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Local adaptation across a complex bioclimatic landscape in two montane bumble bee species

Jason M. Jackson[1] | Meaghan L. Pimsler[1] | Kennan J. Oyen[2,3] | James P. Strange[4] | Michael E. Dillon[2] | Jeffrey D. Lozier[1]

[1]Department of Biological Sciences, The University of Alabama, Tuscaloosa, AL, USA

[2]Department of Zoology & Physiology and Program in Ecology, University of Wyoming, Laramie, WY, USA

[3]Department of Biological Sciences, University of Cincinnati, Cincinnati, OH, USA

[4]Department of Entomology, The Ohio State University, Columbus, OH, USA

Correspondence
Jeffrey D. Lozier, Department of Biological Sciences, The University of Alabama, Tuscaloosa, AL, USA.
Email: jlozier@ua.edu

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Abstract

Understanding evolutionary responses to variation in temperature and precipitation across species ranges is of fundamental interest given ongoing climate change. The importance of temperature and precipitation for multiple aspects of bumble bee (*Bombus*) biology, combined with large geographic ranges that expose populations to diverse environmental pressures, make these insects well-suited for studying local adaptation. Here, we analyzed genome-wide sequence data from two widespread bumble bees, *Bombus vosnesenskii* and *Bombus vancouverensis*, using multiple environmental association analysis methods to investigate climate adaptation across latitude and altitude. The strongest signatures of selection were observed in *B. vancouverensis*, but despite unique responses between species for most loci, we detected several shared responses. Genes relating to neural and neuromuscular function and ion transport were especially evident with respect to temperature variables, while genes relating to cuticle formation, tracheal and respiratory system development, and homeostasis were associated with precipitation variables. Our data thus suggest that adaptive responses for tolerating abiotic variation are likely to be complex, but that several parallels among species can emerge even for these complex traits and landscapes. Results provide the framework for future work into mechanisms of thermal and desiccation tolerance in bumble bees and a set of genomic targets that might be monitored for future conservation efforts.

KEYWORDS

bumble bee, climate adaptation, genetic variation

1 | INTRODUCTION

Understanding how bioclimatic pressures shape genetic variation is of critical importance for conservation as ranges shift under global climate change (Franks & Hoffmann, 2012). Species with large ranges are exposed to diverse climatic conditions that drive local adaptation and shape phenotypic and genetic diversity across their distributions. Temperature and precipitation are among the most ubiquitous selective forces in nature, influencing processes from molecular structure and function to species geographic ranges (Bozinovic, Calosi, & Spicer, 2011; Siepielski et al., 2017). Species with montane distributions inhabit landscapes where the environment changes rapidly over short distances and gradually over larger distances (e.g., temperature with latitude and altitude), and may thus be particularly useful for studying climate adaptation (Fitzpatrick & Keller, 2015; Keller, Alexander, Holderegger, & Edwards, 2013; Verheyen, Tüzün, & Stoks, 2019).

We investigated local adaptation to climate variation using bumble bees (*Bombus*) from montane regions of western North America. Temperature, in particular, has been instrumental in the evolution of *Bombus* (Hines, 2008; Martinet et al., 2018; Williams,

1998; Williams, Lobo, & Meseguer, 2018). Their abundance in cool temperate, arctic, and alpine zones, largely driven by a capacity for facultative endothermy, suggests temperature is an important selective pressure (Heinrich, 2004). However, bumble bee populations experience a range of temperature extremes throughout the year because of their annual colony cycle, from queen diapause in winter cold to worker foraging in summer heat (Woodard, 2017). So while bumble bees are remarkable in their ability to generate heat for activity at cold temperatures (Heinrich & Kammer, 1973), species may also have adaptations to prevent overheating (Heinrich, 1976). The role of moisture as a driver of local adaptation is less well-studied (Woodard, 2017); however, bee species ranges and activity periods can be driven more strongly by precipitation than by temperature (e.g., Jackson et al., 2018; Nicolson, 2009; Williams, Bystriakova, Huang, Miao, & An, 2015; Willmer & Stone, 1998). As temperature and precipitation vary across mountains, evidence for adaptation to these variables in widespread *Bombus* species will be informative for understanding climate-associated selection.

Genome-wide DNA sequencing has revolutionized our ability to uncover signatures of selection in wild populations (Matz, 2018), and available genomic resources in bumble bees facilitate such eco-evolutionary studies (Lozier & Zayed, 2017; Woodard et al., 2015). Adaptations to thermal variables are of particular interest because of the long history of study in bumble bees (Heinrich, 2004) and knowledge of thermal biology in *Bombus* and other insects provides clear hypotheses about targets of selection. Genes relating to thoracic muscle function are probably targets of selection from temperature variation across species ranges, as muscles are important both for flight and shivering thermogenesis in *Bombus* (Heinrich, 1975; Heinrich & Kammer, 1973; Hosler, Burns, & Esch, 2000). Genes related to the nervous system are generally important for maintaining activity at temperature extremes (Robertson, Spong, & Srithiphaphirom, 2017), with those involved in neuromuscular function obvious candidates because of the importance of flight muscle (Esch & Goller, 1991; Goller & Esch, 1990; Kammer & Heinrich, 1972; MacMillan et al., 2016). Finally, loss of nerve and muscle excitability at temperature extremes, especially cold, is associated with failure of membrane channels to maintain ion homeostasis, so ion channel genes are also likely candidates (Andersen, MacMillan, & Overgaard, 2015; Overgaard & MacMillan, 2017; Robertson et al., 2017). Less is known about effects of aridity on *Bombus*, but from other insects we might expect desiccation tolerance to be an important selection target (Chown, Sørensen, & Terblanche, 2011), including genes related to cuticle composition and minimizing water loss from respiration (e.g., tracheal or spiracle development) (Gibbs, Chippindale, & Rose, 1997; Gibbs, Fukuzato, & Matzkin, 2003; Telonis-Scott, Gane, DeGaris, Sgró, & Hoffmann, 2012). Water balance and ion homeostasis are also important for desiccation resistance, and physiological and genetic analyses have identified roles for the digestive and excretory system (e.g., Malpighian tubules), metabolism, and signalling (Liu, Luo, Carlsson, & Nässel, 2015; Telonis-Scott, Sgró, Hoffmann, & Griffin, 2016).

To investigate targets of selection we performed genome scans in two species, *Bombus vancouverensis* (which was recently redescribed, and corresponds to the prior name *Bombus bifarius* subspecies *nearcticus*; Ghisbain et al., 2020) and *Bombus vosnesenskii*, that are good models because of their broad latitudinal and elevational ranges in the Sierra-Cascade regions of California, Oregon, and Washington, U.S.A. (Jackson et al., 2018; Stephen, 1957). We employed restriction site-associated DNA sequencing (RADseq), a powerful tool for detecting selection in wild populations (Andrews, Good, Miller, Luikart, & Hohenlohe, 2016; Catchen et al., 2017). We used environmental association analysis (EAA) as a statistical framework for detecting single nucleotide polymorphisms (SNPs) under selection from bioclimatic variables (i.e., non-neutral "outlier" SNPs) while controlling for demography (Ahrens et al., 2018; Frichot, Schoville, Bouchard, & François, 2013; Günther & Coop, 2013; Rellstab, Gugerli, Eckert, Hancock, & Holderegger, 2015; Schoville et al., 2012). We previously performed a landscape genetics study of spatial and environmental factors that shape demography in these species (Jackson et al., 2018). Both have fairly weak population structure, but diversity and differentiation are shaped by differences in thermal and precipitation niche breadth of the species, with abiotic conditions generally causing *B. vancouverensis* to occupy somewhat higher mean elevations at the same latitudes (Jackson et al., 2018). This study aimed to expand our prior work by uncovering local adaptation to these variables and discovering genes involved in processes like thermal tolerance and desiccation resistance consistent with our physiological hypotheses discussed above. Because the two species both occur across similar elevation and latitude gradients, and may thus be exposed to similar environmental pressures, we also aimed to identify shared adaptations between species to test the hypothesis that genes show evidence of evolutionary convergence from parallel selection (Soria-Carrasco et al., 2014; Yeaman et al., 2016). Recent work has documented the importance of climatic constraints on current and future bumble bee species distributions (Kerr et al., 2015), and examining local adaptation in species with similar ranges but somewhat distinct niches will be useful for revealing both common and unique processes targeted by selection in montane *Bombus*.

