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ORIGINAL ARTICLE

Cold acclimation increases Asian citrus psyllid *Diaphorina citri* (Hemiptera: Liviidae) survival during exposure to freezing temperatures

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Abstract The Asian citrus psyllid, *Diaphorina citri*, is the vector of the pathogenic bacteria that causes Huanglongbing, the most devasting disease affecting citrus worldwide. As cultivation of citrus tends to expand northward, the tolerance of psyllids to freezing temperatures needs to be investigated. While mortality of *D. citri* to cold temperature has been previously studied, much less is known regarding the acclimation potential of psyllids to cold temperatures. We first evaluated cold resistance of *D. citri* depending on color morph and sex. Subsequently, we compared mortality of *D. citri* at −4 °C for 10 hours between unacclimated psyllids, psyllids that were gradually acclimated to cold over 1 or 2 weeks, and psyllids intermittently acclimated also for 1 or 2 weeks. We did not find major differences in cold resistance between color morphs and sex. We found that after 1 week of gradual acclimation, the survival of psyllids at −4 °C increased to 86%, compared to 20% in the control group. Survival did not increase after the second week of gradual acclimation. Although intermittent acclimation improved survival compared to the control group, it was less effective than gradual acclimation with a survival at 30% and 70% after 1 and 2 weeks of acclimation, respectively, although this difference with gradual acclimation was not significant at week 2. These data show that gradual cold acclimation allows *D. citri* to survive brief periods below freezing. It may serve as a mechanism responsible for increasing northern establishment of this pest.

Key words cold acclimation; cold hardy citrus; cold resistance; citrus greening; *Diaphorina citri*; overwintering

Introduction

As climate change progresses, warmer global temperatures and change in precipitation patterns will impact the current distribution of crops worldwide (Sloat *et al.*, 2020). This is the case for citrus (*Citrus* spp.) which, while traditionally limited in the US to Southern California and central Florida, is expanding into the panhandle of Florida, Northern California, or Georgia (Martini & Andersen, 2018). Similarly, in Asia, citrus is now cultivated in Korea (Kim *et al.*, 2021) and is also expanding in northern Japan (Nagaoka-Nakazono *et al.*, 2020). The movement of citrus cultivation northward was made possible by warmer temperatures, less freezing events but also as a result of more cold-hardy varieties and progress made in cold protection, especially with the development of micro-sprinklers. However, as the area of citrus cultivation is expanding, there is a potential for citrus pests also to follow the same trend (Bebber *et al.*, 2013).

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is the vector of the bacterial pathogen, *Candidatus* Liberibacter asiaticus (*C*Las)

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(Rhizobaliales: Rhizobiaceae), the causal agent of the disease Huanglongbing (HLB), also called citrus greening (Grafton-Cardwell *et al*., 2013; Hall *et al*., 2013). The disease is considered the most destructive of citrus crops worldwide (Bové, 2006). Trees infected with *C*Las produce small, bitter-tasting fruit, suffer significant fruit drop, and may die 3–5 years after infection without intervention (Wang & Trivedi, 2013). Since its first detection in Florida in 1998, *D. citri* has spread from the Miami-Dade area up to Gainesville in central Florida (Halbert & Núñez, 2004). It has also spread to other states along the Gulf of Mexico in Alabama, Louisiana, and Texas as well as along the Atlantic coast up to South Carolina (Howle, 2009). However, in North Florida (i.e., the panhandle and lands above 30 parallel North) and in Georgia, *D. citri* densities are still low and HLB is not yet established in citrus groves (Martini *et al*., 2020). The psyllid was also found in California in 2008, and it is moving north, with first records in Sacramento, Marin, Contra Costa, and San Francisco county. Furthermore, detections of *D. citri* in Santa Clara county in Northern California have increased and there is a threat of *D. citri* movement into the San Joaquin Valley, the largest citrus growing region (University of Califronia, Division of Agriculture & Natural Resources, 2021). As ACP detections in backyard citrus in northern California increase, and cold hardy citrus cultivation grows in northern Florida, and also in Georgia, there is a critical need in evaluating *D. citri*'s susceptibility to cold temperatures.

