

# On mating, motherhood, and mortality

The entangled evolution of butterflies, eggs, and enemies.

Liana Owitz Greenberg

# Propositions

1. Ecological selection drives the evolution of post-mating odors.
   (this thesis)

2. Biological control is doomed to fail when it ignores the value of variation within species.
   (this thesis)

3. Misjudging insect maternal choices reveals the narrow lens of human-defined success.

4. Shared first authorship is a signature of independence and collaborative excellence.

5. Imposter syndrome improves scientific rigor.

6. Enthusiasm strengthens science.

7. The corporate university model threatens fundamental research that benefits society.

8. Equity in academia remains tokenistic without structural change to elevate women into scientific leadership.

Propositions belonging to the thesis, entitled

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## The entangled evolution of butterflies, eggs, and enemies

**Liana Owitz Greenberg**

**Thesis**

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CHAPTER 1



# General Introduction:
# Mating, motherhood, and mortality intertwined

## 1.1 Introduction

The lifeforms we see on Earth today look and behave as they do because, generation after generation, their inherited forms and behaviors allowed them to survive and reproduce. Every organism is the product of an unbroken lineage in which each ancestor not only reached reproductive age but did so under environmental pressures that demanded navigating complex trade-offs. They mated, reproduced, avoided predators, endured heat, cold, drought, and disease. Crucially, they did so successfully enough that their offspring could do the same. The cabbage white butterflies (*Pieris* spp., Lepidoptera: Pieridae) and their enemies, tiny egg parasitoid wasps (*Trichogramma* spp., Hymenoptera: Trichogrammatidae), form the central study system of this thesis (Figure 1.1). Though drastically different in size, behavior, and life history, their evolutionary fates are closely intertwined.

The path from birth to reproduction is fraught with complexity. Danger is ever-present: enemies that seek to consume or outcompete, climates that scorch or starve, habitats that shift beneath their wings. For a butterfly, this might mean choosing a sun-warmed leaf on which to bask, evading the flicker of a shadow that could be a bird, or laying a vulnerable egg where an enemy might not find it. For an egg parasitoid wasp, it might mean locating that same egg with astonishing precision and laying its own egg inside the butterfly's egg before the caterpillar can develop. It seems remarkable that any life persists, let alone flourishes.

Nevertheless, not only has life persisted, but it has also diversified. Across generations, slight differences in their form or behavior can determine whether an individual survives long enough to reproduce. A subtle difference in mate choice, choosing a more concealed

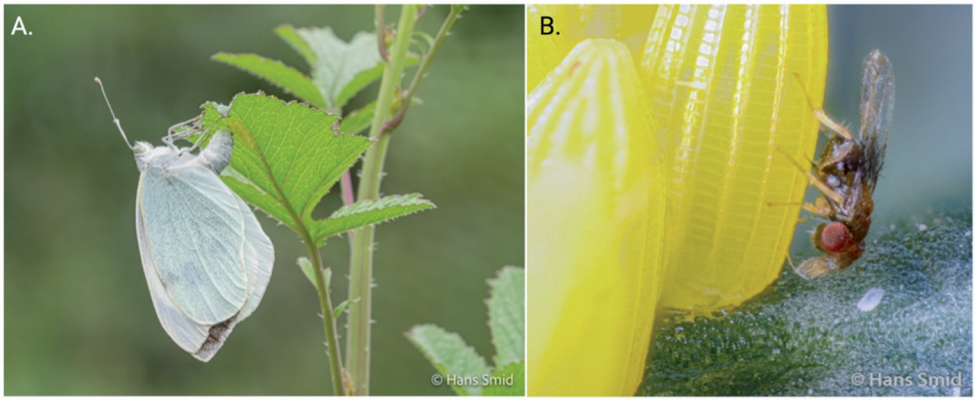



**Figure 1.1)** Study system **A**) Large cabbage white butterfly, *Pieris brassicae*, laying egg clutch on black mustard, *Brassica nigra*. **B**) *Trichogramma evanescens* parasitizing eggs of *P. brassicae*. Photos by Hans Smid.

place to lay eggs, or a heightened ability to evade an enemy can mean the difference between success and failure. In one place, evading predators may be the most pressing challenge, perhaps where birds lurk among cabbage fields, watching for movement. Surviving heat may be the most pressing challenge in another place. These butterflies may shift their activity to cooler mornings or favor host-plants that shade their eggs. An egg parasitoid population in one region might hone its host-finding skills with chemical cues. At the same time, another population may rely more on visual detection, adapted to the light and color of its native landscape. Over time, these local pressures drive populations down different evolutionary paths. Through accumulated variation within species (**intraspecific**) and divergence between them (**interspecific**), the vast, tangled web of biodiversity takes shape.

## 1.2 Study System: *Pieris* and *Trichogramma* in a tangled web

***Pieris*** butterflies are herbivores that specialize on plants in the crucifer family, **Brassicaceae**[1] (Figure 1.2). Several species, especially ***P. rapae*** and ***P. brassicae***, are major agricultural pests. They damage crops such as cabbage, broccoli, kale, and mustard, and are therefore frequent targets of pest management strategies[2,3]. The small cabbage white, *P. rapae*, is a globally invasive species that has spread across North America and other continents[2]. Similarly destructive, the large cabbage white *P. brassicae* lays large clusters of eggs that often result in heavy larval damage, particularly in parts of Europe and Asia[3]. In contrast, ***P. cheiranthi***, the Canary Islands large white, the sister species of *P. brassicae*, is a non-pest species restricted to shrinking humid habitats in the Canary Islands. It lays its egg clutches on wild Brassicales and is threatened by habitat loss[4].

The green-veined white, ***P. napi***, is also not a significant pest species and lays its single eggs mainly on wild Brassicaceae and in moister habitats[5,6]. It is increasingly studied for its remarkable evolutionary plasticity across a range that spans from Morocco to the Arctic Circle, rapidly adapting to these divergent climates[7–9]. ***Pieris bryoniae***, the sister species of *P. napi*, is found at higher altitudes across Europe and shares ecological traits with high-latitude subspecies of *P. napi*, ***P. napi adalwinda***, in Scandinavia. Both have darker wings, possibly a thermoregulatory adaptation to absorb more solar radiation in cooler climates[10,11]. Notably, *P. napi adalwinda* was previously classified as *P. bryoniae*, reflecting the taxonomic challenges within this rapidly evolving species complex[4,8,12]. Both forms are **univoltine** (with one generation per year), unlike other *Pieris* species, which are **multivoltine** (having two to five generations annually)[4].

The final *Pieris* species currently found in the Netherlands is the southern small white, ***P. mannii***[13]. It is a recent arrival in northern Europe and is not considered an agricultural

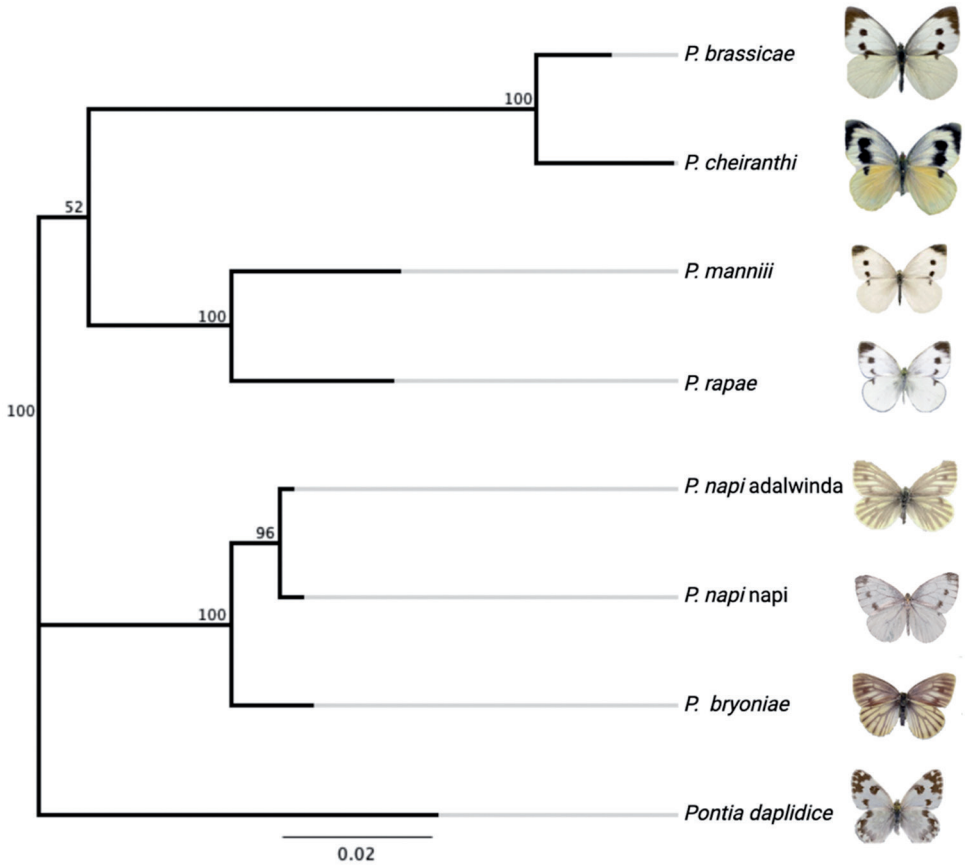



**Figure 1.2)** Phylogeny of *Pieris* (sub)species included in this thesis. CO1 barcodes downloaded from BOLD system's public barcode database (v3.boldsystems.org), aligned with MAAFT for neighbor joining tree with 100 bootstraps constructed on Geneious Prime with default settings. The consensus support percentage is indicated at each node. *Pontia daplidice* is used as an outgroup.

pest, as it primarily feeds on ornamental garden plants[14]. *Pieris mannii* is gaining attention for its rapid and recent range expansion: from isolated mountain populations, it has spread across much of Western Europe in just a decade[13–16].

In this thesis, ***Trichogramma*** wasps, some of the smallest insects in the world, play the role of natural enemies. As **egg parasitoids**, they lay their eggs inside the eggs of insects, mostly Lepidoptera, killing the host embryo before it can hatch[17]. Several *Trichogramma* species are widely used in the **biological control** of Lepidopteran pests**.** *Trichogramma evanescens* and *T. japonicum* are among the most frequently utilized species in commercial and agricultural programs across Europe and Asia[17,18]. These wasps are valued for their ability to suppress pest populations in a targeted and environmentally sustainable

way, often reducing the need for chemical insecticides[18]. Meanwhile, their taxonomy requires revision, with incongruence between morphological and molecular species-level classifications, stemming from the difficulty in morphologically distinguishing species and the perpetuation of inaccurate DNA barcode classifications in public databases[19,20].

At the heart of this story is a central fact: the evolutionary fates of interacting butterflies and egg parasitoids, as well as their pressures of mating, motherhood, and mortality, are inextricably entwined. Natural selection and sexual selection do not pull in separate directions. Instead, they twist around each other, shaping and constraining the evolutionary possibilities for these organisms. In the remainder of this introduction, I briefly explore the potential selection pressures on mating behaviors, oviposition choices, and enemy avoidance in both butterflies and wasps. In the chapters that follow, I examine how these forces shape the reproductive and survival strategies of cabbage white butterflies and their parasitoid enemies, and what this reveals about the evolution of insect life histories across ecological contexts.

## 1.3 Mating: sexual signals and evolutionary stakes

**Sexual selection**, the evolutionary force acting on traits that influence mating success, can drive dramatic divergence, even between closely related populations [21]. Since Darwin's original definition in 1871, to explain the evolution of traits that do not appear to improve fecundity[22], the concept has been refined. It is now generally understood as selection arising from differences in fitness associated with non-random success in the competition for access to gametes[23]. Traits that confer an advantage in attracting mates, such as pheromone production, courtship displays, or phenology (e.g. timing of emergence), can evolve rapidly under sexual selection, particularly when local preferences or environmental conditions differ[21].

Over time, such divergence can contribute to **reproductive isolation**, when populations no longer interbreed. Reproductive isolation can occur through behavioral isolation, where individuals fail to recognize or accept one another as potential mates due to changes in mating cues or preferences. One route by which this can occur is through **assortative mating**, a deviation from random mating where individuals preferentially choose phenotypically similar (or dissimilar) partners[24]. Assortative mating can evolve through active preferences, but also incidentally through constraints during courtship or spatial clustering of different phenotypes[24].

In some cases, traits subject to divergent natural selection that also facilitate mate choice, so-called **magic traits**, lead to reproductive isolation[21,25]. For example, the wing color

pattern in some butterfly species serves both as a mating signal and as a warning to predators[25]. Where populations join different mimicry rings, disruptive natural selection can drive divergence in wing patterns, which in turn reinforces reproductive isolation via assortative mating, and ultimately leads to speciation[25].

However, mating success involves more than attraction. In many insect systems, **mate limitation**, whether due to low dispersal, skewed sex ratios, or spatial separation, means that even reproductively mature individuals may never find a partner or be fertilized[26]. This constraint is particularly relevant in species with short adult lifespans or limited mobility, such as *Trichogramma* wasps. Moreover, even when individuals do encounter potential mates, the act of mating is shaped by a complex sequence of decisions that extend beyond the moment of copulation.

These decisions span both **pre-mating** and **post-mating** stages, also known as pre- and post-copulatory mate choice, respectively. Before mating, individuals may evaluate potential partners based on chemical, visual, or behavioral cues[24]. After mating, further selection can occur through **sperm competition**, where the ejaculates of multiple males compete to fertilize a female's eggs, and **cryptic female choice**, where females exert influence over which sperm is used, often via physiological or biochemical mechanisms[27–30]. These extended processes highlight that mate choice is not a single act, but a dynamic and multilayered filter.

The costs of reproduction add further complexity to mating strategies[31]. Egg production is energetically expensive, and mating itself can carry physical or ecological risks, such as increased exposure to predators or pathogens. As a result, females often evolve choosiness in mate selection[32]. This selectivity is not static: females can exhibit **phenotypic plasticity** in their mate preferences, adjusting either the speed with which they respond to potential mates or the order in which they rank prospective partners[32]. In contrast, males typically adopt strategies that maximize their expected fertilization success from each mating[33]. These include persistent courtship, mate guarding, or mechanisms that reduce the likelihood of female remating[27,33].

Of course, these dynamics do not occur in isolation. Mating strategies are shaped by broader ecological pressures: where and when offspring are laid, the likelihood of their survival, and the selective landscape into which they hatch. Even so, it is helpful to begin with mating as a discrete lens. In the following subsections, by examining mating behaviors and adaptations, we begin to uncover the evolutionary pressures behind who mates with whom and why.

### 1.3.1 Mating in *Pieris*

In butterflies of the genus *Pieris*, mating strategies reflect a complex interplay of chemical signaling, visual cues, and physiological investment. Similar to many butterflies, *Pieris* rely on **pheromone**s, typically (though not always) species-specific chemical signals that mediate reproductive interactions[34,35]. These cues attract mates, signal receptivity, or repel subsequent suitors[35], often acting in concert with visual traits such as wing color and flight displays[36,37]. In *Pieris*, courtship involves both pheromones and visual cues for species recognition and assessment of mate quality[36,37]. Males release **aphrodisiac** pheromones during close-range flight displays, helping females evaluate potential mates[36,38]. To secure paternity and reduce sperm competition, male *P. napi*, *P. rapae*, and *P. brassicae* are known *to* transfer **anti-aphrodisiacs** during copulation[39]. These chemicals reduce female attractiveness to other males, discouraging harassment and acting as a form of chemical **mate guarding**[40,41]. I explore the evolution of these post-mating odors in greater detail in Chapter 6.

Mating systems in *butterflies* range from **monandry** (females mate once) to **polyandry** (females mate with multiple males). *Pieris* spp. are typically polyandrous, with *P. napi* exhibiting striking variation in female mating frequency across its range[29,42–44]. In Scandinavian populations, females are largely monandrous, whereas those in southern regions often remate[42,43]. This variability appears driven by ecological factors such as season length, population density, and host-plant availability, which shape both mating opportunities and the fitness consequences of remating[44]. These dynamics make *P. napi* a compelling model for studying the evolutionary ecology of mating systems.

Polyandry in *Pieris napi* can increase female fitness by providing access to multiple **spermatophores**, which serve not only as sperm sources but also as **nuptial gifts**, nutritional and physiological resources that enhance female fecundity, longevity, and immune function[45–48]. These gifts, transferred during copulation, can constitute a substantial portion of the male's reproductive investment and may influence both female receptivity and reproductive output[49]. Larger or more nutrient-rich spermatophores can delay female remating and significantly contribute to lifetime reproductive success[29,45,48,50]. However, mating is not without cost; for example, each copulation increases the individual's exposure to predators[51]. The fitness benefits of polyandry vary depending on seasonal constraints[41,43,53]. In bivoltine *P. napi*, polyandrous females tend to have higher lifetime fecundity but lower early reproductive output[54]. Under short-season conditions, this delay can reduce the likelihood that their offspring complete development before diapause. In contrast, monandrous females, who reproduce earlier and with higher initial fecundity, are more likely to produce an additional generation when time is limited[54]. These results suggest that season length can counter-select against polyandry by favoring early reproduction, maintaining variation in mating strategies across populations.

### 1.3.2 Mating in *Trichogramma*

Mating in *Trichogramma* wasps is shaped by their tiny size, limited dispersal, and unique reproductive biology. In many species, mating occurs within minutes or hours of emergence, often among siblings and near their shared host egg[55]. This local mating is facilitated by **protandry**, where males emerge before females[56,57], creating strong constraints on mate availability and driving diverse reproductive strategies.

As with all Hymenoptera, *Trichogramma* are **haplodiploid**: fertilized eggs develop into diploid females, while unfertilized eggs develop into haploid males[56]. Haplodiploidy enables **facultative parthenogenesis**, or optional asexual reproduction. In some lineages, **thelytokous asexual reproduction,** where unmated females produce female offspring from unfertilized eggs, has become the norm, often driven by *Wolbachia* endosymbionts[58,59]. At least 18 species (~9% of the genus) are known to carry *Wolbachia* strains that induce parthenogenesis[58].

When sexual reproduction occurs, mating dynamics are shaped by protandry, sex ratio, spatial structure, and limited dispersal[57]. Local mate competition also leads to female-biased sex allocation, as seen in *T. pretiosum*, where offspring sex ratios are less variable than expected under random allocation[60].

As active dispersal is extremely limited and weather-dependent [61], some species have evolved **phoresy,** riding on host insects to reach otherwise inaccessible habitats[62–64]. Phoresy enhances female access to mates and oviposition sites beyond the patch where they emerge[65,66], and may influence population genetic structure, although its effects remain underexplored. While males engage in phoresy less frequently[62], females may arrive already mated or mate locally[67]. Once nearby, *Trichogramma* can use chemical and vibrational cues to locate mates, including pheromones in some species[68].

In short, *Trichogramma* reproduction ranges from sex to obligate asexuality. These strategies are shaped by ecological factors, namely dispersal limitation, encounter rates, endosymbiont infection, local competition, and the physiological constraints of small size. The consequences of limited dispersal are not only behavioral but also genetic, structuring populations and potentially reducing diversity. These dynamics are particularly relevant in biological control, where genetic variation can influence efficacy. The implications of phoresy for *Trichogramma* genetics and control success remain largely unexplored. I return to these themes in Chapter 2 and 7.

## 1.4 Motherhood: Choosing where life begins

There is no such thing as a baby butterfly or wasp. Butterflies and wasps undergo complete metamorphosis, or **holometaboly**, meaning that their juvenile stages, egg, larva, and pupa, are dramatically different from their adult, or imago, form. A newly emerged *Pieris* butterfly bears no resemblance to the leaf-chewing caterpillar it once was. Similarly, *Trichogramma* wasps bear no resemblance to their unseen egg, larval, and pupal forms, which develop entirely inside other insects' eggs. As adults, both feed on nectar, fly to locate mates, and perform the most consequential acts for the survival of their species: **oviposition**, the act of laying eggs.

For both *Pieris* and *Trichogramma*, motherhood comes down to a single act: the careful placement of a tiny egg. However, the contexts in which this act occurs are very different. The butterfly lays her egg on a plant she cannot eat, using chemical signals to choose the right host-plant from a distance on behalf of a chewing larva she will never meet. The wasp inserts her egg into the egg of another insect, relying on chemical signals to find a suitable egg for oviposition. Both mothers make these decisions without the benefit of feedback. In both systems, oviposition is a point of evolutionary convergence where behavior, ecology, physiology, and past selection pressures align in a single decision. Whether tasting leaves with her feet or eavesdropping on another species' mating pheromones, each mother plays a high-stakes game, gambling her reproductive success on a well-placed egg.

A central idea in the study of oviposition behavior is the **"mother knows best" hypothesis**, which posits that females should preferentially oviposit in a way that maximizes the fitness of their offspring[69]. This **preference-performance hypothesis** assumes a tight link between where a mother chooses to lay her eggs (preference) and how well her developing offspring will perform in that environment (performance)[69]. In both *Pieris* and *Trichogramma*, this logic suggests that females should evolve strong preferences for host-plants or host eggs that lead to higher survival, faster development, or greater fecundity of their young. However, reality is often complex and full of trade-offs and constrained by phylogeny[70–73]. In *Pieris*, factors such as prior experience, sensory limitations, or environmental variability may decouple preference from actual larval performance.

Furthermore, competition and reproductive interference with congeners may lead to shifts in host-plant use between closely related species[74]. In *Trichogramma*, similarly, females may encounter constraints imposed by volatile cue reliability, egg accessibility, or time pressure, which force compromises between optimal and available hosts[75], reviewed in Chapters 2 and 3. **Natural selection** should favor females whose choices align with the

success of their offspring, even if this match is not always perfect[69]. I further discuss the preference-performance hypothesis in Chapter 7.

### 1.4.1 Oviposition in *Pieris*

For butterflies, the moment of oviposition is a critical point of maternal investment. Embryos in their eggs are immobile, defenseless, and entirely dependent on their placement. Upon hatching, the larvae will be largely confined to the plant their mother selected. These host-plants serve as both habitat and food for the caterpillars. *Pieris* larvae feed on plants in the Brassicaceae family, and as such, this is where the butterflies lay their eggs[1].

Adult butterflies lead very different lives from their young and have entirely different dietary needs. Many herbivores feed on the same plants as adults as they did as larvae, such that a plant the adult finds palatable and nutritious is likely a good choice for depositing its young, in contrast, butterflies do not share their larvae's diets. Butterflies no longer consume leaves as adults, and their straw-like probosces, adapted for sipping floral nectar, are useless for evaluating leaf quality. Nonetheless, the gravid female butterfly must choose a suitable plant for her offspring, with suitability depending on physical and chemical properties she cannot directly consume to test. The gap between an adult's experiences and the needs of its offspring makes the oviposition decision in butterflies especially remarkable. The adult must assess the quality of leaves not through ingestion, but through chemosensory receptors on her tarsi[76]. In other words, she tastes with her feet. By drumming on the leaf surface, she detects surface compounds with her feet and releases volatile compounds from within the plant that inform her about its chemical makeup, detected by **olfactory receptors** on her antennae[77–79]. In this way, she not only identifies host-plants from the correct taxa but also gains insight into the plants' quality, including previous egg deposition and their anti-herbivore chemical defenses[77,80,81].

*Pieris* butterflies must assess the presence of chemicals known as **glucosinolates**, a class of secondary compounds found in Brassicales[76]. These bitter compounds are characteristic of many crops humans preferentially consume, such as cabbage and mustard. While many herbivores are deterred or poisoned by glucosinolates, *Pieris* caterpillars are specialists on these compounds, equipped with enzymes that detoxify them[82]. The presence of glucosinolates, which signifies that the plant belongs to the Brassicales, stimulates their oviposition. However, not all glucosinolates are equal. Some varieties are more toxic than others, or interact differently with larval physiology, meaning that oviposition decisions must finely discriminate among suitable hosts[81]. There is also variation among *Pieris* populations in their host-plant preferences and performance[15]. For instance, *P. napi* populations from different regions vary in their readiness to accept particular host species[83], with some favoring locally abundant plants while others avoid them. This intraspecific variation reflects both local adaptation and phenotypic plasticity and offers a compel-

ling example of how fine-scale ecological pressures shape oviposition behavior and the potential for population divergence.

Across the Pieridae family, there is more diversity in host-plant use. While *Pieris* species and their relatives in the Pierinae subfamily tribe feed exclusively on plants in the order Brassicales, the family's ancestral food plant was legumes, the Fabaceae[82]. Species in basal genera like *Colias* and *Leptidea* still utilize these Faboid host-plants[82]. The Pierinae were able to switch to feeding on the relatively toxic Brassicales due to the evolution of a special enzyme which breaks down the toxins in these plants, glucosinolates, by evolving genes for gut proteins known as **nitrile specifier proteins (NSP)**, which allowed a rapid expansion of this clade on this newly colonized host-plant through **adaptive radiation**[1,84]. Differences in these genes between species relate to differences in host-plant preferences and continue to drive divergence between species[5]. The genus *Aporia* has again switched hosts from its Brassicaceae feeding ancestral state, now feeding on Rosaceae[1]. Indeed, the evolution and speciation of butterflies are closely tied to the plants on which they feed[85].

Remarkably, larval experience may shape adult preference. During metamorphosis, much of the body is remodeled, but specific structures organized into morphologically distinct clusters, called imaginal discs, persist and give rise to adult tissues[86,87]. These fragments from larvae thus contribute to the adult form[88,89]. It has been shown that exposure to specific plant chemicals as a caterpillar can influence adult host preference, allowing the adult to "remember" the larval preferences[88,89]. Nevertheless, this only solves part of the problem. Choosing a plant that nourishes the larva is one dimension of the oviposition challenge, but the egg itself faces separate, and often severe, risks.

Before hatching, the egg is vulnerable to desiccation, microbial infection, natural enemies, and direct and indirect **plant-mediated defenses**[17,90]. Some Brassicaceae, including commonly used hosts like *Brassica nigra*, can detect egg deposition and respond with **hypersensitive response-like (HR-like) cell death,** a type of tissue death that isolates and kills the developing embryo[92,93]. These egg-killing responses are highly variable among plant species and even among genotypes within a species, posing a powerful, often overlooked selection pressure on oviposition[90]. It has been proposed that some butterflies may avoid these egg-killing responses by laying eggs on specific host-plants without these responses, or on certain tissues, including stems and flowers, which do not exhibit egg-killing responses[93,94]. I test this hypothesis for counter-adaptation to egg killing in the Pieridae in Chapter 4.

Other considerations for an ovipositing butterfly include the plant's location, size, the community it already hosts, and the microclimate it provides[95]. Plants must be large

enough to feed developing larvae, or in large enough patches of suitable plants, should not have too much competition from conspecifics or other herbivores consuming the same plants[96]. Therefore, **oviposition deterrents** and **oviposition-induced plant volatiles (OIPVs)**, which differ between egg-infested and uninfested plants, also help butterflies avoid already infested plants[80] (Chapter 7). The plants should also not already be plagued with natural enemies, including parasitoids or predatory insects that consume eggs[97]. Furthermore, the plants must be near enough to water for the plant not to die of drought, or for the microclimate not to be so dry that eggs desiccate[98,99].

Once a plant and specific tissue on the plant is selected, the butterfly must also select how many eggs to lay in each place. Some species are solitary, always laying single eggs, while others are gregarious, laying clutches of variable size. Choosing to lay a single egg *versus* one hundred eggs is a significant difference, and the selection pressures underlying why particular species choose one strategy or another are still debated[100–105]. Within species, individuals may also vary in the size of their egg clutch[106]. The evolution of gregariousness, which has occurred multiple times in unrelated lineages[100], has been hypothesized to relate to **aposematism**, or warning coloration[107]. Gregarious eggs develop into large groups of crawling, smelly caterpillars, which remain together during early instars. They are quite apparent to enemies that may wish to eat them, and therefore, many gregarious larvae are toxic and benefit from aposematism[107]. *Pieris brassicae,* for example, is gregarious, and larvae have bright colors. In line with this, solitary *Pieris* spp. have cryptic green larvae which can instead just hide from predators. However, other ecological interactions may also select for gregariousness at the egg stage. For example, egg clutches are less vulnerable to egg-killing plant responses[108].

On the other hand, egg clutches are very attractive resources for egg parasitoids. Gregarious larvae are also more susceptible to pathogen infection and increase intra-specific competition[105,109]. Increased host-plant specialization has also been linked to increased likelihood of gregariousnesss[110]. I further discuss the current debate and hypotheses for the repeated evolution of gregariousness in Chapter 7.

The sources of danger to eggs mentioned here are discussed further below, but all play into this crucial oviposition decision. Female butterflies cannot recall these threats from past experiences, and yet, through evolution, butterflies must learn to avoid them. Through this lens, the female's oviposition decision becomes a window into the selective pressures that have shaped her entire evolutionary history. The fleeting contact between the ovipositor and leaf represents a careful decision for offspring survival.

### 1.4.2 Oviposition in *Trichogramma*

*Trichogramma* do not place their eggs on plants. Instead, they inject their eggs directly into the eggs of other insects, usually Lepidoptera. As **idiobionts**, the parasitoids' own development arrests that of their hosts, making the butterfly's eggshell its own capsule. The host egg serves as both shelter and a food source: the wasp larva develops entirely inside, consuming the host embryo and emerging as a fully formed adult. The choice of host egg is thus a complete maternal investment: once the female has laid her egg, she does not return and cannot compensate for a poor decision. I review what is known about how *Trichogramma* seek out tiny, hidden host eggs using chemical host locating cues in Chapter 2. In Chapter 3, I discuss how chemical cues can also help *Trichogramma* assess the suitability of host eggs.

These decisions are not made blindly. Female *Trichogramma* rely on a complex suite of sensory cues to locate host eggs at the right time and place. One of the most powerful of these cues comes not from the host insect itself, but from the plant on which the host oviposited. Plants often respond to insect egg-laying with subtle shifts in chemistry, releasing oviposition-induced plant volatiles (OIPVs), chemical signals that can attract natural enemies such as *Trichogramma*[75,111]. These volatiles are species and context-specific, offering females a reliable cue to the presence of fresh host eggs nearby, even before the host caterpillar hatches[112].

In some *Trichogramma* species, however, the problem of host location is solved in an entirely different, and rather ingenious way: by letting the host do the work. Phoresy not only enables *Trichogramma* to meet mates and reach new patches of host-plants, but hitching a ride on an adult mated butterfly as it flies in search of oviposition sites, leads it directly to freshly laid eggs[66]. Once the butterfly lays its eggs, the wasp dismounts and can immediately start parasitizing. In this way, phoresy extends the wasp's effective dispersal range, increases her precision in host targeting, and bypasses the need for costly long-range search behavior. The cue that triggers phoretic behavior is a product of sexual communication in the host. Some *Trichogramma* species have evolved to detect anti-aphrodisiac pheromones left on female moths during mating[63,64,66]. These chemicals, intended to repel rival males, are exploited by wasps as **kairomones**, interspecific **infochemicals** that in this case are easily detectable and reliable signals indicating that the female butterfly is both recently mated and likely to fly to oviposit on a host-plant soon[63]. *Trichogramma* are very sensitive to differences in the pheromones of their hosts[113–115]. Thus, the male butterfly inadvertently sets the stage for parasitism, creating a complex ecological interaction where mating behavior in one species facilitates host-seeking in another. Because *Trichogramma* adults are so short-lived and dispersal is limited, these tactics, whether chemical eavesdropping or phoretic transport, are vital for homing in on suitable eggs. This strategy is further discussed in Chapters 2, 6 and 7.

As in *Pieris*, variation among populations and species of *Trichogramma* shapes these strategies. Some specialize on particular hosts or respond to specific chemical cues[114,116], while others are generalists. Populations with greater dispersal ability or adapted to phoresy may experience different selective pressures on oviposition timing and host selection[75]. These patterns have consequences not only for evolutionary ecology but also for applied biological control, where host range, habitat specificity, precision, and environmental cues all affect the success of *Trichogramma* as pest-suppression agents[117], as discussed in Chapters 2 and 3. In all cases, however, the story is the same: once the egg is laid, the mother's role is over. There is no provisioning, no nest defense, no rescue from predation. Success depends on detecting the proper signal, arriving at the correct time, and embedding her future into the egg of another.

## 1.5 Mortality: Navigating the dangers of a hostile world

The lives of most insects are short, and often brutally cut shorter. From the moment of fertilization, each life stage of holometabolous insects, egg, larva, pupa, and adult, is exposed to distinct mortality pressures, both abiotic (e.g., temperature, humidity, wind) and biotic (e.g., predators, parasitoids, host defenses). The selective landscape shifts dramatically across stages and species, shaping behavior and morphology in response.

Interestingly, some butterflies are remarkably long-lived, and their longevity is closely tied to their food choice, mating behavior, and predator avoidance. Mark–recapture experiments in Uganda have documented butterfly life spans of nearly 300 days, far exceeding previous expectations[118]. Butterfly longevity has been linked to feeding ecology: genera such as *Heliconius* that feed on pollen[119] and *Euphedra* that feed on fruit[118] live significantly longer than those relying solely on nectar. The nutrient richness of pollen and fruit likely contributes to this extended lifespan, however, further investigation is needed to clarify this link[119]. Predator avoidance is also a key factor influencing longevity, although the relationship between predator-avoiding behaviors and life span remains unclear[118]. For example, pollen-feeding in *Heliconius* enhances female butterfly toxicity in a sex-specific manner, linking longevity to predation risk[120]. Longevity is further intertwined with mating strategies, as pollen-feeding butterflies also exhibit delayed reproductive maturity, which may result in trade-offs with the benefits of longer lifespans[120].

*Pieris* butterflies, on the other hand, live relatively short lives. The time from oviposition of the egg until the death of the adult is approximately two months[121]. This thesis focuses on mortality during the week-long egg stage of *Pieris*, a window of exceptional vulnerability. Here, we explore how natural selection balances different threats, including enemies, environmental stress, and the plants themselves, which affects the survival of butterflies

and wasps. By extension, these risks shape the decisions they make about where, when, and how to reproduce.

### 1.5.1 Threats to *Pieris*

The egg of a butterfly may seem small and insignificant, but for predators and parasitoids, it is a high-value target, and for the butterfly mother, a fragile investment left behind. *Pieris* eggs are laid on the surface of host-plants, often under leaves, fully exposed to environmental conditions and a suite of enemies.

**Abiotic threats** are immediate and unforgiving. Butterfly eggs must not desiccate but must not drown either. Each egg breathes through **aeropyles**, tiny pores that can clog or collapse under extreme heat or moisture[122]. They are also exposed to water loss through **micropyles**, pores which allow egg fertilization inside the butterfly but remain open after egg laying[123]. On hot days, sun-exposed leaves can dry out, shriveling beneath the eggs and thereby increasing the risk of desiccation. On rainy days, water films can suffocate or dislodge them. Climate extremes, especially heatwaves and drought, may destroy eggs, particularly when females oviposit in unsuitable microhabitats with harsh microclimates[99]. I test the hypothesis that climate drives *Pieris* egg pore morphology in Chapter 5.

**Biotic threats** add additional complexity, with predators and parasitoids killing eggs[97]. Egg parasitoids often cause significant mortality in *Pieris* eggs, sometimes killing nearly all eggs in field studies[124]. Some even follow female butterflies in flight, eavesdropping on chemical cues to hitchhike to where they will lay their eggs[62](Chapters 2 and 6). Certain host-plants, such as *Brassica nigra*, mount a direct counterattack by triggering egg-killing responses after perceiving *Pieris* oviposition, but selecting tissues for oviposition carefully may help them avoid this threat[93] (Chapter 4).

Egg-laying and mating are inherently risky behaviors. Butterflies, particularly when stationary during copulation or oviposition, are vulnerable to predators such as birds[51,125]. *Pieris brassicae* females, which lay eggs in large clutches of up to 100 eggs, are particularly at risk, as only 4-5 eggs are laid per minute[126], and thus oviposition may take up to 20 minutes during which the butterfly remains exposed to predators on its host-plant. Mating also poses dangers, as paired butterflies remain together for hours[127], significantly reducing their mobility and increasing their predation risk[51]. During these vulnerable periods, aposematic signaling may serve as a crucial defense mechanism, deterring predators from attempting to eat the butterflies. Interestingly, the evolutionary origins of aposematism remain uncertain[128]. A recent review suggests that bright coloration may have initially evolved to signal an individual's health in the face of parasites and pathogens, with these immunity-linked visual cues later co-opted as warning signals to deter predators[128].

### 1.5.2 Threats to *Trichogramma*

At first glance, the *Trichogramma* egg, laid safely inside the egg of another insect, appears well-protected. However, this protection is conditional and often illusory. This development inside another egg offers some shelter, but not immunity, from environmental and ecological hazards. Every threat that endangers the host egg now affects the wasp's future. If the butterfly chooses a poor location for oviposition, for example, too hot or too dry (Chapter 5), the wasp's egg may also die inside the desiccated host egg[129](Chapter 2). Selecting egg clutches to parasitize that are more resistant to desiccation in general[130] and specifically to desiccation due to egg-killing plant defenses[130,131], may aid egg parasitoids in resisting harsh abiotic conditions.

The immune response of the host egg can sometimes arrest parasitoid development, particularly in more mature eggs[132]. Eggs that are too old will not be preferred by parasitoids and do not support development as well as younger eggs[75] (Chapter 3). Toxins released by the parasitoid also shape the conflict between a host embryo and its egg parasitoid. *Trichogramma* venom contains proteases and phosphatases, enzymes that facilitate the breakdown of the host embryo and promote yolk digestion[132]. At extremely high temperatures, *Trichogramma* emergence from parasitized eggs is significantly reduced, possibly due to detrimental effects of high temperatures on the parasitoid embryo itself, or due to the host embryo developing so quickly that the parasitoid embryo cannot overcome its host[133].

Furthermore, the same egg-killing response that destroys *Pieris* eggs (Chapter 4) can indirectly harm the wasp developing within, especially when necrosis causes the egg to fall off the leaf before the wasp can complete development[93]. Plants that exhibit synergistic direct and indirect defense, both egg-killing and parasitoid-attracting volatiles, could potentially reduce parasitoid success even while appearing to support wasp recruitment[116,134]. On the other hand, perhaps eggs which are already killed by plants may benefit wasps due to their lack of immune response potential[134].

Egg predators, such as ants, may also consume a parasitized egg containing *Trichogramma*[135]. While adult Trichogramma may seem safe from predation, as few predators would consider consuming a tiny adult *Trichogramma*, wind, rain, and changing weather are much more formidable enemies for the wasps than the butterflies[136].

## 1.6 Mating, motherhood, and mortality intertwined

Although *Pieris* butterflies and *Trichogramma* wasps differ dramatically in form, life history, and ecology, they are bound by the same ultimate challenge: surviving long enough

to reproduce and doing so in a way that gives their offspring the best chance of doing the same. Both taxa face intense selection on mating behaviors, oviposition strategies, and stage-specific mortality risks, and in both, these forces interact in complex and sometimes unexpected ways (Figure 1.3).

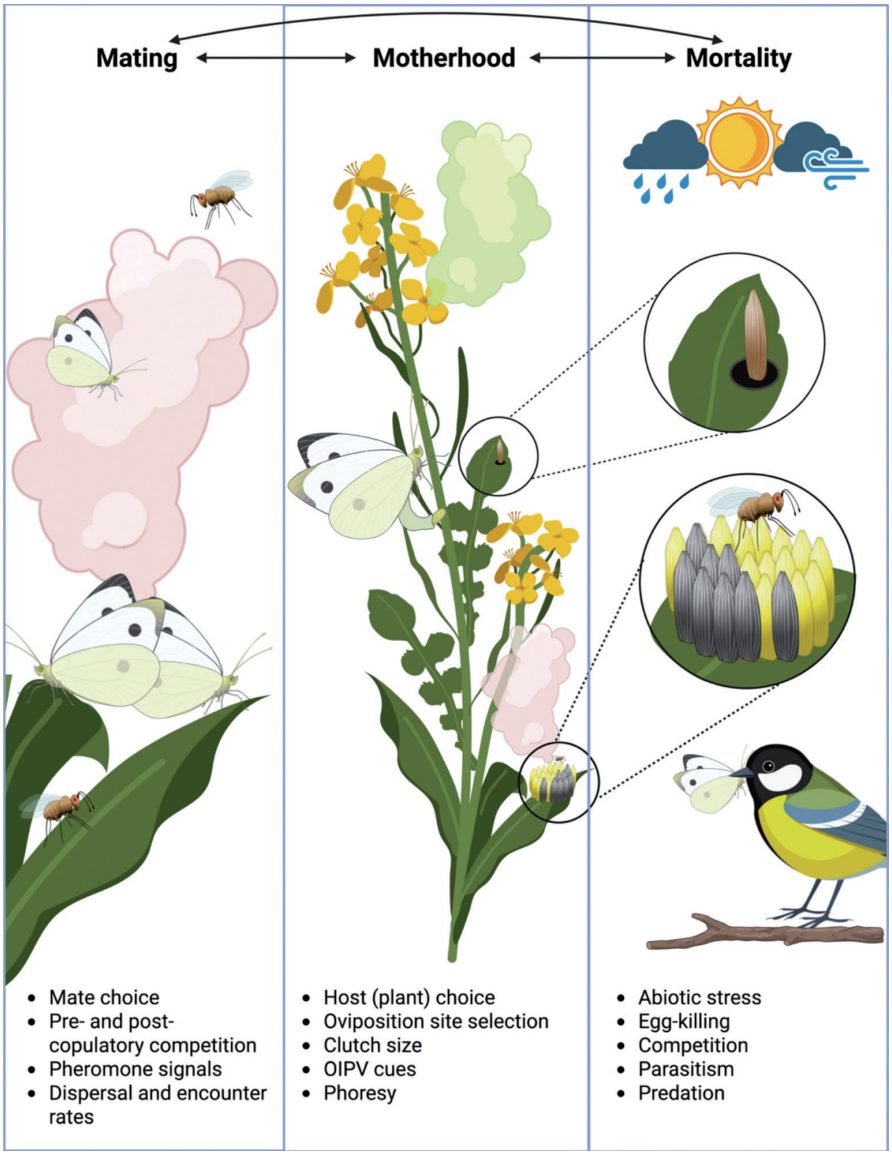



**Figure 1.3)** *Pieris* butterflies and *Trichogramma* wasps co-evolved by balancing their respective selection pressures on mating, motherhood, and mortality. The costs and benefits of decisions at each life stage depend on trade-offs with decisions in other life stages and changing interactions with each other.

In both systems, **mating** decisions are shaped by partner availability and courtship traits, as well as the downstream consequences of those choices. For butterflies, the decision to mate once or multiple times influences nutrient acquisition, egg-laying rate, and exposure to predation. For wasps, mating may not even occur at all in parthenogenetic lineages. However, when it does, success depends on finding a mate in a brief window, often through highly localized strategies, mating immediately upon emerging from the host egg, or relying on phoretic strategies to travel to distant patches and find more genetically diverse mates.

**Motherhood**, too, looks different between the two. A butterfly lays her egg on a plant she does not eat, tasting with her feet and smelling with her antennae to find the right chemistry for a larva she will never meet. A wasp injects her egg into the egg of another insect, using her antennae and ovipositor to find and assess the quality of her hosts. Nevertheless, both are making an irreversible decision that encompasses food, shelter, enemy avoidance, and microclimate, all compressed into a single act of egg placement.

And then there is **mortality**: ever-present, stage-specific, and unforgiving. In *Pieris*, the egg lies exposed to rain, sun, parasitoids, and predators. In *Trichogramma*, the egg is inside a host egg, but it remains vulnerable to the host's immune defenses, plant responses, abiotic conditions, and egg predators. Even successful parasitism can fail when the host egg falls off the leaf or desiccates before the wasp can emerge.

Across both organisms, the boundaries between mating, motherhood, and mortality blur**.** The act of mating affects where and when eggs are laid; the risk of death shapes the cues used for oviposition; the environment selected for the next generation filters back into adult behavior and physiology. What may appear as isolated life stages or behaviors are, in fact, parts of a single evolutionary loop. Selection on any one component echoes across the others.

This topic summons a classic example from the chemical ecology literature that defies categorization into either mating, motherhood, or mortality, exemplifying the entanglement of these phases and organisms. *Trichogramma* avoid parasitizing the eggs of Danaid (Nymphalidae) butterflies that are laden with toxic pyrrolizidine alkaloids[137]. The first eggs laid by the butterflies contain lower concentrations of these toxins than those laid later[137]. How do these toxins accumulate in the eggs? They are acquired with each successive mating, transferred from male to female butterfly[35,138]. As such, a female butterfly's choice of mate depends in large part on his pyrrolizidine alkaloid content[35].

Furthermore, what determines the male's toxin levels? It is partially influenced by where his mother laid her eggs, since different host-plants provide varying levels of pyrrolizidine

alkaloids to the larvae[139]. These larvae grow into adult males whose toxin stores influence their mating success, which then shapes the toxin content of butterfly eggs, which ultimately guides the parasitoid wasp's choice of host[35, 137]. Fortunately for adult male butterflies, nectar feeding may allow them to adjust their alkaloid stores[140]. Here, we observe the entangled evolution of butterflies and their parasitoids, where mating, oviposition, and predator avoidance are interwoven.

In the chapters that follow, I explore these dynamics across both interspecific and intraspecific levels, focusing on how subtle variations in behavior, chemistry, and environment shape and are shaped by evolutionary pressures. By examining *Pieris* and *Trichogramma* side by side, we can begin to understand not just how organisms survive, but also how they evolve through the entangled forces of mating, motherhood, and mortality.

## 1.7 Unraveling Complexity: What We Still Don't Know

Despite decades of research on host choice, mating behavior, and insect–plant interactions, key questions remain unresolved. On a detailed level, this thesis addresses several specific knowledge gaps: How do *Trichogramma* wasps locate suitable host eggs, and how variable are these strategies across and within species (Chapters 2 and 3)? How do *Pieris* butterflies choose oviposition sites in the face of egg-killing plant defenses, and is there evidence for counter-adaptations (Chapter 4)? Can climate influence butterfly egg morphology in ways that affect survival (Chapter 5)? Are butterfly post-mating odors shaped primarily by sexual selection, or by ecological pressures from predators and parasitoids (Chapter 6)? Each of these questions targets a piece of the broader puzzle: how selection acts across multiple stages of an insect's life history.

At a conceptual level, this thesis addresses a significant gap in evolutionary biology: the artificial distinction between sexual and natural selection. While often considered distinct forces, or even in conflict, the evolution of organisms such as butterflies and their parasitoid enemies reflects continuous feedback between these pressures. Mating strategies, oviposition choices, and survival tactics are not shaped in isolation, but in concert, and their effects ripple across life stages and ecological contexts. Nevertheless, few studies attempt to synthesize these forces over the full course of an organism's development. This reduction in complexity is understandable because research requires isolating variables to detect patterns and infer causation. However, in doing so, we risk overlooking the entangled nature of selection as it is experienced in the wild. By combining multiple approaches, life stages, and taxa, this thesis aims to contribute to a more integrated understanding of how complex evolutionary strategies unfold in real time, across real lives.

## 1.8 Thesis objectives and outline: Untangling the Threads of an Entangled System

My thesis examines how evolutionary pressures on mating, oviposition, and mortality influence the behavior and biology of cabbage white butterflies (*Pieris* spp.) and their egg parasitoid enemies, *Trichogramma* wasps. While each chapter investigates a distinct facet of this system, they all return to a central question: how do organisms make reproductive decisions in a world full of enemies and environmental stress? By considering present-day variation in strategies between and within species, I aim to gain insight into this question**.**

In **Chapter 2**, I examine how egg parasitoid wasps find the right host egg in a vast and variable world. I review how chemical cue use by egg parasitoids is highly **variable,** not only across species but within them, shaped by individual experience, evolutionary history, and ecological context. This variation has direct implications for both evolutionary ecology and the applied use of egg parasitoids as biological control agents, where nuanced host-finding strategies may enhance effectiveness. This chapter emphasizes that oviposition by egg parasitoids is deeply reliant on eavesdropping across trophic levels, with oviposition decisions filtered through chemical signals from both the host and the host-plant.

**Chapter 3** builds on this review, reporting that ***Trichogramma* use oviposition-induced plant volatiles (OIPVs) to assess the quality** of host eggs from a distance. Parasitoids integrate plant-emitted cues into their reproductive strategy, such that their preference aligns well with their performance. This nuanced cue-use could be harnessed to further enhance biological control strategies.

In **Chapter 4**, I turn to *Pieris* butterflies and the plants that fight back. I investigate whether **oviposition strategies across Pieridae** may counteract a potent plant defense: **egg-killing** via HR-like cell death. Despite strong selection pressure, I find no evidence of counter-adaptations in oviposition site choice across multiple *Pieris* species. Instead, mothers continue to oviposit on tissues vulnerable to HR response, raising questions about constraint, trade-offs, or yet-unknown fitness benefits of these decisions.

**Chapter 5** explores **egg pore morphology** in *Pieris*, demonstrating that even the often-overlooked microstructures of egg, aeropyles and micropyles, reflect **abiotic selection**. I show that egg pore morphology differs more between *P. napi* populations from different climates than between species, suggesting that climate shapes even the most minute features of reproduction**.** These results highlight how abiotic stress may select for changes in egg pores, traits that affect embryo survival before a predator or parasitoid ever arrives.

In **Chapter 6**, I revisit mating, demonstrating that **post-mating odors in *Pieris*** are shaped not only by **sexual selection** but also by **natural selection** from predators, parasitoids, and host-plants. These odors, long assumed to be anti-aphrodisiacs, are multifunctional signals: they repel males, deter birds, attract parasitoids, and modulate oviposition. Variation in these odors among populations correlates not with mating frequency but with parasitoid pressure, suggesting that mating and mortality are deeply entangled in the evolution of chemical communication**.**

Finally, **Chapter 7** offers a synthesis in which I reflect on how our assumptions about maternal choice are often too narrow. Traditional **preference–performance studies may miss critical layers of ecological complexity**. A "poor" choice for larval growth might benefit offspring immunity, visibility, or long-term survival in ways we do not yet measure. By viewing reproductive decisions through the entangled lenses of mating, motherhood, and mortality, I argue that what may appear as maladaptive may, in fact, be evolutionarily wise**.**

From these chapters, a clear message emerges that reproductive strategies are not shaped by isolated pressures, but by interdependent trade-offs across life stages and generations. Each egg laid carries the outcome of millions of years of selection.

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CHAPTER 2

# 2

# Finding an egg in a haystack: variation in chemical cue use by egg parasitoids of herbivorous insects

This chapter is based on:

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## Abstract

Egg parasitoids of herbivorous insects use an interplay of short- and long-range chemical cues emitted by hosts and host-plants to find eggs to parasitize. Volatile compounds that attract egg parasitoids can be identified via behavioral assays and used to manipulate parasitoid behavior in the field for biological control of herbivorous pests. However, how and when a particular cue will be used varies over the life of an individual, as well as at and below species level. Future research should expand taxonomic coverage to explore variation in chemical cue use in more natural, dynamic settings. More nuanced understanding of the variability of egg parasitoid host-finding strategies will aid in disentangling the underlying genetics and further enhancing biological control.

## 2.1 Introduction

The challenge for egg parasitoids to find an herbivore's inconspicuous egg in a complex environment is akin to trying to locate a needle in a very large and ever-changing haystack. Remarkably, these miniscule insects have evolved to be highly successful at host-finding. Their ability to seek out and kill an herbivore's egg, before it has developed into a voracious larva, is highly valuable for agriculture. The diverse host-finding strategies used by these idiobiont endoparasitoids have been thoroughly reviewed in the past[1,2] as have the ways in which these innate and learned behaviors can be exploited to improve biological control[3,4] . Thus far, research has primarily focused on species within several genera of parasitoids known to target economically important pests, namely the genera *Trissolcus* (Scelonidae), *Telenomus* (Platygastridae), and *Trichogramma* (Trichogrammatidae). As the number of species and their taxonomic coverage investigated continues to grow, so does our ability to find phylogenetic patterns in their use of chemical cues for host-finding.

Egg parasitism has evolved approximately eighteen times within Hymenoptera. Egg parasitoids have been found to predominantly parasitize herbivorous insects that lay their eggs on plants, and of these, primarily from the orders Hemiptera and Lepidoptera[5]. In this review, we focus on hymenopteran parasitoids of these herbivorous species and the chemical cues that they use to find host eggs. Egg parasitoids have antennae that can detect specific volatile organic compounds (VOCs) or non-volatile cues by contact. Many respond to chemical cues emitted directly from their hosts or indirectly induced by the host-plant on which the herbivore is found and on which they then home in on their host eggs. We highlight some of the most recent research on the chemical ecology of egg parasitoid host-finding, and how the parasitoids use an interplay of chemical cues emitted by the host insect, the host-plant, and potentially microbes. We then discuss how dynamic host-finding strategies can be within and between individuals, populations, and species.

## 2.2 Finding hosts using chemical cues

### 2.2.1 Using chemical cues from the host insect

At each developmental stage of an herbivorous insect, chemical volatiles are emitted that have the potential to betray the host's location and inform parasitoids to find a suitable egg for oviposition. Egg parasitoids primarily use VOCs from the adult stage, however, there are examples of egg parasitoid attraction to larval frass volatiles, which may indicate the location of a host[6]. In short-range searching, egg-derived compounds have been shown to act as contact kairomones for several species[7]. Chemical traces left on plants

by ovipositing females can serve as a more conspicuous source of close-range cues[1]. For example, the footprints of Hemiptera have been found to be highly informative contact kairomones for egg parasitoids from several genera, and especially well studied for species of *Trissolcus*[8-10]. Kairomones from scales, i.e. cuticular hydrocarbones like tricosane, shed from the body and wings of adult Lepidoptera can also serve as close-range cues[11,12] (Figure2.1a). Pheromone residues that are absorbed by these scales or deposited directly onto the egg or leaf surface may also be attractive short-range cues[13,14]. When pheromones are emitted directly by an adult insect, these relatively concentrated pheromone plumes may act as highly detectable long-range cues to which many egg parasitoids have been found to be highly sensitive[15-18]. For example, antiaphrodisiac pheromones, which are emitted by mated females, have been found to lure *Trichogramma* to *Pieris* butterflies[19]. Antiaphrodisiacs provide valuable information to an egg parasitoid because these compounds indicate that the host is likely gravid and also signal the presence of




**Figure 2.1)** Examples of *Trichogramma* wasps using different modalities of host-finding. **(a)** Artificially colored scanning electron microscope image of butterfly scales near and on eggs (credits: Hans M. Smid), **(b)** phoresy: hitchhiking *Trichogramma* female with a female butterfly emitting antiaphrodisiac pheromones (credits: Nina E. Fatouros), **(c)** close-range plant cues such as changes in epicuticular waxes reliably indicate host eggs in close vicinity (credits: Nina E. Fatouros), **(d)** *Pieris* butterfly egg parasitized by *Trichogramma* inducing an egg-killing leaf necrosis, representing the double-defense line in which indirect and direct defense work in concert (credits: Lotte Caarls).

a potential 'ride' on the host to fresh eggs and a new patch, known as phoresy[20] (Figure 2.1b). Phoresy has been found in approximately thirty species of egg parasitoids, which is far more prevalent than in any other parasitoids[21]. Further studies are needed to determine the prevalence and specificity of the combination of pheromonal espionage and phoresy across egg parasitoids and their hosts.



### 2.2.2 Using chemical cues from the host-plant

Records of plant volatiles induced by herbivore egg deposition (Oviposition-induced plant volatiles (OIPVs)) attracting egg parasitoids are adding up across different plant species and herbivorous hosts[12]. Both changes in plant volatiles and epicuticular leaf waxes induced by herbivore oviposition are exploited by egg parasitoids and reliably indicate egg deposition in a habitat and/or on a host-plant (Figure 2.1c). OIPVs have been shown to be host-specific[34], host egg age-specific[56], and location-specific[28]. OIPVs may work in concert with direct egg-killing leaf necrosis[57] (Figure 2.1d), depend on the mating status of the egg-laying host[30], and can indicate host quality and whether eggs are already parasitized[31]. Simultaneous caterpillar feeding by (non)hosts or egg deposition by invasive insects can alter egg parasitoid preferences to OIPVs and can even disrupt the signal. For example, the egg parasitoids *Telenomus podisi* and *Trissolcus basalis* are only attracted to OIPVs emitted by plants when induced by eggs in combination with feeding damage of the local host. However, the wasps were neither attracted to OIPVs induced by the invasive pest nor to OIPVs of plants concurrently infested by both the local and invasive host[32,33]. The lack of response toward the invasive species might be due to the absence of a coevolutionary history between the interacting species. Responding only to cues from suitable coevolved hosts probably reduces egg parasitoids' time and energy costs[34].

Attraction of egg parasitoids by OIPVs should benefit plants and selective breeding for OIPVs could be a valuable addition to biocontrol and use of semiochemical tactics[35,36]. First attempts toward breeding for crop resistance to pests by utilizing parasitoid attraction to OIPVs have now been made by testing natural variation in commercial, transgenic, and/or landraces of maize[37-39]. Farmer-selected maize landraces were shown to emit OIPVs induced by egg deposition of the stemborer *Chilo partellus* that are more attractive to *Trichogramma* and *Cotesia* parasitoids than commercial hybrids[39]. A genome-wide association mapping with different maize genotypes revealed >100 SNP molecular markers associated with parasitoid attraction to OIPVs, including a receptor gene that is potentially involved in the recognition of the egg elicitor[37]. Although far from application, identification, and validation of candidate genes for parasitoid attraction and subsequent introgression into commercial hybrids, could eventually lead to higher parasitism rates and reduction in the stemborer pest on maize.

### 2.2.3 Using chemical cues from, or induced by, microbes?

In recent years, there has been an increasing interest in understanding the role of microorganisms in the foraging behavior of insect parasitoids[81,82]. Extracellular microorganisms vertically transmitted from parents to offspring and inoculated onto the host eggshell[12,83] could be potentially exploited by foraging parasitoids as long-range or short-range cues. When insect host eggs are deposited on leaves, plants may perceive and respond to egg-associated microbes and recruit egg parasitoids. In this mode of action, egg-associated microbes are hypothesized to act as microbial elicitors of OIPVs that attract egg parasitoids. However, a recent study using a lepidopteran species failed to show such an eliciting role of egg-associated microbes[84]. This lack of plant response could be due to the absence of core-resident microbes in Lepidoptera[81]. It would be interesting to investigate the role of microbes in other herbivorous insects that vertically transmit symbionts via egg inoculation, such as herbivorous stink bugs (Hemiptera) that smear symbiont-containing fluids from the anus on the eggs whereby gut symbionts are transferred to the next generation[85]. As the main parasitoids of stink bugs are species that attack the egg stage — particularly members of the Scelionidae — stink bugs and their parasitoids are ideal organisms to investigate the role of egg-associated microbes in plant-mediated parasitoid attraction. Extracellular microorganisms may also be exploited by insect parasitoids as short-range or contact cues, when wasps are in close vicinity or encounter a potential host. Many egg parasitoids extensively inspect with their antennae the surface of potential host eggs with which they recognize the host identity. However, there is no evidence yet in the literature that the presence of specific, extracellular microorganisms on host eggshells can be reliable host identity cues for egg parasitoids.

## 2.3 Variability in the use of chemical cues over time and space

With the multitude of chemical cues available to aid a parasitoid searching for eggs, the following question remains: when do parasitoids use a particular cue? The environment in which egg parasitoids live and search for host eggs is dynamic, and the availability and relative concentrations of chemical cues are ever changing along with the background odors. Recent reviews of the neuroscience and ecology of insect olfaction in complex environments have shown how multiple cues can be used simultaneously. Differences in the relative concentrations of cues against different background odors will alter a parasitoid's response to a given cue[40-42]. Often, mixtures of kairomones elicit stronger responses than kairomones presented on their own[4]. Plant and host cues may work in concert and affect the parasitoids' foraging behavior in field conditions[43,44]. Which chemical cues are used likely depends on nuances of spatial and temporal context.

### 2.3.1 Intraindividual variation

Over the life of an egg parasitoid, variation in its physiological state and past experience impact how the parasitoid searches for eggs[45]. For example, transcriptional changes after mating affect parasitoid behavior[46], as do changes in egg load[47] and feeding status[48]. Learning from experiences also greatly impacts which cues parasitoids find most attractive[49,50,51]. Memory formation is complex, dependent on not only conditioning but also on slight differences in reward value[52]. The interplay between innate and learned cues used for host-finding is of great interest[19,53]. 'Maladaptive learning' can occur when a new species is introduced, and there is a mismatch between cues and preferred species for oviposition, such as *Telenomus podisi* associatively learning cues of an unsuitable host[54,55]. The learning abilities of parasitoids can be used via parasitoid olfactory conditioning to enhance the efficiency of parasitism in the field[3].

The suitability of the environment in which the emerged or released egg parasitoid finds itself will also greatly impact which cues it seeks. The variability in landscape characteristics is often overlooked, despite several studies indicating that these factors greatly affect the biology and behavior of egg parasitoids, and thus parasitism rates[47,56,57]. Based on the quality of the patch, and which host species are readily available, an egg parasitoid must decide whether to stay and seek eggs, or whether to find a new patch. To make this assessment, entirely different chemical cues and host-finding modalities may be used (Figure 2.2). As the number and nuance of factors known to affect an individual's host-finding behavior continues to grow, computer modeling will likely be a valuable tool for predicting parasitoid responses and thus in designing dynamic biocontrol programs[45].