2 | MATERIALS AND METHODS

2.1 | Sample collection

Many bioclimatic variables change over geographic space. To separate spatial and environmental effects as much as possible for EAA we attempted to sample bees from the broadest range of possible elevations across a latitudinal span from California, Oregon, and Washington, USA (Figure 1; Table S1; Jackson et al., 2018). The goal was to fully sample environmental variation for each species in a way that captured both rapid and gradual transitions between localities (Dillon & Lozier, 2019; Verheyen et al., 2019). *Bombus vosnesenskii* and *Bombus vancouverensis* workers were collected as previously reported (Jackson et al., 2018), although some new localities and samples are included here that were previously excluded for small

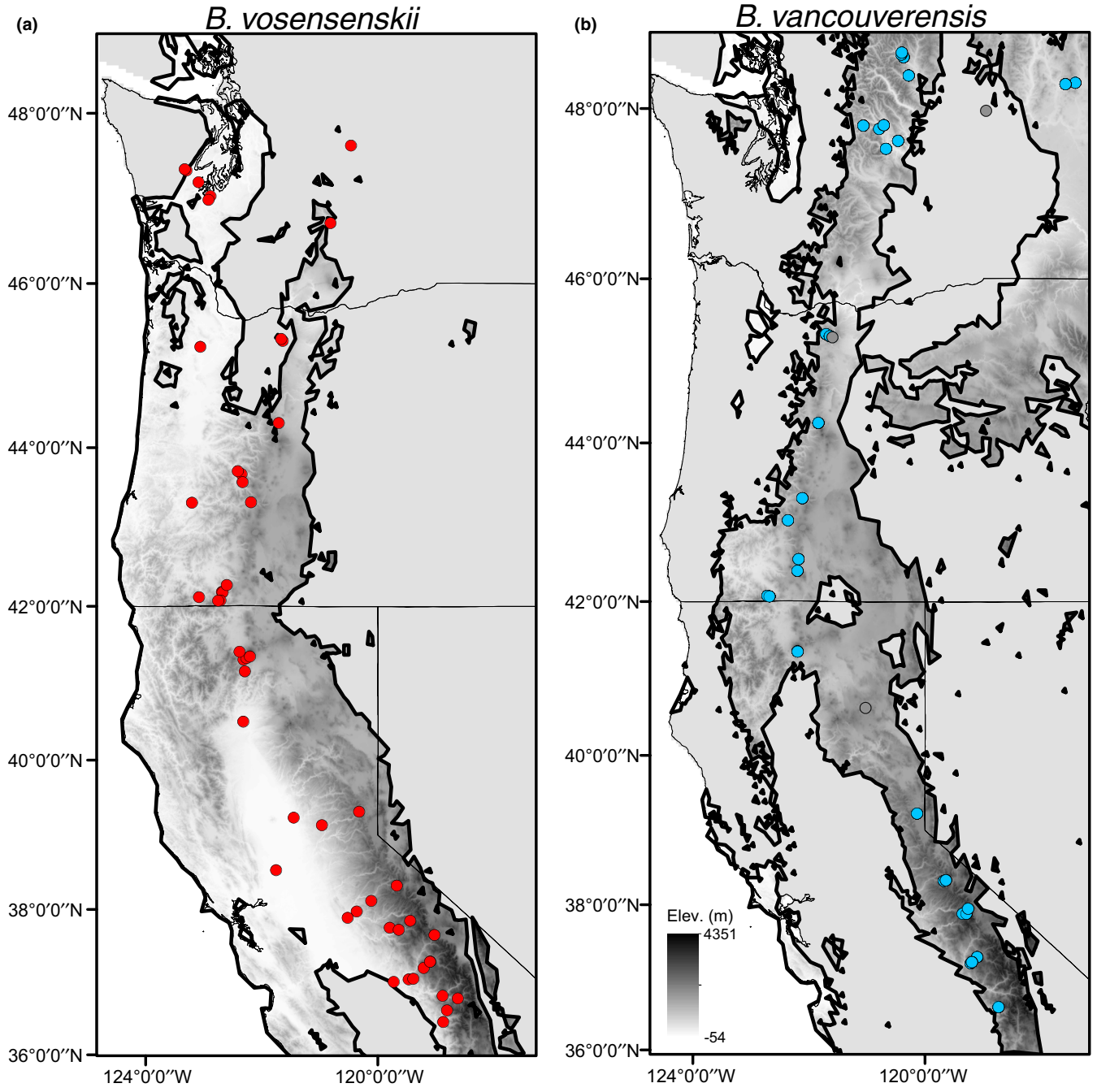


FIGURE 1 Sampling locations of (a) *Bombus vosnesenskii* and (b) *Bombus vancouverensis* along the Sierra-Nevada and Cascade mountain ranges in California, Oregon, and Washington U.S.A. Cutout outlines represent species distribution estimates in the study region from models in Jackson et al. (2018), shading reflects elevation, and points show sample locations [Colour figure can be viewed at wileyonlinelibrary.com]

sample sizes (Table S1). Importantly, the species name *B. vancouverensis* was recently resurrected for the *Bombus bifarius* subspecies *nearcticus* lineage included in this study, and our samples represent the new species *B. vancouverensis* subspecies *nearcticus* (Ghisbain et al., 2020). Samples were filtered to retain one bee per colony as in Jackson et al. (2018). The final data set contained 383 *B. vancouverensis* from 42 sites (9 ± 4 *SD* bees per site) and 587 *B. vosnesenskii* from 50 sites (12 ± 4 *SD* bees per site), similar to recommended EAA study designs that maximize power and reduce false positives (Selmoni, Vajana, Guillaume, Rochat, & Joost, 2020) (Figure S1 for locality characteristics).

2.2 | DNA extraction, sequencing, and sequence processing

We used single-end *PstI* RADseq data generated for Jackson et al. (2018), which contained 18,700 and 37,474 SNPs in *B. vosnesenskii*

and *B. vancouverensis*, respectively, at an average depth of ~21× per SNP per bee in both species. We refer readers to Jackson et al. (2018) for details but, briefly, library preparation and single-end Illumina HiSeq sequencing was conducted by Floragenex, Inc, 100 bp reads were quality filtered and trimmed with Stacks 1.42 (Catchen, Hohenlohe, Bassham, Amores, & Cresko, 2013) and cutadapt 1.13 (Martin, 2011) to 79 bp to remove barcode and restriction sequence. Reads were mapped to the *Bombus impatiens* 2.0 (BIMP2.0) genome (Sadd et al., 2015) using bwa-mem 0.7.15 (Li, 2013). All three species belong to the subgenus *Pyrobombus* Dalla Torre and diverged within the last ~4–6 MYA (Cameron, Hines, & Williams, 2007; Hines, 2008). We utilized SNPs called by Samtools mpileup 1.2 (Li et al., 2009), which performed best for these data (Jackson et al., 2018). After initial quality filtering with vcftools 0.1.16 (Danecek et al., 2011) (map quality ≥ 30, minimum SNP coverage ≥ 8 per sample per locus, genotype quality ≥ 20, missing data ≤ 20% per locus), we removed rare alleles using a minor allele frequency of 5% (full allele frequency spectrum shown in Figure S2). To estimate genome coverage for all sequence data matching the above quality criteria (excluding minor allele frequency filter) we used mpileup to count bases in each BAM mapping file. To estimate coverage representing the final SNP set with the 5% minor allele frequency filter we retained one SNP per 158 bp (the length of both RADseq loci associated with a cut site), multiplied the thinned SNP total by 158bp, and divided by the total length of the BIMP2.0 genome (249 Mb). SNP annotation used SNPeff 4.3 (Cingolani et al., 2012). Population genetic statistics calculations (average F_{ST}, H_E) were performed with hierfstat (Goudet, 2005).

2.3 | Outlier detection and cross-validation across EAA methods

Temperature is critical for bumble bee biology, from individual physiology, to current species ranges, to historical biogeography (Heinrich, 2004; Hines, 2008; Kerr et al., 2015). Because colonies persist from spring to fall and queens must survive winter diapause, it is important to capture effects from annual means, extremes, and variation in climate data (Dillon & Lozier, 2019; Wang & Dillon, 2014). We selected five bioclimate variables with these properties from the Worldclim 2.0 data set at 30 s resolution (Fick & Hijmans, 2017): Annual mean yemperature, maximum temperature of the warmest month, minimum temperature of the coldest month, isothermality, and temperature seasonality. Precipitation is also likely to have direct (e.g., desiccation pressure) or indirect (e.g., availability of floral resources) effects on bee fitness (Nicolson, 2009). Previous studies have also found that precipitation variables can be the most informative for predicting bumble bee ranges (Jackson et al., 2018; Koch, Looney, Hopkins, Lichtenberg, & Walter, 2019). As with temperature, we considered variables capturing means, extremes, and variability: Annual precipitation, precipitation of the wettest/driest months, and precipitation seasonality. We evaluated Pearson's correlations between variables (Figure S1). Correlations do not impact univariate EAAs (all variables associated with SNPs are presented), but were used to reduce the variable set for multivariate analysis (see below).

To detect SNPs under selection or linked to selection targets within each species, we used several EAA methods that test for signatures of adaptation and control for demographic structure in different ways. This allowed us to refine levels of stringency for particular analyses, including relatively low stringency for detecting parallels between species (Yeaman et al., 2016), to combining multiple methods (i.e., "cross-validation") for identifying top candidate outliers within species with high stringency (Lotterhos & Whitlock, 2015; Nadeau, Meirmans, Aitken, Ritland, & Isabel, 2016; de Villemereuil, Frichot, Bazin, François, & Gaggiotti, 2014). As a primary method, we used latent factor mixed modelling (LFMM; Frichot et al., 2013) in the R package LEA 2.7.0 (Frichot & François, 2015). This approach uses individual-level genotype data and controls for demographic structure using *K* latent factors when testing environmental associations; LFMM was generally our most liberal method (LFMM-only = low stringency). As recommended (Frichot & François, 2015; Frichot et al., 2013), we chose *K* from results of the clustering algorithm of sNMF (Frichot, Mathieu, Trouillon, Bouchard, & François, 2014), which indicated $K = 2$ as the optimal number of clusters for *B. vancouverensis* and $K = 1$ for *B. vosnesenskii* (see also Jackson et al., 2018; Lozier, Strange, Stewart, & Cameron, 2011). LFMM requires imputation of missing data, which was performed with the "mode" method in LEA based on most likely genotypes from sNMF, and *p*-values were adjusted using the genomic inflation factor, or small adjustments, following (François et al., 2016), to fit the appropriate "flat" uniform distribution that indicates proper calibration (Figure S3). We corrected for false discovery using the R package qvalue (Storey, Bass, Dabney, & Robinson, 2015). As candidate loci were subsequently filtered by additional statistical methods, we used a liberal false discovery threshold of $q \leq 0.1$. However, most SNP associations reported are at $q \leq 0.05$, as these were more likely to be cross-validated (see Table S1 for detailed results).

