On the Genomic Basis of Mosaic Coevolution in a Social Host-Parasite System

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Maide Nesibe Macit

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Zusammenfassung

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Antagonistische Wirt-Parasit-Interaktionen resultieren oft in ein evolutionäres Wettrüstens, welche durch reziproke Selektionsdrücke vorangetrieben wird. Die daraus resultierenden (Gegen-)Anpassungen erfolgen über breite geographische Räume entlang ihrer Verbreitungsgebiete und unterliegen somit variablen (a)biotischen Einflüssen. Ein integrativer Forschungsansatz ist daher notwendig, um koevolutionäre Prozesse über Verbreitungsgebiete hinweg zu erfassen, wird jedoch bislang aufgrund methodischer Komplexität selten umgesetzt. Besonders bei ektothermen Organismen, deren Physiologie und Verhalten stark von Temperatur und Luftfeuchtigkeit abhängen, können klimatische Faktoren Wirt-Parasit-Interaktionen entscheidend beeinflussen. Sozialparasiten bieten hierfür ein geeignetes Modellsystem: Als ektotherme Insekten reagieren sie empfindlich auf Temperatur, und parasitieren gemäß Emerys Regel (1990) meist nahverwandte Wirtsarten, wodurch beide interagierenden Partner den gleichen klimatischen Selektionsdrücken ausgesetzt sind. In den eusozialen *Hymenopteren*, zu denen Bienen, Wespen und Ameisen zählen, hat sich Sozialparasitismus mehrfach konvergent entwickelt und zielt auf die Ausnutzung des Sozialverhaltens seiner Wirte aus. Besonders auffällig sind dulotische, ‚sklavenhaltende' Ameisen, die in jährlichen Raubzügen Wirtspuppen entführen, aus denen Arbeiterinnen schlüpfen, deren Verhalten ausgebeutet wird. Diese Raubzüge erzeugen starken Selektionsdruck auf Wirtsarten und fördern die Entwicklung spezifischer Abwehrmechanismen. Im Zentrum dieser (Ko-)Anpassungen stehen zwei Phänotypen: das chemische Profil, als Kommunikationsmittel sozialer Insekten, und das Verhalten während eines Raubzugs, meist in Form von Aggression. Parasiten unterlaufen Wirtserkennung beispielsweise durch chemische Mimikry oder Transparenz, während Wirte weitläufige Gegenstrategien entwickeln können. Zugleich dient das chemische Profil als Austrocknungsschutz und muss folglich an lokale Klimabedingungen angepasst sein. Die gleichzeitige Optimierung beider Funktionen kann zu biochemischen Zielkonflikten führen, die zu sogenannten selektiven Interferenzen führen können.

Ziel dieser Dissertation ist es, die molekularen Grundlagen der Koevolution zwischen einem dulotischen Ameisenparasiten und seinem Wirt zu untersuchen. Im Fokus stehen dabei konkurrierende Selektionsdrücke, die gegensätzlichen Anpassungen hervorrufen können, sowie potenzielle pleiotrope Wechselwirkungen in selektierten

Genen. Analysiert wird, inwiefern Koadaptationen und phänotypische Variationen auf spezifische genomische Grundlagen zurückzuführen sind, welche Gene und Funktionen unter Selektion stehen und wie klimatische Faktoren mit diesen Prozessen interferieren. **Kapitel 1** gibt einen Überblick über molekulare Anpassungen sozialer Parasiten und ihrer Wirte innerhalb der *Hymenoptera*. Solche Anpassungen sind mehrfach unabhängig entstanden und zeigen sowohl artspezifische als auch konvergente Muster. Zudem wird die Rolle molekularer Daten, ob genomisch, transkriptomisch oder epigenetisch, für das Verständnis von Koevolution sowie offenen Forschungsfragen hervorgehoben. **Kapitel 2** präsentiert die erste genomweite Assoziationsstudie (GWAS) im Ameisenwirt *Temnothorax longispinosus*, in der genetische Varianten mit der lokalen Prävalenz seines Sozialparasiten *Temnothorax americanus* in zehn Populationen untersucht wurden. Um klimatische Störeinflüsse zu kontrollieren, wurde Klima als unabhängiger Umweltfaktor in die Analyse integriert – mit dem überraschenden Ergebnis, dass klimatische Bedingungen stärkere genomische Signale hervorriefen als die Parasitenprävalenz. Da beide Selektionsfaktoren stark korrelieren, wurden überlappende Kandidatengene gezielt ausgeschlossen, um parasitenspezifische Signale zu isolieren. Dabei zeigte sich, dass insbesondere Immun-Gene stark mit der Parasitenprävalenz assoziiert sind, ein Muster bekannt in klassischen Parasiten, jedoch bislang kaum beschrieben im Sozialparasitismus. Auch Geruchs- und Geschmacksrezeptorgene sowie Gene zur Synthese und Modifikation des chemischen Profils zeigen signifikante Varianten und deuten auf eine genomische Kopplung zwischen chemischen Signalen und ihrer Wahrnehmung in Bezug auf Parasit-induzierter Selektion hin. **Kapitel 3** knüpft an die zuvor identifizierte Korrelation zwischen Klima und Parasitenprävalenz an und untersucht, wie beide Faktoren das Verhalten und das chemische Profil von *T. longispinosus* und *T. americanus* beeinflussen. Es zeigt sich, dass insbesondere das Verhalten stärker mit klimatischen Bedingungen als mit der Prävalenz des Parasiten verknüpft ist: In wärmeren Regionen flüchten Wirte, während Parasiten erhöhte Aggression zeigen, wobei in kühleren Regionen sich dieses Muster umkehrt. Chemische Profile variieren ebenfalls, wobei beim Wirt das Klima, beim Parasiten jedoch seine lokale Prävalenz den stärkeren Einfluss ausübt. Die größere chemische Distanz zwischen sympatrischen Paaren in wärmeren Regionen in standardisierte Haltung aller Kolonien

deuten auf lokale Anpassungen hin. **Kapitel 4** integriert mehrere GWAS-Analysen sowie Genexpressionsdaten und verbindet chemische und verhaltensbezogene Merkmalsausprägungen aus Kapitel 3 mit den ökologischen Selektionsfaktoren aus Kapitel 2, unter Einbezug beider Arten. Damit erlaubt dieses Kapitel neue Einblicke in die molekularen Grundlagen der Koevolution im gemeinsamen ökologischen Kontext. Es zeigt sich, dass Klima und Parasitenprävalenz artspezifisch unterschiedliche Selektionssignale hervorrufen: Beim Wirt stehen erneut Immun-Gene und konstitutiv-transkribierte Abwehrmechanismen assoziiert mit der Parasit-Prävalenz im Fokus, während beim Parasiten Selektion auf Genen erfolgt, die vermutlich mit seinem Raubzugsverhalten verknüpft sind, und zeigt allgemein größere Klima-geprägte Genexpressionsmuster. Gleichzeitig stehen in beiden Arten Gene parallel unter Selektion, die mit Verhalten, Gedächtnis, Zeitwahrnehmung, Toxinproduktion und chemischer Kommunikation assoziiert sind. Die Ergebnisse verdeutlichen, dass reziproke Anpassungen im Rahmen der Koevolution sowohl durch artspezifische als auch konvergente evolutionäre Pfade erfolgen und durch biotische wie abiotische Selektionsdrücke moduliert werden.

Insgesamt zeigt diese Dissertation, dass die Kombination aus Genomik, Verhaltensökologie und Umweltanalysen einen geeigneten Rahmen bietet, um die Komplexität antagonistischer Interaktionen im Kontext der Koevolution zu erfassen. Besonders deutlich wird dabei, dass klimatische Faktoren insbesondere in ektothermen sozialparasitären Systemen einen zentralen Einfluss auf Anpassung und Phänotypen beider Interaktionspartner ausüben können. Dies unterstreicht die Relevanz, Umweltvariabilität systematisch in zukünftige Koevolutionsanalysen einzubeziehen, vor allem im Zuge des anthropogenen Klimawandels.

Summary

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Antagonistic host-parasite interactions can lead to evolutionary arms races driven by reciprocal selection pressures. The resulting (counter)adaptations occur across broad geographic ranges along their shared habitats and are shaped by variable biotic and abiotic factors. Studying these dynamics across these large spatial distribution areas requires an integrative approach, yet such studies remain rare due to methodological complexity. Ectothermic organisms, whose physiology and behaviour are susceptible to temperature and humidity, are particularly affected by climatic variation. Social parasites offer a suitable model system in this regard: as ectothermic insects, their life-history traits are temperature-dependent, and following Emery's rule (1909), they typically parasitise closely related host species, where both partners are consequently exposed to similar climatic selection pressures. In eusocial *Hymenopterans*, including bees, wasps, and ants, social parasitism has evolved repeatedly and convergently, targeting the exploitation of host social behaviour. A particularly striking form is found in dulotic, 'slave-making' ants, which conduct annual raids to abduct host brood that emerge as enslaved workers and perform all colony tasks. These raids impose strong selection pressures on hosts, promoting the evolution of specific defence mechanisms. Two key phenotypes lie at the centre of (co)adaptive processes in both species: the chemical profile, as the communication system of social insects, and behaviour during raids, especially aggression. Parasites can bypass host recognition via chemical mimicry or transparency, while hosts develop diverse counterstrategies. The chemical profile also acts as a desiccation barrier and must therefore adjust to local climatic conditions. Since communication and desiccation protection involve distinct compound classes, their dual optimisation can result in biochemical conflicts and potentially selective interferences during coevolution.

This dissertation investigates the molecular basis of coevolution between a dulotic ant parasite and its host. It focuses on competing selection pressures that may drive divergent adaptations, and on pleiotropic interactions that influence trait expression at the genomic level. Specifically, it explores whether phenotypic variation and coadaptation can be traced to specific genomic architectures, identifies genes and functions under selection, and examines how climatic factors interact with these processes. **Chapter 1** reviews molecular adaptations in social parasites and their hosts within the *Hymenoptera*,

highlighting species-specific and convergent evolutionary signatures. It also outlines how genomic, transcriptomic, and epigenetic data can help address key questions in coevolutionary biology. **Chapter 2** presents the first genome-wide association study (GWAS) in the host *Temnothorax longispinosus*, associating genetic variants with the local prevalence of its parasite *Temnothorax americanus* across ten populations. Climate was incorporated as an independent environmental variable to account for potential confounding effects, revealing that climatic conditions produced stronger genomic signals than parasite prevalence. As both factors were highly correlated, overlapping candidate genes were excluded to isolate parasite-specific signals. This revealed that immune genes were particularly associated with parasite prevalence, a pattern well documented in classical parasite systems but rarely described in social parasitism. Further signals were detected in olfactory and gustatory receptors and in genes involved in synthesising and modifying chemical profiles, suggesting a genomic coupling between chemical signalling and parasite-induced selection on perception. **Chapter 3** builds on the previously identified correlation between climate and parasite prevalence, examining how both factors shape behaviour and chemical profiles in *T. longispinosus* and *T. americanus*. Behaviour was more strongly linked to climate than parasite prevalence: in warmer regions, hosts flee while parasites act aggressively, while in cooler regions, this pattern is reversed. Chemical profiles also varied, with climate being the dominant factor in the host and parasite prevalence in the parasite. Greater chemical distance between sympatric host-parasite pairs in warmer regions under uniform laboratory conditions across all colonies indicates that these differences may reflect local adaptation. **Chapter 4** integrates multiple GWAS analyses and gene expression data, combining behavioural and chemical traits from Chapter 3 with the ecological selection factors from Chapter 2, and does so for both species. This chapter offers novel insights into the molecular basis of coevolution in a shared ecological context. While climate and parasite prevalence produced distinct, species-specific genomic signals, host selection was centred on immune genes and constitutively transcribed defences linked to parasite pressure. The parasite, by contrast, showed selection on genes likely associated with raiding behaviour and exhibited stronger climate-related gene expression. However, several genes were under parallel selection in both species, associated with behaviour,

memory, circadian rhythm, venom production, and chemical communication. These findings show that reciprocal adaptation in this system proceeds through species-specific and convergent evolutionary pathways shaped by biotic and abiotic selection.

This dissertation demonstrates that combining genomic, behavioural, and environmental data provides a robust framework for investigating the complexity of antagonistic interactions within a coevolutionary context. It further highlights the critical role of climate in shaping the evolution of phenotypes in ectothermic social parasite systems, emphasising the importance of incorporating environmental variations, especially under accelerating anthropogenic climate dynamics, into future coevolutionary research.

General Introduction

1. Host-Parasite Coevolution across Populations

Coevolution describes the reciprocal evolutionary responses between interacting organisms and species, driven by selection pressures imposed upon one another (Sattenspiel, 2015). These interactions can occur within or between species, and may be mutualistic, predatory, or parasitic. In the latter two, a key distinction lies in the fitness outcomes for the prey/host: Predators eliminate prey fitness, whereas parasites reduce host fitness, allowing for prolonged interactions and evolutionary feedback with subsequent variations in host fitness across their interaction range (Lafferty & Kuris, 2002). The complex interplay of host-parasite interactions can be reduced to the reciprocal adaptation of a few key traits: The level of *infectivity* of the parasite is defined by its success in infecting hosts, while its *virulence* indicates the extent of inflicted damage (Anderson & May, 1982). The host may evolve counteradaptive resistance strategies to *avoid* or *overcome* parasitism, or *tolerate* infection by developing mechanisms that mitigate the impact of parasite virulence (Råberg et al., 2009; Ayres & Schneider, 2012). Accordingly, in the study of host-parasite coevolution, the *population structure of both species* sets the stage for potential adaptive responses, such as the *genetic basis of infection* and host *resistance* (Buckingham & Ashby, 2022). This reciprocal competition between parasite survival and host defences fosters the selection of, for instance, immune genes in hosts and immune evasion genes in parasites (Shivahare et al., 2023) or host behavioural adaptations to reduce contact points with parasites (Gibson & Amoroso, 2022).

Antagonistic host-parasite interactions are marked by dynamic coevolution, involving parasites' continuous reciprocal adaptation and host counter-adaptation (Dawkins & Krebs, 1979; Hamilton, 1982; Nash et al., 2008; Tellier et al., 2014). These interactions frequently result in patterns of local adaptation, where coevolving species pairs from the same locality (sympatric pairs) often exhibit higher fitness than allopatric combinations (Penczykowski et al., 2016; Laine & Tylianakis, 2024). Such local adaptation can be driven by a locally common parasite strategy within a host population. If hosts fail to ‘respond’ with a counteradaptation, this imbalance may lead to severe ecological

consequences, such as host extinction and host switching (Thompson, 2005; Nuismer & Thompson, 2006; Soler, 2014). However, a common parasite strategy within a population will often trigger counteradaptations in the host, initiating a feedback loop that may escalate into a coevolutionary arms race requiring adaptive responses across multiple biological levels and evolutionary time (Dawkins & Krebs, 1979; Hamilton, 1982; Nash et al., 2008; Tellier et al., 2014). Population structure and factors shaping it play an important role in these arms-race dynamics, since they can substantially influence the strength and direction of selection: A strong population structure, characterised by distinct genetic differentiation, can foster local adaptation, resulting in local hosts and parasites evolving into characteristics suited to their reciprocal partner within a particular environment (Greischar & Koskella, 2007). Strong gene flow can aid in the distribution of genetic variations across populations, where strong positive selection for a beneficial allele can instigate selective sweeps with rapid fixation within a population (Durrett & Schweinsberg, 2005). This, in turn, can reduce the genetic variation that could otherwise fuel the ongoing arms race between hosts and parasites. Further, migration can introduce maladaptive alleles that do not suit a new environment (Gupta & Vadde, 2019), resulting in potential restrictions in reciprocal adaptations to local hosts or parasites. Population sizes can further influence or amplify the effects of random events. In particular, genetic drift impacts small and/or fragmented populations more significantly than larger ones, diminishing genetic diversity and limiting adaptive potential, increasing the likelihood of neutral or deleterious alleles being fixed randomly (Whitlock, 2004). This becomes especially relevant since host and parasite population sizes may change dynamically with (co)evolution and time, similar to the Lotka-Volterra model proposed in predator-prey dynamics (Volterra, 1928; Lotka, 1932). These changes, especially in host population sizes, have consequences for the parasite, as they are ecologically constrained by host availability and often depend on a single or few host species for survival and reproduction (Papkou et al., 2016). The interaction among these factors shapes the evolutionary path of coevolving species, affecting their ability to adapt to their shared environment and each other.

2. The Geographic Mosaic of Coevolution

Spatial heterogeneity is a key factor shaping the outcome of coevolutionary interactions. The Geographic Mosaic Theory of Coevolution (GMTC) posits that coevolution unfolds across landscapes shaped by spatial variation in both biotic (e.g., species communities) and abiotic (e.g., climate) conditions (Gandon, 2002). As a result, selection pressures may vary drastically across locations, creating 'hot spots' of intense reciprocal selection and 'cold spots' with one-sided or absent reciprocity (Gomulkiewicz et al., 2000; Nuismer et al., 2003). From a metacommunity perspective, factors such as geographic distance, dispersal limitation, habitat overlap, species abundance, and environmental variability can all shape coevolutionary trajectories at each and in between each mosaic piece (Thompson, 2005; Urban et al., 2008; Laine, 2009; Laine & Tylianakis, 2024). Such spatially structured selection can lead to genetic mismatches, interrupted feedback cycles, or heightened trait diversity across a species' range (Walter, 2006; Tigano & Friesen, 2016). Understanding how these dynamics play out requires considering traits under selection and the genomic architecture through which coadaptation proceeds. One important framework here presents genotype × genotype × environment (G x G x E) interactions, which describe how phenotypic traits emerge through the interplay between host and parasite genotypes and their shared environment. These interactions are especially relevant in ectothermic species where environmental factors such as temperature and humidity directly affect parasite fitness and host defences (Aaltonen et al., 1997; Oorebeek & Kleindorfer, 2008). G x G x E interactions can lead to 'trait remixing', the reorganisation of phenotypic expression due to variable environments, which can facilitate rapid but context-dependent adaptation (Gomulkiewicz et al., 2000; Thompson, 2005). Traits involved in such dynamics often evolve under multiple, potentially conflicting or interfering selective pressures. Pleiotropy, where a single gene influences multiple traits (Lobo, 2008), can further complicate this process: Pleiotropic genes can exert opposing effects on traits under divergent selection, such as immunity and reproduction (Williams, 2001; Schwenke et al., 2016), with potential effects on coevolutionary interactions. This antagonistic pleiotropy can create trade-offs that constrain adaptive potential, especially across heterogeneous environments (Clarke &

Fraser, 2004; Wolinska & King, 2009; Wisz et al., 2013; Oppold et al., 2016; Mahmud et al., 2017; Amandine et al., 2022). The challenge in analysing G x G x E interactions is to sufficiently identify which of the observed changes in phenotypic traits result from selective pressure from reciprocal partners in their arms race (i.e. coevolution), which ones are caused by climatic selection pressures, and their level of linkage or independence through (antagonistic) pleiotropy (Thompson, 1999, 2005). The importance of studying host-parasite coevolution across broad geographical scales is underscored by the lack of research incorporating, for instance, easily accessible environmental variables, such as climate data. The importance of such studies, however, is evident when comparing the substantial body of scientific work demonstrating the strong influence of, for instance, temperature on various parasite traits across taxa (Macnab & Barber, 2012; Leicht & Seppälä, 2014; Franke et al., 2017; Scharsack et al., 2021; Mironova et al., 2022). The number of such studies including environmental factors, specifically climate, is likely to increase in the near future, driven by two major developments: (1) the growing accessibility of large-scale genome sequencing, and (2) rising interest in the effects of climate change on biodiversity and species interactions (see Haunschild et al., 2016).

3. Genomic Mechanisms and Molecular Signatures of Coevolution

Genomic adaptation to (co)evolutionary pressures encompasses a range of structural and mutational mechanisms. These include gene duplications that create functional redundancy, allowing one gene copy to diverge and adopt novel roles (Innan & Kondrashov, 2010), as well as gene loss events, which streamline genomes, particularly in specialist parasites that no longer require certain traits (Albalat & Cañestro, 2016). These broad genomic changes provide the substrate for fine-scale adaptation within coevolutionary contexts. Such processes have been formalised in models like the gene-for-gene framework, which posits a tight evolutionary coupling between host resistance genes and corresponding parasite virulence genes (Flor, 1971). Genomic studies have since extended these ideas to identify coevolved loci across entire genomes (Dexter et al., 2023).

Mutations are at the core of these processes, providing the raw material for natural selection to act upon. Among these, single-nucleotide polymorphisms (SNPs), and

particularly non-synonymous SNPs (nsSNPs) that alter amino acid sequences, can lead to substantial changes in protein structure and function (Nei, 2005; Shastry, 2009). Even a small number of SNPs can dramatically influence organismal phenotypes. For example, variation in odour perception in *Drosophila* was linked to just four to six nsSNPs within perception genes, which in turn affected behavioural responses upon exposure to specific chemical compounds (Rollmann et al., 2010; Wang et al., 2010). Such examples highlight how minimal genetic variation at the molecular level can significantly alter traits and potentially species interactions. However, genetic variants may evolve too slowly or lack the flexibility to keep pace with fluctuating environments. In such cases, phenotypic plasticity provides a rapid and reversible response mechanism to biotic and abiotic stressors, often mediated by gene expression changes (West-Eberhard, 1989). In host-parasite systems, this plasticity enables organisms to dynamically adjust traits in response to parasite presence, environmental cues, or social conditions. These short-term changes can also act as a 'stepping stone' to adaptation, whereby genotypes responsible for beneficial plasticity become genetically assimilated under selection within a few generations (Ghalambor et al., 2015; Campbell-Staton et al., 2017; Rivera et al., 2021; Raju et al., 2023). This may further occur through mutations in regulatory regions, such as promoters or introns, that render formerly plastic expression to become constitutive and adaptive (Cooper, 2010).

To detect the genomic basis of such traits, especially those involved in coevolutionary dynamics such as host resistance or parasite infectivity, genome-wide association studies (GWAS) can be used to link trait variation with specific SNPs while accounting for confounding factors such as population structure (Yates & Sternberg, 2013). Statistical models used in GWAS range from simple linear regressions to mixed-linear models to more complex and computationally intensive Bayesian and machine learning-based approaches. The latter two methods enable the detection of both major-effect and subtle variants in polygenic traits, where many small-effect loci contribute to a phenotype (Visscher et al., 2017). This contrasts with monogenic traits, where a single gene explains most of the phenotypic variation, a pattern often assumed in early gene-for-gene models but increasingly recognised as an exception rather than the rule in natural systems (Badano & Katsanis, 2002). The utility of GWAS has been demonstrated across diverse

host-parasite systems, including humans, where SNPs were linked to resistance or infectivity to HCV and HIV in specific 'populations' (Shevchenko et al., 2021; Märkle et al., 2023). Beyond its application in biomedical contexts, GWASs offer a powerful tool for natural host-parasite systems. It can uncover how coevolutionary pressures shape the genetic architecture of traits, capturing even subtle genomic variation that may shift the evolutionary trajectory of entire interactions.

4. Sociality in Insects and the Emergence of Social Parasites

Major evolutionary transitions mark fundamental shifts in biological organisation, where formerly autonomous units integrate into higher-level entities, such as the transition from unicellular to multicellular life, or from a solitary to a social lifestyle (West et al., 2015). Each transition is characterised by the emergence of a novel system for storing and transmitting information across generations, referred to as 'codes of life' (Kun, 2021). While early transitions were driven by genetic innovations such as the origin of the genetic code, the emergence of eusociality in insects was centred on the transmission of behavioural information. In this context, behaviour becomes a critical medium for organisation, underpinning complex social systems through mechanisms like cooperative brood care and division of labour. This lifestyle transition involved significant genomic changes, including the expansion of perception genes that regulate chemical communication (Engsontia et al., 2015; Zhou et al., 2015; McKenzie et al., 2016; Gautam et al., 2024), similar to the development of human language (Kun, 2021). These perception genes are crucial for social insects in nestmate recognition, task allocation within their division of labour, and defences against intruders (Dani et al., 2001; Blomquist & Bagnères, 2010; Lorenzi et al., 2011).

The emergence of the social lifestyle has also fostered the development of its exploitation: social parasites have developed mechanisms to infiltrate and manipulate hosts or entire castes within host colonies, exploiting their social behaviour and avoiding the costs of performing these behaviours themselves (Rabeling, 2021). Insects' social parasitism within social *Hymenopteran* species, including bees, wasps and ants, has evolved multiple times independently (Buschinger, 2009). A characteristic is their convergent loss of key traits typically found in free-living social species, such as

independent colony founding, a functional worker caste, and brood care (Sumner et al., 2004; Cervo, 2006; Buschinger, 2009). Following Emery's Rule (Emery, 1909), social parasites tend to exploit closely related species, reflecting their shared evolutionary origins and the use of their common chemical language as a medium for manipulation. The shift from worker-related tasks to host exploitation minimises the need for sensory input in social parasites, reducing selective pressure on olfactory perception, leading to a detectable reduction of odorant receptor genes (Schrader et al., 2021; Jongepier et al., 2021; Gautam et al., 2024). While facultative social parasites may retain some ability to found independent colonies, obligate social parasites depend entirely on their hosts. These parasites exhibit different strategies of host exploitation, ranging from inquilinism, where a parasitic queen cohabits with the host colony without producing workers, to dulotic parasitism, in which parasitic workers raid host nests to permanently 'enslave' foreign brood (Deslippe, 2010). Dulotic ants represent a remarkably evolutionarily distinct form of social parasitism unique to ants (Hölldobler & Wilson, 1990; D'Ettorre & Heinze, 2001). Unlike inquilines, dulotic ants retain their worker caste, which expresses a novel raiding behaviour, where parasitic workers raid and abduct host pupae to replenish their foreign, 'enslaved' workforce. Dulotic parasite species conducting highly destructive raids may reduce local host reproduction and growth, to entire colony collapses, making these 'slave makers' a potent selective force on their hosts (D'Ettorre & Heinze, 2001). The severity of these attacks has driven the evolution of many counter-adaptations in hosts, including aggression as a behavioural defence, nest evacuation, and chemical defences to overcome chemical manipulation (Alloway, 1979; Pohl & Foitzik, 2011; Koenig & Moreau, 2024a and b). Unlike hosts of other 'classic' parasites, enslaved hosts cannot directly regain fitness by resisting their parasites (Gladstone, 1981). Benefits may arise from so-called 'slave rebellions', where enslaved hosts destroy parasite brood (Achenbach & Foitzik, 2009; Pamminger et al., 2011), but front-line strategies during initial encounters can help to avoid host 'enslavement' altogether, similar to avian hosts' rejection of parasitic eggs upon recognition (Langmore et al., 2011). Within this limited yet pivotal timeframe of host-parasite interactions, coadaptive traits are contested, with outcomes depending on the success of defensive and offensive strategies.

5. Coevolving Traits Pivotal in Social Host-Parasite Interactions

Two phenotypes lie at the heart of host-parasite coevolution in eusocial insects: the chemical profile, which underpins nestmate recognition, and behaviour, which governs the response to social intruders. These traits are tightly interlinked, as behavioural responses can follow from detecting chemical cues from intruders via odorant receptors and their subsequent interpretation in the brain. Chemical communication in insects is mediated by cuticular hydrocarbons (CHCs), a diverse group of compounds synthesised via a conserved biosynthetic pathway (Chertemps et al., 2006; Chung & Carroll, 2015; Buellesbach et al., 2022). The colony-specific composition of CHCs plays a central role in nestmate recognition, reproductive division of labour, task allocation, and hygiene (Monnin, 2006; Jongepier & Foitzik, 2016). Chemical cues are exchanged via frequent contact, generating a shared but dynamic colony odour, shaped not only by genetics but also by environmental factors such as diet and temperature (Lenoir et al., 2001). Recognition depends on the detection of CHCs by odorant receptors in antennal sensilla (Hölldobler & Wilson, 1990; Ozaki et al., 2005), comparing incoming chemicals with an internal colony odour template typically acquired early in life (Crozier & Pamilo, 1996; Signorotti et al., 2015). Greater chemical mismatches, often linked to genetic distance, trigger stronger aggressive responses (Scharf et al., 2011; Von Beeren et al., 2011). Parasites may circumvent this system by mimicking host CHC profiles or by reducing their chemical cues to become chemically insignificant (Lenoir et al., 2001; Kleeberg et al., 2017), giving them an advantage in infiltrating host colonies during their raids. These strategies can be accompanied by the use of secretions from the Dufour's gland, so-called 'propaganda' substances, to manipulate host defence responses (Brandt et al., 2006).

CHCs serve a vital physiological role as waterproofing agents: linear *n*-alkanes are more effective at preventing desiccation, while methyl-branched *n*-alkanes and alkenes encode more information for communication and recognition (Bonavita-Cougourdan et al., 1987; Sprenger & Menzel, 2020). This dual role can lead to selective interferences: environmental pressures selecting for waterproofing may reduce recognition accuracy (Gibbs, 1998, 2002; Menzel et al., 2019). This was recently shown in an experimental study in *Lasius* ants, where heat-adapted colonies increased their linear CHC production,

improving desiccation resistance but compromising their recognition efficacy (Wittke et al., 2022). As such, climate-induced shifts in chemical composition may also affect behavioural responses of hosts to intruding parasites due to putative reduced recognition abilities. Similarly to the dual function of CHCs, behaviour in social insects can serve both defensive and thermoregulatory purposes (Kadochová & Frouz, 2014; Ng et al., 2017; Ma et al., 2018). While aggressive behaviour is a key first-line defence against parasitism, behavioural thermoregulation may buffer climatic stress. How these overlapping functions might interfere with each other remains unclear, but the potential for conflict between climatic adaptation and coevolutionary responsiveness on the chemical and behavioural level warrants further investigation.

Chemical profiles and behaviour thus represent the two principal coevolving traits in social host-parasite systems, shaped by coevolutionary pressures and potentially modulated by environmental heterogeneity. Social hosts and parasites are phylogenetically and consequently physiologically close, allowing both partners to potentially experience selection on homologous genes and pathways, particularly those involved in CHC biosynthesis and behavioural regulation (Cini et al., 2015). This shared ancestry and physiological similarity may facilitate the identification of genes associated with trait variation by providing a common genetic and ecological background, reducing confounding effects. It may help disentangle whether specific signatures of selection reflect coevolutionary pressures or broader environmental factors such as climate, as both species are likely to respond similarly to the same abiotic conditions. The dual role of key traits in social signalling and physiological adaptation further introduces evolutionary trade-offs and creates potential for selective interference, where adaptation to one selective force (e.g., desiccation) may indirectly shape coevolutionary outcomes (e.g., chemical signalling). Ultimately, accounting for both trait function and environmental context is essential to accurately infer the genomic basis of coadaptation in social parasite systems.

6. Study Species

The monophyletic genus *Temnothorax* is a diverse taxon found in the Northern Hemisphere (Prebus, 2017). The focal host, *Temnothorax longispinosus*, and its obligate

social parasite, *Temnothorax americanus*, share their geographical distribution across deciduous forests in the northeastern parts of North America (Mackay, 2000), with parasite densities being reported to be 0%, so parasite-free, in northern regions and up to 15% in more southwestern regions (Jongepier et al., 2014; Kaur et al., 2019). Both species inhabit sticks, acorns, and hickory nuts on the forest floor (Foitzik et al., 2009). *T. longispinosus* is a small black ant, measuring 2-3 mm in length. Young workers typically emerge in July and August and initially serve as brood carers until changes in gene expression with age cause them to switch to forager tasks (Caminer et al., 2023). While asexual reproduction via male-destined worker-laid eggs is possible in the absence of a queen, this species generally has low worker reproductive output (Konrad et al., 2012). 'Enslaved' host workers are abducted as pupae by dulotic parasites during raids and undertake virtually all colony maintenance tasks upon emergence within parasitic colonies as they would in their natal colony (Wesson, 1939; Del Rio Pesado & Alloway, 1983). *T. americanus* is slightly larger than its host and exhibits a brown to black colouration. Newly mated parasitic queens usurp local host colonies and replenish their enslaved worker numbers by parasitic workers typically conducting five to ten raids per season (Foitzik & Herbers, 2001; Blatrix & Herbers, 2004; Pamminger et al., 2011). While *T. americanus* can exploit three species (*T. longispinosus*, *T. curvispinosus*, and *T. ambiguous*) (Bolton, 2003), it shows a preference for *T. longispinosus* (Brandt & Foitzik, 2004). During the raiding season in late summer, *T. americanus* workers scout for suitable colonies, conduct tandem runs to recruit sisters and 'enslaved' host workers, and carry out raids. Outside the raiding season, parasitic workers remain within the nest, predominantly engaging in self-grooming and begging for food (Wesson, 1939). Cross-fostering experiments demonstrated that the parasite performed better with sympatric than allopatric hosts (Foitzik et al., 2009), indicating adaptation to a local host. Conversely, studies have not provided evidence for counteradaptation to a local parasite in *T. longispinosus* (Foitzik et al., 2001; Brandt & Foitzik, 2004).

Intense selective pressure from parasites, especially in areas of high parasite prevalence, has led to diverse host and parasite adaptations, with parasite prevalence often used as a predictor of trait expression. For example, host colonies tend to adopt a polydomous structure in heavily parasitised regions, splitting into smaller subunits that

are less attractive to scouting parasites (Alloway, 1979; Pohl & Foitzik, 2011). Interestingly, *T. americanus* is also facultatively polydomous, with colony splits typically occurring at the end of the raiding season, resulting in multiple raiding units that can act independently, further increasing local parasite pressure (Wesson, 1939; Del Rio Pesado & Alloway, 1983; Foitzik & Herbers, 2001). On the chemical level, host CHC profiles were found to diversify across populations, which may hinder parasitic chemical mimicry (Brandt et al., 2005). However, findings were inconsistent across studies. While Jongepier & Foitzik (2016) reported high interpopulation variation in host chemical signatures, Kleeberg et al. (2017) found more uniform host CHC profiles. The latter study also showed that parasitic ants exhibit a shift from methyl-branched to linear *n*-alkanes, reducing their recognisability, a convergent strategy found across multiple slave maker lineages. Notably, host workers carried more complex, caste-specific CHC signatures likely tied to their need for accurate recognition. This reduced reliance on specific recognition cues in parasites may help explain the convergent loss of odorant receptor genes observed across slave maker species, as nuanced social sensing becomes less critical when colonies are infiltrated through stealth and manipulation rather than communication. On the behavioural side, Jongepier et al. (2014) demonstrated that host defence portfolios shift with local parasite prevalence: colonies from unparasitised areas rely more heavily on collective aggression as a frontline defence when faced with intruders, whereas colonies from populations with high parasite prevalence more often employ nest evacuation. This suggests a trade-off between defence strategies, possibly reflecting learned behaviours and previous raiding experience (Pamminger et al., 2011; Koenig & Moreau, 2024b). Segev et al. (2017) added a climatic dimension to this picture, showing that aggression levels also vary along a temperature gradient, with more aggressive host colonies found in colder areas and less aggressive ones in warmer areas. This finding aligns with predictions from the ‘pace-of-life hypothesis’ and raises the possibility that climate may shape host behaviour rather than or alongside parasite prevalence. Together, these studies reveal consistent spatial variation in host and parasite phenotypes.

While parasite prevalence has traditionally been considered the dominant selective force behind these patterns, the potential role of climate or other environmental gradients

has received less attention. Whether some of these previously described associations may be partially driven or confounded by abiotic conditions remains an open question. Exploring this possibility could provide valuable insights into how environmental and coevolutionary pressures interact, especially when both host and parasite traits are shaped by shared ancestry and similar physiological constraints.

7. Objectives

This thesis investigates the genomic and phenotypic foundations of coevolution in an ant social host-parasite system. Focusing on the coadaptive traits of behaviour, chemical profile, and gene expression, it examines how these phenotypes vary across heterogeneous environments, particularly in response to parasite prevalence and climatic conditions, and explores their putative genomic bases. It addresses three central questions: (1) How does host-parasite coevolution manifest at the genomic level across spatially and ecologically diverse populations?, (2) Which genes are associated with variation in key coevolving traits? and finally (3) Are the same genes or pathways under selection in both species, or are genomic targets of selection largely species-specific? **Chapter 1** establishes a conceptual foundation by reviewing current studies on the molecular basis of social parasitism and coevolution in *Hymenoptera*. **Chapter 2** investigates population-level genomic differentiation in the host *Temnothorax longispinosus* across ten natural populations differing in parasite prevalence and climate. Using a genome-wide association study (GWAS), it aims to identify associated genomic variants and further examine population-level variation in CHC composition and antennal gene expression. **Chapter 3** characterises colony-level behavioural and chemical variation in both *T. longispinosus* and *T. americanus* through standardised raiding assays and chemical analyses, aiming to statistically disentangle the effects of parasite prevalence and climate on phenotypes pivotal in social host-parasite coevolution. **Chapter 4** presents a comparative genomic analysis of both host and parasite at the individual level using whole-genome resequencing and gene expression data, with trait measures acquired from Chapter 3. It combines these to conduct several GWASs using environmental and phenotypic data to investigate how these forces shape genomic variation in each species, and investigates transcriptomic activity associated with

environmental factors. This integrative approach aims to disentangle coadaptation signals and assess whether host and parasite evolve through shared or species-specific genetic pathways.

Chapter 1.

Molecular (Co)Evolution of Hymenopteran Social Parasites and Their Hosts

Marah Stoldt, Erwann Collin, **Maide Nesibe Macit**, Susanne Foitzik

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Abstract

Social parasitism describes a fascinating way of life in which species exploit the altruistic behaviour of closely related, social species. Social parasites have repeatedly evolved in the social *Hymenoptera*, including ants, bees, and wasps. The common ancestry and shared (social) environment with their hosts facilitates the study of molecular adaptations to the parasitic lifestyle. Moreover, when social parasites are widespread and virulent, they exert strong selection pressure on their hosts, leading to the evolution of defence mechanisms and triggering a coevolutionary arms race. Recent advances in sequencing technology now make it possible to study the molecular basis of this coevolutionary process. In addition to describing the latest developments, we highlight open research questions that could be tackled with genomic, transcriptomic, or epigenetic data.

Highlights:

- Social parasites are characterised by a loss of odorant receptor genes
- Different genes underlie the strategies used by different social parasites
- Wasps and bees adapt to a parasitic lifestyle by re-shaping existing gene-regulatory networks
- Novel molecular approaches could shed light on social parasite-host coevolution

Chapter 1.

Introduction

Social parasites exploit the altruistic behaviour of social species to raise their offspring (Davies & Brooke, 1989; Brandt et al., 2005; Thorogood et al., 2019), similar to avian brood parasites. This form of parasitism evolved several times independently, in particular in the social *Hymenoptera* (Hölldobler & Wilson, 1990; Hines & Cameron, 2010; Borowiec et al., 2021) and there in all three clades: Social bees, wasps, and ants. These host-parasite relationships typically evolve from common ancestors, with social parasites and their hosts being close relatives, a phylogenetic pattern previously described by Emery (Emery, 1909). Evidence from parasitic bees suggests that parasites commonly exploit their sister species (strict definition Emery's rule) at emergence, while targeting increasingly more distant and diverse hosts over time (loose definition; Sless et al., 2022). The lifestyles of social parasites vary in their degree of host dependency. Facultative social parasites can live on their own, but often use hosts to establish new colonies. Queens of the Cape honeybee *Apis mellifera capensis* can found their own colony and live independently of their host, yet, their parasitic workers disperse to *A. m. scutellata* colonies to exploit their social behaviour and to reproduce via thelytokous parthenogenesis (Neumann & Hepburn, 2002). Obligate social parasites are dependent on their host for their entire life, or at least some stages of it. In the ant *Lasius umbratus*, freshly mated parasitic queens temporarily depend on their host during the founding phase to raise their worker offspring, which later completely replace the host workers (Buschinger, 1986).

A recent phylogenetic study of ants has shown that such temporary parasitic lifestyles can evolve into permanent social parasitism, including dulosis (Borowiec et al., 2021). Parasitic inquiline species found in wasps, bumblebees and ants, have convergently lost the worker caste and instead these queens use the host's workforce to raise their sexual offspring (Dronnet et al., 2005; Cervo, 2006; Buschinger, 2009). A less extreme, but still partial loss of worker function is seen in dulotic ants (sometimes referred to as 'slave-making'), where workers have lost the ability to feed themselves and to perform nursing or foraging tasks. Consequently, they permanently depend on host

workers for their survival (Foitzik et al., 2001; Cervo, 2006; Jongepier et al., 2014), but also acquired novel behavioural traits such as the ability to conduct raids or to reproduce in the queen's presence.

Most social parasites are rare. Yet, dulotic ants and many socially parasitic wasps can be so widespread and virulent that they trigger a perpetual antagonistic cycle of host adaptation and parasite counter-adaptation (Foitzik et al., 2001; Brandt et al., 2005; Ortolani & Cervo, 2009, 2010), called coevolution. The escalation dynamics driven by strong reciprocal selection are known as co-evolutionary arms races (Thompson, 1989, 2005). Differences in parasite prevalence among communities and limited gene flow between populations may trigger a geographic mosaic with coevolutionary hot and cold spots (Thompson, 2005; Bauer et al., 2009; Jongepier et al., 2014; Kleeberg et al., 2015; Kaur et al., 2019). Given the close phylogenetic relationship and shared environment of social parasites and their hosts, they are ideal to study coevolutionary dynamics with fewer confounding factors than seen in other systems, which is important for identifying the expression and regulation of genes associated with social parasitism and host defences (Emery, 1909). In this review, we first summarise which changes in gene expression, genes and genomes have contributed to the evolution of socially parasitic lifestyles. We then discuss studies that investigate the molecular basis of social parasite-host coevolution. Finally, we propose how recent advances in omics technologies can be used to uncover not only molecular adaptations to social parasitism but also to illuminate the coevolutionary dynamics between social parasites and their hosts. In this regard, approaches used in human-pathogen systems could be applied to social insect systems to open new avenues in the study of coevolution between social parasites and their hosts.

Genomic Changes with Social Parasitism

Relaxed selection on traits that lose importance in a parasitic lifestyle (Kirkness et al., 2010; Tsai et al., 2013; Chang et al., 2015; Benoit et al., 2016) could lead to signs of gene loss to extreme genome reduction in obligate parasites of all types (Slyusarev et al., 2020). Thus, genomes of highly specialised social parasites may be expected to contain fewer genes when host exploitation renders some social traits, such as foraging and brood care,

obsolete (Cervo, 2006; Buschinger, 2009). A recent study on *Acromyrmex* inquiline ants provided evidence for genome erosion and relaxed selection in these workerless social parasites and suggested that gene loss was triggered by genomic rearrangements and fusion (Schrader et al., 2021). In contrast, a comparative study on the parasitic bumblebees of the subgenus *Psithyrus* observed increased chromosome numbers and genome sizes comparable to that of the common non-parasitic ancestor, while loss of eleven odorant receptor genes was also detected (Sun et al., 2021).

In dulotic ants, Jongepier et al. likewise reported the convergent loss of odorant receptor genes and a severe reduction of gustatory genes in their genomes across multiple independent origins of dulosis, however, without reduction in genome size (Jongepier et al., 2021). In social insects, these olfactory receptors are important for nestmate recognition and communication, but especially for the detection of volatiles such as sex pheromones or food cues (H. Yan et al., 2020). These gene families were found to have expanded massively during the evolutionary transition to insect eusociality (Robertson & Wanner, 2006; Zhou et al., 2012; Sadd et al., 2015; Zhou et al., 2015; Legan et al., 2021). Notably, many of these olfactory receptors belong to the *9-exon* subfamily, which plays an important role in chemical communication through the detection of cuticular hydrocarbons (Engsontia et al., 2015; Zhou et al., 2015; McKenzie & Kronauer, 2018). A loss of these genes suggests that selection on genes important for olfaction in social parasites is relaxed, probably caused by a reduction in social tasks that require close communication. In contrast, no evidence of gene loss in *Pogonomyrmex* and *Vollenhovia* inquilines was found, and regulatory changes within multifunctional genomes were proposed to be associated with the transition towards social parasitism in these ants (Smith et al., 2015).

Genes Associated with Social Parasitism

A first step in elucidating how social parasitism evolved is to identify genes or allelic variants associated with or causing parasitic behaviour, which are expected to be under strong selection (Fouks & Lattorff, 2016; Wallberg et al., 2016; Feldmeyer et al., 2017). Rather than identifying ‘genes responsible for social parasitism’, empirical studies have

discovered genes associated with specific parasitic traits. For example, in dulotic ants of the genus *Temnothorax*, which are active only during summer raids, genes related to circadian rhythms are under positive selection, possibly explaining the divergent activity patterns of these parasites (Feldmeyer et al., 2017). Accordingly, changes in circadian rhythms have also been found in parasitic paper wasps (Ortolani & Cervo, 2009). Furthermore, genes involved in the synthesis of cuticular hydrocarbons are also under selection in dulotic ants (Feldmeyer et al., 2017). Again, this could be related to their lifestyle, as many social parasites such as dulotic ants avoid detection by carrying fewer cuticular hydrocarbons used in host recognition (Jongepier & Foitzik, 2016; Kleeberg et al., 2017; Kaur et al., 2019). Interestingly, only a single gene from a dataset of raiding dulotic ants was found to be commonly under selection in all three species, likely explained by the divergent raiding strategies employed by these dulotic ants (Feldmeyer et al., 2017). Several studies on the socially parasitic Cape honeybee *Apis mellifera capensis* have identified genomic regions with signatures of selection (Wallberg et al., 2016; Aumer et al., 2019), which were suggested to be associated with thelytoky, a form of asexual reproduction used by parasitic workers (Wallberg et al., 2016; Aumer et al., 2019; Christmas et al., 2019). A recent genome-wide association study (GWAS) failed to confirm that the loci identified by these studies are indeed associated with thelytoky, with the exception of a sole gene, for which specific allelic variants were exclusively found in thelytokous *A. m. capensis* (Yagound et al., 2020). These studies and their diverse outcomes suggest that more work is needed to confirm genomic regions or genes selected for thelytoky and social parasitism in the Cape honey bee (Wallberg et al., 2016; Aumer et al., 2019; Christmas et al., 2019; Yagound et al., 2020).

Transcriptomic Changes with Social Parasitism

Differences in phenotype, especially in closely related species, are not always due to novel alleles, genes, or genomic rearrangements, but may also be caused by phenotypic plasticity and underlying changes in gene-regulatory networks (West-Eberhard, 1989). Since host and parasite pairs share a common non-parasitic ancestor, the evolution of social parasitism may have been facilitated by altering the expression of pre-existing

genes in a new parasitic context, a less costly strategy compared to the evolution of completely new sets of genes. For example, social insects have evolved two distinct female phenotypes, queens and workers, which mostly arise via differential gene expression (Sumner, 2006; Smith et al., 2008). Thus, in the transition to a socially parasitic lifestyle, plastic pathways could be used to adapt, facilitating initial steps that are later hardwired through genetic assimilation (Price et al., 2003). In systems where social parasites are highly reproductive in host colonies, this high fecundity is achieved either by upregulation of queen-specific genes such as *transferrin* or *vitellogenin*, as in the Cape honeybee (Aumer et al., 2018; Korb et al., 2021) or by downregulation of worker-biased genes as seen in the paper wasp *Polistes sulcifer* (Cini et al., 2015).

In another host-parasite system, both the host ant *Temnothorax longispinosus* and its social parasite *Temnothorax americanus* use tandem-running behaviour, a recruitment strategy, during nest relocation and raiding, respectively. RNA-seq experiments revealed that different genes are associated with these homologous tandem-running behaviours, as the expression of orthologous genes was not similarly explained by behaviour (Alleman et al., 2019). This suggests that the parasite has recruited novel genes that result in a phenotype similar to that of the host. However, caution should be exercised when comparing gene expression across species, as transcriptomic differences could be explained in part by differences in tissue composition of the same organs, such as in the brains of parasitic paper wasps and their hosts (Rozanski et al., 2022).

A Host Perspective

Most of the molecular studies discussed to date have focused on the transcriptomic and genomic changes associated with the transition to a socially parasitic lifestyle (see Fig. 1), while the molecular traces of the escalating arms race on hosts have received less attention. Recent evidence from wasp social parasite systems suggests that hosts may also use pre-existing phenotypic plasticity to defend themselves. Cini et al. showed that parasitised workers upregulate a gene with fertility function that is normally overexpressed in queens (Cini et al., 2014, 2015, 2020). Since worker reproduction is usually costly but increases host fitness when in a parasite nest, one could argue that the

possibility of neutral evolution of these regulatory changes is unlikely. In fact, there is additional evidence that this host response is triggered by the presence of the parasite: *Polistes dominula* workers do not begin to reproduce when a conspecific unrelated queen replaces their mother (Monnin et al., 2009). Further studies should unravel whether this response represents a form of rebellion or even benefits the parasite in some way.

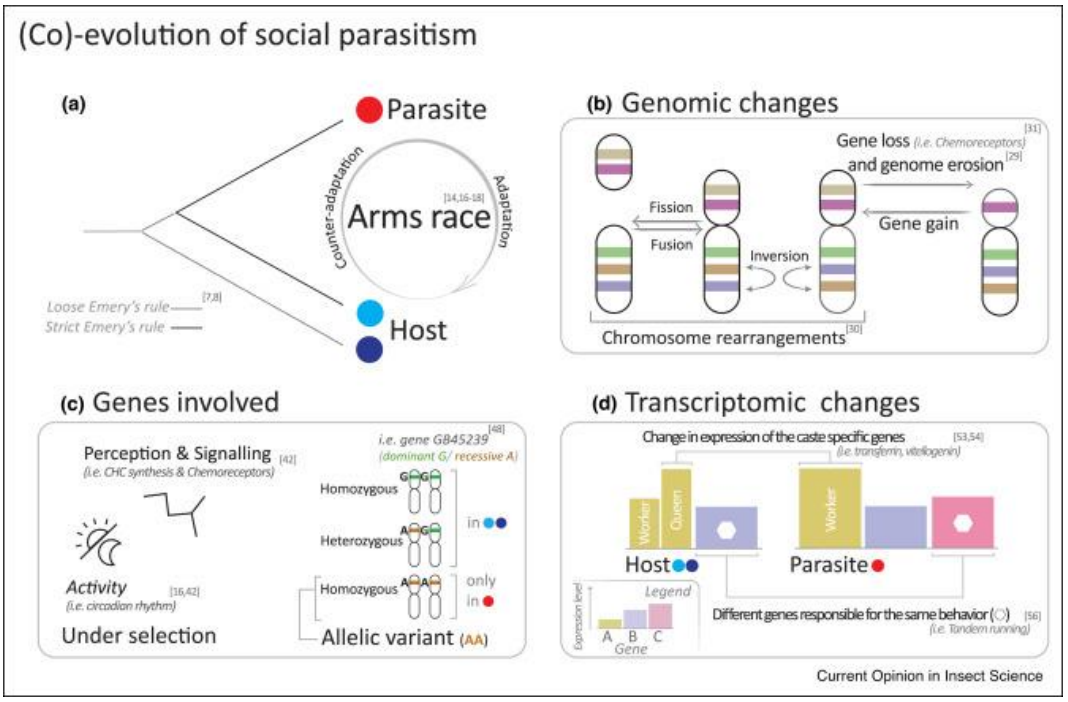

Figure 1. Molecular (co)evolution of social parasites. Schematic overview of the molecular (co)evolution of social parasitism including illustration of some examples. **(a)** Social parasites emerge from a common ancestor with their hosts from which they are more or less closely related (Strict/Loose Emery's rule; (Emery, 1909; Sless et al., 2022). When parasite prevalence and virulence are high, interactions can escalate into a coevolutionary arms race of parasite adaptations and host counter-adaptations (Foitzik et al., 2001; Brandt et al., 2005; Ortolani & Cervo, 2009, 2010). Evidence of molecular changes have been found on three levels: **(b)** Genomic evolution in parasites due to chromosome rearrangements (Sun et al., 2021), the loss of gene families (Jongepier et al., 2021) or genome erosion (Schrader et al., 2021). **(c)** Genes related to the specific lifestyle/infection strategy are under selection in parasites for example, genes involved in circadian rhythm or signalling (Ortolani & Cervo, 2009; Feldmeyer et al., 2017). The allelic variant identified to be causal for social parasitism in the Cape honeybee is recessive so that parasites are always homozygous for this allele (Yagound et al., 2020). **(d)** Genes can lose their plastic expression in social parasites and be expressed across social environments as seen in parasitic paper wasps or honeybees (Aumer et al., 2018; Korb et al., 2021) or new genes can cause homologous phenotypes to the ones observed in hosts (Alleman et al., 2019).

Another interesting avenue for research is to investigate how varying selection pressures may drive the expression of genes underlying offensive and defensive traits in parasites and hosts. Parasite prevalence in the host population has been linked to the expression of phenotypic traits in ant and wasp hosts (Ortolani & Cervo, 2009, 2010; Jongepier et al., 2014; Kleeberg et al., 2015; Jongepier & Foitzik, 2016). These traits include increased aggressiveness, altered defence strategies, colony demography and cuticular hydrocarbon diversity (strategies reviewed in Grüter et al., 2018). A first study by Kaur et al. compared the transcriptomic response of *T. longispinosus* hosts when facing their parasite *T. americanus* as a function of parasite prevalence in the community (Kaur et al., 2019). This revealed that gene expression in the host brain depends on the origin of the parasite they encounter, as dulotic workers from high prevalence populations gained undetected access to the host colony (and thus did not trigger aggressive defences), indicating that the parasite leads the coevolutionary arms race. Table 1 provides an overview of known host defence traits in combination with hypotheses on their underlying molecular bases. These testable predictions could be considered by social insect researchers to investigate the molecular coevolution of social parasite-host systems.

Table 1. Molecular basis and mode of selection on host-defence traits. If selection pressure exerted by social parasites on host populations is high (as seen especially in some dulotic ants and paper wasp social parasites), hosts will develop behavioural, physiological or demographic defences (for a summary of host defences see Aumer et al., 2018). The molecular basis and mode of selection on these coevolutionary adaptations is not well understood and here we provide some predictions to guide future research.

Parasite trait	**Host defence trait**	**Examples**	**Suggested molecular mechanism | Mode of selection**
Tracking host recognition cues via mimicry or camouflage	Increased diversity of recognition patterns to undermine host-matching	Positive correlation between parasite pressure and intercolonial CHC diversity (Martin et al., 2010; Jongepier & Foitzik, 2016)	Molecular changes (e.g. balancing/relaxed selection, increase in mutation rate, introgression, or de-canalisation) on CHC synthesis genes leading to higher variation in chemical cue diversity
Display of overt aggression by parasite to invade/raid host colonies during specific times of the year	**Increase in aggression and/or optimisation of fighting strategies in response to attacks**	Increased aggression in hosts from highly parasitised populations, especially during seasons with frequent parasite attacks (Brandt et al., 2005; Jongepier et al., 2014)	Gain of and selection on genes linked to aggression and recognition abilities, for example, odorant receptors
			Phenotypic plasticity in aggression/recognition traits via differential gene expression in response to seasonal variation in attack risks (Alleman et al., 2018)
	Plastic behavioural defences in response to selection pressure by parasites	Collective defence portfolios shift with parasite pressure (Jongepier et al., 2014)	Behavioural plasticity linked to parasite-induced changes in gene expression

	Increase in body size and/or colony size to improve nest defence	Increase in host body size in the presence of parasites (Ortolani & Cervo, 2010)	Genetic or transcriptional changes in genes related to individual and colony development
Parasitic queens invading host colonies to seek adoption	Strict monogyny (in ants) facilitating the rejection of additional queens, including parasitic ones; Generally, improvement of queen recognition	Link between monogyny and high parasite pressure (Herbers & Foitzik, 2002)	Selection on and expression changes in genes related to social structure and recognition
Manipulations induced by parasites to facilitate host invasion or cohabitation	Unresponsiveness to parasitic manipulation	Positive correlation between parasite prevalence and the ability of the parasite to manipulate the host (Foitzik et al., 2003; Jongepier et al., 2015)	Resistance to manipulation due to alteration of odorant perception/ processing

Novel Molecular Avenues

We propose additional strategies to decipher the molecular basis of social parasite evolution. On the genomic level, several studies have identified gene loss in parasites. However, as social parasites also exhibit novel morphological and behavioural traits, such as saber-shaped mandibles or raids, repurposing of existing genes or gene gains should also play a role. Gains have been previously described in parasitic nematodes, where gene families involved in detoxification expanded, likely to facilitate parasite survival in the presence of defensive chemicals released by the host (Zhang et al., 2020). Future genomic studies should therefore focus more on gene gains and innovations associated with the socially parasitic lifestyle. So far, we have considered genes associated with social parasitism as separate entities, but they could also be linked and inherited as supergenes. Indeed, alternative reproductive strategies were found to be regulated via large non-recombining elements in several ant species (Wang et al., 2013; Purcell et al., 2014; Lagunas-Robles et al., 2021; Brelsford et al., 2020; Z. Yan et al., 2020) and it is similarly possible that the switch to a novel socially parasitic strategy might be linked to a parasitic supergene (Linksvayer et al., 2013). In addition to studying the genomes and transcriptomes, epigenomes should be sequenced to investigate signs of epigenetic regulation that may promote rapid adaptation to interaction partners (Jablonka, 2017). In the greater wax moth *Galleria mellonella* and the red flour beetle *Tribolium castaneum,* epigenetic factors including DNA methylation, histone acetylation and the activity of miRNAs were found to contribute to rapid adaptation in response to bacterial infections (Vilcinskas, 2016; Mukherjee et al., 2019). Epigenetic studies have already been performed on social insects and should be easily transferable to social parasite systems (Simola et al., 2016; Collins et al., 2017; Cardoso-Júnior et al., 2021; Sieber et al., 2021; Morandin & Brendel, 2022). While RNA-seq has been used extensively in hymenopteran social parasites, proteomic approaches are less frequently performed but are necessary to investigate possible post-transcriptional changes that may play a role during social parasite evolution (Hegde et al., 2003). Whether similar mechanisms contribute to social parasite evolution remains to be investigated.

