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Supercooling points of freeze-avoiding bumble bees vary with caste and queen life stage

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A R T I C L E I N F O

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A B S T R A C T

Bumble bees thrive in cold climates including high latitude and high altitude regions around the world, yet cold tolerance strategies are largely unknown for most species. To determine bumble bee cold tolerance strategy, we exposed bumble bees to a range of low temperatures and measured survival 72 h post-exposure. All bees that froze died within 72 h while only one bee died without freezing, suggesting that bumble bees are generally freeze-avoiding insects and may be slightly chill susceptible. We then assessed whether temperatures that cause internal ice formation (supercooling points, SCP) varied among bumble bee castes (drones, workers, and queens), or across queen life stages, collection elevation, species, or season. Males froze at significantly lower temperatures than workers or queens. Queens in pre-overwintering or overwintering states froze at significantly lower temperatures than queens stimulated to initiate ovary development by CO_2 narcosis (i.e., “spring” queens). We also tested whether the presence of water (i.e., wet or dry) or ramping rate affected SCP. As expected, queens inoculated with water froze at significantly higher temperatures than dry queens. SCP tended to be lower, but not significantly so, at faster ramping rates (0.5 °C/min vs 0.25 °C/min). We also found no differences in SCP between queen bumble bees collected in spring and fall, between queens collected at two sites differing in elevation by 1100 m, or between three field-caught bumble bee species. Bumble bees appear to have relatively high, invariable SCPs, likely making them highly susceptible to freezing across all seasons. As bumble bees are not freeze-tolerant and appear to lack the ability to prevent freezing at temperatures much below 0 °C, they may rely on season- and caste-specific micro-habitat selection to thrive in cold climates.

1. Introduction

Low temperatures are primary determinants of insect distributions (Andersen et al., 2015; Sunday et al., 2012). Studies of cold tolerance can therefore yield insights into what insects live where and how they weather challenges of low temperatures both during the growing season and during winter in temperate, polar, and alpine habitats. How insects deal with cold depends on their cold tolerance strategy: insects are typically chill-susceptible, freeze-avoidant, or freeze-tolerant (Bale, 1996; Sinclair et al., 2015). Chill-susceptible insects can be killed by exposure to low temperatures that do not cause internal ice formation. Freeze-tolerant insects survive internal ice formation and often encourage freezing at relatively high temperatures. Freeze-avoidant insects are killed by internal ice formation but otherwise survive low temperatures; they are often marked by an ability to supercool wherein they depress their freezing point well below that of pure water (Salt, 1961). For example, Alaskan beetle larvae (*Cucujus clavipes punieceus*) supercool to temperatures below −70 °C before they freeze (Sformo et al., 2010). Other freeze-avoidant insects are buffered from extreme cold in overwintering microhabitats (e.g., underground or tree crevices); they supercool, but only to a small degree (e.g., Hoshikawa et al., 1988). For instance, air temperatures in Yakutia, Russia can reach −50 to −55 °C, but freeze-avoiding insects in the region supercool to only −31 to −38 °C, and therefore must take advantage of buffered microclimates

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Abbreviations: SCP, supercooling point, the temperature at which internal ice forms, usually measured by observing the exotherm associated with the latent heat of fusion; CT_{min}, critical thermal minima, the temperature at which organisms lose neuromuscular function, typically measured by their ability to right themselves when exposed to low temperatures.

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to survive (Li, 2016).

Bumble bees (*Bombus* spp.) are critical pollinators in temperate (including extreme high latitudes) and montane ecosystems, but little is known about the cold tolerance strategy they employ to deal with extreme cold conditions during the growing season (e.g. cold snaps in spring and fall) or during winter (but see Owen et al., 2016, 2013). Those bumble bees so far measured (*B. terrestris audax* and *B. terrestris dalmatinus*) at first appeared to be chill-susceptible (Owen et al., 2013) as they were killed by temperatures that did not cause internal ice formation. However, these experiments were done by immediate transfer to low temperatures, an approach that can strongly decrease survival (Nguyen et al., 2014). In follow-up experiments, pre-exposure to low temperatures and slow ramping increased post-exposure survival to control levels (Owen et al., 2013). These data suggest that, under more ecologically-relevant conditions, bumble bees are likely freeze avoidant: they have high survival at low temperatures near their SCP, but do not survive internal ice formation, even well before reaching equilibrium ice content (Owen et al., 2013, 2016; Sinclair et al., 2015). However, aside from these measurements on subspecies of *B. terrestris*, we are unaware of other assessments of bumble bee cold tolerance strategy; this is surprising for a group that is so strikingly associated with cold climates (Cameron et al., 2007; Hines, 2008; Woodard, 2017).

Bumble bee castes—workers (non-reproductive females), drones (males), and gynes (reproductive females, “queens”)—may experience different temperatures given their distinct life histories, which may result in cold tolerance differences (Owen et al., 2013; Oyen et al., 2016, 2021). Although workers and males do not overwinter, they nevertheless experience low temperatures. Foraging workers may risk exposure to extreme cold during weather fronts which have been shown to have pronounced effects on other organisms (Stroud et al., 2020). Bumble bee males often experience variable temperatures as they remain outside of the colony after emergence (Heinrich, 2004). Several studies suggest that males either have reduced (Oyen et al., 2021) or similar (Oyen et al., 2016) cold tolerance relative to workers. For *B. vosnesenskii* reared in common-garden conditions from multiple source populations, males generally took longer to recover from chill coma (i.e., had reduced cold tolerance) than workers from the same colonies (Oyen et al., 2021). However, field-collected male and worker *B. huntii* and *B. sylvicola* had similar critical thermal minima (CT_{min}, indicated by loss of righting response) (Oyen et al., 2016). Queens overwinter underground where they are chronically exposed to low temperatures. After emergence in spring, queens face temperature fluctuations and cold snaps while foraging to provision their first brood (after which they remain in underground thermoregulated nests; Alford, 1969). One way to cope with this temperature variability is through shifts in cold tolerance. Thermal tolerance can shift with age (Bowler and Terblanche, 2008) and with previous thermal experience, often associated with seasonal temperature shifts (Block et al., 1990; Morrissey and Baust, 1976; Vrba et al., 2017; Zhou et al., 2011). For instance, the SCP of Antarctic midge larvae decreases significantly during winter compared to the summer, changing from −9.8 °C to −15.1 °C (Kawarasaki et al., 2014). Aside from seasonal shifts, shorter acclimation treatments can influence SCP and even cold tolerance strategy, which can vary given thermal history, cooling rate, and other ecological conditions (Hawes and Bale, 2007). In bumble bees, effects of acute cold exposure on SCP have been variable: SCP decreased in worker *B. t. dalmatinus* exposed to 0 °C for 1 h, whereas SCP of *B. t. audax* exposed to 0 °C for 8 h did not shift (Owen et al., 2013, 2016). These data indicate a hardening response, but limited measurements of bumble bee cold tolerance across seasons and life stages limit our understanding of thermal physiology of these critical pollinators.

