

A phenotypic, transcriptomic, and genomic characterisation of the response to predicted climate change conditions in overwintering *Osmia*

Dissertation
Zur Erlangung des Grades

Doktor der Naturwissenschaften

Am Fachbereich Biologie
der Johannes-Gutenberg-Universität Mainz



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geb. am 05.02.1994 in
Münster, Nordrhein-Westfalen

Mainz, 2026

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Mündliche Prüfung 08.05.2026

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Abstract

Climate change poses substantial challenges to wild bees, particularly through warmer winters that affect the critical overwintering state of temperate species, a physiological state characterised by non-feeding and, thus, heavily reliant on reduced ambient temperatures for limited energy consumption. To characterise the effects of warmer winters on overwintering mason bees across multiple biological levels, this thesis integrates physiological experiments with transcriptomics and population genomics in the two species *Osmia bicornis* and *Osmia cornuta*.

In Chapter 1, bees were exposed to field-realistic, elevated overwintering temperatures, reflecting two major climate change scenarios outlined by the Intergovernmental Panel on Climate Change (SSP2-4.5 and SSP5-8.5). In both species, emergence timing advanced by three to six weeks and weight loss increased by 17–60%, yet survival rates during the experiment were unaffected. These phenotypic responses demonstrate sensitivity to warming winters with potential fitness costs through reduced energy reserves at diapause termination and through potential phenological mismatches.

In Chapter 2, the first chromosome-level genome assembly and annotation for *O. cornuta* was generated, and together with a preliminary population genetic dataset, it was used to reveal low DNA methylation and reduced genetic diversity compared to social bees and other solitary bees. In addition, the employed long-read sequencing-based genome generation approach was characterised by cost-effectiveness while achieving high-quality assembly and annotation results and could, thus, serve as a guide to other de novo genome assembly efforts in non-model organisms.

In Chapter 3, the molecular responses to increased overwintering temperatures were characterised through RNA-sequencing of brain tissue sampled from the physiological experiment across multiple time points (Chapter 1). Both species exhibited extensive transcriptional reorganisation affecting metabolic pathways, protein homeostasis, and developmental programmes, with responses highly dynamic across time—from several hundred differentially expressed genes after one week to thousands of genes after seven and fourteen weeks. Genes associated with oxidative phosphorylation and other metabolic processes shifted from suppression to activation under prolonged warming, highlighting the importance of accumulated

transcriptomic changes. In addition, large population genomic datasets for both species, with male samples from across all of Germany, revealed panmictic population structure and signatures of recent positive selection among temperature-responsive genes with species-specific patterns.

Together, these results suggest that while mason bees possess potential to respond to warming winters through phenotypic and transcriptomic plasticity, fitness costs might still manifest after overwintering due to increased weight loss and prioritisation of metabolic transcriptional programmes over developmental programmes, suggesting that climate change impacts extend beyond direct mortality. As the two species investigated exhibited remarkably similar phenotypic and transcriptomic responses despite differences in selection patterns and two-fold differences in genetic diversity, other related species might harbour a similar potential for plastic responses to warmer winters. While there were sex differences in the physiological response to elevated temperatures, there was only very limited evidence of sex differences in the brain transcriptome response, suggestive of either differences in post-transcriptional regulation or tissue-specificity of sex differences. This integrative approach, combining experimental, molecular, and genomic methods, provides mechanistic understanding of thermal responses necessary for scientifically-informed conservation of wild bees and their pollination services.

Zusammenfassung

Der Klimawandel stellt Wildbienen vor erhebliche Herausforderungen, insbesondere durch wärmere Winter, die den sensiblen Überwinterungsphänotyp von Arten in gemäßigten Breiten potentiell stark beeinflussen. Um die Auswirkungen wärmerer Winter auf überwinternde Mauerbienen über mehrere biologische Ebenen hinweg zu charakterisieren, integriert diese Arbeit daher physiologische Beobachtungen mit transkriptomischen und populationsgenomischen Analysen in den beiden Arten *Osmia bicornis* und *Osmia cornuta*.

In Kapitel 1 wurden die Bienen feldrealistischen, erhöhten Überwinterungstemperaturen ausgesetzt, die zwei Klimawandelszenarien des Intergovernmental Panel on Climate Change (SSP2-4.5 und SSP5-8.5) widerspiegeln. Bei beiden Arten verschob sich der Schlupfzeitpunkt um drei bis sechs Wochen nach vorne und der Gewichtsverlust erhöhte sich um 17–60 %, während die Mortalitätsrate unbeeinflusst blieb. Diese phänotypischen Reaktionen demonstrieren eine Sensibilität gegenüber sich erwärmenden Wintern mit potentiellen Fitnesskosten durch reduzierte Energiereserven zum Schlupfzeitpunkt und mögliche phänologische Verschiebungen.

In Kapitel 2 wurde die erste Genomassemblierung und -annotation auf Chromosomenebene für *O. cornuta* vorgestellt und zusammen mit einem populationsgenetischen Datensatz genutzt, um eine verringerte genomweite DNA-Methylierung und reduzierte genetische Diversität im Vergleich zu sozialen Spezies aufzudecken. Der angewandte Ansatz auf Basis von Long-Read Sequenzierdaten führte dabei zu hochqualitativen Ergebnissen bei gleichzeitiger Kosteneffizienz und könnte, daher, wegweisend für weitere De-novo Assemblierungsprojekte in Nicht-Modellorganismen sein.

In Kapitel 3 wurden die molekularen Reaktionen auf erhöhte Überwinterungstemperaturen durch RNA-Sequenzierung von Hirngewebe charakterisiert. Beide Arten zeigten eine umfassende transkriptionelle Reorganisation, die Stoffwechselwege, Proteinhomöostase und Entwicklungsprogramme betraf. Dabei waren die transkriptomischen Veränderungen über die Zeit hochdynamisch – von mehreren hundert differenziell exprimierten Genen nach einer Woche bis zu mehreren Tausenden nach sieben und vierzehn Wochen. Gene der oxidativen Phosphorylierung wechselten von Suppression zu Aktivierung unter anhaltender Ausgesetztheit gegenüber wärmeren

Überwinterungstemperaturen, was die Bedeutung akkumulierter transkriptomischer Veränderungen unterstreicht. Darüber hinaus offenbarten populationsgenomische Datensätze mit männlichen Individuen aus ganz Deutschland eine panmiktische Populationsstruktur in beiden Spezies und Signaturen rezenter positiver Selektion innerhalb der differenziell exprimierten Gene.

Zusammenfassend legen diese Ergebnisse nahe, dass Mauerbienen zwar das Potenzial besitzen, auf wärmere und wechselhaftere Winter durch phänotypische und transkriptomische Plastizität zu reagieren, dem gegenüber stehen jedoch potentielle Fitnesskosten durch erhöhten Gewichtsverlust und die Priorisierung von metabolischen Signaltransduktionswegen gegenüber Entwicklungsprogrammen. Da beide Arten ähnliche phänotypische und molekulare Reaktionen zeigten – trotz Unterschieden in den rezenten Selektionsmustern und der genetischen Diversität – ist davon auszugehen, dass solche plastischen Reaktionen auch in artverwandten Spezies auftreten können. Während die Geschlechter in beiden Spezies auf phänotypischer Ebene unterschiedlich auf die Temperaturexposition reagierten, zeigte sich nur wenig Differenzierung auf der Ebene der transkriptionellen Genregulation, was auf die Bedeutung post-transkriptioneller Regulationsprozesse oder Gewebespezifität hindeutet. Der hier vorgestellte integrative Ansatz liefert ein mechanistisches Verständnis der molekularen Grundlage thermischer Reaktionen bei überwinternden Wildbienen, welches für den Schutz dieser Arten sowie ihrer Bestäubungsleistungen hochrelevant ist.

Acknowledgements

I am sincerely grateful to my supervisor Joe for the guidance, expertise, and continuous support. His insightful feedback, scientific rigor, and trust in my independence throughout this project have greatly shaped my development as a researcher and have contributed significantly to the realization of this work.

I would like to express my deepest gratitude to my wife Miriam for her unwavering love and support throughout this journey. Her encouragement, understanding, and trust were constant sources of strength across all stages of my PhD and have had a profound impact on my personal development.

I would also like to thank everyone else who influenced and supported me along the way—fellow PhD students, Technicians, PostDoc researchers, Group Leaders and Professors. Through discussions, collaborations, shared ideas, and mutual support, each of you contributed in meaningful ways to both this work and my personal growth as a scientist.

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General Introduction

1.1 Pollinator Decline and Climate Change: An Ongoing Crisis

Pollinators represent one of the most ecologically and economically important groups of terrestrial animals, providing essential pollination services that underpin both natural ecosystem functioning and global food security (Kremen et al., 2007; Kleijn et al., 2015; Potts et al., 2016). Approximately 87.5% of flowering plant species depend on animal pollination (Ollerton et al., 2011), with wild bees serving as the dominant pollinators in most terrestrial ecosystems (Garibaldi et al., 2013). The economic value of insect pollination services has been estimated at €153 billion annually for global agricultural production alone (Gallai et al., 2009; Potts et al., 2016) and whilst the ecological value of maintaining plant community diversity and ecosystem stability is difficult to quantify, pollinator losses could trigger cascading extinctions (Nasrollahi et al., 2023) and substantial ecosystem degradation (Aslan et al., 2013; Valiente-Banuet et al., 2015). Despite their importance, mounting evidence from across the globe indicates that pollinator populations are experiencing significant declines (Potts et al., 2016; Hallmann et al., 2017; Powney et al., 2019; Reilly et al., 2020). These declines have been documented across multiple taxonomic groups, geographical regions, and habitat types, raising concerns about the long-term security of pollination services and the broader implications for biodiversity conservation (Winfree et al., 2009; Winfree et al., 2011; Lippert et al., 2021).

There are multiple, interacting drivers of pollinator decline, including habitat loss and fragmentation due to land-use change (Kremen et al., 2007; Seibold et al., 2019; Winfree et al., 2009), agricultural intensification (Kremen et al., 2002), pesticide exposure (Woodcock et al., 2017; Goulson et al., 2015), pathogens and parasites (Cameron et al., 2011; Fürst et al., 2014), as well as climate change (Bartomeus et al., 2011; Vanbergen and the Insect Pollinators Initiative, 2013; Harvey et al., 2023). Among these stressors, climate change represents a particularly challenging threat due to its progressively accumulating effects, complex interactions with other stressors, and the difficulty in predicting long-term ecological consequences (Walther et al., 2002; Harvey et al., 2023). In addition, unlike habitat destruction or pesticide

exposure, which can potentially be mitigated through targeted conservation efforts or policy changes (Dicks et al., 2016), climate change operates at global scales and its effects are long-lasting, with changes predicted to persist for centuries to millennia, even after a complete cessation of human emissions (Solomon et al., 2009). Lastly, it affects all aspects of an organism's biology simultaneously, from physiological processes to geographic distributions and ecosystem community interactions (Parmesan, 2006; Scaven and Rafferty, 2013).

For pollinators, as for other insects, the impacts of climate change may be substantial due to their ectothermic physiology (Bale and Hayward, 2010). As ectotherms, bees are heavily reliant on ambient temperatures for the regulation of fundamental biological processes, including metabolism (Sinclair et al., 2012), development (Buckley et al., 2017), locomotion (Terblanche et al., 2024), and the timing of critical life-history events such as emergence from dormancy, foraging activity, and reproduction (Kellermann and Heerwaarden, 2019; González-Tokman et al., 2020). Consequently, even modest changes in temperature regimes could have effects on individual fitness and population dynamics (Deutsch et al., 2008). Whilst some species may benefit from warming through extended activity periods or expanded geographic ranges, many temperate species might face increased thermal stress, phenological mismatches with floral resources, and disruption of essential dormancy periods (Scaven and Rafferty, 2013; Kingsolver et al., 2013). Therefore, there is an urgent need to understand how pollinator species respond to ongoing and predicted future climatic change.

1.2 Seasonal Dormancy: A Vulnerable Life-History Stage

For insects inhabiting temperate and higher latitude regions, seasonal overwintering represents one of the most critical life-history adaptations, enabling survival through resource-scarce periods and adverse environmental conditions (Tauber et al., 1986; Danforth et al., 2019). For most temperate insect species, unless they can remain active throughout the cold season in warm microhabitats or due to specific behavioural (Stabentheiner et al., 2003) or physiological adaptations (Walters et al., 2009), seasonal overwintering commonly manifests as dormancy (Tauber et al., 1986), a physiological state characterised by developmental arrest, metabolic suppression and immobility, that can be subdivided into diapause and post-diapause quiescence (Kostál, 2006). Diapause is both initiated and terminated by hormonal processes in

response to predictive environmental cues such as changes in ambient temperature and photoperiod (Denlinger et al., 2012), allowing insects to synchronise the timing of diapause to the availability of critical resources (Danforth et al., 2019). It can be further subdivided into several phases such as diapause induction, preparation, initiation, maintenance and termination (Kostál, 2006). Even after diapause termination, however, insects can remain in a state of post-diapause quiescence, which is often phenotypically indistinguishable from diapause, as individuals will still remain in locomotive and metabolic suppression (Kostál, 2006; Diniz et al., 2017). Yet, in contrast to diapause, during post-diapause quiescence, individuals are more receptive to environmental changes, such as increases in ambient temperatures, which can trigger the termination of seasonal overwintering (Kostál, 2006) and, thus, the emergence into the active season, characterised by foraging, reproduction and nest-building (Danforth et al., 2019).

For pollinator species in temperate regions, seasonal overwintering represents the longest continuous period of the annual life cycle, often spanning five to eight months (Danforth et al., 2019). The vast majority of these species spend the cold season in dormancy (Danforth et al., 2019), meaning individuals must survive without feeding, relying entirely on energy reserves accumulated during the preceding active season whilst simultaneously maintaining cellular and physiological integrity at low temperatures (Hahn and Denlinger, 2007). Therefore, the successful navigation of this dormant period is a critical demographic bottleneck for most pollinators, with dormancy survival rates directly influencing the size and persistence of populations (Williams et al., 2015). Mortality during dormancy can generally result from multiple factors, including predation, pathogen infection, desiccation, or, most relevant to climate change, depletion of energy reserves due to elevated metabolic rates (Sinclair, 2015; Irwin et al., 2001).

The relationship between temperature and metabolism during dormancy is of particular concern in the context of climate change. Metabolic rate typically scales exponentially with temperature, approximately doubling for every 10°C increase ($Q_{10} \approx 2$) in many ectotherms (Gillooly et al., 2001), although this relationship can vary depending on the thermal sensitivity of the organism and its physiological state (Sinclair et al., 2012). Still, as a consequence, even modest increases in overwintering temperatures can substantially elevate metabolic rates, increasing the rate at which stored nutritional reserves, such as lipid, are consumed (Irwin et al., 2001). If reserves become depleted before spring conditions permit emergence and foraging, individuals would face starvation and even if individuals survive, reduced energy reserves could have negative effects on post-emergence fitness through reduced longevity, impaired reproductive capacity, or decreased ability to provision offspring.

(Bosch and Kemp, 2004; Schenk et al., 2018b). Alternatively, elevated winter temperatures or transient warm spells could disrupt the regular progression through different physiological diapause subphases (Kostál, 2006), potentially leading to premature emergence followed by exposure to lethal cold temperatures, or by a lack of floral resources required for survival and reproduction (Kehrberger and Holzschuh, 2019).

The regulatory mechanisms governing dormancy physiology have been shaped by thousands of generations of selection under historical climatic conditions (Tauber et al., 1986). These mechanisms integrate multiple environmental cues—particularly photoperiod and temperature—to coordinate the timing of dormancy entry, maintenance, and termination with seasonal cycles of resource availability and favourable weather conditions (Denlinger, 2022; Kostál, 2006). However, the pace of contemporary climate change, with some regions experiencing winter warming at rates exceeding 0.5°C per decade (Intergovernmental Panel on Climate Change, 2021), substantially exceeds recent historical rates of climatic change over the past millennia and may challenge the capacity of organisms to respond to these changes through phenotypic plasticity or evolutionary adaptation (Hoffmann and Sgrò, 2011). This raises fundamental questions about whether the dormancy-regulating mechanisms in temperate pollinators are capable of recalibrating to future environmental conditions, or whether rapid warming could exceed the tolerance limits and adaptive capacity of these systems (Bale and Hayward, 2010), highlighting the importance of studying the effects of elevated temperatures on this particularly sensitive life stage.

1.3 Genomics and Transcriptomics: Molecular Approaches to Climate Change Research

Whilst organismal-level studies documenting phenotypic responses to climate change have provided invaluable insights into the impacts of warming on pollinators (Scaven and Rafferty, 2013; Kingsolver et al., 2013), such studies have inherent limitations in elucidating the underlying mechanisms driving observed responses or predicting longer-term evolutionary trajectories (Hoffmann and Sgrò, 2011). To address these limitations, there has been increasing recognition of the need to integrate molecular and genomic approaches into climate change research (Hoffmann and Sgrò, 2011; Sgrò et al., 2011). These approaches offer several complementary advantages (DeBiasse and Kelly, 2016): they can reveal cryptic physiological changes

occurring at the molecular level before they manifest as measurable organismal phenotypes, they can identify the specific genes, pathways, and regulatory networks mediating plastic responses to environmental variation (López-Maury et al., 2008) and they enable assessment of genetic variation and signatures of selection, providing insights into the evolutionary potential for adaptation to selection pressures posed by environmental changes (Ellegren, 2014).

Transcriptomics, the genome-wide quantification of gene expression, has emerged as a particularly powerful approach for characterising molecular responses to environmental stressors, including thermal stress (López-Maury et al., 2008; DeBiasse and Kelly, 2016). By measuring the expression levels of thousands of genes simultaneously, transcriptomic analyses reveal a comprehensive molecular phenotype that captures an organism's integrated response to its environment (DeBiasse and Kelly, 2016). Transcriptional plasticity—the ability to modulate gene expression in response to environmental variation—represents a fundamental mechanism enabling organisms to maintain homeostasis under non-optimal conditions (Chevin et al., 2010). When exposed to elevated temperatures, organisms may upregulate stress-response pathways, control metabolic pathways to adjust energy consumption, alter membrane composition to maintain fluidity, and activate or suppress developmental programmes (Sørensen et al., 2003; Kültz, 2005; Teets and Denlinger, 2013; Sinclair and Marshall, 2018).

The transcriptional response to thermal stress in general has been characterised in several insect taxa, revealing both conserved responses shared across diverse lineages and lineage-specific or even population-specific patterns of gene expression (Sørensen et al., 2003; DeBiasse and Kelly, 2016). Common features of thermal stress responses include upregulation of molecular chaperones (particularly heat shock proteins of the Hsp70 and Hsp90 families) that prevent protein misfolding and aggregation, activation of antioxidant defence systems to manage oxidative stress generated by elevated metabolic rates, and modulation of energy metabolism pathways (Bukau et al., 2006; Kültz, 2005; King and MacRae, 2015; Joannis and Storey, 1996). However, the specific pathways involved as well as the magnitude of expression changes can vary substantially among taxa, developmental stages, tissues and environmental contexts (Kingsolver and Buckley, 2020; Torson et al., 2017). In the context of dormancy, transcriptomics offers unique insights into the molecular mechanisms maintaining dormant states and the ways in which these mechanisms may be disrupted by environmental stressors (Ragland et al., 2010). Diapause is accompanied by characteristic transcriptional signatures, including widespread downregulation of developmental genes, modulation of metabolic pathways towards lipid catabolism and activation of stress response mechanisms (Ragland

et al., 2010; Košťál et al., 2017; Hahn and Denlinger, 2007; Kučerová et al., 2016). Understanding how elevated temperatures alter these transcriptional programmes can reveal whether warmer overwintering conditions simply passively accelerate normal dormancy progression, or trigger more actively regulated changes in metabolic and developmental pathways to cope with the environmental stressor (Bale and Hayward, 2010; Kostál, 2006).

Complementing transcriptomics, population genomics provides essential insights into the genetic diversity, population structure, and signatures of selection that underlie a population's capacity for evolutionary adaptation (Ellegren, 2014). Climate change represents a powerful selective force, and populations may respond through shifts in allele frequencies at loci influencing stress responses, developmental progression, metabolic regulation or other traits relevant to responding to thermal changes (Hoffmann and Sgrò, 2011; Exposito-Alonso et al., 2023). Detecting genomic signatures of such selection can identify genes and pathways that have been targets of recent or ongoing adaptation, providing candidate loci that may determine population persistence under continued warming (Hoban et al., 2016). Furthermore, assessing genome-wide patterns of genetic diversity is essential for assessing adaptive potential, as standing genetic variation provides the necessary raw material upon which selection can act (Frankham, 2005), leaving populations with low genetic diversity potentially more vulnerable to rapid environmental change (Frankham, 2005; Ellegren, 2014).

Integration of transcriptomic and population genomic data offers particularly powerful insights by connecting phenotypic plasticity, revealed through transcriptional responses, with evolutionary potential, revealed through genetic diversity and selection signatures (Hoffmann and Sgrò, 2011; Sgrò et al., 2011). For example, genes showing strong transcriptional responses to thermal stress may overlap with regions of elevated genetic diversity and signatures of recent selective sweeps, suggesting that evolutionary adaptation at these loci is both possible and has occurred relatively recently on evolutionary timescales (Merilä and Hendry, 2014; Hoban et al., 2016). Conversely, if thermally responsive genes show no signatures of selection and reduced genetic diversity, this may indicate that phenotypic plasticity is the main coping mechanism that is available for the molecular functions associated with these genes (Merilä and Hendry, 2014).

Despite the valuable insights that can be gained from employing multi-omics methods, studies on the molecular and genomic basis of organismal and ecological traits of pollinators, such as thermal responses, are scarce. This highlights the need for

additional research in this area, particularly in the face of the rapidly progressing crisis of climate change.

1.4 Integrating Phenotype, Transcriptome, and Genome

A more complete understanding of how organisms respond to climate change requires integration across multiple levels of biological organisation (Hoffmann and Sgrò, 2011; Sgrò et al., 2011). For example, while the organismal phenotype reveals the fitness-relevant responses to thermal stress during overwintering, the transcriptomic phenotype illuminates the molecular mechanisms and potential for plasticity underlying these responses (DeBiasse and Kelly, 2016) and population genetic datasets inform on the evolutionary history, genetic diversity, and adaptive potential determining longer-term population trajectories (Ellegren, 2014). In fact, the relationship between phenotypic plasticity and genetic adaptation in the context of climate change represents a fundamental question in evolutionary biology with important conservation implications (Chevin et al., 2010; Hoffmann and Sgrò, 2011). Plasticity can facilitate population persistence in novel environments by enabling individuals to maintain fitness across a broader range of conditions and plastic changes can later become permanently fixed through natural selection (genetic accommodation) (West-Eberhard, 2005; Chevin et al., 2010). However, plasticity can also be maladaptive, for example if the costs of plastic responses outweigh their benefits (Ghalambor et al., 2007; Kvist et al., 2022). Plasticity and adaptation may interact in complex ways, with high plasticity potentially reducing selection intensity and thus slowing adaptive evolution, or alternatively, facilitating range expansions into new environments and exposing previously cryptic genetic variation to selection (West-Eberhard, 2005; Crispo, 2007; Paaby and Rockman, 2014; Fox et al., 2019b).

More specifically, for pollinators exposed to elevated overwintering temperatures, several alternative scenarios are possible regarding the interplay of plasticity and adaptation (Chevin et al., 2010; Merilä and Hendry, 2014). First, observed transcriptional responses to elevated temperatures might represent adaptive plasticity, with individuals successfully modulating gene expression to maintain homeostasis and minimise fitness costs under warmer conditions (Chevin et al., 2010; Kingsolver and Buckley, 2020). If individuals differ in terms of this phenotypic plasticity due to underlying genetic variation, selection could then act to genetically stabilise the most fitness-preserving plastic phenotypes (genetic accommodation (West-Eberhard, 2005)). Alternatively, plastic transcriptional responses might be non-adaptive or

even maladaptive, representing disruptions to fine-tuned metabolism- and dormancy-regulating pathways rather than compensatory adjustments (Bale and Hayward, 2010). In this case, the prevalence of sufficient genetic variation among genetic loci responsible for thermal responses is particularly crucial to allow selection to restore more appropriate phenotypes under the new thermal conditions, for example through changes to thermal sensitivity of the involved regulatory pathways or better adapted thresholds for dormancy entry and exit (Denlinger, 2022). To be able to distinguish between these scenarios and predict population-level responses to elevated overwintering temperatures, an integrative approach, combining measurements of fitness-relevant phenotypic changes with the identification of the underlying molecular pathways and the genetic diversity levels and signatures of recent or ongoing selective sweeps among these pathways is thus of vital importance (Frankham, 2005; Hoban et al., 2016).

1.5 Mason Bees: Understanding the Effects of Climate Change in Wild Bees

Wild bees are the most ecologically and economically important pollinators in terrestrial ecosystems (Garibaldi et al., 2013) and within the broader context of wild bee diversity, a group that warrants particular attention are solitary bees. They comprise the vast majority of bee diversity, representing over 85% of the approximately 20,000 described bee species worldwide (Danforth et al., 2019), split into seven extant families. In addition, solitary bees exhibit tremendous variation in life-history traits such as foraging, nesting and overwintering (Danforth et al., 2019). For example, in terms of foraging, species range from generalists (polylectic species) that collect pollen from diverse plant families, to extreme specialists (oligolectic species) that depend on pollen from a single plant family, genus or even species (Danforth et al., 2019; Minckley and Roulston, 2006). Similarly, nesting strategies vary from generalists that utilise diverse substrates—including soil, pre-existing cavities, or plant stems—to specialists restricted to using particular nesting substrates such as specific soil types, empty snail shells, or nests they built from leaves of particular plant species (Danforth et al., 2019; Westrich, 2019). This ecological diversity makes solitary bees both important contributors to pollination services across varied ecosystems, but also potentially vulnerable to environmental change, particularly for specialist species whose survival depends on the continued availability of specific host plants or nesting substrates (Minckley and Roulston, 2006; Ziska et al., 2016). In addition, unlike social relatives, such as honeybees and bumblebees, solitary bees

lack the buffering effects of colonial organisation, leaving individuals more directly exposed to environmental stressors without the protection of nest thermoregulation, coordinated behavioural responses, or the ability to redistribute resources amongst colony members (Straub et al., 2015; Jones et al., 2004). Despite their increased susceptibility and ecological importance, however, solitary bees remain substantially understudied compared to social species such as honeybees (Genus: *Apis*) (Colgan et al., 2026).

Within the diverse assemblage of solitary bees, mason bees of the genus *Osmia* have emerged as a particularly valuable group for investigating the impacts of environmental stressors, including climate change, on wild bee biology (Bosch, 1994; Bosch et al., 2001). The genus *Osmia* comprises approximately 350 described species distributed predominantly across the temperate regions of the Northern Hemisphere (Danforth et al., 2019), with centres of diversity in the Mediterranean basin, western North America, and East Asia (Rightmyer et al., 2013). Most *Osmia* species exhibit a univoltine life cycle, completing one generation per year, and utilise pre-existing cavities in wood, plant stems, or rock crevices for nesting (Banaszak and Romasenko, 1998; Praz et al., 2008). The life cycle of temperate *Osmia* species follows a characteristic pattern that makes them particularly suited to experimental manipulation and relevant for climate change research (Bosch et al., 2001). Adults emerge in spring, with males typically emerging one to two weeks before females (protandry) (Bosch, 1994). Following mating, females provision a series of linear nest cells within cavities, each containing a supply of pollen and nectar and a single egg (Danforth et al., 2019). Eggs develop through five larval instars, consuming the provision mass before spinning a cocoon and undergoing metamorphosis to the adult stage (Danforth et al., 2019). Critically, adults complete development by mid to late summer but, throughout autumn and winter, they remain within their cocoons in a dormant state, first entering diapause and later transitioning to post-diapause quiescence, overwintering as fully formed imagos (Splitt et al., 2022). This extended adult dormancy period, typically lasting five to eight months depending on species and latitude, represents the longest continuous life stage and exposes individuals to the full spectrum of winter environmental conditions (Bosch, 1994). Several characteristics make *Osmia* species particularly valuable as model systems for climate change research (Bosch et al., 2001). For example, as cavity nesters, their cocoons can be readily collected and subjected to controlled environmental conditions in laboratory settings (Bosch, 1994). This ease of handling enables experimental testing of physiological responses to temperature variation, and the consequences of different climate scenarios in ways that would be either difficult to implement or not reflective of field conditions (due to the effects of soil thermal

buffering) with ground-nesting species (Williams et al., 2015; Schenk et al., 2018b). In addition, their relatively short univoltine generation time (one year) allows for multi-generational studies and assessment of longer-term population responses (Bosch et al., 2001). Lastly, as important commercial pollinators, understanding their responses to climate change has direct applied relevance for agricultural sustainability and pollination security (Gruber et al., 2011).

Two mason bee species of particular importance for European research are *Osmia bicornis* (formerly *O. rufa*) and *Osmia cornuta* (Bosch et al., 2001). *O. bicornis* has a broad distribution across much of Europe and temperate Asia, occupying diverse habitats from agricultural landscapes to urban gardens and forest edges (Westrich, 2019). *Osmia cornuta* typically exhibits a more Mediterranean distribution but has been expanding its range northwards in recent decades, increasingly establishing populations in urban areas of central Europe, including Germany (Westrich, 2019). More recently it has also been reported to establish populations in Canada (Getz et al., 2024), likely through accidental introduction as a result of cocoon trading for agricultural pollination services. Both species are important oligolectic pollinators, visiting a wide range of plant families (Bosch et al., 2001), and have been increasingly employed in commercial pollination of fruit crops, due to their efficiency as pollinators and ease of management (Gruber et al., 2011; Bosch et al., 2006).

Previous research on *Osmia* species has demonstrated that these bees exhibit considerable phenotypic plasticity in response to thermal variation during dormancy. Experimental exposure to elevated overwintering temperatures has been shown to advance emergence timing, reduce body mass at emergence due to accelerated consumption of lipid reserves, and in some cases increase post-emergence mortality (Bosch, 1994; Bosch and Kemp, 2004; Fliszkiewicz et al., 2012; Schenk et al., 2018b; Kemp and Bosch, 2005). Whilst these studies have been performed using experimental conditions of little resemblance to field conditions, these phenotypic responses nevertheless demonstrate that *Osmia* species are sensitive to changes in thermal conditions during overwintering and suggest that contemporary warming trends may be influencing their population dynamics (Kehrberger and Holzschuh, 2019).

For mason bees, despite their ecological and economic importance and their suitability as model systems for climate change research, studies providing molecular and genomic insights into the mechanisms underlying thermal responses have been limited (Albacete et al., 2023). The majority of research into the molecular basis of thermal responses in bees has focused on social species, particularly the western honeybee (*Apis mellifera*), in addition to several other honeybee (*Apis* spp.) and

bumblebee species (*Bombus* spp.), with far less attention paid to solitary bees (Colgan et al., 2022; Webster et al., 2023; Colgan et al., 2026). Among solitary bees, the most extensive molecular work has been conducted on the alfalfa leafcutter bee (*Megachile rotundata*), for which several transcriptomic studies have examined responses to thermal stress and diapause progression finding changes in expression levels of distinct groups of genes, such as immune genes (Xu and James, 2012; Torson et al., 2015b; Torson et al., 2017; Yocum et al., 2018). However, these studies have typically focused on acute or short-term thermal exposure regimes rather than sustained warming scenarios reflective of projected climate change, and have not integrated population genomic data to assess genetic diversity or selection signatures. In summary, this highlights an increased need for transcriptomic and population genetic genome-wide analyses of thermal responses in mason bees.

1.6 Thesis Aims and Structure

The overarching aim of this thesis is to provide a multi-level characterisation of how solitary wild bees respond to elevated overwintering temperatures, integrating organismal phenotypic responses with underlying molecular mechanisms and evolutionary potential. Using two closely related mason bee species, *Osmia bicornis* and *Osmia cornuta*, as model systems, this research aims to address fundamental questions about the capacity of wild bees to respond to predicted and ongoing climate change through phenotypic plasticity and genetic adaptation. The work combines experimental manipulations exposing bees to field-relevant thermal scenarios predicted under IPCC climate change scenarios and measurements of fitness-relevant phenotypic traits with transcriptome-wide characterisation of gene expression responses and population genomic analyses of genetic diversity and selection signatures.

This integrative approach enables several key questions to be addressed. First, how do prolonged elevations in overwintering temperature including field-realistic diurnal and seasonal variation affect the emergence timing, physiological condition, and survival of mason bees? Second, what molecular mechanisms underlie these phenotypic responses, and how do transcriptional responses vary across the overwintering period as bees progress through different dormancy phases? Third, do male and female bees, which differ in emergence timing and potentially in thermal exposure due to nest positioning, also differ in their organismal and molecular responses to elevated temperatures? Fourth, do closely related species, which overlap in distribution but differ in natural phenology and in their historical ranges and recent range dynamics, exhibit similar or divergent responses to such thermal challenges? Fifth,

what levels of genetic diversity exist in contemporary populations of these species, and are there signatures of recent positive selection at genes involved in thermal responses, indicating potential evolutionary adaptation to warming conditions?

The thesis is structured into three chapters, each addressing a different level of biological organisation whilst contributing to the integrated understanding of thermal responses. Chapter 1 presents an experimental study investigating how exposure to elevated overwintering temperatures, designed to reflect current climate change scenarios (SSP2-4.5 and SSP5-8.5), affects the emergence phenology, weight loss and survival during overwintering in *O. bicornis* and *O. cornuta*. This chapter, thus, establishes the fitness-relevant phenotypic consequences of warming winters and provides the organismal context for subsequent molecular investigations.

Chapter 2 addresses a critical methodological prerequisite for molecular studies by presenting the first chromosome-level genome assembly and genome annotation for *O. cornuta*. This high-quality reference genome, combined with methylation-level and population genetic datasets, provides essential infrastructure for downstream analyses including transcriptome mapping, gene identification, population genetic variant calling and comparative genomic analyses. Beyond its utility as a resource, this chapter provides insights into the genome architecture, repetitive element landscape, DNA methylation patterns, and genetic diversity of *O. cornuta*.

Chapter 3 integrates transcriptomic and population genetic datasets to characterise the molecular responses of *O. bicornis* and *O. cornuta* to sustained and field-realistic elevated overwintering temperatures. Using brain tissue sampled at different time points during the experiment in Chapter 1, this chapter provides insights on the molecular changes in response to elevated temperatures across experimental time. In addition, population genomic analyses from both species provide estimates of genetic diversity as well as insights on population structure and selective pressures underlying temperature-sensitive genes.

Collectively, these three chapters provide a comprehensive view of how solitary wild bees respond to warming winters, spanning from observable phenotypes through molecular mechanisms to evolutionary potential. The findings have important implications for predicting the responses of wild bee populations to continued climate change and informing conservation strategies aimed at maintaining pollination services in a rapidly changing world. More broadly, by integrating experimental, molecular, and genomic approaches in a single system, this work also contributes to the understanding of the respective roles of phenotypic plasticity and genetic adaptation in the response to novel environmental conditions, such as increased overwintering temperatures.