It has long been suggested that *D. citri* establishment in the northern part of the country may be impacted by *D. citri* cold susceptibility. One previous study on *D. citri* cold tolerance found 100% mortality for psyllids placed 6 hours at −5 °C, 4 hours at −6 °C, 2 hours at −8 °C and between 70% and 80% mortality for 8 hours at −4 °C (Hall *et al*., 2011), while another found adult survival up to −6 °C, with 50% surviving 0 °C for 2 days and 5 °C for 4 days (El-Shesheny *et al*., 2016). A field experiment conducted in North Florida showed 20% survival of adults during winter despite a decrease in temperature to −5.5 °C for three consecutive days. Interestingly, survival was in the same range the next winter despite milder temperatures (no days < 0 °C), indicating that the freezing events had little effect on the survival of psyllids (Martini *et al*., 2020). This discrepancy between lab and field results suggests cold acclimation is possibly occurring in *D. citri*.

To our knowledge, there is only one other study that investigated cold acclimation of *D. citri* (Hall *et al*., 2011). The authors compared the survival of lab-reared psyllids to psyllids maintained outdoors from December to March. During this acclimation period, average temperature was between 9 and 11 °C. In these conditions, a slight increase of survival from <1% to 16% was observed when psyllids were exposed at −7 °C for 4–6 hours. However, the difference was not significant at $\alpha = 0.05$ for two out of three trials. Importantly, cold acclimation patterns were not investigated.

In the present study we aimed to investigate the extent to which cold acclimation may affect acute survival of *D. citri* adults at freezing temperatures using three lab-based experiments. We did not conduct experiments on cold resistance or cold acclimation for immature stages (eggs or nymphs) as *D. citri* oviposition and nymphal development only occur on young, emerging leaves (also called "flush") (Grafton-Cardwell *et al*., 2013). These flushes are destroyed by freezing temperature with no chance for *D. citri* eggs and nymphs to survive (Zekri *et al*., 2016). Only *D. citri* adults are found during wintertime in Florida (Martini *et al*., 2020). First, we assessed cold tolerance depending on sex and color morph to determine if these factors affected survival and should be addressed separately. Psyllids display two main color morphs: blue-green and gray. The blue-green color morph is associated with an overall better fitness than gray morphotype with higher flight capability (Martini *et al*., 2014), greater body mass, higher fecundity with faster oocyte development (Wenninger & Hall, 2008; Wenninger *et al*., 2009; Ibanez *et al*., 2019). A third yellow-orange color morph exists as well and is associated with reduced fat body combined with heavy egg load in females and larger testes in males (Stockton *et al*., 2017). To our knowledge, the effect of *D. citri* color morph on psyllid cold tolerance has never been studied. This experiment served as a first step to establish differences in cold tolerance between males and females and the two main color morphs to select individuals that do not divert significantly in the cold acclimation experiments.

In our final two experiments, we more directly addressed the question of cold acclimation and survival. Gradual acclimation has been shown to increase cold tolerance among other arthropods of tropical origin, including *Drosophila* (Stockton *et al*., 2018), presumably by allowing the physiology of the insect time to adapt to the surrounding environment, thereby lowering the limits of survival to extreme abiotic conditions. Indeed, otherwise cold intolerant insects may survive biologically significant periods at or below freezing if the cooling window extends over a period of hours or days (Clark & Worland, 2008). Furthermore, other factors such as the pattern and frequency of cooling events over time can affect survival outcomes (Colinet *et al*., 2015). For example, daily warming periods followed by cooling at night may be associated with improved cold tolerance compared to those insects held constantly held at cool temperatures. This may be because brief or intermittent warming periods allow for cold stress recovery (Colinet *et al*., 2015). For this reason, during our cold acclimation experiments we tested two types of acclimation: one consisting of a gradually cooling (hereafter referred to "gradual cold acclimation"), and another one mimicking a drop of temperature only during nighttime (hereafter referred to "intermittent cold acclimation"). Following each acclimation program, we assessed acute survival when exposed to a freezing temperature of −4 °C for 10 hours.

Materials and methods

Insect colony

Diaphorina citri used in these experiments were from a colony that originated from adults collected in Franklin County, FL, in summer 2017, and maintained in a climate-controlled chamber on curry tree, *Murraya koenigii* (L.) Spreng. The growth chamber conditions were 26 °C and 50% RH with 10 : 14 dark : light photoperiod.

