Journal of Agricultural Science and Technology (JAST), 29(3)
In Press, Pre-Proof Version

Comparative Cold Tolerance Mechanisms in Acorn Pests from Iran and Switzerland: Thermal Hysteresis and Hemolymph Elemental Dynamics

Maryam Atapour[1*], and Ahmad Bahmani[2]

Abstract

Oak forests in Iran's Zagros region are undergoing severe decline due to environmental stress and increasing pest outbreaks. This study examined the overwintering survival mechanisms of two major acorn pests, the beech moth *Cydia fagiglandana* (Zeller) and an acorn weevil (*Curculio* sp.), by comparing populations from stressed Zagros forests with those from stable oak forests in Switzerland. We evaluated two key biomarkers of cold tolerance during the coldest months: thermal hysteresis, measured using differential scanning calorimetry (DSC), and hemolymph trace element concentrations (Fe, Cu, Zn, Mg), quantified by ICP-OES. Additionally, EDS–SEM analysis was conducted to assess the spatial distribution of selected internal elements semi-quantitatively. A distinct thermal hysteresis of approximately 4 °C between melting and freezing points was observed exclusively in Zagros *Curculio* larvae. In most samples, metal ion concentrations increased in January, with Zn showing a nearly fourfold increase in the Zagros acorn weevil (170–585 ppb). Elemental mapping by EDS–SEM further showed higher Cu and Zn contents and a shift from uniform to aggregated elemental distribution in overwintering larvae. This study provides new insights into the overwintering physiology of two major acorn-feeding insects under contrasting climatic conditions. These findings contribute to predicting pest responses to environmental change and support future conservation and management of oak forest regeneration.

Key words: Acorn pests, *Cydia fagiglandana, Curculio* sp., DSC, Hemolymph elements, ICP-OES, Overwintering, Thermal hysteresis.

Introduction

Overwintering represents a critical yet often underutilized target in pest management, as suppression of insects during this stage can substantially reduce subsequent spring populations

[1] Department of Plant Production and Protection, Institute of Agriculture, Iranian Research Organization for Science and Technology (IROST), P. O. Box: 33535111, Tehran, Islamic Republic of Iran.
[2] Department of Advanced Materials and New Technologies, Iranian Research Organization for Science and Technology (IROST), P. O. Box: 33535111, Tehran, Islamic Republic of Iran.
*Corresponding author; e-mail: atapour@irost.org

below economic thresholds (Lee, 2010; Storey & Storey, 2012). To survive low-temperature stress, insects employ diverse behavioral, physiological, and biochemical strategies, including diapause, sheltering, migration, enhanced cold hardiness, and synthesis of cryoprotective compounds (Block, 1990; Lee, 2010). Based on their capacity to tolerate internal ice formation, insects are broadly classified as freeze-avoiding or freeze-tolerant, although both strategies involve overlapping mechanisms that differ in function and efficiency (Lee, 2010).

Among the molecular adaptations associated with cold hardiness are ice-binding proteins (IBPs), including antifreeze proteins (AFPs), recrystallization inhibition proteins (RIPs), and ice-nucleating proteins (INPs) (Duman & Newton, 2020). AFPs and RIPs generate thermal hysteresis (TH), a non-colligative depression of the freezing point relative to the melting point, thereby enhancing cold tolerance (Duman, 1979; Duman & Newton, 2020). As TH reflects IBP activity, its measurement in hemolymph provides a useful indicator of cold adaptation, although it is technically challenging in small insect samples. Differential scanning calorimetry (DSC) has been successfully applied to quantify TH in overwintering insects (Storey & Storey, 2012; Lee, 2010; Rozsypal *et al*., 2018; Duman, 2001; Gabbott, 2008).

In addition to IBPs, ion homeostasis is essential for cold survival. Cold-hardy insects maintain more stable Na^+, K^+, and Ca^{2+} levels, whereas freezing stress can disrupt ionic balance and increase toxicity (Zachariassen *et al*., 2004; Missirlis & Oliveira, 2022). Trace metals such as Fe, Cu, Zn, Mn, and Ni are important enzymatic cofactors but may also contribute to oxidative stress (Dow, 2017). Because these elements occur at very low concentrations, their seasonal quantification requires highly sensitive analytical methods. Inductively coupled plasma (ICP) techniques, including ICP-OES and ICP-MS, provide accurate multi-element analysis for small biological samples (Hou & Jones, 2000; Montaser, 1998; Beauchemin, 2010; Profrock & Prange, 2012; Thomas, 2013).

Elemental distribution in insect tissues plays an important role in physiological regulation, biomineralization, and stress adaptation. Field-emission scanning electron microscopy coupled with energy-dispersive X-ray spectroscopy (FE-SEM–EDS) enables micro-scale mapping of macro- and trace elements within tissues such as the cuticle, gut, and excretory organs. Unlike bulk techniques such as ICP, FE-SEM–EDS provides spatial localization of elements including Mg, Cu, Zn, and Ca, and has been widely used to investigate metal accumulation, detoxification, and cuticular composition (Steinbrecht, 1993; Fialkowski *et al*., 2013; Polilov *et al*., 2021).

Acorn-feeding insects are among the important pests of oak forests in the Zagros region of Iran, where they disrupt natural regeneration by destroying developing seeds (Sadeghian *et al*., 2007, Zargaran *et al*., 2018). Acorn weevils (*Curculio* spp., Coleoptera: Curculionidae) and the beech moth *Cydia fagiglandana* (Zeller) (Lepidoptera: Tortricidae) are particularly destructive. Both overwinter as larvae, primarily in soil or within acorns, completing one generation annually in Zagros forests (Sadeghian *et al*., 2007; Zargaran *et al*., 2018). However, knowledge of their overwintering physiology remains limited. *Curculio glandium* Marsham has been shown to employ a freeze-avoidance strategy, although its relatively high supercooling point (−6 to −7 °C) indicates limited cold tolerance (Udaka & Sinclair, 2014). Soil depth may buffer larvae from extreme temperatures, reducing selective pressure for deeper supercooling. Other *Curculio* species exhibit deeper burrowing behavior or prolonged diapause as alternative cold-avoidance strategies (Semel & Anderson, 1988; Vélez-Gavilán, 2022). Information on *C. fagiglandana* is scarce, although related species overwinter within acorns or soil (Sokolova, 2024). In contrast, the codling moth *Cydia pomonella* (L.) shows pronounced freeze avoidance, with supercooling points -the temperature at which spontaneous freezing occurs in the body fluids of an organism, as indicated by the release of latent heat (the exotherm) during cooling- reaching −22 to −26 °C and high winter survival associated with cryoprotectant accumulation (Khani *et al*., 2007; Rozsypal *et al*., 2013). Despite the ecological importance of acorn-feeding insects, their overwintering physiology remains poorly understood, particularly in drought-stressed Zagros oak forests where increasing environmental stress may alter cold-hardiness strategies. Moreover, no previous study has integrated thermal hysteresis analysis, hemolymph trace element profiling, and tissue elemental mapping to investigate cold adaptation in these economically important pests under contrasting climatic conditions. Therefore, this study compared overwintering larvae of *Curculio* sp. and *Cydia fagigl andana* collected from drought-stressed Zagros oak forests (Iran) and climatically stable oak forests in Landquart (Switzerland). Specifically, we quantified thermal hysteresis using differential scanning calorimetry (DSC), determined seasonal changes in hemolymph trace elements (Fe, Cu, Zn, and Mg) by ICP-OES, and examined the spatial distribution of selected elements using FE-SEM–EDS. By integrating these complementary physiological and analytical approaches, this study provides new insights into the mechanisms of cold adaptation in acorn-feeding insects. It improves our understanding of how contrasting environmental conditions may influence