Chapter One: Simulated climate warming scenarios lead to earlier emergence and increased weight loss but have no effect on overwintering survival in solitary bees

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Based on:

Journal of Insect Physiology (2025), Volume 163

2.1 Abstract

Insect pollination is critical for both wildflower stability and agricultural yields, with solitary bees being a group of pollinators of fundamental importance. However, documented declines in populations, exacerbated by environmental pressures, including climate change, pose significant threats to the provision of ecosystem services. Exposure to elevated temperatures during periods of dormancy, such as overwintering, is predicted to lead to phenological shifts, changes in condition, and impacts on survival. However, we currently lack studies that inform how such aspects are affected in future climate change scenarios. Using simulated temperature regimes informed by predictions of the Intergovernmental Panel on Climate Change, we exposed overwintering mason bees (*Osmia* species) to three field-relevant temperature profiles based on either present-day overwintering temperatures or future temperatures predicted under two major climate warming scenarios (SSP2-4.5 and SSP5-8.5) and measured how temperature exposure affected emergence timing, weight loss, and survival. We found that exposure to temperatures under intermediate and worst-case climate warming scenarios led to earlier emergence by approximately three and six weeks, respectively, with increasing divergences in timing of emergence between the sexes of *Osmia bicornis*, which may lead to intraspecific phenological mismatches. While we found no effect of temperature on overwintering survival rates, we observed increased weight loss prior to emergence but found that in contrast to other studies, it only mildly mediated shifts in emergence timing brought about by temperature exposure, suggesting that weight loss is unlikely to play a major role as a trigger of emergence timing in mason bees. Our study contributes to the growing literature highlighting the impact that temperatures under climate change models will have on the timing of key life events for essential pollinators, which may have consequences at the population and community levels.

2.2 Introduction

Insect pollination is widely regarded as an essential ecosystem service (Kremen et al., 2007), being not only crucial for wildflower stability and biodiversity (Potts et al., 2016; Kremen et al., 2007; Kleijn et al., 2015) but also contributing to agricultural yields (Kremen et al., 2004; Reilly et al., 2020), with worldwide economic benefits estimated at over €150 bn annually (Gallai et al., 2009). Despite these benefits, reported population declines of insect pollinators have raised concerns over the long-term provision of pollination services and the associated implications for both natural ecosystems and food security (Lippert et al., 2021; Klein et al., 2007). Several environmental factors have been suggested as key contributors to declines, including habitat loss and fragmentation, increased pesticide exposure, disease, invasive species, as well as climate change (Cameron et al., 2011; Potts et al., 2016; Seibold et al., 2019; Powney et al., 2019; Hallmann et al., 2017). In particular, climatic change has increasingly been highlighted as negatively impacting pollinators (Walther et al., 2002; Vanbergen and the Insect Pollinators Initiative, 2013; Scaven and Rafferty, 2013; Harvey et al., 2023) through heightened exposure to elevated frequencies of and increased variation in temperature and weather extremes, which can affect key biological processes, such as development, metabolism, and nervous system functioning and can lead to permanent shifts in range distribution (McCain and Garfinkel, 2021) and phenology (Memmott et al., 2007; Vasiliev and Greenwood, 2021; Rahimi and Jung, 2024).

For insects, the timing of major life stage events, such as development, growth, reproduction, and dormancy, is highly influenced by environmental conditions (Tauber and Tauber, 1976). With respect to dormancy, strategies have evolved to allow taxa to avoid temporary periods of harsh climatic conditions with different stages of dormancy, including initiation, maintenance, and termination, tightly regulated by the endocrine system in response to environmental cues, such as photoperiod and temperature (Denlinger et al., 2012). For example, in temperate climates, overwintering is a prevalent dormancy strategy and can occur during the adult stage, where metabolism is reduced or during developmental stages, where, additionally, development is halted (Denlinger, 2022). In most cases, overwintering represents a prolonged period of non-feeding, leaving dormant insects reliant on nutritional resources acquired before dormancy entry (Hahn and Denlinger, 2007). Thus, in such states, overwintering insects take advantage of cooler ambient temperatures as observed during winter months in temperate climates to assist in metabolic suppression for prolonged periods of time when resources are unavailable and energetic reserves are limited (Gillooly et al., 2001). Many dormant insects coordinate their

overwintering duration to correspond with the availability of resources and mates in their local environment and are reliant on environmental cues, such as increasing environmental temperatures, to initiate emergence (Denlinger, 2022). Given the dependence of insects on environmental temperatures for dormancy maintenance and termination, it is hypothesised that during periods of dormancy, insects may be particularly vulnerable to shifts in environmental conditions, in particular temperature, due to changes in climate conditions (Bale and Hayward, 2010; Freimuth et al., 2022; Fründ et al., 2013). As a consequence of ongoing climate warming, elevated overwintering temperatures may place additional pressure on already finite resources leading to changes in body condition, as evident by increased weight loss during dormancy (Hahn and Denlinger, 2007). Such changes may potentially impact the ability of organisms to maintain dormancy, leading to reductions in survival, or limiting available resources for energetically-costly post-dormancy activities, such as foraging and mating, thus, contributing to predicted losses in fitness. Increases in winter temperatures can also lead to shifts in emergence timing, which may potentially have detrimental consequences due to intraspecific and interspecific phenological decouplings (Gordo and Sanz, 2005; Schenk et al., 2018a; Freimuth et al., 2022). This is particularly true for species where both sexes go through seasonal dormancy but differ in their timing of emergence as phenological shifts induced by climate warming might differentially affect the sexes, leading to potential mismatches in gonadal development or mate availability (Zonneveld, 1996). Similarly, for insect pollinators, which are reliant on the availability of floral resources, which in turn are reliant on insect-mediated pollination for reproduction, interspecific phenological mismatches are predicted to adversely affect fitness of both interacting parties, with detrimental consequences for ecosystem stability (Kudo and Ida, 2013; Renner and Zohner, 2018).

Concerns about phenological shifts have been explored in solitary bees (Bartomeus et al., 2011), which are an important group of ecological and commercial pollinators (Hoehn et al., 2008; Woodcock et al., 2019). Despite accounting for more than 85% of documented bee species (Danforth et al., 2019), solitary bees are generally understudied in contrast to social bees (Wood et al., 2020), where dormant states, such as diapause, have either been lost or reduced (e.g., honeybees) or are limited to one sex (e.g., bumblebees) (Santos et al., 2019; Colgan et al., 2019; Zapata-Hernández et al., 2024). Furthermore, as colony-level actions of social bees, such as nest thermoregulation, are predicted to help mitigate and reduce the damaging effects of environmental challenges (Straub et al., 2015), studies on thermal tolerance in such species may not be transferable to or representative of bees as a whole. Solitary bees may have increased susceptibility to environmental challenges given that, in many

temperate species, both sexes must successfully undergo and complete overwintering before post-dormancy copulation occurs. Given that sexes differ in many aspects of their biology, including behaviour, physiology, morphology, and life-histories, differential responses to environmental stimuli may lead to phenological mismatches between the sexes. For example, many solitary bee species display protandry - a life-history trait where males emerge before females - meaning earlier emergence as a consequence of temperature exposure may result in males emerging into even less suitable environments. In addition, the haploid nature of males means they automatically express alleles they carry (Joseph and Kirkpatrick, 2004) meaning that males are potentially more prone to environmental pressures if they carry and express deleterious alleles (O'Donnell and Beshers, 2004) albeit evidence is still inconclusive (Ruiz-González and Brown, 2006; Friedli et al., 2020; McAfee et al., 2022).

A common group of solitary bees that have been increasingly used to study the effects of environmental pressures during and after overwintering are mason bees (genus: *Osmia*) (Bosch and Blas, 1994; Bosch and Kemp, 2004; Fliszkiewicz et al., 2012; Schenk et al., 2018b; Albacete et al., 2023). Mason bees are a taxon-rich group with approximately 350 described species, which are widely distributed in temperate climates (Banaszak and Romasenko, 1998) and have gained increased attention due to their potential as efficient pollinators in agriculture, particularly for fruit crops (Gruber et al., 2011; Sedivy and Dorn, 2014; Fliszkiewicz and Giejdasz, 2023). Most mason bees are univoltine and undergo seasonal diapause in the summer as larvae, before spinning a cocoon, completing metamorphosis and entering a winter dormancy as adults, with emergence, mating, nest building and offspring provisioning taking place in the following spring when conditions become more favourable (Splitt et al., 2022). During winter dormancy, overwintering adults are solely reliant on resources provided to them prior to dormancy entry (Bosch and Kemp, 2000). As most mason bees are above ground cavity nesters (Bosch et al., 2001; Praz et al., 2008), temperature has a stronger influence on overwintering duration compared to other environmental factors, such as photoperiod (Beer et al., 2019). Elevated constant or monthly stepwise increases in temperature exposure can affect the duration of dormancy, weight loss and survival in overwintering mason bees, such as *Osmia bicornis*, *Osmia cornuta*, and *Osmia lignaria* (Bosch and Blas, 1994; Bosch and Kemp, 2004; Fliszkiewicz et al., 2012; Schenk et al., 2018b; Albacete et al., 2023). However, as above ground cavity-nesting bees, *Osmia* species are exposed to diurnal and seasonal fluctuations across their overwintering period. Therefore, there is a need to improve our understanding of how these organisms respond to temperature regimes informed by field-based recordings,

which incorporate the variation in thermal exposures that are more reflective of what bees encounter during overwintering in their natural landscapes. In addition, determining their reactions to thermal patterns as expected under current climate change scenarios is also urgently warranted (Paaijmans et al., 2013). Indeed, exposure to fluctuating temperatures during the prepupal larval stage has been shown to accelerate development in mason bees prior to diapause entry compared to constant temperature exposure (Bosch and Kemp, 2000; Radmacher and Strohm, 2011), highlighting the impact temperature regime can have on key ontogenetic events. In addition to development, elevated temperature is proposed to directly affect phenological shifts in terms of emergence timing of *Osmia* species yet our understanding of emergence patterns is limited as previous studies either artificially stimulated emergence through prolonged exposure at elevated temperatures (Kemp and Bosch, 2005; Fründ et al., 2013) or used stepwise exposure regimes (Schenk et al., 2018b). However, sudden stepwise increases of temperature can lead to mass emergence events, as during late-stage periods of dormancy, such as post-diapause quiescence, bees may be more sensitive to environmental temperature changes. In general, our understanding of the temperatures at which mason bees naturally emerge, whether it occurs at a critical thermal threshold in response to the cumulative effects of prolonged temperature exposure, or is connected with changes in intrinsic aspects of body condition, is still poorly studied. In addition, information on how sexes, which naturally differ in phenology but are required to synchronise emergence to maximise individual fitness (Strobl et al., 2019), respond to current and future predicted overwintering temperature, is also still lacking. Similarly, many previous studies used bees from a single local source, which likely underestimates the phenotypic variability in thermal responses found in mason bees (Bosch and Kemp, 2000; Kemp and Bosch, 2005; Radmacher and Strohm, 2011; Fründ et al., 2013; Schenk et al., 2018b).

To address these limitations in our current understanding, we exposed mason bees to field-recorded temperature regimes, incorporating both diurnal and seasonal fluctuations, which were informed by projections for two potential climate change scenarios, which form the basis of the sixth and latest assessment report of the Intergovernmental Panel on Climate Change (Intergovernmental Panel on Climate Change, 2021). According to these projections, surface air temperatures over land in Central Europe - relative to the 1995-2014 reference period - are projected to rise by around 3°C by 2100 under the intermediate-emission scenario SSP2-4.5 and by around 6°C under the worst-case emission scenario SSP5-8.5 (Intergovernmental Panel on Climate Change, 2021; Tebaldi et al., 2021). Using an experimental approach, we investigated how temperatures based on such models affect dormancy

emergence timing, condition (measured by relative weight loss) and survival in both sexes of two common mason bees, *O. bicornis* and *O. cornuta*, sourced from four geographically distinct locations per species. Given the accelerative effect that temperature exposure is predicted to have on overwintering bees in terms of emergence and conditional change, we predict that increased temperature exposure will correspond with earlier emergence, elevated weight loss, and lower survival rates (Fig. 2.1). Additionally, to understand the relationship between temperature exposure, weight loss, and emergence timing, we hypothesised that, if emergence is dependent on the depletion rate of nutritional reserves, shifts in emergence timing caused by temperature exposure would be strongly mediated by increased weight loss prior to emergence.

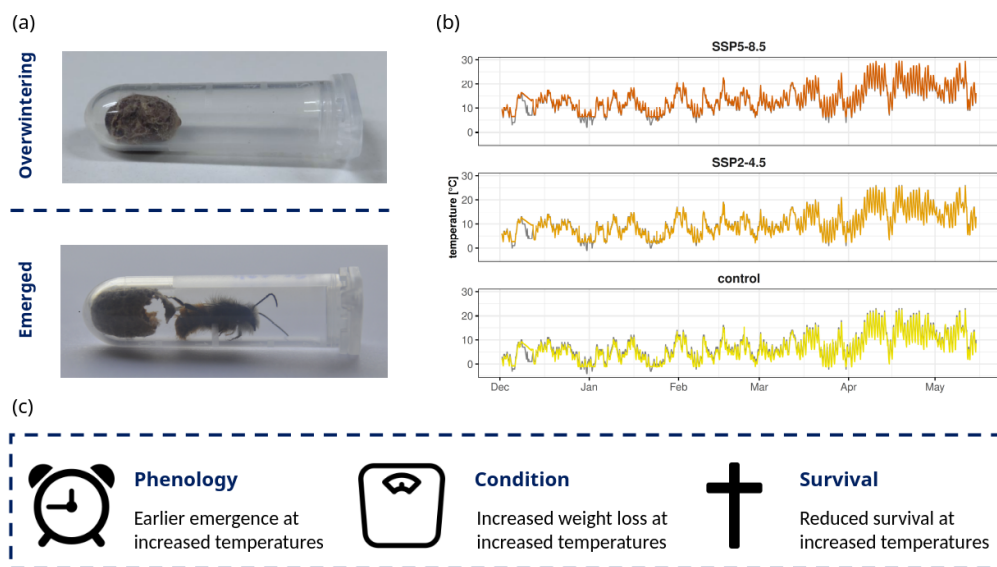


Figure 2.1: Experimental set-up and simulated temperature profiles based on current and predicted climate change models. (a) Images of individuals of the European orchard bee (*Osmia cornuta*) overwintering in a cocoon (upper image) or freshly emerged after overwintering (lower image). (b) Three line graphs depicting an overlay of the three temperature profiles simulated in our experiment (grey) that mason bees were exposed to and their achieved implementation in the form of logged temperatures for each temperature treatment (yellow = control, orange = SSP2-4.5, red = SSP5-8.5). The lower plot facet includes the control group temperature profile, representing surface air temperatures measured 2m above the ground at the weather station of the German National Meteorological Service in Mainz-Lerchenberg, Germany in the winter of 2019/2020. The upper two plots include the same profile, with each time point increased by a fixed 3°C and 6°C relative to the control group profile, representing the climate change scenarios SSP2-4.5 (“intermediate scenario”) and SSP5-8.5 (“worst-case scenario”), respectively, that were laid out by the Intergovernmental Panel on Climate Change in their sixth assessment report. (c) Conceptual portrayal of the three main predictions that we made in relation to how bees respond to increased temperature scenarios.

2.3 Results

Early emergence under elevated temperatures

To determine the effects of temperature on shifts in emergence timing, we implemented three field-based overwintering temperature regimes that included seasonal and diurnal variation, to each of which we exposed females and males of both *Osmia bicornis* and *Osmia cornuta*. We observed significantly earlier emergence under both elevated-temperature scenarios, a pattern which was consistent across all combinations of species and sex (Linear Mixed Effect Model (LMM); Tukey-adjusted $P < 0.05$; Figure 2.2). For *O. bicornis*, mean difference (MD) in date of emergence was 39.48 days earlier in the worst-case emission group and 17.61 days earlier in the intermediate emission group, while for *O. cornuta* mean differences were 40.64 and 19.62 days earlier, respectively. An interaction effect of temperature, species and sex was significant (LMM; Type-III Wald X^2 test; Bonferroni-Holm-adjusted $P < 1e-9$), prompting us to explore species and sex differences in more detail. As expected, due to known protandry in these systems, in the control group and in both elevated temperature groups, males, on average, emerged more than one week earlier than females (LMM; Tukey-Kramer test: Tukey-adjusted $P < 0.05$). However, in *O. bicornis* the difference in emergence timing between the sexes was increased by an additional 5.91 and 8.18 days in both treatment groups, respectively, compared to the control group. In contrast, for *O. cornuta*, we did not observe this effect as sex differences were inconsistent across treatment groups with respect to timing of emergence (Table 2.1, Figure 2.2). In relation to species differences, *O. cornuta* individuals, on average, emerged 3.5 weeks before *O. bicornis*. In addition, we observed that for *O. bicornis*, individuals emerged on days around the same mean temperature (Table 2.1) with no significant differences between treatments (LMM; Tukey-Kramer test: Tukey-adjusted $P > 0.05$), while for *O. cornuta* mean temperature at day of emergence varied significantly with temperature treatment (LMM; Tukey-Kramer test: Tukey-adjusted $P < 0.05$).

Increased weight loss with elevated temperature exposure

As weight loss due to a reduction in nutritional reserves has been posited as a factor associated with earlier emergence, we also investigated the effect of temperature exposure on weight loss. We observed a significant increase in weight loss for both elevated temperature exposure groups across all species and sex combinations (LMM; Tukey-Kramer test: Tukey-adjusted $P < 0.05$, Figure 2.3) with the strongest effect observed in the worst-case emission group where mean weight loss at emergence was 1.43 times higher compared to the control group (Table 2.1) in *O. bicornis*

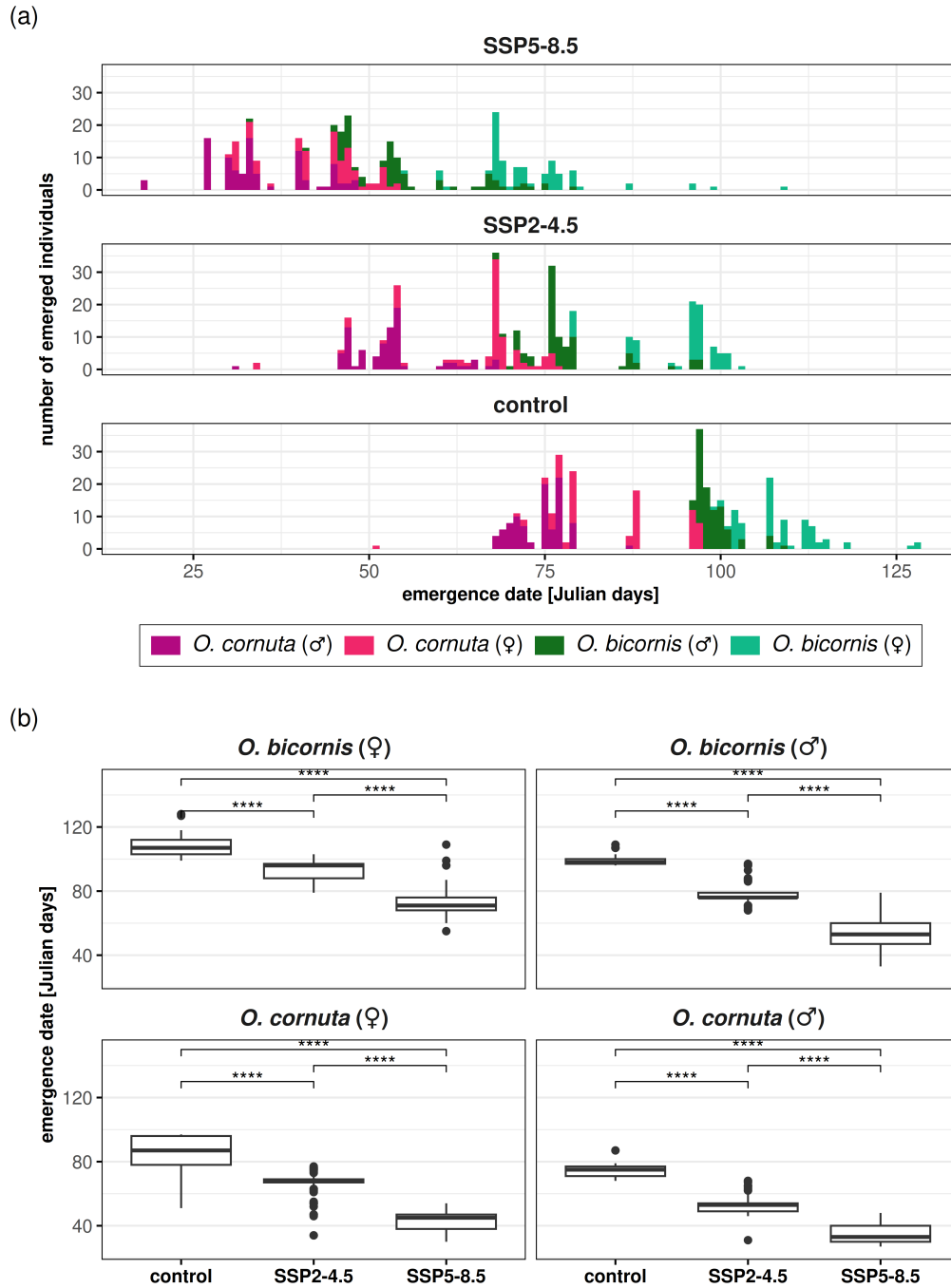


Figure 2.2: Earlier emergence with increasing overwintering temperature exposure.

(a) Stacked bar plots showing for all species and sexes the number of daily emerged individuals (y-axis) in relation to the time of emergence (x-axis), as measured in ordinal days since 1st of January. The lower plot facet depicts the emergence patterns observed with the control group temperature profile, while the upper two facets show emergence patterns under climate change scenarios SSP2-4.5 and SSP5-8.5, respectively. Each species and sex is designated by an individual colour. (b) Box plots representing distributions of emergence times (y-axis) for each species and sex (facets) within each temperature treatment (x-axis). For each species and sex, pairwise differences between temperature treatments are shown with level of significance indicated by number of asterisks (LMM, pairwise contrasts of estimated marginal means using Tukey-Kramer test, adjusted for multiple testing: ns = not significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$).

Table 2.1: Experimental group contrasts in emergence timing, weight loss, and emergence temperature in response to increased overwintering temperatures. For each species and each experimental group, the sample size (n), mean and standard error of different response variables are shown.

Species	Variable [unit]	Control			SSP2-4.5			SSP5-8.5		
		n	Mean	Std. error	n	Mean	Std. error	n	Mean	Std. error
<i>bicornis</i>	Emergence timing [ordinal days]	166	103	0.51	159	85.7	0.82	156	63.9	1
<i>bicornis</i>	Sex differences in emergence [days]	83	9.68	0.74	79	15.57	1.07	78	17.86	1.43
<i>bicornis</i>	Mean temperature at emergence [°C]	166	16.4	0.1	159	16.3	0.2	156	16	0.2
<i>bicornis</i>	Mean weight loss at emergence [%]	166	9.43	0.18	159	11.0	0.24	156	13.5	0.26
<i>cornuta</i>	Emergence timing [ordinal days]	171	79	0.65	169	59.3	0.76	173	38.3	0.63
<i>cornuta</i>	Sex differences in emergence [days]	85	10.58	1.09	84	12.81	1.17	86	8.3	1.09
<i>cornuta</i>	Mean temperature at emergence [°C]	171	11	0.2	169	11.7	0.2	173	14.7	0.2
<i>cornuta</i>	Mean weight loss at emergence [%]	171	6.13	0.18	169	7.26	0.21	173	9.83	0.21

and 1.6 times higher in *O. cornuta*. In comparison, increased weight loss was also observed in the intermediate emission group but at a more moderate level compared to the control group in both *O. bicornis* and *O. cornuta* (1.17 and 1.18 times higher, respectively). In addition to comparing total relative weight loss, during the experiment, we also tracked changes in weight for a subset of bees (75.72%, n = 873 individuals) each week to determine when changes in weight loss occur. We found increasing differences in weight loss across time when comparing elevated temperature groups to the control group. These differences became significant after two weeks of weight tracking in the worst-case emission group. In the intermediate emission group, significant differences were observed after ten weeks (Generalised

Additive Mixed-Effects Model; overlap in 95% CIs). This highlights that not only did exposure to elevated temperatures result in elevated weight loss but the observed timing of the effect was considerably earlier in the worst-case scenario group.

Initial body weight and size do not predict emergence timing and have only a weak effect on weight loss

It has also been suggested in the literature that larger individuals emerge significantly earlier than smaller individuals in both *O. cornuta* and *O. bicornis* as earlier emergence might be beneficial in terms of avoiding competition over resources. We, thus, investigated the relationship between body size, initial body weight before the start of the experiment and weight loss, as well as emergence timing, to understand if differences in response to temperature exposure are predicted by body weight or size. We found that initial body weight and body size were significantly associated with weight loss at emergence but not with timing of emergence (LMM; Type-III Wald X^2 tests: Bonferroni-Holm-adjusted $P < 0.05$). Specifically, we found that in both species heavier, larger individuals tended to emerge with decreased relative weight loss compared to lighter, smaller individuals in response to increased temperatures (Supplemental Figure S1). Overall, however, the contribution of initial body weight in explaining variation in weight loss at emergence measured in terms of change in coefficient of determination (ΔR^2) was extremely low as removing body weight from the model reduced the variation explained by only 0.1% (full model: $R^2 = 94.72\%$; reduced model: $R^2 = 94.62\%$). In line with that, the reduced model had a lower AIC score. The same results were obtained when exchanging initial body weight for body size. Together, this indicates that the initial body condition of individuals does not contribute to emergence timing but contributes to the variation in weight loss, however the effect of its contribution is likely small or negligible.

Effect of temperature on emergence timing is only partially mediated by weight loss

As the role of weight loss has been suggested as a potential contributor to emergence in overwintering insects, we examined if, and by how much, early emergence in response to temperature exposure was mediated by weight loss. We found a significant mediation effect for weight loss at emergence (Moderated Mediation Analysis, Average Mediated Effect = 9.90, Average Direct Effect = 51.33, $P < 10^{-16}$), however, the proportion of the effect of temperature exposure on emergence timing mediated by weight loss amounted to only 23.9%, indicating that the effect of temperature on emergence timing is mediated by weight loss only to a limited extent.

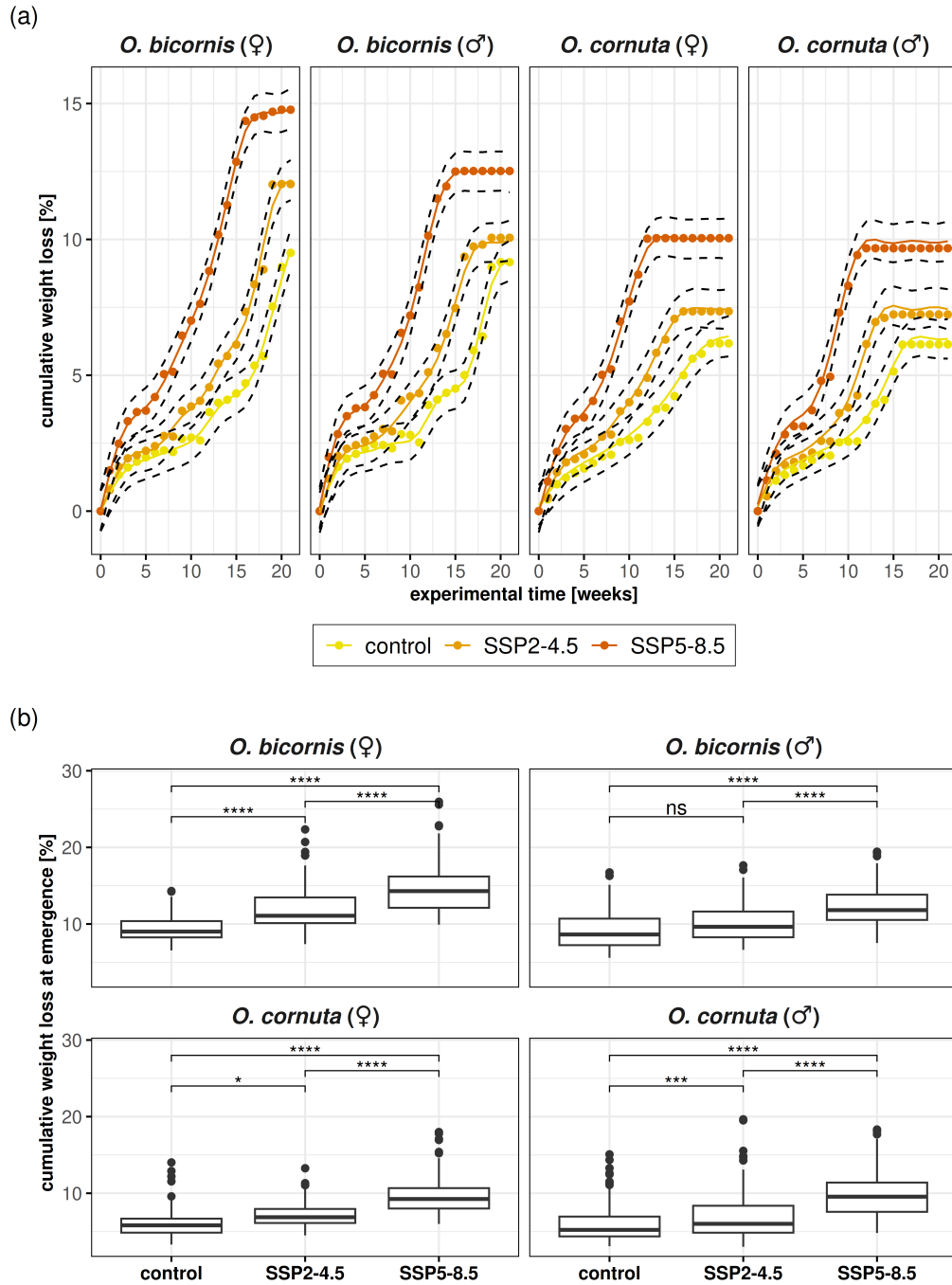


Figure 2.3: Increased weight loss in response to elevated winter temperatures. (a) Line plots showing the accumulation of weight loss (x-axis) over experimental time in weeks (y-axis) for each species and sex (facets) and temperature treatments (yellow = current, orange = SSP2-4.5, red = SSP5-8.5). Coloured dots represent weekly mean weight loss, averaged across individuals within the same group, while coloured lines represent approximations of the raw data fitted using generalised additive mixed-effects models. Dotted black lines mark the upper and lower bounds of the 95% confidence intervals. (b) Box plots showing distributions of cumulative weight loss at emergence (y-axis) for all treatments (x-axis) and species and sexes (facets). For each species and sex, pairwise differences between treatments are shown with level of significance indicated by asterisks (LMM, pairwise contrasts of estimated marginal means using Tukey-Kramer test, multiple testing adjusted: ns = not significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$).

Survival rates during overwintering were not generally affected by elevated temperatures

While earlier emergence and weight loss may have indirect effects on fitness, we also recorded survival rates for all bees in our experiment. Using all bees, we found no significant difference in survival rates across temperature treatments (Generalised Linear Mixed Effect Model; Tukey-Kramer test: Tukey-adjusted $P > 0.05$) highlighting the ability of both species to at least survive and emerge from prolonged elevated thermal exposure. Despite this, irrespective of temperature exposure, we found that *O. bicornis* bees generally had a significantly lower survival rate (87.3%) than *O. cornuta* (91.3%, Fisher's Exact Test, $P = 0.033$, Figure 2.4). Furthermore, within *O. bicornis*, males (84.3%) had a significantly lower survival rate compared to females (90.8%, Fisher's Exact Test, $P = 0.028$). In *O. cornuta*, we also observed a lower survival rate in males but this result was not statistically significant (males: 89.2%; females: 93.8%; Fisher's Exact Test, $P = 0.071$).

2.4 Discussion

In the face of ongoing climatic change, understanding the responses of organisms to increasing elevated temperatures represents one of the most pressing and timely challenges. While advancements are being made in understanding thermal tolerance, we urgently need to assess the capacities of ecologically essential species, such as wild bees, to cope with current field-relevant temperatures and temperatures predicted under future climate scenarios. We, thus, exposed overwintering mason bees to temperature regimes informed by recent historical records and models developed by the Intergovernmental Panel on Climate Change to determine how elevated temperature exposure during dormancy affects timing of emergence, survival, as well as weight loss. We found that exposure to temperatures predicted under intermediate and worst-case scenarios associated with climate change had strong effects on phenology with both species emerging earlier with increasing elevated temperature exposure. Elevated temperature also led to increased divergence in emergence timing between male and female *O. bicornis*, indicating potential intraspecific phenological mismatches. In addition, bees also lost increasingly more weight at higher temperatures yet despite these losses, both weight loss and body size, were weak predictors of premature emergence. While elevated weight losses may have consequences for post-dormancy survival and fitness, we found no decrease in survival rates across temperature regimes highlighting the capacity of mason bees to at least survive overwintering at prolonged temperature exposure.

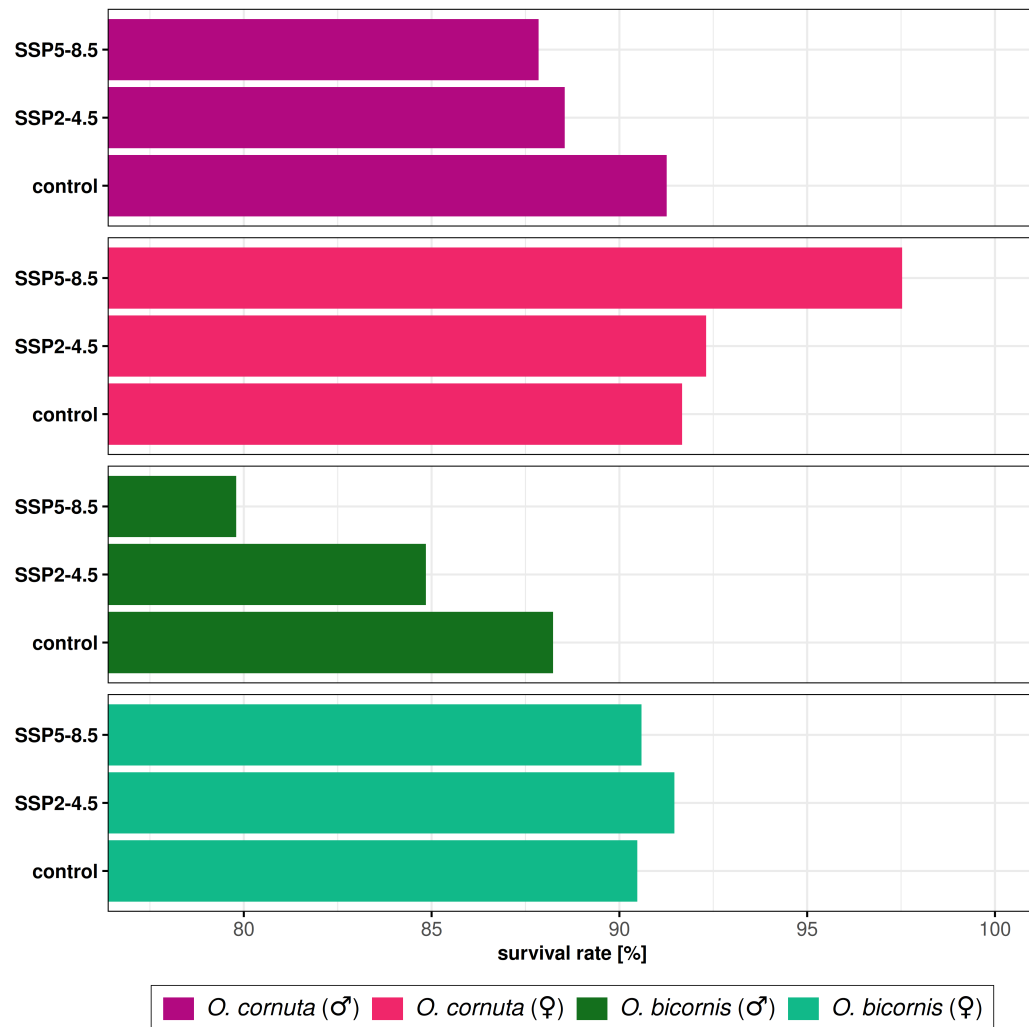


Figure 2.4: Differences in survival in response to elevated overwintering temperatures. Bar plot showing survival rates (x-axis) across all temperature treatments (y-axis) for each species and sex. Survival rates across temperature treatment groups were not significantly affected by elevated temperatures (Generalised Linear Mixed Effect Model: Bonferroni-Holm adjusted $P = 0.53$), however, survival rates in *O. bicornis* (87.3%) were significantly lower than in *O. cornuta* (91.3%, Fisher's Exact Test, $P = 0.033$) and were also significantly lower in *O. bicornis* males (84.3%) compared to *O. bicornis* females (90.8%, Fisher's Exact Test, $P = 0.028$).

