Acute cold stress and supercooling capacity of Mediterranean fruit fly populations across the Northern Hemisphere (Middle East and Europe)

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ABSTRACT

The Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae), holds an impressive record of successful invasion events promoted by globalization in fruit trade and human mobility. In addition, *C. capitata* is gradually expanding its geographic distribution to cooler temperate areas of the Northern Hemisphere. Cold tolerance of *C. capitata* seems to be a crucial feature that promotes population establishment and hence invasion success. To elucidate the interplay between the invasion process in the northern hemisphere and cold tolerance of geographically isolated populations of *C. capitata*, we determined (a) the response to acute cold stress survival of adults, and (b) the supercooling capacity (SCP) of immature stages and adults. To assess the phenotypic plasticity in these populations, the effect of acclimation to low temperatures on acute cold stress survival in adults was also examined. The results revealed that survival after acute cold stress was positively related to low temperature acclimation, except for females originating from Thessaloniki (northern Greece). Adults from the warmer environment of South Arava (Israel) were less tolerant after acute cold stress compared with those from Heraklion (Crete, Greece) and Thessaloniki. Plastic responses to cold acclimation were population specific, with the South Arava population being more plastic compared to the two Greek populations. For SCP, the results revealed that there is little to no correlation between SCP and climate variables of the areas where *C. capitata* populations originated. SCP was much lower than the lowest temperature individuals are likely to experience in their respective habitats. These results set the stage for asking questions regarding the evolutionary adaptive processes that facilitate range expansions of *C. capitata* into cooler temperate areas of Europe.

1. Introduction

Dispersal into new environments is considered to be a key process in the response of insect populations to rapidly changing environmental conditions (Ben-Yosef et al., 2021). Once a species is introduced to climatically novel habitats, they have to survive and reproduce before dispersing to new and/or adjacent areas (Blackburn et al., 2011; Hill et al., 2017). Insects may survive this introduction event by having greater environmental tolerance or by rapidly adjusting performance (i. e. phenotypic plasticity) in that new environment to maximize fitness, or perhaps by some combination of both. Phenotypic plasticity – a trait's response to environmental conditions or a gene-by-environment inter-action – may vary among different populations and in response to different environmental characteristics (Gray, 2013), and is a major

element that allows insects to cope with more extreme temperatures (Angilletta, 2009; Bowler and Terblanche, 2008). Hence, insects typically adjust their phenotype in response to environmental cues (Rodrigues and Beldade, 2020). In the case of thermal stress, insect populations can shift their thermal niche substantially by variation in their phenotypic plasticity to similar conditions (Bujan et al., 2020). Understanding how insects adapt to changing environments is important to assess their ecological and evolutionary dynamics, and predict population resilience to climate change (Oostra et al., 2018). Moreover, it is clear that insects can rapidly adapt through genetic processes (e.g. assimilation, mutation) to novel conditions (Garnas, 2018) to have greater fitness in what would have otherwise been sub-optimal conditions (Hoffmann and Sgrò, 2011).

Thermal acclimation is a form of plasticity that can enhance

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resistance to environmental stress (Terblanche and Hoffmann, 2020), although the potential fitness benefits depend on a range of factors, including the predictability and timing or duration of environmental change (Angilletta, 2009; DeWitt and Scheiner, 2004). Even small changes in a few traits could produce enhanced fitness in certain environments. When temperature changes are predictable and endure for some time, insects may use external cues to change their internal condition (Niehaus et al., 2012). Hence, seasonal adjustments (e.g. acclimatization such as overwintering) would be beneficial when insects occur in an environment where the extent of change is predictable with some reliability (Gabriel et al., 2005), and the "costs" are relatively limited. On the other hand, genotypes that express a generalized phenotype will be preferred when environmental temperature varies unpredictably (Gabriel et al., 2005). From the above, it is clear that adaption to different environmental (thermal) conditions can involve changes in plasticity among individuals, populations, species and life stages (Kingsolver and Buckley, 2018).

Temperature affects a range of biochemical and physiological processes, and is one of the most important environmental factors, affecting insects population dynamics (Li et al., 2021). Insects from temperate, polar and/or high-altitude environments must survive the low temperatures they periodically encounter in their habitat in order to persist and thrive (Sinclair et al., 2015). At low temperatures, most insects lose their ability for coordinated movement, and after this, they enter a state of complete neuromuscular functional deficiency called chill coma (MacMillan and Sinclair, 2011). After re-warming from chill coma, insects can recover from this state with no evidence of injury, but in cases where cold exposure or time spent in chill coma is prolonged, non-freezing related cold injuries can accumulate and lead to behavioral or neuromuscular defects (Overgaard and MacMillan, 2017). The cold hardiness of insects may help classify species into the major categories of chill susceptible, freeze avoidant and freeze tolerant (Sinclair et al., 2015). Parameters used to estimate the tolerance of organisms to extreme temperatures include upper and lower critical thermal limits for activity (CTmin & CTmax), lethal thermal limits (LTL), chill coma recovery (CCR), and supercooling point (SCP). These parameters can be evaluated under chronic or acute conditions using diverse methodology (Beitinger and Lutterschmidt, 2011; Lee et al., 1987; Marshall and Sinclair, 2012; Sinclair et al., 2003). Chill susceptible species succumb to physiological effects of low temperatures well above their SCP (Andersen et al., 2015), while freeze tolerant species tolerate freezing, and freeze avoiding species avoid freezing by suppressing their SCP. Hence, the relationship among the SCP and injury is critical to determine the cold tolerance of insects (Sinclair et al., 2015). Cold tolerance traits frequently correlate strongly with insect geographic distributions such that those with broader distributions may possess greater cold tolerance (Andersen et al., 2015).

