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Source: *The Journal of Arachnology*, 52(2) : 143-150

Published By: American Arachnological Society

URL: <https://doi.org/10.1636/JoA-S-23-006>

Cold tolerance strategy, supercooling, and cold hardening in three populations of the jumping spider *Phidippus audax* (Araneae: Salticidae)

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Abstract. Cold temperatures can cause injury and death, and thermal tolerance influences organismal abundance, distribution, and physiology. We characterized cold tolerance and supercooling point (SCP) in the broadly distributed jumping spider *Phidippus audax* (Hentz, 1845). We found that *P. audax* is freeze-avoidant, surviving chilling but not freezing. Freeze-avoidant organisms can reduce harm by lowering their supercooling point (SCP), the temperature at which they freeze. We assayed fall/winter SCP in field-acclimated spiders from Michigan and compared lab-acclimated spiders from Texas, Oklahoma, and Michigan. Field-acclimated Michigan spiders decreased their SCP from -4.7°C in September to -12.9°C in November. In the lab, Michigan spiders had the lowest SCP (-7.65°C) followed by Oklahoma (-6.21°C) and Texas (-5.50°C). In December and January, nearly all the spiders from Texas and Oklahoma died in the simulated freezing conditions while the Michigan spiders survived at high rates, further suggesting that the Michigan spiders were hardiest followed by Oklahoma and Texas. The lab-acclimated spiders did not consistently reduce their SCP from October to December. Smaller spiders had lower SCPs than larger spiders, and the Michigan spiders were significantly smaller than those from Texas or Oklahoma, suggesting that smaller overwintering size or delayed molting to mature size/sexual maturity may be favored by selection in colder climates. This could help explain differences in size and life cycle between northern and southern *P. audax*, as well as a previously described trend for smaller spiders to be more common in colder areas or Europe.

Keywords: Freezing, freeze-susceptibility, overwintering
<https://doi.org/10.1636/JoA-S-23-006>

In colder climates, organisms may experience injury or death resulting from exposure to chilling and freezing temperatures (Tanaka 2001; Sinclair et al. 2015; Toxopeus & Sinclair 2018). These costs can strongly shape species distributions, their use of microhabitats, their abundance, and their utilization of resources. Thus, chilling and freezing can have important ecological and economic implications, particularly in light of a rapidly changing climate (Bale & Hayward 2010; Schneider et al. 2022). Characterizing the effects of cold temperatures on organisms as well as the strategies they employ to resist thermal challenges, is essential to understanding fundamental ecological concepts such as the niche, and to make predictions regarding habitat and range shifts resulting from climate change (Kearney & Porter 2004).

Cold-tolerance strategy describes the relationship between chilling, freezing, and injury for a given species (Bale & Hayward 2010; Sinclair et al. 2015). Chill-susceptible organisms are harmed when chilled even when no freezing occurs, for example through the loss of enzyme function, loss of cellular homeostasis, or reduced membrane integrity (Sinclair et al. 2015; Bayley et al. 2018; Toxopeus & Sinclair 2018). Freeze-avoidant (i.e., freeze-susceptible) organisms tolerate chilling, but die if their body fluids freeze, often as a result of the mechanical disruption of cells or the rapid freeze-concentration of solutes (Denlinger & Lee 2010; Sinclair et al. 2015; Toxopeus & Sinclair 2018). Freeze-tolerant species tolerate ice formation in their bodies, most often by limiting crystal growth intracellularly, even preemptively facilitating ice formation at higher temperatures and in extracellular spaces such as the gut, to limit damage (Sinclair et al. 2015; Bayley et al. 2018; Toxopeus & Sinclair 2018).

Freeze-avoidant organisms have behavioral and physiological adaptations to reduce the probability of freezing when environmental temperatures fall. For example, they may move to warmer microhabitats such as leaf litter or under snow cover (e.g., Bayram & Luff 1993b), or they may lower their supercooling point (SCP), the temperature at which their body tissues freeze (Sinclair et al.

2015). The SCP of body fluids is typically suppressed below 0°C because dissolved solutes lower the melting point, and because small volumes of fluids, as in cells or interstitial spaces, tend to supercool, or freeze at temperatures below the melting point of the fluid (Lee 2010). Freeze-avoidant organisms may further suppress their SCP in a variety of ways, including the concentration of solutes via desiccation to reduce the melting point of body fluids further, or through the elimination of ice-nucleating particles that can promote the initiation of ice crystals. In some instances, they may even produce cryoprotectant molecules such as poly-ols, which further lower the melting point, or antifreeze proteins (AFPs), which suppress the initiation and growth of ice crystals (Bale & Hayward 2010; Lee 2010; Vrba et al. 2022).

We characterized geographic variation in the cold tolerance of the broadly distributed jumping spider *Phidippus audax* (Hentz, 1845). It is found across North America from coast to coast and from southern Canada to northern Mexico (Edwards 2004) and is polymorphic within and among populations, varying in the extent of characteristic body markings and the color of those markings (Taylor & Peck 1974; Hill 1978; Edwards 2004). Spiders from the southern portion of their range are also larger than more northern spiders. For example, Taylor & Peck (1974) reported that spiders from Texas (12 to 14 mm on average) were about 15% larger than a population in Missouri (10–12 mm on average), while Edwards (2004) reported that spiders from Texas and Mexico may reach sizes up to 20 mm.