We used Bayenv2 as the second statistical approach to cross-validate outliers, requiring that a given SNP met significance thresholds for both LFMM and Bayenv2 for the same environmental variable (LFMM + Bayenv2 = medium stringency). Bayenv2 uses population allele counts, rather than individual genotypes, and thus did not require the added uncertainty of imputation. Bayenv2 controls for population structure differently than LFMM by employing a correlation matrix of genetic similarities based on the X^TX statistic (analogous to F_{ST}) (Günther & Coop, 2013). Correlation matrices were generated from "neutral" data sets in each species by selecting intergenic SNPs (as discussed in Coop, Witonsky, Di Rienzo, & Pritchard, 2010; Rellstab et al., 2015) thinned to one SNP per 1,000 bp, and then all SNPs were tested for associations with each variable. In Bayenv2, a Bayes Factor (BF) given for each SNP as evidence for support of the model with the environmental parameter added over the neutral model. As suggested by Günther and Coop (2013), we also evaluated the transformed rank statistic (ρ). SNPs with high BFs or ρ further from zero are likely to be non-neutral, but the statistics may detect

slightly different signals in the data (Gunther & Coop, 2013). Thus, as suggested in the Bayenv2 manual (Gunther & Coop, 2018), SNPs in the top "*x*%" of BF and "*y*%" of $|\rho|$ ($x < y$) were considered robust candidates. We used thresholds of 1% for BF and 5% for $|\rho|$ (Abebe, Naz, & Léon, 2015; Cushman et al., 2018). Because of the way in which outliers are determined based on rank order percentiles, we used this approach largely as a mechanism for cross-validating candidates from LFMM (see Tables S1 and S2).

Lastly, we used redundancy analysis (RDA) (Forester, Lasky, Wagner, & Urban, 2018) to create the most stringently filtered outlier set, which we used to focus discussion on outlier SNPs that were sufficiently unusual to pass three EAA thresholds (LFMM + Bayenv2 + RDA = high stringency). As a multivariate method, RDA is also useful for identifying the strongest bioclimatic predictor for each SNP. RDA does not incorporate population structure, but still performs well for discriminating true selection targets, especially under isolation by distance scenarios, and has good false positive/negative rates under weak genetic differentiation like that observed here (e.g., $F_{ST} < 0.05$) (Forester et al., 2018). We ran RDA in vegan 2.5 (Oksanen et al., 2018) using a subset of bioclimatic variables with correlations to <0.75 and variance inflation factors < 5 (vif.cca function) (Figure S4). The number of significant axes to retain for outlier analysis was determined with permutation tests (999 replicates with ANOVA.cca; four axes retained for *B. vancouverensis* and three for *B. vosnesenskii* at $p < .01$). We followed published methods and R code (Forester et al., 2018) to identify outliers (±3 *SD* of loading value distribution on each axis) and identify correlations with the best predictor.

After examining initial results, in particular the large number of outliers associated with isothermality that appeared in clusters throughout the scaffolds for *B. vancouverensis*, we were interested in evaluating possible haplotype structure around adaptation candidates. We tested whether linkage disequilibrium was greater between pairs of outlier SNPs relative to nonoutliers, which might indicate regions could be targets of selective sweeps. Linkage disequilibrium (r^2) between SNPs was calculated in vcftools (geno-r2 function). Distance between SNPs used in analyses of linkage disequilibrium decay come from distances in the *B. impatiens* genome. We were interested in testing whether r^2 for loci in relatively close proximity, e.g., 0–50 kb or 0–100 bp where SNPs may be within the same or adjacent genes, was significantly elevated for SNP pairs containing outliers. We generated a null expectation for the genome by resampling all SNPs (with replacement) for the same numbers of SNP pairs observed in the distance bin of interest (0–50 kb, 0–100 kb) for the category of SNP pair (both outliers, at least one outlier) and determined the mean r^2. This process was repeated to generate 10,000 resampled means representing all SNP pairs.

2.4 | Gene ontologies

We analyzed outlier SNPs using gene ontology (GO) annotations (Ashburner et al., 2000). GO enrichment is not necessarily informative about the importance of particular loci for local adaptation but can provide information about outlier functions that might correspond with predictions from knowledge of insect physiology. GO terms are not available for the NCBI BIMP2.0 genes used here, so we performed reciprocal best hits BLAST to analyze enrichment using *Drosophila melanogaster* GOs. BLAST databases were created from BIMP2.0 and *D. melanogaster* assembly Release six protein data using NCBI BLAST+ 2.9.0. BlastP was used to identify genes with best scoring hits in reciprocal subject-query analyses (e-value threshold ≤ 10^{-5}) (Table S3). GO overrepresentation tests for genes (using the BIMP2.0 NCBI gene symbol LOC #) associated with significant cross-validated SNPs were performed using GOfuncR 1.5.1 (Grote, 2018) using a hypergeometric test ($p \leq .05$), with LOCs sequenced in our data used as the background set and annotations from the org.Dm.eg.db 3.8.2 *D. melanogaster* annotation set (Carlson, 2019). GO overrepresentation analyses are presented with the medium stringency cross-validation set (LFMM + Bayenv2), as high stringency SNPs are presented in full in Results. Only intragenic SNPs were included in the analysis. Because some terms associated with biological processes were redundant, we collapsed terms using REViGO (http://revigo.irb.hr/) (Supek, Bošnjak, Škunca, & Šmuc, 2011) (Table S3).

2.5 | Parallels between species

To examine possible convergence to parallel pressures we tested for shared outliers between species. Few SNP parallels were evident so we only present gene level analyses. Because outlier genes shared between species should not be identically influenced by population structure and are thus likely to be truly convergent (Yeaman et al., 2016), we first evaluated the low stringency LFMM-only set (intragenic SNPs only). Although it has been suggested that analyses without population structure control should be sufficient for interspecific comparisons of adaptation parallels (Yeaman et al., 2016), we still aimed to reduce the risk of interspecies false positives arising from intraspecific false positives using at least one EAA method. We also evaluated the intragenic LFMM-Bayenv2 cross-validation set. Significance of overlap was determined with hypergeometric tests (phyper function in R), with the background gene set only including LOCs with sequence data in both species.

3 | RESULTS

RADseq data were consistent among samples and species, producing 3.40 (0.56 *SD*) Mb and 3.82 (0.33 *SD*) Mb per sample of aligned sequence in *Bombus vosnesenskii* and *Bombus vancouverensis* (previously *Bombus bifarius*), respectively, corresponding to approximately 1.4%–1.5% of the total genome sequence (Table S2). After filtering SNPs to a minor allele frequency of 5%, we analyzed 6,998 SNPs in, or adjacent to, 2,199 genes (4,885 intragenic SNPs within 1,492 genes) for *B. vosnesenskii* and 16,398 SNPs within or adjacent to

TABLE 1 Expected heterozygosity (H_E), minor allele frequency (MAF), and F_{ST} for the full data sets (used in all analyses) and the neutral (1 intergenic SNP per kb) data sets used to generate the correlation matrices in Bayenv2

Species	Data set	H_E (95% CI)	MAF (95% CI)	F_{ST} (95% CI)
Bombus vancouverensis	Full	0.269 (0.267–0.271)	0.182 (0.181–0.184)	0.0213 (0.0206–0.0219)
	Neutral	0.261 (0.256–0.267)	0.174 (0.169–0.179)	0.0229 (0.0208–0.0252)
Bombus vosnesenskii	Full	0.250 (0.247–0.253)	0.165 (0.162–0.168)	0.0028 (0.0025–0.0031)
	Neutral	0.249 (0.242–0.257)	0.163 (0.157–0.169)	0.0029 (0.0021–0.0036)

3,124 genes (11,091 intragenic SNPs within 2,177 genes) for *B. vancouverensis*. This corresponds to approximately 0.32% of the genome in *B. vosnesenskii* and 0.63% of the genome in *B. vancouverensis* covered by RADseq data containing high frequency SNPs. Of the 11,600 genes in the BIMP2.0 genome, ~13% in *B. vosnesenskii* and ~19% in *B. vancouverensis* contained at least one SNP. Population structure was low, with F_{ST} = 0.003 and 0.02 for *B. vosnesenskii* and *B. vancouverensis*, respectively (Table 1).

Latent factor mixed modelling to control for population structure (low stringency approach) detected loci with significant associations for all environmental variables in both species (Figure 2; Tables S1 and S2). For all variables, the genomic inflation factor appropriately calibrated *p*-value distributions (Figure S3). In *B. vosnesenskii*, 204 unique SNPs with signals of selection were detected. In *B. vancouverensis*, 712 unique outlier SNPs were detected. The medium stringency outlier set based on the two EAA methods that model population structure (LFMM + Bayenv2) included 79 unique cross-validated SNPs (57 intragenic SNPs in 54 genes) for *B. vosnesenskii* and 191 (139 intragenic SNPs in 116 genes) for *B. vancouverensis* across variables (Figure 2; Figure 3a; Table S2). In most cases, there were similar numbers SNPs across variables, except Isothermality was a notable outlier in *B. vancouverensis*. However, no more than 0.45% of all SNPs were medium stringency outliers for any variable in either species (~1% across all variables; Figure 3a). For both species, the number of outlier SNPs shared between variables was related to the degree of correlation between the variables themselves (Figure S5), but most SNPs were uniquely associated with temperature or precipitation variables, with little overlap (Figure 2).