### 2.3.2 Intrapopulation variation

Even within a single population of egg parasitoids, differences in host-finding behavior will often exist between individuals. For example, experiences before emergence may influence host preferences, i.e., pre-imaginal learning. The choice of host by the parent may alter the preferences of the offspring after only a single generation[58], possibly a result of cues gathered from antennation before the parasitoid hatches from the egg. In other cases, rearing parasitoids for multiple generations on an alternative host does not seem to create a preference for the volatiles of the alternate host over its natural host[59]. Seasonal changes, including changes in the population dynamics of hosts, may also be associated with differences in egg parasitoid cue use within or between generations. An early season parasitoid may prefer the cues associated with a host that has an egg stage early in the season, matching its phenology. Seasonal changes in host use by egg parasitoids have been found[60], as well as seasonal changes in the attraction to the host-specific chemical cues between generations[61]. For example, *Ooencyrtus pityocampae* parasitizes eggs of the pine processionary moth (PPM) but emerges after overwintering in PPM eggs well before the next generation of these moths will fly. Instead, the para-

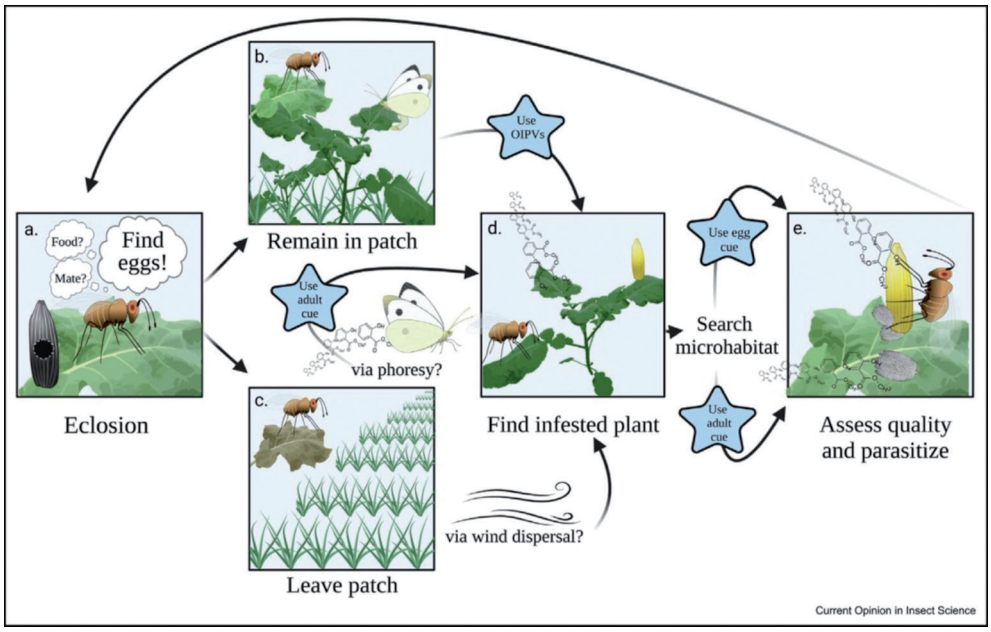



**Figure 2.2)** Schematic representation of how an egg parasitoid variably uses chemical cues to find host eggs after eclosion depending on the context in which it ecloses. **(a)** Which cue is used may depend on the physiological state of the egg parasitoid (altered by, e.g. previous feeding and mating experiences), and on the quality of the patch in which it ecloses (e.g. the proximity to fresh host eggs). **(b)** If the parasitoid remains in the patch, it may use OIPVs to locate an infested plant. **(c)** If the current patch does not provide sufficient oviposition opportunities, the parasitoid may use phoresy or wind dispersal to find a more suitable patch. Adult host pheromones may act as a short-range cue for a phoront to find a host on which to ride. **(d)** Once at an infested plant, short-range cues from the egg itself or left behind by ovipositing females may guide the parasitoid to eggs. **(e)** Once host eggs are found, the parasitoid assesses their quality and decides whether to accept them for oviposition. When the offspring emerge, the process repeats as the quality of the patch and availability of different host species changes over the season. The parasitoid's response to all cues shown may be innate or learned, thus, successive host searches may be influenced by the successes or failures of earlier attempts.

sitoids use the eggs of *Stenozygum coloratum*, the caper bug, as an alternate host in the spring and summer. These spring and summer wasps do not display attraction to the sex pheromone of the PPM, while the later generations do. DNA barcoding confirmed that these parasitoids are of the same species and are not genetically differentiated[61]. DNA barcoding is an especially useful tool for accurate species identification for many taxa of minute egg parasitoids that are difficult to distinguish morphologically and/or have suffered from inaccurate and ever-changing systematics, such as species of *Ooencyrtus* or *Trichogramma*[62]. It is clear that this species varies in host and cue use seasonally. So far, seasonal variation in egg parasitoid cue use has not been considered in designing biological control programs.

### 2.3.3 Intraspecific variation

While it is tempting to generalize at the species level, in many cases, strains of the same species have been found to respond differently to both host- and plant-derived odors[27,29,63,64]. For application in biological control, it is essential that findings with one strain of parasitoid are not necessarily generalized to the entire species. For example, the attraction of one strain of a generalist species to particular host volatiles does not necessarily mean the same preference will be found in a geographically distant population, where it may experience cues of different hosts. DNA sequencing that can identify genetic differentiation can help to discover variation in behavior below the species level, also by clarifying host associations more accurately[65]. Furthermore, discovering the genetic basis of parasitoid preferences allows for selection and breeding for these traits[66,67]. Investigating genetic variation between populations that evolved with different community interactions will give insight into the microevolutionary processes of cue use by egg parasitoids[68,69] and the coevolution of signalers and receivers[70]. It would also be interesting to investigate whether and when exploitation and natural selection on host cue use by egg parasitoids may select for changes in host (plant) chemical communication.

### 2.3.4 Interspecific variation

On a larger timescale, we can consider how host cue use has evolved between parasitoid species. While chemical cue use is highly context dependent, some aspects of the biology of parasitoids point toward their likelihood to use a certain cue. For example, inherent differences in flight capabilities between species likely influence the propensity for an egg parasitoid to use long-range cues. For a species with strong flight capabilities, long-range host cues, such as those from calling virgin moths, may be used to locate and fly to an egg patch. On the other hand, parasitoids that have typically been found to rely on downwind flight for undirected dispersal[71], such as many in the families Trichogrammatidae and Mymaridae, especially benefit from phoretic behavior[21,72]. Parasitoids with weak ovipositors, such as *Telenomus*, are also often phoretic as they require freshly laid eggs of which the chorion has not yet hardened[21]. In *Trichogramma*, most known examples involve the use of long- and short-range cues derived from the host with fewer examples of cues derived from plants. Other taxa seem less reliant on phoresy and using host-derived cues for long-range host-finding. For *Trissolcus*, it seems that plant-derived compounds are the main cues utilized to find a patch, but upon contact with the plant, cues from their stinkbug hosts become increasingly important for their ultimate oviposition decision. Differences in dietary specialization between species may also impact cue use. For example, more specialist egg parasitoids respond innately to chemical cues while generalists require associative learning[73]. However, a lack of accurate host range data precludes generalization.

Divergence of cue use between egg parasitoid species may be strongly tied to divergence in their host use. Investigating the evolution of genes associated with olfaction, such

as those for olfactory receptors and odorant-binding proteins (OBPs), via comparative genomics and transcriptomics coupled with electrophysiology and behavioral assays, can lead to exciting insights into the coevolution of egg parasitoids and their hosts. The rapidly growing availability of high-quality genomes, and tissue- and sex-specific transcriptomic data, allows for better annotation and more accurate comparison of olfactory genes[74-77]. For example, identification of OBPs in *Trissolcus* has revealed lineage-specific expansions as well as orthologs in other Hymenoptera[78]. The presence of large receptor families in trichogrammatids and pteromalids indicates the evolution of high complexity in olfaction in these parasitoids, while relatively simple genetics underlying chemosensation was found for a species of Mymaridae[79]. Furthermore, identification of olfactory-associated genes in parasitoids may also reveal coevolution with host genes that synthesize the compound that the egg parasitoids use as a cue. For example, two pairs of OBPs were found to be similar between *Telenomus podisi* and its preferred host *Euschistus heros*. These OBPs have no known orthologues in other Hymenoptera, suggesting that their possible independent evolution and convergence allowed *T. podisi* to use *E. heros'* semiochemicals[80]. Investigating the timing of such events can help to elucidate the coevolution of egg parasitoids and their hosts.

## 2.4 Future aims:

- Accumulate more information on population differences in chemical cue use in the field.
- Use DNA barcoding to obtain more accurate taxonomic coverage on which parasitoids use which hosts, and to compare genotypic and phenotypic variation between individuals, populations, and species.
- Elucidate the genetic basis underlying variation in cue preference between parasitoid strains and species.
- Investigate the (co)evolutionary effects of selection by eavesdroppers on host cues and signals.

## 2.5 Conclusions

Host finding by egg parasitoids is variable by nature, and this dynamic nature is a fundamental aspect of the parasitoids' strategy to overcome the challenges of finding an egg under variable spatial and temporal circumstances. The oversimplification of host-finding in complex habitats can result in ill-advised release strategies leading to failure of biological control programs. While it is necessary to use laboratory assays to evaluate the physiological possibility for parasitism of a given host by a particular parasitoid, this infor-

mation says little about the likelihood of the wasp to reliably seek out and parasitize the host's egg in the field. Many factors affect field parasitism rates, which cannot be directly deduced from parasitism assays in the lab. Future research efforts should elucidate a) whether the parasitoids are attracted to chemical cues emitted by a particular host population and/or cues induced from a particular host-plant in the context of ever-changing background odors, and b) whether or not the parasitoid can physically travel distances to the host egg in a given landscape and under variable biotic and abiotic conditions. Investigating phenotypic and genotypic variability between individuals, populations, and species will give a more complete understanding of how egg parasitoid host finding has evolved, and how it can be exploited to enhance biological control. It is important to accept that context is highly important to how and when egg parasitoids use chemical cues. Application of egg parasitoids in biological control will require more nuanced consideration of the dynamics of a given crop and parasitoid system, and therefore a more 'personalized' approach.

## Authorship

**LOG**: Conceptualization, Writing – original draft. **MEH**: Writing – review & editing. **ATG**: Writing – review & editing. **AC**: Conceptualization, Writing – original draft. **NEF**: Conceptualization, Writing – original draft.

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CHAPTER 3

# 3

# It’s about time for oviposition-induced cue use

This chapter is based on:

**Greenberg, L. O**. & Fatouros, N. E. (2025). Agroecology: It’s about time for oviposition-induced cue use. *Current Biology*, 35(13), pR651-R654

## Abstract

Egg parasitoids face significant challenges in locating and selecting suitable hosts, often relying on plant-emitted chemical cues to guide oviposition decisions. This review examines recent findings by Tian and Wang et al., which reveal that *Trichogramma japonicum* utilizes oviposition-induced plant volatiles (OIPVs) not only to locate host eggs but also to assess their quality from a distance—an ability not previously documented. The study further demonstrates that OIPV profiles change with the age of the deposited eggs, with certain volatiles peaking at optimal times for parasitoid development. These novel insights are discussed in the broader context of insect–plant–parasitoid interactions, highlighting the ecological and evolutionary significance of time-sensitive and host-specific chemical signaling. The review also explores the practical implications of these findings for biological control, including the manipulation of volatile cues through synthetic application, elicitor use, and crop breeding strategies. Together, the reviewed literature underscores the potential of leveraging parasitoid sensory ecology to improve the precision and efficacy of pest management in agricultural systems.

For a wasp the size of a grain of sand, finding the right egg to parasitize is a monumental task (Chapter 2)[1]. Amidst a jungle of leaves bombarded with chemical cues, parasitoids must not only find the right habitat patch and the right plant, but also the right egg, and within a very brief window of time when it's suitable for their offspring. Their small size and short lives limit dispersal opportunities, so there is little room for error in patch selection. As the egg itself does not reveal itself with long-range cues, how exactly do the wasps accomplish this? It is suggested that egg parasitoids of herbivorous hosts use plant-related cues, such as oviposition-induced plant volatiles (OIPVs), to home in on host eggs (Chapter 2)[1-3]. Recent work by Tian and Wang *et al.*[4] uncovers the surprising fact that egg parasitoids also use OIPVs to assess the quality of host eggs from a distance. They investigate the preferences and performance of *Trichogramma japonicum*, an important egg parasitoid used against rice pest moths, with the rice leaf folder *Cnaphalocrocis medinalis*. Their results reveal that the tiny wasps use OIPVs to efficiently assess the suitability of host eggs from a distance, conserving time and energy. *Trichogramma* wasps' sophisticated host-finding strategies deepen our understanding of plant–herbivore–parasitoid interactions and suggest avenues to fine-tune biological control strategies in agriculture.

Many plants, including valuable crops like rice, are plagued by infestations of herbivorous insects. Before larvae begin chewing on plant leaves, there is often a warning for the plant when the adult herbivores deposit eggs onto the plant's tissues. Plants evolved to detect this earliest cue of an incoming attack and defend themselves before the relatively harmless egg hatches into a voracious larva[5] . These defenses can include direct responses, such as hypersensitive responses, which kill eggs by causing them to desiccate or fall off the plant[6]. Responses can also be indirect, for example by recruiting natural enemies, such as egg parasitoids and predators, who in turn kill the eggs (Chapter 2)[1,3]. These enemies are typically recruited via volatile compounds, or smells, emitted by the plant in response to the pest eggs. Sometimes, direct and indirect defenses are most effective in combination, like the so-called double defense line, whereby egg-killing and egg parasitoid recruitment occur synergistically[6].

Plant volatile compounds travel far and wide, carrying information about the plant's current state to distant insects. Which smells the plant emits, and in what ratio, is shaped by a myriad of factors, such as abiotic stressors like drought[7] and biotic interactions with insects and microbes[8], both above- and belowground[2,9] (Figure 3.1A). Of particular use to parasitoids, whose offspring develop inside herbivorous insects' eggs, are the plant volatile blends induced by their hosts' eggs. These OIPVs are highly variable. The nuanced information conveyed by OIPVs helps egg parasitoids locate the most suitable host eggs for oviposition in a given temporal and spatial context (Chapter 2)[1]. Previous studies have demonstrated that parasitoids respond to and use OIPVs to find hosts and select suitable plants[2,10-12]. OIPVs, and responses to them, can vary with different plant strains[5], different

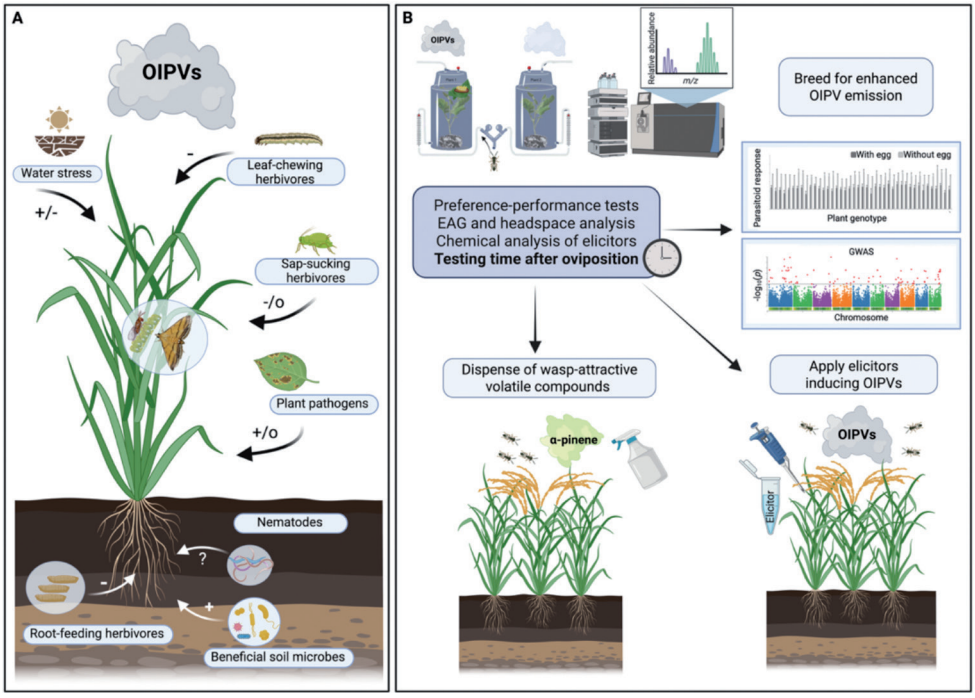



**Figure 3. 1**) Drivers of oviposition-induced plant volatile (OIPV) specificity and paths to application in biological control. (**A**) Emission of OIPVs can be altered by abiotic factors and biotic interactions, including with herbivores and microbes above- and below-ground (revised from Fatouros *et al.*[2]). These drivers can enhance volatile emission (+), reduce volatile emission (-), have a neutral effect (O), or have not been investigated (?). (**B**) Analyzing preference and performance of parasitoids for eggs of various ages, and the volatiles these different eggs induce, can be combined to find the most attractive OIPVs. These compounds can be dispensed in fields, for example, by spraying; induced in plants, for example, by applying elicitors; or considered in plant resistance breeding, for example, by using genome-wide association studies to identify the genetic mechanism of attractive OIPV emission.

herbivore species[13], and even with the time since the egg was deposited[14]. Now we also know that OIPVs aid in parasitoids' host quality assessment, indicating the presence and suitability of host eggs from afar[4].

It is rare for studies to clearly link parasitoid preferences with performance, or to identify the specific plant volatiles used in host location. Tian, Wang, and colleagues addressed this by testing preferences of *T. japonicum* for, and the performance on, eggs of different ages to understand whether they can efficiently make use of particular OIPVs to assess the quality of eggs from the rice pest *C. medinalis*[4]. They used a Y-tube olfactometer and field cages to test preferences for OIPVs, and measured developmental time and eclosion rates to evaluate performance. The results showed that *T. japonicum* females prefer vola-

tiles from rice plants with two-day-old eggs, parasitizing more of these eggs. These were also found to be the most suitable eggs for parasitoid development. Two-day-old eggs induced significantly higher levels of two terpenes emitted from rice plants, D-limonene and α-pinene. Interestingly, previous studies have also noted increased α-pinene emission two days post-oviposition in other systems, also attracting *Trichogramma* parasitoids[14]. The authors further demonstrated that these two volatiles alone can guide wasps to the most suitable eggs in an olfactometer. Using knockout rice plants that lacked these monoterpenes, they showed that wasps were no longer attracted. However, applying synthetic D-limonene and α-pinene restored this attraction. These findings highlight the role of time-sensitive volatile cues in host location and quality assessment. This sophisticated strategy in *Trichogramma* wasps offers promising avenues for enhancing biological control through targeted use of plant traits.



While these wise mother wasps make very successful parasitoids, their pickiness can pose challenges for farmers who wish to harness their pest-suppressing abilities in crops[1]. The pest in this study, *C. medinalis*, is a migrating moth whose larvae occur in large outbreaks which devastate rice crop yields[15]. As the climate warms, the range of this pest is expanding, and the threat it poses to rice cultivation rapidly grows[15]. Therefore, efficient biocontrol of *C. medinalis* is imperative, and needed now more than ever. While Tian and Wang et al.[4] have demonstrated the nuances in cue use by *T. japonicum* that facilitate its ecological success, this nuance may not translate directly to successful use in agriculture. For example, should a more suitable egg be deposited nearby, these more attractive volatiles could lure the biological control agents out of the field we intend for them to manage.

*Trichogramma*-based biological control in rice is well established in China, with its application steadily expanding each year[16]. Trichogramma japonicum has been proven to be an effective agent against lepidopteran pests of rice[4], achieving parasitism rates exceeding 80%[16]. These parasitoids are relatively easy to mass-rear. A single production line can yield two billion *T. japonicum* annually, making them ideal for inundative releases in augmentative biological contro[l16]. Because *Trichogramm*a are haplodiploid and multiple strains can be reared in limited space, it's possible to preserve within-species diversity by maintaining iso-female lines created from individual females (Chapter 2)[1,17]. This is important because *Trichogramma* species exhibit intra-specific diversity in their use of host-finding cues[6,12]. By limiting genetic variability within a line through iso-female rearing, adaptation to lab conditions can be minimized, allowing valuable genetic diversity to be maintained even over many generations[17]. However, since *T. japonicum* is more costly to rear than other *Trichogramma* species, due to their dependence on smaller factitious host eggs in rearings[16],we must continue to optimize this promising and effective species' efficiency in agricultural settings.

To leverage parasitoids' ability to discriminate chemical cues, we can manipulate cue emission[18] (Figure 3.1B). For example, farmers can exploit parasitoid-attracting cues to retain biocontrol agents in desired patches, regardless of the wasps' natural preference[19]. Fields can be made attractive by continuously emitting cues signaling the presence of optimally aged eggs, even if only older or younger eggs are available. However, care must be taken to ensure these volatiles do not attract additional pests[18,19]. Attractive chemical cues can also be applied through targeted spraying alongside parasitoid releases to retain target enemies, which is especially effective when combined with rewards[19]. Alternatively, substances that induce OIPVs (elicitors) can be used, which may enhance both indirect and direct plant defenses[18]. Breeding crops for enhanced egg defenses offers further potential, especially using wild relatives that are often more attractive to enemies because they retain their natural strong herbivore defences[5]. Additionally, genome-wide association studies (GWAS) can identify the genetic make-up behind OIPV production[20], enabling genetic modification of crops to constitutively emit attractive volatiles[5,18], independent of egg presence or age. Although primarily tested with larval parasitoids and predators, these strategies are also applicable to egg parasitoids and OIPVs.

This early warning system that plants utilize to recruit natural enemies via OIPVs has rarely been exploited for biological control[2]. However, manipulation of plant defenses against herbivore eggs is increasingly possible. Therefore, it is about time we utilize egg parasitoid's sensitive use of chemical cues in agriculture against pest eggs[2,13]. We learn from Tian and Wang et al[4] that increasing the efficiency of biological control agents may also be about time, or timing. Incorporating the time-calibrated decision-making of egg parasitoids into a more nuanced system of biological control may be the key to harnessing the pest-suppressing power of these wise wasps.

## Authorship

**LOG**: Conceptualization (equal), Writing – original draft, Writing- reviewing and editing.
**NEF**: Conceptualization (equal), Writing- reviewing and editing, Visualization.

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CHAPTER 4



# Oviposition strategies in Pieridae butterflies and the role of an egg-killing plant trait

This chapter is based on:

Peters, D. H.*, **Greenberg, L. O**.*, & Fatouros, N. E. (2024). Oviposition strategies in Pieridae butterflies and the role of an egg-killing plant trait therein. *Ecology and Evolution*, *14*(7), e11697.

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## Abstract

Most herbivorous insects are host-plant specialists that evolved detoxification mechanisms to overcome their host-plant's toxins. In the evolutionary arms-races between Pieridae butterflies and Brassicaceae plants, some plant species have evolved another defense against the pierids: egg-killing. Underneath the eggs, leaves develop a so-called hypersensitive response (HR)-like cell death. Whether some butterflies have evolved oviposition strategies to counter-adapt against egg-killing remains to be studied. In this study, we assessed the oviposition site location of Pieridae butterflies on their natural host-plants. We described the plant tissue on which we located the eggs of the most common Pieridae in the Netherlands: *Gonepteryx rhamni*, *Anthocharis cardamines*, *Pieris rapae*, *P. napi*, *P. brassicae* and *P. mannii*. Additionally, we assessed expression of HR-like cell death in response to the deposited butterfly eggs. We found that both *A. cardamines* and *G. rhamni* mainly oviposited on the floral stem and the branch, respectively, and oviposited on host-plants from lineages not expected to kill pierid eggs. Accordingly, no HR-like cell death was seen. All *Pieris* eggs found were located on leaves of their host, the only tissue found to express HR-like cell death. Furthermore, each *Pieris* species was found to at least occasionally oviposit on *Brassica nigra*. This was the only plant species in this survey that expressed HR-like cell death in response to the eggs of *P. rapae*, *P. napi* and *P. brassicae*. Our observations demonstrate that HR-like cell death remains an effective defense strategy against these *Pieris* species and as such did not find evidence for the hypothesized counterstrategies. Surveying certain key species and disentangling the micro-evolution of oviposition strategies within a species would allow us to further investigate potential counter-adaptations that evolved against HR-like cell death. This study provides the basis for further investigation of potential counter-adaptations to egg-killing defenses.

## 4.1 Introduction

In the arms-race between insects and plants, different plant families evolved specific toxins against herbivores[1]. In response, some herbivores evolved mechanisms to detoxify the chemical defenses of their host-plants[2]. This results in a coevolutionary arms-race between the plant and attacker that drives the diversification of plant defense chemicals and, in parallel, drives the evolution of new detoxification mechanisms, host shifts and speciation[3–5]. A well-studied example of arms-races between insects and plants are between Pieridae butterflies and Brassicaceae plants. Brassicaceous plants produce secondary metabolites, so-called glucosinolates, to defend themselves against herbivore attackers[6]. Meanwhile, caterpillars from the Pierinae subfamily have repeatedly evolved gut proteins called nitrile-specifier proteins (NSP) that allow them to detoxify the evolving glucosinolates of their host-plants[2,7]. While Pierinae larvae are clearly capable of overcoming their hosts' defenses, detoxification is only a valuable adaptation if there is an opportunity to feed. An understudied adaptation of plants to escape these specialist herbivores is defense against their egg stage[8].



Plants can recognize and respond to herbivore eggs before hatching[9], which prevents herbivore damage to the plant[8]. Plant defense mechanisms can cause egg desiccation, dropping, crushing and killing by ovicidal substances[9,10]. So far, egg-killing traits have been documented in more than 30 different plant species across 12 plant families[11]. Until now, research has mainly focused on toxins against the feeding stages of herbivores and the adaptations developed by these herbivores against them, namely detoxification mechanisms. As a result, interactions between insect eggs and plants have been understudied[8].

The killing of herbivorous insect eggs by the development of leaf cell death has been shown for several species within the Brassicaceae and Solanaceae families[11]. Shapiro and DeVay[12] first described this egg-killing trait against *Pieris* butterflies in *Brassica nigra*. Eggs laid by *P. rapae* on *B. nigra* were found discolored, shriveled and dead due to a necrotic zone in the plant tissue underneath the eggs. This necrotic zone is caused by a hypersensitive response (HR)-like cell death which resembles HR against pathogens, causing the cytoplasm of cells to become disorganized and granulated followed by cell death. In a later study conducted by Fatouros *et al.*[13], induction of HR-like cell death by *P. brassicae* egg clutches was found in *B. nigra* and some eggs in these clutches did not hatch. Later, Griese *et al.*[14] found that gregariously laid *P. brassicae* eggs were less affected by HR. More *P. brassicae* eggs died on necrotic plants when the females were forced to lay single eggs as opposed to their natural oviposition of large egg clutches. Induction of HR-like cell death by *P. rapae* in *B. nigra* led to egg desiccation and resulted in fewer caterpillars hatching, especially when the cell death responses was very strong[15]. Recent research by

Griese et al.[8] showed that HR-like cell death was expressed by other Brassicaceous species closely related to *B. nigra* when induced by a wash made of *P. brassicae* eggs. Only plant species in the tribe Brassiceae and a certain *Aethionema* sp. express HR in response to butterfly eggs[8]. Moreover, only egg wash from different Brassicaceous feeding species of the Pierinae subfamily, including *P. brassicae*, *P. napi*, *P. rapae* and additionally *P. mannii* and *Anthocharis cardamines* induced cell death, while wash of non-brassicaceous feeding Pieridae such as *Gonepteryx rhamni* (subfamily: Coliadinae), *Colias* spp. (subfamily: Coliadinae), *Aporia crataegi* (subfamily: Pierinae) and *Leptidea sinapis* (subfamily: Dismorphinae) induced a lower or no HR-like cell death[8].

Pieridae butterflies may have found ways to overcome mortality due to egg-killing HR-like cell death by, for example, clustering their eggs, shifting to other host-plants not expressing HR or ovipositing on inflorescences instead of leaves [8]. Some Pierinae species have been found to oviposit on inflorescences (e.g. *A. cardamines* or *P. krueperi*) and so far no signs of cell death have been found on inflorescent organs[8,16]. Therefore, it seems unlikely that HR-like symptoms develop on inflorescences. Whether these proposed strategies are used in effect in nature can be investigated by exploring natural patterns of oviposition and the expression of HR in the field.

In this study, we thoroughly assessed the different host-plants of Pieridae butterfly species for egg deposition, as well as the responses of these plants to the deposited eggs. To the best of our knowledge, this is the first study that carefully describes the plant part on which eggs were deposited per plant species and cell death response to eggs for Pieridae species. We screened species common to the Netherlands, namely *G. rhamni*, *A. cardamines*, *P. rapae*, *P. napi*, *P. brassicae* and *P. mannii*. We hypothesize that HR will only be expressed by host-plants in the tribe Brassiceae and only in their leaves. Thus, species found to oviposit on other host-plants or plant tissues will not induce HR-like cell death.

## 4.2 Materials and methods

### 4.2.1 Plants and insects

Eggs of the following Pieridae species were included in this survey. We also list the host-plants that these species are most likely to use in the Netherlands.

- *Species of Brassicaceae that are in the lineage with most species that can express HR-like cell death in response to butterfly eggs (Tribe: Brassiceae) are denoted with a single asterisk[8].
- **The species that have been previously tested and found to express strong HR-like cell death in response to butterfly egg wash have been denoted with two asterisks[8].

***Gonepteryx rhamni*** (Subfamily: Coliadinae, Tribe: Gonepterygini)
Female Brimstone butterflies lay their eggs mainly on juvenile trees of *Rhamnus frangula* (Rhamnaceae). The eggs usually laid not only on the abaxial side of the first fully expanded *R. frangula* leaf within the bud[17], but may also be on a not yet expanded leaf[18]. Occasionally, they may be oviposited near the young leaves on the tip of the branch[19] or on the adaxial side of the leaf [18]. Although eggs are laid singly, eggs may be found together as a result of different females laying on the same leaf or a single female returning several times to the same leaf[18]. *Gonepteryx rhamni* egg wash did not induce HR-like cell death in *B. nigra*[8].

***Anthocharis cardamines*** (Subfamily: Pierinae, Tribe: Anthocharini)
Females of the orange-tip butterfly lay their eggs on the inflorescences of larval food plants, mainly wild Brassiceae. Overall, females oviposit single eggs near the siliques or flowers; however, observations of more eggs per plant and on different sites have been made[20].The eggs are initially straw-coloured and turn bright orange after 2–3 days. As the larvae are cannibalistic, normally only one larva completes its development on a single inflorescence[21]. Commonly used host-plants include *Alliaria petiolate, Cardamine pratensis* and *Arabidopsis thaliana* (Brassicaceae)[21,22]. Additionally, *Sisymbrium officinale*, *Sinapis arvensis**, *Hesperis matronalis, Arabis hirsute*, *Lunaria annua*, *C. amara*, *Brassica rapa** and *Barbarea vulgaris* may be used [18]. Egg wash of *A. cardamines* induced HR-like cell death in *B. nigra* [8].

***Pieris napi*** (Subfamily: Pierinae, Tribe: Pierini)
Females of the green-veined white butterfly lay their eggs on the leaves of several brassicaceous plants in moist habitats, mostly on wild Brassicaceae. They typically lay eggs singly on the abaxial side of a leaf of a small plant that is partially in the shade[23]. In the Netherlands, commonly used host-plants are *Alliaria petiolata, Cardamine pratensis, C. amara, S. arvensis, Raphanus raphanistrum**, *Lunaria rediviva*, *S. officinale, Nasturtium officinale, Brassica oleracea* var. oleracea, *B. nigra*** and *Diplotaxis tenuifolia**[18,23–27]. Plants with conspecific eggs are not necessarily avoided[28]. *Pieris napi* (egg wash) is known to induce HR-like cell death in *B. nigra*[8,12].

***Pieris rapae*** (Subfamily: Pierinae, Tribe: Pierini)
Females of the small white lay their eggs on the leaves of various brassicaceous plant species, both on crop and wild plants. The eggs are laid singly on plants and plants with conspecific eggs or larvae are not avoided[42]. Host-plants that are commonly used by *P. rapae* in the Netherlands are: *A. petiolata, C. pratensis, B. oleracea***, *B. napus***, *B. nigra***, *S. arvensis**, *R. raphanistrum**, *Reseda lutea, Tropaeolum majus, S. officinale, Lepidium draba* and *D. tenuifolia**[18,23–27]. *Pieris rapae* (egg wash) is known to induce HR-like cell death in *B. montana, B. rapa, B. nigra, H. incana, S. arvensis* and *R. sativus*[8,12,14].

***Pieris brassicae*** (Subfamily: Pierinae, Tribe: Pierini)
Females of the large cabbage white are gregarious and lay egg clutches containing 40–100 eggs underneath leaves[23]. *Pieris brassicae* uses different host-plants based on the season, including both wild and crop plants. In the spring and early summer, they use *B. rapa**, during the summer *S. arvensis** and in late summer *B. nigra***[41]. Other host-plants can be *B. oleracea**, *B. napus***, *R. raphanistrum** and *Tropaeolum majus*[27]. Of these host-plants, some of their most commonly used are known to express HR- like cell death, such as *B. nigra*. *Pieris brassicae* eggs (or egg wash) is known to induce HR-like cell death in several species, including *B. montana*, *B. nigra*, *Hirschfeldia incana*, *S. arvensis* and *R. sativus*[8,13,14,29].

***Pieris mannii*** (Subfamily: Pierinae, Tribe: Pierini)
Southern small white females lay single eggs predominantly on the underside of leaves from different Brassicaceous host-plants. Recently, this species underwent a rapid range expansion. In its native region, the dominant host-plants are in the native *Iberis* genus[30]. *Pieris mannii* populations from more recently colonized areas, such as in neighbouring Germany, were found to have reduced specialization and readily oviposited on a wide range of plants including *Alaria petiolata* and *D. tenuifolia*[30]. In the Netherlands, eggs are often laid on *Iberis* spp. that are frequently planted as ornamentals in gardens. Apart from *Iberis* plants, in the expanded ranges, eggs are also known to be laid on *Alyssoides utriculatum*, *L. graminifolium*, *L. pinnata*, *C. impatiens*, *Lobularia maritima* and *Aubrieta deltoidea*[16]. Egg wash of *P. mannii* induced HR-like cell death in *B. nigra*[8].

## 4.2.2 Field survey methods

Fieldwork for this research was conducted in the provinces Gelderland and South Limburg in the Netherlands between April and July 2021 (Table S4.1). Locations were selected based on anecdotal reports of a high abundance of butterfly and host-plant species. Eggs of *G. rhamni* were found at five locations in Renswoude, Wageningen and Wijchen. These locations were near roads and in forests. Eggs of *A. cardamines* were found at 12 locations in Wageningen, Wijchen, Arnhem and Vijlen. All locations were near streams or along paths. All Pieris eggs were found along paths, in fields and gardens at 16 locations in Wageningen and Wijchen. All host-plants were checked as they were found in the search locations. Therefore, some host-plants were found more often than others. For all herbaceous plants, all parts of the plants were checked for eggs. Egg searching on *Rhamnus frangula* is described below. The position of each egg was recorded by the following categories: branch, leaf and flower bud. Additionally, the presence of HR-like cell death was carefully observed with a magnifier or camera with a macrolens. Cell death was scored as described below. A photograph was taken of almost all found eggs.

### 4.2.3 Host-plant and egg identification

*Rhamnus frangula*, the host-plant of *G. rhamni* was identified in the field based on its morphology. All leaves and twigs on branches within a height of ±2 m to the ground were checked for eggs. The eggs were identified by morphology. The trees and eggs were found at five locations (Table S4.1) between mid-April and the beginning of June 2021. Eggs laid anywhere on the leaf bud, leaf or petiole were combined into the category 'leaf'. Other categories were 'flower bud' and 'branch'.

Three main host-plants of *A. cardamines,* namely *C. pratensis, A. petiolata* and *S. officinale*, were identified in the field from their characteristic morphologies. Eggs of *A. cardamines* were identified by their distinctive morphology and presence of adults at the field site. The plants and eggs were found at 12 locations between end of April until the beginning of June in 2021 (Table S4.1). Eggs laid directly on the bud were categorized as 'flower bud' and eggs on any other part of the inflorescence were categorized as 'flower stem'. Eggs on stems of siliques were categorized as 'fruit stem' and the other categories for these plants were 'stem' and 'leaf'.

As *P. brassicae*, *P. rapae* and *P. napi* have several overlapping host-plants, the following plants were simultaneously checked for all of their eggs: *B. nigra, B. napus, B. rapa, C. pratensis, A. petiolata, B. oleracea, R. raphanistrum, S. officinale, D. tenuifolia* and *S. arvensis*. Using morphological differences between *Pieris* eggs described by Frohawk[31], we tried to distinguish *P. rapae* and *P. napi* eggs by counting the number of longitudinal ribs that run along the sides of the eggs from the top to the base (S4.1). Ultimately, this method proved unreliable, and thus, the analysis for all solitary *Pieris* eggs was combined.

*Pieris brassicae* eggs were identified by morphology and their appearance in a clutch. Egg clutches on *B. nigra* were found at two locations in Wageningen from the end of June until the end of July in 2021 and egg development was documented over time.

Similar to *P. rapae* and *P. napi*, *P. mannii* is a solitary egg laying *Pieris* species. Two host-plants were primarily checked for the eggs of *P. mannii*: *Iberis* spp. and *D. tenuifolia*. On both plants, however, occasionally the eggs of the other solitary *Pieris* species could be found. When possible, eggs from these plants were observed for several days to observe the hatching larvae. *Pieris mannii* larvae are distinguishable from *P. napi* and *P. rapae* by their black head capsule. Furthermore, using this feature, several occurrences of *P. mannii* larvae hatching from eggs on other host-plants are also reported in the results.

### 4.2.4 Scoring of HR-like cell death

Cell death was scored according to Griese et al. (2017), with its severity scored from 1 to 3 and 0 meaning no cell death.

### 4.2.5 Literature survey methods for phylogenetic character mapping

The Pieridae phylogeny published by Wiemers *et al.* (2020)[32] was adapted to include only the species that occur in the Netherlands. Mapped traits were as follows: high likelihood of host-plant to express HR according to Griese *et al.* (2020)[14] and (2021)[8], Pieridae eggs induce HR in *Brassica nigra* according to Griese *et al.* (2021)[8], oviposition on leaves, inflorescence or other plant tissue, and HR observed in the field. For the commonly occurring species we surveyed, characters were mapped using our data from the field. For the remaining species, oviposition traits and natural host-plants were retrieved from the guide to the butterflies of Brittain and Europe[23], Eurobutterflies (http://www.eurobutterflies.com) and UK butterflies (https://www.ukbutterflies.co.uk).

### 4.2.6 Statistics

Chi-square tests were performed to examine the relation between the number and position of eggs laid by *G. rhamni* and *A. cardamines*. Chi-square tests were performed in R (version 2022.12.0) using the R package 'stats'[33] with function 'chisq.test'[34] .

## 4.3 Results

### 4.3.1 Oviposition of Coliadinae species on natural host-plant

***Gonepteryx rhamni***

A total of 21 *R. frangula* trees with deposited *G. rhamni* eggs were sampled. In total, 83 eggs were found on these trees. The number of eggs found per position on the trees was significantly different ($X^2$ (2, N = 3) = 59.1, p < .001), with the most eggs found on leaves (71.1%), followed by the branches (25.3%) and finally flower buds (3.6%) (Figure 4.1a–g). No visible HR-like cell death was observed beneath the eggs monitored on any of the 21 *R. frangula* trees.

### 4.3.2 Oviposition of Anthocharini (Pierinae) species on natural host-plants

***Anthocharis cardamines***

A total of 272 *A. cardamines* eggs were found on three host-plants, showing no induction of HR-like cell death. Most eggs were found on *C. pratensis* (66.5%), followed by *A. petiolata* (32.5%), and only two eggs were found on *S. officinale* (1%).

Significant differences were found for the number of eggs observed on four different positions on *C. pratensis* (X2 (3, N = 4) = 462.98, p < .001). Eggs were mainly found on the

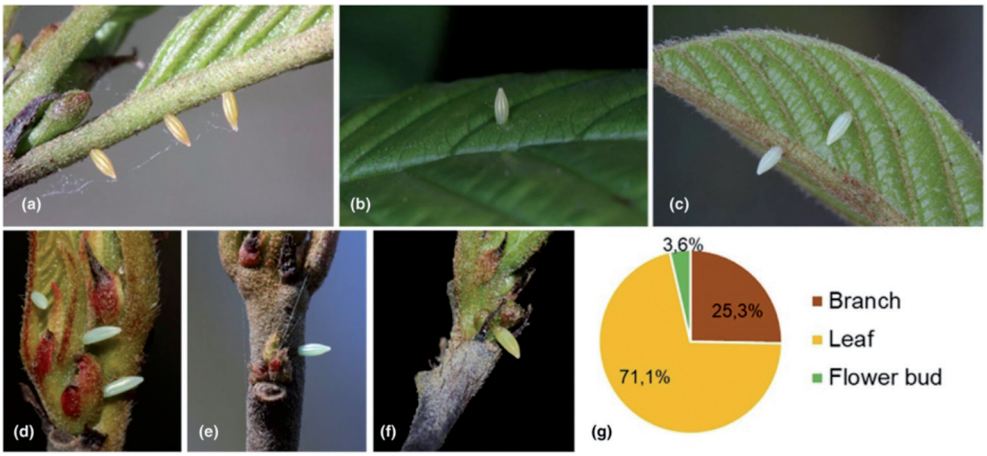



**Figure 4.1)** *Gonepteryx rhamni* eggs on *Rhamnus frangula*. (**a**) petiole and midrib, (**b**) adaxial side leaf, (**c**) abaxial side leaf and midrib, (**d**) leaf bud and midrib, (**e**) branch, (**f**) flower bud and (**g**) pie chart of the percentages of eggs found per position category.



flower stem (94.5%). Occasionally eggs were found on the fruit stem, stem or flower bud (1.7%, 2.2% and 1.7%, respectively) (Figure 4.2a–f).

On 93.3% of the examined *A. petiolata* plants, a single egg was found per plant. In addition, a few plants with two eggs were found and one with three or five eggs (3.4%, 1.1% and 1.1%, respectively). There was one exceptional plant with 12 *A. cardamines* eggs (1.1%), and two unidentified white eggs. These 14 eggs were spread over the lateral and terminal inflorescences and shoots. Significant differences were found for the number of eggs observed on five different positions on *A. petiolata* ($X^2$ (4, N = 5) = 167.35, p < .0001). Overall, most eggs were found on the flower stem and secondly on the fruit stem (74.2% and 14.6%, respectively). Further eggs were found on the stem, leaf or flower bud (5.6%, 2.2% and 3.4%, respectively) (Figure 4.2g–l).

On the two *S. officinale* plants examined, each had one egg on the flower stem of a lateral shoot.

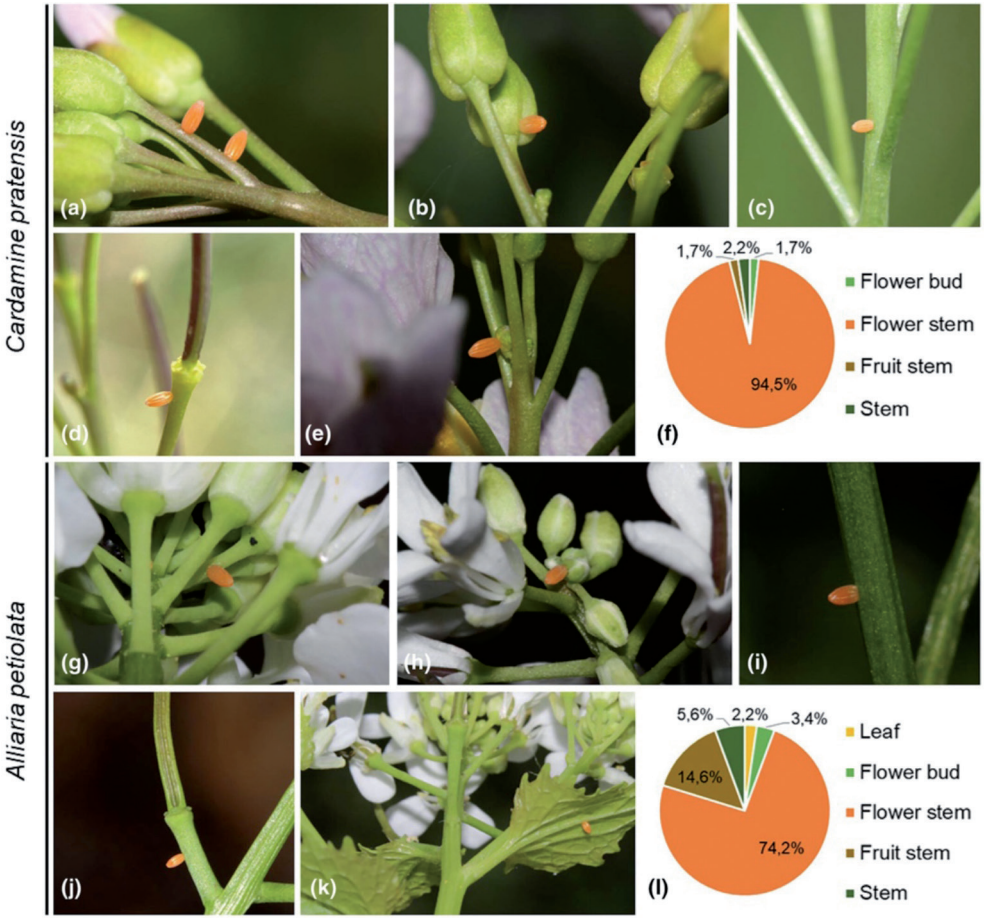



**Figure 4.2)** *Anthocharis cardamines* eggs on two host-plants. *Cardamine pratensis*: (**a**) flower stem, (**b**) flower bud, (**c**) stem, (**d**) fruit stem, (**e**) flower bud and (**f**) pie chart of the percentage of eggs per position category. *Alliaria petiolata*: (**g**) flower stem, (**h**) flower bud, (**i**) stem, (**j**) fruit stem, (**k**) leaf and (**l**) pie chart of the percentage of eggs per position category.

### 4.3.3 Oviposition of different Pierini (Pierinae) species on natural host-plants

***Pieris brassicae***

Six egg clutches of *P. brassicae* were found on two *B. nigra* plants (from now on referred to as clutch A to F), each of which induced varying degrees of cell death. Two of those clutches (A and B) were observed on a single plant over a period of ten days (Figure 4.3). Clutch A consisted of 30 eggs (Figure 4.3a), clutch B of 20 eggs (Figure 4.3b) and both showed no cell death on the first day they were found (score 0). A severe HR-like response (score 3) developed underneath both clutches after six days. No eggs from clutch A, and




**Figure 4.3)** Two *Pieris brassicae* egg clutches on *Brassica nigra*, one with 30 eggs (Clutch A) and one with 20 eggs (Clutch B), both monitored and photographed over a period of 10 days. The upper row shows the abaxial side of the leaf; the lower row shows the adaxial side. The number at the bottom right of photos indicates the number of days after the first observation. Cell death was initially scored as non-existent (score 0) and eventually scored as severe (score 3).



from clutch B, only four caterpillars hatched. The other eggs did not survive, probably as a result of HR-like cell death.

Clutch C consisted of 21 eggs and showed initially no cell death (score 0). After two days, the cell death had developed and was scored intermediate (score 2). After five days, 14 of the eggs had turned black due to parasitization, the remaining seven shriveled. Clutch D was found on the same plant as clutch C, containing 45 eggs and showing no cell death (score 0) on the first day. Three of these eggs were almost hatching and one caterpillar had already hatched. After one day, cell death developed and was scored with intermediate (score 2). By that time three additional caterpillars hatched, two eggs disappeared, and two eggs shriveled. After two days, only seven eggs were left, which were damaged. The other eggs had disappeared.

Clutch E with 79 eggs did not show any cell death (score 0) on the day it was found. After examining the pictures of the clutch, an unidentified species of *Trichogramma* wasp was found parasitizing on the eggs. After three days, cell death developed and was scored intermediate (score 2). Four days after discovering the clutch, the cell death was scored as severe (score 3), but all the eggs had disappeared, leaving no remaining eggshells. Clutch F was found on the same plant as clutch E and had 20 eggs that showed no cell death (score 0) on the first day. After four days, the cell death was scored with intermediate (score 2). This clutch was not further monitored as the plant was mowed. Clutches E and F were found on the same *B. nigra* plant as clutch A and B, but a month later.

**Solitary *Pieris* spp**

A total of 321 solitary *Pieris* eggs were found in the field on the following host-plants: *A. petiolata* (0.3%), *B. nigra* (17.1%), *B. oleracea* (13.1%), *B. rapa* (4.4%), *D. tenuifolia* (2.8%), *R. raphanistrum* (3.7%), *S. arvensis* (0.3%), *S. officinale* (0.9%) and *Iberis* spp. (57.3%) (Figure 4a–e,g). We could not discriminate singly laid *Pieris* eggs using the method of Frohawk (1924)[31] as the number of ribs was overlapping between *P. napi* and *P. rapae* eggs (S4.1). The eggs were all found on various parts of the leaves (Figure 4a-f). Twelve eggs found on *B. nigra* induced cell death in the leaves with a score varying from mild to severe (score 1–3), thus 22% of solitary *Pieris* eggs on *B. nigra* induced cell death (Figure 4.4a,b,g). Of those, two eggs, whose cell death was scored intermediate (score 2) and severe (score 3) were missing after a few days, but no caterpillar was found. Out of one egg (cell death

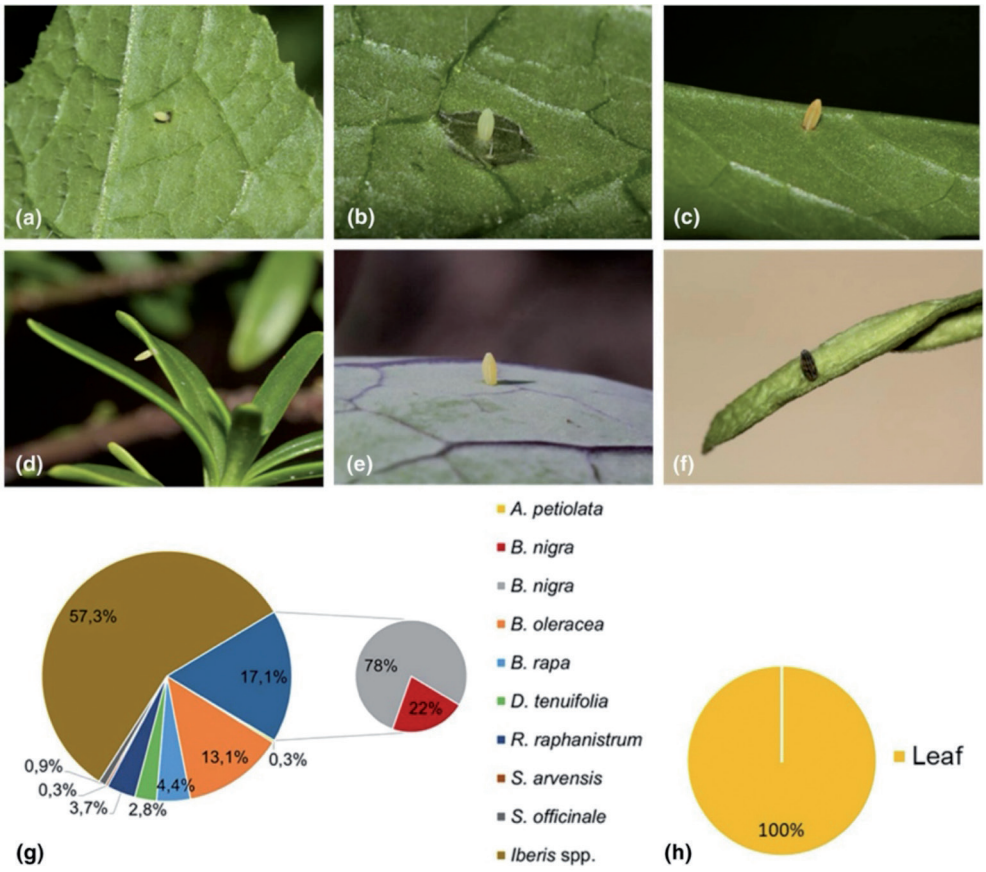



**Figure 4.4)** Solitary *Pieris* eggs on different host-plants. (**a, b**) on *Brassica nigra* inducing HR-like cell death, (**c**) on *B. nigra* not inducing HR-like cell death, (**d**) on *Iberis* spp., (**e**) on *B. oleracea*, (**f**) on *Iberis* sp. and parasitized by a *Trichogramma* sp., (**g**) pie chart of the percentage of eggs per host-plant, for *B. nigra* split into the percentage of plants showing cell death (red) and no cell death (grey) and (**h**) pie chart of the percentage of eggs per position category.

scored with a 2), a caterpillar hatched. Other eggs were not monitored over time. All other eggs found on *B. nigra* did not induce HR-like cell death and neither did eggs found on all other host-plants.

Nearly all eggs found on *Iberis* spp. (Figure 4.4d) that could be identified as larvae were found to be *P. mannii* (>90%, personal observation). This accounted for most of the *P. mannii* observed in this study; however, several *P. mannii* eggs were found on a *Diplotaxis* sp., and notably, also on *B. nigra* on two occasions. No eggs on *Iberis* spp. or *Diplotaxis* sp. were found to have induced HR and of the 12 solitary eggs that did induce HR on *B. nigra*, none could be confirmed as *P. mannii* or otherwise.

### 4.3.4 Mapping host-plant usage, the presence of oviposition and host-plant traits on Pieridae phylogeny

The literature-based expectations and field-based observations for the species studied in this system, as well as the theoretical expectations for the other most commonly found Pieridae species in the Netherlands, are reported below (Figure 4.5). *Gonepteryx rhamni* eggs are expected to be found on Rhamnaceae hosts, and as such, should not and did not induce HR in the field. *Anthocharis cardamine* eggs can be found on a range of brassicaceous hosts; however, the dominantly used species are not likely to express HR and the dominantly used plant tissues, flowers and stems, are also not expected to express HR. As such, in our study, the species found with *A. cardamine* eggs were *C. pratensis* and *A. petiolata* and no occurrences of HR expression were documented. *Pieris brassicae* eggs were found on *B. nigra*, a host-plant known to express strong HR-like cell death in response to *Pieris* eggs. All six clutches found indeed had strong HR expression. *Pieris rapae* and *P. napi* eggs could not be distinguished, but both were found on the leaves of the expected range of host-plants. Only on *B. nigra*, known to express HR in response to solitary *Pieris* eggs, did we observe HR-like cell death. *Pieris mannii* is expected to use *Iberis* plants, which do not express HR. Most *P. mannii* were found on *Iberis* and indeed no HR was expressed. They are also known to occasionally oviposit on inflorescence, which was not observed in our survey. Additionally, as known for their expanded range, *P. mannii* eggs were found on alternate host-plants, including *D. tenuifolia* and *B. nigra*. We have no known instances of *B. nigra* expressing HR in response to *P. mannii* eggs. On *B. nigra*, the vast majority of eggs were either *P. rapae*, *P. napi* or *P. brassicae*, and thus, it is not expected that any of the 12 eggs that did induce HR were likely to be *P. mannii*. Of the species we did not survey, neither eggs from *Leptidea sinapis* (Dismorphiinae) nor *Colias croceus* (Coliadinae) induce HR in *B. nigra* or their natural host-plant. Wash made from *Aporia crataegi* eggs did not or only slightly induce cell death in *B. nigra* (Figure 4.5). Whether eggs of the Western bath white *Pontia daplidice*, which lays its eggs on inflorescences, induce HR in their host-plants remains to be studied.

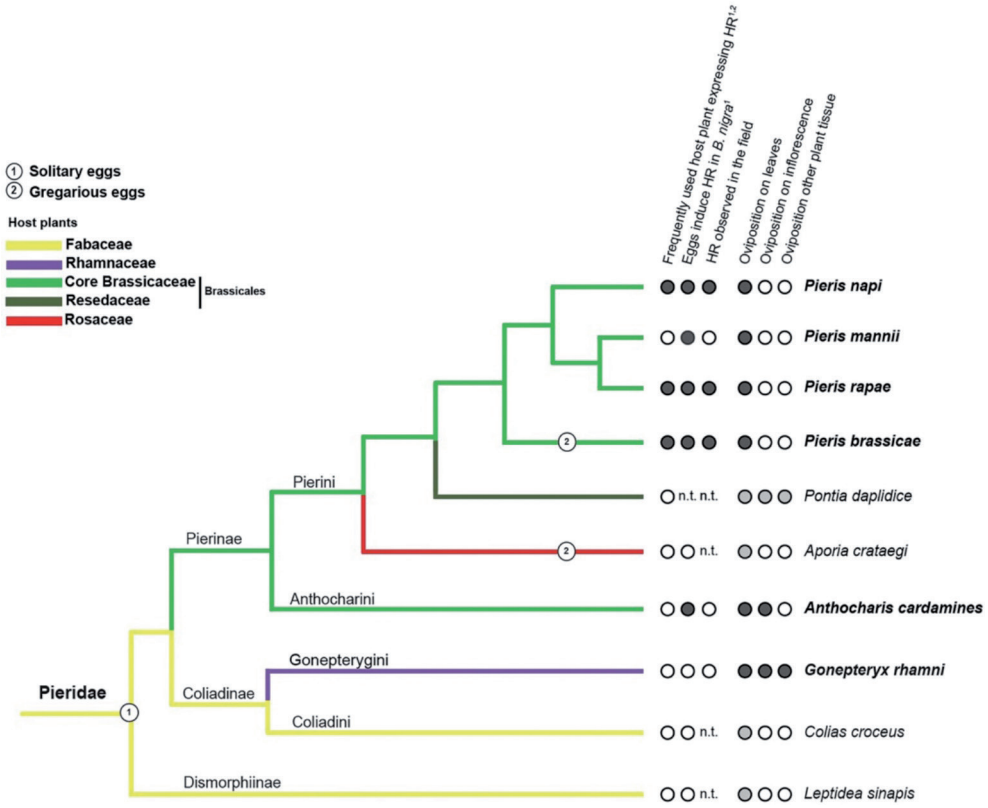



**Figure 4.5)** Mapping host-plant usage, the presence of oviposition and host-plant traits on a recently published Pieridae phylogeny by Wiemers *et al.* (2020). We focused only on species that can be found in the Netherlands, and those which occur commonly and were screened in this study are written in bold (Lewington, 2016). Filled dark circles denote presence observed in this study, empty circles absence according to this study and/or literature and filled grey circles presence according to the literature. n.t. not tested. The branches in the Pieridae phylogeny are colored to primary host-plant associations of the various lineages: Fabaceae (yellow), Rhamnaceae (purple), core Brassicaceae (bright green) and Resedaceae (dark green)—the latter two belonging to the Brassicales, and lastly Rosaceae (red). Mapped traits were as follows: High likelihood of host-plant to express HR according to Griese *et al.* (2020)[1] and Griese *et al.* (2021)[2], Pieridae eggs induce HR in *Brassica nigra* according to Griese *et al.* (2021)[1], oviposition on leaves, inflorescence or other plant tissue and HR observed in the field. Oviposition traits and natural host-plants were retrieved from Tolman and Lewington[23], Eurobutterflies (http://www.eurobutterflies.com) and UK butterflies (https://www.ukbutterflies.co.uk). Most Pieridae species are solitary and lay single eggs (1) with a few exceptions namely *Pieris brassicae* and *Aporia crataegi* which are gregarious species and lay clustered eggs (2).

## 4.4 Discussion

In this field survey, we found 891 Pieridae eggs and for each egg recorded the host-plant and plant tissue on which it was laid, as well as whether the plant showed HR-like cell death underneath the egg. Neither eggs of *G. rhamni* nor *A. cardamines* induce HR-like cell death in their natural host-plants, and their eggs were mainly laid on plant parts other than the leaves. For Pieris species, eggs were always laid on the leaves and cell death was only observed on *B. nigra* leaves but not on *A. petiolate*, *B. oleracea*, *B. rapa*, *D. tenuifolia*, *R. raphanistrum*, *S. arvensis*, *S. officinale*, nor *Iberis* spp. Cell death was never observed on other plant tissues than leaves.

***Gonepteryx rhamni*** belongs to the Coliadinae subfamily of the Pieridae, which does not feed on Brassicaceae. Unlike the other Fabaceae feeding Coliadinae, the *Gonepteryx* butterflies use Rhamnaceae as host-plants[17] (Figure 4.1). In this study, all eggs of *G. rhamni* were found on *R. frangula*. To the best of our knowledge, no Rhamnaceae are known to express HR-like cell death in response to any insect eggs[8]. Eggs were mainly laid on the branches, young leaves and yet unfolded leaves as previously described[17,19]. Multiple eggs were found on the same leaves and branches, most likely due to different females laying on the same leaf or a single female returning[18]. As expected, no cell death was found in any of the tissue types where eggs were laid, including leaf tissues.