Cold tolerance test setup

To assess cold tolerance of psyllids, 5 *D. citri* adults (4–11 days postemergence) were placed in a 50 mL Eppendorf vial containing one mature leaf from Owari Satsuma mandarin, *Citrus unshiu*. We used Satsuma mandarin leaves as food source during the freeze test as detached leaves from *Murraya koenigii* tend to desiccate rapidly. The citrus leaves used were sampled from mature trees and did not receive insecticidal or fungicide treatment in the 6 months before use. The vials were placed vertically in a rack within a refrigerated incubator (Thermo Fisher Scientific, Waltham, MA). For statistical purposes, one tube of five adults was considered a single replicate. Temperature was monitored with a Hobo data logger (Onset, Cape Cod, Massachusetts) for the duration of the experiment. In the incubator, the psyllids were exposed to a fixed temperature for 6 or 10 hours, as recorded for each experiment. After the exposure period, the psyllids were removed from the tube, and mortality was assessed. Psyllids were given ten minutes to acclimate to room temperature (approx. 22 °C), and immobile psyllids were prodded to determine if dead or alive. Psyllids that did not move after stimulation were considered dead.

Experiment 1: Effects of color morph and sex on Diaphorina citri cold tolerance

Psyllids aged 4- to 11-day old postemergence were sampled from the laboratory rearing and were used in the experiments. Psyllids were sorted by sex and by color morph (green-blue or gray) at the beginning of the experiment. For each of the four sex and color morph combinations, the psyllids were exposed to either 22, −1, −2.5, or −4 °C for 6 or 10 hours. For each of these 32 treatment combinations (color morph × sex × temperature × exposure time), there were four replications for a total of 128 replications with a total of 640 *D. citri* assayed.

Experiment 2: Effect of gradual cold acclimation on Diaphorina citri cold tolerance

Psyllids aged 4- to 11-day old postemergence were sampled from the laboratory rearing for use in the experiments. As results from Experiment 1 showed no significant effect of sex and color morph on cold tolerance at −4 °C after 10 hours exposure (see results), *D. citri* used in this experiment were not sexed and not sorted depending on abdominal color. Before the experiment, psyllids in the cold acclimation treatment were isolated on a potted citrus "swingle" plant in an incubator at 24 °C. The temperature of the incubator was decreased by 3 °C three times a week. Thus, the temperature was at 15 °C after 1 week, and 6 °C after 2 weeks (Fig. 1A). The control group was kept on citrus "swingle" plant at a steady temperature of 24 °C in a separate incubator. After 1 week of cold acclimation, *D. citri* from the control and cold acclimation treatments were separated in groups of five in sample vials and tested for cold tolerance as described above for 10 hours at −4 °C. This temperature and time of exposure was chosen as it corresponded to approximately 80% mortality during the first experiment (see result section). The experiment was repeated with different psyllids after 2 weeks of acclimation. There were six replicates (30 psyllids per treatment, 60 total) for the 1-week acclimation assay and nine (45 psyllids per treatments, 90 total) for the 2-week acclimation assay.

Experiment 3: Effect of intermittent cold acclimation on Diaphorina citri *cold tolerance*

Psyllids aged 4- to 11-day old postemergence were sampled from the laboratory rearing for use in the experiments. As before, psyllids were not sexed or separated

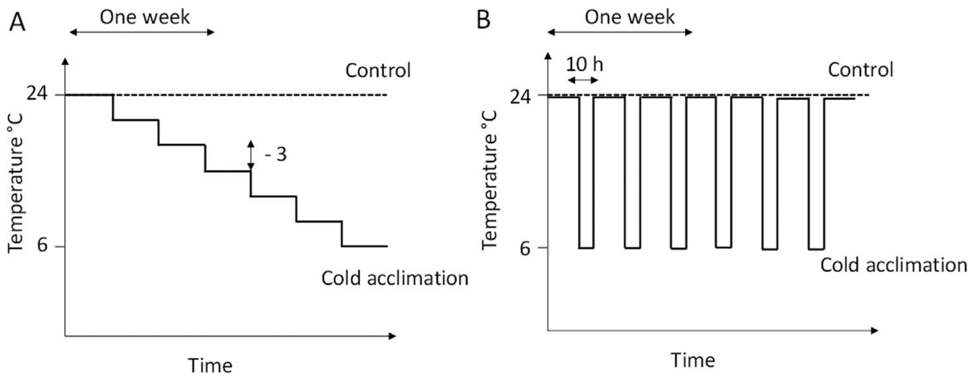

Fig. 1 Schematic design of the protocols for cold acclimation of Asian citrus psyllid *Diaphorina citri*. (A) in the first experiment, *D. citri* in the gradual cold acclimation treatment were submitted to decremental decrease of temperature of −3 °C three times a week (on Monday, Wednesday, and Friday), from 21 to 6 °C in 2 weeks. (B) In the second experiment, *D. citri* in the intermittent cold acclimation treatment were exposed to overnight drop of temperatures from 21 to 6 °C, three times a week (on Monday, Wednesday, and Friday)

