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Research Article

Eco-physiological adaptations to increase survival during winter in the lime butterfly, *Papilio demoleus* (Lep: Papilionidae)

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Abstract: The overwintering conditions and cold tolerance of the Lime butterfly, *Papilio demoleus* L., are relatively unclear. In the present study, overwintering pupae were collected over several months, and changes in supercooling point (SCP), cold-hardiness, and body color were investigated. Also, some eggs were collected and reared in the laboratory, and then pupae were kept for 1-4 weeks at 0, 5, and 15 °C, and the changes in SCPs were studied. Shelters were prepared and installed in the field to check the overwintering sites. Also, the digestive tract was investigated before and at the beginning of the overwintering phase. The results showed that the SCP gradually decreased from -9 to -13 °C during the autumn months, and in lab-reared pupae treated at 0-5 °C, the SCP was reduced to -13 °C. None of the pupae survived at temperatures below the SCP, so this species uses a freeze avoidance strategy. As the weather gets cold, the color of most of the pupae (76.19%) tends to brown, while in the summer months, more than 80% of the pupae are green. The overwintering pupae emptied their ingested foods, and the contents of their digestive system before emptying contain *Pseudomonas* and *Enterobacter* species, both of which can act as ice nucleators. Ultimately, the relationship between these factors in adapting the pest to low temperatures was discussed.

Keywords: Lime butterfly, overwintering, supercooling point, pupal color change, cold tolerance

Introduction

Seasonal temperature changes, especially low temperatures, are one of the most critical challenges for insects during their life cycle. Insects adapt to these changes through behavioral, ecological, biochemical, and physiological methods (Denlinger, 1991; Kostal, 2006). During the cold winter months in temperate regions, a few species can remain active under and even on the snow cover. Still, most insects enter a developmental arrest and become inactive during this time (Lee, 2010). Understanding the abilities of insects and the changes that occur under low temperatures can be used to complete the information on the biology and their optimal management in pest control programs.

The lime butterfly is one of the citrus pests in the north and south of Iran. This insect attacks citrus trees, and its larvae feed on young branches and leaves, mainly newly grown buds.

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The damage caused by the pest is severe in the nurseries and newly built gardens, so feeding on the edges of the leaves makes the trees bare. This pest mainly attacks oranges and sour oranges and then limes and tangerines. It spends 3-5 generations per year in different regions; each generation has five larval stages, and finally, the pupae of the last generation enter the overwintering stage (Kholghi-Eshkalak *et al*., 2017; Islam *et al*., 2019).

In many citrus-growing regions, the weather conditions are mostly moderate and without severe winters, as seen in the southern parts of Iran. However, citrus trees in the northern provinces face low and sub-zero temperatures several days a year, and pupae have adapted well. This adaptation can be caused by behavioral, ecological, biochemical, and physiological changes. The previous studies conducted in lepidopteran pests have shown that in some species, the lipid or glycogen reserves increase up to 1.5 times during the winter diapause. For example, the diapause larvae of the European corn borer, *Ostrinia nubilalis* Hübner (Beck and Hanec, 1960) or the rice stem borer, *Chilo suppressalis* (Walker) (Atapour *et al*., 2008).

In some species, internal biochemical changes lead to external manifestations, which may cause better camouflage in winter shelters, absorption of more light and heat from the environment, etc. For example, in some moths of the Noctuidae family, pigmented stemmata appear in pupae during winter diapause (Hackett and Gatehouse, 1982). The change in the color and shape of wintering insects is more noticeable in butterflies. For example, in the *Papilio xuthus* L., diapausing pupae are brown and smaller than non-diapause green pupae (Tanaka and Tsubaki, 1984). Similarly, a short photoperiod induces diapause and brown color in overwintering pupae in some swallowtail butterflies, such as *Papilio polyxenes* F. and *Papilio troilus* L. (Hazel and West, 1983). Also, during the study of Kaneko and Katagiri (2006), it was found that the diapause pupae of *Pieris brassicae* L., due to the smaller internal cavity between the thorax and abdomen, have a specific gravity above 0.1. As a result, they drown in water; the non-diapausing pupae have a larger cavity, and their specific gravity is less than one. Therefore, they float on the water, and it was proved that this method could be used as a reliable way to detect the diapause status of this insect (Atapour, 2016). Although sometimes the reason for these changes and their benefits for the insect is unclear, it is probably an adaptive solution to pass through the severe conditions of the winter months.