For many overwintering species, the entry into and maintenance of dormant states allows for avoiding harsh environmental conditions where unfavourable climate may potentially limit mating opportunities (Denlinger, 2002) and food availability (Wilsterman et al., 2021; Denlinger, 2002). For overwintering insects, emergence timing is generally influenced by environmental factors, such as photoperiod (Adkisson, 1964) and temperature (Tauber et al., 1986), meaning that shifts in seasonality as predicted under climate change may lead to shifts in overwintering termination with organisms emerging into potentially more inhospitable environment conditions that they are ill-prepared for (Van Dyck et al., 2015). Here, we found that the timing of emergence for both *O. cornuta* and *O. bicornis* was affected by increased temperature exposure. In particular, the strength of the effect of temperature was proportional to the temperature regime with bees exposed to temperatures predicted under scenarios SSP2-4.5 and SSP5-8.5 emerging nearly three and six weeks earlier, respectively. Previous studies on mason bees also found that elevated temperature exposure during overwintering led to earlier emergence from winter dormancy yet such studies either used constant (Kemp and Bosch, 2005) or step-wise monthly temperature increases (Schenk et al., 2018b), which lack the thermal variation that overwintering bees encounter in their natural environment, and which is predicted to increase the sensitivity of insects to climate change (Paaijmans et al., 2013). Similarly, a common procedure used in research on solitary bees involves the artificial stimulation of emergence through incubation of overwintering bees at elevated temperatures (e.g., 17-26°C ((Bosch and Blas, 1994); Bosch and Kemp 2003, 2004; (Kemp and Bosch, 2005); (Fründ et al., 2013); (Albacete et al., 2023))), which may possibly contribute to or exacerbate detrimental effects on post-dormancy performance. While such stimulation temperatures allow for regulation of the timing of emergence for the timed use of mason bees in agricultural practices (e.g., pollination in orchards (Bosch and Blas, 1994)), it limits our understanding of phenotypic variation in terms of emergence, which is required when determining potential ecological mismatches at the intra- and interspecific levels, due to climate-mediated phenological shifts. Indeed, in our experiment, we found that bees routinely emerged at temperatures on average lower than artificial termination temperatures routinely used in the literature, highlighting that lower temperatures may be more appropriate as a stimulant for initiation of post-dormancy activities although further research is required to determine consequences that might exist.

While earlier emergence may be compensated for by the polylectic nature of mason bees (Bartomeus et al., 2011), the biodiversity-associated buffering capacity of an ecosystem (Bartomeus et al., 2013), complementary phenological shifts in floral resources (Rafferty and Ives, 2011), or coordinated timing of mate emergence

(Zonneveld, 1996), the predicted lower diversity of plants under climate change scenarios (Harrison, 2020) may still dampen mitigative actions. For example, earlier emerging bees may compensate by choosing a less optimal or new floral resource, which may have implications for long-term survival and fitness. Indeed, a previous semifield-based study found that even a three day phenological mismatch between mason bee emergence and floral blooming resulted in fitness losses (Schenk et al., 2018a) underlining the negative connotations earlier emergence may have on mason bees. The concerns over potential interspecific mismatches are further highlighted by observations of earlier flowering in response to climate change-induced temperature increases (Menzel, 2003; Gordo and Sanz, 2010; Amano et al., 2010; Renner et al., 2021). Predictions from German-based studies (Renner et al., 2021), which are likely more relevant to our findings given our use of bees originating from sites in Germany and Austria, translate to earlier flowering by 9.6-12.6 days and 19.2-25.2 days under the SSP2-4.5 and SSP5-8.5 scenarios, respectively. Together with our findings of earlier emergence by a mean 19 days under the SSP2-4.5 scenario and a mean 41 days under the SSP5-8.5 scenario, this suggests that mismatches may be potentially greater than previously assumed (Bartomeus et al., 2011; Thackeray et al., 2016). Although a formal examination is required and the temperature profiles that we used relies on a specific year and site, the resulting mismatches of about one and two weeks under the intermediate and worst-case emission scenarios, respectively, would exceed mismatches that were previously found to be detrimental in terms of fitness for mason bees (Schenk et al., 2018a), adding to concerns about phenological mismatches in response to climate change. In addition to differences in emergence timing across different temperature exposure regimes, within *O. bicornis*, we found evidence of the sexes responding differently to elevated temperatures. More specifically, the difference in emergence timing between males and females increased significantly from 10 days in the control group to 16 and 18 days in the intermediate and worst-case emission scenarios, respectively. Previous reports for sex differences in emergence timing vary between four and 11 days for mason bees (Bosch and Blas, 1994; Raw, 2009) but with considerable variation depending on population or ecotype examined (Tasei and Picart, 1973). The monandrous nature of mason bees where females are generally single-mated (Paxton, 2005), places additional pressure on males to emerge earlier, forage, and locate a mate as in such systems, mating with multiple females is a strategy for increasing fitness (Seidelmann, 2014). Mason bees also have complex behavioural repertoires during copulation meaning males must invest time and energy in differentiating receptive and non-receptive females (Strobl et al., 2019), meaning earlier male emergence could be advantageous. However, as emergence timing is predicted to be tightly linked between the sexes, intraspecific divergences, such as those observed here,

may lead to predicted elevated sexual antagonism, which may result in fitness costs (Ekrem and Kokko, 2023). Similarly, male emergence has been more strongly linked with flowering patterns (Bosch and Blas, 1994) meaning that in situations where phenological mismatches occur between plants and pollinators, males may be disproportionately affected. Our findings indicate that under worst-case scenarios, intraspecific divergence in phenology may occur, which may have consequences at the population size. While such differences were not identified in *O. cornuta*, the species-specific responses may be a consequence of *O. bicornis* naturally emerging later from overwintering, and, therefore, being subjected to higher temperature exposure for a more prolonged period. While later emergence is also found naturally in the wild, it may indicate that later-emerging species may face more problems due to higher temperature exposure during overwintering.

Exposure to elevated temperature before and during winter dormancy has been associated with fitness losses, including increased mortality (Radmacher and Strohm, 2011), reduced longevity post-emergence, and increased pesticide sensitivity (Albacete et al., 2023). Therefore, under the predicted climate change scenarios, lower survival rates would be predicted with increasing temperature exposure. Contrary to this prediction, we found no evidence that increased temperature exposure leads to higher mortality during overwintering, a finding in line with a previous study that exposed mason bees to different constant overwintering temperatures and found that survival was unaffected (Fründ et al., 2013). However, post-dormancy longevity was reduced in *O. cornuta* exposed to elevated overwintering temperatures that overlap with our intermediate scenario (Albacete et al., 2023), highlighting that non-lethal observations reported in our study may still have long-term consequences post-emergence in terms of longevity and associated fitness. Thus, while our finding indicates the capacity of overwintering mason bees to survive prolonged exposure at elevated temperatures, further research is required to determine if such exposure results in long-term fitness consequences after emergence.

Our study highlights the strong influence elevated temperature exposure can have on timing of emergence yet the exact triggers leading to dormancy emergence are likely to be a combination of extrinsic and intrinsic factors. Abiotic factors, such as temperature and photoperiod, affect the timing of winter dormancy emergence, yet, given the cavity-nesting nature of overwintering mason bees, the influence of photoperiod on emergence is considered to be less strong compared to temperature (Beer et al., 2019). In comparison, how intrinsic factors, such as changes in body weight, contribute to timing of emergence are less well understood. Here, we found exposure to elevated temperatures predicted under climate change scenarios SSP2-4.5 and SSP5-8.5 resulted in considerable increases in weight loss, with measurable losses

detectable after just two weeks of exposure in bees designated to the worst-case scenario group. Although our measurements are based on whole-bodied individuals, including their cocoons, previous studies on mason bees have demonstrated that exposure to elevated temperatures leads to loss of fat reserves (Fliszkiewicz et al., 2012), while during overwintering, essential compounds, such as carbohydrates and lipids decrease (Dmochowska et al., 2013; Wasielewski et al., 2013) indicating that increased metabolic activity in response to elevated temperature may contribute to the weight loss described in our present study. Given the importance of such reserves to an individual's post-dormancy survival and fitness, it is highly probable that elevated weight loss is negatively associated with fitness as has been described in previous studies that employed constant temperature exposures (Bosch and Kemp, 2004). It is important to highlight, however, that we have not looked at the effect of cocoon degradation on weight loss in this study. A previous study (Bosch and Vicens, 2002) has found cocoon weight to amount to about 15% of total weight (of adults prior to overwintering including their cocoons) in *Osmia cornuta* suggesting that cocoon weight loss might have contributed to the weight loss of samples that we observed in this experiment.

As reductions in energetic resources may contribute to premature emergence (Short and Hahn, 2023), we examined the relationship between temperature exposure, weight loss, and early emergence using a mediation model and found that, contrary to what we had hypothesised, weight loss was only a weak mediator of the effect of temperature exposure on emergence timing. Although this analysis does not replace the need for experimental studies to definitively assess this relationship, our results suggest that weight loss is unlikely to be the main physiological process driving emergence. While other environmental factors, such as changes in photoperiod, are associated with emergence in other insects from overwintering, the influence is predicted to be less in mason bees given they overwinter in cocoons within cavities (Wasielewski et al., 2013), and is instead more influential on post-emergence activities (Beer et al., 2019). In contrast, ontogenetic factors and pre-wintering temperatures (Bosch et al., 2010), likely contribute more to phenotypic variation observed within our analysis. Similarly, differences in phenotypic variation may be explained by genetic differences within and across source sites as has been found in other systems (Pruisscher et al., 2018) and represents a future avenue of research to understand the genetic bases of phenological variation across natural populations.

Aside from environmental factors and weight loss, it has been suggested that body condition, including initial body weight and body size, is an important predictor of variation in emergence timing and weight loss across individuals with heavier individuals suggested to emerge before lighter individuals in response to temper-

ature exposure (Schenk et al., 2018b). Earlier emergence in larger individuals has been hypothesised as a strategy to avoid competition over resources with heavier individuals possessing greater energy reserves needed to emerge earlier but also survive in potentially colder environments. We formally tested this hypothesis, finding that initial body weight was both associated with weight loss, yet contrary to what has been reported prior, we found a pattern suggestive of lighter individuals emerging with lower relative weight loss than heavier individuals. Overall, the effect size of body condition as a predictor of emergence timing was very weak. In line with this, an earlier study that examined the effect of temperature exposure on overwintering *O. cornuta* also included body weight as a predictor variable in their analysis (Bosch and Kemp, 2004) but did not find a significant effect on emergence timing and significant effects on survival and longevity only in males, suggesting that body condition is unlikely to play a major role in explaining emergence timing differences.

2.5 Conclusions

With increasing environmental temperatures predicted under anthropogenic-mediated climate change, understanding the responses of organisms to changing environmental conditions is urgently required. Here, using field-informed temperature regimes, we demonstrate how elevated temperature exposure, reflective of intermediate and worst-case climate change scenarios, significantly influences the phenology and condition of two ecologically and commercially important solitary bees. Our study also underscores the complexity of predicted climatic change-associated impacts on solitary bee species, highlighting intra- and interspecific variation in terms of thermal responses within species that share similar nesting ecology. Information on solitary bee thermal responses, in general, will greatly benefit from examination of more species that differ in terms of nesting ecology, developmental stage of dormancy, seasonal timing of dormancy, as well as duration of dormancy. In addition, further insight into the responses of contemporary populations will be gained through the application of field or semi-field experiments that can help to provide more ecologically relevant information on thermal responses (CaraDonna et al., 2018). Similarly, further understanding changes and responses at the physiological and molecular levels will provide improved insights into the phenotypic plasticity underlying thermal responses. Collectively, our findings contribute to the growing literature highlighting the impact that elevated temperature will have on the timing of key life stage events, which may have implications at the individual level through

emergence timing and increased weight loss, at the population level through intraspecific phenological mismatches between the coordinated emergence timing of sexes (Tauber and Tauber, 1978) and potentially consequences at the community level through interspecific mismatches between pollinators and floral resources (Visser and Both, 2005). Through understanding such processes, mitigation strategies can be developed and implemented to ensure the conservation of essential pollinator species and the maintenance of essential ecosystem services.

2.6 Materials and Methods

Sampling and handling of individuals

We ordered 1153 mason bee cocoons, composed of cocoons of *Osmia bicornis* (n = 573) from four different commercial suppliers in Germany and Austria (Germany: Konstanz [n = 143], Stahlberg [n = 144], Greifswald [n = 146]; Austria: Schärding [n = 140]) and cocoons of *Osmia cornuta* (n = 570) from three different commercial suppliers (Germany: Konstanz [n = 147], Stahlberg [n = 145]; Austria: Schärding [n = 147]), complemented by cocoons of *O. cornuta* extracted from nesting boxes in Rottleberode, Germany (n = 141; approximate site coordinates are provided in Supplemental Table ??). The inclusion of bees from multiple sources allowed us to incorporate increased genetic and phenotypic variation. All cocoons were received in October 2022. Upon reception, the cocoons were placed outside in a water-proof, outdoor cabin until the start of the experiment on December 1st, 2022. Before starting the experiment, cocoons were washed to remove nesting debris under running tap water and dried overnight in the laboratory at 25°C to avoid weight loss bias from debris sticking to the cocoon surface. In line with their natural life cycle, all mason bee cocoons were presumed to contain overwintering adult individuals.

Experimental setup

To simulate overwintering temperatures including predicted temperature increases under climate change, we used three programmable climate cabinets of the same model (RUMED Type 3201, Germany). Each climate cabinet was assigned one of three temperature profiles. The temperature profiles consisted of a baseline profile representing current, field-relevant winter temperatures (as measured by the German National Meteorological Service (DWD) weather station in Mainz-Lerchenberg, Germany between December 2019 and June 2020) and two experimental treatment profiles based upon projections of surface air temperature increases over land in

Central Europe by the year 2100 (Intergovernmental Panel on Climate Change, 2021; Tebaldi et al., 2021) under two Shared Socioeconomic Pathway climate warming scenarios (intermediate emissions: SSP2-4.5, worst-case emissions: SSP5-8.5), which we approximated by implementing a fixed 3°C and 6°C temperature increase relative to the baseline temperature profile, respectively. Similarly to other studies (Ostap-Chec et al., 2021; Ruddle et al., 2018; Nicholls et al., 2017; Szentgyörgyi et al., 2017; Coudrain et al., 2016; Peters et al., 2016; Kehrberger and Holzschuh, 2019), we used reference temperatures (and temperature projections), measured at 2m above ground, as both *O. bicornis* and *O. cornuta* nest above-ground in a variety of pre-existing nesting substrates (Danforth et al., 2019). We programmed the climate cabinets to adjust the temperature every 3 hours according to the underlying weather data at a resolution of 1°C. We independently measured temperature and humidity in all three climate cabinets using individual data loggers (TFA DOSTMANN LOG32TH, Germany) throughout the duration of the experiment. An overview of the simulated temperatures used in each treatment group, including the temperatures recorded by the data loggers, across the duration of the exposure experiment, is depicted in Figure 2.1 (recorded data are provided in Supplemental Table ??). While photoperiod is an important environmental cue influencing diapause initiation and post-overwintering activities, we did not implement a daily illumination cycle, as prior research has demonstrated emergence to be independent of photoperiod in *Osmia bicornis* (Beer et al., 2019).

Before the temperature exposure experiment, we weighed a 1.5ml microtube for each sample, which contained ventilation holes in the lid. The weight of these tubes was assumed constant throughout the experiment. Subsequently, a cocoon was placed inside each tube and measurements were repeated. After initial measurements were completed, samples (tubes containing cocoons) were assigned to the three climate cabinets using block randomisation, ensuring species, sexes and source sites were uniformly distributed across the three climate cabinets. Within each climate cabinet, samples were assigned onto 80-well microtube racks, again using block randomisation, ensuring a uniform distribution of species, sex, and source site sources across racks, thus preventing a positional bias inside climate cabinets (further details on the randomised assignment of individuals is provided in the Supplemental Methods S1). In addition, the positions of the microtube racks inside the climate cabinets were rotated weekly during weight measurements to further avoid potential positional biases.

Weight measurements and emergence tracking

The experiment was started on December 1st with the initial measurements of sample weights, which served as the first time point of the experiment. As noted above, samples were not weighed directly, but instead cocoons and microtubes were weighed together with tube weight subtracted later during data analysis. For a subset of samples ($n = 873$, total = 1153), we measured weight weekly on the same day each week to track when divergences in weight occurred between treatment groups while for the rest of the samples, weight was measured only at the start and end of the experiment (after hatching).

Upon the first hatching, which was recorded in January 2023, all cocoons were checked once per day every day until the completion of the experiment. Upon hatching, which was observable by the complete emergence of the adult from their cocoon, we recorded the date of hatching and weighed each bee on their respective emergence day (each emerged bee was measured inside their respective microtube, which also included the remnants of their cocoon). Using both the starting weight and the weight at emergence, we calculated the total weight loss for each bee across the experiment. In addition to weight, we also recorded and confirmed sex and species based on morphological characteristics.

On May 15th (23 weeks into the experiment), as hatching had drastically decreased over the preceding weeks and with over 85% of bees emerged, the experiment was stopped and temperatures were raised to 26°C across all three climate cabinets for 24 hours to stimulate the emergence of remaining, alive individuals (Bosch and Blas, 1994). Subsequently, all unhatched cocoons were opened via excision using microscissors. For each excised individual, we recorded species, sex, developmental stage (pupa/prepupa/imago), survival status at retrieval (alive/dead) and if applicable, parasitoid presence and genus. As expected from univoltine species, none of the unhatched mason bees were alive upon excision.

Statistical analysis

Once all data were collected, we pre-screened the data and removed outliers if emergence weight was recorded to be higher than initial weight, which resulted in the removal of seven samples, or if a sample had a sudden increase in weekly measured weight, which resulted in the removal of another six samples. In both cases, manual errors introduced during data collection were assumed. One sample was removed because the cocoon was empty upon excision and 19 samples were removed because they contained one of two parasitoids (*Anthrax anthrax* or *Monodontomerus* sp.) that had removed the bees from the cocoons completely. No other parasites were observed. Thus, in total, 33 samples were removed during pre-screening (2.86%, total = 1153), leaving a dataset of 1120 samples of which 273 were weighed only

at the start and end of the experiment and 847 were additionally weighed weekly throughout the experiment. Group-level sample sizes can be found in Table 2.1.

In our analysis to investigate the effect of elevated temperature exposure on weight loss and emergence, we further reduced the pre-screened dataset by including only samples that represented individuals that had hatched alive before the end of the experiment (87.20%, $n = 997$, total = 1120). We fitted two linear mixed-effects models (R package lme4; (Bates et al., 2014)) to investigate the effect of temperature on the continuous variables emergence timing - which we defined as number of ordinal days since 1st of January at the time of hatching of a given individual - and weight loss - defined as percent of body mass lost relative to starting body mass - respectively. In the model that included total weight loss as the response variable, we defined weight loss as the difference in percentage of body weight at emergence relative to body weight at the start of the experiment to account for natural differences in body weight between individuals, sexes, and species.

Originally, in both models, temperature treatment, species and sex and either initial body weight or body length were included as four-way interaction variables and source site was included as a random effect. However, model selection based on Akaike Information Criterion (AIC), as well as evaluation of statistical significance and effect size, led to the removal of both initial body weight and body length from these models. To test, when differences in weight loss became noticeable, we used the subset of 847 weekly-weighed samples and employed a Generalised Additive Mixed-Effects Model (R package mgcv; (Wood, 2010)), which allowed us to fit the non-linear response of weight loss over time, with weight loss as a dependent variable, temperature treatment, species, and sex as independent three-way interaction variables and source site as a random effect.

To test differences in survival rate between temperature treatment groups, we used the full pre-screened dataset including both alive and dead individuals and employed a generalised linear mixed-effects model (R package lme4; (Bates et al., 2014)) to fit a binomial distribution with survival as the response variable, and temperature, species, and sex included as interaction variables and source site as a random effect. Here, we did not include initial body weight as an additional independent variable to test relationship with survival, as it was unknown to us when dead individuals had died, which may have occurred before the start of the experiment - and thus before recording of initial body weight. This means that we were unable to disentangle the effect of initial body weight on survival from death before the experiment on weight loss due to decomposition. Therefore, we avoided testing this relationship.

We also tested whether weight loss as a mediator variable explained the effect of increased temperature treatments on emergence timing. To this end, we implemented a moderated mediation analysis (R package *mediate*; (Yimer et al., 2023)) that compares a full model that includes the proposed mediator variable against a reduced model that lacks the mediator variable (Hayes, 2017). The full model contained temperature treatment as an independent variable, emergence timing as a dependent variable and weight loss as a mediator. Analogously to the linear models, all experimental group combinations of species and sex were additionally included as interaction effects, which in this context are also referred to as moderator variables (Preacher et al., 2007).

For all models, differences within and across experimental groups were assessed using Tukey-Kramer post-hoc tests on estimated marginal means (R package *emmeans*; (Searle et al., 1980)) and corrected for multiple testing using the Tukey adjustment method and the statistical significance of interaction effects was evaluated using Type-III Wald X^2 tests (R package *car*; (Fox and Weisberg, 2018)) and corrected for multiple testing using the Bonferroni-Holm's adjustment method. For all models, model diagnostics were performed using a simulation-based approach (R package *DHARMA*; (Hartig, 2024)). In doing so, mild heteroskedasticity in the form of decreased variation towards the upper end of the distribution in the model that explained relative weight loss at emergence, was detected.

For verification, validation, and reuse, all steps of the statistical analysis were annotated and are provided in Supplemental Script 2 and raw data for the statistical analysis are provided in Supplemental Table ??.

Data Availability Statement

All data and scripts required for reanalysis are retrievable via the following link: <https://ars.els-cdn.com/content/image/1-s2.0-S0022191025000654-mmc1.zip> (alternative mirror: <https://drive.google.com/file/d/1tczAjKwQ2BQsLN8uCBVB-YZdIYqVsCk3/view>)

Acknowledgments

We would like to thank the German Federal Environmental Foundation (Deutsche Bundesstiftung Umwelt, DBU) for supporting JSM with a doctoral scholarship (20021/743). In addition, we would like to thank the Chinese Scholarship Council (202206170038) for supporting the doctoral scholarship of HX. We also thank the support of the Eva Crane Trust (ECTA_20210915), which provided financial support for this project. Lastly, we would like to thank Dr. Eckart Stolle for the provision of nest boxes.

Author Contributions

JSM, LL and TJC conceived the ideas and designed methodology; JSM, LL, DH, IK, HX and TJC collected the data; JSM analysed the data; JSM and TJC led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

2.7 Supplementary Material

Supplemental method S1: Random assignment of cocoons and sex estimation

Randomised assignment of cocoons into different temperature treatments and microtube racks was performed using a custom R script and a table with individual ID, species, source site, weight and size (Supplemental Script 3). As sex was not known before hatching of individuals during, and at the end of the experiment, we used the fact that females are generally bigger than males for both species and predicted sex from weight and size using a k-means classification algorithm ($k = 2$, Supplemental Script 4). Weight was measured via the weighing setup (Materials and Methods: Weight measurements and emergence tracking). Size was inferred programmatically from images taken via a fixed camera setup with the lens pointing downwards onto a white background with a constant height between lens and background and fixed lighting conditions. Cocoons were placed on the white background in an equidistant manner and photos were taken, 10 individuals at a time. Contours and sizes of cocoons in the images were then analysed using a contour detection algorithm from the Python library OpenCV and a custom Python script (Supplemental Script 5). The inferred sizes in pixels were then converted to millimetres based on a fixed conversion value [mm/pixel] specific to the camera setup but kept constant over the course of the measurements.

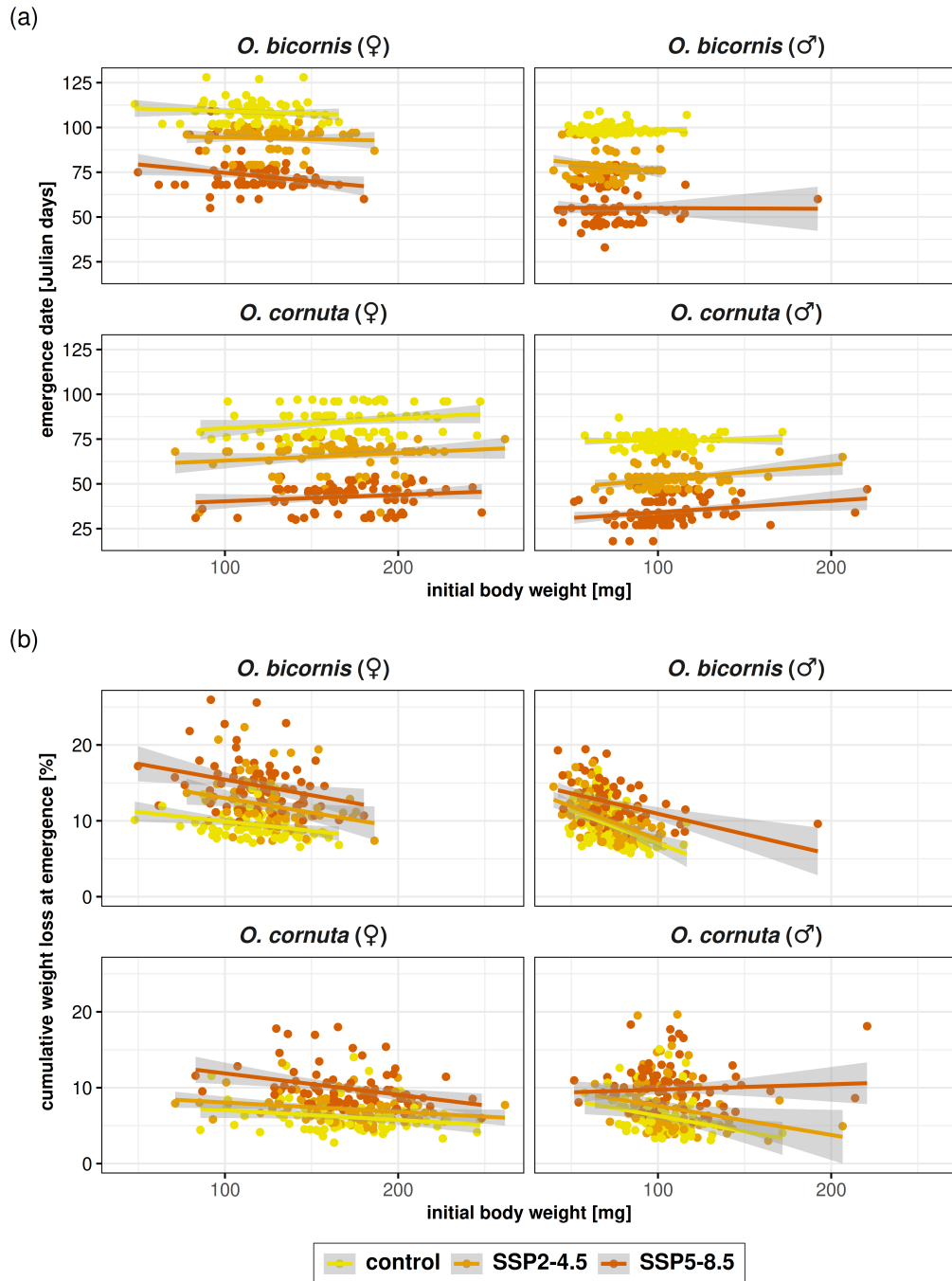


Figure S1: Effect of initial body weight on emergence timing and weight loss at emergence. (A) Dot plots showing for each species/sex combination the emergence date (y-axis) dependent on initial body weight (x-axis). Lines represent linear models fitted to the data with surrounding grey areas depicting the standard error of fitted lines. (B) Dot plots showing for each species/sex combination the weight loss at emergence (y-axis) dependent on initial body weight (x-axis). Lines represent linear models fitted to the data with surrounding grey areas depicting the standard error of fitted lines.

Chapter Two: A chromosome-level genome assembly and resequencing data reveal low DNA methylation and reduced diversity in *Osmia cornuta*

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Based on:

Genome Biology and Evolution (2026), Volume 18, Issue 1

3.1 Abstract

Bees provide essential pollination services that contribute to ecosystem stability, as well as the sustainability of economic crop yields. Due to concerns over global and local declines, improving our understanding of these ecologically and commercially important species is crucial for determining their capacity to respond and adapt to environmental challenges. The European orchard bee (*Osmia cornuta*) is a solitary bee of increasing agricultural importance due to its role in the pollination of fruit crops, yet lacks genomic resources. Using cost-effective Nanopore-only long-read sequencing, we report the first genome assembly for *O. cornuta*, spanning 647.56Mb across 727 contigs (N50=3.94Mb) at a high level of completeness (99.88% BUSCO complete). In line with the expected number of chromosomes in this species, 16 major scaffolds were assembled to chromosome-level. Also, we provisionally investigated the epigenomic architecture of *O. cornuta*, finding low numbers of CG dinucleotides that were either 5'-methylated or 5'-hydroxymethylated, providing additional evidence for the limited role methylation plays in gene regulation in Hymenopterans. To generate improved gene annotations, we combined transcriptomic- and orthology-based approaches, leading to the prediction of 12,144 genes and 25,946 proteins, showing exceptionally high BUSCO completeness (99.64%). Lastly, through whole-genome resequencing of a representative dataset, we provisionally find patterns of reduced nucleotide diversity and lower recombination rates within *O. cornuta* compared to other bee species. Collectively, our study provides a novel insight into the genome architecture of a key pollinator, providing an important resource to facilitate further genomic studies.

3.2 Introduction

Improving our fundamental understanding of the biology of ecologically and commercially important pollinators is particularly important considering they provide key ecosystem services that are crucial for ecosystem stability, biodiversity, and sustainability of agricultural yields (Kremen et al., 2007; Potts et al., 2016; Gallai et al., 2009). Despite this, declines in natural populations have raised concerns about the long-term viability of such pollination services (Hallmann et al., 2017; Powney et al., 2019; Lippert et al., 2021). Key factors that are proposed to contribute to declines include habitat loss and fragmentation, pesticides, parasites and pathogens, invasive species, and changes in climatic conditions (Cameron et al., 2011; Potts et al., 2016; Seibold et al., 2019; Goulson et al., 2015). In addition, for certain pollinators, such as solitary bees, the lack of social buffering commonly found in social bee colonies may mean they are predicted to be even more susceptible to environmental challenges that contribute to declines (Straub et al., 2015). It is, thus, of vital importance to improve our understanding of the biology of solitary bees to inform on their capacity to respond and adapt to environmental challenges.

An important solitary bee of ecological and commercial importance is the European orchard bee *Osmia cornuta* (Latreille, 1805), which is native to most of Central and Southern Europe with its range extending south to Northern Africa and east to Central Asia (Banaszak and Romasenko, 1998). It belongs to a taxon-rich genus of over 300 species (Praz et al., 2008), which are part of the family Megachilidae. Due to their high pollination efficiency in fruit orchards, mason bees, such as *O. cornuta*, have seen increasing commercial use, which has also led to an increased presence in scientific studies (Gruber et al., 2011; Sedivy and Dorn, 2014; Fliszkiewicz and Giejdasz, 2023). However, they also contain certain traits, such as a univoltine life cycle, generalist foraging patterns, and generalist nesting behaviour (Splitt et al., 2022), which make them an increasingly suitable model to study and understand aspects that are more common to the biology of solitary bees (Vicens and Bosch, 2000; Fauria and Campan, 1998; Strobl et al., 2019). *O. cornuta* has also raised recent ecological concerns over its impact through accidental introductions into locations where it is non-native. For example, cocoons of *O. cornuta* have been traded to North America and other places outside of Europe where recent reports have confirmed the presence of feral populations that have begun to establish, such as in British Columbia, Canada (Getz et al., 2024).

Recent advances in sequencing technologies and the accompanying development of genomic tools to analyse these data have facilitated the rapid generation of

genetic data that has informed on the genomic architecture of non-model organisms, including solitary bees (Colgan et al., 2022; Beadle et al., 2019; Barribeau et al., 2015; Kapheim et al., 2015; Santos et al., 2025; Stolle et al., 2023; Yang et al., 2025), which has the potential for application in the fields of agriculture, as well as conservation and wildlife management approaches (Allendorf et al., 2010; Kelemen and Rehan, 2021; Trapp et al., 2017). For example, through the generation of whole-genome sequencing data, it is now possible to accurately determine millions of nucleotide bases providing a solid basis to allow for estimating genetic parameters relevant for ecological modelling and conservation biology, such as genetic variation, effective population size and population connectivity, or identify regions of the genome with signatures of selection, helping to understand the adaptive potential of these species (Webster et al., 2023; Eizaguirre and Baltazar-Soares, 2014). Similarly, transcriptomic-based methods enable the inference of accurate gene models as the basis for the identification of candidate genes and molecular pathways involved in responses to environmental stressors, such as climate change and pesticide exposure (Beadle et al., 2019; Colgan et al., 2022).

In order to leverage these approaches, however, the availability of a high-quality chromosome-level genome assembly, including accurately predicted gene models, is of utmost importance as it underpins all genetic and transcriptomic analyses performed. For a few members of the genus *Osmia*, genome assemblies are already available (Beadle et al., 2019), however, some of these were assembled using short-read data, meaning they are likely less contiguous and more fragmentary, and may suffer from lower quality gene model predictions, limiting their utility compared to long-read generated chromosome assemblies (Ouyang et al., 2025; Stolle et al., 2023). Therefore, for *Osmia* species, such as *O. cornuta*, improvements in the quality of genome assembly, which can be generated to chromosome-level using long-read sequencing data, are urgently warranted to improve our understanding of the genetic architecture of these species, as well as provide sufficient resources to profile the genetic variation harboured in natural populations and resolve aspects of their recent demographic history. Here, we present a genome-based analysis of *O. cornuta*, for which we sequenced an individual male to high sequencing depth using Oxford Nanopore long-read sequencing technology, which we subsequently assembled to chromosome-level and generated high-quality gene model predictions using a combination of transcriptomic- and orthology-based gene annotation approaches. We also provide insights into the epigenomic landscape of *O. cornuta* through examination of DNA methylation levels, genome complexity through analysis of repeat diversity, as well as perform provisional analyses of nucleotide diversity and

recombination rates through whole-genome sequencing of individuals sampled from a European population.