according to abdominal color. Before the experiment, *D. citri* in the cold acclimation treatment were isolated on a potted citrus "swingle" plant in an incubator at 24 °C. The temperature of the incubator was decreased to 6 °C for 10 hours three times a week and subsequently put back at 24 °C (Fig. 1B). The control group was kept on a citrus "swingle" plant at a steady temperature of 24 °C in a separate incubator. After 1 week of cold acclimation, *D. citri* from the control and cold acclimation treatments were separated in groups of five in sample vials and tested for cold tolerance as described above for 10 hours at −4 °C. The experiment was repeated with different *D. citri* after 2 weeks of acclimation. There were six replicates (30 psyllids per treatment, 60 total) for the 1- and 2-week acclimation trials.

Statistical analyses

Survivorship of *D. citri* during the first experiment was analyzed with a generalized linear model (GLM) with binomial distribution. Factors included temperature, exposure time, color morph (green-blue or gray), and sex. The full model contained all two-term interactions; however, nonsignificant interactions ($\alpha = 0.10$) were removed by stepwise deletion (Crawley, 2007). Difference between treatment was assessed with paired chi-square test followed by Bonferroni correction. For experiments 2 and 3, survivorship of each cold acclimation treatment was compared to their respective control with a GLM with binomial distribution.

Table 1 Generalized linear model (GLM) with binomial distribution of *Diaphorina citri* cold resistance depending of sex and color morph. Nonsignificant interactions ($\alpha = 0.10$) were removed by stepwise deletion.

	df	χ^2	*P* value
Temperature	1	331.83	<0.0001
Duration	1	3.46	0.0629
Color morph	1	9.76	0.0018
Sex	1	2.69	0.1013
Duration × temperature	1	5.36	0.0206
Temperature × color morph	1	4.97	0.0257

Results

Experiment 1: Effects of color morph and sex on Diaphorina citri cold tolerance

None of the psyllids in the control treatments (22 °C for 6–10 hours) died during the experiment. As expected, survival of *D. citri* decreased with the temperature and with the time exposed at low temperature (positive interaction between duration and temperature) (Table 1, Fig. 2A). We found a significant interaction between color morph and temperature. At −2.5 °C mortality was significantly higher for gray morphotypes compared to green-blue ones ($\chi^2 = 8.59$, $P < 0.05$ after Bonferroni correction) (Table 1, Fig. 2B). However, at −4 °C this difference was not significant anymore. Finally, sex did not have any effect on psyllid survival (Table 1, Fig. 2C).

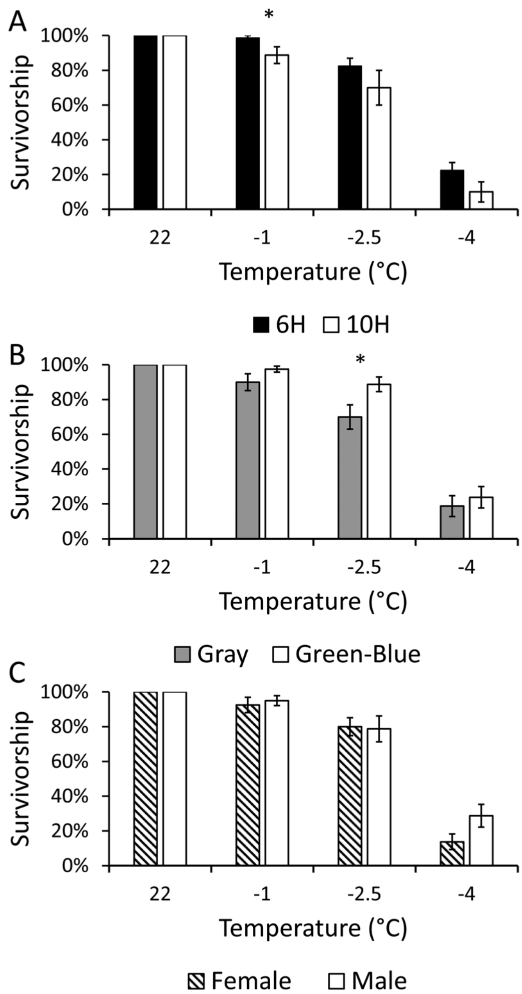

Fig. 2 Average (± SE) survivorship of Asian citrus psyllid *Diaphorina citri* exposed to 22, −1, −2.5, and −4 °C, depending on (A) exposure time (6 or 10 hours), (B) color morph, and (C) sex. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Experiment 2: Effect of gradual cold acclimation on Diaphorina citri cold tolerance