Genes with cross-validated SNPs were enriched for a diverse array of GO terms (Table S3). In *B. vosnesenskii*, thermal outliers were associated with terms including neural and axon development, tracheal system development, locomotion, tissue regeneration/wound healing, and ion channel activity, while precipitation outliers were associated with tracheal and respiratory system development, ion transport, cell and tissue homeostasis, cell adhesion, and tissue organization and regeneration. In *B. vancouverensis*, some terms of interest for temperature variables were related to stress responses, neuron development and growth, ion binding, signalling, responses to hormones and hormone signalling, tracheal development, and heart development and haemolymph coagulation. For precipitation variables, overrepresented terms included regulation of fibroblast growth factor receptor signalling, trachea morphogenesis, several cuticle and chitin development processes, Malpighian tubule development, and ion transporter activity.

3.1 | A focus on top candidate SNPs from high stringency cross-validation

To focus on top candidate SNPs, we used RDA as a third cross-validation method (Table 2; Table S3; Figure S6). RDA reduced the outlier set (51 unique SNPs for *B. vancouverensis* and 14 for *B. vosnesenskii*), but generally retained the most significant SNPs detected by the other methods (Table 2). The top-correlated predictors identified by RDA were largely consistent with significant variables from LFMM + Bayenv2, although there were some discrepancies because of the reduced variable subset. Although it is not possible to discuss all SNPs in detail, there are several key results from this high stringency set.

Bombus vancouverensis has greater genetic diversity than *B. vosnesenskii* (Jackson et al., 2018) and had a larger number of outlier regions, including several clusters of high-ranking SNPs in genes with functions consistent with GO terms above (Table 2). Six high stringency outliers were detected in a ~200 kb region on NT_176515.1 including LOC100741930 and LOC100746249, with even more SNPs detected by individual analyses (Tables S1 and S2). Both genes code for immunoglobulin domain containing proteins: LOC100741930 annotated as *sax-3* in BIMP2.0 (closest homology to *dpr20* in *D. melanogaster*); LOC100746249 is a cell adhesion protein gene annotated as *hmcn1* (homologous to *D. melanogaster side-VI*). The two genes may play a role in synapse formation involving motor neurons. Another notable cluster of outlier SNPs in *B. vancouverensis* is on scaffold NT_176739.1, several of which are in the region of LOC100747727–LOC100747447. In BIMP2.0 LOC100747727 is *CADM2*-like, while LOC100747447 is not annotated, but both are also immunoglobulin domain containing proteins that show homology to *D. melanogaster beat-IIIc* (blastp E-value = 3e-102) and may also be involved in axon guidance at the neuromuscular junction. Other related outliers possibly involved in neural/neuromuscular development include SNPs in LOC100740312 (*NCAM2*; homologous to *side-VII* in *D. melanogaster*) and LOC100749182 (*ABI2*). Three SNPs in the LOC100741585-LOC100741702 region (*Octβ1R*-like and *Octβ3R*-like), were also candidates across methods for *B. vancouverensis* (Table 2), which is particularly interesting because the region harboring the octopamine receptors belongs to a highly divergent and seemingly adaptive region associated with elevation gradients in other bees (*Apis mellifera*)

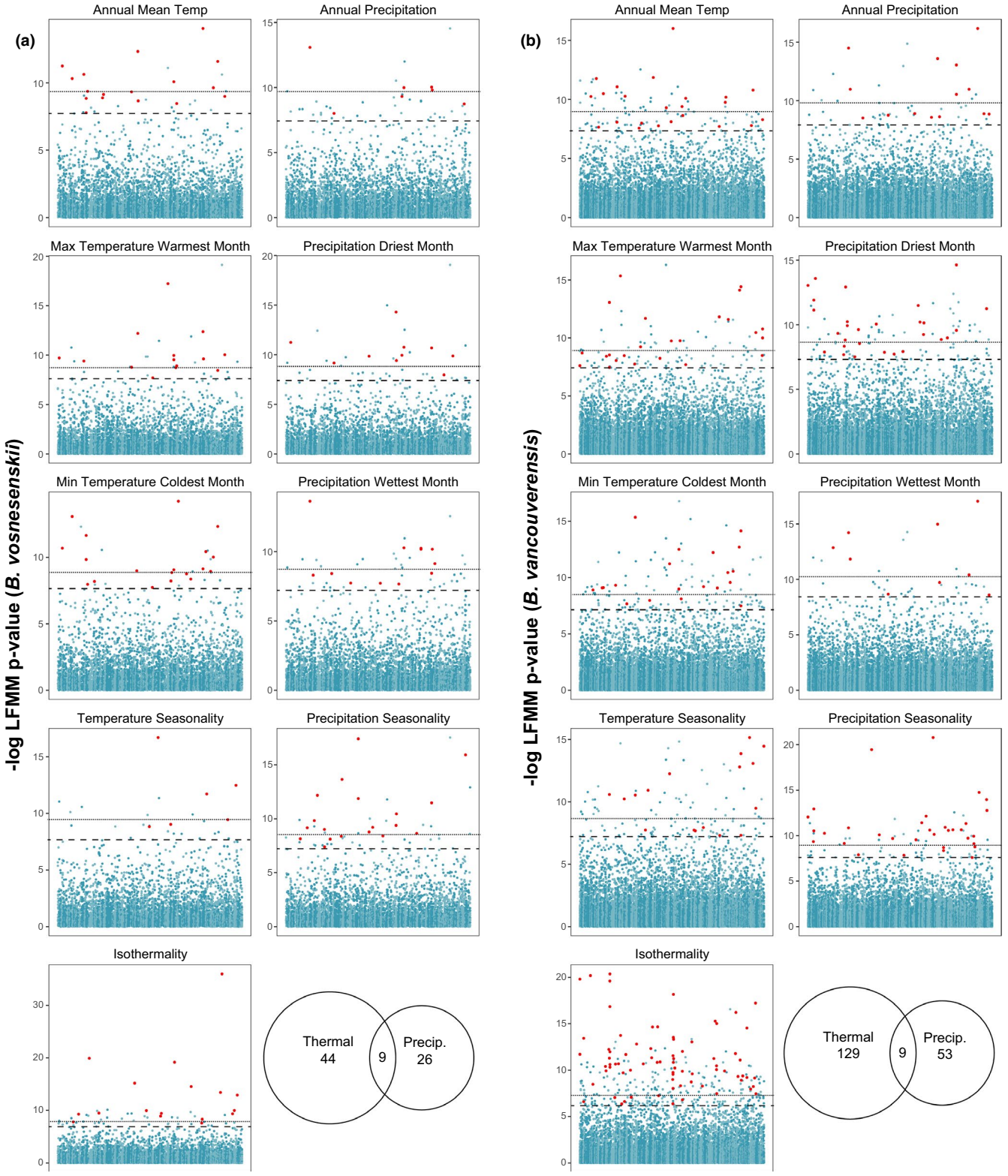
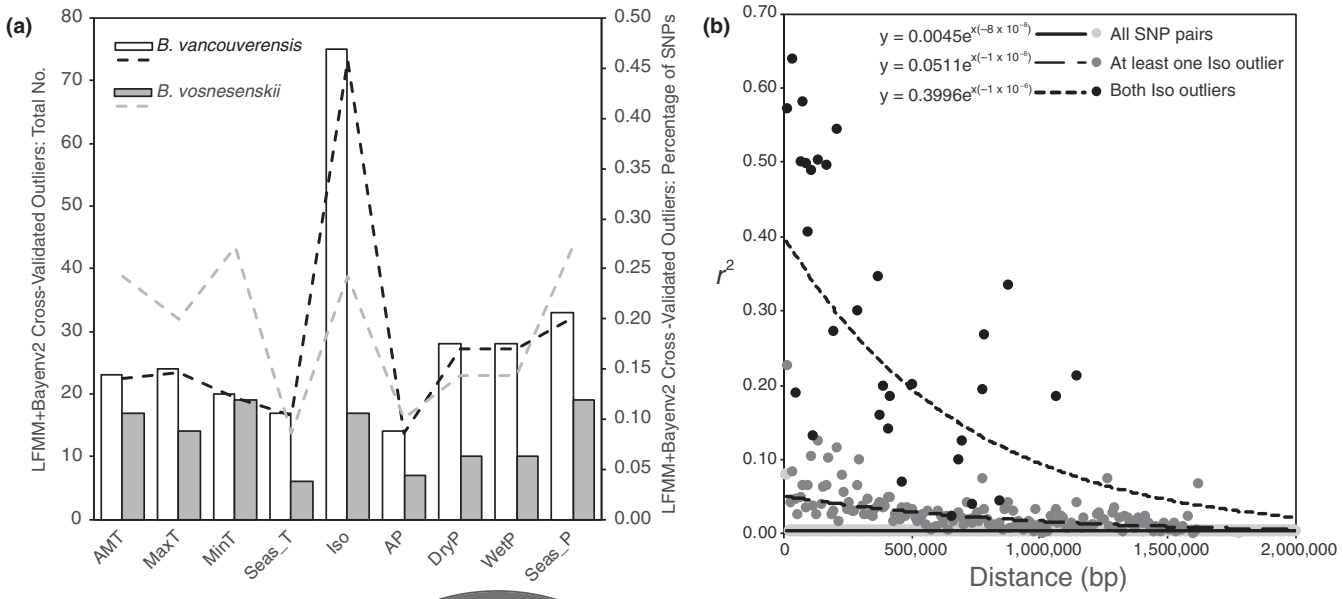
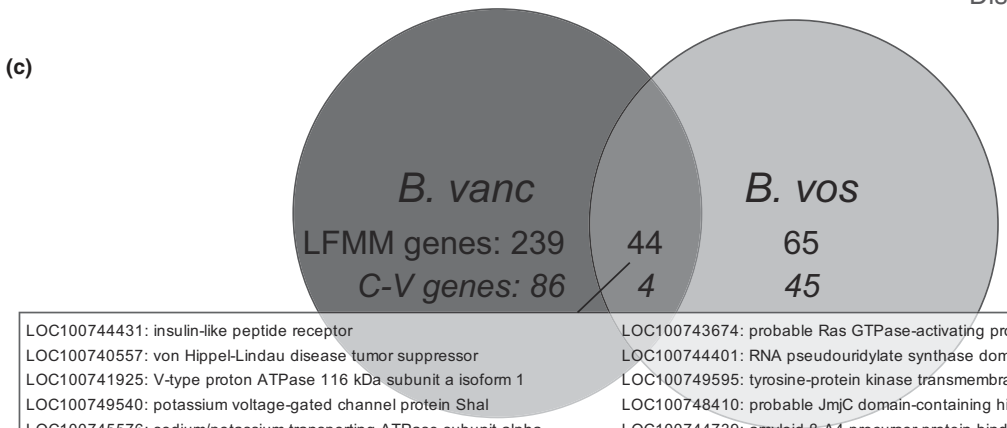