Of the Pierinae subfamily, which all feed on Brassicaceae, we surveyed *A. cardamines* and four species of *Pieris* namely: *P. brassicae*, *P. rapae*, *P. napi* and *P. mannii*. Of these, two were not found to induce HR-like cell death in their brassicaceous host-plants:

***Anthocharis cardamines***: Species in the Anthocharini tribe also feed on brassicaceous plants; however, their NSPs evolved separately from *Pieris*[1] and their preferred host-plants are also slightly shifted from the other Pierinae in this study (Tribe: Pierini). Eggs of *A. cardamines* were mainly laid on the flower stems of *C. pratensis*, *A. petiolata* and *S. officinale*, and occasionally, eggs were found on the fruit stem, stem, flower bud or leaves, which corresponds with previous findings[2,3]. The main host-plant used in our study, *C. pratensis*, has very small leaves that were never found to have eggs. On hosts with bigger leaves, such as *A. petiolata*, *A. cardamine* eggs were occasionally found on leaves, but never found to induce HR-like cell death. While egg wash of *A. cardamines* induced HR-like cell death in the leaves of *B. nigra*[8], it is not surprising that eggs did not induce a necrotic response in the leaves of *A. petiolata* nor *C. pratensis*, as both belong to clades where expression of HR-like cell death is not known[8]. Thus, the fact that most *A. cardamines* eggs were laid on tissues that do not express HR cannot be attributed in these cases to a counterstrategy to avoid HR responses, as none would be expected. Nonetheless, the potential hosts of *A. cardamines* do include some plants that have the potential to express HR, and on these

plants, *A. cardamines*' propensity to oviposit on tissues that should be refuges from HR may still be a benefit.

***Pieris mannii***: The solitary *Pieris* eggs found on *Iberis* plants are likely to be laid by *P. mannii*; however, it cannot be excluded that some of these eggs were *P. rapae*. Nonetheless, HR-like cell death was never observed on *Iberis* sp. plants in this survey, and therefore, it can be concluded that *P. mannii* does not induce HR-like on *Iberis* spp. However, previously we have shown that egg wash made from *P. mannii* eggs induced a strong HR-like cell death and high pathogenesis-related (PR1) gene expression in *B. nigra*[8]. Thus, *P. mannii* eggs are likely to contain the elicitor inducing cell death. The chemical nature of this elicitor is currently under investigation[4]. One species belonging to the genus *Iberis* has so far been tested (*I. amara*) and likewise does not show HR-like cell death[8]. *Iberis* species belong to lineage II outside the Brassiceae. Unlike the Brassiceae, tribe, this lineage did not go through a genome triplication event which is hypothesized to have enabled plants to evolve an HR-like cell death[8]. Together with the findings in this study, it seems unlikely that any *Iberis* spp. shows HR-like cell death. This lack of egg-killing defence could have been a benefit for *P. mannii*, leading to specialization on this genus as opposed to the use of other brassicaceous hosts with egg-killing responses used by its sister species *P. rapae*. However, the species *P. mannii* is rapidly expanding its range throughout Europe. It has been hypothesized that this is due to an expansion in host-plant use, so P. *mannii* is no longer exclusively found on *Iberis* plants[5]. Our observations support this switch, as we have seen *P. mannii* laying eggs on *D. tenuifolia* and *B. nigra*. Interestingly, a *P. mannii* female was observed bending her abdomen towards a flower bud of *I. umbellifera* as if she wanted to lay an egg. The actual eggs were not noticed; however, an egg was previously found on a flower bud of a *Diplotaxis* spp.[6] . If *P. mannii* is shifting to plants that do express HR, alternate strategies such as oviposition on flowers may be selected for. Also of note was the presence of several parasitized eggs on Iberis plants, that are presumed to be *P. mannii* eggs. *Trichogramma* spp. emerged from approximately 10% of these eggs, an association which to the best of our knowledge has not yet been reported.

Of the remaining solitary *Pieris* eggs, eggs were found on *A. petiolata, B. nigra, B. oleracea, B. rapa*, *D. tenuifolia, R. raphanistrum, S. arvensis* and *S. officinale* host-plants. *Pieris rapae* and *P. napi* eggs could not be identified to species level, as they were morphologically indistinguishable. Additionally, it cannot be excluded that *P. mannii* had also laid eggs on these alternate hosts, as discussed above. Moreover, host-plants cannot be used to identify which butterfly species laid the eggs as we have seen both *P. mannii* and *P. rapae* hatching from eggs laid on the same host-plant. Therefore, all solitary *Pieris* eggs on these plants were grouped for analysis. All eggs were laid on leaves, as expected for these species. Some eggs induced cell death, but only those found on *B. nigra*. The HR-like cell death showed to be an effective defence mechanism in some cases, but one

caterpillar hatched from the egg, nevertheless. No clear strategies to overcome HR-like cell death (e.g. ovipositing on the inflorescence) were identified. Interestingly, eggs from the Noctuidae family were found on six *B. nigra* plants and never induced a necrotic response. This confirms previous observations made for *Mamestra brassicae* (also from the Noctuidae family) which neither induced any HR response in *B. nigra* [4,7,8]. Three times, the *Pieris* eggs on the same plant induced a cell death response, even when the noctuid eggs did not. This suggests that the cell death response in *B. nigra* is specific to *Pieris* eggs. HR-like cell death was not found in *B. oleracea*, which was not expected as Griese et al.[8] found enhanced expression in 20%–40% of their plants. However, in this study, eggs were found on nine plants, of which seven were found at the same farm and were, therefore, most likely laid on the same genotype. It could be that the *B. oleracea* plants found in this study were not of a genotype that expresses HR-like cell death. HR-like cell death was not expressed for any of the other host-plants in this study.



All ***P. brassicae*** egg clutches were found on *B. nigra*. HR-like cell death was induced by all six clutches and was an effective defence against three clutches where most of the eggs did not hatch. Interestingly, the research by Griese et al.[14] showed that HR-like cell death was not as effective due to slowed-down desiccation caused by the eggs being laid in a clutch. It has been suggested that clustering of eggs may increase parasitism and predation, and in this study, both egg parasitism and HR-like cell death for the same egg clutch were observed, suggesting a double defense mechanism. Previously, it was found that eggs inducing HR-like cell death in *B. nigra* showed higher parasitism rates than eggs that did not induce cell death[15]. One *P. brassicae* egg clutch was parasitized within 5 days after the first observation, and on another clutch, a parasitoid wasp was found (*Trichogramma* sp.) while parasitizing the eggs. The latter egg clutch was found missing after 4 days, just as a part of another egg clutch. These 'missing' eggs were most likely predated upon by other arthropods. Thus, we found that *P. brassicae* egg clutches were highly vulnerable to both HR and egg parasitism in our study.

The induction of HR in Pieridae butterflies evolved in the Pierinae tribe in concordance with the colonization of the Brassicaceae and the appearance of glucosinolates detoxification[1]. It appears that only eggs of Pierinae species feeding on Brassicaceae elicit cell death on the leaves of plants in the Brassiceae tribe[8]. Exceptions within the Pierinae include the Black-veined white *A. crataegi*, which stopped using Brassicales some 37 Ma years ago[1], and egg wash does not or only slightly induce cell death in *B. nigra*[8]. So far, *A. crataegi* eggs have not been observed to induce HR in its natural host-plants belonging to the Rosaceae. Certain key species remain unsurveyed, such as *Pontia daplidice* whose natural host-plant species *Reseda luteola* belongs to the Resedaceae[9], a family within the Brassicales that is not expected to respond with HR[1]. Another key species to survey which does not occur in the Netherlands is *Pieris ergane*, which lays solitary eggs on host-plants of the

distantly related *Aethionema* genus. Other than *P. mannii*, which specialized on *Iberis* spp. until recently, *P. ergane* is the only *Pieris* to be a specialist on plants outside of the tribe Brassiceae. As one *Aethionema* species was previously found to express HR-like necrosis to *Pieris* egg wash[8], it is also one of the only Brassicaceous plants that is distantly related to the tribe Brassiceae known to do so. Whether eggs of the *P. ergane* or *P. daplidice* induce HR in their host-plants in nature remains to be studied.

Furthermore, it is possible that micro-evolution of elicitors, oviposition strategies and receptor genes in butterflies and plants is occurring at the population level. For example, we have observed certain alpine populations of the rapidly evolving species *P. napi* with a propensity to lay eggs on flowers. It would be highly informative to investigate these differences in oviposition tissue selection on the population level to assess whether these populations laying on flowers avoid egg-killing responses more successfully than the leaf-laying population we studied. It remains unknown whether the butterflies can assess their host-plant's likelihood of expressing egg-killing HR. Behavioral choice assays using genotypes of plants that do or do not express HR will allow us to investigate this detection ability of the butterflies. However, regardless of the butterflies' ability to directly assess the chemical differences signaling the likelihood egg-killing, natural variation in butterflies' egg-laying strategies allows for the selection for counteradaptations. If many *Pieris* eggs do not survive on certain host-plant tissues or lineages with high egg-killing, host shifts could eventually occur due to the survival advantage of butterfly genotypes favoring oviposition on safer plant tissues and species.

Overall, our field observations and literature search support that HR-like cell death in response to butterfly eggs evolved specifically against the Pierinae and in the Brassiceae plants they use most often. Specifically, we found that the genus *Pieris* remained at risk of egg-killing by their host-plants, where oviposition on flowers is not utilized as a counter-adaptation in the specific population we studied. Also unique to *Pieris* was their exclusive deposition of eggs on leaves. It is interesting to consider why this genus is so likely to lay eggs on leaves when leaves are the only tissue that can effectively kill their eggs. This is especially interesting considering that species such as *P. brassicae* are known florivores, eventually moving to the flowers to feed[38], but still oviposit exclusively on leaves. One explanation could be that, compared to the other species in our study that use more visually apparent hosts, *Pieris* use less apparent plants and therefore rely on the use of gustatory chemical cues while assessing sites for oviposition, as seen by frequent alighting on non-host-plant leaves[39]. Thus, the chemical cues in the leaves seem particularly important to guide *Pieris* egg deposition compared to the other species in our study, which could explain their propensity for oviposition on leaves. This is also evidenced by the tarsal gustatory receptors of adult *P. brassicae* that detect glucosinolates in their hosts' leaves[40]. Therefore, it could be that only in these plants that are most subjected to

the leaf-based oviposition by the *Pieris* butterflies (e.g. the Brassiceae tribe and certain *Aethionema* species) that selection pressure would be consistently strong enough for the plants to evolve a mechanism to kill eggs on leaves. In other words, for example, *Anthocharis* butterflies distribute their eggs across multiple plant tissues, including flowers and stems where cell death does not occur. Therefore, the benefit of cell death would be greatly reduced in their hosts and their hosts may not face the consistent strong selection pressure necessary to drive the evolution of egg-killing in the arms-race between Pieridae and Brassicaceae.

## 4.5 Conclusion



In the arms-race between brassicaceous plants and Pieridae butterflies, several counteradaptations to egg-killing HR-like cell death have been suggested. We provide a first attempt to show evidence of this hypothesis in the field. Based on our observations, there is no evidence that Pieridae butterflies try to avoid HR-like cell death by switching to other host-plants, ovipositing on plant tissues that do not respond to eggs with HR-like cell death, or laying egg clutches that are more resistant to the consequences of cell death in egg mortality. Our survey of Pieridae in the Netherlands revealed that the species vulnerable to egg-killing, *Pieris* spp., are most likely to lay eggs on tissues and plants that would express HR, namely on leaves of *B. nigra*. We have seen several cases of successful egg-killing by plant cell death, including egg clutches. It seems most likely that the arms-race between eggs from Pierinae species and Brassicaceous plants is still ongoing. Future studies should collect similar data from an increased number of butterfly and plant species in the field and survey natural variation in oviposition choices between butterfly populations, for example, including *P. napi* populations with a propensity to lay eggs on flowers. With such data, it would be possible to trace these characters onto a phylogeny with increased resolution and to further understand the evolution of egg-killing HR in the Brassicaceae and counteradaptations in the Pieridae.

### Author contributions

**DHP**: Conceptualization (equal); data curation (lead); formal analysis (lead); funding acquisition (equal); methodology (lead); writing – original draft (lead); writing – review and editing (equal). **LOG**: Conceptualization (equal); formal analysis (supporting); funding acquisition (equal); methodology (equal); supervision (equal); writing – original draft (supporting); writing – review and editing (equal). **NEF**: Conceptualization (equal); funding acquisition (lead); methodology (supporting); project administration (lead); supervision (equal); writing – review and editing (equal).

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## Data availability

The data that support the findings of this study are openly available in DRYAD, https://datadryad.org/stash/share/utqGtJtGZS7DP2bxj6ZCW1P5jI3aPkjTw6sjH9qZpHQ.

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CHAPTER 5

# 5

# Butterfly egg plasticity in physiologically and reproductively relevant traits: Adaptation to climate?

This chapter is based on:

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## Abstract

Embryos of terrestrial animals developing inside immobile eggs face variable environmental conditions. Insect egg pores, critical for gas exchange (aeropyles) and fertilization (micropyles), are ecologically important yet understudied. While aeropyles likely adapt to climatic respiratory demands, and micropyles may respond to environmental pressures, their inter- and intra-specific variation remains poorly studied. Therefore, we compared egg pore morphology of three closely related *Pieris* butterflies sampled in the Netherlands. In addition, we compared egg traits of different *P. napi* populations sampled along a 4,000-km latitudinal gradient from northern to southern Europe and linked these traits with available climatic data. After several generations under identical greenhouse conditions, egg pore traits remained highly variable within and between species. Remarkably, populations from divergent climates differed more in pore number and size than did co-occurring species. Aeropyle number and width were strongly associated with climatic variables such as maximum temperature, independent of egg size. Micropyle width varied with the temperature seasonality. Additionally, we observed more old eggs with a coverage on the micropylar region than fresh eggs. Our findings indicate that insect egg pores are traits that may be shaped by local climate.

## 5.1 Introduction

In most insects, parental care is limited to the choice of oviposition site, after which egg survival depends on egg traits[1,2]. Being immobile and unable to relocate to preferred conditions, eggs represent a vulnerable life stage for insects[2–4]. Additionally, insect eggs are tiny, with a large surface area relative to volume, making the thin eggshell a critical mediator between the embryo and its external environment[5,6]. The insect eggshell is typically waterproof, resistant to UV radiation and desiccation, while balancing the competing demands of respiration and water conservation[5]. For many insects, respiration and fertilization are mediated by minute pores on the outer eggshell, known as aeropyles and micropyles, respectively[5,7]. Although these pores are essential for embryonic development, they remain relatively understudied and somewhat overlooked.

While it is well established that environmental variation drives genetic and phenotypic adaptation in many insect egg traits[8], the extent to which this applies to egg pores remains poorly understood[9]. Despite the large interspecific diversity in pore morphology[6,9], most literature has focused on pore morphogenesis[7,10–12], their role in pesticide permeability[13–15], or their utility for taxonomic classification[16,17], rather than the evolutionary pressures shaping their diversification[18]. It has been hypothesized that the remarkable diversity in egg pore structure reflects functional divergence related to adaptation to environmental conditions, thereby mediating embryo development[5,6,19]. More broadly, the drivers of egg morphological diversification, such as egg size, have recently received renewed attention[20,21]. However, only a handful of studies have investigated how insect egg pore traits respond to abiotic stresses or divergent local condition[6,9,19,22].



Aeropyles are specialized structures that allow airflow between the external environment and the spongy chambers of the inner exochorion[5]. In many species, covering the aeropyles with a non-permeable substance causes embryo mortality, highlighting their critical role in respiration[5,22]. All terrestrial insects and their eggs face a trade-off between permitting gas exchange to facilitate metabolic processes, and preventing water loss[23]. This trade-off is highly temperature-dependent because, at higher temperatures, development is faster and metabolic demands increase. Therefore, embryonic oxygen requirements increase, but the rate of water loss and risk of desiccation are also higher[24]. Insufficient oxygen exchange may lead to anoxic regions in the developing embryo[5,23,25]. Thus, the number and size of aeropyles likely play a role in regulating this trade-off. For example, a smaller total aeropyle area (either by fewer or smaller aeropyles or both) may conserve water but limit oxygen availability. In contrast, greater aeration (either by more numerous or larger aeropyles or both) may facilitate rapid growth but increase the risk of desiccation.

Egg size is another factor that may influence the trade-off between respiration and water conservation during embryonic development[26,27], and it, too, varies with latitude and climate. Larger eggs typically contain larger embryos, which require more oxygen[26,27], and egg size often increases with latitude in several insect taxa[27–30]. This trend aligns with Bergmann's rule, formulated initially for endotherms[31], which has been observed in many, but not all, insect species along latitudinal gradients[32–34]. An alternative, the "canteen hypothesis," proposes that larger eggs buffer against desiccation in hot, dry climates by storing more water[26,35]. However, empirical support is mixed, and other factors such as clutch size may better explain variation in desiccation risk. Grouped eggs tend to lose less water than solitary ones[27,36], and clutch size often correlates with egg size[27,28,34]. Recent large-scale analyses of insect eggs suggest that ecological factors, particularly oviposition environment, better explain variation in egg morphology than do universal allometric or physiological rules[38]. Consistent with the finding that ecological factors explain egg morphology, we hypothesize that aeropyle area, rather than egg size per se, shows climate-linked variation. To clarify the role of egg size and whether selection on egg size interacts with the evolution of aeropyles and micropyles[20,26,28] we require studies that simultaneously examine both egg size, egg number, and egg pore traits across diverse climates[20,26,28].

Micropyles are narrow channels that traverse both the exo- and endochorion of the insect eggshell and function as conduits through which sperm reaches the vitelline membrane during fertilisation[5,10,11,14.]. Although micropyle number varies widely among insect species, it is typically conserved within species[9]. These structures may also mediate post-copulatory sexual selection, including sperm competition and cryptic female choice, where multiple micropyles allow the chance for fertilization by multiple sperm[39]. Phylogenetic studies further suggest a relationship between micropyle number and the degree of polyandry or mating frequency across species, with promiscuous species having more micropyles[9]. Despite this knowledge, drivers of divergence in micropyle size between or within species have not yet been investigated. Beyond their role in fertilization, micropyles may also contribute to embryo survival in ecologically meaningful ways. A recent phylogenetic analysis found that micropyle number is positively correlated with both aeropyles and annual precipitation: species from drier climates tend to have fewer micropyles and aeropyles, potentially reducing egg vulnerability to desiccation[40]. Gas exchange can indeed occur through micropyles[41,42]. Beyond their role in fertilization, micropyles may also contribute to embryo survival in ecologically meaningful ways. A recent phylogenetic analysis found that micropyle number is positively correlated with both aeropyles and annual precipitation: species from drier climates tend to have fewer micropyles and aeropyles, potentially reducing egg vulnerability to desiccation[40].

We expect to find differences in micropyle traits both within and between species, which are associated with divergent egg-laying strategies and climates. In addition to the adaptive features of micropyles, a cover may protect the egg from environmental stress by coating the micropyle. For instance, in large cabbage whites (*Pieris brassicae*), the yolk retracts from the anterior pole post-oviposition, and an oily layer covers the micropyles[14]. Since this oily material can prevent water loss[43,44], and gas exchange can occur through micropyles[41,42], sealing the micropylar region may represent an important adaptive mechanism against desiccation. Very few studies have documented or described these egg-derived coating substances in butterfly eggs[14].

Here, we use a common garden approach to investigate variation in egg pore traits in cabbage white butterflies (*Pieris* spp.), focusing on both interspecific and intraspecific patterns. We assess (i) interspecific differences in egg size, aeropyles, and micropyles among three co-occurring species (*P. napi*, *P. rapae*, *P. brassicae*); (ii) intraspecific variation in these traits across a 4,000-km climatic gradient in *P. napi*; and (iii) post-oviposition changes in micropylar coverage. We hypothesise that aeropyle and micropyle traits vary with local climate factors. Specifically, we predict that egg pore traits, more than egg size, reflect bioclimatic pressures. Consistent with this, we find that pore morphology diverges most strongly in *P. napi* populations at climatic extremes, supporting a role for local adaptation.



## 5.2 Materials and methods

### 5.2.1 Insects and plants

*Pieris spp.* butterflies (Lepidoptera: Pieridae) are common all over Europe and can be found where their crucifer (Brassicales: Brassicaceae) host-plants occur. Species differ in their preferred microclimate selected for oviposition, with *P. brassicae* and *P. rapae* generally ovipositing in similar conditions, often in drier habitats, including open fields[45,46]. *Pieris napi* instead selects a moist habitat, often near water when in drier environments[45,47]. Females of *P. napi* and *P. rapae* lay eggs singly, while *P. brassicae* females lay eggs in clutches[47]. These three species all prefer brassicaceous leaf tissue for oviposition. Their eggs, less than 1.5 mm in length, are laid on the lower side of leaves, where the leaf boundary layer provides a moist microclimate and a buffer from high temperatures[2,48,49].

To explore interspecific diversity, we analysed 14 egg clutches from *P. brassicae* (in total 69 eggs), 17 eggs from *P. rapae*, and 25 eggs from *P. napi*. Butterflies were lab-reared and originated from the surroundings of Wageningen, the Netherlands (51°97'91.19"N, 5°68'52.59"E). Butterflies were first collected in 2020 and the rearings were annually supplemented with new wild butterflies. All species were reared on black mustard (*Brassica nigra*) and/or Brussels sprouts plants (*Brassica oleracea* var. *gemmifera*) in greenhouses

(Unifarm) at Wageningen University (18–20°C, 50–70% RH, photoperiod L16: D6). Individual butterfly populations were separately caged and comprised 20-30 adults, fed with a 20% honey/water solution, and allowed to mate freely.

To explore intraspecific diversity in *P. napi* egg traits, we used six additional populations, wild-caught in 2022 and 2023 across Europe. Butterflies were reared for a minimum of three to a maximum of ten generations under greenhouse conditions. From northernmost to south, we analysed eggs from populations originating from (i) Abisko, Sweden (68°34'76.10"N, 18°82'97.70"E), 25 eggs; (ii) Stockholm, Sweden (59°36'44.70"N, 18°03'11.50"E), 25 eggs; (iii) Wageningen, the Netherlands (51°97'91.9"N, 5°68'52.59"E), 25 eggs; (iv) Jura, France (46°55'57.7"N, 6°08'49.60"E), 22 eggs; (v) Callas, France (43°59'03.0"N, 6°53'12.66"E), 25 eggs; (vi) Pyrenees, Spain (42°32'28.3"N, 1°06'42.98"E), 13 eggs; and (vii) Costa brava, Spain (42°26'49.5"N, 3°06'90.01"E), 20 eggs (Figure 5.1*b*). In the wild, the populations from Abisko and Stockholm are univoltine and bivoltine, respectively. Under greenhouse conditions, the butterflies naturally enter diapause after a few generations. We considered a pupa to be diapausing if it did not emerge within a month. These pupae were transferred to a refrigerator (4°) for three to six months and then returned to greenhouse conditions for emergence. Therefore, the number of generations kept in captivity differed between populations. For cryo-scanning electron microscopy, eggs still in position on their host leaf were obtained from adult butterflies kept in the greenhouse and ovipositing freely on black mustard (*Brassica nigra*) plants in February 2023 and April 2024.

### 5.2.2 Cryo-scanning electron microscopy

We imaged a total of 241 eggs of *Pieris* spp. containing 427 micropyles and 10195 aeropyles. *Pieris* eggs of one to three days of age, still in position on the *Brassica nigra* leaf they were laid on, were attached to a sample holder using a very thin layer of Carbon adhesive glue (Leit-C, EMS, Washington, PA, USA). Samples were frozen by plunging them into liquid nitrogen and subsequently placed in a cryo-preparation chamber (MED 020/VCT 100, Leica, Vienna, Austria). To sublimate any water vapour contamination (ice) from the surface, the samples were placed for 10 minutes at -92°C. Samples were then sputter coated with an 8 nm layer of tungsten from three angles and transferred under vacuum to the field emission scanning electron microscope (Magellan 400, FEI, Eindhoven, the Netherlands) onto the sample stage at -120°C. The images were taken with the secondary electron detector set at 2kv, 13pA. For each egg, a minimum of three cryo-scanning electron microscopy (cryo-SEM) images were captured; the whole egg for size measurements (Figure 5.1*a*ii), the apical egg tip to visualise aeropyles (Figure 5.1*a*iii), and the micropylar pit to measure micropyle number and width (Figure 5.1*a*iv). We used general tools from the FIJI platform to measure egg pores on the cryo-SEM images[50].

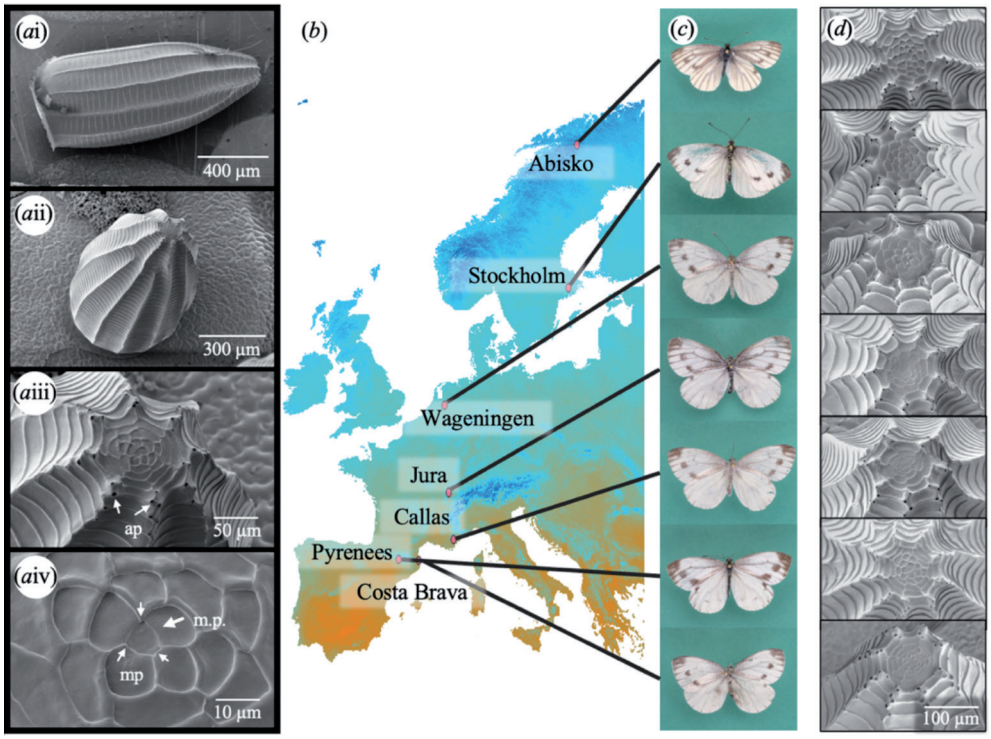



**Figure 5.1)** *Pieris napi* populations collected from North to South Europe and their egg and butterfly wing morphology. (***ai*-iv**) Cryo-scanning electron microscopy images (cryo-SEM) of egg shapes and pores taken from a Stockholm *P. napi* egg. (***ai***) Whole egg image, showing full length. (***aii***) Whole egg image, showing the abaxial leaf surface and the apical side of the egg. (***aiii***) Closeup of egg tip showing the aeropyles (ap; arrows). (***aiv***) Micropylar pit (m.p.), showing the openings of three micropyles (mp; small arrows). (***b***) Map showing the origin of butterfly populations. Colors indicate the maximum temperature of the warmest month, with orange representing high and blue representing low temperatures. (***c***) Variety in butterfly wing melanization across the seven *P. napi* populations. (***d***) The SEM images from the different populations with the egg ridges leading up to the apical egg tip, where the egg pores are located. Aeropyles are located on ridges, and micropyles are in the center of the egg tip (not visible).

### 5.2.3 Local climate data at field sites

The bioclimatic variables from WorldClim[51] are widely used to study climatic effects on insects and other animals. We selected three variables: temperature seasonality (BIO4, the annual standard deviation of monthly mean temperatures), maximum temperature (BIO5, the average maximum temperature of the warmest annual month), and annual precipitation (BIO12). These variables are of biological significance: temperature and humidity directly influence the trade-off between insect gas exchange and water loss[23], and precipitation was previously found to correlate with micropyle number in insect eggs[40]. Temperature seasonality reflects the variation of temperature across an insect's lifetime

and could thus directly (e.g. by a relatively cold start of the season) or indirectly (via other life stages) affect insect egg development[52,53]. Furthermore, we selected the length of the insect growing period, as this affects generation length and, thus, potentially constrains the developmental time of eggs and larvae. Data on the length of the growing period is referred to as 'growing days', which is the number of days when the mean daily temperature exceeded 10°C. This temperature is often used as the minimum threshold for growing degree days in Lepidoptera[54.] These data were obtained from the public dataset of the Food and Agriculture Organization of the United Nations[55] (available at https://gaez.fao.org/).

The freely accessible bioclimatic variables from the WorldClim dataset span 1970-2000. To account for recent changes in the local climate, we calculated the same variables for a more recent timespan, 2018-2021, using the 'biovars' function of the R package *dismo*[56]. This package requires monthly values for precipitation and minimum and maximum temperatures, which we extracted from the 2.5 minutes spatial resolution data from the WorldClim historical monthly weather dataset CRU-TS 4.06[57], downscaled with WorldClim 2.1[51], at our field site coordinates (Table S5.3).

### 5.3.4 Statistical analyses

To test for phenotypic adaptation, we analyzed five different egg characteristics: egg width, micropyle number, micropyle width, aeropyle number, aeropyle width, and presence/absence of a micropylar pit cover. We found egg width to be a suitable proxy for egg size (Figure S5.1, Table S5.1). For each treatment, we constructed either a linear model (*lm*) using base functions in *R*[58] or a mixed effect model (*lmer*) using the *R* package *lme4* when accounting for random effects[59]. As there are multiple aeropyles and micropyles per egg, we treated each measured pore width as an individual data point and included egg ID as a random effect. *Pieris brassicae* butterflies lay eggs in clutches. Therefore, we imaged five eggs per clutch and included clutch ID as a random effect in the models for this species. We separately analysed interspecific differences (three species obtained from Wageningen, n = 46 eggs/clutches) and intraspecific differences (seven populations of *P. napi*, n = 156 eggs). We could not measure all traits from all eggs (e.g. due to a micropylar cover or due to the cryo-SEM angle; Table S5.2).

We tested the model fits using the *DHARMa* package[60]. To analyse categorical data, we compared nested models with the *R* base function 'Anova'. We performed pairwise comparisons using estimated marginal means (EMMs) obtained using the 'emmeans' function from the *emmeans* package with the Tukey method for p-value adjustment[61]. We tested whether *P. napi*'s egg width was associated with aeropyle number, aeropyle width, micropyle number, and micropyle width. Egg width did not correlate with egg pore traits, therefore, we did not correct for egg width in our comparative models. We used eggs

from all species and populations to analyse the increase in micropylar cover with egg age, and constructed a general linear model (*glm*) with the *binomial* family using base functions in R[58]. Here, we excluded 14 eggs that could not be assigned to an age group (remaining n = 217) and included both species and population as random effects.

### 5.3.5 Structural equation model construction

We used a piecewise structural equation model (pSEM) to resolve the relationship between egg pore traits and egg size using the *R* package *piecewiseSEM*[62]. In the model, we included four climatic variables: temperature seasonality, the maximum temperature of the warmest month, annual precipitation, and growing degree days (n = 7 locations). Furthermore, we included five egg variables: egg width (n = 152 eggs), micropyle number (n = 64 eggs), individual micropyle width (n = 165 micropyles), aeropyle number (n = 94 eggs), and individual aeropyle width (n = 4361 aeropyles). Plotting the data indicated the possibility of a polynomial relationship between aeropyle traits and maximum temperature; therefore, we calculated the orthogonal polynomial of maximum temperature and included it as a variable (Supplementary 5). For model selection, we followed the procedure outlined in Boisseau and Woods 2024[20] and described model selection in Supplementary 5. Briefly, the initial model encompassed all possible paths from climatic to egg variables and among egg variables. Then, the most parsimonious model was identified by comparing CIC values, dropping pathways one by one until the CIC value of the main model was lower than that of any of its reduced model versions. In the next step, we tested against the alternative direction of association between variables. To test the assumptions, all individual linear models were investigated using the *simulationResiduals* function of *R* package *DHARMa*[60].



## 5.3 Results

### 5.3.1 *Pieris* egg characteristics

Eggs are shaped approximately as half an ellipsoid, with the anterior end being slightly extended and narrow (Figure 5.1*a*i, *a*ii). The posterior end is blunt and glued to the plant surface, usually on the abaxial surface of a leaf. Eggs are bright yellow to orange and typically contain 12 to 16 ridges, often extended along the complete length of the egg. Aeropyles are generally located on nodes on the anterior end of these ridges (Figure 5.1*a*iii). The ridges extend into a relatively flat crater. The micropylar pit, containing between one and six micropyles, is located roughly in the centre of this crater (Figure 5.1*a*iv).

### 5.3.2 Interspecific differences

Species varied in their egg width (Anova: $X^2 = 99.7$, df = 2, $p < 0.001$). Clustered *P. brassicae* eggs were significantly wider than the singly laid *P. rapae* (estimate = 113 ± 8 µm, $t = 14.5$,

$p < 0.001$) and *P. napi* eggs (estimate = 112 ± 7µm, $t = 15.9$, $p < 0.001$), while *P. rapae* and *P. napi* were similar in width (estimate = 1 ± 8 µm, $t = 0.1$, $p = 0.990$; Table S5.2; Figure S5.2).

Aeropyle number did not significantly vary between species (Anova: $X^2 = 5.1$, df = 2, $p = 0.079$; 5.2*a*i), but aeropyle width did vary (Anova: $X^2 = 25.3$, df = 2, $p < 0.001$; Figure 5.2*b*i). *Pieris napi* and *P. rapae* were similar in aeropyle number and width (*P. napi - P. rapae* aeropyle number: estimate = 0.39 ± 3.76; aeropyle width: estimate = -0.11 ± 0.18 µm, $t = -0.60$, $p = 0.830$). *Pieris brassicae* tended to have more aeropyles (n.s., see Anova results above), and they were significantly smaller (*P. brassicae-P. napi*: estimate = -0.74 ± 0.17 µm; $t = -4.30$, $p < 0.001$, *P. brassicae - P.rapae*: estimate = -0.85 ± 0.16 µm, $t = -5.30$, $p < 0.001$; Figure 5.2*b*i; Table S5.2).

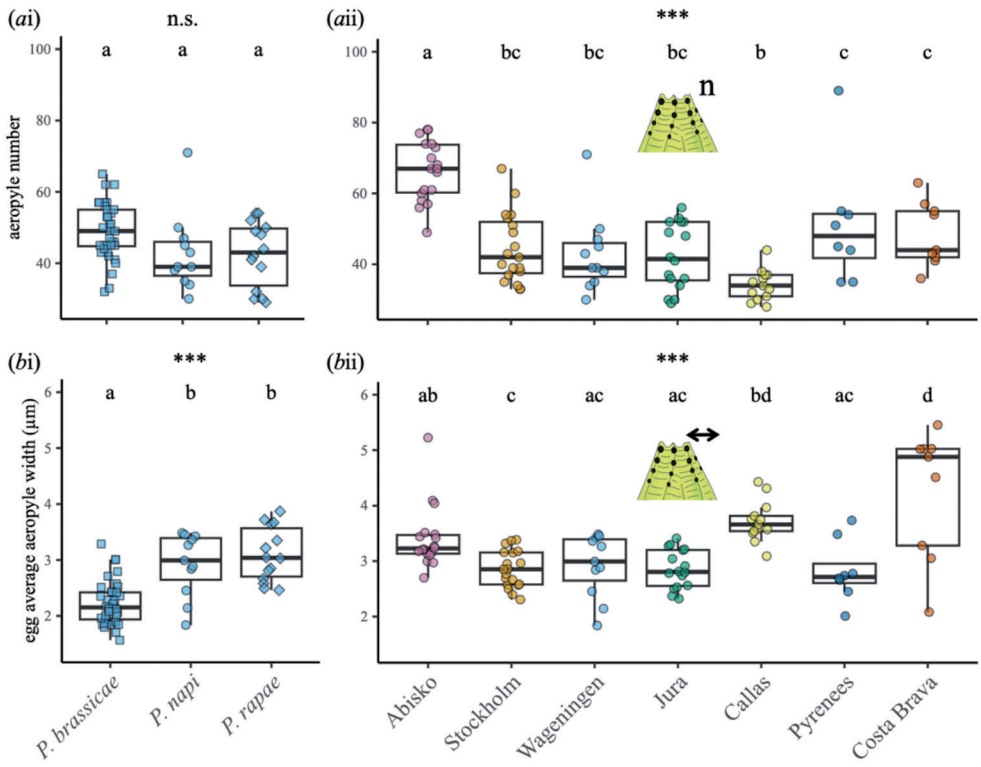



**Figure 5.2)** Comparison of egg aeropyle characteristics (***ai, bi****)* interspecifically between *Pieris spp.* and (***aii, bii****)* intraspecifically between *P. napi* populations**.** (***ai, bi***) Aeropyle number and average aeropyle width between species (n = 61 eggs and n = 2830 aeropyles, respectively). (***aii, bii***) Aeropyle number and average aeropyle width between populations (n = 94 eggs and n = 4411 aeropyles, respectively; Table S5.2; Table S5.5; Table S5.6). Plotted points represent individual eggs. Asterisks indicate the overall significance between compared groups, obtained with an Anova test (n.s. = not significant; *** $p < 0.001$). Different letters indicate significant differences (*post hoc* using EMMs, $p > 0.05$).

Species differed in micropyle number (Anova: $X^2 = 17.1$, df = 2, $p < 0.001$; Figure 5.3*a*ii), with *P. napi* having fewer micropyles (*P. napi - P. brassicae*: estimate = -1.19 ± 0.29, $t = -4.10$, $p < 0.001$; *P. napi - P. rapae*: estimate = -1.30 ± 0.40, $t = -3.25$, $p = 0.005$), and *P. rapae* having a similar number of micropyles as *P. brassicae* (estimate = 0.11 ± 0.36, $t = -0.31$, $p = 0.950$; Figure 5.3*a*i). The width of micropyles also significantly differed between species (Anova: $X^2 = 27.1$, df = 2, $p < 0.001$; Figure 5.3*b*i), with *P. napi* having smaller micropyles (*P. napi - P. brassicae*: estimate = -0.46 ± 0.08 µm, $t = 5.57$, $p < 0.001$; for *P. napi - P. rapae*: estimate = -0.55 ± 0.14 µm, $t = -4.00$, $p < 0.001$), while there was no significant difference between *P. brassicae* and *P. rapae* (estimate = -0.09 ± 0.13 µm, $t = -0.64$, $p = 0.801$; Figure 5.3*b*i; Table S5.2).

### 5.3.3 Intraspecific differences between *Pieris napi* populations

Egg width, used as a proxy for egg size, significantly differed between the populations of *P. napi* (Anova: $F = 20.8$, $p < 0.001$; Table S5.2; Figure S5.2). Eggs from Abisko tended to be larger than all other populations. Mainly, eggs from Wageningen, Jura, Callas, and Costa Brava were significantly smaller (Table S5.2; Figure S5.2; Table S5.9).

Between the seven populations, there was a significant difference between both the aeropyle number (Anova: $F = 16.57$, $p < 0.001$; Figure 5.2*a*ii; Table S2; Table S5) and the individual aeropyle width (Anova: $X^2 = 1068$, df = 7, $p < 0.001$; Figure 2*b*ii; Table S5.2; Table S5.6).

The number of micropyles was found to vary between populations (Anova: $F = 9.52$, $p < 0.001$; Figure 5.3*a*ii; Table S5.2; Table S5.7), as did micropyle width (Anova: $X^2 = 20.7$, $p = 0.002$; Figure 5.3*b*ii; Table S5.2; Table S5.8).

### 5.4.4 Climatic effects on intraspecific egg trait variation

We used a piecewise structural equation model (p-SEM) to test whether local climate could explain variation in egg pore traits found across the European latitudinal gradient. We modelled all possible associations between climatic variables and egg traits, as well as all possible associations between egg traits one another. This initial model included 31 paths, 10 of which were identified as significant after model selection. This analysis revealed that climate was a better predictor of egg traits than egg traits among one another (Figure 5.4). None of the egg traits were associated with one another (Figure 5.4). Although all the tested variables improved model fit (reduced the CIC value), maximum temperature and growing days had stronger effects than precipitation or temperature seasonality (Figure 5.4 and Table S5.4).

### 5.4.5 Cover of micropylar pit

For a proportion of the eggs, the micropyle area was covered by an unknown substance (Figure 5.3*c*). This covering was found for eggs of all three *Pieris* spp. and for all seven

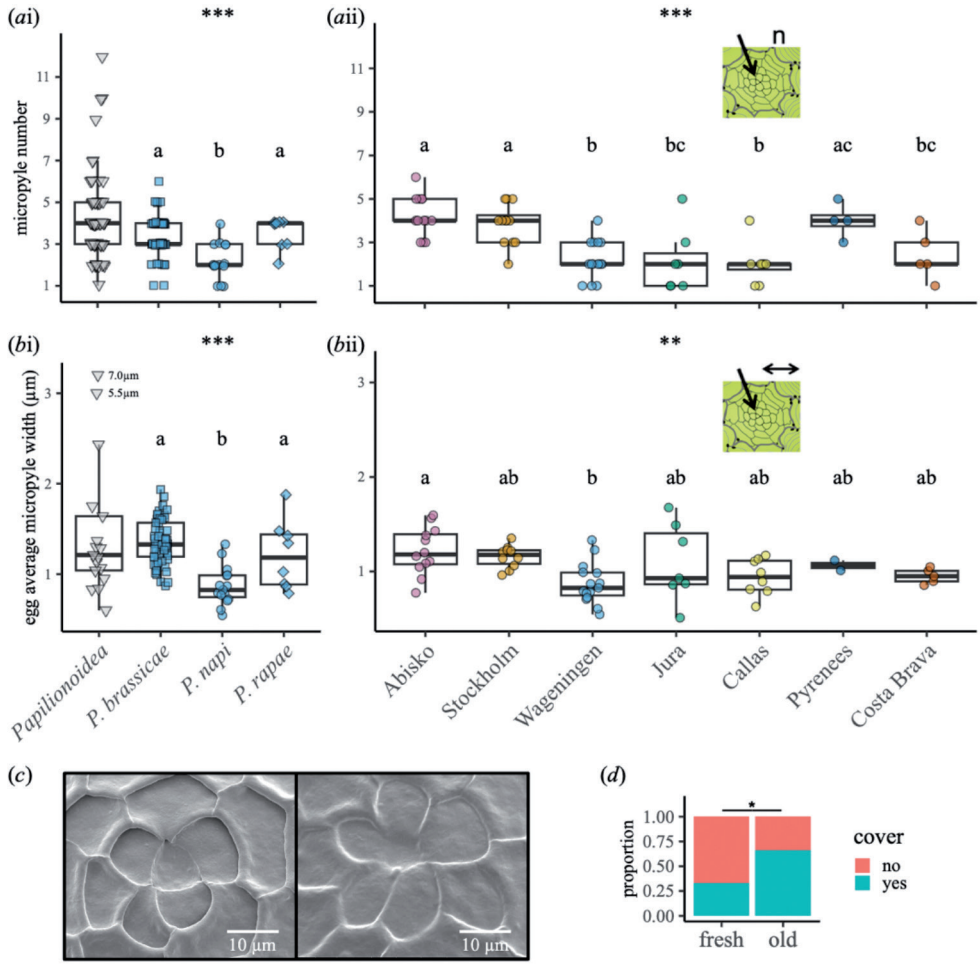



**Figure 5.3)** Comparison of egg micropyle characteristics (***ai, bi***) interspecifically between *Pieris spp.* and species of butterflies (Papilionoidea) and (***aii, bii***) intraspecifically between *P. napi* populations. (***ai, bi***) Micropyle number and average micropyle width between species (n = 81 eggs and n = 255 micropyles, respectively). (***aii, bii***) Micropyle number and average micropyle width between populations (n = 64 eggs and n = 165 micropyles, respectively; Table S5.2; Table S5.7; Table S5.8). (***c, d***) Coverage of the micropyle area. (***c***) Cryo-SEM images show examples of an uncovered micropyle area (left) and a fully covered micropyle area (right). Plotted points represent individual eggs or averages of individual species for Papilionoidea. For the analysis of micropyle width, we used the values of individual egg pores. (***d***) Stacked bar plot showing the proportion of eggs of which the micropyle area was covered to at least some extend for both fresh eggs (laid less than 2 hours before imaging) and older eggs (laid 4 hours or more before imaging; n total = 217 eggs). Data on Papilionoidea micropyles were obtained from Iossa, 2022[40]. Asterisks indicate the overall significance between compared groups (Papilionoidea was not included in comparisons), obtained with an Anova test (** $p <$ 0.01; *** $p < 0.001$). Different letters indicate significant differences (*post hoc* using EMMs, $p > 0.05$).

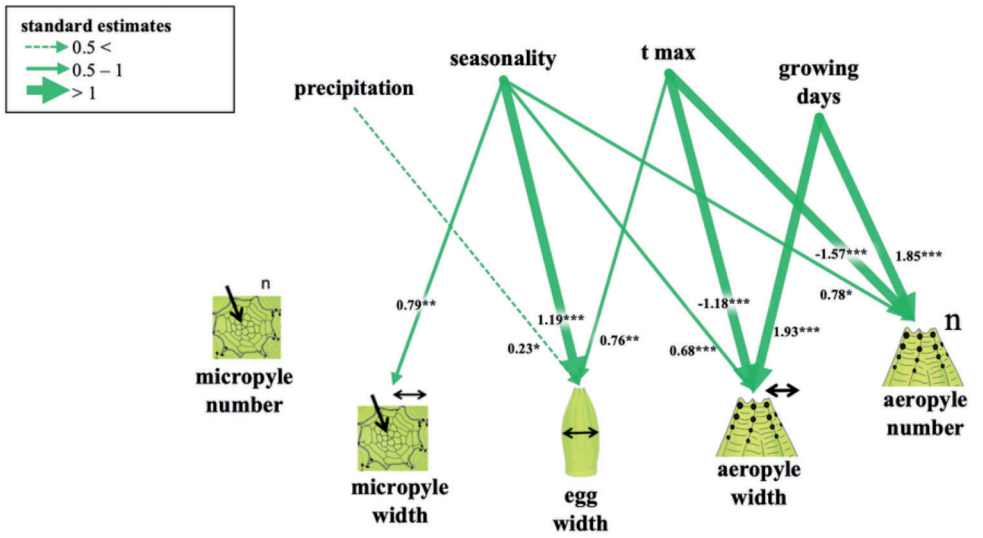



**Figure 5.4)** Piecewise structural equation model (p-SEM) indicating significant associations between the climatic variables and the egg traits. The model includes all measured eggs from the seven populations of *Pieris napi* (n = 155 eggs, n = 165 micropyles, n = 4361 aeropyles). Numbers are standard estimates, and asterisks indicate statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Table S5.4).



populations of *P. napi*. The cover varied between fully enveloping the micropylar pit, making all features indistinguishable, to a thin or partial layer. Eggs that were imaged less than two hours after egg deposition were significantly less often covered than eggs older than four hours (*glm*: estimate = 1.56, *se* = 0.58, *z* = 2.17, *p* = 0.030; Figure 5.3*d*).

## 5.6 Discussion

Our study on egg pore traits (aeropyles and micropyles) in *Pieris* butterflies showed inter- and intra-specific variation in both the number and size of aeropyles and micropyles. Interestingly, *P. napi* populations from divergent climates displayed even greater differentiation than between species, suggesting that climate-associated selection may play a role in shaping these egg pores that are associated with respiration and reproduction. However, given the complexity of potential selective pressures, we emphasize that climate is only one possible driver of this divergence.

An intraspecific comparison of *P. napi* eggs from various populations revealed that climatic variables were significantly associated with both aeropyle and micropyle width, independent of egg size in the intraspecific comparison. Moreover, we observed that

*Pieris* eggs often show a secretion covering the micropylar region within hours of oviposition. This covering may serve to protect against water loss, though its functional role remains speculative. Taken together, our findings suggest that egg pore traits vary within and between *Pieris* species and are potentially responsive to environmental variation. However, determining whether climate causally drives morphological change requires further experimental and genetic work. These egg pores, especially in the context of varying environmental pressures, deserve further attention in studies of insect adaptation to environmental conditions.

### 5.6.1 Drivers of interspecific variation of egg pores in *Pieris*

We explored the interspecific morphological variation of egg pores in *Pieris* by comparing aeropyles and micropyles between three closely related species: *P. brassicae, P. rapae,* and *P. napi*. These species co-occur in Europe, all using Brassicaceae host-plants, but differ in their oviposition behavior, thermosensitivity, and microhabitat use, which likely influences egg exposure to microclimates[45,47,63]. Eggs laid in clutches by the gregarious *P. brassicae* were significantly larger and had slightly more but smaller aeropyles than the solitary eggs laid by *P. rapae* and *P. napi*. The latter two species laid eggs of similar size with aeropyles of a similar number and width. This result aligns with broader findings on insect egg morphology, where interspecific variation includes differences in aeropyle shape, number, and spatial distribution[5–7]. Although prior studies confirmed the presence of aeropyles in these species, quantifications were lacking[10,14,64]. While aeropyles are assumed to evolve under abiotic selection, their precise functional role remains underexplored[5, 65]. Developmental plasticity may also play a role, as aeropyle number and width have been observed to change during embryogenesis[65]. In our case, this did not play a role, as all eggs were 3 days old.

Aeropyles facilitate gas exchange, whereas micropyles mediate sperm entry and may also contribute to environmental adaptation[40]. In contrast to aeropyle patterns, micropyles were more similar between *P. brassicae* (gregarious) and *P. rapae* (solitary), while *P. napi* (solitary) eggs had fewer and smaller micropyles, thus not correlating with egg size or oviposition strategy. Micropyle variation, a common feature across Insecta, is often used for taxonomic classification[5,66,67]. Micropyle number has been linked to differences in precipitation experienced across insects[40]. In Lepidoptera, higher levels of polyandry are associated with more micropyles[9]. In this case, all three *Pieris* species are polyandrous[68,69], though *P. napi* varies in mating frequency across its range[70]. Our data show that *Pieris* species possess micropyle traits typical of other Papilionoidea[40], and in this case micropyle number did not vary with climate and may instead reflect greater influence from phylogenetic or reproductive history.

Although all individuals were reared under the same greenhouse conditions, the ecological difference between species in thermosensitivity and oviposition suggests that their eggs evolved experiencing different climate conditions in the field[45,47,63]. For example, *P. napi* is strongly thermosensitive and prefers cool, moist oviposition sites, potentially reducing heat and desiccation stress on its eggs relative to migratory *P. rapae,* whose eggs can be found in more variable habitats[45]. Field-experienced microclimates, especially those at the leaf boundary layer, likely differ substantially by oviposition strategy[49]. These microhabitat differences could influence adaptive egg pore traits. Recent studies find that both egg size and shape are best explained by the environment in which eggs are laid, such as in aquatic environments or within hosts[38]. The findings suggest that ecological context, rather than fixed scaling rules, shapes the evolution of insect eggs[38]. Nevertheless, our design only considers macroclimatic differences, which may also be mitigated by different oviposition strategies. For example, differences in oviposition tissue selection may buffer differences in macroclimate by adjusting microclimates[90] (Chapter 4). Future work incorporating field microclimate data would help clarify the relative influence of macroclimatic vs. microclimatic pressures.

Ultimately, interspecific comparisons, especially among only three species, are correlational and constrained by confounding factors such as phylogenetic history, oviposition behavior, host-plant use, and developmental timing[71]. Broader phylogenetic analyses suggest some egg trait variation may be explained by phylogenetic signal rather than adaptive divergence[6,20]. Investigating morphological variation within species, such as rapidly evolving *P. napi*, offers greater potential for disentangling ecological from phylogenetic drivers.



### 5.6.2 Drivers of intraspecific variation of egg pores in *P. napi*

*Pieris napi* is particularly suited for examining intraspecific divergence, as its non-migratory, locally adapted populations span a broad latitudinal range[72–75]. In our study, egg pore traits showed even greater divergence among *P. napi* populations than between *Pieris* species, suggesting rapid trait evolution. Notably, the extreme populations at the leading and trailing edges of the population range, the northern Abisko and southern Costa Brava, exhibited the most divergent egg pore traits, while mid-range populations were more similar to one another. Egg pore differences among *P. napi* populations persisted after multiple generations in greenhouse conditions, indicating a heritable basis. This persistent difference across generation makes phenotypic plasticity an unlikely sole explanation. Nonetheless, plasticity cannot be excluded entirely. Edge populations, both northern and southern, often show greater plasticity than central populations[76], raising the possibility that Abisko and Costa Brava eggs exhibit more plastic responses to compensate for being reared under suboptimal conditions. Divergence at the range edges

could therefore reflect either adaptive divergence or evolved differences in plasticity depending on the bioclimatic context from which the species was collected[76].

Genetic drift is a potential explanation for divergence in geographically isolated populations, particularly in species like *P. napi* with limited dispersal. Under a model of isolation-by-distance, we would expect genetic and phenotypic differences to increase gradually with geographic distance due to restricted gene flow among distant populations[77]. Similarly, postglacial recolonization from southern refugia after the last glacial maximum typically results in gradual, clinal patterns of genetic variation along latitudinal gradients, reflecting sequential founder effects during northward expansion[77]. However, our results reveal a non-linear, polynomial pattern of egg pore trait divergence, where both northernmost (Abisko) and southernmost (Costa Brava) populations show the most extreme phenotypes, while central populations are more similar to one another (Figure 5.5). This pattern is inconsistent with a simple isolation-by-distance model or unidirectional recolonization, both of which would predict more gradual change [77,78]. Instead, the observed pattern suggests that local adaptation or selection-driven divergence, possibly interacting with evolved plasticity, is a more likely driver of trait variation at the range edges. Recent genomic analyses provide support for climate-associated selection, identifying summer temperature and precipitation as key drivers of genetic differentiation in *P. napi*, with Iberian populations (like Costa Brava) and the subspecies *P. napi* adalwinda (like Abisko) as genetic outliers[78]. The same populations show the most pronounced divergence in egg pore morphology in our study. While this strengthens the case for climate-mediated selection, it remains correlative. Experimental approaches, such as testing trait performance under variable climates or associating trait variation with climate-linked loci, are needed to establish causality and adaptive evolution of egg pore traits.

Aeropyle number and width varied markedly among *P. napi* populations and correlated with thermal extreme, even more than between the species. Costa Brava eggs, exposed to the hottest climates, had the widest aeropyles, potentially to support increased oxygen demand under elevated metabolic rates, similar to patterns observed in the seed beetle *Callosobruchus maculatus*, where higher larval metabolism correlates with larger aeropyles[22,79]. In contrast, Abisko eggs had the most aeropyles, which may reflect relaxed selection against water loss in its cool, humid environment. These patterns suggest that thermal conditions drive divergence in pore morphology through dual constraints: oxygen demand and desiccation risk. Total gas exchange capacity appears maintained across populations via different trait combinations, indicating functional trade-offs: many small aeropyles (Abisko) versus fewer large ones (Costa Brava).

Micropyle traits also varied among populations, with Abisko eggs having the most and largest micropyles. While intraspecific micropyle variation is less common than interspe-

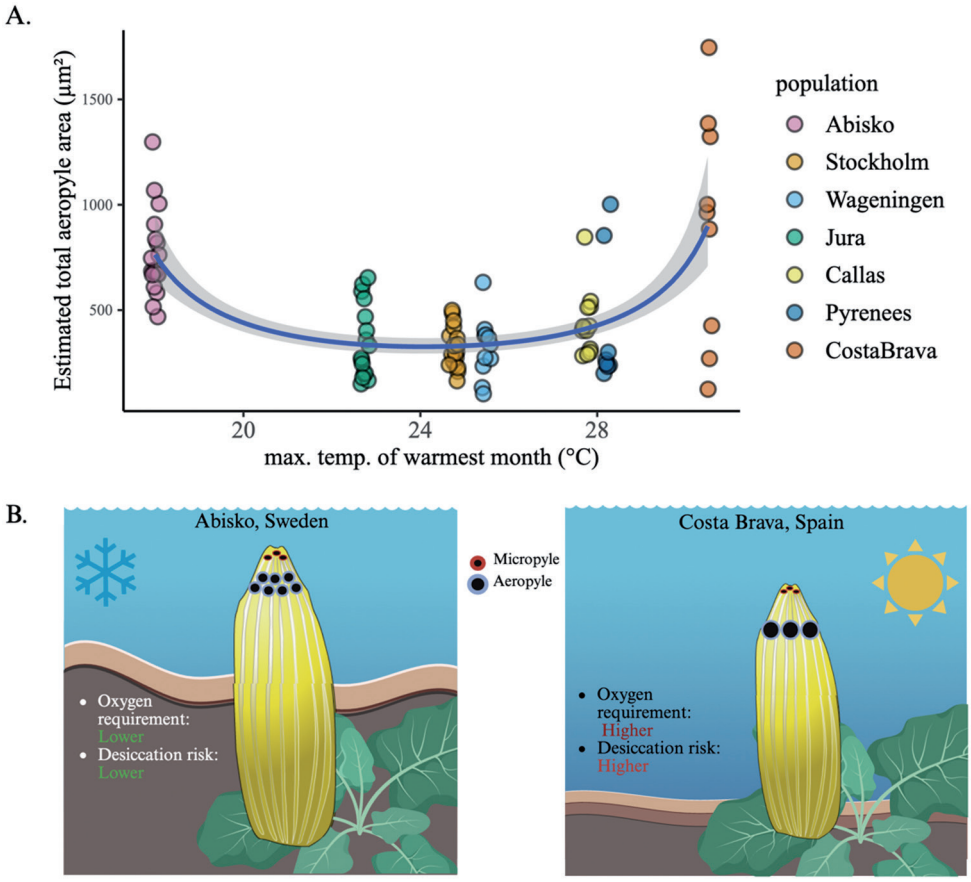



**Figure 5.5)** Divergent egg pore morphology in *Pieris napi* across climatic extremes reflects a trade-off between aeropyle number and size. **(A)** Aeropyle area (width × number) plotted against the maximum temperature of the warmest month shows a non-linear divergence, with the greatest area in northern (Abisko) and southern (Costa Brava) populations, which occupy the coldest and hottest parts of the species' range, respectively. The trendline was plotted using a glm with the formula y~poly(x,2) and Gamma distribution. **(B)** Schematic summary: in Abisko, increased aeropyle area results from more, smaller aeropyles and is paired with larger eggs. In Costa Brava, larger but fewer aeropyles likely meet higher respiratory demands while minimizing water loss; eggs are laid on plants near water to buffer against heat and dryness.

cific divergence[40,80], *P. napi* populations displayed divergence on par with that among closely related *Pieris* species, suggesting greater evolutionary flexibility than previously appreciated. However, only micropyle width, not number, was significantly associated with climatic variables, namely temperature seasonality. Overall, micropyles exhibited less climate-linked divergence than aeropyles, possibly due to stronger functional constraints or differing selective pressures related to reproduction.

Egg size also varied, with Abisko eggs significantly larger than those from other populations, consistent with Bergmann's rule and patterns seen in other Lepidoptera[28,81]. This contradicts the "canteen hypothesis," which predicts larger eggs in dry environments to buffer against desiccation, a hypothesis developed in *Drosophila* (Diptera) and so far, largely unsupported in Lepidoptera[26]. Instead, colder temperatures likely favor larger eggs for developmental benefits; for example, butterflies experimentally shifted to colder conditions have been shown to lay larger eggs, improving offspring survival[81]. Despite size differences, aeropyle area did not scale with egg size. Instead, aeropyle traits were more strongly predicted by climatic variables, particularly maximum temperature and growing degree days, factors known to drive divergence traits such as larval respiration rates in other Lepidoptera[29]. This decoupling suggests that eggs may compensate for environmental demands not by scaling, but through structural or physiological adaptations such as altered shell permeability or enhanced gas exchange via the vitelline membrane or serosa[23,82].

The absence of extreme egg traits in *P. napi* mid-range populations aligns with findings that populations near the center of their thermal niche often experience more stable environments and exhibit reduced physiological divergence[76]. Still, while climate appears to be a major driver of variation, other ecological variables, such as host-plant traits, predation pressure, or microhabitat variation, could also shape egg trait evolution[83]. Moreover, protective egg structures like the serosa, which helps prevent desiccation[83], may interact with changes in egg pore morphology in ways not yet understood. Demonstrating adaptive significance will ultimately require fitness-based experiments across environmental gradients.

### 5.6.3 Micropyle Coating: A Potential Strategy Against Abiotic and Biotic Stress

We found that several hours after oviposition, micropyles were no longer visible on many eggs. By comparing freshly laid eggs to those at least four hours old, we observed that this covering was dynamic and changed over time. The covering may have been secreted several hours after oviposition, or it was already deposited as a transparent substance at the time of oviposition and hardened over time, becoming visible. In contrast to most insect eggs, the inner surface of *P. brassicae* eggs is lined with a layer of unsaturated oil over the region surrounding the micropyle[14]. This oil layer flows into the area of the micropyles within four hours of oviposition and is later reabsorbed before eclosion[14]. Experimental studies show that *P. brassicae* eggs are only susceptible to water-soluble poisons during this brief window, suggesting that the oily coating plays a protective role that the eggshell alone cannot provide[14]. It is likely that the gradual micropyle covering we observed corresponds to the previously described oily layer.

In addition to blocking toxin uptake, non-volatile oily materials may interfere with respiration by obstructing the air spaces in the shell[14]. Our findings, together with prior work, suggest that micropyle traits vary with climate, potentially influencing how embryos interact with environmental conditions[40]. If micropyles indeed mediate climatic effects, this temporary coating may serve to shield embryos from environmental variation. Analogous systems support this interpretation: hydrogel-based egg coatings protect against drought[44] and heat stress[8], while oil layers may reduce water loss through micropyles that would otherwise remain open. Notably, insect eggs can exchange gases through micropyles[41,42], and coatings could help regulate this process. Similar protective layers have been described in aquatic eggs of fish[84] and insects[43], and a waxy chorionic layer in terrestrial Hemiptera also serves a waterproofing function[11,85]. These parallels suggest convergent evolution of protective coatings in diverse taxa. Whether the oily layer in *Pieris* functions analogously remains an open question deserving further investigation.