Survival at or after low temperature exposure is another aspect of an insect's cold hardiness response (Matsukura et al., 2014), and can be especially informative if coupled with measures of SCP. Factors that affect cold hardiness encompass developmental life stage, behavior, thermal history (e.g. acclimation status, or seasonal acclimatization), age, body size and feeding status (Andreadis and Athanassiou, 2017). For example, acclimation at low temperatures for few days can enhance cold hardiness and enable insects to cope with conditions that would otherwise be lethal (Kristensen et al., 2008).

The SCP measures the temperature at which an insect's body fluids begin to freeze (Denlinger and Lee, 2010; Maes et al., 2012). Typically, insects exhibit seasonal patterns of supercooling capacity as they adjust physiologically for winter (Lee et al., 1996). Although the SCP helps identifying physiological boundaries (Renault et al., 2002), its real ecological value remains ambiguous, especially for tropical and chill susceptible species. Although tropical species are not able to survive more than few hours at temperatures lower than 0 °C, they have successfully colonized temperate regions. According to Renault et al. (2002), two hypotheses should be considered a) SCP has no ecological

significance in chill susceptible species, and b) SCP in introduced species is linked to the native characteristics of the species. This is in agreement with Andersen et al. (2015), where for *Drosophila* species, the SCP was a poor predictor of latitudinal distribution and environmental conditions compared to other cold tolerance measures such as critical thermal limits. From the above its clear that different cold tolerance measures suit the species-specific cold tolerance strategy.

The Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), is a highly invasive agricultural pest that has expanded its geographic range from the ancestral habitats of the sub-–Saharan Africa to almost all temperate and tropical areas of the globe in <200 years (Gomulski et al., 2008). In recent years the species is frequently detected in cooler temperate areas of the Northern Hemisphere. Climate change has been suggested as a factor that may change the geographic limits of this pest, allowing its expansion into temperate areas of Europe (Szyniszewska and Tatem, 2014). On the other hand, physiological, morphological and behavioral attributes enable medfly to survive and reproduce in a wide range of climatic conditions and habitats. Diamantidis et al. (2011), determined that medfly populations originating from geographically isolated regions exhibit a high variation in their life history traits such as longevity or fecundity, which may influence establishment and spread. Furthermore, Papadopoulos et al. (1996), demonstrated that in northern Greece, medfly prolongs its larval development inside infested apples to cope with cold winter conditions. This overwintering capacity of introduced medfly populations can determine whether they can establish and grow in colder more temperate areas (Moraiti et al., 2022). Understanding the factors that allow *C. capitata* to adjust to such an enormous range of temperature variation may shed additional important light in elucidating the factors that promote its invasion success in cooler areas of central Europe (Nyamukondiwa et al., 2010).

In recent years, a substantial amount of work has been conducted regarding the thermal tolerance of *C. capitata*, which has mainly focused on the adult stage (Pieterse et al., 2017; Terblanche et al., 2010; Weldon et al., 2018). Acclimation to low temperatures generally increases low temperature tolerance, whereas high temperature acclimation improves high temperature tolerance (Whitman and Agrawal, 2009). Nyamukondiwa et al. (2010), demonstrated positive effects of thermal acclimation on the thermal limits of activity in adults (i.e. critical thermal limits). Nevertheless, there are no studies examining how acclimation to a range of low temperatures can enhance cold tolerance of *C. capitata* in subzero temperatures. Furthermore, little is known regarding the response to thermally stressful conditions of geographically distributed *C. capitata* populations, and nothing is known for those populations in the Northern Hemisphere that originate from areas close to the species northern limits of its distribution (Nyamukondiwa and Terblanche, 2009; Weldon et al., 2018). Regarding SCP, to the best of our knowledge, there is only one study estimating the SCP among different developmental stages (Nyamukondiwa et al., 2013). Although, medfly is characterized as a chill susceptible species, information on the SCP of different developmental stages and different populations broadens the biological knowledge for this important agricultural pest.

To gain insight into whether cold tolerance attributes have changed during the invasion of *C. capitata* in Europe, we determined acute cold stress survival and the SCP of populations obtained from the Northern Hemisphere but reared under 'common-garden' (i.e. similar) conditions. To assess the phenotypic plastic capacity in these populations the effect of acclimation to low temperatures on acute cold stress survival in adults was also examined. We also discuss these data in the context of local climate conditions for each population.

2. Materials and methods

2.1. Populations

To represent better the climatic variability across the Northern

Hemisphere, we used seven *C. capitata* populations originating from Israel (South Arava), Crete (Chania, Heraklion), central and northern Greece (Volos, Thessaloniki), the coastal area of Croatia (Zaton) and Vienna (Austria) (Fig. 1), ranged from relatively cool and humid (e.g. Vienna), to hot and arid (e.g. South Arava; Table 1). The climatic data of the sites used to collect the different populations are given in Supplementary Table S1.

2.2. Insect rearing

The experiments were conducted in the laboratory of Entomology and Agricultural Zoology at the University of Thessaly during September 2019–August 2021 at 25 ± 1 °C, 45–55% relative humidity and 14:10 L: D photoperiod (photo phase started at 07:00 h). Light was provided by daylight fluorescent tubes and by natural light with the intensity inside the rearing cages ranging from 1500 to 2000 Lux. Pupae from Croatia, Austria and Israel were transported by courier to our laboratory in Volos, Greece. Approximately 500–1000 pupae from each location were used to establish experimental colonies in the laboratory. The F1–F6 generations were used to execute the laboratory trial. Within the first six generations there is little variation in biological traits (e.g. response to thermal stress) of "domesticated" populations; however, longer adaption to the lab conditions may affect the response to environmental stress. The Greek populations were derived from field-infested peaches collected around Thessaloniki, and infested oranges from Volos, Chania and Heraklion. The South Arava (Israel) colony was derived from field-infested pomegranate fruits collected in Yotvata, Israel in December 2020, and augmented (F1) on persimmon. The Croatia colony derived from field infested figs from Zaton, (close to Zadar), and the Austria colony derived from field infested pome fruit collected from Vienna.