In addition to increasing our knowledge of the molecular mechanisms underlying the social parasite and host phenotype, future studies should aim to determine how parasite and host populations shape each other at the molecular level. This can be done by studying genes and allelic variants that influence the outcome of the coevolutionary arms race. It is likely that the expression of such genes changes in response to the prevalence of the parasite and that alleles alter their frequency accordingly. Furthermore, by using methods such as GWAS, it is possible to relate genomic positions to varying parasite load, and in the case of social parasites, to the prevalence of social parasites within a population, which are likely to drive adaptations to the interacting partner (Hirschhorn & Daly, 2005). Thus, we are in need of more population genomic studies to detect variation in genotypes linked to parasite presence. In addition, human pathogens have been studied using natural co-GWAS, in which host and infecting parasite are sampled from natural populations, to investigate whether the genomes of both interacting partners determine the infection outcome (Ansari et al., 2017; Lees et al., 2019). We believe that a similar approach could be used to study hymenopteran social parasites and their hosts. By sampling hosts and parasites from the same parasite colony, it is possible to determine whether the phenotype and fitness of a parasitic wasp, bee, or ant might be influenced not only by her own genome, but also by the genome of the host colony in which she grew up or which she exploits as an adult. Such genotype-to-genotype interaction has already been observed in social insects outside the context of social parasitism: In bumblebees, the expression of immune genes depends on both the genotype of the bumblebee and the genotype of the trypanosome parasite with which it is infected (Barribeau et al., 2014).

Outlook

Based on the above suggestions, we propose here that ideal model systems for studying the molecular coevolution of hymenopteran social parasites and their hosts should meet the following requirements: (1) Their genomes need to be sequenced with a corresponding annotation of good quality for detecting any genomic rearrangements, but also to study the epigenome using methods such as chromatin immunoprecipitation

sequencing (ChIP-seq), bisulfite sequencing or ATAC-seq (Assay for Transposase-Accessible Chromatin using sequencing). (2) Ideally, the species should belong to a clade with multiple convergent origins of parasitic behaviour to study gains and losses associated with a parasitic lifestyle. (3) If parasite prevalence varies across communities, the coevolutionary trajectory leading to different adaptations could be studied in more detail. Thanks to the effort of the Global Ant Genomics Alliance and others, more and more high-quality ant genomes are being published, revealing multiple independent origins of social parasitism (Boomsma et al., 2017; Jongepier et al., 2021). Systems with coevolutionary arms races, such as those described before (Foitzik et al., 2003; Ortolani & Cervo, 2010), offer excellent possibilities to advance our knowledge of the molecular mechanisms driving host and parasite adaptations. In the long run, parallels to other host-parasite systems, such as avian brood parasites, will allow us to determine whether similar coevolutionary dynamics shape the evolutionary outcomes not only on a behavioural level but also at the molecular level (Hölldobler & Wilson, 1990).

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Conflict of Interest Statement

Nothing declared.

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Chapter 2.

Genomic Basis of Adaptation to Climate and Parasite Prevalence and the Importance of Odorant Perception in the Ant *Temnothorax longispinosus*

Maide Nesibe Macit[1], Erwann Collin[1], Markus Pfenninger, Susanne Foitzik[2], Barbara Feldmeyer[2]

[1] shared first authors

[2] shared last authors

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Abstract

A co-evolutionary arms race ensues when parasites exhibit exploitative behaviour, which prompts adaptations in their hosts, in turn triggering counter-adaptations by the parasites. To unravel the genomic basis of this coevolution from the host's perspective, we collected ants of the host species *Temnothorax longispinosus*, parasitised by the social parasite *Temnothorax americanus*, from ten populations in the northeastern United States exhibiting varying levels of parasite prevalence and living under different climatic conditions. We conducted a genome-wide association study (GWAS) to identify single-nucleotide polymorphisms (SNPs) associated with both prevalence and climate. Our investigation highlighted a multitude of candidate SNPs associated with parasite prevalence, particularly in genes responsible for sensory perception of smell including odorant receptor genes. We further focused on population-specific compositions of cuticular hydrocarbons, a complex trait important for signalling, communication, and protection against desiccation. The relative abundances of *n*-alkanes were correlated to climate, while there was only a trend between parasite prevalence and the relative abundances of known recognition cues. Furthermore, we identified candidate genes likely involved in the synthesis and recognition of specific hydrocarbons. In addition, we analysed the population-level gene expression in the antennae, the primary organ for odorant reception, and established a strong correlation with parasite prevalence. Our comprehensive study highlights the intricate genomic patterns forged by the interplay of diverse selection factors and how these are manifested in the expression of various phenotypes.

Keywords: Social parasitism, dulosis, local adaptation, PoolSeq, cuticular hydrocarbons, odorant perception

Introduction

Parasites often exert significant selection pressure on their hosts, leading to the evolution of counteradaptations in the host aimed at minimising the parasite's impact. This dynamic fuels an arms race, resulting in reciprocal co-adaptations in both interaction partners (Dawkins & Krebs, 1979; Tellier et al., 2014). The stage for this coevolutionary arms race is set by the prevailing environmental conditions, which vary in time and space, playing a crucial role in shaping the evolutionary trajectories of sympatric host and parasite populations (Clarke & Fraser, 2004; Gregory, 2009; Wisz et al., 2013; Oppold et al., 2016). Adaptive traits can thus evolve in response to biotic and abiotic selective forces in parallel (Briscoe Runquist et al., 2020), and may even favour opposite trait expressions, similar to antagonistic pleiotropy (Garland et al., 2022). With resulting physiological, morphological and/or behavioural adaptations induced by these interacting forces, any analysis that seeks to understand the evolutionary forces acting on a host species through a parasite needs to consider abiotic and other biotic factors alike.

A prime example of a fundamental trait serving both abiotic and biotic factors are the cuticular hydrocarbons (CHCs) of insects. These CHCs primarily safeguard the body of arthropods against desiccation (Edney, 1977; Blomquist & Bagnères, 2010; Berson et al., 2019), with an additional role in communication. Here, they can for instance signal the fertility status (Venard & Jallon, 1980; Jallon, 1984) and, in social insect societies, regulate the hierarchy and division of labour (Monnin, 1999; D'Ettorre & Heinze, 2001; Leonhardt et al., 2016; Honorio et al., 2019). The precise function of all CHC compounds remains largely unknown, and we are only in the early stages of comprehending their specific or multiple roles. Two important classes of CHCs are the linear *n*-alkanes, which tightly layer on insect cuticles as protection against desiccation, and the methyl-branched *n*-alkanes, mainly serving as recognition cues in the discrimination of nestmates and intruders (Dani et al., 2001; Van Zweden & d'Ettorre, 2010; Lorenzi et al., 2011).

This dual function of CHCs introduces an intriguing conflict since the compounds required for protection against desiccation may be at odds with those serving in communication, as methyl-branching decreases desiccation protection capacities (Gibbs

& Pomonis, 1995; Gibbs, 1998, 2002). Depending on the position, adding a methyl-branch to the carbon chain can significantly reduce the melting temperature (Gibbs & Pomonis, 1995), which renders these communication signals more volatile in warmer climates. Consequently, environmental parameters such as temperature were shown to influence the composition of CHC profiles in social insects, indicative of climatic adaptation (Wagner et al., 1998, 2001; Martin & Drijfhout, 2009; Menzel et al., 2018; Sprenger et al., 2019). Simultaneously, the organisational structures of social insects hinges on their ability to produce and recognise these nuanced cues, which act like chemical key cards, allowing individuals to access their native colony (Martin et al., 2008; Krasnec & Breed, 2013). Their diverse functions may result in a tug-of-war for these two classes of CHCs to find an optimum or balance to properly serve both functions sufficiently. In the context of social parasitism, cuticular hydrocarbons play an intriguing role, since social insect parasites break into the fortress of insect societies by overcoming said chemical barrier (Lenoir et al., 2001). During the course of their evolution, these parasites developed and employed various strategies to subvert their hosts' chemical recognition processes and disrupt host defences, for instance by using glandular secretions as a type of chemical warfare (Allies et al., 1986; Bauer et al., 2009), or by reducing their chemical profile in a strategy known as 'chemical insignificance' (Lenoir et al., 2001). The ultimate goal of the parasite is to coerce host workers into brood care of parasitic offspring and colony maintenance.

The small, acorn-dwelling ant *Temnothorax longispinosus* is the main host of the dulotic or 'slavemaking' parasite *T. americanus* (Herbers & Foitzik, 2002), which relies on the host workforce to perform virtually all colony tasks (Wesson, 1939). The geographical distribution of the host extends from Quebec, Canada, in the north to Mississippi, USA, in the south and from Nova Scotia in eastern Canada to Minnesota, USA, in the west (Mackay, 2000) and consequently covers a diverse range of climates. Its parasite coinhabits these areas, albeit in higher densities in warmer regions compared to the host (Jongepier et al., 2014, 2015; Kaur et al., 2019). Parasite prevalence, i.e., the relative abundance of social parasites to host colonies, reflects the parasite pressure on the host, and, in this system, is higher in the southwestern populations. Along their widespread habitats, the differences in abiotic conditions such as climate could be

reflected in various influences on the evolutionary trajectories of the local host populations and consequently the chemical trait expression of CHCs (Blomquist & Bagnères, 2010).

The chemical defences of *T. longispinosus* against 'enslavement', chemical manipulation, and consequent infiltration strategies of the social parasite have been intensively studied. For example, the parasite's ability to be chemically insignificant is based on the loss of a number of methyl-branched *n*-alkanes (Lenoir et al., 2001; Kleeberg et al., 2017). In fact, parasites from areas with high prevalence carry even lower concentrations of these recognition cues and are less likely to be attacked by their hosts (Kaur et al., 2019). Further, the CHC profile of *T. americanus* seems to track that of its host populations (Brandt et al., 2005), likely leading to more successful raids and keeping the aggression of exploited host workers towards parasite pupae in check (Achenbach et al., 2010). As coevolutionary arms races can trigger host diversification (Hamilton, 1982) one of the reported host counteradaptations is indeed the diversification of chemical profiles in host populations from areas where the parasite is present (Jongepier & Foitzik, 2016, but Kleeberg et al., 2017). The resulting unique chemical composition makes it impossible for the parasite to adapt chemically to all local hosts. Another chemical weapon used by the parasite is the secretion of its Dufour's gland, manipulating its hosts during raids, resulting in defenders attacking nestmates rather than intruders (Brandt & Foitzik, 2004). Host populations under strong parasite pressure have thus counter-evolved a lower perception of these chemical manipulation tactics (Jongepier et al., 2015), and at the same time often increase their levels of aggression after a previous encounter with a social parasite (Pamminger et al., 2011; Scharf et al., 2011) as an *a posteriori* defence for future attacks. 'Flight' can also be a successful behavioural strategy in response to social parasites, and the 'fight versus flight' defence portfolio has been observed to shift with parasite prevalence in the field (Hermann, 1984; Hölldobler & Wilson, 1990; Jongepier et al., 2014). Similarly, in areas with low to high parasite prevalence, there is a shift from mono- to polygyny, and an increase in colony size (Foitzik & Herbers, 2001; Foitzik et al., 2009), reducing preferability to the parasite. Although these chemical, behavioural and social-organisational shifts with parasite prevalence are well documented, there are

no studies yet to show the extent to which they have a genetic basis or are in fact largely plastic.

In this study, we aimed to identify signals of selection in the genome of the host ant *T. longispinosus* by two selective forces – climate and parasite prevalence – using a whole-genome sequencing approach on a population level. We further investigated potential links between the population-level expression of two phenotypes – the chemical profile involved in host signalling, and antennal gene expression as an indicator of perception capabilities – to the genome and the environment. We collected 100 independent *T. longispinosus* colonies each from ten different communities in the northeastern USA, including previously studied and new sites, varying in their parasite prevalence and local climate. Our approach included (i) a genome-wide association study (GWAS) to identify single nucleotide polymorphisms (SNPs) associated with either local parasite prevalence or climate, (ii) an investigation of population-specific differences in chemical profiles, (iii) a population-level antennal gene expression study to investigate whether transcriptional activity shifts along the parasite prevalence gradient, and (iv) a GWAS to identify loci associated with the abundance of specific CHC compounds. Since the two analysed phenotypes were shown to have a significant level of plasticity depending on various (a)biotic factors (Feldmeyer et al., 2016; Menzel et al., 2018; Segev & Foitzik, 2019), all experimental colonies were kept under controlled standard laboratory conditions to generate a phenotype expression free from environment-induced variability. Our goal was to uncover the genomic footprints associated with adaptations observed and described in prior studies, and putting forward potential candidate genes for exploration in future studies.

Material and Methods

Ant Collection and Maintenance

Our focal species, *Temnothorax longispinosus*, has black, small, 2-3 mm long workers in facultatively polygonal colonies of several dozen to a hundred workers. It is widespread in deciduous forests in the northeastern USA and southeastern Canada. The colonies inhabit crevices, sticks, acorns or hickory nuts on the forest floor (Foitzik et al., 2009).

Host colonies were collected from July to September 2021 across ten different locales in the north-eastern USA, with minimum distances of 100-200km between collection sites (Fig. 1a and Table S1 and S2). The collection sites were chosen based on previous sampling efforts to ensure long-term information on parasite prevalence and host colony traits (Kaur et al., 2019), with additional sites being selected based on geographical location, forest composition and focal tree densities, namely oak and hickory (U.S. Forest Service, 2021). Per site, collection was further divided in several sub-sites within a 5x5km grid (2 - 10 subsites per population; Table S2) to capture local genetic diversity. Colonies were mainly collected in hollow acorns, less frequently in hickory nuts or rock crevices, and then moved into plastic bags, fed, and stored at 4 °C before being transported to Mainz, Germany. There, they were placed in three-chambered nest boxes with an artificial nest site consisting of acrylic glass (3 mm high) with an entrance embedded between two microscope slides (7.5×2.5×0.5 cm), darkened with red foil. As both, transcriptional activity and the cuticular hydrocarbon profile, can be highly dependent on environmental factors in ants (Feldmeyer et al., 2016; Menzel et al., 2018; Segev & Foitzik, 2019), natural confounding factors were reduced by standardising temperature to 18 °C, controlling humidity (80%), and light cycles of (12h:12 h dark:light) for all colonies. Ants were fed once per week, following the 'Bhatkar diet' (Bhatkar & Whitcomb, 1970) with water given ad libitum, until dissections in mid-November 2021.

Estimating Parasite Prevalence and Climate

For a strong estimation of parasite prevalence, that is the percentage of parasitic colonies in the whole *Temnothorax* community (consisting of *T. longispinosus* and *T. americanus*), we chose previously studied sampling sites (Herbers & Foitzik, 2002; Brandt & Foitzik, 2004; Foitzik et al., 2009; Jongepier et al., 2014), ensuring stable long-term data, since social parasites are irregularly distributed (Foitzik & Herbers, 2001; Herbers & Foitzik, 2002). *Temnothorax* ants have a long generation time of 5-15 years, and their large population sizes suggest relatively stable parasite prevalence over time. Since we focused on *T. longispinosus* colonies and the impact of local parasite pressure, we excluded parasitised colonies with only *T. curvispinosus* host workers (co-occurring with the focal host species in Massachusetts, New York South, Ohio, and West Virginia), a less preferred

host species (Brandt & Foitzik, 2004) from our calculations. Mixed colonies with both exploited *T. longispinosus* and *T. curvispinosus* workers were weighted based on the ratio of workers from each host species (for instance, a 1:1 ratio of both hosts in a parasitic nest is counted as 0.5 instead of 1). To investigate local adaptation to the climate, we retrieved climate data from the CHELSA bioclim database (1981-2010, v.2.1; Supplement, Table 'Climate_data'), which consisted of ten temperature and eight precipitation values. PCA plots were created using *fviz_pca_biplot()* from the *FactoMineR* package (Lê et al., 2008) in *R* v.4.2.2 (R Core Team, 2021). Using a probabilistic broken stick model, Principal Component 1 (PC1) was identified to explain more variances than expected by chance. Thus, PC1 climate eigenvalues were used for our genome-environment-association analysis.

Sample Preparation and DNA/RNA/CHC Extractions

To investigate local adaptation on population level, we took two workers from 90 to 100 independent, predominantly single queen colonies per population (100 in all populations except in New Hampshire with 90, Maryland with 99, and West Virginia with 97 colonies). From each colony, the first two workers that ran to the entrance upon nest disturbance were removed and anesthetised on ice. This procedure allows to collect workers with similar behaviour and transcriptional activity in a standardised way (Kohlmeier et al., 2017, 2018, 2019). From one ant, two legs were dissected for the population genomics analysis (PoolSeq) and transferred to an empty tube, and both antennae were dissected for a pooled gene expression analysis (PoolRNASeq), submerged in 100 µL of Trizol and incubated overnight. Samples were stored at -80 °C until extraction. Dissections were split into 4x25 batches per population, randomly distributed over a two-week span to avoid batch effects, with equal representation of colonies from all subsites. The second ant was taken for pooled gas-chromatography and mass spectrometry (GC-MS) to identify cuticular hydrocarbons (CHCs) and determine the population-level CHC profile. Individual ants were pooled in a glass vial with ants from the same population and stored at -20 °C until CHC extraction. For DNA extraction, the four batches per population were merged into a single tube containing 45 µL ATL buffer and four ceramic beads (ø 1.4 mm). Tissue was then lysed at 30.0 Hz for 9 min

using QIAGEN Tissue Lyser II Retsch MM400. DNA was isolated using the Dneasy Blood & Tissue Kit (Qiagen) following the manual for insect tissue. Pooled whole-genome sequencing was conducted by Novogene with 450 bp insert size to a mean coverage of 48x. For RNA extraction of pooled antennae, all four batches per population were merged in a new tube containing eight ceramic beads (ø 1.4 mm). Tissue was lysed for a total of 12 min at 30.0 Hz using QIAGEN Tissue Lyser II Retsch MM400. RNA was extracted using the Direct-zol RNA Microprep Kit (Zymo). Sequencing of 2x150 bp reads was conducted by Novogene using Illumina NovaSeq. For CHC extraction, pooled ants were air dried for 5 min on a clean dust-free tissue paper under the fume-hood and then transferred back to their original glass vial. Each glass vial was then filled with 350 µl mixed with 50 µl of *n*-octadecane internal standard solution concentrated at 0.01 µg/µL, and then incubated for an additional 10 min. Ants were carefully removed from the glass vial without removing the solute. The volume of the solution was then concentrated using constant nitrogen flow, and transferred into an inlay stored in the glass vial at -20 °C before proceeding to the gas chromatography on Agilent Technologies 7890A and mass spectrometry.

PoolSeq Analysis

Whole-genome sequencing data were trimmed using *Trimmomatic* v.0.39 (Bolger et al., 2014) with a 10 bp head crop, and quality-checked with *FastQC* (Andrews et al., 2015). Overlapping reads in pooled whole-genome sequencing data were identified using *pear* v.0.9.11 (Zhang et al., 2014) and separately mapped and later-on merged to the non-overlapping reads. Mapping of trimmed reads was performed using *BWA mem* (Li & Durbin, 2009) with a minimum seed length (k) of 30 and using an unpublished reference genome of *T. longispinosus* (Boomsma et al., 2017), which resulted in a mean mapping rate of 95.6% (± 1.0%). *Samtools* v.1.10 (Li et al., 2009) *flagstats* was used to determine mapping rate and average coverage. We used *Picard* v.2.20.8 (Broad Institute, 2018) *markDuplicates* to mark and remove duplicate sequences. We then sorted and converted to bam using *samtools* and removed mappings with low quality using *samtools view* (-q 20 -f 0x0002 -F 0x0004 -F 0x0008). Files were combined using *samtools mpileup* to a single file and then converted to a sync file using the *mpileup2sync.jar* script provided by

PoPoolation2 v. 1.201 (Kofler et al., 2011a). We determined linkage disequilibrium using *LDx* (Feder et al., 2012), which showed convergence at around 300 to 400 bp at R^2 of 0.3 in all populations, meaning that SNPs within this range ought to be linked (Fig. S1a). Thus, for further analyses a window and step size of 1 kB were used. We created individual pileup files using *samtools mpileup*, identified and removed indels using *PoPoolation1* v.2.2 (Kofler et al., 2011b) *identifygenomic-indel-regions.pl* and *filterpileup-by-gtf.pl*, respectively. SNPs with quality < 20 were filtered using *subsample-pileup.pl*. To investigate genetic diversity within a population, we used *variance-sliding.pl* and calculated Tajima's π for nucleotide diversity and Watterson's estimator θ for genetic diversity with window and step size of 1 kB. Plots were generated using the *boxplot()* function in *R*. To investigate interpopulational differences, we piled up all populations and the reference files again using *samtools mpileup*, converted to a sync file, and identified and removed indels as previously described. We estimated population divergence as F_{ST}, and their corresponding FET values using *fst-sliding.pl* and *fisher-test.pl* from *PoPoolation2*, respectively. We compared allele frequencies between populations with extreme differences in conditions using the Cochran-Mantel-Haenszel (CMH) test statistics, as we did not expect linearity in loci associated with either of our factors and rather assumed a multigenic trait expression. We conducted CMH tests between the two highest-ranking populations (West Virginia and Pennsylvania) with the lowest-ranking ones (Maine and New Hampshire) in regard to parasite prevalence and irrespective of other factors like local climate (Ohio was excluded due to its unique patterns in allele frequencies; Fig. S2a). We used *PoPoolation2's cmh-test.pl* and *cmh2gwas.pl*, setting the significance level at $p \leq 0.0001$. To account for putative biases from the choice of pairs, analysis was performed twice with reciprocal pairs. With 92% overlaps in SNPs in both runs, non-overlapping SNPs were excluded from further analyses. For PC1 climate eigenvalues, we selected population pairs with the greatest pairwise climate differences, comparing Massachusetts versus Maine, and Vermont versus New York South, with a 95% overlap in both reciprocal runs. Our findings revealed a strong correlation between PC1 climatic eigenvalues (henceforth PC1 climate), explaining 42.1 % of the variance and parasite prevalence (Pearson's cor.: $r = -0.78$, $p = 0.008$; Fig. S2b). We therefore decided to overlap the outliers associated with parasite

prevalence and climate to obtain parasite-prevalence-exclusive SNPs, CMH_{para}, and climate-exclusive SNPs, CMH_{clim}, while discarding overlapping SNPs associated with both conditions. Significant SNPs were categorised as within genes, exons, introns, 2 kB around of genes, or outside of genes using *bedtools intersect* (Quinlan & Hall, 2010). SNPs resulting in amino acid changes were identified using *CROXA/tbg-tools* v.2.0 (Schönnenbeck et al., 2021) and individually investigated using *igvtools* v.11.0.2. (Robinson et al., 2011).

PoolRNASeq Analysis

Trimming and quality checks of PoolRNASeq reads were performed as described above. Mapping of reads was done using *HISAT2* v.2.1.0 (Kim et al., 2015) with the aforementioned reference genome, which resulted in a mean mapping rate of 93.7% (±1.1%). Using the *htseq-count* function from *HTSeq* v.2.0.2 (Putri et al., 2022), we created a transcript read count table, which was used as input for *DESeq2* (Love et al., 2014). Genes with less than ten counts in at least five samples were removed. We tested for differentially antennal expressed genes (AEGs) using parasite prevalence as a continuous variable. Transcripts were variance stabilised before performing a PCA, for which the broken stick model showed Principal Component 1 to explain more variances than expected by chance.

Pooled CHC Analysis

Extraction of chemical profile

The CHC extracts were analysed using a gas chromatograph (7890A, Agilent Technologies, Santa Clara, CA, USA) equipped with a Zebron Inferno ZB5- HT column (Phenomenex Ltd., Aschaffenburg, Germany). Temperature was kept at 60 °C for 2 min, then increased by 60 °C/min up to 200 °C and subsequently by 48 °C/min to maximum of 320 °C and were kept constant for 10 min. Helium was used as carrier gas at a flow rate of 1.2 mL/min. Hydrocarbon molecules were then transferred to a mass selective detector (5975 C, Agilent Technologies) and fragmented with an ionisation voltage of 70 eV. Fragments in the range of 40-550 m/z were detected and subsequently used for substance identification based on the retention times and diagnostic ions (Carlson et al., 1998). We quantified substances using a total ion count from the chromatogram peaks

that we manually integrated using MSD ChemStation (E.02.02.1431, Agilent Technologies). Relative proportions were calculated in Microsoft Excel from which we filtered out i) non-hydrocarbon substances, ii) substances at the end of the profile that could not be reliably integrated, and iii) substances from which the maximum value for a sample stood below 0.5% of the total profile. The overall profiles were then adjusted to 100%. We furthermore identified substances based on the ion mass spectrum from each peak, and classified them as *n*-alkanes or methyl-branched recognition cues previously identified (Jongepier & Foitzik, 2016). A nonmetric multidimensional scaling was used on relative abundances to establish population coordinates for all identified compounds using the *metaMDS()* function from the *vegan* package (Oksanen et al., 2022) in *R*.

CHC association study

We performed a 'phenotype' genome-wide association study (GWAS) using the *BayPass* v.2.2 (Gautier, 2015) covariant model, for which we assumed linearity in CHC trait expression, and aimed to determine SNPs in genes for recognition cues known to be associated with aggression from a previous study on this species (Jongepier & Foitzik, 2016). The goal was to identify genes that might play a role in the synthesis of those CHCs, for which we have experimental evidence that they are used in recognition and aggression. For this, we uniformed coverage to a minimum of 20 using subsample-synchronized.pl and then calculated major and minor allele frequencies by using snp-frequency-diff.pl from the *PoPoolation2* toolkit. The output was transformed to *BayPass* format for pooled samples. SNPs with a Bayes Factor ≥ 20 were deemed significant. Similar as in the CMH analysis, significant SNPs associated with any recognition cue, henceforth $\text{BayPass}_{\text{CHC}}$, were categorised as within genes, exons, introns, 2 kB around of genes, and outside of genes. SNPs resulting in amino acid changes were identified using *CROXA/tbg-tools* (Schönnenbeck et al., 2021).

Annotation and GO-Enrichment Analysis

For all available genes found in the *T. longispinosus* genome, genes were annotated by running a *BlastP* (Altschul et al., 1990) search against the nonredundant nucleotide invertebrate database (retrieved January 2023). To further characterise gene functions, we ran *InterPro* (Paysan-Lafosse et al., 2023) to retrieve Gene Ontology (GO) information,

which was used to perform an enrichment analysis using *topGO* (Alexa & Rahnenfuhrer, 2017) for genes that contain CMH_{para}, CMH_{clim}, and $BayPass_{CHC}$ SNPs, as well as AEGs. For candidate genes, we searched for similarly named genes/proteins in UniprotKB (The UniProt Consortium, 2023) for model organisms such as *Drosophila melanogaster* to obtain their putative function. Candidate genes were characterised as genes containing non-synonymous SNPs identified in the GWAS, and/or had overlaps with candidate genes from previous studies.

Results

Population Structure and Link between Environmental Traits

Intrapopulation nucleotide (Tajima's π) and genetic diversity (Watterson's θ; min = 0.0, max = 0.06, mean = 0.003 for both parameters) were generally low (Fig. S1b and c, respectively). Allele frequency differences F_{ST} were low amongst all populations (min = 0.03, max = 0.049, mean = 0.04; Figure S1d). However, a weak trend of isolation-by-distance was detected (Mantel-test: $r = 0.264$, $p = 0.064$; Fig. 1c). Similarly, a principal component analysis (PCA) based on allele frequencies at all SNP loci indicated Principal component 2, explaining 12.3% of variations, to be linked to the geographical distances between populations (Mantel-test: $r = 0.30$, $p = 0.049$; not depicted). Further, Principal Component 1 (PC1), explaining 14.2 % of observed variation, showed the population Ohio to be an outlier in comparison to the other populations (Fig. S2a). Our extensive sampling of ten populations across the species range revealed strong differences in parasite prevalence ranging from 2.3% to 15.6% (Fig. 1a; Table S1). Moreover, we detected a strong correlation between parasite prevalence and Principal Component 1 of climate data (Pearson's cor.: $r = -0.78$, $p = 0.008$; Fig. S2b), which coincides with the social parasite *T. americanus* occurring at higher relative frequencies in warmer climates. This interaction between parasite prevalence and climate makes it difficult to identify SNPs in the host that are associated with either of the two intertwined environmental parameters. By selecting and contrasting populations in two separate analyses that differed more strongly in either parasite prevalence or climate, and discarding their overlaps, we tried to separate the two factors as much as possible.

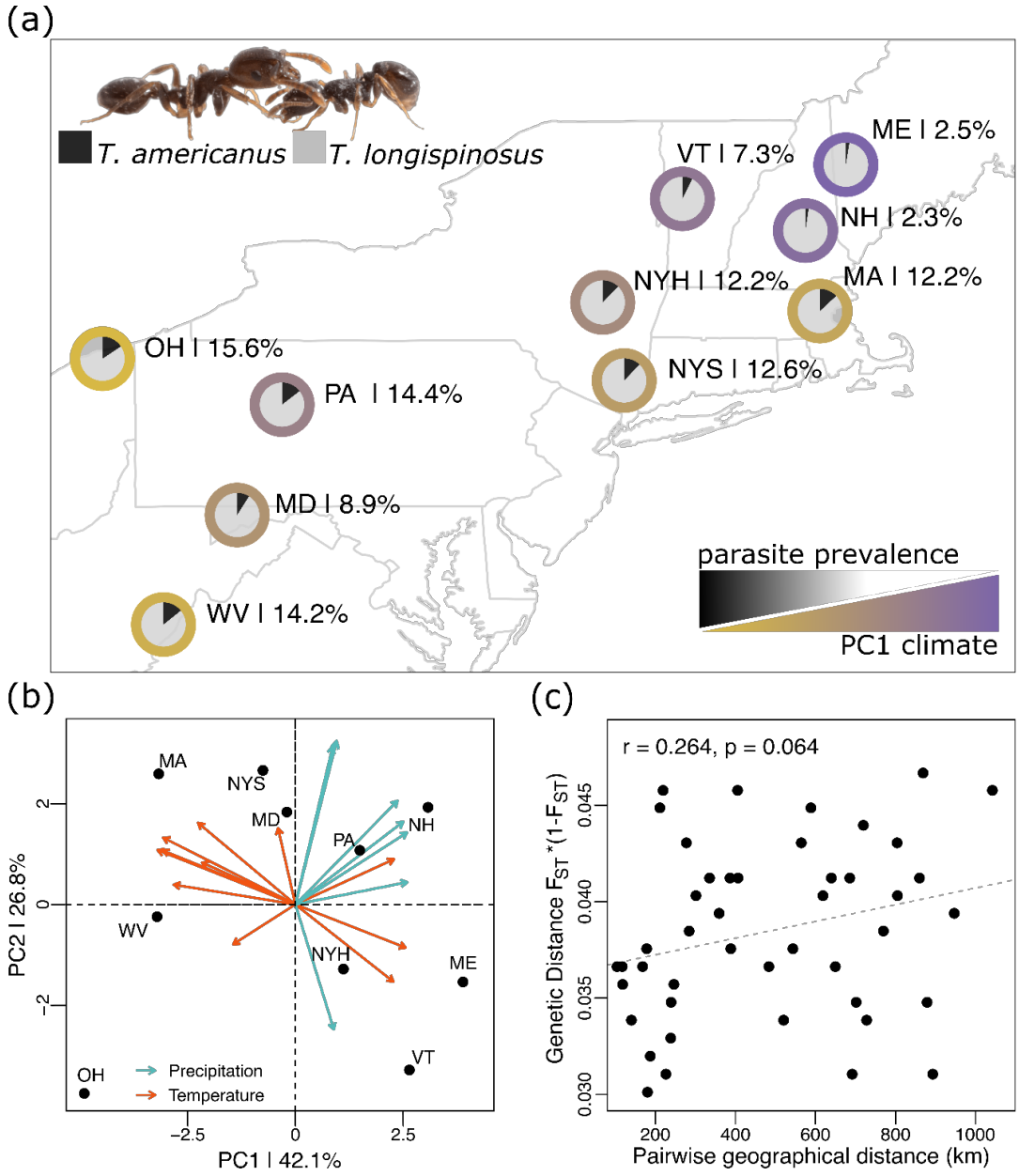

Figure 1. (a) Map of collection sites showing the varying degrees of parasite prevalence, i.e., the percentage of *T. americanus* colonies (see Table S1 for state abbreviations and population names) and their differences in climate indicated by PC1 climate eigenvalues (See Figure 1b). Photo ©Romain Libbrecht (b) A Principal component analysis of climate conditions at our study sites (Table S1) based on CHELSA bioclim data base variables (Supplement, Table 'Climate_data'), with warm and dry populations on the left to cold and wet populations on the right. (c) Pairwise geographical distances of populations (x-axis) versus genetic distances based on F_{ST} values. A trend in isolation-by-distance between populations, as indicated by a Mantel-test, was observed.

Parasite Prevalence Associated SNPs and Functional Properties of Candidate Genes

Using CMH test statistics, we contrasted allele frequencies of two populations with the highest and the lowest parasite prevalence (West Virginia and Pennsylvania versus Maine and New Hampshire, excluding Ohio due to its unique allele frequency pattern; Fig. S2a). This resulted in 1,614 SNPs significantly associated with parasite prevalence ($p \leq 0.0001$), henceforth CMH_{para} SNPs, located in 1,076 different 1 kB windows (Fig.S3a). In total, 754 SNPs were located within introns (46.7%), 90 SNPs were located within exons (5.6%), 208 within 2 kB around a gene (12.9%) and the remaining 562 SNPs outside of genes (34.8%). In summary, 52.3% of all CMH_{para} SNPs were within 288 unique genes, of which 263 were annotated (Supplement, Table 'CMHpara'; Fig. 2).

A GO-enrichment analysis for all CMH_{para} candidate genes revealed biological functions such as 'peptidoglycan catabolic process' and 'sensory perception of smell' as two of the eight significantly enriched GO-terms ($p \leq 0.05$; Fig. 2; Supplement, Table 'topGO_analysis'). For further analyses, we focused on CMH_{para} SNPs within exon regions that resulted in a non-synonymous substitution, with a special emphasis on those leading to changes in biochemical properties of the translated amino acid, also known as a radical substitution (e.g., amino acid with negatively charged side chain changes to one with a hydrophobic side chain). This resulted in 15 candidate genes of which eight contained such radical substitutions (Table 1). Of these, three could be identified as *odorant receptor gene 43a-*, *43a-like* and a non-annotated one, as well as *gustatory receptor gene 28a*. Furthermore, the gene *probable cytochrome P450 6a20* contained a SNP resulting in a nonsense mutation.

Climate Associated SNPs and Functional Properties of Candidate Genes

A PCA using climate variables retrieved from the CHELSA bioclim database revealed PC1 to explain 42.1% of observed variance in temperature- and precipitation-related values, while PC2 explained 23% of the observed variance linked to precipitation-related terms (Fig. 1b; Supplement, Table 'Climate_data'). The climatic landscape varied with geographical distance between populations (Mantel-test: $r = 0.36$, $p = 0.03$; not depicted; raw data Fig. S1d). Within the investigated range, host populations in the southwest are exposed to warmer and drier climates (e.g., Ohio and West Virginia; equivalent to lower

PC1 climate eigenvalues) versus colder and more humid climates in the northeast (e.g., Maine or New Hampshire; equivalent to higher PC1 climate eigenvalues; Fig. 1a, indicated by yellow to purple gradient).

Due to the aforementioned correlation between climate and parasite prevalence, we contrasted pairs of populations with the highest difference in their PC1 climate eigenvalues as a proxy for the largest difference in climatic conditions (ergo Maine and Vermont versus Massachusetts and New York South, excluding Ohio), while avoiding contrasting the same pairs used in the CMH_{para} analysis. This resulted in 3,024 climate associated SNPs, henceforth called CMH_{clim} SNPs, located in 1,998 different 1 kB (Fig. S3b). Of these, 133 SNPs were located within exons (4.4%), 1,492 within introns (49.3%), 287 within 2 kB around a gene (9.5%) and the remaining 1,114 SNPs outside of genes (36.8%). In summary, 53.7% of all CMH_{clim} SNPs were located within 421 unique genes, of which 407 were annotated (Supplement, Table 'CMHclim'; Fig. 2). Genes with the greatest number of SNPs within exons were in *protein mesh isoform X1* with a total of 10 SNPs within exon region and 25 within intron region.

We additionally detected genes with a high number of SNPs solely in intron regions such as *neurobeachin isoform X9* (109 SNPs), *calcium/calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase 1A-like* (102 SNPs), *semaphoring-2A isoform X1* (64 SNPs and 1 SNP in exon region) and *teneurin-m isoform X1* (64 SNPs). In a GO-enrichment analysis for all CMH_{clim} candidate genes, we found biological functions such as those related to meta- and catabolic processes ('collagen metabolic process' and 'glycine catabolic process') and transport and signalling ('transmembrane transport', 'neurotrophin TRK receptor signaling pathway', 'late endosome to vacuole transport', 'intrinsic apoptotic signaling pathway') to be among the 13 significantly enriched GO-terms (partially in Fig. 2; Supplement, Table 'topGO_analysis').

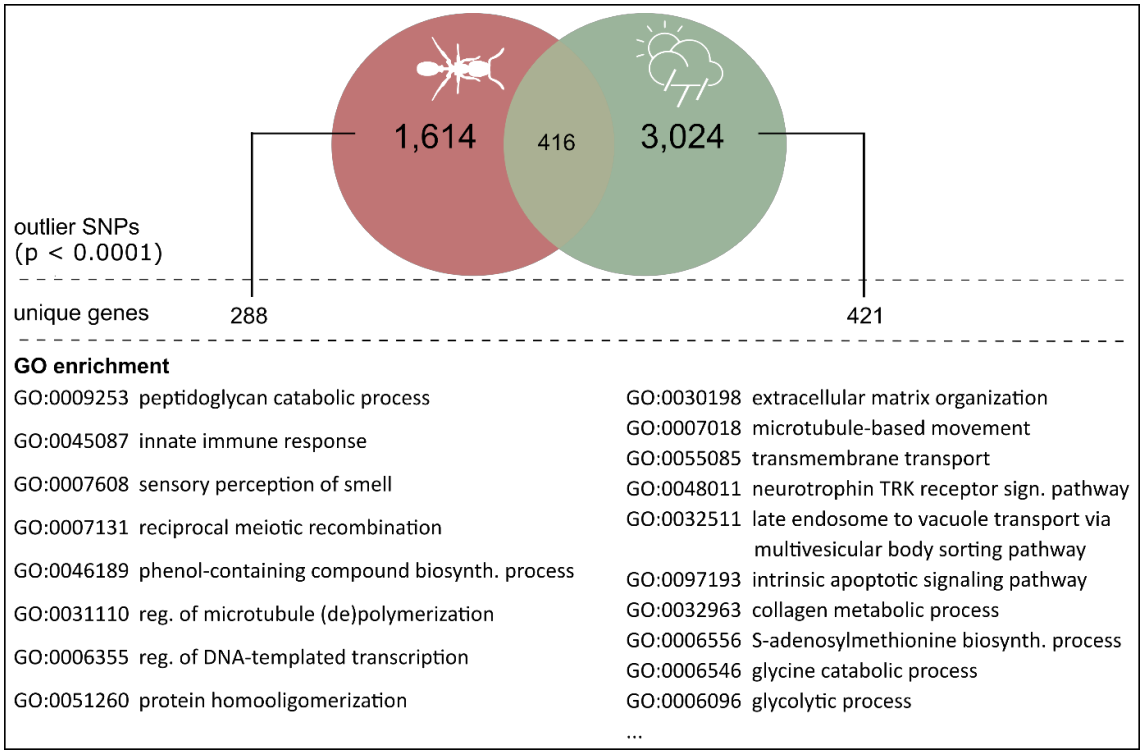

Figure 2. Venn Diagram of single-nucleotide polymorphisms (SNPs) identified using Cochran-Mantel-Haenszel test statistics with populations with high versus low parasite prevalence (red) and high versus low PC1 climate eigenvalues (green) ($p < 0.0001$). Lower part of the figure lists enriched GO-terms of the genes containing outlier SNPs ($p \leq 0.05$; Supplement, Table 'topGO_analysis'; Only top10 shown for CMH_{clim}). Corresponding Manhattan-plots can be found in Fig. S2a and b.

Table 1. Summary of candidate genes containing non-synonymous SNPs associated with parasite prevalence identified by a GWAS using Cochran-Mantel-Haenszel test statistics, contrasting major allele frequencies of two population with highest (PA and WV) versus the lowest (ME and NH; Table S1) parasite prevalence. Additionally, the average percentage of occurrence of the alternate variant is given for both high and low parasite prevalence populations in comparison to the reference genome.

Gene		Amino acid | Base			% alternative variant (average)	
ID	**Name (blastp)**	**In ref ***	**Substitute**	**Base change**	**High pp**	**Low pp**
TlonOR279	non-annotated odorant receptor gene	I	V	T→C	33.5	0.0
Tlon_g00518_i1	*protein claret segregational-like*	A	T **	C→T	55.0	6.0
Tlon_g16880_i1	*trichohyalin-like isoform X1*	P	S **	T→C	49.5	1.5
TlonOR210N	*odorant receptor 43a-like isoform X1*	A	G **	G→C	44.0	2.0
		M	V	T→C	41.0	4.0
		I	V	T→C	44.0	4.5
TlonOR227	*odorant receptor 43a*	V	I	C→T	41.5	2.0
		V	F	C→A	55.0	2.5
TlonGR91	*putative gustatory receptor 28a*	I	L	T→G	66.5	36.0
Tlon_g09433_i1	*BEN domain-containing protein 5-like*	T	A **	A→G	49.0	9.5
Tlon_g00523_i1	*cytochrome c1-2, heme protein, mitochondrial*	P	L **	C→T	74.5	25.5
Cytochrome_P450_Tlon_g04108_i1	*probable cytochrome P450 6a20, partial*	V	A	T→C	0.0	41.0
Hsp70_Tlon_g14341_i1	*heat shock 70 kDa protein cognate 4-like, partial*	N	K **	A→C	42.0	86.0
Tlon_g00451_i1	*HEAT repeat-containing protein 1*	R	K	G→A	18.0	44.0
Peptidase_M13_Tlon_g17006_i1	*endothelin-converting enzyme 2-like*	N	S	A→G	9.0	70.0
Cytochrome_P450_Tlon_g04105_i1	*cytochrome P450 6k1-like, partial*	Q	stop **	C→T	4.5	61.5
Tlon_g06262_i1	*F-box/LRR-repeat protein 4-like*	R	H	G→A	1.5	29.5
Tlon_g11443_i1	*receptor-type tyrosine-protein phosphatase mu-like isoform X1*	P	L **	T→C	2.0	47.0

* in reference genome (based on individuals from New York Huyck)
** radical substitution (change in amino acid biochemical properties)

Population-Level Chemical Profiles and Genome-Wide Associations of Recognition Cues

We identified 54 cuticular hydrocarbons (CHCs), of which nine were previously identified as important recognition cues in *T. longispinosus* (Jongepier and Foitzik, 2016), and six as linear *n*-alkanes important for protection against desiccation in our pools of whole-individuals (Supplement, Table 'CHC_profiles'). We used a nonmetric multidimensional scaling of all 54 CHCs to visualise population-level differences (Fig. S4a) and established a positive correlation of MDS1 to PC1 climate (Pearson's cor.: r = 0.76, p = 0.01; Fig. S4b). We split the dataset in two CHC classes, the six identified linear *n*-alkanes, henceforth *n*-alkanes, and the nine focal recognition cues. We detected a positive correlation between the relative abundances of *n*-alkanes and PC1 climate, with relatively more *n*-alkanes found in the CHC profiles in colder, more humid habitats (Pearson's cor.: r = 0.78, p = 0.008; Fig. 3a), while we detected a negative correlation with parasite prevalence (Pearson's cor.: r = -0.66, p = 0.039; Fig. S5a). We identified non-significant trends of the relative abundances of all recognition cues increasing with parasite prevalence (Pearson's cor.: r = 0.58, p = 0.08; Fig. 3b) and decreasing with PC1 climate (Pearson's cor.: r = -0.57, p = 0.088; Fig. S5b). We further tested for correlations between each individual recognition cue against parasite prevalence but found no significant correlation after FDR-correction. Instead, we identified recognition cue 11-15-DiMeC31 to be significantly correlated with climate (Pearson's cor.: r = -0.91, p = 0.002, Fig. S6).

We conducted a GWAS using *BayPass* to identify SNPs associated (Bayes Factor ≥ 20) with each of the nine focal recognition cues. Overall, we found associations with a total of 231 unique SNPs of which 10 were within exon (4%), 106 in intron (46%), 20 in 2 kB around genes (9%) and 95 outside of genes (41%). In summary, 46% of all recognition cue associated SNPs, henceforth $BayPass_{CHC}$ SNPs, were within 113 unique genes, of which 82 were annotated (Supplement, Table 'BayPassCHC'). The majority of these SNPs (85%) were associated with three recognition cues (11,15-;13,17-DiMeC31, 3-MeC27, and 3-MeC29; Fig. S4c-e). Four of the genes associated SNPs resulted in non-synonymous substitutions, with three of the genes annotated as *UDP-*

glucuronosyltransferase-like, *Meckel syndrome type 1 protein-like,* and a non-annotated OR gene, respectively (Table S3). Gene expression patterns of antennae-pools across populations

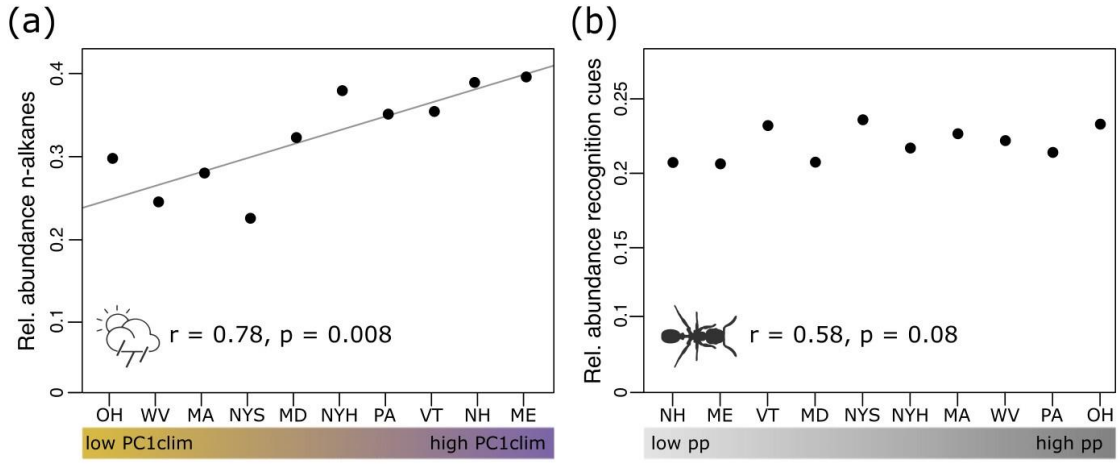

Figure 3. (a) Positive correlation of relative abundance of linear *n*-alkanes with PC1 climate eigenvalues. Populations are sorted according to their PC1 eigenvalue, from warm/dry to cold/humid populations. (b) Relative abundance of recognition cues (as described in Jongepier and Foitzik, 2016), with populations sorted from low to high parasite prevalence, showing a non-significant trend for a positive correlation.

Gene Expression Patterns of Antennae-Pools across Populations

A PCA of pooled antennal transcriptome data revealed that PC1 explained 61% of observed variation (Fig. S7a) and is negatively correlated with parasite prevalence (Pearson's cor.: r = -0.85, p = 0.002; Figure 4a), and weakly, but non-significantly correlated with climate (Pearson's cor.: r = 0.63, p = 0.051; Fig. S7b). A *DESeq2* analysis of pooled antennal transcript data using parasite prevalence as a continuous variable revealed 2,569 significantly differentially expressed transcripts (henceforth referred to as antennal expressed genes (AEGs), $p_{adjust} \leq 0.05$; Supplement, Table 'AEGs'). Among the top10 log_2foldChange (log_2FC) of significant AEGs, we identified three genes directly (*GSK-3-binding protein* and *histamine H2 receptor*) or indirectly (*PHD finger protein rhinoceros*) involved in post-translational modifications (PTM). Furthermore, we found 33 genes involved in epigenetic processes such as histone and methylation modifications and 58 genes involved in ubiquitination. Amongst the top10 -log_2FC of AEGs, we primarily found genes such as *nose resistant to fluoxetine protein 6*, which also contained

two CMH_{para} SNPs in intron regions, and *kelch-like protein 5*, which had each two SNPs in CMH_{para} and CMH_{clim} in intron regions. A GO-term enrichment analysis of all significant AEGs revealed biological functions related to translation and transduction to be highly enriched ('translation' and 'negative regulation of translation', 'signal transduction' and 'regulation of signal transduction'), as well as functions related to sleep and growth ('sleep' and 'transforming growth factor beta receptor signaling pathway'). Moreover, we found three functions to be enriched that are directly relating to PTM ('protein prenylation', 'protein ubiquitination' and 'protein dephosphorylation'; Fig. 4b; Supplement, Table 'topGO_analysis').

To identify links between genetic variants and transcriptional activity, similar to an eQTL approach, we looked at overlapping genes with CMH_{para}, CMH_{clim} and $BayPass_{CHC}$ SNPs and AEGs (Supplement, Table 'Overlap_genes'; Figure S7c). We found the highest number of exclusively shared genes between the AEG dataset and CMH_{clim} (57 genes), followed by $BayPass_{CHC}$ (32 genes) and CMH_{para} (31 genes). All SNPs found in those genes were in intronic regions, except for three within 2 kB of a gene. Although we have specifically investigated amino acid changes for SNPs in exon regions, other loci, not investigated in detail here, could of course also play a role, especially in gene regulation, which could then lead to expression differences. Transcriptional shifts could also allow ants to cope with differences in climate and parasite prevalence.

We further identified six AEGs that were previously described in behavioural transcriptome studies of our study species (Supplement 1, 'AEGs'). For instance, we identified five genes such as *trypsin-1*, *cytoskeleton-associated protein 5* and *DNA-binding protein 8A* that were previously found to be under positive and relaxed selection in *T. longispinosus* (Feldmeyer et al., 2017). We also identified one AEG, *RNA-binding protein 8A*, which was found to be differentially expressed in *T. longispinosus* during a parasitic raiding event (Alleman et al., 2018).

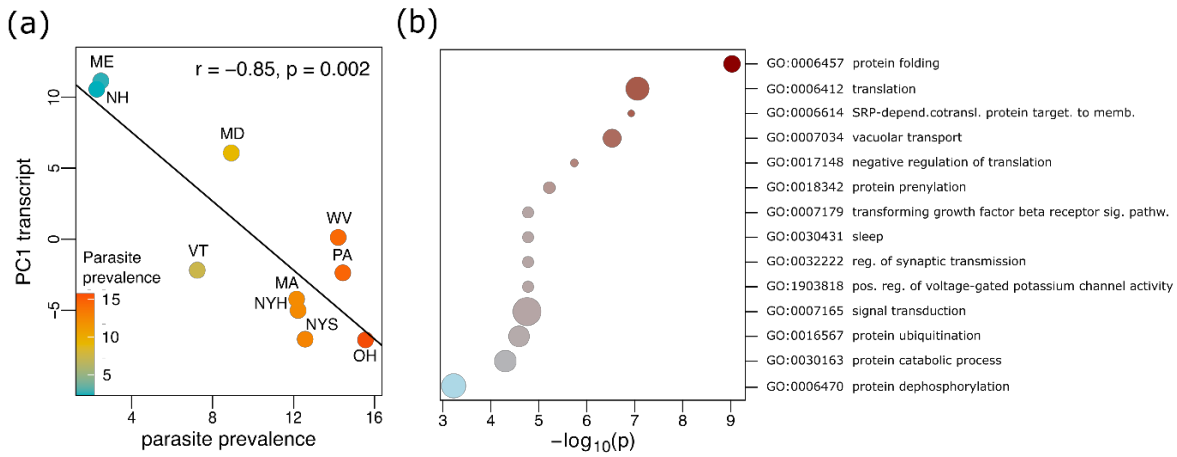

Figure 4. (a) Negative correlation of PC1 of antennal transcriptome data to parasite prevalence (r = -0.85; p = 0.002). We further identified a weak correlation between PC1 antennal transcriptional activity and PC1 climate (r = 0.63; p = 0.051; Figure S7b). (b) GO-enrichment analysis of AEGs linked to parasite prevalence (Supplement, Table 'topGO_analysis'). Three functions relating to post-translational modification, 'protein prenylation', 'protein ubiquitination' and 'protein dephosphorylation' were identified. Bubble size denotes number of found AEGs linked to an enriched function and colours denote p-values of enrichment.

Discussion

The main objective of this study was to assess genomic footprints of selection on the host species *T. longispinosus* potentially exerted by the social parasite *T. americanus* and the local environmental conditions using climate as a proxy. Our approach involved a genome-wide association study (GWAS) with pools of host ants sampled from 100 independent colonies at ten different sites in the northeastern USA. These ten sites were characterised by diverse climatic conditions and different levels of parasite prevalence, i.e., the percentage of parasitised colonies in a population, and both were considered crucial environmental factors. We also investigated the interplay of two phenotypic traits critically related to these factors, i) the cuticular hydrocarbon profile as an indicator of signalling and ii) genes expressed in the antennae as an indicator of cue recognition. We discovered an as-of-yet unknown correlation between local climate and parasite prevalence, further highlighting the complexity and probable limitations of mechanisms driving host-parasite coevolution in regard to their shared environment. We demonstrate

that both ecological parameters were not only associated with genomic signatures of selection but also linked to the expression of both chemical signalling and cue recognition traits.

Weak Population Structure indicating Gene Flow

Despite significant differences in local climate and the extensive sampling area, our study indicates weak population differentiation in *T. longispinosus*. This aligns with a previous microsatellite study, reporting similarly low differentiation in allele frequencies among 14 populations, half of which overlapped with populations from this study (Pennings et al., 2011). The same study also found no patterns of isolation-by-distance, while we identified a non-significant trend (Fig. 1c). Low population differentiation has also been reported in other ant species such as *Formica fusca*, *Crematogaster levior*, *Cardiocondyla obscurior*, and *Temnothorax nigriceps*, attributed to their high dispersal rates (Johansson et al., 2018; Errbii et al., 2021; Hartke et al., 2021; Cordonnier et al., 2022). Similarly, the weak population structure in *T. longispinosus* may be explained by a recent expansion and/or high dispersal rates in this species. As the large population size of *T. longispinosus* (Herbers & Foitzik, 2002; Brandt & Foitzik, 2004; Pennings et al., 2011) prevents genetic drift, and both males and queens conduct large-scale nuptial flights with males in particular contributing strongly to gene flow (Pennings et al., 2011) we suggest high gene flow among populations as the main factor driving the observed low population differentiation.

Genomic Associations to Parasite Prevalence and Climate

Climate parameters used for this study were more stable and accurately measured in comparison to parasite prevalence, which was estimated on the basis of a single or multiple collections per population over several years. In this study, we identified a close correlation of both factors, local climate and parasite prevalence, for which we hypothesise that their interplay ultimately lead to the geographical variation in phenotypic trait expression, already shown (Jongepier et al., 2014, 2015; Jongepier & Foitzik, 2016) and further demonstrated here. While we tried to disentangle both factors in our analysis as much as possible, it should be noted that complete separation of their effects may not be achievable due to their close interconnectedness.