Beyond preventing water loss, egg coatings may also protect against solar radiation and associated temperature or UV stress[86]. Intriguingly, they may also offer defense against biotic threats. Egg parasitoid wasps locate hosts via chemical cues[89] (Chapter 2), and coatings might mask or disrupt these cues or form a physical barrier to oviposition[44]. Whether such coatings influence the risk of egg parasitism in *Pieris* remains unexplored. While speculative, these hypotheses highlight a potentially underappreciated layer of egg adaptation. Future work should investigate whether micropylar coatings vary across climates and whether they contribute to egg survival under different abiotic or biotic stresses.



## 5.7 Conclusion

Our study reveals that egg pores in *Pieris* butterflies are highly variable traits shaped by a complex interplay of ecological, behavioral, and climatic factors. Across both inter- and intraspecific comparisons, we found variation in aeropyle traits, and to a lesser extent, in micropyles, with particularly pronounced divergence among *P. napi* populations from climatically extreme regions.

These differences persisted across multiple generations in greenhouse conditions, indicating a heritable component. Correlations with climate variables such as temperature and growing season length support the hypothesis of local adaptation. Recent phylogeographic genomic analyses identifying climate-associated population divergence in *P. napi* genomes strengthen the case that climatic selection may play a role[78]. The observation that Abisko and Costa Brava populations differ most both in climate and in egg pore morphology suggests that egg traits may be part of a broader adaptive response to local

environments. However, genetic drift, developmental plasticity, or other ecological pressures may also contribute to the observed patterns.

Our results also highlight a potentially under-recognized layer of egg adaptation: the coating that forms over the micropylar region. This secretion may help buffer embryos against desiccation, temperature stress, or parasitism, particularly in climates where such pressures are acute. The potential interaction between micropyle width, environmental exposure, and post-oviposition sealing represents a promising avenue for future research.

Together, these findings emphasize that insect egg traits are not merely static or taxonomic features but may be dynamic, responsive elements of the reproductive phenotype. Understanding their evolution will require integrative approaches that link morphology, physiology, microhabitat selection, and climatic context. As climates continue to shift, such traits may play an increasingly important role in shaping reproductive success and species persistence.

## Authorship

**LOG**: Conceptualization, Investigation, Visualization, Writing - Original Draft Preparation, Writing - Review & Editing. **TdJ**: Data curation, Investigation, Writing - Original Draft Preparation, Writing - Review & Editing. **GI**: Conceptualization (lead), Supervision, Writing – Review & Editing. **NEF**: Conceptualization, Supervision, Writing – Review & Editing.

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CHAPTER 6

# 6

# Enemies, not sex, as a driving force of butterfly post-mating odor evolution

This chapter is based on:

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## Abstract

Not all odors influencing mating behavior evolve as sex pheromones. Female butterflies' post-mating odors have been considered species-specific anti-aphrodisiac pheromones shaped by sexual selection but may also serve broader ecological roles shaped by natural selection. Males transfer odors to females that repel rivals, yet the widespread use of these compounds across phyla makes them targets for eavesdropping, such as by phoretic egg parasitoids. We show that in cabbage white butterflies (*Pieris* spp.), the composition of these compounds is highly variable and attract parasitoids, deter predators, and influence oviposition. Using gas chromatography and electroantennography, we demonstrate that odor emission and perception lack species-specificity: compounds once thought unique to *P. brassicae* and *P. rapae* are shared across Pieridae. In *P. napi*, odor variation among populations correlates with parasitoid pressure, but not with latitude, genetic distance, or mating frequency, suggesting ecological rather than sexual drivers. In *P. brassicae*, CRISPR/Cas9 disruption of odor perception alters oviposition and increases susceptibility to parasitism. Moreover, these odors render females unpalatable to birds. Together, our results show that post-mating odors in *Pieris* butterflies may act as aposematic signals. We provide evidence that these signals evolve under multiple selective pressures, balancing deterrence of mates and predators, parasitoid avoidance, and host-plant interactions. These findings suggest that chemical signals should be viewed as integrating ecological and reproductive pressures, rather than being interpreted solely through the lens of sexual communication.

## 6.1 Introduction

Chemosensory cues are essential for species recognition and mate location across animal phyla[1–3], simultaneously conveying valuable information to potential mates and enemies alike[4,5]. Darwin developed the theory of sexual selection to explain how conspicuous traits, such as the peacock's tail, can evolve despite imposing survival costs[6]. Sex pheromones epitomize this tension: while they facilitate mate attraction and reproductive isolation[7], they can also be exploited by predators and parasitoids[8–10]. This dual role has made sex pheromones a central focus in studies of speciation and the balance between sexual and natural selection[6,11].

Among sex pheromones, post-mating odors are a distinctive class, transferred from males to females during copulation. First described as "post-mating female odors" in *Heliconius erato* (Nymphalidae)[12], these cues have since been identified in multiple *Pieris* (Pieridae) species and are now commonly termed anti-aphrodisiac pheromones, a type of post-mating sex pheromone that deters additional courtship[13,14]. The three components of *Pieris* anti-aphrodisiacs are benzyl cyanide (*P. brassicae*), methyl salicylate (in *P. rapae* and *P. napi*), and indole (in *P. rapae*). Males synthesize these compounds *de novo* from nutrients acquired during feeding[15]. These compounds are transferred to females during copulation via the spermatophore, along with sperm and nutrients[16]. The same compounds occur as products emitted by the Brassicaceae host-plants of *Pieris* butterflies both constitutively[17] and/or as herbivore[58] or oviposition-induced volatiles[18,19].



While these odors signal mating status and repel further courtship, they also expose mated individuals to heightened risk from natural enemies that exploit these cues to locate hosts or prey[20] or assess prey palatability[21]. These odors act as kairomones, directly attracting parasitoids to butterflies and facilitating phoretic transport to oviposition sites[20,22–24]. They can also induce plant defenses that further recruit parasitoids[18,25–29].

The rapid diversification of these odors has primarily been attributed to (intra)sexual selection, driven by male competition to secure paternity through increasingly effective repellents[30,31]. Females would then benefit as well through reduced male harassment during oviposition, but this remains unsubstantiated. In *Pieris*, females gain nutrients from remating[32] and tend not to accept copulation until they are receptive, meaning that males quickly abandon unreceptive females even in the absence of anti-aphrodisiac odors[33]. In addition, in *P. napi,* population-level variation in polyandry does not correspond to differences in spermatophore-derived compounds[34,35]. Rapid population-level divergence has also been observed in the post-mating odors of *Heliconius*[31] and divergence between species does not correspond with mating system[36]. Thus, the assumed sexual conflict over anti-aphrodisiac use as a driver of evolution of these odors shows limited explanatory

power[36–38]. These findings suggest that antagonistic coevolution between sexes alone is unlikely to account for odor variation[39]. Accordingly, we refer to these compounds more broadly as post-mating odors, recognizing their diverse ecological roles and evolutionary pressures.

Signal exploitation by natural enemies may accelerate divergence: when populations face different predators or parasitoids, selection to evade exploitation can drive shifts in signaling traits and reinforce reproductive isolation[8]. Such interactions between natural and sexual selection can promote speciation when mating preferences align with ecologically selected traits[6]. The possibility for natural selection on post-mating odors is especially likely when cues are broadly detectable, attract natural enemies, and confer little direct sexual benefit. In such cases, natural selection may outweigh sexual selection, leading to the reduction or loss of such signals[8,40,41]. Post-mating odors in *Pieris* butterflies provide a compelling example of this dynamic[24,39].

Despite some evidence of species-specificity[14,16], the components of post-mating odors in *Pieris* butterflies are environmentally widespread, being emitted by both insects and their host-plants[42]. This spatial ubiquity suggests overlap among species and vulnerability to exploitation[43], with population-level variation in odor profiles possibly shaped more by ecological than reproductive factors[5]. We hypothesized that natural enemies, such as egg parasitoids (*Trichogramma* spp.), exert stronger selection on post-mating odor blends than mates, as these cues can increase the risk of egg parasitism[20,23,24], and changes in these blends within a species can drive differential responses by *Trichogramma*[44]. Such antagonistic pressures may favor the reduction or modification of odor emission to evade detection[4,8], while in other contexts the emission of specific compounds, such as benzyl cyanide, may function as aposematic signals that deter predators by signaling chemical defense[21]. Given that these same odors also mediate oviposition behavior and elicit defense responses in host-plants[17–19], we further hypothesized that disruption of post-mating odor cues could cascade into altered plant–insect interactions. Collectively, these hypotheses predict that post-mating odors mediate diverse ecological interactions and that their evolution is primarily shaped by natural selection, extending well beyond their traditional role in sexual communication.

## 6.2 Post-mating odor compounds are shared between species

Using direct-contact PDMS fiber sampling[45], we detected four principal post-mating compounds in mated *P. napi* females, with negligible emission in unmated controls (Figure 6.1A, B). Methyl salicylate is the established anti-aphrodisiac in *P. napi*, while benzyl cyanide and indole serve this function in the related species *P. brassicae* and *P. rapae*,

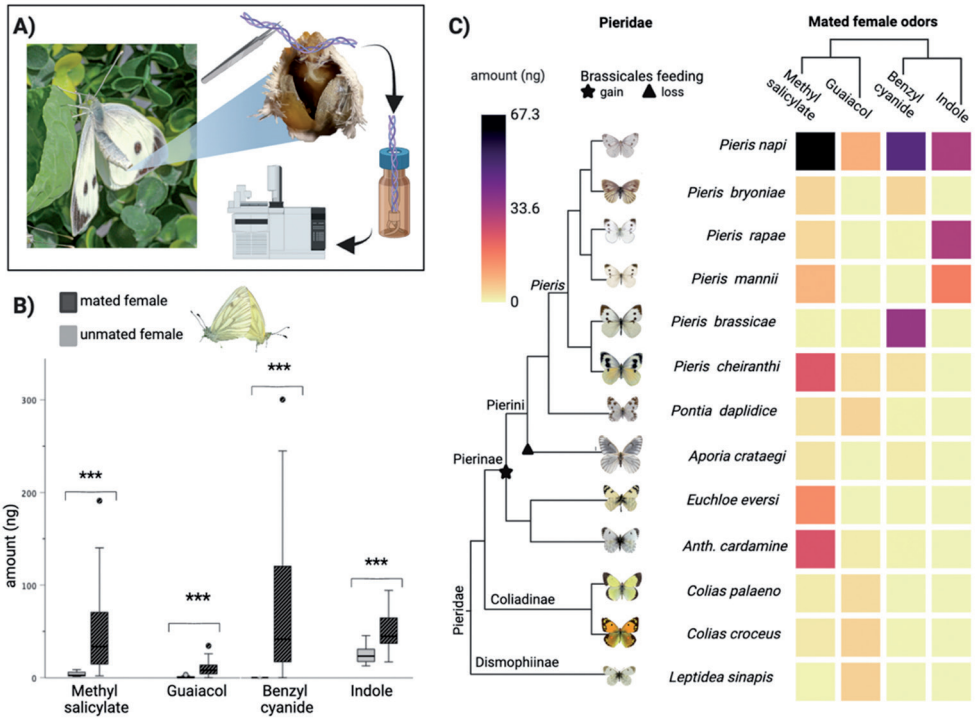



**Figure 6.1)** Post-mating odor emission is not species-specific in Pieridae**. (A)** Schematic of odor collection from wild-caught mated females using PDMS fibers applied to abdominal structures[45]. Inset: mate-refusal posture of a female (photo mate-refusal posture: Hans Smid, magnified female abdomen: Jonne Bonnet); collection with PDMS fibers; elution in hexane; analysis with gas chromatography. **(B)** Emission of four post-mating compounds (ng ± s.e.) in unmated and mated *Pieris napi* females from a Dutch population. Boxplots show data for 5-day-old females, either unmated or mated 24–48 hours prior. Asterisks indicate significance (***$p < 0.001$; statistical details in supplementary methods and Table S6.2). **(C)** Emission profiles of the four components of post-mating odors, namely methyl salicylate, guaiacol, benzyl cyanide and indole across 14 wild-caught mated Pieridae species. Left: phylogeny (after Wiemers et al.[46]). Right: heatmaps show compound quantities (ng) relative to internal standard (pentadecane). Compounds are clustered by chemical distance. Statistical details in supplementary methods and Table S6.3.



respectively[14]. While methyl salicylate alone has been shown to deter male *P. napi*, the behavioral effect of benzyl cyanide and indole in this species remains untested despite their established anti-aphrodisiac role in congeners. We included guaiacol in our chemical analysis because of its prior detection in *P. napi* headspace collections. We did so even though previous studies considered the amount of the compound to be insignificant and likely a by-product of pheromone biosynthesis[15] because the presence of minor compounds may still have significant behavioral effects[42], and guaiacol may modulate or amplify the activity of methyl salicylate[47]. Thus, the post-mating odor profile of *P. napi*

incorporates compounds known to function as anti-aphrodisiacs in congeners. Benzyl cyanide, entirely absent in virgins, and indole, present only at low levels in virgins, likely share a male-transferred origin, though this has only been established in congeners[14,15].

Our analysis showed that all four compounds, including those previously considered possible biosynthetic by-products[15], are robust and reliable indicators of mating in *Pieris napi* because they are present in much higher quantities in mated versus virgin females. Notably, our analysis used Dutch populations of *P. napi* and direct-contact volatile sampling, in contrast to earlier studies that relied on headspace collection. As headspace methods capture volatiles emitted during active mate refusal, while our method samples directly from the relevant tissue, both qualitative and quantitative differences may arise[45]. Manipulation of the butterflies during handling for collection may also enhance the release of certain compounds, as observed in other insects, such as locusts, which emit more benzyl cyanide when disturbed by predators[21].

Across 14 wild-caught Pieridae species, these compounds were consistently present in mated females, with substantial overlap among species (Figure 6.1C). This pattern contrasts with the high species-specificity typical of most lepidopteran sex pheromones and challenges previous assumptions of species-specific anti-aphrodisiacs[14], suggesting that post-mating odor profiles are shaped by shared ecological factors rather than divergent sexual selection[40]. Methyl salicylate was the most widespread, detected in most species, consistent with its broad use as a chemical cue among Lepidoptera[49–51]. Indole showed the highest specificity, restricted to *P. napi*, *P. rapae*, and *P. mannii*. Benzyl cyanide was limited to Pierini, although this may reflect sampling bias; given that cyanogenesis occurs in other Pieridae and distantly related lepidopterans[52,53], a broader evolutionary origin is likely. As expected for compounds derived from common metabolic precursors, such as phenylalanine, the synthesis of methyl salicylate and benzyl cyanide appears independent of host-plant chemistry[14]. Indole, synthesized from tryptophan[14] represent a derived biosynthetic trait, as it appears restricted to a single clade within *Pieris*. While methyl salicylate alone has been shown to deter male *P. napi*, the behavioral functions of benzyl cyanide and indole in this species remain untested[14].

Taken together, the widespread occurrence of these volatiles across species indicates that post-mating odors are environmentally ubiquitous signals that are likely subject to selection pressures from natural enemies more so than conspecific mate recognition. While pheromone specificity between species often depends on differences in ratios and blends, not compound presence or absence[7], high variability within species and populations would indicate that these odors are not reliable for species recognition. Furthermore, because these odors are emitted only after mating has already occurred, there is little reason to expect them to exhibit species specificity despite previous assumptions

of stable differences between *Pieris* species. Unlike aphrodisiac pheromones, which can function in pre-mating species recognition to prevent hybridization[1,7], species-specific post-mating odors would offer no such selective advantage.

## 6.3 Intra-specific variation in post-mating odors corresponds with parasitoid pressure but not geographic, genetic distance, or mating rates.

Marked population-level differences in post-mating odor emission across seven *Pieris napi* complex populations were stable over two years (Figure 6.2A, B). Strikingly, sites with butterflies with low or trace emissions (JUR, BRY, PYR) showed no evidence of egg parasitism despite the presence of *Trichogramma* parasitoids on sympatric lepidopterans (Figure 6.2C, D, Supplement). In contrast, populations with high emissions (COB, WAG, STK, ABS) experienced frequent parasitism and produced distinct blends of methyl salicylate, benzyl cyanide, or indole, though rarely all three. Y-tube olfactometer assays confirmed that *T. cacoeciae*, the dominant phoretic parasitoid on *P. napi* and the only species tested that showed innate attraction to mated *P. napi* (Figure 6.2F), was attracted to volatiles from mated females in high-emitting populations, but not to odors from low-emitting populations (Figure 6.2G). These results demonstrate that post-mating odors in *P. napi* function as long-range attractants for phoretic parasitoids and that emission levels correspond with local parasitism risk.



Odor variation was not associated with geography, genetic structure, mating rates, or host-plant. This pattern held both in our own dataset and when compared to previously published findings on *P. napi,* which included more detailed genomic, host use, and mating analyses[39,54-56]. For example, genetically divergent *P. napi* adalwinda (ABS) and *P. napi* napi (STK, WAG, COB) exhibited similar blends, while distinct species *P. napi* and *P. bryoniae* from the same site (JUR and BRY) shared low emission levels (Figure 6.2A, B). Only *P. bryoniae*, which emits little post-mating odor, showed high mating rates, while all other populations exhibited average remating frequencies regardless of odor emission (Figure 6.2E). This suggests that post-mating volatiles are not primarily constrained by mating system or genetic drift, consistent with previous findings that divergence in *P. napi* and *Heliconius* polyandry does not correspond with differences in spermatophore derived compounds[36].

Emissions of methyl salicylate and benzyl cyanide were strongly correlated and inversely related to indole, indicating a metabolic trade-off or alternative regulatory mechanisms (Figure 6.2B, E). Methyl salicylate and benzyl cyanide derive from phenylalanine, while indole is synthesized from tryptophan[14], representing alternative biosynthetic pathways.

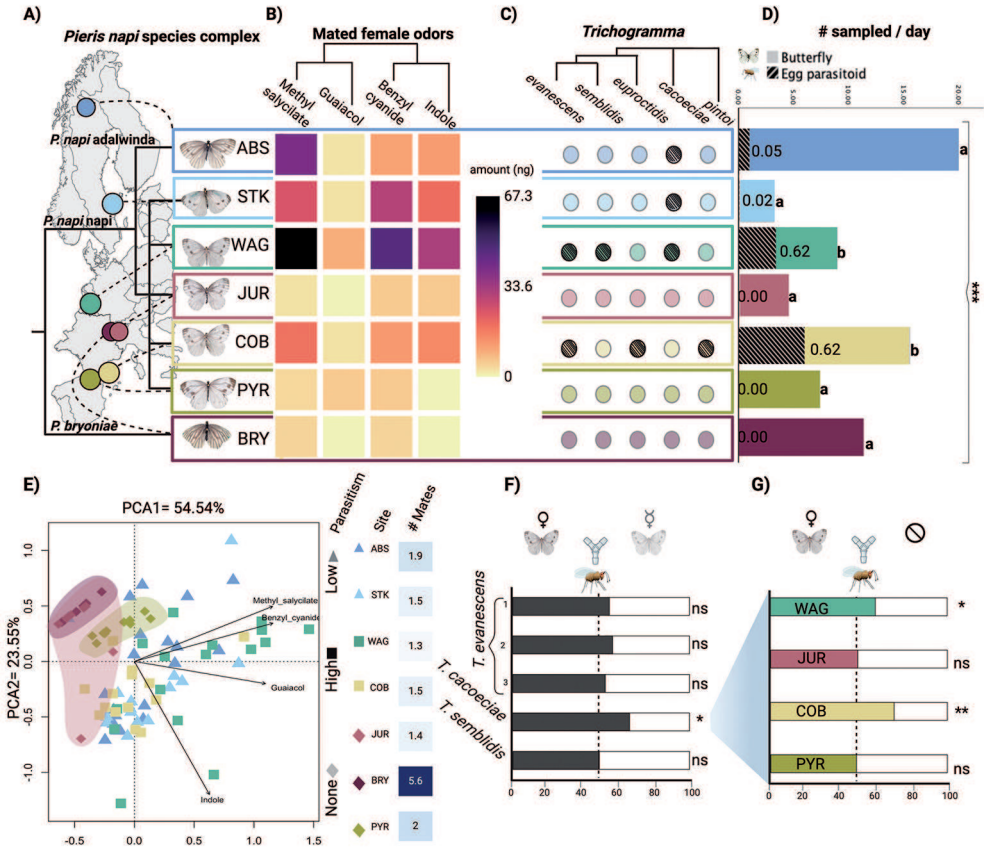



**Figure 6.2)** Intraspecific variation in *Pieris napi* post-mating odors is shaped by parasitoid pressure rather than geography or genetic distance. Asterisks indicate significance (*p < 0.05, **p < 0.01, ***p < 0.001, ns = p > 0.05). **(A)** Sampling locations and COI phylogeny of *P. napi* complex populations across a European latitudinal gradient sampled over two consecutive years (2022 & 2023). Circles on the map indicate collection site, details in supplementary methods. **(B)** Heatmap showing mean emission (ng) of four post-mating volatiles per population across two years, with compounds clustered by chemical distance. Sample sizes in in Table S6.4. **(C)** *Trichogramma* species detected in butterfly eggs and hitchhiking on butterflies by population; wasp phylogeny inferred from COI barcodes. Darkened circles indicate presence. **(D)** Daily capture rates of *P. napi* and *P. bryoniae* butterflies (color bars) and *Trichogramma* wasps (grey slashes) per site; bar labels indicate egg parasitism rates. Letters denote statistically distinct groups. Sample sizes in table S6.5. **(E)** Principal component analysis (PCA) of individual odor profiles by population (colors from panel A). Shapes indicate parasitism level: diamonds (none), triangles (low), squares (high). Blue boxes show the mean spermatophore number per population. Clusters JUR, BRY, and PYR were highlighted with hand drawn ellipses to encompass all points in their respective colors. **(F)** Y-tube olfactometer assays: proportion of naïve *Trichogramma* attracted to mated vs. unmated *P. napi* (WAG). Black bars indicate a preference for mated females. Statistical details in table S6.6. **(G)** Y-tube olfactometer assays, *T. cacoeciae* choice between clean air and mated females from four populations (colors from panel A); colored bars indicate butterfly choice. Statistical details in table S6.7.

Methyl salicylate can also arise from benzyl cyanide, as caterpillars fed benzyl cyanide incorporate it into methyl salicylate, though *P. napi* males can also synthesize methyl salicylate *de novo* from larval precursors and replenish it via nectar feeding[87]. Notably, indole does not attract other parasitoids[57], possibly due to its toxicity to insects[58,59], which may confer an ecological advantage and help maintain odor variation within populations[7,60].

These data indicate that while anti-aphrodisiac use is widespread and conserved across Pieridae, its expression within *P. napi* is highly variable and ecologically contingent. In the absence of detectable effects on mating behavior, high levels of post-mating odor production likely incurs ecological costs via parasitoid attraction. Populations emitting low or no volatiles may escape detection, consistent with the idea that post-mating signal divergence is shaped by enemy-mediated selection rather than mate recognition. Alternatively, parasitoid preferences and cue use may themselves evolve in response to local odor availability, potentially driving reciprocal adaptation in this system. Time-calibrated analyses of biosynthetic and olfactory gene evolution in both butterflies and parasitoids could further illuminate the dynamics of this diffuse coevolutionary interaction[61].

## 6.4 Conserved cross-species and cross-sex olfaction of post-mating odors



Post-mating odors have traditionally been considered species-specific signals, with benzyl cyanide thought unique to *P. brassicae*[62]. However, given the widespread emission of methyl salicylate, benzyl cyanide, and indole across *Pieris* species (Figure 6.1), we tested whether antennal sensitivity to these compounds is similarly conserved.

Using electroantennogram (EAG) recordings, we measured dose-response curves in males and females of four sympatric *Pieris* species (*P. brassicae*, *P. napi*, *P. rapae,* and *P. mannii*) from the Netherlands. Contrary to expectations of species- or sex-specific detection, all species and sexes exhibited remarkably consistent antennal responses to benzyl cyanide, methyl salicylate, and indole (Figure 6.3A). Benzyl cyanide elicited the strongest responses, exceeding those to plant volatiles. In contrast, sensitivity to methyl salicylate and indole resembled responses to other common host-plant cues. This conserved detection likely reflects ancestral olfactory capabilities co-opted for post-mating signaling[63], as these compounds also serve as oviposition cues in host-plants[64-66]. The similar sensitivity across species, including for *P. napi,* that was not previously known to use benzyl cyanide, challenges the notion of strict species-specificity in anti-aphrodisiac reception.

Comparative genomic and antennal transcriptome analyses[67] indicate that *Pieris* chemoreception is conserved across species, with differences arising primarily from expression-

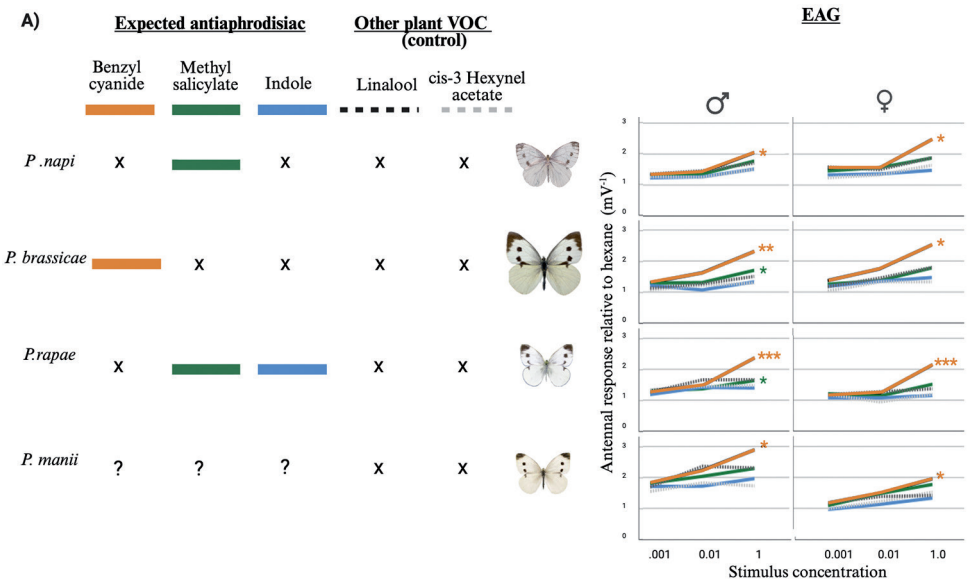



**Figure 6.3 A**) Interspecific variation and genetic basis of olfactory responses to post-mating odors. Electroantennogram (EAG) dose-response curves of males and females from *P. brassicae*, *P. napi* (WAG), *P. rapae*, and *P. mannii* to conspecific and heterospecific anti-aphrodisiac compounds (benzyl cyanide, methyl salicylate, indole) and control plant volatiles (cis-3-hexenyl acetate, linalool). Colored lines indicate compound identity as indicated in the top legend; "X" marks compounds not known as anti-aphrodisiacs in that species. Responses are normalized to solvent control. Asterisks indicate significance effect of dose on response for each compound (*p < 0.05, **p < 0.01, ***p < 0.001, ns = p>0.05). Statistical details can be found in tables S6.8 and S6.9.

level variation. *OR45b* stands out as a *Pieris*-specific and conserved odor receptor in this genus (Figure 6.3B). Knockout of *OR45b* indicates this receptor as a candidate partially mediating post-mating odor detection. CRISPR/Cas9 knockout of *OR45b* in *P. brassicae* significantly reduced antennal responses to these compounds, especially benzyl cyanide and methyl salicylate, without affecting responses to other plant volatiles (Figure 6.3C), confirming its functional role.

The similar antennal sensitivity in males and females is notable. Females have been thought to lack control over anti-aphrodisiac emission[37], with odor titers decreasing steadily with each emission event when the mate-refusal posture is adopted[16,48, 68]. However, females' ability to detect these compounds may serve to regulate remating timing and avoid autotoxicity from benzyl cyanide, which metabolizes to dangerous hydrogen cyanide[21]. Potential female self-regulation of anti-aphrodisiac levels warrants further investigation**.** In other Pieridae, such as wood whites (*Leptidea* spp.), only females can discriminate between con- and hetero-specific mates, which only differ in subtle amounts of chemical cues[69-71]. That male *P. napi* tailor ejaculate is dependent on female

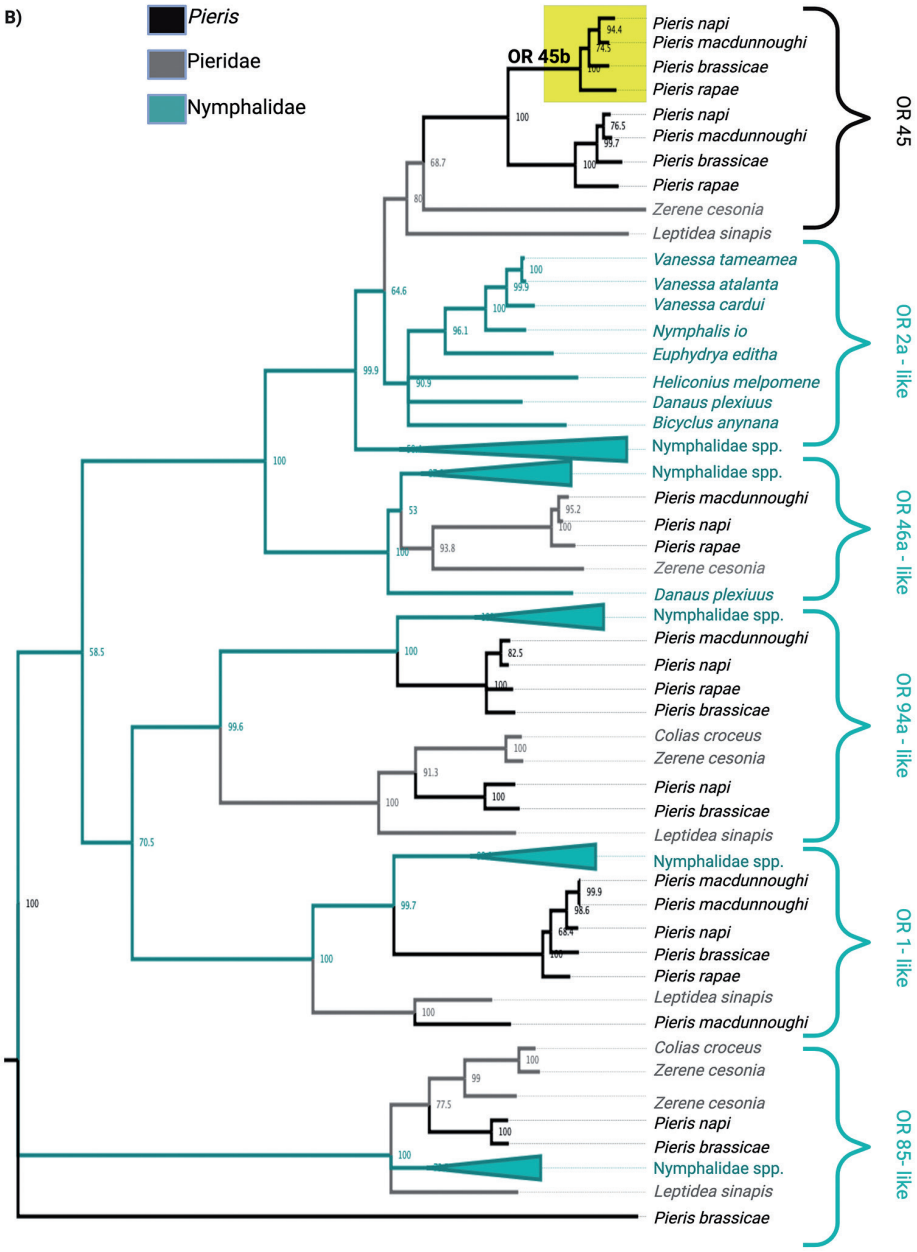



**Figure 6.3 B**) Interspecific variation and genetic basis of olfactory responses to post-mating odors. Maximum likelihood phylogeny of OR45b and homologs in Pieridae and Nymphalidae, rooted on *P. brassicae* ORCO, highlighting conservation of this receptor unique to *Pieris*.

anti-aphrodisiac emission[72] may also provide a degree of protection from dangerous anti-aphrodisiac accumulation or emission.

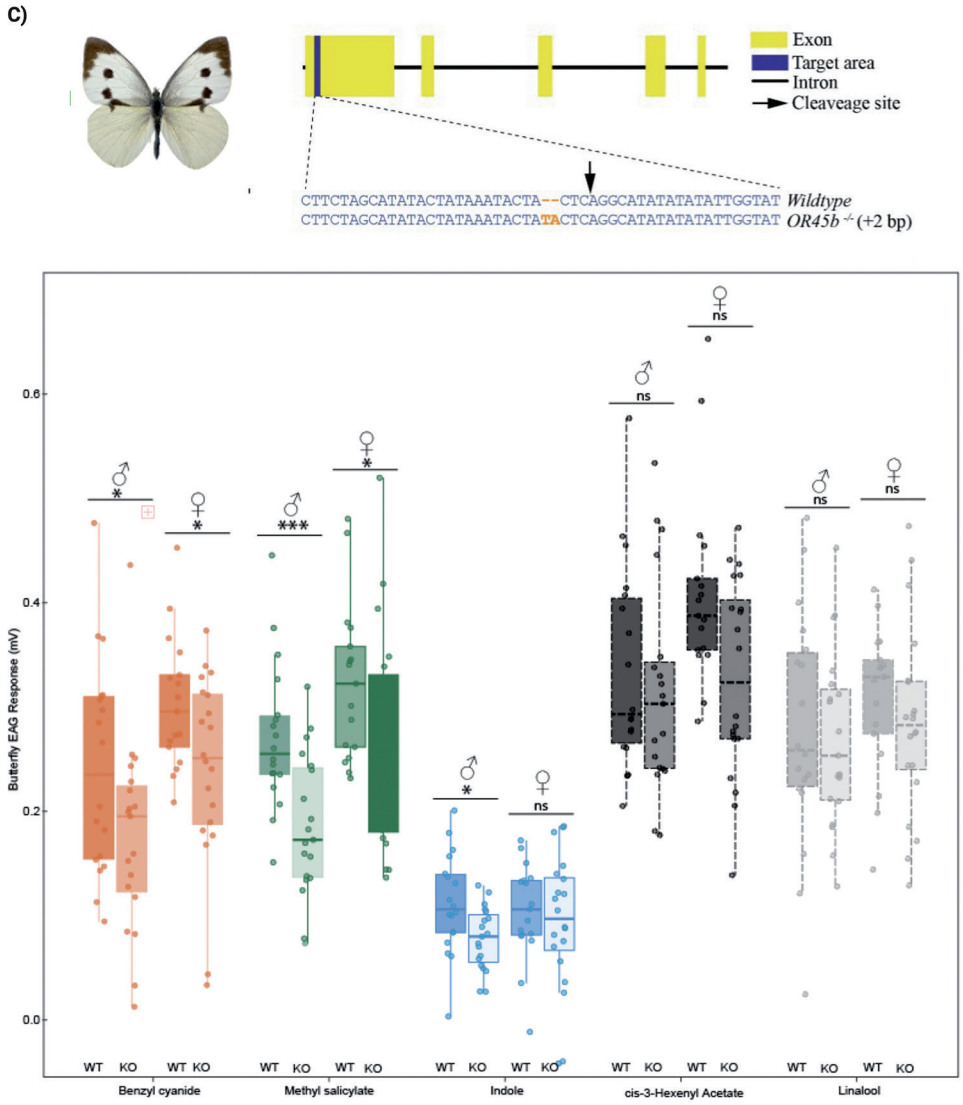



**Figure 6.3 C**) Interspecific variation and genetic basis of olfactory responses to post-mating odors. Schematic of CRISPR/Cas9 knockout of OR45b in *P. brassicae* and EAG responses of wild-type (WT) and OR45b knockout (KO) butterflies volatiles from panel A, demonstrating decreased post-mating odor detection in KO males and females. Asterisks indicate significance: significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns = $p>0.05$). Statistical details for these five compounds are found in table S6.9 and full EAG panel results are in Figure S6.3.

## 6.5 Functional consequences of post-mating odor perception

If post-mating odors are not essential for mating and increase parasitoid attraction, why do butterflies use them at all? To address this question, we examined the functional consequences of post-mating odor perception and emission in *P. brassicae*.

Disruption of the *Pieris*-specific odorant receptor OR45b[67] in *P. brassicae* reduced antennal responses to post-mating odors (Fig 6.3C). OR45b knockout females laid significantly more eggs on previously infested host-plants. They showed reduced avoidance of oviposition-induced plant volatiles compared to wild-type females (Fig. 6.4A, B, supplement), both indicating impaired host discrimination. Methyl salicylate, benzyl cyanide, and indole are implicated in host-plant selection[18,19,73], and benzyl cyanide is deposited with *P. brassicae* eggs[28,74]. A reduced olfaction of these compounds likely impaired the ability of the knockout butterflies to detect heavily egg-laden plants, which would have been poor choices for oviposition because larval competition would be high. Knockout females were also more attractive to *Trichogramma* parasitoids than wild-type females (Fig. 6.4C). We expect that this altered parasitoid response is due to knockout females also emitting higher levels of benzyl cyanide due to a loss of feedback regulation, though this remains to be tested. If wild-type butterflies can reduce emissions when environmental titers of this risky compound exceed a certain threshold, e.g. by avoiding adopting the mate-refusal posture[48], this would further suggest that post-mating odor detection enables females to regulate emissions, thereby optimizing both host selection and enemy avoidance. Future studies should investigate odor emission and adoption of the mate-refusal posture between these wild-type and mutant butterflies.



## 6.6 Benzyl cyanide as an olfactory aposematic signal

Recent findings in locusts have shown that benzyl cyanide functions as an olfactory aposematic signal, warning predators of toxicity rather than serving strictly as an anti-aphrodisiac[21]. In our system, where butterflies emit even lower amounts of benzyl cyanide, this hypothesis is supported by behavioral assays with great tits (*Parus major*): while naïve birds attacked benzyl cyanide-treated and untreated butterflies equally, experienced birds reliably avoided treated individuals (Fig. 6.4D). This learned aversion enables them to avoid ingesting benzyl cyanide, which is converted to hydrogen cyanide upon attack, poisoning predators[21,52,53].

Classic experiments showed that mice injected with homogenates of *P. brassicae* and *P. napi* died rapidly, even when the butterflies were reared on glucosinolate-free diets, whereas *P. rapae* homogenates were non-lethal[75]. Consistent with the *de novo* synthesis

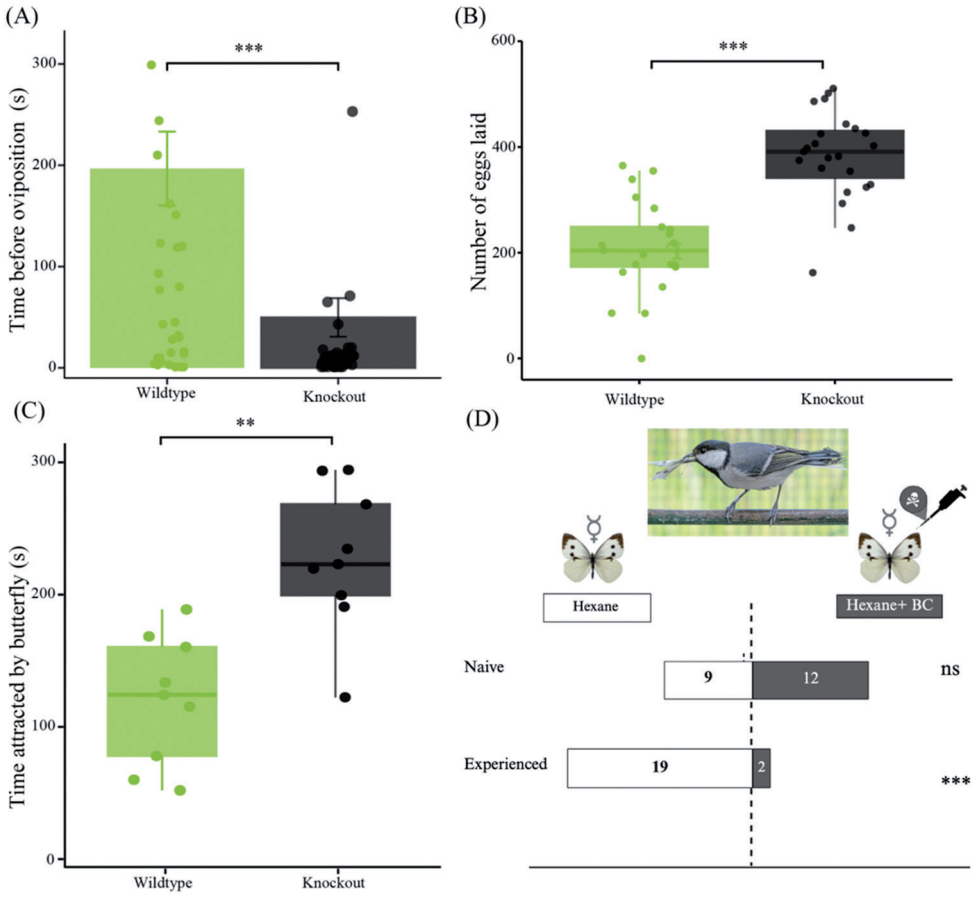



**Figure 6.4)** Functional consequences of post-mating odor perception and emission in *Pieris brassicae***.** All statistical tests and sample sizes are provided in supplementary methods. Asterisks indicate significance: *p < 0.05, **p < 0.01, ***p < 0.001, ns = p > 0.05. **(A)** Time spent by wild-type (WT) and OR45b knockout (KO) females selecting host-plants. **(B)** Total number of eggs laid by WT and KO females on a single plant (not replaced) over one week. **(C)** Time spent by *Trichogramma* wasps above virgin and mated WT or KO females. **(D)** Naïve great tits (*Parus major*) given a choice between two virgin *P. brassicae*, one treated with 1–10 ng benzyl cyanide. The first attack choice was recorded after 10 minutes and repeated with the same birds in a second trial, now experienced. Statistical details in Table S6.10. Inset: photo of *P. major* eating *P. brassicae* by Hans Smid.

of post-mating odors[46], toxicity was maintained even when larvae were reared on artificial diets without glucosinolates[75]. While this finding puzzled the researchers, the endogenous nature of benzyl cyanide in *Pieris* explains toxicity without dependence on plant-derived toxin sequestration. This finding also suggests that *P. rapae* may benefit from mimicry, gaining protection from predators by resembling the more toxic congeners without incurring the physiological costs of compartmentalizing benzyl cyanide itself.

Under high parasitoid pressure, such mimicry may allow species to retain anti-predator benefits while reducing the risk of parasitoid attraction, either by emitting lower levels of benzyl cyanide (and methyl salicylate) or by shifting to alternative compounds such as indole. *P. brassicae*, with its high benzyl cyanide concentrations, is among the most unpalatable members of a putative mimicry ring, supported by its distinctive black-and-white warning coloration[75,76]. However, prior studies on mimicry and aposematism in cabbage whites often failed to account for sex or mating status, typically using dead models or not specifically including mated females that emit warning odors when alive, which may explain inconsistent findings regarding palatability[75-78]. Our results suggest that the full ecological and evolutionary significance of these chemical defenses may only be revealed when considering live, mated females in the mate-refusal posture.

## 6.7 Synthesis and conclusions

Our findings overturn the long-standing view that post-mating odors in *Pieris* function and evolve primarily as sex-specific anti-aphrodisiac pheromones[14-16]. Instead, we reveal that these compounds, once thought to be private sexual signals, exhibit unexpectedly low interspecific variation in both emission and detection. These so-called "pheromones" are, in fact, ubiquitous volatiles, easily intercepted by a broad range of organisms. Our results show that both males and females of multiple *Pieris* species detect and respond to these compounds, challenging the idea of strict species or sex-specificity and suggesting a deeper evolutionary conservation of the underlying sensory pathways. Meanwhile, these odors exhibit high intraspecific diversity. This diversity does not track drift or mating behavior but instead aligns with ecological pressures: populations facing higher parasitoid risk emit less, while predator interactions and host-plant use further shape odor profiles. Whether this variation reflects underlying genetic divergence or phenotypic plasticity remains unresolved, but the latter may offer a particularly adaptive response to shifting ecological pressures.

Why then do butterflies persist in using such broadly available cues, despite the clear costs of parasitoid attraction? Our data support a new framework: these volatiles act as multifunctional infochemicals shaped by balancing trade-offs of ecological selection pressures. Detection of post-mating odors aids females in host-plant selection and may mediate feedback regulation of emission, allowing individuals to optimize the trade-off between reproductive signaling and enemy avoidance. Notably, benzyl cyanide also serves as an deterrent against great tits, indicating protection against vertebrate predators. This dual role is also observed in other insects, such as locusts, where benzyl cyanide serves as an aposematic signal, which may explain its evolutionary persistence in *Pieris* despite the risks of parasitoid eavesdropping.

Our results further suggest that the evolutionary interplay between toxicity, mimicry, and chemical signaling has shaped the diversification of post-mating odors in cabbage whites. Species like *P. rapae*, which emit no benzyl cyanide, may benefit from mimicry, gaining predator protection by resembling more toxic relatives while minimizing the costs of parasitoid attraction. In contrast, high-emitting species such as *P. brassicae* occupy the core of a mimicry ring, supported by both chemical and visual aposematism.

More broadly, our study calls for a rethinking of insect pheromones that co-opt common plant volatiles. Rather than functioning as isolated sexual signals, these compounds operate at the intersection of mating, oviposition, predation, and ecological community dynamics. In *Pieris*, post-mating odors reflect not only reproductive status but also the evolutionary entanglement of sexual selection, host adaptation, and enemy-mediated selection. This multifunctionality may be the rule rather than the exception for broadly detectable chemical signals in complex ecological networks.

## 6.8 Methods

Complete protocols, reagents, and additional analyses are provided in the Supplementary Methods S6.

**Study system and sampling.** We studied seven populations of the *Pieris napi* species complex and 12 other *Pieris* species across a European latitudinal gradient in 2022 and 2023. Adult butterflies were collected in the field and reared under controlled conditions. Individuals were captured in containers to check for phoretic *Trichogramma* and parasitized eggs were collected from host-plants to assess parasitism rates and rear parasitoids for identification. Mating frequency was estimated by counting spermatophores in dissected wild females. Sampling details are provided in Supplementary Table S1.

**Insect and bird rearing.** Butterflies were reared on *Brassica nigra* under standardized greenhouse conditions (25 °C, 30% RH, 18:6 h light:dark). *Trichogramma* were maintained on *Ephestia kuehniella eggs*. Birds (*Parus major*) were reared at the Netherlands Institute of Ecology (NIOO-KNAW) under ethical approval.

**Insect identification and phylogenies.** Species identification for *P. napi* species complex and parasitoids was based on CO1 barcodes, sequenced following Chelex DNA extraction and PCR with Folmer primers[118]. Sequences were aligned using MAFFT, and maximum likelihood trees were constructed in Geneious Prime (100 bootstraps, default settings).

**Chemical analyses.** Post-mating odors were collected from live females who were found ovipositing on plants in the field using direct sampling with PDMS fibers[85] by rubbing fibers against the abdomens of females found laying eggs. Samples were eluted in hexane and analyzed via gas chromatography using synthetic standards. Emission rates of benzyl cyanide, methyl salicylate, indole, and guaiacol were quantified relative to internal pentadecane standards. Virgin vs. mated *P. napi* were compared using lab-reared individuals. Chemical distances for clustering were calculated with ChemmineTools.

**Electrophysiology.** Electroantennogram (EAG) recordings were performed on excised antennae from three-day-old butterflies (n = 15–20 per group) exposed to synthetic odorants. Responses of four *Pieris* species were measured against three anti-aphrodisiac compounds and two control volatiles. Dose-response curves were generated using three dilutions. For *P. brassicae*, EAGs were recorded for wild-type and OR45b knockouts using 47 odorants (Fig. S3).

**Genetic and phylogenetic analyses of odorant receptor.** Odorant receptor gene candidates were identified by comparative genomic analysis using protein BLAST on NCBI against all Pieridae and Nymphalidae. Maximum likelihood phylogenies of odorant receptor genes were constructed using protein alignments. For genetic analyses, CRISPR/Cas9 was used to generate OR45b knockout lines in *P. brassicae*. Guide RNAs targeting the first exon of OR45b were designed using Geneious Prime and synthesized via in vitro transcription. Newly laid eggs were microinjected with sgRNA/Cas9 complexes, and resulting caterpillars were reared to adulthood. Knockout individuals were identified by PCR and sequencing of leg tissue, with non-edited siblings serving as wild-type controls.



**Behavioral assays.** Oviposition assays involved greenhouse trials with wild-type and knockout females exposed to host-plants and allowed to mate freely. Mating rates were determined (Fig. S4), and egg-laying was monitored daily for seven days (Fig. S4). Crosses among wild-type and knockout individuals tested for sex-specific effects (Fig. S4). *Trichogramma* responses were assessed using olfactometers: static assays compared *P. brassicae* knockout vs. wild-type, and Y-tube assays tested the attraction of naïve wasps to reared *P. napi* odors. Bird assays used naïve and experienced great tits to test preference between benzyl cyanide-treated and control butterflies (1–10 ng). Predator choice was recorded over sequential morning and afternoon trials.

**Statistical analysis.** Statistical analyses, sample sizes, and significance thresholds are detailed in the Supplementary Methods and Tables S2–S4. Analyses were conducted in R and SPSS.

## Authorship

**LOG**: Conceptualization (lead), Project administration, Data collection curation, Experiment execution, Visualization, Writing – Original Draft Preparation, Writing –Review & Editing

**QW**: Conceptualization, Data collection curation, Experiment execution , Writing –Review & Editing,

**JB**: Data collection and curation, experiment execution. **EtLB**: Data collection and curation, experiment execution. **JvB**: Data collection and curation, experiment execution. **TF**: Data collection and curation, experiment execution **JF**: Data collection and curation, experiment execution. **NP**: Data collection and curation, experiment execution. **AR**: Data collection and curation, experiment execution **EvT**: Data collection and curation, experiment execution. **JV**: Data collection and curation, experiment execution. **MD:** Supervision, Writing –Review & Editing, **AG**: Methodology, Resources, Writing –Review & Editing, **KvO**: Methodology, Resources. **MES**: Supervision, Writing –Review & Editing. **BJZ**: Supervision, Writing –Review & Editing. **AH**: Conceptualization, Supervision, Writing –Review & Editing. **BAP**: Conceptualization, Supervision, Writing –Review & Editing. **NEF**: Conceptualization, Supervision, Writing –Review & Editing

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CHAPTER 7

# 7

# Synthesis: Mother *does* know best

## 7.1 Introduction

This thesis focused on the evolutionary ecology of oviposition, egg survival, and post-mating behavior in butterflies (*Pieris* spp.) and their egg parasitoids (*Trichogramma* spp.). It explored how maternal decisions are shaped by, and help shape, interactions with host-plants and enemies. By integrating behavioral assays, morphological analyses, chemical ecology, and field surveys, I examined how these insects navigate the conflicting demands of mating, motherhood, and mortality. Whether we consider a female butterfly selecting a host-plant or a parasitoid seeking an egg to exploit, the ultimate mother's choice of oviposition will reverberate across the life cycle.

At the heart of this discussion lies the preference–performance hypothesis: mothers choose oviposition sites in ways that enhance the performance and survival of their offspring. In other words, the mother knows best[1]. It is an elegant and intuitive concept that has also been contested. While some studies uphold it as a guiding principle in insect ecology, others highlight mismatches between maternal choice and offspring success, especially when viewed through a narrow lens, focusing on a single measure of success or a single life stage. What if these apparent mismatches arise not from maternal failure but from overlooking the full spectrum of threats and trade-offs faced by mothers and their offspring? Have we really been observing all that drives the evolution of these traits and choices?

Drawing on data from across the thesis and touching upon several related experiments that did not become chapters, in this discussion I explore how maternal choices are influenced not just by food quality but by climate, morphology, chemical signaling, and the constant pressure of predators and parasitoids. I consider these dynamics from the perspectives of both the butterfly and the egg parasitoid. Both butterfly and parasitoid mothers face a world where danger comes in many forms and success depends on interpreting subtle environmental cues that may not directly threaten her.

In this chapter, I argue that maternal decision-making is not only adaptive and strategic but also balances current constraints with evolutionary possibilities. From the intricate chemical cues guiding *Trichogramma* to the adaptable eggs of *Pieris*, mothers have evolved to make the best of a dangerous world in ways we are only beginning to understand. I highlight some of these significant open questions and future research which may reveal further insight into this remarkable complexity.

## 7. 2. The preference-performance hypothesis

### 7.2.1 Mothers choosing wisely: Evidence for the preference–performance hypothesis

In plant-herbivore systems, alignment between maternal choice and offspring success is widely supported: females typically oviposit on host-plants that support the best larval development[1-4]. Meta-analyses confirm that offspring tend to grow larger and faster and survive better on plants where females lay the most eggs[1]. Similarly, parasitoids tend to lay eggs in hosts which will best support their offspring and adjust the number of eggs laid per host to maximize offspring success[5].

Throughout this thesis, I demonstrate that both *Pieris* butterflies and *Trichogramma* parasitoids make remarkably fine-tuned oviposition decisions, utilizing environmental chemical cues, which appear to be shaped to enhance offspring outcomes. These maternal choices are flexible and context-sensitive, influenced by the dynamic and variable cues found in complex habitats.

In **Chapters 2 and 3**, I show that *Trichogramma* rely on reliable and detectable chemical cues, including oviposition-induced plant volatiles (OIPVs) and host pheromones, to detect suitable host eggs. These cues are ephemeral and highly variable, yet parasitoids exhibit sophisticated discrimination for cues that drive their oviposition behavior. They adjust their choices depending on habitats and individual experience and can even distinguish subtle differences in host quality down to egg age.

Oviposition preferences and offspring performances also depend on egg structure. In **Chapter 5**, I demonstrate that *Pieris* eggs differ in aeropyle and micropyle morphology, traits essential for gas exchange and fertilization. These traits vary by species and geography and are associated with climatic variables. This correlation suggests that females lay eggs that are most likely to function optimally in their specific environments, reinforcing the alignment between maternal behavior, embryonic success, and local environmental conditions. We still do not know how much control females have over egg structures, or whether these morphological differences are heritable or plastic. Nonetheless, we now know that these structures are dynamic and correlate with climactic divergence, and their potential as adaptive traits should be further investigated.

In **Chapter 6**, I find that *Pieris* females make mating behavior and oviposition choices shaped by parasitoid and predator avoidance. When we disrupted chemosensation of *P. brassicae* by CRISPR/Cas9 knockout of a single olfactory receptor, females could no longer distinguish between optimal host-plants (undefended and competitor-free) and suboptimal ones (already induced and likely to harbor conspecific eggs). Females also appear

to modulate their odor emissions after mating, reducing post-mating odors that attract parasitoids, thereby minimizing the risk of enemy recruitment to their freshly laid eggs. Some species also use post-mating odors that are toxic, protecting them from predation and allowing gravid females to lay eggs safely.

Together, these findings demonstrate that differences in oviposition strategies are not only species-specific but also influenced by a suite of ecological pressures. Females optimize more than just larval nutrition: they weigh trade-offs between food, enemies, climate, mating systems, and the broader chemical landscape into which their offspring are born.

While most studies of the preference–performance hypothesis focus on herbivore maternal choice among host-plants, a bi-trophic interaction, the complexity of maternal strategy increases substantially when considering parasitoids of herbivores[1]. In these tritrophic systems, parasitoid wasps must select between host eggs laid on different plant species, integrating cues from both the herbivore and the plant [6,7]. In such cases, host preference is often highly adaptable to local habitat conditions, where the availability or reliability of host-finding cues varies across environments[8,9].

Egg parasitoid wasps like *Trichogramma* have evolved to exploit a particularly ideal resource: nutrient-rich, immobile eggs that other insect mothers have carefully laid on appropriate host-plants[10]. These eggs cannot defend themselves behaviorally, and when their mothers have not provided structural or chemical defenses, hiding eggs becomes the herbivore's primary strategy to avoid parasitism[10]. For the wasp, the challenge is finding these hidden eggs (**Chapter 2**). Successfully locating and parasitizing such tiny, hidden targets requires morphological and behavioral adaptations that distinguish egg parasitoids from those targeting larvae, pupae, or adult hosts. They must be even tinier than other parasitoids to develop inside miniscule eggs and detect egg-specific cues that will guide them quickly to these ephemeral eggs before the chorion hardens or the caterpillar hatches. Understanding the evolution of egg parasitism can illuminate broader themes in the evolution of specialization and maternal preference. The host shift to targeting eggs represents a significant transformation in life history strategy and maternal investment, yet the mechanisms and evolutionary pathways behind it remain largely unexplored.



### 7.2.2 Future Directions: The Origins of Egg Parasitism

From an evolutionary standpoint, insect eggs are ephemeral but ideal hosts, necessitating rapid and highly accurate detection by parasitoids. The challenge of finding appropriate eggs to parasitize raises a more profound question about the evolutionary

origins of egg parasitism: **How did egg parasitism evolve?** Recent phylogenetic analyses estimate that egg parasitism has evolved independently at least 18 times within Hymenoptera[10]. Many of these parasitoids especially target the eggs of Lepidoptera and Hemiptera[10]. The prevalence of egg parasitoids using herbivorous hosts suggests eggs on plants are particularly vulnerable to egg parasitoids, perhaps because many egg parasitoids make use of plant-derived cues[10].

Fourteen of the egg parasitoid lineages fall within the Chalcidoidea, where several families are exclusively egg parasitoids[11]. It has been proposed that egg parasitism is ancestral within this superfamily, with ancestors being minute wasps that parasitize eggs and later evolve into larger species adapted to other host stages[11]. However, newer phylogenies and taxonomic revisions challenge this view, showing that even within Chalcidoidea, egg parasitism in Trichogrammatidae and Mymaridae may have evolved independently[12]. Mapping all known egg-parasitic taxa onto the hymenopteran tree reveals a surprising diversity of their repeated origins (**Figure 7.1**). Future studies should ask:

- Did egg parasitoids evolve from ancestors that parasitized plants (i.e. gall-formers) or other host stages (i.e. larval or pupal or imago parasitoids)?
- Are egg-larval parasitoids, which lay eggs in host eggs but do not immediately kill their host to later emerge from host larvae, a derived or ancestral state of egg parasitism?
- What behavioral and sensory innovations enabled wasps to exploit such elusive and ephemeral hosts?
- What ecological or competitive pressures favored this highly specialized niche?

### 7.2.3 When Mother's Choices Appears "Wrong": Rethinking Contradictory Findings

Maternal choices sometimes appear puzzling. While most herbivore studies support the preference–performance hypothesis[1], many others, especially in non-herbivorous insects, report a decoupling of maternal preference from offspring success[1,15-21]. This apparent mismatch may reflect (1) ecological or life-history variation that modifies the relationship, (2) non-adaptive maternal behavior, or (3) adaptive behavior not linked directly to food quality[1].

