume containing the entire scanned individual, we used different medical data applications: ParaView (Kitware, USA) and AMIDE (UCLA University, USA). This kind of software also divides the selected volume into a large number of small volume units called voxels, as many as the system resolution allows, and exports their numerical data. These numerical data provide, for each voxel, its tomographic density in Hounsfield units (HU). By analyzing the distribution of these densities, we identified the main morphological components: inner air, hemolymph, low- and high-density tissues. Following to the procedure described by León-Quinto et al. (2020), we

then quantified the volume of each component by integrating the number of voxels over its corresponding density range.

2.3. Digital Holographic Microscopy

The possible cold-induced changes in the outer morphology of larvae were analyzed through DHM. This is an interferometric technique that provides a fast measurement of the 3D structure of an object's surface (Rappaz et al., 2005; Kim, 2011). For DHM measurements, we took integument samples from 45 anesthetized larvae (15 per temperature group). We then captured their holographic images, 2–3 for each individual, with a DHM-R2100 holographic microscope (Lyncée Tec, Lausanne, Switzerland) and its associated Koala software (Cuche et al., 2009). We used a lens 20*x* and wavelength of $\lambda = 684.9$ nm. This setup provides 3D images that, in the plane of view, covered an area of 330 × 330 μm^2 with a resolution of 0.45 μm. Perpendicular to this plane, the resolution was 5 nm in the center but degrades to 20 nm at the edges of the image.

Fig. 1 summarizes our procedure for computing the surface roughness. Since DHM images generally covered areas with different slope and roughness (Fig. 1a–b), we divided the central part (high resolution) of each image into 16 × 16 subzones. For each subzone, the surface roughness was computed as the maximum distance from the subzone surface to the local best-fit plane, which corresponds to the amplitude of the surface fluctuations around the local mean plane (Fig. 1c–d). Subzones with a correlation factor smaller than 0.96 for the local best-fit plane were rejected. Therefore, the final number of subzones considered for roughness measurements was 3996, 3987 and 3570 for 23 °C, 5 °C and 1 °C groups, respectively.

2.4. Low molecular weight biochemical substances

In order to measure glucose, trehalose and glycerol levels, we extracted hemolymph from 45 larvae (15 per temperature group). Each hemolymph sample was centrifuged and the supernatant was stored at −80 °C until use.

For all these metabolites, the concentration in hemolymph was measured by spectrophotometry and enzymatic assay kits. More specifically, the amounts of glucose and trehalose were quantified with Merck/Sigma-Aldrich (MAK263) and Megazyme (K-TREH) assay kits, respectively. Glycerol was measured by an enzymatic procedure with free glycerol reagent (F6428 Merck/Sigma-Aldrich) applied to the samples and to a pure glycerol standard (G7793, Merck/Sigma-Aldrich). Absorbance was measured (two replicates per sample) at 570 nm, 340 nm, and 540 nm to quantify glucose, trehalose, and glycerol levels, respectively. In all cases, the sample preparation and the calculation procedure were performed according to the instructions provided by the manufacturer.

2.5. Statistical analysis

All statistical tests were performed using the XLStat package (Addissoft, New York, USA) and the R software, version 4.3.1. To detect statistical differences, all analyzes considered a $P < 0.05$ significance level. In the study of morphological and biochemical responses to cold stress, several dependent variables (cuticle roughness, concentration of

