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The Effect of Pupal Cold Treatment on Chill-Coma Recovery Time And Reproductive Success in Two *Drosophila Suzukii* (Diptera: Drosophilidae) Population

İki *Drosophila Suzukii* (Matsumura, 1931) (Diptera: Drosophilidae) Populasyonunda Pupa Evresinde Soğuk Uygulamasının Soğuk Komasından Kurtulma Süresi ve Üreme Başarısı Üzerine Etkisi

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ABSTRACT

Drosophila suzukii (Matsumura, 1931) is an invasive species causing significant economic losses. Effective control techniques for this species fall within a broad scope of study. However, due to population-specific differences based on local adaptations, population-specific approaches in effective control are crucial for sustainability. This study, we examined chill coma recovery time (CCRT) and reproductive success in two temperate zone *D. suzukii* populations, utilizing differentiated treatments involving cold exposure during both pupal and adult stages. While no significant differences were found in CCRT between populations, notable variations were observed among treatment groups and sexes. Cold treatment during the pupal stage enabled faster recovery from chill coma in adults of both populations. However, this effect exhibited variation based on population and sex. The control group and the Pupa + Adult and Adult cold-treated groups had significantly different reproductive success. In contrast to CCRT, differences in reproductive success due to cold treatment during the adult stage resulted in opposite effects in populations. These findings suggest that strategies for combating this species should be tailored according to the local adaptations that populations acquire.

Key Words

Cold tolerance, *Drosophila suzukii*, reproductive success, invasive pest.

ÖZ

Drosophila suzukii (Matsumura, 1931) ekonomik kayıplara neden olan istilacı bir türdür. Bu tür ile etkin mücadele teknikleri geniş bir alanın çalışma kapsamına girmektedir. Ancak lokal adaptasyonlara bağlı populasyonlar arası farklılıklar nedeniyle etkin mücadelede populasyona özgü yaklaşımlar sürdürülebilirlik bakımından önem arz etmektedir. Bu çalışmada iki ılıman kuşak *D. suzukii* populasyonu kullanarak pupa ve ergin evre soğuk uygulama grupları ile bu populasyonların soğuk komasından kurtulma süresi (SKKS) ve sonrası üreme başarıları ölçülmüştür. SKKS bakımından populasyonlar arasında farklılık bulunmazken, uygulama grupları ve eşeyler arasında anlamlı farklılıklar ölçülmüştür. Pupa evresinde soğuk uygulaması her iki populasyonda erginlerin soğuk komasından daha hızlı çıkmalarını sağlamıştır, ancak bu etki populasyona ve eşeye bağlı varyasyon göstermiştir. Kontrol grubu ile Pupa + Ergin ve Ergin soğuk uygulama grupları arasında üreme başarısı bakımından farklılıklar ölçülmüştür. SKKS aksine üreme başarısındaki farklılıklarda ergin dönem soğuk uygulamasının populasyonlarda zıt yönlü bir etki ile sonuçlandığı gözlenmiştir. Sonuçlar, bu tür ile belirlenecek mücadele stratejilerinin populasyonların kazandıkları lokal adaptasyonlar kapsamında şekillenmesi gerektiğini göstermektedir.

Anahtar Kelimeler

Soğuk toleransı, *Drosophila suzukii*, üreme başarısı, istilacı zararlı.

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INTRODUCTION

Invasive insect species exert significant impacts on ecosystems, leading to ecological, environmental, and economic harm [1]. These species directly affect biodiversity by feeding on native plants, preying on or serving as hosts for various species, or acting as parasitoids on local organisms [2]. Moreover, invasive insects can carry diseases that may infect local species or compete for vital resources and habitats. Their effects are not confined to the aforementioned consequences; they also contribute to the spread of infectious human diseases, contribute to forest loss, and hinder efforts to mitigate climate change [3].

Insect populations' growth and colonization speed depend on life-history traits, such as reproduction capacity and development time [4]. Consequently, invasive pests present valuable opportunities for researchers to comprehend population biology mechanisms and devise management strategies. Advancing our understanding of life-history traits will allow us to predict and address the potential severe impacts of these pests and identify critical life-history stages during species management. Additionally, the development of population dynamics and population genetics theories will contribute to the implementation of efficient control methods for invasive species [5].