FIGURE 2 Manhattan plots of LFMM −log(*p*-values) by scaffold position for each environmental variable for (a) *Bombus vosnesenskii* and (b) *Bombus vancouverensis*. Scaffold breaks are highlighted by alternating shades, with medium stringency outliers cross-validated by LFMM and Bayenv2 highlighted red. Dashed/dotted lines reflect q = 0.10/0.05 FDR thresholds. Venn diagrams show numbers of medium stringency cross-validated SNPs associated with thermal variables, precipitation variables, or both [Colour figure can be viewed at wileyonlinelibrary.com]

(Wallberg, Schöning, Webster, & Hasselmann, 2017). Two SNPs (~3 kb apart) that were among the most significant in the *B. vancouverensis* data set were also associated with the insulin hormone system in LOC100744431 (*ILPR*). One last outlier worth mentioning (NT_176439.1:1211546) was associated with precipitation variables and fell within LOC100742243 (*hdc*), which is important for





LOC100744431: insulin-like peptide receptor	LOC100743674: probable Ras GTPase-activating protein
LOC100740557: von Hippel-Lindau disease tumor suppressor	LOC100744401: RNA pseudouridylate synthase domain-containing protein 2-like
LOC100741925: V-type proton ATPase 116 kDa subunit a isoform 1	LOC100749595: tyrosine-protein kinase transmembrane receptor Ror
LOC100749540: potassium voltage-gated channel protein Shal	LOC100748410: probable JmjC domain-containing histone demethylation protein 2C
LOC100745576: sodium/potassium-transporting ATPase subunit alpha	LOC100744739: amyloid β A4 precursor protein-binding family B member 1-interacting protein-like
LOC100748598: suppressor of lurcher protein 1	LOC100740344: epidermal growth factor receptor
LOC100742360: integrin alpha-PS2	LOC100741037: homeobox protein homothorax
LOC105680549: connectin	LOC100743731: putative uncharacterized protein DDB_G0271606
LOC100744578: muscle M-line assembly protein unc-89	LOC100745386: uncharacterized MFS-type transporter C09D4.1
LOC100741329: peroxidasin-like	LOC100748080: uncharacterized LOC100748080
LOC105681212: lachesin	LOC100742880: uncharacterized LOC100742880
LOC100747772: semaphorin-2A	LOC100744200: uncharacterized LOC100744200
LOC100744926: neurexin-3b-like	LOC105680252: uncharacterized LOC105680252
LOC100747745: neurexin-1-like	LOC100745168: uncharacterized LOC100745168
LOC100749771: neurobeachin	LOC100740629: uncharacterized LOC100740629
LOC100741492: leucine-rich repeat-containing protein let-4	LOC100743152: uncharacterized LOC100743152
LOC100747801: CCR4-NOT transcription complex subunit 6-like	LOC100743267: uncharacterized LOC100743267
LOC100743853: poly(rC)-binding protein 3	LOC100744905: uncharacterized LOC100744905
LOC100742861: 3-phosphoinositide-dependent protein kinase 1	*LOC100741930: protein sax-3*
LOC100744170: xaa-Pro aminopeptidase 1	*LOC100745294: uncharacterized LOC100745294*
LOC100740002: coiled-coil domain-containing protein 137	*LOC100746960: ankyrin repeat domain-containing protein 50*
LOC100748441: carbonic anhydrase-related protein 10	*LOC100750116: polypeptide N-acetylgalactosaminyltransferase 2*

FIGURE 3 (a) Number (bars) and percentage (lines) of SNPs detected as medium stringency outliers for each variable. (b) The relationship between linkage disequilibrium (r^2) and pairwise distance for all intrascaffold *Bombus vancouverensis* SNP pairs, pairs where at least one SNP was detected as a high stringency Isothermality outlier, or pairs where both SNPs were outliers, with lines fit using an exponential decay function. (c) Venn diagrams of unique and overlapping gene IDs (LOCs) for LFMM-only and medium stringency analyses. Numbers are presented only for genes with sequence in both species (1,176 genes). All overlapping gene IDs are listed, with the four cross-validated LOCs in italics. Results are summarized across all variables, but with each gene being counted only once (see Table S2 for specific comparisons)

regulating tracheal branching, consistent with the GO results above. *Bombus vosnsesnskii* had fewer SNPs overall and fewer cross-validated SNPs (*N* = 14), so there were no genes or regions containing high outlier densities (Table 2), although there were still some notable candidates. One of the these, SNP NT_176521.1:155783 in LOC100749230 (calcium-activated potassium channel *slo*), was especially interesting, given the role of this gene in maintaining calcium activated potassium current in muscles and neurons.

TABLE 2 High stringency SNPs (LFMM, Bayenv2, and RDA), with annotation information (gene products flanking intergenic SNPs separated by "/") and associated bioclimatic variables

Species	Scaffold	Position (bp)	Effect	BIMP2.0 Genes	Gene product annotation(s)[a]	Gene size (bp)	Cross-validated variables (RDA predictor and correlation)[b]
Bombus vancouverensis	NT_176429.1	60679	Intron	LOC100744431	Insulin-like peptide receptor	32,474	$ISO^{L=32,B=6}$ (ISO = 0.684)
	NT_176429.1	63457	Synonymous	LOC100744431	Insulin-like peptide receptor	32,474	$ISO^{L=1,B=17}$ (ISO = 0.536)
	NT_176439.1	700997	Intron	LOC100742611	Uncharacterized LOC100742611	194,031	$SEAS\_P^{L=41,B=61}$ (SEAS_P = 0.140)
	NT_176439.1	1211546	Intron	LOC100742243	Headcase protein	171,335	$DRYP^{L=6,B=48}$,$SEAS\_P^{L=15,B=42}$ (ISO = 0.285)
	NT_176508.1	802818	Intron	LOC100748080	Uncharacterized LOC100748080	274,960	$WETP^{L=6,B=19}$ (AMT = 0.198)
	NT_176515.1	892102	Intron	LOC100744401	RNA pseudouridylate synthase domain-containing protein 2-like	181,401	$SEAS\_T^{L=22,B=145}$,$MAXT^{L=5,B=51}$ (AMT = 0.151)
	NT_176515.1	1423320	Intron	LOC100741930	Protein sax-3	238,113	$ISO^{L=1,B=2}$ (ISO = 0.739)
	NT_176515.1	1492709	Intergenic	LOC100741930-LOC100746249	Protein sax-3/hemicentin-1	NA	$ISO^{L=7,B=4}$ (ISO = 0.698)
	NT_176515.1	1516920	Intergenic	LOC100741930-LOC100746249	Protein sax-3/hemicentin-1	NA	$ISO^{L=1,B=1}$ (ISO = 0.772)
	NT_176515.1	1546354	Intergenic	LOC100741930-LOC100746249	Protein sax-3/hemicentin-1	NA	$ISO^{L=30,B=7}$ (ISO = 0.654)
	NT_176515.1	1581863	Intergenic	LOC100741930-LOC100746249	Protein sax-3/hemicentin-1	NA	$ISO^{L=65,B=16}$ (ISO = 0.636)
	NT_176515.1	1616576	Intergenic	LOC100741930-LOC100746249	Protein sax-3/hemicentin-1	NA	$ISO^{L=57,B=10}$ (ISO = 0.665)
	NT_176526.1	434528	Intron	LOC100748280	Basement membrane-specific heparan sulfate proteoglycan core protein	186,092	$AMT^{L=8,B=13}$,$DRYP^{L=54,B=93}$ (AMT = 0.196)
	NT_176532.1	207053	Intergenic	LOC100747436-LOC100747552	Somatostatin receptor type 2-like/uncharacterized LOC100747552	NA	$ISO^{L=54,B=71}$ (ISO = 0.514)
	NT_176532.1	1037178	Intron	LOC105680252	Uncharacterized LOC105680252	97,779	$DRYP^{L=18,B=67}$ (ISO = 0.291)
	NT_176533.1	1565064	Intron	LOC100745000	Ras-responsive element-binding protein 1-like	108,193	$ISO^{L=15,B=33}$ (ISO = 0.514)
	NT_176550.1	705302	Intron	LOC100745605	Uncharacterized LOC100745605	82,622	$ISO^{L=254,B=42}$ (ISO = 0.532)
	NT_176561.1	86384	Intergenic	LOC100745207-LOC100741419	SKI family transcriptional corepressor 2	NA	$DRYP^{L=58,B=94}$ (AP = 0.167)

(Continues)

TABLE 2 (Continued)