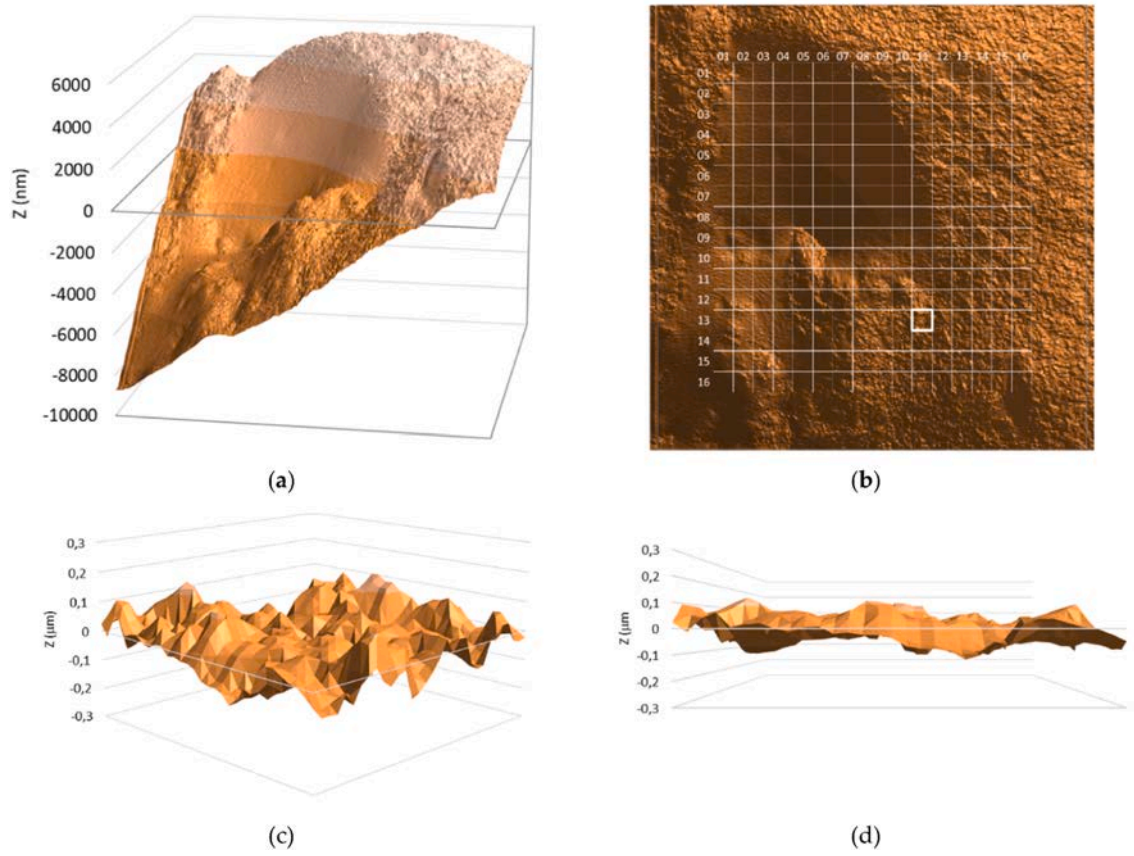

Fig. 1. Example of the roughness measurement: (a) 3D holographic representation of an integument sample; (b) zenithal view of the same holographic image as in the previous panel, division into 16 × 16 subzones; (c) 3D representation of one of these subzones (marked in panel b by a thick-lined square), the XY plane corresponds to the local best-fit plane; (d) edge view of this subzone, where the cuticle roughness was computed as the amplitude of fluctuations around the local best-fit plane.

different substances and density components) were studied in experimental situations determined by the following factors (and their interaction): temperature treatment, with nominal levels "23 °C", "5 °C" and "1 °C", and collection season, with nominal levels "Spring" and "Autumn". Since we focused only on last instar larvae, all animals had very similar masses and, therefore, we did not include the larval mass as an additional factor. Indeed, three-way MANOVA showed that larval mass had no significant effect on either the biochemical response, $P = 0.146$, or the density components, $P = 0.494$ (see Supplementary Material).

The effect of temperature treatment and collection season on the levels of different metabolites, as well as on the density components, was investigated using principal component analysis (PCA) to reduce dimensionality, and then analyzing the data projections on the most relevant principal axes (those with eigenvalues greater than 1) through two-way MANOVA with interaction. If only one principal axis was selected, we used instead two-way ANOVA with interaction. Difference in cuticle roughness of larvae across temperature treatments was studied using Welch one-way ANOVA. All these analyses, both univariate and multivariate, included Tukey post-hoc tests for pairwise comparisons. When the samples did not meet the requirements of normality and homoscedasticity, we used instead Games-Howell post-hoc tests for pairwise comparisons.

To show the robustness of our conclusions, we also carried out comparisons among groups through other statistical analyses: two-way MANOVA without dimensionality reduction and, in the case of tissues and inner components, through Dirichlet regression. This latter procedure is especially suitable (Rencher, 1997; Ferrari and Cribari-Neto, 2004; Maier, 2014) for the analysis of compositional data, which are subjected to a constant sum constraint. These other statistical tests are included as Supplementary Material.

3. Results

3.1. Insect behavior during and after low temperature treatments

Although there are no previous studies for *Paysandisia archon* with a precise measurement of its chill coma onset temperature (CTmin), we observed that all cold-treated larvae, without exception, stopped feeding and entered chill coma at the two temperatures studied (1 °C and 5 °C). Therefore, we can say that these two temperatures are below CTmin and, consequently, that the animals of these two groups experienced some degree of cold stress. Larvae subjected to 5 °C generally recovered mobility a few minutes after returning to warm temperatures, while those subjected to 1 °C needed about 2 h to recover coordinated mobility.

One week after the end of cold treatment, we found a survival rate of 100 % (15/15) at 5 °C and 93 % (14/15) at 1 °C. Therefore, this Neotropical species exhibited a remarkable tolerance to these two low temperatures.

3.2. Inner morphology: X-Ray CT

Fig. 2 shows some representative 2D slices of the inner morphology of *Paysandisia archon*, from transverse, coronal and sagittal images obtained with the X-Ray CT system. The color scale represents the tomographic density in HU units, so that yellow and red regions correspond to the densest tissues: the cephalic capsule, the digestive tract, most of the integument and some small organelles. Green and blue regions correspond instead to areas of low tomographic density: mainly fat body and hemolymph, which are spatially mixed, and part of the integument (its outermost envelope and some inner areas). Finally, the air contained in the tracheal structure appears as black areas. The 3D visualization of this same example is shown in Fig. 3.

In order to quantify the volume of the above morphological components, Fig. 4 shows our results for the average distribution of tomographic densities in the three groups of larvae. We see that all histograms have a peak that covers a density range from 120 to 244 HU. This peak corresponds to *High-density tissues*: the cephalic capsule, most part of the integument and also the digestive tract, with high opacity to X-rays. *Low-density tissues*, essentially the fat body, appear instead as a smooth peak covering a wide range of densities, from −200 to 60 HU, while inner voxels with density < −200 HU corresponds to *inner air* (tracheal system). Within the low-density region, we also observe a prominent peak from −32 to 0 HU rising above the general distribution. This latter range of tomographic densities corresponds to liquid substances, mainly hemolymph in the case of insects. Its contribution can be computed by fitting the background distribution (soft tissues) to a cubic polynomial. The remaining interval, from 60 to 120 HU, corresponds to a *border* of very small volume (small number of voxels) between high and low density tissues. In the present work, we used the central value of the border region, 90 HU, as the density that separates high (>90 HU) and