Cold tolerance often varies geographically, with insects from colder climates having increased cold tolerance both within and among species (Addo-Bediako et al., 2000; Gaston and Chown, 1999; Overgaard et al., 2015; Pimsler et al., 2020; Sunday et al., 2012). For bumble bees, species from higher elevations have lower CT_{min}, i.e., increased cold tolerance (Oyen et al., 2016). Across populations, CT_{min} correlates with local climate across broad altitudinal and latitudinal gradients (Pimsler et al., 2020), but chill coma recovery time does not (Oyen et al., 2021). For other insects, SCP can vary among populations (e.g., linden bugs, stoneflies, and leafminers; Chen and Kang, 2004; Ditrich et al., 2018; Hotaling et al., 2021). In other instances, SCP is conserved within and among species (Schoville et al., 2015). Whether SCP varies among species or populations of bumble bees is, to our knowledge, unknown.

Here, we first determined cold tolerance strategy of bumble bees by measuring survival and SCP of *B. impatiens* across a range of low temperatures. We then asked whether SCP varied among castes (drones, workers, queens) and across life stage (pre-overwintering, overwintering, and after stimulation of ovary development by CO_2 treatment, i.e. post-overwintering or “spring”) for queen *B. impatiens*. To identify the influences of abiotic factors on cold tolerance, we measured SCP across season, with manipulated moisture levels (i.e., wet vs dry), and with altered cooling rates. Lastly, we measured SCP across three *Bombus* species collected at two elevations to ask whether cold tolerance varies with local climate.

2. Materials and methods

2.1. Animals – lab-reared

Commercial colonies of *B. impatiens* (Koppert Biological Systems, Howell, MI, USA) were maintained at 28 °C on a 12:12 L:D cycle within an incubator (Model #I36VL, Percival Scientific, Perry, IA, USA). Colonies had ad libitum access to the commercial nectar shipped with each colony via a wick and reservoir, and were provided 3 tsp of ground pollen (Bee Pollen, Prairie River Honey Farm, Grand Island, NE, USA) every other day. Colonies ship with workers and the founder queen, with new queens and males typically emerging after several weeks to a month or more. For SCP measurement, males were either taken directly from colonies or from worker microcolonies (Klinger et al., 2019). New queens were either tested directly (“pre-overwintering”), exposed to low temperatures to stimulate overwintering (“overwintering”) (Woodard et al., 2019), or stimulated to bypass overwintering by several bouts of CO_2 narcosis, which triggers ovary development and egg production, leading to a “spring” queen (Amsalem and Grozinger, 2017; Röseler, 1985; Watrous et al., 2019). Without exposure to CO_2, bumble bee queens do not mature ovaries until 5–10 days after they emerge from overwintering (Amsalem and Grozinger, 2017). Overwintering queens were ramped in an incubator from 28 °C to 4 °C over 7 days, with the simulation of winter cooling leading to reduced activity and resulting in 100% survivorship after 6 weeks at 4 °C. *B. impatiens* is a commercially-managed species with its native distribution encompassing much of the eastern US (Williams et al., 2014). Although commercial rearing may alter life history and physiology of these animals (Gibbs, 1999), measurements of cold tolerance are nevertheless relevant not only for optimizing winter cold storage in commercial settings but also for comparison with cold tolerance of wild-caught bees. Furthermore, use of bees from lab-reared colonies allowed us to minimize variability in responses due to thermal history, age, relatedness, and other factors that could not be controlled for in wild-caught bees.

2.2. Animals – field-caught

From July 2019 to May 2021, we collected wild bumble bee queens by net during spring and fall activity periods. Bees were kept in individual ventilated vials within a cooler on ice until they were returned to the lab and allowed to warm to room temperature (~22 °C) prior to SCP measurements, which were performed on the same day. We collected *B. huntii* in Laramie, WY USA (2190 m, 41.31, −105.58), and *B. flavifrons* and *B. frigidus* in the Medicine Bow Mountains (3290 m, 41.34, −106.29) 40 miles west of Laramie, WY, USA (under United States Forest Service Medicine Bow-Routt National Forest special use permit to UW).

2.3. Determining cold tolerance strategy

To determine cold tolerance strategy, we exposed *B. impatiens* workers (n = 16 or 20 per treatment) and newly emerged queens (n = 5 or 6 per treatment) from two colonies to one of a series of cold treatments (−2, −4, −6, −8, −10, or −12 °C), monitored ice formation, and assessed survival (Owen et al., 2013, 2016; Sinclair et al., 2015). Prior to cold treatments, we weighed bees (0.001 g; AccuLab ALC-210.4, Sartorius Group, Göttingen, Germany) and placed them in individual 1.5 mL Eppendorf tubes (Fisher Scientific, Waltham, MA, USA) capped with a breathable cotton wick, which held bees in place such that their abdomens stayed in contact with a 30–36 gauge T-type thermocouple. Tubes (with bees and thermocouples) were placed into holes in an aluminum block affixed to a thermoelectric cooler (TEC) controlled by a proportional integrative derivative (PID) controller (Auber Instruments, Inc., Alpharetta, GA, USA), with the hot side of the TEC affixed to a heat sink submerged in a cold water bath. For each temperature treatment, bees were first held at 22 °C for 5 min, then cooled to the designated treatment temperature (see above) at a rate of 0.5 °C per min, where they were held for 15 min before being ramped back to 22 °C at 0.5 °C per min (Owen et al., 2013, 2016). For each trial we measured whether each bee froze (see *SCP Measurements* below for description) and whether it survived for 72 h after temperature exposure. After cold exposure, we transferred bees to microcolony containers, with bees from each temperature treatment and colony held together. Bees were provided nectar ad libitum, 1 tsp of ground pollen, and a piece of comb from their source colony. We monitored bees for survival at 24, 48, and 72 h after cold exposure; we considered bees alive if they were able to walk or respond to gentle prodding with forceps.