In addition to appearance changes, behavioral adaptations can be seen in some insects in winter. In some species of ladybird beetles, bugs, or moths of the Noctuidae family, collective behavior can be seen in winter shelters. Such behavior can be caused by the lack of shelter and suitable space to spend this period or be related to reproductive behaviors, or even in the direction of greater protection against enemies and predators. Making a cocoon is one of the same behavioral adaptations seen, for example, in some species of moths in the Tortricidae, Pyralidae, and Saturniidae families during winter (Danks, 2006; Denlinger, 2002).

Also, stopping feeding and emptying the contents of the digestive system are among the behavioral adaptations that can be seen in some insects before entering the overwintering phase. It seems that this action leads to the removal of food particles and bacteria known as INA and can accelerate the formation of ice crystals inside the digestive system, which will cause death. Ice nucleating active (INA) bacteria uniquely form ice crystals at temperatures close to -1 to -2 °C. These bacteria, which are generally Gram-negative and are located in the genus *Pseudomonas*, *Xanthomonas*, *Pantoea*, *Comamonas*, and *Erwinia*, were found mainly on the leaves and in the marginal parts, especially older leaves, and can act as a catalyst to cause frostbite in the leaf, as well as the insects that feed on them (Lee *et al*., 1993, Steigerwald *et al*., 1995). For example, the behavior of emptying the digestive system can be seen in the overwintering larvae of the rice stem-borer larvae, which increases the cold tolerance in winter by stopping feeding and emptying the contents of the digestive canal in autumn (Tsumuki *et al*., 1992).

Considering the little information about the overwintering conditions of this species, the present study was carried out so that the information related to the biology of this pest, especially this part of the life cycle, will be clarified. This information can be used to make decisions about its optimal control, such as predictions related to mortality in pest population prediction models.

Materials and Methods

Collecting and insect rearing in the laboratory

Collection of last instar larvae:

During August, September, October, and November, 45 - 50 last instar larvae, along with citrus leaves (for feeding) from sour orange trees located near the Entomology Laboratory of the Iranian Research Organization for Scientific and Technology (35.62 °N, 51.18 °E, 1250 m a.s.l) were randomly collected and placed inside a container. The insects were kept outside the laboratory until pupation. Rearing containers (23 L × 15 W × 10 H cm) were checked and cleaned daily, and fresh leaves were replaced with used leaves. Due to the difficulty of accessing and collecting pupae in sufficient numbers for experiments, this work was done. For this purpose, the last instar larvae were collected and pupated inside the containers about 7-10 days after collection, and their coloring percentage (Fig. 1) and their supercooling point were measured 48 hours after the emergence of the pupae. After conducting preliminary tests and determining the supercooling point, a temperature of -15 °C was chosen, and at least 30 pupae were tested after 6, 12, and 24 hours of treatment at this temperature. Then, the survival rate was checked based on the possibility of moving the end part of the body.

Collection of eggs:

In this experiment, eggs were collected from sour orange trees in September and transferred to the laboratory. All were incubated in plastic containers at 25 ± 2 °C, 60 ± 5% RH, and photoperiod of 16L: 8D until the emergence of pupae. Then, six different temperature treatments were performed on pupae according to Fig. 2. Fresh citrus leaves were used to feed the larvae. In these six treatments, one or four weeks of storage at 15, 5, and 0 °C were investigated.

Supercooling point measurement

Each pupa was affixed with sticky tape to a thermocouple (NiCr-Ni probe) attached to an automatic recorder (Tes 1384, Data logger 4 Input Thermometer, Taiwan) to measure the supercooling points (SCPs) of the whole body. Specimens were placed in the test chamber whose temperature was decreased from +25 to -25 ºC at a rate of 1 ºC /min. The cooling rate was recorded at 20-second intervals. The SCP was taken as the lowest temperature before the temperature increase caused by the latent heat of crystallization (Neven 1999).

Figure 1 Comparison of the color difference of *Papilio demoleus* pupae collected in August (right) and November (left).