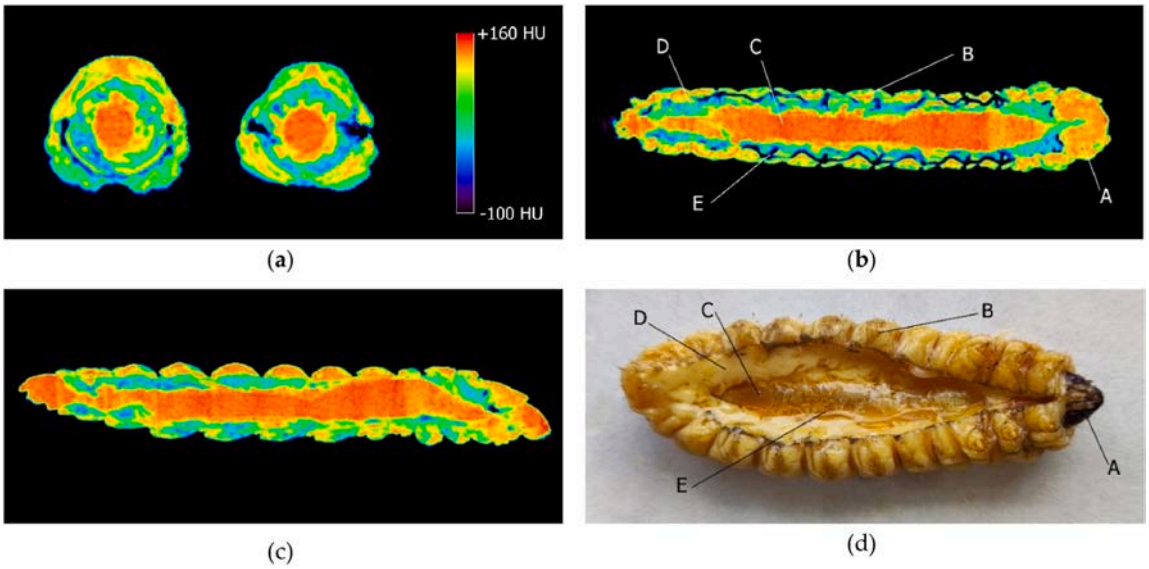

Fig. 2. X-ray CT slices of the inner morphology of *Paysandisia archon* from (a) traverse (2 slices), (b) coronal and (c) sagittal views. For comparison, (b) and (d) label some relevant morphological components: A. cephalic capsule, B. integument, C. digestive tract, D. fat body and E. tracheal structure (almost translucent).

Fig. 3. Three-dimensional X-Ray CT image of the inner structure of *Paysandisia Archon*. High-density (ρ > 90 UH) components appear as yellow-red areas, and low-density (ρ ≤ 90 UH) components appear as blue-green areas. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

low (≤90 HU) density tissues. The volume of each component was then obtained by integrating the number of voxels (volume units) over the corresponding density range.

Once the density intervals that define each internal component were identified, we measured their relative volumes for each larva. We then carried out a principal component analysis (PCA) for analyzing possible effects of temperature treatment and collection season on these internal density components (see Supplementary Material 1 for details). We found (Fig. 5a) that high-density tissues, low-density tissues and hemolymph were the main contributors to PC1 (when the former increases, the other two tend to decrease), while inner air was the main contributor to PC2. We also found that data obtained at different seasons had overlapping ellipses and, therefore, the collection season had no significant effect on the density components (Fig. 5b and Table 1). In contrast, temperature treatment had significant effect on data clustering (Fig. 5c). In particular, the data corresponding to 1 °C appeared clearly separated from the other two towards higher values of PC1 (towards higher percentages of high-density tissues and lower percentages of low-density tissues and hemolymph), while 5 °C and 23 °C had indistinguishable effects and formed a homogeneous group.

The above conclusions, obtained through PCA, were identical to those found from other statistical analyses without dimensionality reduction (Supplementary Materials 2 and 3 describe the results from MANOVA and Dirichlet regression, respectively). These additional tests also allowed us to identify which specific density components (inner air, hemolymph, low- and high-density tissues) had significant changes with temperature. We found (Table 2) that, when exposed to moderately low temperatures (5 °C), none of these components had significant differences compared to the control group (23 °C). On the contrary, larvae exposed to 1 °C had a significant reduction in all low-density components (air, hemolymph, and other low-density tissues), as well as a significant increase in the percentage of high-density tissues compared to the control group. The most notable change was an almost 4-fold decrease, with respect to the basal level at 23 °C, in the hemolymph volume of animals exposed to 1 °C.

To better understand the above results, we visualized the internal distribution of the different density components (Fig. 6 shows characteristic cross-sectional images for the three groups of larvae). We observed that, under warm (23 °C) and moderately low (5 °C) temperature conditions, the integumentary system presented a heterogeneous density, with abundant areas of low-density tissue almost mixed with other areas of high-density tissue. At a temperature of 1 °C, the larvae had instead an integumentary system with a thicker and more dominant dense tissue. Since the volume of the digestive tract (about 9 % of the total) did not exhibit apparent alterations, the above changes on the integumentary system seemed to be the primary factor contributing to a higher proportion of dense tissues at 1 °C. Parallel to the volumetric increase of dense tissues at 1 °C, low-density tissues (fat body) and fluid components (hemolymph) decreased in volume. Consequently, the ratio of high to low density tissues increased as a morphological response of *Paysandisia archon* to adverse cold environments.