The Drosophila genus, which some species have been used as a model organism in many studies since the early 1900s, has nearly 1600 species [6]. Unlike other known *Drosophila* species, *Drosophila suzukii*, commonly known as spotted wing drosophila (SWD), is a pre-harvest pest that harms healthy fruits. Adult females oviposit through serrated ovipositor to healthy blackberries, cherries, peaches, grapes, and various fruits [7-9]. The larvae that develop from the egg cause major agricultural damage by feeding on the soft tissue of the fruit [7,10].

Drosophila suzukii, originally from Southeast Asia, rapidly spread throughout the entire Asian continent [11-18], to Europe [19-23], to North and South America [24-28] and to North Africa [29] due to its strong adaptive capabilities. Currently we know that this species has a worldwide distribution. With the large spread of this invasive species, *D. suzukii* has acquired the features of a cosmopolitan species like other *Drosophila* species [19, 30].

Drosophila suzukii has gained global recognition as an invasive species due to its rapid physiological and biological responses to various environmental conditions [31]. An in-depth understanding of the genotype and phenotype of invading insect populations in regions of invasiveness or potential invasiveness can lead to effective management methods [32]. For instance, thermal tolerance plays a crucial role in comprehending their ecological niches and invasion success [33], enabling researchers to grasp population dynamics and enhance management strategies.

Many studies have described *D. suzukii* as a species sensitive to cold [34-38]. Studies on cold tolerance vary concerning examined traits such as survival [35,36,38-40], developmental time [35,41], thermal acclimation [33], reproductive success [35, 38], and aging [38]. In addition, there are various methods to measure cold tolerance in insects, and one of them is the Chill Coma Recovery Time (CCRT) defined by David et al. [42]. CCRT refers to the duration required for motor activity to recover after an insect experiences a temporary loss of movement due to exposure to cold temperatures. This phenotypic trait, which assesses the population's ability to tolerate cold conditions, is valuable in determining their cold thermal tolerance [43]. Furthermore, it represents one of the phenotypes that provide adaptation to changing environments in insects' adult stages [44]. This assesses cold tolerance in *D. suzukii* by measuring the recovery time from chill coma [33,38,40,45-47]. However, no study focusing on population comparisons and inter-population variability has been conducted in these investigations.

In the control of harmful species, a crucial factor is the reproduction of the species. Therefore, there are numerous studies aimed at reducing reproduction, such as the technique of creating sterile individuals. Despite the existence of more environmentally friendly methods of control, like biological control, the sterile insect technique, or RNA interference, chemical pesticides, which are more economical and easier to implement, are often preferred as the dominant control strategy. To minimize the environmental impact of chemical pesticides, lower doses and fewer applications are recommended. For this purpose, conducting chemical applications when the population of the invasive species in the target area is low and/or weak can reduce the chemical pressure on the environment. Therefore, identifying the periods when populations have low reproductive rates can be crucial for an effective control strategy.

Reproductive success is influenced by various factors, including genotype, mating success, courtship behavior, sperm load, ovariole number, age, body size, postzygotic survival, and environmental variables such as light, temperature, and nutrition, among others. Isolating the specific effects of each variable can be challenging. However, the ultimate and most critical outcome is the number of offspring that successfully survive. Temperature is considered an important environmental variable that significantly affects species' reproductive success [48]. Reproductive success is often regarded as the most comprehensive measure of overall fitness [49], making it a key trait of special interest in studies related to thermal adaptation.

In nature, particularly temperate zone populations are exposed to low temperatures due to diurnal temperature fluctuations. This study focuses on the cold tolerance and reproductive success of two populations found in the temperate zone were measured by a comparative method with and without short-term cold treatment at the pupal stage.

MATERIALS and METHODS

Natural populations of *D. suzukii* was collected from Giresun, Turkey (40.93°N, 38.24°E), and Yeşilöz, Ankara, Turkey (40.30°N, 32.34°E) in 2018. The annual climatic variables for Ankara and Giresun are given in Fig. 1. Populations were maintained at 25°C and 60-65% humidity on a 12-h light:12-h dark condition on a standard cornmeal-agar-sugar-yeast medium.