overwintering survival, with implications for predicting pest outbreaks and supporting sustainable oak forest management under climate change.

Materials and Methods

The Insects

Larvae of pests were collected from naturally fallen acorns on the ground beneath oak trees during the autumn and winter months of 2024–2025 from the Zagros oak forests in Darreh Somagh, Chegeni County, Lorestan Province (33.56°N, 48.40°E, 1496 m a.s.l.) and, in 2025, from the Landquart forests in Switzerland (46.96°N, 9.53°E, 550 m a.s.l.). The collected larvae were transferred to the laboratory, along with a small amount of soil and/or acorns, where all subsequent experiments were conducted.

A small incision was made at the posterior end of each larva using a sterile disposable glass Pasteur pipette, and the exuding hemolymph was collected (Butolo *et al*., 2020). Samples were transferred into 0.2-mL microtubes and stored at −80 °C until subsequent experimental procedures.

Thermal Hysteresis Measurement

To assess the potential presence of ice-binding proteins (IBPs) in the hemolymph samples, thermal hysteresis (TH) was determined using differential scanning calorimetry (DSC). The procedure was performed following the methods described by Kostál *et al*. (2011) and Mao *et al*. (2011), with minor modifications. Briefly, 5 µL of hemolymph was loaded into a 40-µL sealed aluminum DSC pan and analyzed using a DSC instrument (NETZSCH DSC 200 F3 Maia, Germany). Samples were first equilibrated at 30 °C for 1 min, then cooled to −30 °C at a rate of 10 °C min^{-1}. After holding at −30 °C for 1 min, the samples were reheated to 30 °C at the same rate. The melting and freezing points were determined from the thermograms, and thermal hysteresis was calculated as the difference between these two temperatures.

The experiment was conducted on four groups: Iranian/Swiss acorn weevils and Iranian/Swiss beech moths. Specimens were collected in November and January. For each treatment, three biological replicates were analyzed. The hemolymph collected from a single larva was considered one biological replicate. To ensure sufficient sample availability, hemolymph was extracted from at least 10 larvae per treatment. Approximately 30–50 µL of hemolymph was obtained from each

larva and transferred directly into 0.2-mL microcentrifuge tubes without the addition of any buffer. The samples were then stored at −80 °C until further analyses.

Determination of Hemolymph Trace Element Concentrations by ICP-OES

Total concentrations of Fe, Zn, Cu, and Mg in hemolymph were determined by ICP-OES using inductively coupled plasma optical emission spectr oscopy (ICP-OES; Agilent 5900, USA) following procedures adapted from Kokot and Matysiak (2008), Hansen *et al*. (2013), and Gao *et al*. (2016) with minor modifications. Briefly, 50 µL of hemolymph from each sample was digested in two sequential steps with a total of 4 mL of 2% nitric acid (prepared from 65% ultrapure nitric acid) and incubated in a water bath at 120 °C for 60 min. After digestion, samples were centrifuged three times at 18,000 rpm for 15 min at 4 °C, and the clear supernatant was used for elemental analysis. Selected elements: iron (Fe), zinc (Zn), copper (Cu), and magnesium (Mg), were quantified. Calibration standards (10, 50, 200, and 500 ppb; ICP Calibration Standard, CPAchem) and a 2% nitric acid blank were used. Calibration standards and procedural blanks were analyzed together with the samples to monitor instrumental performance throughout the analytical sequence. Instrumental settings included axial viewing mode with optimized pump speed, sample uptake, RF power, and gas flows to ensure stable plasma conditions.

The experimental groups, sampling months (November and January), and number of biological replicates (n = 3 per group, Sabo *et al*., 2024) were identical to those described in the previous experiment.

Semi-quantitative elemental analysis (FE-SEM–EDS)

To visualize the relative spatial distribution of selected elements (C, N, O, Mg, Cu, and Zn) within overwintering insects, semi-quantitative elemental analysis was performed using a field emission scanning electron microscope (FE-SEM; TESCAN MIRA \ LMU, Czech Republic) equipped with an energy-dispersive X-ray spectroscopy detector (EDS; SAMx DXP-X10P, France). The entire larval body was analyzed without tissue dissection. FE-SEM–EDS was employed to obtain semi-quantitative information on the relative elemental composition and distribution of the selected elements rather than their absolute concentrations.

Samples were freeze-dried for 24 h (LEYBOLD-HERAEUS, LYOVAC GT3) to ensure complete dehydration and subsequently transferred to the electron microscopy laboratory. Before analysis,

samples were coated with a 15 nm aluminum layer to enhance surface conductivity and minimize charging effects (Polilov *et al*., 2021; Steinbrecht, 1993).This aluminum coating was applied solely to improve surface conductivity and minimize charging effects during imaging. Because aluminum was not among the target elements and its characteristic X-ray peaks do not overlap with those of the analyzed elements, no significant interference with elemental analysis was expected.

EDS measurements were carried out at an accelerating voltage of 20 kV and a beam current of 10 nA at 5000× magnification. X-ray spectra were acquired for 60 s over a 0–20 keV energy range using a silicon detector (10 mm^2 active area). FE-SEM–EDS analyses were performed on representative larvae of the Iranian acorn weevil and beech moth collected in November and January (n = 3 per group), selected according to specimen availability and preservation quality.

Data Analysis

All statistical analyses, including mean comparisons, were conducted using SPSS version 24 (IBM Corp., Armonk, NY, USA), and graphical representations were prepared with Microsoft Excel.

