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ORIGINAL PAPER

Body mass and sex, not local climate, drive differences in chill coma recovery times in common garden reared bumble bees

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Abstract

The time required to recover from cold exposure (chill coma recovery time) may represent an important metric of performance and has been linked to geographic distributions of diverse species. Chill coma recovery time (CCRT) has rarely been measured in bumble bees (genus *Bombus*) but may provide insights regarding recent changes in their distributions. We measured CCRT of *Bombus vosnesenskii* workers reared in common garden laboratory conditions from queens collected across altitude and latitude in the Western United States. We also compared CCRTs of male and female bumble bees because males are often overlooked in studies of bumble bee ecology and physiology and may differ in their ability to respond to cold temperatures. We found no relationship between CCRT and local climate at the queen collection sites, but CCRT varied significantly with sex and body mass. Because differences in the ability to recover from cold temperatures have been shown in wild-caught *Bombus*, we predict that variability in CCRT may be strongly influenced by plasticity.

Keywords *Bombus vosnesenskii* · Cold tolerance · Allometry · CCRT · Thermal ecology

Introduction

For many invertebrates, prolonged exposure to low temperatures results in chill coma, a reversible state of paralysis (Hazell and Bale 2011; MacMillan and Sinclair 2011; Mellanby 1939; Overgaard and MacMillan 2017). Chill coma is both a physiological and an ecological threshold. With loss of central nervous system (CNS) and muscle function at low temperatures, organisms are no longer able to feed (Harrington and Taylor 1990), reproduce (Larsson 1989), or evade predation (Hughes et al. 2010). Therefore, the time required for recovery of muscle and CNS function following chill coma, chill coma recovery time (CCRT), often represents an important physiological metric of performance, particularly for species that are distributed across broad environmental gradients (Gibert and Huey 2001; Castañeda et al. 2005; Sisodia and Singh 2010). Cold tolerance metrics often shift in parallel in many species (Andersen et al. 2015; Sunday et al. 2012; Terblanche et al. 2006). For example, both critical thermal minimum (CT_{min}, the temperature at which an organism loses critical muscle function) and CCRT are lower in *Drosophila* from cooler temperate climates relative to *Drosophila* from lower latitudes (Gibert and Huey 2001; Hoffmann et al. 2002; David et al. 2003;

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Ayrinhac et al. 2004; Sisodia and Singh 2010). Although CT_{min} and CCRT often vary in parallel and are both used to estimate cold tolerance, their underlying physiological mechanisms are different. The onset of chill coma is driven by temperature-dependent spreading depolarization in the central nervous system (CNS) usually followed by muscle membrane depolarizaion; recovery from chill coma requires reestablishment of extracellular and intracellular ion homeostasis necessary for the restoration of both CNS and muscle function (Overgaard and MacMillan 2017; Andersen and Overgaard 2019).

Following CT_{min}, which is characterized by immediate silencing in the CNS, prolonged chill coma leads to a redistribution of ions throughout the body (Findsen et al. 2014; Des Marteaux and Sinclair 2016; Robertson et al. 2017). At benign temperatures, ion and water balance are sustained by the dynamic interplay between secretion by the Malpighian tubules and reabsorption in the hindgut. Prolonged exposure to low temperatures reduces the capacity of temperature-sensitive ion pumps, leading to ion imbalance (MacMillan et al. 2014, 2015; Gerber and Overgaard 2018). This temperature-driven redistribution of ions is caused by leakage of extracellular Na^+ into the gut with water following, which reduces hemolymph volume and subsequently concentrates extracellular K^+ (hyperkalemia; Koštál et al. 2004; Andersen et al. 2017a; Overgaard and MacMillan 2017). As concentrations of extracellular ions (particularly K^+) increase, muscles depolarize, potentially opening voltage-sensitive Ca^{2+} channels, leading to increased intracellular Ca^{2+} levels which initiate apoptotic pathways and eventual cell death (Bayley et al. 2018). More recent data suggest that apoptosis is less indicative of whole-animal chilling injury than is hemolymph hyperkalemia, suggesting a direct link between ion imbalance and cold-induced cell death (Carrington et al. 2020). Therefore, the ability to defend intra- and extracellular ion concentrations and quickly re-establish ion homeostasis following cold exposure directly impacts adaptation to low temperatures in insects (MacMillan et al. 2015; Andersen and Overgaard 2020). Enhanced function of osmoregulatory organs (both the Malpighian tubules and the hindgut) may therefore facilitate faster recovery from chill coma (lower CCRT) in cold-adapted insect populations (MacMillan et al. 2015; Andersen et al. 2017a, b; Andersen and Overgaard 2020).