Chill coma recovery time and reproductive success assay

The experiment was depicted in Fig. 2, where the CCRT was measured in two treatment groups, and reproductive success was measured in one control group and two treatment groups for each population.

In each group, pupae were collected when they were close to completing metamorphosis, and sex determination was feasible through the presence of male sex combs. At this stage, pupae were segregated by sex and, 10 pupae per vial were transferred.

The Pupa + Adult cold-treated group contains a 'pupal cold treatment' and was carried out by treating the pupae in empty glass vials to 0 ± 0.5°C for 3 hours. After the 3-hour cold exposure, pupae were transferred to vials with medium and placed in incubators at 25 ± 0.5°C for 48 hours to allow the emergence of adult flies. Surviving individuals aged 1-2 days were transferred to empty glass vials and then exposed to another 3 hours at 0°C to measure Chill Coma Recovery Time (CCRT) (Fig. 2). Following the cold treatment, the flies were returned to room temperature (25 ± 0.5°C), and the time of recovery from cold shock was recorded as the time it took for each fly to stand on all six legs.

The cold-treated and recovered flies were anesthetized using CO_2 to measure reproductive success after cold treatment. Five females and five males were paired per vial, resulting in 15 mating vials for the Giresun population and 16 mating vials for the Ankara population. These vials were placed in an incubator set at 25 ± 0.5°C. The parents were transferred to a fresh medium every 24 hours and removed from the vials after 72 hours. Reproductive success after cold treatment was assessed based on the count of pupae and emerged adults.

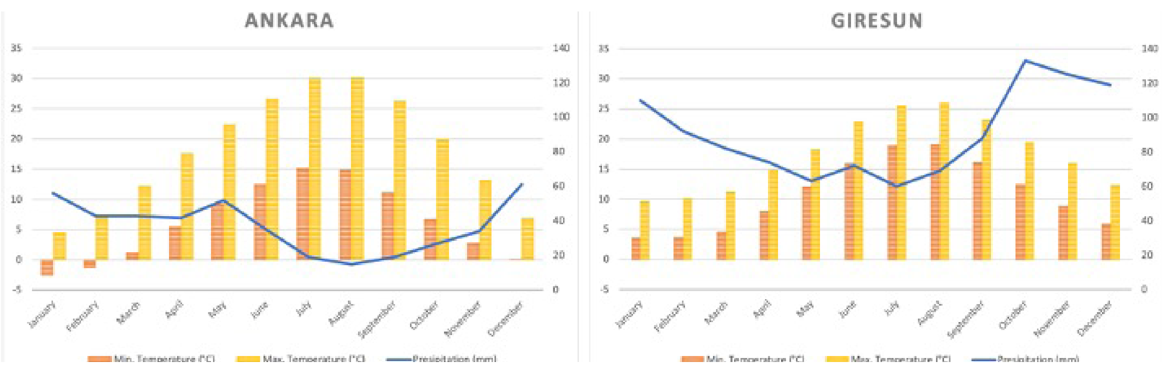

Figure 1 Annual climatic variables for Ankara and Giresun province.