Results

Thermal Hysteresis in the Studied Samples

The melting points, freezing points, and corresponding thermal hysteresis values of the examined samples are presented in Table 1. Among all groups, only the Iranian oak seed weevil specimens exhibited a melting–freezing point difference greater than 1 °C. The mean thermal hysteresis was 1.7±0.1 °C in November, which nearly doubled to 4.1±0.7 °C in January. Significant differences were observed in the melting and freezing points of acorn weevils ($F_{3,8} = 55.93$, $P < 0.001$ and $F_{3,8} = 26.37$, $P < 0.001$) and the beech moth ($F_{3,8} = 862.38$, $P < 0.001$ and $F_{3,8} = 862.38$, $P < 0.001$) collected from the two locations in both sampling months.

Trace Metal Element Concentrations in Hemolymph

The hemolymph concentrations of Cu, Fe, Zn, and Mg in the four studied treatments (Iranian/Swiss acorn weevils and Iranian/Swiss beech moths) collected in November and January are presented in Table 2. Overall, significant differences in the concentrations of the four analyzed elements were observed for each insect species between the two sampling locations and across the November and January. The concentrations of all examined ions were higher in samples collected in January compared to November. Among the elements, Zn exhibited the most pronounced increase,

particularly in the Zagros acorn weevil (approximately fivefold) and the Zagros beech moth (approximately twofold) during January.

Distribution patterns of selected elements

The distribution patterns of C, N, O, Mg, Cu, and Zn in weevil larvae collected in November 2024 and January 2025 are shown in Figure 1. EDS elemental maps were interpreted qualitatively as relative spatial distributions and were not used for absolute elemental quantification. In the larvae collected in November, the elements were distributed relatively uniformly, whereas in the overwintering samples, C, O, and Mg exhibited aggregated patterns within the tissues. Consistent with the ICP analysis results, a relatively higher signal intensity/distribution of Cu and Zn were higher in the overwintering weevil larvae (Table 3). The elemental distribution patterns in acorn moth larvae collected in November and January are presented in Figure 1: c and d. The changes in the overwintering moth larvae were less pronounced than those observed in the weevil larvae.

Discussion

In this study, samples were collected from two geographically and climatically contrasting regions; The Zagros oak forests of Darreh Somagh (Lorestan Province, Iran) are characterized by a semi-arid Mediterranean climate with lower annual precipitation, greater seasonal water deficit, and more frequent drought events than the temperate oak forests of Landquart, Switzerland. In contrast, the Swiss site experiences a cooler and more humid climate with higher annual precipitation and more stable moisture availability. According to long-term climatic datasets, the two localities differ substantially in annual mean temperature, precipitation regime, and winter thermal conditions, indicating that insects inhabiting the Zagros forests experience considerably greater environmental stress (IPCC, 2023). These contrasting climatic conditions provide a suitable natural framework for comparing overwintering physiological adaptations in acorn-feeding insects.

The Iranian population is referred to herein as Curculio sp. because molecular evidence demonstrated that it is distinct from C. glandium, whereas formal species delimitation requires additional morphological and integrative taxonomic analyses and is beyond the scope of the present study. Therefore, the observed differences should be interpreted as reflecting both geographic/environmental influences and potential species-specific variation, rather than geographic effects alone.

In a study of the acorn weevil (*C. glandium*) in Canada by Udaka and Sinclair (2014), the authors demonstrated that this pest employs a freeze-avoidance strategy. However, the SCP of the larvae was relatively high compared with most freeze-avoiding species (approximately −6 to −7 °C). Exposure of larvae to 0 °C for 2–4 weeks reduced the SCP to approximately −11 °C. Our findings revealed that the seasonal pattern of SCP variation and the cold-tolerance strategy of *C. glandium* populations from Canada and Switzerland are consistent. In contrast, the Zagros species exhibited an increasing trend in SCP during the cold season, and low-temperature acclimation did not result in a reduction of SCP. Notably, larvae were capable of surviving internal ice formation at temperatures below their SCP, suggesting that this species likely employs a freeze-tolerance strategy. Similarly, in the beech moth, a seasonal reduction in SCP was observed during colder months; however, individuals were unable to survive temperatures below their SCP, indicating a freeze-avoidance strategy (data in press).

Differential scanning calorimetry (DSC) analysis of residual heat release demonstrated that the thermal hysteresis was less than 1 °C in the Swiss weevil and in both Iranian and Swiss beech moth populations in November and January. In contrast, this difference in the Zagros weevil species was approximately 2 °C in November and increased to nearly 4 °C in January. This pattern may reflect physiological mechanisms associated with enhanced freeze avoidance, potentially including the action of ice-binding proteins (IBPs), although their presence and activity were not directly assessed in this study.

Two groups of ice-binding proteins create a difference between the melting point and the freezing point: antifreeze proteins (AFPs) and recrystallization inhibition proteins (RIPs), which play distinct roles in insects with different cold-tolerance strategies. AFPs are primarily found in freeze-avoidant species, lowering the freezing point of body fluids to prevent ice formation, whereas RIPs are more common in freeze-tolerant species, inhibiting the growth of existing ice crystals to reduce cellular damage. However, both protein groups can occur in insects employing different cold-tolerance strategies, potentially serving separate physiological functions, such as osmotic regulation and cellular protection at low temperatures (Duman, 2015; Duman & Newton, 2020).

A comparison of meteorological data, particularly the average minimum temperature in January, between the Central Zagros region (the sampling site in Iran) and Landquart, Switzerland, shows that this parameter is approximately −4 °C in Landquart, whereas it ranges from −6 to −12 °C in the Zagros region (Arsalani *et al*., 2023; MeteoSwiss, 2025). In addition to higher elevation and

lower temperatures, another important factor is the difference in humidity between the two regions. Due to the lower humidity in the Zagros region and the occurrence of recent droughts, a more pronounced nocturnal temperature drop is observed, especially during nighttime hours. In other words, diurnal temperature fluctuations in this region are greater than those in the more humid climate of Landquart. Therefore, in general, the level of environmental stress imposed on overwintering insects in the Zagros region is considerably higher than that in Switzerland. Since overwintering larvae of the beech moth reside within cocoons and often inside acorns, their microhabitat conditions may partially mitigate these environmental stresses.

In contrast, for the Iranian acorn weevil (a species different from the Swiss counterpart), which overwinters without a protective cocoon and at a depth of approximately 5 cm in the soil, the adoption of a freeze-tolerance strategy appears to be ecologically justified, and it appears that the production of RIPs is one of the strategies employed to survive this period.