Gradual cold acclimation dramatically increased cold tolerance of *D. citri* ($\chi^2 = 72.240$, df = 1, $P < 0.001$). Interestingly, survivorship did not increase between 1 or 2 weeks of acclimation (no interaction between week and treatment, $\chi^2 = 1.295$, df = 1, $P = 0.2551$). While only 20% of psyllids in the control group survived after 6-hour exposure at −4 °C, 93% survived after 1 week of acclimation, and 80% after 2 weeks of acclimation. In both cases, the increase of survivorship compared to psyllids in the control treatment was highly significant ($\chi^2 = 40.847$, df = 1, $P < 0.001$; $\chi^2 = 32.148$, df = 1, $P < 0.001$; for week 1 and week 2, respectively) (Fig. 3A).

Experiment 3: Effect of intermittent cold acclimation on Diaphorina citri cold tolerance

Intermittent acclimation increased cold tolerance of *D. citri* ($\chi^2 = 12.161$, df = 1, $P < 0.001$). There was a difference in survival between week 1 and week 2 ($\chi^2 = 16.970$, df = 1, $P < 0.001$). In the first week, survival increased from 7% in the control group to 30% in the cold-acclimated group ($\chi^2 = 5.822$, df = 1, $P = 0.0158$) and in the second week to 23%–70% ($\chi^2 = 8.268$, df = 1, $P = 0.004$). The difference in survival in the intermittent versus gradually acclimated psyllids was significantly lower than the survival of psyllids under gradual acclimation after 1 week ($P < 0.001$), whereas after 2 weeks, psyllids exposed to either acclimation methods did not differ in survival ($P = 0.144$) (Fig. 3B).

Discussion

As climate change is altering the current distribution of crops worldwide, it is important to evaluate the potential acclimation of associated pests to their new environment. We first tested if sex or color morph had any impact on cold tolerance. We found no difference between cold resistance between males and females. In another study, male and female *D. citri* tended to have similar performances for other physiological functions such as flight performance (Martini *et al*., 2014). We found a higher cold resistance of blue-green morphotype as compared to gray when exposed at −2.5 °C; however, this difference disappeared at −4.0 °C. Blue-green color morph in *D. citri* is due to the presence of pigmented cells of the fat body and to an increased level of hemocyanin (Hosseinzadeh *et al*., 2019), a copper-binding oxygen transport protein that causes blue coloration of hemolymph of other arthropods when oxygenated (Coates & Nairn, 2014). Higher levels of hemocyanin associated with a higher body mass for blue-green morphotypes may play a role in cold resistance.

Subsequently, we compared two cold acclimation treatments. The first included gradually decreasing temperature and the second included temperatures that only decreased at night. We found that *D. citri* can quickly acquire cold tolerance when exposed to cold temperature and gradual acclimation was significantly better in enhancing *D. citri* survival in cold temperatures than discrete acclimation. Nevertheless, acclimation during nighttime only was still enough to significantly increase survival of *D. citri* compared to unacclimated individuals. In Diptera, exposure to cold stress for few hours also significantly increased cold tolerance. Interestingly,

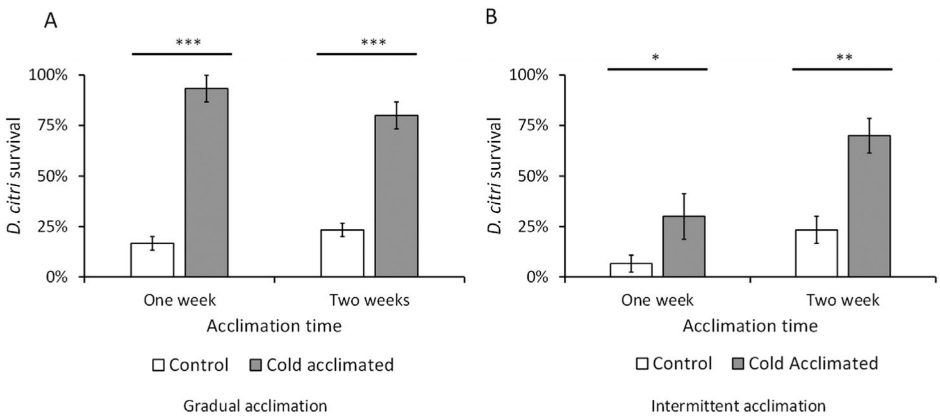

Fig. 3 Average (± SE) survivorship of Asian citrus psyllid *Diaphorina citri* exposed to −4 °C, for 10 hours (A) after gradual cold acclimation or after (B) intermittent cold acclimation. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

this phenomenon called "rapid cold hardening" has been discovered in cold-susceptible and cold-resistant insects (Clark & Worland, 2008).