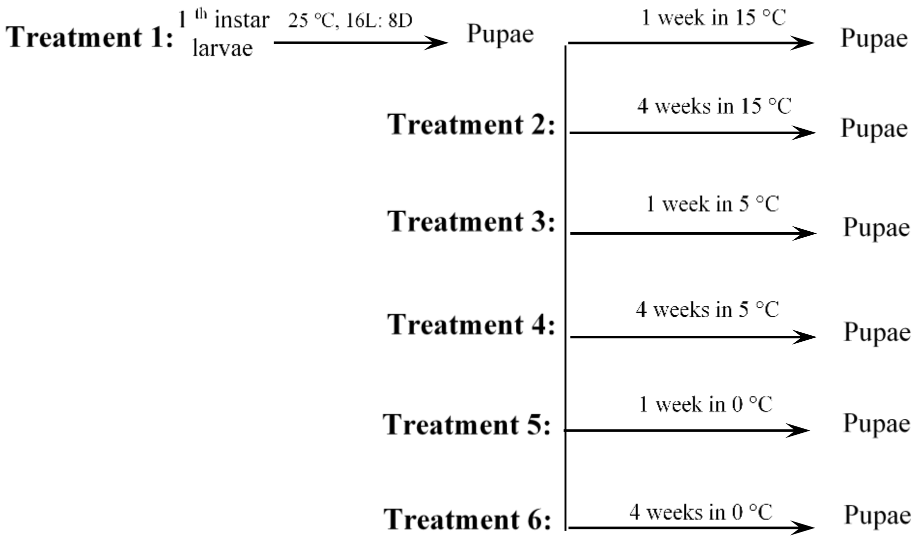

Figure 2 Different temperature treatments on *Papilio demoleus* pupae in the laboratory.

Installation of shelters to determine the overwintering sites of the pupae

Special shelters were installed on the trees to investigate the overwintering places of the pupae (Fig. 3). The pupae of some butterflies spend the winter in shelters on non-host trees. During the past years, no overwintering pupae were found on the citrus trees, so during the summer months, these shelters were installed to check whether the pupae of this pest or other species take refuge in these places during the winter. Different parts were built using natural materials, such as pinecones, hollow stems of plants, straw /stubble, corrugated cardboard, etc. in these shelters. In April of the second year of installation, the shelters were examined.

Examining the contents of the alimentary canal and bacterial flora

Dissections to investigate the alimentary tract were performed on either the last instar larvae collected in August or November. The dissection tools and plates were washed in 70% ethanol before dissection. The larvae were individually placed into 70% ethanol, gently shaken for 3 min, washed three times with distilled water for surface sterilization, transferred to filter paper, and were anesthetized by keeping them at 0 °C for 15 min (Yaman *et al.*, 2017). Larvae were individually transferred onto a Sylgard-lined dissection dish with a clear silicone pad (150 mm × 20 mm) and prepared for alimentary tract dissection.

Figure 3 The shelter installed on the tree to investigate the possible overwintering site of the *Papilio demoleus* pupae

The larvae were pinned through the sides of the prothorax and the last abdominal segment to hold it ventral side down with the body straight. Then, the cuticle was cut along the dorsal midline anteriorly using scissors, and the whole part was separated from the digestive system (Fig. 4, Torson *et al*., 2020). The alimentary tract was mixed with 1 ml of sterile normal saline solution into a sterile 1.5 ml microtube and placed in a microcentrifuge (Tomos, China) for 3 minutes to completely homogenize the contents of the digestive tract with normal saline.

A drop of the prepared sample was taken from the dissected insect, diluted from 10-1 to 10-5 with sterile water, and spread on nutrient agar plates. The plates were incubated at 30 °C for five days. After incubation, the plates were examined, and bacterial colonies were selected. The selected colonies were purified by subculturing on plates. Different colony types of bacteria were selected and purified on nutrient agar plates by subculturing. All bacterial isolates were initially stained with Gram's dye to identify gram-negative bacteria. Then, the OF test (oxidative-fermentative test) was performed to check the state of bacterial metabolism. Based on the fermentative or non-fermentative metabolism, biochemical tests were performed to identify a gram-negative bacteria genus (Castrillo *et al*., 2000 with some modifications; Yaman *et al*., 2017). The isolates bacterial strains were preserved for long-term storage in nutrient broth with 15% glycerol at –80 °C for further studies at Persian Type Culture Collection (PTCC), Iranian Research Organization for Science and Technology (IROST).

Data analysis

Analysis was performed using the SPSS version 16.0. All data were expressed as mean ± SE. Differences between treatments were compared by one-way analyses of variance (ANOVAs), followed by a Tukey's test for multiple comparisons at $P < 0.05$, and the graphs were drawn using Excel 2013 software.