Differences in cold tolerance traits, including CT_{min} and CCRT, often correlate with the geographic distributions of diverse organisms (Sunday et al. 2012; Pimsler et al. 2020). For many species, CCRT decreases with increasing altitude and latitude (Angert et al. 2011; Sinclair et al. 2012). Links between CCRT and geographic distributions are most compelling in *Drosophila*, which have been measured in both common garden (demonstrating adaptation) and field experiments (possibly encompassing both acclimation and adaptation) across multiple geographic gradients (Hoffmann et al. 2001, 2003; David et al. 2003). CCRT of *Drosophila melanogaster* decreased by nearly 20 min along a 28° latitudinal gradient in Eastern Australia (Hoffmann et al. 2002), and by 45 min in *Drosophila ananassae* across a latitudinal gradient in India (Sisodia and Singh 2010). These studies suggest local adaptation in CCRT in flies as they were reared in common-garden conditions prior to testing. Similar geographic variation in CCRT has been shown in other organisms, including woodlice (Castañeda et al. 2005), butterflies (Zeilstra and Fischer 2005), triatomine bugs (Vega et al. 2015), and damselflies (Stoks and Block 2011). Given the clear links between geographic gradients and CCRT in diverse species, bumble bees (genus *Bombus*), which are broadly distributed throughout the Northern Hemisphere (Williams et al. 2014) and show pronounced geographic variation in CT_{min} (Pimsler et al. 2020), may also show geographic variation in CCRT. Further, analyses of bumble bee specimen records and associated climate data suggest that recent range shifts and population declines may reflect mismatches between changing temperatures and thermal tolerance limits (Kerr et al. 2015; Soroye et al. 2020). Direct measurements of geographic variation in bumble bee thermal tolerance will likely enable more robust predictions of the impacts of climate change on these important pollinators (Woodard 2017).

Building on a long history of research on thermal biology of bumble bees (Heinrich 1972, 1974, 1975,1979,1993), more recent work has measured thermal tolerance in the group (Owen et al. 2013; Martinet et al. 2015; Oyen and Dillon 2018) and begun to document variation in thermal physiology across environmental gradients (Oyen et al. 2016; Hamblin et al. 2017; Pimsler et al. 2020). The temperature at which bumble bees lose their righting response and the temperature at which they recover from chill coma co-vary with altitude among species, with those from high altitude having lower CT_{min} and chill coma recovery temperatures (Oyen et al. 2016). However, this study was conducted on wild bees tested within 2 h of their collection in the field, so it is unclear if these differences in cold tolerance reflect adaptive evolution or are the product of adult phenotypic plasticity, developmental, or even epigenetic effects. More recently, Pimsler et al. (2020) found pronounced variation in CT_{min} among bumble bees reared in common-garden conditions from queens captured across latitudinal and altitudinal gradients. Bees with origins in the coldest environments (high latitude, high altitude sites) tolerated temperatures nearly 10 °C colder than did bees from the warmest environments (low latitude lowlands), providing compelling evidence for local adaptation in CT_{min} (and epigenetic effects may also be at play). Because CCRT can be easily measured (Sinclair et al. 2015), varies with altitude and latitude in many insects (Gibert and Huey 2001; David et al. 2003), can respond to selection (Anderson et al. 2005), and has a different underlying mechanism relative to CT_{min}, it may provide a useful metric (complementary to CT_{min}) for estimating the influence of plasticity and local adaptation in thermal tolerance on current and future distributions of bumble bees (Goulson et al. 2008).

Male bumble bees are often overlooked when considering the ecology and physiology of social pollinators because female workers are primarily responsible for brood care, cell building, hive maintenance, and foraging (Goulson 2003). However, given the considerable life history differences between male and female bumble bees, the advantage of broad thermal tolerance is potentially greater for males than females. Upon emergence, male bumble bees leave the nest in search of queens with which to mate. In contrast to females which spend nights in thermoregulated nests, male bumble bees rarely return to the nest, instead spending their nights exposed to potentially cold conditions (Goulson 2003). On cool mornings, typically reported between 0 and 2 °C, drones are often found completely inactive, hanging by their mandibles from leaves and twigs (Heinrich 1979). Throughout the geographic range of bumble bees measured in our study, ambient temperatures can dip below − 6 °C, even during growing seasons which can extend from April to September, depending on seasonality at various sites. Given the cool conditions that male bumble bees likely experience while overnighting outside the nest at high altitudes and latitudes, male bumble bees may require increased tolerance to cold exposure as compared to females.

We measured CCRT of male and female *Bombus vosnesenskii* reared in common garden conditions from queens collected at sites across western North America (Fig. 1). We hypothesized that variation in climate experienced by these populations would be matched by variation in CCRT, allowing bumble bees to cope with local climate conditions. We predicted that bumble bees from colder climates would recover from cold exposure more quickly (have shorter CCRT) than those from warmer climates. We also predicted that, given their different life history, males would be more cold-tolerant (have shorter CCRT) than workers (females).