2.4. SCP measurements

We measured SCP of wild and commercial bumble bees using established methods (Sinclair et al., 2015). Bees were placed into individual Eppendorf tubes as described above (see *Determining cold tolerance strategy*). Temperatures of as many as 16 bees at a time were monitored via TC-08 thermocouple readers and associated software (Pico Technology, Tyler, TX, USA). Using the apparatus described above (see *Determining cold tolerance strategy*), bees were ramped at a constant rate (0.25 or 0.5 °C/min) from a starting temperature, with SCP taken as the temperature at which the trace showed the characteristic exotherm indicating ice formation (Sinclair et al., 2015). We adjusted starting temperature to reflect the thermal history of measured bees: workers, males, and non-overwintering queens were started at room temperature (22 °C), whereas overwintering queens were started at 4 °C, the temperature at which they were held for six weeks prior to measurements. In addition to these measurements of “dry” SCP, we also measured wet SCP for a subset of animals by adding a drop of water to the Eppendorf tube prior to placing the bee in the tube. Water can act as a potent ice nucleator, triggering freezing at warmer temperatures (Sinclair et al., 2015) and bees in natural conditions may be in contact with water during cold exposure.

2.5. Analyses

We analyzed the effects of ramping rate (0.5 °C or 0.25 °C/min), moisture (wet or dry), season (spring or fall), altitude (2190 or 3290 m), caste (male, worker, queen), and species using ANCOVA with mass as a covariate (body size can influence SCP; Hahn et al., 2008). Prior to running models, data were tested for normality (Shapiro-Wilk’s test) and homogeneity of variances (Fligner-Killeen test) with α set to 0.05. Where appropriate, we used TukeyHSD for post-hoc comparisons among groups. All statistical analyses were performed in R 4.0.5 (R Core Team, 2021).

3. Results

3.1. Cold tolerance strategy

B. impatiens workers (n = 138) and queens (n = 34) had similar responses to cold exposure. When ramped from 22 °C to −2, −4, −6, or −8 °C at 0.5 °C/min, no bees froze. Conversely, all bees froze when ramped to −10 and −12 °C (see Fig. 1). All workers were alive 72 h after exposure to −2, −4, −6, and −8 °C. All queens survived exposure to −2 and −4 °C and only one queen died in each of the −6 and −8 °C treatments. All but one worker and all queens died within 72 h of exposure to −10 °C, and all workers and queens died within 72 h of exposure to −12 °C. Logistic fits to these survival data confirmed that lower lethal temperatures coincide closely with temperatures that cause internal ice formation (SCP) for both worker and queen *B. impatiens* (Fig. 1; LLT_{50} = −9.0, −8.5 for workers and queens, respectively).

3.2. Ramp rate and moisture

We measured potential effects of ramp rate and the presence of water on SCPs for field-caught *B. huntii* queens. Ramp rate did not significantly alter SCP estimates (−5.0 ± 1.1 °C at 0.25 °C/min vs −5.9 ± 1.0 °C at 0.50 °C/min; Tables 1 and 2; Fig. 2), but wet SCP (−4.2 ± 1.2 °C) was significantly higher than dry SCP (−5.9 ± 1.0 °C; Tables 1 and 2; Fig. 2). Mass did not significantly affect SCP for either ramp rate or moisture analyses (both $P > 0.385$; Table 2).

3.3. Variation in SCP among castes and across queen life stage

SCPs differed among *B. impatiens* castes (Fig. 3A) with both queens (−7.2 ± 1.5 °C) and workers (−6.8 ± 1.1 °C) freezing at significantly higher temperatures than males (−10.0 ± 1.9 °C; Table 2, Fig. 3A). Mass did not significantly affect SCP variation among castes (P = 0.710;

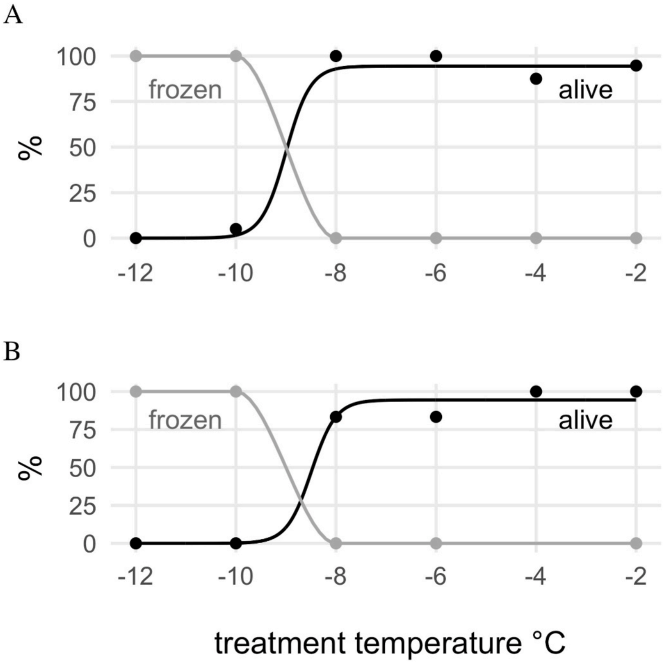

Fig. 1. Bumble bees (*B. impatiens*) survive cold exposure as long as they don’t freeze. Groups of worker (A) and queen (B) bumble bees were ramped and then held at low temperatures for 15 min before ramping back to 22 °C and monitored for survival for 72 h. All but one bee (a worker) that froze died, whereas nearly all bees survived exposure to cold temperatures that did not cause internal ice formation, suggesting that bumble bees are freeze-avoiding and may be chill-susceptible. Lines are logistic fits (see text for details).