To determine the SCPs of pupae collected during four months, each insect was used as a replicate, and at least 30 replicates were used. During different temperature treatment tests, 20 insects were examined as 20 replicates in each treatment. To check the contents of the digestive system, at least four active last instar larvae one day before pre-pupation (collected in August, day 14 of the larval period) and four last-instar larvae of the last generation overwintering one day before pre-pupation (collected in November, day 21 of the larval period) and each larva was considered as a replicate. The data were statistically analyzed using SPSS 16.0 software. The graphs were drawn using Excel 2013 software.

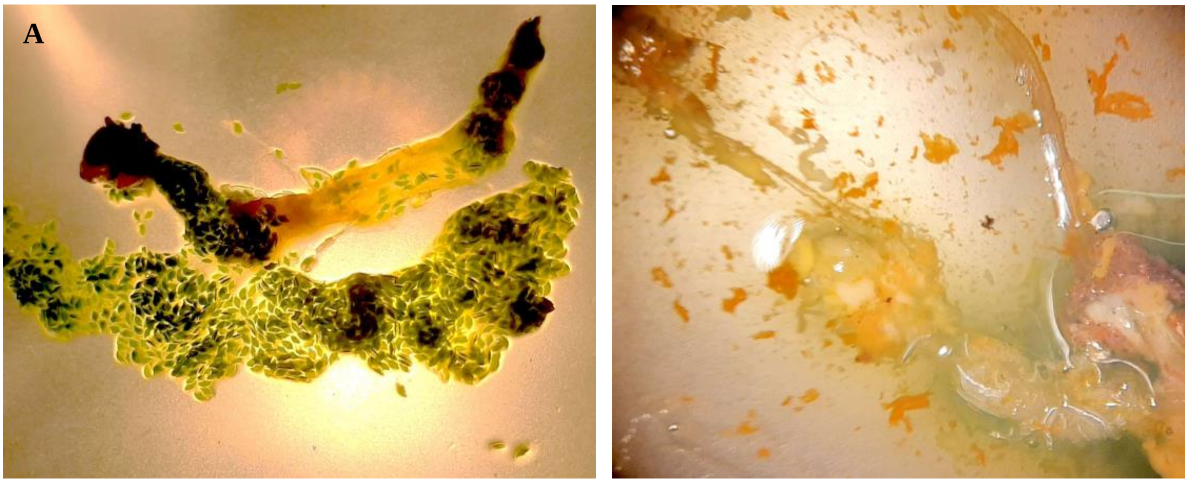

Figure 4 Alimentary canal and its contents in a sample of last instar larva with normal pupation period (A) compared to last instar larva with overwintering pupation (B) of *Papilio demoleus.*

Results

Supercooling point of the whole body

The changes in SCPs of pupae collected from August to November are shown in Fig. 5. Generally, the SCPs decreased significantly from about -9 °C to -13 °C. The SCP was almost constant during the two months of summer, but it declined significantly in October and especially in November.

The SCPs of the treated pupae that were kept for 1 or 4 weeks at 15 °C were the same as the pupae collected in summer (about -9 °C) but significantly decreased to -12 to -13 °C when pupae were kept at 5 or 0 °C for 1 or 4 weeks (Fig. 6).

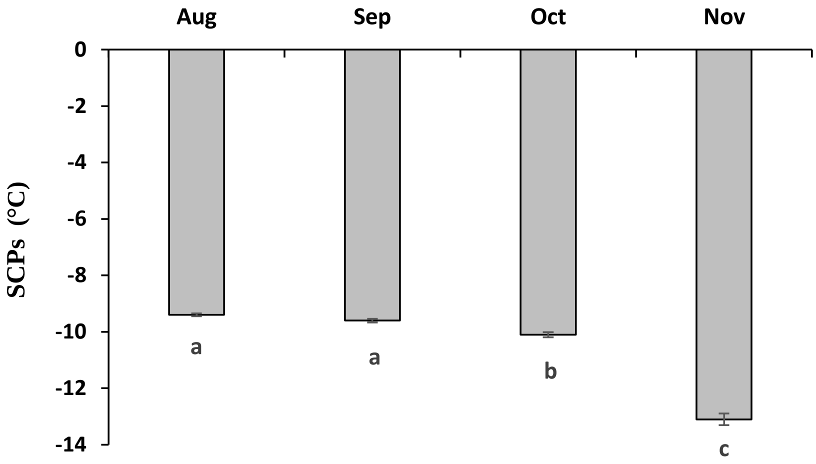

Figure 5 Changes of the supercooling points (SCPs) of *Papilio demoleus* pupae collected from August to November. Means followed by the same letter in each column are not significantly different using Tukey's test at $P < 0.05$ in each column.