3.3. Outer morphology: digital holographic microscopy

Using DHM we obtained the results shown in Fig. 7 for cuticle roughness in the three temperature groups. Although the mean values appeared to be quite similar (0.10 ± 0.07 μm at 23 °C; 0.11 ± 0.07 μm at 5 °C; 0.14 ± 0.08 μm at 1 °C), the roughness distributions had different shapes and the Welch ANOVA analysis (see Table 3) determined that such differences were all statistically significant (see Supplementary Material 4 for details). Indeed, in the control group (23 °C), the roughness distribution had a very pronounced maximum at low values (~0.04 μm) and then decreased abruptly with a very smooth secondary maximum at higher roughness (~0.17 μm). In contrast, in animals exposed to low temperatures (1 °C), the roughness distribution appeared clearly shifted towards higher values: the maximum at ~ 0.04 μm was considerably less pronounced than in the control group, while the maximum at ~ 0.17 μm contained most of the analyzed subzones. This was also observed in the graph (Fig. 7d) of cumulative frequencies (percentage of subzones with a roughness smaller than a certain value). We found that about 60 % of subzones at 23 °C had roughness values smaller than 0.1 μm, while about 70 % of subzones at 1 °C had roughness values greater than 0.1 μm. The 5 °C group presented an intermediate behavior, although much closer to the Control than to the 1 °C group.

3.4. Biochemical response: glucose, trehalose and glycerol

The possible effects of temperature treatment and collection season on the observed metabolite levels were investigated using a principal component analysis (PCA). Glycerol and glucose levels mainly contributed to PC1, while trehalose level mainly contributed to PC2 (Fig. 8a). We found no significant separation between the centroids of data obtained in different seasons (Fig. 8b and Table 4). In contrast, the temperature treatment had significant effect on the levels of potentially cryoprotective substances (Fig. 8c). In particular, the 1°C-group was separated from the other two towards higher values of PC1 (higher glycerol and glucose levels), while 5 °C and 23 °C had indistinguishable effects and formed a homogeneous group.

The above conclusions, derived from PCA, were identical to those found from other statistical analyses without dimensionality reduction

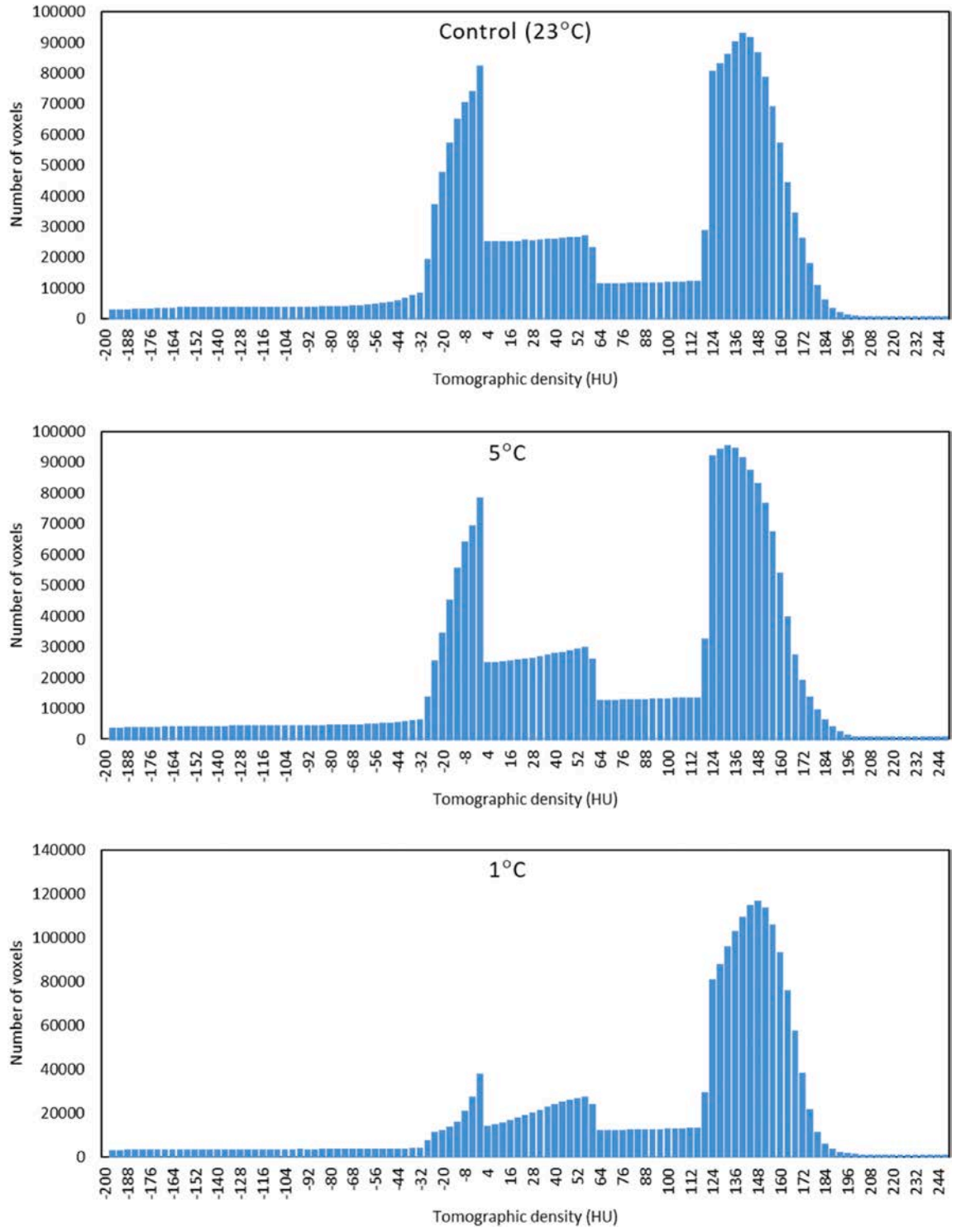

Fig. 4. Average distribution of tomographic densities in the three temperature groups (n = 15 larvae per group): controls (23 °C) and those subjected to low (5 °C and 1 °C) temperatures. The tomographic density axis was divided into intervals of 4 HU and, for each interval, we averaged the number of voxels over each group.