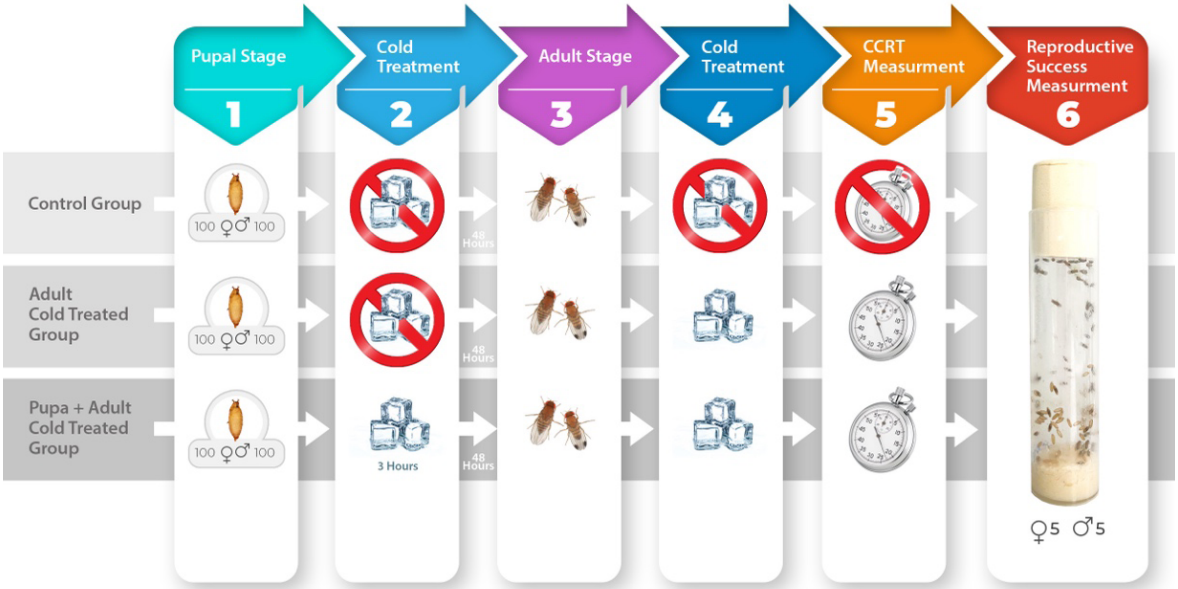

Figure 2 Depiction of the protocol to measure CCRT and reproduction success.

In the Adult cold-treated group, CCRT was assessed in 1-2-day-old adult flies after they emerged from the pupal stage. This assessment involved exposing the flies to a 3-hour cold treatment at 0± 0.5°C, without prior cold exposure during the pupal stage (Fig. 2). CCRT and reproductive success were evaluated using the same protocol as in the first treatment group. Reproductive success was assessed in 19 vials for the Giresun population and 18 vials for the Ankara population.

Cold treatment was not administered to the last group, which served as the "control group" for reproductive success assessment (Fig. 2). The identical protocol for collecting the flies was employed. Reproductive success was evaluated in 18 vials per population.

Statistical Analyses

All analyses were performed in R [50]. We initially tested the normality of data using Shapiro–Wilk's normality test. To determine the significant difference between the treatment groups for CCRT for population, we fitted a generalized linear mixed- model (Gamma family with a log link) using the 'glmer' function in the 'lme4' package [51], with population and treatment group as fixed effects and 'vial' as a random factor to account for any uncontrolled variability among the vials. We used a Likelihood ratio test to analyze the significance of fixed effects.

Divergences in the average reproductive success among the groups were analysed using a GLMM like above. In these models, the count of pupae and adults served as dependent variables, with "population", "treatment group" and 'hours after cold treatment' and their interaction as independent variables, while vials were incorporated as a random factor, a Poisson family (best fitted distribution of the data) with a log link function.

The statistical significance of each variable was determined by an analysis of deviance via the 'Anova' function implemented in the 'car' package [52], function emmeans() with Tukey adjustment in the 'emmeans' package was used for pairwise comparisons for CCRT and reproductive success [53].

RESULTS

Chill Coma Recovery Time

Chill coma recovery time was measured for both populations. The effect of sex on CCRT was significant (Likelihood ratio test between sexes $\chi2 = 87.41$, $P < 0.0001$), where males have a longer mean CCRT than females in both treatment groups by about 248.56 ± 25.32 seconds. The mean CCRT for the Ankara population is 901.55 seconds in females and 1105.76 seconds in males in the Pupa + Adult cold-treated group and 900.26 seconds in females, and 1193.13 seconds in males in the Adult cold-treated group. Besides, the mean CCRT for the Gi-