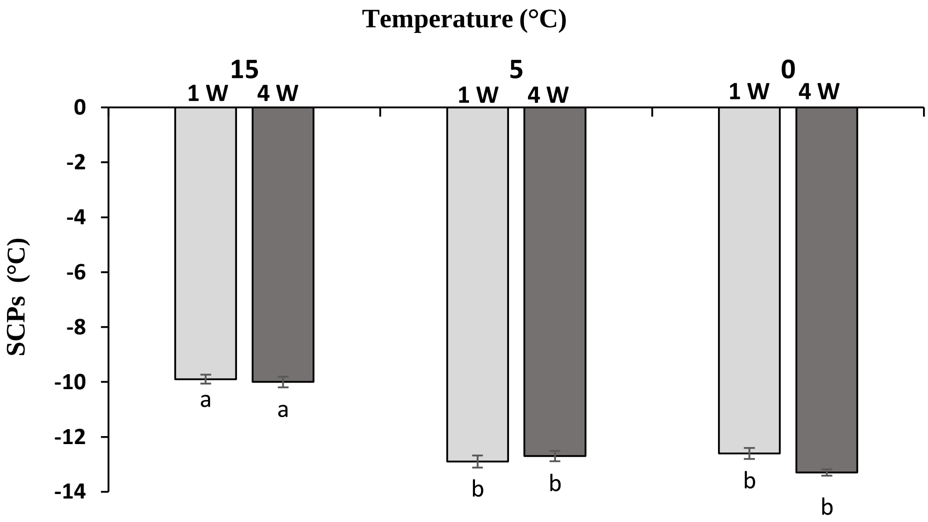

Figure 6 Changes of supercooling points (SCPs) of *Papilio demoleus* pupae treated at 15, 5 or 0 °C for one or four weeks (1 W, 4 W). Means followed by the same letter in each column are not significantly different using Tukey's test at $P < 0.05$ in each column.

However, no significant difference was observed between acclimated temperatures, and the time of one or four weeks indicates that exposure to 5 °C or below for one week can reduce the SCPs to -13 °C.

Color changes of pupae

Examining the color of the pupae collected from citrus trees showed that during the two months of summer, more than 80% of the pupae were green, and less than 20% were brown, while in the next two months, about 84% of the pupae turned brown. The rest were green (Fig. 7). Therefore, exposure of immature stages (larvae) to low temperatures and possibly other environmental factors such as photoperiod can induce these color changes.

Overwintering shelters

Previous studies have shown that at least one year is needed for insects to recognize the shelters and settle in them for overwintering, so in the spring of the second year, the shelters were examined. Although no pupae of the lime butterfly were found in these shelters, many valuable and predatory insects had sheltered in these places to spend the winter. Insects such as spiders, yellow jacket bees, some cockroaches, earwigs, and some species of beetles were found in these shelters. Further investigations revealed that the overwintering pupae of this insect (often brown) are formed on the branches of trees near citrus trees - often in places where the density of branches is higher – also the other structures with the same color as themselves (connection with the silk belt, Fig. 8).

Figure 8 Attaching the overwintering pupae to the structures of the same color near citrus trees with a fine silk girdle.

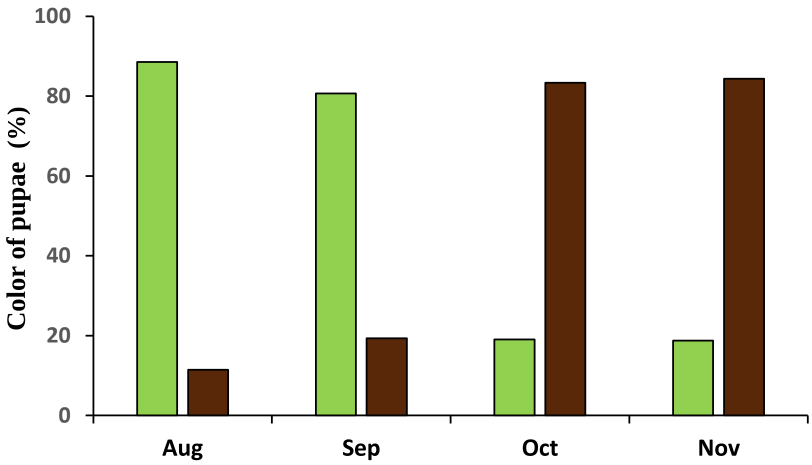

Figure 7 Percentage of body color changes (green-brown) of *Papilio. demoleus* pupae collected from August to November. Green and brown colors indicate nondiapausing and diapausing pupae.