Colonies from the different populations were maintained in wooden

Table 1
Köppen-Geiger climate classification of different *Ceratitis capitata* collection sites. For each collection site, average temperature (mean, min, max) and humidity is given.

Population	Climate	Average Temperature (°C)			Average Humidity (%)
		Mean	Min	Max	
Vienna	Temperate oceanic	10.80	6.61	14.95	71.83
Zaton	Warm temperate	15.04	11.43	18.3	70.75
Thessaloniki	Cold semi-arid	14.53	11.22	18.68	67.58
Volos	Mediterranean	15.01	11.75	18.28	69.16
Chania	Mediterranean	16.50	14.96	18.41	69.92
Heraklion	Mediterranean	17.75	19.99	21.95	67.66
South Arava	Hyper-arid	20.77	14.30	27.15	41.41

frame, wire-mesh cages under constant density (100 adults per 30 × 30 × 30 cm cage) at 25 ± 1 °C, 45–55% relative humidity and 14:10 L:D photoperiod (photo phase started at 07:00 h). Flies were provided with water and a standard adult diet, consisting of a mixture of yeast hydrolysate, sugar, and water (YS) at 1:4:5 ratio, respectively. Females were allowed to lay eggs on red, hollow, perforated plastic domes (5.5 cm-diameter) that was fitted in the lid of a plastic petri dish. The base of the petri dish was partially filled with water to maintain humidity, and 0.5 ml cup filled with orange juice as an oviposition stimulator. Domes were exposed in the rearing cages for 24 h, and the collected eggs were used to establish and maintain the respective colony. Eggs (50–100) were seeded in the artificial diet (50 g sugar, 50 g brewer's yeast, 25 g soybean flour, 1 g salt mixture, 4 g ascorbic acid, 4 g citric acid, 0.75 g sodium propionate and 250 ml water).
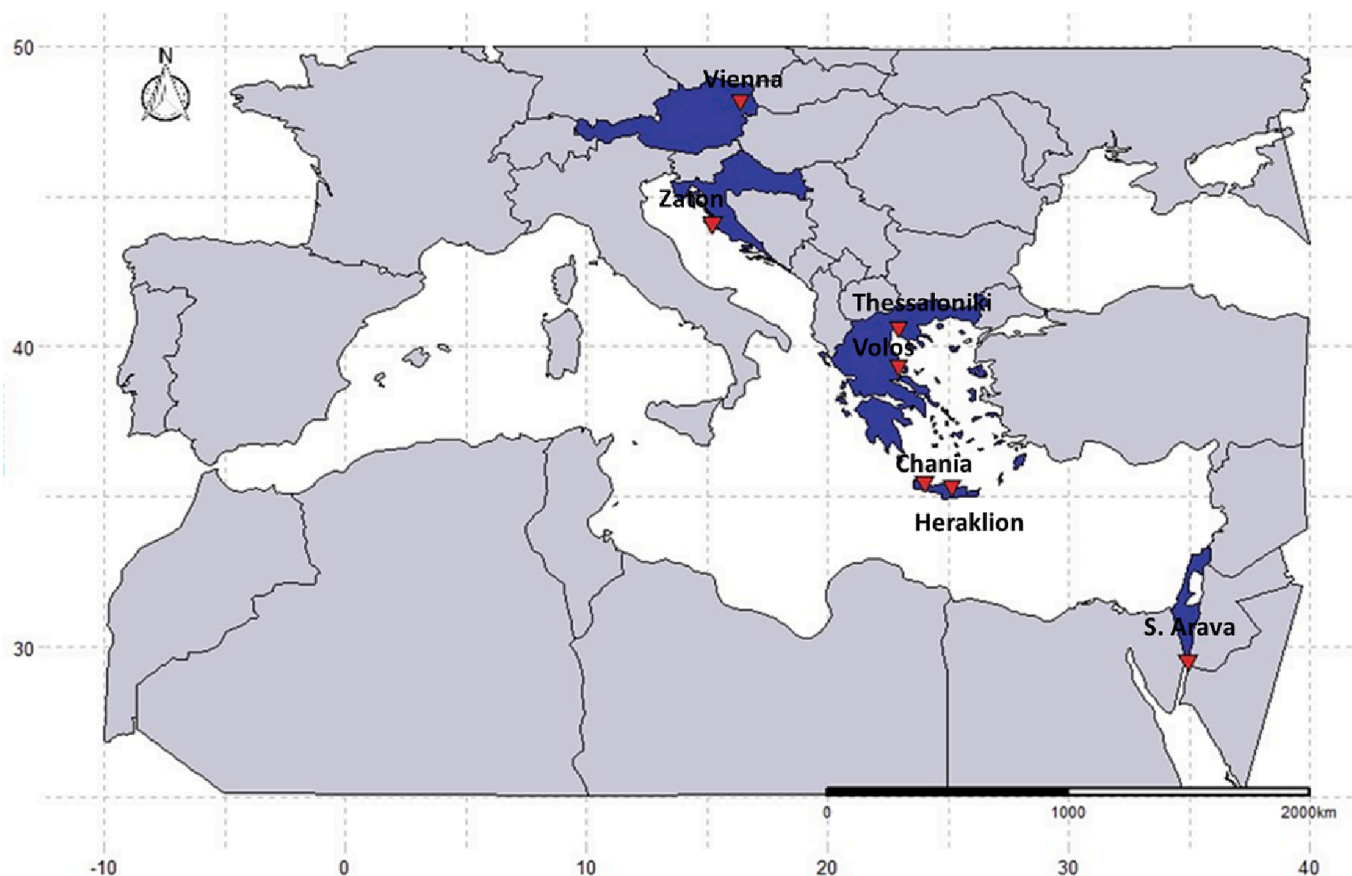

Fig. 1. Locations in the Mediterranean and Central Europe where *Ceratitis capitata* populations used in the current study originated.