(Supplementary Material 2 describes the results from MANOVA). These additional tests also allowed us to identify which specific metabolites (glycerol, glucose and trehalose) had significant changes in response to temperature. We found (Fig. 9) that trehalose had average levels (22 ± 7 mM at 23 °C, 24 ± 7 mM at 5 °C, 21 ± 6 mM at 1 °C) without significant changes with the environmental temperature. In contrast, the glucose levels of larvae exposed to a temperature of 1 °C had a significantly higher average value (9 ± 5 mM), almost a 7-fold increase compared to the control group (23 °C). Indeed, all individual glucose levels at 1 °C were higher (or much higher) than 3 mM, while individual glucose levels in the control group never exceeded 2 mM, with an average value of 1.3 ± 0.3 mM. Larvae exposed to a temperature of 5 °C had an average glucose concentration of 2 ± 1 mM, with no statistically significant differences compared to the control group. Finally, we also

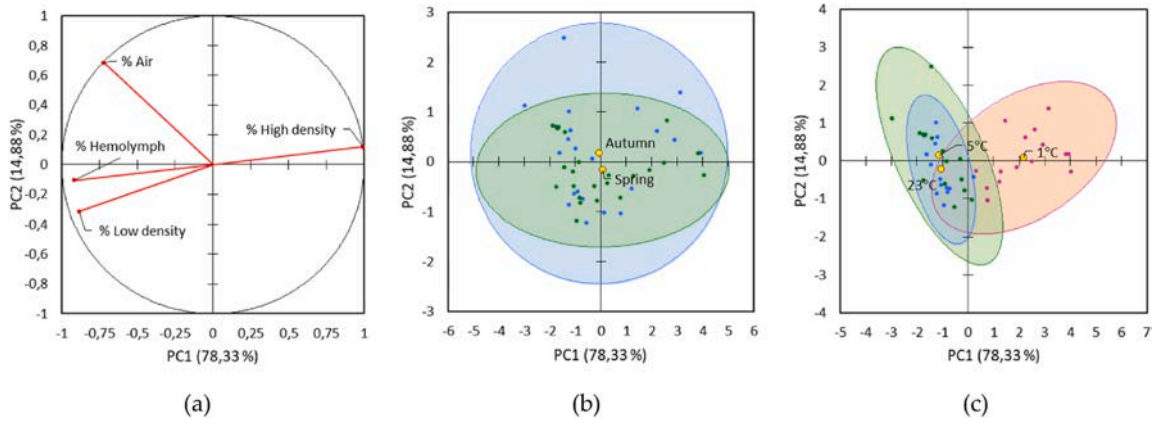

Fig. 5. Principal component analysis (PCA) of density components. (a) Factor loading plot of each density component in the PC1-PC2 plane, (b) PCA for the two collection seasons: autumn (blue) and spring (green), (c) PCA for the three temperature treatments: 1 °C (red), 5 °C (green) and 23 °C (blue). Ellipses denote 95 % confidence intervals. The eigenvalues of PC1 and PC2 were 3.13 and 0.59, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Density components: Two-way ANOVA with interaction for PC1 and post-hoc tests.

Factor	ANOVA		Games-Howell post-hoc test (P values)		
	F	P	1 °C vs 5 °C	1 °C vs 23 °C	5 °C vs 23 °C
Temperature	63.09	**< 0.001**	**< 0.001**	**< 0.001**	0.969
Season	0.01	0.907	–	–	–
Interaction	0.13	0.879	–	–	–

Table 2
Volume percentage (mean ± standard deviation) of different components in the three temperature groups.

Group	Inner Air	Hemolymph	Low-density tissues	High-density Tissues
23 °C	7 ± 1 %[a]	15 ± 2 %[a]	29 ± 2 %[a]	49 ± 2 %[a]
5 °C	8 ± 1 %[a]	13 ± 2 %[a]	30 ± 2 %[a]	49 ± 4 %[a]
1 °C	6 ± 1 %[b]	4 ± 1 %[b]	22 ± 6 %[b]	68 ± 7 %[b]

Different letters identify groups with significant ($P < 0.05$) differences according to both beta regression and Tukey post-hoc tests (see Supplementary Material 2 and 3).

found that the glycerol concentration in hemolymph (Fig. 9c) of control animals (42 ± 12 mM at 23 °C) had no significant difference from that of animals exposed to moderately low temperatures (50 ± 15 mM at 5 °C). In contrast, larvae exposed to a temperature of 1 °C had a significantly higher level of glycerol than in the other two groups. On average, we found that the glycerol concentration (88 ± 16 mM at 1 °C) in larvae subjected to severe cold stress had a two-fold increase compared to the control group.