The contents of the alimentary canal and bacterial flora

The contents of the alimentary canal of late-instar larvae with normal pupation period (collected in August) and late-instar larvae with overwintering pupation period (collected in November) were examined (Fig. 4). It was found that in normal larvae, one day before pre-pupation, the digestive system was still full of food contents (cut leaves), in contrast, in the larvae of the overwintering generation, the contents of the digestive system were completely emptied one day before pre-pupation and also, not much feeding was done in the last days of the larvae. Due to the prolonged larval period in this generation (average 22 days in larvae collected in November compared to an average of 15 days in normal larvae, unpublished data), there seems to be enough time to stop feeding and empty the digestive tract in these larvae.

A total of 19 colonies were formed among the examined samples, of which 15 were gram-positive and were seen in the digestive system of larvae collected in August and November. Only 4 Gram-negative colonies were observed in the contents of the digestive tract of larvae with normal pupation (collected in August). Considering that known ice nucleating active (INA) bacteria are generally Gram-negative, therefore gram-positive colonies were omitted, and according to the results of the of and biochemical tests, two genera of *Pseudomonas* and *Enterobacter* were identified, although accurate species identification required molecular tests, which were not performed in this study.

Discussion

It was considered that the SCPs of the pupae that were gradually exposed to low temperatures outside and lab-reared pupae, acclimated at low temperatures, decreased, and none of the pupae survived at a temperature lower than SCPs. It seems these pupae use freeze avoidance cold-hardiness strategy.

Most of the studied insects, especially in temperate regions, use the freezing avoidance strategy. For example, in previous studies in Iran, the alfalfa weevil *Hypera postica* (Gyllenhal) similarly reduces its supercooling point from -8 to -13 °C during the cold months (Saeidi and Moharramipour, 2017). With a similar pattern, the codling moth, *Cydia pomonella* (L.) (Khani and Moharramipour, 2010), elm leaf beetle, *Xanthogaleruca luteola* (Müller) (Soudi and Moharramipour, 2011), potato tuberworm, *Phthorimaea operculella* (Zeller) (Hemmati *et al*., 2014), beet moth, *Scrobipalpa ocellatella* Boyd. (Ganji and Moharramipour, 2017), Large cabbage white butterfly, *P. brassicae* (Atapour, 2016, 2017), and others also use a similar strategy.

Freeze avoidance is the most common adaptation to cold temperatures compared to the freeze tolerance strategy. Freeze-susceptible insects lower their SCP by producing antifreeze or heat-shock proteins and accumulating cryoprotectants to prevent freezing. Also, they empty their bodies of ice nucleators such as food particles, fungi spores, or some bacteria in the digestive tract. In freeze-susceptible species, SCP decreases are correlated with more severe winter temperatures. Mortality can still occur in freeze-susceptible insects if temperatures go below the SCP. While most freeze-tolerant insects freeze at relatively high temperatures to avoid the rapid formation of ice crystals that can cause injury, these species try to increase the SCPs as much as possible, and it seems that this process requires spending more energy compared to lowering of SCPs in the freeze avoidant strategy. In addition, some researchers believe insects in the northern hemisphere have mostly adapted freeze avoidance strategies because the winter months are more prolonged, colder, and more extreme. In contrast, freeze-tolerant insects dominate the southern hemisphere, where cold temperatures are less durable and intensive (Colinet *et al*., 2015; Kostal, 2006; Sinclair, 1999).

The observations showed that most of the pupae had turned brown with the decrease in temperature. A recent study on this species in Bangladesh has also indicated similar results. In the larvae that were reared in conditions with low temperatures, most of the pupae (76.19%) turned brown, while most of the pupae whose larvae were grown at higher temperatures (65.38%) turned green (Islam *et al*., 2019). Such appearance changes have been seen in some other overwintering insects. For example, in another butterfly of the same genus, *P. xuthus*, diapausing pupae are brown and smaller than non-diapausing pupae, which are green (Tanaka and Tsubaki, 1984). In previous studies on the relationship between photoperiod and color and pupation site of some species of butterflies, it has been found that diapause and pupal color in some species such as *P.polyxenes* and *P.troilus* are strongly affected by larval photoperiod and short photoperiods eliciting brown diapausing pupae although in some species such as *Battus philenor* (L.) and *Eurytides marcellus* F. pupal color is not affected by larval photoperiod, and it seems this phenomenon is related to their ecological characteristics and natural population site in winter (Hazel and West, 1983; Hazel *et al*., 1998). It has also been determined that the green or blue photoreceptors of the larval eyes (stemmata) of some species of swallowtail butterflies, caused by the secretory vesicles in the cells of the ventral ganglia of the brain, cause the release of the neuropeptide hormone, PMRF, which ultimately leads to the change of pupal color to green or brown (Mellencamp *et al*., 2007).