2.3. Survival of adults after acute cold stress

To determine the lower lethal temperature that kills 50% of individuals (LLT50) we conducted preliminary trials at a range of low temperatures (-8, −6, −5, −4, −2, −3 °C) in a programmable water-cooling bath. In each trial, flies were subjected to one of the above temperatures for one hour. After exposure, survival was recorded after 24 h. Using a probit analysis, the LLT50 was estimated at −5.5 °C. To estimate the LLT50 after cold treatments, a laboratory strain (Benakeio) kept under the same laboratory conditions for more than 30 years, was used.

To estimate the acute cold stress, 5-day-old adults (males and females) that were acclimated at either 10, 20, or 30 °C for 5 consecutive days were subjected to a standard cold stress of −5.5 °C for 1 h. We used the following populations: Thessaloniki-Greece (F2-F3), Heraklion-Greece (F3) and South Arava-Israel (F4). Flies from each population and acclimation regimes were randomly distributed in 10 replicates of 10 individuals each (5 males and 5 females) and were subjected to acute cold stress (-5.5 °C for 1 h) using 10 glass vials for each replication. Following exposure at −5.5 °C for 1 h, adults were transferred back to 25 °C, placed in plexiglass cages with access to food and water, and checked for survival 24 and 48 h later. Survival after 24 and 48 h was defined as the ability of flies to respond to mild prodding using a thermally inert object (e.g. paintbrush) or expressing other normal behaviors such as walking, flying and feeding.

2.4. Supercooling point

To determine the SCP, the lowest temperature before an exothermic reaction indicating the freezing of body fluids, third instar larvae, pupae (1-day and 5-day old) and adults (5-days old) were assessed. A wild population originated from field infested fruits was used from the area of Thessaloniki. The F1-F6 laboratory generations of the other six populations were used (the South Arava corresponded to the F2-F6). To determine the SCP the protocol of Hou et al. (2009), was followed. Briefly, 30 individuals of each population and life stage were individually loaded into 1.5 ml microcentrifuge tubes. In each tube we inserted a type-T thermocouple sensor (36 SWG) assuring that the sensor, with the help of a cotton-wool wick, got in direct contact with the body of the loaded individual. All tubes were placed into a plastic bag and then into a refrigerated, circulating programmable bath (Polystat, Cole-Parmer) containing silicon oil. Thermocouples were connected to two 8-channel Picotech TC-08 (Pico Technology, Cambridge, UK) thermocouple interfaces that precisely recorded temperature at 1 Hz using the PicoLog software. Experiments were initiated at 15 °C, held for 5 min to equilibrate, and then the temperature programmed to decrease at a rate of 0.23 °C/min.

2.5. Data analysis

Data were checked for normality and equality of variance using the Shapiro-Wilk and the Levene test, respectively. The effects of population, thermal acclimation treatment, sex and recovery time period (survival after 24 and 48 h) and their interactions on adult survival after acute cold stress were assessed by logistic regression model, with a binomial distribution and logit-link function for proportions outcome (i. e. number of dead/alive flies). ANOVA was used to analyze effects of life stage, populations, sex, and age on the SCP. Bonferroni's test was applied to adjust for multiple comparisons at $\alpha = 0.05$.

3. Results

3.1. Response after acute cold stress

Adult response after acute cold stress (mortality rates after exposure at-5.5 °C for 1 h) was affected by the origin of the *C. capitata* population

and acclimation (Table 2). In addition, the recovery time period (24 and 48 h) was a significant predictor of adult mortality rates as well as the interactions between population and acclimation (Table 2). The lowest survival rates were observed in flies originating from South Arava (Israel) acclimated at 30 and 20 °C, after 24 h. The survival rates of flies that had been acclimated at 10 °C were higher than that of flies that had been acclimated at 30 °C, regardless of population (Fig. 2A, B). Pairwise comparison revealed that, regardless of the acclimation, sex and the recovery time period (24 and 48 h), adults from the South Arava population had the lowest survival rates, followed by those of Heraklion, Thessaloniki (*P* < 0.036, Bonferroni test) with no differences between the last two (*P* = 0.292, Bonferroni test).

Plastic responses were significantly different among the tested populations. In particular, the Greek populations followed similar patterns in response to acclimation, while in the population originating from South Arava, both sexes expressed higher plasticity (Fig. 2B). Logistic regression revealed that in South Arava death probability was significantly higher with the increasing of the acclimation temperature compared to Thessaloniki (OR = 0.803 (0.774, 0.832); *P* < 0.01), and Heraklion (OR = 0.844 (0.814, 0.876); *P* < 0.01) (Supplementary Table S2).