Northern and southern populations also experience distinct climates. In northern regions, extended harsh winters feature routine freezing at much lower temperatures, while in southern regions freezing is far less common with typically mild-to-warm winters. Where freezing winter temperatures are a regular occurrence, *P. audax* exhibits a highly coordinated annual life cycle. Spiders overwinter in a sexually immature stage and molt to sexual maturity in the spring. Mature spiders reproduce and die in the spring and summer, and offspring grow until fall when they overwinter again as immature

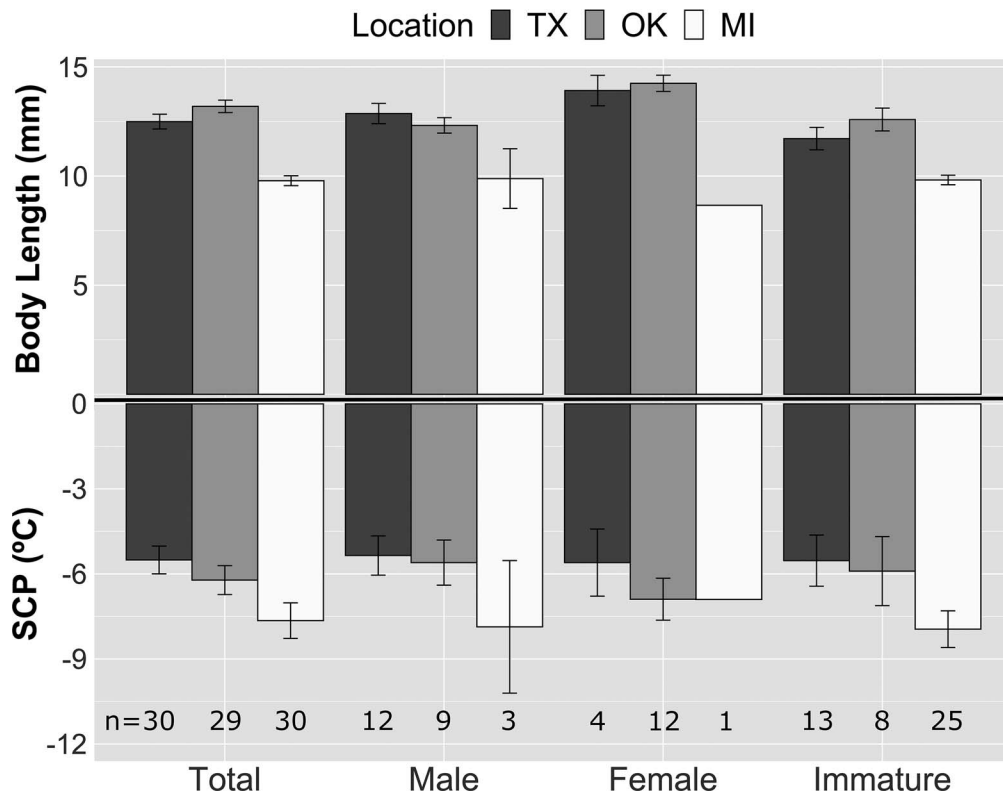


Figure 1.—Mean (\pm SE) body length (mm), SCP ($^{\circ}$ C) and sample sizes for all spiders, males, females and immature (indeterminate) spiders from Texas (TX), Oklahoma (OK) and Michigan (MI). In TX and MI each, there was one spider for whom SCP was measured, but photos were not taken for size measures and sex determination. Sample sizes for each group are provided.

spiders (Edwards 2004). In contrast, in warmer regions, diverse age classes may be found throughout the year (Edwards 2004), suggesting that the life cycle is less structured with a shortened, or even absent, overwintering period.

Together, these phenotypic and ecological differences suggest that populations may have been shaped uniquely, and that differences in cold hardiness could exist between northern and southern populations. To assess whether populations differ in cold hardiness, we determined the cold-tolerance strategy for *P. audax*, assessed how SCP changed seasonally, and compared the cold hardiness of spiders from three locations with different average winter conditions: western Michigan where the average winter minimum is -6.1° C (Station USW00094860), southern Oklahoma with an average winter minimum of -1.3° C (Station USC00345563), and south/central Texas with an average winter minimum of 8.3° C (Station USC00419559; 2006–2020 Winter Averages from National Centers for Environmental Information; Palecki et al. 2021). We assessed whether there were differences in SCP, or in the capacity to lower SCP in response to seasonal cues of the onset of winter. Based on previous studies of insects and spiders in similar temperate continental habitats (Bayram & Luff 1993a, b; Tanaka 1996; Murphy et al. 2008; Turnock & Fields 2005; Murphy et al. 2008), we predicted that all populations would exhibit a freeze-avoidant cold-tolerance strategy and that they would suppress their SCP when exposed to cold temperatures and shortened day length. In addition, we predicted that the Michigan population would exhibit the greatest resistance to freezing, followed by Oklahoma, and finally Texas.