4. Discussion

This work addressed for the first time the morphological and biochemical responses of *Paysandisia archon* to low temperatures. Morphological changes were measured through a combination of two modern imaging techniques. On the one hand, we evaluated inner changes by means of X-Ray Computer Tomography together with a procedure that provides a precise and automatic measurement of air content, hemolymph, and tissues of different densities (León-Quinto et al., 2020). On the other hand, we used Digital Holographic Microscopy to evaluate outer changes, such as the cuticle roughness. From the first of these techniques, we found significant cold-induced changes in the inner morphology of this species, but only when the environmental temperature was remarkably low (1 °C). For moderately low temperatures (5 °C), *Paysandisia archon* did not show morphological evidence of cold stress.

Among the morphological responses at 1 °C, we found a cold-induced increase in the volumetric content of high-density tissues. This increase was mainly due to the fact that the integumentary system becomes thicker and denser and, therefore, it involves the outermost areas in direct contact with the environment. On the contrary, the content of low-density tissues (mostly fat body) significantly decreased with cold. This latter result is consistent with previous studies on the fat body content of overwintering insects. Such studies found that many insects increase their fat body reserve before the arrival of the cold season, and then use this reserve as a major source of energy (Arrese and Soulages, 2010; Sinclair and Marshall, 2018), as well as a source for protein redistribution (Csikós et al., 1999), during the winter months. In our

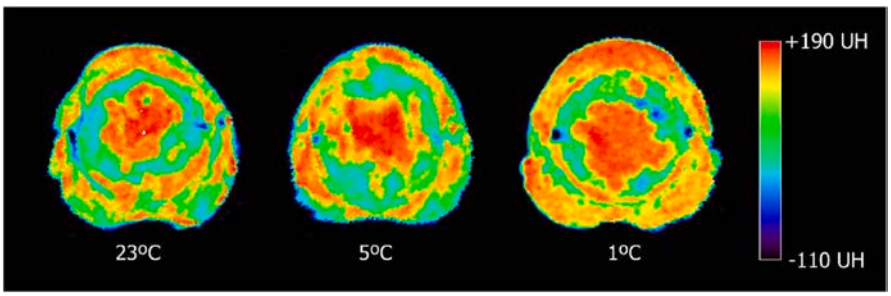

Fig. 6. Representative cross-sectional X-Ray CT images of the three groups of larvae: controls (23 °C) and those subjected to moderately low (5 °C) or very low (1 °C) temperature.

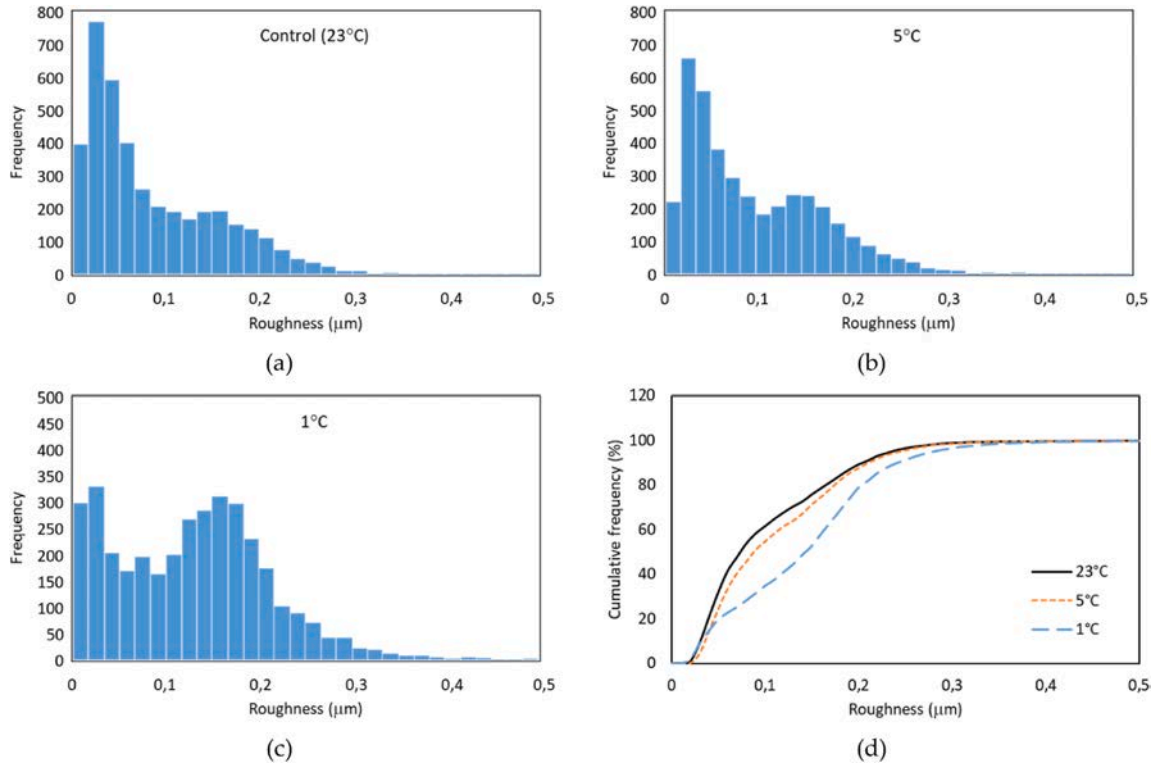

Fig. 7. Histograms (a, b, c) and cumulative frequency (d) of cuticle roughness in the three groups of larvae: controls (23 °C) and those subjected to moderately low (5 °C) or very low temperature (1 °C).

Table 3
Welch ANOVA and pairwise comparisons of roughness data for temperature treatments.