Our GWAS identified half the number of SNPs associated with parasite prevalence in comparison to climate, and accordingly half the number of candidate genes. We found eight enriched biological functions in these candidate genes associated with parasite prevalence, for example 'sensory perception of smell'. A successful first line of defence for the host is the proper recognition of intruders, followed by an appropriate behavioural response, providing the greatest benefit for host colonies (Jongepier et al., 2014). We therefore suspected high selection pressure on recognition and signalling capabilities, and anticipated SNPs in genes associated with parasite prevalence relating to chemical recognition, such as *odorant* and/or *gustatory receptor* genes, as well as genes associated with the synthesis of CHCs, such as *fatty-acid-synthase* (*FAS*) genes (Chung et al., 2014; Wicker-Thomas et al., 2015; Pei et al., 2019; summarised in Holze et al., 2021). Indeed, we identified non-synonymous SNPs occurring exclusively in highly parasitised populations within three *odorant receptor* (*OR*) genes (TlonOR210N, -27and -279) and one gustatory receptor gene (TlonGR91). Changes in odour perception due to specific SNPs within *OR* genes have previously been observed in *Drosophila melanogaster* (Wang et al., 2010), in which some SNPs were further associated with behavioural shifts due to reduced olfactory abilities (Rollmann et al., 2010). These variants in the OR genes might lead to a diversity in recognition abilities along the parasite prevalence (and climate) gradient. Additionally, we identified non-synonymous SNPs associated with parasite prevalence within *fatty-acid synthase*-like and *catalase* genes. *FAS* are involved in the synthesis of CHCs and have been linked to the protection against desiccation in insects (Holze et al., 2021). Known for its role in combating reactive oxygen species (Nandi et al., 2019), *catalase* might be linked to changes in host stress levels along the parasite prevalence gradient. We further unveiled distinctive variants within two *Cytochrome P450* genes, *6a20* and *6k1*, with the latter one even containing a nonsense mutation, which might greatly impact the functionality of its protein. We also discovered variants within the gene *trichohyalin-like* associated with parasite prevalence. This gene has previously been associated with cuticle-related functions in *D. melanogaster* and in the social bumblebee *Bombus terrestris* (Feng et al., 2022; Liu et al., 2022). While it remains speculative, it is plausible that this candidate

gene could alter the cuticle of hosts, i.e., lead to a harder cuticle with better defence abilities, favourably in highly parasitised areas.

For our analysis of the effect of climate, we identified enriched GO functions associated with catabolic, metabolic, transport and signalling related functions for climate-associated candidate genes, which could be reflective of adjusting, managing, and responding to external climate challenges. As for SNPs within genes associated with climate, we identified two genes, *protein mesh* and *neurobeachin*, containing large numbers of SNPs. *Protein mesh* had been previously linked to the proper development of Malpighian tubes in *D. melanogaster* (Jonusaite et al., 2020), an organ important for homeostasis of ions and water, therefore important for cold resistance in this insect model (Andersen et al., 2017). Similarly, *neurobeachin* was found to be important in heat stress response in mammals (Howard, 2006; Igoshin et al., 2019).

Population-Level Cuticular Hydrocarbon Profiles and their Genome Associations

Cuticular hydrocarbons play vital roles in social insects, serving both as waterproofing agents and as recognition cues in intra- and interspecific biotic exchange of information (Sprenger & Menzel, 2020). However, it remains elusive if specific compounds fulfil specific task, or whether they are multifunctional. Several studies indicate that methyl-branched *n*-alkanes often function as behavioural and recognition cues (Ginzel et al., 2003; Lacey et al., 2008; Guédot et al., 2009; Ruther et al., 2011; Silk et al., 2011; Sakata et al., 2017; Awater-Salendo et al., 2020). These methyl-branched *n*-alkanes are likely influenced by climate due to their increased volatility in warmer temperatures as a result of their lowered melting points (Gibbs & Pomonis, 1995). Given the significance of accurate recognition, particularly in the context of social parasitism, it is reasonable to infer that the abundance of methyl-branched recognition cues could be influenced and perhaps constrained by the local climate. This, in turn, could lead to a trade-off between climate adaptations and proper parasite recognition abilities.

We hypothesised that chemical profiles will be dependent on both abiotic and biotic factors and will likely show patterns shaped by local climate and parasite prevalence alike. In our population-level CHC analysis we found such a correlation between the proportion of linear versus methyl-branched alkanes with climate and

parasite prevalence. Methyl-branched alkanes are commonly used for recognition purposes, while *n*-alkanes may be particularly good for protection against desiccation due to their linear structure (Dani et al., 2001; Van Zweden & d'Ettorre, 2010; Lorenzi et al., 2011). The relative abundance of *n*-alkanes increased from populations in warm/dry environments to those in cold/wet regions, which directly contrasts findings from *T. longispinosus* temperature acclimation experiments in which *n*-alkane abundance increased in high temperatures compared to moderate ones, showcasing quick plastic responses in the chemical profile to drastic temperature changes (Menzel et al., 2018). As colonies from all populations were maintained under the same stable laboratory conditions for several months before measuring CHC profiles, the observed differences between warmer and colder adapted populations likely stem from local adaptation to the original habitat rather than plastic responses to the standard laboratory conditions.

While we could not detect a correlation of parasite prevalence and the sum of all recognition cues, we observed a significant correlation between the relative abundance of the single recognition cue 11,15-;13,17-DiMeC31 and climate. This particular cue, previously linked to desiccation resistance in aphids (Yang et al., 2022), explained a large portion of the aggressive behaviour in our study species towards intruders (referred to as 11,15-DiMeC31 in the study, though it is a mixture with 13,17-DiMeC31, Jongepier & Foitzik, 2016).

With our CHC-GWAS we identified associated SNPs within six different *odorant receptor* genes (*OR*) and two *FAS*-like genes. Unlike *FAS*-like genes that may directly be linked with the synthesis of specific compounds, *ORs* are not involved in CHC biosynthesis, but are important for perception of smell and might thus naturally appear amongst the associated candidate genes. Notably, the majority of significant SNPs in this analysis were generated by only five of the nine recognition cues, including the previously mentioned cue 11,15-DiMeC31. Consequently, this cue met several criteria: i) It accounted for the majority of the host's aggressive behaviour towards the parasite as shown in behavioural assays (Jongepier & Foitzik, 2016), ii) its abundance, even under laboratory conditions, was correlated with local environmental factors, and iii) we were

able to identify particular candidate genes associated with it. These candidate genes now warrant closer investigation in future studies.

Antennal Expressed Genes and their Correlation to Parasite Prevalence

Exposure to a social parasite had been shown to induce differential gene expression in the antennae (Stoldt et al., 2023), and more risk-taking tasks such as foraging are accompanied with an increase in antennal gene expression, even more so than brain gene expression (Caminer et al., 2023), showcasing the importance of this peripheral organ in enemy recognition. Variation in gene expression, especially in olfactory receptor genes, could potentially influence the ability to discriminate between nestmates and enemies in social insects. However, perception also depends on the stability of cues on the cuticle, influencing their evaporation rate, further influenced by the local climate.

Our analysis of population-specific patterns of transcriptional activity in host antennae showed a strong correlation of differential transcriptional expression to the local parasite prevalence, and a considerably weaker one to climate. This strong effect of parasite prevalence on gene activity in the antennae (henceforth antennal expressed genes, AEGs) could theoretically be attributed to plasticity of gene expression in response to parasite presence, which is questionable, as on average only half of natural host colonies ever encounter a parasite, even in highly parasitised populations (Foitzik et al., 2001). Our AEG dataset was also generated without the colonies being experimentally exposed to social parasites, and some workers might have even hatched in captivity. This suggests that the population-specific differences in transcript expression are probably due to local adaptation rather than plasticity, and/or that local adaptation allows for a wider palette of possible phenotypes. To be able to better detect a parasite one could imagine hosts to increase the transcriptional activity of *OR* genes. However, we did not detect differential expression of *odorant* nor *gustatory receptor* genes, but instead showed an enrichment of biological functions relating to post-translational modifications. Another gene expression study in brains of the same species investigating the effect of parasites on hosts revealed a similar overrepresentation of two GO-terms ‘protein dephosphorylation’ and ‘protein ubiquitination’ (Kaur et al., 2019). This might indicate the importance of epigenetic influences on AEGs shaped by local parasite prevalence, playing a more long-term and

heritable role on baseline gene expression, in contrast to the short(er)-term quick shifts in gene expression upon encounter with a parasite (Feldmeyer et al., 2016).

We found four *Cytochrome P450* genes (*6k1-like*, *6a14*, *9e2-like*, and *4C1-like*) in our AEGs, with one of them also appearing in the parasite prevalence GWAS. The differential expression of certain *P450* genes was observed to be indicative of disease, parasitic infections, but also CHC synthesis (Nadin et al., 1995; Samanta et al., 2003; Moustafa et al., 2008; Lin et al., 2019; Morgan et al., 2020; Holze et al., 2021). While these functions can be more generally stated to be a stress response induced by increased parasite presence, the function in CHC synthesis might play a more impactful role in parasite recognition abilities and might help to create a nuanced and unique host chemical profile.

Conclusion

In this study, we combined environmental- and trait-association analyses to gain deeper insights into adaptive patterns associated with parasite prevalence and climate in the host species *T. longispinosus*. We found evidence for selection on a number of *odorant receptor* genes and differences in cuticular hydrocarbon profiles, which are both relevant in the perception of the parasite. However, we also discovered a strong link between parasite prevalence and climate across the sampled populations with varying impact of both environmental factors on allele frequencies and phenotypic traits. For example, the transcriptional activity in the antennae was highly linked to parasite prevalence, while CHC compositions were more tightly associated with climate. We identified several interesting candidate genes likely playing a role in host adaptation, for which we plan to corroborate in detailed functional analyses on colony-level. Our study allows to gain deeper insights into the many facets of host-parasite coevolution and the importance of eco-evolutionary dynamics.

Author Contribution

SF and BF conceived the study. MNM, EC, SF and BF designed the experimental set-up and collected ant colonies. MNM and EC sampled colonies and performed dissections.

MNM performed DNA- and RNA-extractions. EC performed CHC extractions, integrated and identified compounds. MNM with help from MP and BF performed PoolSeq, PoolRNASeq and CHC analysis. MNM wrote first draft of the manuscript and all authors revised it.

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Competing Interests

The authors declare no conflict of interest.

Data Availability Statement and Benefit-Sharing

The following supplementary material is available under open access: Supplement (containing Tables ‘Climate_data’, ‘CMHpara’, ‘CMHclim’, ‘BayPassCHC’, ‘AEGs’, ‘CHC_profiles’, ‘topGO_analysis’, ‘Overlap_genes’) and Supporting Information (including Supplementary Tables and Figures). Raw sequence data was uploaded at the European Nucleotide Archive (ENA) and are accessible under the study accession PRJEB68328.

Supplementary Material

Supplement Tables

Table S1. Summary of collection sites. States and their abbreviations, coordinates, and number of collected colonies of host and parasite and their long-term parasite prevalence as well as their prevalence based solely on collections for this study (see Methods and Methods 2.2. for more information about the estimates)

Population	Location	Coordinates	*T. longispinosus*	*T. americanus*	Parasite prevalence*	Parasite prevalence 2021
Massachusetts (MA)	Beaver Brook North and Rock Meadow Reservation	42.407, -71.204	501	69.86	12.233	13.024
Maryland (MD)**	New Germany State Park	39.611, -79.134	112	11	8.943	8.943
Maine (ME)	Bethel Community Forest	44.413, -70.851	504	13	2.515	4.375
New Hampshire (NH)	Belknap Mountain State Forest	43.510, -71.402	423	10	2.309	8.333
New York Huyck (NYH)	Huyck Preserve	42.525, -74.160	5634	780	12.161	18.863
Newy York South (NYS)**	Fahnestock State Park	41.455, -73.868	178	25.62	12.583	12.583
Ohio (OH)	Private Property***	41.753, -80.967	573	105.58	15.558	13.427
Pennsylvania (PA)**	S.B. Elliot State Park	41.124, -78.523	314	53	14.441	14.441
Vermont (VT)	Branbury State Park	43.945, -73.076	1787	140	7.265	10.460
West Virginia (WV)	Watoga State Park	38.110, -80.136	843	139.65	14.211	16.977

Table S2. Detailed location and coordinates of all subsites.

State	Subsite	Coordinates	Location
MA	a	42.410836, -71.208085	Beaver Brook North Reservation
	b1	42.410836, -71.208085	Rock Meadow Conservation Area
	b2	42.399000, -71.194361	
ME	a	44.406120, -70.861389	West Bethel Park
	b	44.407185, -70.866737	Bethel Community Forest- West
	c	44.415182, -70.857712	
	d	44.421951, -70.819382	Bethel Community Forest – East
MD	a	39.629078, -79.145439	New Germany State Park
	b	39.514118, -79.156654	
	c1	39.570572, -79.201034	Big Run State Park
	c2	39.627922, -79.107284	Roadside
	d	39.627922, -79.107284	Private Property
	e	39.638573, -79.105064	behind Graveyard
	f	39.628662, -79.125107	Acorn Loop Hiking Trail, New Germany State Park
	g	39.650993, -79.122215	Meadow Mountain Trail, New Germany State Park
NH	a	43.500557, -71.413171	Private Property
	b	43.516384, -71.379341	Belknap Mountain State Forest
	c	43.513794, -71.413536	Private Property
NYH	a1	42.520329, -74.146408	Huyck Preserve
	a2	42.515518, -74.161789	
	b	42.515518, -74.161789	
	c	42.528486, -74.160333	
	d	42.532192, -74.162888	
	e	42.533791, -74.164268	
	f	42.525547, -74.170349	
	g	42.530544, -74.151466	
NYS	a	41.445290, -73.865179	Fahnestock State Park
	b	41.450997, -73.857043	
	c	41.480529, -73.917455	Private Property
	d	41.443157, -73.867508	Stone Garden at Fahnestock State Park
	e	41.454262, -73.833420	Tree Lake Trail at Fahnestock State Park
OH	a	41.759503, -80.965593	Private Property
	b	41.759514, -80.966522	
	c	41.758891, -80.966794	
	d	41.758312, -80.965341	
	e	41.755195, -80.946406	
	f	41.757405, -80.942399	
	g	41.749222, -80.948235	
	h	41.743347, -80.958672	

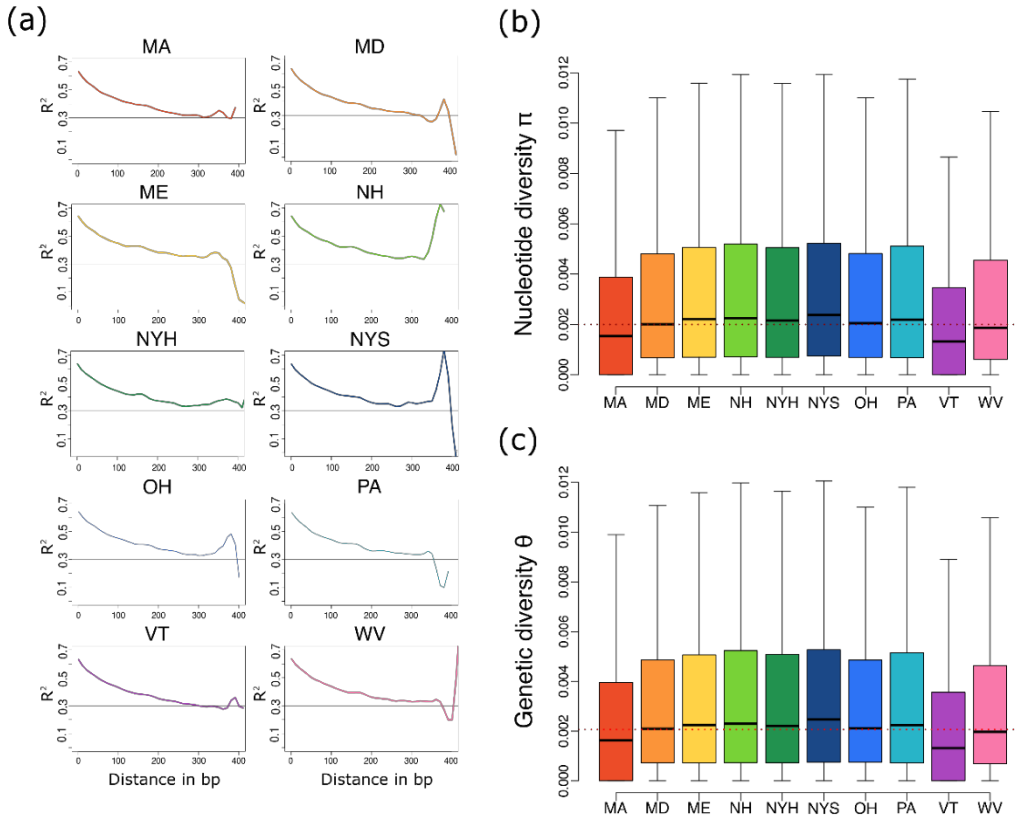
	i	41.744363, -81.031441	
	j	41.748822, -80.974843	
	k	41.747403, -80.968187	
PA	a	41.137976, -78.516263	S.B. Elliot State Park
	b	41.111185, -78.528163	
	c	41.102461, -78.526507	
	d	41.118119, -78.518040	
	e	41.137276, -78.520605	
	f	41.126563, -78.528959	
	g	41.136524, -78.505280	
	h	41.139987, -78.503009	
	i	41.118080, -78.516296	
	j	41.113468, -78.571419	
VT	a1	43.965164, -73.076485	Branbury State Park
	a2	43.972078, -73.072761	
	b	43.944454, -73.102104	
	c	43.972078, -73.072761	
	d	43.971760, -73.086716	
	e	43.979463, -73.064017	
	f	43.973076, -73.059197	
	g	43.969719, -73.083763	
	h	43.924728, -73.096313	
	i	43.882782, -73.063850	
	j	43.842194, -73.060585	
WV	a	38.124519, -80.114105	Watoga State Park
	b	38.106472, -80.129555	Ann Bailey Trail, Watoga State Park
	c	38.107899, -80.131996	
	d	38.107495, -80.134593	
	e	38.119175, -80.154663	Watoga Park Road, Watoga State Park
	f	38.102936, -80.145622	Ann Bailey Trail, Watoga State Park
	g	38.102665, -80.141525	
	h	38.106857, -80.136772	

Table S3: Summary of candidate genes containing non-synonymous SNPs in exon regions identified by *BayPass* (Bayes Factor ≥ 20) associated with relative abundances of focal recognition cues

Recognition cue	Gene		Amino acid | Base		
	ID	**name**	**In ref**	**Variant**	**Base change**
11,15-;13,17-DiMeC31	Tlon_g03574_i1	*UDP-glucuronosyltransferase-like*	K	N*	A→ C
3-MeC29	Tlon_g10572_i1	*Meckel syndrome type 1 protein-like*	F	I	A→ T
5-MeC27	TlonOR277	*odorant receptor*	A	P*	C→ G
3-MeC27, 3-MeC29	Tlon_g03216_i1	NA	E	Q*	C→ G

* Radical substitution

Supplement Figures



(d)

	MA	MD	ME	NH	NYH	NYS	OH	PA	VT	WV
MA		732	224	123	243	244	809	623	231	897
MD	0.035		864	774	525	488	282	173	696	192
ME	0.048	0.043		109	340	410	873	724	183	1,046
NH	0.037	0.040	0.038		251	306	808	644	145	951
NYH	0.034	0.035	0.043	0.037		122	569	393	185	706
NYS	0.036	0.038	0.048	0.042	0.038		593	391	289	654
OH	0.042	0.045	0.049	0.045	0.045	0.047		216	690	411
PA	0.042	0.038	0.046	0.043	0.039	0.043	0.047		548	364
VT	0.032	0.032	0.039	0.035	0.031	0.040	0.043	0.039		883
WV	0.032	0.033	0.048	0.041	0.036	0.038	0.043	0.041	0.036	

Distance (km)

F_{ST} values

Figure S1. (a) Results of Linkage disequilibrium analysis, showing convergence at around 300 bp at $R^2 = 0.3$ (b) Nucleotide diversity π and (c) Genetic diversity Θ over 1 kB window sizes. (d) Mean pairwise F_{ST} between populations in 1 kB windows and pairwise geographical distance (see Fig. 1c for IBD).

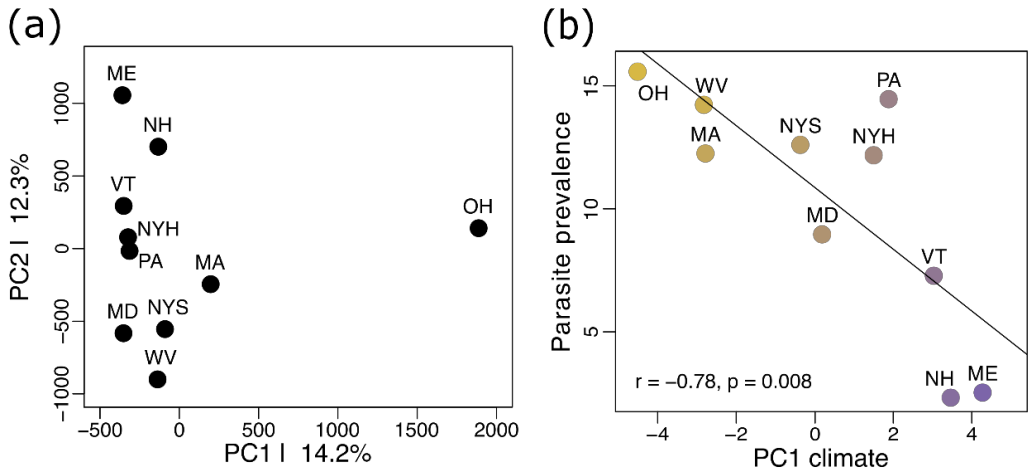


Figure S2. (a) Principal component analysis of all allele frequencies, showing Ohio as a clear outlier. (b) Correlation of parasite prevalence and PC1 climate eigenvalue, showing strong positive correlations.

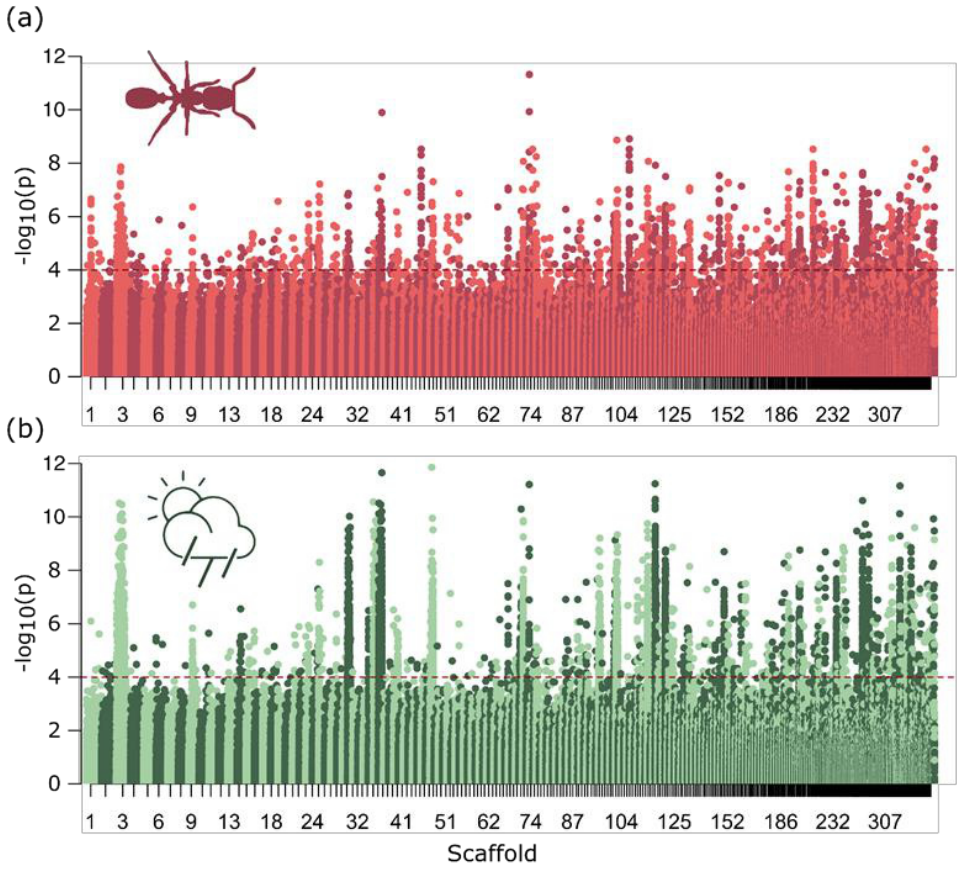


Figure S3. Manhattan plots of all SNPs identified using Cochran-Mantel-Haenszel test statistics associated with (a) parasite prevalence and (b) climate over all Scaffolds. Significance threshold was set at $p \leq 0.0001$.

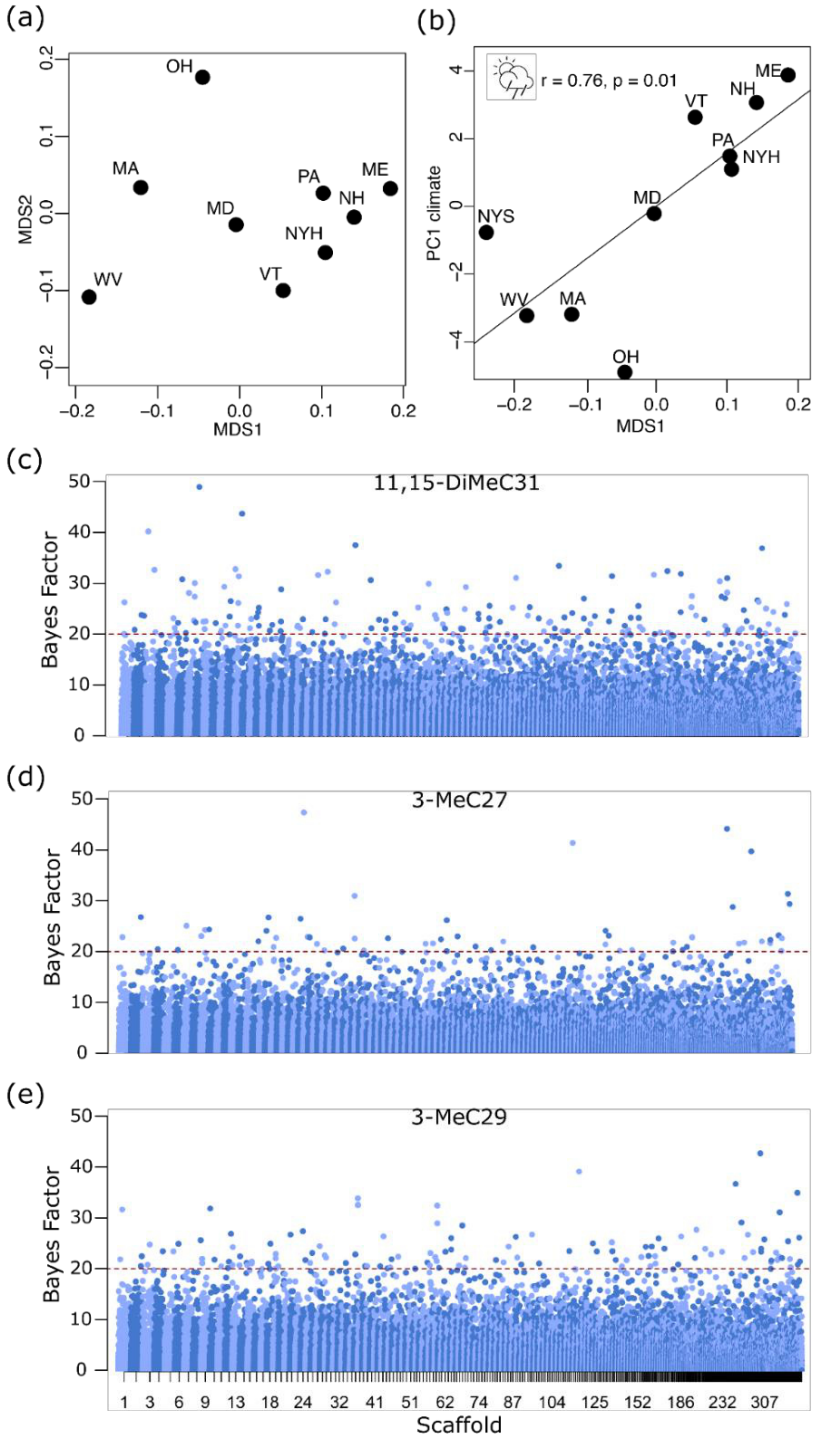


Figure S4. (a) MDS plots of relative abundances of all *n*-alkanes and recognition cues. (b) Positive correlation of MDS1 and PC1 climate values. (c) Manhattan plot of all SNPs associated with recognition cue 11,15-DiMeC31 (shortened from 11,15-;13,17-DiMeC31), (b) 3-MeC27, and (c) 3-MeC29 with significant SNPs characterised by a Bayes Factor ≥ 20.

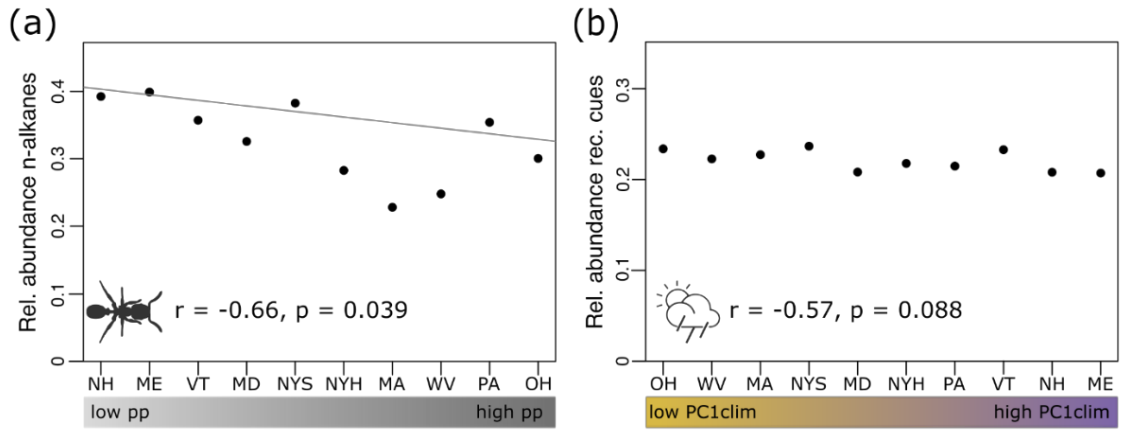


Figure S5. Correlations of relative abundances of (a) linear *n*-alkanes against parasite prevalence, sorted by increasing parasite prevalence, which showed to be negatively correlated with p = 0.039, and (b) recognition cues against climate PC1 values, sorted from low (warm/dry) to high (cold/wet), which was tendentially negatively correlated with p = 0.088. Correlations with the reciprocal environmental trait can be found in Figure 3.

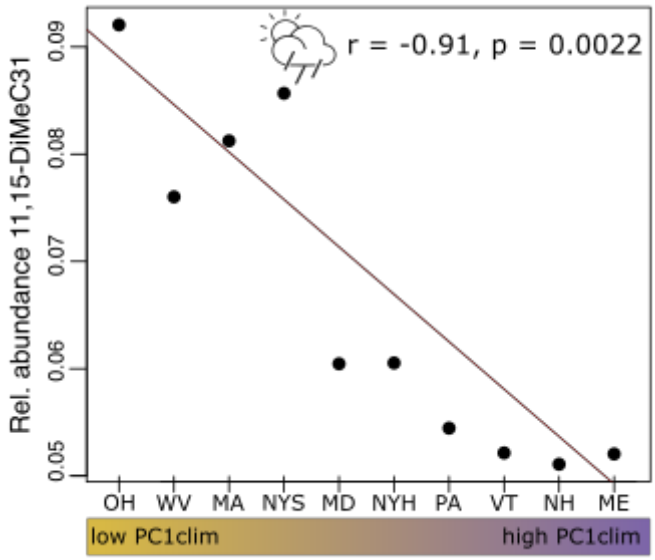


Figure S6. Correlation of relative abundance of recognition cue 11,15;13,17-DiMeC31 (shortened to 11,15-DiMeC31) and climate PC1 values, sorted from low (warm/dry climate) to high (cold/wet) values, for which we identified a significant negative correlation.

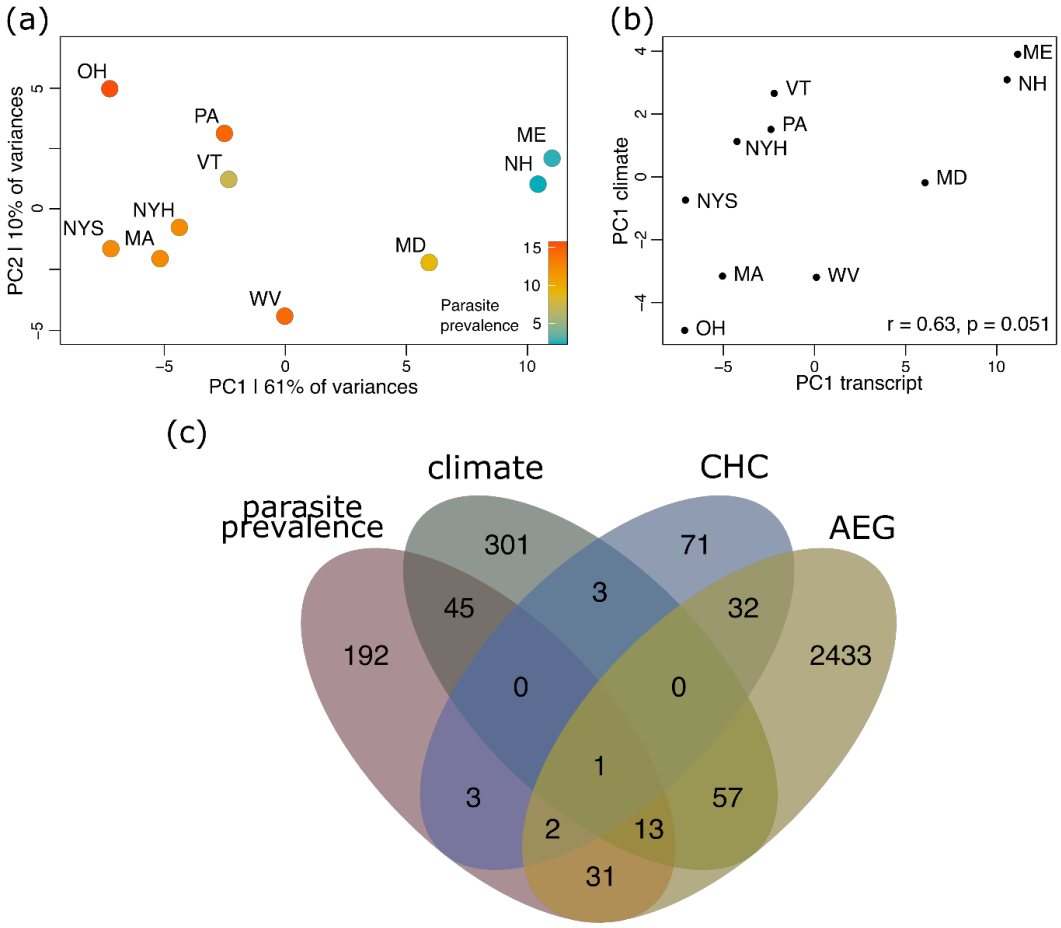


Figure S7. (a) Principal component analysis of pooled antennal gene expression. B) Venn-Diagram of all genes with SNPs associated with parasite prevalence, climate (both identified using CMH statistics), the chemical profile (identified using *BayPass*) as well as antennal expressed gene (AEG) retrieved from pooled antennal samples.

Chapter 3.

Climate and Parasite Pressure Jointly Shape Traits Mediating Interactions Between an Ant Host and Its Social Parasite

Erwann Collin[1], **Maide Nesibe Macit[1]**, Marti Wittke, Chris Hörrmann, Claudio Haase, Laura Heil, Maria Litto, Florian Menzel, Barbara Feldmeyer, Susanne Foitzik

[1] shared first authors

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Abstract

Host–parasite relationships are often characterised by coevolutionary arms races. While abiotic influences on host-parasite interactions are already acknowledged, integrating both biotic and abiotic factors remains essential for understanding coevolution in the context of environmental change. In this study, we analysed the interactions of the obligate social parasite *Temnothorax americanus*, a dulotic ant, and its primary host, the ant *T. longispinosus*, focusing on behavioural and cuticular hydrocarbon traits that govern parasite invasion and host defence. We studied the link between these traits and local climate as well as parasite prevalence. Our results revealed that behavioural interactions were more strongly associated with climate than parasite prevalence. Hosts from warmer, drier regions exhibited reduced aggression during parasite encounters, opting to pick up the brood and flee, while parasites from these regions displayed heightened aggression and activity. Enemy recognition in these ants is mediated by cuticular hydrocarbons (CHCs). These were linked to the local climate in the host but linked to its local prevalence in the parasite. As all colonies were maintained under standardised conditions for a year, we attribute the observed phenotypic traits to evolutionary adaptation rather than phenotypic plasticity. Our findings suggest that both abiotic and biotic factors play critical roles in shaping co-evolved traits, sometimes leading to unexpected trait patterns that would potentially be overlooked when considering only a single factor. These insights provide a framework for understanding how climate influences coevolution of interacting species.

Keywords: coevolutionary arms race, bioclimatic gradient, climate, nestmate recognition, social parasites.

Introduction

Climate influences the structure and function of ecosystems, often exerting spatially heterogeneous effects that differentially impact species phenology, geographic distribution, as well as community composition and dynamics (Walther et al., 2002). Species interactions cannot be fully understood without accounting for the environmental conditions in which they occur (Ockendon et al., 2014; Poisot et al., 2017). For example, song sparrow nests experienced lower predation rates in wetter years, highlighting how climate indirectly affects prey by altering predator-prey interactions (Chase et al., 2005). Similarly, while parasites exert selection pressure on their hosts, their interactions are also influenced by climate (Manlik et al., 2023). This is particularly evident in ectothermic hosts and parasites, where interaction dynamics depend on the physiological and behavioural adaptations of each partner to key environmental factors such as temperature (Franke et al., 2019; Ragonese et al., 2024). Moreover, these factors may interact in complex ways influencing host-parasite relationships. For instance, while drought and elevated temperatures independently enhance parasitoid-control of aphid populations, their combined effects negatively impact parasitoids (Romo & Tylianakis, 2013). Therefore, assessing influential abiotic factors is essential for understanding the coevolutionary dynamics of host-parasite interactions. Insects protect themselves from desiccation with a layer of cuticular hydrocarbons (CHCs) on their cuticle (Sprenger & Menzel, 2020), which represents a crucial adaptation that serves multiple functions. Water retention is thought to be primarily achieved by hydrocarbons with high melting points such as linear alkanes (*n*-alkanes), which typically convey little informational content (Bonavita-Cougourdan et al., 1987). However, CHCs also play a key role in communication, particularly in social insects, mediating nestmate recognition and influencing behavioural interactions. Nestmate recognition relies on structurally more complex molecules including methylated alkanes and alkenes, which encode information on individual and colony identity. As these substances have lower melting points, they are less effective at preventing water loss (Gibbs, 1995; Menzel et al., 2019). At the colony level, a relatively uniform colony odour is generated through the mixing of individual chemical profiles via mutual grooming (Lenoir et al., 2001). This chemical

signature is learned early in life, stored, and updated as a neural template in the ant's brain, enabling individuals to distinguish outsiders with qualitatively and/or quantitatively distinct CHC profiles (Blomquist & Bagnères, 2010). When two ants meet, they compare each other's CHC profiles to assess whether the other one is a nestmate. The likelihood of aggression (i.e. recognition as foreign) increases with chemical distance between their profiles (Sturgis & Gordon, 2012). Since nestmate recognition and waterproofing rely on CHC classes with opposing properties, social insects may not optimise both functions simultaneously. In environments where both are crucial, CHC composition likely reflects trade-offs.

Despite their ability to recognise foes, social insects remain vulnerable to invasion by closely social parasites that mimic their CHC profiles (Lenoir et al., 2001). Social parasitism has evolved several times independently in ants, accounting for up to one-third of all species in temperate zones (Kutter, 1969). Dulosis is a form of ant social parasitism where parasitic workers specialise in raiding allospecific host colonies to abduct their worker brood, whose social behaviour they later exploit as adults (D'Ettorre & Heinze, 2001). While some dulotic species are facultative parasites, most are obligate and have lost essential abilities such as brood care and foraging. Their frequent and destructive raids, which peak during summer at high temperatures, exert strong selection on free-living host colonies, driving the evolution of defensive strategies (Buschinger et al., 1980; Brandt & Foitzik, 2004). Dulotic ants manipulate host behaviour and evade recognition through various chemical strategies, including chemical mimicry, which involves synthesising chemical cues that resemble the host's profile, and chemical insignificance, characterised by the absence or reduction of recognition cues used by hosts to identify enemies. Parasites often engage in aggressive interactions with hosts during raids (D'Ettorre et al., 2002; Brandt et al., 2006; Jongepier et al., 2015). When hosts encounter dulotic parasites, rapid recognition and a collective defence are crucial to repel or escape the threat (Pamminger et al., 2011).

Our study focuses on the coevolving obligate dulotic social parasite *Temnothorax americanus* and its primary host *Temnothorax longispinosus* (Foitzik et al., 2001, 2009; Jongepier et al., 2014) for which the degree of escalation in their co-

evolutionary arms race varies geographically (Foitzik et al., 2001). For instance, in regions with high parasite prevalence, *T. americanus* carries fewer chemical recognition cues, facilitating its invasion into host colonies (Kleeberg et al., 2017; Kaur et al., 2019). Additionally, *T. americanus* secretes substances from its Dufour's gland that induce fights among host workers. This manipulative tactic, particularly prevalent in parasites from high-prevalence areas, may enhance parasite effectiveness (Brandt et al., 2005; Jongepier et al., 2015). In response to parasite pressure, *T. longispinosus* populations alter their social colony structure (Herbers & Foitzik, 2002), shift their behavioural strategy from fighting to fleeing, and display increased aggression after enemy encounters (Jongepier et al., 2014; Kleeberg et al., 2014). Hosts also respond by increasing the diversity of chemical recognition cues between colonies, potentially impairing the parasite's ability to match host profiles (Jongepier & Foitzik, 2016). Simultaneously, as ectotherms, their physiology and behaviour are strongly affected by temperature and humidity, which putatively impacts their interactions. For instance, *T. longispinosus* responds to drier conditions by dynamically increasing the proportion of *n*-alkanes in its CHC profile (Menzel et al., 2018). With potential trade-offs in functions of *n*-alkanes and recognition cues, impairment in recognition abilities due to these dynamic shifts might be possible. This study investigates how local climate and parasite pressure shape host-parasite interactions by influencing traits related to perception, signalling, and defence. In northern regions, we hypothesise that hosts, known for heightened aggression against conspecifics (Segev et al., 2017), will also exhibit stronger defences against parasites. As parasite raids prioritise quantity over quality of host colonies (Miller, 2021), and the raiding period is shorter in colder climates, we therefore expect parasites from these regions to exhibit more aggressive behaviour to ensure that their raids are successful. In southern regions with warmer, drier climates, both species are expected to increase *n*-alkanes for desiccation resistance (Sprenger & Menzel, 2020), potentially conflicting with the host's need for methylated alkanes to recognise nestmates. To test these predictions, we analyse ten populations (up to 1,000 km apart) across varying climates and parasite prevalence, assessing behavioural interactions, cuticular hydrocarbons, and colony structure.

Methods

Ant Species, Collection, and Maintenance

T. longispinosus, a facultatively polygynous and seasonally polydomous ant, is widely distributed in deciduous forests of northeastern North America. Colonies typically contain several dozen workers inhabiting preformed cavities in hollow acorns, sticks, or rock crevices on the forest floor (Herbers, 1989). It often occurs in sympatry with its related social parasite *T. americanus* (Foitzik et al., 2009), which conducts annual raids during summer, where parasitic workers raid host nests to abduct worker brood (Foitzik & Herbers, 2001; Foitzik et al., 2009). While parasite colonies are monogynous, many are also polydomous, each containing a few parasitic workers alongside captive hosts (Herbers & Foitzik, 2002). *T. americanus*, though capable of exploiting three different *Temnothorax* species, prefers *T. longispinosus* as a host, resulting in higher prevalences and colony sizes in high-density populations of this host (Brandt & Foitzik, 2004).

We collected ants from July to September 2021, during the peak reading season, at ten locations in the northeastern USA, each separated by at least 100 km, typically over 200 km. Site selection was based on previous collection data, geographic location, and information on forest composition and density obtained from US Forest Service data. At each site, sampling occurred at 2–10 subsites within a 5 km radius to ensure reliable parasite prevalence estimates while maintaining genetic homogeneity within populations and geographic isolation between them (Table S1, Jongepier et al., 2014; Kaur et al., 2019). Host colonies were collected at a minimum distance of 70 cm and had to contain at least one queen to maximise the likelihood of sampling independent colonies. Upon collection, ant colonies were provisioned and transported under cooled conditions before being transferred to artificial nest sites for acclimatisation in the laboratory. Each colony was then housed in three-chambered plastered nest boxes with food and water, maintained under a 12h:12h light-dark photoperiod at 21 °C.

Parasite Prevalence and Climate

We estimated parasite prevalence as the percentage of *T. longispinosus* colonies containing *T. americanus* queens and/or workers (Brandt & Foitzik, 2004; Foitzik et al.,

2009; Jongepier et al., 2014). Given temporal and spatial variation in prevalence, we incorporated data from previous collections for more accurate long-term estimates (Foitzik & Herbers, 2001; Herbers & Foitzik, 2002). Parasite prevalence (PP) served as a proxy for parasite pressure on hosts and the ecological success of *T. americanus* (with an empirical maximum of 15-20%), as successful parasites are more likely to establish daughter colonies (Kaur et al., 2019). *Temnothorax* ants have long generation times, with life expectancies of workers from temperate climates ranging from 2-3 years up to 7 years (Plateaux, 1986). Paired with their large populations, prevalences remain relatively stable over time. We use the same method as approached by Macit et al. (2024) to calculate parasite prevalence, incorporating long-term data (Herbers & Foitzik 2002; Jongepier et al. 2014; Kaur et al. 2019), which allowed us to calculate long-term average prevalence estimates. To adjust these estimates for regions where *T. americanus* exploits several host species, we calculated parasite prevalence based on the number of *T. americanus* colonies with *T. longispinosus* hosts, excluding those with only *Temnothorax curvispinosus* host workers, a less preferred host (found in MA, NYS, OH, and WV; Table S1, Brandt and Foitzik, 2004). For mixed-species parasitic colonies, we applied a correction factor based on the ratio of *T. longispinosus* to *T. curvispinosus* exploited workers. For instance, a parasitic nest containing equal numbers of both host species was counted as 0.5 rather than 1. This adjustment reflects the assumption that, in mixed-species nests, only half of the raids likely targeted *T. longispinosus* colonies, thereby reducing the inferred parasite pressure on this host.

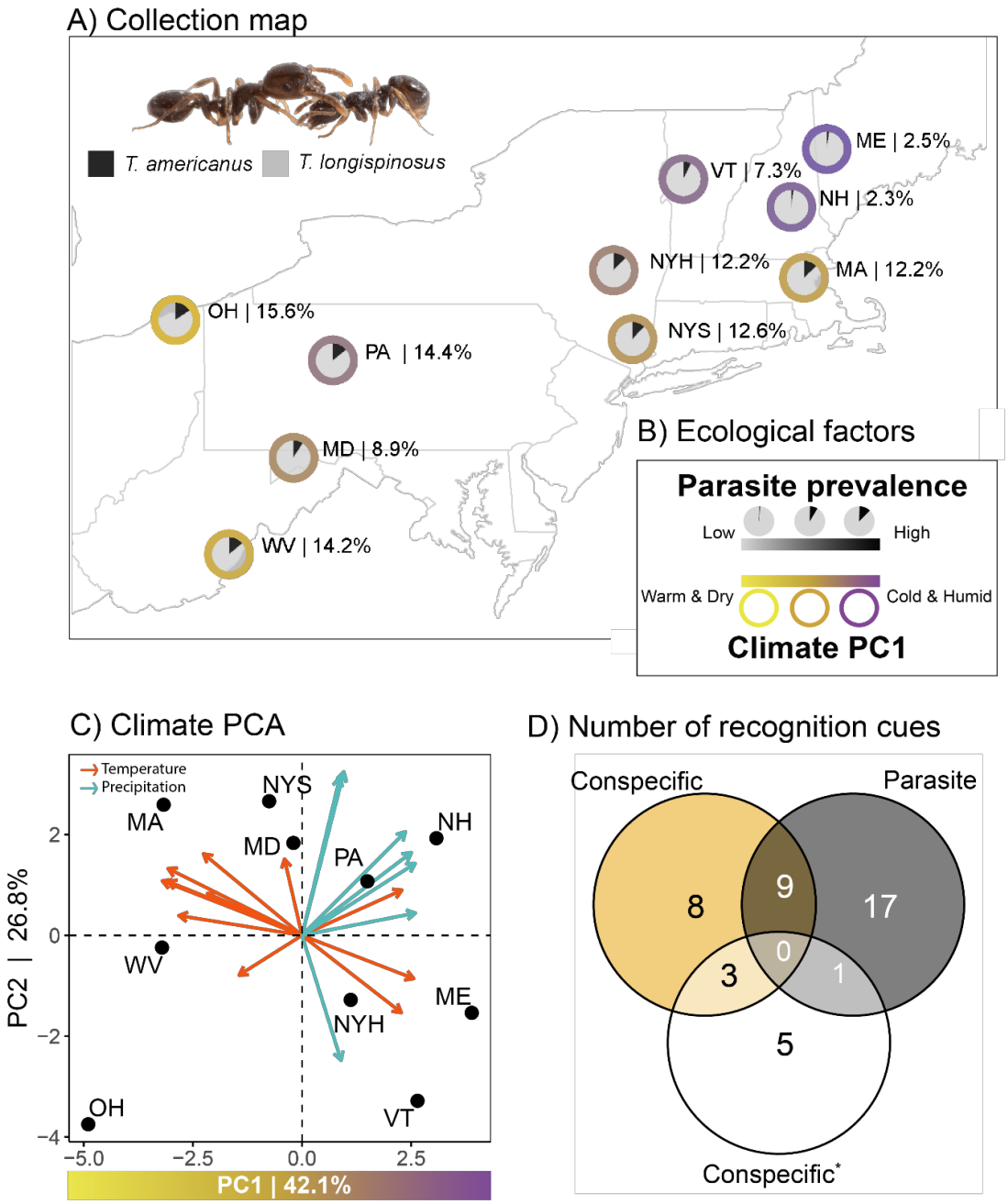

Figure 1. A) Map of collection sites showing parasite prevalence (% *T. americanus* colonies among host colonies) and climate variation indicated by PC1 climate values (x-axis in B) (Table S1 for state abbreviations and population names, map adapted from Macit et al., 2024); Photo ©Romain Libbrecht. B) Ecological factors included in the analyses throughout the study. C) Principal Component Analysis (PCA) of climate conditions at study sites (Table S1). The arrows represent the contribution and direction of individual climate variables to the two principal components (PC1 and PC2) based on CHELSA bioclim variables (Macit et al., 2024), aligning populations from warm/dry

climates on the left and cold/wet climates on the right. D) Venn diagram comparing recognition cues (CHC compounds associated with aggression) across studies. Blank circles and '*' denote recognition cues identified by Jongepier and Foitzik (2016) for *T. longispinosus* conspecific aggression. Orange and black circles indicate recognition cues associated with *T. longispinosus* aggression toward conspecifics and *T. americanus*, respectively, from this study. Climate also influences *Temnothorax* traits (Bengston & Dornhaus, 2014; Segev et al., 2017; Segev & Foitzik, 2019). We collected ants across a broad geographic range from Maine to West Virginia and obtained climate data from the CHELSA Bio-database (1981–2010, v. 2.1) (Karger et al., 2017), which includes ten temperature and eight precipitation variables. A principal component analysis (PCA) was conducted, and climate-PC eigenvalues were used for further analyses based on previously published data (Macit et al., 2024). Principal Component 1 (PC1) explained 42.1% of the variance and was associated with *T. americanus* prevalence, with higher prevalence linked to higher temperatures and lower precipitation (Pearson's correlation: $r = -0.78$, $p = 0.008$). This suggests that the social parasite prefers or performs better in warmer, drier climates. Additionally, the strong climate-prevalence link complicates independent analyses of their effects on trait expression (Macit et al., 2024).

Behavioural Assays

On average, 155 host and 34 parasite colonies were collected per site to perform sympatric behavioural interactions under standardised conditions. From this collection, we tested the responses of 15 *T. longispinosus* colonies per population ($N = 150$) to the introduction of *T. longispinosus* and *T. americanus* workers originating from the same site one year post-collection (Table S2), which contrasts with the previous full-factorial approach, involving fewer populations and including staged allopatric intrusions (Kaur et al., 2019). In the previous study, ant colonies were maintained for only a few weeks, whereas we kept colonies for over one year to ensure that the majority of host workers had no prior experience with environmental conditions at their site of origin, including direct interactions with parasites. A week before the assays, these *T. longispinosus* colonies were used to establish *T. longispinosus* standardised subcolonies ($N = 150$) comprising 10 workers, a queen (if present), five larvae, and a few eggs (details in the supplement), placed in an arena. Trials consisted of the introduction of three types of sympatric workers in separate trials: *T. longispinosus* nestmates, *T. longispinosus* workers from sympatric colonies of the same pool involved in the experiments, and sympatric *T. americanus* workers. After 10 min of acclimation, a previously marked intruder was introduced with sterile forceps, and interactions were recorded for another 10 min (4K Sony FDR-AX33).

Each day, 50 subcolonies underwent randomised trials, followed by a one-week recovery period to control for prolonged physiological and behavioural shifts resulting from prior parasite encounters by *T. longispinosus* workers (Pamminger et al., 2011; Koenig & Moreau, 2024a and c). Behavioural experiments were conducted three days per week, allowing all subcolonies to be observed within three weeks (Fig. S1A). Each subcolony was randomly assigned an observation time of day, and this assignment remained consistent across the three trials. Observations took place from 8:30 to 16:30. Of the 450 recorded assays, 396 (nestmate: 134, conspecific: 138, parasite: 124) were eligible for behavioural analyses after excluding assays after which an uncontrolled increase in worker numbers (drift) before the onset of the recording was observed, and assays in which the number of workers from the subcolony was fewer than five.

We analysed both subcolony and intruder behaviours during their interactions. Each trial consisted of 27 scans at 20-second intervals, totalling nine minutes of interaction. The number of workers from the subcolony displaying a particular behaviour was recorded for each scan, as well as the behaviours of the intruders. For the final analyses, behaviours were added up across scans to obtain one number per behaviour per subcolony. Finally, behaviours were grouped into categories such that aggressive behaviour included mandible opening, biting, stinging, pulling, dragging, carrying and pinning (only for the subcolony), and sociopositive behaviour included antennation, grooming and trophallaxis (Table S3). Additionally, we recorded brood carrying behaviour, as a proxy for flight response for subcolonies (Jongepier et al., 2014), and passive behaviours (e.g., curled-up posture, immobility) in intruders which were frequently observed. We performed beta-binomial generalised linear mixed models (GLMMs) with the *glmmTMB* package (Brooks et al., 2017) in *R* v.4.2.3 (R Core Team, 2021) with response variables aggression, sociopositive, brood carrying or passive behaviour, respectively. We included as fixed effects intruder type, parasite prevalence, climate (PC1), queen number, and the pairwise interactions of intruder type with parasite prevalence, queen number and climate, respectively (details in Supplement Table S4). Site variation was not included in the model to limit the number of parameters and reduce the risk of overfitting. We accounted for potential pseudo-replication by including 'climate' as a fixed factor, which captures site-specific variation, and by incorporating

subcolony ID and intruder ID as random effects. Collinear factors were sequentially removed, and final models were selected via stepwise automatic reduction based on the Akaike information criterion (AIC; Table S4). Pairwise comparisons were adjusted using Bonferroni corrections.

Cuticular Hydrocarbon Analyses

We analysed the cuticular hydrocarbon (CHC) profiles of individual *T. americanus* (n = 129) workers and pairs of *T. longispinosus* nestmates (n = 144) from behavioural trial colonies (see collection table in supplementary material). Workers were immersed in 350 µL hexane for 10 min, and extracts were concentrated under nitrogen flow. CHC analysis was conducted using a gas chromatograph (7890A, Agilent Technologies, USA) with a Zebron Inferno ZB5-HT column (Phenomenex Ltd., Germany) and a mass selective detector (5975C, Agilent Technologies; details in the supplement). Substances were quantified via single-ion monitoring using MSD ChemStation (E.02.02.1431, Agilent Technologies). We considered hydrocarbons longer than C20, excluding non-hydrocarbons and compounds with an average proportion below 0.1% or a maximum relative abundance below 0.5%. Hydrocarbons were treated as functional traits (Menzel et al., 2017) and categorised as *n*-alkanes or methylated alkanes. Following Jongepier and Foitzik (2016), we identified CHC sets associated with colony aggression toward conspecifics (n = 118) and social parasites (n = 105). Using a stepwise reductive algorithm in *R* (based on Wittke et al., 2022), we identified CHCs whose quantity differences in the profile correlated with aggression, separately for conspecifics and parasites. For each species, we performed PERMANOVAs using *adonis2* from *vegan* (Oksanen et al., 2022) to examine links between the composition of overall CHC profiles, *n*-alkanes, or recognition cues, and parasite prevalence or climate. Ordinations of individual CHC profiles were done with non-metric multidimensional scaling (NMDS; Bray-Curtis distances; package *vegan*). Associations between chemical composition and environmental factors were visualised using *ordisurf* (*vegan*). A principal component analysis (PCA) was conducted to link CHC relative abundance to climate and parasite prevalence. To analyse intrapopulation variability in CHCs, *n*-alkanes, and recognition cues, we used the *betadisper* function (*vegan*). We then tested whether centroid values

per species varied with climate and prevalence using Spearman's rank correlations. Interspecific differences in the abundance of *n*-alkanes and recognition cues were analysed using beta GLMMs (*glmmTMB* package). Chemical distances between sympatric and allopatric host-parasite pairs were calculated using Bray-Curtis distances and assessed with a Mantel test (Pearson's correlation, 2,000 permutations). Lastly, we examined how chemical distances among sympatric pairs varied along climate and parasite prevalence gradients using Spearman's rank correlation test.