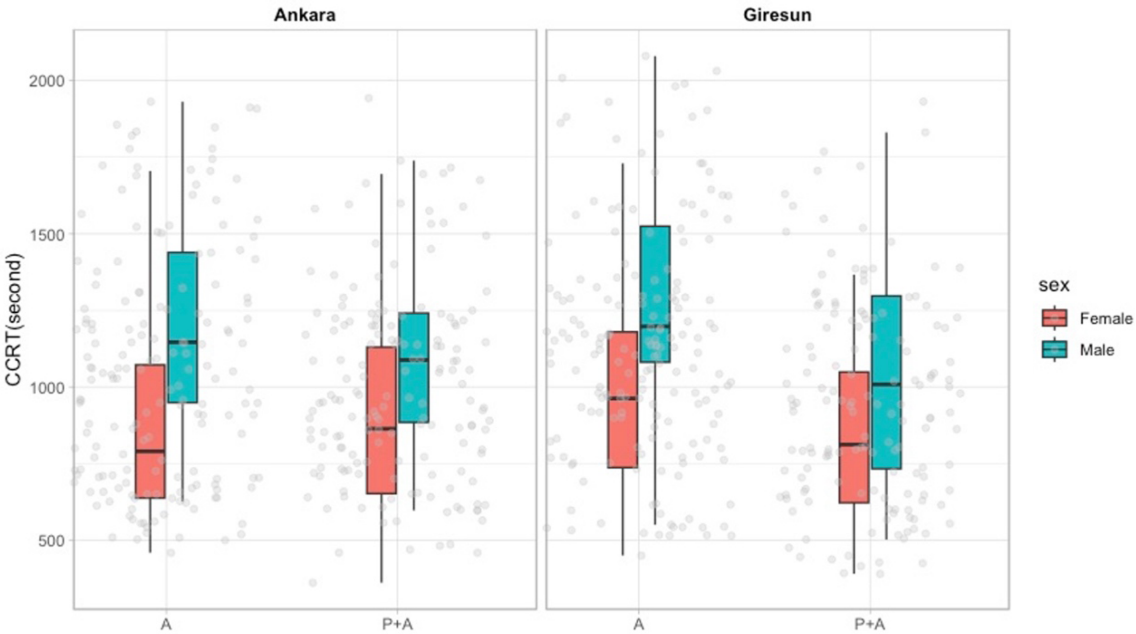

Figure 3 Chill coma recovery time (CCRT) of Drosophila suzukii after cold exposure. Left graph Ankara population, right graph Giresun population. A: Adult Cold-Treated Group, P+A: Pupa + Adult Cold-Treated Group.

resun population is 864.28 seconds in females, 1039.79 seconds in males in the Pupa + Adult cold-treated group, and 975.56 seconds in females, and 1289.85 seconds in males for the Adult cold-treated group.

The preadult cold treatment at the pupal stage has an impact on CCRT in pairs of males ($F_{1,\ 158}$ = 21.303, p<0.0001) and females ($F_{1,\ 164}$ = 4.80, p<0.05) in the Giresun population, emphasizing faster recovery from chill coma (Fig. 3). Pupal cold treatment has also an advantage for faster recovery from chill coma in males of the Ankara population, but it is statistically non-significant. Furthermore, there is no significant difference between the mean CCRT of the populations (Likelihood ratio test between populations χ2 = 0.17, P =0.679).

Reproductive success after cold treatment

To measure reproductive success, we counted the number of pupae and adults in each group every 24 hours. We fitted a linear model to predict the variation between pupae and adults and the model explains statistically not significant and very weak proportion of variance ($F_{1,616}$ = 0.09, p=0.76). Therefore, all analyses were done with the number of pupae.

Fig. 4 shows the average pupae counts observed in the treatment groups. The population (χ2 =7.626, df = 1, P < 0.01) and the treatment groups (χ2 = 24.748, df = 2, P < 0.0001) both had significant effects on reproductive success. Furthermore, there was no significant interaction between these two factors.

Pairwise comparisons between treatment groups of the Ankara and Giresun populations are given in Fig. 4. Control groups significantly differ from the Pupa + Adult cold-treated groups (Tukey test results for Ankara and Giresun populations p<0.0001 and p<0.05 respectively), where the control group has a higher number of pupae. Whereas comparisons between Adult cold-treated and Pupa + Adult cold-treated groups show significant differences only in the Giresun population (Tukey test, p<0.01).