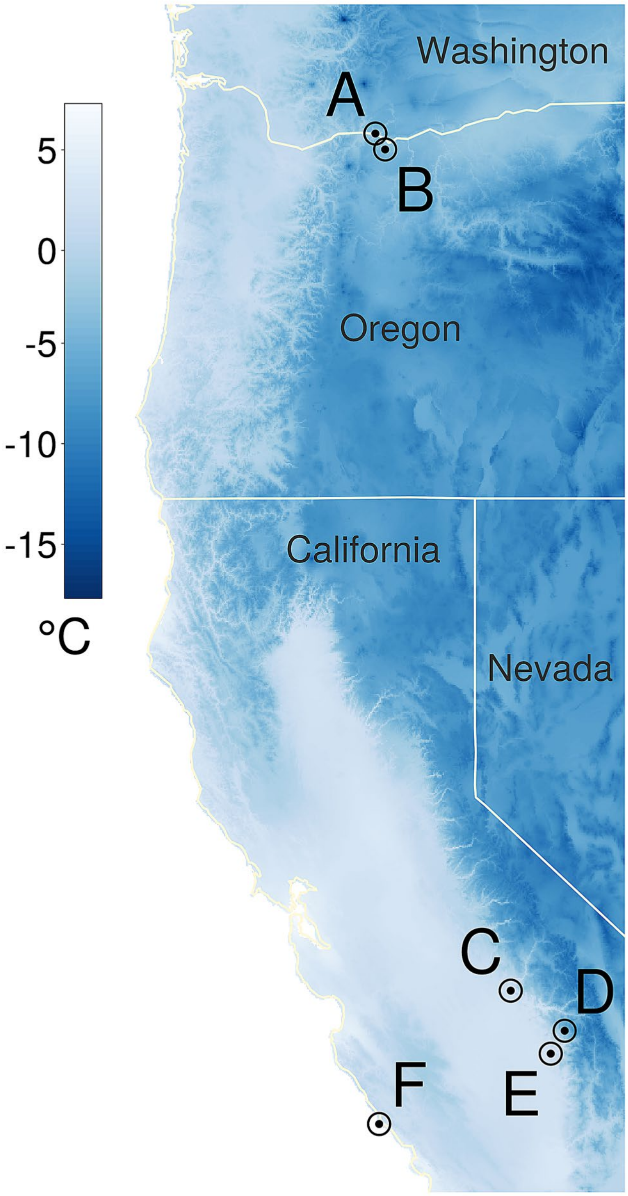

Fig. 1 *Bombus vosnesenskii* queen collection sites (A–F, see Table 1) with map color scale indicating minimum annual temperatures

Materials and methods

Bumble bee collection and husbandry

Bombus vosnesenskii queens were collected in the spring of 2016 during queen emergence from winter dormancy. A total of six sites across three regions were sampled (Fig. 1, Table 1): four sites at low and high elevation in central California, USA (LCA and HCA, respectively, average latitude of 36.581 ± 0.459° N) and two sites at low elevation in Oregon, USA (LOR, average latitude of 45.258 ± 0.568° N). Low elevation queens from both CA and OR were collected at an average elevation of 371 m (± 311 m), and high elevation queens from CA were collected at 2154 m (Table 1). Queens were collected by net while foraging or flying, then kept in vials in a cooler for transport to the USDA-ARS Pollinating Insect Research Unit's (PIRU) bumble bee rearing facility (Logan, Utah USA).

At PIRU, queens were induced to produce colonies following Strange (2010) with modifications. Each queen was given a unique identification code and placed in a 2.25 L

Table 1 Collection localities of queen *B. vosnesenskii* from which colonies were reared in common garden conditions

Site	Region	State	Latitude	Longitude	Elevation (m)	Collection date	January, July minimum temperatures (°C)
A	LOR	OR	45.696	− 121.339	70	1-Apr-16	− 1.7, 13.9
B	LOR	OR	45.535	− 121.208	441	2-Apr-16	− 4.3, 9.6
B	LOR	OR	45.535	− 121.208	441	4-Apr-16	− 4.3, 9.6
B	LOR	OR	45.535	− 121.208	441	3-Apr-16	− 4.3, 9.6
B	LOR	OR	45.535	− 121.208	441	3-Apr-16	− 4.3, 9.6
C	LCA	CA	37.036	− 119.527	351	26-Feb-16	1.7, 17.4
D	HCA	CA	36.631	− 118.804	2154	13-May-16	− 4.6, 9.3
D	HCA	CA	36.631	− 118.804	2154	13-May-16	− 4.6, 9.3
D	HCA	CA	36.631	− 118.804	2154	13-May-16	− 4.6, 9.3
E	LCA	CA	36.400	− 118.991	225	25-Feb-16	2.5, 17.6
F	LCA	CA	35.689	− 121.288	7	24-Feb-16	4.2, 9.9

Region abbreviations indicate elevation and state, with LOR and LCA indicating low elevation sites in Oregon and California, respectively and HCA representing high elevation sites in California

plastic queen initiation box (Biobest, Leamington, ON) with approximately 500 mg pollen and sugar solution provided in a 60 mL plastic reservoir ad libitum. The initiation boxes were then placed in a dark, climate-controlled room that was kept at 27 ± 1 °C and 55–60% humidity (Strange 2010). Queens were checked daily for nesting signs such as wax secretion, honey pot construction, and presence of brood. Once five workers had eclosed, the small colony was moved into a 7.75 L plastic hive box (Biobest, Leamington, ON). Once colonies had more than 20 workers, they were transported to the University of Wyoming for physiological experiments. From nest initiation through to their use in experiments, queens and colonies were checked daily for the presence of disease and pests, fed as needed, and managed to maximize colony growth.