METHODS

Spider collection & maintenance.—We measured thermal tolerance in “field-acclimated” *P. audax* spiders from Michigan (MI), as well as “lab-acclimated” *P. audax* spiders from Michigan (MI), Oklahoma (OK) and Texas (TX) to better understand the factors shaping thermal performance, to assess any geographical differences, and to determine whether lab-acclimatization influenced thermal performance. All spiders were collected primarily from fences, as well as other man-made structures and vegetation. From September 2017 to January 2018, we collected 42 field-acclimated spiders near Allendale, MI (mean location: 42.9732° N, 85.8873° W) and measured SCP within 5 hours of collection to limit acclimatization to lab conditions. In August 2019, we collected 171 spiders which were acclimated to lab conditions for at least 5 weeks before SCP measurement. These lab-acclimated spiders included 56 spiders from near Kingston, OK (mean location: 33.7267° N, 96.8670° W), 60 spiders from Sinton, TX (mean location: 28.1149° N, 97.3967° W), and 55 spiders from western MI (mean location: 41.9147° N, 85.1726° W). All spiders in both studies were sexually immature at the time of collection, but some molted to sexual maturity in the lab (see Fig. 1).

Lab-acclimated spiders were housed individually in round 16 oz plastic containers nested within a 32 oz container containing tap water (Gordon Food Service). Containers had a foam stopper in the lid, a 3-inch PVC tube for structure, and a cotton roll through the bottom of the 16 oz container which wicked water from the lower container, humidifying the upper chamber and providing a water source. While spiders were still active (until the end of

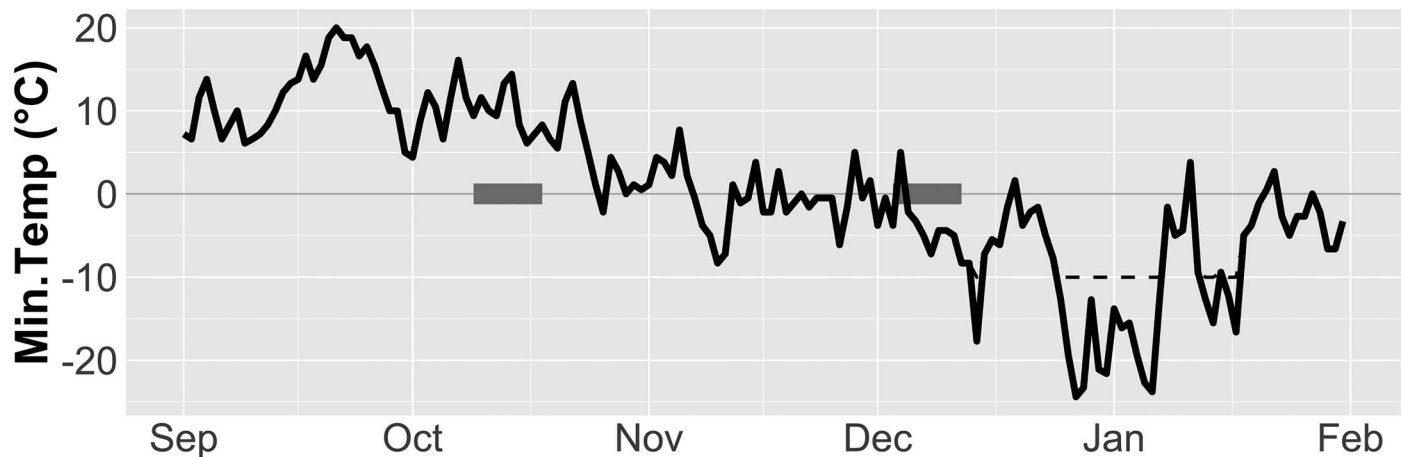


Figure 2.—Daily low air temperatures in Allendale, Michigan during the period of the study in 2017. In 2019, spiders were housed in growth chambers that matched these same low temperatures from day to day. Dashed lines show dates when the minimum possible temperature of -10°C was met in the growth chamber. SCP measurement periods in 2019 are indicated with gray bars.

October), they were fed once or twice a week with size equivalent or smaller crickets (*A. domesticus*; Ghann's Cricket Farm). Their containers were cleaned periodically by picking out dead insects, wiping the container down with wet paper towels, and replacing the cotton wick when soiled.

Beginning on 1 September 2019, spiders from all three locations were maintained in a growth chamber that regulated daylength and temperature to provide seasonal cues of the onset of winter. Sunrise and sunset times as well as the daily high and low air temperatures were matched to the same dates in 2017 for Allendale, Michigan (see Fig. 2 for daily low air temps). Temperature data were obtained from the Weather Underground historical dataset (Historical Weather, online at <https://www.wunderground.com/history>). The highest daily temperature was set to occur at 3 pm, and the lowest daily temperature was set to occur at dawn. Temperature ramped linearly between these set points. The minimum temperature possible in the chamber was -10°C . Each shelf within the growth chamber contained spiders from all three populations to minimize shelf effects.