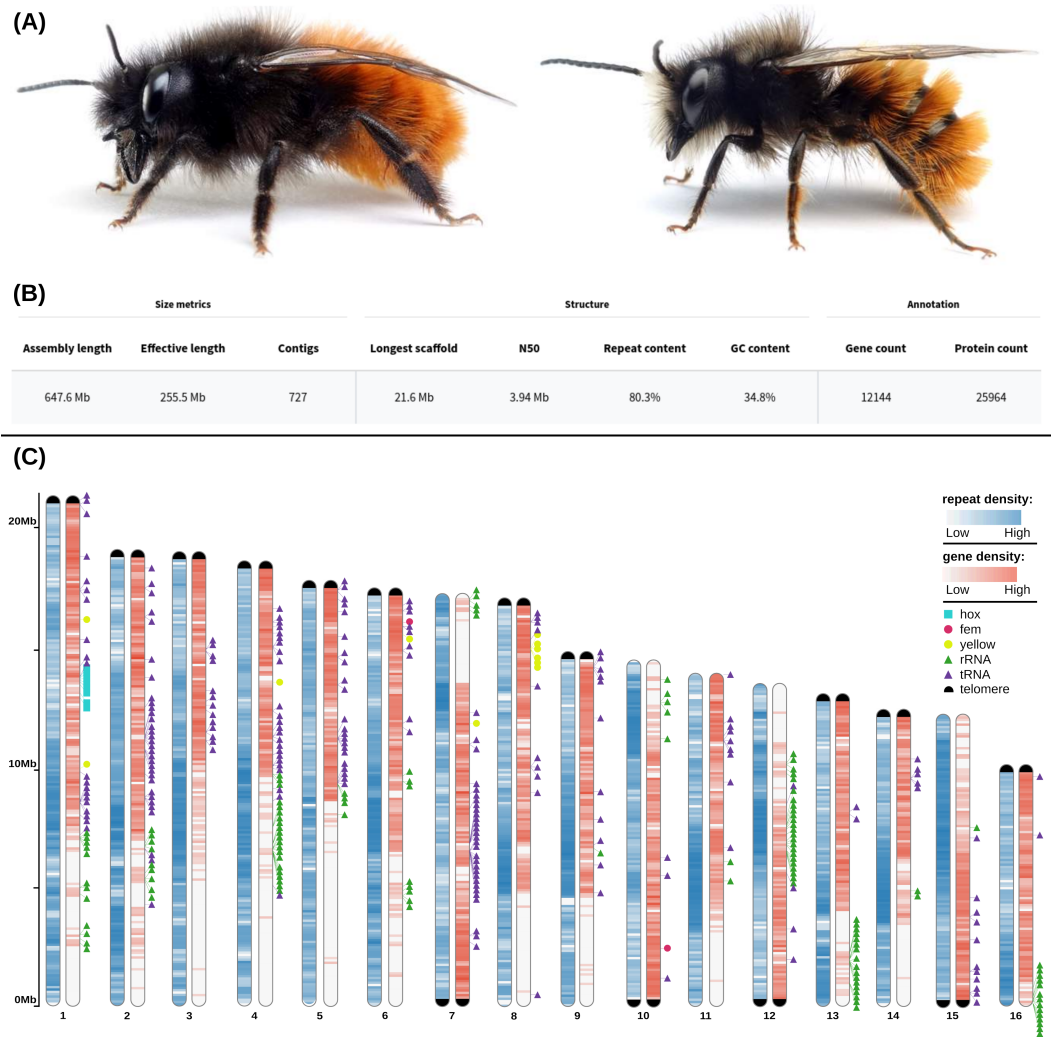


Figure 3.1: Habitus, overview statistics and schematic chromosomal karyogram. (A) Habitus of *Osmia cornuta*, depicting both a female (left) as well as a male (right) individual (image copyright:). (B) Assembly and annotation summary statistics, classified into size-related, assembly structure-related, and annotation-related metrics. (C) A schematic karyogram that depicts for each of the 16 chromosomes regions of increased or reduced gene- and repeat-density using colour spectra. Depicted pairs of chromosomes do not represent separate, diploid chromosome homologues but copies of the same haploid chromosome, each copy highlighting the density of a different set of genetic features. In addition, the presence and location of some representative gene families, which tend to cluster in specific regions of the genome, are depicted using coloured symbols. An additional, black symbol is used to mark telomeres at chromosomal ends. The size of this symbol is uniform and does not represent the actual length of these telomeric regions.

3.3 Results and Discussion

High-completeness, chromosome-level genome assembly from long-read sequencing data

From a single *Osmia cornuta* male (Figure 3.1A), we generated 1,338,458 highly accurate, filtered reads on approximately half of a Nanopore PromethION flow cell at a total length of 26.12Gb (mean length = 19.52kb; maximum length = 930.51kb). Filtered reads were then corrected using HERRO, resulting in 1,110,145 reads at a total length of 20.94Gb (mean length = 18.86kb; maximum read length = 139.28kb). Using these reads, we generated 11 candidate assemblies, six of which were generated using flye and five were generated using hifiasm. Based on variation in contiguity, completeness, and the presence and extent of telomeres, we selected an assembly generated using hifiasm for further research and publication. This assembly initially contained 777 contigs (total length = 654.189Mb), but was reduced by 44 contigs following purging of putative duplicate haplotigs. It had an excellent merquy QV score of 54.86, corresponding to a very low error rate (estimated error rate = 1 error per 306,000 bases), indicative of a high-quality, highly accurate assembly. Similarly, screens of foreign DNA did not classify any contigs as being contaminants. A scan for mitochondrial sequences suggested that six contigs were putative mitochondrial in origin. After removal of mitochondrial-derived contigs, the final assembly contained 727 contigs, without gaps, and a total length of 647,555,333bp (Figure 3.1B) with a BUSCO completeness score of 99.78% (duplicated: 0.12%, fragmented: 0.03%, missing: 0.07%) and a GC content of 34.8%. The longest contig spanned 21.64Mb, the mean length of contigs was 890.72kb and N50, N70, and N90 lengths were 3.94Mb (n=29 contigs), 1.35Mb (n=91) and 0.34Mb (n=271), respectively. By mapping the reads back to the genome assembly, we inferred a mean coverage of 40.22X (SD=32.14), whereas less repetitive parts of the largest primary contigs (n=16) showed a coverage of approximately 55.14X. The high completeness of our assembly was further supported by the identification of 15 complete telomeres (AACCT) among the 16 largest contigs, which represent the 16 putative chromosomes previously described via karyotyping (Kerr and Laidlaw, 1956). Another nine putative telomeres were found on shorter (<1.5Mb) contigs. Additionally, we assembled the mitochondrion contig to a length of 35,525bp, containing the expected two ribosomal RNA (rRNA), 24 transfer RNA (tRNA), and 13 protein-coding genes. A comprehensive annotation of repetitive elements determined that 80.3% of the genome was composed of repeats (Figure 3.2C), of which the majority were LTR retrotransposons (34.29% of the assembly) and simple repeats (29.78%), while other types were much less common (DNA transposons:

7.25%, LINE: 6.67%, Rolling Circle: 0.6%, and Unclassified Repeats 1.7%). At 648Mb, the length of this assembly is on the higher end, yet still very comparable to the length of publicly available high-quality genome assemblies in the same Genus (*Osmia bicornis*: 223Mb, *Osmia lignaria*: 498.5Mb) and Family (*Megachile rotundata*: 613.4Mb, *Coelioxys conoideus*: 417.6Mb). Analogously, the BUSCO completeness score of 99.78% is similar, and even slightly higher, compared to high-quality assemblies of closely related species (*Osmia bicornis*: 98.5%, *Osmia lignaria*: 97.9%, see Supplemental Table S2). Together, the identification of the presence and extent of telomeric regions and the exceedingly high BUSCO completeness suggest a highly contiguous genome assembly, comparable with high-quality PacBio and Hi-C genome assemblies of other bee species (Toth et al., 2024; Wallberg et al., 2019; Stolle et al., 2023).

High-quality structural annotations generated using a combination of protein-, transcript- and orthology-based evidence

We generated gene model predictions for the assembly using two independent approaches: first, using transcript- and protein-based evidence (implemented with BRAKER3); and second, using inferred orthology with the closely related *O. bicornis* (implemented with TOGA). We then combined predictions from these two approaches to generate a more complete set of gene models. The combined set of predictions comprised 12,144 genes, which is comparable to gene counts reported for long-read based assemblies of related species (*O. bicornis*: 12,706; *O. lignaria*: 13,795; Supplemental Table S2). The inferred gene set is predicted to encode for 25,946 protein-coding transcripts. The resulting protein-coding transcript predictions showed a BUSCO completeness score of 99.64%, whereas proteins from the individual annotation approaches resulted in completeness scores of 97.20% and 97.51%, respectively. Compared to the completeness score of 99.88% for the assembly (Figure 3.2A), our high completeness score for the annotation suggests that, using this combined approach, we were able to predict gene models for nearly all genes that were assembled. The high completeness of the structural annotations was further highlighted by a high percentage (97.64%) of matches against conserved and taxonomically ‘consistent’ hierarchical orthogroups (HOGs) from the Orthologous Matrix (OMA) database, as well as a low percentage (3.63%) of matches against HOGs that are considered taxonomically ‘inconsistent’ with the species of interest (Figure 3.2B). These numbers are highly comparable to the structural annotation of the closely related *O. bicornis* (<25 million years of divergence (Cardinal and Danforth, 2013; Gonzalez, 2011), 97.68% completeness, 3.67% ‘inconsistency’ of proteome), which has been annotated via the NCBI Eukaryotic Genome annotation pipeline, indicating that our structural annotation has achieved a similar level of

quality. In line with the high completeness of our gene annotations, we successfully confirmed the presence of several marked gene clusters and genes (Figure 3.1C), among which the *yellow* (chromosome 8), *hox* (chromosome 1) and *osiris* clusters (chromosome 4), as well as the *feminizer* gene (chromosome 6), which forms part of the sex determining cascade. As our annotation approach did not predict non-coding gene models, we additionally scanned the chromosomal scaffolds of our assembly for tRNA and rRNA genes. In doing so, we found 195 tRNA genes with particularly pronounced clusters on chromosomes 2, 4, and 7, as well as 126 rRNA genes with strong clustering on chromosomes 4, 12, 13, and 16. Lastly, we also examined synteny of our genome assembly with that of the closely related *O. bicornis* via comparison of predicted proteomes. Summarised to gene level, we found that 77.43% of genes were collinear between the two species. Using this synteny-based approach, we further identified 16 chromosomal homologues to those described in *O. bicornis*, providing evidence of the high quality and completeness of the *O. cornuta* genome assembly. Additionally, we also found evidence of inverted segments on several chromosomes within the *O. cornuta* genome assembly, reflecting putative chromosomal rearrangements since the latest common ancestor (Figure 3.3). In summary, our combined annotation approach produced a highly complete set of gene predictions for *O. cornuta*, successfully identifying key gene clusters, and demonstrating strong syntenic conservation with the closely related *O. bicornis*.

Evidence of genome-wide methylation patterns

A key advantage of Nanopore technology is its ability to detect modified bases, such as methylated cytosine, during sequencing, allowing for a direct examination of the epigenomic landscape. Methylation levels have previously been described as low in members of the Hymenoptera, including other bee species (Bewick et al., 2017; Provataris et al., 2018; Glastad et al., 2014), with studies employing bisulphite sequencing identifying approximately 1% of genome-wide cytosines reported as methylated in the honeybee *A. mellifera* (Harris et al., 2019), and the bumblebee *B. terrestris* (Pozo et al., 2021). We, therefore, examined whether the genome of *O. cornuta* contained similar levels of methylated cytosines. By extracting base modification information directly from Nanopore data through basecalling, we found that the *O. cornuta* genome assembly contained no 6'-methyladenosine (6mA), 5'-methylcytosine (5mC), or 5'-hydroxymethylcytosine (5hmC) modifications, but low numbers of 5'-methylcytosine and 5'-hydroxymethylcytosine in the context of CG dinucleotides (5mCG, 5hmCG). For 5mCG, out of 24,194,970 called sites, 192,079 (0.84%) were considered methylated, being supported by at least one read, while 51,437 (0.23%) sites showed methylation within more than 20% of reads. For 5hmCG, out of 24,194,970 called sites, we identified 201,054 (0.9%) sites that

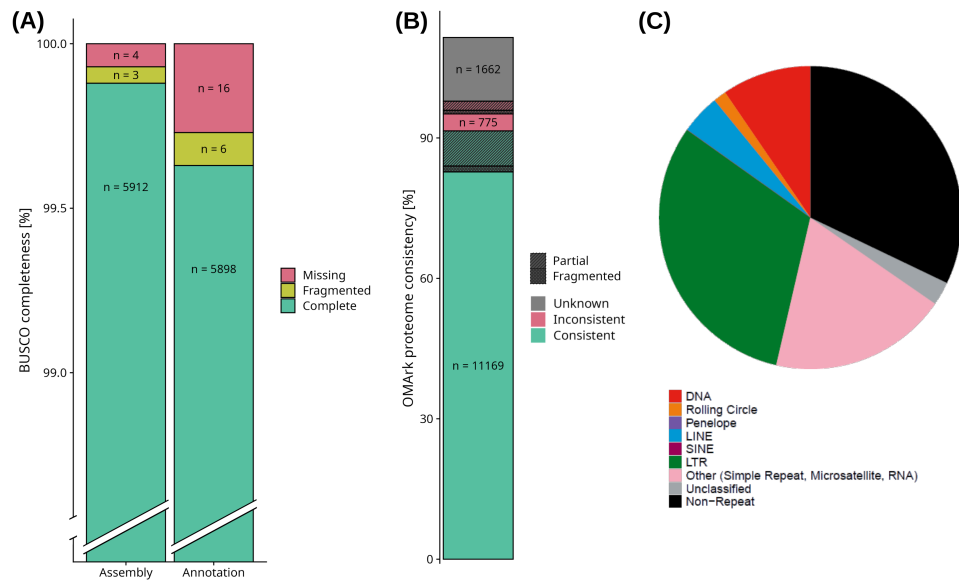


Figure 3.2: High assembly- and annotation completeness and overview of genomic repeat architecture. (A) Stacked bar charts showing the results of the overlap of the assembly (left) and the predicted protein models of the annotation (right) with single-copy orthologs from the hymenopteran dataset (n=5919) of the OrthoDB (v12) database. Within each bar chart, the level of overlap for three subcategories is colour-coded: “Complete”, green, refers to an assembly region or a protein fully overlapping with an ortholog in the reference database; “Fragmented”, yellow, refers to an assembly region or a protein that partially overlaps with a reference ortholog; and “Missing”, red, which refers to a reference ortholog that did not overlap with any region of the assembly or predicted protein set. (B) A bar chart showing the annotation consistency of the predicted gene models when compared against hierarchical orthogroups (HOGs) of the OMA database with subcategories either colour-coded or patterned: “Consistent”, green, referring to a gene model has been placed in the correct lineage; “Inconsistent”, red, referring to a gene model that has been placed in an incorrect, unspecific lineage; “Unknown”, gray, which refers to a gene model has not been placed in any of the tested lineages; “Partial”, stripes, meaning not all parts of a gene model match a HOG; and “Fragmented”, checkered, meaning a gene model does not match all parts of a HOG. (C) A pie chart showing the genomic composition by repeat type and the fraction of non-repeat regions. Collectively, more than three quarters of the genome are made up of repeat sequences with long terminal repeats (LTR) being the most common. Legend: DNA = DNA transposon; Rolling Circle = rolling circle transposon; Penelope = Penelope transposon; SINE = short interspersed nuclear element; LTR = long terminal repeat; and RNA = retrotransposon. (D) Four histograms, each depicting the distribution of the kimura 2-parameter distance in base pairs for a specific type of repeat. Within each histogram, the repeat type is broken up into subtypes, each displayed at a different colour.

were considered methylated, being supported by at least one read, while 14,125 sites (0.06%) showed methylation in more than 20% of reads. To understand how 5'-methylation of CpG loci varied across different regions and features of the genome,

we inferred for each investigated genomic feature type, the percentage of sites that were considered weakly methylated, finding evidence of considerable variation in methylation patterns across the genome (Table 3.1). In particular, exons showed by far the highest proportion of 5'-methylated CpG sites (2.53%), while introns and intergenic regions had a relatively lower proportion of 5'-methylated CpG sites (intron: 0.88%, intergenic: 1.02%). Strikingly, in low complexity and repeat regions, the frequency of 5'-methylated CpG sites was up to 100x reduced compared to all other genomic feature types (low complexity: 0.01%; repeats: 0.03%). The same overall patterns were observed for 5'-hydroxymethylcytosine CpG modifications. Collectively, we found that DNA methylation levels within the genome of *O. cornuta* are low, however comparable to levels previously reported for other hymenopterans (Glastad et al., 2017; Oldroyd and Yagound, 2021; Standage et al., 2016). As epigenetic modifications, such as DNA methylation, are linked to the adaptive potential of species (Ashe et al., 2021; Stajic and Jansen, 2021), e.g. through their effect on phenotypic plasticity (Romero-Mujalli et al., 2024; Oostra et al., 2018; Fox et al., 2019a), our findings of low methylation levels in this pollinator species raise concerns about its potential to adapt to future environmental challenges, such as climate change. In fact, this species' previously reported increased susceptibility to pesticides after exposure to increased temperatures (Albacete et al., 2023), might be related to the herein reported low levels of methylation via their effect on phenotypic plasticity. However, the investigation of this hypothesis requires additional data and it is noteworthy that this species is currently undergoing population- and range-expansions (Westrich, 2019), and thus, does not seem to be limited by the herein reported epigenetic limitations which raises interest in the genetic potential that allows this species to thrive.

Evidence of reduced genetic diversity, low recombination rates and reduced effective population size around the last glacial maximum

As a preliminary examination of the population genetics of *O. cornuta* and as it has been reported that many groups within the Hymenoptera have relatively low genetic diversity and recombination rates compared to other insect groups (Weyna and Romiguier, 2021), for example due to haplodiploidy (Sun and Hernandez, 2023), we investigated genetic diversity, linkage disequilibrium and recent changes in effective population size from whole-genome resequencing data for 10 females sampled from across Germany (sampling sites in Supplemental Table S1). We identified 594,857 high-quality, filtered SNPs (biallelic: 99.7%) across the 16 chromosomal scaffolds of our assembly (255.5Mb effective length), yielding an overall variant density of 2.33 SNPs per kilobase. Variant density varied across chromosomal scaffolds, ranging from 1.73 (Chromosome 3) to 3.22 SNPs per kilobase (Chromosome 15).

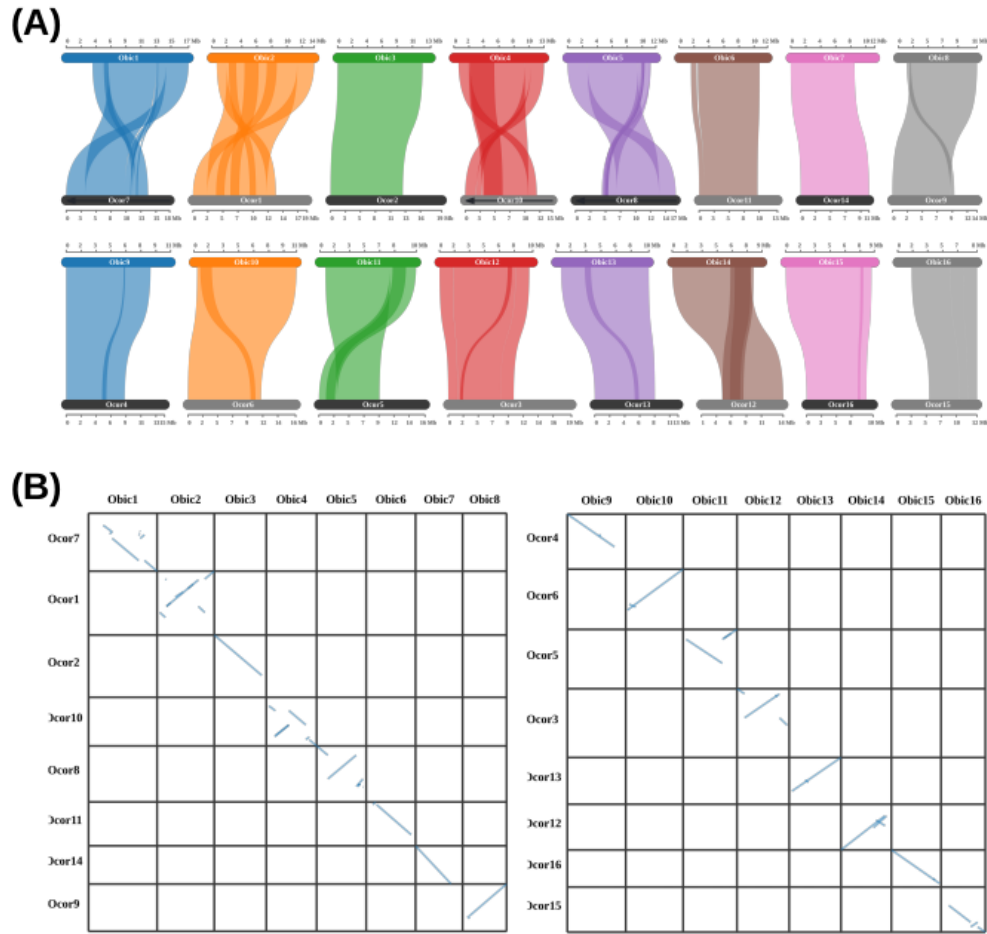


Figure 3.3: Synteny comparison with *Osmia bicornis*. (A) A two-row parallel link plot giving an overview of syntenic regions between putative chromosomal scaffolds of *O. bicornis* (Obic; top of each row) and *O. cornuta* (Ocor: bottom of each row) based upon comparisons of predicted proteomes. The markers show the genomic coordinates for each putative chromosome in megabases (Mb). (B) Dot plots displaying a more detailed examination of synteny between the two species. The binned x-axis represents the position along identified chromosomes in *O. bicornis* with one chromosome per bin. Similarly, the y-axis represents the position along chromosomes in *O. cornuta* with one chromosome per bin. A dot represents a syntenic region that is shared between the two species at the respective chromosomal loci.

Structural annotation revealed that the majority of variants (94.9%) were positioned in non-coding regions, such as intronic, intergenic, and regions upstream/downstream of genes. Among protein-coding regions, 74.5% of variants (n=22,116) were synonymous (silent) polymorphisms preserving amino acid sequences, while 25.3% (n=7,517) were nonsynonymous (missense) variants, predicted to alter the

Table 3.1: Genome-wide patterns of methylation across different genomic features. A table showing for each genomic feature considered in the analysis, the percentage as well as the number of CpG sites for which we found evidence of either a 5'-methylcytosine modification (5mCG) or 5'-hydroxymethylcytosine (5hmCG) modification. In addition, the total number of potentially methylated CpG sites for each genomic feature is shown in a separate column.

Genomic Feature	5mCG			5hmCG		
	Methylated sites (%)	Methylated Sites (n)	Methylatable Sites (n)	Methylated Sites (%)	Methylated Sites (n)	Methylatable Sites (n)
Exon	2.53	23,738	936,051	1.79	16,781	936,051
Intron	0.88	37,240	4,190,504	1.11	46,615	4,190,504
Intergenic	1.02	83,839	8,198,788	1.17	95,525	8,198,788
Low Complexity	0.01	8,654	7,448,992	0.02	12,157	7,448,992
Repeat	0.03	54,052	15,547,674	0.06	89,611	15,547,674

amino acid sequence of the resulting protein. Protein-truncating variants, such as early stop codons, were rare, with nonsense mutations attributed to only 0.14% (n=42) of variants within protein-coding regions and critical splice site disruptions caused by only 21 variants genome-wide. The overall missense-to-silent ratio of 0.34 likely reflects the expected purifying selection acting against amino acid-altering mutations.

Using our set of called variants, we calculated genome-wide estimates of nucleotide diversity in non-overlapping 10kb-sized windows finding a median diversity of $\pi=0.0012$ (Figure 3.4A) as well as a median Watterson's theta of $\vartheta_w=0.001$ across all 16 chromosomal scaffolds. This is considerably lower than whole-genome sequencing based published estimates of nucleotide diversity in eusocial species of the genera *Apis* and *Bombus* (Leroy et al., 2024; Heraghty et al., 2023) which range from $\pi=0.0014$ to 0.0025 but is also lower than the estimate inferred for another solitary species of the same family (Megachilidae: *Megachile rotundata*) at $\vartheta_w=0.003$ (Jones et al., 2019). However, we are aware of the possibility that our relatively low sample size might capture less genetic variation and thus underestimate genetic diversity compared to other studies of higher sample size. We also identified heterogeneity in the chromosomal distributions of nucleotide diversity (Figure 3.4B). Two chromosomal scaffolds (9 and 15) showed significant overrepresentation among the top 5% of windows in terms of elevated nucleotide diversity compared to the genome-wide background (Fisher's exact test, Benjamini-Hochberg adjusted $p < 0.01$). We

also assessed nucleotide diversity differences across specific genomic regions and found that median nucleotide diversity was significantly elevated in both intronic ($\pi=0.0017$) and repeat regions ($\pi=0.0016$) compared to the rest of the genome ($\pi=0.0012$, Wilcoxon rank-sum test, $p < 1 \times 10^{-16}$), but was not significantly elevated or decreased in intergenic ($\pi=0.0012$) or low complexity ($\pi=0.0014$) regions. In contrast, exonic regions ($\pi=0.0006$), were significantly decreased in diversity compared to the rest of the genome (Wilcoxon rank-sum test, $p < 1 \times 10^{-16}$), which, especially compared to the three times higher diversity estimates found in introns, may be interpreted as potential evidence of different selective pressures acting on coding and non-coding regions of the genome (Figure 3.4A). Linkage disequilibrium (LD) analysis revealed comparatively slow LD decay across the genome (Figure 3.4C). The correlation coefficient (r^2) stayed at values above 0.3 for distances below 100bp and converged to around 0.1 by 10kb of distance separating linked loci, whereas in another study on another species from the same family (Megachilidae: *M. rotundata*) LD decay reached values as low as around 0.05 by 10kb of distance (Jones et al., 2019). In the same study, the recombination rate for *M. rotundata* was reported to be 1.02cM/Mb. In contrast, the advanced eusocial western honeybee (Apidae: *Apis mellifera*) reached LD levels as low as 0.05 already at distances below 100bp due to its extremely high recombination rate of 23.94cM/Mb (Jones et al., 2019). Together, our preliminary assessment provides evidence of reduced nucleotide diversity, albeit with peaks of elevated genetic diversity (Figure 3.4), and relatively low rates of recombination both at shorter and longer physical distances in *O. cornuta* compared to social honeybees (23.94cM/Mb), bumblebees (4.76-8.7cM/Mb), stingless bees (9.3–12.5cM/Mb) and solitary leafcutter bees (1.02cM/Mb) (Liu et al., 2017; Waiker et al., 2021; Stolle et al., 2011; Jones et al., 2019).

From our WGS data, we also inferred historic effective population sizes for *O. cornuta*, which suggest a decline towards and after the last glacial maximum (Figure 3.5). For more recent historic times, the effective population sizes were consistent for samples between 10,000 and 30,000 with exception of two samples. Given that *O. cornuta* is currently undergoing an expansion towards the north of Germany (Westrich, 2019), it is possible that the herein observed low genetic diversity in *O. cornuta* is attributable to reduced effective population size commonly observed at the forefront of a populations range expansion (Hartl and Clark, 2007). Similarly, the reduced effective population size due to a historic population bottleneck, is an additional possible explanation for the observed reduced genetic diversity. However, these hypotheses need to be tested against data with higher sample size and from more established populations with less variable effective population size, such as those from glacial refugia. Furthermore, effective population size estimates are

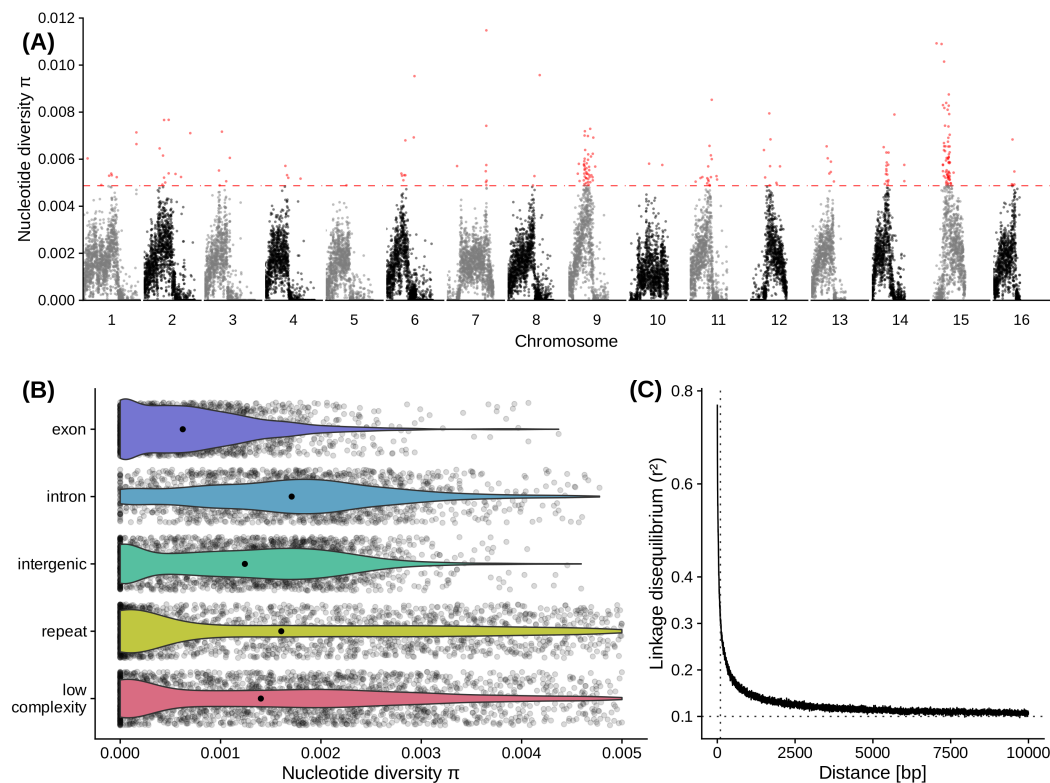


Figure 3.4: Low nucleotide diversity and low linkage disequilibrium decay inferred from whole-genome-resequencing. (A) A Manhattan plot showing the distribution of nucleotide diversity estimates, as measured in π , along each of the 16 chromosomes of the *O. cornuta* genome assembly. To achieve a higher resolution, each dot represents a 10kb window instead of the 100kb window that we used to estimate the values reported in the text. Coloured dots above the horizontal dashed line represent the top 5% most diverse windows across the whole-genome. (B) Violin plots showing the distributions of nucleotide diversity estimates across primary genomic feature types with each feature type on a separate row and indicated by an individual color. The single black dots in the middle of each violin plot mark the median while transparent dots in the background show the raw data points (10kb windows). (C) A line graph showing the logarithmic decay of linkage disequilibrium (y-axis) with increasing distance between loci (x-axis). Linkage disequilibrium was computed in terms of r^2 . A dotted horizontal line marks the value of 0.1, which is the value of convergence for this curve, whereas a vertical dotted line marks a distance of 100bp that can be used to compare LD decay across species. Here, at 100bp, LD has decayed to a value of around 0.3.

sensitive to demographic parameters, such as recombination rate and mutation rate, which are typically derived from closely related species. Given this reliance on reference data from other species, species-specific parameters for *Osmia cornuta* would substantially improve the reliability of population size estimates.

Conclusion

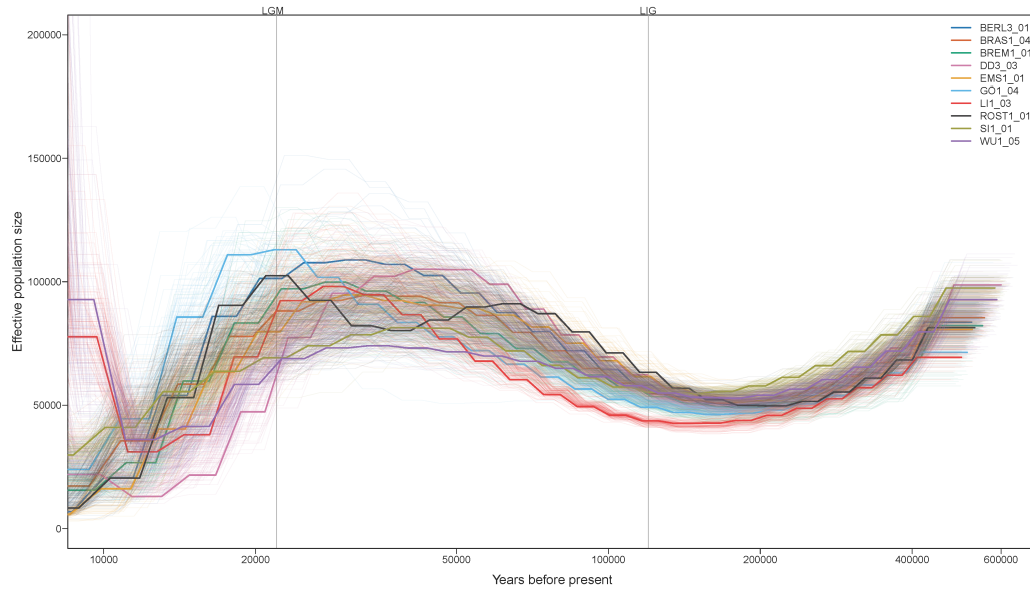


Figure 3.5: Effective population size is estimated to have declined since the Last Glacial Maximum. Line plot showing inferred demographic history of ten female *O. cornuta* sampled across Germany. The y-axis represents effective population size, while the x-axis indicates time in years before present. Each individual is represented by a distinct colour, with faint lines illustrating the variation in effective population size estimates based on 100 bootstrap iterations. Vertical grey lines correspond to the Last Glacial Maximum (LGM) and Last Interglacial (LIG) period, respectively.

Our study presents a highly contiguous, chromosome-level genome assembly, complete with a high-quality genome annotation for the ecologically and commercially important solitary bee *O. cornuta*. Given the lack of genomic resources that exist for *O. cornuta*, as well as other mason bees, this genome assembly provides a valuable resource for future genetic and genomic analyses to inform on how evolutionary processes are shaping their capacity to adapt to environmental pressures. We provide a demonstration of this potential by detailing preliminary measures of genetic variation, recombination rates, genome-wide methylation, as well as demographic changes within a European population. For example, using this dataset we found evidence of reduced genetic variation and reduced DNA methylation levels, which, together with this species' current expansion of range and population levels, raises interesting questions about its potential for adaptation to future environmental challenges. Our project also demonstrates the feasibility to generate chromosome-level assemblies of high completeness *and* genome-wide DNA methylation profiles using as little as half of an Oxford Nanopore PromethION flow cell, which has an estimated cost of ~€500, providing growing support to how improvements in sequencing costs and throughput, base calling accuracy, and assembly algorithms, have increased the

utility of Nanopore-sequenced long reads alone, to generate high-quality genome assemblies. Additionally, our study shows that a combined approach to genome annotation employing both transcriptomic- and orthology-based methods can improve the completeness of genome annotations. By providing both a resource for future analyses on an important solitary bee species but also novel, cost-effective approaches to genome assembly and annotation, we aim to contribute to an urgently needed, growing research interest in the genomics of understudied pollinators.