Based on the results of ICP-OES, the concentration of Mg in the hemolymph of all examined samples was markedly higher than that of the other measured ions. This pattern is consistent with previous studies indicating that Mg is typically one of the dominant inorganic cations in insect hemolymph, often reaching concentrations several-fold (up to ~10-fold) higher than other divalent ions due to its central role in ATP-dependent enzymatic processes and metabolic regulation (Nation, 2002; Chapman, 2013). Although alternative explanations, such as analytical artifacts or sample contamination, cannot be completely excluded, all samples were processed under identical experimental conditions using the same analytical protocol. Therefore, the consistent differences observed between populations are more likely to reflect biological variation than methodological bias.

Overall, all metal elements (Mg, Zn, Fe, and Cu) exhibited an increase during January in nearly all samples, except Cu in the beech moth larvae. A general winter-associated elevation of hemolymph ionic concentrations has been widely reported in insects and is commonly linked to cold acclimation processes, including adjustments in osmoregulation, cryoprotection, and maintenance of cellular homeostasis under low temperature stress (Overgaard & MacMillan, 2017; MacMillan *et al*., 2015).

Notably, the magnitude of ion increase was consistently higher in specimens collected from Iran, particularly in the Zagros populations of the acorn weevil, compared to those from Switzerland (Landquart). This suggests a stronger physiological response to environmental stressors in the

Iranian populations. Insects inhabiting more thermally variable or harsh environments are known to exhibit enhanced plasticity in ion regulation as part of their adaptive response to abiotic stress (Bowler & Terblanche, 2008).

Given the established roles of Mg, Zn, Fe, and Cu in antioxidant defense and redox homeostasis, the elevated concentrations of these elements observed during winter may be consistent with physiological adjustments associated with cold acclimation. Cold exposure has been reported to increase reactive oxygen species (ROS) production in insects, potentially increasing the demand for metal-dependent antioxidant systems, including Cu/Zn-superoxide dismutase (Felton & Summers, 1995; Xikeranmu *et al*., 2019). However, because oxidative stress markers and antioxidant enzyme activities were not directly measured in the present study, these interpretations should be regarded as hypotheses rather than direct evidence of enhanced oxidative stress responses.

The stronger ion elevation observed in the Zagros populations is consistent with an adaptive response to more severe thermal fluctuations and environmental stress, although the present study does not establish a causal relationship between environmental conditions and the observed physiological changes. Similar geographic differences in ionoregulatory and cold tolerance strategies have been documented in other insect species, where populations from harsher climates tend to exhibit greater physiological adjustments in hemolymph composition and ion balance (Koštál *et al*., 2004; MacMillan & Sinclair, 2011). However, because this study was based on naturally occurring populations, the observed associations should be interpreted as correlational rather than causal. Controlled experimental studies will be required to determine the relative contributions of environmental conditions and intrinsic population differences to the observed physiological responses.

Interestingly, Cu showed a divergent pattern in the acorn weevil, where its concentration increased markedly in Zagros samples but remained relatively stable or slightly decreased in Beech moth. This may reflect species-specific differences in copper-dependent enzymatic activity or variation in antioxidant defense strategies, particularly involving Cu-dependent superoxide dismutase isoforms (Li *et al*., 2011). These findings suggest that seasonal and geographic variation in hemolymph ion composition is closely associated with environmental stress intensity, particularly thermal stress, and reflects adaptive physiological mechanisms that enhance survival under winter conditions.

Based on the FE-SEM–EDS analysis, the overwintering larvae of both the weevil and moth species exhibited a higher atomic percentage of carbon compared with pre-wintering individuals. This pattern may be consistent with physiological changes associated with overwintering, including alterations in body composition and reduced water content, as reported in previous studies (Denlinger, 2002; Košťál, 2006). In contrast, both the atomic and weight percentages of oxygen were lower in overwintering samples. Although this observation is compatible with the metabolic suppression commonly associated with diapause or quiescence (Hahn & Denlinger, 2011; Rozsypal & Košťál, 2010), the present study did not directly measure body water content, respiratory activity, or metabolic rate. Therefore, these interpretations should be regarded as plausible explanations rather than direct evidence of the underlying physiological processes.

A pronounced decrease in nitrogen content was observed particularly in overwintering larvae of the weevil species. This pattern may indicate reduced protein turnover and a metabolic shift away from nitrogen-rich compounds, alongside a relative increase in lipid utilization and storage, which is a well-documented feature of insect overwintering physiology (Hahn & Denlinger, 2011). Additionally, magnesium levels were generally higher in active (non-overwintering) larvae than in overwintering ones. Given the central role of Mg^{2+} in ATP-dependent enzymatic reactions and metabolic regulation, this reduction likely reflects the overall decline in metabolic activity during diapause, when energy-demanding biochemical processes are strongly suppressed (Grewal, 2012; Storey & Storey, 2012).

In contrast, trace elements such as zinc and copper showed higher atomic and weight percentages in overwintering larvae compared with active individuals, so the elemental changes in the tissues showed a similar pattern to the variations of these ions within the hemolymph, based on ICP-OES analyses. This pattern is consistent with their known involvement in oxidative stress regulation and antioxidant defense systems. Zinc and copper are essential components of key enzymes, such as superoxide dismutase (SOD), which plays a central role in mitigating the accumulation of reactive oxygen species (ROS) under environmental stress conditions, including cold exposure and diapause (Storey & Storey, 2012; MacMillan *et al*., 2015). Similar increases in trace-metal involvement during overwintering have been reported in other insect species, in which elemental redistribution is considered part of a broader physiological strategy to enhance stress tolerance (Vukašinović *et al*., 2024).

Overall, these results align well with previous studies showing that insect overwintering is accompanied by coordinated biochemical and elemental remodeling. This includes reduced levels of metabolically active elements such as nitrogen and magnesium, alongside increased representation of stress-related trace elements such as zinc and copper. Such changes reflect the integrated physiological strategy of insects to survive prolonged cold periods through metabolic depression and enhanced oxidative stress management (Denlinger, 2002; Košťál, 2006; Hahn & Denlinger, 2011). Beyond the physiological differences identified in this study, the ecological significance of these two pests further emphasizes the importance of the present research. Acorn weevils and the beech moth are among the principal acorn-feeding pests of oak forests, causing substantial losses in acorn viability and thereby limiting natural regeneration. In the Zagros forests, where *Quercus* species constitute the dominant forest trees and these ecosystems are increasingly threatened by drought, climate change, and widespread oak decline, such levels of acorn infestation may further compromise regeneration capacity. Likewise, in European oak forests, acorn-feeding insects are recognized as important ecological factors influencing seed recruitment and forest dynamics. Understanding the cold-hardiness strategies and overwintering physiology of these pests is therefore not only important for elucidating their adaptation to contrasting climatic conditions but also for predicting their persistence, distribution, and potential population outbreaks under future climate change scenarios. Such knowledge provides a physiological basis for improving pest forecasting and developing more effective management strategies aimed at conserving oak forest regeneration in both regions.