3.2. Supercooling point

The SCP differed among the developmental stages (*F* = 117.404, df = 4, *P* < 0.001). Regarding larvae (L$_3$) the SCP varied greatly among populations (*F* = 10.724, df = 6, *P* < 0.001) (Fig. 3A, Table 3). Pairwise comparisons revealed that SCP of larvae (L$_3$) from Zaton was lower (-21.09 ± 0.4 °C) than that from all other populations (*P* < 0.001, Bonferroni test). On the other hand, larvae from Thessaloniki had the highest SCP (−17.12 ± 0.5 °C) compared to all tested populations (Fig. 2A). Larvae from Zaton had significant lower SCP than those of larvae from Volos (*P* < 0.001, Bonferroni test), S. Arava (*P* = 0.012, Bonferroni test) and Heraklion (*P* = 0.003, Bonferroni test), and marginally lower than that of larvae from Vienna (*P* = 0.063, Bonferroni test) (Fig. 3A).

For pupae, SCP among populations differ marginally only between Thessaloniki and South Arava (*P* = 0.048) while pupae age (*F* = 374.489, df = 1, *P* < 0.001) and their interaction (*F* = 11.709, df = 1, *P* < 0.001) were significant predictors of SCP (Fig. 3B, Table 3). Regardless of the population, 1-day old pupae had higher SCP compared to 5-days old ones, except for pupae from S. Arava. The SCP of young pupae (1d old) ranged between −19.71 ± 0.2 °C (South Arava) and −17.94 ± 0.1 °C (Thessaloniki), while that of old pupae (5d old) from −21.08 ± 0.1 °C (Heraklion) to −19.99 ± 0.1 °C (South Arava) (Fig. 3B).

The supercooling points (SCPs) of adults is shown in Fig. 3C. There was a significant difference in SCP among populations (*F* = 4.125, df = 6, *P* = 0.001), but not between males and females (*F* = 0.243, df = 1, *P* = 0.623) (Table 3). Flies from Vienna had significantly higher SCP than

Table 2
Results of the logistic regression model testing the effects of population, acclimation, sex and recovery time period (24 and 48 h) on survival rates of *Ceratitis capitata* adults following an acute cold stress at −5.5 °C for 1 h.

| Source of variation	Wald x^2	df	*P*
Intercept	146.533	1	<0.001
Population	87.832	2	<0.001
Acclimation	186.304	1	<0.001
Sex	0.124	1	0.724
Recovery time period (24 and 48 h)	28.157	1	<0.001
Population*Acclimation	140.333	2	<0.001
Population*recovery time period (24 and 48 h)	4.684	2	0.096
Population*sex	20.350	2	<0.001
Acclimation*recovery time period (24 and 48 h)	8.069	1	0.005
Acclimation*sex	1.332	1	0.249
Acclimation*sex*population	28.379	2	<0.001
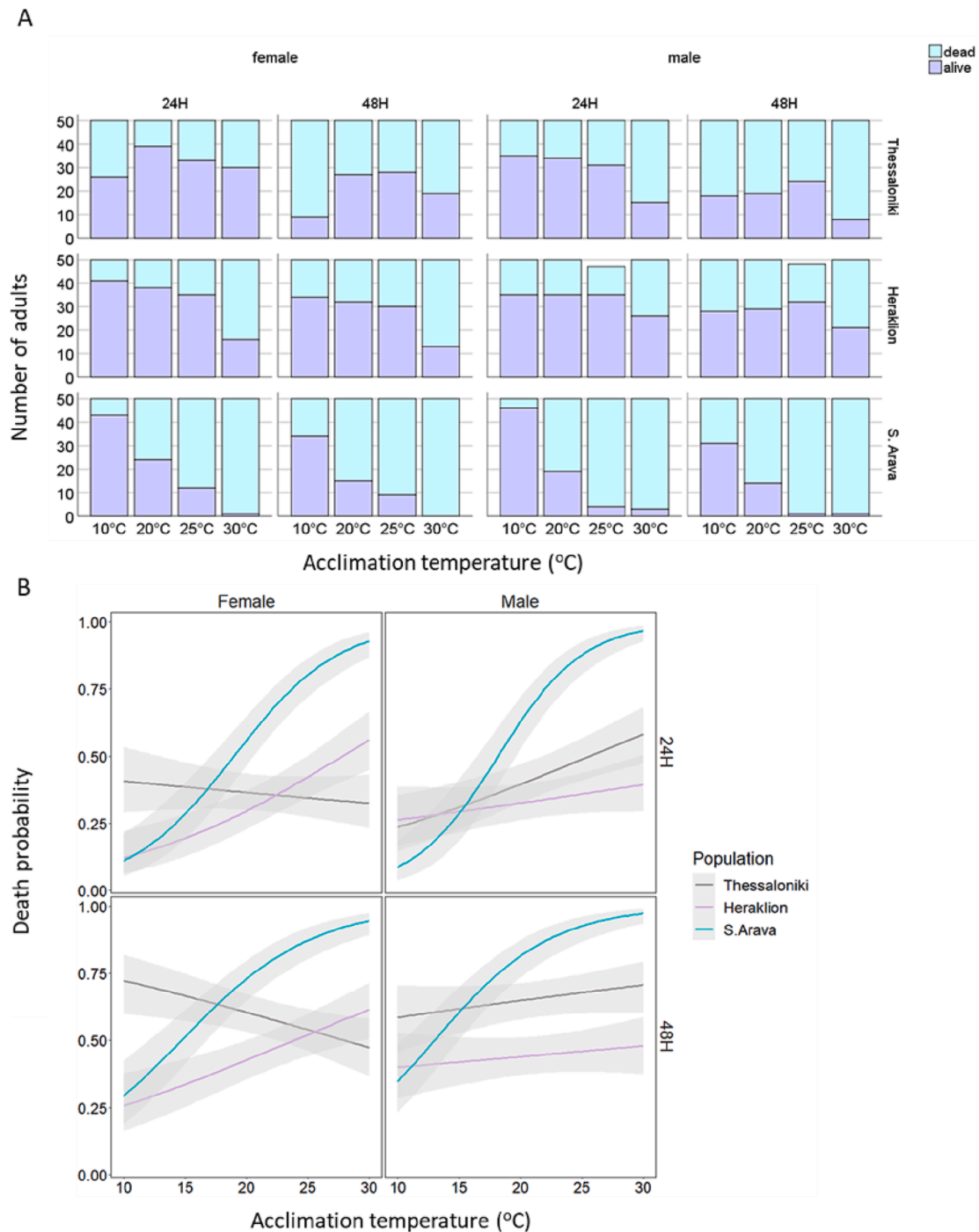
Fig. 2. (A) Response after acute cold stress of adults from three *Ceratitis capitata* populations following different acclimation regimes (30, 20, 10, 25 °C [control] for 5 days). **(B)** Predicted death probabilities following a GLM logistic regression analysis (see Table 2). Acclimated and control flies were subjected to −5.5 °C for 1 h. Mortality was assayed at 24 and 48 h after the cold shock. For each acclimation treatment 50 females and 50 males were used.