Measurement of CCRT

To measure CCRT, bumble bees were removed from colonies with forceps and weighed to the nearest mg. Once weighed, 12 bumble bees at a time were placed individually in five dram plastic vials, which were submerged in a circulator chiller (Lauda Brinkmann RC6, Division of Sybron Corporation, Westbury, NY). Pilot experiments revealed that over 50% of bees held at − 6 °C for 4 h did not fully recover motor function within 5 h, suggesting that these conditions caused chilling injury (Sinclair et al. 2015). Bees held at 0 °C for 4 h recovered motor function within 1–3 min, suggesting that these conditions did not fully induce chill coma (Macdonald et al. 2004; Sinclair et al. 2015). Therefore, to avoid chilling injury and ensure that all bees were below the chill coma threshold, we exposed bees to − 4 °C for 2 h; all bees exposed to these conditions were unresponsive to perturbations for several minutes after being returned to room temperature but also fully recovered from chill coma within ~ 20 min. Further, these are likely ecologically realistic temperature exposures, given that bumble bees begin foraging early in the morning, even at high latitude sites (Stelzer and Chittka 2010), when daily minimum temperatures are often below 0 °C.

Following the 2-h exposure, bumble bees were removed from the water bath and placed individually into the 12 wells of tissue culture microplates (CELLTREAT, USA, Houston TX) on the benchtop at room temperature (22 ± 0.05 °C; Omega Multi-Channel Digital Thermocouple Thermometer, Omega Engineering Inc., Norwalk, CT, USA). The clear microplate lid permitted easy visualization of each bumble bee during recovery. The microplate was placed on a white sheet of paper and filmed from above with a digital camcorder (Handycam CX405, SONY Corporation, Tokyo, Japan) on a tripod. A stopwatch started immediately after bumble bees were removed from the cold was placed within the camera view to record the time at which bumble bees recovered from chill coma, as indicated by coordinated fore- and hindleg movement, which was clearly distinguishable from twitches of the limbs observed during rewarming (MacMillan et al. 2012).

Climate data

Mean temperatures (annual, minimum, maximum) and bioclimatic variables (Bioclim1-19) within a 1 km radius of each queen collection site (Table 1) were estimated using ‘WorldClim’, a global climate database with high spatial resolution produced through interpolation of average monthly climate data from weather stations on a 30 arc-s resolution grid (Version 1.4, www.worldclim.org; Hijmans et al. 2005).

Analyses

ANOVA was used to compare mass and CCRT among sites and between sexes. Pairwise analyses revealed significant collinearity among many individual climate variables and bumble bee mass; smaller bumble bees were typically from warmer sites. Because mass strongly influenced CCRT and varied among sites and colonies, we used the residuals from the regression of CCRT on mass for climate model comparisons.

To identify factors affecting recovery rates for bumble bees, we performed linear mixed effects regression analyses using the R package *nlme* (Pinheiro et al. 2013). We evaluated the relationships between (1) body mass, sex, and CCRT; and, (2) the regressed residuals of CCRT on mass, sex, and climate variables. CCRT was evaluated with respect to multiple factors: site (a fixed factor), body mass (a fixed factor), sex (a fixed factor), the interaction between body mass and sex, colony (a random factor), and January minimum temperature or July minimum temperature or annual mean temperature or annual precipitation or temperature seasonality (all fixed factors, see below). During model selection, Akaike's information criterion (AIC), biological significance of fixed effects, and parsimony were used to distinguish among competing models (Warren and Seifert 2011; Lancaster et al. 2015).

Because climate variables were highly correlated, we used cluster analysis in the R package *psych* (Revelle 2020) to choose representative temperature and Bioclim factors for inclusion in models. Item cluster analysis is an algorithm that hierarchically clusters predictors based on the extent that they covary (Revelle 1979). The resultant proximity matrix reflects Pearson correlations where the most correlated factors fall closest within the matrix. Two metrics, α and β, provide estimates of reliability within each cluster, with α representing the highest correlations between variables within the cluster and β being based upon the two least correlated items within each cluster. To show the most conservative estimates of clustering reliability, we report minimum β values.

Minimum temperatures clustered into two groups whereas Bioclim variables clustered into three groups (Supplemental Figs. 1 and 2). Based on clusters, we selected July minimum temperature, which clustered tightly with August minimum temperature ($\beta = 1.0$), and January minimum temperature, which clustered tightly with ten other minimum temperatures ($\beta = 0.96$). Together, July minimum temperature and January minimum temperature characterize the annual temperature variability at our sites and were selected to represent cool summer and winter temperatures experienced by bumble bees in these regions. We also selected annual mean temperature (BIO 1), which clustered with seven other variables ($\beta = 0.68$), annual precipitation (BIO 12), which clustered with three other variables ($\beta = 1.0$), maximum temperature of the warmest month (BIO 5), which clustered with three variables ($\beta = 0.95$), and temperature seasonality (BIO 4), which clustered with two other variables ($\beta = 0.92$). Together these variables capture site differences in temperature and precipitation, which have both been shown to influence cold tolerance metrics (Whitford and Ettershank 1975; Terblanche et al. 2006; Vega et al. 2015).