The mean pupa rations for the control groups in Ankara and Giresun population are 1.278 per vial and 1.296 per vial respectively, over a period of 72 hours. Population control groups show no significant differences. The mean pupa ratio for Adult cold-treated groups is statistically significant (p<0.05) between populations, with a ratio of 0.627 per vial for Ankara and 1.614 per vial for Giresun. Pupa + Adult cold-treated groups have a very low reproductive output in both populations with a ratio of 0.292 per vial for Ankara and 0.578 per vial for Giresun population where comparisons between populations are not significant (Fig. 4). While the viability from pupa to adult generally ranged from 93% to 100%

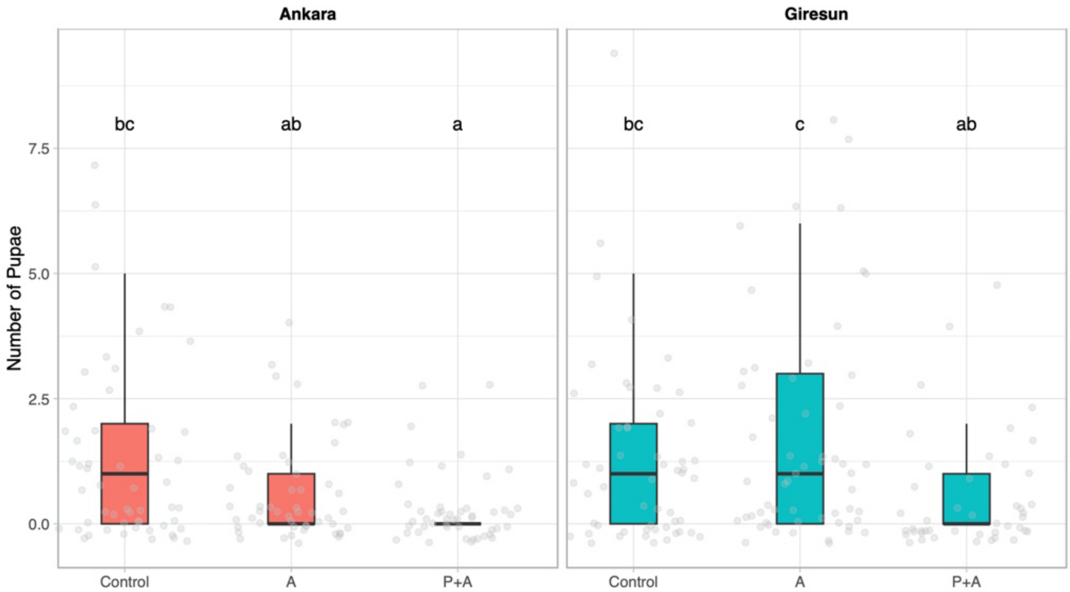

Figure 4. Pupae number of Drosophila suzukii after cold exposure. Left graph Ankara population, right graph Giresun population. Control: Control Group, A: Adult Cold-Treated Group, P+A: Pupa + Adult Cold-Treated Group. Boxes with different letters indicate differences among the groups based on a Tukey post hoc test, P ≤ 0.05.

the results did not change significantly between pupa and adult rations.

When viewed on a daily basis, the highest number of matings occurred on the third day, between 48-72 hours, and this pattern was consistent across both control and treatment groups.

In the initial 24 hours, reproduction rates were notably low across all groups, ranging between 0 and 0.278 pupae per vial. Moving to the second 24-hour period, the control group exhibited pupae ratios of 1.389 per vial in the Ankara population and 1.333 per vial in the Giresun population. For flies exposed to cold twice, once during the pupal stage and again during the adult stage, reproduction rates were 0.188 per vial in the Ankara population with a decrease of 86% compared to the control group and 0.333 per vial in the Giresun population with a decrease of 75% compared to the control group during the second 24-hour period. During the second 24-hour period for the Adult cold-treated group, the number of pupae observed per vial was 0.294 in the Ankara population and 1.579 in the Giresun population. There was a significant 79% decrease in the Ankara population compared to the control groups, while the Giresun population showed an approximate 18% increase compared to the control group. In the third 24-hour period, the control group in the Ankara population had a count of 2.167 per vial, the Adult cold-treated group had 1.588 per vial, and the Pupa + Adult cold-treated group exhibited 0.688 pupae per vial. This represents a 27% decrease in the Adult cold-treated group and a substantial 68% decrease in the Pupa + Adult cold-treated group. On the other hand, in the Giresun population, the control group was observed at a ratio of 2.444 per vial, the Adult cold-treated group exhibited a ratio of 3.000 per vial, and the Pupa + Adult cold-treated group had a pupa ratio of 1.400 per vial. The reproductive rate increased by approximately 23% in the Adult cold-treated group, while the Pupa + Adult cold-treated group experienced a reproductive decrease of 43% compared to the control. The effects of cold treatment at the adult stage seem to be acting in opposite directions in these populations. Reproductive success was negatively affected in the Ankara population after cold exposure, whereas it was positively influenced in the Giresun population. The differences between populations and groups was observed mainly during the third 24-hour period (Fig. 5).