In non-herbivores, mismatches are more common. For instance, the parasitoid *Aphidius matricariae* could discriminate among aphid hosts but chose suboptimal plant–aphid complexes in complex habitats[21]. Similarly, wormlion (Diptera: Vermileonidae) females did not prefer optimal larval habitats, resulting in less effective pit traps[20]. Such outcomes may reflect constraints or trade-offs in maternal strategies that prioritize overall fitness,

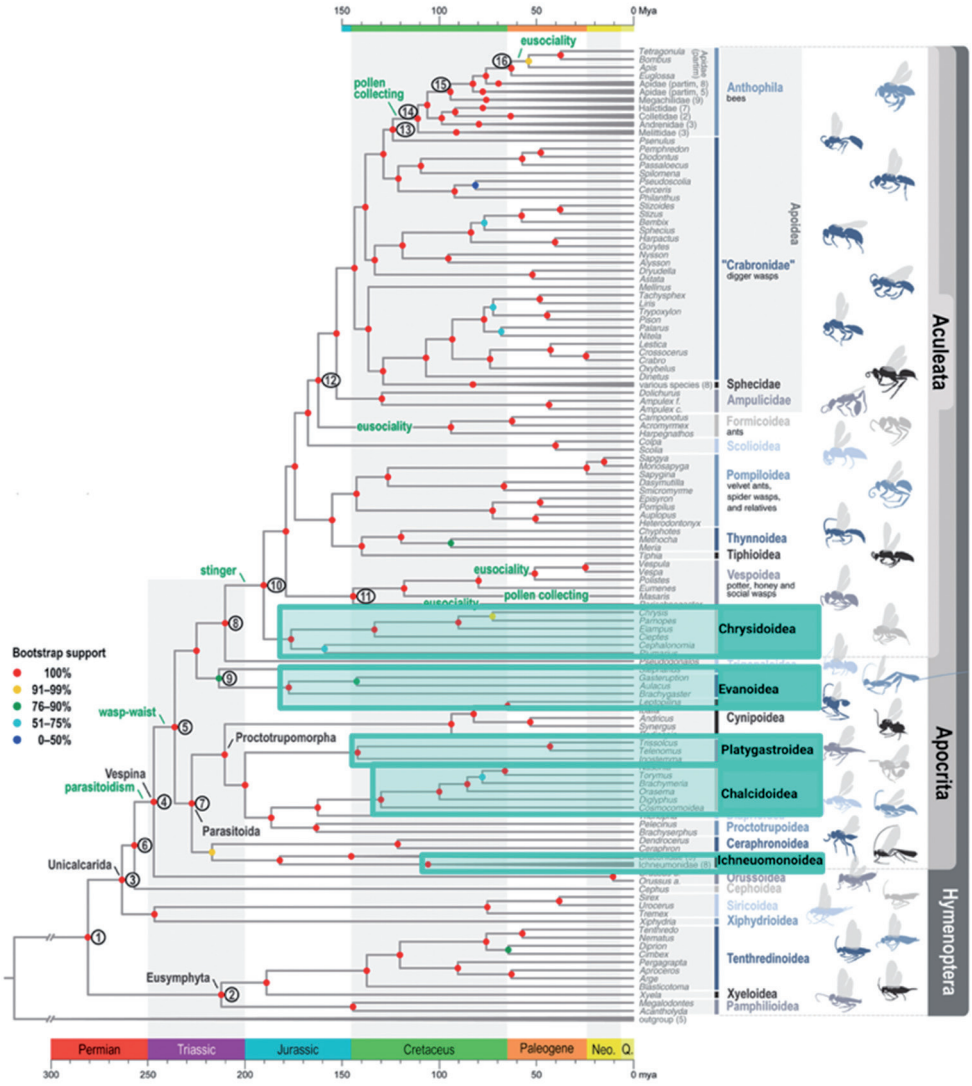



**Figure 7.1)** Evolution of egg parasitism in Hymenoptera**.** Hymenopteran phylogeny adapted from Peters *et al.*[13]. Teal boxes indicate taxa containing egg parasitoids according to Fatouros *et al.*[10].

even when offspring performance metrics (e.g., growth or development rate) are compromised. This suggests that environmental complexity can disrupt the expected preference–performance alignment. Mismatches may serve adaptive purposes. For example, Colorado potato beetles, solanaceous specialists, laid more eggs on poor-quality *Solanum immite*, despite poor larval growth on these plants[17]. This conflict may be explained by bet-hedging, where mothers spread risk across host options to buffer against environmental uncertainty[17]. Herbivores that can feed on a diversity of plants in a genus may

expand their host repertoire and spread risk across plants[56]. Thus, evidence conflicting with the preference–performance hypothesis may represent adaptive risk distribution across variable host environments[17].

While studies considering bi-trophic interactions often find support for the preference-performance hypothesis, excluding multitrophic interactions is a significant limitation. In more realistic and more complex systems, nutritionally marginal plants may reduce exposure to enemies, offer better microclimates, or support mutualistic endosymbionts. Maternal decisions and offspring outcomes do not occur in isolation but rather within rich ecological contexts that include predators, parasitoids, mutualists, and microclimatic variability. Thus, apparent mismatches may reflect more comprehensive maternal strategies that prioritize offspring survival across all selective pressures, not just larval growth or size, but total offspring fitness across ecological conditions. For example, a study in *Pieris* shows parasitism pressure on larvae by the wasp *Cotesia glomerata* and the tachinid fly *Epicampocera succincta* can shape food plant preferences in *Pieris* butterflies[4]. Although all tested host-plants were nutritionally suitable, species like *P. napi* preferred nutritionally inferior hosts (e.g., *Arabis*) likely to avoid parasitoids in long-lasting habitats where enemies are abundant[4]. Larvae which grow for longer periods are also more likely to face predation[22]. Females will even avoid laying eggs entirely when predators are present[23,24]. These findings highlight how host-plant choice reflects a trade-off between food quality and parasitoid avoidance, underscoring the need to include natural enemies when testing the preference–performance hypothesis.

A compelling example of maternal behavior that may seem "wrong" without crucial ecological context comes from Monarch butterflies. When infected with the protozoan parasite *Ophryocystis elektroscirrha* (OE), monarch mothers can pass the infection to their offspring via their eggs[25]. Infected larvae cannot switch host-plants after hatching, so they rely entirely on their mother's oviposition choice to provide any protection. Some milkweed species contain high levels of cardenolides, plant toxins on which monarchs specialize that reduce OE virulence[25]. Infected females have been shown to lay eggs on these medicinal plants preferentially[26]. Although these plants may not enhance larval growth or size, they improve survival under infection. This maternal "self-medication" highlights how oviposition choices can optimize offspring performance in pathogen-rich contexts, even when the selected plant is suboptimal by conventional nutritional metrics. Indeed, studies show that plant toxins can reduce weight gain while increasing survival[27], underscoring the pitfalls of equating larval growth with fitness.

Another example where a mother's counter-intuitive behaviors may be explained by protecting offspring from OE-like parasites comes from *Heliothis virescens*, where female moths exhibit striking intraspecific variation in their sex pheromone blends[28]. While

most moths use highly conserved signals to attract mates in the dark, some *H. virescens* females produce "rare" blends that are less attractive to males and only succeed in attracting mates when common-blend females are nearby[28].

This intraspecific variation appears maladaptive until parasites and parasitoids are taken into consideration. Rare-blend females show higher resistance to OE-like parasites under lab conditions[29] and are less attractive to *Trichogramma* wasps that mount females to hitchhike, as only the common pheromone blend lures them[30]. Thus, although rare-blend females may attract fewer mates, they may gain survival advantages, suggesting that natural selection helps maintain variation in this sexually selected trait as a form of bet-hedging, where different strategies may confer fitness advantages under fluctuating parasite pressure. As we found in **Chapter 6**, even mating cues may evolve under selection pressures to protect offspring, which are equally or arguably more potent than pressures emerging from interactions with mates.

Interestingly, not all females can produce these rare pheromone blends, as they are genetically determined. The change in pheromone ratio in the "rare" blends is due premature stop codon in the delta-11-desaturase gene[31]. While some of this variation may be selectively maintained, it is also possible that aspects of signal diversity persist due to drift or stochastic founder effects, especially in small or isolated populations. However, variation in these pheromone blends appears stably maintained in multiple populations, which may reflect negative frequency-dependent selection, where a trait's fitness benefits increase as the trait becomes more rare[32]. In this context, rarer pheromone signals confer a selective advantage by evading detection by parasitoids or pathogens. However, as these rare blends become more common, their advantage may wane, allowing other variants to rise in frequency. Such dynamics are characteristic of host–parasite coevolution, where shifting enemy pressures continually reshape the adaptive landscape[32]. This evolutionary cycling helps explain the persistence of seemingly maladaptive traits and supports the idea that maternal behavior reflects long-term balancing strategies shaped by multiple selective forces. The maintenance of variable blends of post-mating odors seen in **Chapter 6**, for example, where some individuals which rely more on the compound indole instead of methyl salicylate and benzyl cyanide, could also be an example of this dynamic, discussed further in section four of this discussion. Overall, we can conclude that mother's mating behaviors may be explained by factors beyond mate interactions.

From this perspective, many perceived maternal mistakes are artifacts of narrowly defined fitness metrics. In **Chapter 4**, I describe how *Pieris* butterflies oviposit on *Brassica nigra* leaves, despite those tissues and that species strongly expressing HR-like egg-killing defenses. While seemingly maladaptive at first glance, this may reflect trade-offs. Egg

placement under leaves could shield eggs from UV and high temperature[33], rain[34], and predators[35,36], or enhance larval access to defense compounds[37] or adults' pheromones' precursors[31]. Larvae might benefit from starting life on a leaf, rather than navigating hostile surfaces from flowers or stems. Meanwhile, we describe that some populations that we could not include in that study do show avoidance of leaves, laying eggs on stems and flowers that do not express HR, and these counter adaptations may be found in some populations and not others.

A further dimension often overlooked in preference–performance studies is the condition of the mother herself. According to the Trivers–Willard hypothesis**,** mothers in better condition may adjust the sex ratio of their offspring for higher potential reproductive returns, while those in poorer condition may invest conservatively[38]. This implies that oviposition decisions might vary not only with external environment but also with maternal state, shaping choices that appear maladaptive when maternal condition is not considered. Similarly, the silver spoon effect suggests that offspring performance can be strongly influenced by maternal condition, independent of the environment the mother chooses[39]. In such cases, two mothers making identical oviposition choices could yield very different offspring outcomes simply because of differences in their physiological or developmental state. While offspring may simply be constrained by poor state of the mother, it is also possible that this scarcity shapes offspring's phenotype to be most well suited for a potentially similarly scarce environment[39]. These possibilities reinforce the idea that maternal "mistakes" may instead reflect condition-dependent strategies that are contextually adaptive, even when they do not optimize traditional performance metrics like larval weight or survival in isolation.

Maternal oviposition choices often reflect multidimensional optimization, as mothers must balance complex trade-offs among offspring number, size, and survival across variable environments[40,41]. Rather than maximizing a single performance metric, such as larval weight or immediate survival, mothers may select oviposition sites that minimize overall reproductive risk, maximizing total fitness for offspring, even if these sites are suboptimal for one particular life stage as long as lifetime reproductive fitness is maximized[42,43]. This complexity is well documented in the literature, where maternal effects, differences in water uptake, sibling competition, or predation risk often complicate the classic egg size–number trade-off[1,40,41].

This aligns with the concept that organisms do not act as idealized fitness-maximizing machines, so-called "Darwinian demons", who have optimized each and every trait to be the most fit[43]. Instead, evolution shapes whole-organism strategies within ecological and genetic constraints, often resulting in apparent maladaptations when traits are viewed in isolation[43]. The persistence of specific cue-based responses suggests that, histori-

cally, such cues were reliable predictors of offspring fitness, even if they are now filtered through ever-changing environmental noise.

Therefore, apparent contradictions to the preference–performance hypothesis often stem from narrow definitions of performance[1]. True reproductive success integrates direct and indirect fitness across life stages and variable environments[42,44]. Experimental evolution in semi-natural or fluctuating environments offers a promising approach to test how maternal strategies perform under realistic ecological scenarios, as such experiments have demonstrated the evolution of deterministic maternal effects and trade-offs in response to environmental unpredictability[45]. Until such approaches are standard, deviations from the preference-performance hypothesis should be interpreted with caution.

Importantly, "mistakes" may seed innovation. In fieldwork, we observed butterflies ovipositing on non-typical hosts[46]. *Pieris mannii*, for instance, has expanded beyond *Iberis* spp. to plants like *Diplotaxis*[91,92]. Though *Iberis* spp. lacks HR defenses, they are not enemy-free, as we report the first record of *Trichogramma* parasitizing *P. mannii* eggs on these hosts[46] (**Chapter 4**). The switch to more common, albeit HR-inducing hosts, has provided broader habitat access and abundant larval resources aiding their rapid expansion across western and northern Europe in just a decade[47,48]. Such exploratory oviposition may set the stage for host shifts. If a larva tolerates the new plant, it may survive, and over time, this could lead to the diversification of the species. Host use variability has been linked to insect radiations, including in Pieridae[49,50,51]. In *Pieris* butterflies, repeated maladaptive egg-laying on novel host-plants has, over time, led to larval adaptation. For instance, *Pieris oleracea* has evolved the ability to develop on the invasive mustard *Alliaria petiolata* during its bolting stage after decades of poor larval performance[49]. Similarly, population genomic studies in *P. macdunnoughii* show genetic signatures of selection in response to the novel host *Thlaspi arvense*, particularly in genes related to metabolism and larval development, indicating ongoing adaptation for larval survival[49].



### 7.2.4 Future Directions: From Mismatch to Radiation

Recent comparative work shows that both host-switch events and ongoing probing of novel hosts contribute to diversification in butterflies. While major radiations often follow host switches, much of the diversity is maintained by variability in host use itself. This dynamic probing of alternative hosts provides the raw material for adaptation and potentially for the emergence of new insect–plant associations[50]. Thus, variability in host use is essential for butterfly diversification, with subsequent radiations following colonization of a new host leading to species radiations[50,51].

Genomic studies show that both chemosensory and detoxification gene families have evolved rapidly in herbivorous insects, particularly in Lepidoptera[17]. In some species, expansions of olfactory and gustatory receptor genes, especially those expressed in females, point to a direct role in shaping oviposition behavior through plant chemical detection[52]. Simultaneously, adaptations such as nitrile-specifying proteins (NSPs) in *Pieris* enable the detoxification of chemically defended host-plants[53,54]. Host shifts appear to involve coordinated changes across genes for plant detection, digestion, and detoxification[52]. Although most functional analyses have been limited to a small number of candidate genes, there is growing evidence that gene expansions and possibly positive selection on olfactory and detoxifying pathways may facilitate host shifts. Notably, microbial partners can also modulate plant metabolism and insect responses, and can be important players in larval detoxification of new plants, highlighting the broader ecological context[52].

In *Pieris macdunnoughii*, repeated oviposition on the invasive and toxic plant *Thlaspi arvense*, despite poor larval performance, has led to detectable evolutionary responses[49]. Transcriptomic analyses show that larvae feeding on *T. arvense* express genes related to stress responses, apoptosis, and accelerated development, while lacking expression of specialized detoxification enzymes. In contrast, larvae on native hosts show higher expression of genes for detoxification and adaptive feeding. Interestingly, adult females with differing oviposition preferences show no differential gene expression in gustatory or olfactory tissues but do show significant genetic differentiation in signal transduction genes, suggesting a heritable basis for oviposition behavior[49]. Population genomic studies further reveal selection signatures in *P. macdunnoughii* populations exposed to *T. arvense*, especially in genes related to metabolism and larval development, highlighting how persistent preference-performance mismatches can drive adaptive genetic changes over time[49].

Together, these findings suggest that while chemosensation and detoxification may not be co-regulated at the expression level, they are genetically and evolutionarily linked through selection on maternal behavior and larval physiology. This perspective supports exciting evolutionary questions:

- How frequently do oviposition "mistakes" occur, and how often do they lead to adaptation?
- Can selection on larval survival drive indirect changes in oviposition behavior through genetic correlations?
- Could experimental manipulation of maternal preference and larval performance accelerate or replicate host shifts?

### 7.2.3 Take home message on "preference-performance"

Maternal oviposition decisions are context-dependent and shaped by complex ecological trade-offs. While female preferences often align with offspring performance, mismatches can reflect hidden selective pressures, such as enemy avoidance, microclimate buffering, or bet-hedging strategies. What may seem maladaptive may represent multidimensional optimization. Future work should broaden the scope of how "performance" is measured, integrate multi-trophic interactions and life stages, and explore the genetic and sensory architecture underlying maternal choice. By exploring the genetic and behavioral architecture of maternal preference and offspring performance, we may uncover how evolution turns maternal error into opportunity and how a single egg on the "wrong" plant can reshape adaptive landscapes.

## 7.3. Host-finding Strategies: Why do only some egg parasitoids hitchhike?

Hitchhiking, or phoresy, is one of the most striking strategies seen in egg parasitoids. By riding butterflies, *Trichogramma* wasps gain direct access to freshly laid eggs, bypassing the challenge of locating. While the benefits seem obvious, phoresy is not universal, and even among phoretic species, its expression is variable and context-dependent (**Chapter 2**). Though phoresy provides immediate access to freshly laid eggs, slightly older eggs may sometimes enhance parasitoid success (**Chapter 3**), challenging assumptions about its universal benefit. *Trichogramma* also use volatile cues like oviposition-induced plant volatiles (OIPVs) to locate host patches, a sophisticated alternative that may outperform phoresy when wasps are already emerging in a suitable patch. In this section, we explore what is known about phoresy in *Trichogramma*, the reasons for our current knowledge gap, and the steps we can take to gain further insight.



### 7.3.1 Challenges of phoresy surveys

First and foremost, the scarcity of studies on egg parasitoid phoresy stems from the simple fact that it remains challenging to quantify. Tiny hitchhiking wasps are easily missed in the field, especially since they often dismount when butterflies are disturbed. Catching butterflies in nets with small containers, which we immediately close, can help capture phoronts[55]. Nonetheless, we have seen them escape from these containers before they can be closed[56]. Moreover, wasps are not always present on butterflies. For example, when a butterfly has just laid an egg, the wasp may have already dismounted onto the host-plant before the butterfly is caught. While many more wasps can be collected from parasitized eggs in the field, it is impossible to distinguish between parasitoids that arrived via phoresy and those that reached the eggs independently, whether by walking, flying, or being wind-swept. With this container-in-net method, one must catch nearly

100 butterflies before a single data point on phoresy can be collected[55,57]. Thereby, documenting and disentangling phoresy patterns remains very challenging.

To work around these limitations, we aimed to develop a technique to determine whether a wasp had been on a butterfly at any point in the recent past using environmental DNA (eDNA) for meta-barcoding. Preliminary eDNA results show promise: using *Trichogramma*-specific primers we designed, *Trichogramma* DNA can be recovered from a glass vial that contained a live *Trichogramma* for as little time as one hour[56] (**Figure 7.2**). Later tests reveal that *Trichogramma* DNA could also be recovered from butterfly wings[56]. However, issues with contamination in negative controls imply that further testing is required in more sterile environments. These eDNA results opens exciting possibilities for studying phoresy, allowing surveys of wasps which had once been hitchhiking by amplifying parasitoid eDNA left behind, even if they had dismounted by the time the butterfly was

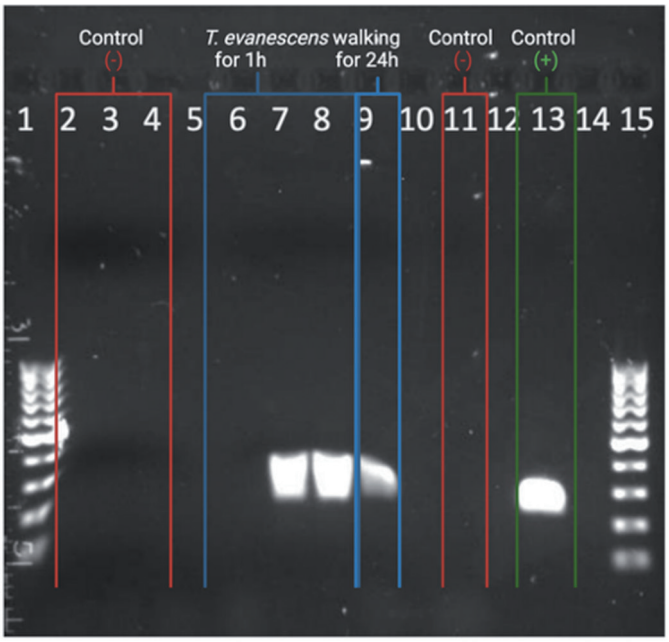



**Figure 7.2)** eDNA reveals traces of *Trichogramma* left behind, figure adapted from Palmen 2024[56]. Gel electrophoresis picture (1% EtBr gel) of a PCR performed on DNA extracted from glass vials which had a *Trichogramma evanescens* in them for different amounts of time. PCR was performed using the TevaCOI primers targeting part of the COI gene of *T. evanescens*. Lane 1 and 15: 100bp ladder. Lanes 2-4: Negative controls - red (empty glass vials). Lane 5: Empty. Lanes 6-8: Tubes which had *T. evanescens* in them for 1 hour- blue. Lane 9: Tube which had *T. evanescens* in it for 24 hours- blue. Lane 10: Empty. Lane 11: Negative PCR control-red. Lane 12: Empty. Lane 13: Positive PCR control with DNA isolated from crushed *T. evanescens*- green. Lane 14: Empty.

captured. Applying this technique to large samples of field-caught butterflies will help reveal and refine patterns of phoresy.

### 7.3.2 Emerging phoresy patterns

Our fieldwork in Europe and Panama reveals that phoresy is widespread among egg parasitoids but environmentally modulated. Across both Heliconiini (Panama) and *Pieris* (Europe) butterflies, we captured an unexpectedly diverse range of hitchhiking Hymenoptera (**Figure 7.3A**). In surveys in Panama in past years, previously undescribed *Ooncyrtus* and *Trichogramma* species were identified parasitizing and hitchhiking on *Heliconius* butterflies[58,59]. *Heliconius* egg parasitoids seem reliant on OIPVs for host-finding because parasitism rates were far lower on bait eggs placed next to host-plants compared to bait plants laid on host-plants which could induce OIPVs[60] (**Figure 7.3B**). The egg parasitoids of *Heliconius* also reveal high generalization in their use of host-plants and butterflies[60] (**Figure 7.3C**).

Notably, in the Netherlands, we caught *Pieris* butterflies with wasps from the Mymaridae family, known as fairy wasps, which are even smaller than *Trichogramma*[57]. These wasps are exclusively egg parasitoids, but are not known to parasitize *Pieris*, and we have not observed them emerging from *Pieris* eggs in our extensive surveys. However, DNA barcoding revealed the presence of Mymarid (*Anagrus*) DNA inside a solitary *Pieris* egg[61]. Whether such taxa are regularly phoretic on butterflies remains to be determined. Even when not all hitchhikers we found are egg parasitoids, other wasps may still benefit from butterfly-assisted dispersal into suitable habitats, particularly since many wasps we found attack larvae and pupae associated with the butterflies' Brassicaceae host-plants[57].

Our phoresy survey data, albeit limited in sample size, is beginning to reveal that environmental variation in weather and habitat strongly impact phoresy behaviors and the likelihood of detection[57,63,64], as predicted in **Chapter 2**. For example, we found phoresy was impacted by:

- Weather: Phoronts were frequently captured immediately after rain[57], possibly because sheltering butterflies were easier to board or because wasps had not yet dismounted at oviposition sites.
- Season: Phoresy peaked in September, when *Trichogramma* numbers were highest due to cumulative population growth over multiple *Pieris* generations[57,63].
- Habitat quality: Hitchhiking was more common in depleted habitats, such as late-season rapeseed fields where butterflies eclosed but fresh foliage and eggs were absent[64]. In these sites, with no OIPVs to guide them or nearby suitable eggs, *Trichogramma* may hitchhike to reach better habitat. Conversely, in high-quality patches with abundant eggs, phoresy was rare, likely because local host-finding via OIPVs, walking, or short flights sufficed (**Chapter 2**).

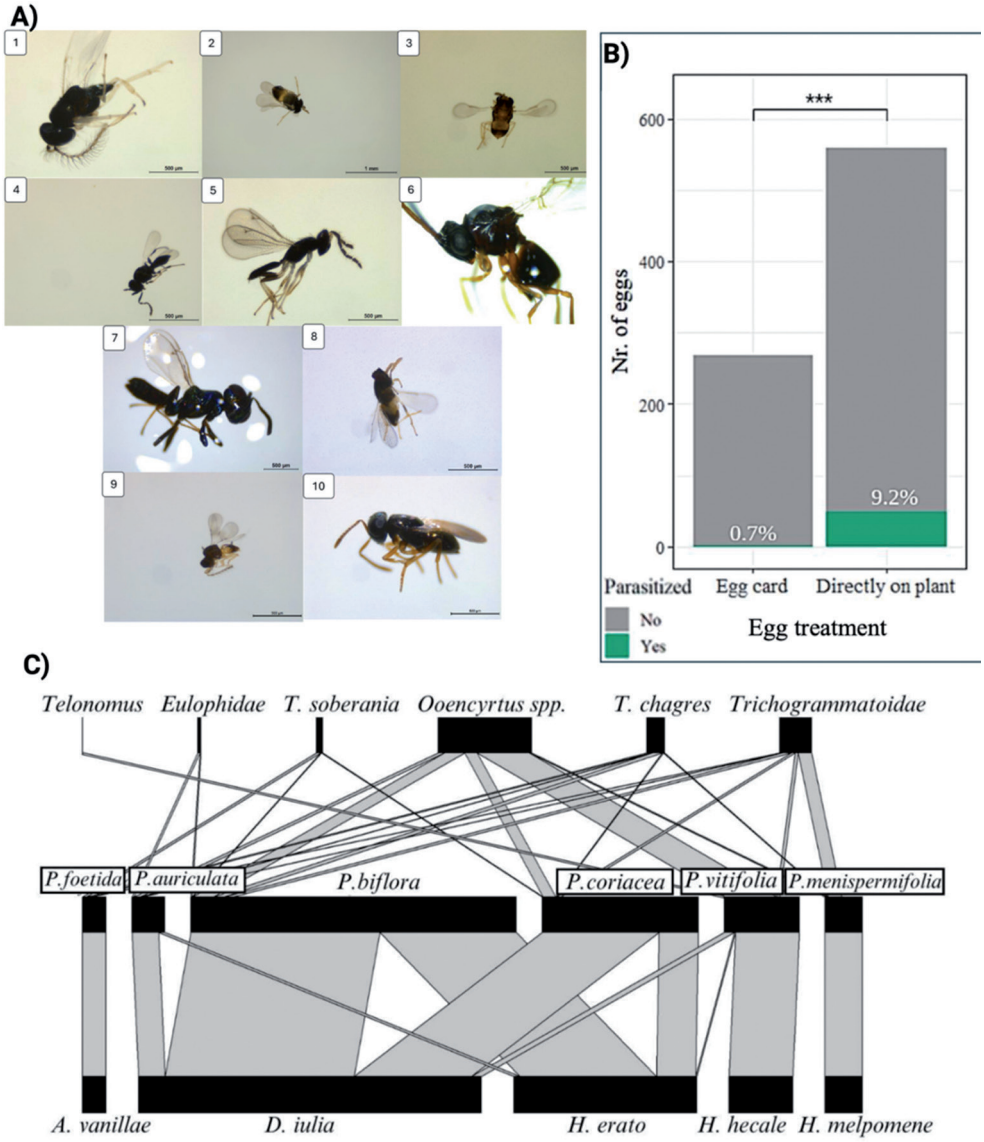



**Figure 7.3)** Egg parasitoids of Heliconiini species in Gamboa, Panama. **A)** Diverse hitchhikers sampled from *Heliconius* in 2024[62]: 1. *Ooyncertus* sp, 2. Aphelinidae sp., 3. Aphelinidae sp., 4. Platygastroidea sp., 5. Eulophidae sp., 6.Cynipoidea sp., 7. Platygastroidea sp, 8. Aphelinidae ep, 9. Chalidoidea sp., 10. *Ooncyrtus* sp. **B)** Number of (parasitized) eggs on bait plants with naturally laid eggs directly on the plant versus eggs placed on egg cards, which lacked OIPVs[99]. Kruskal-Wallis test, *** = P<0.001, N=600. **C)** Tripartite network of interactions between parasitoids (top bars), *Passiflora* (middle bars) and Heliconiini eggs (lowest bars)[60]. Thickness of grey bars indicates the strength of the interaction. N=1076. T.=*Trichogramma*, H.=*Heliconius*, D.=*Dryas*, E.=*Eueides*.

Unexpectedly, male wasps were also frequently found hitchhiking, including on male butterflies, a behavior rarely reported previously[55]. In our observations, male phoronts were nearly as common as females[57,63], raising questions about their dispersal strategies, mate-finding, or other pressures shaping this behavior. Similar to females, males must locate suitable patches and potential mates, especially in contexts where sib-mating is unlikely due to poor quality hosts in which mainly male eggs are laid[65]. In other taxa, it is known that phoresy by males increases mating encounters or allows escape from competition[66–68]. While male butterfly odors do not attract *Trichogramma* wasps as strongly as those of mated females, they remain more attractive than control air[69], suggesting chemical cues may still play a role.

We conducted preliminary olfactometer mounting assays that further support phoresy by male wasps and on male butterflies. Female *Trichogramma* readily mounted male butterflies, doing so as often and even faster than on females (**Figure 7.4A–B**). Male *T. pintoi*

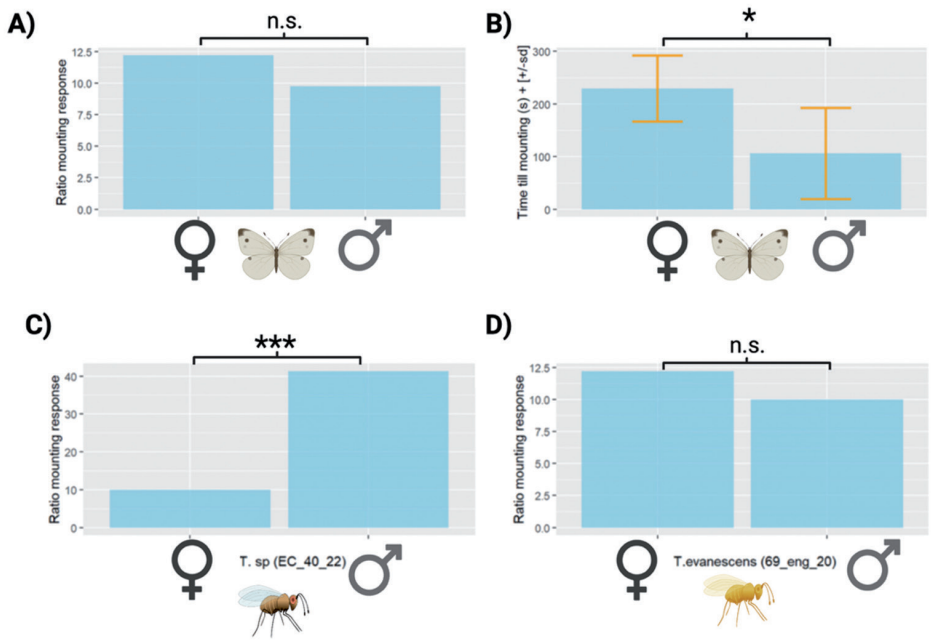



**Figure 7.4)** Mounting assay reveals female *Trichogramma* readily mount male *Pieris* and male *Trichogramma* readily mount mated female *Pieris*. Figures adapted from van Buul[111]. **A)** Mounting rates of female *T. evanescens* (line 69_eng_20) on mated female vs. male *P. brassicae* (Pearson's chi-squared test, p=7.239, N = 41). **(B)** Time (seconds) until female *T. evanescens* (line 69_eng_20) mounted mated female or male *P. brassicae* (t-test for equal variances, p=0.041, N= 41). **C)** Mounting preference of *T. pintoi* (EC_40_22) males and females in response to mated female *P. brassicae* (Pearson's chi-squared test, p< 0.001, N= 19). **D)** Mounting behavior of *T. evanescens* (69_eng_20) males and females in response to mated female *P. brassicae* (Pearson's chi-squared test, p=0.753, N = 40).

also mounted female butterflies more often than their female wasp counterparts (Figure 7.4C), while mounting rates between male and female *T. evanescens* were similar (Figure 7.4D). These results open the possibility that hitchhikers use male butterflies and that male wasps hitchhike, possibly to reach resource-rich habitats[55] or to transfer to reach females during butterfly mating. The use of male-specific cues by *Trichogramma*, such as citral, the distinctive pheromone of male *P. napi*, should be investigated[70,71].

### 7.3.3 Genetic and morphological evidence of hitchhiker diversity between and within species

In order to clarify phoretic patterns, distinguishing *Trichogramma* species and strains is essential, as host-finding strategies vary not only between species but also within them[73] (e.g., **Figure 7.4C and D**; **Chapter 2**). However, species classification in *Trichogramma* remains notoriously difficult.

DNA barcoding, though widely used, faces challenges in *Trichogramma*. DNA barcodes, which are short, species-specific sequences with minimal within-species variation, are a powerful tool for rapid ID, particularly for taxa like *Trichogramma* with minute or cryptic morphology[74]. However, errors in public reference databases, due to the difficulty of morphological ID, can perpetuate misidentifications[75]. Further complications arise from molecular phenomena such as nuclear copies of mitochondrial DNA (NUMTs) and heteroplasmy, multiple mitochondrial genomes in one organism. While both occur in Hymenoptera[76–79], NUMTs are the likely culprit in *Trichogramma* (as in other chalcids)[80]. We observed NUMT signatures in our own COI sequences of *T. evanescens*, consistent ambiguous base calls from double peaks at the same sites across strains in individual wasps (**Figure 7.5**). These artifacts can mask real mitochondrial variation, especially with standard primers[79,81], though NUMTs themselves may hold evolutionary insights[82]. These issues, combined with incomplete and sometimes erroneous public databases, undermine species-level resolution. NUMTs in *Trichogramma* must be further investigated, as part of refinement of their taxonomy[75].

To resolve such ambiguities, molecular and morphological data must be integrated[75,83]. Yet even morphology poses difficulties: species-level traits depend on dissecting male genitalia (**Figure 7.6A-F**), often impossible for *Wolbachia*-infected or female-only lines. In our samples, mismatches occurred between barcode and morphometric identification (**Figure 7.6G**). For example, *T. evanescens* features for many individuals overlap with other species, and two lines identified as *T. semblidis* are clearly distinguished as separate species morphologically. For one of these *T. semblidis* lines, 7_eng_20, while the ITS2 sequences clearly matches publicly available *T. semblidis* ITS2 sequences, its COI sequence is very different from other *T. semblidis*, and more closely matches the COI of the species *T. platneri*. Database errors, NUMTs, or even hybridization or biparental inheritance, may all

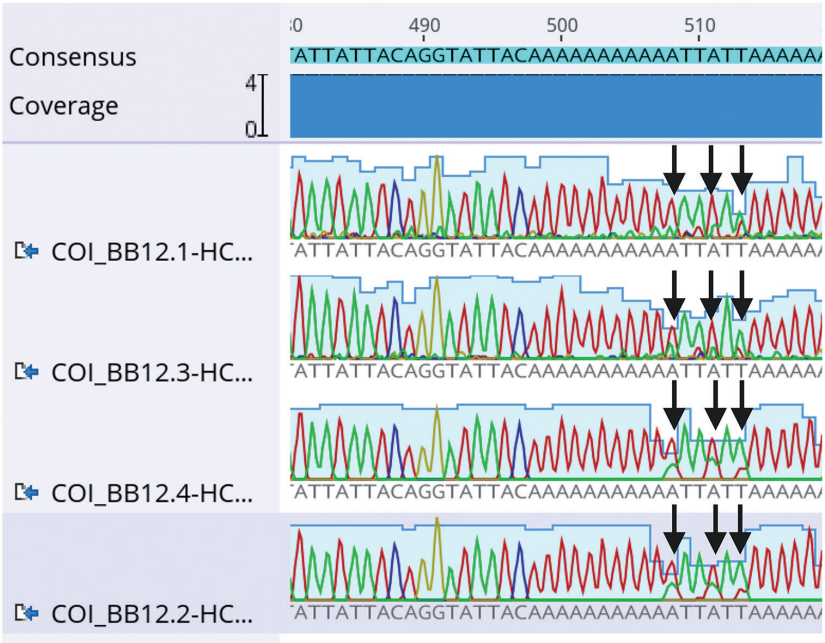



**Figure 7.5)** Evidence for NUMTs (nuclear mitochondrial pseudogenes) in *Trichogramma evanescens*. COI barcodes from individual *T. evanescens* wasps, amplified with the standard barcoding Folmer primer set, show consistent ambiguous base calls at the same nucleotide positions across multiple strains. One such site is highlighted in the black rectangle. Chromatogram from representative strains, with black arrows indicating clear double peaks at the same ambiguous sites across strains, despite each sample coming from a single individual. Since mitochondrial genomes are haploid and maternally inherited, heterozygous positions should not occur. Downstream of position 520, sequences become unreadable due to overlapping signal, likely from co-amplification of mitochondrial COI and NUMTs. These patterns persisted across PCRs with varying annealing temperatures meant to fine tune specificity, always in the same locations in various strains, suggesting they are not artifacts of suboptimal conditions but indicative of NUMT interference in *T. evanescens*.



contribute to this confusion [75,84]. Moreover, global spread of biocontrol agents may blur species boundaries, as species that were once isolated only by geography may now co-occur and lack barriers to hybridization[85]. Meanwhile the taxonomic expertise required to parse them is dwindling[75]. These results highlight the urgent need to reassess *Trichogramma* taxonomy, including whether commonly used markers (COI, ITS2) are sufficient for this taxon[86].

Furthermore, cryptic species may have been missed in earlier classifications, and diversity below the species level may supersede divergence between currently named species[75,84]. Within-species diversity also matters. In *T. evanescens*, a widely used biocontrol agent, we detected substantial cryptic haplotype variation linked to collection from different hosts and locations around Wageningen, Netherlands[63,73] (**Figure 7.7A**). For example, some

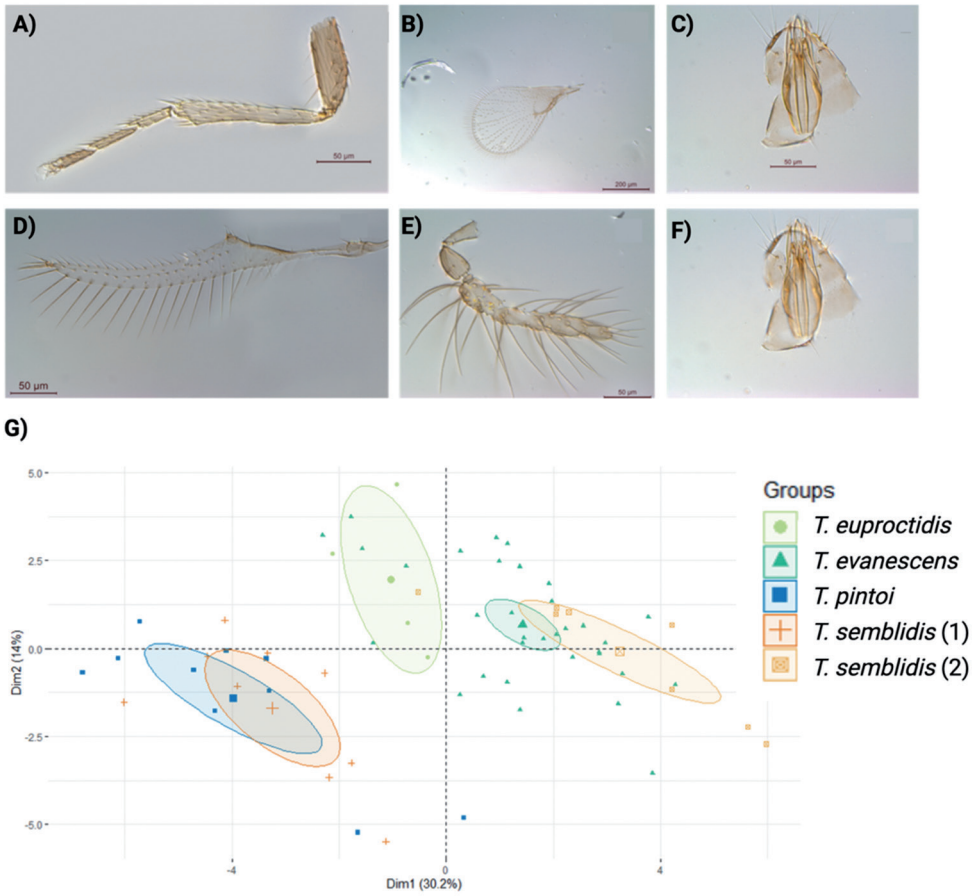



**Figure 7.6)** Incongruence between morphological and barcode identification of *Trichogramma* species. Adapted from van Beeck, 2025**. A-F)** Representative images of dissected body parts for morphological ID of *T. evanescens* male from 15-WUR-20. **A)** Hind leg, **B)** Hindwing, **C)** Forewing**, D)** Antennae, **E)** Dorsal side genital, **F)** Ventral side genital **A)** Hind leg, **B)** Hindwing, **C)** Forewing, **D)** Antennae, **E)** Dorsal side genital, **F)** Ventral side genital **G)** PCA plot with lines and species from rearing. Data ordinated based on combined morphometric features, colored by line of origin determined by ITS2 barcodes and BLAST on NCBI. Ellipses represent the 95% confidence interval of the centroid of each laboratory line.

strains respond to HR-plant volatiles, while others collected nearby do not[73], differences that could influence field success under varying environmental conditions (**Chapter 2**).

We have begun investigating whether this diversity translates to differences in biocontrol success. In several pilot field releases, a single release of a single strain boosted *Pieris* egg parasitism from 0% to 60% (**Figure 7.7B**). Our next step is to test haplotype-specific performance using iso-female lines, which preserve genetic variation until release and

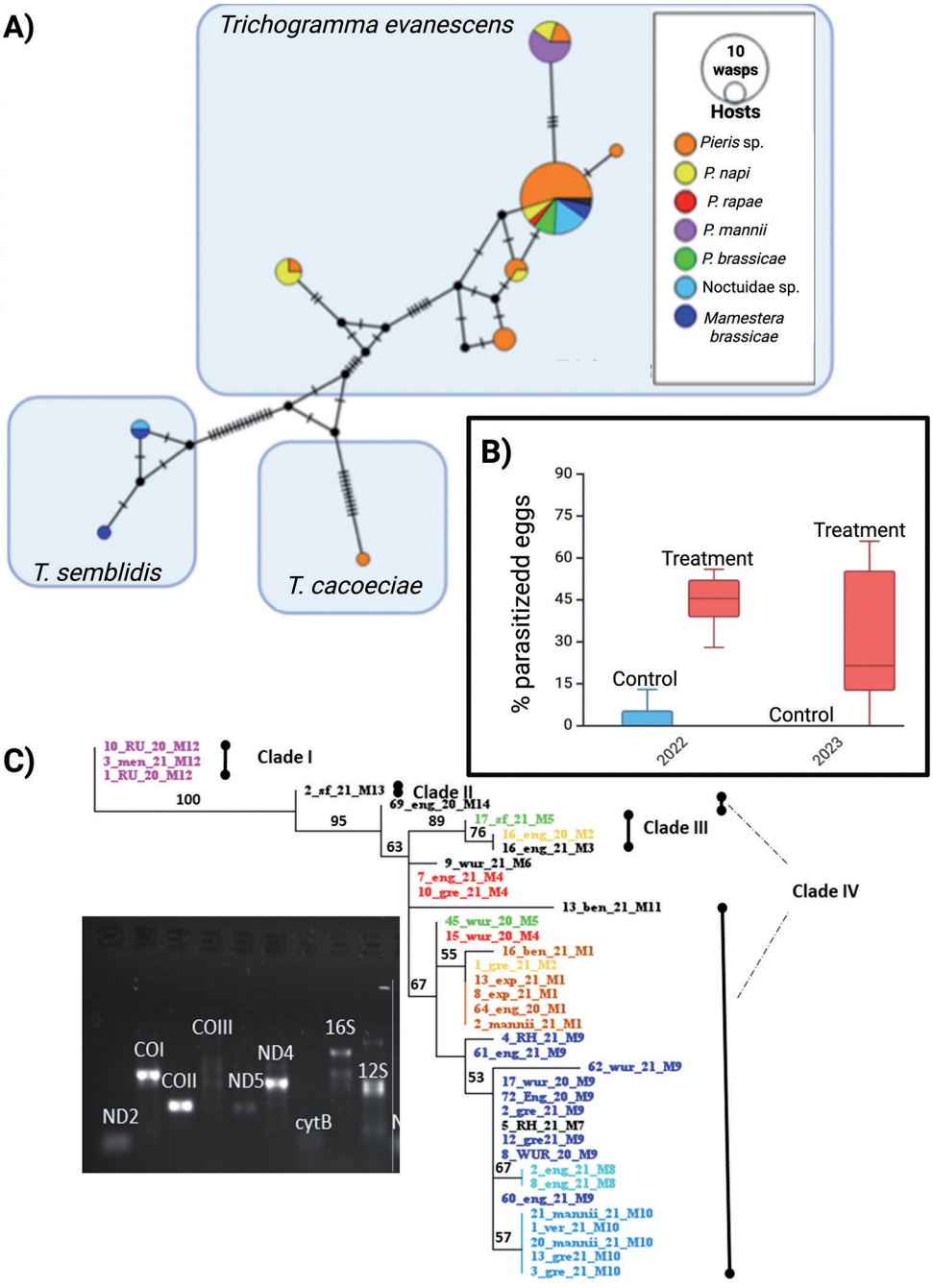
reduce adaptation to rearing conditions, while maintaining unique barcodes[87]. Their progeny can be traced back to specific haplotypes using an extended barcode system we designed that includes concatenated mitochondrial, ribosomal, and nuclear loci beyond standard COI or ITS2 barcodes[83] (**Figure 7.7C**).

In addition to behavioral variation, hitchhiker lineages may also show morphological adaptations. In other taxa, phoresy is supported by specialized tarsal structures that help the parasitoid cling to its host. Using SEM, we examined *Trichogramma* tarsi and observed a previously unreported inflation mechanism that enhances surface grip[72] (**Figure 7.8**). While our small sample did not reveal clear differences between lines, we must consider that wasps found emerging from eggs may also have hitchhiked, even if not caught on butterflies. Interestingly, in a morphometric clustering analysis, females of the only confirmed hitchhiking *T. evanescens* line stood out for its antennal morphology. Its antennae were particularly large compared to other lines[75,83], however, only male features are used for classification, and such differences in females, likely highly relevant to oviposition and cue use adaptations, are entirely ignored in morphological keys and analyses[75]. These morphological distinctions among haplotypes, and between hitchhikers and non-hitchhikers, merit further investigation.

### 7.3.4 Future Directions: Mapping, Studying, and Managing the Invisible Travelers

I have highlighted many unknowns which warrant further study. For methodological improvement, *Trichogramma* phoresy surveys and identification methods require improvement. Accurate identification and larger data sets will reveal phoresy patterns. Enhanced understanding of which *Trichogramma* are phoretic, and when, should be followed by efforts to improve open field biological control, with an emphasis on understanding the functional consequences of intraspecific diversity. In summary, I find these to be the most urgent research goals:

- **Refine eDNA methods** to detect hitchhiking parasitoids on butterflies. Improve sensitivity and specificity to reduce false positives and enable broader-scale field surveys.
- **Investigate male phoresy**, determine whether males are dispersing randomly, seeking mates, or engaging in behaviors linked to habitat quality or host patch colonization.
- **Identify conditions that trigger a shift between OIPV use and phoresy**. Manipulate environmental conditions in controlled experiments and conduct theoretical modeling studies to analyze the risks and benefits of dispersal by phoresy under variable conditions, including different densities of males, females, and hosts.


A)
Trichogramma evanescens
10 wasps
Hosts
Pieris sp.
P. napi
P. rapae
P. mannii
P. brassicae
Noctuidae sp.
Mamestera brassicae
T. semblidis
T. cacoeciae
B)
% parasitizedd eggs
90
75
60
45
30
15
0
Treatment
Control
Treatment
Control
2022
2023
C)
10_RU_20_M12
3_men_21_M12
1_RU_20_M12
Clade I
100
2_sf_21_M13
Clade II
69_eng_20_M14
95
89
17_sf_21_M5
76
16_eng_20_M2
16_eng_21_M3
63
Clade III
9_wur_21_M6
7_eng_21_M4
10_gre_21_M4
Clade IV
13_ben_21_M11
45_wur_20_M5
15_wur_20_M4
55
16_ben_21_M1
1_gre_21_M2
13_exp_21_M1
8_exp_21_M1
64_eng_20_M1
67
2_mannii_21_M1
4_RH_21_M9
61_eng_21_M9
62_wur_21_M9
53
17_wur_20_M9
72_Eng_20_M9
2_gre_21_M9
5_RH_21_M7
12_gre21_M9
8_WUR_20_M9
67
2_eng_21_M8
8_eng_21_M8
60_eng_21_M9
21_mannii_21_M10
1_ver_21_M10
57
20_mannii_21_M10
13_gre21_M10
3_gre_21_M10
COIII
16S
COI
ND4
COII
ND5
12S
ND2
cytB

- **Reassess *Trichogramma* species-level taxonomy** by integrating high-resolution barcoding, refining barcode markers, and always including morphological validation. Address issues related to NUMTs, heteroplasmy, and database errors.
- **Expand SEM-based analyses** to detect potential structural adaptations for phoresy (e.g., tarsal inflation, specialized claws, antennal enlargement). Broader sample sizes may uncover lineage-specific traits.
- **Bridge phenotype to genotype, understand functional diversity** by testing whether genetically distinct haplotypes vary in ecologically relevant traits such as host-finding behavior, cue preference (e.g., OIPVs), and field performance. Link these traits to success in natural settings using open-field biocontrol experiments.
- **Conduct open-field biocontrol trials** that compare the efficacy of different *T. evanescens* haplotypes or hitchhiker lineages under real-world conditions, with success linked back to genetic and behavioral profiles by tracking haplotypes

### 7.3.5 Take home message on "Phoresy"

Phoresy offers egg parasitoids a direct route to freshly laid eggs, but it is not universally used. Our research reveals that hitchhiking is widespread but modulated by ecological context, wasp strain, and host availability. New methods such as eDNA detection and haplotype tracking are uncovering hidden diversity in host-finding strategies, while mounting assays and SEM suggest overlooked morphological and behavioral adaptations. Understanding the evolutionary drivers and consequences of phoresy, especially within species with high intraspecific diversity such as *T. evanescens,* will be key to improving biocontrol and grasping the full spectrum of parasitoid dispersal strategies.



**Figure 7.7)** Intraspecific variation within mitochondria of *Trichogramma evanescens*. **A)** Median joining network of *Trichogramma* species collected from different lepidopteran eggs in Wageningen reveals high diversity within *T. evanescens***.** Size of circle represents number of samples with shared haplotype, color represents host sources, dashes between circles represent number of nucleotide differences between haplotypes**. B)** Field release of *T. evanescens* greatly improves *Pieris* parasitism rates. Using four open fields of Brussel sprout (*Brassica oleracea* var. gemmifera), all fields were experimentally infested with *Pieris* eggs. Three plots had *T. evanescens* (strain 69_eng_20) released approximately 1000 individuals (treatment, red). One plot did not have any wasps released (control, blue). Plots had 20m distance between them, and wasps were released in the center of the treatment plots. Baseline parasitism rate before experiment began was 0%, and treatment significantly increased parasitism: higher in treatment fields (up 60%) while control fields never exceeding 15%. Repeated twice per year in two years (N=4). **C)** 12 unique barcodes from concatenated genes used to identify *T. evanescens* haplotypes[83]. Agarose gel (1%) electrophoresis image of PCR product of different genes sequenced to develop the unique haplotypes, represented by *T. evanescens* line 60_eng_21. Maximum likelihood analyzed phylogenetic tree concatenated unique barcodes (N=37)**.** Each tip label shows name of line_haplotype. Nodes indicate consensus support from 100 bootstraps.

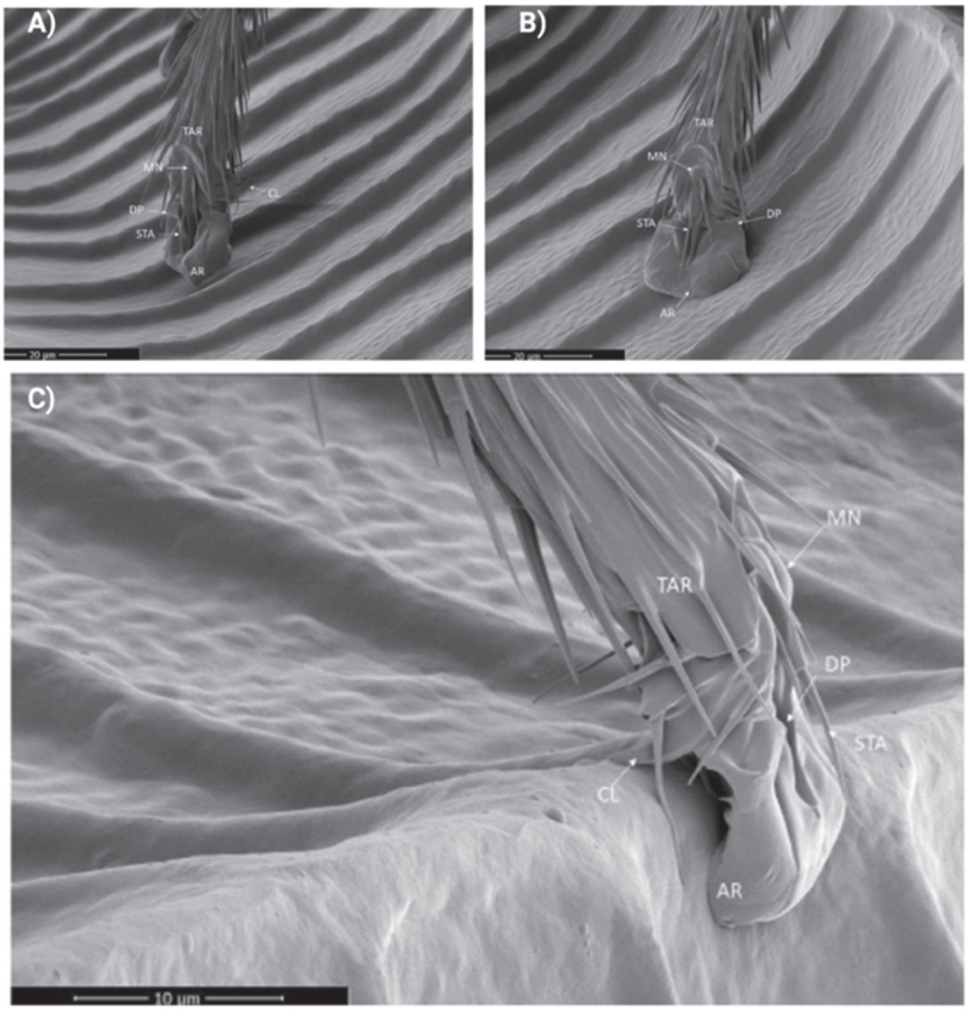



**Figure 7.8)** Cryo-SEM reveals tarsal adaptations of *Trichogramma* may facilitate phoresy by enhancing grip**.** The inside of the pretarsi shows the claws (CL) and the flexible distal (PL-D) and rigid proximal (PL-P) plates of the planta, underlying the arolium (AR). The distal plates of the planta are unfolded when the arolium is everted (Figure 6A). The structures that can be identified when looking at the outside of the leg, are the terminal tarsomere (TAR), arolium (AR), manubrium (MN), dorsal plates (DP) and the setae (STA). **A-B)** *Trichogramma semblidis* imaged on *P. napi* egg. **A)** When the pretarsus of the middle leg of *T. semblidis* is not touching the surface the shape is pointed with less everted arolium (AR) (3500x). **B)** *Trichogramma* increase the arolium size of their pretarsus upon touching a surface, allowing increased surface area for gripping (3500x). **C)** The pretarsal elements of a female *T. cacoecieae*, a phoretic parasitoid of *P. napi*, imaged walking on a *P. napi* egg. The claw (CL) allows gripping of the egg ridge (6500x).

## 7.4. Risky Sex: Why use post-mating odors?

Throughout this thesis, I have moved away from the term anti-aphrodisiacs to more broadly refer to post-mating odors. Post-mating odors is how the odors were first coined when they were discovered in *Heliconius erato*[88]. Why? I have found that they are more than just (anti-) aphrodisiac pheromones.

For many butterflies, sex does not end with copulation. In species such as *Pieris*, males transferred post-mating odors, chemical signals that render them less attractive to other males. These post-mating odors have been thought to function as a kind of chemical chastity belt, reducing harassment and saving the female from repeated mating costs. However, in *Pieris*, we have known for a while that these same compounds come with a catch: they attract egg parasitoids, particularly *Trichogramma*. What began as a solution to one problem introduces another, a classic evolutionary trade-off. We also find evidence that, instead of simply a tool for male control over female mating, these odors likely serve as valuable anti-predator protection for gravid females, over which females themselves likely exert some control.

In **Chapter 6**, I showed that for *Pieris* females, emitting and detecting post-mating odors has at least four consequences:

1. **Deterring males**; reducing male harassment when already mated and not receptive to remate.
2. **Attracting *Trichogramma* wasps**; likely because parasitoids have evolved to exploit these reliable cues of host availability.
3. **Repelling an avian predator**; functioning as aposematic warnings warning of benzyl cyanide.
4. **Informing about host-plants**; indicating identity and quality of host-plant, and whether already infested with eggs and/or induced to directly or indirectly defend against butterfly eggs.



Why use an anti-aphrodisiac at all when it increases the risk of parasitism? The answer lies in the balancing trade-offs, the multifunctionality of chemical signals, and how selective pressures vary depending on the enemies involved. Perhaps the primary benefit of using these odors is that predators are deterred from valuable gravid females who emit these odors. Only this function is likely to serve as a signal, which evolved to be used by signaler and receiver, as opposed to a cue, evolved for one participant, and eavesdropped on by another[89]. Both the butterfly, which will not be eaten, and the bird, which will not eat a poisonous food, benefit and would select for the evolution of this as a signal used by both emitter and receiver (**Figure 7.9**). The benefits of informing mates seem minimal in comparison to informing predators, and studies have shown in multiple

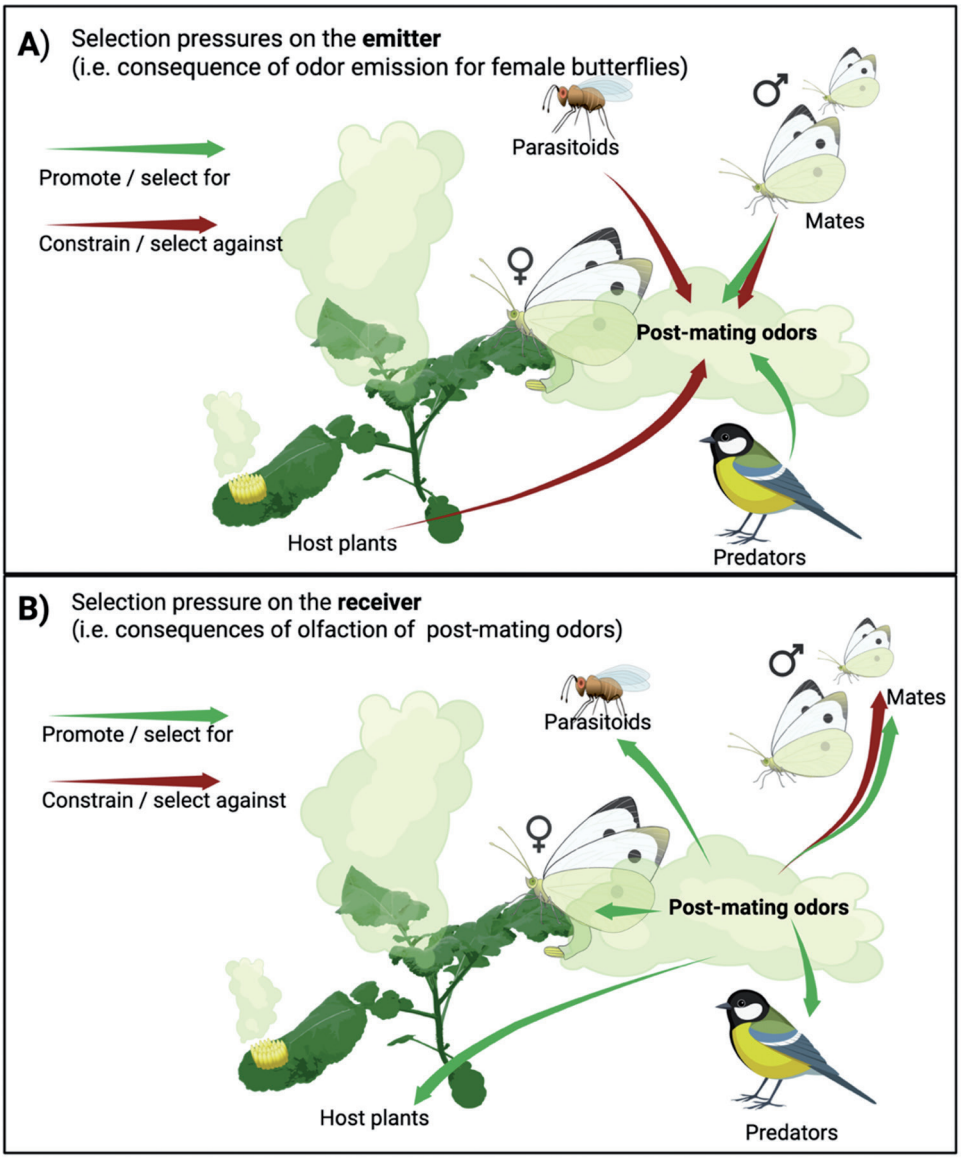



**Figure 7.9)** Conceptual model of pressures on post-mating odors in *Pieris brassicae*, demonstrating predator avoidance likely exerts the strongest evolutionary pressure to act as a signal due to benefit for both emitter and receiver**. A)** Selection pressure on the emitter (mated female butterfly) to emit post-mating odors by the receivers. Host-plant = negative, induces direct and indirect defense; predators = positive, prevents predation of butterfly, parasitoids = negative, attracts parasitoids; mates = positive or negative, depending on receptivity of the female. **B)** Selection pressure on receivers to be perceptive of post-mating odor cues (female butterfly = positive, can reduce emission to prevent danger; host-plant = positive, can detect presence of herbivore; predators = positive, can avoid intoxication from unpalatable prey; parasitoids = positive, can find a phoretic host and host eggs; male mates = positive and negative, depending on honest signaling of receptivity by female).

instances (including **Chapter 6**) that using these odors does not reliably alter mating frequency[90-96]. Whether or not it is worthwhile to inform predators may depend upon the pressure that eavesdropping *Trichogramma* impose, measured by the variable parasitism rates the butterflies' eggs incur.

### 7.4.1 Managing risk with behavior, mimicry and chemical shifts

Post-mating odors in butterflies are unique among pheromones in that males synthesize the compounds, while females emit them. As a result, selection can act on biosynthesis in males and on emission in females. While genetic changes in male biosynthetic pathways may be more obvious, females likely exert control as well (**Chapter 6**).