Variable	Welch ANOVA		Games-Howell post-hoc test (P values)		
	F	P	1 °C vs 5 °C	1 °C vs 23 °C	5 °C vs 23 °C
Cuticle roughness	290.4	< **0.001**	**< 0.001**	**< 0.001**	**< 0.001**

Table 4
Metabolite levels: Two-way ANOVA with interaction for PC1 and post-hoc tests.

Factor	ANOVA		Tukey post-hoc test (P values)		
	F	P	1 °C vs 5 °C	1 °C vs 23 °C	5 °C vs 23 °C
Temperature	67.10	**< 0.001**	**< 0.001**	**< 0.001**	0.969
Season	3.28	0.078	–	–	–
Interaction	0.104	0.901	–	–	–

case, the animals had no chance to accumulate fat body before cold treatment, so they directly used their basal reserve.

Concerning the volumetric content of hemolymph, we found that such a component had an almost 4-fold decrease when the environmental temperature had values (1 °C) close to 0 °C. In addition, a reduction in water content could also have occurred to some extent in other components or tissues. In this way, our analysis using holographic microscopy revealed cold-induced changes in the 3-D structure of the outer integumentary surface. More specifically, we found that the cuticle roughness significantly increased at low temperatures, which could be due to a certain degree of dehydration of the integument. A reduction in water content was previously observed in some other species as a morphological adaptation against possible damage by freezing. Indeed, in some extreme cases, cryoprotective dehydration was identified as the central strategy to avoid freezing in certain small invertebrates: nematodes (Wharton et al., 2005), springtails (Clark et al., 2009) and Antarctic midges (Kawarasaki et al., 2014). A partial reduction in the water content was also reported for some overwintering Coleoptera (Lundheim and Zachariassen, 1993) and Lepidoptera (Kojic et al., 2010) as a way to reduce damage from freezing of biofluids, as well as to increase the concentration of cryoprotective substances. However, in more severe cold exposures than those considered in the present study, a reduction in water content could also appear as part of the progression of chilling injury. In this sense, a reduction in hemolymph volume was reported, along with a similar increase in gut water content, in insects subjected to almost lethal cold exposure (MacMillan and Sinclair, 2011). Such a migration in water content from hemolymph to gut was associated to chilling injury, rather than a protective response to cold. Although we cannot rule out such a possibility, in our study we did not detect any increase in gut volume, and cold exposure was far from lethal as larvae recovered mobility and restarted their feeding without apparent chill injuries.

The above cold-induced morphological changes were accompanied by an increase in the concentration in hemolymph of various low-molecular-weight cryoprotective substances. In particular, we found that *Paysandisia archon* had a relatively high basal level of glycerol compared to that reported in the literature for most other tropical lepidopterans from warm areas (Park and Kim, 2013, 2014; Vatanparast and Park, 2022). Such a high basal glycerol level in *Paysandisia archon* could explain, at least in part, the absence of increase in this polyol when larvae were subjected to 5 °C. In other tropical lepidopterans, such a moderately low temperature already produced sufficient cold stress to cause an increase in glycerol concentration (Singh et al., 2010). We found, however, that *Paysandisia archon* only requires a significant (2-fold) increase in glycerol levels at temperatures as low as 1 °C, where the risk of freezing begins to be close.

Regarding glucose concentration, it presented an almost 7-fold increase with respect to the basal level but, just as for glycerol, only when

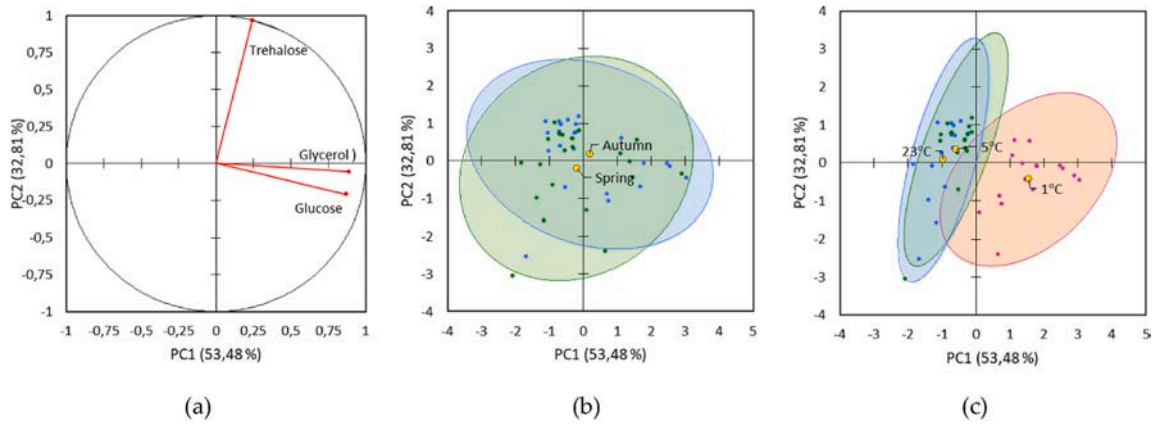

Fig. 8. Principal component analysis (PCA) of metabolite levels. (a) Factor loading plot of each metabolite in the PC1-PC2 plane, (b) PCA of metabolite data for the two collection seasons: autumn (blue) and spring (green), (c) PCA of metabolite data for the three temperature treatments: 1 °C (red), 5 °C (green) and 23 °C (blue). Ellipses denote 95 % confidence intervals. The eigenvalues of PC1 and PC2 were 1.60 and 0.98, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