Following model selection, pairwise analyses confirmed no collinearity between variables included in models (all variance inflation factors < 3). A Tukey HSD test for unequal sample size was used a posteriori. Prior to analyses, we checked assumptions of normality and homoscedasticity of raw data using *Q*–*Q* plots and Bartlett tests, respectively. Post hoc analyses revealed normal distribution of model residuals, and a Fligner–Killeen test confirmed homogeneity of model residuals. All analyses and figures were done in R version 3.1.3 (R Development Core Team 2017).

Results

CCRT was measured for 509 *B. vosnesenskii* including 284 females and 225 males. The bees came from 11 colonies reared from queens collected from six sites spanning from ~ 35 to 45° N latitude and from 7 to 2154 m in elevation (Table 1; Fig. 1). Across all bees, CCRT ranged from 5.7 to

Table 2 Variation in body mass and chill coma recovery time (CCRT) among populations and between sexes for bumble bees reared in common garden conditions from queens collected at six different sites (Fig. 1, Table 1)

Region	Site	Females			Males		
		n	Mass (mg)	CCRT (min)	*n*	Mass (mg)	CCRT (min)
LOR	A	48	111.6 ± 30.9^{a}	12.6 ± 2.5	–	–	–
LOR	B	72	150.7 ± 31.7^{b}	11.4 ± 1.8	60	$143.1 \pm 32.0^{*}$	13.1 ± 2.2
LCA	C	–	–	–	53	$89.4 \pm 24.5^{\sim}$	11.2 ± 2.6
HCA	D	70	126.4 ± 28.8^{c}	11.0 ± 1.9	83	$131.5 \pm 27.8^{*}$	11.0 ± 1.9
LCA	E	34	60.9 ± 16.0^{d}	11.5 ± 1.4	–	–	–
LCA	F	60	101.8 ± 27.8^{a}	10.6 ± 1.7	41	$113.5 \pm 23.0^{\dagger}$	11.6 ± 1.8

Values are mean ± SD. Significant differences in mass are indicated by letters (female) or symbols (male). See text for statistical details

Table 3 Effects of body mass and sex on CCRT among all bees and among bees within each site with mass:sex interaction for those sites with both sexes represented

Effect on CCRT	Site A Females only		Site B* Females and males		Site C Males only		Site D* Females and males		Site E Males only		Site F Females and males		Overall**		
	F (d,f)	*P*	*F* (d,f)	*P*	*F* (d.f)	*P*	*F* (d.f)	*P*	*F* (d.f)	*P*	*F* (d.f)	*P*	*F* (d,f)	*P*	AIC
Mass	4.17 (1,46)	**0.047**	0.96 (1,126)	0.328	3.01 (1,39)	**0.091**	1.05 (1,147)	0.307	11.22 (1,32)	**0.002**	5.86 (3,97)	0.337	5.50 (1,514)	**0.019**	2246.74
Sex	–	–	15.44 (1,126)	**< 0.001**	–	–	0.16 (1,147)	0.691	–	–	5.86 (3,97)	**0.018**	9.93 (1,509)	**0.002**	2222.22
Mass* sex	–	–	7.86 (1,126)	**0.006**	–	–	0.02 (1.147)	0.876	–	–	5.86 (3,97)	**0.003**	2.69 (1,507)	0.102	2212.68

Results are from linear mixed effects models with mass, sex and CCRT as fixed effects and colony as a random effect for sites B and D in which multiple colonies were tested. We did not assess the effect of colony for sites A, E, or F because 2 or fewer colonies were represented at those sites (Table 1). We did not assess effect of sex for sites A, C, and E, which only had either males or females represented. Significant effects in bold

*Colony was included as a random effect because bees came from more than two colonies

**Includes all bumble bees at all sites

19 min. Because mass may significantly influence thermal tolerance traits (Baudier et al. 2015; Oyen et al. 2016), we assessed whether CCRT varied with mass and whether mass varied between sexes or among sites (Tables 2 and 3). Overall, males and females did not differ significantly in mass ($F_{1,519} = 2.27$, $P = 0.133$), but mass, sex, and their interaction influenced CCRT of bees from some sites (Table 3).

Overall, a linear mixed effects model with colony as a random effect and mass and sex as fixed effects showed that CCRT significantly increased with mass for all bees ($F_{1,514} = 5.50$, $P = 0.02$) (Table 3). For every 100 mg increase in mass, CCRT was approximately 6.7 min longer. Furthermore, CCRT varied significantly between the sexes, driven by a tendency for males to recover from cold more slowly than females across all sites ($F_{1,513} = 13.60$, $P = 0.0002$). Overall, female CCRT increased approximately 2.2 min for every 100 mg increase in body mass, and male CCRT only increased approximately 1 min for every 100 mg increase in mass. However, the effect of mass on CCRT for both sexes varied among sites (Fig. 2; Table 3). CCRT increased with mass for females at sites LOR-A, LOR-B, and LCA-E and for males at site LCA-F, but did not change significantly with mass at other sites (Table 3). CCRT only differed significantly between the sexes at site LOR-B, with males recovering from chill coma significantly more slowly than their female counterparts (Table 3).