To date, post-mating odors have only been described in two genera. In *Heliconius*, the effect is so pronounced that even a human nose can detect whether a female has mated, due to the strong "witch hazel" scent of *H. erato*. Comparisons with *Heliconius* offer useful context: in this genus, females use specialized structures known as "stink clubs" to emit post-mating odors[97]. *Pieris* may possess analogous structures (**Figure 7.10**), and variation in these could influence emission, for example, open reservoirs in these structures function to dissipate volatiles, and the number and size of these reservoirs vary between species, which may drive variation in odor emission speed and strength[97,98]. Disturbing butterflies, such as by a predator (e.g. shaking locusts[99]) or handling for odor collection (e.g. **Chapter 6**), may also release these compounds and needs to be investigated. In addition, behavioral mechanisms such as changes in the mate-refusal posture may allow females to modulate emission, as each adoption of the mate-refusal posture has been found to subsequently reduces odor titres[160]. We found that wild-type females appeared to reduce emission when volatile titers were high in glass olfactometer assays, such that mutant butterflies who could not detect titers continued emitting odors and thus became highly attractive to *Trichogramma*. This suggests that they may suppress the mate-refusal posture, or adjust anatomical features involved in emission, in response to context. Future studies should analyze the difference in behavior and odor emission between mutant and wild-type butterflies to test this explanation for the observed change in parasitoid behavior.

A potential microbial component adds further complexity. Microbes are known to alter pheromone chemistry and could be housed within these odor-emitting structures[100,101], and their own microbial volatiles also acting as insect semiochemicals[102]. Many of the volatiles associated with post-mating odors are commonly produced or modified by microbial metabolism[103–107] (**Figure 7.11**). Guaiacol synthesis is not known in animals, and may occur via microbes, and its synthesis is unrelated to the other known post-mating odors[106] (**Figure 7.11**). In other insects, such as male fruit flies, specific rectal bacteria

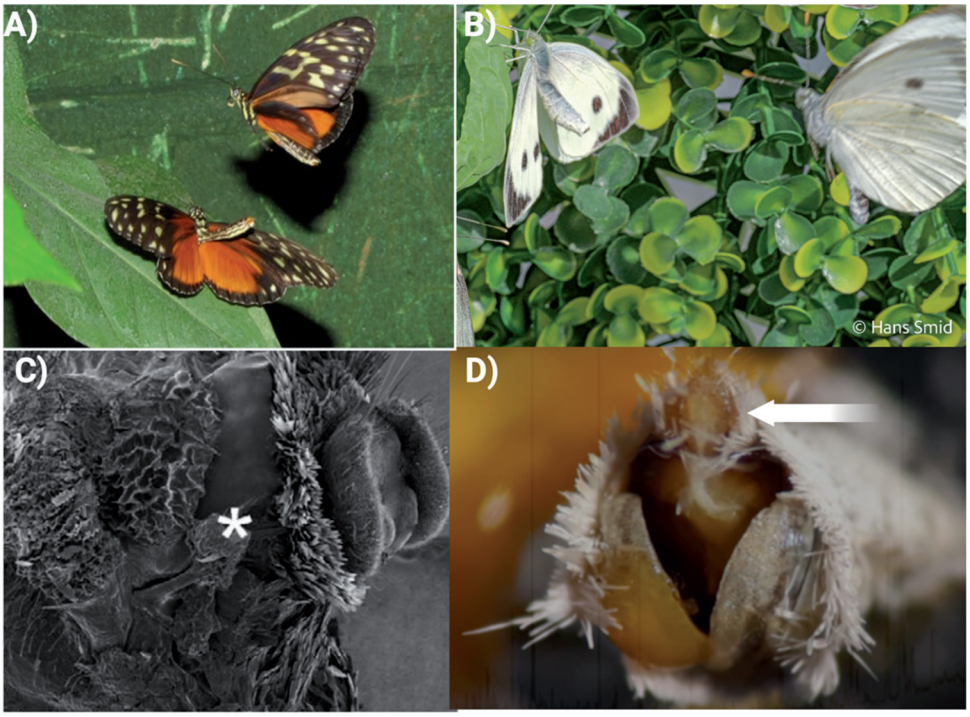



**Figure 7.10)** Post-mating odor emission in *Heliconius* and *Pieris***.** Females emit post-mating odors when approached my male butterflies (or enemies) while adopting the "mate-refusal posture" in both **A)** *Heliconius* (photo by Eric Eaton) and **B)** *Pieris* butterflies (photo by Hans Smid). **C)** In *Heliconius,* post-mating odors are stored and emitted from female-specific structures called stink clubs, located at the tip of the abdomen and inflated during the mate-refusal posture, seen here in SEM and identified by a white asterisk. (from Borges *et al.*)[97] **D)** In *Pieris*, the storage and emission organs of post-mating odors have not been identified, but we suspect the pilose structure indicated with the white arrow is analogous to stink clubs and only exposed during the mate-refusal posture. The purpose of this structure, to the best of our knowledge, has not been described so far (photo by Jonne Bonnet).

are entirely responsible for producing attractive pheromones, suggesting that microbial contributions to chemical signaling can be fundamental, not merely modulatory [108].

While the production of these compounds may involve microbial metabolism, whether such microbial contributions are adaptively regulated or stochastically acquired remains an open question. Nevertheless, such variation may still shape semiochemical profiles across species and populations[108], particularly if females exert some degree of control over microbial colonization, directly or via host-plant choice. While direct evidence for maternal transmission of pheromone-producing microbes in butterflies is limited, other insects do show maternal control over microbiota inheritance[109], especially where the

microbes affect key fitness traits like defense or communication. This raises the possibility that oviposition site choice or egg-associated microbial transfer could influence the semiochemical profiles of offspring in an evolutionarily meaningful way.

If, for example, guaiacol is from a microbial origin[106,110], and its production may amplify the butterflies' own male-synthesized methyl salicylate[111], variation in microbiomes may alter emission profiles in different species and populations, as differences in host-plant choice may drive differences in microbiome[112]. Furthermore, microbes may also be involved in the conversion between methyl salicylate and benzyl cyanide occurring within the females[110] (**Figure 7.11**). While adult butterfly microbiomes are considered less diverse than other life stages and other adult insects[113], the differences in microbiome between male and female abdomens should be investigated with tissue specific explorations. Whether butterflies can influence or selectively retain beneficial microbial partners remains largely unexplored, but emerging studies across insect systems suggest this possibility deserves greater attention in future work.

Many compounds in post-mating odors play roles in both sexual communication and ecological defense (**Chapter 6**). More compounds than expected make up the post-mating odor bouquet of *P. napi*. The role of these compounds in *P. napi* might have been underestimated due to methodological limitations in collection (e.g., headspace vs. abdominal extracts) or instrument sensitivity. Compounds such as guaiacol, which show only faint signals in GC chromatograms, may be easily overlooked without targeted analysis.

Methyl salicylate and benzyl cyanide are chemically related, made from the same precursors and converted between each other within *P. napi* males (**Figure 7.11**). This is also seen by their strong correlation in our analysis in **Chapter 6**, where individuals with high quantities of one also had high quantities of the other, and *vice versa*, while they were negatively correlated with the amount of indole. Individuals with more indole had less of both methyl salicylate and benzyl cyanide. Both methyl salicylate and benzyl cyanide attract *Trichogramma*, while only benzyl cyanide is known to act as a warning signal to predators, methyl salicylate is used to lure insect predators to fields for biological control[114]. Whether these compounds can evolve separately remains unknown but seems likely that their fates are tied. Intriguingly, *P. brassicae* manages to produce only Benzyl cyanide, and its close relative *P. chieranthi* seems to dominantly use methyl salicylate. *Pieris rapae* on the other hand is not known to emit benzyl cyanide. What genetic changes underly these differences would be interesting to disentangle.

One compound stands out for its dual evolutionary potential: indole. Synthesized from tryptophan by *P. rapae* males and found in the shikimate acid pathway in plants, indole

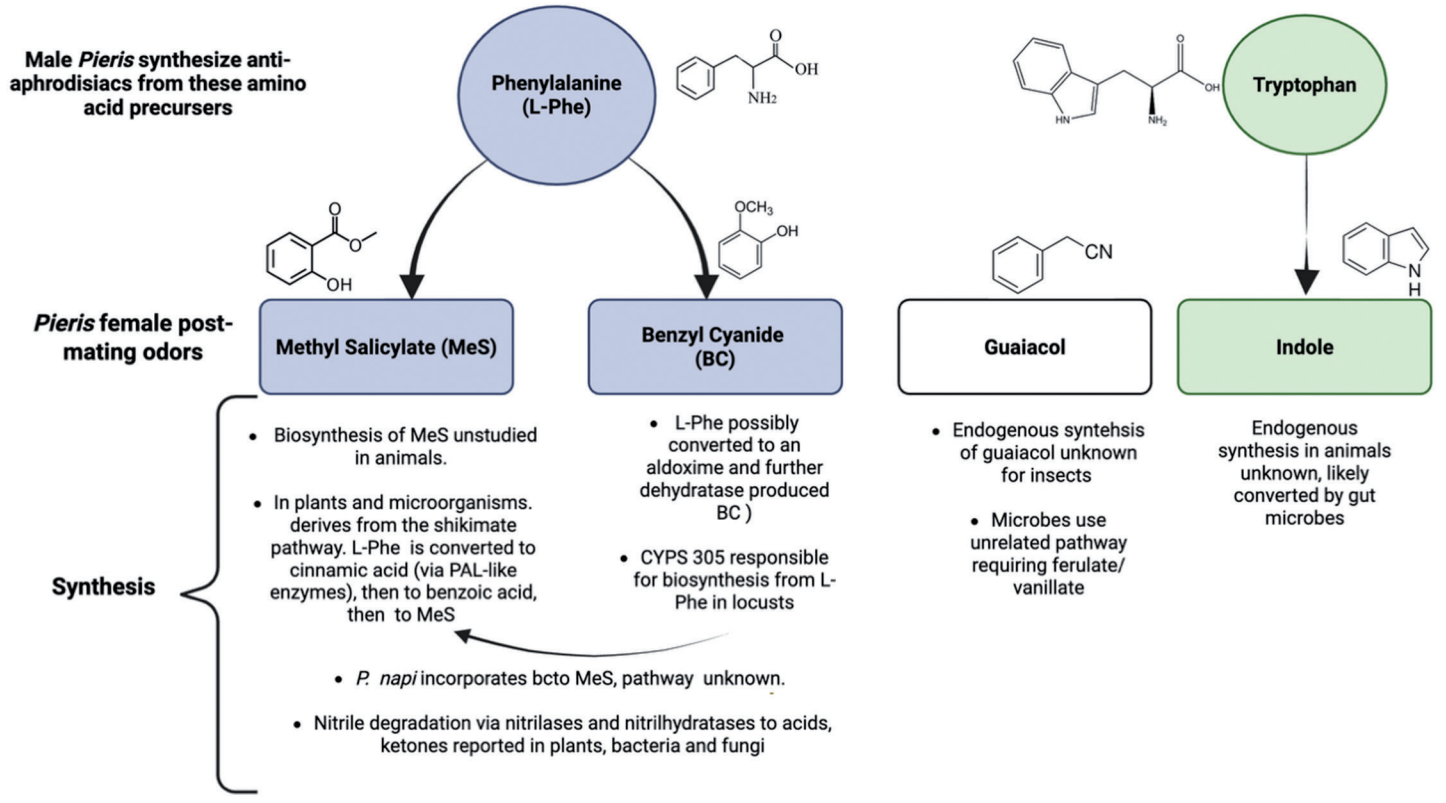



**Figure 7.11)** What is known and remains unknown about biosynthesis of *Pieris* post mating odors. Four post-mating odors, all found in *Pieris napi*, in rectangles: methyl salicylate (MeS), guaiacol, benzyl cyanide (BC), and indole, and their precursors for *de novo* synthesis in males in circles. MeS and BC synthesized by males, known respectively in *P. napi* and *P. brassicae*, from phenylalanine (l-Phe) and *P. rapae* males are known to synthesize indole from tryptophan[110, 116]. In *P. napi* it is known that BC is also incorporated into MeS[110]. Biosynthesis known from other organisms is described in text[99,103,106,110].

is a common component of herbivore-induced plant volatiles (HIPVs) (**Figure 7.11**). Synthesis by animals remains undescribed and may well occur via microbes in the butterfly gut[103]. Unlike other anti-aphrodisiac components that attract parasitoids, indole may not function as a parasitoid kairomone. In fact, it is toxic to parasitoids[115], which may suggest this compound would not be a wise choice for a recruitment cue from the parasitoid's perspective. This toxicity could make indole a relatively "safe" chemical choice for butterflies, offering protection against parasitoid exploitation.

This raises an intriguing evolutionary possibility: indole may serve as a protective chemical switch. By co-opting a plant-derived compound already embedded in ecological signaling systems, *P. rapae* may have evolved a way to maintain anti-aphrodisiac function while avoiding parasitoid detection. In the conceptual figure (**Figure 7.9**), I speculate that bird avoidance likely exerts the strongest selective pressure**.** From this, I propose that the evolution of indole-based signaling may represent a strategic compromise: it allows escape from parasitoids while simultaneously deterring avian predators using more conspicuous warning signals, potentially through mimicry of toxic species such as *P. brassicae*[117]. Species such as *P. rapae* and *P. mannii* I consider convincing mimics, with only subtle features such as the angles of their wing tips as main clues for humans to their identity **(Figure 7.12A)**.

This dual ecological function positions indole as a pivotal evolutionary switch, a compound that retains its role in post-mating signaling while avoiding parasitoids and benefiting from visual aposematism. This dual action creates space for chemical convergence and mimicry, where unrelated species could evolve similar odor profiles to share these adaptive benefits. *Pieris rapae* is a ubiquitous and cosmopolitan species. From a human perspective, it is considered a major pest and has spread across continents more than any of its congeners. This may have been driven by its heavy reliance on indole signaling while maintaining near-perfect mimicry of *P. brassicae*.

We see in *P. napi* populations that some individuals use benzyl cyanide and methyl salicylate together, or use indole, but not both. This dichotomy could reflect a tradeoff between the two, as indeed they may interact chemically (**Figure 7.11**). But also, these individuals using indole may represent variation upon which selection can occur, eventually becoming a favored compound for the species that use it (e.g. *P. napi* and *P. rapae*). Mimicry works when certain individuals still poison the enemy who eats them, and warning colors are still often enough honest[118].

However, such mimicry may be imperfect. For example, the canary island white *P. cheiranthi*, who emits methyl salicylate and not benzyl cyanide, may be a less convincing mimic (**Figure 7.12 B**). Evolving in the Canary Islands, where there are no *P. brassicae* or *P. napi*,

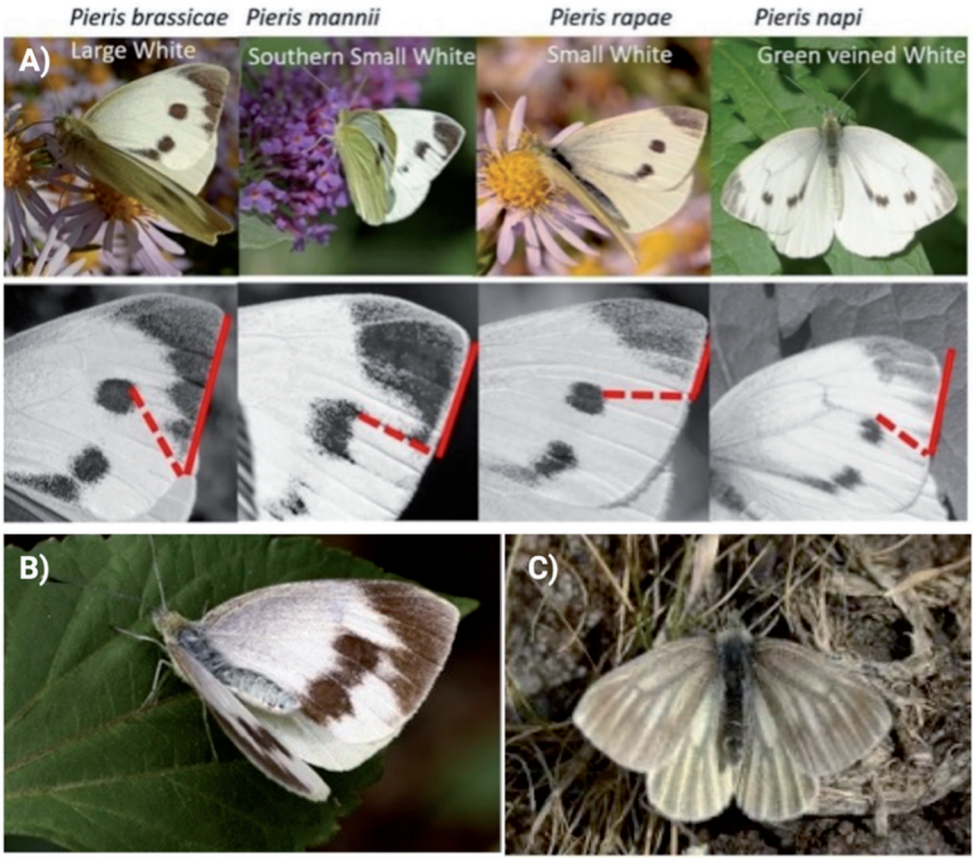



**Figure 7.12)** Mimicry in *Pieris*? Convincing and less convincing mimics**. A)** Difficult to distinguish common species, where the angle of spots on wing tips is the main distinguishing difference. Figure by Chris van Swaay, Butterfly-conservation.org. **B)** *P. cherianthi*, sister of *P brassicae*, whose spots seem to be blurring and does not co-occur with toxic *P. brassicae* on these islands and may not benefit from mimicry, thus relaxed selection (photo from eurobutterflies.org). **C)** *P. napi* adalwinda, subspecies of *P. napi*, similar to all cold climate and high altitude napi who have increased melanization for sun absorption but become less convincing mimics of other *Pieris*.

there are no honest toxic signalers to mimic. Interestingly, it appears as if this butterfly's spots are blurring, losing their refined shapes, and with them, its resemblance to the toxic *P. brassicae*. Mimicry only works when the toxic model species remains present for predators to learn from[118,149], and with no protective benefit for intermediated phenotypes[14]. Thus, mimicry does not seem to benefit this species isolated on these islands. Relaxed constraint on mimicry would be expected in such a setting, and relaxed constraints on wing pattern may represent this weakened selection.

*Pieris napi* also becomes a less convincing mimic when it is found at high altitudes or latitudes (**Figure 7.12C**), such as *P. napi* adalwinda and *P. bryoniae*, where melanic wings likely help the butterfly absorb sun in colder environements[119], and this may overwhelm the benefit of looking like the bright white *P. brassicae*. It can also be expected that avian predators pose less of a threat at high altitudes and latitudes[120,121]. However, the eyes of human observers may be deceiving, and the UV reflectance that these butterflies and their predators see could complicate the story[122,123].

### 7.4.2 Why Not Be Social? Evolution of gregariousness in relation to post-mating odors

Intriguingly, gregariousness and the use of post-mating odors may also be related to post-mating odors. The decision to lay eggs singly or in clusters reflects a complex trade-off shaped by ecological pressures. Gregarious oviposition, observed in butterflies such as *P. brassicae* and many *Heliconius* species, offers benefits such as enhanced larval co-operation, improved thermoregulation, and synchronized hatching to overwhelm plant defenses[124-126]. However, these advantages are counterbalanced by increased vulnerability to parasitoids and pathogens, as clustered eggs present larger visual and chemical targets[127].

Gregariousness is often linked to aposematism, where clustered larvae benefit from collective warning coloration[128,129]. For example, *P. brassicae* larvae exhibit bright coloration and toxicity, unlike solitary *Pieris* species that rely on camouflage. This strategy aligns with observations that gregarious larvae frequently feed on toxic host-plants, leveraging plant-derived defenses for protection[128,129]. However, clustering also increases competition and disease transmission among larvae, creating a delicate balance between defense and demographic costs[130,131].



Egg clustering may mitigate plant defenses, such as desiccation due to egg-killing plant defenses by creating microhabitats that protect internal eggs[132] (**Chapter 4 and 5**). However, clutches also become highly attractive targets for egg parasitoids. When a parasitoid encounters a clutch of 30–50 eggs, it has the potential to produce many offspring, sometimes over a hundred. In contrast, a solitary *Pieris* egg typically supports the development of only 2–3 *Trichogramma* individuals (personal observation). Interestingly, *P. cheiranthi* lays loosely spaced clutches, potentially reducing desiccation benefits[133] (**Figure 7.13**). Just as the spots on *P. chieranthi* seem to blur as this isolated species evolves away from its sister species *P. brassicae*, its egg clutches are losing their neat structures and large size.

A similar pattern of variation in egg clustering is seen outside Pieridae, notably in the nymphalid butterfly *Chlosyne lacinia*. Experimental work has shown that larger, multilayered egg batches have significantly higher hatching success than smaller, monolayered




**Figure 7.13)** Inter-specific variation in egg- laying strategies in *Pieris*. **A)** Solitary egg of *P. rapae*, the small cabbage white (photo from the Field Guide to the Insects of Tasmania). **B)** Loosely laid egg clutch of *P. chieranthi*, the Canary Islands large cabbage white, whose eggs still have spaces between them (photo by Wolfgang Wagner, Pyrgus.de), and **C)** neatly laid and larger egg clutch of P. *brassicae*, the large cabbage white, with little to no gap between eggs (photo by Holger TK).

clutches, particularly under low humidity conditions[134]. The protective effect of layering and density appears to reduce egg desiccation, supporting the idea that clustering itself can be an adaptive response to environmental stress. However, natural populations of *C. lacinia* still exhibit wide variation in clutch structure, suggesting that desiccation resistance trades off against other factors, such as sibling cannibalism within dense clusters[134]. These findings offer a useful comparative context for interpreting the loose and increasingly irregular egg clutches of *P. cheiranthi*. Like tropical Nymphalids, which tend to lay eggs singly or in loose monolayers, *P. cheiranthi* may be shifting away from tightly packed clusters due to ecological or evolutionary pressures that outweigh the benefits of desiccation protection.

Earlier studies on phoresy in *Pieris* proposed that phoretic parasitoids may preferentially target gregarious butterflies, since these offer greater reproductive potential and allow more time for mounting and dismounting as the female lays eggs over several minutes rather than just a few seconds. While phoresy is indeed more common on gregarious species overall, field studies show that *P. brassicae*, a gregarious species, was not particularly burdened by hitchhiking *Trichogramma*. Instead, the more abundant *P. rapae*, a solitary egg layer, was most often found with hitchhikers. Although this may partly reflect sampling bias due to the relative rarity of *P. brassicae*, it also suggests a possible tradeoff between egg clutch size and host abundance[55]. Additionally, laying egg clutches can be time-consuming, exposing the mother to predators[135].

When studying the evolution of gregariousness in butterflies, *Heliconius* (Nymphalidae) offers a compelling comparison to *Pieris*, as gregariousness has evolved repeatedly across this genus and varies within species[129]. For instance, *H. charithonia* exhibits facultative clustering among populations, indicating behavioral plasticity in response to local ecological pressures. This intraspecific variation, along with contrasts in clutch structure between *P. brassicae* and *P. cheiranthi* (**Figure 7.13**), underscores that egg-laying strate-

gies are dynamic adaptations which have the potential to be shaped by predator and parasitoid pressures.

The correlation between gregariousness and parasitism rates may underlie an emerging link between gregariousness and post-mating odor evolution in *Heliconius*. With their many independently evolved gregarious species, *Heliconius* are well-suited to test whether phoretic parasitoid selection constrains post-mating odor complexity. The genus has long intrigued evolutionary biologists[136], inspiring hypotheses about their behavioral divergence, rapid sympatric speciation despite high gene flow, and more recently, the diversification of their pheromones[136,137]. Reproductive strategies vary, with some species mating at pupae with immature monandrous females, others as adults with typically polyandrous females[129]. Clutch size also varies, from solitary eggs to clutches of over 100 eggs[115], with further variation in clutch size and mating frequency among recently diverged populations[129,138]. Supporting this system is an extensive genomic toolkit (24 sequenced species), well-resolved phylogenies, and detailed analyses of pheromone chemistry and many available genomes[136,139–141].

However, while much is known about *Heliconius* butterfly pheromones, their natural enemies remain largely uncharacterized, unlike in *Pieris* butterflies, where parasitoid interactions have been central to ecological research. While predator avoidance has been linked to traits such as wing color and toxicity[142,143], little is known about their parasitoids, and nothing is known about whether they impose selection on chemical communication. Addressing this gap is crucial for understanding the selective forces shaping post-mating signaling. Our preliminary data from the Panamanian lowlands reveal consistent parasitism (11–16% of eggs) in passion-vine butterflies and frequent phoresy (11.3% of butterflies captured), with the rate of both significantly higher in gregarious species[60,62,144].



Interest in *Heliconius* butterfly pheromones continues to grow. Both qualitative and quantitative variation in post-mating odor blends have been documented across and within species[90,145,146]. Morphological differences in abdominal storage structures may also contribute to variation in post-mating odor emission[98]. A breakthrough was the discovery of a terpene synthase gene responsible for the synthesis of a (E)-β -ocimine, prominent post-mating odor in *H. melpomene*, absent in its sister species *H. cydno*[147]. Comparative genomic analysis indicates this difference results from ancestral gene duplications and lineage-specific losses of function[145,147].

The evolution of these chemical signals appears linked to gregarious behavior. Initial studies found that polyandrous, non-pupal-mating species had more complex and rapidly evolving post-mating odor bouquets, which was interpreted as a response to male–male competition[145]. However, this study inadvertently confounded mating system

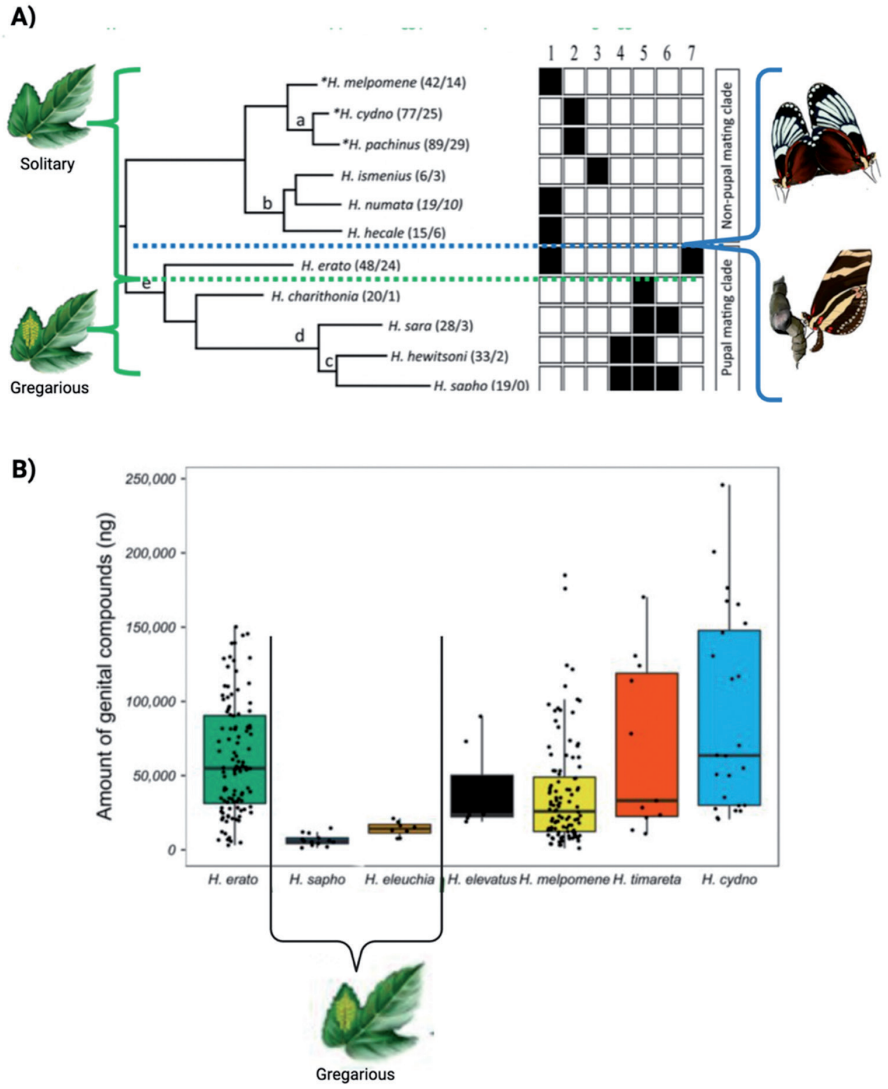



**Figure 7.14)** Gregariousness and post-mating odors in *Heliconius* butterflies. **A)** Figure from Estrada *et al.*[146] showing blend composition of *Heliconius* spp. The black squares represent the presence of one of the seven selected compounds. The numbers in parentheses show the total number of compounds / the number of compounds unique to this species. The blue line indicates the paper's authors' proposed hypothesis, where divergence patterns are grouped by mating system (pupal or non-pupal mating) and the green line indicates my alternate hypothesis grouping divergence by egg clutch sizes, where species laying large clutches are more likely subject to natural selection by phoretic egg parasitoids that constrains their AA divergence. This is supported by the the similarity of *Heliconius erato*, a butterfly that is in the pupal mating clade, to the blend composition of the non-pupal mating clade, possibly because it also lays single eggs and faces little phoretic parasitoid selection pressure. **B)** Gregarious species (with black bracket) produce far less quantity of post mating odors than solitary species. Adapted from Darragh *et al.*[145].

with gregariousness, because non-pupal maters were solitary, whereas pupal maters were gregarious (**Figure 7.14A**). Another study with a slightly different species samples, again revealed that gregarious species produce simpler, less abundant post-mating odor blends [145] (**Figure 7.14B**).

My reevaluation of both data sets with gregariousness in mind (**Figure 7.14**) reveals that species grouping by gregarious *versus* solitary explains variation in odor complexity as well, or better than mating system. For instance, *H. erato*, the only solitary butterfly tested from the pupal-mating clade, has an odor bouquet that resembles that of non-pupal maters who are also solitary. This supports the hypothesis that selection by egg parasitoids, placing far greater selection pressure on the gregarious species, may constrain post-mating odor use. With such strong mimicry rings in *Heliconius*[143,148], it may not be a risk for these gregarious butterflies to avoid using these odors.

More recent work further challenges the idea that mating strategy is the primary driver of odor evolution[90]. Focusing on a larger sample of 36 species of Heliconiini butterflies, the researchers again tested whether post-mating odors diversified more rapidly in polyandrous species due to male-male competition. Contrary to expectations, they found no correlation between mating strategy and the diversification rate of these chemical blends. Instead, most species rely on one of four dominant volatiles, including (E)-β-ocimene. The study also revealed that the presence of certain compounds, such as 4-hydroxycyclopent-2-en-1-one in *H. sara*, depends on larval diet, linking host-plant use to reproductive signaling in contrast to *Pieris* species for which post-mating odors are synthesized in pre-cursors available regardless of which host-plant is selected. Comparisons in host-plant use between Nymphalidae and Pieridae have previously yielded reconciliations of alternative hypotheses for host-plant use diversification[50], and likely will continue to be valuable for comparison on this topic.



In line with my own hypotheses linking post-mating odors to gregariousness, gregarious *Heliconius* species in this large study entirely lack the prominent post-mating odor compound (E)-β-ocimene (**Figure 7.15**). This compounds absence is particularly intriguing as (E)-β-ocimene functions as an antiaphrodisiac in several *Heliconius* species. The genetic underpinnings of (E)-β-ocimene production provide a promising avenue for investigating the co-evolution of chemical signaling and ecological interactions, especially in relation to gregariousness and parasitism risk[147].

Unfortunately, current sampling lacks key gregarious or semi-gregarious species from clades such as *Melpomene* and *Sylvaniform*. Including these would greatly clarify whether the absence of (E)-β-ocimene is a general feature of gregariousness or an artifact of taxonomic bias. Expanding this work will also allow tests of how gregariousness and larval

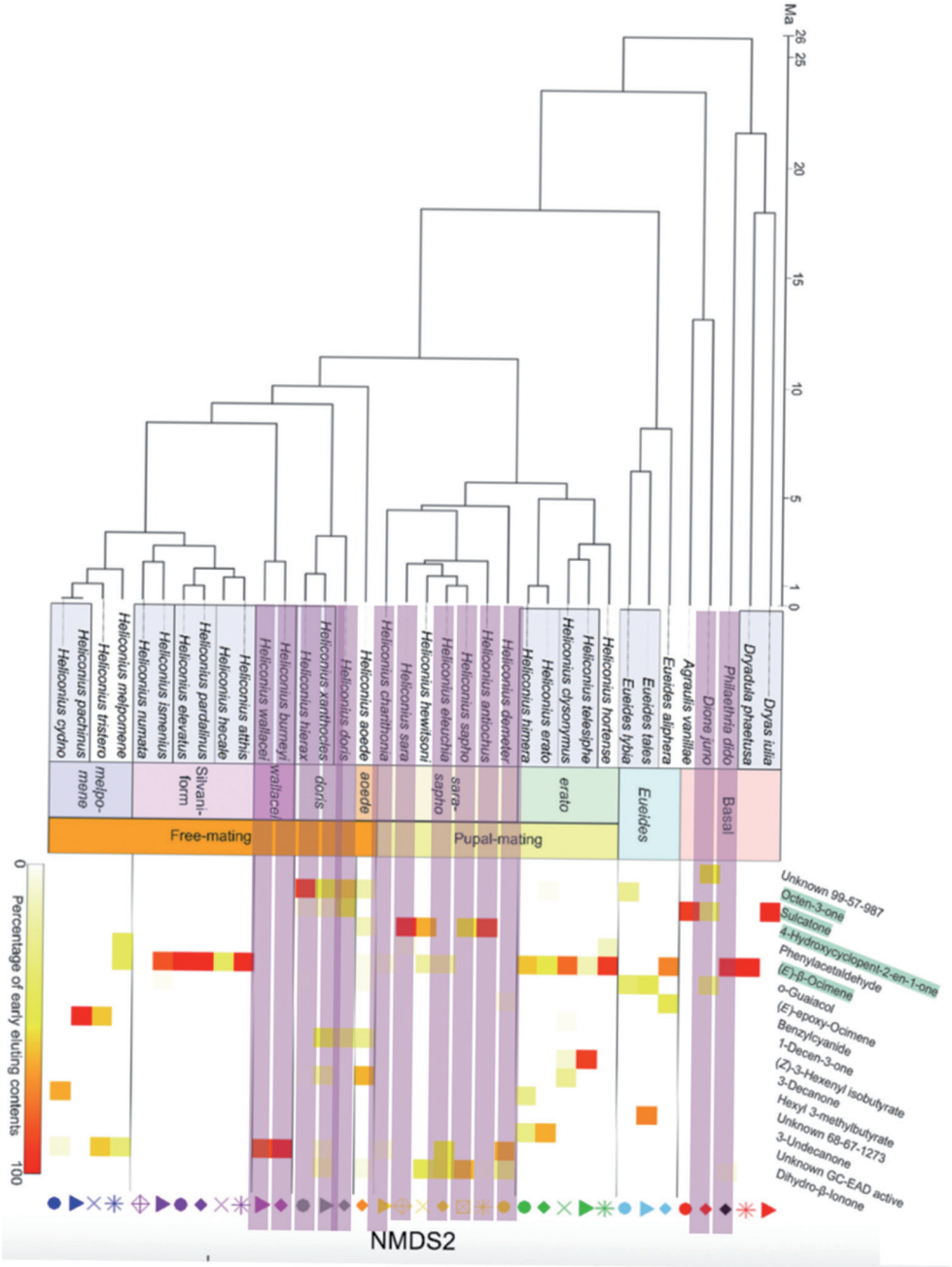



**Figure 7.15)** Figure adapted from Cama *et al.*[90]. A wider sample of *Heliconius* post-mating odors, with intriguing lack of (E)-β-ocimene in nearly all gregarious species in the genus, which are shaded in purple. Wider sampling including gregarious, and facultative gregariousness, and the melpomene and Silvaniform clade would help further elucidate these patterns[90]. The most prominent early eluting compounds across the dataset, are highlighted in green. Only (E)-β-ocimene is confirmed as anti-aphrodisiac across several species, and the genetic basis for emission has been elucidated[147].

defense chemistry interact with post-mating evolution, and including phoretic parasitoid assays and testing their behavior would further elucidate the potential link between these factors.

This direction holds promise. As we know from *Pieris* species that egg parasitoids exploit post-mating odors to locate hosts and conspicuous post-mating odors increase risk, selection may favor chemical inconspicuousness in gregarious butterflies. At the same time, *Heliconius* species are renowned for their chemical defenses derived from toxic host-plants and their aposematism to warn predators of their toxicity. Whether these defenses can counteract the costs of conspicuous post-mating odors, or interact with them, is largely unknown. Comparative work across species differing in toxicity and social behavior and parasitism rates could shed light on how natural selection balances reproductive and defensive signaling. This interplay between sociality, chemical signaling, and enemy pressure is a rich area for future research, particularly at the interface of evolutionary genomics and behavioral ecology.

### 7.4.3 Future Directions: Post-Mating Odors, Gregariousness, and Chemical Ecology in *Pieris* and *Heliconius*

Understanding the evolution of post-mating odors in butterflies requires a comparative approach, especially between *Pieris* and *Heliconius*, which differ in mating systems, social behavior, and ecological pressures such as predation and parasitism. By examining how these factors interact with gregariousness, a trait that influences both parasitoid risk and predator avoidance, we can uncover the ecological drivers shaping chemical signaling in these groups.

- **Biosynthetic Pathways and Genetic Basis**: Identify the genes, tissues, and mechanisms responsible for post-mating odor production in both *Pieris* and *Heliconius* species, using dissection to locate storage tissues and transcriptomic profiling to uncover similarities and differences. Explore the role of microbes in odor synthesis by sequencing microbiome, in vitro assays, and manipulating microbe presence in butterflies.
- **Predator and Parasitoid Assays**: Conduct experiments comparing responses of predators and parasitoids to mated versus virgin butterflies and assess how odor blends and visual signals interact to deter or attract natural enemies. Compare odor blends emitted by butterflies in response to mates or predators and in the presence of parasitoids.
- **Chemical Trade-Offs and Host-plant Effects**: Test whether compounds like indole in *Pieris* or (E)-β-ocimene in *Heliconius* serve dual roles in mate deterrence and enemy avoidance and manipulate host-plant chemistry to determine its influence on odor composition and function.

- **Clutch Size:** Investigate whether gregariousness correlates with odor strength, clutch size, and parasitism rates, and explore how social context or environmental cues influence egg-laying strategies in both genera.
- **Comparative Phylogenetic Studies**: Expand analyses to include solitary, gregarious, and (intra-specific variation in) facultatively gregarious species across different clades and mating systems, aiming to clarify the evolutionary relationships among chemical signaling, sociality, and ecological risk.
- **Theoretical Models of Odor Evolution and Sociality:** Develop models to explore how mating systems, gregariousness, and ecological risk interact to shape the evolution of post-mating odor signaling. By incorporating variables such as clutch size, predation, and parasitism pressure, such models could yield testable predictions about when chemical deterrents evolve, persist, or are lost.

### 7.4.4 Take home message on post-mating odors

Post-mating odors in butterflies are not merely anti-aphrodisiacs, they are multifunctional signals shaped by competing evolutionary pressures. While these male-transferred compounds deter further mating and reduce harassment, they can also attract egg parasitoids like *Trichogramma*, turning a protective cue into a potential liability. Some compounds, such as benzyl cyanide, may additionally function in predator deterrence, suggesting that selection by predators, not just mates or parasitoids, can shape chemical signaling and switches to using new compounds.

These odors reflect complex trade-offs: males control synthesis, females control release, and social behavior like gregariousness further modulates selection. Gregarious species may suppress volatile emission to avoid detection, whereas solitary ones can afford stronger signals.

*Heliconius* butterflies offer a powerful contrast to *Pieris*: while we know more about *Heliconius* genomics, chemical pathways, and aposematism, its parasitoids remain understudied, where *Pieris* excels. Together, they form a classic and complementary pair across Nymphalidae and Pieridae to test longstanding theories in evolutionary ecology, including the co-evolution of chemical signaling, sociality, and enemy pressure.

## 7.5 Beyond the Egg: Why Lay One at All?

Throughout this thesis, I have explored the remarkable complexity of oviposition strategies in butterflies and their parasitoids, highlighting the diverse behaviors evolved to maximize the survival of a single egg. These strategies are shaped by host cues, climate,

predators, and chemical warfare, yet the egg remains a profoundly vulnerable life stage, exposed to environmental variability it cannot escape. This raises a fundamental question: if live birth (viviparity) has evolved repeatedly in animals, including insects, why does oviparity remain so widespread?

Viviparity has evolved independently across major animal groups, mammals, reptiles, amphibians, and several insect lineages such as aphids, tsetse flies, and cockroaches, suggesting strong selective forces can favor internal development under certain conditions[150-153]. However, viviparity remains rare in insects, suggesting that significant constraints limit its widespread adoption.

Recent genomic and transcriptomic studies reveal that transitions to viviparity involve convergent changes in reproductive anatomy and gene regulation. These include remodeling of the female reproductive tract, immune tolerance of embryos, and increased maternal provisioning[150]. In tsetse flies, this shift has exerted strong selection on male reproductive gene[152]. Aphids, by contrast, demonstrate reproductive plasticity: alternating between sexual (egg laying) and asexual (parthenogenic) modes, their viviparity is tied to a highly adaptable genetic program responsive to environmental cues[151].

Kalinka *et al.*[153] proposed that viviparity may arise not from selection to protect eggs directly, but as a byproduct of selection on reduced fecundity or postcopulatory processes, such as internal sperm competition. In this view, maternal adaptations, such as internal fertilization sites, can enable internal development as a side effect, later co-opted for offspring protection. Work in *Drosophila* has revealed that female reproductive-tract secretions, specifically those produced by large secretory cells lining the spermathecae, play a crucial role in post-mating physiology[154]. Ablation of these cells not only impairs sperm storage but also leads to ovoviviparity, the retention and internal development of fertilized eggs, demonstrating that subtle changes in female reproductive tract function can induce a shift toward live birth. This finding provides a mechanistic link between reproductive tract physiology and the evolution of viviparity and suggests that the transition to live birth may sometimes arise from changes in female, rather than male, reproductive adaptations.



While live birth has evolved independently numerous times, similarities in genes needed to accomplish this exist across distant taxa. Studies on how the placenta evolved in the livebearing fish *Heterandria formosa* compared its genome to that of closely related non-placental species. The authors identified 17 genes under positive selection and five gene duplications unique to *H. formosa*[155]. Notably, many of these genes are involved in placental functions in mammals, such as endometrial tissue remodeling and cell prolif-

eration. The findings suggest that adaptive changes in these genes may have been key in driving the evolution of the placenta in this fish lineage.

From an evolutionary perspective, viviparity may not always be "adaptive" for the offspring, but rather favored when maternal fitness is optimized by retaining control. As Gavrilov-Zimin argues, live birth often arises when oviposition becomes incompatible with a lineage's anatomy or lifestyle[156]. This reflects a key theme across this thesis: natural selection shapes maternal strategies to maximize total fitness, not necessarily offspring survival at every stage.

Despite the clear risks faced by insect eggs, the evolution of viviparity is limited by substantial physiological, ecological, and evolutionary constraints. The transition to live birth requires extensive anatomical and physiological remodeling, can reduce fecundity, and may impose costs on maternal mobility and survival[150,152,153]. Indeed, live-bearing imposes significant costs: reduced fecundity, limited mobility, and physiological burdens on the mother[157]. In many insects, especially mobile taxa like butterflies or parasitoids, laying eggs likely offers a fitness-maximizing strategy by distributing risk, minimizing maternal exposure, and preserving maternal survival. In this context, oviparity may better serve maternal interests, and her ability to continue laying eggs, even when it increases individual egg vulnerability.

Yet, from the offspring's perspective, internal development offers obvious benefits: stable conditions, predator protection, and enhanced provisioning. This creates a classic mother–offspring conflict: mothers may favor risk-spreading via oviposition, while selection on the offspring would favor retention. That this conflict is often resolved in favor of the mother reflects that maternal fitness is ultimately the target of natural selection.

Viviparity may be more likely in insects with low mobility and strong site fidelity, such as aphids, where maternal and offspring interests most closely align. Aphid viviparity allows clonal expansion under stable conditions, where high reproductive output offsets viviparity's constraints[151]. In contrast, butterflies traverse fragmented landscapes and must make complex oviposition decisions under uncertainty. The flexibility of oviparity allows them to fine-tune egg placement based on microclimates, host quality, and predator cues, an ability tightly linked to their reproductive success. For such lineages, perhaps **"**mother knows best**"** not because oviparity protects each egg, but because it enables mothers to balance the entire fitness equation across environments. Even so, this balance can shift. In some systems, viviparity may become adaptive if selection pressures align, e.g., if females become sedentary, or environmental predictability increases. Future studies could explore what ecological or genetic changes allow certain lineages to cross this threshold, while others remain egg-layers.

Ultimately, mothers must decide how much to invest in their own bodies vs. their offspring. Theoretical work on maternal effects further deepens our understanding of the evolutionary dynamics shaping reproductive strategies. Internal provisioning allows mothers to exert fine-scale control over offspring development by adjusting investment based on their own physiological state and environmental context[158]. Such maternal effects can influence key offspring traits, including lifespan, by mediating trade-offs between maternal somatic maintenance and reproductive output, particularly under resource limitation[158]. However, the evolution of maternal control is not unconstrained. Parent–offspring conflict places limits on the extent to which maternal effects align with offspring interests[159]. When traits such as dispersal or developmental timing carry costs, offspring may evolve strategies that diverge from maternal optima[159]. These models highlight that the evolution of live birth, or any form of maternal control, is not simply a matter of its potential benefits for offspring survival, but also of the ecological, physiological, and evolutionary conditions that modulate the balance of interests between mother and offspring. As this thesis section has, the persistence of oviparity in many insect lineages may ultimately reflect a resolution of this conflict in favor of maternal fitness: where the flexibility and risk distribution of egg-laying optimize maternal success, even at the cost of increased offspring vulnerability.

### 7.5.1 Future Directions: Exploring the Evolutionary Limits of Oviposition

Several key questions remain unresolved. The intermediate steps between egg retention and full viviparity are not well understood, and the genetic mechanisms underlying these transitions are only beginning to be uncovered[189,193]. The ecological drivers, such as predation, parasitism, host association, and maternal behavior, that shape the evolution and maintenance of viviparity across insect lineages remain a rich area for investigation. Future research should:

- Integrate comparative genomics, experimental manipulations, ecological and behavioral studies, and phylogenetic analyses to clarify the evolutionary pathways and constraints shaping reproductive strategies in insects**.** Focus on species with facultative egg retention or ovoviviparity, and **mapping reproductive modes onto phylogenies**, will be especially valuable for understanding the selective pressures and genetic changes involved in the emergence of live birth.
- **Experimentally manipulate egg retention** in model insects (e.g., through ablation of reproductive structures[154]) to observe physiological and developmental consequences.
- **Compare reproductive tract architecture and gene expression** between closely related oviparous and viviparous species

- **Explore microbiome involvement** in maternal provisioning or immunity during internal development (as hinted in aphids and tsetse flies).
- **Incorporate life-history trade-offs**—especially fecundity, maternal mobility, and dispersal—into **theoretical models** of viviparity evolution. Building on previous models[158,159]**,** model the evolution of maternal effects and control under varying degrees of parent–offspring conflict**.** Such models could explore when maternal provisioning strategies evolve to favor maternal vs. offspring optima, and how constraints (e.g., cost of trait expression, information asymmetries) shape transitions toward viviparity or complex oviposition behavior, especially in the context of enemy pressures.

### 7.5.2 Take home message on oviparity

Viviparity has evolved repeatedly across the animal kingdom, including in some insects, yet it remains rare among them. Most insects retain oviparity, laying eggs that develop externally, which offers key advantages: high fecundity, low maternal investment after laying, and the flexibility to spread risk across environments. Although live birth can enhance offspring survival in hostile conditions, it requires extensive physiological remodeling and imposes costs on maternal mobility and survival.

Insects often favor strategies that maximize maternal fitness across variable conditions, even if individual offspring face higher risks. The rarity of viviparity in insects reflects not just developmental constraints but also a reproductive strategy that allow mothers to optimize total fitness rather than protect each egg. In this light, oviparity is not simply an ancestral state, but a highly successful, evolutionarily stable solution, that suits butterflies well.

## 7.6 Conclusions: Strategic motherhood in a multi-enemy world

Laying an egg may seem simple, but as this thesis has shown, it is anything but. It is a complex act shaped by evolution, ecology, and strategy that balances mobility with protection, fecundity with investment, and certainty with risk. The fact that it persists, despite all the vulnerabilities it introduces, is itself a testament to the necessity of evolutionary compromise. Across both *Pieris* butterflies and *Trichogramma* parasitoids, maternal behavior emerges as a central force in shaping reproductive success. Oviposition is not just about placing an embryo on food; it is about navigating a maze of enemies and environments, where the wrong decision can be fatal, and the right one can reverberate across generations.

In **Chapter 2 and 3**, we saw how parasitoids use a nuanced interplay of short- and long-range chemical cues, including oviposition-induced plant volatiles (OIPVs), to locate hidden hosts, an elegant sensory strategy shaped by enemy ecology.

In **Chapter 4**, we confronted the seeming paradox of maladaptive oviposition, such as laying on egg-killing plants, and revealed it as an evolutionary trade-off, where mothers may prioritize egg survival, microclimate, or future developmental benefits over short-term larval growth. This tension between appearance and function echoes throughout the thesis: what seems "wrong" may in fact be strategic, risk-aware motherhood.

**Chapter 5** revealed that even the morphology of the egg, the number and size of gas-exchange pores, is under maternal control and climate-dependent, reflecting an often-overlooked axis of adaptation. Mothers do not just choose where to lay; they lay eggs that are structurally suited to the world their offspring will face.

In **Chapter 6** *Pieris* butterflies use post-mating odors to repel males and predators while unintentionally luring parasitoids, showing how a single trait can carry overlapping, often conflicting functions that the butterflies must manage. By using mimicry, or switching to new compounds, they can maximize benefits while mitigating consequences.

In **Chapter 7**, this synthesis has connected these threads into a broader view: that **"**mother knows best" is not a rule, but a consequence of complexity. Maternal behavior reflects selection pressures operating not just at the larval stage, but across development, generations, and ecological interactions.

This thesis also raises new questions that stretch beyond traditional oviposition ecology. Could host-switching in butterflies be driven by genetic coupling between maternal preference and larval compatibility? Why do some parasitoids hitchhike while others rely on plant volatiles? Do post-mating odors link mating strategies and egg laying-strategies via predator and parasitoid pressures? And finally, why hasn't live birth evolved in more insects?



Ultimately, these findings do more than explore a set of behaviors. They argue for an expanded understanding of strategic motherhood, a framework that sees mothers not as passive reproducers, but as ecological decision-makers, shaped by and shaping their environments. In a world of shifting climates, evolving enemies, and ecological uncertainty, the future of insect reproductive success depends on these maternal decisions. And if we listen closely to what their choices are telling us, we may learn not only about insects, but about the deep evolutionary wisdom embedded in life itself.

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# Summary

Insects reproduce in landscapes where they encounter plant toxins, climatic extremes, predators, and parasitoids. My thesis asks how butterflies and their egg-parasitoid enemies navigate this maze and what those decisions reveal about their intertwined evolution of mating, oviposition, and mortality. Focusing on *Pieris* butterflies and *Trichogramma* wasps, I combine broad literature syntheses with targeted experiments to trace how selection operates across life stages and trophic levels, and how today's variation between and within species can reveal selection pressures from the past. In doing so, I treat each reproductive decision as the product of an ecological balancing act stretching from egg to adult and across generations.

I begin by developing a conceptual framework in which mating, oviposition, and enemy avoidance are inextricably entangled, between life stages and between herbivores (*Pieris*) and their enemies (*Trichogramma*). This opening review (Chapter 1) argues that the behaviour of mothers cannot be understood without simultaneously considering the sexual and natural selection imposed by their mates and their enemies.

Building on that foundation, Chapter 2 surveys how egg parasitoids locate hosts that are hidden, sparse, and short-lived. This review explores how egg parasitoids use host- and plant-derived chemical cues to locate hidden host eggs. I show that cue use is highly variable across taxa and even among individuals, variation that reflects tuning to specific ecological contexts.

Chapter 3 narrows in on oviposition-induced plant volatiles (OIPVs). This review focuses on oviposition-induced plant volatiles and shows that parasitoids use these reliably for long-distance host egg quality assessment. These reviews again emphasizes that cue-based strategies are **c**ontext-dependent and labile.

The thesis then pivots to the butterflies themselves. Chapter 4 tests whether pierids can sidestep a potent plant defence—hypersensitive response-like (HR) necrosis that kills their eggs. I found that all surveyed *Pieris* species lay eggs on HR-expressing leaves, and that HR-like cell death was frequently triggered, with no evidence of behavioral counter-adaptation in our sampled populations. In contrast, non-*Pieris* pierids preferentially oviposit on host tissues or species that do not express HR. These findings suggest that HR-like egg-killing remains an effective plant defense against *Pieris* butterflies, and that, at least in the populations studied, no clear behavioral counter-adaptations have evolved. This points to potential asymmetry in the evolutionary arms race and underscores the value of examining microevolutionary variation within species to detect emerging or context-dependent strategies of resistance.

In Chapter 5, I scale down to the microscopic architecture of eggs. This chapter investigates egg pore morphology in *Pieris* species and populations along a climatic gradient. I found that egg pores, aeropyles and micropyles that are key to gas exchange and fertilization, differed more between *P. napi* populations than between *Pieris* species. These traits correlated with the climates of origin rather than with egg size or phylogeny, suggesting local adaptation to abiotic stressors. This chapter highlights that even microscopic traits exhibit adaptive variation within species and may evolve rapidly in response to environmental pressures.

Chapter 6 revisits butterfly mating and incorporates the threat of egg parasitoids and predators as drivers of mating behaviors. This chapter focuses on *Pieris* post-mating odors and reframes them not only as anti-aphrodisiacs but as multifunctional ecological signals. These odors repel males, but also attract parasitoids, deter predators, and affect oviposition decisions. I found substantial variation within and among species in odor profiles, variation that tracks parasitoid pressure across populations rather than mating frequency or latitude. Disruption of odor detection altered oviposition behavior and susceptibility to parasitoids. These findings show how complex selection across interactions and environments can maintain chemical diversity within species.

Finally, Chapter 7 synthesizes these strands, arguing that what looks like maladaptive maternal behavior under a narrow lens often proves wise when viewed through the full, tangled web of selection. Maternal behavior in insects, and the (mis)alignment of preference and performance, reflects a dynamic compromise among competing pressures**.** These strategies are not static nor uniform. Variation within and between species, in behavior, morphology, and chemical signaling, offers a window into evolution in action, revealing the selective pressures insects face and the constraints they navigate. Looking ahead, this synthesis identifies several key directions for future research.

By combining reviews with novel data, and by highlighting intra- and interspecific variation, this thesis contributes to a more nuanced understanding of insect reproductive evolution. It shows that oviposition traits are rich with evolutionary complexity, making them ideal traits for studying adaptation, constraint, and trade-offs in a changing world. Ultimately, the findings reinforce a central message: mother does know best, and what she knows is shaped by the evolutionary legacy of diverse enemies, changing environments, and complex trade-offs.

# Appendices

## S4. Supplement for Chapter 4

## Oviposition strategies in Pieridae and the role of an egg-killing plant trait

### S4.1 Attempts at morphological egg identification

A morphological identification method was tested to see if *Pieris napi* and *P. rapae* eggs are distinguishable. Eggs from both species look very much alike as they are similar in size and colour. This newly used method was based on the number of longitudinal ribs that run along the sides of the eggs from the top to the base. Frohawk (1924) describes that *P. napi* has fourteen or fifteen ribs, of which nine or ten run from top to bottom, and the other five start a little under the top and run to base. For *P. rapae* there are twelve ribs, of which ten run from top to bottom, and two start a little under the top and branch from the other ribs (Frohawk, 1924). Based on this information it was expected that eggs with twelve ribs or less would be *P. rapae* and eggs with thirteen to fifteen ribs would be *P. napi*. To be able to count the ribs of the eggs, pictures were taken with a phone and magnifier of the top of the egg so all ribs would be pointing out. The ribs were then carefully counted. However, it was found that this method could not be used reliably. There were numerous cases where an egg was laid by a known species, and when the number of ribs on the egg was subsequently checked, it did not match expectations. Therefore, we found that the number of ribs on *P. napi* and *P. rapae* eggs considerably overlapped using our counting method such that this method could not be used to distinguish species. The number of ribs on *P. mannii* eggs was not checked. Therefore, all solitary *Pieris* eggs were grouped for analysis.

**Table S4.1:** Locations of host plants and eggs per butterfly species.

| ***Gonepteryx rhamni*** | | | | |
|---|---|---|---|---|
| **Location** | **Coordinates** | **Host plant** | **#trees** | **Number of eggs** |
| De Oude Tol, Wageningen | 51°59′12.7″N 5°40′47.6″E | *R. frangula* | 8 | 35 |
| Hernense bos, Hernen | 51°49′43.7″N 5°41′39.7″E | *R. frangula* | 3 | 6 |
| Kavelpad, Wijchen | 51°49′08.7″N 5°43′25.4″E | *R. frangula* | 1 | 5 |
| Randweg, Wijchen | 51°49′08.0″N 5°43′33.0″E | *R. frangula* | 7 | 32 |
| Wittenoordseweg, Renswoude | 52°04′47.6″N 5°31′32.9″E | *R. frangula* | 2 | 5 |
| **Total** | | | **21** | **83** |
| ***Anthocharis cardamines*** | | | | |
| **Location** | **Coordinates** | **Host plant** | **#plants** | **Number of eggs** |
| Van Cittersweg, Balgoij | 51°47′34.8″N 5°42′15.1″E | *C. pratensis* | 112 | 148 |
| Akkermaalsbos, Wageningen | 51°58′54.3″N 5°39′23.8″E | *C. pratensis* | 28 | 32 |
| Veerdam, Wageningen | 51°57′45.9″N 5°41′16.2″E | *A. petiolata* | 6 | 6 |
| De Blauwe Bergen, Wageningen | 51°58′55.6″N 5°39′46.3″E | *A. petiolata* | 1 | 1 |
| Dijkgraaf, Wageningen | 51°58′48.6″N 5°39′22.6″E | *A. petiolata* | 14 | 16 |
| Bornsesteeg, Wageningen | 51°59′07.4″N 5°39′42.1″E | *A. petiolata* | 1 | 1 |
| Bakenbergseweg, Arnhem | 52°00′38.4″N 5°52′19.6″E | *A. petiolata* | 40 | 56 |
| Zwanensingel, Wijchen | 51°49′02.6″N 5°43′52.1″E | *A. petiolata* | 3 | 3 |
| Geertjesweg, Wageningen | 51°58′30.6″N 5°41′16.7″E | *A. petiolata* | 3 | 5 |
| Dolderstraat, Wageningen | 51°58′36.7″N 5°41′20.2″E | *A. petiolata* | 1 | 1 |
| Zoomweg, Wageningen | 51°58′49.7″N 5°41′27.4″E | *S. officinale* | 2 | 2 |
| Cottessen, Vijlen | 50°45′50.8″N 5°56′38.7″E | *C. pratensis* | 1 | 1 |
| **Total** | | | **212** | **272** |

**Table S4.1:** *Continued*

| ***Solitary Pieris eggs*** | | | | |
|---|---|---|---|---|
| **Location** | **Coordinates** | **Host plant** | **#plants** | **Number of eggs** |
| Honingblokpad, Wageningen | 51°58′47.2″N 5°41′07.2″E | *S. officinale* | 2 | 2 |
| WUR Student Farm, Wageningen | 51°58′53.9″N 5°39′26.1″E | *B. oleracea* | 7 | 11 |
| WUR Student Farm, Wageningen | 51°58′53.9″N 5°39′26.1″E | *D. tenuifolia* | 4 | 4 |
| WUR Student Farm, Wageningen | 51°58′53.9″N 5°39′26.1″E | *R. raphanistrum* | 1 | 12 |
| Unilever, Wageningen | 51°59′03.6″N 5°40′00.2″E | *B. nigra* | 11 | 17 |
| Veenhof, Wijchen | 51°49′11.0″N 5°44′15.7″E | *S. arvensis* | 1 | 1 |
| Radix, Wageningen | 51°59′11.2″N 5°39′50.5″E | *B. nigra* | 19 | 31 |
| Radix, Wageningen | 51°59′11.2″N 5°39′50.5″E | *S. officinale* | 1 | 1 |
| Unifarm, Wageningen | 51°59′15.6″N 5°39′47.2″E | *B. nigra* | 1 | 1 |
| Orion, Wageningen | 51°59′04.7″N 5°39′56.9″E | *B. nigra* | 1 | 1 |
| Aalsburg, Wijchen | 51°48′31.3″N 5°42′43.8″E | *B. oleracea* | 1 | 22 |
| Aalsburg, Wijchen | 51°48′31.3″N 5°42′43.8″E | *B. rapa* | 1 | 14 |
| Uiterwaarden, Wageningen | 51°57′51.3″N 5°40′59.1″E | *B. nigra* | 1 | 5 |
| Campus Plaza, Wageningen | 51°58′57.4″N 5°39′50.9″E | *B. oleracea* | 1 | 9 |
| De Blauwe Bergen, Wageningen | 51°58′55.6″N 5°39′46.3″E | *A. petiolata* | 1 | 1 |
| Saltshof, Wijchen | 51°49′26.4″N 5°43′47.2″E | *Iberis* sp. | 1 | 4 |
| Veenhof, Wijchen | 51°49′10.9″N 5°44′14.3″E | *Iberis* sp. | 1 | 2 |
| Veerweg, Wageningen | 51°57′52.9″N 5°40′43.0″E | *Iberis* sp. | n.a. | 113 |
| Ritzema bosweg, Wageningen | 51°58′12.4″N 5°40′32.5″E | *Iberis* sp. | n.a. | 20 |
| Diedenweg, Wageningen | 51°58′09.3″N 5°41′02.1″E | *Iberis* sp. | n.a. | 9 |
| Mennonietenweg, Wageningen | 51°57′59.8″N 5°39′18.9″E | *Iberis* sp. | n.a. | 14 |
| Rijnveste, Wageningen | 51°58′41.1″N 5°38′56.8″E | *Iberis* sp. | n.a. | 19 |
| Leeuweriksweide, Wageningen | 51°58′31.3″N 5°39′17.7″E | *Iberis* sp. | 3 | 3 |
| Veerweg, Wageningen | | *D. tenuifolia* | n.a. | 5 |
| **Total** | | | **55** | **321** |

| ***Pieris brassicae*** | | | | |
|---|---|---|---|---|
| **Location** | **Coordinates** | **Host plant species** | **# of plants** | **Number of eggs** |
| Radix, Wageningen | 51°59′11.2″N 5°39′50.5″E | *B. nigra* | 1 | 149 |
| Unifarm, Wageningen | 51°59′15.6″N 5°39′47.2″E | *B. nigra* | 1 | 66 |
| **Total** | | | **2** | **215** |

## References S4

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# S5. Supplement Chapter 5

# Butterfly egg plasticity in physiologically and reproductively relevant traits: Adaptation to climate?

## S5.1 Egg width as proxy for volume

To validate the use of egg width as a proxy for egg size, we measured the width and length of 72 eggs from four *P. napi* populations (Abisko, Wageningen, Jura, and Costa Brava). Butterflies were reared as described in 'Material and Methods' section 'insects and plants', and eggs were collected in February 2025. Eggs were photographed using a Leica DM i1 microscope at five times magnification.