that of flies from Thessaloniki ($P = 0.003$, Bonferroni test) and S. Arava ($P = 0.006$, Bonferroni test). SCP ranged from -17.47 ± 0.2 °C (Vienna) to -18.95 ± 0.1 °C (Thessaloniki) (Fig. 3C).

4. Discussion

Our results demonstrate that adult survival after acute cold stress differed among the four populations tested suggesting that geographical origin of population may affect its cold tolerance. The South Arava population was the least tolerant to cold stress, but the most plastic one compared to the other tested populations. Moreover, there was a strong

association between the acclimation regime and cold tolerance of the different medfly populations with populations acclimated in low temperatures being more cold tolerant than those acclimated in warmer temperatures. Furthermore, the SCP differed among developmental stages, and among populations sourced in the Northern Hemisphere. Adults have the highest SCP and pupae (1d old) the lowest one. Older pupae and adults seem to have similar SCP in all studied populations.

4.1. Effect of acclimation on acute cold stress

The extent to which insects exhibit thermal acclimation is affected by
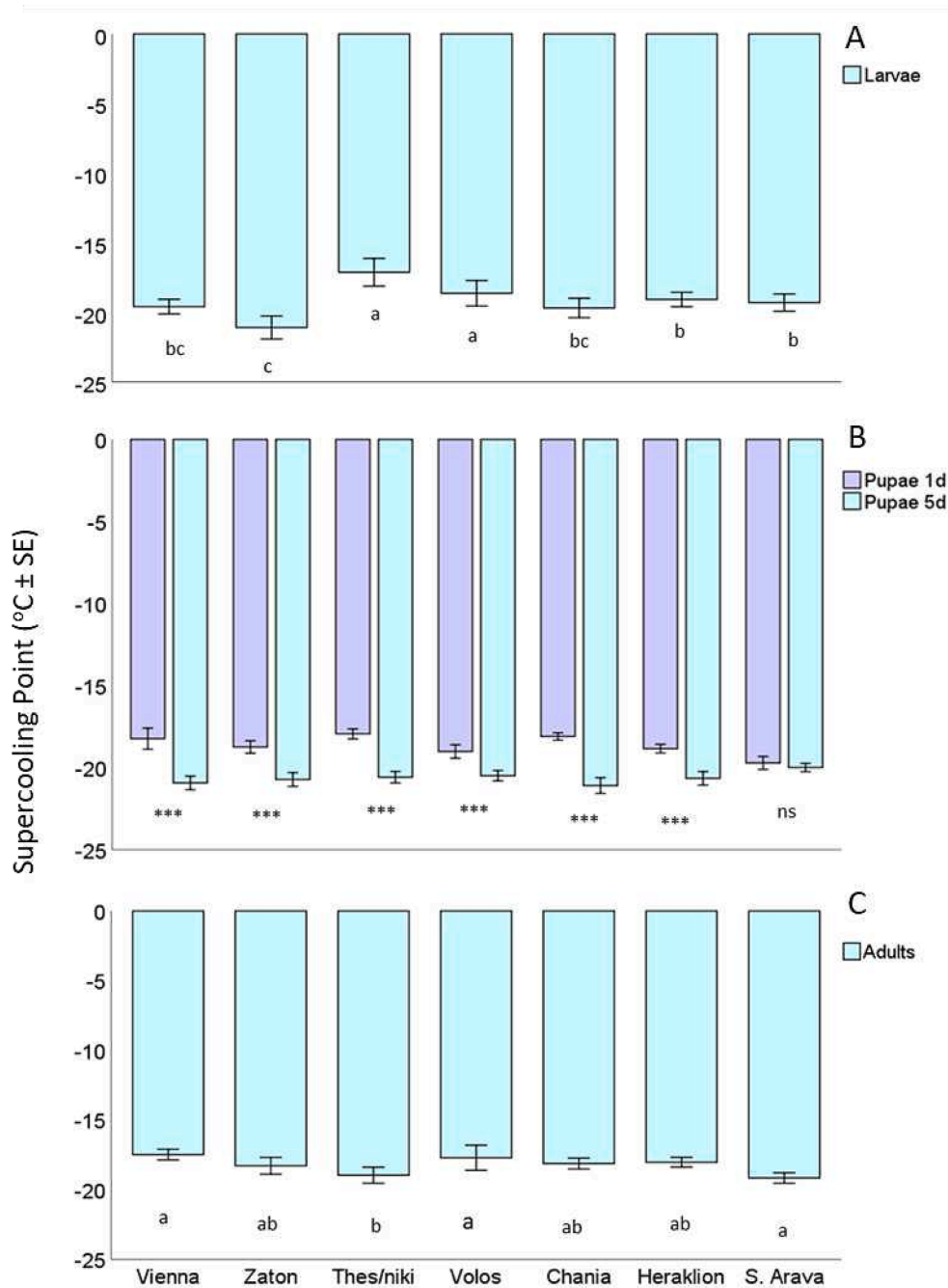
Fig. 3. Average SCP of L$_3$ larvae (A), pupae (B) and adults (C) of different *Ceratitis capitata* populations. Different letters stand for significant difference ($P < 0.005$; Tukey). *P* value <0.001 are marked with three (***) asterisks. *P* value more than 0.05 is designated with (ns). For each population and life stage 30 individuals were used.