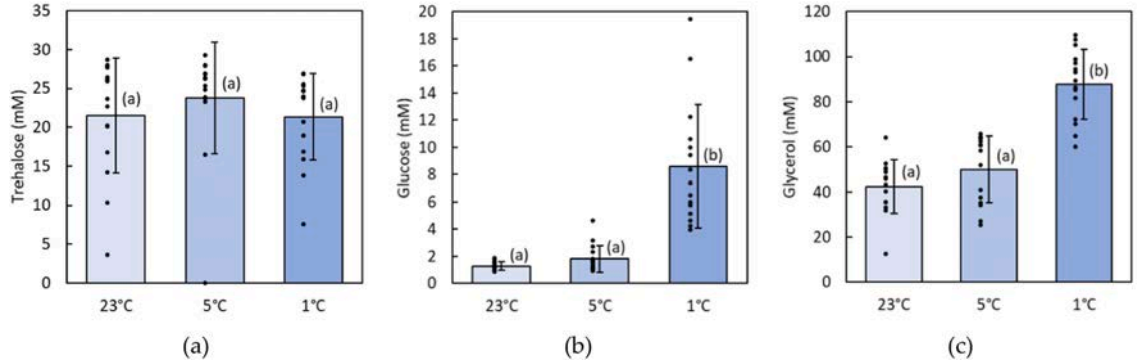

Fig. 9. Average (error bars give the standard deviation) and individual values (circles, $n = 15$ per group) of the concentration in hemolymph of (a) trehalose, (b) glucose and (c) glycerol for different temperature treatments. Different letters identify groups with significant ($P < 0.05$) differences according to Tukey and Games-Howell post-hoc tests (see Supplementary Material 2).

larvae were exposed to a temperature (1 °C) with close risk of freezing. Accumulation of glucose in hemolymph was previously found in the pine caterpillar, *Dendrolimus tabulaeformis*, a chill tolerant insect (Zeng et al., 2008). However, in our study, this increase was much higher than in the pine caterpillar, which could perhaps be interpreted on the basis that glucose plays in *Paysandisia archon* a more relevant role as a cryoprotectant than in the pine pest. Such a relevant role of glucose to deal with low temperatures was previously shown in other insects native to tropical and warm regions, like the cockroach *Gromphadorhina coquereliana* (Chowanski et al., 2015, 2017) and the red palm weevil *Rhynchophorus ferrugineus* (León-Quinto et al., 2020; León-Quinto and Serna, 2022).

In the current study we also found that trehalose was the most abundant sugar in hemolymph, although it remained at nearly the same level at the three temperature groups. A nearly constant level of trehalose with a cold-induced accumulation of either glucose or glycerol were previously found for the cryoprotective response of other insect species. For instance, a cold-induced increase of glucose level with unaltered trehalose content was reported in several species of insects inhabiting warm and tropical areas (Overgaard et al., 2014; Chowanski et al., 2015; León-Quinto and Serna, 2022). In the same way, a glycerol accumulation with constant level of trehalose during cold stress was observed in the moth *Plutella xylostella* (Park and Kim, 2014), as well as in the oriental corn borer, *Ostrinia furnacalis* (Goto et al., 2001), a freeze-tolerant species. In this latter case, the overwintering trehalose levels were similar to those found in this work for *Paysandisia archon*, a Neotropical insect. This suggests that, in *Paysandisia archon*, the nearly constant level of trehalose during cold stress is probably due to its already high basal level.

In summary, the present study showed for the first time that *Paysandisia archon* is able to deal with sudden non-seasonal moderately low temperatures. Indeed, it seemed to tolerate a temperature of 5 °C with no apparent need to develop complex changes, neither at the morphological nor biochemical level. Its behavior in vivo at this temperature went in the same way, with no mortality found and with the recovery of mobility a few minutes after the end of cold stress. This tolerance to moderately low temperatures (5 °C) could be facilitated by the basal levels found for glycerol and trehalose, high as compared to those reported for other Neotropical lepidopterans, probably acting along with other substances such as heat shock proteins or aquaporins. Only a temperature as low as 1 °C, probably close to a risk of freezing, seemed to generate in this pest insect a cold-induced response both at the morphological and biochemical level. These observed changes suggest that, when faced with a period of low temperatures, this species deploys a morphological response that consists of a reduction in the liquids most susceptible to freezing (hemolymph), the progressive consumption of fat body probably as a source of energy and proteins, while the integument, in direct contact with the environment, becomes thicker, denser and rougher. In parallel, the main low-molecular-weight biochemical substances acting as cryoprotectants in Lepidoptera were increased,

particularly glucose and glycerol, reaching values similar to those observed in some insects native to cold areas. We also found that trehalose was the most abundant sugar, but it did not increase at low temperatures, probably because its basal level was already high.

Our overall results suggest that *Paysandisia archon* could overwinter in areas with lower temperatures than previously considered. This is especially relevant in the context of global warming, where the spread of this pest to new regions could occur as some cold places are becoming less adverse. In order to develop more effective control strategies, geographic distribution models should then incorporate parameters related to its resistance to low temperatures. In addition, knowledge of the specific morphological and biochemical changes developed by this species in response to cold is also useful to try to prevent them. In this way, it would be interesting to study in future research how to block the biological processes leading to the thickening of the integument or the accumulation of cryoprotective biochemical substances.

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CRediT authorship contribution statement

Trinidad León-Quinto: Writing – original draft, Visualization, Supervision, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Roque Madrigal:** Methodology, Investigation, Data curation. **Esteban Cabello:** Formal analysis. **Antonio Fimia:** Methodology, Investigation, Data curation. **Arturo Serna:** Writing – original draft, Visualization, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2024.103795.

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