Species	Scaffold	Position (bp)	Effect	BIMP2.0 Genes	Gene product annotation(s)[a]	Gene size (bp)	Cross-validated variables (RDA predictor and correlation)[b]
	NT_176563.1	668352	Intron	LOC100745368	Mannosyl-oligosaccharide alpha-1,2-mannosidase isoform B-like	66,819	$MAXT^{L=81,B=133}$, $AP^{L=42,B=27}$ (AP = 0.293)
	NT_176636.1	2432457	Intron	LOC100740616	Fanconi anemia group J protein homolog	168,881	$SEAS\_P^{L=28,B=147}$ (SEAS_P = 0.173)
	NT_176687.1	253407	5′ UTR	LOC100747926	Solute carrier family 28 member 3	4,606	$SEAS\_P^{L=32,B=54}$ (ISO = 0.534)
	NT_176715.1	201136	Intergenic	LOC105680664-LOC100742593	Golgin subfamily A member 6-like protein 2/brain tumor protein	NA	$DRYP^{L=78,B=148}$ (ISO = 0.357)
	NT_176739.1	247240	Missense	LOC100741223	CXXC-type zinc finger protein 1-like	3,637	$ISO^{L=104,B=8}$ (ISO = 0.603)
	NT_176739.1	616766	Intron	LOC100747727	Cell adhesion molecule 2-like	9,082	$ISO^{L=34,B=38}$ (ISO = 0.535)
	NT_176739.1	618218	Intron	LOC100747727	Cell adhesion molecule 2-like	9,082	$ISO^{L=148,B=86}$ (ISO = 0.517)
	NT_176739.1	618284	Intron	LOC100747727	Cell adhesion molecule 2-like	9,082	$ISO^{L=77,B=44}$ (ISO = 0.554)
	NT_176739.1	618307	Intron	LOC100747727	Cell adhesion molecule 2-like	9,082	$ISO^{L=87,B=107}$ (ISO = 0.485)
	NT_176739.1	656559	Intergenic	LOC100747727-LOC100747447	Cell adhesion molecule 2-like/uncharacterized LOC100747447 (homologous to *beat-III*)	NA	$AMT^{L=1,B=74}$ (ISO = 0.273)
	NT_176739.1	1018267	Intron	LOC100749763	Cordon-bleu protein-like 1	87,818	$ISO^{L=46,B=30}$ (ISO = 0.568)
	NT_176739.1	1025390	Intergenic	LOC100749763-LOC100750002	Cordon-bleu protein-like 1/tyrosine-protein kinase PR2	NA	$ISO^{L=16,B=11}$ (ISO = 0.640)
	NT_176739.1	1110117	Synonymous	LOC100741668	Low-density lipoprotein receptor-related protein 2	38,403	$ISO^{L=5,B=3}$ (ISO = 0.672)
	NT_176739.1	1297632	Intron	LOC100749292	N66 matrix protein-like	12,788	$ISO^{L=17,B=39}$ (ISO = 0.536)
	NT_176739.1	1381929	Missense	LOC100745294	Uncharacterized LOC100745294	80,926	$ISO^{L=36,B=45}$ (ISO = 0.584)
	NT_176744.1	68849	Intron	LOC100744062	Dipeptidase 1-like	64,871	$SEAS\_P^{L=65,B=18}$ (AMT = 0.158)
	NT_176801.1	918827	Intergenic	LOC100741585-LOC100741702	Octopamine receptor beta-1R-like/octopamine receptor beta-3R-like	NA	$ISO^{L=74,B=26}$ (ISO = 0.475)
	NT_176801.1	1018318	Intron	LOC100741702	Octopamine receptor beta-3R-like	101,281	$ISO^{L=28,B=55}$ (ISO = 0.549)
	NT_176801.1	1020514	Intron	LOC100741702	Octopamine receptor beta-3R-like	101,281	$ISO^{L=236,B=32}$ (ISO = 0.554)
	NT_176882.1	1338089	Intergenic	LOC100749839-LOC100749415	Soma ferritin-like/troponin I	NA	$SEAS\_T^{L=89,B=23}$ (SEAS_T = 0.216)
	NT_176913.1	255590	Intron	LOC100744184	Uncharacterized LOC100744184	174,684	$SEAS\_T^{L=76,B=120}$, $AP^{L=41,B=26}$ (AP = 0.201)
	NT_176917.1	114866	Intron	LOC105681005	Uncharacterized LOC105681005	2,942	$MINT^{L=42,B=112}$ (ISO = 0.255)

(Continues)

TABLE 2 (Continued)

Species	Scaffold	Position (bp)	Effect	BIMP2.0 Genes	Gene product annotation(s)[a]	Gene size (bp)	Cross-validated variables (RDA predictor and correlation)[b]
	NT_176967.1	465578	Intron	LOC100743152	Uncharacterized LOC100743152	173,883	$AP^{L=37,B=123}$,$WETP^{L=15,B=80}$ (AP = 0.221)
	NT_176967.1	2518893	Intron	LOC100741473	Solute carrier family 12 member 4	102,347	$MINT^{L=9,B=126}$ (AP = 0.182)
	NT_177001.1	1619601	Missense	LOC100744620	Protein prickle-like	14 149	$ISO^{L=11,B=5}$ (ISO = 0.615)
	NT_177072.1	136858	Intergenic	LOC100746942-LOC100748583	Protein phosphatase methylesterase 1/protein quiver	NA	$ISO^{L=72,B=35}$ (ISO = 0.577)
	NT_177271.1	262144	Intron	LOC100747022	NADPH--cytochrome P450 reductase	19,508	$ISO^{L=31,B=24}$ (ISO = 0.584)
	NT_177303.1	38611	Intergenic	LOC100742319-LOC100742444	Pollen-specific leucine-rich repeat extensin-like protein 4/dynein heavy chain 17, axonemal-like	NA	$ISO^{L=9,B=49}$ (ISO = 0.529)
	NT_177318.1	1875696	Intergenic	LOC100742801-LOC105681407	UPF0489 protein C5orf22 homolog/UPF0489 protein C5orf22 homolog (withdrawn)	NA	$SEAS\_P^{L=14,B=145}$ (SEAS_P = 0.148)
	NT_177421.1	18808	Intergenic	LOC100749182	abl interactor 2	3,353	$SEAS\_T^{L6=,B=1}$,$MAXT^{L=3,B=3}$,$MINT^{L=5,B=101}$, $AP^{L=9,B=2}$,$WETP^{L=12,B=9}$ (AP = 0.182)
	NT_177563.1	263373	Intron	LOC100740312	Neural cell adhesion molecule 2	307,886	$SEAS\_P^{L=42,B=158}$ (SEAS_P = 0.275)
	NT_178085.1	580706	Intergenic	LOC105681685-LOC100742359	Sodium-potassium-calcium exchanger 6, mitochondrial-like/protein timeless homolog	NA	$ISO^{L=101,B=125}$ (ISO = 0.546)
	NT_179893.1	332895	Intron	LOC100746284	Irregular chiasm C-roughest protein-like	158,663	$AP^{L=26,B=28}$,$WETP^{L=33,B=40}$ (AP = 0.165)
Bombus vosnesenskii	NT_176463.1	1286504	Intergenic	LOC100742851-LOC100743090	Chymotrypsin-1-like/transcription factor AP-2-epsilon	NA	$ISO^{L=35,B=13}$ (ISO = 0.201)
	NT_176499.1	289575	Intron	LOC100744435	Uncharacterized LOC100744435	64,963	$AP^{L=2,B=4}$,$WETP^{L=1,B=5}$ (AP = 0.173)

(Continues)

TABLE 2 (Continued)

Species	Scaffold	Position (bp)	Effect	BIMP2.0 Genes	Gene product annotation(s)[a]	Gene size (bp)	Cross-validated variables (RDA predictor and correlation)[b]
	NT_176521.1	155783	Intron	LOC100749230	Calcium-activated potassium channel slowpoke	107,317	$ISO^{L=2,B=2}$, $SEAS\_P^{L=6,B=3}$ (ISO = 0.202)
	NT_176552.1	1105723	Intron	LOC100746960	Ankyrin repeat domain-containing protein 50	218,904	$WETP^{L=24,B=25}$ (AP = 0.141)
	NT_176736.1	502387	Synonymous	LOC100749679	Slit homolog 1 protein-like	5,426	$ISO^{L=8,B=38}$ (ISO = 0.171)
	NT_176737.1	932793	Intron	LOC100746817	Homeobox protein prospero	43,060	$SEAS\_T^{L=12,B=6}$ (SEAS_T = 0.176)
	NT_176808.1	34817	3′ UTR	LOC100747566	Putative transcription factor capicua	40,779	$ISO^{L=12,B=10}$ (ISO = 0.230)
	NT_176861.1	362035	Intron	LOC100742395	Protein NDRG3	110,620	$MINT^{L=22,B=52}$ (SEAS_T = 0.090)
	NT_176882.1	2652440	Intron	LOC100750008	Glutathione S-transferase 1-like	2,330	$MAXT^{L=19,B=5}$,$AP^{L=6,B=5}$,$WETP^{L=3,B=4}$, $DRYP^{L=8,B=9}$ (AP = 0.168)
	NT_176897.1	1851541	Intron	LOC100743674	Probable Ras GTPase-activating protein	261,821	$MINT^{L=1,B=1}$ (AMT = 0.109)
	NT_177066.1	915104	Intergenic	LOC100748226-LOC100740426	Microtubule-associated serine/threonine-protein kinase 3/prolyl 3-hydroxylase 1-like	NA	$MAXT^{L=13,B=1}$,$AP^{L=5,B=1}$,$WETP^{L=24,B=1}$, $DRYP^{L=7,B=1}$, $SEAS\_P^{L=9,B=1}$ (SEAS_P = 0.252)
	NT_177487.1	288453	Intron	LOC100744344	Uncharacterized LOC100744344 (withdrawn)	9,826	$ISO^{L=1,B=1}$ (ISO = 0.232)
	NT_178641.1	2102576	Intergenic	LOC100747071-LOC105681808	Neo-calmodulin-like/broad-complex core protein isoforms 1/2/3/4/5-like (withdrawn)	NA	$SEAS\_T^{L=2,B=42}$ (SEAS_T = 0.161)
	NT_178834.1	7817	Intron	LOC100747745	Neurexin-1-like	74,845	$ISO^{L=7,B=4}$ (ISO = 0.169)

Note: Variables cross-validated for LFMM + Bayenv2 are shown with their rank order significance, and the best RDA predictor and its correlation coefficient are given in parentheses.