The average egg width per population was very similar to the average egg width measured from the cyro-Scanning Electron Microscope images, indicating that egg size remains relatively constant between generations (Table S5.1).

Egg volume was estimated by using the formula for an ellipsoid; *Volume = (π/6)*egg_length*egg_width^2*[28]. We found egg width to be a good proxy for estimated egg volume (*lm()* not correcting for population; Adjusted R-squared = 0.91; see Figure S5.1).

**Table S5.1.** Comparing photograph-measured egg width to cyro-Scanning Electron Microscopy (SEM) measured egg width per population ($\bar{x}$ ± SD (n)). Averages per population were similar, indicating there is only limited plasticity in egg size between *P. napi* generations. All photographs were taken in 2025. The SEM-measured eggs were either taken in 2023 (Wageningen, Jura and Costa Brava populations) or in 2024 (Abisko population).

| Population | Egg length (µm) – photograph-measured | Egg width (µm) – photograph-measured | Egg width (µm) – SEM-measured |
|---|---|---|---|
| Abisko | 1220 ± 49 µm (24) | 558 ± 39.8 µm (14) | 531 ± 15 (25) |
| Wageningen | 1150 ± 67 µm (17) | 477 ± 31.1 µm (14) | 463 ± 24 µm (25) |
| Jura | 1066 ± 57 µm (18) | 495 ± 25.0 µm (11) | 489 ± 29 µm (21) |
| Costa Brava | 1185 ± 48 µm (13) | 503 ± 16.4 µm (11) | 488 ± 25 µm (19) |

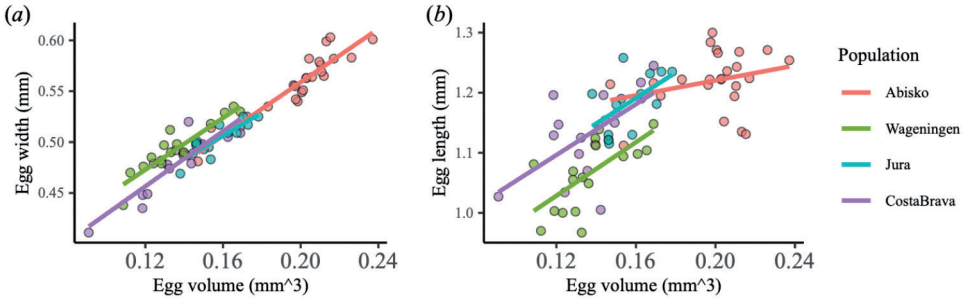



**Figure S5.1)** Egg width (*a*) and length (*b*) as proxy of egg volume in *Pieris napi*. Egg width showed a good fit to the egg volume of 72 eggs from four *P. napi* populations. Egg volume was estimated by the formula for an ellipsoid: *Volume = (π/6)*length*width^2* [28].

## S5.2 Summarized egg-measurements per *Pieris* rearing

**Table S5.2.** Measurements for egg characteristics ($\bar{x} \pm$ SD (n)) for the three *Pieris* spp. (location = Wageningen) and the seven *P. napi* populations. Numbers in brackets are sample sizes (eggs). For *Pieris brassicae*, one sample consists of one egg clutch, measured as five random eggs from the clutch.

| | Egg width (µm) | Aeropyle number | Aeropyle width | Micropyle number | Average micropyle width |
|---|---|---|---|---|---|
| **A) *Pieris* Species** | | | | | |
| *P. brassicae* | 575 ± 22 µm (14) | 47 ± 8.3 (14) | 2.16 ± 0.31 µm (14) | 3.4 ± 0.65 (14) | 1.35 ± 0.18 µm2 (14) |
| *P. rapae* | 462 ± 26 µm (17) | 42 ± 9.2 (14) | 3.24 ± 0.58 µm (17) | 3.5 ± 0.76 (8) | 1.21 ± 0.38 µm2 (8) |
| *P. napi* (Wag.) | 463 ± 31 µm (25) | 43 ± 11.1 (11) | 2.73 ± 0.47 µm (25) | 2.2 ± 0.86 (15) | 0.87 ± 0.22 µm2 (15) |
| **B) *P. napi* Population** | | | | | |
| Abisko | 531 ± 15 (25) | 66 ± 8.5 (18) | 3.39 ± 0.54 µm (22) | 4.2 ± 0.93 (13) | 1.22 ± 0.25 µm (12) |
| Stockholm | 514 ± 24 (25) | 45 ± 9.8 (19) | 2.87 ± 0.32 µm (21) | 3.8 ± 0.94 (12) | 1.16 ± 0.12 µm (10) |
| Wageningen | 463 ± 24 (25) | 43 ± 11.1 (11) | 2.73 ± 0.47 µm (25) | 2.2 ± 0.86 (15) | 0.87 ± 0.22 µm (15) |
| Jura | 489 ± 29 (21) | 42 ± 19.4 (16) | 2.86 ± 0.31 µm (22) | 2.1 ± 1.46 (7) | 1.09 ± 0.41 µm (7) |
| Callas | 482 ± 38 (24) | 34 ± 4.4 (13) | 3.63 ± 0.33 µm (25) | 2.0 ± 0.93 (8) | 0.94 ± 0.19 µm (8) |
| Pyrenees | 532 ± 25 (13) | 51 ± 17.2 (8) | 2.85 ± 0.49 µm (10) | 4.0 ± 0.81 (4) | 1.06 ± 0.08 µm (2) |
| Costa Brava | 488 ± 25 (19) | 48 ± 9.1 (9) | 3.82 ± 1.18 µm (19) | 2.4 ± 1.14 (5) | 0.95 ± 0.08 µm (5) |
| *P. napi* total average | 498 ± 35 (152) | 48 ± 13.9 (94) | 3.18 ± 0.70 µm (144) | 3.02 ± 1.34 (64) | 1.04 ± 0.26 µm (59) |

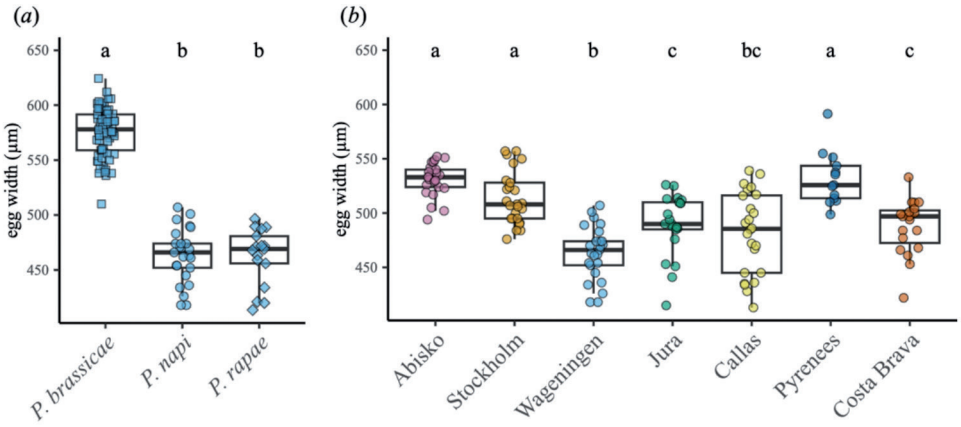



**Figure S5.2.** Comparison of egg width (*a*) interspecifically between Pieris spp. and (*b*) intraspecifically between P. napi populations. Points represent individual eggs (n = 111 and n = 152 eggs, respectively; Table S5.2; Table S5.9). Different letters indicate significant differences (*post hoc* using EMMs, $p > 0.05$).

## S5.3 Details of the seven *P. napi* populations

**Table S5.3.** Summarized conditions for each of the European populations

| population | latitude | longitude | Collected from field | seasonality | Max temp | precipitation | growing days | host plant | local conditions at host plant site |
|---|---|---|---|---|---|---|---|---|---|
| **ABS** | 68.34761 | 18.82977 | 2023 | 864.4 | 18.0 | 369.4 | 59 | *Arabidopsis* spp. and*Barbarea* spp. | Any present host plants near buildings and roads |
| **STK** | 59.36447 | 18.03115 | 2023 | 751.4 | 24.8 | 503.5 | 136 | *Alaria* spp. and *Bunias* spp. | Obtained from a park, not exclusively near water |
| **WAG** | 51.97919 | 5.685259 | 2022 | 576.4 | 25.5 | 824 | 179 | various crop plants | Obtained near the river Eng, where conditions were cool and moist |
| **JUR** | 46.55577 | 6.08496 | 2022 | 651.8 | 22.8 | 1348.3 | 130 | *Cardamine heptaphylla* | Moist and cool rocky ledges in a forested area |
| **CAL** | 43.5903 | 6.531266 | 2022 | 601.7 | 27.8 | 829.4 | 202 | *Alaria petiolata* | Host plants in a relatively hot and dry area |
| **PYR** | 42.32283 | 1.064298 | 2023 | 650.2 | 28.3 | 812.6 | 189 | *Alaria petiolata* | A dry and warm mountain regio with salty soil and little vegetation, where ovipositioning only occured near water sources |
| **Costa Brava** | 42.26495 | 3.069001 | 2022 | 592.4 | 30.5 | 567.7 | 275 | *Lepidium draba* | Obtained from host plants that were shaded by trees in a dry agricultural area |

## S5.4 Correlation plots between egg traits

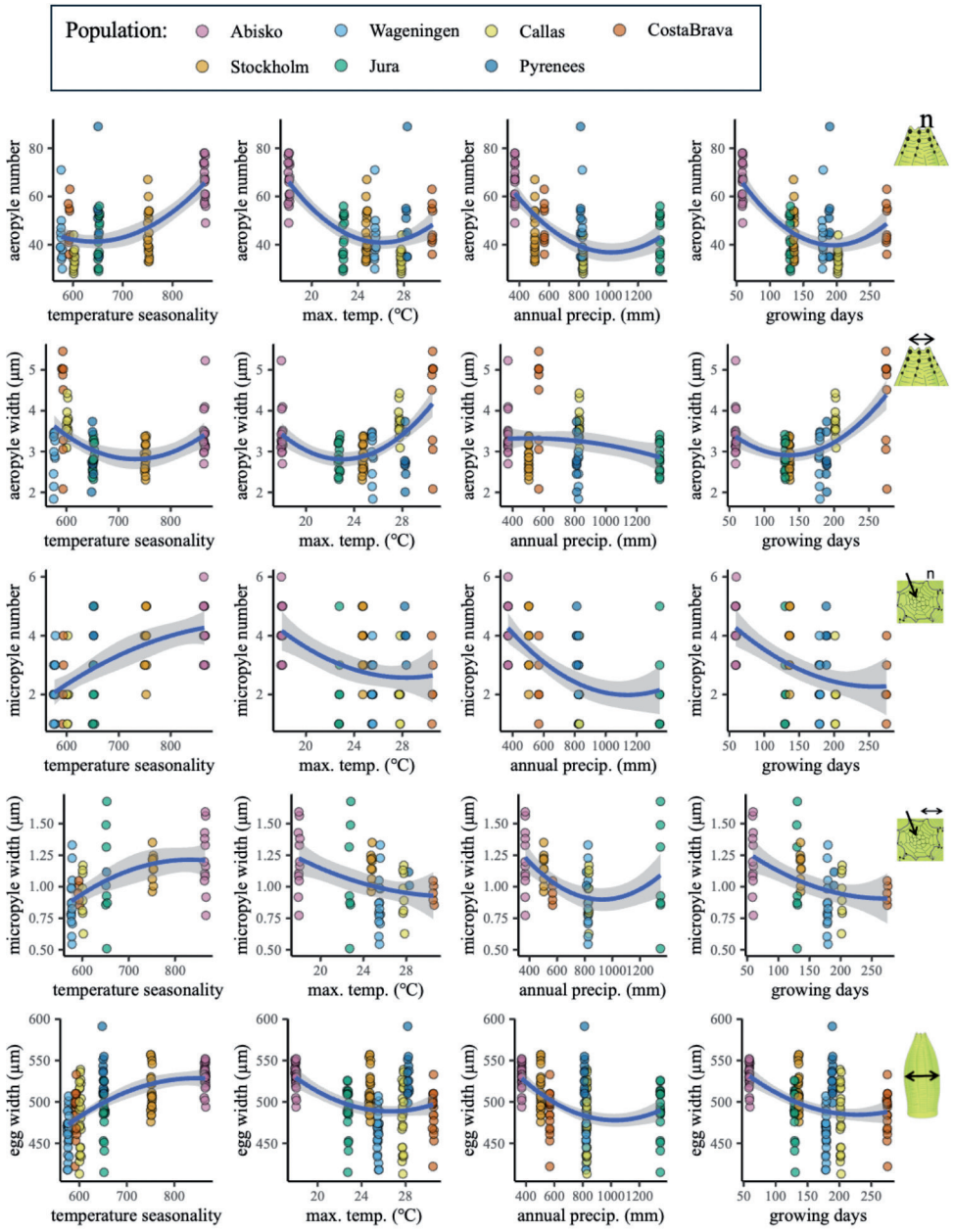



**Figure S5.3.** Relations between climatic variables and egg pore traits. (aeropyle number: n = 61 eggs, aeropyle width: n = 2830 aeropyles, micropyle number: n = 81 eggs, micropyle width: n = 255 micropyles; Table S2).

## S5.5 Piecewise Structural Equation Model (pSEM) selection

Our initial model included every possible association between egg traits and climatic variables (20 associations), as well as every possible association between egg trait to egg trait (nine associations, Figure S5.4). Plotting the data indicated the possibility of a polynomial relation between aeropyle traits and maximum temperature. We manually calculated the orthogonal polynomial of maximum temperature (Gautschi, 1996). and included this as an explanatory variable for aeropyle width and number (two associations).

The directionality of associations between egg traits were based on assumed biological relevance. The associations between climatic variables were incorporated in the model as random effects (six associations possible). To select the most parsimonious model that best explains the relationship among variables, we followed the procedure outlined in Boisseau and Woods (2024). In the first phase of model selection, individual associations were dropped step by step, testing which removed association led to the greatest reduction in the C statistic Information Criterion (CIC) (Gonzales-Voyer and von Hardenberg, 2014), equivalent to d-sep determined Akaike Information Criterion or AIC from the current model. The current model is considered the best model if further dropping associations no longer leads to a decrease in CIC (see Figure S5.4). In the second phase of model selection, we tested alternative hypotheses on the directionality, and thus causal relation, of associations. Model fit was considered equivalent if $\Delta CIC \leq 2$, in which case the equivalent models should be averaged. Naturally, the causal relation between egg traits and climate is only in one direction. We further argued that the causal relation between egg width and micropyle number is one-way, as egg size is related by ecological and female traits (Boisseau and Woods, 2024; Church et al., 2019). There was only one alternative directionality possible after our model phase one: the pathway between the micropyle number and aeropyle number (Figure S5.5). As we used transposed datasets for aeropyle and micropyle width (every egg-pore value having its own data point), the directionality of their associations could not be reversed. Of the reduced model from phase one, only two alternative causal hypotheses could be tested (Figure S5.5). The alternative causal hypothesis did not improve model fit, Figure S5.5. Therefore, the reduced model (Figure S5.4*b*; Figure S5.5*a*), was selected as the final model. The model summary can be found in Table S5.4.

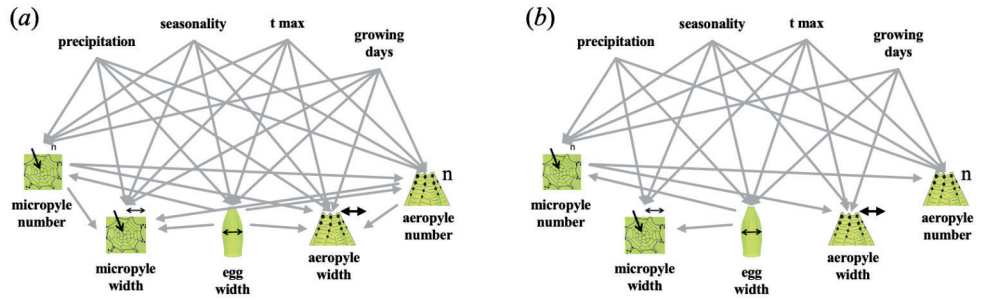



**Figure S5.4)** Phase one of model selection, model reduction. In the first phase, the initial structural model (*a*) including all possible associations was reduced step by step by dropping the association that led to the greatest increase in model fit (reduction in C statistic Information Criterion or CIC). Both polynomial relations (between both aeropyle traits and maximum temperature) did not contribute to model fit. The first phase resulted in a reduced structural model (*b*). The initial model had a CIC of 97.42, and the reduced model had a CIC of 73.16

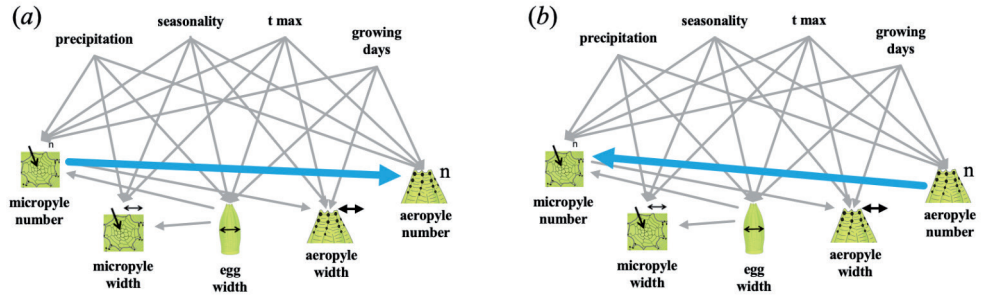



**Figure S5.5)** Phase two of model selection testing alternative causal hypotheses. In the second phase, the directionality of the pathway between the micropyle number and aeropyle number of the reduced structural model (*a*) was reversed to an alternative structural model (*b*). This reversion did not lead to improved model fit. The C statistic Information Criterion (CIC) of the reduced model = 73.16, and the CIC of the alternative model = 84.94. ΔCIC > 2 and thus the reduced model (*a*) was selected as final model.

**Table S5.4.** Standard estimates, standard errors, degrees of freedom, critical values and p-values for the final selected piecewise-SEM.

| association | standard estimate | se | df | Crit.Value | p |
|---|---|---|---|---|---|
| **egg width <-- t. seasonality** | **1.187** | **0.203** | **147** | **5.849** | **<.001** |
| **egg width <-- t. max.** | **0.762** | **0.257** | **147** | **2.970** | **0.004** |
| **egg width <-- precipitation** | **0.231** | **0.111** | **147** | **2.083** | **0.039** |
| egg width <-- growing days | -0.149 | 0.312 | 147 | -0.477 | 0.634 |
| micropyle number <-- t. seasonality | 0.327 | 0.314 | 57 | 1.044 | 0.301 |
| micropyle number <-- t. max. | 0.565 | 0.422 | 57 | 1.341 | 0.185 |
| micropyle number <- precipitation | -0.198 | 0.172 | 57 | -1.154 | 0.253 |
| micropyle number <-- growing days | -0.569 | 0.531 | 57 | -1.071 | 0.289 |
| micropyle number <-- egg width | 0.199 | 0.119 | 57 | 1.672 | 0.100 |
| **aeropyle number <-- t. seasonality** | **0.779** | **0.367** | **33** | **2.120** | **0.042** |
| **aeropyle number <-- t. max** | **-1.572** | **0.380** | **33** | **-4.139** | **<.001** |
| aeropyle number <-- precipitation | -0.117 | 0.205 | 33 | -0.570 | 0.573 |
| **aeropyle number <-- growing days** | 1.853 | 0.512 | 33 | 3.618 | 0.001 |
| aeropyle number <- micropyle number | 0.225 | 0.146 | 33 | 1.541 | 0.133 |
| **aeropyle width <-- seasonality** | **0.685** | **0.145** | **36.40** | **22.274** | **<.001** |
| **aeropyle width <-- t. max** | **-1.182** | **0.223** | **35.59** | **28.216** | **<.001** |
| **aeropyle width <-- growing days** | **1.935** | **0.261** | **35.05** | **54.964** | **<.001** |
| aeropyle width <-- micropyle number | -0.135 | 0.079 | 36.61 | 2.899 | 0.097 |
| **micropyle width <-- t. seasonality** | **0.791** | **0.256** | **56.24** | **9.482** | **0.003** |
| micropyle width <-- t. max | 0.265 | 0.203 | 50.89 | 1.691 | 0.199 |
| micropyle width <-- precipitation | 0.227 | 0.155 | 61.23 | 2.130 | 0.150 |
| micropyle width <-- egg width | -0.135 | 0.120 | 63.93 | 1.269 | 0.264 |

## S5.6 Pairwise comparisons for population egg traits

**Table S5.5.** Aeropyle number compared between the seven *Pieris napi* populations. *Post hoc* using EMMs. Abbreviations: pop) population, est.) estimate, n) number, SE) standard error, df) degrees of freedom, t) t score, p) probability. ($\alpha$: $p > 0.05$).

| pop | pop | est. (n) | SE | df | t | p |
|---|---|---|---|---|---|---|
| **Abisko** | **Callas** | **32.18** | **3.58** | 87 | 8.98 | **<.001** |
| **Abisko** | **CostaBrava** | **18.00** | **4.02** | 87 | 4.48 | **<.001** |
| **Abisko** | **Jura** | **24.02** | **3.38** | 87 | 7.10 | **<.001** |
| **Abisko** | **Pyrenees** | **15.33** | **4.19** | 87 | 3.66 | **0.008** |
| **Abisko** | **Stockholm** | **21.81** | **3.24** | 87 | 6.73 | **<.001** |
| **Abisko** | **Wageningen** | **23.52** | **3.77** | 87 | 6.24 | **<.001** |
| **Callas** | **CostaBrava** | **-14.18** | **4.27** | 87 | -3.32 | **0.022** |
| Callas | Jura | -8.16 | 3.68 | 87 | -2.22 | 0.297 |
| **Callas** | **Pyrenees** | **-16.85** | **4.43** | 87 | -3.81 | **0.005** |
| Callas | Stockholm | -10.37 | 3.55 | 87 | -2.93 | 0.064 |
| Callas | Wageningen | -8.66 | 4.03 | 87 | -2.15 | 0.335 |
| CostaBrava | Jura | 6.02 | 4.10 | 87 | 1.47 | 0.763 |
| CostaBrava | Pyrenees | -2.67 | 4.79 | 87 | -0.56 | 0.998 |
| CostaBrava | Stockholm | 3.81 | 3.99 | 87 | 0.96 | 0.962 |
| CostaBrava | Wageningen | 5.52 | 4.43 | 87 | 1.25 | 0.874 |
| Jura | Pyrenees | -8.69 | 4.26 | 87 | -2.04 | 0.399 |
| Jura | Stockholm | -2.21 | 3.34 | 87 | -0.66 | 0.994 |
| Jura | Wageningen | -0.51 | 3.86 | 87 | -0.13 | 1.000 |
| Pyrenees | Stockholm | 6.47 | 4.15 | 87 | 1.56 | 0.708 |
| Pyrenees | Wageningen | 8.18 | 4.58 | 87 | 1.79 | 0.560 |
| Stockholm | Wageningen | 1.71 | 3.73 | 87 | 0.46 | 0.999 |

**Table S5.6.** Aeropyle width compared between the seven *Pieris napi* populations. *Post hoc* using EMMs. Abbreviations: pop) population, est.) estimate, n) number, SE) standard error, df) degrees of freedom, t) t score, p) probability. ($\alpha$: $p > 0.05$).

| pop | pop | est. (µm) | SE | df | t | p |
|---|---|---|---|---|---|---|
| Abisko | Callas | -0.287 | 0.203 | 88.8 | -1.42 | 0.792 |
| **Abisko** | **CostaBrava** | **-0.820** | **0.226** | 85.0 | -3.63 | **0.008** |
| Abisko | Jura | 0.558 | 0.191 | 87.1 | 2.93 | 0.064 |
| Abisko | Pyrenees | 0.609 | 0.235 | 85.1 | 2.59 | 0.143 |
| **Abisko** | **Stockholm** | **0.579** | **0.181** | 85.5 | 3.20 | **0.031** |
| Abisko | Wageningen | 0.509 | 0.212 | 86.5 | 2.40 | 0.212 |
| Callas | CostaBrava | -0.533 | 0.245 | 89.1 | -2.18 | 0.318 |
| **Callas** | **Jura** | **0.845** | **0.213** | 92.2 | 3.97 | **0.003** |
| **Callas** | **Pyrenees** | **0.896** | **0.253** | 88.8 | 3.54 | **0.011** |
| **Callas** | **Stockholm** | **0.866** | **0.204** | 91.3 | 4.24 | **0.001** |
| **Callas** | **Wageningen** | **0.796** | **0.232** | 90.8 | 3.43 | **0.016** |
| **CostaBrava** | **Jura** | **1.378** | **0.235** | 88.0 | 5.87 | **<.001** |
| **CostaBrava** | **Pyrenees** | **1.429** | **0.272** | 86.2 | 5.25 | **<.001** |
| **CostaBrava** | **Stockholm** | **1.399** | **0.227** | 87.0 | 6.16 | **<.001** |
| **CostaBrava** | **Wageningen** | **1.329** | **0.252** | 87.4 | 5.26 | **<.001** |
| Jura | Pyrenees | 0.051 | 0.244 | 87.7 | 0.21 | 1.000 |
| Jura | Stockholm | 0.022 | 0.192 | 89.9 | 0.11 | 1.000 |
| Jura | Wageningen | -0.049 | 0.222 | 89.7 | -0.22 | 1.000 |
| Pyrenees | Stockholm | -0.029 | 0.236 | 86.9 | -0.12 | 1.000 |
| Pyrenees | Wageningen | -0.100 | 0.261 | 87.3 | -0.38 | 1.000 |
| Stockholm | Wageningen | -0.070 | 0.214 | 88.8 | -0.33 | 1.000 |

**Table S5.7.** Micropyle number compared between the seven *Pieris napi* populations**.** *Post hoc* using EMMs. Abbreviations: pop) population, est.) estimate, n) number, SE) standard error, df) degrees of freedom, t) t score, p) probability. ($\alpha$: $p > 0.05$).

| pop | pop | est. (n) | SE | df | t | p |
|---|---|---|---|---|---|---|
| **Abisko** | **Callas** | **2.23** | **0.45** | 57 | 4.99 | **<.001** |
| **Abisko** | **CostaBrava** | **1.83** | **0.52** | 57 | 3.50 | **0.015** |
| **Abisko** | **Jura** | **2.09** | **0.47** | 57 | 4.48 | **<.001** |
| Abisko | Pyrenees | 0.23 | 0.57 | 57 | 0.41 | 1.000 |
| Abisko | Stockholm | 0.40 | 0.40 | 57 | 1.00 | 0.953 |
| **Abisko** | **Wageningen** | **2.03** | **0.38** | 57 | 5.39 | **<.001** |
| Callas | CostaBrava | -0.40 | 0.57 | 57 | -0.71 | 0.992 |
| Callas | Jura | -0.14 | 0.52 | 57 | -0.28 | 1.000 |
| **Callas** | **Pyrenees** | **-2.00** | **0.61** | 57 | -3.28 | **0.028** |
| **Callas** | **Stockholm** | **-1.83** | **0.45** | 57 | -4.04 | **0.003** |
| Callas | Wageningen | -0.20 | 0.44 | 57 | -0.46 | 0.999 |
| CostaBrava | Jura | 0.26 | 0.58 | 57 | 0.44 | 0.999 |
| CostaBrava | Pyrenees | -1.60 | 0.67 | 57 | -2.40 | 0.219 |
| CostaBrava | Stockholm | -1.43 | 0.53 | 57 | -2.71 | 0.115 |
| CostaBrava | Wageningen | 0.20 | 0.51 | 57 | 0.39 | 1.000 |
| Jura | Pyrenees | -1.86 | 0.62 | 57 | -2.98 | 0.061 |
| **Jura** | **Stockholm** | **-1.69** | **0.47** | 57 | -3.57 | **0.012** |
| Jura | Wageningen | -0.06 | 0.46 | 57 | -0.13 | 1.000 |
| Pyrenees | Stockholm | 0.17 | 0.57 | 57 | 0.29 | 1.000 |
| **Pyrenees** | **Wageningen** | **1.80** | **0.56** | 57 | 3.22 | **0.033** |
| **Stockholm** | **Wageningen** | **1.63** | **0.39** | 57 | 4.24 | **0.002** |

**Table S5.8.** Micropyle width compared between the seven *Pieris napi* populations**.** *Post hoc* using EMMs. Abbreviations: pop) population, est.) estimate, n) number, SE) standard error, df) degrees of freedom, t) t score, p) probability. ($\alpha$: $p > 0.05$).

| pop | pop | est. (µm) | SE | df | t | p |
|---|---|---|---|---|---|---|
| Abisko | Jura | 0.121 | 0.106 | 53.3 | 1.14 | 0.913 |
| Abisko | Callas | 0.278 | 0.102 | 56.7 | 2.72 | 0.113 |
| Abisko | CostaBrava | 0.271 | 0.116 | 50.4 | 2.35 | 0.244 |
| Abisko | Pyrenees | 0.150 | 0.154 | 40.2 | 0.97 | 0.958 |
| Abisko | Stockholm | 0.063 | 0.086 | 38.6 | 0.73 | 0.990 |
| **Abisko** | **Wageningen** | **0.330** | **0.082** | 47.5 | 4.00 | **0.004** |
| Jura | Callas | 0.157 | 0.123 | 65.4 | 1.28 | 0.859 |
| Jura | CostaBrava | 0.151 | 0.134 | 58.3 | 1.12 | 0.919 |
| Jura | Pyrenees | 0.029 | 0.169 | 45.4 | 0.17 | 1.000 |
| Jura | Stockholm | -0.058 | 0.110 | 52.7 | -0.53 | 0.998 |
| Jura | Wageningen | 0.209 | 0.107 | 61.4 | 1.96 | 0.453 |
| Callas | CostaBrava | -0.007 | 0.131 | 60.9 | -0.05 | 1.000 |
| Callas | Pyrenees | -0.128 | 0.166 | 46.3 | -0.77 | 0.987 |
| Callas | Stockholm | -0.215 | 0.106 | 55.8 | -2.03 | 0.407 |
| Callas | Wageningen | 0.052 | 0.103 | 66.0 | 0.50 | 0.999 |
| CostaBrava | Pyrenees | -0.122 | 0.175 | 44.9 | -0.70 | 0.992 |
| CostaBrava | Stockholm | -0.209 | 0.119 | 50.1 | -1.76 | 0.584 |
| CostaBrava | Wageningen | 0.058 | 0.116 | 56.6 | 0.50 | 0.999 |
| Pyrenees | Stockholm | -0.087 | 0.157 | 40.3 | -0.55 | 0.998 |
| Pyrenees | Wageningen | 0.180 | 0.155 | 42.8 | 1.16 | 0.904 |
| Stockholm | Wageningen | 0.267 | 0.087 | 47.3 | 3.08 | 0.050 |

**Table S5.9.** Egg width compared between the seven *Pieris napi* populations**.** *Post hoc* using EMMs. Abbreviations: pop) population, est.) estimate, n) number, SE) standard error, df) degrees of freedom, t) t score, p) probability. (*α*: $p > 0.05$).

| pop | pop | est. (µm) | SE | df | t | p |
|---|---|---|---|---|---|---|
| **Abisko** | **Callas** | **48.8** | **7.6** | **145** | **6.45** | **<0.001** |
| **Abisko** | **CostaBrava** | **42.9** | **8.1** | **145** | **5.32** | **<0.001** |
| **Abisko** | **Jura** | **41.6** | **7.9** | **145** | **5.30** | **<0.001** |
| Abisko | Pyrenees | -1.1 | 9.1 | 145 | -0.12 | 1.000 |
| Abisko | Stockholm | 17.0 | 7.5 | 145 | 2.27 | 0.265 |
| **Abisko** | **Wageningen** | **67.3** | **7.5** | **145** | **8.98** | **<0.001** |
| Callas | CostaBrava | -5.9 | 8.1 | 145 | -0.73 | 0.991 |
| Callas | Jura | -7.2 | 7.9 | 145 | -0.91 | 0.970 |
| **Callas** | **Pyrenees** | **-50.0** | **9.1** | **145** | **-5.47** | **<0.001** |
| **Callas** | **Stockholm** | **-31.8** | **7.6** | **145** | **-4.20** | **<0.001** |
| Callas | Wageningen | 18.5 | 7.6 | 145 | 2.44 | 0.190 |
| CostaBrava | Jura | -1.3 | 8.4 | 145 | -0.16 | 1.000 |
| **CostaBrava** | **Pyrenees** | **-44.0** | **9.5** | **145** | **-4.61** | **<0.001** |
| **CostaBrava** | **Stockholm** | **-25.9** | **8.1** | **145** | **-3.21** | **0.027** |
| **CostaBrava** | **Wageningen** | **24.4** | **8.1** | **145** | **3.02** | **0.046** |
| **Jura** | **Pyrenees** | **-42.7** | **9.4** | **145** | **-4.57** | **<0.001** |
| **Jura** | **Stockholm** | **-24.6** | **7.9** | **145** | **-3.13** | **0.034** |
| **Jura** | **Wageningen** | **25.7** | **7.9** | **145** | **3.28** | **0.022** |
| Pyrenees | Stockholm | 18.2 | 9.1 | 145 | 2.00 | 0.417 |
| **Pyrenees** | **Wageningen** | **68.4** | **9.1** | **145** | **7.55** | **<0.001** |
| **Stockholm** | **Wageningen** | **50.3** | **7.5** | **145** | **6.71** | **<0.001** |

## References S5

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# 6. Supplement for Chapter 6

# Enemies, not sex, as a driving force of butterfly post-mating odor evolution

## Methods

### S6.1 Field surveys

#### *S6.1.1 Sites*

To assess variation in post-mating odors of *Pieris napi* and other Pieridae and their interactions with *Trichogramma* in geographically distant locations, we sampled six different populations of *P. napi*, its subspecies, and sister species across Europe. Sites were selected based on citizen science data indicating that *Pieris napi* and its close relatives were abundant. Sources included the Catalonian butterfly monitoring scheme (CatalanBMS.org), Obervations.org, INaturalist.com, and Artportalen.SE. At each site we also collected other Pieridae species we encountered.

Samples were collected in 2022 and 2023 from May to July in natural and cultivated sites. Each sampling site consisted of 2-5 transects within a area with a 50km radius (figure S1). Each transect was visited at least twice per sampling year, with sampling at least once in the morning and once in the afternoon. The coordinates listed below are the CenterPoint of each sampling area.

The Abisko (**ABS**) site is located in Northern Sweden (68° 2'21.95"N, 19°26'46.16"E). The sites around Abisko were characterised by big patches of flowering host plants like Barbarea vulgaris and various *Arabidobsis* species. From the *Pieris* genus only the subspecies *Pieris napi* adelwinda Fruhstorfer was present, which occurs in one generation per year. At this site, we also collected *Colias palaeno*.

The Stockholm (**STK**) site is located in Southern Sweden (59°12'3.61"N, 17°45'21.31"E). We collected *Pieris napi* napi in city parks of Stockholm and Tovetorp biological station. In the city, *Pieris* were found mainly on *Bunias orientalis* and *Allaria petiolata*. Around Tovetorp, the landscape was characterised by forest interspersed with flowering meadows. *P. rapae* and *P. brassicae* were also present, and the three *Pieris* species generally have two generations per year and are generally found on sparsely occurring *Barbarea vulgaris*. At this site we also collected *Leptidea sinapis*.

The Wageningen (**WAG**) site is located in the center of the Netherlands (51°54'41.53"N, 5°39'14.48'E). Around Wageningen the field sites were characterized by cultivated pastures with crops (many varieties of *Brassicae oleracea*) and wild-flower strips with *Raphanus raphanistrum*, *Sinapis arvensis* , and *Brassica nigra*. Here, three to four generations of *P. napi* napi, *P. rapae*, *P. brassicae*, and *Pieris mannii* co-occur and were all collected. We also collected *Anthocharis cardamine*, which were using *Cardamine pratensis*.

The Jura (**JUR**) site is located in the Jura Mountains on the French side of the border between France and Switzerland. (46°31'2.74"N, 6° 4'55.76"E). The field sites around Belfontaine, in the Jura mountains were characterised by meadows with flowering plants. At this site, both *P. napi* napi and *Pieris bryoniae* Ochsenheimer (Lepidoptera:Pieridae) were abundant and found using the host plant *Cardamine heptaphylla*. *P. bryoniae* (**BRY**) is a very close relative of P. *napi*, and in some sites where they co-occur they are known to hybridize. We also collected *Aporia crataegi* at this site.

The Costa Brava (**COB**) site is located in the north-eastern coast of Catalonia, Spain (42°13'22.91"N, 3° 0'50.31"E). The field sites in this area were characterised by grazing pastures interspersed where *P. napi*, *P. rapae*, and *P. brassicae* were all abundant and generally have four to five generations per year. *P. napi* was found near streams at the shaded edges of fields using the plant *Lepidium draba* and *Brassica nigra.* At this site, we also collected *Colias croceus*.

The Pyrenees (**PYR**) site is located on the Spanish side of the Pyrenees mountains, bordering France and (42°23'46.59"N, 1° 3'49.78"E). We collected mainly around Gerri de la sal, a dry mountainous area characterized by salt flats and high biodiversity. *Pieris napi* napi were less common, and found alongside streams on infrequent host plants such as *Allaria petiolata*. Here, we also collected *Pieris rapae*, *Leptidea sinapis* and *Pontia daplidice*.

Apart from our sampling of *Pieris napi* populations, further sampling of *Pieris chieranthi*, *Euchloe eversi*, and *Pieris rapae* was conducted in Tenerife and La Palma, in the Canary Islands, at 13 sampling sites selected in coordination with the ICAE Tenerife Agricultural Center. *Pieris napi* and *Pieris brassicae* do not occur there. *Pieris chieranthi* were collected in La Palma.




**Figure S6.1)** Sampling sites across Western Europe. The overlaid circles represent the sampling areas, with a radius of 50 km. ABS= Abisko, STK = Stockholm, COB= Costa Brava, WAG = Wageningen, JUR= Jura mountains, COB= Costa Brava, and PYR = Pyrenees mountains.

### *S6.1.2 Phoresy and parasitism rates*

Sample sizes for butterflies and wasps per location can be found in table S6.5. Eggs were collected from hostplants at the six different locations. Eggs were checked for signs of parasitism in the field. When eggs are parasitized they turn black within approximately 5 days since parasitiation. Eggs that still appeared yellow (unparasitized) were collected in plastic petri dishes with moistened filter papers and observed until either turning black or caterpillars hatched, at which point they were recorded as parasitized or unparasitized. Caterpillars that hatched from unparasitized eggs were put back in the environment of collection. Some caterpillars were kept for starting a rearing of the population when back in Wageningen.

Parasitized eggs were isolated in small tubes to allow capture of hatching of the wasps. Empty eggshells were stored in 96% ethanol for DNA analyses to identify the host species when both *P. rapae* and *P. napi* were present, as they use similar host plants and their eggs cannot be morphologically distinguished (Chapter 4). However, identification of eggs to species label was not always possible and parasitism rates are calculated as the proportion of parasitized versus unparasitized eggs for all solitary Pieris eggs.

Emerged wasps were relocated to a plastic tube and provided with a droplet of biological honey. The sex of parasitoids was established by looking at the antennae. When a female wasp was present, the wasps were provided with reared on *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) obtained from Koppert Biological Control (The Netherlands) to start a rearing. Dead specimens were stored in 96% ethanol for DNA analyses.

Butterflies were caught to survey for phoretic *Trichogramma* following the method described by Fatouros & Huigens (2012), using a butterfly net (Ø 40cm, Vermandel, The Netherlands). At the bottom of the net, a plastic container (Volume 250ml) was secured with a rubber band. In one fluid sweep of the butterfly net, the butterflies were caught in the air without touching the vegetation surrounding, to minimize bycatch. After the butterfly entered the container, the container was closed immediately with a lid to maximize the chance of capturing all possible hitchhikers present on the butterfly. After every catch, the container was replaced by a new empty container. After every three catches, a control sweep was performed, in which the same sweeping motion was used. For the control catches, instead of catching a butterfly, only air was sampled, and the container was checked for presence of bycatch. The control sweeps did not yield any *Trichogramma* wasps.

Phoresy rate was determined in the field by catching *Pieris napi* and checking them for possible hitchhikers. The butterflies were thoroughly checked, two times with a ±30 minutes interval. Special attention was given to the head, thorax and wings of the butterfly.

When a possible hitchhiker was spotted the container with the butterfly was retrieved to the lab. The containers were put in the fridge at 4°C for 5-10 minutes to slow down the butterfly and possible hitchhikers. Possible hitchhikers were separated from the butterfly and put in a plastic test tube with a drop of biological honey and eggs to parasitize. Depending on the sex of the butterfly, it was released (male) or kept for pheromone collection (female). Possible hitchhikers were reared and brought back to Wageningen. Laboratory populations were established and reared on *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs. Dead specimens were stored in 96% ethanol for DNA analyses.

***S6.1.3 Parasitoid identification***

Wasps were dried at 30 °C to minimize the amount of ethanol present in the samples. Sterile glass pestles for grinding samples were made by melting the tip of glass Pasteur pipettes into a rounded ball, with a Bunsen burner. Single *Trichogramma* wasps were grinded with a sterile glass pestle in a 0.5 ml Eppendorf tube. For each sample a new sterile pestle was used. 50 µl of 5% Chelex X-100 and 4 µl Proteinase K (20 mg/ ml) was added to each sample using a pipette and centrifuged to spin all content to the bottom of the Eppendorf tube. Samples were incubated for a minimum of 6 hours at 56°C. Samples were centrifuged for 1 minute and subsequently heated for 10 minutes at 95°C to inactivate Proteinase K. Finally, samples were spun down, and DNA extract was used for PCR. A polymerase chain reaction to amplify the Folmer barcode region (Folmer et al., 1994)[79] was performed using forward primer LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' and reverse HCO2198: TAAACTTCAGGGTGACCAAAAAATCA-3'. The reaction was conducted in a total volume of 25 µl containing 20.5 µl of PCR Master Mix (12.5 µl 2X mix Phusion flash, 1.0 µl 10 mM forward primer, 1.0 µl 10 mM reverse primer, 6.0 µl H2O) and 4.5 µl of DNA template. The PCR cycling program consisted of a starting cycle of 10 sec at 98°C followed by 30 cycles of denaturation for 5 seconds at 98°C, annealing for 10 seconds at 58°C and polymerisation for 30 seconds at 72°C with a final elongation cycle of 60 sec at 72°C after the last cycle. Amplicons were run on a 1.5% agarose gel in 0.5 TAE at 100 V for approximately 30 minutes. The gel was stained with ethidium bromide (0.5 µl EtBr per 100 ml gel) and visualized with UV light. Trichogramma amplicons detected by electrophoresis with the expected band size (550 bp in size) were prepared for sequencing bidirectional Sanger sequencing by Eurofins genomics. Sequences were prepared using Geneious prime 2023.0.4. De novo assembly of forward and reverse reads, trimming, and generation of consensus (error probability limit 0.05) were performed with default settings. A consensus sequence was made using default settings for all sequences. Identification of the wasps was achieved by comparing the obtained FASTA-sequences with the Hymenoptera collection (nr/nt) database using BLASTN (NCBI interface). The identification of the FASTA-sequences was based on the E-value (>0.01), Percentage Identity (>95) and Max score. A phylogenetic tree was built by performing a multiple alignment of obtained *Trichogramma* sequences on Geneious prime 2023.0.4. Alignment was made

with MAFFT (Katoh et al., 2009) using default settings. A maximum likelihood tree was generated using PHYML with 100 bootstraps. Geneious consensus tree builder was used to make a consensus tree.

## S6.2 Rearing

***S6.2.1*** *Pieris napi*

Larvae of *P. napi* collected from eggs laid by 5- 10 wild butterflies during field work from May-July, 2022 and 2023 were reared at the greenhouses of Unifarm, Wageningen University & Research. Rearings were maintained under greenhouse conditions in ventilated nylon cages (adults: 120 cm3, larvae: 60 cm3) at 21°C, 60% relative humidity and a 18:6 h light:dark photoperiod. Adult butterflies were provided with a cotton wad soaked in 10% honey water solution and a *Brassica nigra* plant for egg-deposition. Every second day the cotton wad was refreshed and eggs were collected to maintain the population. Once populations were established with approximately 30 adults at a time. Approximately 100 eggs were collected every other week. Larvae were reared on *B. nigra* plants.

***S6.2.2*** *Pieris brassicae*

Seeds of cabbage plants (*Brassica oleracea* var. *gemmifera* cv. Cyrus; Brussels sprouts) were planted in individual pots and grown for four weeks after germinating. The cabbage plants were cultivated under glasshouse conditions with 22 ± 3 °C, RH 50-80 % and 16 h light: 8 h dark cycles. *Pieris brassicae* (Lepidoptera: Pieridae) caterpillars and butterflies were taken from the laboratory colony maintained at the Laboratory of Entomology, Wageningen University & Research, The Netherlands. The caterpillars were reared on four-week-old *Brassica oleracea* var. *gemmifera* cv. Cyrus, Brussels sprouts plants at 22 ± 3 °C, RH 60-80 % with a 14h light : 10h dark photoperiod until pupae. The male and female butterflies were fed with 5 % sugar water under the same environmental conditions as the caterpillars. The genetically modified insects are reared in a glasshouse compartment at Unifarm, Wageningen University & Research at 22 ± 3 °C, RH 60-80 % with a 14h light : 10h dark photoperiod. Mutant insects were supplied with the same food as wildtypes.

***S6.2.3 Wasps***

*Trichogramma* wasps collected during fieldwork from May-July, 2022, were reared under laboratory conditions in glass tubes (Ø 1cm) at 18°C, 50-70% relative humidity and a 16:8 h light:dark photoperiod at the Laboratory of Genetics of Wageningen University & Research. Adults were reared on egg-cards ( 50 x 150mm) made from *Ephestia kuehniella* eggs sourced from Koppert in The Netherlands secured to the cards with double-sided tape. The wasps were provided with a small droplet of organic honey. After emergence, male and female parasitoids were kept together for mating. Fresh egg cards were given

every three weeks. For the experiments, females were isolated in glass tubes before being tested.

For bioassays with mutant butterflies, the egg parasitoid species used in this experiment was *Trichogramma evanescens* (strain 69_eng_20) (Hymenoptera: Trichogrammatidae). This iso-female line was reared from a single female wasp collected in Wageningen, The Netherlands and has since been as described above.

#### *S6.2.4 Birds*

For this study we will use great tits (*Parus major*) (n=17) housed at the Animal Ecology laboratory at the Netherlands Institute for Ecology (NIOO-KNAW), Wageningen. The birds are a F2 population and were born and raised in captivity. The birds originated from a population of urban birds. They were individually housed in cages of 90 x 50 x 30 cm under a photoperiod of 14:10 L:D and were fed on a diet of mealworms, songbird seed mix and apple pieces. Drinking water and bathing trays were freely available. Five sides of the cage are covered so the influence of external disturbances is as low as possible. The birds are used by esearchers and have been used in exploratory and behavioural studies before. They did not have any prior experience with Pierid butterflies or with benzyl cyanide.

## S6.3 Odor samples

#### *S6.3.1 Collection in the field*

Per population 13-27 females of *P. napi* and per population and 2-10 individuals of other Pierid species were collected for pheromone collection (described in S6.1.1). To ensure we collected odors from mated females, we sampled from wild females that we witnessed laying eggs on host plants.

To determine the composition of post-mating odors, we collected odor samples with PDMS fiber rubs. A study by Lievers & Groot (2016) showed that pheromone composition collected with PDMS rubs closely resembles the pheromone composition collected with solid-phase micro-extraction (SPME), used by most previous studies into AA of *Pieris* (Lievers & Groot, 2016). All tools used during pheromone collection were thoroughly cleaned before and between each sample collection in ethanol and hexane.

Pheromone rubs were collected following the method described by Lievers & Groot. For the fiber rub method, fused silica optical fibers coated with a 100 µm PDMS layer (Polymicro Technologies Inc., Phoenix, AZ, USA) were cut into 20 mm pieces. The fibers were washed by gently shaking them in a bottle with hexane. Each fiber was subsequently wrapped air-tight in aluminum foil and stored in an aluminum foil envelope until use in

the field. Pheromones were collected from female butterflies 2-6 hours after collection in the field. Pseudo mate-refusal posture was achieved by gently squeezing the abdomen of the butterfly between thumb and index finger, whereby the abdominal structures exposed during the mate-refusal posture were also exposed during this handling. With clean forceps, the fiber was rubbed over the abdominal structures, extruded by the female, for ±3 minutes. Each fiber was subsequently placed in a 0.05 mL clear glass micro-insert in a 2 mL clear glass crimp-cap vial. Odor collection vials consisted of inserts that were placed in a spring (36 x 5 mm) in a 2 mL clear glass crimp-cap vial (32 x 12 mm) and capped with a crimp cap with a PTFE (tetrafluoroethylene) septum. The vials were pre-filled with 50 ul of hexane and 200 ng of pentadecane as an internal standard. Fibers were then rinsed in the vials by gently tilting the crimp cap vials a couple of times. After 30-60 minutes, fibers were rinsed again and removed from the insert and sealed vials were stored at -20°C until further analysis.

***S6.3.2 Mated versus unmated P. napi***

To assess which of the pheromones are transferred by the males to the females, we compared the pheromones of virgin and mated females from the greenhouse rearing of the Wageningen population. To achieve this, we sampled 10 mated females and 20 virgin females per population following the PDMS fibre rub method described above. Virgin females were obtained by separating the butterflies by sex as pupae. After the emergence of the butterflies, 10 females were kept separated to assure virginity, the rest were combined with the male butterflies for mating. Butterflies were observed and all mating couples were directly isolated in separate nylon cages (15 cm3). When mating was finalized and the pairs separated, females were isolated in individual containers until odor collection to avoid them emitting odors when adopting mate refusal postures, which also occurs in response to other female butterflies, thereby keeping them from reducing titers. PDMS rubs were taken 36 hrs after mating. At the time of sampling, butterflies of 5-8 days old.

***S6.3.3. Odor analysis***

Pheromones were analyzed following the method described by Lievers & Groot, 2016 . For pheromone analysis, the volume of all extracts was reduced to 1–2 µl under a gentle stream of nitrogen. To prevent evaporation after this, the 2 µl samples were taken up together with 1–2 µl octane (Anhydrous 99+%) with a 10 µl glass syringe (701SN needle; Hamilton). The total volume of 2–4 µl was placed in a 50 µl glass insert, which was placed in a metal spring (35x5mm) within a 2 ml glass crimp vial, capped with an 11 mm aluminum crimp cap and a PTFE (tetrafluoroethylene) septum.

The pheromone extracts were injected with an Agilent 7693A Automatic Liquid Sampler into a splitless inlet of a 7890A gas chromatograph. Between samples, the syringe of the

automatic liquid sampler was cleaned by flushing 10 times with acetone and 10 times with hexane. The GC was equipped with an Agilent DB-WAXetr column of 30 m x 0.25 mm x 0.25 µm coupled with a flame ionization detector (FID) at 250°C. The program was as follows: 2 minutes on 60°C then temperature was increased by 30°C/minute to 180°C, followed by an 5°C/minute increase of temperature to 230°C. Between samples, the column was heated to 245°C for 15 minutes. To check the retention times and identify the compounds in the extracts, 2–4 µl of a blend of authentic standards of all pheromone compounds (1:1:1:1) was injected before and after every series of injections. Authentic standards used were methyl salicylate (Reagent Plus® ≥99% GC, Sigma Aldrich, US), benzyl cyanide (analytical standard >98%, Sigma Aldrich, US), 2-methoxyphenol (guaiacol) (pure reference standards (1 gram) in solid form, Sigma Aldrich, US), and indole (pure reference standards (1 gram) in solid form, Sigma Aldrich, US). Guaiacol and indole were first dissolved in hexane before injected. Areas under the pheromone peaks were determined using Agilent ChemStation (version B.04.03) .

For every pheromone sample, net amount of each compound was calculated relative to 200 ng pentadecane internal standard. The quantity from all virgin and mated females from Wageningen *P. napi* were used to construct box plots in SPSS v28(Mann Whitney U tests, Table S6.2) For the heat maps of both species and populations, we calculated the average quantity for each compound using all individuals sampled (Table S6.3 and S6.4), and heatmaps were visualized by Biorender. The phylogenies of the *P. napi* populations were constructed using the CO1 barcodes of representatives from each population downloaded from BOLD systems analysis. The accessions used for each population were: Abisko - MW502229; Stockholm - EULEP6041-20; Wageningen- GU669668; Jura- MN141461; bryoniae- MN141756; Costa Brava- GU669673; and Pyerenese - GU669672. Chemical hierarchical clustering was performed in ChemMine tools (2011) using AP Tanimoto values for each compound and hierarchical clustering was performed with default settings.

To create the PCA of the wild-caught *P. napi* from different populations, the quantities of each of the four compounds for each individual were used (Table S6.4). The Principal Component Analysis (PCA) in R using the `vegan` package (Dixon et al, 2003) was used with the grouping variable was coded as a factor. The PCA was conducted using the `rda()` function with the arguments `center = TRUE` and `scale = TRUE`. Two criteria were used to determine the number of principal components retained for interpretation: Eigenvalues greater than 1 were considered significant and retained. A biplot of the PCA was created using `ordiplot()` and `points()`, displaying sites (observations) with symbols and colors representing group membership. Additionally, we fitted vectors of the odor compounds onto the PCA plot using the `envfit()` function with 10,000 permutations, visualizing only those variables with a significance threshold of $p < 0.001$.

**Table S6.2** Mated versus virgin *P. napi* Wageningen samples

| Odor | Mated mean +- sd ng | N mated | Virgin mean +- sd ng | N virgin | Test | U | P-value |
|---|---|---|---|---|---|---|---|
| Methyl salicylate | 69.14 +-63.17 | 10 | 19.88 +- 26.97 | 21 | Mann-whitney U | 12 | <0.001 |
| Guaiacol | 11.69 +- 10.49 | 10 | 4.21 +- 6.52 | 21 | Mann-whitney U | 9.5 | <0.001 |
| Benzyl cyanide | 102.15 +- 108.37 | 10 | 25.7 +-43.72 | 21 | Mann-whitney U | 6.5 | <0.001 |
| Indole | 40.57 +- 30.21 | 10 | 23.71 +- 23.42 | 21 | Mann-whitney U | 38 | 0.001 |

**Table S6.3)** Pieridae post-mating odors samples

| Species | Locations | N | Mean ng +- SD MesA | Mean ng +- SD Guaia | Mean ng +- SD BC | Mean ng +- SD Indole |
|---|---|---|---|---|---|---|
| *Pieris napi* | WAG | 16 | 67.29 +- 72.8 | 9.84 +- 9.44 | 49.34 +- 64.47 | 33.17 +- 48.33 |
| *Pieris bryoniae* | JUR | 13 | 4.21+- 3.17 | 0 | 4.44+- 3.51 | 0 |
| *Pieris rapae* | WAG, COB, PYR | 5 | 3.97 +- 4.25 | 0 | 0.79+-0.83 | 32.9 +- 22.8 |
| *Pieris mannii* | WAG | 8 | 9.89+-12.38 | 0 | 0 | 15.86+-10.96 |
| *Pieris brassicae* | WAG | 4 | 0.82 +- 0.99 | 0.85 +- 1.71 | 36.99 +- 48.7 | 0 |
| *Pieris cheiranth-* | La Palma | 4 | 25.19 +- 9.99 | 3.29 +- 1.9 | 2.9 +- 2.87 | 0 |
| *Pontia daplidice* | PYR | 6 | 2.86 +- 5.81 | 4.62 +- 2.90 | 0.88 +- 1.61 | 0 |
| *Anthocharis cardamine* | WAG | 2 | 25.84 +- 16.45 | 1.65 +- 0.74 | 0.94 + 1.32- | 0 |
| *Euchloe eversi* | Tenerife | 2 | 15.72 +-1.79 | 0 | 0.43 +- 0.24 | 0 |
| *Aporia crataegi* | JUR | 3 | 2.43 +- 1.99 | 0 | 1.81 +-1.411 | 0 |
| *Colias croceus* | COB | 7 | 1.99 +- 2.57 | 4.84 +- 3.48 | 0 | 0 |
| *Colias palaeno* | ABS | 4 | 1.81 +- 2.72 | 3.48 +- 6.30 | 0 | 0 |
| *Leptidea sinapis* | STK, JUR | 11 | 0.65 +- 0.93 | 5.01+- 3.88 | 0 | 0 |

**Table S6.4***) Pieris napi* populations post-mating odors

| Locations | N odor samples | N mating rate | Mean ng +- SD MesA | Mean ng +- SD Guaia | Mean ng +- SD BC | Mean ng +- SD Indole |
|---|---|---|---|---|---|---|
| ABS | 18 | 25 | 42.01+- 58.29 | 2.63+- 1.4 | 11.68+- 15.39 | 12.77 +- 10.89 |
| STK | 15 | 2 | 25.87 +- 75.35 | 2.75 +- 2.31 | 31.65 +- 57.31 | 20.87 +- 11.12 |
| WAG | 16 | 16 | 67.29 +- 72.8 | 9.84 +- 9.44 | 49.34 +- 64.47 | 33.17 +- 48.33 |
| JUR | 8 | 12 | 2.78 +- 1.07 | 0 | 5.88 +- 8.35 | 6.12 +- 14.2 |
| BRY | 13 | 5 | 4.21 +- 3.17 | 0 | 4.44 +- 3.51 | 0 |
| COB | 14 | 18 | 19.74 +- 49.13 | 2.86 +- 3.3 | 13.02 +- 17.65 | 15.93 +- 8.85 |
| PYR | 10 | 3 | 4.04 +- 2.61 | 6.27 +- 3.15 | 6.23 +- 6.09 | 0 |

**Table S6.5)** *Pieris napi* and *Trichogramma* sampled per location

| Location | 2022 | | | | 2023 | | | | Phoretic *trichogramma*? | Total #wass / P. napi |
|---|---|---|---|---|---|---|---|---|---|---|
| | # P. napi adults | # eggs | # *Trichogramma* | Sampling days | # P. napi adults | # eggs | # *Trichogramma* | Sampling days | | |
| ABS | 224 | 630 | 7 | 9 | 31 | 85 | 5 | 3 | N | 0.047 |
| STK | 32 | 0 | 1 | 9 | 26 | 46 | 1 | 2 | Y (1) | 0.017 |
| WAG | 62 | 119 | 32 | 11 | 7 | 0 | 13 | 2 | Y (3) | 0.65 |
| JUR | 10 | 10 | 0 | 2 | 21 | 81 | 0 | 5 | N | 0 |
| BRY | 19 | 10 | 0 | 2 | 61 | 81 | 0 | 5 | N | 0 |
| COB | 115 | 355 | 59 | 9 | 38 | 214 | 25 | 5 | Y (2) | |
| PYR | 26 | 14 | 0 | 5 | 26 | 10 | 0 | 2 | N | 0 |

## S6.4 Genome editing and mutant screening

### *S6.4.1 OR45b identification and phylogeny*

The identification of OR45b as a conserved receptor in *Pieris*, described in detail in Wang *et al.* (2023). The protein sequence of OR45b used in this study was obtained from Wang *et al. (2023)*, who identified olfactory receptor (OR) genes in *Pieris brassicae* through transcriptomic analysis of larval head tissues. Briefly, they performed de novo RNA sequencing of L3 caterpillar heads and quantified OR expression across developmental stages using qPCR. Total RNA was isolated from pooled larval and adult antennae, and transcriptomes were assembled by mapping reads to the *P. brassicae* genome (GCA_942653925) using Hisat2 and StringTie. Candidate chemoreceptors were identified through homology searches (DIAMOND) using known OR sequences from related *Pieris* species, and expression levels were calculated as transcripts per million (TPM). OR45b was among the deorphanized receptors identified in this dataset. The amino acid sequence for OR45b retrieved from this dataset was used as the reference in our BLAST searches, phylogenetic analyses, and subsequent CRISPR knockout experiments.