SCP measurement.—In 2017, we measured SCP as soon as possible following collection (within 1–5 hours of collection). In 2019, spiders from each population were randomly assigned to three groups for SCP measurement at one of three timepoints: 9–18 October 2019, 3–12 December 2019, and 24 January to 1 February 2020. However, due to near total mortality of the OK and TX spiders prior to the January period, no SCP measurements were collected in January. We measured SCP in 15 spiders from each population at each timepoint, except for OK at the December timepoint, when we only measured 14.

During SCP measurements, spiders were secured, 1 to 6 at a time, to the lid of a polystyrene 12-well culture plate using thin strips of aluminum foil tape across the pedicel (the constriction between the cephalothorax and abdomen) as well as the front legs. We also placed a small amount of thermal paste (TE Technologies, Inc. TP-1 Thermal Paste) on the sternum of the cephalothorax to improve thermal conduction with the cooling block. The bottom of the culture plate, with holes to facilitate access, was then placed over the spiders, enclosing them in the wells, and the assembled plate was placed on a programmable Peltier cooling block (TE Technologies, Inc. TC-720 controller and CP-036 block). The holes

in the culture plate were covered with plastic coverslips to limit condensation during cooling, and the plate and block were insulated with foam. The block was cooled at $0.25^{\circ}\text{C}/\text{minute}$ from room temperature ($\sim 20^{\circ}\text{C}$) until an exotherm, the release of heat associated with the formation of ice crystals, was observed (Sinclair et al. 2015).

The temperature of each spider was measured during cooling with a 36-gauge K-type thermocouple (Omega 5TC-TT-K-36-36) that passed through a small opening in the coverslips and was adhered to the carapace with a small amount of thermal paste. Thermocouple measurements were logged on a LabJack U6 data logger and CB-37 terminal board. The data logger collected 10 measurements per second, and measurements were smoothed by averaging 5 measurements in a sliding window. The SCP was estimated as the coldest average temperature recorded prior to the exotherm.

To assess how SCP changed over time in 2017, we fit a linear regression model in SPSS v. 25.0 (IBM Corp.) predicting log transformed SCP temperatures ($\ln[20+\text{SCP}]$) from date. To assess how body size, population and month influenced SCP in 2019, we fit a Generalized Linear Model using Proc GenMod in SAS 9.4 (SAS Institute Inc.). The model predicted log transformed SCP temperatures ($\ln[20+\text{SCP}]$) from collection location (TX, OK, MI), month of SCP measurement (OCT, DEC), and log transformed body length. The model estimated the effects of the OK and MI locations relative to TX, and the effect of measurement in DEC relative to OCT. The overall significance of the effect of collection location was assessed with a Wald test (SAS Institute Inc.), which tested the hypothesis that both parameters for location (OK relative to TX and MI relative to TX) were not different than 0. A model with interaction terms (QIC: 91.7, QICu: 93.0) did not improve model fit over one without interactions (QIC: 91.7, QICu: 91.0), so the model without interactions was retained.

Body size, sex and maturity.—Immediately prior to SCP measurements, we photographed each spider to determine the sex, maturity, and total body length for each spider. Spiders were placed inside a small petri dish and their dorsal and ventral surfaces were photographed with a 1 mm standardized grid. Three to five images were focus-stacked using Helicon Focus (Helicon Soft Ltd., Ukraine) to ensure clear pictures. We measured body length of the dorsal surface, from the posterior tip of the abdomen to the anterior edge of

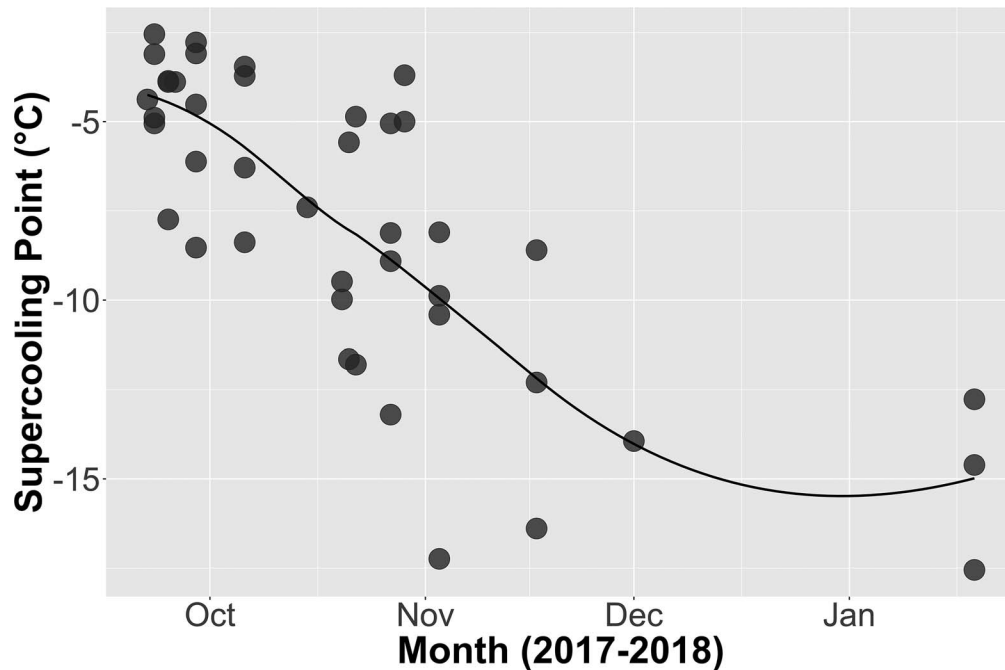


Figure 3.—The relationship between the supercooling point (SCP) and date for field-acclimated spiders.