Table 1
Variation in bumble bee supercooling points (SCP) across caste, species, and season. All values (mean ± sd) are for measurements of dry SCP at a ramp rate of 0.5 °C/min unless otherwise specified. Sample sizes indicated in parentheses. For *B. impatiens*, "fall" values are for queens 1 week post emergence from commercial colonies and "spring" values are for post-emergence queens treated with CO_2 to induce ovary development. "Winter" values are for *B. impatiens* queens from commercial colonies after being held at 4 °C for 6 weeks. See Table 2 for statistical comparisons among groups.

species (elevation)	males	workers	queens		
			fall	winter	spring
Bombus impatiens (2190 m)	−10.0 ± 1.9 (19)	−6.8 ± 1.1 (23)	−7.9 ± 0.4 (6)	−8.4 ± 0.5 (7)	−5.7 ± 1.2 (8)
Bombus huntii (2190 m)	–	–	−5.0 ± 1.1 (8)[a]	–	−5.0 ± 0.3 (8)[a] −5.9 ± 1.0 (8) −4.2 ± 1.2 (16)[b]
Bombus flavifrons (3290 m)	–	–	–	–	−5.5 ± 0.7 (7)
Bombus frigidus (3290 m)	–	–	–	–	−5.2 ± 1.2 (8)

[a] Ramp rate of 0.25 °C/min.
[b] Wet supercooling point.

Table 2
Summary statistics for models comparing supercooling points of bumble bees across ramp rate, moisture, caste, life stage, species, elevation, and season. See Table 1 for SCP values and Figs. 2–5.

Figure	Model effects	*F* (df)	*P*
2	ramp rate (0.25, 0.5 °C/min)	3.10 (1,13)	0.102
	mass	0.01 (1,13)	0.936
2	moisture (wet, dry)	12.17 (1,21)	**0.002**
	mass	0.79 (1,21)	0.385
3A	caste (male[a], worker[b], queen[b])	27.08 (2,59)	**<0.001**
	mass	0.14 (1,59)	0.712
3B	queen life stage (pre-overwintering[a], overwintering[a], CO_2[b])	21.29 (2, 17)	**<0.001**
	mass	0.29 (1,17)	0.597
4	species	1.16 (2,19)	0.334
	mass	3.58 (1,19)	0.074
4	elevation (2190m, 3290m)	1.93 (1,20)	0.180
	mass	3.15 (1,20)	0.091
5	season (fall, spring)	0.21 (1,13)	0.657
	mass	0.13 (1,13)	0.724

Table 2).

Queen SCP varied with life stage, with CO_2-treated ("spring") queens (−5.7 ± 1.2 °C) having significantly higher SCP than both pre-overwintering (−7.9 ± 0.4 °C) and overwintering (−8.4 ± 0.5 °C) queens, which did not differ significantly in SCP (Table 2, Fig. 3B). Mass did not significantly affect SCP variation among queen life stages ($P = 0.712$; Table 2).

3.4. Elevation, species, and seasonal differences in SCP

Bumble bee SCPs were not significantly different for queens collected at low (2190 m; −5.9 ± 1.0 °C) and high (3290m; −5.4 ± 1.0 °C) elevation sites (Table 2, Fig. 4), despite pronounced differences in local climate (see Oyen et al., 2016), nor did SCP differ significantly among queens of three bumble bee species (Table 2; Fig. 4). For *B. huntii*, SCP of spring queens (−5.0 ± 1.1 °C) was indistinguishable from that of fall queens (−5.2 ± 0.3 °C), but SCP of spring queens was significantly more variable (Fligner-Killeen test; $\chi^2{}_1 = 5.73$, $P = 0.0167$; Fig. 5). Mass was not a significant covariate in elevation ($P = 0.091$), species ($P = 0.074$), or seasonal analyses ($P = 0.724$).

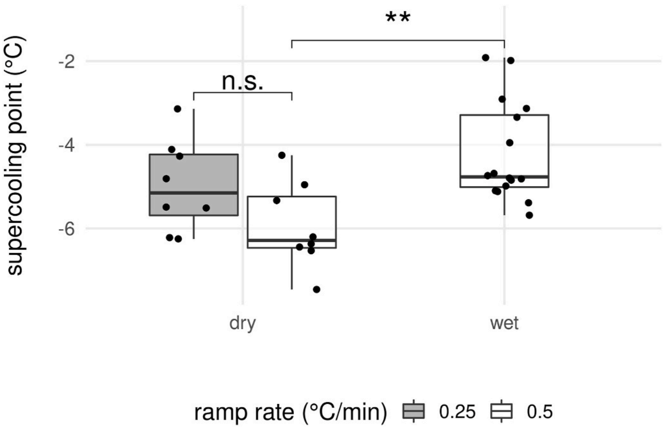

Fig. 2. Bumble bee queens (*B. huntii*) freeze at higher temperatures when wet. Bees ramped at 0.5 °C/min had significantly higher SCPs when wet (i.e., with a droplet of water) vs when dry ($P = 0.004$; see Table 1 and text for details). Bees ramped more slowly (0.25 °C/min) tended to freeze at higher temperatures, but this effect was not significant ($P = 0.375$).

4. Discussion

In general, bumble bees survive low temperatures as long as they don't freeze (Fig. 1). Our data provide strong evidence that *B. impatiens*, like *B. terrestris* (Oyen et al., 2016) and likely other bumble bees, are freeze-avoidant (Sinclair et al., 2015). While most bumble bees survived exposure to temperatures just above their SCPs, a few did not, suggesting that bumble bees may be chill-susceptible. Characterizing the exposure durations at which bees can survive temperatures before ice nucleation may further illuminate strategies bumble bees use to withstand cold conditions.

Male bumble bees had significantly lower SCPs than workers and queens (Fig. 3), an unexpected finding given other comparisons of thermal tolerance between the sexes. A meta-analysis of terrestrial ectotherms found little evidence for differences in thermal tolerance between males and females (Pottier et al., 2021). Similarly, CT_{min} did not differ between males and females of three bumble bee species (Oyen et al., 2016). The lower SCP of male bumble bees documented here may reflect their distinct life history. During the growing season, workers leave the nest to forage but return to the thermoregulated colony at night and during inclement weather and queens remain within the colony, so neither caste likely experiences prolonged exposure to below freezing temperatures prior to fall (Heinrich, 2004). Males, on the other hand, leave the colony for good shortly after eclosion; because they remain above-ground, they are more likely to experience freezing temperatures, particularly during fall cold snaps. Furthermore, cold snaps often include precipitation, which may further increase risk of freezing given higher SCPs in the presence of water (Fig. 2). As males are exposed to more adverse conditions than workers or queens in the growing season, their lower SCP paired with microhabitat selection may be critical for their survival.