DISCUSSION

This study focused on cold tolerance and the ability of reproductive success after cold exposure in the pupal and adult stages. It is known that *D. suzukii* cannot survive for more than 42 days at temperatures below 1°C [35]. In nature, fluctuating temperatures that are not constant contribute to insects' ability to withstand cold

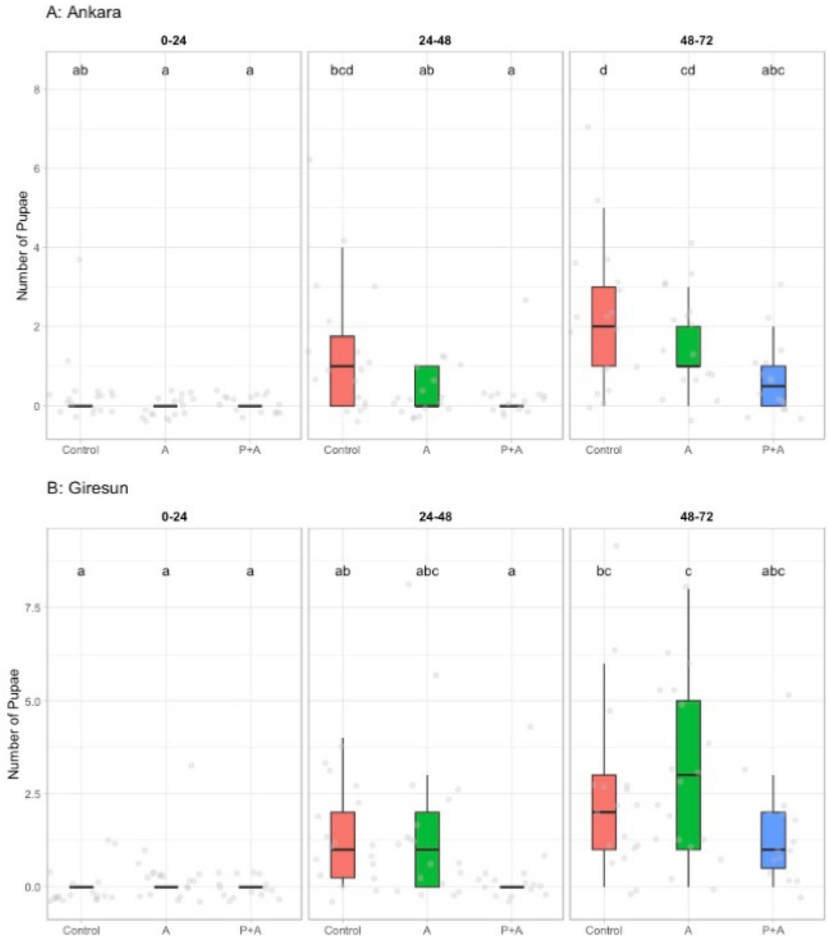

Figure 5. Drosophila suzukii pupae number after cold treatment in hourly intervals. A: Ankara population, B: Giresun population. Control: Control Group, A: Adult Cold-Treated Group, P+A: Pupa + Adult Cold-Treated Group. Boxes with different letters indicate differences among the groups based on a Tukey post hoc test, P ≤ 0.05.

temperatures and successfully overcome winter conditions [54]. In this study, the populations we used were collected from regions with annual average temperatures of 12°C for Ankara and 14.5°C for Giresun. These populations will likely acclimate to temperature conditions by facing temperature fluctuations during the winter months (Fig. 1), enabling their survival. However, considering the distinct climatic variables in these two regions, it is anticipated that the populations from each region have been subjected to different selection pressures regarding cold tolerance, leading to local adaptation. Our results indicate that there was no expected difference among populations in terms of recovery time from cold coma due to local adaptation. However, we observed variation among populations concerning reproductive success after cold treatment.