the carapace between the primary eyes, using ImageJ v. 1.52 image-analysis software (U.S. National Institutes of Health). We assessed the sex and maturity of the spiders by determining whether they possessed mature pedipalps (males), or a visible epigynal opening (females). Spiders were classified as male, female, or of indeterminate sex (immature). Comparisons between populations for body size, proportions of sexes and ages were conducted in SPSS v. 25 (IBM Corp.) using ANOVAs and the LSD correction for multiple comparisons in post-hoc tests.

Cold tolerance strategy.—We determined whether *P. audax* was chill-susceptible, freeze-avoidant or freeze-tolerant by assessing mortality for spiders that had frozen, and those that had been chilled only. We cooled 17 spiders from TX ($n = 9$) and MI ($n = 8$), as described above, in groups of 2 to 4 until we observed exotherms in approximately half of the spiders (indicating that they had frozen). The other spiders were deemed to have been chilled but not frozen. We then stopped cooling and allowed spiders to return to room temperature naturally, with no additional warming or cooling. We assessed survivorship of frozen and chilled spiders 1 hour, 1 day, and 1 week following the cooling treatment. If *P. audax* is chill susceptible, then chilled spiders should die, if freeze-avoidant, then only spiders that froze should die, and if freeze-tolerant, then even spiders who froze should survive.

RESULTS

Cold tolerance strategy.—A total of 8 of the 17 spiders in the cold tolerance strategy study reached their supercooling point and froze (3 TX spiders and 5 MI spiders) at an average temperature of -4.7 °C (range: -0.1 to -13 °C). All the spiders that froze were dead immediately upon warming while all the spiders who did not freeze were alive and actively feeding 1 week later. In addition, none of the spiders who were frozen as part of the SCP study recovered following freezing. These observations indicate that *P. audax* is not

chill-susceptible, nor can it tolerate freezing, and thus, has a freeze-avoidant cold tolerance strategy.

Field-acclimated spiders.—Field-acclimated *P. audax* from MI cold-hardened, reducing their average SCP between September 2017 and January 2018 ($\ln\text{SCP} = -0.012 \cdot \text{Date} + 2.76$, $F = 46.42$, total $df = 41$, $P < 0.001$, $R^2 = 0.537$). The SCP decreased linearly from 22 September 2017 to 30 November 2017 and then reached an asymptote between the end of November and January (Fig. 3). The SCP dropped from an average of -4.6 ± 0.48 °C in September (mean \pm SE), to -7.5 ± 0.75 °C in October, to -12.9 ± 1.0 °C from November to January. The minimum SCPs observed were between -15 °C and -18 °C.

Lab-acclimated spiders.—Spiders from MI, OK and TX differed in size, as well as the proportion of immature spiders (Fig. 1). The MI sample had a much greater percentage of immature spiders of indeterminate sex (83%) than either TX (43%) or OK (27%; $\chi^2 = 26.62$, $P < 0.001$). In addition, the MI spiders had smaller body lengths (9.79 ± 0.23 mm; ANOVA: $F = 38.817$, $P < 0.001$) than either TX (12.54 ± 0.35 mm; $P < 0.001$) or OK (13.19 ± 0.28 mm; $P < 0.001$), but TX and OK did not differ ($P = 0.125$). MI spiders remained smaller than TX or OK even when comparing mature (ANOVA: $F = 12.255$, $P < 0.001$) or immature spiders alone (ANOVA: $F = 17.780$, $P < 0.001$). Within locations, males, females, and spiders of indeterminate sex (immature) did not differ in size in either TX or MI (both $P > 0.1$), but in OK, females were larger (ANOVA: $F = 6.61$, $P = 0.005$) than either males ($P = 0.003$) or indeterminate sex spiders ($P = 0.01$).

Overall, the SCP of the lab-acclimated spiders was -6.5 ± 0.33 °C (SE). There were no differences in the overall mean SCP between males, females and immature/indeterminate sex spiders (ANOVA, $F = 1.44$, $P = 0.24$). Spiders from MI had a lower SCP (-7.65 ± 0.63 °C; ANOVA $F = 4.832$, $P = 0.002$) than spiders from OK (-6.2 ± 0.51 °C; $P = 0.013$) or TX (-5.5 ± 0.49 °C; $P < 0.001$), but the SCP in TX and OK did not differ ($P = 0.40$; see Fig. 4).