the type of the trait tested and the environment at which insects develop (temperate, tropical). Until now, numerous studies have shown how acclimation to low temperatures can increase different metrics of cold hardiness and cold tolerance of diverse insects, such as supercooling point and critical thermal limits (e.g. limits to activity or loss of neuromuscular function) as well as cold tolerance (Enriquez and Colinet, 2019; Jakobs et al., 2015). In the present study, acclimation at low temperatures before cold exposure at −5,5 °C for 1 h, seems to benefit only populations originating from Heraklion and South Arava, with the most benefited one being the S. Arava. In environments with low seasonal variation in temperature, the acclimation ability of insects can be reduced (Ghalambor et al., 2006). In South Arava, adults are captured all year round (Moraiti et al., 2022), so flies are exposed to seasonal

variation in temperature resulting in high acclimation ability. In Heraklion, *C. capitata* overwinters at all stages due to mild winters, and adults during winter can be found at low density so acclimation ability of adults is lower than those of adults in S. Arava. In contrast, in Thessaloniki *C. capitata* overwinters as larvae (particularly 1st and 2nd instars) inside the fruits, and flight period lasts for few months (summer), when there is no high temperature variation (Papadopoulos et al., 2001). This may explain why in the present study acclimation to low temperatures, seems to have little impact on adults from Thessaloniki that were exposed at −5,5 °C for 1 h.

Table 3
ANOVA table on the effects of life-stage, (i) population on SCP of larvae, (ii) population, age and their interactions on SCP of pupae and (iii) population, sex and their interactions on SCP of adults of different populations of *Ceratitis capitata*.

Source of variance	Sum of squares	df	*F*	*P*
Stage	926.438	4	117.404	<0.001
(i) *Larvae*				
Between Population	258.668	6	10.724	<0.001
Within Population	816.043	203		
Error	1074.711	209		
(ii) *Pupae*				
Intercept	162393,685	1	147237.627	<0.001
Population	13.656	6	2.276	0.056
Age	413.038	1	374.489	<0.001
Population*age	77.486	6	11.709	<0.001
Error	448.895	407		
(iii) *Adult*				
Intercept	69229.033	1	30,541	<0.001
Population	56.102	6	4.125	<0.001
Sex	0.548	1	0.242	0.623
Population*sex	7.477	6	0.550	0.770
Error	442.013	195		

4.2. Response of different populations to acute cold stress

Our results revealed that medfly populations originating from different geographic areas respond differently after one hour exposure at −5,5°C. Differences among geographic populations in their tolerance to subzero temperatures may be related to the selection of physiological and metabolic traits appropriate to the prevailing climatic regimes in the area of origin. Greek populations (Thessaloniki, Heraklion) were most tolerant to cold treatment when compared to South Arava flies. Of interest is that the Thessaloniki population (the more Northern population in Greece) and the Heraklion population (the most Southern population in Greece) did not show significant differences in their tolerance to subzero temperatures. Papadopoulos et al. (1996), have studied the overwintering capacity of medfly in northern Greece for three winters, and the results showed that *C. capitata* overwinters almost exclusively in the larval stage followed by pupae in early spring. Hence, low winter temperatures limit adult activity in Thessaloniki. Likewise, the period of flight activity becomes longer in warmer, more southern areas (Katsoyannos et al., 1998; Papadopoulos et al., 2001). In southern Greece a small number of adults might be active during winter. Thus, the population from Heraklion might experience low winter temperatures in the adult stage and may be more or equally cold tolerant compared to that from Thessaloniki that experience low temperatures at the larval stage. This plasticity may be influenced by larval rearing conditions as well as the thermal experience of adults (Rako and Hoffmann, 2006; Ransberry et al., 2011). A global *meta*-analysis of critical thermal limits has shown that lower limits are generally more plastic than upper critical limits, and that development may be a key factor in enhancing thermal-acclimation induced plasticity in insects (Weaving et al., 2022). Weldon et al. (2018), studied the geographic variation in climate stress resistance among *C. capitata* populations from southern Africa. The results revealed that temperature seasonality seemed to be positively related with lower thermal limits, while upper thermal limits were not affected by bioclimatic variables.

The difference in cold resistance among populations originated from different climates and latitudes is consistent with results for other species too (Hoffmann and Watson, 1993). Ayrinhac et al. (2004), studied whether population origin determines the cold adaption of *Drosophila melanogaster*. The results showed that recovery after a cold shock at 0 °C, was faster for populations living at high latitudes under temperate climates than tropical populations. Populations from southern areas in the Northern Hemisphere, are expected to have experienced warmer conditions compared to those from northern latitudes. Mild temperatures in south temperate habitats result in higher winter mean temperatures (Sinclair and Chown, 2005). Also, populations coming from higher latitudes tend to have broader thermal tolerances than those originating from lower latitudes (Chown et al., 2002).