Additionally, differences in diet between castes may influence SCP. Pollen in the gut can serve as an ice nucleator, increasing SCP (Owen et al., 2013). Queens and workers consume pollen and nectar and regurgitate the mixture to feed developing offspring; whether male

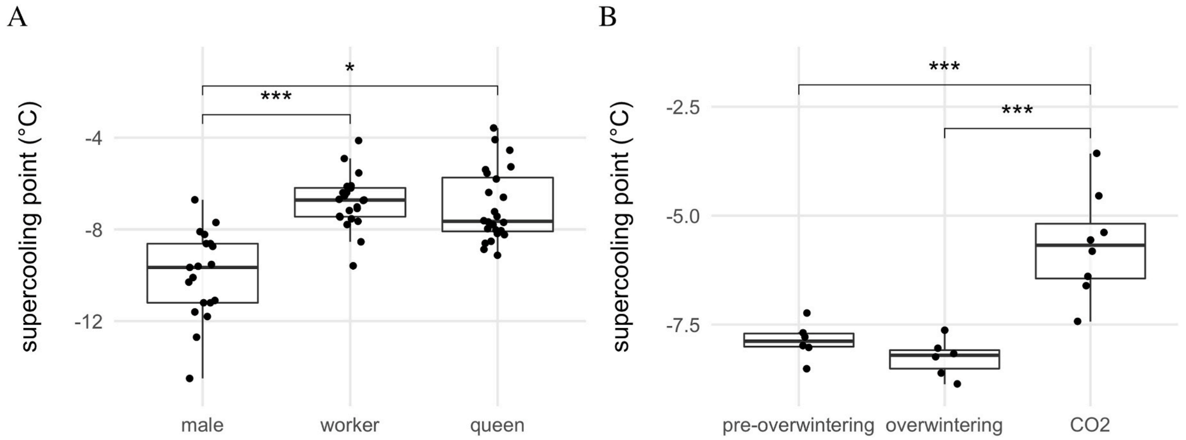

Fig. 3. Bumble bee (*B. impatiens*) SCP varies by caste and queen life stage. (A) Males had significantly lower SCPs than both workers ($P < 0.001$) and queens ($P = 0.010$). (B) Newly eclosed queens ("pre-overwintering") and queens induced to overwinter and held at 4 °C for 6 weeks ("overwintering") had significantly lower SCPs than queens stimulated to initiate ovary development by exposure to CO_2 ($P < 0.001$ for both comparisons). See Table 1 and text for details.

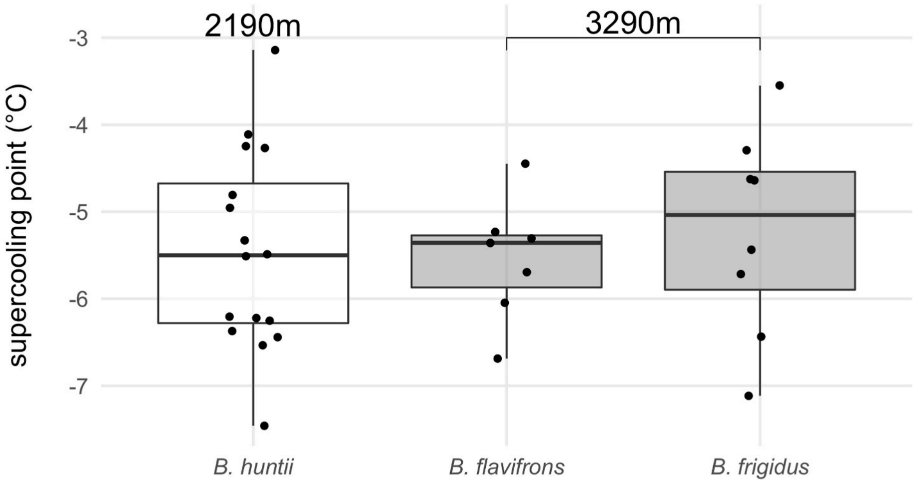

Fig. 4. SCP did not vary with collection altitude or species. Queen bumble bees were collected by net from two sites (2190 and 3290 m asl; see text for details) and kept on ice prior to SCP measurement (within ~3 h of capture). SCP was not significantly different across species or collection altitude (all $P > 0.385$).

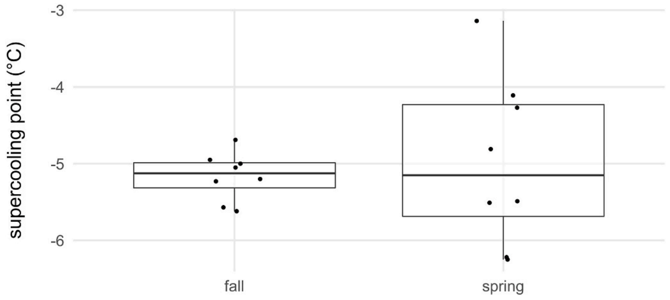

Fig. 5. SCP did not vary with season for wild-caught *B. huntii* queens. Queens were collected by net in Laramie, WY (2190 m asl) in September after emergence from natal colonies and prior to overwintering ("fall") and in July after "spring" emergence (which can occur late in this high elevation site).

bumble bees consume pollen is unclear. If males contribute to brood care for several days before leaving the colony (Belsky et al., 2020), males could consume pollen when feeding from honey pots (a mixture of pollen and nectar), even if limited evidence suggests that they do not directly feed on pollen outside of the colony (Fowler et al., 2020). If males largely abstain from eating pollen, they may reduce the risk of ice nucleation, depressing SCP.

Life stage affected queen SCP (Fig. 3B), with queens in pre-overwintering (3–7 days old) and overwintering states (held at 4 °C for 6 weeks in the lab) freezing at significantly lower temperatures than CO_2-treated queens (that had likely initiated ovary development; Amsalem and Grozinger, 2017). As the CO_2 treated *B. impatiens* queens were not significantly different from wild caught *B. huntii* spring queens, our results suggest ovary development may play a role in the loss of cold tolerance between overwintering and spring queens. These queens may shift investment from sequestration of polyols or other cryoprotectants (Clark and Worland, 2008; Denlinger et al., 2012; Ghosh et al., 2017), towards egg production (Votavová et al., 2015); loss of cold tolerance at this stage may have minimal consequences given that once queens are laying eggs, they remain underground in thermoregulated nests (Goulson, 2003). Alternatively, the pre-overwintering and overwintering queens may have little to no pollen in their guts as they stop feeding on pollen several days before they enter their overwintering state (Pridal and Hofbauer, 1996). This lack of pollen in the gut may help explain the low SCPs of pre-overwintering and overwintering queens compared to CO_2 treated queens which are actively feeding on pollen to feed developing offspring. The similarity in SCPs between commercially-reared and wild-caught queens (Figs. 3 and 4; Table 1) suggests little effect of commercial rearing on SCP in bumble bees; in fact, wild-caught queens were less cold tolerant than newly emerged and overwintering *B. impatiens* queens. But measurements of SCP of wild-caught *B. impatiens*, ideally across the geographic range, will be necessary to better understand potential effects of commercial rearing on thermal biology of bumble bees.