Colony Structure Analyses

Using Poisson and quasibinomial GLMMs (*glmmTMB* package in *R*), we incorporated colony-level traits by including the number of individuals within a colony. Specifically, we counted the number of *T. longispinosus* queens and workers in free-living colonies, the number of *T. americanus* workers in parasitic colonies, and the proportion of *T. americanus* workers relative to captive host workers. These variables were analysed in relation to climate and parasite prevalence.

Results

Subcolonies Behaviour towards Intruders

T. longispinosus workers were twice as likely to react aggressively to parasitic intruders as to foreign conspecifics, while aggression toward nestmates was rare (beta-binomial GLMM- intruder type: $\chi^2 = 268.17$, $p < 0.001$; Fig. 2A). Overall, aggression probability increased in colonies from colder/wetter climates (beta-binomial GLMM- climate: $\chi^2 = 8.79$, $p < 0.01$; Fig. 2A), and tended to be higher in those from high parasite prevalence sites (beta-binomial GLMM- PP: $\chi^2 = 2.88$, $p = 0.09$; Fig. 2A). Workers picked up brood more frequently in response to social parasites than to conspecific intruders (beta-binomial-GLMM- intruder type: $\chi^2 = 75.96$, $p < 0.001$; Fig. 2B), particularly in colonies from warmer/drier climates (beta-binomial GLMM- climate-intruder type interaction: $\chi^2 = 6.57$, $p = 0.04$; Fig. 2B). Sociopositive behaviours were most frequent toward nestmates and less common toward conspecifics or parasites (beta-binomial GLMM- intruder type: $\chi^2 = 84.92$, $p < 0.0001$), with these behaviours remaining unaffected by environmental factors (Table S6A).

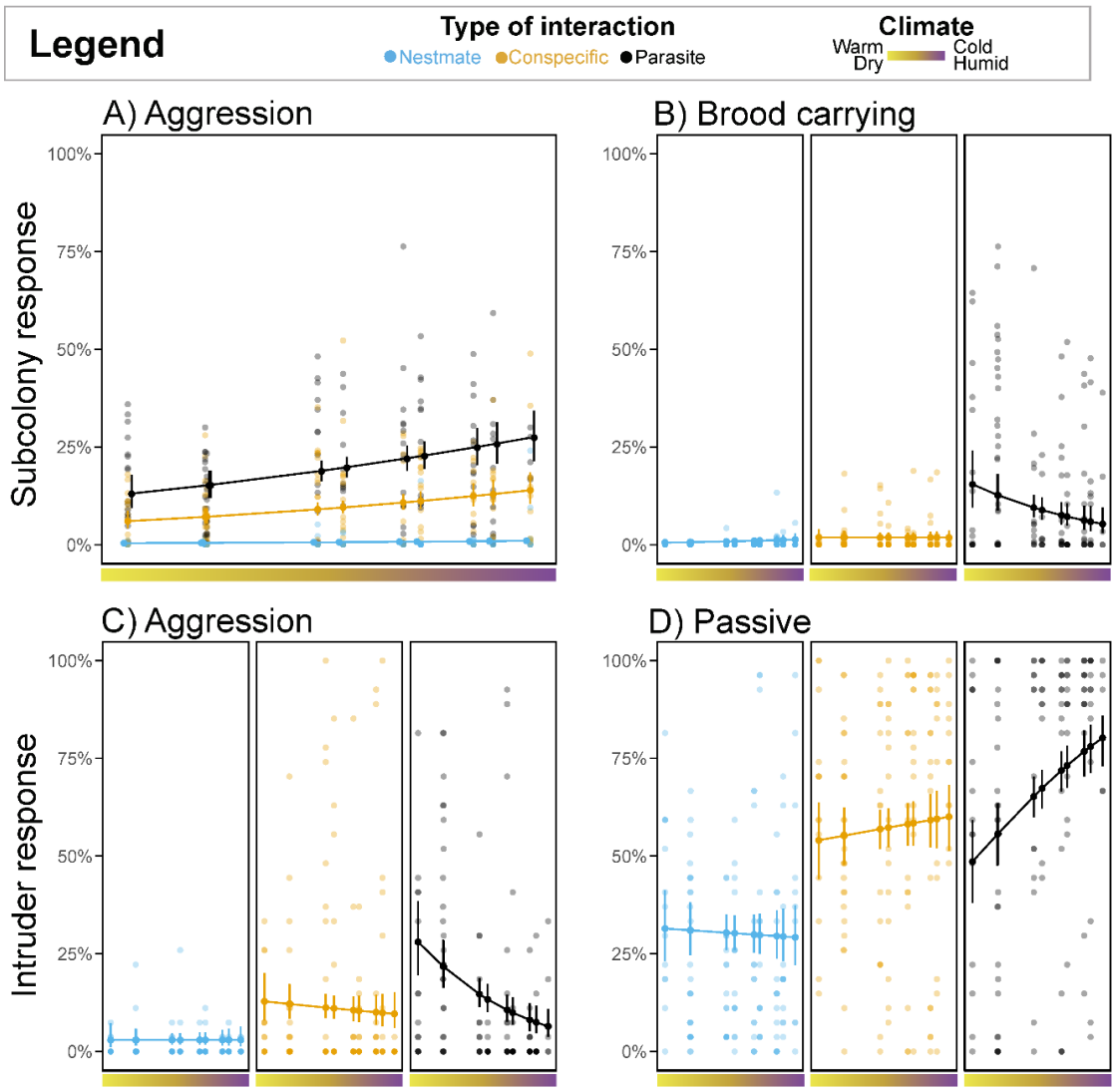

Figure 2. Scatterplots showing associations between climate and behavioural responses of subcolonies toward each intruder type (A, B) and intruder responses toward subcolonies (C, D). Transparent points represent raw data, means across the climate gradient are connected by a line, and predicted means are indicated by solid dots (± 95% CI). Interaction types are colour-coded based on the intruder type. Subplots are used where interaction effects are detected (B, C, D). Data seem to be arranged in columns as data points per colony stem from 10 populations according to their climate value. PA and NYH share quasi-identical climate values and can thus not be discriminated in the figure.

Intruder Behaviour towards Subcolonies

Intruder aggression varied significantly by type, with conspecifics and social parasites reacting more aggressively than nestmates (beta-binomial GLMM- intruder type:

$\chi^2 = 35.65$, $p < 0.0001$; Fig. 2C). Parasites from colder/wetter climates were less aggressive than those from warmer/drier regions (beta-binomial GLMM- climate-intruder type interaction: $\chi^2 = 6.91$, $p = 0.03$; Fig. 2C). Nestmates were the most active intruders, whereas conspecifics and social parasites remained passive in over half of the observations (beta-binomial GLMM- intruder type: $\chi^2 = 102.80$, $p < 0.0001$; Fig. 2D). Passivity increased in colder/wetter climates, particularly among parasites (beta-binomial GLMM- climate: $\chi^2 = 7.22$, $p = 0.007$; climate-intruder type interaction: $\chi^2 =$, $p = 0.005$; Fig. 2D). Furthermore, parasites were the least likely to engage in sociopositive interactions with subcolony members, compared to nestmates and conspecifics (beta-binomial GLMM- intruder type: $\chi^2 = 179.98$, $p < 0.0001$, Fig. S1C). Sociopositive responses also decreased as subcolony size increased (beta-binomial GLMM- number of workers: $\chi^2 = 4.56$, $p = 0.03$; Fig. S1B). Only conspecifics showed a higher tendency for sociopositive responses in warmer/drier climates (beta-binomial GLMM- climate-intruder type interaction: $\chi^2 = 5.72$, $p = 0.06$; Fig. S1C).

Identification of Recognition Cues linked to Host Aggression

The chemical profiles of *T. longispinosus* contained 72 CHCs, 52 of which overlapped with the 63 CHCs found in *T. americanus* (Fig. S1D). Using the reduced set of recognition cues, a strong positive correlation emerged between aggression and chemical distance in both conspecific and parasite interactions (Spearman's rank correlations conspecific- $\rho = 0.45$, $p < 0.0001$; parasite: $\rho = 0.54$, $p < 0.001$; Fig. S4C, D), which was not detected when considering all CHCs (Spearman's rank correlations- conspecific: $\rho = 0.15$, $p = 0.11$; parasite: $\rho = 0.02$, $p = 0.81$; Fig. S4A, B). Stepwise reduction identified 27 CHCs in *T. americanus* and 20 in *T. longispinosus* as 'recognition cues' triggering aggression in *T. longispinosus*, with nine compounds overlapping (Fig. 1C). Three of these cues overlapped with those identified by Jongepier and Foitzik (2016) for conspecific aggression (5-MeC27, 4-MeC28, and 5-MeC29), and one for parasite aggression (7-MeC31). None of the recognition cues were *n*-alkanes, despite these comprising 53% and 70% of the CHC relative abundance in *T. longispinosus* and *T. americanus*, respectively. All conspecific aggression cues were methylated alkanes, except for one alkene (C29-ene) found only in *T. longispinosus*. Seven of these were absent from *T. americanus*

profiles (Fig. S3). Similarly, all parasite recognition cues were methylated, with seven exclusive to *T. longispinosus* and four to *T. americanus*.

Host and Parasite CHC Profiles and Association to Climate and Parasite Prevalence

T. longispinosus worker CHC composition was strongly linked to local climate but not parasite prevalence (PERMANOVA- all CHCs- climate: df = 1, R^2 = 0.11, F = 17.04, p < 0.001; PP: df = 1, R^2 = 0.007, F = 1.19, p = 0.27; Fig. 3A). Similar patterns were found for *n*-alkanes, conspecific, and parasite recognition cues, all of which were shaped by climate but unaffected by parasite prevalence (PERMANOVA- *n*-alkanes- climate: df = 1, R^2 = 0.06, F = 8.47, p < 0.001; PP: df = 1, R^2 = 0.005, F = 0.73, p = 0.44; conspecific recognition cues- climate: df = 1, R^2 = 0.08, F = 12.55, p < 0.001; PP: df = 1, R^2 = 0.006, F = 0.92, p = 0.49, parasite recognition cues- climate: df = 1, R^2 = 0.10, F = 16.50, p < 0.001; PP: df = 1, R^2 = 0.005, F = 0.81, p = 0.56; Fig. 3B, C, D). However, intercolonial variability in these CHC subsets was independent of both factors (Table S7).

In contrast, *T. americanus* CHC composition, particularly its *n*-alkane profile, tended to shift with parasite prevalence but not climate (PERMANOVA- all CHCs- climate: df = 1, R^2 = 0.01, F = 1.33, p = 0.22; PP: df = 1, R^2 = 0.02, F = 2.28, p = 0.07; *n*-alkanes- climate: df = 1, R^2 = 0.003, F = 0.36, p = 0.70; PP: df = 1, R^2 = 0.05, F = 6.38, p < 0.01; Fig. 3E, F). However, recognition cue composition was unaffected by climate and parasite prevalence (PERMANOVA- conspecific recognition cues- climate: df = 1, R^2 = 0.01, F = 1.32, p = 0.26; PP: df = 1, R^2 = 0.01, F = 1.70, p = 0.13; parasite recognition cues- climate: df = 1, R^2 = 0.01, F = 1.37, p = 0.19; PP: df = 1, R^2 = 0.006, F = 0.79, p = 0.60). Intercolonial variability in *T. americanus n*-alkanes and conspecific recognition cues was higher in southern sites with high parasite prevalence and warmer/drier climates (Spearman's rank correlations- *n*-alkanes- climate: rho = -0.65, p = 0.05; PP: rho = 0.70, p = 0.03; conspecific recognition cues- climate: rho = -0.73, p = 0.02; PP: rho = 0.54, p = 0.11; Fig. 3G, H).

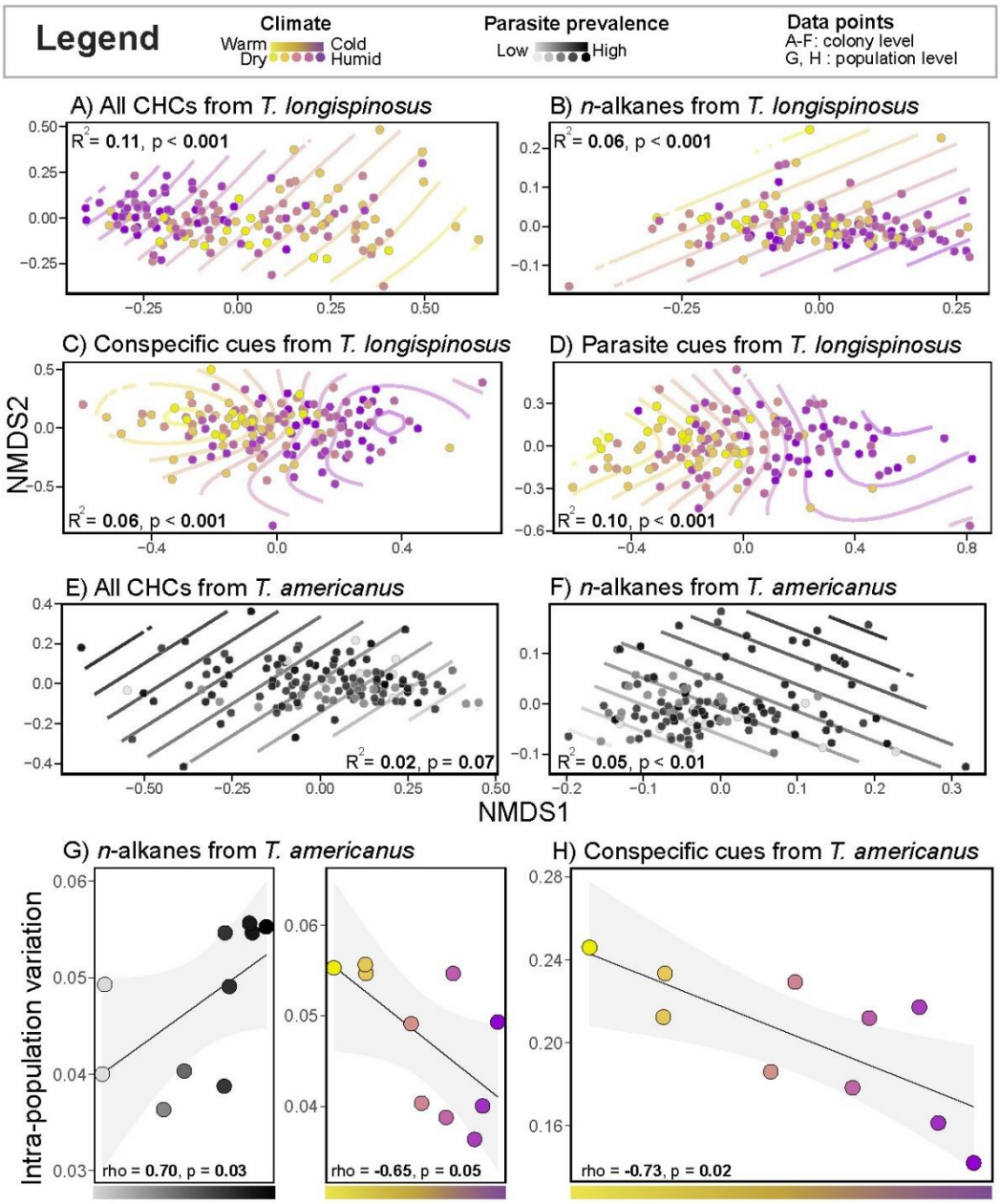

Figure 3. NMDS plots showing significant PERMANOVA associations between colony CHC composition, climate (colour-coded dots), and parasite prevalence (grayscale contours). A) All CHCs in *T. longispinosus*, B) *n*-alkanes in *T. longispinosus*, C) conspecific recognition cues in *T. longispinosus*, D) *T. americanus* recognition cues in *T. longispinosus*, E) all CHCs in *T. americanus*, and F) *n*-alkanes in *T. americanus*. G) Scatterplot of *T. americanus n*-alkane variability (± SE), positively associated with parasite prevalence but negatively with climate. H) Scatterplot of *T. americanus* recognition cue variability (± 95% CI), used by *T. longispinosus* for conspecific recognition, in relation to climate.

T. longispinosus chemical profiles contained a lower relative abundance of *n*-alkanes compared to *T. americanus (*beta GLMM- species: $\chi^2 = 128.70$, $p < 0.0001$; Fig. 4A). In *T. longispinosus*, *n*-alkane abundance decreased in warmer/drier climates, while *T. americanus* showed no climatic shifts (beta GLMM- species-climate interaction: $\chi^2 = 3.67$, $p = 0.06$; Fig. 4A). Additionally, *T. longispinosus* populations from cold/wet climates exhibited higher relative abundances of all seven *n*-alkanes and four of the five identified 3-monomethyl alkanes (Fig. S2C). *T. longispinosus* profile contained more recognition cues related to conspecific and parasite aggression than *T. americanus* (beta GLMM- conspecific recognition cues- species: $\chi^2 = 127.48$, $p < 0.0001$; parasite recognition cues- species: $\chi^2 = 157.95$, $p < 0.0001$; Fig. 4A, B). In both species, the relative abundance of recognition cues linked to conspecific and parasite aggression was higher in warmer/drier climates (beta GLMM- conspecific recognition cues- climate: $\chi^2 = 3.90$, $p = 0.05$; parasite recognition cues- climate: $\chi^2 = 5.59$, $p = 0.02$; Fig. 4A, B). Moreover, parasite recognition cues tended to increase with parasite prevalence in both species (beta GLMM- parasite recognition cues- PP: $\chi^2 = 3.70$, $p = 0.05$; Fig. 4B).

Chemical Distance between *T. americanus* and *T. longispinosus* Sympatric and Allopatric Profiles

CHC distances between free-living hosts and parasites in sympatry and allopatry were similar (Pearson's correlation- $r = 0.09$, $p = 0.16$). However, sympatric host-parasite pairs from warmer/drier regions showed greater chemical divergence than those from cold/wet climates (Spearman's rank correlation- climate: rho = -0.79, $p = 0.01$; Fig. 4D). In contrast, chemical distance between sympatric hosts and parasites was unlinked to parasite prevalence (Spearman's rank correlation- PP: rho = 0.50, $p = 0.14$).

Colony Structure

Free-living *T. longispinosus* colonies in regions of high parasite prevalence and a cold/wet climate contained more workers and queens (generalised Poisson GLMM-PP: workers: $\chi^2 = 30.09$, $p < 0.001$, Fig. S5A; queens: $\chi^2 = 7.44$, $p < 0.01$, Fig. S5B, Climate: workers: $\chi^2 = 19.07$, $p < 0.001$, Fig. S1A; queens: $\chi^2 = 66.07$, $p < 0.0001$, Fig. S5B). The number of *T. americanus* workers within parasite colonies increased with parasite prevalence (generalised Poisson GLMM- PP: $\chi^2 = 4.62$, $p = 0.03$, Fig. S5B), but was unaffected by

climate (Table S2). However, the proportion of *T. americanus* workers relative to captive host workers was unlinked to parasite prevalence or climate (Table S8).

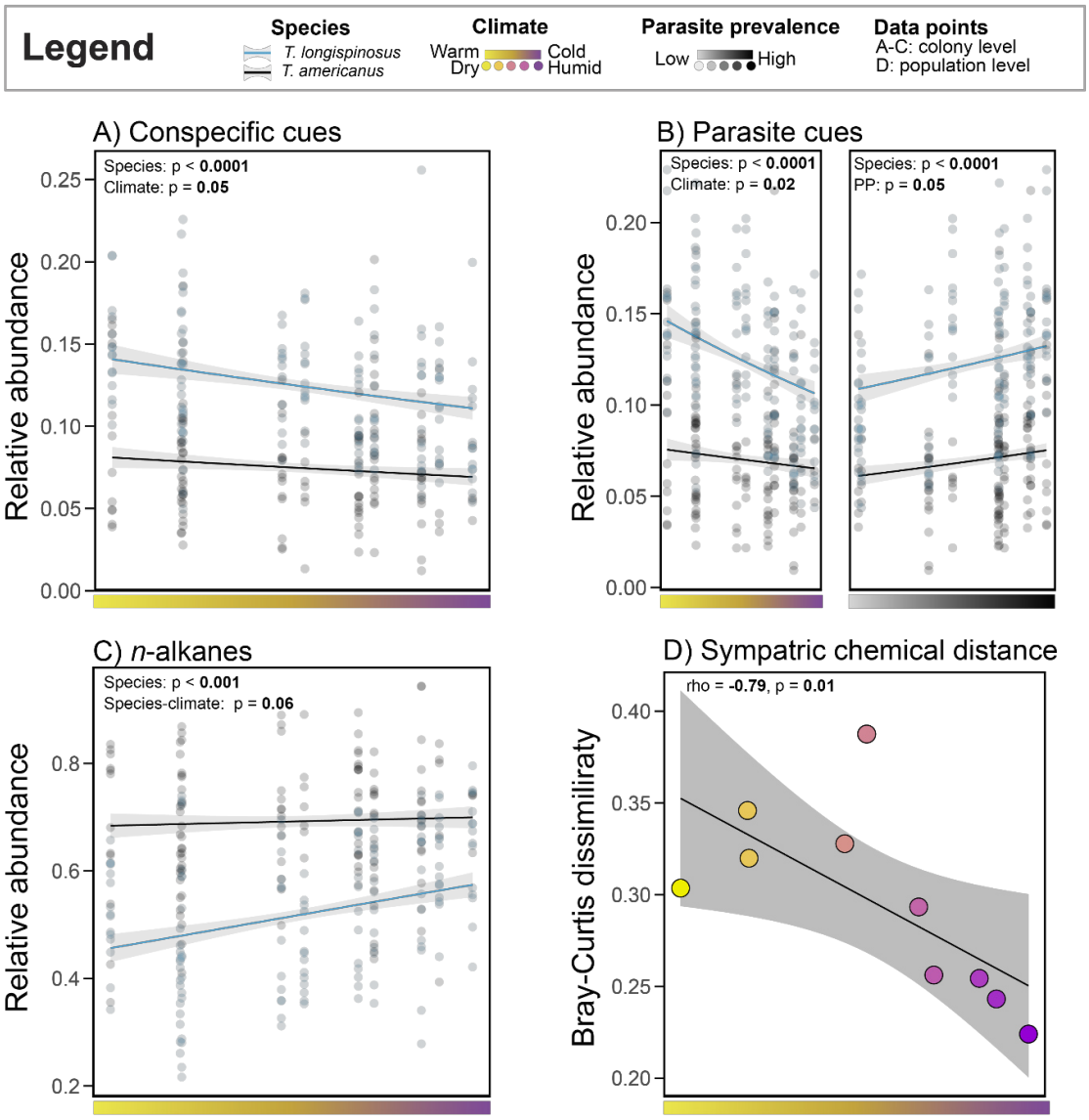

Figure 4. Scatterplots of estimated relative abundances (± 95% CI) of A) host recognition cues, B) parasite recognition cues, and C) *n*-alkanes in hosts (blue) and parasites (black) across climate gradients. D) Scatterplot showing decreasing CHC profile dissimilarity (± 95% CI) in sympatric host-parasite pairs along the climate gradient.

Discussion

The coevolutionary arms races between parasites and hosts are of great interest to evolutionary biology due to their reciprocal and continuous dynamics. Biotic and abiotic effects on host-parasite coevolutionary dynamics have been intensively investigated (Byers, 2021; Johnson and Haas, 2021; Peacock et al., 2022), in particular in disease ecology (i.e., Morales-Castilla et al., 2021; Gsell et al., 2023), but more empirical data are required to grasp the underlying mechanism. Climate, for example, has a major impact on insects, affecting their behaviour, physiology and chemical profiles (Menzel et al., 2017; Manlik et al., 2023; Couper et al., 2024). Our results show strong differences in various traits associated with climate. As these differences persisted despite several months of controlled laboratory conditions, this indicates that climate influences host and parasite co-evolutionary strategies. Geographical variation in behaviour suggests that parasites outperform hosts in warmer climates, which may be caused by factors such as higher host availability and/or local adaptation to warm, dry conditions. Our study further revealed that climate and parasite prevalence are differentially related to host and parasite CHC profiles. Climate was significantly associated with host CHC composition, while the parasite CHC profile was linked to parasite prevalence. Unexpectedly, hosts from warmer regions exhibited a lower proportion of *n*-alkanes and expressed more methylated alkanes, suggesting a trade-off between waterproofing and parasite detection in the CHC profiles. We identified recognition CHCs associated with host aggression toward parasites, including parasite-specific cues that may act as undesirable markers and *T. longispinosus*-specific cues that could function as desirable signals for nestmate recognition.

Influence of Climate and Parasite Prevalence on Behaviour

In this system, it has previously been shown that hosts employ behavioural, chemical, and morphological defences to prevent or minimise parasite attacks (Jongepier et al., 2014; Grüter et al., 2018). In *T. longispinosus*, workers primarily use collective aggression against parasitic intruders but shift to nest evacuation under increasing parasite pressure (Jongepier et al., 2014; Kleeberg et al., 2015; Segev et al., 2017). Here we further investigate these dynamics, but this time additionally examine how climate and parasite

prevalence influence *T. longispinosus* and *T. americanus* behaviour during interactions. We assumed that hosts and parasites from northern regions are generally more aggressive, as both are likely under selection for more efficient resource acquisition and defence due to the shorter active season. Previous studies have shown that hosts from colder northern regions display increased aggression toward conspecifics (Segev et al., 2017), and that parasite decision-making during raids may be constrained by limited time (Miller, 2021). In line with this, hosts from colder climates displayed greater aggression, suggesting that abiotic factors primarily drive this behavioural trait rather than parasite pressure. For example, higher host aggression may also evolve in environments with lower resource availability, such as shorter growing seasons, to protect limited resources like food and brood (Sorvari & Hakkarainen, 2004; Cristaldo et al., 2016). This aligns with seasonal aggression patterns in *Formica exsecta* and *Plagiolepis pygmaea* (Katzerke et al., 2006; Thurin & Aron, 2008) and reduced recognition abilities in *Formica xerophila* at lower temperatures, leading to increased aggression (Tanner, 2009). In contrast, parasite workers from cold, wet climates were less aggressive and more passive during host colony invasions, potentially eliciting stronger defensive responses from the hosts. A cross-fostering study (Kaur et al., 2019) has shown that the behavioural and chemical strategies of parasites can influence host responses during intrusions, including gene expression in the brain, indicating that parasite strategies can modulate both host behaviour and neural activity. While parasite behaviour is often shaped by selection for successful raids, their passivity in northern regions may indicate lower tolerance to colder climates. Despite a constant temperature regime in our experimental design, our findings could be caused by thermal mismatch differences between hosts and parasites, with parasites potentially having lower thermal limits that may exceed winter temperatures in northern latitudes. Cold winters at high latitudes might restrict parasite populations, and as a result limit prevalence, while summer heat in the South could enhance their aggressive behaviour (Bradshaw & Holzapfel, 2006). For instance, in terrestrial ectotherms, including ants, temperature strongly influences behavioural activity, with upper thermal limits varying little and lower limits decreasing with latitude (Sunday et al., 2010). Temperature-driven changes in metabolic activity may also alter behavioural interactions (Krapf et al., 2023; Menges et al., 2023). For example, in *T. curvispinosus*,

another *T. americanus* host, warmer winters show positive effects on running speed (Diamond et al., 2018), which could similarly improve raiding efficiency in the parasite. We predicted that flight responses in the host, characterised by larva carrying, as an essential behaviour during parasite invasion, would increase with parasite pressure (Jongepier et al., 2014). However, climate emerged as the primary predictor, with hosts from warmer regions displaying higher levels of this behaviour. Notably, flight responses increased in warmer climates while aggression decreased, suggesting that the host's collective defence strategies, ranging from fight to flight (Jongepier et al., 2014), may be shaped by local climatic conditions rather than parasite prevalence.

Impact of Climate and Parasite Prevalence on Cuticular Hydrocarbon Trait Expression

CHCs are essential for both waterproofing and recognition, but these functions rely on compounds with opposing biophysical properties, creating a potential trade-off. We analysed CHC composition and functional classes in relation to climate and parasite prevalence. Our results confirm that parasites were chemically similar to their hosts but had fewer methylated alkanes, consistent with previous studies (Kleeberg et al., 2017). In populations from warmer, drier climates, host and parasites showed greater chemical divergence, likely resulting in quick parasite discrimination and more effective nest evacuation (D'Ettorre et al., 2002). While parasite prevalence did not directly affect chemical distance, higher prevalence in these climates may have driven hosts to increase the proportion of methylated alkanes. Greater divergence could also result from parasites adopting generic chemical profiles to exploit multiple host species, as *T. curvispinosus* is also parasitised in warmer regions (Brandt et al., 2005).

In *T. americanus*, *n*-alkane composition was not linked to climate, suggesting that the level of *n*-alkanes in this species is either suitable across their distribution range or that this species is genetically constrained and cannot adjust its profile to better suit cold climate conditions. The high *n*-alkane levels may provide sufficient waterproofing while also aiding parasites in evading detection by diluting host recognition cues (Lorenzi & d'Ettorre, 2020). Greater variability in *n*-alkane composition was observed in regions with high parasite prevalence and warm, dry climates, possibly due to genetic diversity, host diversity, or microclimatic variation, which may hinder hosts from learning parasite

CHC profiles. Contrary to expectations, parasite prevalence did not affect *n*-alkane proportion but did influence composition as such, with unclear effects on host recognition.

In hosts, *n*-alkane composition varied with climate, but in the opposite direction of expectations. The relative abundance of *n*-alkanes and monomethylated alkanes, important for waterproofing, increased in colder, wetter climates where desiccation risk is lower (Wagner et al., 2001; Menzel et al., 2018). This suggests an evolutionary trade-off, where hosts may prioritise recognition cues over waterproofing in warmer, drier regions if parasite pressure or other biotic pressures are high, potentially compromising optimal behavioural defence against parasites. Ants have exceptional olfactory abilities crucial for distinguishing nestmates from foes (Sturgis & Gordon, 2012). Recognition relies on detecting either undesirable outsider cues or the absence of desirable cues, or both (Sherman et al., 1997; Guerrieri et al., 2009; Neupert et al., 2018). In *T. longispinosus*, methylated alkanes play a key role in conspecific recognition, and their increased variability in parasite-exposed populations suggests a defensive function (Jongepier & Foitzik, 2016). This latter study therefore assumed that parasite recognition relied on the same compounds as conspecific recognition, but our findings suggest otherwise. We identified that host aggression towards conspecifics or parasites was primarily associated with a distinct set of methylated alkanes. Some of the cues related to parasite aggression were exclusively found on parasites, while others were unique to hosts. This suggests that hosts may recognise parasites by comparing both the presence and absence of specific compounds against their internal recognition template (Neupert et al., 2018).

The composition of parasite recognition cues carried by *T. longispinosus* was linked to climate. Both species exhibited higher recognition cue abundance in warmer climates, possibly to counter increased evaporation rates (Menzel et al., 2019). This was unexpected, as reliable recognition is crucial for detecting intruders. However, with limited knowledge of odour perception, it remains unclear how recognition templates evolve within a colony. In contrast, parasite recognition cue composition was unaffected by climate or parasite prevalence, indicating minimal geographic variation. Parasites also

had lower recognition cue abundance than hosts, which probably contributes to them not being detected.

Variation in Colony Size and Social Structure with Climate and Parasite Prevalence

Our study found that climate and, to a lesser extent, parasite prevalence had opposing effects on host colony structure: colonies in high-prevalence and colder regions had more queens and workers, contrasting with previous findings (Herbers & Foitzik, 2002; Foitzik et al., 2009). Polygyny, especially when combined with polydomy, may function as a bet-hedging strategy to protect host colonies against raids, particularly by reducing the risk associated with queen loss. In colder climates, polygyny may also eliminate the risky independent founding phase (Bourke & Heinze, 1997), and larger colonies may enhance winter survival. In high-prevalence regions, *T. americanus* colonies also had more workers (Herbers & Foitzik, 2002), which could potentially lead to larger colonies through a greater investment in reproductives. However, parasite colony size was unaffected by climate.

Conclusions

Abiotic factors have been shown to play an important role in shaping coevolutionary interactions between hosts and parasites, though their complex and multifactorial effects on these systems require further investigation (Manlik et al., 2023; Couper et al., 2024; Ragonese et al., 2024). Understanding the relationship between climate and ectotherm host-parasite interactions is increasingly critical for conservation, given the profound effects of abiotic factors on their physiology and behaviour (Franke et al., 2019; Ragonese et al., 2024). Our findings demonstrate that the climatic landscape is clearly bound with the geographic variation of hosts and parasite traits during their interactions. This adds to growing evidence that climate is a major player involved in host-parasite dynamics (Dziuba et al., 2023; Manlik et al., 2023; Couper et al., 2024), and underscores the need to systematically integrate abiotic factors into coevolutionary research.

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Conflict of Interest Statement

The authors declare no conflict of interest.

Supplementary Material

Additional Information on Parasite Prevalence

We tested whether long-term and our collection data differed in estimating parasite using a paired Wilcoxon signed-rank test, which is appropriate for small sample sizes. The test did not reject the null hypothesis ($p = 0.08$), suggesting that parasite prevalence estimates are relatively stable over time. Therefore, we combined both data to increase the reliability of our estimates (Table S1).

Additional Information on Ant Maintenance

Colonies were maintained under controlled laboratory conditions at 21 °C (temperature) and 70% relative humidity (RH) with a 12:12h light/dark cycle to simulate natural conditions. Ants were housed in artificial nests consisting of plaster-based chambers placed within plastic foraging arenas (dimensions: 10 × 10 × 4 cm), allowing for easy access and observation.

Additional Information on Behavioural Assays and their Statistical Analyses

We investigated the behavioural responses of 15 *T. longispinosus* colonies per population ($N = 150$) to the introduction of *T. longispinosus* or *T. americanus* workers, one year after collection. A week before the tests, a standardised subcolony was created from each colony, comprising two workers from the nest entrance, eight workers near the brood (1st

week, median = 10 workers, variance = 1.21, Table S2), the queen (if present), five larvae, and a small pile of eggs, transferred to a cylindrical arena (D = 37 mm, H = 32 mm). The ants were fed the same diet as before, with food removed during the tests. Arenas were labelled with unique random numbers to ensure observer blindness during testing and video analysis. We introduced three types of sympatric workers in separate trials: nestmates of *T. longispinosus* from the same colony as the subcolony, *T. longispinosus* workers from different colonies but from the same population, and parasitic *T. americanus* workers from the same site. *T. longispinosus* workers were consistently taken from their nest entrance, while entrance workers were not always available for parasitic *T. americanus* workers due to their low numbers per nest. Each worker was marked with orange paint on the first segment of the gaster at least a day before the test and again marked with blue paint after the experiment to prevent reuse. Workers were taken from an average of five colonies per population to balance colony effects based on average the stock of parasite colonies, except in Maine and New Hampshire where fewer parasitic colonies were available. Experimenters were unaware of the opponents' origin but could not be blind to species identity due to morphological differences.

Behavioural experiments were conducted three days per week for three weeks. Each day, 50 subcolonies were tested with a randomised trial, either involving nestmates, foreign conspecifics, or parasitic workers. Thereafter, subcolonies rested for a week before the next trial. Videos of trials were recorded using four identical 4K cameras (Sony FDR-AX33 camera) for 20 min. For the first ten minutes, subcolonies acclimated to the light while being recorded. Afterwards, marked workers were introduced into the arenas using sterile forceps while recording for another ten minutes. That second recording part was used for behavioural analyses. We analysed the behaviour of the subcolony towards different intruder types and the behaviour of the intruders themselves. Each trial consisted of 27 scans with 20-second intervals, conducted over nine minutes. The number of workers displaying specific behaviours was recorded during each scan, considering the number of workers in the subcolony. To quantify intruder behaviour, we added the number of scans in which a particular behaviour occurred to obtain quantitative scores. Behaviours were categorised as aggressive (including biting, stinging, pulling) and sociopositive (including antennation, grooming, trophallaxis; for details see Table S3).

For the subcolony, we also quantified brood-carrying behaviour and for the intruder passive behaviour (referring to curled-up posture, immobility).

We performed generalised linear mixed models (GLMMs) using the *glmmTMB R* package (Brook et al., 2017) and ensured the correct distribution of the models with the diagnostic tools provided by the *DHARMa* package (Hartig, 2022). In models, we included behaviours and factors including intruder type, parasite prevalence, climate (PC1), presence of a queen, and their interactions. Colony origin of subcolony and intruders were considered as a random factor and only included in the absence of singularity, checked with the function *check_singularity* from the *performance R* package (Lüdecke et al., 2021). We did not include information on subsites from which a colony was sampled (see 'colony_collection.xlsx') as a factor in our analyses, as we considered other random effects more relevant and aimed to avoid potential overfitting. We conducted a variance inflation factor (VIF) analysis using the function *check_collinearity* to sequentially eliminate collinear factors (Table S4) and assessed models using a stepwise automatic model reduction approach, using the *buildmer* package (Voeten 2024), based on the Akaike information criterion (AIC) to produce a final model. We used the *joint_tests* function from the *emmeans R* package (Lenth et al., 2024) to conduct combined tests on the adjusted means of the factors included in the models. Our primary focus was to evaluate which effects persisted across model reduction and whether the stepwise model reduction effectively eliminated non-significant terms (Table S5). We back-transformed model estimates from logit scale to proportion scale estimates for interpretation using the code type = 'response' in *emmeans* (Table S6). We used the *emmeans* function to identify the model estimates related to categorical factors (i.e. type of intruder) and the *emtrend* function with continuous factors (i.e. climate gradient) and adjusted the tests with a conservative Bonferroni correction for pairwise comparisons.

Additional Information on the Cuticular Hydrocarbon Analyses

We analysed the cuticular hydrocarbon (CHC) profiles of single *T. americanus* workers and pairs of *T. longispinosus* nestmate workers from colonies involved in behavioural trials. Workers were flash-frozen on dry ice, stored at -20 °C, then brought back to room temperature and immersed in approximately 350 µl hexane for 10 minutes. After

removal, the hexane extracts were concentrated under a nitrogen flow. Samples were analysed using a gas chromatograph (7890A, Agilent Technologies, Santa Clara, CA, USA) equipped with a Zebron Inferno ZB5-HT column (Phenomenex Ltd., Aschaffenburg, Germany). The temperature was initially held at 60 °C for two minutes, then increased by 60 °C/min to 200 °C and subsequently by 48 °C/min to a maximum of 320 °C, which was maintained for ten minutes. Helium was used as the carrier gas at a flow rate of 1.2 mL/min. Hydrocarbon molecules were transferred to a mass selective detector (5975 C, Agilent Technologies) and fragmented with an ionisation voltage of 70 eV. Fragments in the range of 40–550 m/z were detected and subsequently identified based on their retention indices and diagnostic ions (Carlson et al., 1998). We quantified substances using single-ion monitoring by manual integration in the software MSD ChemStation (E.02.02.1431, Agilent Technologies). We considered all hydrocarbons longer than C20 but excluded non-hydrocarbons (which were rare) and compounds with an average proportion below 0.1% and a maximum abundance below 0.5% of the total profile. Hydrocarbons were treated as functional traits following Sprenger and Menzel (2020) and categorised into *n*-alkanes and methyl-branched alkanes, with the same classification applied to the recognition cues identified in this study. Non-identified CHCs were assumed to be methyl-branched alkanes, as all *n*-alkanes present in both species were identified, and alkenes are generally rare in *Temnothorax* species, accounting for less than 0.9% in *T. longispinosus* (Sprenger & Menzel, 2020).

Aggression in *T. longispinosus* towards foreign conspecifics can be predicted based on abundance differences of nine hydrocarbons (Jongepier & Foitzik, 2016). Here, we aimed to identify the CHC compounds linked to aggression of hosts toward both conspecifics and social parasites. We applied a reductive algorithm based on Wittke et al. (2022), which initially includes the full set of CHCs and determines the correlation with aggression. The algorithm then sequentially removes compounds that do not improve the correlation strength while standardising the relative abundance to 1 after each removal. The process stops when no further compound can be omitted without reducing the correlation, yielding a final set of recognition cues that best explain the observed aggression patterns. This analysis was performed separately for conspecific and parasite cues, allowing us to identify distinct sets of CHCs relevant for recognition of conspecifics

and parasites. To examine the relationship between chemical composition and ecological predictors, we conducted a PERMANOVA using the *adonis* function in the *vegan R* package (Oksanen et al., 2022).

For each species, we included either the overall CHC profile, *n*-alkanes, or the recognition cues identified in this study, testing their association with parasite prevalence and climate. To visualise individual CHC composition in two-dimensional space, we performed non-metric multidimensional scaling (NMDS) with the ORD function using 1,000 permutations based on Bray-Curtis distances. The *ordisurf* function was used to assess linearity in associations between chemical composition and environmental factors. Additionally, we conducted a principal component analysis (PCA) using the *prcomp* function, considering all CHCs, to investigate the relationship between the relative abundance of each compound and climate or parasite prevalence in both species. Using the *vegan* package, we applied the *betadisp* function to extract centroid values, estimating CHC variability between individuals within populations for all CHCs, *n*-alkanes, and recognition cues. We then tested whether centroid values varied across climate gradients and parasite prevalence using Spearman's rank correlation tests. All analyses were performed in *R* v.4.2.3 (R Core Team, 2021).

Supplement Figures

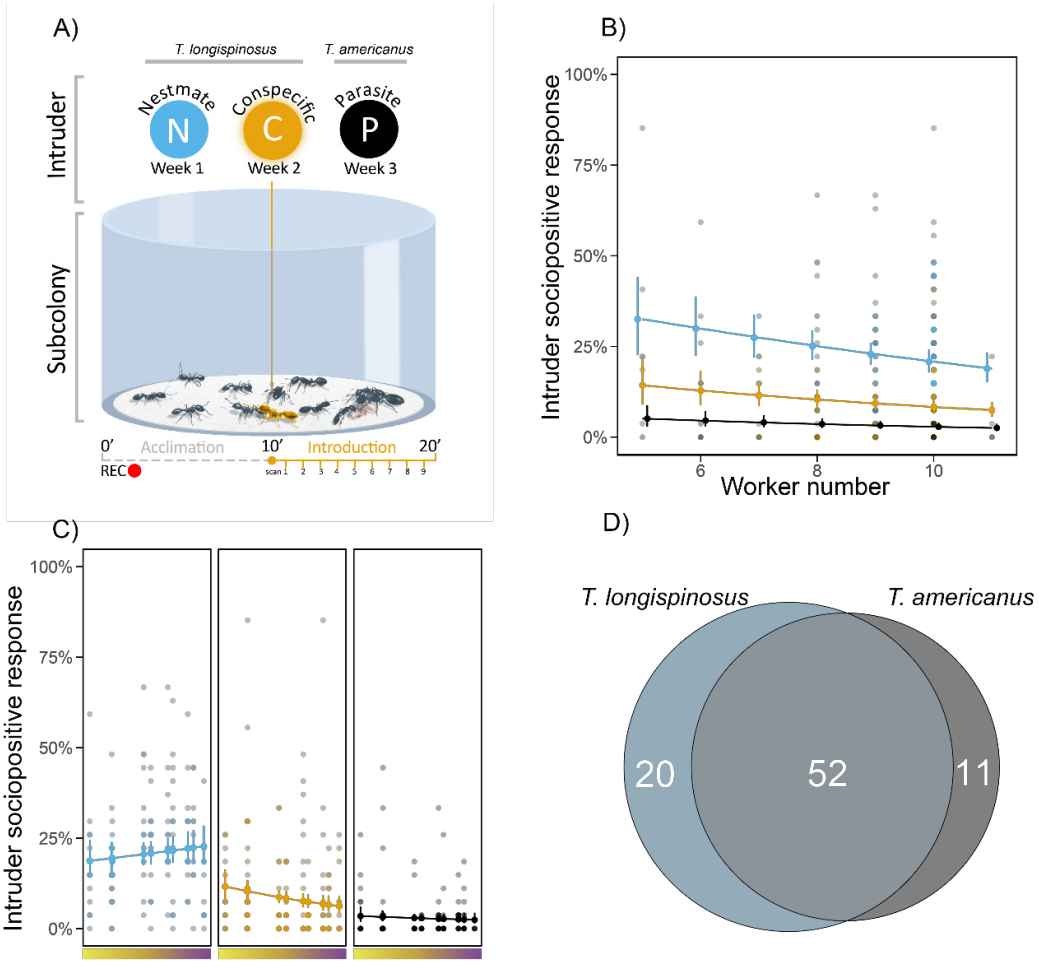

Figure S1. A) Schematic representation of the experimental design for behavioural assays. Once per week, over a period of three weeks, *T. longispinosus* subcolonies were randomly assigned to an encounter with either a nestmate, a sympatric conspecific, or a sympatric *T. americanus* parasite. Following a ten-minute acclimation period under recording conditions, the intruder was introduced, and the behaviour of both the subcolony and the intruder was observed in nine scans over the course of the trial. B–C) Scatterplots illustrating the association between sociopositive behavioural responses of intruders and subcolony size (B) or climate (C). Model mean estimates are shown as solid dots with standard error (± SE), while transparent data points represent raw observations. Due to an interaction effect between intruder type and climate, the B plot is split into subplots to facilitate interpretation. D) Venn diagram displaying the number of species-specific CHCs and shared CHCs between *T. longispinosus* and *T. americanus*.

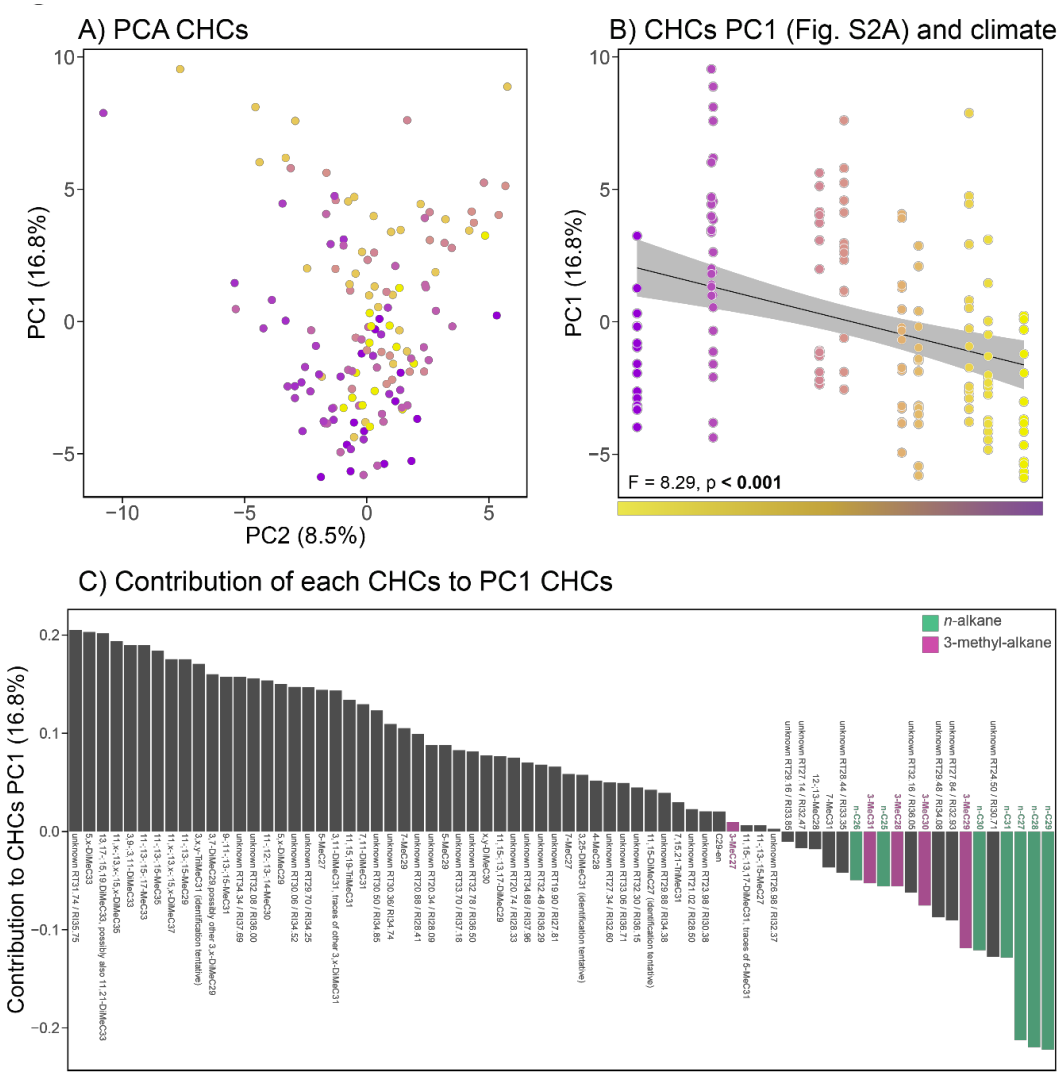

Figure S2. A) Two-dimensional PCA plot (PC1 and PC2) depicting individual CHC composition in *T. longispinosus*, with individuals colour-coded according to the local climate of their origin. B) Scatterplot showing the relationship between PC1 values of individual CHC composition (mean estimates ± SE) and climate, illustrating the association between overall CHC variation and environmental conditions. C) Bar plot displaying the contribution of each CHC to PC1 (y-axis, corresponding to Fig. S2B). *n*-alkanes and 3-methyl-alkanes, which are more abundant in colder/wetter regions, are colour-coded according to the legend. Unknown compounds are annotated by their retention time (RT) and retention index (RI).

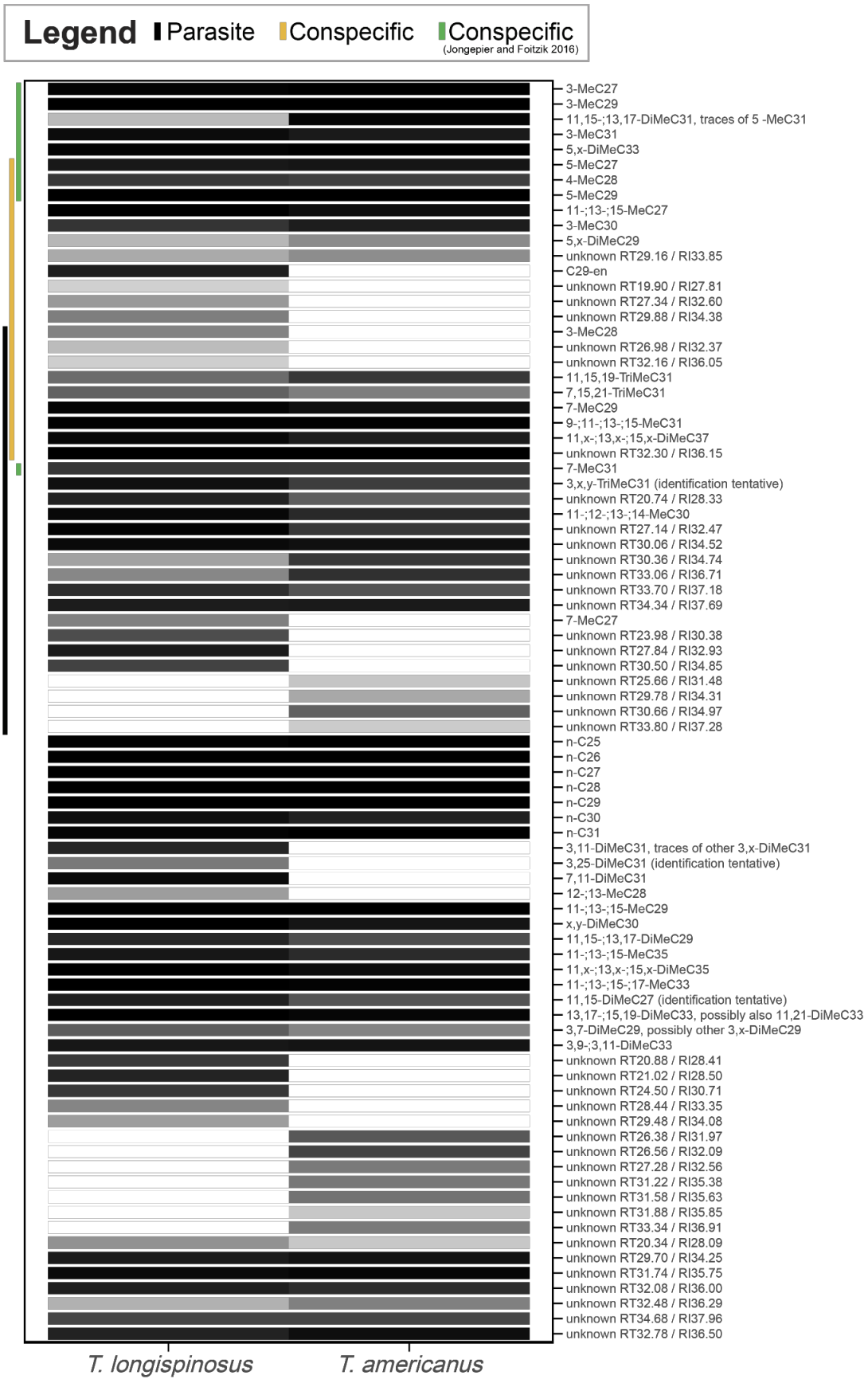
Legend
Parasite
Conspecific
Conspecific
(Jongepier and Foitzik 2016)
3-MeC27
3-MeC29
11,15-;13,17-DiMeC31, traces of 5 -MeC31
3-MeC31
5,x-DiMeC33
5-MeC27
4-MeC28
5-MeC29
11-;13-;15-MeC27
3-MeC30
5,x-DiMeC29
unknown RT29.16 / RI33.85
C29-en
unknown RT19.90 / RI27.81
unknown RT27.34 / RI32.60
unknown RT29.88 / RI34.38
3-MeC28
unknown RT26.98 / RI32.37
unknown RT32.16 / RI36.05
11,15,19-TriMeC31
7,15,21-TriMeC31
7-MeC29
9-;11-;13-;15-MeC31
11,x-;13,x-;15,x-DiMeC37
unknown RT32.30 / RI36.15
7-MeC31
3,x,y-TriMeC31 (identification tentative)
unknown RT20.74 / RI28.33
11-;12-;13-;14-MeC30
unknown RT27.14 / RI32.47
unknown RT30.06 / RI34.52
unknown RT30.36 / RI34.74
unknown RT33.06 / RI36.71
unknown RT33.70 / RI37.18
unknown RT34.34 / RI37.69
7-MeC27
unknown RT23.98 / RI30.38
unknown RT27.84 / RI32.93
unknown RT30.50 / RI34.85
unknown RT25.66 / RI31.48
unknown RT29.78 / RI34.31
unknown RT30.66 / RI34.97
unknown RT33.80 / RI37.28
n-C25
n-C26
n-C27
n-C28
n-C29
n-C30
n-C31
3,11-DiMeC31, traces of other 3,x-DiMeC31
3,25-DiMeC31 (identification tentative)
7,11-DiMeC31
12-;13-MeC28
11-;13-;15-MeC29
x,y-DiMeC30
11,15-;13,17-DiMeC29
11-;13-;15-MeC35
11,x-;13,x-;15,x-DiMeC35
11-;13-;15-;17-MeC33
11,15-DiMeC27 (identification tentative)
13,17-;15,19-DiMeC33, possibly also 11,21-DiMeC33
3,7-DiMeC29, possibly other 3,x-DiMeC29
3,9-;3,11-DiMeC33
unknown RT20.88 / RI28.41
unknown RT21.02 / RI28.50
unknown RT24.50 / RI30.71
unknown RT28.44 / RI33.35
unknown RT29.48 / RI34.08
unknown RT26.38 / RI31.97
unknown RT26.56 / RI32.09
unknown RT27.28 / RI32.56
unknown RT31.22 / RI35.38
unknown RT31.58 / RI35.63
unknown RT31.88 / RI35.85
unknown RT33.34 / RI36.91
unknown RT20.34 / RI28.09
unknown RT29.70 / RI34.25
unknown RT31.74 / RI35.75
unknown RT32.08 / RI36.00
unknown RT32.48 / RI36.29
unknown RT34.68 / RI37.96
unknown RT32.78 / RI36.50
T. longispinosus
T. americanus

Figure S3. The square heatmap illustrates the presence of each cuticular hydrocarbon (CHC) across individuals from each species. Each row represents a specific chemical compound, with colour intensity indicating the proportion of individuals in which the compound is detected, ranging from white (absent in all individuals) to black (present in all individuals). Compounds are arranged from top to bottom in the following order: recognition cues, *n*-alkanes, other identified CHCs, and unknown CHCs. Unknown CHCs are annotated by their retention time (RT) and retention index (RI). The different types of recognition cues are marked by vertical lines on the left, with colour codes provided in the legend.