However, cold tolerance was acquired more quickly if acclimation was made with a gradual decrease of temperature *versus* an intermittent acclimation treatment. This suggests that unlike several other temperate holometabolous species (Colinet *et al*., 2015), colder nights may not be sufficient to induce an optimal cold acclimation in *D. citri*. Survival of *D. citri* should be significantly lower in case of a sudden freezing event not preceded by a decline of ambient temperatures during the day. Nevertheless, our results obtained may explain how *D. citri* can survive to freezing temperature in northern Florida and California where previous data indicated this was unlikely (Hall *et al*., 2011).

Mechanisms of cold acclimation in *D. citri* remain to be fully discovered. El-Shesheny *et al*. (2016) demonstrated that after a single day at 5 °C, ATPase enzyme activity increased significantly. The authors also found a decrease in AMP: ATP and ADP: ATP ratios and a complete change in sugar nucleotides. Studies on other insects suggest that desaturation of fatty acid are a vital component of cold acclimation. Higher proportions of unsaturated fatty acids have been found in hibernating weevils, lepidopterans, and dipterans (Kostal & Simek, 1998). Similarly, lower rearing temperatures have been found to increase the proportion of unsaturated fatty acids in dipterans and cockroaches (Kostal & Simek, 1998). An increased proportion of unsaturated fatty acids in cell membranes preserves their dynamic properties and maintains cell functions. In addition, a previous study demonstrated that *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) exposed to a gradual cooling showed a change in the sugar metabolism with increase in glucose and trehalose. The increase of trehalose stabilizes membranes by interacting with the phosphate of phospholipids (Crowe *et al*., 1992). Further investigations regarding *D. citri* metabolism should be conducted to better understand cold acclimation. For instance, a transcriptomic analyses and RNA sequencing would reveal genes that are up- or downregulated after cold acclimation (Enriquez & Colinet, 2019). Similarly, a metabolomic analysis would help to identify the metabolic pathways altered by cold acclimation (MacMillan *et al*., 2016).

Under field conditions, behavioral strategies may also help psyllids to overcome freezing temperatures. To prevent desiccation, which is a major risk associated with cold (Danks, 2000), some insects will seek shelter during a cold event (Sinclair, 1999; Palmer & Siebke, 2008). Field data suggest that *D. citri* may also select areas in the citrus canopy that have a microclimate with higher temperatures. Survey efforts conducted in Central Florida revealed that the density of *D. citri* was higher in the south-exposed canopy rather than other parts of the citrus tree. Interestingly, temperature in the south-exposed canopy was approximately 1.5 °C higher than on other parts of the canopy (Martini *et al*., 2016) indicating that, during wintertime, psyllids tend to aggregate on the warmer side of the canopy.

Overall, our experiments showed that *D. citri* could resist freezing temperatures at a higher rate than previously thought. The mortality of *D. citri* increased from 20% to >80% following 1–2 weeks of gradual acclimation. Infield conditions, overwintering survivorship of *D. citri* was approximately 20% following a winter with temperatures as low as –5.5 °C (Martini *et al*., 2020). While these results obtained in laboratory conditions may not precisely predict the true survival of *D. citri* in the field where additional mortality may occur due to natural enemies and pathogens, it demonstrated that cold is unlikely to become a barrier to *D. citri* expansion in North Florida and North California.

Our results are important as *D. citri* and HLB are the most destructive pests in citrus worldwide. This new data will help creating more accurate risk model that could predict survival of Asian citrus northward. This information is particularly critical for North America and Asia where citrus cultivation is developing north due to climate change and the development of new cold hardy citrus varieties (Martini & Andersen, 2018; Kim *et al.*, 2021).

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Author contribution

XM, MJR secured funding; XM, DS conceived the experiments; KM conducted the experiments; XM analyzed the data; XM, DS, and MJR wrote the manuscript with inputs from KM.

Disclosure

The authors have no conflict of interest to disclose.

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