Given the differences in SCP between life stages of lab-reared queens (Fig. 3B), we were surprised to find no difference in SCP between spring and fall wild-caught queens (Fig. 5), which not only differ in life stage but also experience different environmental cues. Temperature and photoperiod can profoundly alter physiological responses related to diapause, including cold tolerance (Beck, 1962; Košťál, 2006; Musolin and Numata, 2003; Saunders, 2014). Though these results are puzzling, we speculate that cold tolerance may only shift after fall queens have selected a suitable overwintering habitat where they likely stay for the winter; the wild fall queens we captured were still active above ground, so likely still mating, feeding, and sequestering resources for overwintering (Pridal and Hofbauer, 1996). Furthermore, SCPs of spring queens were significantly more variable than those of fall queens, perhaps because of variability in the status of spring queens: from having just emerged from overwintering to having fully developed ovaries and being in the process of initiating colonies. As such, differences in SCP associated with ovary development (Fig. 3B) may in part explain the variation in spring queen SCP documented here (Fig. 5). Overall, more work investigating whether temperature, photoperiod, life stage, diet, and other factors alter multiple aspects of bumble cold tolerance (CT_{min}, CCRT, and SCP; Oyen et al., 2021; Pimsler et al., 2020) will be critical to better understand how these animals thrive in cold climates (Woodard, 2017).

The sparse available data suggest that bumble bees overwinter 2–20 cm underground (Liczner and Colla, 2019), likely in part to escape freezing temperatures. Measurements of soil temperatures in October in Laramie, Wyoming near the base of a tree (bumble bees have often been found overwintering under trees; Williams et al., 2019) reveal that soil temperatures at the ground surface and 5 cm below ground can fall below −18 and −8 °C, respectively (Supplementary Fig. 1). These measurements suggest that queen bumble bees may face substantial freezing risk, even during the fall in buffered underground hibernacula. And, given that wet bees froze at significantly higher temperatures (Fig. 2), overwintering queens may favor microhabitats that remain dry throughout the winter, further minimizing freezing risk. Where and why queens overwinter where they do requires further investigation, but their relatively high SCPs suggest that winter microhabitat selection is likely critical for overwinter survival of queen bumble bees.

5. Conclusions

Our findings suggest that bumble bees are freeze-avoiding insects and may be slightly chill susceptible. Bumble bee SCPs appear to be conserved among species and across elevation but vary among castes and across queen life stages. Bumble bees have relatively high (i.e., warm) SCPs compared to other bees (Krunić and Stanisavljević, 2006) and insects (Sformo et al., 2010; Sømme, 1982); overwintering queen bumble bees may therefore be more susceptible to projected increased variability in winter temperatures (Williams et al., 2015). More research on cold tolerance of additional bumble bee species in diverse contexts (i.e., geographical, seasonal) will be critical to understanding and mitigating potential threats to these critical pollinators.

Authorship statement

M.E.D, S.A.W., E.C.K. conceptualized the project, M.E.D. acquired funding and resources, all authors conducted experiments and analyzed the data, E.C.K. and S.A.W wrote the initial draft, and all authors edited the final manuscript.

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.jtherbio.2022.103196.

References

Addo-Bediako, A., Chown, S.L., Gaston, K.J., 2000. Thermal Tolerance, Climatic Variability and Latitude 7.

Alford, D.V., 1969. A study of the hibernation of bumblebees (*Hymenoptera: Bombidae*) in southern England. J. Anim. Ecol. 38, 149. https://doi.org/10.2307/2743.

Amsalem, E., Grozinger, C.M., 2017. Evaluating the molecular, physiological and behavioral impacts of CO_2 narcosis in bumble bees *(Bombus impatiens)*. J. Insect Physiol. 101, 57–65. https://doi.org/10.1016/j.jinsphys.2017.06.014.

Andersen, J.L., Manenti, T., Sørensen, J.G., MacMillan, H.A., Loeschcke, V., Overgaard, J., 2015. How to assess *Drosophila* cold tolerance: chill coma temperature and lower lethal temperature are the best predictors of cold distribution limits. Funct. Ecol. 29, 55–65. https://doi.org/10.1111/1365-2435.12310.

Bale, J.S., 1996. Insect cold hardiness: a matter of life and death. Eur. J. Entomol. 93, 369–382.

Beck, S.D., 1962. Photoperiodic induction of diapause in an insect. Biol. Bull. 122, 1–12. https://doi.org/10.2307/1539316.

Belsky, J.E., Camp, A.A., Lehmann, D.M., 2020. The importance of males to Bumble Bee (*Bombus* species) nest development and colony viability. Insects 11, 506. https://doi.org/10.3390/insects11080506.

Block, W., Baust, J.G., Franks, F., Johnston, I.A., Bale, J., 1990. Cold tolerance of insects and other arthropods. Philos. Trans. R. Soc. B 326, 613–633. https://doi.org/10.1098/rstb.1990.0035. Biological Sciences, Life at Low Temperatures.

Bowler, K., Terblanche, J.S., 2008. Insect thermal tolerance: what is the role of ontogeny, ageing and senescence? Biol. Rev. Biol. Proc. Camb. Phil. Soc. 339–355. https://doi.org/10.1111/j.1469-185X.2008.00046.x.

Cameron, S.A., Hines, H.M., Williams, P.H., 2007. A comprehensive phylogeny of the bumble bees (*Bombus*). Biol. J. Linn. Soc. 91, 161–188. https://doi.org/10.1111/j.1095-8312.2007.00784.x.

Chen, B., Kang, L., 2004. Variation in cold hardiness of *Liriomyza huidobrensis* (Diptera: Agromyzidae) along latitudinal gradients. Environ. Entomol. 33, 155–164. https://doi.org/10.1603/0046-225X-33.2.155.

Clark, M.S., Worland, M.R., 2008. How insects survive the cold: molecular mechanisms—a review. J. Comp. Physiol. B 178, 917–933. https://doi.org/10.1007/s00360-008-0286-4.