The mean CCRT shows sexual dimorphism, with females recovering faster from chill-coma, likely due to their larger body size [55] and higher body lipid content compared to males [56]. As expected, our study also found that females emerged from cold coma faster than males in all the groups where the recovery time from cold coma was measured. In our study, where we measured the impact of cold exposure during the pupal stage on adult cold tolerance, we found that the cold treatment during the pupal stage had a population- and sex-specific effect on adult cold tolerance. Our results revealed that in the Giresun population, flies exposed to cold during the pupal stage recovered from chill coma faster, enhancing adult cold tolerance significantly. Furthermore, this effect was more pronounced in males. However, in the Ankara population, although the cold treatment during the pupal stage had a similar impact on adult cold tolerance, this effect was not found to be statistically significant. One possible reason for this result is that the pupal sheath (puparium) contributes to the development of cold resistance in *D. suzukii* but this contribution shows variability by not fully protecting it from cold environmental conditions and this protection indicates inter-population and inter-sexual variation. Enriquez and Colinet [36] results where they measured cold and heat tolerance in *D. suzukii*, support our approach, the adult stage in *D. suzukii* is more cold-resistant than the pupal stage. Our results also indicate that the pupal sheath does not provide complete thermal protection, thus enhancing cold tolerance to a certain extent through acclimatization. In concordance with *D. suzukii*, Krebs & Loeschcke [57] conducted an experimental design with seven *Drosophila buzzatii* populations and showed that exposure to heat during the pupal stage enhanced heat resistance in the adult stage. Where Stockton et al. [37] demonstrate that pupal development and survival in cold conditions can be increased by cold acclimation. This finding supports our results, which indicate that the cold treatment during the pupal stage influences adult cold tolerance in *D. suzukii*. Based on these findings, our results support the idea that cold-treatment during the pupal stage has an advantage over untreated groups in terms of cold tolerance during the adult stages. However, the degree of this advantage varies depending on population and sex.

In the scope of the research, the reproductive success of individuals was examined after exposure to cold during the Pupa + Adult and Adult stages, as well as for those that were not treated to any cold exposure. It was observed that cold exposure during both the pupal and adult stages, and only during the adult stage, had an impact on reproductive success. Additionally, reproductive success showed variations concerning the hours following cold stress. It was observed that the hours that passed after the applied stress increased the reproductive success in all groups. It was determined that almost no reproduction occurred in the first 24 hours in all groups, and this observation was interpreted not only as the recovery period for individuals after thermal stress. This pattern was also observed in the control group, which led to the interpretation of stress due to the individuals being transferred to a new environment. Thermal acclimation has been shown to impact mating success in *Drosophila melanogaster*, with heat acclimation enhancing mating activity at higher temperatures [58], while cold acclimation reduces mating activity [59]. Our results reveal population-specific effects, indicating that there is no general pattern for cold acclimation and its impact on reproductive success, unlike in *D. melanogaster*. Specifically, cold treatment in the adult stage decreased reproductive success in Ankara populations when compared to the control groups. Conversely, in the Giresun population, the Adult cold-treated group exhibited increased reproductive success compared to the control group (Fig. 4). Interestingly, the effect of cold treatment on reproductive success in these two populations contradicts the pattern observed in recovery time from cold coma. This discrepancy suggests that populations have diverged in terms of reproductive success following cold stress. Based on this data, it can be inferred that their local adaptation to cold significantly influences their reproductive success, a crucial aspect of their life history traits.

In this study, the negative impact of Pupa + Adult cold treatment in both populations on reproductive success, suggests a potential weakening of the populations, which could indicate a general trend for this species. However, the differing effects of Adult cold treatment on reproductive success among populations suggest that this phenomenon might be population-specific, highlighting potential variations in cold tolerance between populations. Ultimately, while the weakening of populations exposed to low temperatures is a possibility, offering a potentially opportune time for effective control measures, conducting further research on cold tolerance across multiple populations is likely to yield more robust strategies. The current findings underscore the importance of developing population-specific, local strategies in combating *D. suzukii*.

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