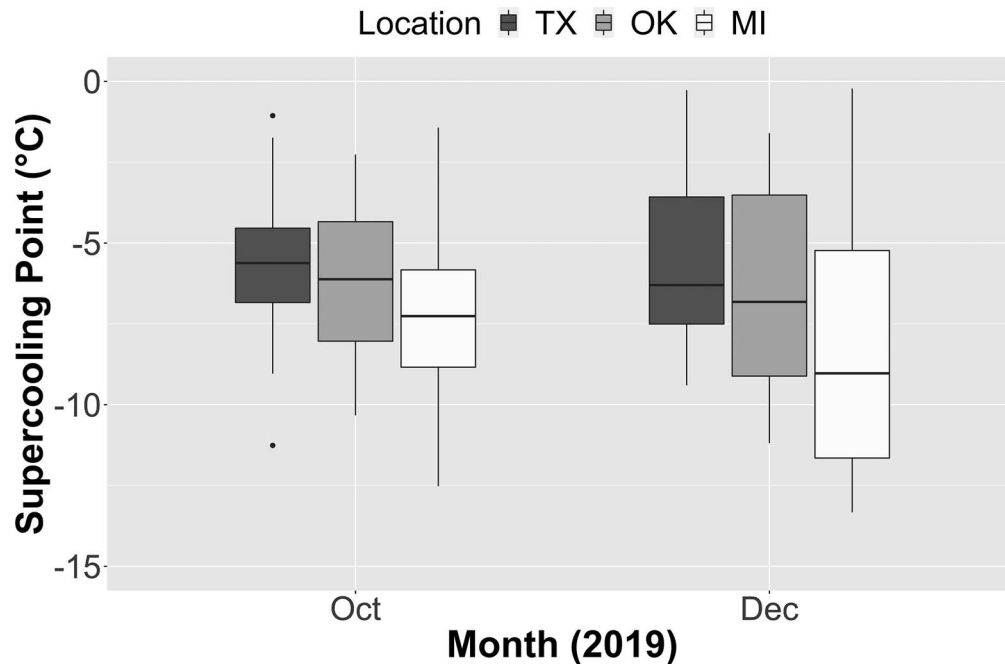


Figure 4.—Mean (\pm SE) supercooling point (SCP) in lab-acclimated spiders from OK, TX, and MI, in October and December 2019.

Collection location affected SCP (Table 1; $P = 0.0102$), but while collection from OK had a significant negative effect on SCP ($P = 0.004$) relative to TX, collection from MI was marginally insignificant ($P = 0.08$), despite being the location with the lowest mean SCP. Body size strongly influenced SCP ($P = 0.0014$), and larger spiders had higher SCPs than smaller spiders across all populations (Fig. 5).

While the mean SCP tended to decrease from October to December in all three populations (Fig. 4), month was not a significant factor (Table 1; $P = 0.45$). Thus, while the average SCP of the MI spiders was comparable between October 2017 (-7.5 ± 0.75 °C) and October 2019 (-7.3 ± 0.70 °C), the lab-acclimated populations in

Table 1.—Generalized linear model relating collection location, month of SCP measurement, and body length to the SCP in 2019 samples. The model estimated the parameter (effect), SE of the parameter, and p-value for OK and MI locations relative to TX, SCP measurement in DEC relative to OCT, and finally, body size. Negative parameters indicate factors that lowered the SCP relative to the reference level. The collective significance of the location parameters (i.e., were there any significant effects of Location overall) was estimated using a Wald test. Significant p-values are indicated in bold.

Variable	Parameter estimate		Parameter p-value	Wald test p-value
	b	SE		
Intercept	1.39	0.40	0.0006	0.0108
Location				
TX	–	–	–	
OK	–0.09	0.03	0.0040	
MI	–0.09	0.05	0.0816	
Month				
OCT	–	–	–	
DEC	–0.05	0.06	0.4495	
ln (body length)	0.52	0.16	0.0014	

2019 did not cold-harden as effectively as the field-acclimated MI population from 2017. However, while the mean SCP did not change significantly in any population from October to December, the variance in $\ln(\text{SCP})$ increased significantly in the MI population (Levine's test, $F_{1,28} = 8.53$, $P = 0.007$), but not in OK ($F_{1,27} = 1.43$, $P = 0.24$) or TX ($F_{1,28} = 0.11$, $P = 0.74$).

Between 1 September and 12 December, spiders from all three populations survived in the growth chambers at high rates with daily mortality of between 0.00 to 0.22%. However, between 12 December 2019 and 24 January 2020, the TX and OK spiders experienced an order of magnitude higher daily mortality of 2.33% and 2.22% respectively, resulting in nearly complete mortality during this period (Table 2). During this same period, the MI spiders continued to experience low mortality (0.23%) and the level of mortality between MI and TX/OK differed significantly ($\chi^2 = 12.9$, $df = 4$, $P = 0.012$).

DISCUSSION

The SCP of arthropods is influenced by diverse factors including the season, their physiological status, microhabitat, feeding history, and life stage (Bowler & Terblanche 2008; Sinclair et al. 2015). Additionally, the specifics of the protocol used to measure SCP, including how they were acclimated, the speed at which temperature was ramped, and features of the measurement apparatus, can affect SCP as well (Sinclair et al. 2015). All of this can make direct comparisons of SCPs across studies challenging. However, the SCPs we measured in *P. audax*, ranging from -0.2 to -17.5 °C, are consistent with previous estimates for temperate spiders, which ranged from about -1 to -20 °C (Cloudsley-Thompson & Constantinou 1992; Bayram & Luff 1993a; Tanaka 1996, 2001; Tanaka & Watanabe 1996; Murphy et al. 2008). These previous estimates only included a single measurement of SCP from a salticid jumping spider (*Euophrys lanigera* (= *Pseudeuophrys lanigera* (Simon, 1871) SCP = -13.7 °C; Cloudsley-Thompson & Constantinou 1992).