Acknowledgements

This work has been supported by the Center for International Science Studies & Collaborations (CISSC), Ministry of Science, Research and Technology of Iran, which utilized the facilities of the Iranian Research Organization for Science and Technology (IROST). The authors gratefully acknowledge the valuable collaboration of Dr. Majid Tavakoli (Agricultural and Natural Resources Research Center of Lorestan) for his significant assistance in specimen collection and species identification. We would also like to express our gratitude for the scientific advice of Professor Brent Sinclair (University of Western Ontario, Canada), who played a particularly important role in designing the framework of this research, especially at its initial stage.

References

1. Arsalani, M., Grießinger, J. and Bräuning, A. 2023**.** Tree-ring-based seasonal temperature reconstructions and ecological implications of recent warming on oak forest health in the Zagros Mountains, Iran. *Int. J. Biometeorol.*, **67(2)**, 317–331.https://doi.org/10.1007/s00484-022-02380-5

2. Beauchemin, D. 2010. Inductively coupled plasma mass spectrometry. *Anal. Chem.*, **82(12)**, 4786–4810.https://doi.org/10.1021/ac100629x

3. Block, W. 1990. Cold tolerance of insects and other arthropods. *Philos. Trans. R. Soc. Lond. B Biol. Sci.*, **326(1237)**, 613–633.https://doi.org/10.1098/rstb.1990.0035

4. Bowler, K. and Terblanche, J. S. 2008. Insect thermal tolerance and climate change. *J. Insect Physiol.*, **54**, 125–136.https://doi.org/10.1016/j.jinsphys.2007.07.009

5. Butolo, N. P., Azevedo, P., Alencar, L. D., Domingues, C. E. C., Miotelo, L., Malaspina, O. and Nocelli, R. C. F. 2020. A high quality method for hemolymph collection from honeybee larvae. *PLoS ONE*, **15(6)**, e0234637.https://doi.org/10.1371/journal.pone.0234637

6. Chapman, R. F. 2013. *The Insects: Structure and Function*. 5th Ed., Cambridge University Press, Cambridge.

7. Denlinger, D. L. 2002. Regulation of diapause. *Annu. Rev. Entomol.*, **47**, 93–122. https://doi.org/10.1146/annurev.ento.47.091201.145137

8. Dow, J. A. T. 2017. The multifunctional *Drosophila melanogaster* Malpighian tubule. *J. Insect Physiol.*, **106**, 44–54.https://doi.org/10.1016/j.jinsphys.2017.05.003

9. Duman, J. G. 1979. Thermal hysteresis factors in overwintering insects. *J. Insect Physiol.*, **25(10)**, 805–810.https://doi.org/10.1016/0022-1910(79)90083-0

10. Duman, J. G. 2001. Antifreeze and ice nucleator proteins in terrestrial arthropods. *Annu. Rev. Physiol.*, **63**, 327–357.https://doi.org/10.1146/annurev.physiol.63.1.327

11. Duman, J. G. 2015. Animal ice-binding (antifreeze) proteins and glycolipids: An overview with emphasis on physiological function. *J. Exp. Biol.*, **218(12)**, 1846–1855. https://doi.org/10.1242/jeb.118503

12. Duman, J. G. and Newton, S. S. 2020. Antifreeze and ice-recrystallization-inhibition proteins in insects. *Curr. Opin. Insect Sci.*, **41**, 1–8.https://doi.org/10.1016/j.cois.2020.04.003

13. Felton, G. W. and Summers, C. B. 1995. Antioxidant systems in insects. *Arch. Insect Biochem. Physiol.*, **29**, 187–197.https://doi.org/10.1002/arch.940290207

399 14. Fialkowski, W., et al. 2013. Elemental microanalysis of insect cuticle using SEM-EDS. *Micron*,
400 **45**, 186–192.https://doi.org/10.1016/j.micron.2013.01.001
401 15. Gabbott, P. 2008. *Principles and Applications of Thermal Analysis*. Royal Society of
402 Chemistry, Cambridge.
403 16. Gao, Y., Li, S., Zhang, Y. and Wang, X. 2016. Determination of trace metals in biological tissues
404 using ICP-OES following nitric acid digestion. *Biol. Trace Elem. Res.*, **172(2)**, 380–
405 388.https://doi.org/10.1007/s12011-015-0579-3
406 17. Grewal, P. S. 2012. Magnesium-dependent metabolic regulation in insects. *Insect Biochem.*
407 *Mol. Biol.*, **42**, 1–10.https://doi.org/10.1016/j.ibmb.2011.10.002
408 18. Hahn, D. A. and Denlinger, D. L. 2011. Energetics of insect diapause. *Annu. Rev. Entomol.*, **56**,
409 103–121.https://doi.org/10.1146/annurev-ento-112408-085436
410 19. Hansen, M., Hyldig, G. and Larsen, E. H. 2013. Quantification of trace metals in biological
411 matrices using ICP-OES. *Food Chem.*, **141(3)**, 1976–1981.
412 https://doi.org/10.1016/j.foodchem.2013.05.050
413 20. Hou, X. and Jones, B. T. 2000. Inductively coupled plasma optical emission spectrometry. In:
414 Meyers, R. A. (Ed.), *Encyclopedia of Analytical Chemistry*, pp. 9468–9485. Wiley, Chichester.
415 https://doi.org/10.1002/9780470027318.a0918
416 21. IPCC. 2023. *Climate Change 2023: Synthesis Report*. Intergovernmental Panel on Climate
417 Change, Geneva, Switzerland.
418 22. Kokot, S. and Matysiak, J. 2008. Determination of trace elements in biological samples by ICP-
419 OES after acid digestion. *J. Anal. At. Spectrom.*, **23(8)**, 1101–1106.
420 https://doi.org/10.1039/b803501a
421 23. Košťál, V. 2006. Eco-physiological phases of insect diapause. *J. Insect Physiol.*, **52(2)**, 113–
422 127.https://doi.org/10.1016/j.jinsphys.2005.09.008
423 24. Košťál, V., Vambera, J. and Bastl, J. 2004. On the nature of pre-freeze mortality in insects:
424 Water balance, ion homeostasis and energy charge in the adults of *Pyrrhocoris apterus*. *J. Exp.*
425 *Biol.*, **207(9)**, 1509–1521.https://doi.org/10.1242/jeb.00923
426 25. Košťál, V., Zahradníčková, H. and Šimek, P. 2011. Hyperactive antifreeze proteins of a freeze-
427 avoiding insect enhance supercooling capacity and prevent ice recrystallization. *Proc. Natl. Acad.*
428 *Sci. U.S.A.*, **108(10)**, 4042–4047.https://doi.org/10.1073/pnas.1017305108