Denlinger, D.L., Yocum, G.D., Rinehart, J.P., 2012. 10 - hormonal control of diapause. In: Gilbert, L.I. (Ed.), Insect Endocrinology. Academic Press, San Diego, pp. 430–463. https://doi.org/10.1016/B978-0-12-384749-2.10010-X.

Ditrich, T., Janda, V., Vaněčková, H., Doležel, D., 2018. Climatic variation of supercooling point in the linden bug *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae). Insects 9, 144. https://doi.org/10.3390/insects9040144.

Fowler, A.E., Stone, E.C., Irwin, R.E., Adler, L.S., 2020. Sunflower pollen reduces a gut pathogen in worker and queen but not male bumble bees. Ecol. Entomol. 45, 1318–1326. https://doi.org/10.1111/een.12915.

Gaston, K.J., Chown, S.L., 1999. Elevation and climatic tolerance: a test using dung beetles. Oikos 86, 584. https://doi.org/10.2307/3546663.

Ghosh, S., Choi, K., Kim, S., Jung, C., 2017. Body compositional changes of fatty acid and amino acid from the queen of Bumblebee, *Bombus terrestris* during overwintering. J. Apic. 32, 11–18. https://doi.org/10.17519/apiculture.2017.04.32.1.11.

Gibbs, A.G., 1999. Laboratory selection for the comparative physiologist. J. Exp. Biol. 202, 2709–2718.

Goulson, D., 2003. Bumblebees: Their Behavior and Ecology. Oxford University Press.

Hahn, D.A., Martin, A.R., Porter, S.D., 2008. Body size, but not cooling rate, affects supercooling points in the Red Imported Fire Ant, *Solenopsis invicta*. Environ. Entomol. 37, 7.

Hawes, T.C., Bale, J.S., 2007. Plasticity in arthropod cryotypes. J. Exp. Biol. 210, 2585–2592. https://doi.org/10.1242/jeb.002618.

Heinrich, B., 2004. Bumblebee Economics. Harvard University Press.

Hines, H.M., 2008. Historical biogeography, divergence times, and diversification patterns of bumble bees (Hymenoptera: Apidae: *Bombus*). Syst. Biol. 57, 58–75. https://doi.org/10.1080/10635150801898912.

Hoshikawa, K., Tsutsui, H., Honma, K., Sakagami, S.F., 1988. Cold resistance in four species of beetles overwintering in the soil, with notes on the overwintering strategies of some soil insects. Appl. Entomol. Zool. 23, 273–281. https://doi.org/10.1303/aez.23.273.

Hotaling, S., Shah, A.A., Dillon, M.E., Giersch, J.J., Tronstad, L.M., Finn, D.S., Woods, H. A., Kelley, J.L., 2021. Cold tolerance of mountain stoneflies (Plecoptera: Nemouridae) from the high Rocky Mountains. West. N. Am. Nat. 81, 54–62. https://doi.org/10.3398/064.081.0105.

Kawarasaki, Y., Teets, N.M., Denlinger, D.L., Lee, R.E., 2014. Alternative overwintering strategies in an Antarctic midge: freezing vs. cryoprotective dehydration. Funct. Ecol. 28, 933–943. https://doi.org/10.1111/1365-2435.12229.

Klinger, E.G., Camp, A.A., Strange, J.P., Cox-Foster, D., Lehmann, D.M., 2019. *Bombus* (Hymenoptera: Apidae) microcolonies as a tool for biological understanding and pesticide risk assessment. Environ. Entomol. 48, 1249–1259. https://doi.org/10.1093/ee/nvz117.

Koštál, V., 2006. Eco-physiological phases of insect diapause. J. Insect Physiol. 52, 113–127. https://doi.org/10.1016/j.jinsphys.2005.09.008.

Krunić, M.D., Stanisavljević, L.Ž., 2006. Supercooling points and diapause termination in overwintering adults of orchard bees *Osmia cornuta* and *O. rufa* (Hymenoptera: Megachilidae). Bull. Entomol. Res. 96, 323–326. https://doi.org/10.1079/BER2006423.

Li, N.G., 2016. Strong tolerance to freezing is a major survival strategy in insects inhabiting central Yakutia (Sakha Republic, Russia), the coldest region on earth. Cryobiology 73, 221–225. https://doi.org/10.1016/j.cryobiol.2016.07.007.

Liczner, A.R., Colla, S.R., 2019. A systematic review of the nesting and overwintering habitat of bumble bees globally. J. Insect Conserv. 23, 787–801. https://doi.org/10.1007/s10841-019-00173-7.

Morrissey, R.E., Baust, J.G., 1976. The ontogeny of cold tolerance in the gall fly, *Eurosta solidagensis*. J. Insect Physiol. 22, 431–437. https://doi.org/10.1016/0022-1910(76)90014-7.

Musolin, D.L., Numata, H., 2003. Photoperiodic and temperature control of diapause induction and colour change in the southern green stink bug *Nezara viridula*. Physiol. Entomol. 28, 65–74. https://doi.org/10.1046/j.1365-3032.2003.00307.x.

Nguyen, C., Bahar, M.H., Baker, G., Andrew, N.R., 2014. Thermal tolerance limits of diamondback moth in ramping and plunging assays. PLoS One 9, e87535. https://doi.org/10.1371/journal.pone.0087535.

Overgaard, J., Kristensen, T.N., Mitchell, K.A., Hoffmann, A.A., 2015. Thermal tolerance in widespread and tropical*Drosophila* species: does phenotypic plasticity increase with latitude? Am. Nat. https://doi.org/10.1086/661780.

Owen, E.L., Bale, J.S., Hayward, S.A.L., 2016. Establishment risk of the commercially imported bumblebee *Bombus terrestris dalmatinus*—can they survive UK winters? Apidologie 47, 66–75. https://doi.org/10.1007/s13592-015-0376-8.

Owen, E.L., Bale, J.S., Hayward, S.A.L., 2013. Can winter-active bumblebees survive the cold? Assessing the cold tolerance of *Bombus terrestris audax* and the effects of pollen feeding. PLoS One 8, e80061. https://doi.org/10.1371/journal.pone.0080061.

Oyen, K.J., Giri, S., Dillon, M.E., 2016. Altitudinal variation in bumble bee (*Bombus*) critical thermal limits. J. Therm. Biol. 59, 52–57. https://doi.org/10.1016/j.jtherbio.2016.04.015.