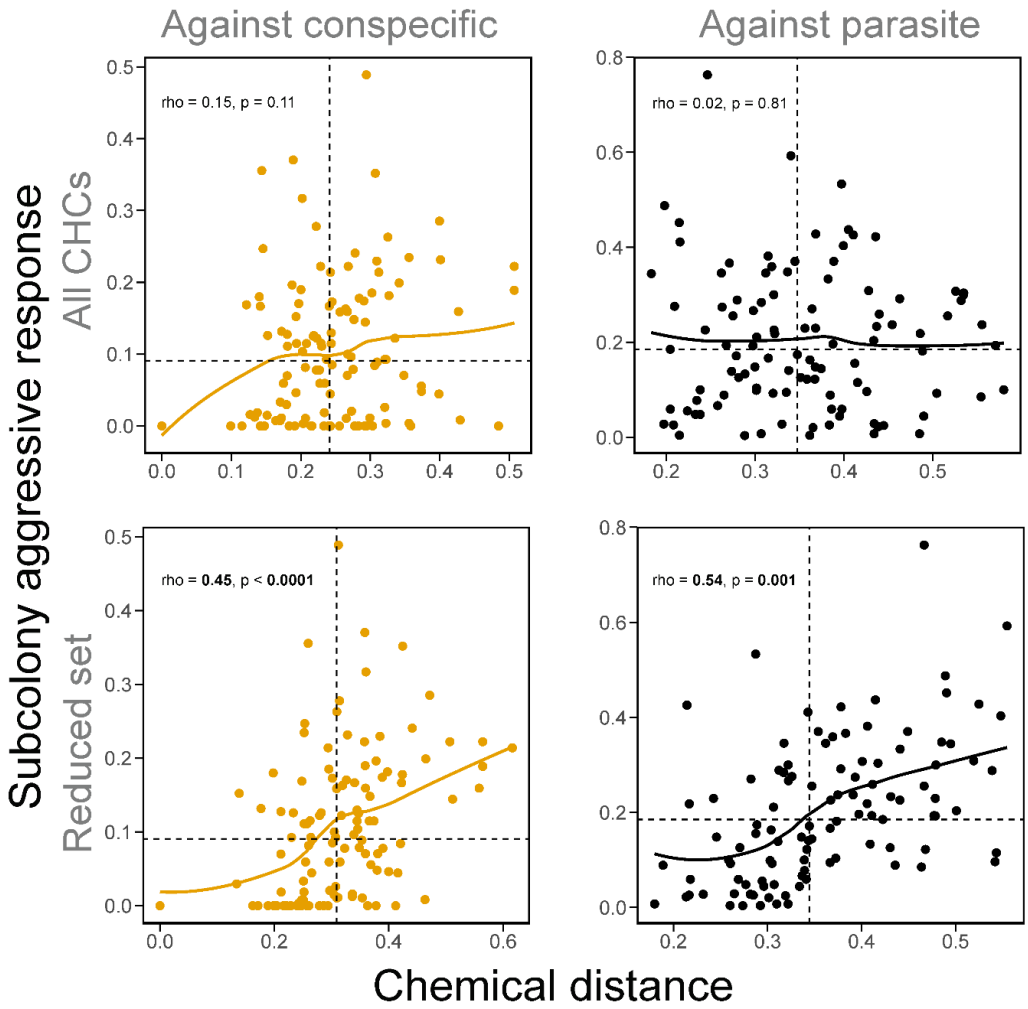

Figure S4. Scatterplots depicting the relationship between chemical distance and subcolony aggression towards intruders. A) and C) (colour-coded in orange) represent subcolony aggression towards conspecific intruders, while B) and D) (colour-coded in black) represent subcolony aggression towards parasite intruders. A) and B) are based on all CHCs, while C) and D) are based on the reduced set of CHCs (conspecific or parasite recognition cues), obtained by the reductive algorithm that are most strongly associated with subcolony aggression.

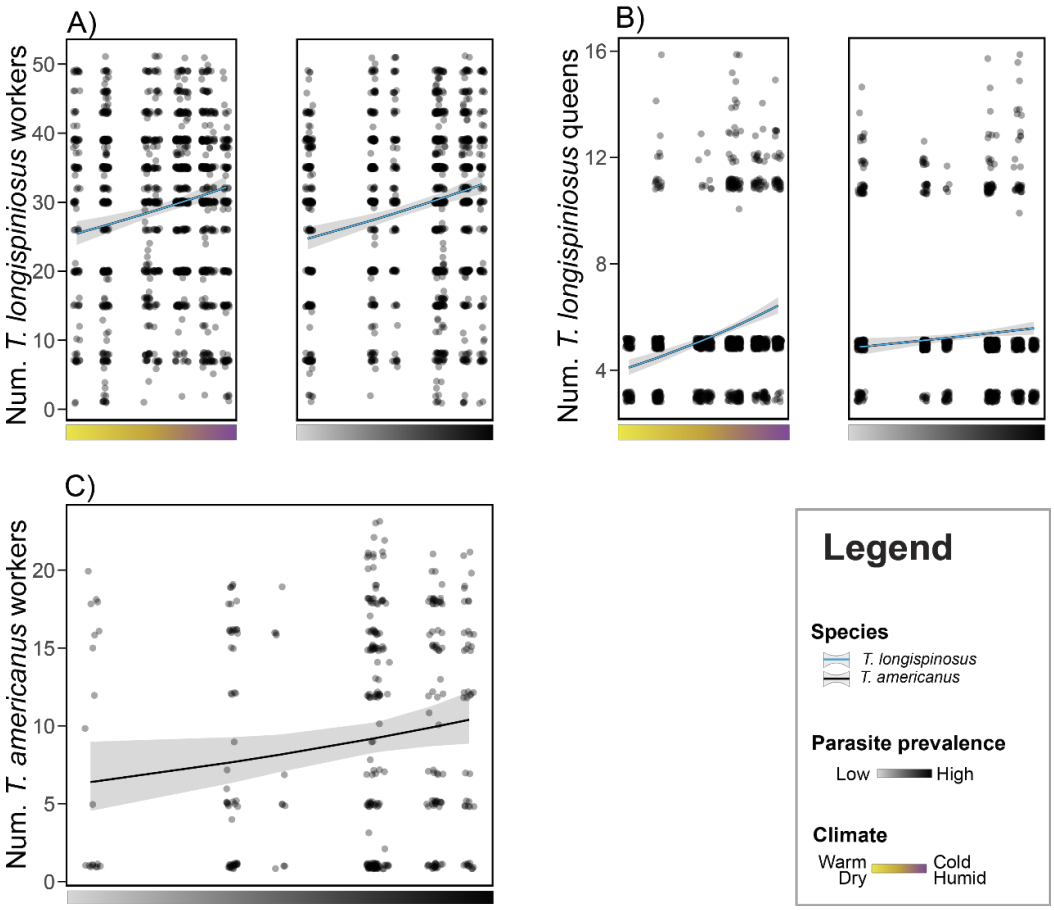

Figure S5. Scatterplots illustrating the relationships between colony composition, climate, and parasite prevalence. A) and B) show the associations between the number of *T. longispinosus* workers (A) and queens (B) with climate and parasite prevalence (see legend for details). C) depicts the relationship between the number of *T. americanus* workers and parasite prevalence.

Supplement Tables

Table S1. Collection sites with long-term colony collection information for each species. Estimates of 'Parasite prevalence*' are based on long-term collection, while 'Parasite prevalence 2021' refers to estimates based on this study collection. '**' refers to the populations sampled for the first time.

Population	Location	Coordinates	*T. long*	*T. amer*	Parasite prevalence*	Parasite prevalence 2021
Massachusetts (MA)	Beaver Brook North and Rock Meadow Reservation	42.407, -71.204	501	69.86	12.233	13.024
Maryland (MD)**	New Germany State Park	39.611, -79.134	112	11	8.943	8.943
Maine (ME)	Bethel Community Forest	44.413, -70.851	504	13	2.515	4.375
New Hampshire (NH)	Belknap Mountain State Forest	43.510, -71.402	423	10	2.309	8.333
New York Huyck (NYH)	Huyck Preserve	42.525, -74.160	5634	780	12.161	18.863
New York South (NYS)**	Fahnestock State Park	41.455, -73.868	178	25.62	12.583	12.583
Ohio (OH)	Private Property	41.753, -80.967	573	105.58	15.558	13.427
Pennsylvania (PA)**	S.B. Elliot State Park	41.124, -78.523	314	53	14.441	14.441
Vermont (VT)	Branbury State Park	43.945, -73.076	1787	140	7.265	10.460
West Virginia (WV)	Watoga State Park	38.110, -80.136	843	139.65	14.211	16.977

Table S2. Summary of the behavioural assays. Number of behavioural assays associated with each type of intruder within each week are colour-coded.

	Week 1 (20-22 July 2022)				**Week 2** (27-29 July 2022)				**Week 3** (3-5 August 2022)				**All weeks**		
Number of behavioural assays	N: 46 C: 48 **P**: 49				N: 47 C: 47 **P**: 43				N: 41 C: 43 **P**: 32				N: 134 C: 138 **P**: 124		
Measure	Max	Mean	Median	Variance	Max	Mean	Median	Variance	Max	Mean	Median	Variance	Mean	Median	Variance
Number of workers/subcolony	11	9.7	10	0.6	11	9.4	10	1.3	10	9.1	9	1.5	9.4	10	1.21

Note: Colour code: N: Nestmate, C: Conspecific, **P**: Parasite. The minimal number of workers required for the analyses for each colony was set at 5.

Table S3. Ethogram of the behaviours used with the scan sampling method for behavioural analyses. Aggression (*) of subcolony towards introduced conspecific and parasite workers was used to determine the chemical compounds most associated with an aggressive response.

Variables	**Var. used in analyses**	**Description**
ID_col_subcol		Unique subcolony ID used during the collection for all colonies (slavemaker and host)
ID_rand_subcol		Subcolony random ID, from 1 to 150
ID_col_intrud		Unique intruder colony ID used during the collection for all colonies (slavemaker and host).
ID_subcol_project		Colony ID from the subcolony used across different datasets. It contains all information of the colony in that form: X (species: SM or L)_XX (population origin)_X (colony number)
ID_intrud_project		Colony ID from the intruder used across different datasets. It contains all information of the colony in that form: X (species: SM or L)_XX (population origin)_X (colony number)
week		The week in which the behavioural assay was recorded (either week 1,2 or 3)
type		Type of intruder placed in the arena
prevalence		Parasite prevalence estimates based on the 10 collection sites
population		Sites from which colonies were collected, in reference to the states in Northern America

PC1_climate		Values of the 1st PC axis from the population climate data spanning over temperature and precipitations
day		The day the behavioural assays was recorded
start_assay		The time of the day the behavioural assay record started
n_queen_subcol		Number of queens in the arena at the beginning of the video (1 is expected, except for queenless colonies)
n_workers_subcol		Number of workers in the arena at the beginning of the video before introduction
subcol_mandibles	Aggression*	Number of host workers that open their mandibles as a threat response around an introduced worker
subcol_biting		Number of host workers that grab with their mandibles the libs of an introduced worker without dragging
subcol_stinging		Number of host workers that bit and curve the thorax and stab with their stinger an introduced worker
subcol_dragging		Number of hosts workers that grab the libs of an introduced worker and drag it in a direction
subcol_carrying		Number of host workers that lift an introduced worker with their mandibles while moving around
subcol_pinning		Number of host workers dragging in opposite directions the legs of an introduced worker
subcol_antennation	Sociopositive	Number of host workers that touch bodyparts of an introduced worker with their antenna
subcol_grooming		Number of host workers that gently use their mouthparts on the introduced worker while touching it with the antenna
subcol_trophallaxis		Number of host workers showing a mutual mouthpart contact with an introduced ant
subcol_brood_carrying	Brood carrying	Number of host workers that carry the brood with their mandibles
intrud_mandibles	Aggression	An introduced worker that opens its mandibles around another worker (threat behaviour)
intrud_biting		An introduced worker that grabs with its mandibles the libs of another worker without dragging
intrud_stinging		An introduced worker that bits and curves its thorax and stab with its stinger another worker
intrud_dragging		An introduced worker that grabs the libs of another worker and drags it in a direction
intrud_carrying		An introduced worker that lifts another worker with its mandibles while moving around
intrud_antennation	Sociopositive	An introduced worker that touches bodyparts of another worker with its antenna
intrud_grooming		An introduced worker that gently uses its mouthparts on another worker while touching it with its antenna
intrud_trophallaxis		An introduced worker showing a mutual mouthpart contact with another ants
intrud_immobile	Passive	An introduced worker that stands on its legs, inactive during an interaction with another worker
intrud_curled		An introduced worker that adopts a pupae like posture, where the legs are back hidden close to the thorax and the antenna close to the head in an interaction with another worker
corpse_begin		Number of dead workers found on the arena floor at the beginning of the video before the introduction of the worker

Table S4. Systematic three-step methodology used for model reduction in the context of the A) subcolony and B) intruder responses.

A)	Aggression					Sociopositive				Brood carrying			
	Full	$Colinear_1$	$Colinear_2$	$Colinear_{red}$	AIC_{red}	Full	Colinear	$Colinear_{red}$	AIC_{red}	Full	Colinear	$Colinear_{red}$	AIC_{red}
Intruder type	4.63e+09	172.61	1.03	1.01	1.01	940.93	224.72	1.01	1.01	625.77	176.64	1.04	1.03
Parasite prevalence	20.91	20.29	2.23	2.23	2.27	5.09	5.07	2.41		7.86	2.41	2.40	
Climate	25.13	24.14	11.72	2.25	2.27	5.19	5.12	3.62	2.32	10.10	5.69	5.65	3.93
Queen number	1.03e+08	1.01	1.01	1.01		2.20	1.02	1.02		6.81	6.80	1.02	
Parasite prevalence * Type	534.11	**520.54**				328.74	**328.27**			**282.29**			
Climate * Type	54.00	51.76	**10.33**			12.68	12.57	2.12	2.33	21.69	4.02	3.95	3.92
Queen presence *Type	**3.78e+09**					**304.95**				232.89	**231.39**		
(1 | subcolony ID)	✓					✓				✓			
(1 | intruder colony ID)	✓									✓			

B)	Aggression					Sociopositive					Passive				
	Full	Colinear 1	Colinear 2	$Colinear_{red}$	AIC_{red}	Full	Colinear 1	Colinear 2	$Colinear_{red}$	AIC_{red}	Full	Colinear 1	Colinear 2	$Colinear_{red}$	AIC_{red}
Intruder type	1.58e+10	7157.04	242.99	1.08	1.08	7930.33	743.23	220.86	1.04	1.04	8769.49	778.25	257.98	1.03	1.02
Parasite prevalence	12.18	12.10	12.46	2.38		3.66	3.67	2.40	2.40		6.31	6.28	2.34	2.35	
Climate	14.94	14.57	15.02	7.70	7.07	3.88	3.89	3.18	3.16	1.66	6.63	6.56	4.21	4.21	3.02
Queen number	7.04e+07	1.03	1.03	1.03		1.57	1.57	1.59	1.03		2.29	2.28	2.28	1.03	
Worker number	5.74	5.81	1.08	1.08		1.55	1.05	1.04	1.04	1.04	2.44	1.06	1.06	1.06	
Parasite prevalence*Type	558.92	551.60	**555.83**			341.52	**338.71**				329.39	**328.62**			
Climate * Type	37.48	35.97	35.80	6.39	7.49	11.49	11.20	1.76	1.69	1.69	15.32	14.78	2.89	2.83	3.06
Queen presence * Type	**3.06e+09**					226.18	227.95	**226.96**			287.95	284.57	**281.51**		
Worker number * Type	5691.42	**5641.23**				**5094.91**					**5588.44**				
(1 | subcolony ID)						✓					✓				
(1 | intruder colony ID)	✓														

Note: First, random factors that caused model singularity were removed (random effect kept: ✓). From then, we estimated the Variance Inflation Factor (VIF) to evaluate the degree of multicollinearity among predictors. Each predictor was colour-coded according to their collinearity level (low [0:5]: green, moderate [5:10]: blue, high [10, +∞]: red). The predictor with the highest VIF was sequentially eliminated (in bold character) from transitory models ($Colinear_n$) until a model that consisted of predictors under a VIF value of 10 ($Colinear_{red}$). We then performed a stepwise model reduction on $Colinear_{red}$ models using the Akaike Information Criterion (AIC) to identify and retain the most relevant predictors (remaining VIF values) and random effects (✓). The resulting model was called AIC_{red} for which we also estimated the VIF.

Table S5. Summary from the analysis of variance-like table for the behavioural response of A) the subcolony and B) intruders for the full, $Colinear_{red}$ and AIC_{red} models. Column related to degree of freedom (df1, df2) is associated to F statistic test (F). Significant associations ($p \leq 0.05$) are shown in bold.

A)

		Aggression						**Sociopositive**						**Brood carrying**					
		Full		$Colinear_{red}$		AIC_{red}		Full		$Colinear_{red}$		AIC_{red}		Full		$Colinear_{red}$		AIC_{red}	
	df1,df2	F	p	F	p	F	P	F	p	F	p	F	p	F	p	F	p	F	p
Intruder type	2, ∞	6.87	**0.001**	125.12	**<0.0001**	134.09	**<0.0001**	7.32	**<0.001**	42.64	**<0.0001**	42.46	**<0.0001**	15.11	**<0.0001**	38.18	**<0.0001**	37.98	**<0.0001**
Prevalence	1, ∞	0.18	0.67	2.45	0.12	2.88	0.09	0.25	0.62	0.29	0.59			0.82	0.37	0.69	0.41		
Climate	1, ∞	2.01	0.16	8.33	**<0.01**	8.79	**<0.01**	0.06	0.81	0.06	0.81	0.97	0.32	0.72	0.40	0.51	0.48	0.08	0.78
Queen presence	1, ∞	0.00	1	0.28	0.60			0.07	0.79	0.27	0.61			0.60	0.44	1.92	0.17		
Prevalence * Type	2, ∞	0.95	0.39					1.62	0.20					0.45	0.64				
Climate * Type	2, ∞	0.67	0.51					3.26	**0.04**	2.63	0.07	2.42	0.09	1.30	0.27	3.14	**0.04**	3.28	**0.04**
Queen presence * Type	2, ∞	0.02	0.98					0.24	0.78					0.59	0.55				

B)

		Aggression						**Sociopositive**						**Passive**					
		Full		$Colinear_{red}$		AIC_{red}		Full		$Colinear_{red}$		AIC_{red}		Full		$Colinear_{red}$		AIC_{red}	
	df1,df2	F	p	F	p	F	p	F	p	F	p	F	p	F	p	F	p	F	p
Intruder type	2, ∞	0.07	0.93	17.36	**< 0.0001**	17.82	**< 0.0001**	26.71	**< 0.0001**	90.17	**< 0.0001**	89.99	**< 0.0001**	16.13	**< 0.0001**	50.64	**< 0.0001**	51.40	**< 0.0001**
Prevalence	1, ∞	0.02	0.90	0.01	0.91			0.07	0.79	0.18	0.67			0.03	0.87	0.05	0.83		
Climate	1, ∞	2.17	0.14	1.90	0.17	4.46	**0.03**	0.98	0.32	0.26	0.61	1.24	0.27	3.77	**0.05**	3.84	**0.05**	7.22	**< 0.01**
Queen presence	1, ∞	0	1	0.16	0.69			0.08	0.78	0.05	0.83			0.04	0.85	0.11	0.74		
Worker number	1, ∞	0.10	0.76	0.17	0.68			5.23	**0.02**	4.67	**0.03**	4.56	**0.03**	1.64	0.20	1.61	0.20		
Prevalence * Type	2, ∞	0.23	0.79					1.63	0.20					0.06	0.94				
Climate * Type	2, ∞	2.21	0.11	3.12	**0.04**	3.46	**0.03**	4.58	**0.01**	2.87	0.06	2.86	0.06	2.64	0.07	5.88	**< 0.01**	5.37	**< 0.01**
Queen presence * Type	2, ∞	0.49	0.61					0.21	0.81					1.51	0.22				
Worker number * Type	2, ∞	0.82	0.44					0.31	0.74					0.12	0.89				

Table S6. Marginal mean estimates of AIC_{red} for each predictor and behavioural response of A) subcolony and B) intruders.

A)	**Aggression**			**Sociopositive**			**Brood carrying**		
	p	Estimate ± SE	p_{types}	p	Estimate ± SE	p_{types}	p	Estimate ± SE	p_{types}
Type	**<0.0001**	0.007 ± 0.002 (N) 0.095 ± 0.008 (C) 0.197 ± 0.013 (**P**)	**<0.0001** (N↔C) **<0.0001** (N↔**P**) **<0.0001** (C↔**P**)	**<0.0001**	0.131 ± 0.007 (N) 0.104 ± 0.006 (C) 0.053 ± 0.005 (**P**)	**0.012** (N↔C) **<0.0001** (N↔**P**) **<0.0001** (C↔**P**)	**<0.0001**	0.009 ± 0.003 (N) 0.018 ± 0.005 (C) 0.088 ± 0.014 (**P**)	**0.008** (N↔C) **<0.0001** (N↔**P**) **<0.0001** (C↔**P**)
Parasite prevalence	0.09	0.004 ± 0.002							
Climate	**0.003**	0.106 ± 0.036		0.32	0.015 ± 0.016		0.779	-0.012 ± 0.043	
Climate * Type				0.09			**0.039**	0.101 ± 0.081 (N) -0.003 ± 0.063 (C) -0.134 ± 0.057 (**P**)	0.210 (N) 0.961 (C) **0.018** (**P**)

B)	**Aggression**			**Sociopositive**			**Passive**		
	p	Estimate ± SE	p_{types}	p	Estimate ± SE	p_{types}	p	Estimate ± SE	p_{types}
Type	**<0.0001**	0.030 ± 0.007 (N) 0.110 ± 0.015 (C) 0.132 ± 0.018 (**P**)	**<0.0001** (N↔C) **<0.0001** (N↔**P**) 0.9126 (C↔**P**)	**<0.0001**	0.220 ± 0.015 (N) 0.089 ± 0.009 (C) 0.031 ± 0.005 (**P**)	**<0.0001** (N↔C) **<0.0001** (N↔**P**) **<0.0001** (C↔**P**)	**<0.0001**	0.302 ± 0.023 (N) 0.573 ± 0.025 (C) 0.675 ± 0.026 (**P**)	**<0.0001** (N↔C) **<0.0001** (N↔**P**) **0.014** (C↔**P**)
Climate	**0.035**	-0.078 ± 0.037		0.266	-0.030± 0.027		**0.007**	0.061 ± 0.023	
Climate * Type	**0.032**	0.0013 ± 0.085 (N) -0.0367 ± 0.049 (C) -0.197 ± 0.050 (**P**)	0.988 (N) 0.455 (C) **0.0001** (**P**)	0.057	0.028 ± 0.030 (N) -0.078 ± 0.038 (C) -0.041 ± 0.058 (**P**)	0.352 (N) **0.038** (C) 0.489 (**P**)	**0.005**	-0.012 ± 0.039 (N) 0.028 ± 0.037 (C) 0.167 ± 0.041 (**P**)	0.758 (N) 0.447 (C) **0.0001** (**P**)
Worker number				**0.033**	-0.012 ± 0.057				

Note: 'p' indicates the p-value related to the effect of a predictor on a behavioural response. ' p_{types}' either represents the Bonferroni adjusted p-values '(↔)' for pairwise comparisons between types of intruders, or the p-values referring to the type of intruder within interaction effects. Colour codes are 'N': Nestmate, 'C': Conspecific; '**P**': Parasite, bold values indicate significant p-values. Significant associations ($p \leq 0.05$) are showed in bold black characters.

Table S7. Inter-colonial variability in the CHC composition depending on the species and according to climate or parasite prevalence.

	CHCs		*n*-alkanes		Conspecific recognition cues		Parasite recognition cues	
	Climate	Parasite prevalence	Climate	Parasite prevalence	Climate	Parasite prevalence	Climate	Parasite prevalence
T. longispinosus	-0.55$^{(0.10)}$	0.26$^{(0.47)}$	-0.35$^{(0.33)}$	0.22$^{(0.54)}$	0.04$^{(0.92)}$	0.15$^{(0.68)}$	0.52$^{(0.13)}$	-0.33$^{(0.35)}$
T. americanus	-0.39$^{(0.26)}$	0.41$^{(0.25)}$	**-0.65$^{(0.05)}$**	**0.70$^{(0.03)}$**	**-0.73$^{(0.02)}$**	0.54$^{(0.11)}$	-0.41$^{(0.25)}$	0.35$^{(0.33)}$

Note: Upper case bracket values represent the p-values associated with the rho Spearman rank correlation coefficient. Significant associations ($p \leq 0.05$) are shown in bold black characters.

Table S8. Summary of the model estimates (± SE) of the association between colony size traits (upper cells) in *T. longispinosus* colonies (left of the bold bar) and *T. americanus* colonies (right of the bold bar) against climate and parasite prevalence.

	Num. of free *T. longispinosus* workers		Num. free *T. longispinosus* queens		Num. *T. americanus* workers		Fraction of *T. americanus* workers	
	Estimate ± SE	p	Estimate ± SE	p	Estimate ± SE	p	Estimate ± SE	p
Climate	0.027 ± 0.006	**< 0.0001**	0.051 ± 0.006	**< 0.0001**	0.009 ± 0.019	0.64	-0.017 ± 0.021	0.43
Parasite prevalence	0.021 ± 0.004	**< 0.0001**	0.010 ± 0.004	**< 0.01**	0.037 ± 0.017	**0.03**	-0.002 ± 0.018	0.88

Note: Significant associations ($p \leq 0.05$) are shown in bold black characters.

Chapter 4.

The Genomic Basis of Social Parasitism: A Geographical Mosaic of Behavioural, Chemical, and Environmental Adaptations in a Widespread Host-Parasite System

Maide Nesibe Macit[1], Erwann Collin[1], Maria Esther Nieto-Blazquez, Marion Kever, Maria Litto, Esther Jaitner, Markus Pfenninger, Barbara Feldmeyer[2], Susanne Foitzik[2]

[1] shared first authors

[2] shared last authors

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Abstract

Coevolutionary dynamics in host-parasite systems are driven by reciprocal selection and environmental pressures. When parasite and host are closely related and have similar evolutionary potentials, evolution may follow parallel trajectories, affecting the same traits and underlying genes. We investigated coevolution and its genomic basis in the dulotic ant parasite *Temnothorax americanus* and its host *T. longispinosus* across a broad climatic gradient using population genomics, genome-wide association and transcriptome analyses. Population genomics revealed a striking contrast: panmictic host populations versus structured parasite populations, consistent with geographic mosaic dynamics. Genomic responses to parasite prevalence were strongly asymmetric: hosts showed strong selection on immune and structural defence genes, potentially with pleiotropic social functions. Parasites exhibited weaker signals, often in regulatory genes linked to behavioural shifts critical for raiding. Both species displayed shared genomic signatures of climate adaptation (e.g., desiccation resistance, stress response), suggesting convergent physiological responses. Genes associated with host-parasite encounters (mechanosensation, circadian rhythms, venom) also showed parallel selection. Behavioural traits such as aggression showed limited genomic signals but potentially higher transcriptional plasticity. Associations with chemical traits revealed shared selection on genes involved in cuticular hydrocarbon biosynthesis and chemosensory perception, indicating evolutionary coupling of signal production and perception. Constitutive gene expression patterns diverged: host expression correlated with parasite prevalence, while parasite expression was more strongly linked to climate, reflecting contrasting regulatory pressures. Our study demonstrates how differing population structures, asymmetric reciprocal selection, and environmental context shape divergent genomic trajectories of coadaptation, reflecting distinct evolutionary architectures across a heterogeneous landscape.

Keywords: coevolution, slave-making, Myrmicinae, *Temnothorax*, GWAS, social parasite

Introduction

Antagonistic interactions between hosts and parasites can lead to dynamic coevolutionary arms races where both parties continuously adapt and diversify in response to one another (Dawkins & Krebs, 1979; Hamilton, 1982; Nash et al., 2008; Tellier et al., 2014). These arms races occur at the population rather than species level, with the independence of local interactions shaped by the extent of gene flow among populations. According to the geographic mosaic of coevolution (Thompson, 1999, 2005), resulting arms races escalate at different rates across each mosaic piece, with variations in environmental conditions and community compositions leading to divergent local adaptations. Some traits may evolve in response to both antagonistic partners and abiotic conditions, where trade-offs may arise when both demands cannot be fulfilled simultaneously (Wolinska & King, 2009; Amandine et al., 2022). This presents two interrelated challenges: i) determining which observed changes in phenotypic traits result from the coevolutionary arms race versus environmental conditions, and ii) inferring the extent to which these processes are interdependent or linked through pleiotropic interactions (Thompson, 1999, 2005). Such interactions can promote or constrain trait expression in antagonistic relationships, occasionally resulting in counterintuitive outcomes (Clarke & Fraser, 2004; Gregory, 2009; Wisz et al., 2013; Oppold et al., 2016; Mahmud et al., 2017). Species population structure, shaped by historical events and ongoing gene flow, influences evolutionary cohesion and the available adaptive genetic variations (Whitlock, 2004; Durrett & Schweinsberg, 2005; Greischar & Koskella, 2007; Gupta & Vadde, 2019). Consequently, it also shapes the adaptive evolutionary trajectories of these species in response to environmental and biotic selection pressures across their geographic ranges (Aaltonen, 2002; Tigano & Friesen, 2016). To determine the genomic basis of these interacting evolutionary forces, a comprehensive analysis must consider them simultaneously.

Brood and social parasitism is a lifestyle characterised by behavioural manipulation and exploitation of host social traits (Hughes et al., 2012; Jamie & Kilner, 2017; Jongepier et al., 2015; Rojas-Ripari et al., 2021). Social parasites evade the costs associated with labour tasks such as brood care, nest construction, and foraging by exploiting their host's workforce (Lenoir et al., 2001; Buschinger, 2009). Hosts can

counter-evolve a range of defences to mitigate the fitness costs of parasitism, including avoidance behaviour, aggressive defences, and tolerance strategies limiting parasite-inflicted damages (Råberg et al., 2009; Feeney et al., 2014; Gibson & Amoroso, 2022). Recognition is a prerequisite for successful counteradaptation to detect and discriminate parasites from conspecifics or benign species (Langmore et al., 2011). Hosts of avian brood parasites may evade parasitism by identifying and immediately rejecting foreign eggs (Soler, 2017; Manna et al., 2019). Similarly, hosts of social insect parasites initiate defence responses upon identifying parasite-specific chemical cues (Delattre et al., 2012). Such cue recognition is facilitated through odorant receptors located in the antennal sensilla of insects (Hölldobler & Wilson, 1990; Ozaki et al., 2005). Odorant receptor genes have undergone expansion during the evolution of insect sociality, along with the emergence of complex chemical communication and the regulation of social organisation (McKenzie & Kronauer, 2018; Gautam et al., 2024). Social parasites have convergently lost many odorant receptor genes, particularly those linked to worker behaviour (Caminer et al., 2023; Harrison et al., submitted) due to relaxed selection on traits less important in the parasitic lifestyle (Jongepier et al., 2021; Schrader et al., 2021; Gautam et al., 2024). These chemoreceptors detect cuticular hydrocarbons (CHCs), a complex mixture whose compositions vary qualitatively and quantitatively. CHCs are critical for social insect communication, especially in nestmate recognition, enabling the discrimination of colony members from parasitic intruders (Dani et al., 2001; Blomquist & Bagnères, 2010; Lorenzi et al., 2011). They also contribute to desiccation resistance by sealing the cuticle and supporting thermoregulation, playing an important role in climate adaptation (Menzel et al., 2018; Sprenger et al., 2019). CHC composition can change dynamically to alleviate drought stress (Sprenger et al., 2018) or to adapt to fluctuating temperatures (Wagner et al., 1998, 2001; Martin & Drijfhout, 2009; Menzel et al., 2018; Sprenger et al., 2019), but may consequently impair detection efficiency (Wittke et al., 2022), hinting at a potential trade-off in fulfilling both functions. This highlights the challenge of optimising enemy recognition and desiccation resistance, especially in species interactions across diverse climatic landscapes.

The myrmicine ant *Temnothorax americanus* is a dulotic social parasite, which raids *Temnothorax longispinosus* colonies to capture worker brood and exploits them for

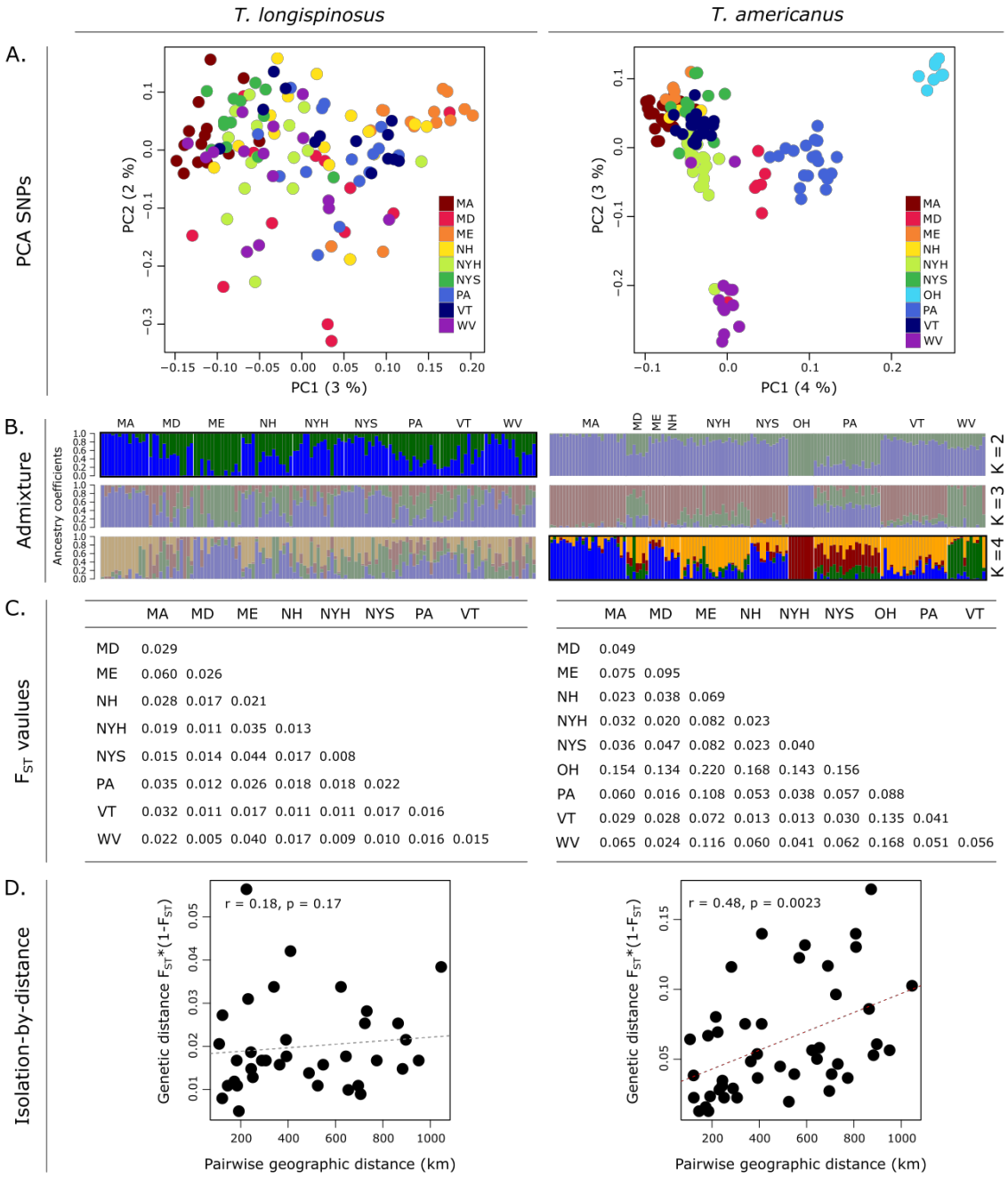
their social behaviours (Wesson, 1939; Hölldobler & Wilson, 1990; Buschinger, 2009; Schmid-Hempel, 2019; Rabeling, 2021). Both species are widely distributed across northeastern North America, with parasites more abundant in southwestern regions (Jongepier et al., 2014; Macit et al., 2024). Geographical variations in parasite prevalence and thus selection pressures on local hosts prompted the evolution of divergent coevolutionary strategies. These include modifications in the CHC profile composition: *T. americanus* employs chemical insignificance by reducing its amount of recognition cues to evade host detection (Lenoir et al., 2001; Kleeberg et al., 2017; Kaur et al., 2019), while its host diversifies its colony-specific CHC profiles in areas where parasites are present, impairing advances at parasite chemical matching with hosts (Jongepier & Foitzik, 2016; Kleeberg et al., 2017; Collin et al., in review). Behavioural strategies in the host exhibit spatial shifts associated with variation in parasite abundance and climate (Jongepier et al., 2014; Segev et al., 2017; Collin et al., in review): In heavily parasitised, warm regions, hosts tend to show reduced aggression towards highly aggressive parasites, whereas in colder regions with low parasite prevalence, hosts display greater aggression towards their less aggressive sympatric parasites (Segev et al., 2017; Collin et al., in review). Following such parasite encounters, hosts temporarily elevate aggression levels and increase venom production (Pamminger et al., 2011; Scharf et al., 2011; Koenig & Moreau, 2024a), possibly in preparation for further attacks. Accordingly, hosts modify their brain transcriptome activity in response to encounters with aggressive parasites, while parasites show dynamic changes in their gene expression before and during attacks, corresponding to shifts in activity levels (Alleman et al., 2018; Kaur et al., 2019). Adding a spatial component, a previous Pool-seq study on the host *T. longispinosus* showed the antennal transcriptome strongly linked to local parasite prevalence (Macit et al., 2024). This study further provided first insights on the genomic basis of population-level host adaptation, identifying strong selection on immune functions and olfactory perception, namely selection on *peptidoglycan recognition protein* and various *odorant receptor* genes. Fundamentally, the previously unknown link between parasite prevalence and local climate conditions may crucially shape the geographic mosaic of coevolution across their heterogeneous habitats.

Here, we present the first study on the genomic basis of co-adaptation in a host and its social parasite by disentangling the effects of biotic and abiotic interactions across populations. We re-sequenced individual ants from over 120 colonies, each of the host *Temnothorax longispinosus* and its parasite *T. americanus*, across ten sites within their shared range in the Northeastern United States. For both species, we investigated: (i) population structure, (ii) genomic differentiations between populations, (iii) genome-wide associations with abiotic and biotic environmental variables (parasite prevalence and climate), (iv) genome-wide associations with key traits relevant to host-parasite interactions (chemical profiles, attack- and defence-related behaviours) obtained from our twin study (Collin et al., in review), and (v) gene expression patterns in head and fat body associated with parasite prevalence and climate. We predict that genes critical in the coevolution of these species are involved in enemy recognition (e.g., odorant receptors), signalling (e.g., cuticular hydrocarbons via fatty acid synthesis), behaviour (e.g., biogenic amines), and immune response (e.g., peptidoglycan recognition proteins). Our integrative approach establishes links between key phenotypic traits and their underlying genomic architecture, providing insights into the complex spatial dynamics of biotic and abiotic factors that shape coevolutionary patterns between social hosts and their parasites.

Results

Population Structure

Fifteen workers from independent colonies of the host *Temnothorax longispinosus* and between 5 and 24 individuals of the parasite *Temnothorax americanus* per population (depending on availability; Suppl. S1) were individually re-sequenced across ten populations (Fig. S1). Population structure in the host species was nearly absent, with very low pairwise F_{ST} values (mean $F_{ST} = 0.021$). In contrast, the parasite exhibited low but more than twice as high F_{ST} values (mean $F_{ST} = 0.055$; Fig. 1C). Two genotype clusters were identified in the host, whereas four distinct clusters were detected in the parasite (Fig. 1B). Isolation-by-distance was stronger in the parasite ($r = 0.48$, $p = 0.0023$) than in the host ($r = 0.18$, $p = 0.17$; Fig. 1D). Additional details are provided in the SI.



	MA	MD	ME	NH	NYH	NYS	PA	VT
MD	0.029							
ME	0.060	0.026						
NH	0.028	0.017	0.021					
NYH	0.019	0.011	0.035	0.013				
NYS	0.015	0.014	0.044	0.017	0.008			
PA	0.035	0.012	0.026	0.018	0.018	0.022		
VT	0.032	0.011	0.017	0.011	0.011	0.017	0.016	
WV	0.022	0.005	0.040	0.017	0.009	0.010	0.016	0.015

	MA	MD	ME	NH	NYH	NYS	OH	PA	VT
MD	0.049								
ME	0.075	0.095							
NH	0.023	0.038	0.069						
NYH	0.032	0.020	0.082	0.023					
NYS	0.036	0.047	0.082	0.023	0.040				
OH	0.154	0.134	0.220	0.168	0.143	0.156			
PA	0.060	0.016	0.108	0.053	0.038	0.057	0.088		
VT	0.029	0.028	0.072	0.013	0.013	0.030	0.135	0.041	
WV	0.065	0.024	0.116	0.060	0.041	0.062	0.168	0.051	0.056

Figure 1. Population Structure Analysis. (A) Principal Component Analysis of SNP data, based on a thinned dataset containing ~50k SNPs per species. (B) Admixture plot depicting the identified genotypes and their proportions for each population. Highlighted are admixture subplots for the lowest cross-entropy identified using *LEAce()* ran 50x (Fig. S2A). (C) Pairwise F_{ST} values. (D) Isolation-by-distance.

Genomic Variations Linked to Population Differentiation

We used *OutFLANK* (Whitlock & Lotterhos, 2015) to identify differentiated loci ($p \leq 0.05$) based on F_{ST} outliers, indicative of local differentiations between populations. We detected 6,018 outlier loci in the host, including 282 non-synonymous SNPs (ns-SNPs) across 461 genes (Fig. 2A-B; Table S3). In contrast, the parasite exhibited only half as many outlier SNPs (3,250), but the number of ns-SNPs (177) and associated genes (405) did not differ from those of its host ($p_{adjust} > 0.05$ for both; Fig. S4A, S5A). Candidate genes in the host included *insulin-degrading enzyme* (*IDE*), *chitinase 10* (*CHT10*), and several *peptidoglycan recognition protein* (*PGRP*) genes (Table S4). In the parasite, notable genes included *cubilin* and *vitellogenin 1-like* and genes involved in cuticular hydrocarbon (CHC) synthesis, such as *fatty acyl-CoA reductase* and *desaturases* (Table S5). Among the 12 enriched biological functions of host genes (Fisher's exact test, $p \leq 0.05$) were immune-related processes, particularly 'peptidoglycan catabolic process'. In the parasite, 20 enriched biological functions of parasite genes included pathways related to lipid metabolism, such as 'lipid metabolic process' (Fig. 2C, Suppl. S2).

Genomic Variations Linked to Environmental Traits

We used *BayPass* v2.2 (Gautier, 2015) with its covariate model to associate genotypes with environmental factors (parasite prevalence and climate) and phenotypic traits (chemical profile and behaviour) in the host and the parasite (summary in Table S3, raw data in Suppl. S2).

Parasite Prevalence

Parasite prevalence, defined as the relative abundance of parasites in relation to host colonies, is a proxy for parasite success and the selective pressure parasites impose on local host populations. In the host, we identified 2,706 significant SNP loci (with Bayes Factor (BF) ≥ 15) associated with population-wide parasite prevalence (including 155 ns-SNPs) across 487 genes. In contrast, we identified fewer SNPs in the parasite (1,690 SNPs, including 54 ns-SNPs), but those were distributed across more genes (525; gene count: $\chi^2 = 10.66$, $p_{adjust} = 0.0019$; Fig. 2A-B; Fig. S5A). The number of ns-SNPs was higher in the host, as were their Bayes Factors, indicating a stronger genomic association with parasite prevalence (ns-SNPs count: $\chi^2 = 15.02$, $p_{adjust} < 0.001$; BF: ANOVA $F = 6.58$,

$p_{adjust} < 0.03$; Fig. S4A-B). Host candidate genes were similar to those identified to be differentiated between populations, including *PGRP* genes (highest BF > 50) and multiple *CHT10* genes (highest BF = 39; Fig. 3A; Table S4). These genes were also identified in a previous host Pool-seq GWAS analysis (Macit et al., 2024; Fig. S6), further supporting their role in parasite resistance/tolerance. We found that heterozygosity of significant loci within *PGRPs* was differentiated between populations (ANOVA F = 2.65, p < 0.007; Fig. S3). In contrast, parasite genes associated with parasite prevalence showed little overlap with those identified to be differentiated among populations. Among the ones with the strongest signals were two *guanine nucleotide-binding protein-like 3* genes (*GNL3L*; highest BF > 50), two *multidrug resistance-associated proteins 4* (*MRP4*; highest BF > 40) and a *fatty acyl-CoA reductase* (*FAR*; highest BF = 21; Fig. 3A; Table S5). Enriched functions differed between host and parasite: In the host, again, immune-related pathways like 'peptidoglycan catabolic process' were among the five enriched functions, while among the five enriched functions in the parasite were those relating to transcriptional regulation, such as 'regulation of DNA-templated transcription' (Fig. 2C, Suppl. S2).

Climate

By using population-specific climate data encompassing various temperature and precipitation variables (see Macit et al., 2024), we identified 3,016 SNPs associated with climate in the host (including 192 ns-SNPs) across 772 genes (Fig. 2A-B). In the parasite, we identified a similar number of associated SNPs (2,582) and genes (774) but fewer ns-SNPs (97; ns-SNP count: $\chi^2 = 9.94$, $p_{adjust} < 0.003$; gene count: $p_{adjust} > 0.05$; Fig. S5A, S4A). Host genes with the strongest genomic associations included two *multidrug resistance proteins* (*MRP4*; highest BF > 40), a *trichohyalin-like* gene (highest BF > 50), and an *odorant receptor* gene (*OR*; highest BF = 23.9; Fig. 3B, Table S4). In contrast, parasite candidate genes showed significantly weaker associations than their host (BF: ANOVA F = 20.41, $p_{adjust} < 0.001$; Fig. S4B). Among the parasite genes with the strongest associations were *MATH and LRR domain-containing protein* (*PFE0570w*; highest BF = 36.0), *x-ray repair cross-complementing protein 5* (highest BF = 27.5), and a *fatty-acid reductase* (*FAR*; highest BF = 21.3; Fig. 3B, Table S5). Host climate-associated genes were enriched for five biological functions, primarily related to gene regulation and

post-translational modifications (‘regulation of DNA-templated transcription’, ‘protein phosphorylation’). In contrast, the parasite’s 14 enriched functions were distinct from its host's, with several related to metabolic and catabolic processes and signalling pathways (‘semaphorin-plexin signalling pathway’; Fig. 2C).

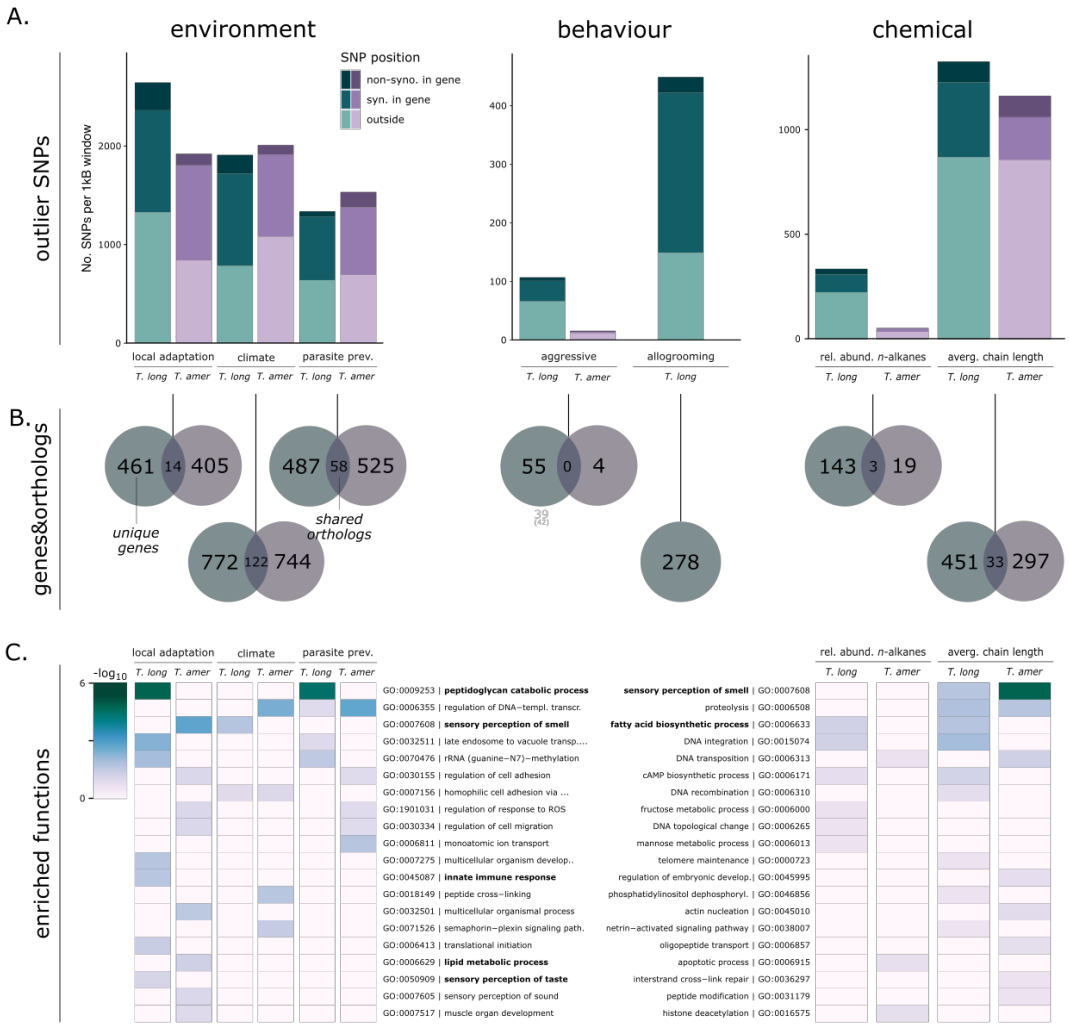


Figure 2. Genome-wide association analyses. (A) Total number of unlinked SNPs (i.e., 1 SNP per 1 kB windows) identified by the different analyses (‘local adaptation’ refers to loci differentiated between populations), divided into non-synonymous SNPs, syn. in-exon SNPs and SNPs outside of gene regions (see Fig. S4A for more information). (B) Number of unique genes in which these SNPs reside, with overlap indicating orthologous SNP-containing genes in both species (see Fig. S5B for more information). (C) Heatmap of a collection of enriched biological GO-terms with functions in bold.

Co-Adaptation

We used *OrthoFinder* v.2.5.4 (Emms & Kelly, 2015) to identify shared genes that were differentiated among populations, climate-adapted or correspond to their coevolution in both species. We identified 14 orthologous genes differentiated between populations in the host and the parasite, including *pumilio homolog 2*, linked to spatial memory in *Mus musculus* (Siemen et al., 2011) and *retinol-binding protein pinta-like*, related to visual responses in *Drosophila* (Wang & Montell, 2005; Table S6). However, the number of shared genes was not higher than expected by chance, indicating population differentiation acting on different genes and pathways in both species (hypergeometric model $p = 0.22$; Jaccard index = 0.021, Fig. S5B). In contrast, for both parasite prevalence and climate, the number of orthologous candidate genes identified in the GWAS was greater than expected by chance, indicating selection on the same genes (both $p < 0.0001$; Jaccard indices: climate = 0.10, parasite prevalence = 0.068). In the GWAS on parasite prevalence, 58 orthologs were found under reciprocal selection in both species. Many of these genes were associated with neural, synaptic, and developmental functions (*neurotrimin*, *semaphorin-1A* and *-2A*, *fasciclin-1, latrophilin/cirl, netrin-A*), lipid modifications and homeostasis (*elongation of very long chain fatty acids protein*, *phosphodiesterase*), circadian rhythm (*timeless*), and venom production (*venom dipeptidyl peptidase 4*; Fig. 3A and Table S6). In the climate GWAS, we identified 122 orthologous genes associated with climate adaptation in both species, twice as many as in the parasite prevalence GWAS. Several of these genes were associated with functions related to neural and photosensory perception (*fascicilin-1*, *semaphorin-2A*, *phospholipase A1*), but also to oxidative and environmental stress management (*oxidation resistance protein 1*, *carboxylic ester hydrolase*), and water retention (*nephrin*; Fig. 3B and Table S6).

Genomic Variations Linked to Behaviour

Interactions between dulotic parasites and their hosts are mediated via behavioural traits, particularly aggression during raids. As hosts typically recognise parasites by their chemical profile, encounters often escalate to overt aggression due to chemical mismatches, suggesting targeted aggressive responses are subject to selection. Here, we

use the number of aggressions after introducing an individual parasite into the host nest as a behavioural proxy (Collin et al., in review). We detected only a weak genomic signal associated with aggressive behaviour in both species. For the host, we identified 131 associated SNPs (including six ns-SNPs) across 55 genes, which included *protein groucho* (BF = 22.7), *calpain-D* (BF = 16.5), and also *insulin degrading enzyme* with several intron SNPs (highest BF = 15.8; Table S4). Those genes were enriched in four biological functions, such as neural repair and perception ('response to axon injury', 'visual perception'). For the parasite, we identified only 15 SNPs (including two ns-SNPs; Table S5) within four genes, significantly less than host candidate genes (gene count: $\chi 2 = 36.42$, $p_{adjust} < 0.001$; Fig. 5A), which included a *juvenile hormone esterase* (BF = 15.6). Due to the low number of genes, no enriched functions could be determined (Table S5, Suppl. S2).

Host workers are often injured during raids, with their nestmates typically reacting by wound grooming. To simulate parasite-induced injuries, we observed nestmate responses to leg removal in host colonies, hypothesising grooming behaviour to be linked to parasite prevalence (see SI). Instead, we found a stronger association with local climate, with higher allogrooming frequencies in colonies from warmer regions ($\chi^2 = 7.36$; $p = 0.007$; Fig. S14), which we explained as preventing bacterial proliferation in warmer temperatures. We identified a strong genomic basis for this behaviour, with 523 associated SNPs (including 27 ns-SNPs) across 278 genes (Suppl. S2). Candidate genes included *neprilysin-4* (BF = 24.3) and *DNA topoisomerase 3-alpha-like* (BF = 19.5; Table S4). Among the twelve enriched biological functions were some involved in transcription and expression ('regulation of DNA-templated transcription', 'regulation of gene expression'; Table S4).

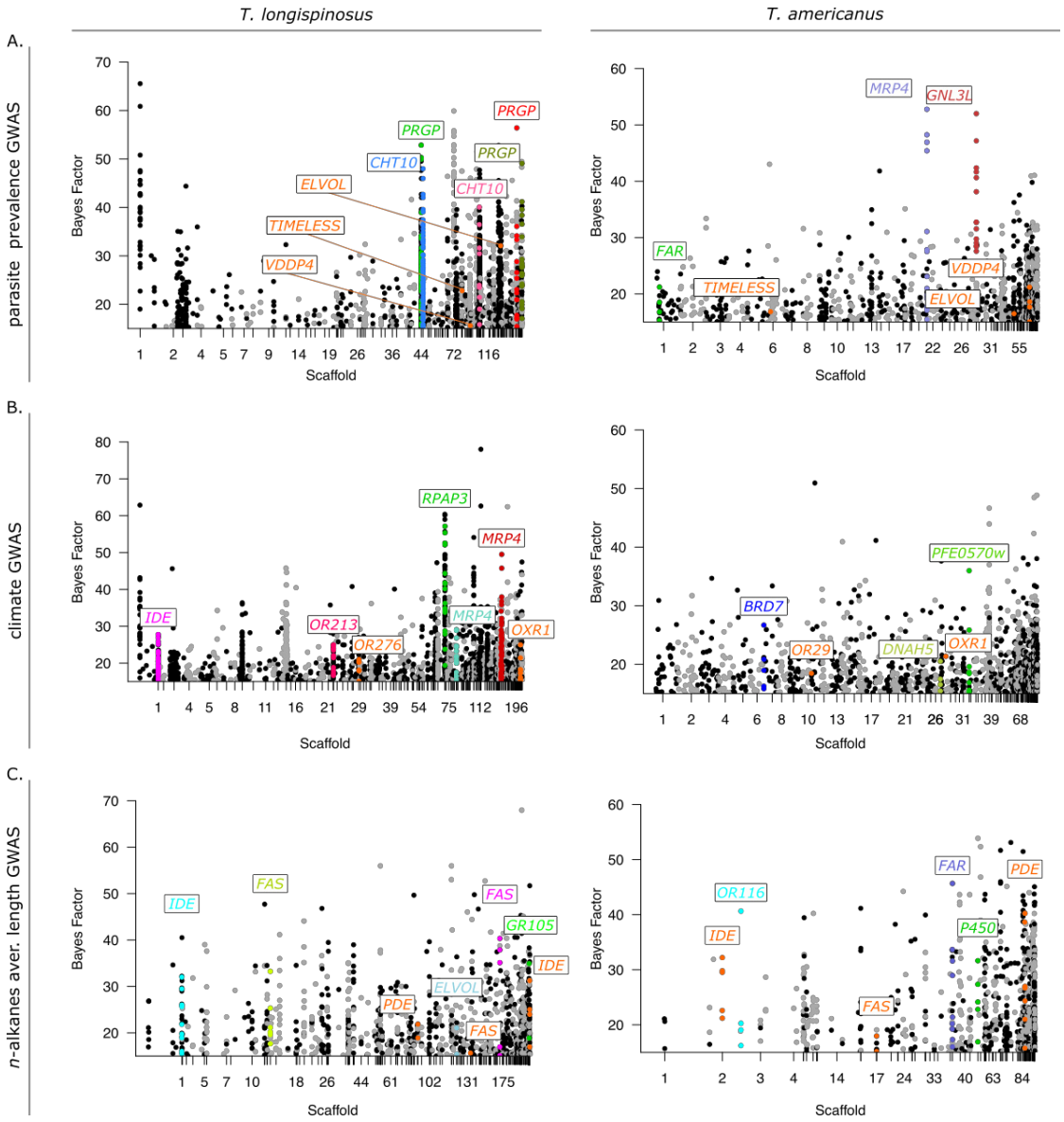


Figure 3. Genome-Wide Association Studies (GWAS) of *Temnothorax longispinosus* and its parasite *T. americanus*. Manhattan plots display significant single nucleotide polymorphisms (SNPs) associated with (A) parasite prevalence, (B) climate, and (C) average chain length of *n*-alkanes. Only SNPs exceeding a Bayes Factor (BF) of 15 and located within or proximal (± 2 kB) to annotated genes are shown. Orthologous candidate genes between the two species are highlighted in orange (Table S6). Candidate genes presented here represent a subset of those exhibiting the high number of non-synonymous SNPs with high BF values. More details can be found in Table S4 and S5).