26. Lee, R. E. Jr. 2010. A primer on insect cold-tolerance. In: Denlinger, D. L. and Lee, R. E. (Eds.), *Low Temperature Biology of Insects*, pp. 3–34. Cambridge University Press, Cambridge.https://doi.org/10.1017/CBO9780511675997.003

27. Li, J. M., Su, Y. L., Gao, X. L., He, J., Liu, S. S. and Wang, X. W. 2011. Molecular characterization and oxidative stress response of an intracellular Cu/Zn superoxide dismutase (CuZnSOD) of the whitefly, *Bemisia tabaci*. *Arch. Insect Biochem. Physiol.*, **77(3)**, 118–133. https://doi.org/10.1002/arch.20428

28. MacMillan, H. A. and Sinclair, B. J. 2011. Mechanisms underlying insect low-temperature tolerance. *J. Insect Physiol.*, **57** (1), 12-20. https://doi.org/10.1016/j.jinsphys.2010.10.004.

29. MacMillan, H. A., Baatrup, E. and Overgaard, J. 2015. Concurrent effects of cold and hyperkalemia cause insect chilling injury. *Proc. R. Soc. B*, **282(1817)**, 20151483. https://doi.org/10.1098/rspb.2015.1483

30. Mao, Y., Liu, X., Wang, Y. and Chen, L. 2011. Measurement of thermal hysteresis activity of antifreeze proteins using differential scanning calorimetry. *Cryobiology*, **63(3)**, 236–242. https://doi.org/10.1016/j.cryobiol.2011.06.005

31. Missirlis, F. and Oliveira, P. L. 2022. Metal metabolism and oxidative stress in insects. *Curr. Opin. Insect Sci.*, **49**, 1–7.https://doi.org/10.1016/j.cois.2021.10.003

32. Montaser, A. (Ed.). 1998. *Inductively Coupled Plasma Mass Spectrometry*. Wiley-VCH, New York.

33. Nation, J. L. 2002. *Insect Physiology and Biochemistry*. CRC Press, Boca Raton.

34. Overgaard, J. and MacMillan, H. A. 2017. The integrative physiology of insect chill tolerance. *Annu. Rev. Physiol.*, **79**, 187–208.https://doi.org/10.1146/annurev-physiol-022516-034142

35. Polilov, A. A., Makarova, A. A., Pang, S., Xu, C. S. and Hess, H. 2021. Protocol for preparation of heterogeneous biological samples for 3D electron microscopy. *Sci. Rep.*, **11**, 2993. https://doi.org/10.1038/s41598-021-83936-0

36. Pröfrock, D. and Prange, A. 2012. ICP-MS for quantitative analysis in environmental and life sciences. *Appl. Spectrosc.*, **66(8)**, 843–868.https://doi.org/10.1366/12-06681

37. Rozsypal, J. and Košťál, V. 2018. Supercooling and freezing as alternative strategies. *J. Insect Physiol.*, **111**, 53–62.https://doi.org/10.1016/j.jinsphys.2018.10.006

458 38. Rozsypal, J., Košťál, V., Zahradníčková, H. and Šimek, P. 2013. Overwintering strategy and
459 cold tolerance in *Cydia pomonella*. *PLoS ONE*, **8(4)**, e61745.
460 https://doi.org/10.1371/journal.pone.0061745
461 39. Sabo, R., Staroň, M., Sabová, L., Jančo, I., Tomka, M. and Árvay, J. 2024. Toxic and essential
462 elements in honeybee venom from Slovakia: Potential health risk to humans. *Heliyon*, **10(20)**,
463 e39282.https://doi.org/10.1016/j.heliyon.2024.e39282
464 40. Sadeghian, B., Dordaei, A. A. and Nikdel, M. 2007. Damage assessment of acorn pests in
465 Arasbaran forests. *Iran. J. For. Range Prot. Res.*, **4(2)**, 113–118.
466 41. Steinbrecht, R. A. 1993. Freeze-substitution for insect studies. *Microsc. Res. Tech.*, **24(6)**, 488–
467 504.https://doi.org/10.1002/jemt.1070240605
468 42. Storey, K. B. and Storey, J. M. 2012. Insect cold hardiness. *Can. J. Zool.*, **90(4)**, 456–475.
469 https://doi.org/10.1139/z2012-011
470 43. Thomas, R. 2013. *Practical Guide to ICP-MS: A Tutorial for Beginners*. 3rd Ed., CRC Press,
471 Boca Raton.
472 44. Vukašinović, E. L., Popović, Ž. D., Ninkov, J., Čelić, T. V., Uzelac, I., Kojić, D. and Purać, J.
473 2024**.** Management of inorganic elements by overwintering physiology of cold hardy larvae of
474 European corn borer (*Ostrinia nubilalis*, Hbn.). *J. Comp. Physiol. B*, **194(2)**, 145–154.
475 https://doi.org/10.1007/s00360-024-01537-5
476 45. Xikeranmu, Z., Abdunasir, M., Ma, J., Tusong, K. and Liu, X. 2019. Characterization of two
477 copper/zinc superoxide dismutases (Cu/Zn-SODs) from the desert beetle *Microdera punctipennis*
478 and their activities in protecting *Escherichia coli* cells against cold. *Cryobiology*, **87**, 15–
479 27.https://doi.org/10.1016/j.cryobiol.2019.03.006
480 46. Zachariassen, K. E., Kristiansen, E. and Pedersen, S. A. 2004**.** Inorganic ion homeostasis in
481 cold-acclimated insects. *J. Exp. Biol.*, **207(23)**, 4215–4223.https://doi.org/10.1242/jeb.01289
482 47. Zargaran, M. Z., Najafi, M. R., Mirab-Balou, M. and Tavakoli, M. 2018. Identification of oak
483 seed insects in Ilam forests. *Iran. J. For.*, **10(1)**, 55–65.
484
485
486
487
488

Journal of Agricultural Science and Technology (JAST), 29(3)
In Press, Pre-Proof Version

مقایسه سازوکارهای تحمل سرما در آفات بذر بلوط از ایران و سوئیس: پسماند حرارتی و پویایی عناصر فلزی همولنف