Oyen, K.J., Jardine, L.E., Parsons, Z.M., Herndon, J.D., Strange, J.P., Lozier, J.D., Dillon, M.E., 2021. Body mass and sex, not local climate, drive differences in chill coma recovery times in common garden reared bumble bees. J. Comp. Physiol. B. https://doi.org/10.1007/s00360-021-01385-7.

Pimsler, M.L., Oyen, K.J., Herndon, J.D., Jackson, J.M., Strange, J.P., Dillon, M.E., Lozier, J.D., 2020. Biogeographic parallels in thermal tolerance and gene expression variation under temperature stress in a widespread bumble bee. Sci. Rep. 10, 17063. https://doi.org/10.1038/s41598-020-73391-8.

Pottier, P., Burke, S., Drobniak, S.M., Lagisz, M., Nakagawa, S., 2021. Sexual (in) equality? A meta-analysis of sex differences in thermal acclimation capacity across ectotherms. Funct. Ecol. 1365–2435. https://doi.org/10.1111/1365-2435.13899, 13899.

Pridal, A., Hofbauer, J., 1996. Laboratory rearing and nutrition of young queens of bumblebee (*Bombus terrestris* L.) from emergence to diapause. Fruit Grow. Res. 14, 125–131.

R Core Team, 2021. R: A Language and Environment for Statistical Computing. Vienna, Austria.

Röseler, P.-F., 1985. A technique for year-round rearing of *Bombus terrestris* (Apidae, Bombini) colonies in captivity. Apidologie 16, 165–170. https://doi.org/10.1051/apido:19850206.

Salt, R.W., 1961. Principles of insect cold-hardiness. Annu. Rev. Entomol. 6, 55–74. https://doi.org/10.1146/annurev.en.06.010161.000415.

Saunders, D.S., 2014. Insect photoperiodism: effects of temperature on the induction of insect diapause and diverse roles for the circadian system in the photoperiodic response. Entomol. Sci. 17, 25–40. https://doi.org/10.1111/ens.12059.

Schoville, S.D., Slatyer, R.A., Bergdahl, J.C., Valdez, G.A., 2015. Conserved and narrow temperature limits in alpine insects: thermal tolerance and supercooling points of the ice-crawlers, *Grylloblatta* (Insecta: Grylloblattodea: Grylloblattidae). J. Insect Physiol. 78, 55–61. https://doi.org/10.1016/j.jinsphys.2015.04.014.

Sformo, T., Walters, K., Jeannet, K., Wowk, B., Fahy, G.M., Barnes, B.M., Duman, J.G., 2010. Deep supercooling, vitrification and limited survival to −100°C in the Alaskan beetle *Cucujus clavipes puniceus* (Coleoptera: Cucujidae) larvae. J. Exp. Biol. 213, 502–509. https://doi.org/10.1242/jeb.035758.

Sinclair, B.J., Coello Alvarado, L.E., Ferguson, L.V., 2015. An invitation to measure insect cold tolerance: methods, approaches, and workflow. J. Therm. Biol. 53, 180–197. https://doi.org/10.1016/j.jtherbio.2015.11.003.

Sømme, L., 1982. Supercooling and winter survival in terrestrial arthropods. Comp. Biochem. Physiol. Part A Physiol. 73, 519–543. https://doi.org/10.1016/0300-9629(82)90260-2.

Stroud, J.T., Mothes, C.C., Beckles, W., Heathcote, R.J.P., Donihue, C.M., Losos, J.B., 2020. An extreme cold event leads to community-wide convergence in lower temperature tolerance in a lizard community. Biol. Lett. 16, 20200625. https://doi.org/10.1098/rsbl.2020.0625.

Sunday, J.M., Bates, A.E., Dulvy, N.K., 2012. Thermal tolerance and the global redistribution of animals. Nat. Clim. Change 2, 686–690. https://doi.org/10.1038/nclimate1539.

Votavová, A., Tomčala, A., Kofroňová, E., Kudzejová, M., Šobotník, J., Jiroš, P., Komzáková, O., Valterová, I., 2015. Seasonal dynamics in the chemistry and structure of the fat Bodies of Bumblebee queens. PLoS One 10, e0142261. https://doi.org/10.1371/journal.pone.0142261.

Vrba, P., Zahradníčková, H., Konvička, M., 2017. Temporal plasticity in cold hardiness and cryoprotectant contents in northern versus temperate *Colias* butterflies (Lepidoptera: Pieridae). Cryoletters 38, 330–338.

Watrous, K.M., Duennes, M.A., Woodard, S.H., 2019. Pollen diet composition impacts early nesting success in queen bumble bees *Bombus impatiens cresson* (Hymenoptera: Apidae). Environ. Entomol. 48, 711–717. https://doi.org/10.1093/ee/nvz043.

Williams, C.M., Henry, H.A.L., Sinclair, B.J., 2015. Cold truths: how winter drives responses of terrestrial organisms to climate change. Biol. Rev. Biol. Proc. Camb. Phil. Soc. 90, 214–235. https://doi.org/10.1111/brv.12105.

Williams, N.M., Mola, J.M., Stuligross, C., Harrison, T., Page, M.L., Brennan, R.M., Rosenberger, N.M., Rundlöf, M., 2019. Fantastic bees and where to find them: locating the cryptic overwintering queens of a western bumble bee. Ecosphere 10, e02949. https://doi.org/10.1002/ecs2.2949.

Williams, P.H., Thorp, R.W., Richardson, L.L., Colla, S.R., 2014. Bumble Bees of North America: an Identification Guide, Bumble Bees of North America. Princeton University Press. https://doi.org/10.1515/9781400851188.

Woodard, S.H., 2017. Bumble bee ecophysiology: integrating the changing environment and the organism. Curr. Opin. Insect. Sci. 22, 101–108. https://doi.org/10.1016/j.cois.2017.06.001.

Woodard, S.H., Duennes, M.A., Watrous, K.M., Jha, S., 2019. Diet and nutritional status during early adult life have immediate and persistent effects on queen bumble bees. Conserv. Physiol. https://doi.org/10.1093/conphys/coz048.

Zhou, Z.-S., Guo, J.-Y., Li, M., Ai, H.-M., Wan, F.-H., 2011. Seasonal changes in cold hardiness of *Ophraella communa*. Entomol. Exp. Appl. 140, 85–90. https://doi.org/10.1111/j.1570-7458.2011.01128.x.