Genomic Variations Linked to the Cuticular Hydrocarbon Profile

Chemical traits play a key role in host-parasite interactions by signalling and recognition mediation via methyl-branched alkanes, as well as climate adaptations by protection against dehydration via linear *n*-alkanes. As cuticular hydrocarbon biosynthesis is mediated via a conserved pathway, similar genes may be subject to selection in both species. However, we found no associated genetic variants in either species with recognition cues identified by Collin et al. (in review). Contrarily, for the relative abundance of linear *n*-alkanes, we identified 424 associated SNPs in the host (including 27 ns-SNPs) across 143 genes, and a considerably weaker genomic basis in the parasite, with only 56 SNPs (including 3 ns-SNPs) across 19 genes. While the host possesses more ns-SNPs, their association values did not significantly differ between species (ns-SNP count: $\chi2 = 14.04$, $p_{adjust} < 0.0005$; BF: ANOVA $p_{adjust} = 0.52$; Fig. S4A-B). Candidate genes in the host included some known to be involved in CHC biosynthesis, such as *fatty acid synthases* (*FAS*, highest BF > 40) and *cytochrome P450* genes (hereafter referred to as *P450*; highest BF = 39), along with olfactory perception genes like an *OR* (highest BF = 16) and a *gustatory receptor* gene (*GR*; BF = 19; Table S4). The parasite exhibited fewer associated genes ($\chi2 = 79.03$, $p_{adjust} < 0.001$; Fig. S5A), including a *FAS* (BF = 25.5) and a *P450* gene (BF = 15.9), but no perception genes (Table S5). Among the eight enriched functions of host candidate genes were processes related to CHC biosynthesis, and meta- and catabolic processes ('fatty acid biosynthetic process', 'fructose/mannose metabolic process'). The parasite had half as many enriched functions, including those relating to gene regulation and cell death ('histone deacetylation', 'apoptotic process'). Two orthologs were found between the species, of which one was annotated as *cullin-3* (Table S6).

The average chain length of linear *n*-alkanes can further influence desiccation resistance properties. We found that the average chain lengths of linear *n*-alkanes differed among populations in both species (host: ANOVA $F = 3.50$, parasite: $F = 3.67$, both $p < 0.01$) but also between species, with the host having longer linear *n*-alkanes (Mann-Whitney U test, $z = 13.62$, $p < 0.00001$; Fig. S10, see SI). Using chain lengths as a parameter, we identified 2,216 SNPs (including 100 ns-SNPs) across 481 host genes in

our GWAS. The parasite had a similar number of associated SNPs (2,114) as well as ns-SNPs (102) with similar association values (ns-SNP count and BF: both $p_{adjust} > 0.5$; Fig. S4A-B). However, the parasite had fewer candidate genes than the host (297; gene count: $\chi^2 = 15.19$, $p < 0.0005$; Fig. S5A). Identified host candidate genes were those known in CHC biosynthesis, such as several *FAS* genes (highest BF > 40), a *very long chain fatty acids protein* (BF = 21.1), and again perception genes such as an *OR* (BF = 15.5) and *GR* (BF = 18.9; Fig. 3C, Table S4). The parasite showed similar genes, such as several *P450* (highest BF = 39.5), *FAS* (highest BF = 18.0), *FAR* genes (highest BF > 40), as well as several *ORs* (highest BF = 33.1; Fig. 3C, Table S5). We identified 15 orthologous candidate genes between the two species, more than expected by chance (Jaccard coefficient = 0.063, $p < 0.001$; Fig. S5B), which included a *FAS* and a *phosphodiesterase* gene (Table S6).

Transcriptional Activity Associated with Parasite Prevalence

Our earlier study on host population pools revealed a strong correlation between global antennal gene expression and local parasite prevalence (Macit et al., 2024). Compared to that earlier study, which was conducted after only a few weeks of standardised ant husbandry, we kept ants used for this study for over eight months, so that workers might have emerged under standard laboratory conditions. We also used individual data from both species to examine whether transcriptional activity in the head or fat body was similarly linked with local parasite prevalence (Suppl. S1). Principal Component Analysis revealed no clustering according to populations (Fig. 4A-B), and none of the principal components, as a proxy for global gene expression, were associated with parasite prevalence in either species (Pearson's correlation in both tissues: $p > 0.05$). Using parasite prevalence as a continuous variable in a *DESeq2* analysis, we identified a greater number of differentially expressed genes in the host compared to the parasite in both tissues (fat body: $\chi2 = 92.19$, head: $\chi2 = 162.91$, both $p_{adjust} < 0.001$). The expression of 462 genes in the host fat body was associated with parasite prevalence, including a *FAR* gene and a *FAS* gene (Fig. 4C). In contrast, only 214 genes in the parasite fat body transcriptome were associated with parasite prevalence, approximately half the number observed in the host. Significant genes included a *FAS*, a *circadian clock-controlled gene*

(*CLOCK*) and a *retinal homeobox protein Rx2* (*RAX*; Fig. 4C). Among the 14 enriched functions of candidate genes in the host fat body were those related to meta- and catabolic processes (Suppl. S1). Fat body expressed genes in the parasites showed 22 enriched functions, primarily associated with neuronal and stress management. In the host head transcriptome, the expression of 167 genes was associated with parasite prevalence, including a *FAS* gene and an *odorant-binding protein* gene (*OBP*; Fig. 4D). Among the 13 enriched functions were primarily those in fatty acid and metabolic processes (Suppl. S1). Only one significant gene was found for the parasite's head transcriptome (*fatty-acid amide hydrolase*; Fig. 4D).

Using the same transcriptome data, we analysed associations between constitutive gene expression and climate. In the fat body, more genes were climate-associated in the parasite (884) than in the host (525). Comparatively, the head transcriptome revealed fewer linked genes, with similar numbers between host (49) and parasite (40). Details, including gene functions, are provided in the supplement (Fig. S11, SI and Suppl. S1).

Parasite-Associated Loci Linked to Differential Gene Expression

Variants within non-coding regions can be important for adaptation if they lead to altered expression of the associated genes (Nourmohammad et al., 2017). We employed an eQTL-like strategy to link SNPs identified in the parasite prevalence GWAS within regulatory regions (i.e. introns or ± 2 kB upstream, referred to as 2 kB-SNPs) to regulatory changes associated with parasite prevalence. We identified two genes, *multidrug resistance protein* (*MRP4*) and *chitinase* 10 (*CHT10*), which are differentially expressed in the host fat body and head transcriptomes, respectively. *MRP4* contained 90 intron SNPs, and *CHT10* contained 68 intron SNPs and two 2 kB-SNPs associated with parasite prevalence. In the parasite head transcriptome, the expression of *fatty acid amide hydrolase* (*FAAH*) associated with parasite prevalence was found to contain ten intron SNPs and one 2 kB-SNP (Suppl. S1).

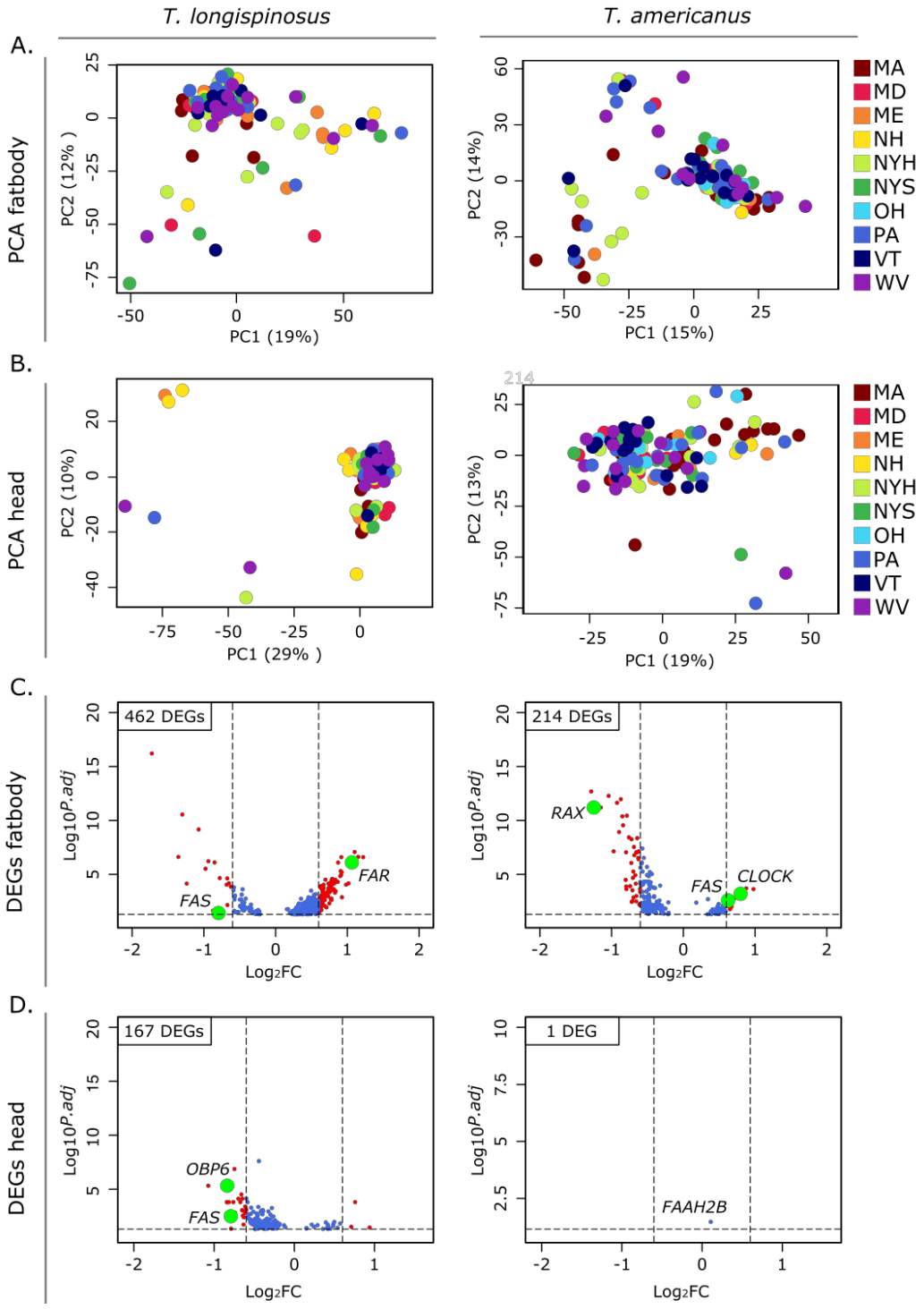


Figure 4. Analysis of transcriptome data. (A) PCA of fat body transcriptome and (B) head transcriptome in both species. (C) Volcano Plot of differentially expressed genes associated with parasite prevalence in the fat body and (D) in the head samples, with curated candidate genes with the highest or lowest log_2FC highlighted.

Discussion

Coevolutionary divergence of key phenotypes in host-parasite interactions results from reciprocal selection pressures (Tellier et al., 2014; Kurtz et al., 2016), paired with selection pressures imposed by their shared environments. In species coinhabiting widespread heterogeneous environments, disentangling the genomic basis of the resulting mosaic of coevolution requires an integrative approach that accounts for the ecological context in which coevolution unfolds. Here, we examined the coevolutionary basis of the dulotic ant *Temnothorax americanus* and its host *T. longispinosus* across a broad climatic gradient in northeastern North America. By combining genome-wide association studies of environmental and phenotypic traits, and investigating gene expression, we explore both the genetic targets and regulatory architecture underlying host-parasite coadaptation.

Disparate Population Structures Shape the Evolutionary Trajectories of Host and Parasite

Population genetic analyses revealed a marked contrast between the near-panmictic host and genetically structured parasite populations, consistent with previous studies based on neutral genetic markers (Brandt et al., 2007; Pennings et al., 2011; Pamminger et al., 2014; Macit et al., 2024). This mismatch supports the geographic mosaic theory of coevolution (Thompson, 2005), where spatially variable selection and gene flow shape coadaptive trajectories differentially. The parasite's structure may further reflect recent range expansions into previously unparasitised regions (Jongepier et al., 2014; Macit et al., 2024) compounded by low dispersal and small effective population sizes, enhancing the effects of drift and local adaptation (Whitlock, 2004; Excoffier et al., 2009). The latter was indeed found in the parasite, showing stronger patterns of adaptations to local hosts, with the lack of hosts adapting to local parasites (Foitzik et al., 2001; Brandt & Foitzik, 2004; Foitzik et al., 2009).

Parasite Prevalence Imposes Strong Selection on the Host

Genome-wide association analyses revealed strong asymmetries in genomic responses to parasite prevalence: host populations showed stronger and more coherent selection

signals, consistent with stronger local selection pressures directly imposed by the parasite. Candidate genes in *T. longispinosus* showed enrichment in immune functions and structural defence functions, notably *peptidoglycan recognition protein*s (*PGRPs*), which typically initiate antimicrobial responses (Dziarski, 2004; Harris et al., 2015; Wang et al., 2019). Some *PGRPs* were also found to modulate behaviour via neuroimmune interactions using the gut-brain axis (Gonzalez-Santana & Diaz-Heijtz, 2020; Fioriti et al., 2024), and modulate egg-laying behaviour via octopaminergic neuronal circuits (Kurz et al., 2017), suggesting pleiotropic links between immunity and socially relevant traits. Such dual functionality may reflect genomic trade-offs or pleiotropic interactions between pathogen defence and behavioural plasticity, warranting further investigation on their precise mechanisms in social insects. Ongoing research suggests an expansion in *PGRP* genes in the genus *Temnothorax,* followed by a secondary reduction in their social parasites (pers. com. Lumi Viljakainen). Additional strong signs of selection on *chitinase 10*, involved in cuticle fortification, might indicate parasite-driven mechanical defences against injuries inflicted during raids (Shahabuddin & Kaslow, 1993; Qu et al., 2021; Rabadiya & Behr, 2024). In contrast, the parasite exhibited weaker and more diffuse selection signatures. Since parasite prevalence serves as a proxy for local population size, it may reflect a more complex, polygenic parameter shaped by interacting factors such as climate and demography. This complexity likely dilutes its role as a direct selective pressure (Vellend et al., 2014), leading to more diffuse associations (Abdellaoui & Verweij, 2021). Enriched functions of parasite prevalence-associated genes were related to gene regulatory function, suggesting dynamic regulatory mediation of adaptation. However, strong signals of selection were still observed in a few candidate genes, for instance, in *multidrug resistance-associated protein 4*, which was previously found to be downregulated during parasite raids (Alleman et al., 2018). As a key regulator of prostaglandin signalling, this gene was previously linked to reproductive and behavioural regulation in solitary and social insects (Stanley, 2006; Stanley & Kim, 2019; McAfee et al., 2024). As such, it may have a role in the behavioural shift from an inactive (queen-like, egg-laying) to an active (forager-like, raiding) lifestyle during raiding season (Blatrix & Herbers, 2004; Pohl & Foitzik, 2011). Another candidate gene with strong selection signals was *guanine nucleotide-binding protein-like 3*, a G-protein associated with

learning, memory, and pheromone production (Guillén et al., 1990; Calkins et al., 2019; Liu et al., 2021), which may facilitate scouting and coordination during raids.

Shared signatures of selection in both species were found in genes related to mechanosensation (*latrophilin/cirl*; Scholz et al., 2015), circadian rhythms (*timeless*), and venom production. These genes may play critical roles in mediating host-parasite encounters by detecting colonies or intruders, coordinating raids and defences, and modulating aggression in response to each other. Selection on time-tracking genes may align with seasonal shifts in raiding and defensive behaviours (Brandt et al., 2005; Pamminger et al., 2011), contributing to the temporal organisation of antagonistic phenotypes. The function of venom-related genes under selection in both species may likely diverge: hosts employ venom in nest defences (Koenig & Moreau, 2024c), while parasites use glandular secretions from the Dufour's gland in offensive behaviours like host manipulation (Brandt et al., 2005; Jongepier et al., 2015). These in parallel evolving genes with potential different functions echo the 'toolkit hypothesis', where coevolving partners repurpose conserved pathways to meet divergent ecological demands (Cini et al., 2015). Such functional overlaps highlight how common ancestry can constrain divergence while enabling asymmetric evolutionary outcomes tailored to specialised lifestyles, warranting further investigation into their specific role in host-parasite interactions.

Climate Adaptation Shows Parallel Genomic Responses

As ectotherms, insects depend on physiological and biochemical adaptations to cope with climatic variation. Both species exhibit significant overlaps in climate-associated selection signatures, where shared genes and pathways mediate convergent physiological responses. These included genes with functions in water retention (*nephrin*; Solanki et al., 2021), stress responses (*oxidation resistance protein*; Wei et al., 2021), but also neuronal development (*semaphorin-2A*; Bates & Whitington, 2007), which may pleiotropically modulate desiccation tolerance and climate-sensitive behaviours (Segev et al., 2017). The intertwined relationship between climate and parasite prevalence is evident in climate-induced behavioural shifts during host-parasite encounters (Collin et al., in review), which can enhance, constrain, or redirect behavioural adaptations central

to their coevolution under varying climatic conditions. Similar patterns have been observed in other systems, where abiotic conditions interact with biotic pressures to influence parasite dynamics via behavioural, ecological, or evolutionary pathways (Zamora-Vilchis et al., 2012; Møller et al., 2013; Bennett et al., 2016; Cable et al., 2017; Dziuba et al., 2023; Gray & Rabeling, 2023). Species-specific climate-adaptation patterns, however, also highlight their distinct life history demands: host-specific genes relate to cuticle maintenance and growth (*trichohyalin-like*, *insulin-degrading enzyme*; Stoppelli et al., 1988; Galagovsky et al., 2014), supporting long-term climatic resilience preferable for foraging, while parasite-specific genes are linked to development, metabolism, and CHC biosynthesis (*elongases*, *reductases*; Day et al., 2005; Sieron et al., 2019; Chougule et al., 2020; Farina et al., 2022), pointing to structural and physiological adjustments for their sedentary lifestyle.

Behavioural Traits Show Weak Genomic Associations

Aggressive behaviour plays a central role in both host defence and parasite offence during raids, yet it exhibits few detectable genomic associations in either species. This likely reflects the complex and indirect genetic architecture of behavioural traits, which can obscure clear links in genotype-phenotype association studies (Abdellaoui & Verweij, 2021). Alternatively, gene expression plasticity may play a greater role than genomic divergence, as evident in substantial expression changes during raids in both species (Alleman et al., 2018). The *insulin-degrading enzyme* (*IDE*) gene was upregulated in hosts during raids (Alleman et al., 2018), and was also among the few aggression-related host candidate genes. Since older foragers are the primary defenders during parasite attacks (Koenig & Moreau, 2024a), and *IDE* has been implicated in age-dependent role shifts in *T. longispinosus* (Caminer et al., 2023), this gene likely links aggression to the transition to defending foragers in hosts. In contrast, among the few parasite-specific aggression genes was *juvenile hormone esterase*, linked to pheromone degradation and odour perception (Wei et al., 2021), likely enhancing sensitivity in detecting host aggression cues, but also caste-shifts (Mackert et al., 2008), similarly linking age-dependent shifts to aggression. In contrast, host-specific allogrooming of injured workers showed a strong genomic basis. This may be explained by its multifunctional role,

including hygiene and social immunity, resulting in strong selection pressures that result in equally strong genomic signatures, similar to those identified in *Drosophila* (Yanagawa et al., 2020). Selection on chemosensory genes (*ORs* and *GRs*) associated with allogrooming underscores the importance of recognition of chemical signalling of (injured) nestmates (Sambandan et al., 2006; Yanagawa et al., 2010, 2014), while enrichments of regulatory functions suggest dynamic mediation of this behaviour, as also seen in honeybees (Hamiduzzaman et al., 2017).

Genetic Basis of Chemical Signalling and Its Link to Chemosensory Perception

The cuticular hydrocarbon (CHC) profile in social insects serves dual functions in desiccation resistance and chemical communication (Sprenger et al., 2019), and is likely shaped by climate and host-parasite coevolution. The abundance of (linear) *n*-alkanes and their average chain lengths, both traits linked to desiccation resistance, showed strong genomic bases in both species, hinting these to be complex polygenic traits. Similar genes and functions were selected for in both species, including *fatty acid synthases*, implicated in the CHC biosynthesis, highlighting the use of convergent genetic pathways. The selection of chemosensory genes such as *ORs* and *GRs* in both species highlights the intricate pleiotropic relationship between chemical signalling and odorant perception. While previous *Hymenopteran* CHC genetic studies did not identify SNPs within perception genes (Buellesbach et al., 2022; Cohen et al., 2023), analyses on desiccation tolerance in *Drosophila* similarly found variants within *OR* and *GR* genes (Griffin et al., 2017; Rajpurohit et al., 2018). This tight evolutionary coupling of perception and signalling in both species could support a signalling function for linear *n*-alkanes previously identified in social wasps (Michelutti et al., 2018).

Using population-level data, we previously identified genomic loci associated with putative recognition cues in the host (Macit et al., 2024). However, we could not reproduce these results using colony-level data for both species, potentially due to high variability in individual recognition cues and limited statistical power from low sample sizes. popGWAS approaches (Pfenninger, in press) could offer improved power for detecting genomic associations in such variable traits and should be considered in future studies.

Transcriptional Activity and Gene Regulation in Host–parasite Interactions

Host defence and parasite raiding behaviours are accompanied by complex transcriptional changes (Alleman et al., 2018; Kaur et al., 2019). To assess population-level variation in constitutive gene expression, we analysed transcriptomes from both species' fat body and head tissues after eight months of standardised laboratory conditions. While this design reduces environmental noise and allows for controlled comparisons, it captures baseline, adaptive expression and may overlook important context-dependent regulatory responses as were identified in previous studies (Alleman et al., 2018; Kaur et al., 2019). Thus, the presented data reflect evolved, genetically encoded expression patterns rather than acute, plastic responses to parasites. The main ecological driver of population-level differences in constitutive gene expression differed between species: in the host, more differentially expressed genes were associated with local parasite prevalence, while in the parasite, more were associated with climate. In *T. longispinosus*, this pattern likely reflects regulatory adaptation to sustained parasite pressure. Transcriptomic analyses of pooled antennae samples showed gene expression strongly correlated with parasite prevalence but not climate (Macit et al., 2024). This is especially relevant since constitutive gene expression is costly and usually evolutionarily constrained (Wagner, 2007). This may hint at the tremendous selective pressure of parasite prevalence on this tissue, where directional selection of genotypes responsible for this beneficial gene expression pattern leads to rapid fixation within highly parasitised populations (Ghalambor et al., 2015; Campbell-Staton et al., 2017; Rivera et al., 2021). Host expressed genes associated with parasite prevalence included *odorant-binding protein* and *fatty acid synthase*, involved in chemical perception and CHC biosynthesis, linking transcriptional variation directly to recognition and signalling, both relevant in host-parasite interactions. In the parasite, the lower number of expressed genes associated with parasite prevalence may reflect both its sedentary lifestyle and simpler CHC profile (Kleeberg et al., 2017; Collin et al., in review), but also the fact that local prevalence likely reflects a weaker selective force on the parasite than on its host. Among these few differentially expressed genes associated with parasite prevalence were *FAAH*, involved in lipid signalling and neural plasticity (Ueda et al., 2000; McKinney & Cravatt, 2005; Sang & Chen, 2006), a circadian regulator linked to time-sensitive behavioural cycles, and *retinal homeobox protein*, implicated in

olfactory learning (Belyaeva et al., 2009; Hofmann et al., 2016). These genes suggest gene regulation in the parasite to be more dynamic, context-dependent, and likely activated during raids. This highlights the role of timed gene expression, as evident in the importance of time-tracking genes in social parasite evolution (Feldmeyer et al., 2017), and points to flexible rather than constitutive regulatory strategies in the parasite.

Conclusion

This study demonstrates how a social ant parasite and its host, despite shared ancestry and ecological overlap, follow divergent genomic trajectories of coadaptation across multiple populations spanning a broad interaction range. Contrasting population structures, near-panmixia in the host and pronounced genetic structuring in the parasite, generate a genomic mosaic in which coevolutionary dynamics and the degree of reciprocity vary across space. While both species exhibited similar genomic responses to prevailing climate, suggesting convergent physiological adaptation to shared environmental conditions, selection signatures linked to their coevolution and traits pivotal in their interactions were largely species-specific and reflective of their distinct lifestyle. The parasite generally showed weaker genomic signals, primarily in genes associated with behavioural shifts underlying its raiding phenotype, whereas the host displayed strong selection on immune-related genes, potentially linked to defences against parasite-induced injuries and pleiotropically the modulation of social behaviours. The difference in the strength of selection signals on the same parameter likely reflects that parasite prevalence acts as a direct selective pressure on the host, but due to its strong link to climate, presents a multilayered selective pressure for the parasite, resulting in more diffuse genetic signals. By integrating genomic, phenotypic, and environmental data, this study unravels how coadaptation arises from the intricate interplay of population structure, reciprocal selective pressures, and ecological context, underscoring the highly context- and role-specific nature of social parasitism's evolution in heterogeneous landscapes.

Materials and Methods

Sample Collection and Estimation of Parasite Prevalence

Colonies of *Temnothorax longispinosus* and its social parasite, *T. americanus*, were collected from ten locations across the northeastern USA (Fig. S1, Table S1 and S2). Sampling occurred near roads and tracks in state parks and on private property, with permission obtained. Ants were brought to Mainz, Germany, and maintained under standard laboratory conditions for eight months before dissection. The accompanying behavioural and chemical data from Collin et al. (in review) were obtained four additional months later. Parasite prevalence, calculated as the percentage of parasite colonies within the local *Temnothorax* community, was determined using long-term collection data (Herbers & Foitzik, 2002; Brandt & Foitzik, 2004; Achenbach & Foitzik, 2009; Jongepier et al., 2014; Kaur et al., 2019; Macit et al., 2024).

Sample Preparation, Sequencing and Pre-Processing

Fifteen independent colonies per population and species were sampled (host: min = 14 max/mean/median = 15; parasite: min = 5, max = 24, mean = 15, median = 18). DNA from the thorax, and RNA from the fat body and head were extracted. Whole-genome sequencing (WGS) and RNA sequencing (RNA-seq) were performed on an Illumina NovaSeq 6000 platform by Novogene. Both were quality-checked with *FastQC* v.0.11.9 (Andrews et al., 2015) and trimmed using *Trimmomatic* v.0.39 (Bolger et al., 2014). WGS data were mapped using *BWA mem* v.0.7.17 (Li et al., 2009), and RNA-seq data were mapped using *HISAT2* v.2.1.0 (Kim et al., 2015) to unpublished reference genomes (Boomsma et al., 2017). For WGS data, variants were called using *BCFtools* v1.16 (Li, 2011) using predetermined filtering parameters, resulting in similar numbers of SNPs in both species (host: 1,677,757 SNPs; parasite: 1,604,099 SNPs). Transcript read count tables were generated using *HTSeq* v.2.0.2 (Putri et al., 2022) from RNA-seq data, and used for *DESeq2* v.1.42.0 (Love et al., 2014) in *R* v.4.3.2 (R Core Team, 2021) using parasite prevalence as a continuous variable. The analysis was repeated using climate PC1 values (obtained from Macit et al., 2024) as a continuous variable and will be reported on in the SI. Further information on candidate genes in both WGS and RNA-seq data was collected using *InterPro* v.5.61.93 (Paysan-Lafosse et al., 2023), retrieving Gene

Ontologies (GO) to perform *topGO* v.2.54.0 (Alexa & Rahnenfuhrer, 2017) and *BlastP* v.2.13.0 (Altschul et al., 1990), searching against the non-redundant invertebrate database (retrieved on NBCI on Jan 2022), and proteomes of *Drosophila melanogaster* and *Apis mellifera* (retrieved on UniProt on Jan 2024; Proteome IDs: UP000000803 and UP000005203; UniProt Consortium, 2018). Any gene names given in this study refer to the best blast hit (based on e-value) generated in *A. mellifera* if not stated otherwise. Orthologs were identified using *OrthoFinder* v.2.5.4 (Emms & Kelly, 2015). Chi-square tests in *R* assessed significant differences in candidate gene numbers (gene numbers: *T. longispinosus*: 16,064, *T. americanus*: 14,128) and ns-SNPs (exon lengths: *T. longispinosus*: 21,924,724, *T. americanus*: 20,133,265). Bayes Factors of ns-SNPs were log-transformed and analysed for differences between species using a one-way ANOVA in *R*. Significant overlaps in orthologous candidate genes were determined using the *hypergamous()* function from *SciPy* (Virtanen et al., 2020). All statistical tests used a significance threshold of FDR-corrected $p \leq 0.05$.

Population Structure, Local Adaptation and GWAS

Population structure was analysed using the *R* package *sambaR* (De Jong et al., 2021), incorporating the *findstructure()* function. The lowest cross-entropy was observed at $k = 2$ for the host and $k = 4$ for the parasite (Fig. S2A). Locally differentiated SNPs ($p \leq 0.05$) were identified using *OutFLANK* (Whitlock & Lotterhos, 2015) within the *selectionanalyses()* function in *sambaR*. Genome-wide association studies (GWASs) were conducted using *BayPass* v2.2 (Gautier, 2015) in its standard covariate mode. The parameters employed to identify their genomic basis included: (i) parasite prevalence, (ii) climate parameters, (iii) chemical profiles (relative abundance of recognition cues, (linear) n-alkanes, and their average chain length) and (iv) aggressive behaviour, important for both parasitic raids and host defences, and allogrooming for the host. Other statistically significant species-specific behaviours, such as host brood-carrying and parasite passive behaviour, were similarly analysed but presented in the SI only. Data for chemical and behavioural analyses largely originated from Collin et al. (in review) from sister ants genotyped in this study. Identified SNPs were considered significant at a Bayes

Factor of ≥ 15. A detailed description of the methods used in this study can be found in the SI.

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Author Contribution

SF and BF conceived the study. MNM, EC, SF, and BF designed the experimental setup and collected ant colonies. MNM and EC sampled colonies and performed dissections. MK performed DNA and RNA extractions. MNM performed all population genomic and transcriptomic analyses, supervised by BF and partially MP. MNM and MENB performed demographic history analysis. EJ and ML conducted injury experiments, which were analysed by EC, and supervised by SF. MNM wrote the first draft of the manuscript, and all authors revised it. The authors declare no conflict of interest.

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Data Availability

The following supplementary material will be available under open access after publication: Supplement S1, S2 and S3. Supporting Information (including Supplementary Tables, Figures, Methods, Results and additional Discussion) will be presented in the following. Raw sequence data were uploaded to the European Nucleotide Archive (ENA) and are accessible under study accession no. PRJEB76961.

Supplementary Information

Table S1. Summary of collection sites. States and their abbreviations, coordinates, and number of collected colonies of host and parasite and their long-term parasite prevalence as well as their prevalence based solely on collections from our preliminary study (Macit et al., 2024) (see M&M section in that study for more information about the estimates).

Population	Location	Coord.	*T. longispinosus*	*T. americanus*	Parasite prevalence*	Parasite prevalence 2021
Massachusetts (MA)	Beaver Brook North and Rock Meadow Reservation	42.407, -71.204	501	69.86	12.233	13.024
Maryland (MD)	New Germany State Park	39.611, -79.134	112	11	8.943	8.943
Maine (ME)	Bethel Community Forest	44.413, -70.851	504	13	2.515	4.375
New Hampshire (NH)	Belknap Mountain State Forest	43.510, -71.402	423	10	2.309	8.333
New York Huyck (NYH)	Huyck Preserve	42.525, -74.160	5634	780	12.161	18.863
Newy York South (NYS)	Fahnestock State Park	41.455, -73.868	178	25.62	12.583	12.583
Ohio (OH)	Private Property	41.753, -80.967	573	105.58	15.558	13.427
Pennsylvania (PA)	S.B. Elliot State Park	41.124, -78.523	314	53	14.441	14.441
Vermont (VT)	Branbury State Park	43.945, -73.076	1787	140	7.265	10.460
West Virginia (WV)	Watoga State Park	38.110, -80.136	843	139.65	14.211	16.977

* long-term data from Macit et al. (2024), Kaur et al. (2019), Jongepier et al. (2014), Foitzik et al. (2009), Brandt & Foitzik (2004), and Herbers & Foitzik (2002)

Table S2.1. Detailed location and coordinates of all subsites (MA-NYS)

State	Subsite	Coordinates	Location
MA	a	42.410836, -71.208085	Beaver Brook North Reservation
	b1	42.410836, -71.208085	Rock Meadow Conservation Area
	b2	42.399000, -71.194361	
ME	a	44.406120, -70.861389	West Bethel Park
	b	44.407185, -70.866737	Bethel Community Forest- West
	c	44.415182, -70.857712	
	d	44.421951, -70.819382	Bethel Community Forest - East
MD	a	39.629078, -79.145439	New Germany State Park
	b	39.514118, -79.156654	
	c1	39.570572, -79.201034	Big Run State Park
	c2	39.627922, -79.107284	Roadside
	d	39.627922, -79.107284	Private Property*
	e	39.638573, -79.105064	Behind Trinity Cemetery
	f	39.628662, -79.125107	Acorn Loop Hiking Trail, New Germany State Park
	g	39.650993, -79.122215	Meadow Mountain Trail, New Germany State Park
NH	a	43.500557, -71.413171	Private Property*
	b	43.516384, -71.379341	Belknap Mountain State Forest
	c	43.513794, -71.413536	Private Property*
NYH	a1	42.520329, -74.146408	Huyck Preserve
	a2	42.515518, -74.161789	
	b	42.515518, -74.161789	
	c	42.528486, -74.160333	
	d	42.532192, -74.162888	
	e	42.533791, -74.164268	
	f	42.525547, -74.170349	
	g	42.530544, -74.151466	
NYS	a	41.445290, -73.865179	Fahnestock State Park
	b	41.450997, -73.857043	
	c	41.480529, -73.917455	Private Property*
	d	41.443157, -73.867508	Stone Garden at Fahnestock State Park
	e	41.454262, -73.833420	Tree Lake Trail at Fahnestock State Park

Table S2.2. Detailed location and coordinates of all subsites (OH-WV)

State	Subsite	Coordinates	Location
OH	a	41.759503, -80.965593	Private Property*
	b	41.759514, -80.966522	
	c	41.758891, -80.966794	
	d	41.758312, -80.965341	
	e	41.755195, -80.946406	
	f	41.757405, -80.942399	
	g	41.749222, -80.948235	
	h	41.743347, -80.958672	
	i	41.744363, -81.031441	
	j	41.748822, -80.974843	
PA	a	41.137976, -78.516263	S.B. Elliot State Park
	b	41.111185, -78.528163	
	c	41.102461, -78.526507	
	d	41.118119, -78.518040	
	e	41.137276, -78.520605	
	f	41.126563, -78.528959	
	g	41.136524, -78.505280	
	h	41.139987, -78.503009	
	i	41.118080, -78.516296	
	j	41.113468, -78.571419	
VT	a1	43.965164, -73.076485	Branbury State Park
	a2	43.972078, -73.072761	
	b	43.944454, -73.102104	
	c	43.972078, -73.072761	
	d	43.971760, -73.086716	
	e	43.979463, -73.064017	
	f	43.973076, -73.059197	
	g	43.969719, -73.083763	
	h	43.924728, -73.096313	
	i	43.882782, -73.063850	
	j	43.842194, -73.060585	
WV	a	38.124519, -80.114105	Watoga State Park
	b	38.106472, -80.129555	Ann Bailey Trail, Watoga State Park
	c	38.107899, -80.131996	
	d	38.107495, -80.134593	
	e	38.119175, -80.154663	Watoga Park Road, Watoga State Park
	f	38.102936, -80.145622	Ann Bailey Trail, Watoga State Park
	g	38.102665, -80.141525	
	h	38.106857, -80.136772	

* Permission granted

Table S3. Summary of local adaptation analysis using *OutFLANK* (Whitlock & Lotterhos, 2015) and GWASs using *BayPass* (Gautier, 2015)

species	type of trait	type of covariant	parameter source	#samples	#SNPs (exon : intron : 2 kB : outside)	#genes (% to all genes)	#enriched functions
T. longispinosus	general local adaptation		Macit et al., 2024	127	6018 (396 : 2822 : 529 : 2271)	461 (2.87)	11
	environment	climate		127	3016 (260 : 1287 : 395 : 1074)	772 (4.81)	5
		parasite prevalence		127	2706 (221 : 1184 : 289 : 1012)	487 (3.03)	5
	behaviour	aggressive	Collin et al., in review	96	131 (9 : 33 : 18 : 71)	55 (0.34)	4
		brood carrying		96	39 (1 : 5 : 4: 29)	10 (0.062)	0
		allogrooming	this study	66	523 (40 : 284 : 40 : 159)	281 (1.75)	12
	chemical	rel. abund. recog. cues	Collin et al., in review	127	0	0	0
		rel. abund. linear *n*-alkanes		127	424 (31 : 91 : 55 : 247)	143 (0.89)	8
		aver. chain length *n*-alkanes		127	2216 (121 : 550 : 282 : 1263)	451 (2.81)	11
T. americanus	general local adaptation		Macit et al., 2024	137	3250 (240 : 1458 : 351 : 1201)	405 (3.44)	20
	environment	climate		137	2582 (124 : 911 : 229 : 1318)	744 (5.27)	14
		parasite prevalence		137	1690 (73 : 728 : 141 : 748)	525 (3.72)	5
	behaviour	aggressive	Collin et al., in review	49	15 (2 : 2 : 0 : 11)	4 (0.028)	0
		passive		49	35 (0 : 5 : 6 : 24)	11 (0.078)	0
	chemical	rel. abund. recog. cues		116	0	0	0
		rel. abund. linear *n*-alkanes		116	56 (4 : 14 : 2 : 36)	19 (0.13)	4
		aver. chain length *n*-alkanes		116	2114 (121 : 389 : 223 : 1381)	297 (2.1)	13

Table S4. Selection of GWAS candidate genes for host species *Temnothorax longispinosus*. In bold red are genes that were also identified as candidates in one of the other GWAS, and in bold orange are genes that have an orthologous candidate gene in the parasite (Table S6). Gene names with asterisks were highlighted in the Manhattan plots in Fig. 3A-C. The full list of SNPs and genes can be found in Suppl. S2.

genomic association		candidate genes (#nsSNP, highest BF)	gene name	selection of enriched functions
local adaptation		**Tlon_g15805** (17)	*unc-45 homolog B*	GO:0009253 peptidoglycan catabolic process GO:0045087 innate immune responses GO:0005975 carbohydrate metabolic process GO:0009081 branched-chain amino acid metabolic process
		Tlon_g11697 (12)	*mesh*	
		Tlon_g00264 (10)	*insulin-degrading enzyme*	
		Tlon_g13447 (6)	*chitinase 10*	
		Tlon_g16880 (6)	*trichohyalin-like*	
		Tlon_g13446 | **g13570** | **g07266** | g07261 (17)	*PGRP*	
parasite prevalence		**Tlon_g13446** | **g13570** | **g07266** | g07269 | g07264 | g07263 | g13445 (18, 52.8)	*PGRP**	GO:0009253 peptidoglycan catabolic process GO:0045087 innate immune responses
		Tlon_g13447 | g13449 | g00818 | g00817 (8, 39.1)	*chitinase 10 (cht10)**	
		Tlon_g15805 | g05389 | g15802 (8, 33.1)	*unc-45 homolog B*	
climate		Tlon_g04123 | g17850 (17, 45.8)	*multidrug resistance-associated protein 4 (MRP4)**	GO:0006355 regulation of DNA-templated transcription GO:0006468 protein phosphorylation
		Tlon_g16886 (7, 52.6)	*RNA polymerase II-associated protein 3 (RPAP3)**	
		TlonOR213 | **OR276** (4, 23.9)	*odorant receptor gene**	
		Tlon_g16880 (10, 60.4)	*trichohyalin-like*	
		Tlon_g00264 (4, 20.5)	*insulin-degrading enzyme*	
aggression		Tlon_g09388 (1, 22.7)	*protein groucho*	GO:0048678 response to axon injury GO:0007601 visual perception
		Tlon_g09021 (1, 16.5)	*calpain-D*	
		Tlon_g11073 (1, 15.1)	*F-box/LRR-repeat protein 4 (FBXL4)*	
allogrooming		Tlon_g04574 (1, 24.3)	*neprilysin-4*	GO:0006355 regulation of DNA-templated transcription GO:0010468 regulation of gene expression
		Tlon_g14038 (1, 18.5)	*RNA exonuclease 1*	
		Tlon_g01170 (2, 19.5)	*DNA topoisomerase 3-alpha-like*	
***n*-alkanes**	**rel. abund.**	TlonOR120 | **OR436** (17, 22.6)	*odorant receptor*	GO:0006633 fatty acid biosynthetic process
	aver. chain	Tlon_g05168 | g08044 | g05010 | g02972 (7, 40.3)	*fatty acid synthase (FAS)**	GO:0006633 fatty acid biosynthetic process GO:0007608 sensory perception of smell
		Tlon_g01986 (2, 21.1)	*elongation of very long chain fatty acids(ELVOL)**	
		Tlon_g00265 | **g13585** (3, 31.3)	*insulin-degrading enzyme (IDE)**	
		TlonOR436 (1,15.5)	*odorant receptor*	
		TlonGR105 (1, 18.9)	*gustatory receptor (GR)**	

Table S5. Selection of GWAS candidate genes for the parasite *Temnothorax americanus*. In bold red are genes that were also identified as candidates in one of the other GWAS, and in bold orange are genes that have an orthologous candidate gene in the host (Table S6). Gene names with asterisks were highlighted in the Manhattan plots in Fig. 3A-C. The full list of SNPs and genes can be found in Suppl. S2

genomic association		candidate genes (#nsSNP; highest BF)	gene name	selection of enriched functions
local adaptation		Tame_g13193 (10)	*cubilin*	GO:0006629 lipid metabolic process GO:0006869 lipid transport GO:0007605 sensory perception of sound
		Tame_g13414 (8)	*fatty acyl-CoA reductase*	
		Tame_g09688 (5)	*cytochrome P450*	
		Tame_g09621 (5)	*vitellogenin 1-like*	
		Tame_g09678 (4)	*long-chain-fatty-acid--CoA ligase*	
		Tame_g00717 (3)	*circadian clock-controlled protein*	
		Tame_g13242 (3)	*acyl-CoA Delta(11) desaturase*	
parasite prevalence		Tame_g08420 (4, 52.0)	*guanine nucleotide-binding protein-like 3 (GNL3L)**	GO:0006355 regulation of DNA-templated transcription GO:0006370 7-methylguanosine mRNA capping
		Tame_g06605 | g06604 (4, 48.2)	*multidrug resistance-associated protein 4 (MRP4)**	
		Tame_g00154 (4, 21.2)	*fatty acyl-CoA reductase (FAR)**	
climate		**Tame_g00154** (2, 21.3)	*fatty acyl-CoA reductase*	GO:0008152 metabolic process GO:0030149 sphingolipid catabolic process GO:0071526 semaphorin-plexin signalling pathway GO:0007166 cell surface receptor signalling pathway
		Tame_g09659 (8, 36.0)	*PFE0570w**	
		Tame_g08104 (3, 20.5)	*dynein heavy chain 5 (DNAH5)**	
		Tame_g13489 (2, 20.8)	*bromodomain-containing protein 7 (BRD7)**	
		Tame_g16378 | g13911 (4, 27.5)	*x-ray repair cross-complementing protein 5 | 6*	
aggression		Tame_g14891 (1, 15.6)	*carboxylic ester hydrolase*	
***n*-alkanes**	**rel. abund.**	**Tame_g04921** (1, 25.5)	*fatty acid synthase*	GO:0016575 histone deacetylation GO:0006915 apoptotic process
		Tame_g14411 (1, 15.9)	*cytochrome P450*	
	aver. chain	Tame_g11443 | **g14411** (7, 39.5)	*cytochrome P450 (P450)**	GO:0007608 sensory perception of smell GO:0006857 oligopeptide transport GO:0031179 peptide modification
		TameOR116 | OR216 | OR319 | OR204 (5, 20.8)	*odorant receptor (OR)**	
		Tame_g06476 | **g04921** | g06477 (4, 18.0)	*fatty acid synthase (FAS)**	
		Tame_g10337 | g10334 (2, 45.7)	*fatty-acyl-CoA reductases (FAR) **	

Table S6. Orthologous candidate genes identified in GWAS of both host *T. longispinosus* and parasite *T. americanus* species. Gene names with asterisks were highlighted in orange in the Manhattan plots in Fig. 3A-C. Details can be found in Suppl. S2.

genomic association		orthoGroup (Tlon | Tame)	gene name
local adaptation		OG0000567 (Tlon_g00462 | Tame_g01233)	*cytochrome P540*
		OG0001201 (Tlon_g03331 | Tame_g03182)	*pumilio homolog 2*
		OG0004851 (Tlon_g12994 | Tame_g05034)	*heterogeneous nuclear ribonucleoprotein U*
		OG0000100 (Tlon_g16890| Tame_g08624)	*retinol-binding protein pinta-like*
parasite prevalence		OG0001670 (Tlon_g18643 | Tame_g15493)	*venom dipeptidyl peptidase 4 (VDP4)**
		OG0000523 (Tlon_g17222 | Tame_g13159, Tame_g13161)	*protein timeless homolog (TIMELESS)**
		OG0000957 (Tlon_g04125 | Tame_g12517)	*elongation of very long chain fatty acids protein (ELVOL)**
climate		OG0006318 (Tlon_g09719 | Tame_g08220)	*oxidation resistance protein 1 (OXR1)**
		OG0000059 (TlonOR272,TlonOR274,TlonOR276 | TameOR29)	*odorant receptor (OR)**
***n*-alkanes**	**rel. abund.**	OG0000233 (Tlon_g13654 | Tame_g00605)	*cullin-3*
	aver. chain	OG0000018 (Tlon_g02972| Tame_g06476)	*fatty acid synthase (FAS)**
		OG0010382 (Tlon_g18775 | Tame_g16435)	*phosphodiesterase (PDE)**
		OG0000036 (Tlon_g13585 | Tame_g05743)	*insulin-degrading enzyme (IDE)**

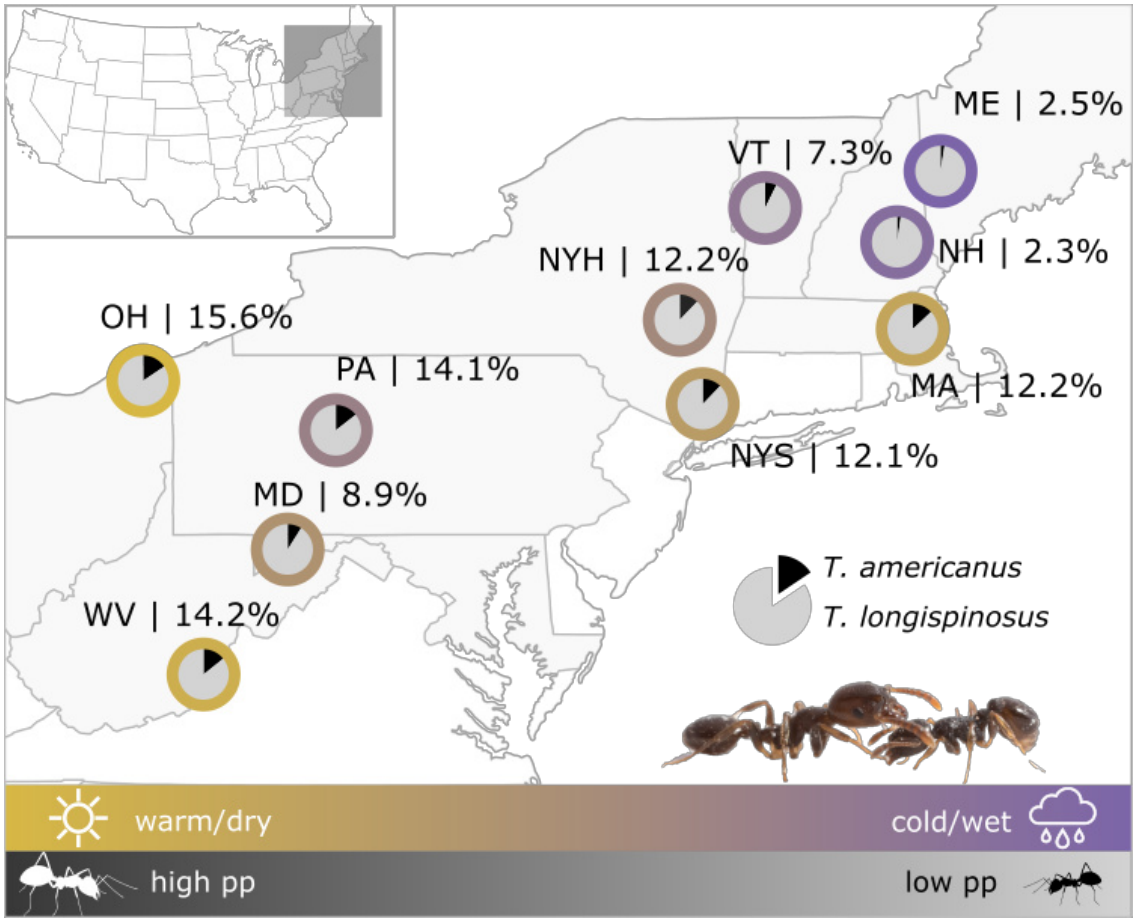


Figure S1. Geographic distribution and sampling of *Temnothorax longispinosus* and its parasite *T. americanus* in the northeastern United States. Sampling sites (see Tables S1 and S2 for details) are depicted using pie charts that indicate local parasite prevalence (percentages shown above) and colour gradients representing local climate conditions, ranging from warm and dry (yellow) to cold and wet (purple), as described in Macit et al. (2024). Photo © Romain Libbrecht.

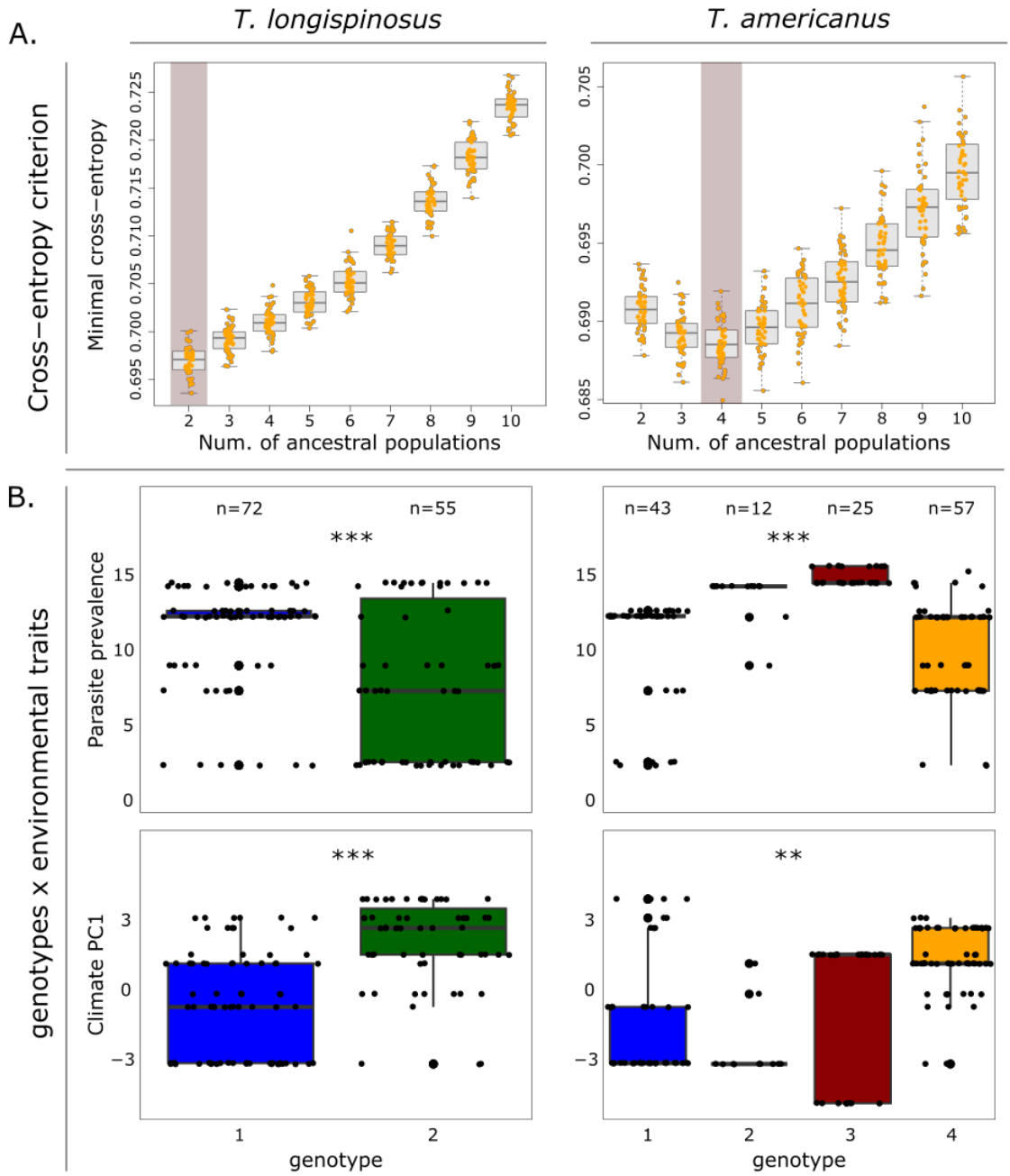


Figure S2. Population structure and environmental associations of genotypes. (A) Determination of the optimal number of ancestral populations (k) based on the cross-entropy criterion for k = 2 for *T. longispinosus* and k = 4 for *T. americanus*. (B) Associations of individual genotypes and environmental variables (parasite prevalence and climate PC1). ANOVA tests revealed significant effects of both parasite prevalence and climate on genotype association in *T. longispinosus* (parasite prevalence: $F = 42.72$, $p < 0.0001$; climate PC1: $F = 30.75$, $p < 0.0001$) and *T. americanus* (parasite prevalence: $F = 46.84$, $p < 0.001$; climate PC1: $F = 9.01$, $p = 0.0032$).