مریم عطاپور، و احمد بهمنی

چکیده

جنگل‌های بلوط منطقه زاگرس ایران به دلیل تنش‌های محیطی و افزایش طغیان آفات، با روند شدید زوال مواجه هستند. در این مطالعه، سازوکارهای بقای زمستان‌گذرانی دو آفت مهم بذر بلوط شامل شب پره بذرخوار بلوط *Cydia fagiglandana* (Zeller) و یک گونه سرخرطومی بلوط (*Curculio* sp.) با مقایسه جمعیت‌های موجود در جنگل‌های تحت تنش زاگرس و جنگل‌های پایدار بلوط در سوئیس بررسی شد.در این پژوهش، دو زیست‌نشانگر کلیدی مرتبط با تحمل سرما در سردترین ماه‌های سال ارزیابی شدند: پسماند حرارتی که با استفاده از گرماسنجی روبشی تفاضلی (DSC) اندازه‌گیری شد و غلظت عناصر با مقادیر خیلی کم موجود در همولنف(Fe، Cu، Zn وMg) با استفاده از دستگاه ICP-OES تعیین گردید. علاوه بر این، آزمون EDS–SEM برای بررسی توزیع برخی عناصر در دن لاروها به‌صورت نیمه‌کمی انجام شد. پسماند حرارتی مشخصی حدود ۴ درجه سلسیوس بین نقطه ذوب و انجماد، لاروهای سرخرطومی زاگرس مشاهده شد. در بیشتر نمونه‌ها، غلظت یون‌های فلزی در دی ماه افزایش یافت؛ به‌طوری‌که میزان عنصر روی در سرخرطومی بلوط زاگرس تقریباً چهار برابر افزایش نشان داد (از ۱۷۰ به ۵۸۵ ppb). بررسی توزیع عناصر با استفاده از EDS–SEM نیز افزایش مقادیر مس و روی و همچنین تغییر الگوی توزیع عناصر از حالت یکنواخت به تجمع‌یافته در لاروهای زمستان‌گذران را نشان داد. این مطالعه دیدگاه‌های جدیدی درباره فیزیولوژی زمستان‌گذرانی دو حشره مهم تغذیه‌کننده از بذر بلوط تحت شرایط اقلیمی متفاوت ارائه می‌دهد. این یافته‌ها می‌توانند به پیش‌بینی پاسخ آفات به تغییرات محیطی کمک کرده و از برنامه‌های آینده حفاظت و مدیریت تجدید حیات جنگل‌های بلوط حمایت کنند.

Table 1. Melting temperature (Tm), freezing temperature (Tf), and calculated thermal hysteresis (TH = Tm − Tf) of hemolymph samples obtained from acorn weevil (*Curculio* spp.) and the beech moth (*C. fagiglandana)* larvae collected in November 2024 and January 2025 from Zagros (Iran) and Landquart (Switzerland).

Pest	Location	Date	Melting point (Tm) °C (mean ±SE)	Freezing point (Tf) °C (mean ±SE)	Thermal hysteresis (Tm − Tf) °C (mean ±SE)
Curculio spp.	Zagros	Nov	-5.9±0.4 ^{b*}	-7.6±0.2 ^{b}	1.7±0.1 ^{b}
		Jan	-2.03±0.5 ^{a}	-6.1±0.5 ^{ab}	4.1±0.7 ^{a}
	Landquart	Nov	-10.1±0.3 ^{c}	-10.4±0.4 ^{c}	0.3±0.1 ^{b}
		Jan	-4.9±0.4 ^{b}	-5.6±0.3 ^{a}	0.6±0.08 ^{b}
C. fagiglandana	Zagros	Nov	-4.4±0.1 ^{A}	-5±0.1 ^{A}	0.6±0.06 ^{A}
		Jan	-6.7±0.1 ^{B}	-7±0.1 ^{B}	0.2±0.08 ^{A}
	Landquart	Nov	-15.3±0.1 ^{C}	-15.9±0.3 ^{C}	0.6±0.2 ^{A}
		Jan	-16.6±0.3 ^{D}	-17.4±0.2 ^{D}	0.8±0.1 ^{A}

*For each insect species, means followed by the same letter within a column are not significantly different according to Tukey's test (P > 0.05). Lowercase letters indicate comparisons among acorn weevil, whereas uppercase letters indicate comparisons among beech moth.

Table 2. Concentrations of copper (Cu), iron (Fe), magnesium (Mg), and zinc (Zn) in the hemolymph of acorn weevil (*Curculio* spp.) and the beech moth (*C. fagiglandana)* larvae collected in November 2024 and January 2025 from Zagros (Iran) and Landquart (Switzerland).

Pest	Location	Date	Cu ppb (m±SE)	Fe ppb (m±SE)	Mg ppb (m±SE)	Zn ppb (m±SE)
Curculio spp.	Zagros	Nov	57.05±0.78 ^{b*}	236.49±31.55 ^{b}	2671.27±298.3 ^{b}	169.14±9.75 ^{b}
		Jan	112.27±12.9 ^{a}	392.06±12.43 ^{a}	5957.5±461.2 ^{a}	585.3±18.9 ^{a}
	Landquart	Nov	33.14±5.79 ^{b}	73.63±7.44 ^{c}	1704.52±234 ^{b}	74.31±13.9 ^{c}
		Jan	36.86±2.68 ^{b}	95.51±3.5 ^{c}	2360.42±279.1 ^{b}	131.28±4.22 ^{bc}
C. fagiglandana	Zagros	Nov	60.61±4.03 ^{A}	214.44±16.07 ^{B}	2280.45±88 ^{A}	215.3±15.9 ^{B}
		Jan	56.55±3.39 ^{A}	318.1±12.45 ^{A}	2765.94±62.3 ^{A}	430.26±17.0 ^{A}
	Landquart	Nov	50.38±3.69 ^{A}	93.78±14.68 ^{C}	1140.36±65.8 ^{B}	86.19±5.67 ^{C}
		Jan	45.55±3.86 ^{A}	157.85±12.20 ^{B}	2022.94±389.9 ^{AB}	185.84±17.4 ^{B}

*For each insect species, means followed by the same letter within a column are not significantly different according to Tukey's test (P > 0.05). Lowercase letters indicate comparisons among acorn weevil, whereas uppercase letters indicate comparisons among beech moth.

Table 3. Elemental composition (Weight Percent (w%) and Atomic Percent (at.%), mean ± SE) of Iranian acorn weevil (*Curculio* sp.) and beech moth (*C. fagiglandana*) larvae collected in November 2024 and January 2025, determined by FE-SEM–EDS analysis. Data are presented for 6 major and trace elements including C, N, O, Mg, Cu, and Zn.