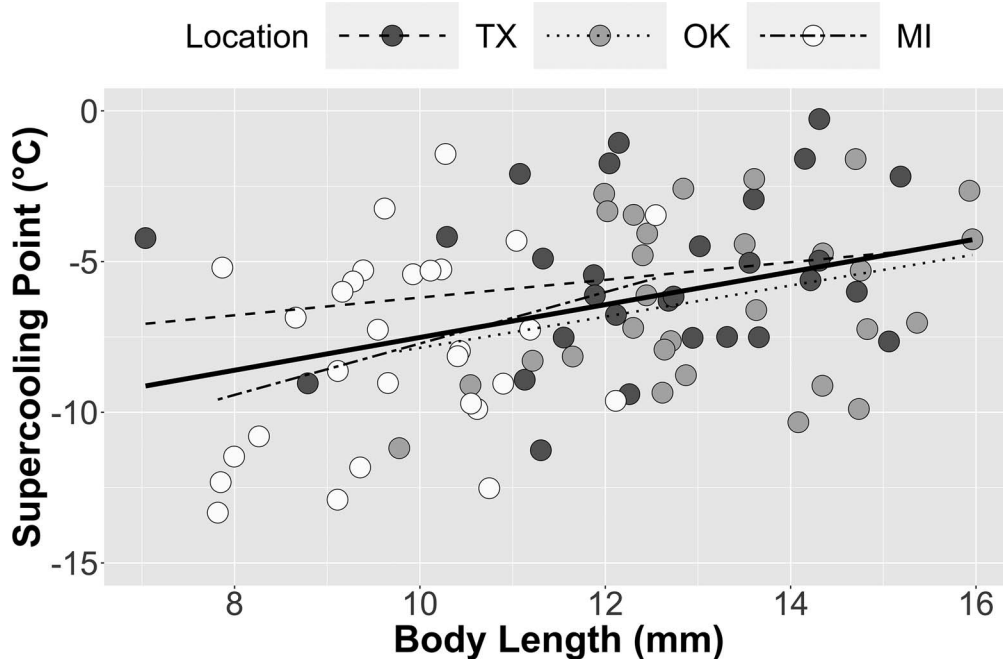


Figure 5.—The relationship between supercooling point (SCP) and body size for all samples (solid line), and within each location (dashed lines).

P. audax demonstrated a freeze-avoidant cold tolerance strategy. Whereas freeze-tolerant species often initiate freezing at warmer SCPs to reduce damage from rapid freezing, freeze-avoidant species typically lower their SCP to avoid mortality (Lee 2010). In accordance with this expectation, the field-acclimated spiders from Michigan reduced their SCP in advance of the onset of the coldest winter temperatures. The coldest winter conditions observed in 2017, a week of minimum temperatures between -19°C and -24°C in late December, surpassed the observed SCPs in our study. Thus, while the temperatures spiders experience in their shelters and microhabitats may be moderated (e.g., Cramer & Maywright 2008), it's clear that SCP suppression is essential to the survival of *P. audax* in Michigan, and that the limits of their ability to resist freezing potentially restrict their distribution.

The lab-acclimated spiders did not reduce their SCP similarly, though the MI spiders did exhibit increased variation in SCP. Thus, while some spiders may have lowered their SCP, the response was inconsistent. We did not assess the mechanisms by which field-acclimated spiders lowered their SCPs, limiting our ability to assess

Table 2.—Summary of mortality in the growth chambers for spiders collected from Texas (TX), Oklahoma (OK) and Michigan (MI) during the three periods between SCP measurements. For each population and time period, the table reports the number of spiders that died during the period, the total number at the start of the period, and the average daily percent mortality during the period. The total at the start of each period is reduced from the end of the previous period because individuals were sacrificed for SCP measurements. Periods of high mortality are bolded.

Dates	TX	OK	MI
Sep 1 - Oct 18	4/60 (0.14%)	0/56 (0.00%)	3/55 (0.11%)
Oct 18 - Dec 12	4/41 (0.18%)	5/41 (0.22%)	2/37 (0.10%)
Dec 12 - Jan 24	22/22 (2.33%)	21/22 (2.22%)	2/20 (0.23%)

Mortality/Starting Population (% Daily Mortality)

why lab-acclimated spiders did not lower SCP consistently. In addition, there are many conditions in the lab that could have influenced SCP, including differences in food/feeding schedules (Tanaka & Watanabe 1996), the smooth ramping between high and low temps in the cold chamber, insufficient seasonal cues of winter temperatures, the inability to seek favorable microhabitats, or the inability to desiccate. Desiccation increases the concentration of solutes in body fluids, lowering the SCP, and has been shown to lower SCP in other spiders (DeVito & Formanowicz 2003; Murphy et al. 2008). It is possible that lab-acclimated spiders held in small, humidified enclosures experienced higher relative humidity than in the field, limiting evaporative water loss. Additionally, while spiders in the field likely curtail feeding with the onset of cold temperatures, lab-acclimated spiders were in small enclosures with prey and a water source, possibly allowing them to feed (or drink) much later in the season during brief windows of warmer temperatures. This would not only limit desiccation, raising the melting point of body fluids, but could also introduce ice nucleating particles into their gut, promoting ice formation at warmer temperatures (Lee 2010; Sinclair et al. 2015).