Apparently, *C. capitata* geographical distribution is largely defined by its ability to stand stressful conditions and to respond to seasonal variation in temperature. Behavioral and physiological strategies at different climatic regions and microhabitats where *C. capitata* populations originated may be different resulting in differential selection pressures for thermal response. Understanding cold adaption of different populations its vital to identify latitudinal selection pressures driving these adaptions (Moraiti et al. 2022).

4.3. Effect of sex on acute cold stress

Response to acute cold stress differed between males and females, regarding the population. Females from Thessaloniki respond differently to acclimation than the rest of the flies. Specifically, acclimation at low temperatures before cold exposure at −5,5°C for 1 h did not benefit females, considering that survival after 48 h was similar to that of females acclimated at 20, 25 and 30 °C. In populations originating from Heraklion and S. Arava, survival after acute cold stress did not differ between males and females. This difference regarding the response of males and females after acute cold stress, suggests that thermal acclimation doesn't affect the two sexes in a similar manner. Intersexual differences in response to unfavorable temperatures are common but difficult to explain (Renault et al., 2002) and as revealed in the present study, considering plastic response, are context specific (Scharf et al., 2019). Males and females differ in a wide range of morphological and physiological aspects (Tarka et al., 2018) that may regulate response to different environmental stresses. To our knowledge there are no studies examining how different morphological (e.g. body weight, size) and physiological aspects can affect the acclimation capacity of insects. Understanding how sexual dimorphism can affect acclimation capacity may help to address biases in prediction of *C. capitata* susceptibility to climate change.

4.4. Differences among developmental stages

In agreement with our results, an earlier study on *C. capitata* from South Africa revealed that SCP differs among life stages. Nyamukondiwa et al. (2013), reported that pupae had the lowest SCP (-19.5 °C), a result that agrees with our findings (SCP of pupae was −20.76 °C). Adults had the highest SCP, followed by larvae and pupae. The content of the alimentary channel (e.g. food, symbiotic bacteria) may be involved in ice formation and hence may explain the differences we found among the developmental stages (Tanaka and Udagawa, 1993). The importance of the gut contents as source of ice nucleators had been demonstrated in other species too (Tanaka, 1994). The complete absence of nucleating agents either in the gut, hemolymph or other fluid compartments, may explain the lower SCP of pupae (Renault et al., 2002). Likewise, the empty gut of the ready to pupate L_3 medfly larvae that used in the current study may justify the lower SCP point than those of adults. The lowest among stages SCP of pupae may be explained considering that bare pupae in the soil is a common overwintering stage of *C. capitata* in cooler areas. Furthermore, hemolymph osmolality plays a great role in modulating the SCP, and cryoprotectants (glycerol, ribitol, sorbitol, myo-inositol etc.) which are synthesized by insects to survive in low temperatures, play a key role as compatible osmolytes (Overgaard et al., 2005; Storey and Storey, 1991). *Ceratitis capitata* may accumulate those cryoprotectants during overwintering to increase the chance of winter survival (Denlinger, 2002). In addition to ice nucleators and osmolality, body water content and, thus dehydration level, can also affect the SCP.

Nevertheless, in the present study there are no data regarding the mass and water content of insects among the different populations and developmental stages.

Sex was not a predictor of the SCP of adults. To the best of our knowledge, there are no studies on Tephritidae examining the effect of sex on SCP. This lack of sex-related variation in SCP may be related with the fact that hemolymph properties were similar between males and females in the different populations. Hence, controlling for sex in SCP assays in these species might not be necessary especially when similar young ages are considered (Renault et al., 2002).

4.5. Difference among populations in SCP

Differences among populations in SCP were only detected in the larval and adult stage. Larvae from the Zaton population (Croatia) exhibited the lowest SCP, and adults from the Vienna one lower SCP than that from Thessaloniki and S. Arava. Hence, there is no conclusive correlation between climatic variation and SCP capacity. One possible explanation is that for populations originating from high/low-latitude range edges, maintaining high fitness is not enough, so adaption at range edges remains challenging (Angert et al., 2020). Moraiti et al. (2022), tested *C. capitata* populations with similar broad climatic variability on chill coma recovery assays. The results revealed that despite the differences at the population level, climatic variability did not seem to affect the recovery time after chill coma assays suggesting that the physiological basis of this phenomena requires further investigation.

5. Conclusions

Understanding the cold tolerance strategy is essential for predicting the potential geographical range of *C. capitata*, particularly in an era of rapid climate change. This study is the first to report geographical patterns of two cold tolerance measures (acute cold stress and SCP) of the widespread pest *C. capitata*. The results revealed that geographically isolated populations of the northern Hemisphere to acute cold stress, respond differently. Plastic responses to acute cold stress were population and sex specific. In addition, there are vast population-level differences regarding cold tolerance, SCP is not a good predictor for chill susceptible insects and has not evolved during the *C. capitata* invasion in Europe. In the future, using more sampling sites within the currently distribution range of *C. capitata* in the Northern Hemisphere, is crucial to achieve a proper correlation to biogeography. Thus, our data provide novel insights into thermal biology of medfly and generate important data for predictive population modeling to estimate range expansion of this notorious pest into cooler more temperate areas of Europe.

CRediT authorship contribution statement

Georgia D. Papadogiorgou: Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Cleopatra A. Moraiti:** Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. **David Nestel:** Methodology, Resources, Writing – review & editing. **John S. Terblanche:** Methodology, Writing – review & editing. **Eleni Verykouki:** Formal analysis, Visualization, Writing – review & editing. **Nikos T. Papadopoulos:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing

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Declaration of Competing Interest

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinsphys.2023.104519.

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