Abbreviations: AMT, annual mean temperature; AP, annual precipitation; DRYP, precipitation of driest month; ISO, isothermality; MAXT, maximum temperature of warmest month; MINT, minimum temperature of coldest month; SEAS_P, precipitation seasonality; SEAS_T, temperature seasonality; WETP, precipitation of wettest month.

[a]Uncharacterized genes marked withdrawn are in BIMP2.0 but not predicted in a recently updated *Bombus impatiens* annotation.

[b]After the variable abbreviation, the superscript values reflect the rank order of the SNP in terms of significance for that variable with L, LFMM and B, Bayenv2 (note that if the same value is given for multiple SNPs, they are tied for that rank). See Table S1 for complete list of statistics for all SNPs, and Table S2 for details on cross-validated loci from different methods.

One clear pattern from all levels of SNP filtering is that many outliers, for *B. vancouverensis* in particular, were associated with Isothermality (Figure 3a, Table 2; Figure S6) and we were interested in more detailed examination of these SNPs. As discussed above, many outliers in *B. vancouverensis* were clustered in the same genome regions (see Results above); many were not closely adjacent SNPs within the same RAD-tag, but dispersed across thousands of bp and multiple RAD sequences. This seemed large given the expected distance decay of linkage expected for bumble bees (Sadd et al., 2015) so we evaluated whether SNPs within putatively adaptive regions (particularly isothermality) exhibited unusually large linkage disequilibrium. Indeed, for the whole genome, average LD (r^2) dropped to near zero beyond 10 kb, consistent with previous results (Sadd et al., 2015). Significantly, larger LD was maintained at longer distances for regions with high stringency isothermality outliers (Figure 3b; Figure S7), suggesting linkage is more extensive than expected in putatively adaptive regions. It thus seems likely that many of the outliers with isothermality are true positives, although we suspect that some of these associations may actually reflect correlations with other factors (see Section 4).

3.2 | Parallels between species

For LFMM, 44 outlier containing genes were shared between species (Figure 3c), representing a significant degree of overlap (hypergeometric test, $p < .0001$). Only one candidate SNP was shared between species in neurexin-1-like (LOC100747745). Overrepresented GO categories for overlapping genes were similar to many GO terms from individual species analysis, including terms consistent with our hypotheses: nervous system development (synapse and neuromuscular junction, as well as memory and learning), respiratory and tracheal system development, hypoxia response, response to temperature stimulus, sodium and potassium transport, and homeostasis (Table S3). With the restriction of medium stringency cross-validation, there were still four genes shared between species for the same variable (Figure 3c). This number of genes was not greater than expected by chance; however, a notable shared outlier is *sax-3*, which is a top outlier region in *B. vancouverensis*. No high stringency LOCs (Table 2) were shared.

4 | DISCUSSION

This study documents genomic adaptation to temperature and precipitation in montane bumble bees, including high-quality intraspecific candidate loci validated with multiple EAA approaches to some general patterns of similarity between species. Like many species facing environmental change, the distributions of numerous bumble bee species are contracting (Cameron et al., 2011; Goulson, Lye, & Darvill, 2008; Kerr et al., 2015), driven in part by mismatches with local climate niches (Kerr et al., 2015). The distribution of adaptive genomic variation within species ranges may be crucial for resilience to climate change (Fitzpatrick & Keller, 2015; Manel et al., 2018), with wild bees of special interest because of the implications for pollination services (Garibaldi et al., 2013). Understanding climate adaptation in bumble bees, and similarities between species, will provide insights relevant for conserving intra- and interspecific diversity in this group.

4.1 | Biological insights from environmental association analysis

4.1.1 | Temperature

Insect thermal tolerance is an important determinant of species distributions and should be an important target of divergent selection when species occupy diverse thermal environments. For many insects, the association between minimum tolerable temperature and the minimum temperature at which species are found suggests an especially important role for cold tolerance (Hazell, Groutides, Neve, Blackburn, & Bale, 2010; Kellermann et al., 2012; Kimura, 2004). This is true for bumble bees, especially in montane regions (Hines & Williams, 2012; Martinet et al., 2018; Williams, 1985; Williams et al., 2018). Facultative endothermy makes bumble bees well suited for low temperatures, with several mechanisms to generate heat that include activation of muscles for nonflight thermogenesis ("shivering") (Esch & Goller, 1991; Heinrich, 1975; Heinrich & Kammer, 1973; Masson, Hedges, Devaux, James, & Hickey, 2017). In insects generally, loss of neuromuscular function at cold temperatures can lead to the loss of coordinated movement and, ultimately, "chill coma" (Kelty & Lee, 2001; MacMillan & Sinclair, 2011; Overgaard & MacMillan, 2017). Our results identified a number of SNPs that support selection on targets related to neural and muscular function. In *Bombus vancouverensis* we identified several clusters of outlier SNPs associated with immunoglobulin domain family members involved in neural processes, including development of motor neurons and the neuromuscular junction, based on homology with *Drosophila melanogaster* and other species (Arzan Zarin & Labrador, 2019; Zallen, Yi, & Bargmann, 1998). Several regions with immunoglobulin superfamily members have a high density of outliers, with LOC100746249-LOC100741930 (*hmcn1* - *sax3*) being particularly notable. Genes in these outlier clusters have homology to Sidestep and Beaten Path encoding genes in *D. melanogaster* (e.g., the LOC100747727-LOC100747447 region with beat-III, LOC100746249 and a second gene LOC100740312 with *sidestep* genes), which is worth further investigation because these proteins directly interact at the motor neuron synapse (Arzan Zarin & Labrador, 2019; Carrillo et al., 2015; Inaki, Yoshikawa, Thomas, Aburatani, & Nose, 2007; Li et al., 2017; Pipes, Lin, Riley, & Goodman, 2001). Finally, a few more general neural genes also contain outliers in both species, such as the neurexin family loci that plays a role in synapse architecture and function (Menon, Carrillo, & Zinn, 2013; Rui et al., 2017).

We also hypothesized that the cell membrane and ion transport could be important selection targets, especially relating to calcium

and potassium ion balance (Cooper, Hammad, & Montooth, 2014; Findsen, Overgaard, & Pedersen, 2016; Overgaard & MacMillan, 2017; Parker et al., 2018; Teets, Yi, Lee, & Denlinger, 2013). Challenges to neural and muscular systems from low temperatures can result from inactivation of voltage-sensitive calcium channels, which drive the action potential upstroke in insect muscle (Andersen et al., 2015; Findsen et al., 2016), as well as changes in extracellular potassium that spreads depolarization across cell membranes (Robertson et al., 2017). Therefore, adaptations to modulate the effects of ion channel thermal sensitivity may be critical to the maintenance of neuromuscular function across environmental temperatures within a species range. We see evidence for selection on genes with functions relating to membrane composition, ion transport and binding, and signalling in both species (Table S3). In *Bombus vosnesenskii*, one of the most significant outliers was in the calcium-activated potassium channel *slo*, which maintains calcium activated potassium current in muscles and neurons (Atkinson, Robertson, & Ganetzky, 1991) and is important for contraction of flight muscle (Keyser, 2005) and responses to environmental stimuli (Yu, Upadhyaya, & Atkinson, 2006). In *B. vancouverensis*, one high stringency outlier was detected in *solute carrier family 12 member 4* (homologous to *kcc* in *D. melanogaster*), which is also involved in potassium-chloride homeostasis of neurons (Sun, Tian, Turner, & Ten Hagen, 2009). In the analysis of species overlap, both species have outlier SNPs in the potassium voltage-gated channel protein *Shal* (LOC100749540; Figure 3c), which regulates potassium permeability of neural membranes (Choi, 2004). Altogether, we hypothesize that outliers suggest an important role for selection on loci involved in maintaining neuron and muscle function, consistent with the role of muscle in *Bombus* thermogenesis and flight and more general physiological expectations related to organismal function under extreme temperatures.

4.1.2 | Precipitation variables

Expectations for genes and traits necessary for adaptation to precipitation are less clear than for temperature, but sensitivity of bees to aridity is not uncommon (Nicolson, 2009; Willmer & Stone, 1998). In insects, selection for desiccation tolerance can reduce water loss, although desiccation resistance is complex, including changes to the cuticle and respiratory system to minimize water loss, or various alterations to increase water storage or retention (Chown et al., 2011; Gibbs et al., 1997; Griffin, Hangartner, Fournier-Level, & Hoffmann, 2017; Telonis-Scott et al., 2016). At the individual bee level, temperature and moisture can be linked (Willmer & Stone, 1998), especially in large bees where water is produced metabolically during thermogenesis and flight. However, evaporative water loss during respiration may be important in certain climates (Heinrich, 1977; Nicolson, 2009). Detection of precipitation-associated outliers in genes relating to chitin and cuticle development in *B. vancouverensis* and to development of the respiratory/tracheal system in both species is consistent with these expectations, although the exact mechanism by which tracheal or cuticle development differences may function to reduce water loss within species needs more research (Chown et al., 2011; Gibbs et al., 2003; Telonis-Scott et al., 2012). Likewise, the detection of precipitation-associated outliers in genes relating to Malpighian tubule development, digestive tract, and tissue/ion homeostasis is consistent with other studies of desiccation in insects (Lemaitre & Miguel-Aliaga, 2013; Liu et al., 2015; Telonis-Scott et al., 2016) and suggests that genes relating to water-solute balance could be important targets of selection from selection across a species ranges. Altogether, results suggest that investigating intraspecific variation in the physiology of water loss or storage in bumble bees could be a valuable avenue for future research, especially given that many of these precipitation associated processes have potentially overlapping functions with thermal tolerance (MacMillan et al., 2016).