Elt.	*Curculio* sp. w% (m±SE)	(Nov) at.%(m±SE)	*Curculio* sp. w%(m±SE)	(Jan) at.%(m±SE)	*C. fagiglandana* w%(m±SE)	(Nov) at.%(m±SE)	*C. fagiglandana* w%(m±SE)	(Jan) at.%(m±SE)
C	35.51±0.09 ^{d*}	42.04±1.2 ^{D}	51.36±0.66 ^{b}	65.86±0.92 ^{A}	43.2±0.77 ^{c}	49.12±0.57 ^{C}	55.12±0.57 ^{a}	59.78±0.88 ^{B}
N	12.83±0.91 ^{b}	13.17±0.23 ^{A}	6.69±0.43 ^{d}	6.88±0.25 ^{C}	15.9±0.38 ^{a}	13.82±0.73 ^{A}	9.47±0.27 ^{c}	9.17±0.44 ^{B}
O	44.37±1.2 ^{a}	39.17±0.12 ^{A}	24.09±0.14 ^{d}	23.34±0.35 ^{D}	35.9±0.95 ^{b}	32.28±0.7 ^{B}	28.26±0.46 ^{c}	27.44±1.51 ^{C}
Mg	6.22±0.23 ^{a}	3.74±0.29 ^{A}	4.84±0.68 ^{ab}	1.42±0.29 ^{C}	4.77±0.25 ^{ab}	288±0.16 ^{AB}	3.32±0.56 ^{b}	2.55±0.23 ^{BC}
Cu	1.18±0.12 ^{bc}	0.27±0.03 ^{B}	5.48±0.12 ^{a}	1.28±0.11 ^{A}	0.74±0.36 ^{c}	0.17±0.01 ^{B}	1.75±0.18 ^{b}	0.21±0.01 ^{B}
Zn	0.48±0.04 ^{c}	0.13±0.01 ^{B}	5.53±0.26 ^{a}	0.32±0.05 ^{A}	0.46±0.03 ^{c}	0.15±0.02 ^{B}	1.54±0.25 ^{b}	0.35±0.04 ^{A}

*For each element, means followed by the same letter within a row are not significantly different according to Tukey's test (P > 0.05). Lowercase letters indicate comparisons among weight percentages, whereas uppercase letters indicate comparisons among atomic percentages in each row.

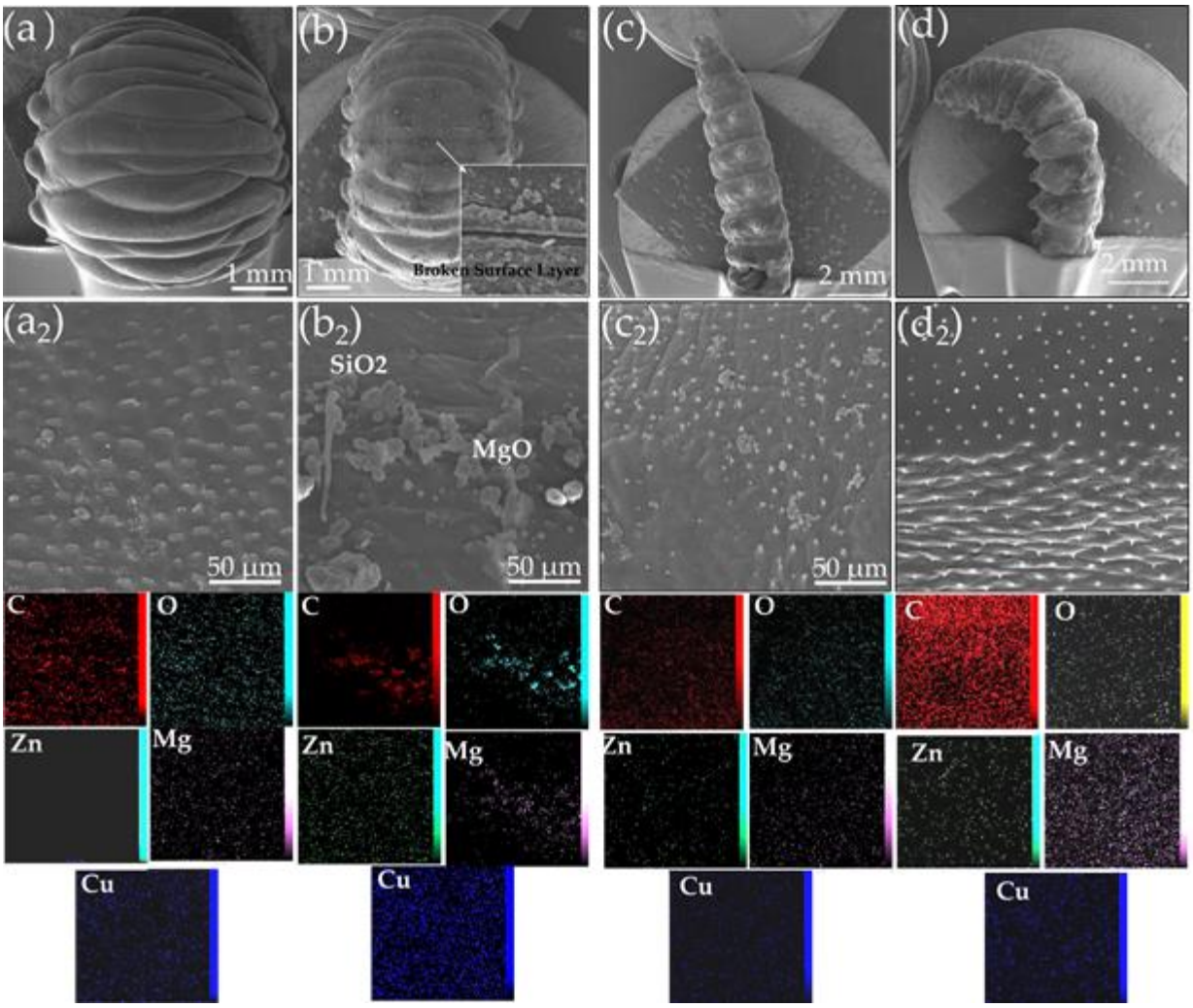

Fig. 1. A representative elemental map of an whole larval body of a sample of Iranian acorn weevil larvae (*Curculio* sp.) collected in November 2024 (**a**) and January 2025 (**b**) and Iranian beech moth larvae (*C. fagiglandana)* collected in November 2024 (**c**) and January 2025 (**d**), obtained by FE-SEM–EDS. Images a_2 - b_2 and c_2-d_2 show a randomly selected area of the dorsal surface of the larval body at 50× and 25× magnification respectively. C: carbon, N: nitrogen, O: oxygen, Mg: magnesium, Cu: copper, and Zn: zinc.