Regarding the overwintering places of the pupae, it seems that the reason for this relocation and the change in the color of the overwintering pupae is ultimately due to more camouflage and shelter from predators. However, more studies in a longer period and wider locations can clarify more complete information on this issue.

The overwintering pupae emptied their ingested foods, and their digestive system contained Pseudomonas and Enterobacter species before emptying. A similar study on the overwintering adults of the elm leaf beetle, *X. luteola* found that during the winter, the contents of the digestive tract were wholly emptied, and the SCP was also reduced. These changes, along with the increase of myo-inositol (as a cryoprotectant polyol), could increase the cold-hardiness of the insect at low temperatures (Soudi *et al*., 2011). It has also been found that *P. syringae* bacteria can significantly affect the SCPs of larvae and pupae of the olive leaf moth, *Palpita unionalis* (Hübner) (Hekmat *et al*., 2015).

Treatment of adults of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), which are freeze intolerant, with a solution containing the Gram-negative bacterium *P. syringae*, increased the SCPs from -7.6 to -3.7 °C, so as a result, mortality increased in low temperatures (Lee *et al*., 1994). The current study showed that in the last-instar larvae of the overwintering generation of the lime butterfly, food particles and ice-nucleating bacteria are emptied from the digestive tract as much as possible. One of the reasons for reducing the freezing point in overwintering pupae can be the discharge of this bacteria from the body.

Conclusion

The fact that the exploration of a suitable overwintering shelter and the production of cryoprotectants are stimulated by some environmental stimuli and controlled by hormones, in addition to the truth that several compounds can be produced and increased during the winter, shows that cold-hardiness is a dynamic phenomenon that is well connected with environmental conditions. This definition differs from the previous concept, which introduced cold-hardiness as a fixed and inflexible stage to protect the organism against low temperatures. Interpretation and understanding of many factors of tolerance to low temperatures require accurate evaluations of the habitat temperature over a long winter period.

In the case of the lime butterfly, citrus orchards are usually located in temperate areas with high humidity, so they are expected not to face very low temperatures. In the southern regions of Iran, this issue is entirely accurate. Still, in the northern areas, there are at least several days with snow, frost, and sub-zero temperatures in the winter. Therefore, it is expected that this species has developed suitable adaptations to tolerate low temperatures in winter. Moving from evergreen citrus trees, with foliage that provides shade, to surrounding deciduous trees or structures that are exposed to sunlight, and besides that, changing the color of the pupae to brown and dark brown for camouflage among these leafless stems, shows a fascinating example of adaptation and intelligence of an insect species.

Such a subtle and intelligent movement was observed in the overwintering larvae of the beet armyworm, *Spodoptera exigua* (Hübner), which also uses the freezing avoidance strategy. While during the regular activity in the spring and summer months, the larvae pupate beneath the soil around the beet bushes, the larvae of the last generation migrate to the sunny side of the ditches and the edges of the fields in late summer or early autumn, despite not harvesting beet bushes in the fields. They pupate inside mud chambers, and in this way, they benefit from the heat of the sun as much as possible, and they avoid low temperatures as much as possible in an ecological way (Atapour *et al*., 2011; Atapour and Moharamipour, 2014).

According to the series of studies that have been carried out in recent years in Iran on cold-hardiness strategies of some insects, it has been found that most of the studied species reduce the SCPs by several degrees during the cold months. Their minimum lethal temperature is also in the same range; therefore, most species use the freeze avoidance mechanism (To review, refer to Atapour and Moharramipour, 2021). Only two species have been known as freeze tolerant (moderate) in Iran, which include rice stem borer, *C. suppressalis* (Atapour and Moharramipour, 2009), and sunn pest, *Eurygaster integriceps* Puton (Araghie Farahani *et al*., 2010, 2014). Unlike other studied species, the SCP in these two species increases during the cold months, and they can tolerate temperatures below the SCPs. These results seem reasonable considering the weather conditions of the country. The studies in this field are still limited, and it is necessary to conduct more studies on other species, especially those active in the country's cold regions.