3.4 Materials and Methods

Sample collection

For the purpose of genome assembly, in March 2023, one male adult was collected and immediately snap-frozen after emergence from pre-placed nest boxes from Rotleberode (Südharz, Saxony-Anhalt, Germany, 51°31'N, 10°57'E). For the purposes of gene model prediction, we sampled from adult and developmental stages and later complemented these with additional publicly available data to increase gene discovery rates, as certain genes may only be expressed during certain stages or tissues. We collected the following individuals: 1) one female adult was sampled from the same nest box as the male used for genome assembly and immediately snap-frozen after emergence; and 2) male and female pupae ($n = 30$) were sampled in 2023 and 2024 from additional pre-placed nest boxes, and immediately snap frozen. All samples were stored at -80°C until DNA and RNA extractions were performed, respectively. Lastly, for the purposes of examining intraspecific genetic variation present within a representative *O. cornuta* population, we sampled female adults from 10 independent sites (more than 50km apart) across Germany (for exact locations see Supplemental Table S1) for whole-genome resequencing ($n=10$ individuals total) and downstream population genetic-based analyses. For sampled individuals, field-based species identification was initially performed based on morphological features (Westrich, 2019), before bees were immediately conserved in absolute ethanol in the field with subsequent storage at -80°C . Species identification for all samples was later assessed and validated using computational techniques (Supplementary Information: Computational validation of species identification).

DNA extraction and long-read sequencing

High-molecular-weight DNA was extracted from the thorax of the single adult male using the New England Biosystems Monarch HMW DNA extraction kit, yielding $7.5\mu\text{g}$ of DNA (260/280nm ratio of 1.7, 230/280nm ratio of 1.0, at a concentration

of 150ng/ μ l according to a Nanodrop ND-1000). A Nanopore sequencing library was generated using a ligation-based kit (SQK-LSK114) from Oxford Nanopore Technologies (ONT). Sequencing was performed on approximately half of the capacity of a PromethION flow cell (v10.4.1 FLO-PRO114M) in two runs of 20 hours and 48 hours, respectively, with each run separated by a flow cell wash. The initial 39.57Gb of raw data (4.86 million reads at N50 of 17kb) were subjected to basecalling a second time with dorado (v0.7.0+71cc744, model sup5.0.0) on a Geforce GTX3090 GPU. Reads were then filtered by quality ($\geq$ Q90) and length ($\geq$ 8kb) (Filtlong v0.2.1, seqtk v1.4-r122), and, additionally, error-corrected with HERRO model_v0.1 for R10.4.1 data (Stanojevic et al., 2024) after which reads less than 5kb were removed.

RNA extractions for transcriptome-facilitated genome annotation

For the prediction of gene models during genome annotation, we generated a transcriptomic dataset for the head tissues of a single *O. cornuta* adult female. To achieve this, from a snap frozen sample, we removed the head from the thorax, and transferred it to a 1.5ml centrifuge tube containing 200 μ l of TRIzol. The sample was manually homogenised using a micropestle until the head was visually disrupted. Total RNA was subsequently extracted using a TRIzol-chloroform-based extraction protocol (Colgan et al., 2011), and then purified using a Monarch RNA purification kit (NEB) before being DNase-treated (TURBO DNase, Qiagen) to remove putative DNA contaminants. RNA was quantified and assessed using the FastGene NanoSpec Photometer (FG-NP01), before being stored at -80°C. DNase-treated RNA was shipped on dry ice to a commercial supplier (Novogene, Germany) where quality was further assessed using an Agilent Bioanalyzer. An mRNA-enriched library was prepared and paired-end sequencing (2*150bp) was performed to produce a minimum of 50 million paired-end reads. For the pupal samples, total RNA was extracted from four different leg tissues (hind tibia, mid tibia, hind basitarsus, mid basitarsus) from 30 samples (15 female, 15 male) using mechanical homogenization (Tissue Lyser LT, Qiagen) and a Zymo DirectZol kit, which included DNase digestion. For the female adult, a mRNA-enriched library was prepared, while for the pupae samples mRNA was extracted from total RNA (poly-T oligo-attached magnetic beads), followed by fragmentation and cDNA synthesis with random hexamer primers. Sequencing (paired end 150 bp) was performed by Novogene (Germany) generating a mean read count of 55,206,130 across samples (min: 12,042,090, max: 162,207,301).

DNA extractions for population genetic analyses

For the population genetic-based analyses, we removed ethanol-preserved samples (n=10) from the -80°C freezer. As abdomens can harbour microbial contaminants,

we removed them using microscissors and the remaining tissue was subsequently washed with three rounds of autoclaved phosphate-buffered saline (PBS) to remove residual ethanol. Tissue homogenisation was performed using micropestles on tissue samples in 1.5ml Eppendorf tubes filled with 200 μ l of PBS. Following this, DNA was extracted using the Monarch Genomic DNA Purification kit, according to the manufacturer's instructions. After extraction, the quality and quantity of isolated genomic DNA were checked using NanoDrop and Qubit photometric measurements, as well as by using low concentration agarose gel electrophoresis at low voltage. Only samples with a minimum total DNA amount of 1.2 μ g, 260nm:280nm absorbance spectrum ratios of at least 1.8, and visible sharp bands in electrophoresis gels were considered for sequencing. Extracted DNA was sent to a commercial sequencing company (Novogene, UK) where further quality assessments using an Agilent Bioanalyzer were performed. After quality checks, for each sample, an individual PCR-free library was produced using a NEB library kit, individually barcoded, and sequenced on an Illumina NovaSeq 6000 (2*150bp) to a minimum sequencing output of 3Gb per sample, resulting in a mean read count of 32,695,578 across samples (min: 26,511,816, max: 38,517,412, Supplemental Table S1), as well as a mean sequencing depth of 19x, based on the total length of chromosomal scaffolds.

Genome assembly

Using a combination of filtered long FASTQ reads and HERRO-corrected FASTA reads, we generated and assessed different *de novo* assemblies using flye v2.9.3 (Kolmogorov et al., 2019), specifically, generating assemblies with variation in minimum overlap (10,000, 8,000, and 6,000bp) with presets for pacbio-hifi (corrected reads), nano-corr (corrected reads), and nano-hq (un-corrected reads). As a complementary approach, we generated a second set of assemblies using hifiasm v0.24.0 (Cheng et al., 2024) using the “-ont” flag with uncorrected reads and with five different parameter sets (see code on GitHub repository for this study). From each resulting assembly, we removed contigs shorter than 5kb using seqtk as long reads used for the assemblies were either 8kb or longer meaning that contigs shorter than this length represent potential sequencing artefacts or are biologically less informative. Each assembly was assessed for summary statistics (assembly_stats, <https://github.com/sanger-pathogens/assembly-stats>), gene completeness (Compleasm v0.2.7 with database hymenoptera_odb12) and the presence of the canonical hymenopteran telomere repeat "AACCT" using tidk, (Brown et al., 2025).

The best assembly was assessed and chosen according to the lowest number of missing or duplicated BUSCO (Manni et al., 2021) genes, the highest number of identified telomeric regions, and the longest contigs. Based on these criteria,

“hifiasm3” was selected as the best assembly, which was generated using hifiasm with the following parameters: “--telo-m AACCT --primary -l 2 -s 0.65 -D 10.0 -N 300 --dual-scaf --ont”. This assembly was polished using medaka v1.12.1 (<https://github.com/nanoporetech/medaka>), followed by the purging of haplotigs and overlaps using purge_dups v1.2.5 (Guan et al., 2020), as well as the sorting by length in a descending order (i.e., longest to shortest) and renaming of contigs using seqkit v2.5.1 (Shen et al., 2016). The quality of the assembly was then again assessed using assembly_stats, Compleasm, and tidk, which collectively provide information on basic summary statistics of genome size and structure, assembly completeness, and potential contaminants. As an additional step, assembly completeness relative to the long reads used for assembly was assessed with merquy (version 2022-09-07 from the github master at <https://github.com/marbl/merquy>, (Rhie et al., 2020)).

The putative mitochondrion was assembled in a separate process. First, we used BLASTN (Camacho et al., 2009) with a set of bee mitogenomes to identify six contigs likely of mitochondrial origin. One of these contigs, representing a putative single mitochondrial genome copy, was selected as a reference so as to allow extraction of Nanopore long reads that matched it after mapping with minimap2 v2.28-r1209 (Li, 2018). These extracted reads were subsequently assembled using flye v2.9.3-b1797 (Kolmogorov et al., 2019) into a single contig of 35,525bp. We used Blastn of contig ends and Minimap alignment to itself to confirm that the ends are not overlapping. This assigned mitogenome was then annotated with mitos v2 (Donath et al., 2019). To confirm that no eukaryotic genes are present on the mitochondrial contig, we used galba (Brůna et al., 2023) and helixer (https://www.plabipd.de/helixer_main.html).

Assessment of possible contamination

We screened the assembled nuclear genome using BlobToolKit (Challis et al., 2020) to identify scaffolds of unusual GC content-to-read coverage ratios, as well as scaffolds assigned to non-target organisms, which likely represent contaminants. In addition, the assembly was further assessed for contamination using the NCBI Foreign Contamination Screen (fcs v0.4.0, (National Center for Biotechnology Information, 2024)) pipeline with NCBI’s *Osmia cornuta* TaxID 185587 specifically selected.

Repeat annotation

Putative repetitive elements in the genome assembly were identified and annotated using EarlGrey v5.1.1 (Grey, n.d.), which incorporates RepeatMasker v4.15 () with

the DFAM v3.7 and RepBase (2018) databases, and was run with clustering and softmasked output. This soft-masked genome assembly was used as the input for gene model predictions ('Gene annotation'). Satellites were identified with Satellite Repeat Finder (), while microsatellites and regions of low complexity were identified using a combination of the following complementary tools: etrf (-m 200 -l 13;), trf-mod v4.10.0, SA-SSR v0.1 alpha (--min-repeats 5 --min-nucs 10 --max-ssr-len 12 --max-seq-len 25000000), ULTRA v1.0.0 beta 12 (--min_unit 4 --min_length 10 --period 300, filtered for minimum length of 100bp), tatan v49 (-f4, filtered for minimum repeat number=5), sdust (-w 64 -t 20, filtered for minimum length=10), and hrn (filtered for minimum length=10, seqtk v1.3-r115-dirty). Low mappability regions were identified using genmap v1.3.0 (parameters: -K 35 -E 2), with subsequent filtering based on mappability below 0.05 and a minimum length of 100bp. From the EarlGrey annotations, we removed entries classified as "Unknown" and "Satellite" to create a set of transposable element (TE) annotations. Putative microsatellite and low complexity regions were merged and overlaps with TE annotations removed using bedtools to create a set of repeat annotations.

Gene annotation

To increase our ability at predicting gene models for tissue-specific or lowly-expressed genes, we complemented our samples of adult head (n=1) and pupal leg (n=30) tissue with publicly available samples of antennal tissue (n=8) generated by the Institute of Applied Genomics (NCBI BioProject: PRJNA185987), as well as a publicly available whole-bodied sample generated through the 1KITE project (n=1, NCBI BioProject: PRJNA252331). Prior to gene model prediction, we performed data quality evaluations for all samples. For each sample, we first examined quality metrics generated using FastQC (Andrews et al., 2010), which provides information on base quality, as well as presence and extent of adapter contamination. As a complementary approach, we investigated the proportion of raw reads mapping to the generated genome assembly using HISAT v2.2.1 (Kim et al., 2019), finding high mapping rates (mean: 96.06%). We then combined and visualised the outputs for both sets of quality assessments using MultiQC v.1.29 (Ewels et al., 2016). Based on the results of the quality assessment, we removed adapter sequences, which can affect mapping rates and quality, and filtered reads by quality (PHRED score ≥ 15) and length (minimum length ≥ 50 bp) using fastp v0.20.1 (Chen et al., 2018).

The filtered reads were then used in conjunction with a protein database comprising arthropod-derived proteins from OrthoDB v12 (Kuznetsov et al., 2023), published hymenopteran proteins (Kapheim et al., 2015), and all proteins from the curated Swiss-Prot database (UniProt Consortium, 2023a) to predict gene models within the

soft-masked genome assembly using BRAKER3 v3.0.8 (Gabriel et al., 2024). Initial predictions from this approach were further processed to identify and correct putative incorrect gene models, such as by splitting up gene models with no overlap among their transcript “children” into separate gene models, and by removing transcripts with internal stop codons or missing start or stop codons. As a complementary approach, gene models were independently predicted via a second approach based upon orthology to the *O. bicornis* reference genome (iOsmBic2.1, GCF_907164935.1) using TOGA v1.1.7 (Kirilenko et al., 2023). The resulting predictions, based upon orthologous matches, were filtered to include only predictions classified as “intact”, “partially intact”, or “uncertain loss”. Genes with no overlap among any of their transcripts were split into separate genes. During gene model prediction, when transcripts from *O. bicornis* were aligned to the *O. cornuta* genome, some transcripts mapped to different chromosomal scaffolds or contigs than the rest of the transcripts from the same source gene. These inconsistently mapped transcripts were removed to make sure that each newly predicted gene model in *O. cornuta* was single-locus. As the orthology approach to gene prediction sometimes generated very short introns of 1-3bp in otherwise correctly predicted transcripts, we joined adjacent exons if they were separated by less than 4bp. Incomplete gene predictions with missing start or stop codons, or internal stop codons, were removed to retain only the highest quality predictions. We then computed overlap statistics between the two sets of annotations and complemented the BRAKER3 gene models with any additional transcript isoforms that were unique to the filtered TOGA predictions.

We inferred functional annotation from our translated, predicted proteins via homology and structural similarity to the publicly available annotation databases InterPro, Egg-Nog, BUSCO, dbCAN, SignalP, and UniProt (Blum et al., 2021; Huerta-Cepas et al., 2019; Sauria et al., 2015; Zheng et al., 2023; Cantalapiedra et al., 2021; UniProt Consortium, 2023b) using Funannotate v1.8.17 (Palmer and Stajich, 2020). Lastly, we quality checked our final genome annotation using Compleasm in “protein” mode (Nevers et al., 2025; Manni et al., 2021) and OMark v0.3.1 (Nevers et al., 2025). To complement our annotation with tRNA and rRNA gene models, we ran tRNAscan-SE (v2.0.12, (Chan et al., 2021) and Barnap (v0.8, eukaryotic mode: -k euk, (Seemann, n.d.), respectively. Finally, putative mitochondrial fragments integrated into the genome (i.e., NUMTs) were assessed by querying the *O. cornuta* nuclear genome assembly using BLASTN with the putative mitochondrial genome sequence previously identified in our assembly.

Methylation analysis

As methylation can play an important role in gene regulation, we examined our Nanopore data for base modifications to identify presence and extent of methylation patterns in the *O. cornuta* genome. We used dorado v0.9.0 (<https://github.com/nanoporetech/dorado>) to basecall the raw Nanopore data (pod5) with the base modifications 5mCG, 5hmCG, 6mA, 5mC, and 5hmC, using the super accurate model (dna_r10.4.1_e8.2_400bps_sup@v4.3.0) on a Geforce RTX3090 (with parameter `--modified-bases-threshold 0.30`). We extracted base modification signals using modkit pileup (v0.4.1, <https://github.com/nanoporetech/modkit>) from the resulting sorted BAM file. To assess how methylation patterns differed among genomic regions and features (e.g., exonic, intronic, intergenic), we first counted, for each of these feature types, the total number of sites that could potentially be methylated using bedtools intersect. Similarly, we then counted for each feature type, the number of sites that were either weakly methylated (sites covered by at least one read containing a methylated base) or highly methylated (sites with at least 20% of reads containing a methylated base). To account for large differences in the mean length of features, we summarised all counts in 100kb windows (bedtools map -o sum) across the genome, before dividing counts of weakly and highly methylated sites by the total number of sites that could potentially be methylated to generate percentages of methylation across all feature types. Throughout this approach, we processed 5mCG and 5hmCG methylation signals separately.

Analysis of synteny with *O. bicornis*

To further assess contiguity and completeness of our genome assembly, we examined collinearity, particularly synteny of chromosomal segments, between our *O. cornuta* genome assembly and a reference genome assembly of the closely related red mason bee, *O. bicornis*. To this end, we extracted the longest predicted protein isoforms from both assemblies and ran a protein-to-protein BLAST (BLASTP) search (Camacho et al., 2009) to identify putative homologues. The result of this BLAST search was then provided as input into MCScanX v1.0.0 (Wang et al., 2012), which identified collinear blocks between the two assemblies that were subsequently visualised using the web application SynVisio (Bandi and Gutwin, 2020).

Calling, filtering, and annotation of variants

Using the same quality control (QC) pipeline as that for the RNA-seq data, we assessed the raw sequencing data for the short-read whole-genome resequencing (WGS) samples for read-based quality metrics, as well as read mapping rates against our annotated genome assembly using bwa-mem v2.2.1 (Li, 2013). Summarisation of mapping statistics was performed using samtools stats (Danecek et al., 2021) and the generation of overview visualisations using MultiQC (Ewels et al., 2016). Raw

reads were quality filtered and adapters trimmed using fastp (Chen et al., 2018) with quality (PHRED score ≥ 15) and length (minimum length ≥ 50 bp) filters. For variant calling, filtered reads were first mapped against the genome assembly using bowtie2 v2.5.4 (Langmead and Salzberg, 2012), followed by variant calling using the BCFtools v1.21 mpileup-call pipeline (Danecek et al., 2021). Variants were filtered using VCFtools v0.1.17 (Danecek et al., 2011) with the following parameters: removal of indels; minimum and maximum mean read depth filters of 5 and 500, respectively; and a minimum quality score of 40. Additionally, a maximum of 20% of samples (i.e., two individuals) per site was allowed to have missing data. After filtering, the mean and median quality (QUAL) scores were 576.2 and 389, respectively. Filtered variants were used as input to a principal component analysis to investigate the genetic relationship between samples (Supplemental Figure S1). Lastly, variants were annotated with SnpEff v5.2f (Cingolani et al., 2012) using protein-coding sequence predictions from our annotation pipeline.

Inference of nucleotide diversity and linkage disequilibrium

Filtered variants were indexed using tabix (Li and Durbin, 2011) before running pixy v1.2.11 (Korunes and Samuk, 2021) to infer nucleotide diversity π and Watterson's θ across all chromosomes in both 100,000 and 10,000bp sliding windows. In addition to a sliding window approach, π was inferred collectively for each investigated genomic feature (exon, intron, intergenic regions, repeats and low complexity regions) to understand how different functional regions of the genome differ in nucleotide diversity. We inferred linkage disequilibrium decay curves from filtered variant calls using PopLDDecay v3.43 (Zhang et al., 2019). Inbreeding coefficients for population genomics samples were calculated using VCFtools -het.

Demography inference

To infer past changes of effective population size, we applied PSMC (Li and Durbin, 2011) to sequencing data from the 10 sequenced female adults (Supplemental Table S1). Variants were called and consensus sequences generated employing a pipeline that consisted of samtools v1.08 mpileup, bcftools v1.10.2 call, and vcfutils.pl (the 'vcf2fq' pipeline). To improve variant detection in low coverage samples we copied and merged forward and reverse reads. For PSMC, we used parameters informed by related species. Based on estimations from the buff-tailed bumblebee (Apidae: *Bombus terrestris*), a mutation rate (μ) of 3.6×10^{-9} (Liu et al., 2017) per base pair per generation was chosen, assuming one generation per year as the species is univoltine (Splitt et al., 2022; Tasei and Picart, 1973). Additionally, the recombination rate estimates for a closely related solitary bee (Megachilidae: *Megachile rotundata*, (Jones et al., 2019)), which is 1.02 cM/Mb and equivalent to a

per base recombination rate of 1.02×10^{-8} , was used, producing a recombination to mutation rate ratio of 2.5 (-r). We applied a maximum TMRCA of -7 (-t) and the atomic time interval pattern was set to “2+2+25*2+4+6” (-p), to increase comparability (Hilgers et al., 2025). To assess variance in effective population size estimates, we performed 100 bootstrap replicates per individual.

Data Availability Statement

Data and scripts required for reanalysis are retrievable from the following link: . The genome assembly and long-read fastq reads will be made publicly available on NCBI under the BioProject accession PRJNA1289724. DNA resequencing and RNA data will be made available under the BioProject accession PRJNA1289047.

Acknowledgments

We would like to thank the German Federal Environmental Foundation (Deutsche Bundesstiftung Umwelt, DBU) for supporting JSM with a doctoral scholarship (20021/743), and the Eva Crane Trust (ECTA_20210915, United Kingdom), for financial contribution to the *Osmia cornuta* population genomics. As part of the GEvol SPP 2349, this study was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – project number 503360601 (ES, XH, TJC, KN), and the GenEvo Research Training Group (GRK2526) – project number 407023052 (EAB, TJC). We thank Dr. Francieli das Chagas for confirmation on the identity of yellow cluster genes. We would also like to thank the environmental offices of Baden-Württemberg, Bavaria, Berlin, Bremen, Hesse, Mecklenburg-Western Pomerania, Lower Saxony and Saxony, and especially Dr. Peer Schnitter (Landesamt für Umweltschutz (LAU), Saxony-Anhalt), for collection permits. For caretaking of a nesting site, logistics and provision of flower resources, we thank Joachim and Petra Stolle. We also thank the molecular lab at the LIB, in particular Anja Bodenheimer, for technical support with ONT sequencing. We would like to thank the HPC facility at JGU for access to computational resources. We also thank the HPC facilities at the LIB for computation support and the LIB for startup-funds for a GPU server (ES). For granting copyright to images of *Osmia cornuta*, we thank Michael and Mandy Fritzsche from Apidarium ().

Author Contributions

ES, JSM, and TJC conceived the study. ES generated the long-read data. JSM and TJC generated whole-genome and RNA-seq data, KN and AH generated RNA-seq data for which ES and XH performed sampling. ES and XH performed the repeat inference. ES performed the methylation detection. ES and JSM did the contamination screening. ES generated the genome assembly. JSM generated the

genome annotation. JSM performed synteny and overlap inferences. JSM performed statistical analyses and generated all figures. JSM performed population genetic analyses with EAB contributing the PSMC analysis. JSM wrote the draft, while ES and TJC contributed to the final manuscript. All authors gave final approval for publication.

3.5 Supplementary Material

Computational validation of species identification

Species identification for all samples was initially performed based on morphological characteristics but later confirmed computationally. To that end, we first identified the locus of the *O. cornuta* cytochrome c oxidase subunit 1 (COX1) sequence within our genome assembly via orthology with the COX1 sequence of the *Osmia bicornis* reference genome (iOsmBic2.1, GCF_907164935.1) using BLASTN (v2.14.1+, (Camacho et al., 2009)). The *O. cornuta* COX1 orthologue sequence was then extracted from this locus using samtools faidx (Danecek et al., 2021) and searched against COX1 sequences housed in the database of Barcode of Life Data Systems (BOLD, queried on the 3rd of October, 2024, (Ratnasingham and Hebert, 2007)), validating that the male, which we used to generate the genome assembly was correctly identified as *O. cornuta*. For all other DNA- and RNA-seq samples, reads were mapped against the *O. cornuta* genome assembly using STAR (v2.7.11a, (Dobin et al., 2013)) and for each sample a consensus sequence of matches against the identified *O. cornuta* COX1 locus was constructed using samtools consensus (Danecek et al., 2021). The resulting COX1 sequences were then searched against BOLD using their web API to confirm species identifications.

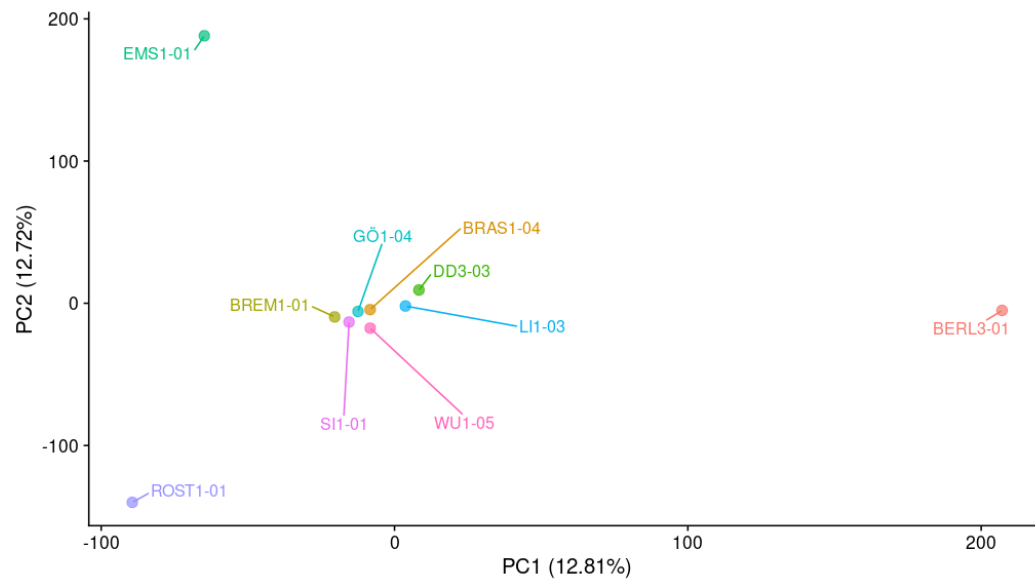


Figure S1: Genetic relationship among sampled *Osmia cornuta*. Scatterplot displaying the first two principal components calculated from a principal component analysis based on single nucleotide polymorphisms called within a population of *Osmia cornuta* sampled across different locations in Germany. The x-axis shows the first principal component and the total variance explained by it, while the y-axis shows the second principal component and the variance it explains. Each coloured and labeled dot represents a single female *O. cornuta*.

Table S1: Sample site information for *Osmia cornuta* used for population genomics. The table is showing for each *O. cornuta* that was considered for the population genomics analyses, the sample identifier, the date of sampling, sampling site coordinates, inbreeding estimates calculated from expected versus observed homozygosity, and the total count of raw reads generated for each sample.

Sample ID	Sampling Date	Latitude	Longitude	Altitude [m]	Inbreeding Coefficient F	Raw Read Count
BERL3-01	12/04/23	52.60	13.40	49	0.21189	38,517,412
BRAS1-04	29/03/23	52.31	10.52	69	0.10376	28,428,320
BREM1-01	10/04/23	53.07	8.80	8	0.19567	29,153,738
DD3-03	05/05/22	50.88	13.68	387	0.18829	36,106,378
EMS1-01	10/04/23	52.36	7.30	41	0.23890	36,025,098
GÖ1-04	23/04/22	48.71	9.63	338	0.11099	26,511,816
LI1-03	02/05/22	50.39	8.05	129	0.12830	31,449,700
ROST1-01	11/04/23	54.09	12.09	26	0.19660	34,304,438
SI1-01	23/04/22	49.22	8.79	197	0.04068	31,255,604
WU1-05	27/04/22	49.77	9.93	233	0.11870	35,203,272

Table S2: Comparison of assembly and annotation statistics with reference species.
The table is showing assembly and annotation statistics across several categories for the herein presented genome for *Osmia cornuta*, in comparison to statistics from pre-published, high-quality RefSeq genomes for *Osmia bicornis* and *Osmia lignaria*.

Category	<i>Osmia cornuta</i>	<i>Osmia lignaria</i>	<i>Osmia bicornis</i>
Assembly level	Chromosome	Chromosome	Chromosome
Genome coverage	40.22	137	93
Assembly length [Mb]	647.7	498.5	223
Contig count	727	236	563
N50 [Mb]	3.94	14.2	9.1
GC content [%]	34.8	39	39.5
Gene count	12144	13795	12706
BUSCO completeness [%]	99.78	97.9	98.6

Chapter Three: Transcriptomic changes and genetic architecture underlying response to elevated overwintering temperatures in *Osmia*

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In preparation

4.1 Abstract

Rising winter temperatures as a consequence of climate change pose a substantial threat to the ability of organisms to maintain essential dormant states. For wild bees, such as solitary bees, which provide essential pollination services yet spend much of their lives in dormant stages, understanding how elevated temperatures affect the dormancy phenotype is critical for predicting population responses. However, the molecular and genomic bases of thermal responses for many solitary bees, especially during overwintering, remain poorly understood. Here, we present the first transcriptome-wide analysis of how sustained exposure to elevated overwintering temperatures informed by current climate change scenarios influences solitary bee gene expression. To achieve this, we exposed *Osmia bicornis* and *Osmia cornuta* females to temperature regimes reflecting IPCC climate projections (SSP2-4.5 and SSP5-8.5) over the 14-week time course. We identified extensive transcriptional changes in the brain with thousands of temperature-responsive genes (*O. bicornis*: 4,959; *O. cornuta*: 3,725), with 44-58% of these genes showing time-dependent responses highlighting the importance of how temperature may affect different points during dormancy progression. Functional analyses of temperature-responsive genes revealed a progressive temporal shift toward elevated oxidative phosphorylation and mitochondrial activity, consistent downregulation of developmental pathways, and altered protein and fatty acid metabolism, indicating that thermal exposure is associated with key changes in metabolism and development. As sexes of mason bees display differences in dormancy duration, which may be impacted by temperature exposure, we also examined males at a single intermediate time point (7 weeks) revealing that sex-specific transcriptional differences were minimal due to both sexes responding to temperature in a conserved manner. To gain further insight into how temperature-responsive genes are evolving in contemporary populations, we performed population genomic-based analyses of 40 *O. bicornis* and 30 *O. cornuta* males revealing panmictic breeding populations with higher nucleotide diversity in *O. bicornis*. Signatures of recent or ongoing positive selection among temperature-responsive genes were detected in both species, however, the relationship between gene expression and signatures of selection differed between the two species whereby a negative correlation was only observed in *O. cornuta* meaning genes with elevated expression generally had lower signatures of genetic adaptation, highlighting the complex nature of how phenotypic plasticity and genetic adaptation may contribute to thermal responses. Overall, our findings provide an important insight into the plastic and evolutionary responses of solitary bee species, which has

implications for the conservation and sustainability of essential ecosystem services provided by these key pollinators.

4.2 Introduction

Wild bees are an important group of pollinators, providing essential pollination services crucial to both ecosystem stability and agricultural productivity (Kremen et al., 2007; Potts et al., 2016; Kleijn et al., 2015; Kremen et al., 2004). However, recent population declines have raised urgent concerns about the long-term security of these services (Gallai et al., 2009; Potts et al., 2016; Lippert et al., 2021; Reilly et al., 2020; Hallmann et al., 2017; Powney et al., 2019). Among the multiple stressors driving these declines, ongoing climatic change is increasingly being highlighted as a major factor due to its progressively accumulating and difficult-to-predict long-term consequences (Walther et al., 2002; Harvey et al., 2023; Vanbergen and the Insect Pollinators Initiative, 2013; Scaven and Rafferty, 2013; Bale and Hayward, 2010).

The effects of ongoing climate change are predicted to be particularly impactful for many temperate species of wild bees, which like other insects, are ectotherms and, therefore, heavily reliant on ambient temperatures for the regulation of important biological processes, including metabolism (Sinclair et al., 2012), development (Buckley et al., 2017), and locomotion (Terblanche et al., 2024), as well as timing of key life stage events (Kellermann and Heerwaarden, 2019; González-Tokman et al., 2020). One such event is seasonal dormancy, which wild bees can use to survive during resource-scarce periods and avoid unfavourable environmental conditions (Danforth et al., 2019; Williams et al., 2015). A prominent component of this seasonal dormancy is diapause, which is a dormant state characterised by arrested development and reduced metabolism (Denlinger, 2002). For bees that overwinter, they are dependent on low ambient temperatures to aid metabolic suppression, and thus, reduce resource consumption during extended periods of non-feeding, when survival hinges on stored reserves accumulated prior to dormancy entry (Gillooly et al., 2001; Hahn and Denlinger, 2007). Due to this reliance, warmer winters and more frequent extreme weather periods are expected to lead to fitness-reducing consequences (Kingsolver et al., 2013; Sales et al., 2021). The regulatory mechanisms governing dormancy physiology, influenced by environmental cues such as ambient temperature, are finely tuned to historical climatic conditions (Denlinger et al., 2012; Denlinger, 2022). However, the pace of contemporary climate change far exceeds historical rates of change (Intergovernmental Panel on Climate Change, 2021), raising critical questions about whether dormant states and associated underlying mechanisms in bees can respond to elevated overwintering temperatures through phenotypic plasticity or whether populations possess sufficient genetic variation to allow for selection to act upon (Sgrò et al., 2011; Hoffmann et al., 2023). With respect to phenotypic plasticity, transcriptional plasticity, which is the

ability to modulate gene expression in response to environmental variation, may provide a first line of defence against thermal stress, allowing organisms to detect and interpret cues, and subsequently adjust the expression of genes underlying key physiological processes and maintain homeostasis under non-optimal conditions to mitigate impact (López-Maury et al., 2008). However, as transcriptional responses are energetically costly (Lynch and Marinov, 2015), and thus may range from insufficient to potentially maladaptive, they may instead accelerate fitness declines (Wang and Zhang, 2011; Wagner, 2005). Therefore, as a first step to understanding plastic responses, identifying which genes and pathways respond to elevated overwintering temperatures can help reveal the physiological systems most affected by thermal exposure (DeBiasse and Kelly, 2016; Logan and Cox, 2020). Moreover, characterizing transcriptional responses provides the foundation for assessing whether natural populations harbour sufficient standing genetic variation in stress-responsive pathways, a prerequisite for evolutionary rescue in rapidly changing environments (Carlson et al., 2014; Supple and Shapiro, 2018). Therefore, understanding both the molecular and genetic bases of thermal responses during dormancy is essential for predicting population persistence under ongoing climate change.

A group of wild bees where our knowledge of molecular biology and genomics requires urgent improvement is solitary bees. Solitary bees comprise over 85% of extant bee species (Danforth et al., 2019), including differences in developmental stage of dormancy, duration of dormancy, as well as the environmental cues that either initiate or terminate dormant states (Santos et al., 2019). In general, our molecular knowledge of these dormant phenotypes in bees is largely limited due to them being understudied in comparison to social taxa where dormancy is either absent or limited to female queens (Santos et al., 2019; Colgan et al., 2019). In addition, solitary bees may be more vulnerable to ambient temperature changes during overwintering as they lack colony-level responses, including coordinated behavioural thermoregulation, which can buffer individuals from thermal extremes in social species (Straub et al., 2015; Jones et al., 2004; Zhao et al., 2021). Research, exploring the molecular basis of thermal responses in overwintering solitary bees is thus urgently required (Colgan et al., 2026).

One group of solitary bees that have received increasing attention for studying environmental pressures during overwintering periods are mason bees (genus: *Osmia*) (Bosch, 1994; Bosch and Kemp, 2004; Fliszkiewicz et al., 2012; Schenk et al., 2018b; Albacete et al., 2023; Möllmann et al., 2025). This taxon-rich group comprises approximately 350 described species that are widely distributed across temperate climates with most species showing a univoltine life cycle and undergoing seasonal summer dormancy as larvae before spinning cocoons and completing

metamorphosis (Banaszak and Romasenko, 1998; Rightmyer et al., 2013). They then enter winter dormancy as imagos, emerging the following spring when favourable conditions return for mating, nest building, and offspring provision (Splitt et al., 2022). As predominantly above-ground cavity nesters (Bosch et al., 2001; Praz et al., 2008), variation or increases in ambient overwintering temperatures are expected to have a particularly strong influence on factors maintaining dormancy (Beer et al., 2019). In addition, mason bees are increasingly being employed as pollinators in agriculture, particularly for fruit crops, adding to the applied requirement to understand how they are affected by elevated overwintering temperatures predicted under climate change due to the impact declines may have on crop production and sustainability (Gruber et al., 2011; Sedivy and Dorn, 2014; Fliszkiewicz and Giejdasz, 2023).