Sex, body mass, site, climate variables, and interactions were included as fixed factors in models, with colony included as a random factor. The best model (minimum AIC) did not include any climate variables (Tables 3 and 4, Fig. 3). All climate variables listed in Table 4 were significantly related to the mass-corrected CCRTs (all $P < 0.003$) in simple linear models without random or interaction effects but no climate variable explained more than two percent of the variability in CCRT (all $R^2 < 0.02$). Models including climate variables with colony as a random effect and sex as an interaction term had significantly lower AIC values than simple linear models without random or interaction effects. However, all models with climate variables had larger AIC values than those without (Tables 3 and 4). Figure 3 shows mean CCRTs of male and female bumble bees in relation to representative climate variables (minimum annual temperature and minimum July temperature); neither of these variables explained significant variation in CCRT for females (minimum annual temperature: $F_{1,3} = 0.24$, $P = 0.67$; minimum July temperature: $F_{1,58} = 8.15$, $P = 0.10$) or males (minimum annual temperature: $F_{1,3} = 0.38$, $P = 0.58$; minimum July temperature: $F_{1,3} = 0.18$, $P = 0.70$).

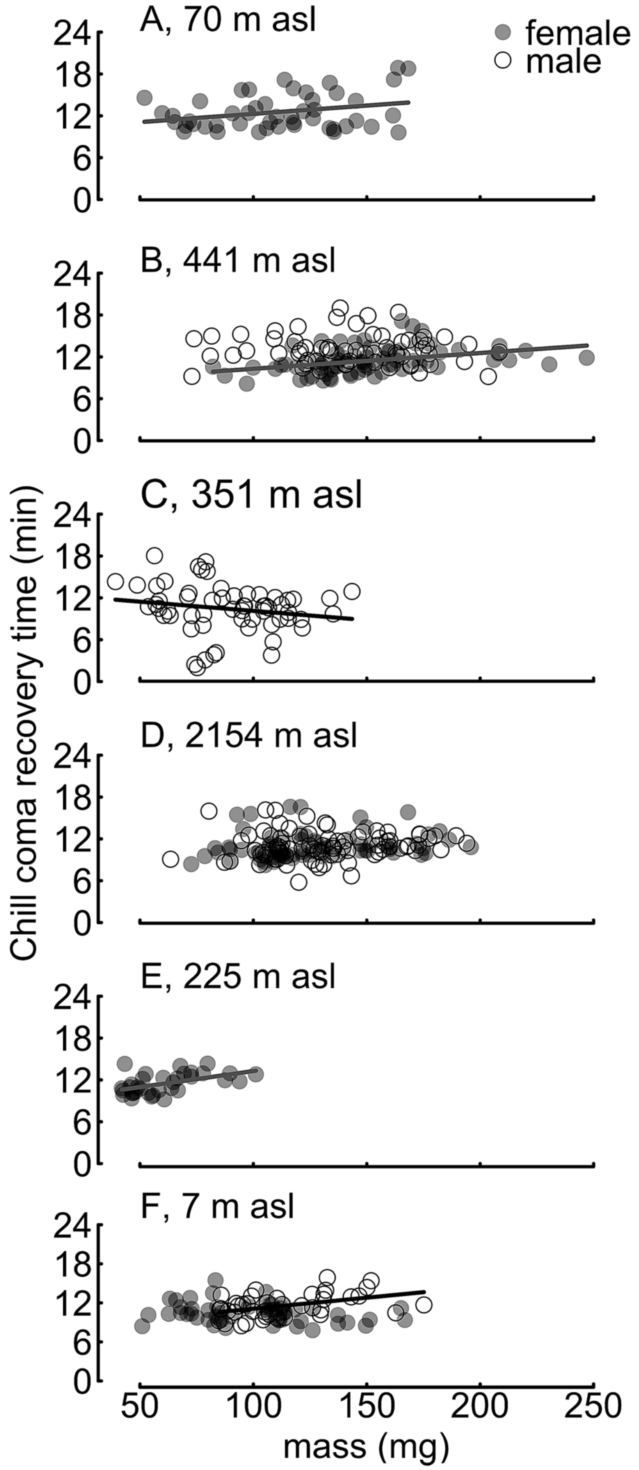

Fig. 2 CCRT varied with mass and sex for bumble bees reared in common garden conditions from queens collected across sites in western North America (see Fig. 1). Significant variation in CCRT with mass for workers (filled circles, $n=248$) and males (open circles, $n=225$) indicated by gray and black regression lines, respectively. Site elevations are given in meters above sea level (asl)

Table 4 Effects of representative climate variables on mass-corrected chill coma recovery time (CCRT), with colony as a random factor (see text for details)

Effect on CCRT	*F* (d,f)	P	AIC
Minimum July Temperature	0.949 (1,9)	0.356	2232.15
Annual mean temperature BIO 1	0.583 (1,0)	0.465	2232.71
Minimum January temperature	0.038 (1,9)	0.850	2233.07
Annual precipitation BIO 12	0.122 (1,9)	0.735	2236.75
Max temperature of the warmest month BIO 5	1.034 (1,9)	0.682	2237.03
Temperature seasonality BIO 4	1.230 (1,9)	0.296	2239.45

We also examined the influence of sex on each model, but in every case AIC values were greater and the interaction between sex and the climate variable was not significant