4.1.3 | Parallels between species

Identifying parallels among related species that experience similar abiotic pressures provide a different approach to identify genes targeted by natural selection, such as through convergence or selection on standing genetic variation (Yeaman et al., 2016). For LFMM, *B. vancouverensis* and *B. vosnesenskii* exhibit significantly greater overlap in the numbers genes with outlier SNPs than expected by chance (Figure 3c), and there were several genes in notable categories that emerged as parallels. For example, *sax-3*, which had numerous outliers in *B. vancouverensis* was shared between species, as were other immunoglobulins related to neural development (LOC100741329, LOC105681212), insulin-like peptide receptor, neurexins (LOC100747745, LOC100744926), and ion transporters/channels (e.g., LOC100749540, LOC100745576). GO terms related to neural organization and function, homeostasis and ion transport, and tracheal/respiratory system development are similar to those identified within species. However, the increased stringency of within-species cross-validation significantly reduced overlap. Clearly more work is required to optimize the balance between false positives and loss of power in both intraspecific and comparative adaptation scans (Ahrens et al., 2018).

4.2 | Complexity of detecting selection in wild populations

In wild populations, environmental selection pressures will usually be somewhat correlated with each other and to spatial factors, which in turn can influence population structure. Thus, employing statistical methods that control for demography is important when looking for adaptation (Ahrens et al., 2018). A related issue is that climate adaptation probably involves complex traits that are difficult to detect (Barghi et al., 2019; Rose, Bay, Morikawa, & Palumbi, 2018, but see Forester et al., 2018). However, beyond these issues that contribute to false positives and negatives, biogeographical correlations mean that even true positives may be correlated with

multiple abiotic predictors or reflect a response to unmeasured driving factors (de Villemereuil et al., 2014). Cross-validation (either with multiple methods within species, or between species) provides confidence that many of our outliers are true positives, but the methods that account for population structure (LFMM, Bayenv2) only test associations with individual variables. Multivariate approaches (e.g., RDA) can identify the top SNP-predictor correlations, but assignments are tentative because several variables may exhibit moderate correlations with candidate SNPs (Table S2). It is reassuring that RDA tended to identify predictors similar to the other methods but teasing apart causal relationships between genetic patterns and individual variables remains a challenge (Figure S5).

Some outliers are probably responding to selection from factors not directly studied. For example, temperature is a major determinant of the active season for bumble bees, so SNPs significantly associated with temperature could indirectly reflect responses to correlated factors like season length, especially for predictors that measure variability. This could explain why variables capturing aspects of variation (e.g., isothermality, precipitation seasonality) had the most outliers and were the driving variables for RDA axes 1 and 2 (Figure S6). Isothermality provides an interesting case study here. Isothermality represents the magnitude of average day-night temperature fluctuations relative to annual fluctuations. In our montane system, southern high elevation sites have large relative daily fluctuations compared to northern and low elevation sites (see also Wang & Dillon, 2014). Isothermality could thus capture any number of selection pressures, such as those relating to aspects of season length, day length, elevational pressures, and so on, which could contribute to the relatively large numbers of outliers. Insulin-like peptide receptor, *ILPR*, (LOC100744431) is the top cross-validated outlier in response to Isothermality in *B. vancouverensis* (and shared between species). In bumble bees and other insects, expression of insulin signalling pathway genes is an important regulator of diapause, and can be influenced by the cold temperatures experienced during queen overwintering (Amsalem, Galbraith, Cnaani, Teal, & Grozinger, 2015). However, insulin signalling is involved in other processes such as the sleep cycle, longevity, and sexual maturation, any of which may respond to aspects of seasonality that correlate with Isothermality (Cong et al., 2015; Post et al., 2018). Although limited in number, other studies of montane adaptation in bees have suggested that the strongest signatures of selection may be in genes associated with olfaction, foraging, and reproduction (Montero-Mendieta et al., 2019; Wallberg et al., 2017). Honey bees from Kenyan elevation gradients show an adaptive sweep in a high-elevation inversion that contains several genes, but most notably several octopamine receptors (Wallberg et al., 2017). We identified parallel adaptive signatures in the *Octβ1R*-like and *Octβ3R*-like region (Wallberg et al., 2017). In addition to possible roles in mediating thermal stress in insects (Chen, Hung, & Yang, 2008; Srithiphaphirom, Lavallee, & Robertson, 2019), octopamine influences worker bee behaviour in *Apis mellifera* (Schulz & Robinson, 2001; Schulz, Sullivan, & Robinson, 2002), and signatures of selection may again reflect an adaptation related to foraging across habitat types, as opposed to Isothermality per se (Wallberg et al., 2017). This is further supported by top GO terms relating to learning and memory in the analysis of species parallels. Given the parallels observed here and in other bees, selection pressures driving differentiation in hormone and neurotransmitter receptors across environments would be worth additional study. Importantly, isothermality correlated outliers were also found in regions of relatively large LD compared to the genome overall, suggesting these adaptive regions are unique relative to the rest of the genome beyond statistical correlations of individual SNPs with abiotic variables. Selective sweeps are expected to produce regions of large LD, so this may not be surprising, but does mean more work will be needed to identify the actual sites targeted by selection across adaptive outlier-containing regions. Ultimately, functional tests will be needed to confirm the biological significance of outlier genes.

One additional caveat to our results that must be investigated with future research is that RADseq may incompletely sample the genome relative to linkage disequilibrium and gene size and thus miss many selected loci (Lowry et al., 2017). Sequence data containing SNPs with MAF >5% covered <1% of the total genome sequence, and there were instances where SNPs in one species were not sequenced in the other, limiting the potential to identify both parallel and species-specific signatures of local adaptation. The small bumble bee genome size, relatively large proportion of intragenic SNPs, and tendency for adaptive regions to exhibit somewhat elevated LD (at least in *B. vancouverensis*) may partially alleviate such problems. However, if RADseq randomly samples the genome, the sequences are also likely to be biased towards larger genes. Indeed, the average gene size of genes containing SNPs (~56.1 kb for *B. vancouverensis*, ~71 kb for *B. vosnesenskii*) is larger than in the BIMP2.0 genome overall (~14.6 kb), and the mean size of genes containing high-stringency intragenic outliers is larger still (~96.7 kb for *B. vancouverensis*, ~85.4 kb for *B. vosnesenskii*). Some of the genes for which we have multi-SNP detection are small (e.g., four SNPs in the 9,082 bp cell adhesion molecule-like), but other genes are large (e.g., *sax-3* is ~238 kb) and contain only one genic outlier SNP. Nonetheless, it seems likely that large genes should generally have more possible targets of selection and thus provide greater power to detect outliers, with this problem exacerbated by genome subsampling approaches that bias sequence data towards large genes. This should not affect the significance of outlier results directly, which are computed on individual SNPs, but the bias toward detecting outliers in large genes and against small genes should be considered during interpretation of data. This may be an important consideration for all RADseq selection scan studies, and one that may often be overlooked. Given the small bumble bee genomes, a shift to whole genome resequencing is feasible, and will hopefully allow us to mitigate such RADseq biases in future studies.

In conclusion, we have uncovered multiple loci that are strong candidates for local adaptation to temperature and precipitation variation in montane *Bombus*. The diversity of genes detected with high confidence support the expectation that responses to climate gradients within and between species is probably complex but do match our hypotheses, including genes related to ion balance and

development and function of the neuromuscular junction as targets of thermal adaptation, and genes associated with interesting traits like respiratory system, cuticle, and digestive tract development related to precipitation. However, even with extensive sampling across environments, more work must be done to untangle the true driving forces of adaptation for candidate loci. In particular, despite some parallelism in genes related to important biological processes between species, evidence for particular genes exhibiting parallel allele frequency shifts was more limited. This could be due to the limitations of RADseq data, but may also be due to the unique pressures faced by each species, even across fairly similar landscapes. Ongoing work testing physiological limits in these species should aid such efforts (Oyen, Giri, & Dillon, 2016), as will whole genome sequencing data. Our results highlight important research avenues into mechanisms by which bumble bees and other insects may adapt to climatic stresses, and thus have ecological and conservation implications. As low elevation organisms move upslope from thermal niche shifts, ranges for montane *Bombus* will probably erode (Koch et al., 2019), but other aspects of climate variation (e.g., seasonality, precipitation) could remain mismatched and lead to complex climate change responses (Dillon & Lozier, 2019; Kerr et al., 2015). Continued observation of this system will give additional clues about species characteristics that ease or hinder adaptation to changing bioclimatic landscapes.

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AUTHOR CONTRIBUTIONS

J.M.J., and J.D.L. conceived the study details and wrote most of the paper. J.M.J. generated data and performed statistical analyses. M.L.P. developed many of the bioinformatics analyses and wrote the relevant Methods sections. All authors conducted field work and made intellectual contributions to development of research questions, study design, and text.

DATA AVAILABILITY STATEMENT

Raw sequence data are available on the Sequence Read Archive (SRP149031). Sample information (locality coordinates, environmental data), SNP data (VCF format), various input data files, command line scripts, and metadata are available on DRYAD (https://doi.org/10.5061/dryad.79cnp5hrt).

ORCID

Jason M. Jackson https://orcid.org/0000-0003-1872-4974
Jeffrey D. Lozier https://orcid.org/0000-0003-3725-5640

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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