Previous studies on mason bee species have demonstrated that elevated overwintering temperatures induce fitness-relevant phenotypic changes, such as early emergence, increased fat reserve depletion and decreased emergence weight, as well as increased post-emergence mortality (Bosch, 1994; Bosch and Kemp, 2004; Fliszkiewicz et al., 2012; Schenk et al., 2018b; Albacete et al., 2023; Kemp and Bosch, 2005; Möllmann et al., 2025). In comparison to increased post-emergence mortality (Kemp and Bosch, 2005), mortality during dormancy was not significantly affected by elevated overwintering temperatures (Möllmann et al., 2025). However, the same study observed that individuals exposed to elevated temperatures emerged considerably earlier, a potential response to offset energy deprivation (Möllmann et al., 2025). Collectively, these results indicate the vulnerability of mason bees to elevated temperatures during dormancy and suggest that plastic traits may serve as a primary response mechanism. However, in comparison, little is known about the molecular pathways underlying these plastic responses, and whether hidden phenotypic changes, unobservable at the organismal level, are occurring. Similarly, it is currently unknown whether mason bees harbour genetic variation to allow for selection to act upon to respond to ongoing and future climatic challenges that may influence their overwintering physiology and strategies. In the closely-related leafcutter bee *Megachile rotundata*, an above-ground nester that spends winter dormancy in the larval stage, molecular-based studies have revealed transcriptomic changes after short-term thermal exposure or after exposure to increased temperature fluctuations during overwintering (Xu and James, 2012; Torson et al., 2015a; Torson et al., 2017; Cambron-Kopco et al., 2022; Yocum et al., 2005). However, these transcriptomic studies were generated to either focus primarily on short-term thermal exposures or perform comparisons between dormant states rather than

sustained warming meaning our knowledge of how overwintering bees respond to field-relevant projected climate change scenarios is currently limited.

To fill this gap, we present the first transcriptome-wide analysis of two mason bee species, *Osmia bicornis* and *Osmia cornuta*, following chronic exposure to elevated temperatures during dormancy. More specifically, we exposed overwintering bees over a fourteen-week time course to temperature regimes, which incorporate diurnal and seasonal fluctuations, and reflect the most recent climate projections (SSP2-4.5 and SSP5-8.5), developed by the IPCC (Intergovernmental Panel on Climate Change, 2021). As part of our analysis, we measured transcriptional changes in the brain, the primary organ involved in the processing of environmental signals, as well as the coordination of the thermal response (Gallio et al., 2011). With over 3,000 temperature-responsive genes identified in brain tissue in both species, our findings reveal extensive transcriptional reorganisation in response to prolonged warming and demonstrate that the molecular consequences of sustained thermal exposure during dormancy are broader than previous short-term temperature exposure studies would suggest. Furthermore, by integrating population genetic datasets for both species, we assessed patterns of genetic variation and signatures of selection on a genome-wide level, as well as in temperature-responsive genes, providing insights into the adaptive potential of these essential pollinators to respond to current as well as future climate change.

4.3 Results

Changes in gene expression linked to increased metabolism, olfactory activation and reduced developmental pathway activity as a result of elevated overwintering temperatures

To investigate transcriptomic changes in the brains of overwintering mason bees due to the effect of elevated temperatures, we exposed 36 overwintering females of *Osmia bicornis* to one of three field-based overwintering temperature regimes (each $n = 12$) that included seasonal and diurnal variation and are informed by current overwintering temperatures as well as those predicted under an intermediate (SSP2-4.5) as well as a more extreme climate change scenario (SSP5-8.5). To additionally highlight changes in temperature responsiveness across the progression of overwintering, we collected individuals from the experiment at one of three time points (after 1 week, 7 weeks or 14 weeks; for each temperature/time point combination: $n = 4$). Using a likelihood ratio test (LRT), we found a large number

($n = 4,959$) of differentially expressed genes (DEGs, False Discovery Rate < 0.05) in response to elevated overwintering temperatures. However, a large portion of DEGs showed a significant interaction with the time point of sampling (44.1%, $n = 2,189$ DEGs), indicating that the effect of elevated temperature differs across experimental time and dormancy progression. Similarly, in a principal component analysis using all expressed genes, a clustering of samples based upon temperature or time was only observed after accounting for variation of the other respective variable (i.e., samples clustered by temperature regime after accounting for time of sampling; Figure 4.1) with a particularly clear separation of the baseline and the higher temperature group based on the first and second principal components, which collectively, explained 40% of the variance.

To gain a more detailed understanding of the thermal response of gene expression at each time point and to account for the large number of DEGs changing expression not only based upon thermal exposure regime but also based upon sampling time point, we analysed the data at each distinct sampling time point independently. We found 447 temperature-sensitive DEGs after one week, with increases to 3,062 and 2,783 DEGs detected after seven and 14 weeks, respectively (LRT, FDR < 0.05). In line with this, hierarchical clustering of variance-stabilised read counts of DEGs showed distinct expression clusters of consistent over- or underexpression at the seven and 14 week time points, which were slightly less pronounced after only one week of temperature exposure (Figure 4.1).

To determine potential functional changes associated with temperature exposure, we then performed gene set enrichment analyses (GSEA), finding a large number of distinct biological terms and pathways over- or underexpressed in response to elevated temperatures at all three time points (Figure 4.2; FDR < 0.05 ; control vs highest temperature treatment). More specifically, we found a number of terms associated with putative metabolic changes across all time points, yet, the exact processes differed between time points. For example, after one week, we found genes with reduced expression as enriched with terms related to cytosolic translation and oxidative phosphorylation, while at the same time point, genes with increased expression were enriched for terms related to ribosome production and protein turnover (Figure 4.2). Additionally, we found expression changes in genes related to fatty acid metabolism. After seven weeks, we found the same pattern that genes with reduced expression were enriched for terms related to cytoplasmic translation, but, in comparison to the previous time point, we found the inverse pattern whereby genes linked to protein degradation and processing were reduced instead of elevated (Figure 4.2). Here, we also found genes with elevated expression enriched for mitochondrial activity. After 14 weeks, genes with reduced expression were again

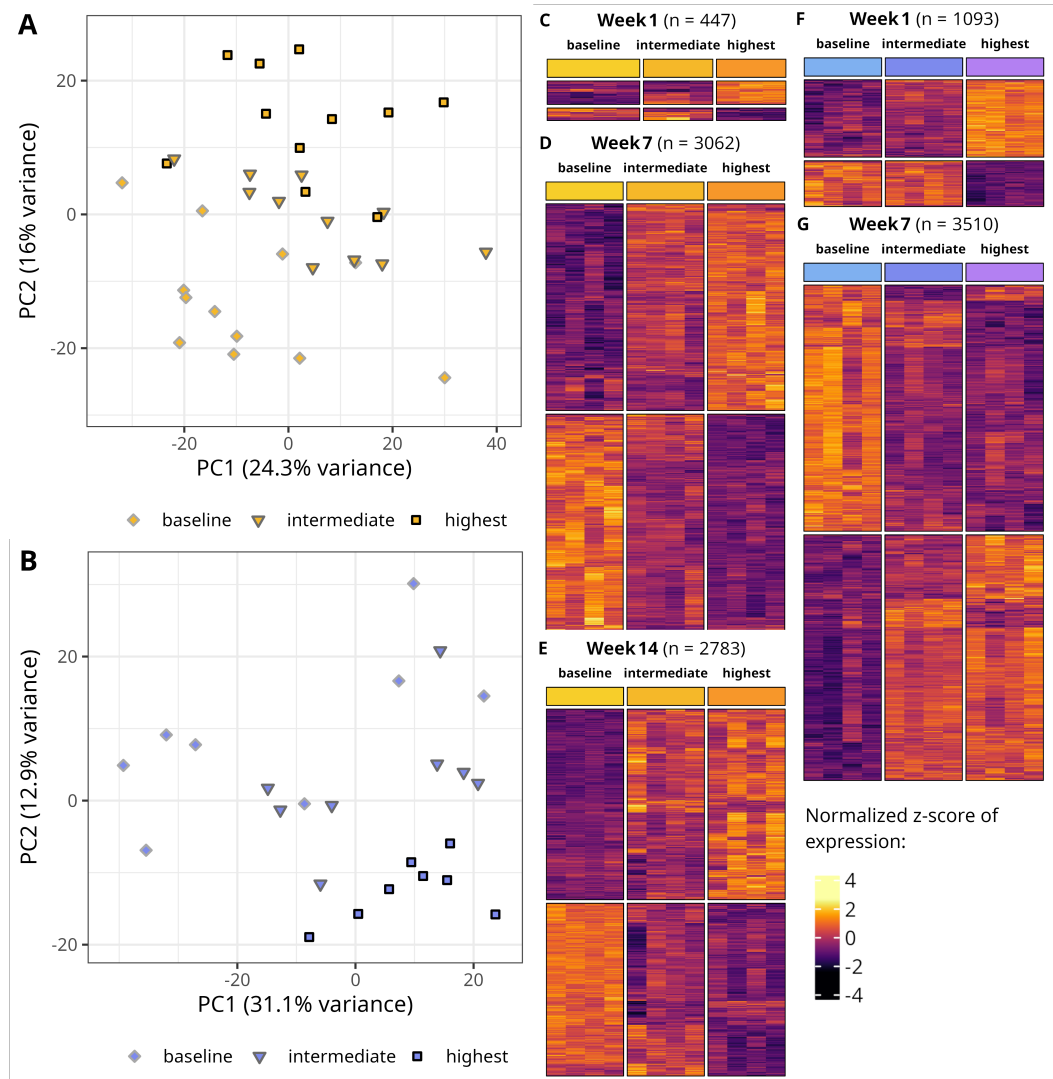


Figure 4.1: Time-dependent gene expression changes in response to elevated temperatures in mason bees. (A, B) Scatter plots depicting for both species (A = *Osmia bicornis*, B = *Osmia cornuta*) expression patterns in response to elevated overwintering temperatures, based upon per-gene read counts, reduced in dimensionality via variance stabilisation followed by reduction of time point-related variation and principal component analysis. The first two principal components (“PC”) in terms of the highest percentage of variance explained are plotted on the x- and y-axes, respectively. Temperature treatment groups are marked using different shapes and contrast strengths. (C-G) Heatmaps showing per-time point expression patterns in response to elevated overwintering temperatures based upon variance-stabilised expression matrices (C-E = *O. bicornis*, F,G = *O. cornuta*), filtered to contain only differentially expressed genes (False Discovery Rate < 0.05) at each time point (C = week 1, D = week 7, E = week 14, F = week 1, G = week 7). Columns represent individual samples and rows represent genes. Column labels and colours depict temperature treatment groups.

enriched for terms related to cytoplasmic translation but additionally, elevated genes were enriched for oxidative phosphorylation and mitochondrial activity. Furthermore, there were also changes in gene expression related to fatty acid metabolism. In addition to patterns that changed across time, we also found several consistent patterns across time points, most notably an enrichment in the expression of neural developmental pathways and an enrichment of olfactory activity within upregulated genes (Figure 4.2). Lastly, some patterns were restricted to individual time points, such as the downregulation of genes associated with oogenesis and a few other reproductive terms after 14 weeks.

Only minor sex-specific differences in the transcriptomic response to elevated overwintering temperatures

As sexes may differ in their response to elevated temperatures, we compared the brain gene expression of female and male *O. bicornis*. For this, we compared 12 females with 12 males, all of which were sampled at the intermediate time point (i.e., 7 weeks) and were part of the same exposure experiment. Individuals of both sexes were exposed to the same three temperature profiles as outlined above with four biological replicates sampled per sex and temperature-profile. We identified only five genes where response to temperature was significantly different across the two sexes, showing mixed direction of expression (LRT, FDR < 0.05). As this gene number is too low for enrichment-based analyses, we attempted to discern functional information for these genes directly from protein-protein sequence similarity-based comparisons with the fruit fly, *Drosophila melanogaster*. Through this approach, we identified these genes as putative homologues of hulk, pastel, cinnabar (encodes a kynurenine 3-monooxygenase), Aasdh (encodes an aminoadipate-semialdehyde dehydrogenase), as well as of an unnamed gene that encodes a triacylglycerol lipase. These genes are related to diverse biological processes, such as lipid catabolism, eye colour determination, long-term memory, and transcriptional regulation.

Observation of similar patterns in closely related *Osmia cornuta*

To determine whether temperature exposure leads to similar transcriptional responses across mason bee species, we analysed the gene expression profiles of brains of the closely related *Osmia cornuta* exposed to the same three temperature regimes. Our dataset consisted of 24 females, sampled at two time points (one and seven weeks post-exposure start), identical to the first two sampling time points of *O. bicornis*. We only used these first two time points for comparison between the species due to *O. cornuta* emerging 4–6 weeks earlier than *O. bicornis* in our experiment (Möllmann et al., 2025), which reflects the natural differences in phenology between these two species. Here, we collectively found 3,725 genes significantly differentially

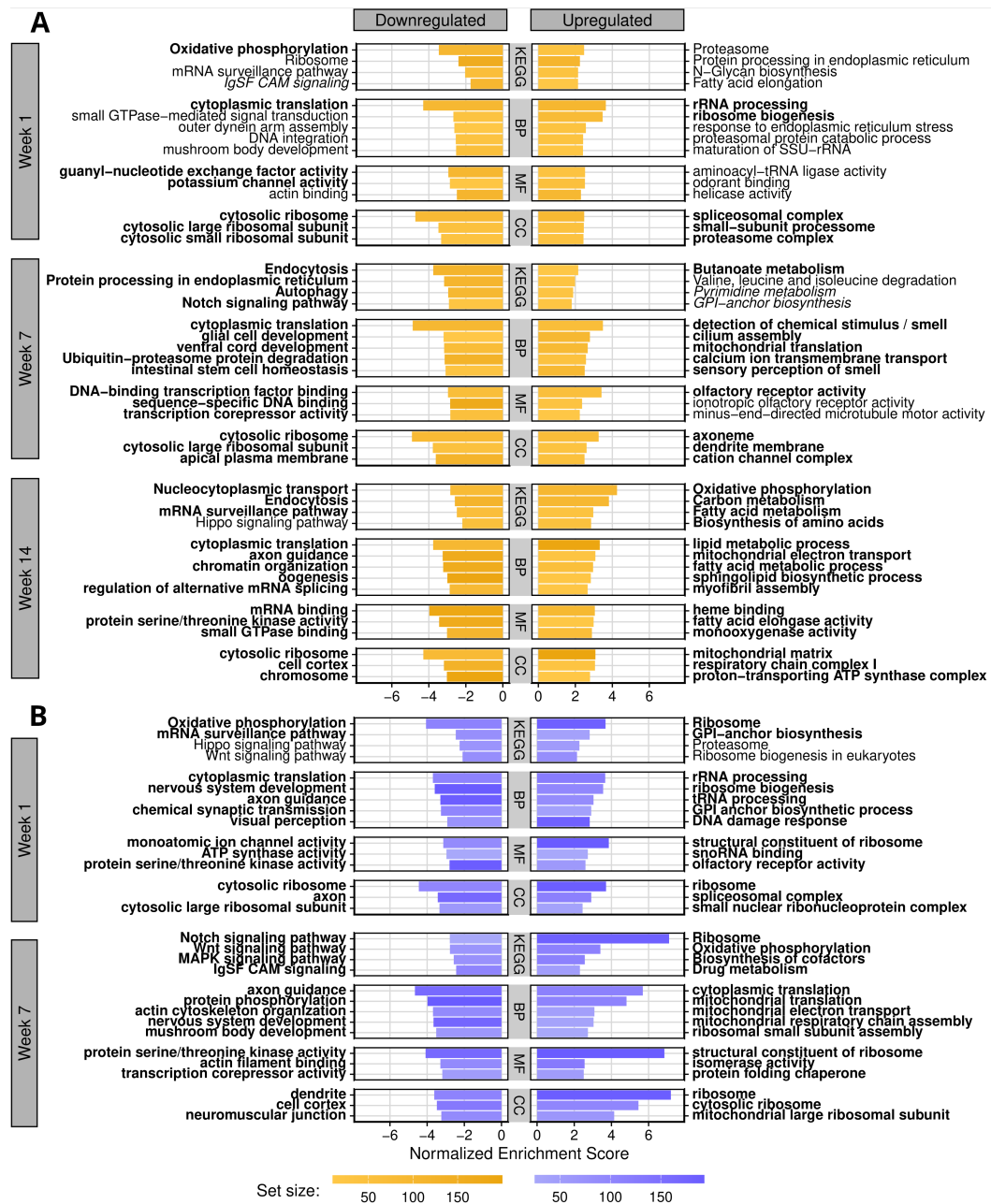


Figure 4.2: Temporal dynamic gene expression response of metabolic and developmental pathways under elevated overwintering temperatures in mason bees. (A, B) For each species (A = *Osmia bicornis*, B = *Osmia cornuta*), the Gene Ontology (GO) terms and KEGG pathways with the most extreme normalised enrichment scores in a gene set enrichment analysis are shown in a multi-faceted bar plot. Individual facet rows represent the type of gene set that was used (BP = biological process, MF = molecular function, CC = cellular component, KEGG = KEGG pathways). Bold terms are significant terms at a False Discovery Rate (FDR) < 0.01, with terms in regular script type significant at FDR < 0.05 and italic terms are significant at FDR < 0.1. The colour gradients depict the number of genes per respective gene set with each species indicated by an individual colour (orange = *O. bicornis*; blue = *O. cornuta*).

expressed in response to elevated temperature exposure using all samples (LRT, $FDR < 0.05$). To test whether the reduced number of DEGs (1,234 fewer DEGs than *O. bicornis*) was due to a smaller sample size, we reran the differential gene expression inference in *O. bicornis* after removal of the third time point and, found a more comparable number of DEGs ($n = 3,379$ instead of 5,000 DEGs) to *O. cornuta*. Similar to *O. bicornis*, we tested for an interaction between temperature and time for *O. cornuta* and found 2,149 DEGs (57.7%) significant for an interaction effect. After correcting for the effect of time, we again found a clear clustering of temperature groups in a PCA using all 24 samples (Figure 4.1B), again prompting us to analyse the time points independently. Using this approach, after one week, we found 1,093 DEGs, which was two times more compared to *O. bicornis*, while after seven weeks, we found 3,510 DEGs, which was slightly more than in *O. bicornis* (Figure 4.1F-G).

To discern putative functional changes that are consistent across species in response to temperature exposure, we also performed a GSEA based on differentially expressed genes in *O. cornuta*. Similar to *O. bicornis*, after one week exposure, we found genes with reduced expression in response to temperature exposure to be enriched for terms related to oxidative phosphorylation and cytoplasmic translation. In addition, we found genes with increased expression enriched for terms associated with ribosome production. Furthermore, after seven weeks, we found that genes with increased expression were enriched for terms related to oxidative phosphorylation, mitochondrial and ribosomal activity, which was similar to enriched terms detected in *O. bicornis* at seven and fourteen weeks post-exposure. We also found that genes involved in developmental pathways were significantly downregulated at both time points in *O. cornuta*. Unlike *O. bicornis*, we did not detect enrichments or depletions for genes linked to olfactory activity in *O. cornuta*. Similar to before, we also investigated sex-biased temperature responses and found only ten genes (LRT, $FDR < 0.05$) where the response to elevated overwintering temperatures significantly differed between the sexes, again with mixed direction of expression. Based on protein-protein orthology, we found no overlap between these genes and the genes in *O. bicornis* where the effect of temperature differed significantly between the sexes.

Whole-genome resequencing supports panmictic populations in both species but species differences in nucleotide diversity and selection patterns

As differences in gene regulation in response to elevated overwintering temperatures may be attributable to genetic differentiation between individuals, we generated whole-genome resequencing datasets for males of both species (*O. bicornis*: $n = 40$; *O. cornuta*: $n = 30$), sampled from across Germany (minimum distance between

sampling sites = 32 km) to test for the presence or absence of population-level differentiation, quantify the genetic variation harboured in thermal-responsive genes, and determine signatures of recent or ongoing positive selection (Figure 4.3A-B). Therefore, we first assessed the population structure of each species using principal component and admixture-based analyses based on linkage-disequilibrium pruned ($LD \leq 0.2$) single nucleotide polymorphisms (SNPs). We found no clear clustering of samples based on the principal component analyses (PCA, first two principal components, which collectively explained 5.9% (*O. bicornis*) and 8.2% (*O. cornuta*), respectively, displayed in Figure 4.3C-D). This finding was further supported by co-ancestry-based analyses using admixture, which found the strongest statistical support for a single ancestral population within each species ($k = 1$, Figure 4.3E), indicating a panmictic breeding population for each species across their sampled ranges.

However, as singular large-scale genetic differentiation signals, such as those from chromosomal rearrangements, are lost during LD-pruning, we also performed unpruned PCAs for each chromosome in both species to determine localised regions of genetic differentiation. We found two chromosomes in *O. bicornis* (chromosomes 4 and 10) with strong differentiation between samples in the top principal component (chromosome 4: 20.8% of variance explained; chromosome 10: 15.1% of variance explained). Notably, this substructure disappeared after LD-pruning on *O. bicornis* chromosome 10, but not on chromosome 4, suggestive of the presence of extended blocks of increased linkage disequilibrium in some individuals within the population (Supplemental Figure S3).

To determine the genetic variation found in temperature-responsive genes, we first calculated genome-wide nucleotide diversity (π) in non-overlapping 10 kb-sized windows. We found a higher median nucleotide diversity in *O. bicornis* ($\pi = 0.0028$, Supplemental Figure S2A) compared to *O. cornuta* ($\pi = 0.0013$, Supplemental Figure S2B). We also found evidence of temperature-responsive genes overlapping with windows of increased or decreased nucleotide diversity in both species. A significant genome-wide association between temperature-responsive genes and windows of extreme diversity (either below 5th or above 95th percentile based on π), however, was detected only for regions of decreased diversity (Fisher's exact tests, $P < 10^{-7}$). For comparisons against pre-existing population genetic datasets in related species, using the same approach, we also calculated Watterson's theta θ_w for 10 kb sliding windows, again finding lower levels in *O. cornuta* ($\theta_w = 0.0013$) compared to *O. bicornis* ($\theta_w = 0.0036$). Homologues in *O. bicornis* of two chromosomes, previously reported to have above-average nucleotide diversity π in *O. cornuta* (chromosomes 9 and 15, (Möllmann et al., 2026)), also showed significantly increased nucleotide

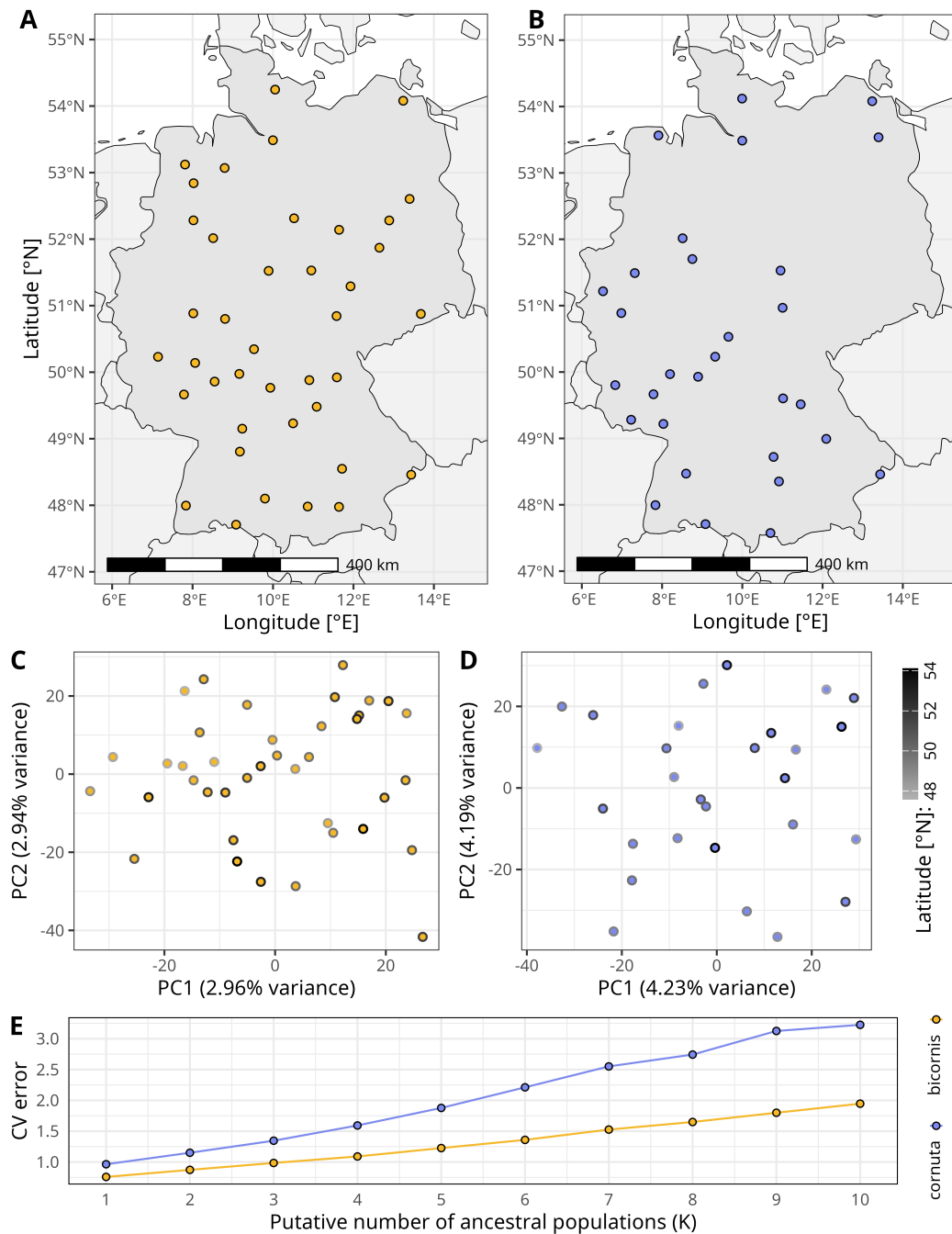


Figure 4.3: Whole genome resequencing of mason bees reveals weak population structure. (A,B) Map of collection sites of wild-caught (A) *Osmia bicornis* and (B) *Osmia cornuta* males. Each data point represents a single sampling site and a single individual. (C,D) Principal component analyses of linkage-disequilibrium pruned ($LD \leq 0.2$) genome-wide SNPs (C = *O. bicornis*, D = *O. cornuta*), depicting the top two principal components (PC) in terms of explained variance. Each data point represents a single, male individual. The greyscale colour surrounding individual data points shows the latitude that an individual was sampled at. (E) A line graph showing for each species the cross-validation (CV) error against the number of putative ancestral populations (K) in an ADMIXTURE-based analysis using linkage disequilibrium-pruned ($LD \leq 0.2$) genome-wide SNPs. Each species is represented by an individual colour (orange = *O. bicornis*; blue = *O. cornuta*).

diversity in *O. bicornis* (chromosomes 8 and 16, Wilcoxon rank-sum test comparing π within focal chromosomes to genome-wide background, $p < 0.01$).

To understand whether temperature-responsive genes show genomic signatures consistent with recent or ongoing positive selection, we first detected and quantified genome-wide loci with signatures of extended haplotype homozygosity, which are produced as a consequence of complete or incomplete selective sweeps. For this, we calculated the number of segregating sites by length (nSL) per polymorphic site, averaged across 10 kb-sized windows by taking the mean of the absolute nSL score per SNP, and identified several regions showing elevated absolute nSL ($|nSL|$) in the genomes of both species (Figure 4.4A-B). We found an overlap of temperature-responsive genes with windows of increased $|nSL|$ (above the 95th percentile), but no significant genome-wide association was detectable (Fisher's exact test, $P > 0.05$). However, through a more detailed investigation, using the maximum $|nSL|$ ($\max(|nSL|)$) score for each gene, we found a significant negative association between $\log_2(\text{Fold Change})$ and $|nSL|$ among temperature-sensitive genes (Figure 4.4C-G), suggesting that genes upregulated in response to elevated temperatures were less likely to be found in genomic regions with signatures of ongoing or recent selective sweeps than those that were downregulated. It should be noted, however, that this trend was only consistently significant across all sampled time points in *O. cornuta* (linear regression, $P < 10^{-8}$), whereas in *O. bicornis*, a statistically significant trend was only observed at the intermediate time point (week 7, linear regression, $P < 10^{-3}$). In addition, the effect size was extremely low in *O. bicornis* ($R^2 = 0.003$) compared to *O. cornuta* ($R^2 > 0.03$), adding to the evidence of species-level differences in recent selection among temperature-sensitive genes.

Signatures of recent selection among temperature-sensitive genes are most strongly linked to signal transduction

To investigate which processes temperature-responsive genes with signatures of recent selective sweeps were associated with, we used the same gene-level $\max(|nSL|)$ scores as before and performed one-sided gene set enrichment analyses. In *O. bicornis*, we found no significant terms ($\text{FDR} < 0.1$) at the first time point (week 1), but several significant terms at the other two time points (week 7 and week 14), whereas in *O. cornuta* all time points (week 1 and week 7) showed significant associations to several terms or pathways (Figure 4.5). In both species, however, among time points with significant terms, we found the terms with the strongest enrichment signals (normalised enrichment score, NES) to be associated with neural signal transduction processes (Figure 4.5). As a notable difference between the species,

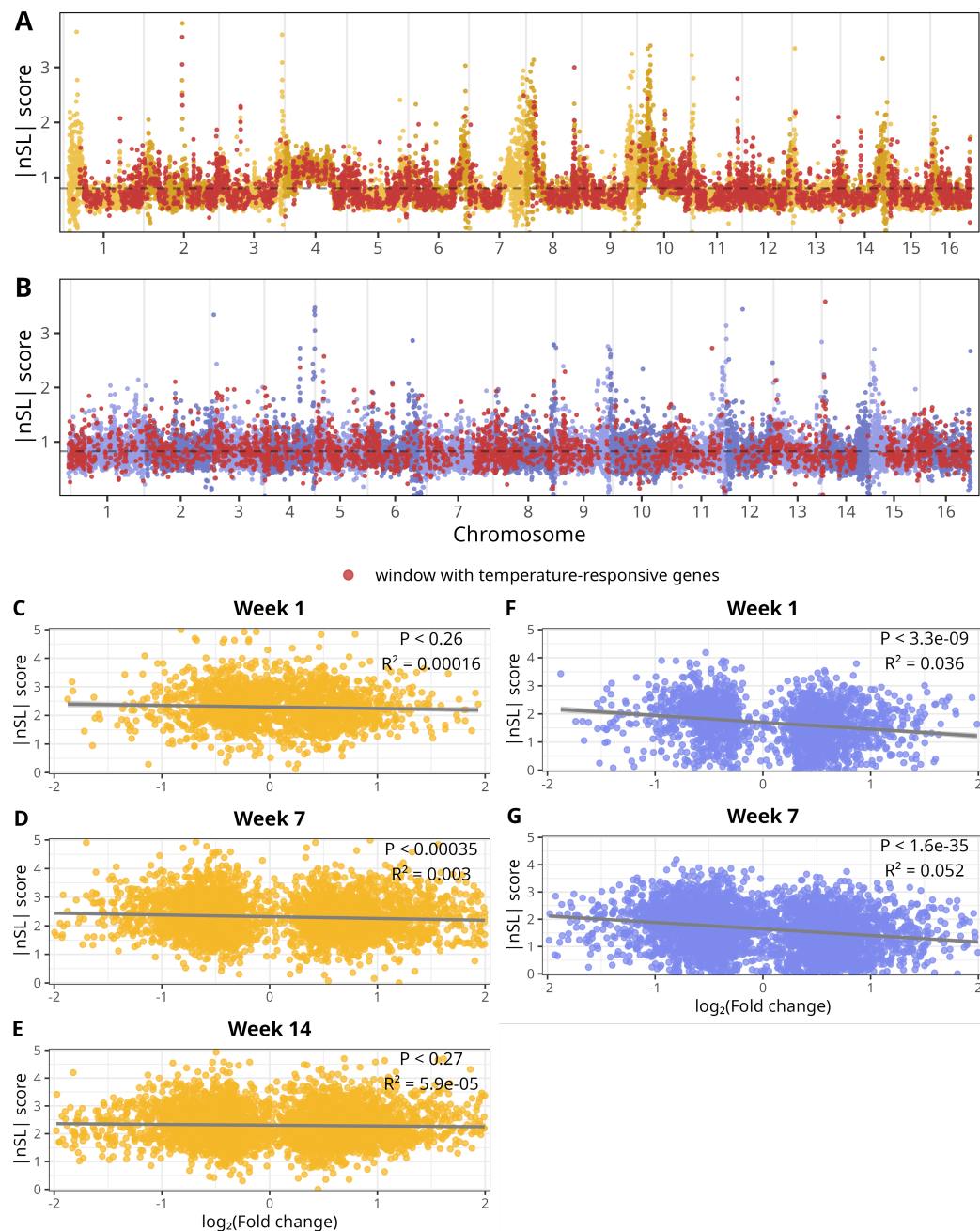


Figure 4.4: Patterns of recent selection in the genomes of *Osmia bicornis* and *Osmia cornuta*. (A,B) For each species (A = *Osmia bicornis*, B = *Osmia cornuta*) and along all 16 chromosomes of their genomes, a manhattan plot depicting extended haplotype homozygosity, measured in terms of normalised |nSL| score and averaged over 10,000 bp windows using the mean. Windows containing temperature-responsive genes (Likelihood ratio test: False Discovery Rate (FDR) < 0.05) are colored in red. The dotted horizontal line represent the genome-wide mean of |nSL|. Chromosomes are size-sorted from largest (1) to smallest (16) in both species, individually, and chromosome indices are, therefore, not directly comparable across species. (C-G) Scatter plots and linear regression models depicting the relationship between gene expression (x-axis) and |nSL| (y-axis) within temperature-responsive genes (baseline vs highest) at each experimental time point for *O. bicornis* (C-E) and *O. cornuta* (F,G). For each gene, the SNP with the maximum |nSL| score was chosen as representative. Horizontal lines (showing the slope of the regression), p-values and R^2 -values were inferred from a linear regression model using $\log_2(\text{Fold Change})$ as predictor and |nSL| score as response variables.

the Wnt signaling pathway was significantly enriched in *O. bicornis* (at weeks 7 and 14), but not in *O. cornuta*.