We collected the vast majority of the spiders in our study from nests in fences, and if less-protected spiders (i.e., higher SCPs) died or moved to more protected, but less accessible, microhabitats as temperatures fell, then sampling bias rather than cold hardening could also explain the increasingly lower SCPs in the spiders collected later. For several reasons, we think this is unlikely. First, we collected 42 living spiders in 2017, and only ever observed 1 dead spider in its silken nest. Thus, mortality in the sites we collected from was low. In addition, through the period when SCP was reduced in 2017, from September to November, the environmental temperatures remained relatively high (typically above 0°C), and thus are unlikely to have killed spiders, even those with higher SCPs. We also observed low mortality in the lab-acclimated Michigan spiders, further suggesting that mortality is limited in the Michigan population. Finally, for much of the period over which

SCP was reduced, temperatures were cold enough that the ability of the spiders to efficiently move into different microhabitats was likely limited, and nearly all the silken nests that we inspected remained occupied, indicating that the spiders nesting in fences had not moved to a new microhabitat.

The populations from MI, OK, and TX differed in their cold hardiness as predicted. The MI spiders had the lowest SCP. The linear model found that body size strongly influenced SCP, and because the MI spiders were so much smaller than those from TX or OK, much of the difference in SCP for the MI spiders was attributed to size. After accounting for size, collection from the OK site remained a significant factor predicting a lower SCP relative to TX. The death of nearly all the TX and OK spiders in December and January, while nearly all the MI spiders survived, further suggests that the MI spiders were much hardier. The TX and OK spiders who died in the growth chambers during this period, which was the coldest period of the experiment, likely died when they exceeded their SCPs, and their body tissues froze. However, while freeze-avoidant individuals typically only die when their body fluids freeze, prolonged exposure to temperatures below freezing can disrupt cellular physiology sufficiently to cause death, even in the absence of freezing (Bale 2002; Toxopeus & Sinclair 2018). In either case, the observed SCPs and patterns of mortality clearly suggest that TX and OK spiders were less cold hardy than spiders from MI.

Our findings were consistent with previous work demonstrating that *P. audax* from southern populations are larger than more northern spiders and exhibit less coordinated annual life cycles, with more diverse age classes in a given season (Taylor & Peck 1974; Edwards 2004). We observed that many of the TX and OK spiders molted to sexual maturity in the fall while most of the MI spiders did not, and that the MI spiders were much smaller than either TX or OK, even when comparing only mature or immature spiders to control for maturity. These differences could reflect ecological factors such as the absence of competition with other species of *Phidippus* CL Koch, 1846 (Edwards 2004), or a longer growing season (Mousseau 1997) in southern regions. This study suggests that increased winter mortality of larger spiders could also favor smaller size in northern *P. audax*. Larger individuals tend to have higher SCPs in a wide range of invertebrates (e.g., Bayram & Luff 1993b; David & Vannier 1996; Hahn et al. 2008). While larger individuals are more resistant to desiccation (Entling et al. 2010), which could be adaptive in drier, hotter regions, this limits their ability to lower SCP via desiccation. In addition, because larger individuals have a greater volume of fluids, there are more ice nucleation sites, increasing the probability of freezing at a given temperature (Bale 2002; Toxopeus & Sinclair 2018). While we did not find evidence that immature spiders had lower SCPs than mature spiders, overwintering in an immature stage and molting to sexual maturity in the spring might enhance winter survival in colder regions, either because immature spiders are smaller, or as a result of a physiological or morphological difference between immature and sexually mature individuals. Since the MI spiders were less likely to molt to sexual maturity, this delayed maturation could be involved in their greater cold hardiness.

This study demonstrates that thermal variation across the range could shape the distribution and body size of *P. audax*, particularly at the extremes of the range. This helps to explain recognized variation in size within *P. audax*, which are smaller in the north. It also suggests a new explanation for interspecific trends in Europe that find smaller spiders in cool, moist regions and larger spiders in

warm, dry locations (Entling et al. 2010). Prior work attributed these patterns to differences in metabolic rates, desiccation resistance, and community interactions, but did not consider cold hardiness (Entling et al. 2010).

ACKNOWLEDGMENTS

We thank the Grand Valley State University (GVSU) Center for Scholarly and Creative Excellence for funding and Sango Otieno from the GVSU Statistical Consulting Center for assistance with the GLM Analysis. We also thank the Welder Wildlife Refuge as well as the University of Oklahoma Biological Station who graciously provided housing and a place to collect. Finally, we thank George McBane and Richard Vallery, for valuable technical assistance developing our methodology for measuring temperatures, Will Bowers for assistance with R visualizations, and Amy Russell for feedback on the manuscript.

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Manuscript received 2 February 2023, revised 6 June 2023, accepted 16 July 2023.