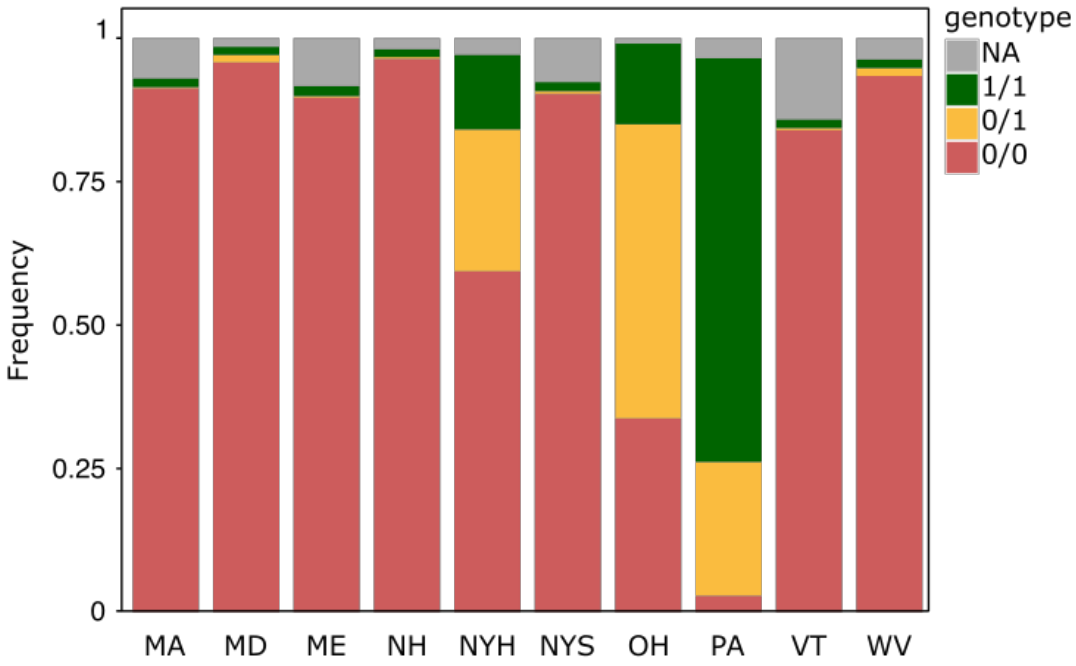


Figure S3. Genotype Frequencies of *T. longispinosus PGRP* loci. Boxplots shows the distribution of genotype frequencies across eleven *PGRP* genes in ten *T. longispinosus* populations. A significant effect of population on genotype frequency was detected (ANOVA $F = 2.651$, $p = 0.0069$).

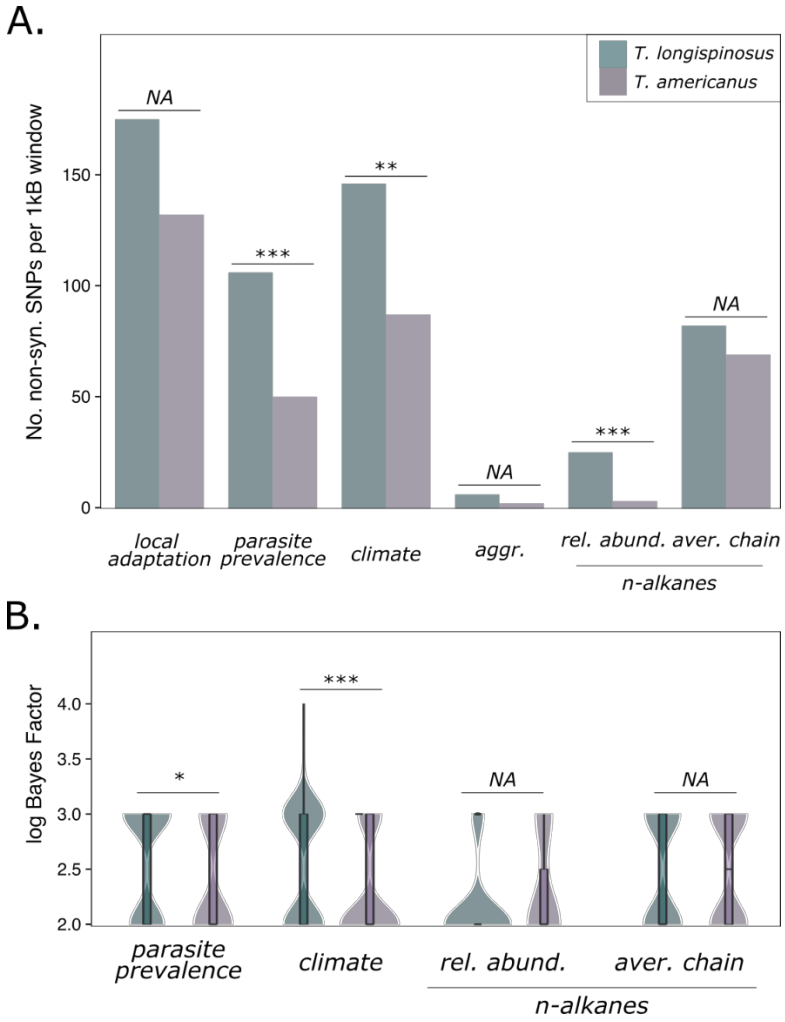


Figure S4. (A) Number of significant non-synonymous SNPs per 1 kB window per analysis and species. The number of highly associated (BF ≥ 15) non-synonymous SNPs (ns-SNPs) within 1 kB windows (i.e. unlinked) identified in each genome-wide association study. The host species *T. longispinosus* exhibited a significantly greater number of unlinked associated ns-SNPs in all analyses ($p_{adjust} \leq 0.05$) except for locally adapted loci, aggression and average *n*-alkane chain length, where both species showed a similar number of ns-SNPs. (B) Distribution of log-transformed Bayes Factors for all ns-SNPs. *T. longispinosus* exhibited significantly higher Bayes Factors for ns-SNPs associated with parasite prevalence ($p_{adjust} = 0.022$) and climate ($p_{adjust} = 0.0025$), but not for the relative abundance of *n*-alkanes and their average chain lengths ($p_{adjust} = 0.52$ for both).

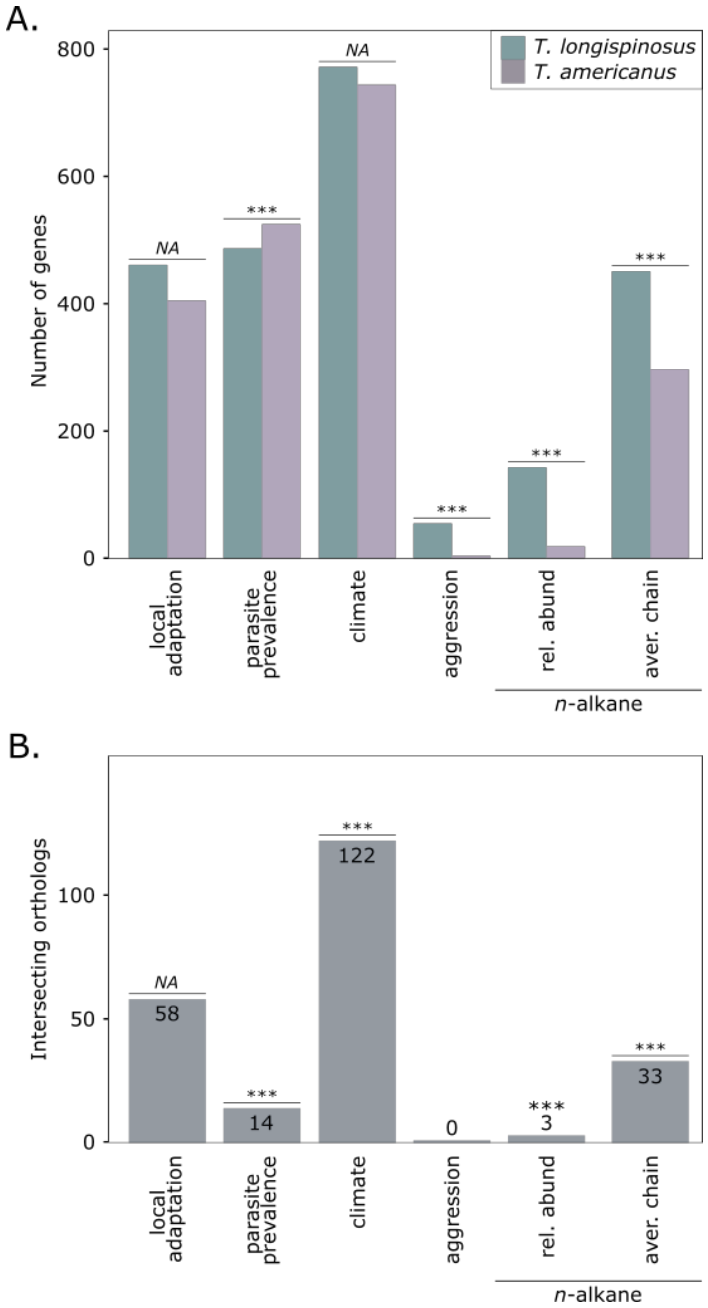


Figure S5. (A) Number of genes containing significantly associated loci per analysis. *T. longispinosus* exhibited significantly more candidate genes than its parasite in all analyses ($p_{adjust} < 0.001$), except for the local adaptation and climate association analysis, where both species showed similar numbers of candidate genes. (B) Orthologous candidate genes. The number of orthologous genes identified for parasite prevalence, climate, and chemical traits was significantly greater than expected by chance ($p < 0.001$), indicating their shared ancestry.

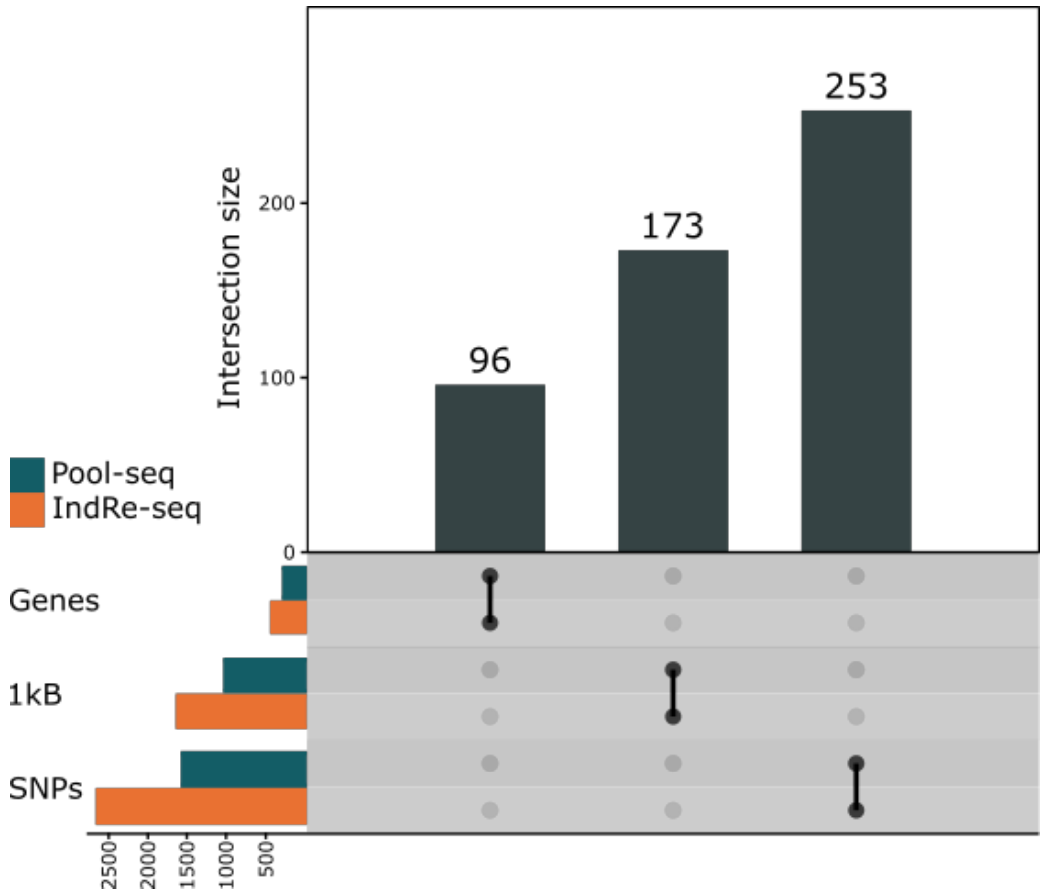


Figure S6. Overlap of *T. longispinosus* candidate genes, genomic regions, and SNPs associated with parasite prevalence from a previous host Pool-seq study. UpSet plot illustrating the intersections of candidate genes, unique 1 kB genomic regions, and specific SNPs identified in the current host-specific parasite prevalence GWAS, as well as those previously identified using Pool-seq data (Macit et al., 2024).

Supplementary Materials and Methods

Sample Collection and Estimation of Parasite Prevalence

The focal host species *Temnothorax longispinosus* inhabits deciduous forests in the northeastern USA and southeastern Canada (Jongepier et al., 2014). Colonies of this tiny black ant are facultatively polygynous, typically containing a few dozen workers, each measuring 2-3 mm in length. The range of this *Myrmicine* ant largely overlaps with that of its obligate social parasite, *Temnothorax americanus*, a slightly larger species with a black to brown colouration. Parasite colonies usually contain fewer than a dozen parasitic workers and fewer than 100 host workers. Colonies were collected from July to September 2021 across ten locations in the northeastern USA, with collection sites spaced 100-200 km apart (Fig. S1, Table S1). At each location, multiple areas within a 5 km radius were sampled to capture local diversity (Table S2). To ensure sampling of independent colonies, for both species, collected colonies had to have a minimum distance of 70 cm, and had to contain a queen. Ant colonies were transported to Mainz, Germany, maintained under standardised conditions (12:12h dark:light photoperiod at 18 °C and 80% humidity), fed once per week with the Bhatkar diet (Bhatkar & Whitcomb, 1970) and given water ad libitum.

Parasite prevalence serves as a measure of local parasite pressure on the host and an estimate of parasite success and was estimated as the percentage of social parasite colonies within the local *Temnothorax* community (*T. americanus* / *T. longispinosus*). Number of colonies of the secondary host, *T. curvispinosus*, found in MA, NYS, OH, and WV (see Table S1 for state abbreviations), were excluded. Parasitic colonies exploiting this secondary host exclusively were also excluded from calculations, and parasitic nest containing a mix of *T. longispinosus* and *T. curvispinosus* hosts were weighted based on the ratio of workers from the focal host species. Calculations were performed on long-term collection data (Table S1) (Herbers and Foitzik 2002; Brandt and Foitzik 2004; Foitzik et al., 2009; Jongepier et al. 2014; Kaur et al., 2019; Macit et al. 2024).

Sample Preparation, Dissections, and DNA/RNA Extractions

We aimed to sample one worker from 15 independent colonies for each of the two focal species in each of the ten populations. This target was met for the host species (except for

VT with 14 samples), but not for the social parasite due to low parasite prevalence in some locales. For *T. americanus,* the number of parasitised colonies sampled per population was uneven, ranging from the minimum of three samples from the NH population to 24 samples from MA (number of parasitic colonies per population: median = 19, mean = 15.4) (Suppl. S1). For the host, if possible, sampled colonies consisted of a single queen with approximately 30 workers. From each host colony, the first worker to exit the opened nest was collected to standardise sampling across the same behavioural caste. For the parasite, colonies with hosts preferably consisting only of *T. longispinosus* were chosen, although this was not always possible. There, a random parasitic worker was sampled. Ants were anesthetised on ice and quickly decapitated. The head, including antennae, was placed in a tube with 100 µl Trizol and six ceramic beads (1.4 mm diameter). The thorax, including limbs, was placed in a separate tube with six ceramic beads, and both samples were lysed for four minutes at 30 Hz using a QIAGEN Tissue Lyser II Retsch MM40. After visual inspection, additional lysis was performed for two minutes if necessary. The head/antennae samples (hereafter referred to as head samples) were then supplemented with an additional 300 µl Trizol to reach a final volume of 400 µl, and mixed to ensure complete submersion of the tissue. The fat body was carefully dissected from the abdomen and submerged in 400 µl Trizol. DNA samples were stored at -20 °C afterwards, while RNA samples were incubated in Trizol at room temperature for 30 minutes before being stored at -80 °C until extraction. DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen), following the insect protocol. RNA was extracted using the Direct-zol RNA Microprep Kit (Zymo) using the standard protocol. Whole-genome sequencing (WGS) and RNA sequencing (RNAseq) of 2x150 bp reads were performed by Novogene using the Illumina NovaSeq 6000 platform.

Whole-Genome Analysis

Sequencing data were trimmed using *Trimmomatic* v.0.39 (Bolger et al., 2014) with a 10bp head crop, and quality-checked with *FastQC* v.0.11.9 (Andrews et al., 2015). Trimmed reads were mapped using *BWA mem* v.0.7.17 (Li & Durbin, 2009) with a minimum seed length (k) of 30 and utilising unpublished reference genomes of *T. longispinosus* and *T. americanus* (Boomsma et al., 2017). This resulted in mean mapping rates of 94.4% (± 2.9%) and 95.3% (± 2.5%) for the two species, respectively.

We used *Picard* v.2.20.8 (Broad Institute, 2018) *markDuplicates* to mark and remove duplicate sequences. We then sorted and converted to bam using *SAMtools* v.1.10 (Li & Durbin, 2009) and removed mappings with low quality using *samtools view* (-q 20 -f 0x0002 -F 0x0004 -F 0x0008). *Samtools flagstats* and *depth*, and *QualiMap* v.2.2.1 (Okonechnikov et al., 2016) were used to determine mapping rate and average coverage (see Suppl. 1). Individual BAM files were merged using *BCFtools* v1.16 (Li, 2011) with *mpileup* (--min-MQ 20 --min-BQ 13 -C50), and variant sites were called using the call function. Indels were removed, and variant sites with a depth of 5 or lower in each sample were masked. To filter out over- and underrepresented variants, the lower and upper 5% of global depth for each species were calculated and applied ($925 \geq DP \leq 2725$ for *T. longispinosus*, and $1100 \geq DP \leq 3114$ for *T. americanus*). Finally, we excluded variants present in less than 90% of all samples (F_MISSING < 0.1) and those with a minor allele frequency below 0.05 (MAF < 0.05). With these parameters, we identified a similar number of SNPs in both species (1,677,757 SNPs for the host and 1,604,099 SNPs for the parasite). Hard-filtered vcf-files were then converted to a MAP and PED file using *VCFtools* v.0.1.17 (Danecek et al., 2011) and further converted to binary files using *PLINK* v.1.90b6.13 (Purcell et al., 2007). For population structure analyses, filtered vcf-files were further thinned by a factor of 5 kB using *vcftools –thin*, resulting in around 50 k SNPs per species. Since linkage disequilibrium in *T. longispinosus* is around 1 kB (Macit et al., 2024), with similar linkage assumed for *T. americanus*, this window size prevented the thinned dataset from containing linked loci. All subsequent analyses were conducted on unthinned SNP data.

Population Structure Analysis

We performed population structure analyses using the *findstructure()* function in *sambaR* (De Jong et al., 2021) on the thinned dataset. This, among other plots, generated PCAs of SNP data by calling the *snprelate()* function (Zheng et al., 2017), and generated admixture plots that are wrapped in the *findstructure()* function. We calculated cross-entropy using the *LEAce()* function (Frichot et al., 2014) and accounted for randomness in cross-entropy calculations by running the function 50x to confidently determine the lowest k. We identified several outlier genotypes in both species and applied specific criteria to determine whether these samples should be excluded. Samples that were consistently

assigned to a unique genotype in the admixture plots, from the optimal k up to k = 10, and exhibited virtually no admixture with other genotypes, were excluded from further analyses. This resulted in the removal of five samples from each species (Suppl. S1, 'sample_info'), originating from different areas, which may represent falsely identified species.

Additionally, divergent genotypes were identified in both species from the Ohio population. In *T. longispinosus*, all 15 samples from Ohio were genetically distinct and thus excluded from further analyses, since their difference from all other populations might overshadow any other population-specific patterns in the GWASs. In *T. americanus*, two genetically distinct clusters were found in Ohio, referred to as parasite OH and parasite OH2 (Fig. S7B). The parasite OH cluster consisted of nine colonies with exclusively or predominantly *T. longispinosus* hosts, which were included in subsequent analyses, since samples from other populations (i.e. PA) showed admixture with this genotype. However, the more genetically distinct cluster, parasite OH2, consisted of ten colonies with exclusively or predominantly *T. curvispinosus* host workers, and was excluded due to the same reason as the exclusion of host OH. The significant genetic differences between these two clusters, despite sympatry, might suggest host specialisation or a cryptic species. Further investigations are currently underway in collaboration with C. Rabeling and M. Prebus. However, we will present population structure analysis, including this outlier OH population, further below, but not in the main text.

The lowest cross-entropy was observed at k = 2 for the host when the host OH population was excluded (Fig. S2A), and at k = 3 when it was included (Fig. S7C). Similarly, for the parasite, the lowest cross-entropy was at k = 4 when the parasite OH2 was excluded (Fig. S2A), and k = 5 when included (Fig. S7C). Based on the admixture plots and the k values corresponding to the lowest cross-entropy, samples for both host and parasite were assigned to their majority genotype group as a categorical variable to determine if this grouping is associated with environmental traits. Pairwise W&C F_{ST} values (Weir & Cockerham, 1984) were calculated using *sambaR*.

Identifying outlier SNPs indicative of local adaptation

We performed *OutFLANK* (Whitlock & Lotterhos, 2015) within the *selectionanalyses()* function in *sambaR* to identify outlier SNPs setting the do_meta flag to TRUE. This setting grouped samples according to their assigned sampled population, which allowed us to identify outlier SNPs ($p \leq 0.05$) as a result of local adaptation/differentiation among population. Outlier SNPs identified using *OutFLANK* will henceforth be referred to as a SNP related to local adaptation.

Genome-wide associations to Environment and Phenotype

We conducted a genome-wide association study (GWAS) using *BayPass* v2.2 (Gautier, 2015) in its standard covariate mode to identify associations between single nucleotide polymorphisms (SNPs) and two environmental traits, climate and parasite prevalence, as well as two colony-level phenotypic traits, raid-related behaviours and the chemical profile. These analyses will be referred to as environmental, behavioural, and chemical GWAS. The exact values for all covariates used to create the environmental file (efile) are provided in Suppl. S2. Details of covariates for each GWAS will be explained below. Allele counts for all SNPs in all samples were extracted using *bcftools query*, specifically extracting the AD field for each sample to generate a genotype file for *BayPass*. Using allele count data rather than genotype data provides stronger association signals (González-Silos et al., 2022). A Bayes Factor (BF) of ≥ 15 was chosen as the significance threshold. Significant SNPs were categorised as within genes, in exons, in introns, 2 kB up- or downstream of genes, and outside of genes using an in-house script, as well as *CROXA/tbg-tools* (Schönnenbeck et al., 2021). Special emphasis was placed on candidate genes with a high number of SNPs, specifically non-synonymous ones, and those with exceptionally high Bayes Factors (i.e., BF > 40).

Environmental GWAS - Climate

For climate, we used the population eigenvalues of Principal Component 1 from an analysis of climate-based data from the CHELSA bioclim database (1981-2010) (Karger et al., 2017), which included ten temperature and eight precipitation values (Macit et al., 2024). These PC values range from populations with warm and dry climates (negative

values) to those with cold and wet climates (positive values; Fig. S1; Fig. 1A-B in Macit et al., 2024).

Environmental GWAS - Parasite prevalence

Parasite prevalence is defined as the percentage of parasitised colonies within the local *Temnothorax* population (*T. longispinosus* and *T. americanus* colonies), ranging from 2.3% to 15.6%. Similar to climate values, parasite prevalence is considered a population-level trait. A strong correlation was identified between parasite prevalence and climate, with warmer climates supporting a higher prevalence of parasites than colder ones. However, sufficient variability exists to distinguish between these factors in association analyses (Macit et al., 2024).

Behavioural GWAS

To identify the genetic basis of variations in behavioural traits relevant to both host and social parasite, we studied the response of host colonies to a sympatric invader in a preliminary study (Collin et al., in review) for which we used pivotal observed behaviours statistically linked to parasite prevalence and/or climate that were obtained around a year after collection, thus around four months after dissections of DNA and RNA samples used in this study. Around the same time, we conducted injury experiments on the host, which will be described in detail below. There, we observed differences in allogrooming behaviour in sister ants to an injured ant depending on the local climate from which the colonies originated. Both behavioural trials were conducted on ants coming from the same colonies as the ants' genotyped in this study, and were thus colony-level traits. In total, investigated behaviours included:

- Host aggression (Jongepier et al., 2014; Kleeberg et al., 2015) and brood-carrying. Only in response to parasitic invaders, host workers were noted to pick up brood and try to flee (Jongepier et al., 2015).
- Parasite aggression and passivity. Some parasites exhibited low aggression and submissive, passive behaviour (Collin et al., in review).
- Allogrooming of injured host workers. During raids, host workers are often injured or even lose limbs (Foitzik et al., 2001). The response of nestmates by

grooming injured host workers covaries with climate (see below) and was thus investigated on a probable genetic basis of this behaviour.

For all behavioural GWASs, we used the total number of each behaviour performed by a colony during the trials as a covariate in the GWAS, which was normalised across the number of behaviour scans performed (27 scans per trial).

Chemical GWAS

The interaction between social parasites and their hosts is closely linked to cuticular hydrocarbons, which play a crucial role in recognition within this system (Collin et al., in review). To investigate these traits and their genetic basis, cuticular hydrocarbons were extracted from two workers of a host colony and one from a social parasite colony, also used in this study, with their chemical profile being analysed by using gas chromatography-mass spectrometry (GCMS). Colony-level association analyses were performed on the following chemical information:

- *Relative abundance of recognition cues:* For both species, some methylated hydrocarbons were found to be important for eliciting aggression, including 23 CHCs for the host and 20 for the parasite (Jongepier & Foitzik, 2016; Collin et al., in review).
- *Relative abundance of (linear) n-alkanes.* Linear *n*-alkanes are generally not used in recognition but serve to protect against desiccation. Social parasites exhibit a profile rich in *n*-alkanes (Kleeberg et al., 2017), which might help them to avoid detection (Kaur et al., 2019).
- *Average chain length of n-alkanes.* Longer-chained *n*-alkanes have better desiccation protection capabilities, as longer chains form longer and tighter layers. Since we identified population- and species-specific differences in their length (Fig. S10), a genomic basis for this chemical trait could be assumed.

The number of samples included in each GWAS varied slightly among the environmental, behavioural, and chemical GWASs, as well as between species, due to the inability to consistently obtain both genotype and phenotype data for all samples (summarised in Table S3).

RNA analysis

Trimming and quality control of RNA-seq data were performed as described above for WGS data. Reads were mapped using *HISAT2* v.2.1.0 (Kim et al., 2015) to the aforementioned reference genome. Mean mapping rates for transcriptome data in *T. longispinosus* were 64.2% (± 33.4%) for fat body samples and 80.1% (± 26.3%) for head samples, and in *T. americanus* 64.9% (± 31.5%) for fat body samples and 80.9% (± 22.7%) for head samples. Using the *htseq-count* function from *HTSeq* v.2.0.2 (Putri et al., 2022), we created transcript read count tables used as input for *DESeq2* v.1.42.0 (Love et al., 2014) in *R* v.4.3.2 (R Core Team, 2021). Transcripts with less than ten counts in at least one-third of all samples were removed. Due to high variances in all four RNAseq datasets, we filtered for outliers using *pca.outlier()* function from the *mt* package v.2.0.1.19 (Lin et al., 2021), which identifies outliers based on Mahalanobis distance of PC1 and PC2 values. Outlier detection was performed twice for all four read count tables, and outlier samples were removed from the original read count table (see Suppl. S1). The read count tables were then used to conduct a PCA. Differentially expressed genes (DEGs) were tested using *DESeq2* with variance stabilised data, with each parasite prevalence (results presented in the main manuscript), climate PC1 eigenvalues and aggression values (results presented here) used as continuous variables in both tissues and both species. We identified DEGs that contain loci in putative promoter and intron regions associated with parasite prevalence, as these may impact expression patterns (Cooper, 2010).

Functional and Comparative Analyses

To further characterise candidate genes (i.e. those containing significant SNPs in any of the analyses), we obtained functional annotations for all genes in the reference genomes using their peptide sequences. First, we ran *InterPro* v.5.61.93 (Paysan-Lafosse et al., 2023) to retrieve Gene Ontology (GO) information, which was used to perform functional enrichment analyses using *topGO* v.2.54.0 (Alexa & Rahnenfuhrer, 2017). Next, we ran a *BlastP* v.2.13.0 (Altschul et al., 1990) search against the non-redundant invertebrate database (retrieved on NBCI on Jan 2022) and also a proteome database consisting of *Drosophila melanogaster* and *Apis mellifera* (retrieved on UniProt on Jan 2024; Proteome IDs: UP000000803 and UP000005203) (The UniProt Consortium, 2023). Any gene

names provided in this study for specific candidate genes will always refer to the best BLAST hit generated in *A. mellifera*, unless stated otherwise. We further retrieved the UniProt entry ID, associated GO terms, function text and associated publications via the UniProtKB entry for hits in *A. mellifera*. Orthologs between both species were identified using *OrthoFinder* v.2.5.4 (Emms & Kelly, 2015), and their GO functions were summarised using *REVIGO* (Supek et al., 2011).

To test whether the number of candidate genes in each GWAS and differentially expressed genes differed between species, we used the built-in chi-square function in *R*, considering the total number of genes in both reference genomes (*T. longispinosus* = 16,064, and *T. americanus* = 14,128). Similarly, to test whether the number of non-synonymous SNPs differed between species, we used the chi-square test, considering the total length of exon regions in both reference genomes (*T. longispinosus* = 21,924,724 and *T. americanus* = 20,133,265). For non-synonymous SNPs, we tested for significant differences in log-transformed Bayes Factors between species using a one-way ANOVA followed by a Tukey HSD post-hoc test to identify pairwise differences. To test if the number of overlapping orthologous candidate genes in each GWAS and differentially expressed genes differed between species, we used the *hypergamous()* function from the Python-based *SciPy* software (Virtanen et al., 2020). For all statistical tests, we chose a significance threshold at $p \leq 0.05$.

Demographic History Analysis

We employed a pairwise sequentially Markovian coalescent model (PSMC; Liu & Hansen, 2017) to detect changes in the effective population size (N_e) over a broad temporal scale. We chose two samples per population per species with the lowest level of admixture (excluding host OH and parasite OH2) (Suppl. S1). We created mpileup files per species and called variances using *bcftools*. Consensus files were generated using *vcfutils.pl vcf2fq* (-d 10 -D 100) and converted to the appropriate data type using *fq2psmcfa*. The PSMC analysis was performed across 64 interval times (-p 6+25*2+4+4) and the upper limit of the TMRCA (-t) set to 10. Plots were generated using *psmc_plot.pl* with generation time set to 10 years (-g 10) as was previously described in the host species (Kaur et al., 2019) and mutation rate set to the one found in *A. mellifera* (-μ 3.4e-9; Liu

& Hansen, 2017; Yang et al., 2015) as was similarly done in *Solenopsis* ants (Cohen & Privman, 2019).

Additional Results and Discussion

Population Structure

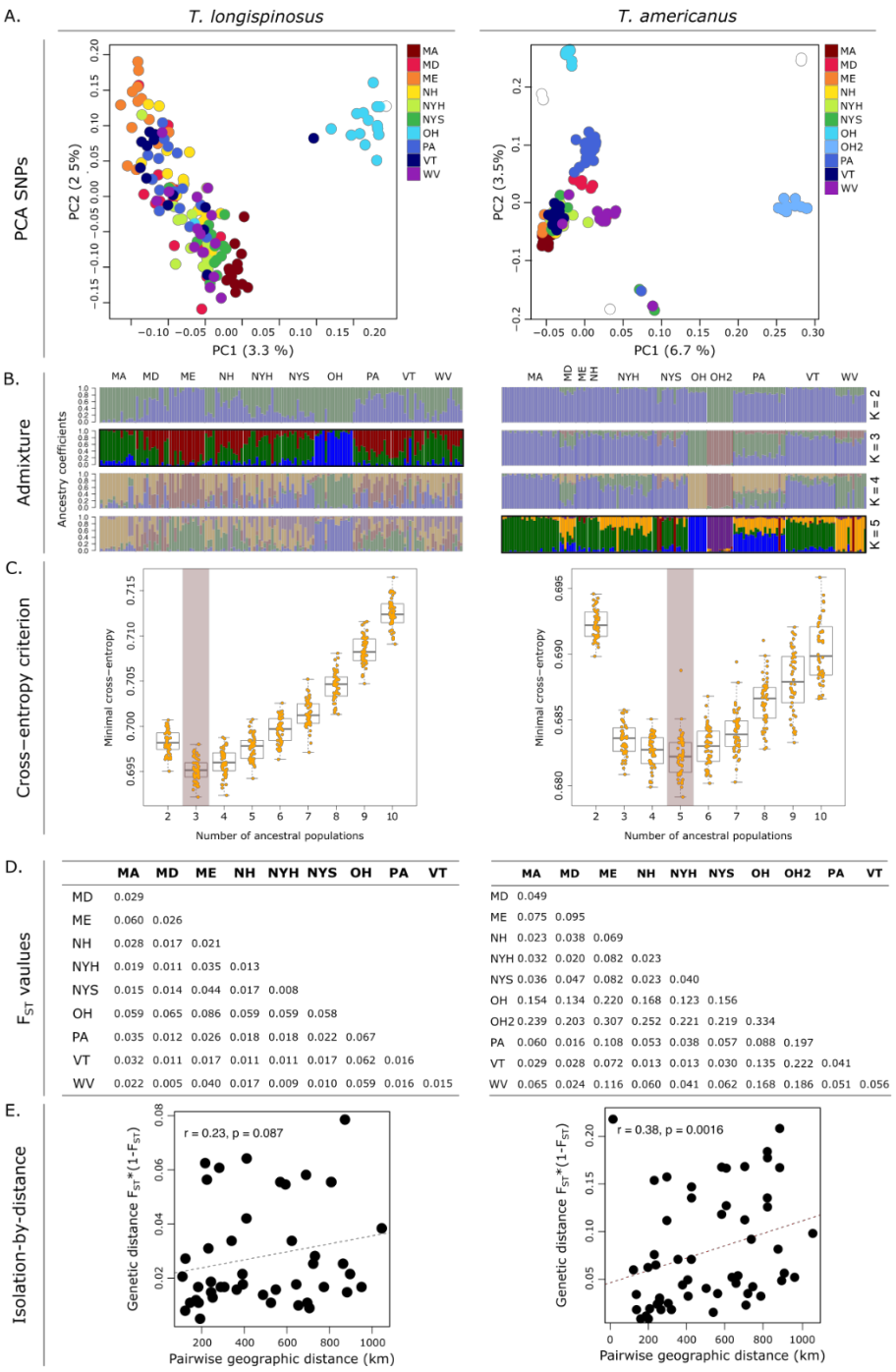
A principal component analysis (PCA) of the thinned SNP dataset for the host revealed no clear clustering by population, although samples from Massachusetts (MA) were distributed toward the far negative side of the PC1 axis, and those from Maine (ME) toward the far positive side (Fig. 1A). Admixture analysis indicated that the lowest cross-entropy occurred at $k = 2$ ancestral group (Fig. S2A). Populations from MA, New York Huyck (NYH), New York South (NYS), and West Virginia (WV) were predominantly characterised by the blue genotype (genotype 1), whereas ME was primarily associated with the green genotype (genotype 2; Fig. 1B). Each sample was assigned to its majority genotype, with these assignments being significantly influenced by local parasite prevalence (ANOVA $F = 42.72$, $p < 0.0001$), climate values ($F = 30.75$, $p < 0.0001$), and their interaction ($F = 6.57$, $p = 0.012$; Fig. S2B). For the parasite, the PCA of thinned SNP data showed clearer population clustering, in particular in samples collected in OH, Maryland (MD), WV, and Pennsylvania (PA; Fig. 1A). Admixture analysis revealed the lowest cross-entropy at $k = 4$ (Fig. S2A), with distinct genotype patterns observed in MA, OH, Vermont (VT), and WV (Fig. 1B). Each sample was assigned to its majority genotype (blue, green, red and orange, Fig. 1B). These assignments were significantly affected by local parasite prevalence (ANOVA $F = 46.84$, $p < 0.001$), climate values ($F = 9.01$, $p = 0.0032$), and their interaction ($F = 29.43$, $p < 0.0001$; Fig. S2B).

Population Structure including outlier Ohio populations

Our population structure analysis of the primary host, *T. longispinosus*, revealed stark differences in all host samples from Ohio (referred to as ‘host OH’ henceforth) compared to those from all other sites. Similarly, in the parasite, samples from OH clustered into two distinct groups (referred to as ‘parasite OH’ and ‘parasite OH2’ henceforth), seen as two distinct clusters in a PCA of thinned SNP data (Fig. S7A). The same grouping was also evident in admixture plots, where parasite OH2 showed a distinct genotype with no admixture in any other samples or population (Fig. S7B). The lowest cross-entropy in the

dataset, with and without host OH and parasite OH/OH2, differed by 1, indicating that host OH and parasite OH2 consistently formed their own ancestral population (Fig. S7C). Pairwise F_{ST} values in host and parasite were slightly higher when host OH and parasite OH2 were included (Fig. S7D). Isolation-by-distance patterns were slightly stronger in the host ($p = 0.089$), and in the parasite ($r = 0.38$, $p = 0.0016$; Fig. S7E). Our metadata revealed that *T. americanus* samples with the parasite OH genotype (blue in Fig. S7B, $k = 5$) and the parasite OH2 genotype (purple) differed in the host species they enslaved: Parasitic colonies with majority parasite OH genotype almost exclusively enslaved *T. longispinosus* hosts, and those with the parasite OH2 genotype enslaved predominantly *T. curvispinosus* hosts or a mix of *T. longispinosus* and *T. curvispinosus* hosts. For the host, we were unable to identify a driver for the unique host OH genotype based on our metadata. We hypothesise that the climate conditions in Ohio, which are comparatively drier than those in neighbouring PA, MD and WV sites, may drive such genomic differences observed. Further research on possible host-driven speciation, or identification of a cryptic species, should increase sample sizes of host and parasite, noting down the host species in parasitised colonies, and expanding sampling sites to the US states of Illinois and Michigan, which contain *T. curvispinosus* in high densities.

Tajima's D values were consistently low and slightly positive across populations in both *T. longispinosus* and *T. americanus*, reflecting a mild excess of intermediate-frequency alleles (Fig. S8). This pattern arises from nucleotide diversity (π) exceeding Watterson's theta (θ) in all populations, suggesting stable population sizes with no recent bottlenecks or expansions. The moderate and tightly clustered π and θ values indicate relatively high genetic diversity and demographic uniformity across regions in both species. The similarity between host and parasite implies shared evolutionary dynamics, possibly due to common environmental pressures or interdependent dispersal histories, though the low magnitude of Tajima's D points to weak or background-level selective forces rather than strong directional or balancing selection.



	MA	MD	ME	NH	NYH	NYS	OH	PA	VT
MD	0.029								
ME	0.060	0.026							
NH	0.028	0.017	0.021						
NYH	0.019	0.011	0.035	0.013					
NYS	0.015	0.014	0.044	0.017	0.008				
OH	0.059	0.065	0.086	0.059	0.059	0.058			
PA	0.035	0.012	0.026	0.018	0.018	0.022	0.067		
VT	0.032	0.011	0.017	0.011	0.011	0.017	0.062	0.016	
WV	0.022	0.005	0.040	0.017	0.009	0.010	0.059	0.016	0.015

	MA	MD	ME	NH	NYH	NYS	OH	OH2	PA	VT
MD	0.049									
ME	0.075	0.095								
NH	0.023	0.038	0.069							
NYH	0.032	0.020	0.082	0.023						
NYS	0.036	0.047	0.082	0.023	0.040					
OH	0.154	0.134	0.220	0.168	0.123	0.156				
OH2	0.239	0.203	0.307	0.252	0.221	0.219	0.334			
PA	0.060	0.016	0.108	0.053	0.038	0.057	0.088	0.197		
VT	0.029	0.028	0.072	0.013	0.013	0.030	0.135	0.222	0.041	
WV	0.065	0.024	0.116	0.060	0.041	0.062	0.168	0.186	0.051	0.056

Figure S7. Population structure analysis including all Ohio samples. (A) Principal Component Analysis of SNP data, based on a thinned dataset containing ~50k SNPs per species. (B) Admixture plot depicting the various genotypes identified and their distribution/proportion across samples in populations. Highlighted is the optimal k. (C) The determination of the optimal number of ancestral populations (k) based on the cross-entropy criterion, revealing k = 3 for *T. longispinosus* and k = 5 for *T. americanus* using *LEAce()* ran 50x. (D) Pairwise F_{ST} values. (E) Isolation-by-distance.

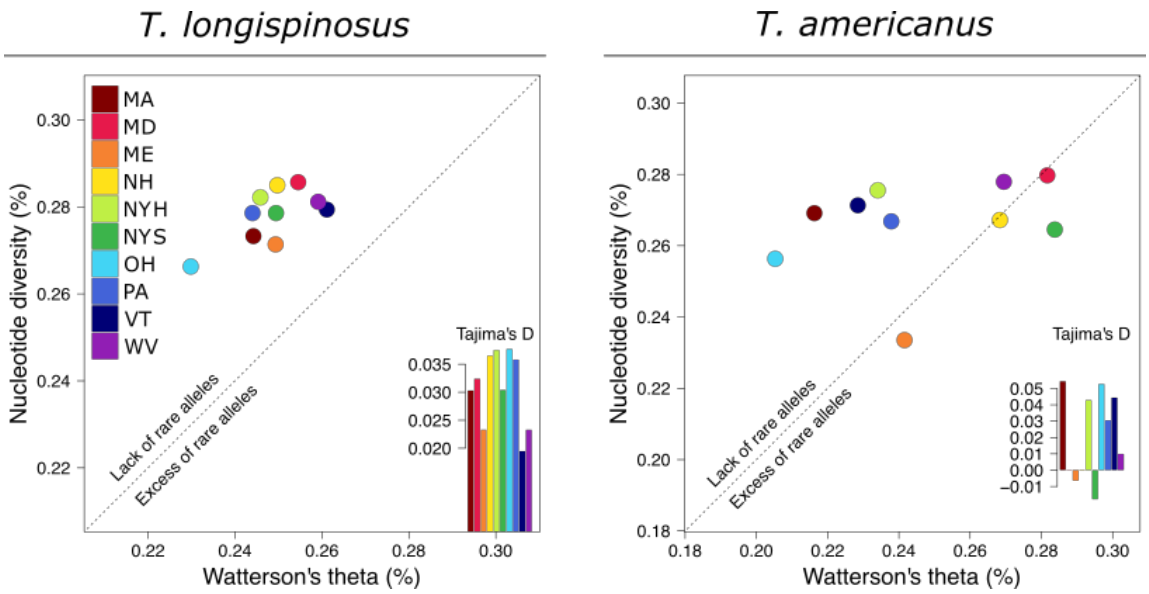


Figure S8. Comparative SNP frequency spectrum. Scatterplots show the relationship between nucleotide diversity (π) and Watterson's theta (θ) for each sampled population in both species. The dashed line represents $\pi = \theta$, along which Tajima's D = 0, with points above the line indicating a relative deficit of rare alleles (positive Tajima's D), while those below indicate an excess of rare alleles (negative Tajima's D). Insets display population-specific Tajima's D values, highlighting consistently positive values in *T. longispinosus* (left) and more variable patterns in *T. americanus* (right), suggesting differences in demographic history or selective pressures between host and parasite, but are likely the result of uneven sampling sizes in the parasite.

Demographic History Analysis

Our PSMC analysis yielded counterintuitive results, with the N_e of the parasitic ant species being higher than that of its host: *T. americanus*, as a dulotic parasite, is usually rare, resulting in lower population sizes, as reflected in its classification as a vulnerable species on the IUCN Red List (IUCN, 2022). While the absolute N_e values may underestimate true population sizes, particularly in haplodiploid social insects where N_e is calculated differently than by PSMC (Hedrick & Parker, 1997; Cohen & Privman, 2019), the observed disparity warrants further discussion. A potential explanation for this discrepancy is the substantially higher worker reproduction rate in the parasite. Host worker reproduction is restricted to queenless colonies (Bourke, 1988). In contrast, it is a regular and significant component of the parasite's reproductive strategy, with studies reporting up to 70% of parasitic males being worker-reared in *T. americanus* (Buschinger

& Alloway, 1977; Foitzik et al., 2001). Given the parasite's prevalence (2-15%), which suggests a comparatively smaller population size, the high rate of worker reproduction could contribute to a higher-than-expected N_e. Furthermore, while the parasite exhibits clear population structuring, potentially leading to an overestimation of N_e, the host appears closer to panmixia, which could lead to an underestimation of N_e (Hilgers et al., 2024).

Both species exhibit a peak in N_e around 2 million years ago, but their trajectories diverge, with the parasite's population sizes dropping seemingly more rapidly than those of the host, both reaching similar values around 100,000 years ago (Fig. S9). The Pleistocene ice age in the Appalachian Mountains likely prompted southward migrations, with certain areas within our sampling range (PA, MD, WV and partially OH) serving as ice-free refugia (Bloom, 2018; Earth@Home, 2024). The subsequent glacial retreat around 11,000 years ago likely facilitated recolonisation and significantly influenced contemporary population structures. These ice-free areas are also those with high parasite prevalence, which may suggest the parasite inhabits these regions for longer compared to northern populations.

Finally, the host and parasite differ in major population traits, with low population structure in the host and strong structuring in the parasite. This may result in problems comparing the results of both species. We acknowledge those and other limitations associated with PSMC analyses, including sensitivity to parameter choice and potential inaccuracies when using scaffold-based genomes as was done here, particularly for recent time scales (Li & Durbin, 2011). Therefore, the PSMC results presented here should be interpreted cautiously.

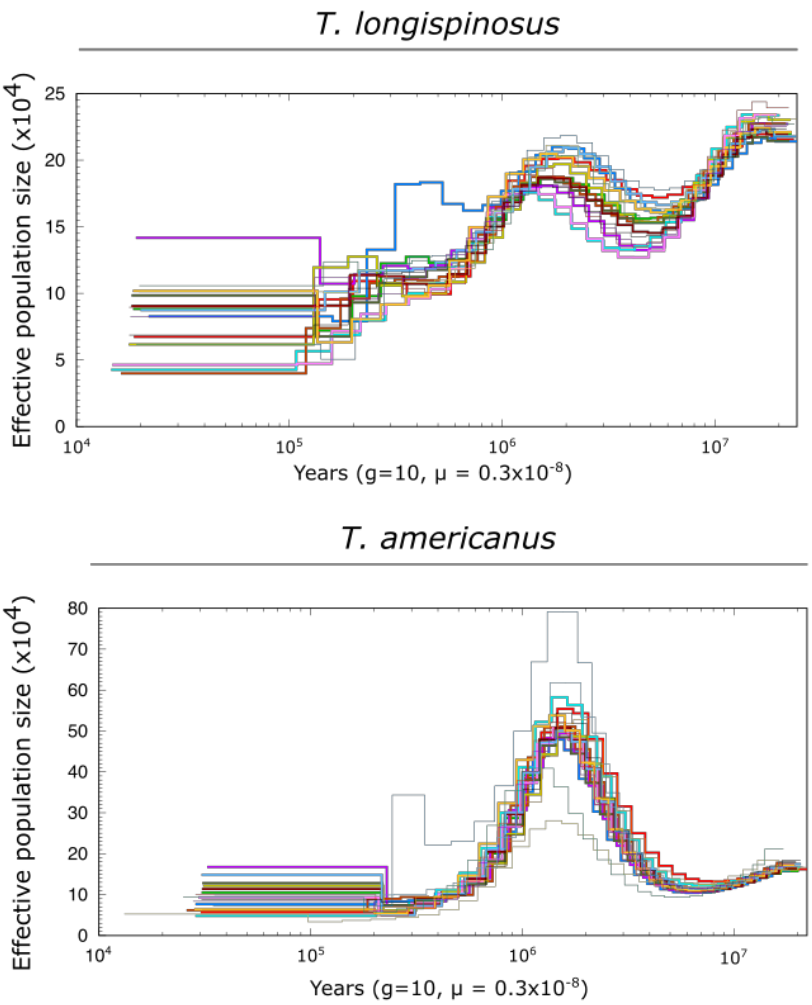


Figure S9. Demographic history analysis. Parameters used were -p 6+25*2+4+4, setting the upper limit of the TMRCA (-t) to 10, generation time to 10 years (-g 10) and mutation rate to 3.4e-9 (-μ). Effective population size (N_e) over time for host and parasite both shows peaks around 2 million years ago, followed by a steeper decline in parasite N_e.

Chemical Characteristics

The chemical composition of the cuticular hydrocarbons in social insects serves a dual function, protecting against desiccation and facilitating communication (Sprenger et al., 2019). The length of linear *n*-alkanes can influence their protection against desiccation properties, with longer linear *n*-alkanes creating tight layers with low viscosity. Conversely, shorter chains allow better fluidity through the cuticle of insects (Menzel et al., 2019). The average chain lengths in both species appear to be similar, with small standard deviations (host: average 27.74, std: 0.16; parasite: 27.28, std: 0.12). We

performed a one-tailed ANOVA test using *aov()* function in *R* and confirmed that variances in average chain lengths of linear *n*-alkanes are significantly different between populations in the host (Df = 8, F = 3.504, p = 0.0012) and in the parasite (Df = 9, F = 3.668, p = 0.00050), and highly differed between species, with the parasite showing shorter chain lengths than the host (Mann-Whitney U test: z = 13.62, p < 0.00001; Fig. S10), which has been reported before in this and other dulotic ant species, and discussed to reduce host detection (Kleeberg et al., 2017). Based on these observed differences, we felt justified in investigating the putative genomic basis of these significant differences in chain lengths and thus performed a GWAS, for which we report the results in the main document.

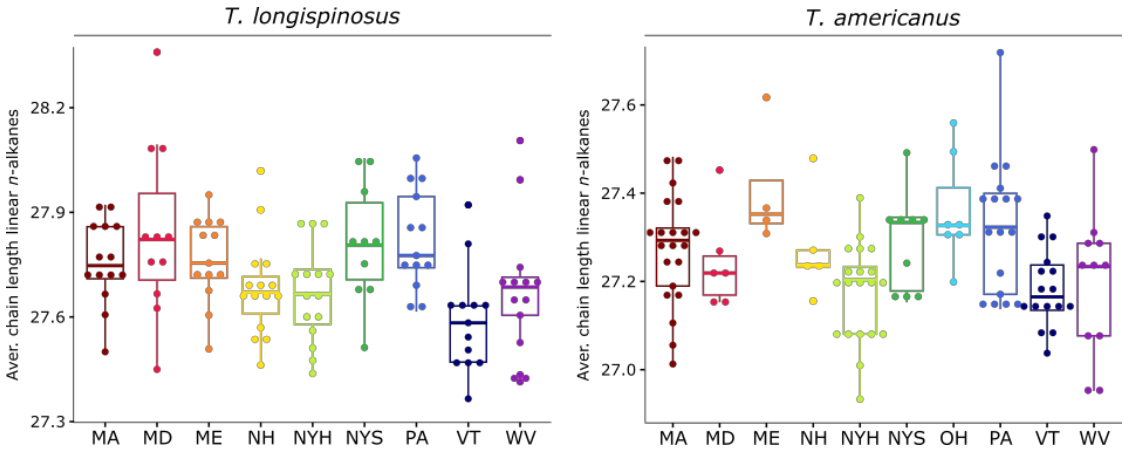


Figure S10. Linear *n*-alkane chain length variations. Boxplots showing the distribution of average chain lengths of linear *n*-alkanes in host and parasite populations. Significant differences in chain length were observed within both host (ANOVA, F = 3.50, p = 0.0012) and parasite (ANOVA F = 3.67, p = 0.0050) populations, as well as between species (Mann-Whitney U test, z = 13.62, p < 0.00001).

GWAS of species-specific behaviours

In Collin et al. (in review), host and parasite ants exhibited specific behaviours during experimental raids that appear adaptive to local environmental pressures. The host ants exhibited aggressive behaviour toward parasites, akin to 'fight', and brood-carrying behaviour, which aligns with 'flight' or protective responses. Parasites, in contrast, showed aggression and passive 'freeze' behaviours, with inactivity potentially serving as a survival tactic. The primary objective of this study was to explore the genetic

underpinnings of co-evolutionary traits in these interactions, with a particular focus on aggression, given the availability of reciprocal data between hosts and parasites. However, given the observed associations between other species-specific behaviours and parasite prevalence, a GWAS was conducted on these behaviours and is reported in the following.

Host - brood-carrying

We identified six genes containing significant loci associated with brood-carrying behaviour in the hosts, including one non-synonymous SNP (ns-SNP) in the *FAS* gene (Suppl. S2). Two other candidate genes were *IDE* and *radish*, which has a role in olfactory long-term memory (Folkers et al., 2006). In the host aggression GWAS, *IDE* was suggested as influential in age-dependent aggression, given its role in other insulin-related genes in the shift from nursing to foraging (Chen et al., 2023). Notably, foragers, rather than nurses, typically engage in parasite defence (Koenig & Moreau, 2024a). Since brood-carrying is primarily a nursing behaviour, the findings imply a complementary role for *IDE* in age-specific host responses to parasitic intruders. Additionally, *radish* and *FAS* may facilitate recognising and memorising parasite-associated chemical cues, initiating brood-carrying to protect vulnerable larvae and pupae.

Parasite - passive

We identified five genes containing significant loci associated with passive 'freeze' behaviours in the parasite, although none were non-synonymous (Suppl. S2). Noteworthy genes include an *odorant receptor* gene, and those involved in transcription and gene expression (*tumor protein p53-inducible nuclear protein 2*, *histone demethylase UTY*, *polyglutamine-repeat protein pqn-41*). The number of candidate genes relating to gene expression and transcription might suggest a stronger emphasis on gene expression patterns for this specific behaviour. Earlier research has shown that parasites exhibit shifts in gene expression when transitioning from a resting to a raiding state (Alleman et al., 2018), underscoring the selective pressure on gene regulatory functions. Further and more generally, passive or inactive behaviour within its parasitic nest was previously deemed to be indicative of 'behavioural water-saving strategies' in support of their strategy of

chemical insignificance suggested by Lorenzi (2021), and might link passiveness to a default behaviour.

Differentially Expressed Genes Associated with Climate and Orthologous Gene Expression

We performed *DESeq2* on fat body and head samples for both species using climate PC1 eigenvalues as a continuous variable. For the host fat body transcriptome, we identified 525 genes to be significantly associated with climate PC1 values, which included several *FAS* genes. For the parasite's fat body transcriptome, we identified significantly more genes (884; gene count: $\chi2 = 95.13$, $p_{adjust} < 0.001$), including candidate genes such as *chitinase domain-containing protein 1 (CHID1)* and a *fatty acyl-CoA reductase (FAR*; Fig. S11, Suppl. S1). The host fat body transcriptome showed 18 enriched functions, with many relating to mitochondrial functions (i.e., 'mitochondrial respiratory chain complex I assembly', 'mitochondrial electron transport') and meta- and catabolic processes (i.e., 'proteolysis involved in protein catabolic process', 'galactose metabolic process'). For the parasite fat body, we identified 20 enriched functions, with most of them being involved in either gene expression itself ('translation', 'mRNA transport', 'protein transport', 'protein folding') or meta- and catabolic processes (i.e., 'cellular metabolic process', 'catabolic process'; Suppl. S1). For the host's head transcriptome, we identified 49 genes significantly associated with climate PC1 values, most notably an *odorant binding protein* (*OBP*) gene and a *phosphodiesterase* (*PDE*). The parasite's head transcriptome showed a similar number of genes (40; gene count: $\chi2 = 0.72$, $p_{adjust} = 0.44$), and similarly included an *OBP* gene (Fig. S11, Suppl. S1). The host head transcriptome showed nine enriched functions, with half of them being involved in metabolic processes (i.e., 'glucose metabolic process', 'lipid metabolic process'). The parasite's head transcriptome showed 10 enriched functions with the most enriched ones relating to gene expression processes (i.e., 'translation', 'regulation of mRNA processing'; Suppl. S1).

Sympatric host-parasite populations are exposed to the same environmental conditions (parasite prevalence and climate). We therefore identified orthologous genes that are differentially expressed in response to these environmental conditions in both

species, indicating a similar utilisation of gene expression pathways. For orthologous expressed genes associated with parasite prevalence in both species' fat bodies, we identified 21 differentially expressed orthologous genes associated with parasite prevalence, which was more than expected by chance (hypergeometric test: $p < 0.001$, Jaccard index = 0.034; Suppl. S1). These included genes with functions in oxidative stress and neuronal activity such as c*uticular protein 14 precursor*, s*uperoxide dismutase*, *proteasome subunit alpha type*, and *clavesin-2*. We used the online tool *REVIGO* to identify commonalities among the associated Gene Ontology (GO) terms of orthologous expressed genes. We found them to be related to core cellular processes ('cellular component organization') and gene regulatory functions (i.e., 'regulation of transcription by RNA polymerase II'). For orthologous expressed genes associated with climate for both species' fat bodies, we identified 77 genes, which was more than expected by chance (Jaccard index = 0.061, $p < 0.001$). Among those were genes with functions in fatty and lipid metabolic processes (*very long-chain-specific acyl-CoA dehydrogenase*, *long-chain-fatty-acid-CoA ligase*), but also cuticle composition (*probable chitinase 10*; Suppl. S1). Using *REVIGO*, we identified commonalities in associated GO terms, of which the vast majority were related to gene regulatory functions (i.e., 'regulation of DNA-templated transcription', 'mRNA processing'), and further some post-translational modifications ('protein phosphorylation', 'protein dephosphorylation'). For both environmental factors, parasite prevalence and climate, we found no associated orthologous expressed genes in head samples.