4.4 Discussion

Global climate change is altering overwintering conditions for insects worldwide, with winter temperatures rising faster than summer temperatures in many temperate regions and with an increasing frequency of warm spells during the cold season (Intergovernmental Panel on Climate Change, 2021; Stergiou et al., 2025). For wild bees, which provide essential pollination services and often spend much of their lives in dormant overwintering stages (Potts et al., 2016; Danforth et al., 2019), understanding how elevated temperatures affect the molecular and associated physiological processes maintaining dormancy is critical for predicting population responses to ongoing climate change (Williams et al., 2015). While the effects of elevated overwintering temperatures on phenological shifts in emergence timing and increased weight loss have been documented (Schenk et al., 2018b; Möllmann et al., 2025), the underlying transcriptomic changes and their potential fitness consequences during overwintering remain poorly understood. Furthermore, whether natural populations harbour genetic variation for increased thermal tolerance during overwintering and whether signatures of recent adaptation to warming winters are detectable remains unexplored, limiting our ability to predict evolutionary responses to future overwintering conditions (Van Heerwaarden Kellermann and Van Heerwaarden, 2019; Webster et al., 2023). Therefore, we characterised brain-related transcriptomic responses of overwintering mason bees exposed to elevated overwintering temperatures and investigated population genetic patterns of diversity and recent selection among temperature-responsive genes. We identified thousands of such temperature-responsive genes in both species, a large share of which changed in response over the course of dormancy, indicating that temperature exposure during overwintering leads to complex transcriptomic changes, dependent not only on the strength of the applied exposure but also on the duration of exposure as well as the dormancy phase that bees were in. Across both species, the primary functional patterns that emerged among temperature-responsive genes included a progressive temporal shift towards elevated oxidative phosphorylation and increased mitochondrial activity as well as a consistent downregulation of developmental pathways. Our population genetic analyses revealed panmictic population structure in both species with higher nucleotide diversity in *O. bicornis* compared to *O. cornuta*. Signatures of recent or ongoing selection were detected among temperature-responsive

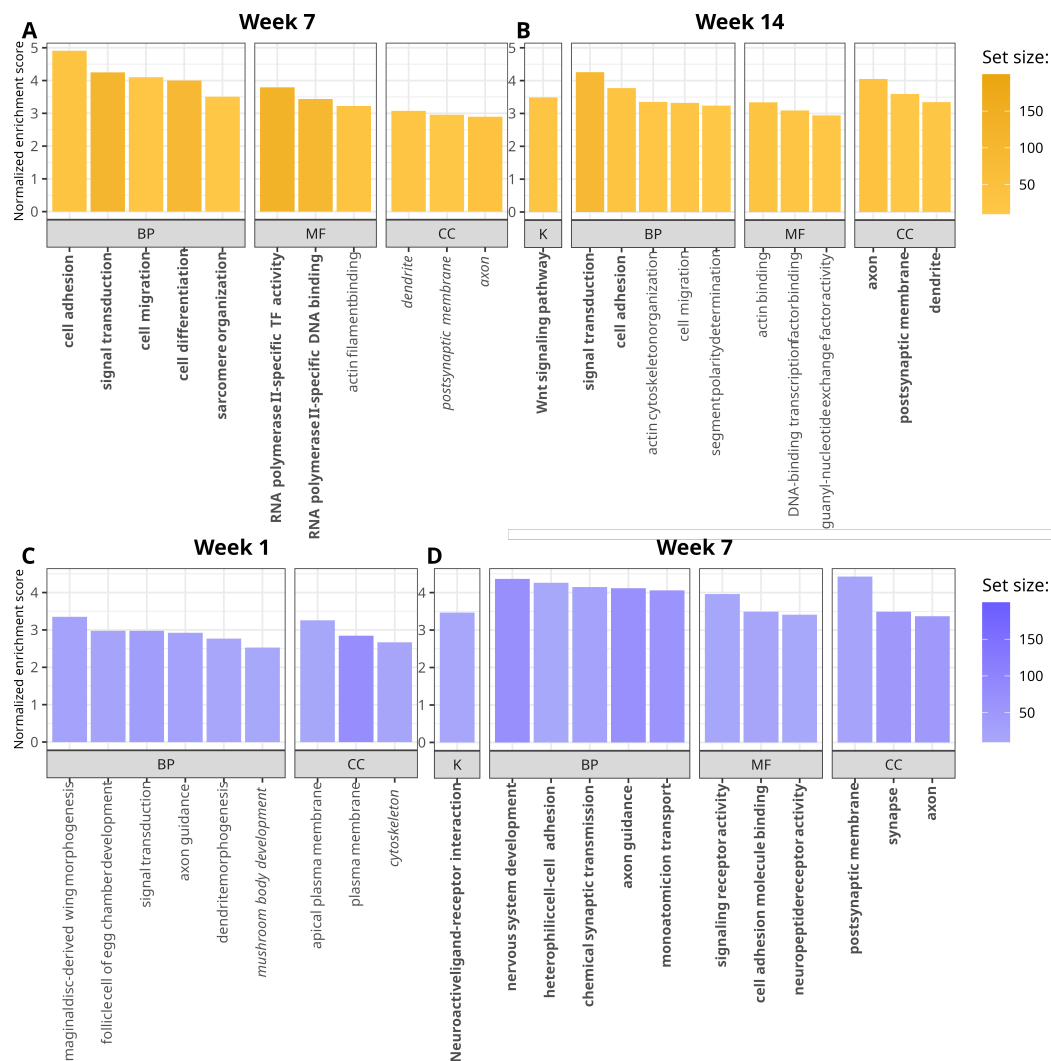


Figure 4.5: Signatures of recent selection among temperature-sensitive genes are most strongly linked to signal transduction. (A-D) Multi-faceted bar plots, depicting for each species (A,B = *O. bicornis*, C,D = *O. cornuta*) the most strongly enriched GO terms and KEGG pathways in terms of highest |nSL| scores among temperature-responsive genes (FDR < 0.05, baseline vs highest) across time points. There was no significant enrichment after week 1 for *O. bicornis*. The y-axis depicts the normalised enrichment score and the x-axis depicts enriched pathways divided into facets of different gene set categories (K = KEGG pathways, BP = biological process, MF = molecular function, CC = cellular component). Terms in bold script type are significant at a False Discovery Rate (FDR) < 0.01, terms in regular script type are significant at FDR < 0.05, and terms in italic script type are significant at FDR < 0.1. The colour gradients depict the number of genes per respective gene set with each species indicated by an individual colour (orange = *O. bicornis*; blue = *O. cornuta*).

genes in both species, however, patterns differed between species with a statistically significant relationship between gene regulation and signatures of selection only detected among downregulated temperature-responsive genes in *O. cornuta*. Overall, because highly similar phenotypic and molecular response to temperature were observed in both species (Möllmann et al., 2025), yet pre-existing genetic variation and patterns of selection differed between them, thermal responses may be driven primarily by phenotypic plasticity rather than ongoing genetic adaptation and the species may differ in their potential for future adaptation to warmer overwintering temperatures.

We observed thousands of temperature-responsive genes in both species, which represents a substantial reorganisation of the brain transcriptome in response to elevated overwintering temperatures. This scale of response suggests that maintaining appropriate physiological states during dormancy under elevated temperatures requires coordinated regulation across multiple molecular systems. Although several previous studies have looked at the immediate molecular effects of elevated temperatures in other wild bees, such as *Megachile rotundata* (Xu and James, 2012; Torson et al., 2015a; Torson et al., 2017; Cambron-Kopco et al., 2022), none have explored the effects of field-relevant long-term thermal exposure during dormancy, reflective of predicted changes in overwintering conditions due to climate change. In line with this, we observed a trend of increasing numbers of differentially expressed genes (DEGs) with increasing duration of thermal exposure, highlighting the difference between an immediate transcriptomic response and an accumulated response. Interestingly, in *O. bicornis*, where an additional sampling time point after fourteen weeks was available, this trend did not continue with similar numbers of DEGs at both the seven weeks and the fourteen weeks time points, suggesting that accumulated responses alone cannot explain the observed changes over time. As dormancy periods consist of different phases, such as diapause and post-diapause quiescence (Košťál et al., 2017), our observed transcriptomic responses may be due to the phase of dormancy the bees were in when sampled, which probably differed between time points. With the experimental setup employed in this study, it was not possible to directly disentangle the two experimental variables dormancy stage and duration of thermal exposure. However, under field-relevant conditions, these two variables would be similarly interlinked with increasing winter temperatures predicted under future climate change influencing overwintering bees across all overwintering stages. Interestingly, the number of DEGs after one week of exposure was much higher in *O. cornuta* ($n = 1,093$) compared to *O. bicornis* ($n = 447$) but converged to similar levels after seven weeks of exposure (both within 3,000 - 3,500 DEGs) potentially indicative of a stronger short-term response in *O. cornuta*.

However, due to natural differences in emergence phenology, the two species might have been at different stages of overwintering (Splitt et al., 2022).

The observed shift from suppressed to elevated expression of genes associated with oxidative phosphorylation across the experimental timeline represents one of the most striking temporal patterns in our data. After one week of exposure to elevated temperatures, we observed reduced expression of genes related to oxidative phosphorylation in *O. bicornis*, consistent with the initial maintenance of metabolic suppression, which is a key characteristic of diapause (Kostál, 2006; Hahn and Denlinger, 2007; Denlinger, 2022). However, by seven weeks in *O. cornuta* and 14 weeks in *O. bicornis*, this pattern had reversed, with clear upregulation of genes linked to oxidative phosphorylation and mitochondrial activity emerging. This temporal progression suggests that prolonged exposure to elevated temperatures may eventually overcome the regulatory mechanisms maintaining metabolic suppression during dormancy, forcing a transition toward more active metabolic states (Hahn and Denlinger, 2011). Such premature metabolic activation could have severe consequences for overwintering fitness, as it would increase energy consumption during a period when foraging is impossible and individuals must rely entirely on stored reserves accumulated during the previous active season. Indeed, this observation may explain previously documented organismal phenotypic responses to thermal exposure, such as fat-body depletion and increased emergence weight loss (Fliszkiewicz et al., 2012; Schenk et al., 2018b; Möllmann et al., 2025).

The contrasting patterns of genes underlying protein metabolism observed across time points further illustrate the dynamic nature of thermal responses during overwintering. At the earliest sampling time point, we observed evidence of possible increased protein turnover, with both ribosome production and protein degradation pathways showing elevated expression. This pattern may reflect a plastic response aimed at maintaining cellular homeostasis through increased quality control mechanisms, perhaps to manage proteins damaged or destabilised by thermal stress (Bukau et al., 2006; Alagar Boopathy et al., 2022). By the intermediate time point, however, this pattern had reversed and protein degradation and processing pathways became downregulated, suggestive of increased maintenance of pre-existing protein pools and reduced protein turnover, a pattern potentially indicating that the response to possible metabolic stress and increased resource scarcity has become more important than the response to acute thermal stress, by this stage (Klaips et al., 2018). Changes in genes related to fatty acid metabolism, observed most prominently at the early and late sampling points, may reflect alterations in energy substrate utilization and membrane remodeling processes. During diapause, insects typically rely heavily on lipid reserves for energy, and the regulation of fatty acid

metabolism is critical for balancing energy production with the preservation of lipid stores needed to sustain the full overwintering period and support post-dormancy development and reproduction (Hahn and Denlinger, 2007; Sinclair, 2015). The changes in fatty acid metabolic genes observed in this study coincide with previously reported increased fat-body depletions in bees exposed to elevated overwintering temperatures (Fliszkiewicz et al., 2012).

Among the most consistent patterns across both species and all time points was the widespread downregulation of developmental pathways, including processes related to morphogenesis, cell differentiation, and tissue development. This finding might suggest that bees need to prioritize addressing the thermal exposure challenge over progressing through late-stage dormancy developmental programs (Schmalensee et al., 2024). However, it might also be simply reflective of a faster physiological shift from dormancy maintenance to dormancy termination programs in response to elevated overwintering temperatures (Košťál et al., 2017; Schmalensee et al., 2024). Sexes differ in timing of emergence with males emerging before females (protandry), a strategy that increases the fitness of males, and is likely both facilitated by the nest positioning of males later in the nesting cavity so they are closer to the entrance, where they are more exposed to increasing environmental temperatures in spring that aid emergence (Strobl et al., 2019). Therefore, it may have been predicted to identify stronger differences between the sexes in the brain, a tissue integral for interpreting and coordinating responses to environmental stimuli (González-Tokman et al., 2020; Gallio et al., 2011). Given the well-documented sexual dimorphism in life-history traits and the potential for sex-specific selection pressures (Teder, 2014; Rogers et al., 2016; Torchio and Tepedino, 1980; Möllmann and Colgan, 2022), the paucity of genes showing sex-biased responses to elevated overwintering temperatures was somewhat unexpected. This would suggest that temperature exposure exerts a similar pressure on both sexes, resulting in similar responses identified at the transcriptional level in both sexes. Moreover, the complete absence of overlap between the sex-responsive genes identified in the two species suggests that these limited sex-specific effects may reflect stochastic variation rather than consistent biological differences in thermal responsiveness. While the reduced differences in the sexes could potentially be explained by our experimental set-up, whereby males and females were kept in individual tubes rather than kept in nest boxes, which may contribute to differential exposure of sexes to ambient temperature as males are laid closer to the entrance, within the same experiment, we observed clear sex differences in emergence timing in response to elevated overwintering temperatures (Möllmann et al., 2025). While these phenological differences between the sexes may seem to contradict the molecular results of

absence of sex differences reported here, it is important to note that our analysis did not encompass transcriptomes of bees immediately prior to emergence. As observed previously (Möllmann et al., 2025), timing of emergence is highly transient both within and across geographical locations meaning the sampling of such a time point is difficult. Timed emergence can be performed using experimental manipulation through sustained elevated temperature exposure (Bosch, 1994), but this would introduce a confound to our experimental design. It is, thus, likely that these previously reported increasing sex differences in emergence phenology in response to elevated overwintering temperatures are a result of transcriptomic changes that occur more closely to the timing of emergence. Essential to the adaptive responses of organisms to environmental factors, including temperature, is genetic variation. Previous work on *Osmia* species has largely been restricted to microsatellite-based analyses, which although informative on population structure (Neumann and Seidelmann, 2006; Van Eeckhoven et al., 2022; Koch et al., 2023), suffer from either low sample sizes, or low resolution. Whole-genome resequencing offers an unbiased insight into polymorphic sites across a population, providing information on genetic variation, as well as population structure, and genomic signatures of ongoing or recent selection (Webster et al., 2023). Our estimate of the genome-wide median nucleotide diversity in *O. cornuta* ($\pi = 0.0013$) is consistent with the genome-wide estimate previously described in Möllmann et al. 2025 ($\pi = 0.0012$; (Möllmann et al., 2026)) from a smaller dataset ($n = 10$) of female individuals sampled across Germany, suggesting that these estimates are an accurate approximation of real population-level genetic diversity. For *O. bicornis*, pre-existing genome-wide estimates of nucleotide diversity were not available as of preparation of this manuscript. However, compared to estimates from related eusocial species of the genera *Apis* and *Bombus* (ranging from $\pi = 0.0014$ to 0.0025 ; (Leroy et al., 2024; Heraghty et al., 2023; Colgan et al., 2022)), the herein described median diversity for *O. bicornis* is relatively high. Similarly, compared to the only pre-existing genetic diversity estimate for a solitary bee species (*Megachile rotundata*: $\theta_w = 0.003$, (Jones et al., 2019)), our findings suggest relatively increased genetic diversity in *O. bicornis* ($\theta_w = 0.0036$). The great differences in genome-wide nucleotide diversity between the two species could reflect differences in effective population size, historical demography, or the intensity of selection acting on these populations. Similarly, our samples included bees from across the geographical range of Germany combined with whole-genome resequencing, which offers advantages in terms of breadth of genome-wide resolution, which may contribute to our ability to detect higher rates of nucleotide diversity. Higher genetic diversity in *O. bicornis* could point to a greater potential for adaptive evolution in response to changing thermal environments, providing the basis for selection to act upon. The finding

that both species appear panmictic across Germany, with no detectable population structure in genome-wide analyses, suggests relatively high gene flow and large effective population sizes, factors that could facilitate rapid adaptation to changing environmental conditions.

While genetic variation as measured in nucleotide diversity can provide an insight into the capacity of a population to respond to environmental challenges, understanding the presence and extent of selection throughout the genome provide important evolutionary insights into adaptation (Eizaguirre and Baltazar-Soares, 2014). Through integrating transcriptomic and population genomic data, our analysis revealed an association between genes with reduced expression in response to elevated overwintering temperatures showing stronger signatures of recent positive selection, as measured by signatures of extended haplotype homozygosity. Conversely, genes with elevated expression had less signatures of experiencing recent selection, suggesting that transcriptional responses may be more essential for the organismal response to environmental temperature. However, this association was only consistent in *O. cornuta*, where it was observed at both sampling time points and explained a modest but highly significant proportion of variation ($R^2 > 0.03$). In *O. bicornis*, the pattern was weak and only statistically significant at the intermediate time point, suggesting potential species-level differences in their recent evolutionary history, which may lead to differences in thermal adaptation. For example, while both species overlap in their native continental range, *O. cornuta* has been increasing its northern range (Getz et al., 2024), especially in Germany (Westrich, 2019), which may lead to greater selection pressure acting on genes linked to environmental pressures, such as temperature. The absence of a consistent linear relationship between genes with elevated expression and extended haplotype homozygosity appears to suggest that increased expression of genes evolving under more relaxed selection pressures may be sufficient for the organism to respond to increased thermal exposure. However, it should be emphasised that this does not mean that all thermal-response genes are neutrally evolving as was evident from our analysis where certain genes with elevated expression also had relatively high $|nSL|$ scores (Figure 4.4C-G). Such genes may represent candidates whereby the adaptive (gene expression) and evolutionary responses are synchronised, which may have key roles in thermal responses, as well as longer term adaptation. However, functional studies would be required to validate the roles, if any, such genes play in thermal responses.

The enrichment of selection signatures among genes involved in neural signal transduction pathways, particularly evident across both species and multiple time points, suggests that the regulation of neural activity and signaling may represent a critical

target of selection underlying thermal adaptation during overwintering. Signal transduction pathways are responsible for detecting and responding to environmental cues, regulating cellular metabolism, and coordinating physiological responses across tissues (Gomes and Blenis, 2015). Fine-tuning these pathways to function appropriately under varying thermal conditions while maintaining dormancy physiology could be essential for successful overwintering across environmentally heterogeneous regions. The specific enrichment of Wnt signaling pathway genes among selection candidates in *O. bicornis*, but not *O. cornuta*, adds another layer of species-level differentiation as, in insects, Wnt signaling is known to promote developmental patterning through cellular communication and cell cycle regulation (Wadsworth and Dopman, 2015; Chen and Xu, 2014) and as it has been shown to regulate thermal responses in other groups of organisms (Risha et al., 2021).

Understanding the capacity of organisms to respond to ongoing climatic change, represents one of the most urgent challenges that society faces. Our findings, here, provide important insights into the molecular and evolutionary responses of mason bees to increased temperature exposure, which have implications for predicting how overwintering insects will respond to climate change-induced warming. The observation that transcriptomic responses vary substantially across the overwintering period suggests that climate change impacts will depend critically on the timing and duration of warm periods during winter. Brief early-season warm spells may elicit different physiological responses and have different fitness consequences compared to sustained warming later in the overwintering period or to overall elevation of average winter temperatures. Current climate projections indicate not only increasing average winter temperatures but also greater temperature variability and more frequent extreme warm events during winter months. Our results suggest that such patterns could be particularly disruptive, potentially triggering inappropriate metabolic activation or depleting energy reserves prematurely. In addition, the comparison of transcriptomic and population genetic results suggests that the observed phenotypic responses to elevated overwintering temperatures are a more direct result of phenotypic plasticity rather than natural selection acting on existing genetic variation.

4.5 Conclusion

This study provides the first comprehensive molecular characterisation of how prolonged exposure to elevated, field-relevant overwintering temperatures affects dormant solitary bees, revealing extensive transcriptional reorganisation, which

intensified over the course of the experiment time. The identification of thousands of temperature-responsive genes in both *O. bicornis* and *O. cornuta*, a major share of which showed time-dependent responses, demonstrates that the molecular consequences of warming winters extend beyond immediate stress responses. The progressive shift from metabolic suppression toward increased oxidative phosphorylation and mitochondrial activity, alongside consistent downregulation of developmental pathways, suggests that previously observed fitness-relevant phenotypic changes, such as early emergence and reduced emergence weight, may be explained by an accumulated transcriptomic response although future work would benefit from examining shifts in gene expression immediately prior to emergence through extensive detailed tracking to determine the contribution of short-term shifts to emergence timing. Our findings also suggest that the timing and duration of warm periods during winter will be particularly important, as transcriptional responses and their physiological consequences vary substantially across the overwintering period and that previously performed short-term thermal exposure experiments might not be sufficient to explain observed phenotypic responses, as accumulated transcriptomic changes are missed.

Our integrative approach combining transcriptomics and population genomics reveals important insights into the adaptive potential of these essential pollinators. Taken together, our results suggest that transcriptional plasticity rather than evolved genetic adaptation may constitute the primary mechanism by which these species currently respond to thermal variation during overwintering. However, the observed species-level differences in patterns of recent or ongoing selection among thermal-responsive genes, suggests that species may differ in their evolutionary potential for adaptation under continued warming. As we did not measure long-term fitness consequences, whether such responses are sufficient to cope with ongoing climatic change, a point of concern for many taxa (Radchuk et al., 2019), remains an open question. Despite this, we believe our findings offer novel insights into how wild bees are responding to field-relevant environmental pressures and will assist in the development of solitary bees as important models for monitoring the impact of climatic change on vulnerable states, which has larger implications for understanding how key life stages will be impacted by ongoing climatic shifts.

4.6 Materials and Methods

RNA sample collection and brain dissections

For the transcriptomic analysis, samples were collected at one of three time points during the same experiment. The experiment, which applied a baseline temperature profile based upon field-relevant overwintering temperatures, as well as two elevated temperature profiles (intermediate, based upon SSP2-4.5 and highest, based upon SSP5-8.5) to overwintering males and females of *Osmia bicornis* and *Osmia cornuta*, is described in detail in a precursor study (Möllmann et al., 2025) that explored the organismal phenotypic responses to prolonged temperature exposure. More specifically, one week after the start of the experiment on the 1st of December, 2022, the first batch of samples intended for RNA sequencing was removed from the experiment and snap frozen immediately in liquid nitrogen with subsequent storage at -80°C. The other two batches were sampled after seven and 14 weeks, respectively. Within each experimental group, samples were then randomly chosen for brain dissections. For a detailed sample size breakdown per experimental group, see Supplemental Table S1. For each sample, brain dissections were performed on ice-cooled, silicone slides under a stereo-microscope while fully submerged in 1x phosphate-buffered saline (PBS). For each sample, the head was first separated from the thorax and immediately transferred to a droplet of PBS (50-100 µl) on top of a silicone slide where they were held in place using micro-forceps. After dorsal opening of the head capsules, followed by orthogonal cuts towards the eyes, whole brains (including the central brain, the antennal and optical lobes, and the subesophageal ganglion) were carefully removed from the head capsules and immediately transferred to ice-cooled 1.5 ml microcentrifuge tubes filled with 100 µl of TRIzol to prevent cellular RNA degradation. Samples were subsequently frozen at -80°C prior to extraction.

RNA extractions, library preparation, and sequencing

Total RNA from TRIzol-stabilised brain samples was extracted using a TRIzol-chloroform-based extraction protocol (Colgan et al., 2011) and subsequently purified using the Monarch RNA purification kit (New England Biolabs) before being DNase-treated using TURBO DNase (Qiagen) to remove putative DNA contaminants. Following extraction and purification, RNA quantity was assessed using the FastGene NanoSpec Photometer (FG-NP01), indicating samples were of high quality and sufficient yields. RNA was stored at -80°C until shipment on dry ice to a commercial sequence service provider (Novogene, Germany). Here, quality was independently assessed using an Agilent Bioanalyzer. After confirmation of sample quality, for each sample, an mRNA-enriched library was prepared. Libraries were individually barcoded, pooled and paired-end sequencing (2 × 150 bp) was carried out to a minimum of 40 million reads (20M read pairs) per sample, resulting in a mean read

count of 52,533,484 across samples after quality filtering (min: 39,282,110; max: 109,830,868).

Differential gene expression analysis

Using the raw sequences, we performed per-sample quality assessments based on raw read quality metrics generated with FastQC (v0.12.1) (Andrews et al., 2010), as well as based on mapping rates using Kallisto (v0.51.1) (Bray et al., 2016). For *O. bicornis*, we used the latest chromosome-level reference assembly and annotation (iOsmBic2.1; GCF_907164935.1), whereas for *O. cornuta*, we used the chromosome-level assembly and annotation, published previously (Möllmann et al., 2026). To generate a summary and combined visualisation of QC reports, we used MultiQC (Ewels et al., 2016). We then processed the raw reads by removing adapter sequences and filtered by quality (Phred quality score ≥ 15) and length (minimum length ≥ 50 bp) using fastp (v1.0.1) (Chen et al., 2018). After filtering, the mean alignment rate of reads was 93.82% for *O. bicornis* and 93.30% for *O. cornuta*. Mapped transcriptome-level reads were then summarised to gene-level using tximport (v1.38) (Soneson et al., 2015) and for each species, a principal component analysis (PCA) was performed using variance-stabilised read counts for further quality assessment. Due to lower mapping rates, lower raw read quality, and outlier clustering in the PCA, two *O. bicornis* samples (KB45: week 1 / intermediate temperature; GB112: week 1 / highest temperature) were removed from all subsequent analyses. Following quality assessments and outlier removal, DESeq2 (v1.50) (Love et al., 2014) was used to infer differentially expressed genes (DEGs, False Discovery Rate (FDR) < 0.05) and $\log_2$ fold changes between experimental groups using likelihood-ratio tests (LRT). In particular, we first performed, for each species, a global LRT using temperature treatment regime, time in weeks, and their interaction as the full model and just time as the reduced model, to capture any genes responsive to increased temperatures. We then reran the LRT using the same full model but with the inclusion of both temperature and time in the reduced model to capture only genes where the effect of temperature changed over time. We then compared the two resulting sets of DEGs to infer the percentage of temperature-responsive genes that also showed an interaction with time. To perform a PCA using all samples (per species), we used the normalised read counts as input to `limma::removeBatchEffect` (v3.66) (Ritchie et al., 2015) to account for variation introduced by the different time points and plotted the subsequent processed counts using ggplot2 (Wickham, 2016). Following the observation of a large proportion of interaction genes, we then decided to perform individual tests at each time point instead of a global test, to be able to accurately differentiate between temperature-related transcriptomic changes at different time points. From each per-time point

test, we then extracted the normalised read counts, the number of DEGs, and the $\log_2$ fold changes. The normalised read counts were used to plot heatmaps using the ComplexHeatmap library (Gu et al., 2016).

DNA sample collections

For the population genetic datasets, we sampled male adults from independent sites across Germany (*O. bicornis*: $n = 40$, *O. cornuta*: $n = 30$) with a minimum distance of 32 km between sites (Figure 4.3). At each sampling site, species identification for each specimen was performed based on morphological features (Danforth et al., 2019), before they were immediately transferred to absolute ethanol for preservation of DNA integrity. For long-term storage, samples were kept at -80°C . Field-based species identifications were later validated computationally from the generated sequencing data (Supplementary Information: Computational validation of species identification).

DNA extractions and sequencing for population genetic analyses

For the purpose of DNA extractions, we first removed the abdomen and the wings of each sample using microscissors as the abdomen can harbour microbial contaminants and as the wings can interfere with later steps of the extraction protocol. The remaining tissue was subsequently washed with three rounds of autoclaved PBS to remove residual ethanol and then homogenised using micropestles in 1.5 ml Eppendorf tubes containing 200 μl of PBS. Subsequent tissue washing and DNA extraction was performed according to the protocol in Möllmann et al. (Möllmann et al., 2026). For library preparation and sequencing, the extracted DNA samples were sent to a commercial sequencing service provider (Novogene, UK). After additional quality verification using an Agilent Bioanalyzer, PCR-free libraries were constructed for each sample using a New England Biosystems library kit. These libraries were individually barcoded and sequenced on an Illumina NovaSeq 6000 platform (2×150 bp), with a minimum target output of 3 Gb per sample. This approach yielded an average of 34,443,038 reads per sample in *O. bicornis* (min: 24,764,756; max: 69,879,612) and 31,819,847 reads per sample in *O. cornuta* (min: 26,498,394, max: 42,191,586).

Population genetic analyses

To quality assess the population genetic datasets, we used a combination of read-based quality metrics, inferred using FastQC (Andrews et al., 2010), as well as genomic mapping rates generated using bowtie2 v2.5.4 (Langmead and Salzberg, 2012) and summarised using samtools stats (Danecek et al., 2021). We used MultiQC (Ewels et al., 2016) to combine and visualise all quality metrics. We then used fastp

(Chen et al., 2018) to filter raw reads based on base calling quality (PHRED score ≥ 20) and minimum length (≥ 50 bp) and to remove adapter contamination. For each species, filtered and processed reads were then mapped against their respective genome assemblies (*O. bicornis*: iOsmBic2.1/GCF_907164935.1; *O. cornuta*: see (Möllmann et al., 2025)) using bowtie2. Subsequently, variant calling was performed on all reads with a minimum mapping quality score of 20 using the BCFtools v1.21 mpileup-call pipeline (Danecek et al., 2021) and resulting variants were filtered for a minimum variant calling quality score of 40, an average read depth between 5x and 20x across samples as well as a maximum percentage of samples with missing calls of 30%. The resulting filtered datasets contained 2,651,539 and 976,544 single nucleotide polymorphisms (SNP) at mean quality scores of 1680.13 and 1894.28 for *O. bicornis* and *O. cornuta*, respectively. As an additional quality control step, we calculated for each sample heterozygosity estimates using vcftools -het (Danecek et al., 2011) to validate expected haploid ploidy of samples. To investigate the presence of population structure, loci with linkage-disequilibrium above 0.2 or with a minor allele frequency below 0.05 were first pruned from the datasets (*O. bicornis*: 280,299 SNPs remaining, *O. cornuta*: 106,233 SNPs remaining) using plink v1.9.0-b.7.8 (Zheng et al., 2012; Chang et al., 2015), before principal component analyses were performed using SNPrelate v1.44.0 (Zheng et al., 2012) and admixture analyses were performed using ADMIXTURE v1.3.0 (Alexander and Lange, 2011). To infer the nucleotide diversity estimates π and Watterson's θ , we indexed our SNP datasets using tabix (Li and Durbin, 2011) and subsequently ran pixy v1.2.11 (Korunes and Samuk, 2021) using 10,000bp-sized windows. Lastly we inferred at the number of segregating sites by length (nSL), a haplotype-based estimate that can be used to detect recent or ongoing selective sweeps, which are the products of positive selection, in a population, using selscan v2.1.0 (Szpiech and Hernandez, 2014). After normalising in one percentage bins using the selscan norm function, nSL scores were summarised to two levels: a) the mean of the absolute nSL scores across 10,000bp-sized windows, as well as b) the absolute maximum nSL score for each gene. Window-based estimates were then visualised in manhattan plots, whereas gene-based estimates were used for gene set enrichment analysis and for correlating against gene expression ($\log_2(\text{Fold change})$) using linear regression.

Gene set enrichment analysis

For the RNA-seq data, after running DESeq2, the inferred $\log_2$ -fold changes of all genes were used as input to fgsea (v1.36) (Korotkevich et al., 2016) to perform a gene set enrichment analysis using a combination of *Drosophila melanogaster* and *O. bicornis* gene ontology (GO) terms for three categories (biological process, molecular function, and cellular component) (Ashburner et al., 2000; Gene On-

tology Consortium et al., 2023) and *D. melanogaster* KEGG pathways (Kanehisa, 2000). GO terms and KEGG pathways from *D. melanogaster* genes were assigned to *O. bicornis* and *O. cornuta* genes via protein-protein orthology generated using OrthoFinder (v2.5.5) (Emms et al., 2025) with the latest RefSeq genomes of *Bombus terrestris*, *Megachile rotundata*, and *Osmia lignaria* included in the orthology detection analysis as outgroups for increased sensitivity. All putative homologues with *D. melanogaster*, including many-to-many relations, were included in the gene set enrichment database. Before gene set enrichment analysis, both the GO terms and the KEGG pathway terms were size-filtered and only gene sets with between 10 and 200 genes were retained to reduce noise and to remove overly general terms. In addition, for increased sensitivity, the parameter gseaParam was set to 0 which equates to performing an unweighted Kolmogorov-Smirnov test instead of the original weighted gene set enrichment algorithm (Subramanian et al., 2005) which is considered to confer relatively low statistical power (Tamayo et al., 2016; Mathur et al., 2018). After testing and p-value adjustment using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995), we removed redundant hits from our list of results by pairwise comparisons of the leading edge sets between all KEGG pathway terms or terms within the same GO category. If two leading edge sets shared 75% or more of their members and had the same direction of enrichment (i.e., positive or negative normalised enrichment score (NES)), only the term with the higher absolute NES was retained for the final presentation of results in the graphical figures. For the DNA-seq data, GSEA was performed with this same approach, using the gene-level absolute maximum nSL scores instead of fold changes. Finally, for the figures, depending on the informativeness of the category, we kept only the three to five most enriched upregulated and downregulated pathways and terms, as indicated by their NES value.

Data Availability Statement

All data and scripts required for reanalysis are retrievable from the following link: <https://drive.google.com/drive/folders/1p53i9DqSZU-let4v4sgzVQkAuDIv0LVZ>. RNA data and population genetic datasets will be made publicly available as NCBI BioProject upon publication.

Acknowledgments

We would like to thank the German Federal Environmental Foundation (Deutsche Bundesstiftung Umwelt, DBU) for supporting JSM with a doctoral scholarship (20021/743), and the Eva Crane Trust (ECTA_20210915, United Kingdom), for financial contribution to the *Osmia cornuta* population genomics. We would also like to thank the environmental offices of Baden-Württemberg, Bavaria, Berlin,

Bremen, Hesse, Mecklenburg-Western Pomerania, Lower Saxony and Saxony, and especially Dr. Peer Schnitter (Landesamt für Umweltschutz (LAU), Saxony-Anhalt), for collection permits. For caretaking of a nesting site, logistics and provision of flower resources, we thank Joachim and Petra Stolle. We would like to thank the HPC facility at JGU for access to computational resources. We would like to thank Jessica Ehlers for helping MB with brain tissue dissections.

Author Contributions

TJC and JSM conceived the study. JSM collected samples for the population genetic datasets and CK contributed several additional samples. MB performed brain tissue dissections on experimental samples. JSM, HX and TJC generated DNA-seq and RNA-seq data from population genetic and experimental samples. JSM performed the transcriptomic analyses. JSM performed the population genetic analyses. JSM performed the statistical analyses and generated all figures. JSM wrote the draft, while TJC contributed to the final manuscript. All authors gave final approval for publication.

4.7 Supplementary Material

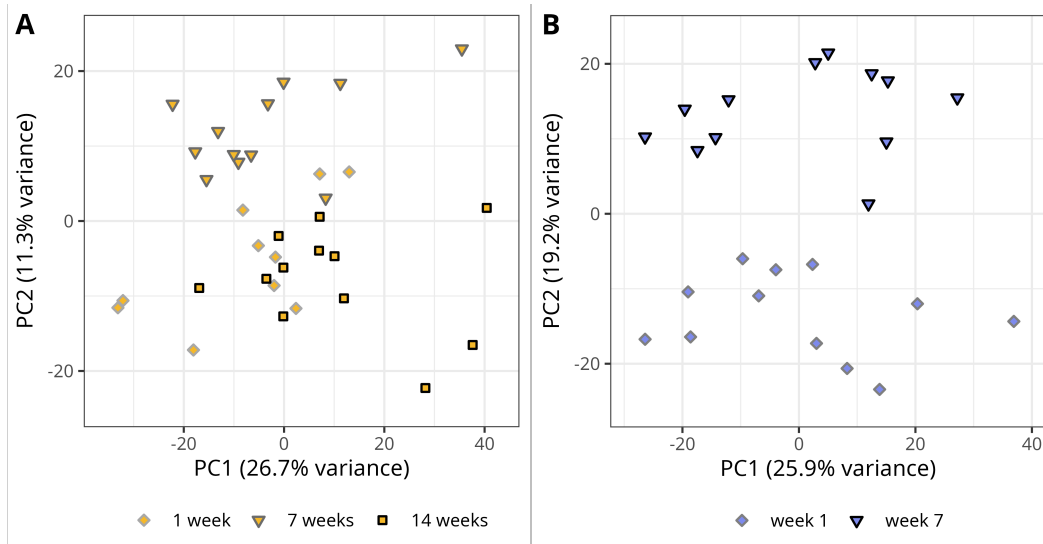


Figure S1: Gene expression patterns across sampling time points. (A,B) Principal component analyses of variance stabilised read counts after reduction of variation attributable to temperature treatment (A = *O. bicornis*, B = *O. cornuta*). The first two principal components (“PC”) in terms of the highest percentage of variance explained are plotted on the x- and y-axes, respectively. Time points of sampling are depicted in different colours and shapes.