Discussion

Contrary to our prediction, variation in CCRTs of bumble bees reared in common garden conditions was not explained by the climate of queen collection sites (Fig. 3). We did, however, observe different trends among sites. For instance, at the most northern sites (LOR-A and LOR-B), female bees tended to have longer CCRTs. Interestingly, females from the coastal site, LCA-F, had relatively short CCRTs given their warmer origins, but this could be driven by abnormally cool summer temperatures (Fig. 1). Site HCA-D, the site with the coldest minimum annual and July temperatures, displayed expected results with short male and female CCRTs. Although some climate variables were related to mass-corrected CCRTs in simple linear regressions, none explained more than two percent ($r^2<0.02$) of the variation in CCRT. Given that our analyses include only six unique sites, we hesitate to interpret these marginally significant results. In contrast, local climate explained nearly 68% of the variation among populations in CT_{min} of common garden reared bumble bees (Pimsler et al. 2020).

Two variables, minimum July temperature and minimum January temperature, captured much of the temperature variability at our sites and serve as proxies for the seasonal cold thresholds that may influence CCRT. However, neither of these were significantly related to CCRT in mixed effects models. Furthermore, annual precipitation, annual mean temperature, and temperature seasonality were also unrelated to CCRT. These inconsistent patterns are difficult

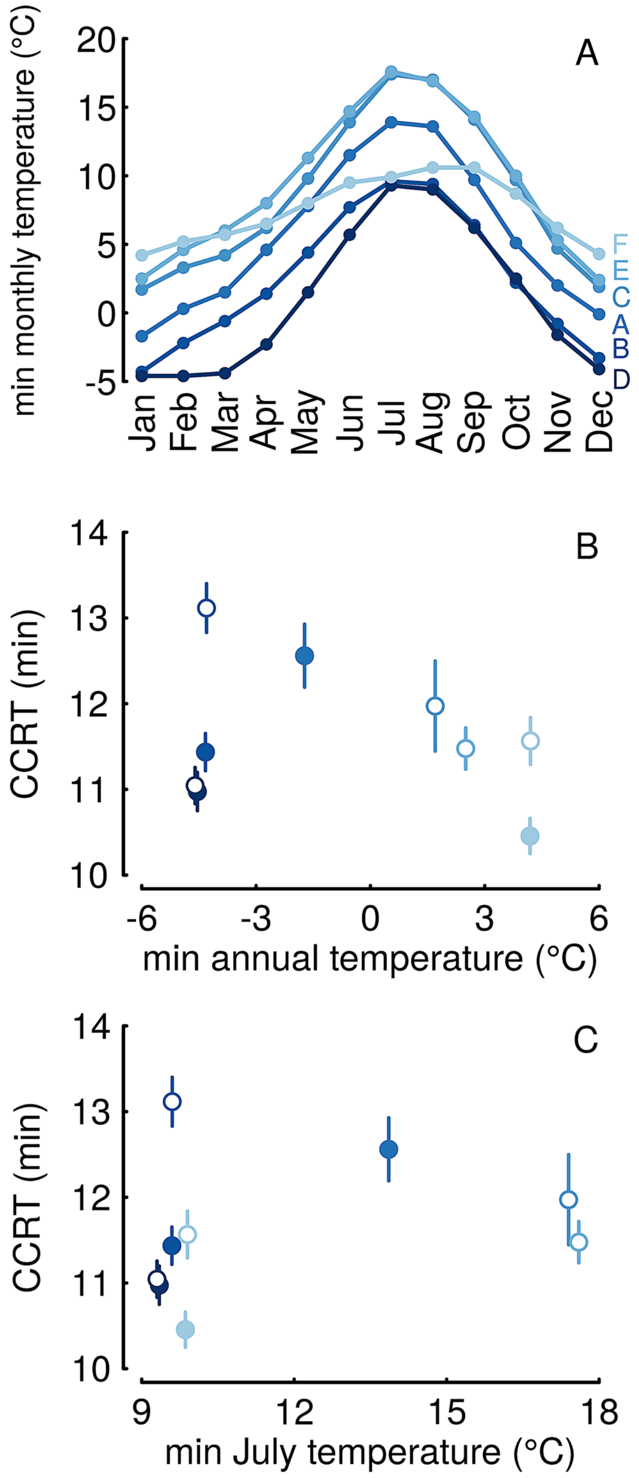

Fig. 3 CCRT did not vary consistently with local climate of queen collection sites. **A** Lines depict minimum monthly temperatures averaged from 1 km buffers (WorldClim1.4; Hijmans et al. 2005) around queen collection sites. Mean CCRT for female (filled circles, $n=248$) and male (open circles, $n=225$) bumble bees compared to minimum annual temperature (**B**) and minimum July temperatures (**C**) at queen collection sites. Point colors in **B** and **C** match colors in **A**, with darker blues indicating colder climates

to explain but may reflect differences in plasticity between common garden bumble bees used in this study and their wild counterparts. This underlines the importance of individual variation as opposed to regional variation and suggests the need to sample more sites, which is unfortunately challenging in this system, given the difficulty of large-scale collection and rearing of queens from many regions.