On the other hand, the freeze-tolerant strategy requires more complex solutions. Also, it consumes more energy (Toxopeus and Sinclair, 2018), so it can be expected that most insect species in Iran use the freeze avoidance strategy. Regarding these last two species, according to their overwintering places (the end of the rice stalks, which are usually placed in water in the winter, in the case of rice stem-borer larvae, or beneath litter under shrubs and trees in high elevations in the case of sunn pest), justifies using of the freeze tolerance strategy.

Along with ecological adaptations, behavioral and physiological changes also occur in overwintering insects. The reduction and cessation of feeding in the last-instar larvae of the overwintering generation of lime butterfly and the emptying of the contents of the digestive system provide the opportunity for the insect to remove a large number of microbes, especially the Gram-negative ice-nucleating active bacteria, from the body. As a result, along with other physiological mechanisms, it reduces the supercooling point by several degrees and thus avoids the possibility of body freezing.

The current study investigated ecological and behavioral strategies for lime butterfly use during cold months. It was generally found that the exposure of larvae to low temperatures (0 to 5 °C) can decrease the freezing point by several degrees. However, emptying the digestive tract also contributes to this multi-degree reduction. Also, changing the place of pupation in the last generation, sheltering in sunnier branches, and changing the color from green to dark brown probably help this species avoid freezing as much as possible. Although various biochemical and physiological changes also occur inside the body of overwintering insects, future studies can clarify this issue and provide a more complete understanding of how this pest overwinters in the northern parts of Iran, which faces relatively colder conditions in winter compared to many areas under citrus cultivation in the word.

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Declaration of conflicting interests

"The Author states that there is no conflict of interest."

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تطابق‌های اکوفیزیولوژیکی جهت افزایش بقا در زمستان در پروانه برگخوار مرکبات، *Papilio demoleus* (Lep: Papilionidae)

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چکیده: اطلاعات کمی در مورد چگونگی زمستان‌گذرانی و تحمل دماهای پایین پروانه برگخوار مرکبات، *Papilio demoleus* در دست می‌باشد. در مطالعه حاضر، شفیره‌های زمستان‌گذران طی چندین ماه جمع‌آوری و تغییرات نقطه انجماد، میزان تحمل به سرما و تغییرات رنگ شفیره‌ها بررسی گردید. همچنین تعدادی تخم جمع‌آوری و در آزمایشگاه پرورش یافت سپس شفیره‌ها به‌مدت یک و یا ٤ هفته در ۳ دمای صفر، ۵ و ۱۵ درجه سلسیوس نگه‌داری شدند و تغییرات نقطه انجماد آنها بررسی شد. جهت بررسی مکان‌های زمستان گذرانی شفیره‌ها، پناهگاه‌هایی آماده و در نواحی مختلف نصب گردید. همچنین وضعیت دستگاه گوارش و فون باکتری‌های موجود نیز قبل و در آغاز مرحله زمستان‌گذرانی مطالعه شد. نتایج نشان داد نقطه انجماد به‌تدریج طی ماه‌های پاییز از ۹- به ۱۳- درجه سلسیوس کاهش یافت و در شفیره‌های آزمایشگاهی تیمار شده در دمای صفر یا ۵ درجه سلسیوس دمای انجماد به ۱۳- درجه کاهش یافت. هیچیک از شفیره‌ها نتوانستند پس از ۲٤ ساعت قرارگیری در دماهای پایین‌تر از نقطه انجماد زنده بمانند و لذا به‌نظر می‌رسد این‌گونه از استراتژی اجتناب از یخ‌زدگی بهره می‌برند. با سرد شدن هوا رنگ بیشتر شفیره‌ها (۷٦,۱۹ درصد) به‌سمت قهوه‌ای متمایل می‌شود درحالی‌که در ماه‌های تابستان بالای ۸۰ درصد شفیره‌ها سبز رنگ بودند. شفیره‌های زمستان‌گذران به‌طور کامل دستگاه گوارش خود را تخلیه کرده بودند و محتویات دستگاه گوارش آن‌ها قبل تخلیه حاوی گونه‌های جنس سودوموناس و انتروباکتر که هر دو می‌توانند به‌عنوان هسته یخ عمل کنند بود. در انتها ارتباط این عوامل در چگونگی تطابق برگخوار مرکبات با دماهای پایین مورد بحث قرار گرفت.

واژگان کلیدی: پروانه برگخوار مرکبات، زمستان‌گذرانی، نقطه انجماد بدن، تغییر رنگ، تحمل به سرما