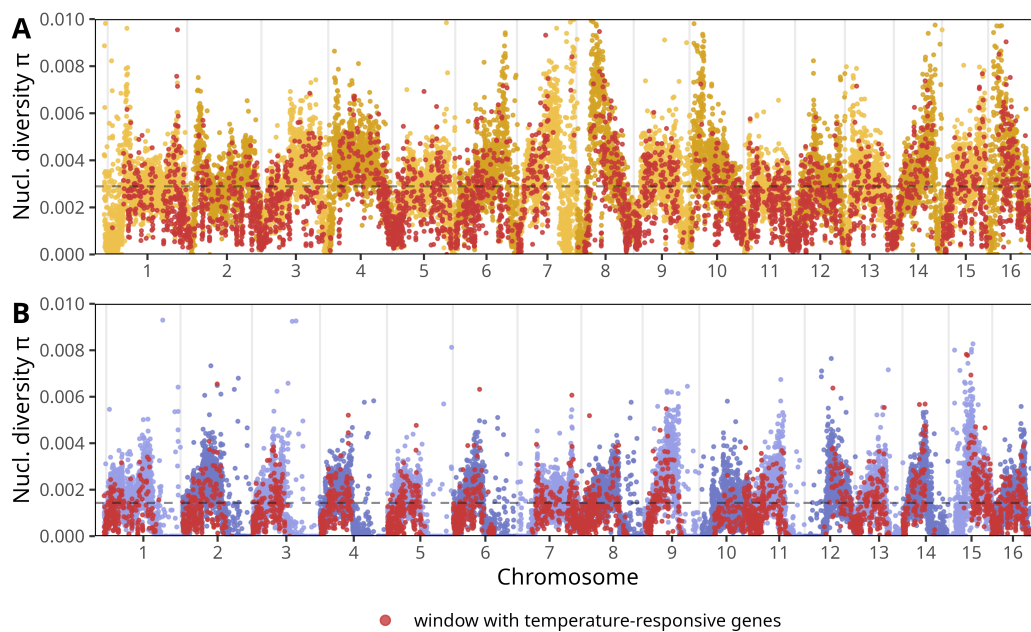


Figure S2: Genome-wide and temperature-response gene specific nucleotide diversity. (A,B) Manhattan plots of mean nucleotide diversity (π) in 10kb windows along the chromosomes of (A) *Osmia bicornis* and (B) *Osmia cornuta*. Windows containing temperature-responsive genes (LRT, FDR < 0.05) are colored red. Horizontal dashed lines represent the genome-wide mean of π . Chromosomes are size-sorted from largest (1) to smallest (16) in both species, individually, and chromosome indices are therefore not directly comparable across species.

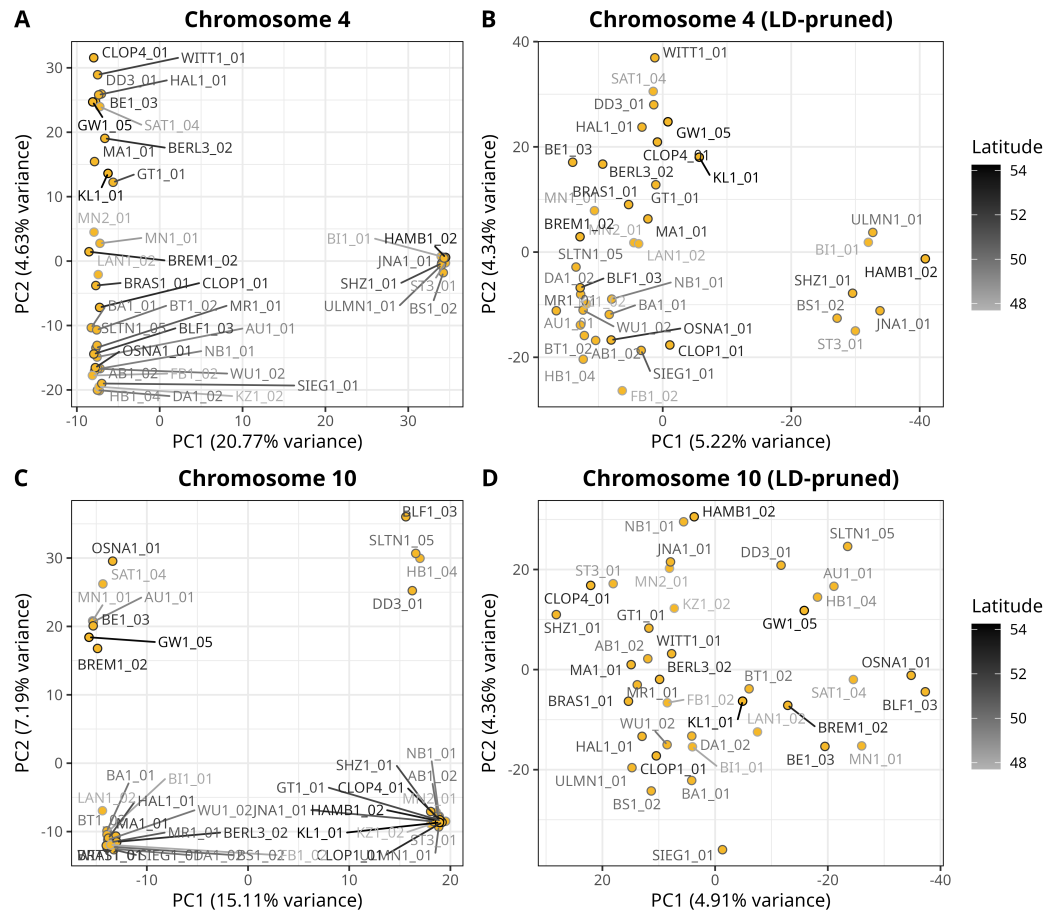


Figure S3: Clustering of samples before and after LD-pruning in *Osmia bicornis*. (A-D) Principal component analyses of Single Nucleotide Polymorphisms located on *Osmia bicornis* chromosomes 4 (A,B) and 10 (C,D) before (A,C) and after (B,D) linkage-disequilibrium pruning ($LD \leq 0.2$). The x- and y-axes show the first and second principal components and their explained variance, respectively. Grey-scale colours represent the latitude that an individual was sampled at.

Table S1: Sample sizes per experimental group for RNA sequencing. Number of biological replicates per species, sex, temperature treatment and sampling time point used in the transcriptomic analysis.

Species	Sex	Time point	baseline	intermediate	highest
<i>Osmia bicornis</i>	female	one week	4	4	4
		seven weeks	4	4	4
		fourteen weeks	4	4	4
<i>Osmia cornuta</i>	female	one week	4	4	4
		seven weeks	4	4	4
	male	seven weeks	4	4	4

Climate change represents one of the defining challenges of the 21st century, affecting multiple levels across biological systems from populations to whole ecosystems (Intergovernmental Panel on Climate Change, 2021; Parmesan, 2006). For wild bees, which provide critical pollination services (Potts et al., 2016; Kleijn et al., 2015) whilst spending much of their lives in thermally sensitive dormancy (Danforth et al., 2019; Williams et al., 2015), understanding responses to warming is both scientifically informative as well as relevant to the conservation of ecosystems and agricultural production. This thesis has adopted an integrative approach to investigate how two closely related mason bee species, *Osmia bicornis* and *Osmia cornuta*, respond to elevated overwintering temperatures predicted under current climate change scenarios. By combining experimental manipulation of ambient temperatures during overwintering with phenotypic measurements, transcriptomic profiling, and population genomic analyses, this work provides a unique multi-level characterisation of putative climate change responses in solitary wild bees.

The three data chapters of this thesis reveal several relevant findings. Chapter 1 demonstrated that exposure to elevated overwintering temperatures reflecting IPCC scenarios SSP2-4.5 and SSP5-8.5 advances emergence timing by three to six weeks, respectively, increases weight loss by 17-60%, but does not increase mortality during dormancy. Chapter 2 provided an essential genomic resource for molecular investigations with the first high-quality chromosome-level genome assembly for *O. cornuta* and revealed evidence of reduced genetic diversity and lower DNA methylation levels compared to social bee species. Chapter 3 integrated the characterisation of transcriptomic responses to elevated overwintering temperatures with population genomic patterns, identifying thousands of temperature-responsive genes in both species, demonstrating extensive transcriptional reorganisation affecting metabolic pathways, protein homeostasis, and developmental programmes, whilst revealing species-specific differences in genetic diversity and signatures of recent selection, including among thermal-responsive genes. This synthesis combines the most relevant findings of all chapters to jointly address questions that range from specific organismal and molecular responses measured at the phenotypic level, to the role of phenotypic plasticity, genetic adaptation, as well as intrinsic sex differences con-

tributing to thermal responses. Lastly, it discusses the broader implications of such findings for wild bee conservation.

5.1 Phenotypic Plasticity and Genetic Adaptation

A fundamental question in evolutionary biology concerns the respective roles of phenotypic plasticity and genetic adaptation in enabling organisms to cope with environmental change (Merilä and Hendry, 2014; Chevin et al., 2010). This question takes on particular urgency in the context of rapid anthropogenic climate change, where the pace of environmental change may exceed the rate at which adaptive evolution can occur (Hoffmann and Sgrò, 2011). This thesis provides several lines of evidence that address this question in the context of the responses of wild bees to elevated overwintering temperatures.

One line of evidence comes from comparing the population genetic data of the two study species (Chapter 3) to the analysis of fitness-related phenotypic responses during the thermal-exposure experiment (Chapter 1). Despite substantial differences in genome-wide nucleotide diversity, with *O. bicornis* exhibiting roughly twice the genetic variation of *O. cornuta*, and differences in signatures of recent selective sweeps among thermal-responsive genes, the patterns of phenotypic and transcriptomic responses to elevated temperatures were highly similar in both species. For example, both species advanced emergence timing by comparable amounts under the same thermal scenarios, both exhibited similar patterns of weight loss, and both showed similar transcriptomic responses, both in terms of quantitative and functional patterns with comparable numbers of differentially expressed genes at similar time points and corresponding regulatory pathways affected. If these phenotypic changes were due to genetic differentiation within each species, we would expect decreased survival rates during the experiment, indicating that selection is filtering out unfavourable genotypes, as well as similar levels of genetic differentiation and haplotype homozygosity among thermal-responsive genes in both species, indicating that selective pressures have been shaping similar levels of differentiation and adaptation (Hoffmann and Sgrò, 2011). The thesis' results, however, show that mortality rates were not affected by overwintering thermal stress, and species differed in their genetic architecture despite similar phenotypic responses, suggesting a reduced role for genetic differentiation compared to phenotypic plasticity in responding to the introduced thermal exposure. It should be noted, however, that genetic diversity is strongly affected by effective population size and that both species differ in their recent demographic history and distribution patterns across Europe (Westrich,

2019). Thus, it cannot be ruled out that observed genetic diversity differences were at least somewhat influenced by ongoing northwards range expansions in *O. cornuta* (Slatkin and Excoffier, 2012), which has only recently established in Northern Germany (Westrich, 2019), although, given the sampling scheme for the two species, this phenomenon is unlikely to explain the full two-fold difference that we observed. While, in addition, our population genetics dataset was slightly smaller for *O. cornuta*, which is another factor that could reduce inferred estimates of genetic diversity, the independent dataset for *O. cornuta*, presented in Chapter 2, confirms the estimates inferred in Chapter 3. Additionally, during the inference of diversity estimates, an algorithm that accounts for differences in sample size was used (Korunes and Samuk, 2021).

The low levels of DNA methylation found in *O. cornuta* (Chapter 2) add an interesting dimension to this discussion. DNA methylation plays important roles in gene regulation and has been implicated in phenotypic plasticity in some systems (Glastad et al., 2014), particularly in social insects where differential methylation contributes to caste determination and behavioural plasticity (Glastad et al., 2014; Glastad et al., 2017). The observation that *O. cornuta* exhibits methylation levels substantially lower than those reported for social bee species raises questions about the molecular mechanisms underlying transcriptional plasticity in solitary bees (Sørensen et al., 2003). If methylation-based regulatory mechanisms are less prominent, other mechanisms, such as transcription factor regulation, histone modifications, or regulation via non-coding RNAs, could potentially play larger roles in mediating the observed plastic changes in response to elevated overwintering temperatures (Kültz, 2005).

While additional evolutionary experiments, such as a common garden experiment (Villemereuil et al., 2016), would have to be performed to strengthen the argument that phenotypic plasticity is the primary driver of observed responses to thermal exposure during overwintering, the reliance on plasticity rather than genetic adaptation would have important implications for predicting long-term responses to continued warming (Sgrò et al., 2011; Hoffmann et al., 2023). Plasticity can facilitate population persistence in novel environments by enabling individuals to maintain fitness despite insufficient genotypic adaptations to the environment, potentially buying time for adaptive evolution to occur (Chevin et al., 2010; Ghalambor et al., 2007). However, plastic responses are limited. They evolved under historical selection pressures, such as the naturally occurring thermal variation within and between overwintering seasons, and may not be able to fully compensate for the more challenging conditions outside of this range predicted under climate change (Forrest, 2016; Crispo, 2007). For example, the observation of widespread transcriptional changes affecting thousands of genes suggests that maintaining homeostasis

under elevated temperatures is accompanied by large-scale transcriptomic reorganisation (López-Maury et al., 2008; Lynch and Marinov, 2015), raising questions about whether plastic responses can be sustained indefinitely or whether they impose hidden costs that only become evident over time, such as over the course of the active season after emergence (Radchuk et al., 2019; Chevin et al., 2010). In fact, the plastic phenotypic responses observed in Chapter 1 could potentially have mid-term or long-term fitness consequences. For example, even though mortality rates were not increased during dormancy, there is evidence from the literature that post-emergence survival rates could be decreased in response to elevated wintering and pre-wintering temperatures (Bosch and Kemp, 2004). In addition, it is unclear how individuals cope with reduced emergence weight and earlier emergence timing after emergence. In theory, these could lead to reduced reproductivity and reduced offspring survival (if adults die before completion of offspring provision). Thus, the overall success of the observed plastic responses in maintaining fitness under the imposed thermal stress is not yet definitively determined and requires additional experiments to more directly test fitness consequences, particularly in terms of reproductive output and offspring survival.

Besides the capacity for phenotypic plasticity as an immediate response mechanism, there is evidence to support the idea that genetic adaptation to increased overwintering temperatures is possible in the long run. The substantial individual variation observed within the plastic responses to thermal stress (Chapter 1)—with some individuals losing less weight and emerging closer to normal timing despite identical thermal exposure—suggests that populations harbour considerable phenotypic diversity upon which selection could act (Hoffmann and Sgrò, 2011) as this variation in thermal sensitivity represents the prerequisite for adaptive evolution (Frankham, 2005; Sinclair et al., 2012). If warming winters impose fitness costs through premature emergence or energetic depletion, individuals exhibiting more moderate responses may enjoy selective advantages, potentially driving evolutionary shifts in thermal reaction norms over subsequent generations (Merilä and Hendry, 2014).

As the capacity for this shift is strongly dependent on genetic differentiation underlying these differences in phenotypic responses, however, and as species differ in the amount of genetic differentiation observed among thermal-responsive loci - with increased genetic diversity in *O. bicornis*, there is reason to believe that *O. cornuta* might be less capable of such mid- to long-term genetic adaptations. Furthermore, the species-specific differences in signatures of ongoing or recent selection suggest that the two species may already differ in their evolutionary trajectories (Hoffmann and Sgrò, 2011). The consistent and significant relationship between thermally-

responsive gene expression and signatures of selection in *O. cornuta*, absent in *O. bicornis*, suggests that these species may be at different stages of adaptation or may have been adapting to different selective pressures related to their distinct demographic histories and native ranges (Westrich, 2019). *Osmia cornuta*'s recently more limited and more Mediterranean distribution and its recent northward expansion may have favored genetic changes at different loci compared to more stable *O. bicornis* populations (Parmesan, 2006).

5.2 Sex-Specific Thermal Responses Despite Transcriptomic Similarity

In Chapter 1, sex-specific phenotypic responses to elevated overwintering temperatures were documented. In *O. bicornis*, the divergence in male and female emergence timing increased from 10 days under control conditions to 18 days under worst-case scenarios. In addition to the increased risk of males emerging into a non-optimal environment, given the monandrous mating system of mason bees, where females typically mate only once (Paxton, 2005), such temporal asynchrony between sexes could reduce mating opportunities, increase male-male competition during the extended period before females emerge, or lead to suboptimal mate quality if females are forced to mate with males that have been active for extended periods, potentially reducing sperm quality or quantity (Strobl et al., 2019). While these reproductive consequences would not necessarily manifest as post-dormancy mortality, they could substantially affect population growth rates (Bosch and Vicens, 2006).

However, despite the magnitude of these observed sex-specific phenotypic differences, transcriptomic differences between sexes were both minimal at the baseline overwintering temperature, as well as after thermal exposure, indicating that the observed phenotypic differences cannot be explained via differences in transcriptional gene regulation in the acute response to thermal exposure or via innate differences in transcriptional regulation of thermal-sensitive genes prior to the exposure. Instead, several alternative mechanisms might explain the observed patterns of sexual differentiation.

First, the employed type of analysis (differential gene expression) does not directly capture post-transcriptional regulatory differences, even though the underlying data is enriched for mature mRNA, meaning that several additional analyses revealing post-transcriptional regulatory mechanisms could be performed using the data. For

example, an analysis of differential exon usage can inform on the prevalence of alternative splicing and on differences between the sexes therein (Anders et al., 2012). Alternative splicing is a widespread mechanism for generating protein diversity from a single gene and has been implicated in sex-specific phenotypes across insects (Verhulst et al., 2010; Salz and Erickson, 2010). Beyond alternative splicing, other post-transcriptional mechanisms that would not be detected by standard differential gene expression analysis include differential mRNA stability and degradation rates, small- and microRNA-mediated translational repression, RNA editing, and alternative polyadenylation (Alberts et al., 2022). Any of these mechanisms could produce sex-specific differences in protein abundance or function without corresponding changes in overall transcript levels. Indeed, in insects, sex determination itself is often regulated post-transcriptionally through alternative splicing cascades, demonstrating the importance of these mechanisms in generating sex-specific phenotypes (Verhulst et al., 2010; Salz and Erickson, 2010). Future analyses of the existing RNA-seq data focusing on these post-transcriptional processes might reveal regulatory differences between the sexes that are invisible to conventional differential expression approaches.

Second, phenotypic differences do not always require transcriptomic signals of similar magnitude. In principle, differential regulation of a single key gene—encoding for example a heat shock protein that could directly mediate the physiological response to elevated temperatures—could be sufficient to produce substantial phenotypic divergence between the sexes. While such subtle but functionally important transcriptomic changes should not be dismissed, this explanation is unlikely to account for the observed patterns. For example, there is no overlap between the sets of genes showing sex-specific thermal responses in *O. bicornis* and *O. cornuta*. If a single key gene was driving sex-specific phenotypic differences in both species, we would expect to find it differentially regulated across species. Moreover, examination of the individual genes with sex-biased thermal responses in *O. bicornis* reveals no clear candidates for a gene that could be expected to be independently responsible for the observed phenotypic change. These five genes, homologues of *Drosophila melanogaster*'s *hulk*, *pastrel*, *cinnabar*, *Aasdh*, and a triacylglycerol lipase, are associated with diverse biological processes that bear little obvious connection to thermal tolerance or emergence timing. Specifically, *hulk* functions in transcriptional regulation during development in *D. melanogaster* but has no clearly established role in thermal stress responses. *Pastrel* is involved in long-term memory formation in the mushroom bodies, a function relevant to learning and navigation but not to the metabolic or developmental processes that govern emergence phenology. *Cinnabar* encodes a kynurenine 3-monooxygenase involved in tryptophan metabolism and

eye pigmentation, a pathway unrelated to thermal physiology. *Aasdh* encodes an aminoadipate-semialdehyde dehydrogenase involved in lysine degradation, again a metabolic function without obvious links to differential thermal sensitivity between sexes. Finally, while the triacylglycerol lipase could theoretically influence energy metabolism during dormancy, lipid catabolism is a conserved process orchestrated by many genes in parallel, and differential regulation of a single lipase seems insufficient to explain the phenological divergence observed between the sexes in response to elevated overwintering temperatures. Thus, the functional heterogeneity of these genes, combined with the lack of cross-species conservation, are more suggestive of them representing minor modulatory effects rather than core drivers of sex-specific thermal responses.

Lastly, the transcriptomics employed in this thesis only captured differences in gene regulation in brains, which means that sex-specific regulatory differences potentially occurring in other tissues would not be captured. While the brain has been selected as the primary tissue of interest due to its central role in receiving environmental signals and coordinating physiological responses (Gallio et al., 2011; González-Tokman et al., 2020), emergence is likely also influenced by processes occurring throughout other tissues such as the fat body, flight muscles, and reproductive organs. For instance, males and females differ substantially in reproductive investment and body composition, and these tissues may respond differently to thermal stress in ways that influence the timing of dormancy termination. However, we would most likely still expect to find a stronger parallel signal of differential gene expression in the brain, in concert with the gene expression differences in such tissues, due to the central role of the brain in coordinating responses throughout the body. Bulk RNA sequencing of whole brains may mask differences specific to individual brain subregions, though, as even within the brain, distinct neuronal networks located in different areas, could exhibit sex-biased thermal responses that might become diluted when averaging across all brain regions. Future studies looking at the transcriptomic response across several tissues or within more defined subregions of the brain, would thus be required to comprehensively assess whether tissue- or network-specific regulatory differences could underlie the observed sex-specific phenotypic responses.

5.3 Temporal Dynamics of Thermal Responses

One of the most important observations within this thesis is the accumulating, but also stage-dependent transcriptomic response of wild bees to sustained elevated

overwintering temperature profiles. This suggests that thermal responses are complex, dynamic processes that vary with the intensity, duration and timing of thermal exposure (Kingsolver and Buckley, 2020; Buckley et al., 2017). This temporal dimension has previously been overlooked in most studies on insect thermal biology, where experiments typically employed constant temperatures or short-term exposure profiles. The field-relevant temperature profiles and time-series sampling employed in this thesis thus reveal dynamics unobservable using simpler experimental designs.

The transcriptomic analyses provide one of the clearest lines of evidence of temporal dynamics (Chapter 3). The number of differentially expressed genes increased substantially with exposure duration—from several hundred genes after one week to over 3,000 genes after seven weeks in both species—suggesting a substantial difference in the more immediate and more accumulated responses to elevated temperatures. Adding even more complexity, this trend did not continue into the fourteen week time point that was additionally available in *O. bicornis*. Instead, the number of differentially expressed genes plateaued after the intermediate time point while the functional pathways affected by differential regulation differed between all time points. For example, genes associated with oxidative phosphorylation were suppressed after one week but increasingly activated after seven to fourteen weeks, whilst patterns of protein metabolism reversed between early and intermediate time points. In addition, olfactory pathway activation was only observable at the intermediate time point in *O. bicornis* and the earliest time point in *O. cornuta*. These temporal dynamics suggest that bees are not responding uniformly to elevated temperatures across overwintering and likely reflect, at least in part, progression through different dormancy and diapause phases (Denlinger, 2002; Kostál, 2006; Košťál et al., 2017). However, the experimental design employed in this thesis, whilst providing valuable temporal resolution, could not disentangle the effects of exposure duration from the effects of dormancy stage and diapause phase, as these variables were inherently correlated when simulating field-relevant conditions. Therefore, the observed temporal dynamics likely reflect both the cumulative effects of prolonged thermal exposure, as well as the changing physiological state of bees as they progress through diapause phases and through post-diapause quiescence (Košťál et al., 2017; Diniz et al., 2017). Disentangling these variables would require molecular markers that reliably indicate diapause phase or experimental designs that control for exposure duration at all time points, for example with thermal exposure in overlapping time windows of fixed length along the time-course of the experiment. However, such molecular markers do not exist yet and the described experimental approach is challenging to implement. In fact, from an ecological perspective, the confounding of these variables may be relatively unproblematic as in

field populations experiencing climate change, elevated temperatures will naturally affect bees across all dormant phases, making the integrated response observed herein directly relevant for predicting population-level consequences.

Interestingly, while transcriptomic changes were highly temporally dynamic, with distinct functional signatures at each time point, the phenotypic changes observed in Chapter 1 followed different patterns. Weight loss accumulated approximately linearly over the course of the experiment for as long as bees remained dormant under elevated temperatures, without a plateau or phase-specific shifts observed in transcriptomic responses. This discrepancy suggests that the relationship between molecular and phenotypic responses is complex. The progressive depletion of energy reserves, indicated by accumulating increases in weight loss (Hahn and Denlinger, 2011; Fliszkiewicz et al., 2012), was not accompanied by a clear transcriptomic signature of metabolic regulation that stayed consistent across all sampled transcriptomics time points. Instead, several metabolic pathways were initially downregulated and later upregulated, for example oxidative phosphorylation or lipid and protein metabolism. On the one hand, this might suggest that these pathways were actively suppressed in the beginning to retain homeostasis of energetic expenditure (Sinclair, 2015), but later this suppression was no longer possible, potentially due to hidden costs, finally resulting in an upregulation of these pathways. On the other hand, these transcriptomic changes might reflect the natural molecular progression of bees through diapause phases, accelerated by elevated temperatures (Gillooly et al., 2001). Results from one of the other physiological variable that was measured, emergence timing, showed that bees emerged earlier under elevated overwintering temperatures with no evidence of a threshold-like behaviour, where for example bees would emerge only above specific temperature thresholds, regardless of exposure duration. Instead, the shift in emergence patterns was more likely to be of accumulative nature, a response to the combination of thermal exposure magnitude and duration. Analogously to the weight loss results, this pattern did not overlap with transcriptomically observed changes, again highlighting that the relationship between these two biological levels is complicated in the context of elevated overwintering temperatures in dormant mason bees.

In summary, integration of the temporal transcriptomic dynamics with phenotypic outcomes reveals that the costs of thermal exposure accumulate progressively but manifest differently across biological levels. Weight loss becomes detectable after just two weeks of elevated exposure (Chapter 1), coinciding temporally with early transcriptomic changes in metabolic pathways (Chapter 3), while by seven weeks, extensive transcriptional reorganisation is evident and functional pathway signatures have shifted. Together, these progressive changes imply that there is no single critical

period of thermal sensitivity during dormancy—rather, thermal effects accumulate progressively, with phenotypic consequences becoming increasingly severe the longer bees experience elevated temperatures. For future research endeavours, as well as conservation and management approaches, this suggests that the full costs of climate change on overwintering bees will only become apparent when considering the entire dormancy period rather than isolated time points.

5.4 Limitations and Future Work

Whilst this thesis provides comprehensive insights into thermal responses across multiple biological levels, several limitations and challenges remain that could inform future research directions. For example, the laboratory-based experimental approach, whilst enabling reproducibility and precise control of the experimental environment, necessarily simplified the complexity of natural environments. Field conditions include additional stressors (such as pathogens, predators, resource variability) and natural thermal variation at finer spatial scales that are not easily replicated in a laboratory environment using climate cabinets. To bridge the gap between reproducibility under controlled experimental conditions and the ecological applicability of results, field experiments and field observations could be performed (CaraDonna et al., 2018). For example, studies tracking emergence phenology and reproductive success, placing groups of individuals from the same population into different, naturally-occurring thermal environments could not only validate the here presented laboratory findings but also further clarify the relationship between phenotypic plasticity and genetic adaptation in wild bees response to elevated overwintering temperatures predicted under climate change (CaraDonna et al., 2018; Villemereuil et al., 2016). The tracking of fitness-related metrics such as reproductive success throughout the active season would be of particular importance to understand if the phenotypic responses to thermal exposure observed in this work have long-term consequences for the fitness of individuals (Kingsolver et al., 2013; Ostap-Chec et al., 2021).

Additionally, the inability to fully disentangle exposure duration from dormancy phase represents a fundamental challenge in studying organisms with complex developmental and physiological progression (Tauber et al., 1986; Kostál, 2006). Whilst the temporal sampling in Chapter 3 provided valuable resolution, definitively attributing transcriptomic changes to specific dormancy phases would require additional experimental manipulations (Košťál et al., 2017), particularly, the exposure of bees to elevated temperatures only during specific phases whilst maintaining control

temperatures at other times. For example, an experimental approach using overlapping windows of fixed thermal exposure duration along the course of overwintering could be employed to determine at which phase individuals are most sensitive to thermal exposure. While such experiments would be technically challenging, they could illuminate critical windows of thermal sensitivity and identify which dormancy phases are most vulnerable to disruption from environmental stressors (Košťál et al., 2017; Denlinger, 2022).

While performing all experiments and analyses on more than one species allowed for some level of cross-species generalisation, the geographic and taxonomic scope of this thesis, focusing on only two species from a single Genus (*Osmia*: Megachilidae), limits generalisability. *Osmia* comprises approximately 350 species globally, exhibiting tremendous diversity in life histories, thermal biology, and ecological contexts (Danforth et al., 2019). Other solitary bee families—Andrenidae, Halictidae, Colletidae, Melittidae and Apidae—differ substantially from Megachilidae in developmental timing, dormancy strategies, and nesting biology (Westrich, 2019). Ground-nesting, overwintering as larvae rather than adults, facultative rather than obligate diapause: each of these life-history characteristics might mean that they respond differently to warming compared to mason bees (Tauber et al., 1986). Expanding comparative studies across this diversity would be valuable for understanding which findings are taxonomically general versus specific to cavity-nesting megachilids overwintering as adults (Ellegren, 2014).

The population-level analyses in Chapters 2 and 3, whilst providing valuable insights into genetic diversity and selection signatures, were based on samples from a single region (Germany). Larger, range-wide sampling would enable more powerful detection of population structure, local adaptation, and geographic variation in genetic diversity (Ellegren, 2014; Hoban et al., 2016). In addition, temporal sampling—comparing historical museum specimens to contemporary populations—could reveal evolutionary changes occurring over recent decades of warming (Hoffmann and Sgrò, 2011). Such historical genomic approaches have proven powerful in other systems and could directly test whether thermal responses are evolving (Ellegren, 2014).

The molecular mechanisms triggering emergence remain incompletely understood despite the insights provided by transcriptomics (Denlinger, 2022). Whilst we observed changes in metabolic genes, developmental genes, and signalling pathways, the causal chains linking thermal exposure to neuroendocrine signalling to diapause regulation require further elucidation (Košťál et al., 2017). Candidate gene approaches targeting known diapause regulatory pathways, combined with func-

tional validation through molecular manipulation, could test specific mechanistic hypotheses and reveal causal relationships (Ragland et al., 2010).

The post-emergence consequences of thermal exposure during dormancy represent a critical knowledge gap (Bosch and Kemp, 2004). Whilst we observed no elevated mortality during dormancy, previous studies document reduced post-emergence longevity, and our findings of extensive transcriptional reorganisation in addition to premature emergence and increased overwintering weight loss suggest that fitness during the subsequent active season could be reduced (Sinclair, 2015; Kingsolver et al., 2013). More long-term experiments, tracking individuals from the larval stage through overwintering, emergence, foraging, reproduction to offspring production would be able to definitively quantify fitness consequences and identify the life stages where fitness costs as a result of thermal exposure would manifest most strongly.

Climate change does not occur in isolation with wild bees facing multiple interacting stressors including habitat loss, pesticides, pathogens, and land-use intensification (Goulson et al., 2015). Understanding how thermal stress interacts with these other stressors is important for evidence-based conservation approaches (Vanbergen and the Insect Pollinators Initiative, 2013). Some questions have already been addressed, such as: Do thermally stressed bees show greater pesticide sensitivity (Albacete et al., 2023)? How strong are phenological mismatches in plant-pollinator interactions due to elevated overwintering temperatures under field conditions (Kehrberger and Holzschuh, 2019)? However, several ecologically important questions remain to be addressed, for example: Can increased floral and nesting diversity buffer against thermal stress? Is the capacity of overwintering bees to respond to pathogens impaired under thermal challenge? Addressing such multi-stressor questions requires complex experimental designs and ideally field-based approaches that capture natural stressor combinations (Goulson et al., 2015).

Finally, the mechanistic insights from this thesis could inform predictive models of population responses to climate change. Mechanistic models incorporating dormancy phases, emergence phenology, energetic costs, and phenological matching could predict population dynamics under various climate scenarios, identify critical thresholds beyond which populations decline, and evaluate potential management interventions (Tennakoon et al., 2024). Linking such demographic models to species distribution models could predict range shifts, identify climate refugia, and prioritise conservation areas (Supple and Shapiro, 2018). However, the construction of such models requires additional empirical data, such as post-emergence fitness consequences and how phenological mismatches translate to reproductive output (Potts et al., 2016).

5.5 Conclusions

This thesis demonstrates an integrative approach for understanding responses of overwintering wild bees to climate change across multiple biological levels: organismal, molecular and genetic. While the organismal level showed which fitness-relevant changes occurred, the molecular data informed on how these changes occurred and the population genetic data revealed the evolutionary context and genetic potential for adaptation (Ellegren, 2014). Integrating across these levels helps in achieving a holistic understanding of wild bees' responses to predicted future challenges posed by global change (Webster et al., 2023; Frankham, 2005; Merilä and Hendry, 2014).

The combined findings in this thesis suggest that mason bees respond to elevated overwintering temperatures through extensive phenotypic and molecular changes mediated primarily by plasticity. While this plasticity enables populations to maintain survival rates during dormancy even under moderate to strong warming, hidden post-emergence costs might manifest through premature emergence, energetic depletion, and potential phenological mismatches. Underlying these responses, we found species differences in genetic diversity and signatures of selection, suggesting differential vulnerability and adaptive potential of taxonomic groups of wild bees. In addition, temporal dynamics were more important on a molecular than organismal level. While transcriptomic responses were not static but differed across phases of dormancy and diapause, organismal phenotypic consequences of warming, such as increased weight loss and premature emergence, accumulated more linearly over the course of the experiment. Lastly, mortality during overwintering was not elevated, however potential long-term hidden fitness costs were discussed, meaning substantial challenges to population persistence under climate change due to accumulated fitness losses cannot be ruled out.

For wild bee conservation, these findings emphasise that climate change poses complex, multi-faceted challenges that similarly require multi-level solutions. The continued availability of thermally variated micro-climates and diverse plant communities with variation in flowering phenology will be particularly important, especially in anthropogenically dominated landscapes to protect species against the consequences of climate change during and after overwintering. This is particularly important for specialist bees with narrow nesting and foraging requirements. Micro-climates would allow species to find colder or more insulated thermal environments for nesting to shield against sustained thermal stress and warm spells during dormancy, whereas the availability of flowering resources early in the season due to plant di-

versity will be crucial for bees to maintain fitness even under premature emergence, as observed under thermally elevated overwintering conditions. Lastly, genomes and population genetic datasets for additional wild bees species will be required to assess and generalise on the potential for genetic adaptation in these important pollinators (Webster et al., 2023; Ellegren, 2014).

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