One reason CCRT may be unrelated to local climate is that bumble bees regulate nest microclimates and may, therefore, be less influenced by ambient temperatures (Weidenmüller et al. 2002). Furthermore, bumble bees could shift activity times to avoid exposure to temperature extremes (as do ants; Guo et al. 2020) and, given sufficient energy reserves, may be able to sustain high body temperatures long enough to return to the nest, thereby avoiding cold extremes. However, several studies have linked local climate with bumble bee thermal tolerance (Hamblin et al. 2017; Oyen et al. 2016; Pimsler et al. 2020) as well as variation in population genetic structuring (Jackson et al. 2018, 2020). Future work investigating the importance of microclimates and behavior will strengthen our understanding of the link between thermal tolerance and bumble bee distributions (see e.g. Braschler et al. 2020).

We predicted that male bumble bees would have shorter CCRTs than females, given that they spend nights outside, rather than in the thermoregulated nest with females. However, at every site, common garden female workers had shorter CCRTs relative to male drones of comparable mass. Another study found a similar difference in cold tolerance for a different species, *B. huntii:* females recovered from chill coma at significantly colder temperatures than their male counterparts (Oyen et al. 2016). And this finding is consistent with sex effects on cold tolerance in other insects: studies in locusts, flies, moths and honey bees have shown that females are generally more cold tolerant than males (Andersen et al. 2017a; Boersma et al. 2018; David et al. 1998; Davidson 1990; Goller and Esch 1990). The mechanisms underlying these consistent differences in cold tolerance between males and females remain unclear. One possibility is that high [Na^+] in ovaries (see Des Marteaux and Sinclair 2016) delays cold-induced ion imbalance in the hemolymph, but this hypothesis has not been explicitly tested to our knowledge.

The strongest trend we found was an increase in CCRT with body mass. Few studies have explicitly measured the effects of body mass on CCRT or other cold tolerance metrics and results are generally mixed: for both woodlice and *Temnothorax* ants, larger individuals recovered from chill coma more slowly (Castañeda et al. 2005; Modlmeier et al. 2012), whereas CCRT was independent of body mass in winter ants (Tonione et al. 2020). Across ectotherm species, a meta-analysis suggests that cold tolerance decreases with increasing body size, as larger ectotherms reach CT_{min} at warmer temperatures (Leiva et al. 2019). By contrast, Oyen et al. (2016) found that larger bumble bees had lower CT_{min}; and core temperature measurements in lab-reared bees suggest that this pattern was not driven by differences in thermal inertia (Oyen and Dillon 2018). However, in the current study, the increase in CCRT with mass may relate to thermal inertia because bees were moved directly from − 4 to 22 °C in contrast to the ramping approach used in the CT_{min} study. An additional possibility is that body size-related differences in CT_{min} and CCRT reflect the allometry of key organs involved in defending ion balance after cold exposure.

Several studies suggest that regaining muscle function during chill coma recovery requires the reestablishment of ion balance in the hemolymph by both the Malpighian tubules and the hindgut (Alvarado et al. 2015; MacMillan et al. 2015; Gerber and Overgaard 2018; Andersen and Overgaard 2020). Although the influence of Malpighian tubule size on CCRT is unknown, individuals with larger osmoregulatory organ to hemolymph ratios may reestablish ion gradients more rapidly than those with smaller ratios. Hymenoptera have unusual organ allometry compared to other insects, with the relative size of the Malpighian tubules decreases with increasing body size (Polilov and Makarova 2017). Larger bees may therefore have relatively smaller osmoregulatory organs. If transport capacity is related to size, relatively large hemolymph volumes and relatively small Malpighian tubules may slow the rate at which larger bees can recover ion balance, leading to longer CCRTs compared to smaller bees. Although allometric relationships of organ size and hemolymph volume in relation to CT_{min} and CCRT are virtually unstudied, both the ability of insects to maintain function of osmoregulatory organs during cold exposure (Andersen et al. 2017b), and the ability to restore ion balance after rewarming are tightly coupled to cold tolerance (Andersen and Overgaard 2020). Differences in these capacities may therefore underly sex and size-related differences in CCRT of bumble bees.

In conclusion, bumble bee CCRTs are strongly influenced by body mass, perhaps due to organ allometry. The influence of body mass and allometry on thermal tolerance metrics is an understudied relationship and may be an interesting avenue for future research. Male bumble bees had significantly longer CCRTs than females which may be related to ovaries being enriched in Na^+, buffering the effects of cold temperatures. Surprisingly, we found no relationship between local climate at the queens' collection sites and CCRT. Given that several other studies have linked local climate to bee thermal tolerance limits (Gonzalez et al. 2020; Hamblin et al. 2017; Oyen et al. 2016; Pimsler et al. 2020), it is possible a low CT_{min} allows bumble bees to avoid chill coma altogether, thus relaxing selection on CCRT. During the growing season, bumble bees only experience cold temperatures outside the nest, where entrance into chill coma increases the risk of predation and reduces foraging time, so a low CT_{min} may be more advantageous than the ability to quickly recover from chill coma. As such, in contrast to other insects, CCRT may not be an ecologically relevant metric of cold tolerance in bumble bees.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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