In our transcriptome analyses, using parasite prevalence as a continuous variable, we consistently observed significantly lower transcriptional activity in the parasite compared to the host (see main text). However, using climate as a factor revealed a partial reversal of this pattern, with the parasite now exhibiting increased constitutive gene expression in its fat body associated with climate than its host. We also found more orthologous expressed genes between the two species related to climate than parasite prevalence, using shared genes as pathways involved in gene regulatory functions. Investments in constitutive gene expression of the head transcriptomes further suggest similar climate-driven transcriptional adaptations in this organ across both species. Functional enrichment analyses revealed the use of similar processes and pathways across

tissues and species: Metabolic and catabolic processes were enriched in the fat body transcriptomes of both the host and parasite, as well as in the host's head. Mitochondrial functions were also enriched in the host fat body, while gene expression-related functions were prominent in the parasite fat body and head transcriptomes. These enriched metabolic/catabolic, and mitochondrial/ATP-related functions may reflect a shared investment in processes that scale with temperature, potentially influencing growth and body size in both species, as is the case in other insects (Bjørge et al., 2018). The identification of *FAS* and *FAR* genes in both species may also reflect climate-adaptive chemical desiccation protection, given their role in the CHC biosynthesis. Intriguingly, *OBP* gene were identified as candidate genes in both species in their head samples: This finding may indicate an adaptive response to increased volatility of chemicals in warmer temperatures, potentially enhancing odorant perception (Biessmann et al., 2010), including, the detection of alarm pheromones (Du & Chen, 2021).

Differentially Expressed Genes associated with Aggression

Using aggression values obtained from Collin et al. (in review) for identifying differential gene expression, we identified 313 DEGS in the host's fat body and none in its head in association with aggression. In contrast, the parasite exhibited 2,701 DEGs in the fat body and two in the head (Suppl. S1). Given the negligible number of DEGs in head samples, subsequent analyses focused exclusively on fat body tissue. There, the parasite displayed more than eight times the number of DEGs associated with aggression compared to its host, a statistically significant difference (gene count: $\chi 2 = 3269$, $p_{adjust} < 0.0001$). Functional enrichment analysis revealed 17 enriched biological GO terms in the host and 32 in the parasite. Both species shared two enriched terms related to gene regulatory processes ('translation', 'translation initiation'). In the host, additional enriched functions included further regulatory processes ('mRNA export from nucleus') and post-translational modifications ('post-translational protein modification'). Nearly all enriched functions in the parasite were related to gene regulation ('mRNA splicing via the spliceosome', 'ribosomal large/small subunit biogenesis') (Suppl. S1). However, due to the low number of aggression data available, this analysis was performed on around 1/3rd of the available samples, and results should be interpreted cautiously.

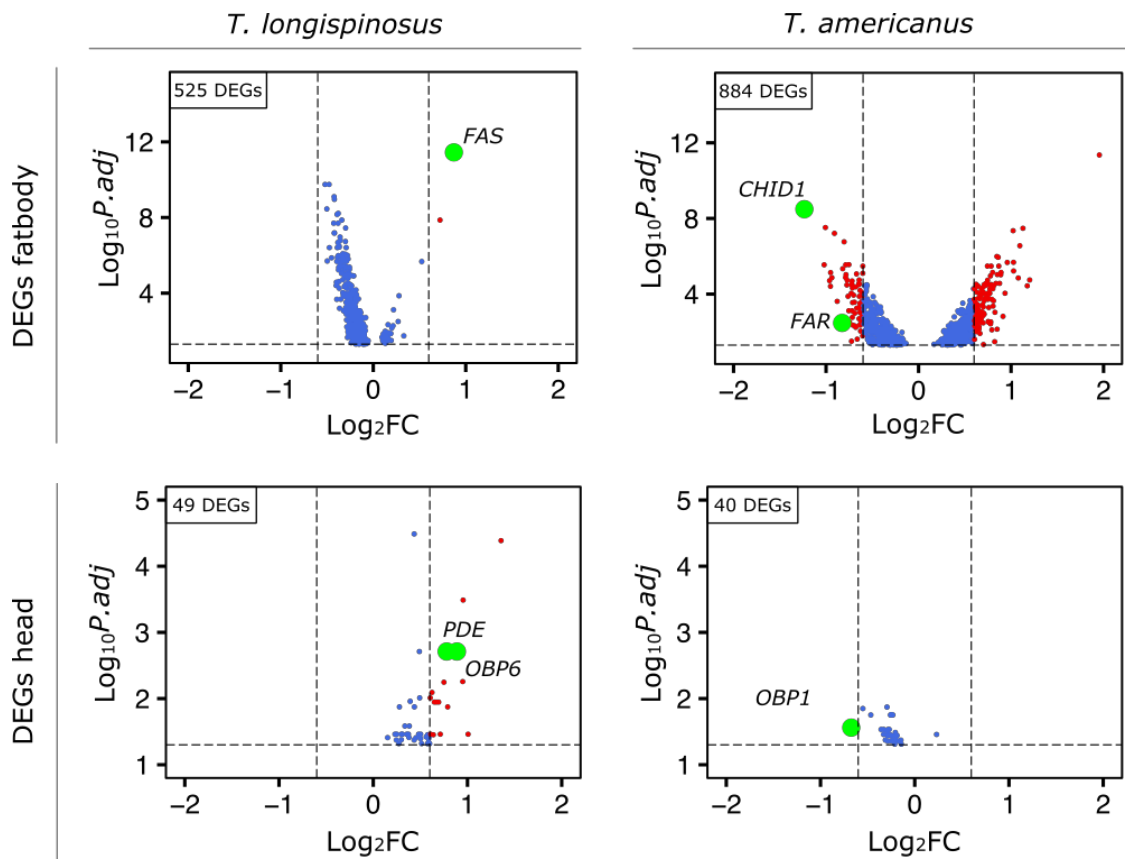


Figure S11. Differential gene expression in *T. longispinosus* and *T. americanus* in response to climate. Volcano plots showing differential gene expression in fat body and head tissues of *T. longispinosus* and *T. americanus* associated with climate PC1. Plotted are significantly differentially expressed genes (DEGs; $p \leq 0.05$) with thresholds drawn at log2FC = 0.7. Selected candidate genes of interest are labelled.

First Aid in Ants: Response of *Temnothorax* Ants to Injury in Relation to Social Parasite Prevalence and Climate

Abstract

Destructive raids by dulotic parasitic ants often injure defending host ants, prompting nestmates to provide wound care to reduce the risk of secondary infections. To test whether this behaviour varies with parasite pressure, we simulated raid-induced injuries in *Temnothorax longispinosus*, host of the dulotic parasite *T. americanus*. Injured ants from warmer climates received more frequent and prolonged allogrooming, suggesting a temperature-driven adaptation to potentially elevated microbial risks. In contrast, grooming decreased in populations with higher parasite prevalence, possibly reflecting a strategic shift toward replacing injured workers instead of caring for them. These results highlight the interaction between climate and parasitism in jointly shaping social care strategies, revealing context-dependent adaptations in host-parasite systems.

Introduction

Injuries can pose high risks by either being fatal or indirectly via subsequent infections (Hart, 2011), with further consequences on colony-level in social insects. Extreme behaviours, such as self-amputation (Emberts et al., 2017) and the amputation of injured limbs by sisters in ants (Frank et al., 2024), have evolved to reduce injury costs or infection risks. The socially parasitic ant *Temnothorax americanus* replenishes its workforce through destructive raids on host colonies, often injuring host workers. Given the varying prevalence of *T. americanus* (2-16%) across the interaction range with its host, *T. longispinosus*, we investigated whether the host's injury response via leg amputation varies with parasite pressure, but given its ecological impact on parasite prevalence, we further included climate as an explanatory factor (Macit et al., 2024; Collin et al., in review). In early 2024, we conducted injury experiments on *T. longispinosus* colonies (min = 1, max = 18, mean/median = 13 colonies per population; Fig. S12) from our existing host-parasite collection. By creating injuries via dissection of a limb and subsequently assessing the behaviour of injured ants and their sisters, we

attempted to examine the relationship between host responses to injured nestmates along a parasite prevalence and climate gradient, as well as in relation to the original host colony size. We predicted that (i) hosts in highly parasitised areas show increased injury care, potentially due to selection on immune genes in these populations (Macit et al., 2024), and (ii) warmer climates might favour increased grooming of injured nestmates to reduce infection risks of highly proliferating bacteria in warmer temperatures (Linder et al., 2008). Ant behaviour was scored using BORIS software (Friard & Gamba, 2016) based on an established ethogram (Table S7).

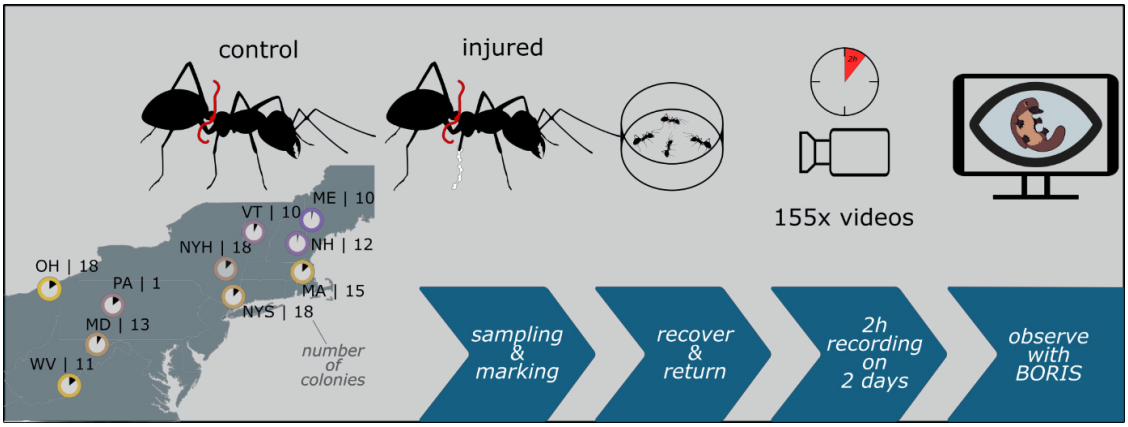


Figure S12. Experimental design for injury assays. Healthy host colonies from each population were selected. A worker ant was randomly selected and marked with a red wire. For the treatment groups (injured and non-control), the middle leg was quickly removed. Following a 5-minute recovery period, both control and injured ants were returned to their original colonies, and their interactions were recorded for a period of 2 hours. This observation was repeated for an additional two hours on the following day if the control or injured ant survived.

Materials and Methods

Colonies were collected and maintained as described in the Materials and Methods section above. In total, one to 18 independent host colonies per population were used (see map in Fig. S12). The number of queen(s), workers and larvae was counted before the experiment. Coloured wire loops were used to mark ants to be injured, and their controls. Controls and experimental ants were briefly sedated with CO_2. For the experimental ants, a single middle leg was cut using sterilised micro scissors, followed by a 5-minute recovery period before reintroduction to the nest for observation. This procedure was

similarly applied to the control ants with no leg amputation. Recordings lasted two hours, with each colony observed for two consecutive days if the injured ant or the control survived the first day. Video analysis was conducted using *BORIS* software (Friard & Gamba, 2016), focusing on the behaviours of injured and control ants as defined in an ethogram (Table S7). We quantified the occurrence of observed behaviours, activities, and interactions, and for a subset of these, their duration was also measured (Suppl. S3). Data analyses involved generalised linear mixed models (*glmms*) using the *glmmTMB* package (Brooks et al., 2017) in *R* v.4.2.3 (R Core Team, 2021). These included the behaviour as dependent variables, the treatment (control vs. injured), parasite prevalence, climate PC1, the interactions between the treatments and each of these ecological factors, and colony size as a random factor. Models were checked for residual fit using the *DHARMa* package (Hartig, 2016), given a distribution that seemed adapted to the type of data. Model reduction was performed based on the assessment of multicollinearity using the *performance* package (Lüdecke et al., 2019) to eliminate parameters causing collinearity, followed by stepwise automatic model reduction using the *buildmer* package (Voeten, 2019) based on the Akaike Information Criterion (AIC) to obtain the final models. The output of the resulting models was extracted using the *emmeans* package (Lenth, 2025) with the *joint_tests()*, *emmeans()*, and *emtrends()* functions to obtain the estimates.

Results

Injured ants engaged in allogrooming more frequently and for longer durations ($\chi^2 = 15.17$; $p = 0.0001$; $\chi^2 = 22.42$; $p < 0.0001$, respectively) and showed a higher occurrence of cowering ($\chi^2 = 7.87$; $p = 0.005$) than control ants. Conversely, injured ants spent less time selfgrooming ($\chi^2 = 4.06$; $p = 0.05$) and engaged in selfgrooming less frequently ($\chi^2 = 4.98$; $p = 0.03$). They were also less frequently moving ($\chi^2 = 13.13$; $p = 0.0003$) or standing ($\chi^2 = 5.6$; $p = 0.02$) compared to control ants (Fig. S13). The duration of allogrooming was linked to an interaction between treatment and climate ($\chi^2 = 7.80$; $p = 0.005$; Fig. S14). Across climatic gradients, allogrooming duration ($\chi^2 = 15.28$; $p = 0.0001$) and allogrooming occurrence ($\chi^2 = 7.36$; $p = 0.007$) were negatively associated with PC1 climate. In contrast, both moving occurrence ($\chi^2 = 3.84$;

$p = 0.05$) and standing occurrence ($\chi^2 = 3.88$; $p = 0.05$) increased with PC1 climate. Higher parasite prevalence was associated with reduced allogrooming duration ($\chi^2 = 12.75$; $p = 0.0004$), while the occurrence of standing increased with parasite prevalence ($\chi^2 = 5.32$; $p = 0.02$).

Table S7. Ethogram of observed behaviours. List of observed and scored behaviours during the experiments, including abbreviations and detailed descriptions of each behaviour.

Behaviour	Abbreviation	Description
Allogrooming	G1	Interaction of sister ant with the injury or the control leg
Selfgrooming	G2	Selfgrooming the injury or the control leg
Antennating	A1	Antennating the injury or the control leg
Activity		
Moving	V1	Moving actively around
Cowering	V2	Laying on the back or side and not moving (shock or dead)
Standing	V3	Standing straight/still
Interaction		
Marker	X1	Interacting with red wire loop

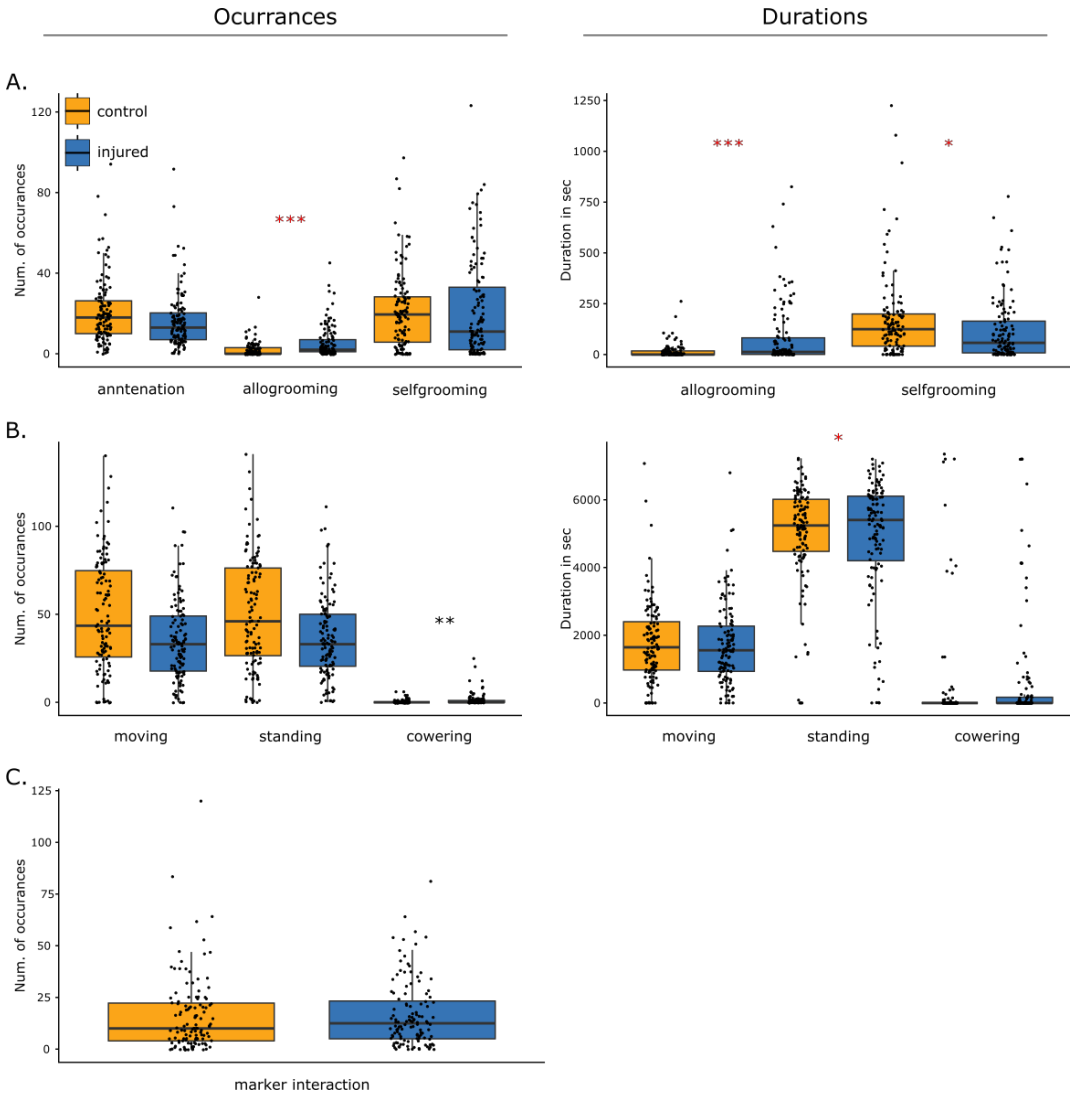


Figure S13. Behavioural responses to injury. Boxplots of occurrences and durations of (A) social and grooming behaviour, (B) active behaviour, and (C) interaction with the wire loop. Significant differences ($p < 0.05$) between control and experimental ants are depicted via red asterisks.

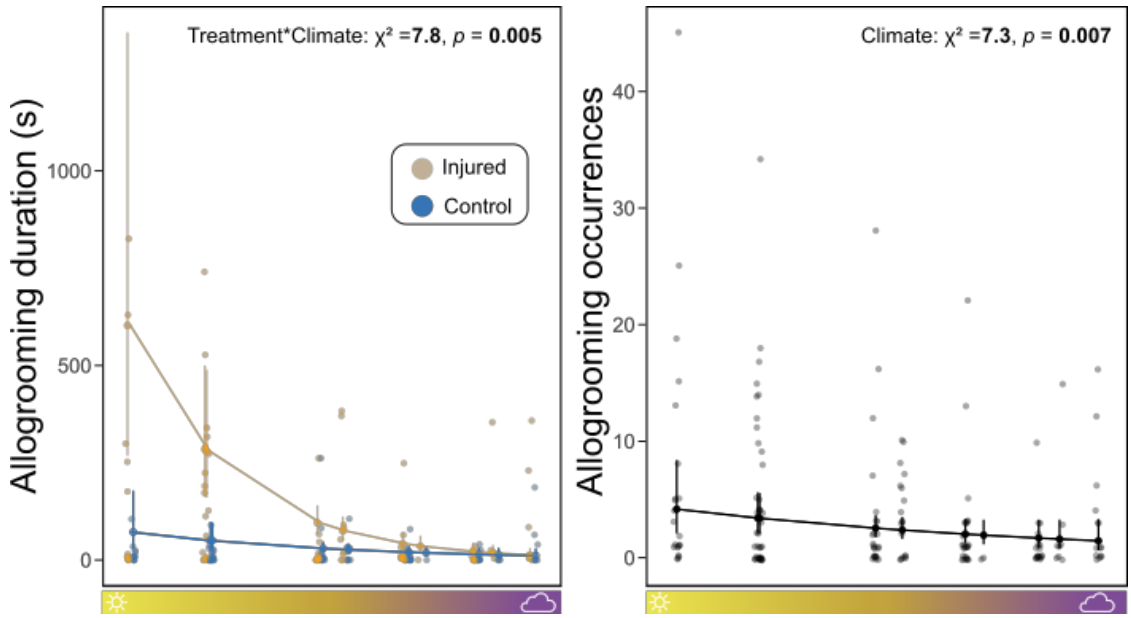


Figure S14. Effects of climate on allogrooming duration and occurrence. Allogrooming duration was linked to an interaction between treatment and climate ($\chi^2 = 7.8$; $p = 0.005$). Across climatic gradients, allogrooming duration ($\chi^2 = 15.3$; $p = 0.0001$) and allogrooming occurrence ($\chi^2 = 7.3$; $p = 0.007$) were negatively associated with PC1 climate.

Discussion and Conclusion

This study examined social and grooming behaviours in response to ant injury, factoring in climate and parasite prevalence while controlling for colony size. Results indicated that ants from warmer climates groomed injured nestmates more frequently and for longer, suggesting temperature positively affects altruistic care. Notably, in warmer regions, injured ants received more allogrooming compared to controls. The combination of parasites and higher average temperatures, which increase pathogen proliferation (Linder et al., 2008), may lead to increased care to minimise worker loss and prevent microbial infections after parasite raids. However, our analysis also revealed that higher parasite prevalence was linked to less allogrooming, suggesting that in highly parasitised environments, colonies may allocate resources differently, opting for brood care over wound care due to increased infection fatality risk.

General Discussion

1. Summary of Key Findings

Understanding how antagonistic species coevolve across heterogeneous landscapes remains a central challenge in evolutionary biology. While behavioural and ecological outcomes of social host-parasite interactions have been studied extensively, the genomic underpinnings of these dynamics are less understood. In this thesis, we investigated the coevolutionary dynamics between the social ant host *Temnothorax longispinosus* and its parasite, *Temnothorax americanus*, integrating genomic, phenotypic, and environmental data to explore how coevolution unfolds across a broad geographic range characterised by distinct biotic and abiotic conditions. **Chapter 1** reviewed omics-level molecular adaptation studies in social host-parasite systems of *Hymenoptera*, showing that social parasites often exhibit a loss of odorant receptor genes. At the same time, distinct genetic and regulatory changes underlie specific parasitic strategies. The chapter also highlights the potential of novel molecular approaches to elucidate the genomic mechanisms of host-parasite coevolution, including genome-wide association studies (GWASs). In **Chapter 2**, we used such an approach with pooled genome sequences of the host populations of *T. longispinosus* across ten locales with various parasite population sizes and distinct climatic conditions. We uncovered a link between the two environmental traits, where warmer and drier climatic conditions positively associate with parasite prevalence. A GWAS identified genomic loci associated with local parasite prevalence and climate, finding double the number of climate-adapted genes than parasite-adapted ones. For the latter, we identified a class of immune genes (*PGRPs*) related to microbial recognition in the host to be under strong parasite-induced selection. We hypothesise that this adaptation counters aggressive tactics of parasites that harm host workers during raids, effectively reducing the risk of secondary infections. However, the specific mechanisms may not be straightforward, necessitating further investigation. In **Chapter 3**, we investigated variations of pivotal phenotypes across environmental gradients through large-scale behavioural and chemical experiments in sympatric host-parasite pairs. Variations in host chemical profiles were shaped by local climate, whereas those of the parasite were shaped by parasite prevalence, suggesting environmental drivers affect both species differently. Climate, rather than parasite prevalence,

significantly impacted the behaviour of both species: parasites' aggression increased with warmer climates, while that of their hosts decreased, resulting in contrasting behavioural strategies across their habitats. In **Chapter 4**, by combining aggression and chemical data from Chapter 3 with methodologies from Chapter 2, we used individual whole-genome data from both species to identify the genomic basis of key phenotypic traits and adaptations to their shared environment. GWASs identified genes evolving in parallel in both species, particularly those related to behaviour and perception, with selection primarily acting on species-specific genes. We confirmed the selection on several *PGRP* genes associated with parasite prevalence in the host. The parasite exhibited less pronounced selection signals, with genes under selection potentially mediating its behavioural transition during raiding season. Interestingly, the chemical profile in both species showed signs of selection on *odorant receptor* genes, connecting signalling to their perception at the genome level. Climate adaptations were found to be facilitated using similar genes, such as those relating to the biosynthesis of chemical compounds relevant to desiccation resistance, indicating similar climate-induced selection pressures in both species. On the population structure, hosts and parasites showed asymmetries: The host was nearly panmictic, while the parasite showed clearer structures, potentially influencing their abilities for specialised adaptation to their local reciprocal partners. This highlights the complex genomic interplay in adapting to environmental factors alongside their coevolution, making it more difficult to pinpoint the source of co-adaptive traits. In the following sections, these findings are placed within a broader framework of host-parasite coevolution across heterogeneous environments. Section 2 examines how spatial population structure, climate gradients, and dispersal asymmetries shape the coevolutionary landscape of both species. Section 3 focuses on the genomic architecture of key behavioural and chemical traits, highlighting the roles of pleiotropy, plasticity, and selective interference in modulating trait expression and adaptation in this social host-parasite pair.

2. Environmental Heterogeneity and the Coevolutionary Landscape

2.1. Geographical Structure of Coevolution - Past and Present

Social parasitism provides a powerful lens through which to study coevolution, as environmental factors can influence interacting species similarly due to shared ancestry. However, selective pressures shaping host and parasite genomes are rarely uniform: differences arise from distinct life history traits, population structures, and genomic architectures shaped by their demographic history, even if species are embedded within the same ecological context. Co-adaptations thus emerge from the interplay between evolutionary history, spatial population structure, and ecological factors of species interactions across environmental gradients (Thompson, 2005; Urban et al., 2008; Laine, 2009; Laine & Tylianakis, 2024). The legacy of post-glacial colonisation, climatic shifts, asymmetric dispersal, and barriers to gene flow creates locally divergent populations that shape the intensity and direction of selection in reciprocal interactions. For instance, the butterfly *Aricia agestis* has undergone a northward range shift of approximately 10 km per year in Britain since the 1990s, attributed to climate warming. Within these newly colonised areas, larval mortality is reduced due to lower parasitism by specialist parasitoids, illustrating an 'enemy release' dynamic at range margins (Menéndez et al., 2008). Huffaker's classic mite experiments demonstrated that spatial heterogeneity alone, even without climatic factors, can enable prey persistence by creating predator-free areas (Huffaker, 1958), restricting and shaping species interactions locally.

In the social parasitic ant *Temnothorax americanus* and its host *T. longispinosus*, post-glacial expansions from southern refugia following the retreat of ice sheets approximately 11,000 years ago are suspected in both species based on geographic and historical data (Chapter 4). However, differential expansion rates, habitat preferences, and physiological constraints may have produced the distinct genetic structures detectable nowadays. While *T. longispinosus* displays a north-south latitudinal differentiation, but still approaches panmixia, *T. americanus* exhibits an additional east-west longitudinal differentiation and shows strong isolation-by-distance, suggesting a more complex biogeographic structuring. These patterns mirror findings in other social insects, where the presence or lack of historical refugia and restricted dispersal shaped contemporary genetic structure

(e.g., *Formica* ants; Johansson et al., 2018). Recent detections of *T. americanus* in previously parasite-free regions support the hypothesis of a recent northward range expansion, potentially facilitated by milder winters and hotter summers under ongoing climate change in non-coastal New England (Chapters 2 and 4) (Earth@Home, 2024). Such expansions generate spatial and temporal variation in parasite prevalence and selection pressures, generating a geographic mosaic of coevolution (Thompson, 2005). Several factors may interact to produce the uneven distribution of host and parasite populations. These mechanisms likely do not operate in isolation but are reinforced by one another across environmental gradients. Below, plausible drivers are summarised:

- **Thermal Tolerances:** Parasites may possess a narrower thermal niche or lower cold tolerance than their hosts, as evident in their higher prevalence in warmer regions, limiting their (reproductive) success in cooler northern climates. Analyses on thermal windows are lacking for both species, but divergences even among closely related ectotherms are not uncommon (Dixon et al., 2009).
- **Climate Constraints and Range-Edge Maladaptation:** Range-edge parasite populations may be limited by insufficient warm summer days to initiate raids. Smaller effective population sizes near range margins may further constrain adaptation to new climate conditions, exacerbating maladaptation (Dybdahl et al., 2014).
- **Contrasting Dispersal Strategies:** The hosts weak population structure may be driven by high gene flow through long-range male-biased dispersals (Pennings et al., 2011) facilitated through prevailing south-west to north-east winds along the Appalachian range (Hagreen & Leslie, 2004; U.S. EPA 2016), a pattern similarly observed in mosquitos (Huestis et al., 2019).
- **Enhanced Host Defences in the North:** Northern host populations exhibit significantly higher aggression due to resource scarcity (Chapter 3, Segev et al., 2017; more in Section 2.3), incidentally reducing parasite success.
- **Avoidance Behaviour:** Hosts may avoid parasite-rich areas, promoting spatial segregation and creating ‘enemy-free spaces’ (Letourneau & Altieri, 1999).

The effect of some or all the listed factors may be reflected in *T. americanus* being more adapted to sympatric host populations (Foitzik et al., 2009), whereas *T. longispinosus* exhibits little difference in its defensive responses toward sympatric versus allopatric parasites (Foitzik et al., 2001; Brandt & Foitzik, 2004). This asymmetry suggests that host defences are more generalised and primarily shaped by local climatic conditions rather than parasite prevalence (Chapter 3). Genomic analyses further reveal species-specific genes differentiated between populations of both species (Chapter 4), indicating that even shared environmental gradients can act on divergent sets of genes in host and parasite, as these differences likely stem from their contrasting life history strategies, demographic histories, and dispersal capacities. These align with broader models of coevolution: the coexistence of tightly matched traits (e.g., immune gene responses in the host; Chapters 2 and 4) and more environmentally modulated traits (e.g., behaviour and CHC profiles; Chapter 3) suggests that pairwise and diffuse coevolutionary dynamics operate concurrently. While pairwise coevolution entails strong reciprocal selection between specific host-parasite genotypes, diffuse coevolution captures broader, indirect adaptations shaped by multiple interaction partners or environmental constraints (Janzen, 1980; Gomulkiewicz et al., 2007).

2.2. Climate as a Coevolutionary Modifier

Climate emerges as a major ecological force in shaping host-parasite coevolution in our system: Climate and parasite prevalence are tightly correlated (Chapter 2), yet climate alone consistently predicted behavioural variation and chemical composition more robustly in both *T. longispinosus* and *T. americanus* (Chapter 3). In terrestrial ectotherms like ants, temperature and behavioural activity levels are linked due to their effect on the metabolic rate (Sunday et al., 2011; Krapf et al., 2023; Menges et al., 2023). However, the pivotal behaviour of aggression showed asymmetric expression in *T. longispinosus* and *T. americanus*, despite both species being exposed to the same climatic conditions. Such asymmetric effects of temperature on key traits in species interaction were found in the perception of chemical cues. For instance, in *Formica* ants, lower temperatures impair nestmate recognition (Tanner, 2009), but in *Lasius* ants, recognition declines with acclimation to higher temperatures (Wittke et al., 2022). Thus, the relationship between

climate and species interactions (via recognition and subsequent behaviour) is complex, likely influenced by multiple interacting factors rather than following a uniform directional trend with climate or temperature. This, however, does not imply that parasite pressure is inconsequential in the expression of these traits. Instead, the stronger predictive power of climate may reflect the continuous nature of climate variables, and the greater reliability of environmental data compared to estimates of parasite prevalence. Consequently, climate must be considered a significant actor in host-parasite coevolution, shaping adaptations that may be equally, or even more profoundly, driven by environmental pressures alongside biotic interactions. In our study species, these patterns are mirrored at the genomic level: despite their contrasting population structures, *T. longispinosus* and *T. americanus* exhibited almost double the numbers of genes under selection to climate than those to parasite prevalence, with substantial overlap in climate-adapted genes and their functions between the two species (Chapter 4). This striking similarity highlights climate as a dominant and spatially pervasive force, shaping genomic adaptation even across species with highly divergent population structures and lifestyles. In social parasite taxa and their hosts, shared ancestry can lead to conserved usage of genes due to experiencing similar environmental pressures (Cini et al., 2015). Climate-adapted genes often reoccurred in other GWASs, predominantly genes associated with CHC biosynthesis, likely due to their desiccation protection properties (e.g., *FAS* and *FAR* genes). Such convergence suggests that selection on shared molecular pathways may arise from the simultaneous pressures of climatic and biotic factors, potentially constraining the routes available for adaptation (more in Section 3.2.). Thus, climate adaptation and coevolution may be intertwined already at the genomic level through pleiotropic effects, mainly of genes involved in the CHC biosynthesis, as they modulate climate adaptation and social communication.

Climate may also influence the seasonal timing of interactions. Climate conditions during key phenological windows, such as breeding or raiding seasons, can decisively affect the outcome of parasitic events. In avian brood parasites, climate change has shifted the migratory arrival of hosts and parasites at their shared breeding sites, resulting in mismatches affecting parasite success (Saino et al., 2009; Mikula et al., 2024). In *Polistes* wasps, parasitic queens preferentially usurp host colonies with large nests and brood in

an advanced stage of development (Cervo & Turillazzi, 1996), synchronising host vulnerability with parasitic attack. *T. americanus* similarly prefers larger host colonies (Alloway, 1979), but mainly targets eggs and young larvae (Brandt et al., 2005). With raiding season coinciding with the emergence of young host workers in late summer, this may hint at synchronised raids with the emergence of naïve host workers, who may lack prior raiding experience. This timing may be reflected at the genomic level as well: In Chapter 4, we identified that parallel-evolving genes associated with parasite prevalence have roles in time tracking, possibly seasonal regulation, pointing to the already described importance of time-tracking genes in the evolution of social parasitism (Feldmeyer et al., 2017). These findings reinforce that climate shapes trait expression, phenology, and behavioural plasticity in ways that critically impact coevolutionary dynamics.

2.3.Dynamic Trait Variations - The Hawk-Dove Game

Across their interaction range, aggression, the key phenotype for parasite offence and host defence, varies in opposite directions between species. Segev et al. (2017) described this as a result of resource limitations in colder regions, supported by climate being the primary explanatory factor in aggression variations in both species (Chapter 3). However, aggression remains a pivotal phenotype in social host-parasite interactions (Jongepier et al., 2014, 2015). In dulotic species, raids resemble episodic social predator hunts, requiring coordinated group behaviour and (chemical) communication for successful prey detection and retrieval (Hall et al., 2010; Lang & Farine, 2017). This cooperative predation involves population-specific tactics and specialised behavioural routines: The strategic reduction of certain chemicals and the use of venom for prey subordination is common in social ant predators (Powell & Clark, 2004; Dejean et al., 2024) and also present in the focal dulotic parasite species (Brandt et al., 2006; Jongepier et al., 2014; Chapter 3). As such, reframing social parasites to predators might help explain divergent behavioural adaptations through mathematical interaction models. Here, the Hawk-Dove game (Maynard Smith, 1976) offers a fitting framework for modelling asymmetric interactions over contested resources of similarly sized individuals. This may help identify the ecological factor that may lie at the intersection of climate and parasite prevalence, explaining the link identified in Chapter 2. In this model, individuals compete

for a resource of value *v* at a cost *c*, adopting either an aggressive (Hawk) or peaceful (Dove) strategy depending on the cost-value ratio. For instance, in the butterfly *Pararge aegeria*, males retreat from sunspots already occupied by rivals, reflecting a Dove strategy when habitat availability lowers the value of the contested resource (Davies, 1978). Consequently, the cost-value landscape might shift in areas where sunspots are rare, resulting in a Hawk strategy. In the focal social host-parasite system, this framework captures the climate-modulated, asymmetric interaction between *T. longispinosus* and *T. americanus*: In the host, *v*, the value of brood, increases in colder climates due to limited foraging time and higher resource scarcity, favouring aggressive, Hawk-like defences as already identified by Segev et al. (2017). In warmer climates, brood may be less valuable relative to the cost of fighting, making Dove-like strategies like nest evacuation[1] more favourable. In contrast, the parasite faces increasing *c*, cost of failure, under parasite-dense conditions: In warmer regions with more parasitic colonies, intraspecific competition intensifies, especially since host colonies become more defensive after already encountering a parasite (Pamminger et al., 2011; Alleman et al., 2018; Koenig & Moreau, 2024c). Each raid thus reduces the availability of unalerted host colonies within a population. Under these conditions, a one-shot, high-aggression strategy may be more optimal, favouring the parasites' Hawk-like tactics in warm, parasite-dense areas. The inverse pattern, reduced aggression in parasites from colder, lowly parasitised areas, may reflect maladaptation rather than local optimisation: In newly colonised populations, Dove-like strategies may persist due to founder effects, genetic drift, or mismatched climate conditions as described before, particularly where selection is weak or interfered by other pressures. Such evolutionary lags are common in expanding or bottlenecked populations (Schlaepfer et al., 2002). Thus, this model supports the 'fight-to-flight' portfolio proposed by Jongepier et al. (2014), but with behavioural tactics shifting in response to changes in the cost-value landscape across climatic gradients.

[1] Nest evacuation, a Dove-like behaviour, was interpreted as a response to parasitism (Jongepier et al., 2014), but similar to aggression, may likely be shaped by climate. Nest relocation is intrinsic to *Temnothorax* ants and serves to evade raids (Koenig & Moreau, 2024b), with potential thermoregulatory functions (Pinter-Wollman & Brown, 2015; Villalta et al., 2020a), supporting a general link between climatic variability and relocation frequency. In *Temnothorax crassispinus*, repeated relocations enhance efficiency in nest relocations (Mitrus, 2016), suggesting a learned component, and may be, if often utilised for thermoregulation in warm climates, more readily utilised during a raid.

3. The Genomic Architecture of Host-Parasite Coevolution

3.1. Adaptive Gene Expression as the Driver of Behavioural Plasticity

Aggression in social insects is a highly context-dependent trait, shaped by a complex interplay of environmental cues, internal physiological states, social signals, and proximate factors such as developmental history and genetic architecture (Sih et al., 2004; Jandt et al., 2014). Extrinsic factors like elevated temperatures can increase aggression by raising metabolic rates (Barki et al., 2022), while intrinsic factors, such as caste, nutritional status, circadian rhythms, and general activity, modulate behaviour according to the colony's internal state (Grover et al., 2007; Knadler & Page, 2009; Jandt et al., 2014; Kamhi et al., 2015). During (social) interactions, aggression can be triggered by detecting non-nestmates via mismatches in their chemical profiles or other chemical cues (Scharf et al., 2011; Von Beeren et al., 2011; Villalta et al., 2020b; Kannan et al., 2022). However, the decision to attack is not a simple reflexive response to sensory cues. Instead, it may be mediated by flexible cost-benefit evaluation, as described in the game-theoretical model above, where the value of the resource is weighed against the likelihood of success and the potential costs of escalation (Enquist & Leimar, 1987). This decision-making landscape is susceptible to shifting ecological and social conditions, including climate variation (Segev et al., 2017; Chapter 3) and parasite prevalence (Jongepier et al., 2014). Consequently, aggression is expected to rely less on fixed genetic predispositions and more on condition-dependent regulatory mechanisms. Consistently low heritability estimates for genes associated with aggressive behaviour support this view (Van Oers et al., 2004; Van Oers & Mueller, 2010) and suggest dynamic modulation instead of genetic hardwiring. At the molecular level, these behavioural dynamics are mediated by changes in gene expression, particularly in the brain, where important neuroendocrine peptides (i.e., serotonin, dopamine, and octopamine) regulate arousal thresholds and responsiveness (Kamhi et al., 2015; Aonuma & Benelli, 2023; Barbero et al., 2023). Thus, transcriptional plasticity allows real-time behavioural adjustments to social and environmental stimuli (Zayed & Robinson, 2012; Renn & Schumer, 2013; Ruiz-Ortiz & Tollkuhn, 2021). For instance, exposure to dulotic parasites alters transcriptomic profiles in the brain and antennae of enslaved ant hosts, suggesting adaptive rewiring of

chemosensory pathways in response to parasitic contexts (Ozaki et al., 2005; Stoldt et al., 2023; Watanabe et al., 2023). Caste-specific transcriptomic shifts further underline the importance of plasticity, as caste differentiation relies on behavioural transitions regulated through both gene expression and epigenetic mechanisms (Howe et al., 2016; Geffre et al., 2017; Opachaloemphan et al., 2018; Sieber et al., 2021). These regulatory pathways enable social insects to rapidly modulate caste-related behaviours, such as foraging and aggression, in response to changing conditions. If such plastic responses consistently confer fitness benefits under stable selective regimes, they may become genetically assimilated over evolutionary time and leave detectable genomic signatures (Price et al., 2003; Ghalambor et al., 2015).

While climate statistically explains more variation in behavioural and chemical phenotypes (Chapter 3), parasite prevalence strongly imprints the underlying gene regulatory and transcriptomic architecture in both host and parasite (Chapters 2 and 4). These findings highlight the evolutionary importance of adaptive gene expression and its plasticity in coevolutionary systems, particularly under persistent biotic pressures such as parasite prevalence. This underscores the need to consider plasticity as a multi-layered process, with trait expression shaped by environmental and evolutionary influences dynamically. For example, previous work has shown that the behavioural and chemical strategies of *T. americanus* can modulate brain gene expression in *T. longispinosus*, thereby altering its behavioural responses (Kaur et al., 2019). Expanding on this, constitutive gene expression in the host fat body is shaped by parasite prevalence (Chapter 4), and global antennal gene expression strongly correlates with local parasite prevalence (Chapter 2). This is particularly relevant given that the transcriptomic data in both Chapters were generated from ants kept under standardised laboratory conditions for several months after collection, reflecting baseline gene expression in the absence of immediate environmental triggers. Therefore, the observed pattern supports a scenario of genetic assimilation, where persistent parasite pressure has selected for stable, population-specific expression profiles, potentially fine-tuning the host's recognition system towards its local parasite. Genes under selection with parasite prevalence in the host further reflect this trend, most notably the *insulin-degrading enzyme* (*IDE*), a caste-specific gene in host foragers (Caminer et al., 2023; Chapter 4). As foragers are the

primary defenders during raids (Koenig & Moreau, 2024a), *IDE* may facilitate caste-biased expression of aggression-related traits in response to parasite threat. In the parasite, selected genes were also implicated in behavioural transitions between raiding and non-raiding states (Chapter 4). Additionally, selection on *juvenile hormone esterase* associated with aggression in the parasite suggests a parallel regulatory mechanism influencing queen-like dominance behaviours (Kelstrup et al., 2014), potentially enhancing raiding success. However, more broadly, genes under selection with parasite prevalence, and gene expression associated with parasite prevalence, showed enrichment for gene regulatory functions in both species. This suggests that selection may predominantly target regulatory control points influencing genes and gene expression to induce behavioural shifts associated with caste in the host and raids in the parasite.

3.2. Selective Interferences in the Chemical Profile

Adaptive traits may evolve under conflicting ecological pressures, where optimising one trait compromises another, a phenomenon known as selective interference (Ghalambor, 2003). Such trade-offs are common across taxa: floral traits that attract pollinators can also draw herbivores (Muola et al., 2022), and melanism in vipers enhances thermoregulation but increases predation risk (Andrén & Nilson, 1981; Martínez-Freiría et al., 2020). In social insects, both desiccation resistance and chemical communication rely on cuticular hydrocarbons (CHCs), creating potential conflicts where climate and temperature adaptation can impair social signalling (Wittke et al., 2022). CHC biosynthesis is controlled by pleiotropic genes (Wang et al., 2025), with different compound classes facing distinct selection regimes: Linear *n*-alkanes form tight, high-melting-point layers that reduce water loss on the insect cuticle, whereas methyl-branched *n*-alkanes facilitate communication, but have lower melting points due to branching, making them more volatile in warm climates (Ikedou et al., 2005; Martin et al., 2013; Wittke et al., 2022). Across ant species, warmer climates are typically associated with an increase in linear *n*-alkanes, though species-specificity in the compositions of the chemical profile is evident (Menzel et al., 2018; Sprenger et al., 2018; Villalta, et al., 2020b; Baumgart et al., 2022). In this species-specific composition, genetic background and lifestyle might select for climate resilience and effective communication

differentially, ultimately aiming for a 'sweet spot' in CHC composition, balancing both functions. Such a balance could be achieved through local adaptation, allowing CHC profiles to evolve in response to inferring population-specific selection pressures (Villalta et al., 2020b). While a near-panmictic population structure (Chapter 4) may limit the potential for regional divergence in the chemical profile, population-specific CHC variation has nonetheless been observed (Jongepier & Foitzik, 2016; Kleeberg et al., 2017; Chapter 3). These host divergences were shaped more strongly by climate than parasite prevalence (Chapter 3), implying that the intense abiotic pressure of climate may overcome high gene flows and potentially override local adaptation to parasites. In contrast, the parasite *T. americanus* shows stronger associations between CHC profiles and its prevalence, while climate plays a secondary role. Limited gene flow may permit such local adaptation in the parasite (Chapter 4). However, increased intrapopulation CHC variance in colder regions (Chapter 3) complicates this interpretation but may be explained by small effective population sizes restricting local adaptation, resulting in the aforementioned maladaptation at range margins.

In the host, the key conflict may lie between maintaining long-chain linear CHCs for desiccation resistance and methyl-branched CHCs for social recognition (Dani et al., 2001; Van Zweden & d'Ettorre, 2010; Lorenzi et al., 2011). Investment in one function limits the other, positioning compound classes under selective interference. In contrast, the parasite may face fewer such constraints. As a dulotic species, *T. americanus* employs chemical insignificance as a chemical tactic to avoid host detection (Kaur et al., 2019). The subsequent selection for higher abundances of linear CHCs may incidentally result in better desiccation resistance, which may lie at the heart of its higher success in warmer regions. Yet, this introduces a different trade-off: maintaining short-chain linear CHCs for chemical invisibility reduces waterproofing, as shorter chains are less effective at preventing desiccation (Gibbs & Pomonis, 1995). In *T. americanus*, a gene annotated as *fatty acid amide hydrolase* (*FAAH*) genetically exemplifies such conflict: It was found to be under selection with both climate and parasite prevalence and was the only gene differentially expressed in head tissue linked to parasite prevalence (Chapter 4). Although not directly involved in CHC biosynthesis (Holzer et al., 2009), a *FAAH* in *Apis mellifera* has been implicated in CHC composition. This gene was found to be expressed in

oenocytes where CHC biosynthesis occurs and is thought to regulate important precursors (Moris et al., 2023). *FAAH* knockdown via RNAi in honeybees led to longer-chained CHCs, suggesting a regulatory role in chain lengths of CHCs. In the focal parasite, the identified *FAAH* may thus act as a switch promoting shorter CHCs to evade host detection (Kleeberg et al., 2017). This would place chain length, rather than compound class, at the centre of selective interference in the parasite, consistent with its strong genomic signals of this trait (Chapter 4). Additional evidence comes from transcriptomic data: several genes mediating fatty acid elongation (*ELOVL*) were found to be downregulated in *T. americanus* prior to raiding events (Alleman et al., 2018), possibly resulting in shorter CHC chain length during host infiltration. This might suggest seasonal adjustments in CHC chain lengths during raiding season, but remains to be investigated, since it is unclear if identified *FAAHs* are orthologous or even retain the same function in honeybees and ants. Selective interferences in the chemical profile may thus operate along distinct axes in the two species: CHC class in the host and chain length in the parasite. These divergent strategies highlight how lifestyle and ecological constraints shape the coevolution through opposing selective forces.

3.3. Selection on *PGRPs* - Immune Investment or Neofunctionalisation

Among the clearest genomic signals of parasite-driven selection in *T. longispinosus* were the selection on *peptidoglycan-recognition protein* genes (*PGRPs*) (Chapters 2 and 4). These immune-associated genes were exclusively under strong selection with parasite prevalence, suggesting a specialised role in host-parasite dynamics, with no signs of selection on *PGRPs* in the parasite genome. *PGRPs* are under positive selection in social insects (Viljakainen et al., 2009) and were found to retain conserved roles in microbial recognition and immune activation in various ant species (Felden et al., 2024; Masson et al., 2024; Zhu et al., 2024). Ongoing research indicates that *PGRPs* are expanded in *Temnothorax* hosts but show signs of gene loss in dulotic *Temnothorax* parasites (Viljakainen, pers. comm.). This pattern resembles the evolution of odorant receptor genes in social insects, where duplication during the transition to eusociality is followed by caste-specific subfunctionalisation and loss in social parasites due to reduced reliance on fine-tuned communication (Zhou et al., 2012; McKenzie et al., 2016; Pask et al., 2017).

Yet, in contrast to odorant receptor genes, the function of duplicated *PGRPs* in social host-parasite systems remains unresolved. While canonical *PGRPs* detect bacterial peptides, sub- or neofunctionalised variants may instead recognise parasite-related compounds during their scouts and raids. These could include non-CHC molecules such as short peptides, volatile pheromones, or manipulation compounds secreted during raids (D'Ettorre et al., 2002), or, additionally, parasite-associated microbial communities. This aligns with findings that immune signalling can modulate social behaviour via gene expression even in the absence of a (microbial) infection ('sterile infection'; Bos et al., 2012; Yanagawa et al., 2017). Even further, infections can lead to persistent changes in behaviour and gene expression upon exposure, as was found in *Drosophila* and other insects (Kodrík et al., 2019; Low et al., 2022; Romano et al., 2022; Vincent et al., 2022). As such, neofunctionalised *PGRPs* may no longer act solely as microbial detectors but as sensors of parasite-induced signals, akin to damage-associated molecular patterns recognised by specific vertebrate receptors (Chen & Nuñez, 2010; Rock et al., 2010). Such a role would not lead to direct changes, consistent with the absence of selection signs of *PGRPs* in behavioural and chemical GWASs. Instead, they may indirectly initiate regulatory cascades that prime transcriptional and physiological defences, or lead to epigenetic alterations following parasite contact (Mukherjee & Dobrindt, 2024), potentially explaining changes in behaviour, gene expression and venom production evident in the host species after parasite encounters (Pamminger et al., 2011; Alleman et al., 2018; Koenig & Moreau, 2024c). Whether duplicated *PGRPs* in *T. longispinosus* retain immune functions or have acquired new sensory roles remains unknown. Their putative duplication, parasite-specific association, and lack of selection in the parasite make them strong candidates for future studies on the molecular basis of coevolved recognition and subsequent defences in social insects.

4. Perspectives

This thesis demonstrates that coevolutionary dynamics between social parasites and their hosts are shaped by a complex interplay of climate and parasite pressure across geographic mosaics of species interaction, paired with their divergent life histories and strategies. Climate consistently emerged as the dominant force influencing behavioural

and chemical phenotypes. Specifically, behavioural asymmetries in host and parasite may stem from divergent, species-specific selection landscapes across their climatically heterogeneous habitats. Different ecological forces shape chemical profiles: Host CHC profiles are mainly shaped by climate, with stronger selection pressure of climate favouring selection for compounds not ideal for the recognition of parasites. The parasite's chemically insignificant strategy and climate adaptations may select in the same direction, namely the production of linear CHCs. The possible trade-off may emerge in CHC chain length since longer chains aid desiccation resistance, while shorter ones may reduce detectability. Accordingly, pleiotropic genes involved in CHC synthesis, modification and perception (*FAS*, *FAAH*, *ORs*, and *GRs*) were repeatedly identified across GWAS and transcriptomic datasets in both species, placing chemical compounds and detection at coevolution's centre. Further, genes under coevolutionary selection were highly species-specific. The strongest selection signal was found in the host for *PGRPs*, where sub- or neofunctionalisation could be assumed, since canonical *PGRPs* in other insects have also been shown to mediate behaviour and gene expression downstream. This aligns with enrichments for gene regulatory functions in many of the analysed traits, highlighting the importance of plasticity and regulatory dynamics in shaping coevolutionary outcomes.

While this thesis provided novel insights into the gnomic basis of social host-parasite coevolution, leading questions are plentiful. The following suggestions might inspire further relevant studies in social host-parasite coevolution.

1.) From Genomic Signals to Functional Validation

Despite evidence of genomic adaptation in social parasites and their hosts (Chapter 1), GWAS remains underutilised in these systems (Rönkä et al., 2024). Their scarcity complicates predictions about genes under selection, especially when parasite-associated traits covary with climate or population structure, as identified in Chapter 3. Candidate genes involved in CHC biosynthesis, chemosensory perception (e.g. odorant receptors), and immune recognition consistently emerged across GWAS and/or transcriptomic datasets or showed strong signs of selection. Their recurrence suggests that social parasite coevolution centres on the molecular architecture of communication and recognition.

Functional approaches, such as CRISPR/Cas (with protocols available for ants; Konu et al., 2023) and RNAi, combined with electroantennography and behavioural assays, could help to link gene functions and SNP variants to variations in aggression, perception, recognition, and decision-making to establish causality.

2.) GWAS Design for Social Parasite Systems: Methodological Considerations

High trait plasticity in social insects complicates genotype–phenotype mapping, especially when phenotypes vary between individuals. In this study, pooling individuals improved signal detection in gene expression analyses (Chapter 2), suggesting that pooled RNA-seq can reduce noise and better capture population-level patterns if constitutive transcriptomes are investigated. Further, using Bayesian or machine-learning GWAS methods (e.g. 'AutoML', Lakiotaki et al., 2023) may increase power to detect small-effect loci underlying coevolved traits, especially if genomic signals are amplified via pooled Whole-Genome-Sequencing (e.g. 'popGWAS', Pfenninger, in press). However, social parasites are often rare and classified as a vulnerable species (IUCN, 2022), making large-scale pooling ethically challenging. This intensifies the 'large p, small n' problem of high-dimensional data with limited samples, potentially leading to diffuse genomic signals of selection as identified in Chapter 4 in the parasite. When Pool-seq is not feasible, computational pooling across timepoints may offer a workaround, enhancing insights into the parasite's genomic adaptation while minimising oversampling.

3.) Climate Change and Trait Divergence

Many traits once considered adaptations to parasitism, such as aggression and chemical profiles, are strongly shaped by climate (Chapter 3). Future work should disentangle the specific climatic drivers (e.g. maximum temperature during raiding season) influencing these traits alongside parasite prevalence (e.g. nest evacuation as identified by Jongepier et al., 2014). For instance, *T. americanus* workers raid more often on exceptionally warm days, and with increasing numbers of hot summer days in the sampled regions (U.S. EPA, 2023), this may expand its raiding window, especially in cooler regions. Both species show comparable numbers of climate-associated genes, yet their adaptive capacities may differ. The parasite's limited dispersal and stronger population structure may constrain its

genomic response relative to the more panmictic host. With generation times of 5–15 years, evolutionary responses may lag behind rapid climate change, especially in smaller populations in the north. As plasticity to adapt to changing climates reaches its limits, mismatches between behaviour and environment could impose asymmetric fitness costs on hosts and parasites. With the climatic data used in Chapters 2 – 4 covering 1981-2010 and parasite prevalence data spanning 2002-2024, their partial overlap may already capture ongoing climate selection, underscoring the need for continued monitoring and updated models.

In sum, this thesis offers novel insights into the genomic basis of social insect host-parasite coevolution, uncovering both expected and unexpected patterns. While we attempt to simplify complexity by studying closely related species, similarity does not guarantee simplicity. The result is a genomic mosaic of parallel paths, convergent strategies, and species-specific detours. While the picture remains incomplete, this work helps frame the edges of a puzzle and hands over a few corner pieces for future research to build upon.

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Curriculum Vitae

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Publications

Macit, M. N., Collin, E., Nieto-Blazquez, M. E., Kever, M., Litto, M., Jaitner, E., Pfenninger, M., Feldmeyer, B., & Foitzik, S. (submitted) The Genomic Basis of Social Parasitism: A Geographical Mosaic of Behavioural, Chemical, and Environmental Adaptations in a Widespread Host-Parasite System. *Molecular Biology and Evolution*.

Collin, E., **Macit, M. N.**, Wittke, M., Hörrmann, C., Haase, C., Heil, L., Menzel, F., Feldmeyer B., & Foitzik, S. (in review) Climate and Parasite Pressure jointly shape Traits Mediating the Coevolution between an Ant Social Parasite and its Host. *Journal of Evolutionary Biology*.

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Stoldt, M., Collin, E., **Macit, M. N.**, & Foitzik, S. (2023) Brain and antennal transcriptomes of host ants reveal potential links between behaviour and the functioning of socially parasitic colonies. *Molecular Ecology*. https://doi.org/10.1111/mec.17092.

Stoldt, M., **Macit, M. N.**, Collin, E., & Foitzik, S. (2022) Molecular (co)evolution of hymenopteran social parasites and their hosts. *Current Opinion in Insect Science*. https://doi.org/10.1016/j.cois.2022.100889.

Negroni, M. A., **Macit, M. N.**, Stoldt, M., Feldmeyer, B., & Foitzik, S. (2021) Molecular regulation of lifespan extension in fertile ant workers. *Philosophical Transactions of the Royal Society B*. https://doi.org/10.1098/rstb.2019.0736.