The phylogenetic tree was constructed in Geneious prime 2023.0.4using the amino acid sequences of OR45b and OR45a from Wang *et al.*, 2023 for *Pieris brasice, P. rapae, P. napi*, and *P. macdonoughii*. *P. brassicae* ORCO was used as the outgroup. The sequence of OR45b from P. brassicae was input into NCBI protein blast filtered for all Pieridae and all Nymphalidae separately. The top 50 matches from both the Pieridae and Nymphalidae results were aligned with the eight Pieris OR45a and b sequences and the ORCO outgroup with MAFFT (Katoh *et al.*, 2009) with default settings, and trimmed to remove any partial sequences and perfect alignment at both ends. A neighbor-joining tree with 1000 bootstraps was used to construct a consensus tree with consensus values at the nodes.

The tree was edited in Figtree to denote the taxonomic classification of the species from which the sequences came, and the clades of olfactory receptors named by the receptors in the clade which had already been de-orphanized in at least one of the species included in the clade.

***S6.4.2 CRISPR***

The first exon of *OR45b* was targeted to knock out the gene. The sgRNA was designed with Geneious Prime v10.0.9 (Geneious, New Zealand) by searching -17bp-NGG sequences. The designated sgRNA was evaluated by Exonerate 2.0 [38] searching against the genome by the est2genome function with a loose cut-off of 50 scores. sgRNA template was synthesized by mixing 5 µl of sgRNA primer (ATTTAGGTGACACTATATTTTTTGGTGTTCCATGGCCGTTTTAGAGCTAGAAATAGCAAG), 5 µl of constant primer (AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGC

CTTATTTTAACTTGCTATTTCTAGCTCTAAAAC), 50 µl of Q5 Hot Start High-Fidelity 2 × Master Mix (NEB, USA) as well as 40 µl of $H_2O$, incubated at 98 °C for 2 min, followed by 35 cycles each at 98 °C for 20 s, 65 °C for 10 s and 72 °C for 10 s; the program ends at 72 °C for 5 min. The cycling product was purified as sgRNA template using QIAquick PCR Purification Kit (Qiagen, Germany) according to the manufacturer's instructions. sgRNA was synthesized using a MEGAscript™ SP6 Transcription Kit (Invitrogen, USA) and incubated at 37 °C for four hours followed by adding 1 µl Turbo DNase and incubating at 37 °C for 15 min. The reaction products were purified with Monarch RNA Cleanup Kit. The concentration of sgRNA was determined by DeNovix (DeNovix, USA).

Before injection, a mixture of 2 µl of 300 ng/µl sgRNA, 1 µl of EnGen Spy Cas9 NLS (NEB, USA), 1 µl of 10 × NEBuffer r3.1 and 6 µl of $H_2O$ was incubated at 25 °C for 10 min. sgRNA/Cas9 mixture was colored with 1 µl food dye as a visual marker for injected eggs before loading the mixture into the glass needle of FemtoJet (Eppendorf, Germany). Newly laid 30-min-old eggs were collected from our laboratory colony and were injected with colored sgRNA/Cas9 complex. The injected eggs were incubated at 25 °C for four-five days until hatching. Around 100 caterpillars were reared on *B. oleracea* until pupation.

***S6.4.3 Screening***

Following eclosion of the butterflies, one leg of each butterfly was then dissected to screen for mutants. The leg samples were lysed and extracted using MyTaq Extract-PCR Kit (Bioline, UK) according to manufacturer's instructions. Two microliters of extract as gDNA template were added into the PCR reaction system of 12.5 µl of 2 × MyTaq Red Mix (Bioline, UK), 2 µl of forward primer (ATGGCTAACATATCTGATACTTTTAATATC) and reverse primer (TTACTGCAATACCTTCTCATAATGCC) each and 6.5 µl of $H_2O$. The reaction system was incubated at 95 °C for 2 min followed by 38 cycles each at 95 °C for 15 s, 55 °C for 15 s

and 72 °C for 10 s; the program ends at 72 °C for 2 min. The PCR products were checked by gel electrophoresis and sequencing (Eurofins, The Netherlands). The transformed butterflies are further referred to as "knockout" or KO and the butterflies from the same rearing that did not undergo genome editing are referred to as "wildtype" or WT. The resulting mutation be found in Figure S6.3.

## S6.5 Electrophysiology

### *S6.5.1 Dose-response analysis*

To assess olfactory sensitivity across species and sexes, we performed electroantennogram (EAG) recordings on adult males and females of *Pieris brassicae*, *P. mannii*, *P. rapae*, and *P. napi*. The excised antenna was mounted between two glass capillaries filled with EAG Ringer solution (as per standard insect saline recipes), with each capillary containing a silver/silver chloride (Ag/AgCl) electrode. One electrode was connected to ground, and the other to a 10× preamplifier (Ockenfels Syntech, Germany). Signals were digitized using an IDAC-2 acquisition controller (Ockenfels Syntech) and recorded with EAG Pro software. The antenna was oriented ventral side up to maximize exposure to odor pulses.

Odor stimuli consisted of five compounds: methyl salicylate, benzyl cyanide, indole, cis-3-hexenyl acetate, and linalool (≥98% purity Sigma Aldrich). Odorants were diluted in HPLC-grade hexane to create a three-step dose series: 0.01, 0.1, and 1.0 (v/v). A 10 µL aliquot of each solution was pipetted onto a strip of filter paper inserted into a disposable pipette tip, which was then loaded into the stimulus cartridge. Each odorant was tested at all three concentrations per individual. Stimuli were presented as 0.5–1.0 s air pulses delivered via a continuous humidified air stream (1 L/min flow rate). The first puff from each newly loaded stimulus cartridge was discarded to avoid solvent front effects, and responses were recorded beginning with the second puff.

Odorant presentation order was randomized across individuals to reduce position and fatigue effects; however, concentrations were always presented in ascending order. At least 20 seconds elapsed between stimulus puffs to allow antennal recovery and baseline stabilization. Each trial included multiple control stimuli: clean air, blank filter paper, filter paper with only hexane, and empty pipette tips. All EAG responses were recorded in mV and baseline-corrected and normalized relative to the individual's response to hexane alone. The effect of dose on relative response for each species, sex, and odor was tested for significance using a linear regression with concentration as a predictor in SPSS version 28.

### *S6.5.2 Electroantennogram (EAG) recording of mutants versus wildtype*

A panel of 47 chemical compounds that are potentially involved in the tritrophic interaction including esters, alcohols, aldehydes, alkene, ITCs, heterocyclics and nitriles (Table S6.1) were selected as candidate ligands (concentration at $10^{-2}$ v/v) of OR45b based on previous literature. EAG responses were investigated for the left antennae of three-day-old males and females (n = 17-20 for both genotypes). Filter papers were inserted into Pasteur pipettes and loaded with 10 µl of a certain chemical diluted in paraffin oil, uncontaminated filter paper and paraffin oil-loaded filter paper were employed as negative controls. The left antennae of butterflies were excised at the basal end and the distal tip was also removed for better conductivity. Both antenna ends were placed in glass capillaries filled with EAG ringer solution. Ag/Ag-Cl wires were inserted into both capillaries, with one connected to a ground electrode and one connected to a 10× pre-amplifier (Ockenfels Syntech, Germany), to form an electric circuit. The electrical signals were converted from analog to digital using an IDAC-2 (Ockenfels Syntech, Germany) and sent to a personal computer. The first puff of all newly loaded chemicals was discarded and responses were recorded starting with the second puff of chemicals, the magnitude of response was recorded in mV. At least 20 seconds passed between odor puffs, to allow the antenna to recover and the signal to return to a stable baseline. Compounds were tested in random orders. The response signals were recorded using EAG Pro software (Ockenfels Syntech, Germany). The response of each chemical was calibrated by subtracting the background signal (response to uncontaminated filter paper). Differences of EAG responses to the chemicals between the two genotypes were tested by using the Student's t-test when the values were normally distributed and had similar variance, or tested by using the Kruskal-Wallis test when the data failed to meet the criteria of the Student's t-test. The results from the full panel can be found in Figure S6.3.

**Table S6.1.** Chemical compounds that were used for the electroantennographically test

| Name | CAS number | Purity | Manufacturer |
|---|---|---|---|
| Benzoic acid | 65-85-0 | ≥99.5% | Sigma-Aldrich |
| (*E*)-Anethole | 4180-23-8 | 99.0% | Sigma-Aldrich |
| 1-Pentanol | 71-41-0 | ≥ 99.0% | Sigma-Aldrich |
| (*Z*)-2-Penten-1-ol | 1576-95-0 | 95.0% | Sigma-Aldrich |
| 1-Hexanol | 111-27-3 | 98.0% | Fluka |
| (*Z*)-3-Hexen-1-ol | 928-96-1 | 98.0% | Sigma-Aldrich |
| 1-Octen-3-ol | 3391-86-4 | 98.0% | Sigma-Aldrich |
| Geraniol | 106-24-1 | 98.0% | Sigma-Aldrich |
| (*E*)-2-Hexen-1-ol | 928-95-0 | ≥ 95.0% | Sigma-Aldrich |
| Linalool | 78-70-6 | 97.0% | Sigma-Aldrich |
| 1,8-Cineole | 470-82-6 | 99.0% | Sigma-Aldrich |
| 1-Penten-3-ol | 616-25-1 | 99.0% | Sigma-Aldrich |

**Table S6.1.** *Continued*

| Name | CAS number | Purity | Manufacturer |
|---|---|---|---|
| Phenylethyl alcohol | 60-12-8 | ≥99.0 % | Fluka |
| 1-Methoxy-2-propanol | 107-98-2 | ≥ 99.5% | Sigma-Aldrich |
| 3-Pentanol | 584-02-1 | 98.0% | Sigma-Aldrich |
| 3-Methyl-1-butanol | 123-51-3 | ≥ 99.0% | Sigma-Aldrich |
| 3-Octanol | 589-98-0 | 99.0% | Sigma-Aldrich |
| Hexanal | 66-25-1 | 98.0% | Sigma-Aldrich |
| (*E*)-2-Hexenal | 6728-26-3 | 98.0% | Sigma-Aldrich |
| Benzaldehyde | 100-52-7 | ≥99.0% | Fluka |
| Phenylacetaldehyde | 122-78-1 | ≥95.0% | Fluka |
| Heptaldehyde | 111-71-7 | 95.0% | Sigma-Aldrich |
| 1-Nonanal | 124-19-6 | 95.0% | Sigma-Aldrich |
| 3-Methylbutanal | 123-51-3 | ≥ 98.5% | Fluka |
| 2-Methylbutanal | 96-17-3 | 95.0% | Sigma-Aldrich |
| (*Z*)-2-Pentenal | 1576-87-0 | 95.0% | Sigma-Aldrich |
| Nonane | 111-84-2 | 99.0% | Sigma-Aldrich |
| Limonene | 5989-27-5 | 97.0% | Sigma-Aldrich |
| *α*-Pinene | 80-56-8 | 98.0% | Sigma-Aldrich |
| *β*-Pinene | 127-91-3 | ≥ 95.0% | Sigma-Aldrich |
| Myrcene | 123-35-3 | ≥ 99.0 | Sigma-Aldrich |
| *β*-Caryophyllene | 87-44-5 | ≥ 98.0% | Sigma-Aldrich |
| Pentyl acetate | 628-63-7 | 99.0% | Sigma-Aldrich |
| Hexyl acetate | 142-92-7 | 99.0% | Sigma-Aldrich |
| (*Z*)-3-Hexenyl acetate | 3681-71-8 | 98.0% | Sigma-Aldrich |
| Methyl salicylate | 119-36-8 | ≥ 98.0% | Sigma-Aldrich |
| Indole | 120-72-9 | ≥ 99.0% | Sigma-Aldrich |
| Benzothiazole | 95-16-9 | ≥ 96.0% | Sigma-Aldrich |
| Allyl ITC | 57-06-7 | ≥ 95.0% | Sigma-Aldrich |
| Benzyl ITC | 622-78-6 | 98.0% | Sigma-Aldrich |
| Methyl ITC | 556-61-6 | 97.0% | Fluka |
| Butyl ITC | 592-82-5 | 99.0% | Sigma-Aldrich |
| Phenyl ITC | 103-72-0 | 98.0% | Sigma-Aldrich |
| 3-Hydroxy-2-butanone | 513-86-0 | ≥ 98.0% | Sigma-Aldrich |
| 3-Octanone | 106-68-3 | ≥ 98.0% | Sigma-Aldrich |
| Benzyl cyanide | 140-29-4 | 98.0% | Sigma-Aldrich |
| Paraffin oil | 8012-95-1 | - | Sigma-Aldrich |

## S6.6 Behavior assays

### *S6.6.1 Y-tube*

To assess the attraction of *Trichogramma* to methyl salicylate, bioassays with three strains of *T. a evanescens*, *T. semblidis*, and *T. cacoeciae*, all collected in Wageningen, were conducted using a Y-tube olfactometer. similar to the one described by Fatouros et al.(2014). Air was filtered through activated charcoal and humidified by passing through a bottle with tap water before entering the system. The air was split into two, each tube leading into a glass container (500 ml) with an odour source of either a virgin or mated female from Wageningen as described in section 1.3.2. From the glass containers containing the odour source a tube led into one of the arms of a glass Y-tube olfactometer (stem cm, arms cm, cm). The Y-tube was mounted on a board with an incline so that the wasps would walk upwards. The airflow was set at 300 mL min−1 in each arm using flow meters. The bioassays were carried out in the laboratory of Wageningen University between 11:00 and 17:00 at room temperature. One diffused uniform light source was situated above the Y-tube set-up and was kept centred directly over the y-tube.

The butterflies were introduced in the glass container which were shut using a Viton O-ring and a metal clamp. After introducing the butterflies in the containers, their odor was allowed to distribute through the system for 2 minutes. Air flow first passed through an activated carbon filter, then through distilled water and finally entered the Y-tube at 300 ml/min. The olfactometer was thoroughly washed with ethanol, rinsed with water, and air-dried at room temperature after every test day.

Naïve female wasps of 3-5 days old were well-fed and had not yet had oviposition experience. 1 hour before testing, female wasps were separated from the rearing and kept in plastic tubes with a drop of honey. Ten female wasps were released in the glass Y-tube olfactometer (Table S6.6). They could select to walk toward the side of the Y-tube with air flow from the glass container housing two mated female butterflies vs. the side of the Y-tube with two virgin females. After 20- 30 minutes, the wasps collected in each of the trapping bulbs were counted. When a wasp did not make a choice within 30 minutes, not crossing the redline indicted in figure S6.1, it was recorded as a “no response” and excluded from the statistical analysis. Each wasp was used only once to test for innate response. To exclude any bias and directional effects, the position of the tubes leading from the glass containers containing the odour source to the Y-tube olfactometer, was exchanged after every 2nd trial.

In the following assays (Table S6.7), these methods were repeated using *Trichogramma cacoeciae* from Wageningen were tested between two odor sources: clean air or mated

butterflies from one of the populations, either WAG, COB, JUR, or PYR. They were tested using the same protocol described above.

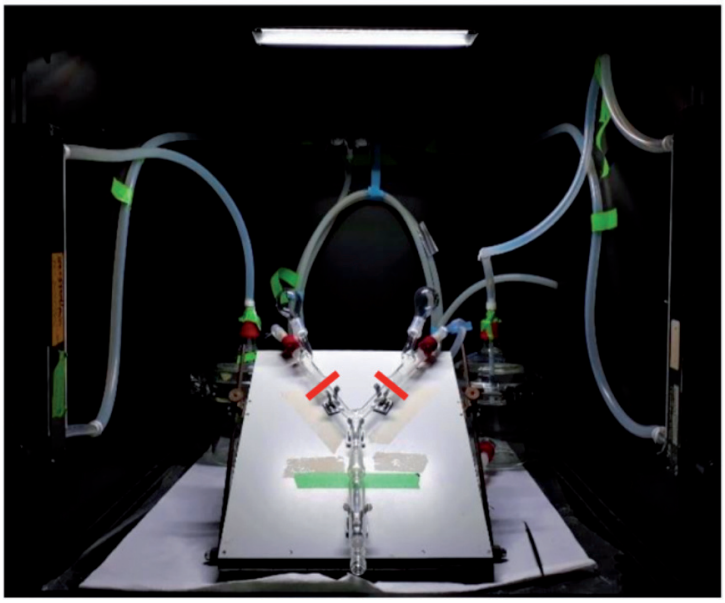


**Figure S6.2)** Y-tube olfactometer set-up. Test odors collect in bulbs, towards which wasps may walk. Red bars represent the divide between "response" and "no response".

### *S6.6.2 Static olfactometer*

In order to explore the influence of *OR45b* on mating frequency and consequently on attractiveness of both butterfly genotypes to *T. evanescens* wasps, the following experiment was conducted: we used either freely mated or virgin WT butterfly females and freely mated or virgin KO butterfly females as odor sources in this experiment. Similar to the oviposition experiment (1), butterflies were allowed to mate freely with others of the same genotype. All butterflies were placed in mesh cages immediately after eclosion. For the mated butterfly treatment, 15 female butterflies were placed in a single cage together with 30 males of the same age and genotype and allowed to mate freely without cabbage plant. For the virgin female treatment, 15 newly eclosed virgin females were placed in a cage together and not allowed to have contact with males for 10 days before testing. Tests were performed 10 days after eclosion with both mated and virgin WT or KO females. Mating rates for butterflies in these tests can be found in Figure S6.9.

To investigate behavioral responses of the wasps to butterfly odors, we used a two-chamber olfactometer (18 cm high × 12 cm in diameter) described previously *(Fatouros et al., 2005, Huigens et al., 2009)*. Two butterflies from one of the four treatments were

placed in one chamber of the olfactometer while the other chamber was kept empty. We specifically used two butterflies; they would fly and trigger each other to exhibit the "mate-refusal posture", potentially emitting benzyl cyanide *(Andersson et al., 2003)*. Data was only used when mated butterflies adopted the mate-refusal posture at least once per wasp trial. Butterflies were placed at the bottom of the chamber and the top was covered with fine mesh. On top of the mesh we placed a single, mated and honey-fed female *T. evanescens* that had eclosed within the previous 3 days and had no prior oviposition experience. To close the olfactometer, a glass lid was placed on top so that the parasitoid was able to walk around on the mesh and on the lid above the two chambers. The wasp was released in the center between the two chambers, and a 300 second timer was started immediately. The number of seconds that the parasitoid spent on the side of the chamber with butterflies within 300 seconds was recorded. Each butterfly pair (n = 9 for both WT and *OR45b* KO mated butterflies, n = 10 for WT virgin and n = 12 for *OR45b* KO virgin butterflies) was tested with three individual egg parasitoids, with each wasp (n = 27-30) being used for only a single trial. Between each of the three trials, the olfactometer was rotated 90 degrees. After three trials, a new butterfly pair was used from a randomly selected treatment. The chamber was opened to allow the air within to be refreshed between butterfly pairs. The difference of egg parasitoid preference to the butterflies among the treatments was tested using one-way ANOVA followed by Tukey's post-hoc test.

#### *S6.6.4 Mating rates in the field*

From each population, 5 – 10 butterflies used for odor collection were frozen and later dissected using a scalpel and pointy forceps. Wings and head were discarded. The abdomen was carefully opened and the bursa copulatrix was isolated. With pointy forceps, the bursa copulatrix was carefully opened, and spermatophores were isolated. Mating frequency was established by counting the number of spermatophores present in the bursa copulatrix.

#### *S6.6.5 Egg laying dynamics of mutant versus wildtype butterflies*

To compare the egg-laying behavior of the two genotypes, we designed three different experiments. The results are in Figure S6.6.

(1) In order to determine whether *OR45b* has an effect on mating and egg-laying behavior, a single pair of newly emerged male and female butterflies of the same genotype were put into individual cages (40 cm × 40 cm ×60 cm) so that the butterflies could mate freely during the experiment (n = 14 for both genotypes). The pairs were supplied with a four-week-old Brussels sprouts plant for oviposition and honey water for food. The butterflies were allowed to mate freely and repeatedly and were exposed to the same plant during the entire experimental period of eight days. The number of eggs per butterfly

pair was counted daily for seven days from Day 0 to Day 7, with the day that butterflies were placed in the cages marked as Day 0. The eggs were allowed to hatch over the following week in order to measure the hatching rate and to determine whether or not the eggs had been fertilized. Immediately at the end of the eighth day, the butterflies were collected and frozen for dissection. The spermatophores were removed and counted from each butterfly to compare the number of times both WT females and KO females mated during eight days. All female butterflies were collected and frozen at -20 °C. The frozen butterflies were then dissected under 20× magnification to count the number of spermatophores present in the bursa copulatrix, as the number of spermatophores is equivalent to the number of successful copulations. The bursa copulatrix was removed from the abdomen and carefully opened so that the spermatophores within it could be counted (Figure S6.8). The fullness of the spermatophore was ranked as follows: a. full, b. partially full, or c. empty. A spermatophore was considered as full (a) when its envelope was taut due to the high volume of its contents which completely filled the envelope. This state indicates that copulation was recent, as spermatophore digestion begins approximately 24 hours post mating. An empty spermatophore (c), for which only the envelope is present and the contents within have been fully digested and thus are no longer visible, indicates that copulation had occurred at least two days prior to dissection. Anything in between these states was considered partially full (b).

(2) In order to determine if the effect of the knockout on oviposition was linked to differences found in mating frequency, we compared the number of eggs laid by couples of the same genotype when butterflies were only allowed to mate once. To control for mating frequency, three-day-old virgin females were put together with three-day-old virgin males in a cage (40 cm × 40 cm ×60 cm). Upon mating, pairs were moved to a new cage, and once they separated, the male was removed entirely. Therefore, each cage contained either a single WT female (n = 17) or KO female (n = 19) that had mated once. Then each mated female butterfly was placed in a cage supplied with a cabbage plant and honeywater as described above. The number of eggs laid per female and the number of newly hatched caterpillars were counted every day.

(3) Finally, in order to determine whether effects on egg laying and mating resulted from changes in the KO female, or the KO male, or both, we further crossed the mutant butterflies and wildtype butterflies and conducted the same experiment as above. Each cage (40 cm × 40 cm ×60 cm) had a single pair that consisted of either a WT female and a KO male (n = 10) or a KO female and a WT male (n = 10), and butterflies were allowed to mate freely during the experiment. The female butterflies were collected and dissected, and the number of eggs per pair and newly hatched caterpillars was counted as we described above.

In all three experiments, differences in egg-laying dynamics between genotypes (WT and KO) or treatments (cross-mating) were tested by using a general linear model (GLM) with negative binominal distribution. Differences of number of eggs hatching, egg hatching rate and number of matings between genotypes (WT and KO) or treatments (cross-mating) were tested by using Student's t-test when the date was normally distributed and has equal variance, otherwise differences were tested using the Wilcoxon rank-sum test when the criteria of the Student's t-test did not apply.

***S6.6.6 Oviposition choice of mutant versus wildtype butterflies***

An oviposition preference assay was conducted to evaluate whether WT and KO female butterflies respond differently to the odors of egg-infested host plants (Figure S6.7). In short, *Brassica oleracea* var. *gemmifera* Cyrus plants were infested with 7 - 10 egg clutches by WT butterflies. Singly mated butterflies of both genotypes were prepared 24-48 hours prior to the behavioral test as described above for oviposition experiment (2). Plants were infested with eggs 72 hours beforehand to obtain infested plants before starting the tests. Just prior to the test, the egg clutches were gently removed from the plants (subsequently called egg-infested) and any possible damage caused by this removal was mimicked on the control plant (called non-infested). The two plants were grown from the same batch of seeds and under the same environmental conditions. A previously egg-infested and a non-infested plant were then placed at the far end of a mesh cage (1.8 m ×1.8 m ×1.8 m). A female butterfly was released from the opposite side of the cage halfway between the two plants. The number of seconds until the butterfly landed on a plant and the plant identity, were recorded. Then, the number of seconds until an egg was laid on a certain plant, was also recorded. If no egg was laid within 15 minutes, the butterfly was considered a non-responder. Butterflies (n = 41 for each genotype) were tested one time on a pair of plants (n = 18), and each pair of plants was used for a maximum of 6 butterflies. The side of the cage which had the treatment or control plant was alternated between every butterfly tested, the genotype of butterflies was also alternated between WT and KO females. The difference in the time that butterflies spent on selecting a cabbage plant for oviposition was tested by Wilcoxon rank-sum test. The difference in butterfly oviposition-site selection between the two genotypes was tested by using a Chi-square test.

***S6.6.7 Predation***

The experiments were conducted in the same cages in which the great tits (*Parus major*) were housed (Figure S6.2). Prior to the start of each trial, external feeder trays and water bathing dishes were removed. A wooden cover plate was inserted and placed on top of the cage bedding to reduce potential external influencing factors (e.g., residual food in the bedding) and to ensure that only the test objects were present in the environment.

To assess whether great tits exhibited a preference for butterflies lacking the odor benzyl cyanide, a two-choice assay was employed. In each trial, birds were presented with one control butterfly (treated with hexane only) and one treated butterfly (hexane plus benzyl cyanide at one of two concentrations). The butterflies used were recently frozen wild-type virgin females from the rearing and treated according to the following protocols: 1. Control: A virgin female pipetted with 10 µL of hexane. 2. Treatment: A virgin female pipetted with 10 µL of a benzyl cyanide solution in hexane. The total amount of benzyl cyanide applied ranged between 0.01 µg and 0.1 µg. All solutions were applied to the thorax and abdomen.

Butterflies were placed next to each other in the cage, equidistant from the back and front walls. Each was positioned in a small petri dish containing non-toxic green polymer oven-bake clay (DécoTime) as a background for contrast and to record peck marks from the birds. The hindwings were gently pressed into the clay to hold the butterfly in an upright position, displaying both fore- and hindwing spots in a typical Pieridae deterrence posture. Upon introduction of the petri dishes into the cage, researchers exited the room to minimize disturbance. Bird behavior was recorded using a GoPro Hero 5 (GoPro Inc., San Mateo, USA) camera at 25 fps and 720p resolution. Each bird was given five minutes to interact with the butterflies. Following the trial, any butterfly remains were removed and stored at –20 °C for later analysis.

Each bird underwent two trials across the course of the experiment, conducted on alternating days using the same setup and treatment combinations. To control for side bias, the positions of the treatment and control butterflies were switched between trials. The bird's first choice was recorded as the first butterfly taken into the beak. Birds that did not make a choice within the trial period were excluded from further analysis. The latency to attack (time until the butterfly was taken into the beak) was recorded as a proxy for repellence.

All procedures were conducted in accordance with ethical standards. No harm was inflicted on the great tits, and no invasive or unethical interventions were performed. Experimental protocols were approved and supervised by Kees van Oers of the Ethics Committee of NIOO-KNAW. The license for the study was issued under number NIOO 23.07 (AVD 80100 2019 9005) / IvD 3050 on 07-11-2023.



**Figure S6.3)** Experimental set up for predator choice assays with *Parus major* and dead, virgin *Pieris brassicae* with or without benzyl cyanide applied to abdomen.

# Supplementary Results

## S6.7 Post-mating odor emission is not species-specific in Pieridae (Figure 6.1)

**Table S6.2** Mated versus virgin *P. napi* Wageningen samples

| Odor | Mated mean +- sd ng | N mated | Virgin mean +- sd ng | N virgin | Test | U | P-value |
|---|---|---|---|---|---|---|---|
| Methyl salicylate | 69.14 +-63.17 | 10 | 19.88 +- 26.97 | 21 | Mann-whitney U | 12 | <0.001 |
| Guaiacol | 11.69 +- 10.49 | 10 | 4.21 +- 6.52 | 21 | Mann-whitney U | 9.5 | <0.001 |
| Benzyl cyanide | 102.15 +- 108.37 | 10 | 25.7 +-43.72 | 21 | Mann-whitney U | 6.5 | <0.001 |
| Indole | 40.57 +- 30.21 | 10 | 23.71 +- 23.42 | 21 | Mann-whitney U | 38 | 0.001 |

**Table S6.3)** Pieridae post-mating odors samples

| Species | Locations | N | Mean ng +- SD MesA | Mean ng +- SD Guaia | Mean ng +- SD BC | Mean ng +- SD Indole |
|---|---|---|---|---|---|---|
| *Pieris napi* | WAG | 16 | 67.29 +- 72.8 | 9.84 +- 9.44 | 49.34 +- 64.47 | 33.17 +- 48.33 |
| *Pieris bryoniae* | JUR | 13 | 4.21+- 3.17 | 0 | 4.44+- 3.51 | 0 |
| *Pieris rapae* | WAG, COB, PYR | 5 | 3.97 +- 4.25 | 0 | 0.79+-0.83 | 32.9 +- 22.8 |
| *Pieris mannii* | WAG | 8 | 9.89+-12.38 | 0 | 0 | 15.86+-10.96 |
| *Pieris brassicae* | WAG | 4 | 0.82 +- 0.99 | 0.85 +- 1.71 | 36.99 +- 48.7 | 0 |
| *Pieris cheiranth-* | La Palma | 4 | 25.19 +- 9.99 | 3.29 +- 1.9 | 2.9 +- 2.87 | 0 |
| *Pontia daplidice* | PYR | 6 | 2.86 +- 5.81 | 4.62 +- 2.90 | 0.88 +- 1.61 | 0 |
| *Anthocharis cardamine* | WAG | 2 | 25.84 +- 16.45 | 1.65 +- 0.74 | 0.94 + 1.32- | 0 |
| *Euchloe eversi* | Tenerife | 2 | 15.72 +-1.79 | 0 | 0.43 +- 0.24 | 0 |
| *Aporia crataegi* | JUR | 3 | 2.43 +- 1.99 | 0 | 1.81 +-1.411 | 0 |
| *Colias croceus* | COB | 7 | 1.99 +- 2.57 | 4.84 +- 3.48 | 0 | 0 |
| *Colias palaeno* | ABS | 4 | 1.81 +- 2.72 | 3.48 +- 6.30 | 0 | 0 |
| *Leptidea sinapis* | STK, JUR | 11 | 0.65 +- 0.93 | 5.01+- 3.88 | 0 | 0 |

## S6.8 Intraspecific variation in *Pieris napi* post-mating odors is shaped by parasitoid pressure rather than geography or genetic distance (Figure 6.2)

**Table S6.4)** *Pieris napi* populations post-mating odors

| Locations | N odor samples | N mating rate | Mean ng +- SD MesA | Mean ng +- SD Guaia | Mean ng +- SD BC | Mean ng +- SD Indole |
|---|---|---|---|---|---|---|
| ABS | 18 | 25 | 42.01+- 58.29 | 2.63+- 1.4 | 11.68+- 15.39 | 12.77 +- 10.89 |
| STK | 15 | 2 | 25.87 +- 75.35 | 2.75 +- 2.31 | 31.65 +- 57.31 | 20.87 +- 11.12 |
| WAG | 16 | 16 | 67.29 +- 72.8 | 9.84 +- 9.44 | 49.34 +- 64.47 | 33.17 +- 48.33 |
| JUR | 8 | 12 | 2.78 +- 1.07 | 0 | 5.88 +- 8.35 | 6.12 +- 14.2 |
| BRY | 13 | 5 | 4.21 +- 3.17 | 0 | 4.44 +- 3.51 | 0 |
| COB | 14 | 18 | 19.74 +- 49.13 | 2.86 +- 3.3 | 13.02 +- 17.65 | 15.93 +- 8.85 |
| PYR | 10 | 3 | 4.04 +- 2.61 | 6.27 +- 3.15 | 6.23 +- 6.09 | 0 |

**Table S6.5)** *Pieris napi* and *Trichogramma* sampled per location

| Location | 2022 | | | | 2023 | | | | Phoretic *Trichogramma*? | Total #wass / P. napi |
|---|---|---|---|---|---|---|---|---|---|---|
| | # P. napi adults | # eggs | # *Trichogramma* | Sampling days | # P. napi adults | # eggs | # *Trichogramma* | Sampling days | | |
| ABS | 224 | 630 | 7 | 9 | 31 | 85 | 5 | 3 | N | 0.047 |
| STK | 32 | 0 | 1 | 9 | 26 | 46 | 1 | 2 | Y (1) | 0.017 |
| WAG | 62 | 119 | 32 | 11 | 7 | 0 | 13 | 2 | Y (3) | 0.65 |
| JUR | 10 | 10 | 0 | 2 | 21 | 81 | 0 | 5 | N | 0 |
| BRY | 19 | 10 | 0 | 2 | 61 | 81 | 0 | 5 | N | 0 |
| COB | 115 | 355 | 59 | 9 | 38 | 214 | 25 | 5 | Y (2) | |
| PYR | 26 | 14 | 0 | 5 | 26 | 10 | 0 | 2 | N | 0 |

**Table S6.6)** *Trichogramma* species comparison in y-tube

| Line | Species | N (10 wasps per trial) | Mean % to mated female | One-sample t test | One sided P-value |
|---|---|---|---|---|---|
| 16_ben_20 | *T. evanescens* | 6 | 55.11 | .817 | 0.226 |
| 64_ eng_20 | *T. evanescens* | 5 | 58.15 | .762 | 0.240 |
| 69_eng_20 | *T. evanescens* | 6 | 55.07 | .725 | 0.248 |
| 18_eng_21 | T. semblidis | 6 | 55.73 | .734 | 0.248 |
| 6_gre_21 | T. cacoeciae | 6 | 66.35 | 2.255 | 0.037 |

**Table S6.7)** *Trichogramma cacoeciae* response to different *Piers napi* populations

| Butterfly population | N (10 wasps per trial) | Mean % to mated female | One-sample t test | One sided P-value |
|---|---|---|---|---|
| WAG | 16 | 62 | 1.76 | 0.04 |
| JUR | 7 | 50 | 0.04 | 0.97 |
| COB | 10 | 73 | 4.46 | 0.002 |
| PYR | 25 | 50 | .063 | 0.475 |

## S6.9 Interspecific variation and genetic basis of olfactory responses to post-mating odors (Figure 6.3)

**Table S6.8** Dose response EAG stats linear regression

| odor | species | sex | N (for three concentrations) |
|---|---|---|---|
| BC | *P. brassicae* | male | 27 |
| | *P. brassicae* | female | 39 |
| | *P. napi* | male | 30 |
| | *P. napi* | female | 36 |
| | *P. rapae* | male | 36 |
| | *P. rapae* | female | 27 |
| | *P. mannii* | male | 18 |
| | *P. mannii* | female | 23 |
| MeSa | *P. brassicae* | male | 27 |
| | *P. brassicae* | female | 39 |
| | *P. napi* | male | 30 |
| | *P. napi* | female | 36 |
| | *P. rapae* | male | 35 |
| | *P. rapae* | female | 27 |
| | *P. mannii* | male | 18 |
| | *P. mannii* | female | 24 |
| Indole | *P. brassicae* | male | 27 |
| | *P. brassicae* | female | 38 |
| | *P. napi* | male | 30 |
| | *P. napi* | female | 36 |
| | *P. rapae* | male | 36 |
| | *P. rapae* | female | 27 |
| | *P. mannii* | male | 18 |
| | *P. mannii* | female | 25 |
| Linalool | *P. brassicae* | male | 27 |
| | *P. brassicae* | female | 39 |
| | *P. napi* | male | 30 |
| | *P. napi* | female | 36 |
| | *P. rapae* | male | 34 |
| | *P. rapae* | female | 27 |
| | *P. mannii* | male | 18 |
| | *P. mannii* | female | 24 |

**Table S6.8** *Continued*

| odor | species | sex | N (for three concentrations) |
|---|---|---|---|
| Cis-3 | *P. brassicae* | male | 27 |
| | *P. brassicae* | female | 39 |
| | *P. napi* | male | 30 |
| | *P. napi* | female | 36 |
| | *P. rapae* | male | 35 |
| | *P. rapae* | female | 27 |
| | *P. mannii* | male | 18 |
| | *P. mannii* | female | 24 |

**Table S6.9)** Coeffieicents table of regression of dose-response EAGS in *Pieris*

| | | | | | B | Std. Error | Beta | | |
|---|---|---|---|---|---|---|---|---|---|
| bc | female | *P. brassicae* | 1 | (Constant) | 0.287 | 0.115 | | 2.484 | 0.018 |
| | | | | Concentration | 0.900 | 0.199 | 0.597 | 4.527 | **0.000** |
| | | *P. mannii* | 1 | (Constant) | 0.709 | 0.294 | | 2.408 | 0.025 |
| | | | | Concentration | 0.692 | 0.496 | 0.291 | 1.394 | 0.178 |
| | | *P. napi* | 1 | (Constant) | 0.384 | 0.214 | | 1.792 | 0.082 |
| | | | | Concentration | 1.117 | 0.369 | 0.461 | 3.026 | **0.005** |
| | | *P. rapae* | 1 | (Constant) | 0.116 | 0.132 | | 0.881 | 0.387 |
| | | | | Concentration | 1.055 | 0.227 | 0.681 | 4.645 | **0.000** |
| | male | *P. brassicae* | 1 | (Constant) | 0.523 | 0.221 | | 2.368 | 0.026 |
| | | | | Concentration | 1.149 | 0.381 | 0.516 | 3.016 | **0.006** |
| | | *P. mannii* | 1 | (Constant) | 0.375 | 0.199 | | 1.883 | 0.078 |
| | | | | Concentration | 0.810 | 0.344 | 0.508 | 2.358 | **0.031** |
| | | *P. napi* | 1 | (Constant) | 0.490 | 0.164 | | 2.993 | 0.006 |
| | | | | Concentration | 0.722 | 0.282 | 0.436 | 2.560 | **0.016** |
| | | *P. rapae* | 1 | (Constant) | 0.169 | 0.083 | | 2.047 | 0.048 |
| | | | | Concentration | 0.984 | 0.156 | 0.735 | 6.316 | **0.000** |

**Table S6.9)** *Continued*

| | | | | | B | Std. Error | Beta | | |
|---|---|---|---|---|---|---|---|---|---|
| ci | female | *P. brassicae* | 1 | (Constant) | 0.029 | 0.053 | | 0.548 | 0.587 |
| | | | | Concentration | 0.102 | 0.091 | 0.180 | 1.116 | 0.272 |
| | | *P. mannii* | 1 | (Constant) | 0.313 | 0.246 | | 1.276 | 0.215 |
| | | | | Concentration | 0.771 | 0.423 | 0.362 | 1.821 | 0.082 |
| | | *P. napi* | 1 | (Constant) | 0.197 | 0.111 | | 1.783 | 0.084 |
| | | | | Concentration | 0.267 | 0.191 | 0.233 | 1.399 | 0.171 |
| | | *P. rapae* | 1 | (Constant) | 0.054 | 0.069 | | 0.774 | 0.446 |
| | | | | Concentration | 0.144 | 0.120 | 0.234 | 1.204 | 0.240 |
| | male | *P. brassicae* | 1 | (Constant) | 0.200 | 0.119 | | 1.682 | 0.105 |
| | | | | Concentration | 0.243 | 0.205 | 0.231 | 1.186 | 0.247 |
| | | *P. mannii* | 1 | (Constant) | 0.252 | 0.198 | | 1.273 | 0.221 |
| | | | | Concentration | 0.164 | 0.341 | 0.119 | 0.481 | 0.637 |
| | | *P. napi* | 1 | (Constant) | 0.360 | 0.143 | | 2.521 | 0.018 |
| | | | | Concentration | 0.280 | 0.246 | 0.210 | 1.135 | 0.266 |
| | | *P. rapae* | 1 | (Constant) | 0.222 | 0.058 | | 3.842 | 0.001 |
| | | | | Concentration | 0.157 | 0.107 | 0.247 | 1.465 | 0.152 |
| in | female | *P. bras* | 1 | (Constant) | 0.096 | 0.070 | | 1.359 | 0.182 |
| | | | | Concentration | 0.276 | 0.125 | 0.347 | 2.217 | **0.033** |
| | | *P. mann* | 1 | (Constant) | 0.290 | 0.274 | | 1.056 | 0.302 |
| | | | | Concentration | 0.584 | 0.482 | 0.245 | 1.211 | 0.238 |
| | | *P. napi* | 1 | (Constant) | 0.259 | 0.102 | | 2.530 | 0.016 |
| | | | | Concentration | 0.165 | 0.176 | 0.159 | 0.938 | 0.355 |
| | | *P. rapa* | 1 | (Constant) | 0.079 | 0.055 | | 1.435 | 0.164 |
| | | | | Concentration | 0.068 | 0.095 | 0.142 | 0.715 | 0.481 |
| | male | *P. bras* | 1 | (Constant) | 0.089 | 0.109 | | 0.820 | 0.420 |
| | | | | Concentration | 0.376 | 0.188 | 0.371 | 1.996 | 0.057 |
| | | *P. mann* | 1 | (Constant) | 0.167 | 0.138 | | 1.209 | 0.244 |
| | | | | Concentration | 0.283 | 0.238 | 0.285 | 1.190 | 0.251 |
| | | *P. napi* | 1 | (Constant) | 0.311 | 0.119 | | 2.608 | 0.014 |
| | | | | Concentration | 0.366 | 0.206 | 0.318 | 1.776 | 0.087 |
| | | *P. rapa* | 1 | (Constant) | 0.121 | 0.060 | | 1.995 | 0.054 |
| | | | | Concentration | 0.185 | 0.114 | 0.269 | 1.626 | 0.113 |

**Table S6.9)** *Continued*

| | | | | | B | Std. Error | Beta | | |
|---|---|---|---|---|---|---|---|---|---|
| li | female | *P. bras* | 1 | (Constant) | 0.109 | 0.071 | | 1.536 | 0.133 |
| | | | | Concentration | 0.456 | 0.122 | 0.524 | 3.743 | **0.001** |
| | | *P. mann* | 1 | (Constant) | 0.630 | 0.346 | | 1.819 | 0.082 |
| | | | | Concentration | 0.468 | 0.597 | 0.165 | 0.784 | 0.441 |
| | | *P. napi* | 1 | (Constant) | 0.299 | 0.119 | | 2.513 | 0.017 |
| | | | | Concentration | 0.496 | 0.205 | 0.383 | 2.420 | **0.021** |
| | | *P. rapa* | 1 | (Constant) | 0.240 | 0.058 | | 4.156 | 0.000 |
| | | | | Concentration | 0.153 | 0.100 | 0.293 | 1.533 | 0.138 |
| | male | P. bras | 1 | (Constant) | 0.186 | 0.118 | | 1.573 | 0.128 |
| | | | | Concentration | 0.466 | 0.204 | 0.415 | 2.282 | **0.031** |
| | | P. mann | 1 | (Constant) | 0.426 | 0.181 | | 2.351 | 0.032 |
| | | | | Concentration | 0.361 | 0.313 | 0.278 | 1.156 | 0.265 |
| | | P. napi | 1 | (Constant) | 0.465 | 0.154 | | 3.026 | 0.005 |
| | | | | Concentration | 0.410 | 0.265 | 0.281 | 1.548 | 0.133 |
| | | P. rapa | 1 | (Constant) | 0.253 | 0.086 | | 2.937 | 0.006 |
| | | | | Concentration | 0.298 | 0.158 | 0.316 | 1.885 | 0.068 |
| me | female | P. bras | 1 | (Constant) | 0.137 | 0.054 | | 2.564 | 0.015 |
| | | | | Concentration | 0.391 | 0.092 | 0.571 | 4.236 | **0.000** |
| | | P. mann | 1 | (Constant) | 0.777 | 0.395 | | 1.965 | 0.062 |
| | | | | Concentration | 0.641 | 0.681 | 0.197 | 0.940 | 0.357 |
| | | P. napi | 1 | (Constant) | 0.323 | 0.105 | | 3.066 | 0.004 |
| | | | | Concentration | 0.488 | 0.181 | 0.419 | 2.687 | **0.011** |
| | | P. rapa | 1 | (Constant) | 0.178 | 0.067 | | 2.674 | 0.013 |
| | | | | Concentration | 0.369 | 0.115 | 0.541 | 3.214 | **0.004** |
| | male | P. bras | 1 | (Constant) | 0.331 | 0.122 | | 2.715 | 0.012 |
| | | | | Concentration | 0.593 | 0.210 | 0.492 | 2.825 | **0.009** |
| | | P. mann | 1 | (Constant) | 0.346 | 0.203 | | 1.710 | 0.107 |
| | | | | Concentration | 0.454 | 0.349 | 0.309 | 1.301 | 0.212 |
| | | P. napi | 1 | (Constant) | 0.425 | 0.148 | | 2.872 | 0.008 |
| | | | | Concentration | 0.451 | 0.255 | 0.317 | 1.768 | **0.088** |
| | | P. rapa | 1 | (Constant) | 0.161 | 0.069 | | 2.323 | 0.026 |
| | | | | Concentration | 0.360 | 0.129 | 0.438 | 2.799 | **0.009** |

## S6.10 Interspecific variation and genetic basis of olfactory responses to post-mating odors (Figure 6.3)

### *S6.10.1 Genome editing and full panel EAG results*

CRISPR/Cas9 was employed to disrupt the gene coding for olfactory receptor OR45b by targeting a region in the first exon with a single guide RNA. The potential off-target sites were searched by using bioinformatic tools (Exonerate 2.0 and online website CHOPCHOP v3) *(Zhu et al., 2014; Labun et al., 2019)*. No off-target binding sites were found in the coding regions. Around 40 % of the injected eggs successfully hatched, and the caterpillars survived to butterflies. Sequencing results showed that in one mutant line two base pairs were inserted in the first exon (Figure S6.4A). Transmembrane domain predictions showed that OR45b has a seven-transmembrane domain while the genome-edited OR45b exhibits an aborted coding sequence led by an early stop codon (Figure S6.3B). A couple of mutant butterflies with the same mutation were collected to generate homozygous mutants, each generation of *OR45b* KO insects was checked by PCR and DNA sequencing to ensure the homozygous colony. The offspring of these homozygous mutants was used in our experiments.

A panel of 47 volatile chemicals were employed to assess the electrophysiological responses of both wildtype and mutant butterflies of both sexes. We found that *OR45b* KO of both males and females showed reduced EAG response to seven chemicals, including the plant volatiles 1,8-cineole, benzaldehyde, limonene, *β*-caryophyllene, methyl salicylate and 3-hydroxy-2-butanone, as well as the anti-aphrodisiac pheromone benzyl cyanide (Figure S6.4C & D). In addition, only males have reduced antennal responses to another seven chemicals: (*E*)-2-hexenal, 2-methylbutanal, *α*-pinene, indole, benzothiazole and butyl isothiocyanate (ITC) (Figure S6.4C) and only females exhibited reduced responses to five chemicals (1-octen-3-ol, 3-octanol, phenylacetaldehyde, (*Z*)-2-pentenal, phenyl ITC) (Figure S6.4D).

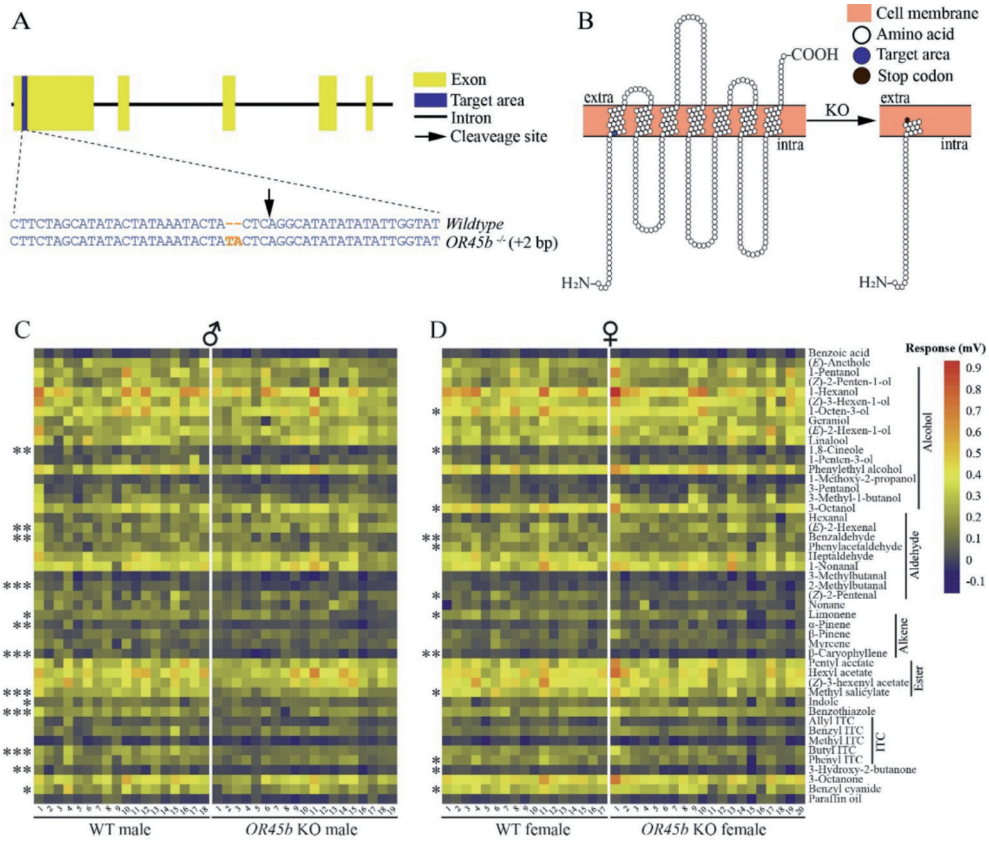



**Figure S6.4)** Odorant receptor gene *OR45b* editing and electroantennogram (EAG) responses in *Pieris brassicae* butterflies. (A) *OR45b* gene structure and mutation site. Yellow blocks indicate exons of *OR45b*, blue block indicates the area that was magnified and mutant sites were highlighted in bold orange. (B) *OR45b* gene transmembrane domain predictions in wildtype (WT) and knockout (KO) butterflies. Orange blocks indicate neuronal membrane; white circles indicate amino acids; blue circle indicates the target site; brown circle indicates the early stop codon; extra and intra are extracellular and intracellular. (C) EAG response of male butterflies from both butterfly genotypes. (D) EAG response of female butterflies from both butterfly genotypes. Chemical names are indicated on the right panel and classified into chemical classes. Significantly different response to chemicals between the two genotypes were indicated by asterisks * ($0.01 < P < 0.05$), ** ($0.001 < P < 0.01$) and *** ($P < 0.001$). Differences between WT and KO butterflies were tested by using a Student's t-test when the data was normally distributed and had similar variance or tested by using the Kruskal-Wallis test when the criteria of the Student's t-test did not apply for the data. The intensity of butterfly antennal responses is indicated by colors from dark blue (lowest) to red (highest), which is shown in the legend. N=17-20 butterflies of each sex for each genotype.

**Table S6.9)** EAG response for 5 compounds of interest in knockout versus wildtype *Pieris brassicae* (N= 17 – 20 butterflies)

| Odor | sex | test | p-value |
|---|---|---|---|
| Benzyl cyanide | Male | T-test | 0.0491 |
| | Female | T-test | 0.0242 |
| Methyl salycilate | Male | T-test | 0.0007 |
| | Female | T-test | 0.0461 |
| Indole | Male | T-test | 0.0149 |
| | Female | T-test | 0.6072 |
| Cis-3-hexynl acetate | Male | T-test | 0.05321 |
| | Female | Kruskal-wallis | 0.0511 |
| Linalool | Male | T-test | 0.7839 |
| | Female | T-test | 0.4082 |

## S6.11 Functional consequences of post-mating odor perception and emission in Pieris brassicae. (Figure 6.4)

### *S6.11.1 Butterfly egg-laying behavior and mating frequency*

To evaluate the effect of impaired olfaction on oviposition, the numbers of eggs laid by both butterfly genotypes were counted for seven days in experiment (1) where a couple of newly enclosed butterflies were placed in a cage and were allowed to mate freely. None of the butterflies deposited eggs on the first two days, Day 0 and Day 1. On Day 2 both genotypes of butterflies started laying eggs although only a few eggs were found on the plants and there was no difference between the two butterfly genotypes. From Day 3 onwards, *OR45b* KO butterflies laid significantly more eggs than WT butterflies ($P < 0.0001$, GLM negative binominal) (Figure S6.6). We further examined the number of eggs hatching between the genotypes: we found that *OR45b* KO butterflies laid significantly more fertilized eggs developing into caterpillars than WT butterflies ($P = 0.0002$, Wilcoxon rank-sum test), while egg-hatching rates were comparable between the two genotypes ($P = 0.0620$, Wilcoxon rank-sum test). Female butterflies were collected and dissected after the experiment. After dissection of the bursa copulatrix we found that *OR45b* KO butterflies tended to have mated significantly more times than WT butterflies ($P = 0.0297$, Wilcoxon rank-sum test). Interestingly, we found two full spermatophores in some of the *OR45b* KO female butterflies, indicating that mating occurred twice in quick succession, before the first spermatophore was fully or even partially digested (Figure S6.6).

We then compared the numbers of eggs laid by butterflies that had mated only once and did so for the comparison between the two butterfly genotypes in experiment (Figure S6.6). *OR45b* KO butterflies laid significantly more eggs on *B. oleracea* plants than WT

butterflies since Day 1 ($P < 0.0001$, GLM negative binominal) (Figure 6.4B). The number of hatched eggs per female was similar for once-mated WT butterflies and once-mated *OR45b* KO butterflies ($P = 0.5323$, Student's t-test). However, the proportion of eggs that hatched was lower for *OR45b* KO butterflies than for WT butterflies ($P = 0.0019$, Wilcoxon rank-sum test).

In order to determine which sex determines the higher number of eggs laid by butterflies, wildtype and mutant butterflies were crossed and butterflies were allowed to mate freely, a cabbage plant was provided as egg-laying substrate in experiment 3. The *OR45b* KO females that mated with WT males laid significantly more eggs on cabbage than WT females that mated with KO males ($P = 0.0015$, GLM negative binomial) (Figure S6.6). WT females mated with KO males and KO females mated with WT males yielded a similar number of hatching eggs ($P = 0.4238$, Wilcoxon rank-sum test) (Figure S6.6) with a similar egg-hatching rate ($P = 0.7318$, Wilcoxon rank-sum test). Female butterflies of both genotypes were then collected and dissected; dissection results showed that the female butterflies in the two combinations had mated a similar number of times ($P = 0.1360$, Wilcoxon rank-sum test).

***S6.11.2 Butterfly host-plant selection and egg parasitoid preferences***

We investigated the behavioral responses of mated female butterflies to previously egg-infested and non-infested plants. WT female butterflies spent significantly more time before the first contact to the plants than *OR45b* KO butterflies ($P = 0.0001$, Wilcoxon rank-sum test) (Figure 6.4A). WT females were slightly more attracted to non-infested plants over egg-infested plants, although this difference was not significant ($P = 0.1599$), while *OR45b* KO females did not show any bias between the two plants ($P = 0.4349$); there is no significant difference in the final host-plant selection between the two genotypes ($P = 0.1217$, Chi-square test) (Figure S6.7).

Next, the behavioral preference of *T. evanescens* wasps was tested to evaluate the role of *OR45b* in tritrophic interactions (Figure 6.4C). The wasps significantly preferred mated female butterflies that were treated differently ($P < 0.0001$, one-way ANOVA). In which, the wasps spent more time in the presence of *OR45b* KO mated females over the other butterflies ($P = 0.0001$, mated KO vs virgin KO; $P = 0.0052$, mated KO vs mated WT; $P < 0.0001$, mated KO vs virgin WT), while they did not prefer mated female WT butterflies over any of the genotypes of virgin butterflies ($P = 0.2445$, one-way ANOVA). To clarify why odors of mated *OR45b* KO female butterflies were more attractive to the egg parasitoids, we analyzed matings of both butterfly genotypes by dissecting the spermatophore. The mated female butterflies of both genotypes harbored a similar number of spermatophores ($P = 0.1396$, Wilcoxon rank-sum test) (Figure S6.9).

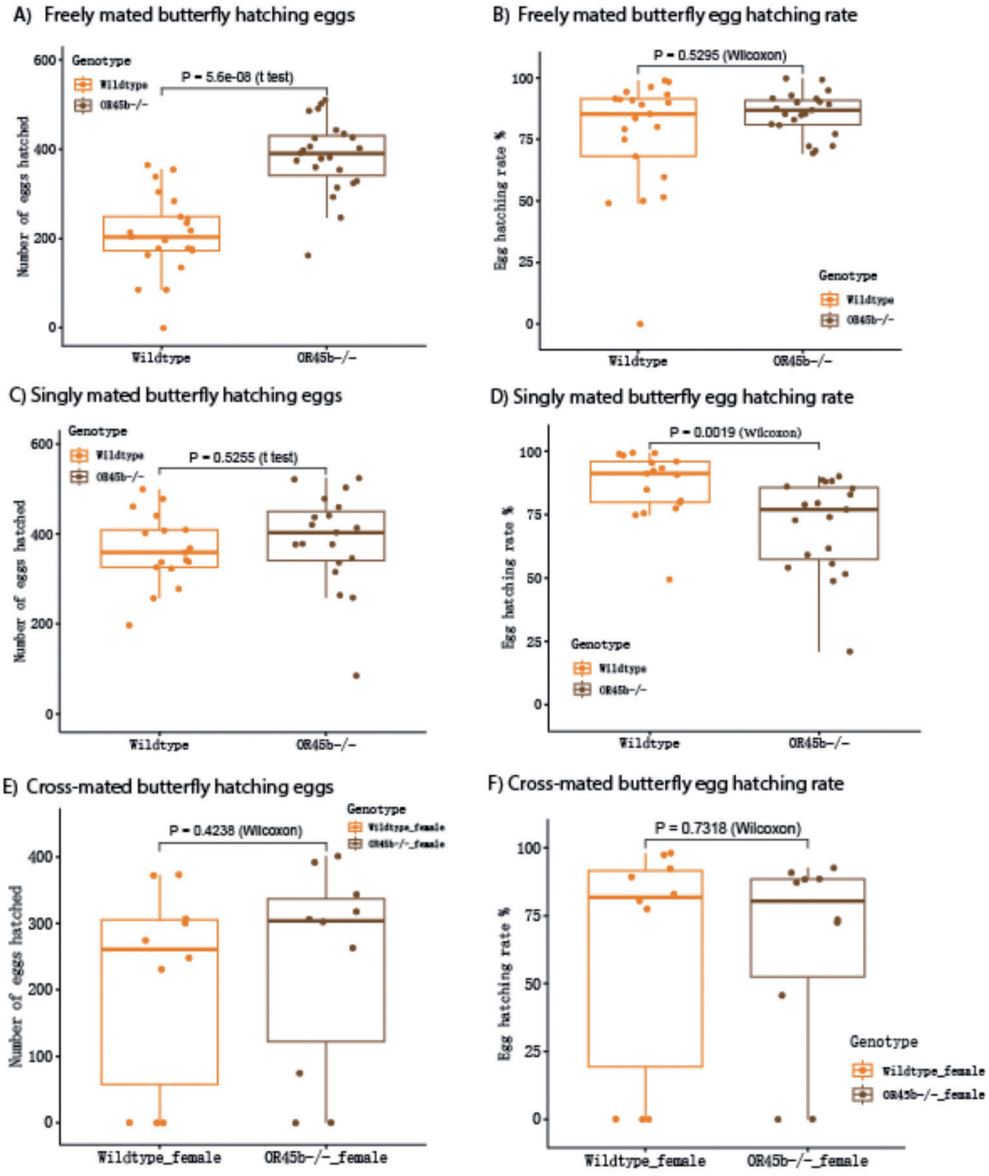



**Figure S6.6)** Comparison between wildtype (WT) and *OR45b* knockout (KO) *Pieris brassicae* in egg-laying behavior, number of matings and egg hatching success and rate. (A) Egg-laying dynamics of both genotypes of butterflies, a significant difference was detected with GLM with negative binomial distribution (n=14 for both genotypes). (B) Number of hatched eggs laid by both genotypes. Wilcoxon rank-sum test (n=14 for both genotypes). C) Number of eggs laid by females which mated one time. D) Number of eggs hatching, laid by females which mated one time. Egg-hatching rates by both genotypes (n=14 for both genotypes). E) Eggs laid in WT versus pair with only the female butterfly as KO but the male is WT. F) Hatching rate of eggs laid by WT versus pair where only the female is KO.

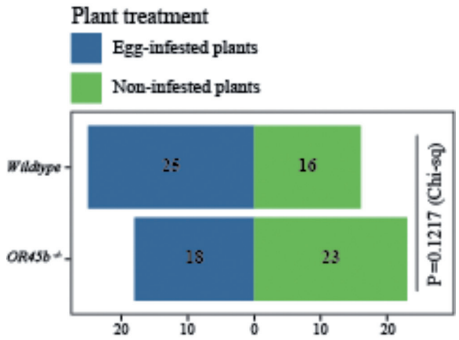



**Figure S6.7)** Oviposition choice of mutant and wildtype *Pieris brassicae*. Bars show the plants that both genotypes of female butterflies oviposited on. Blue and green indicate non-infested and egg-infested plants respectively. Difference was tested by Chi-square test.

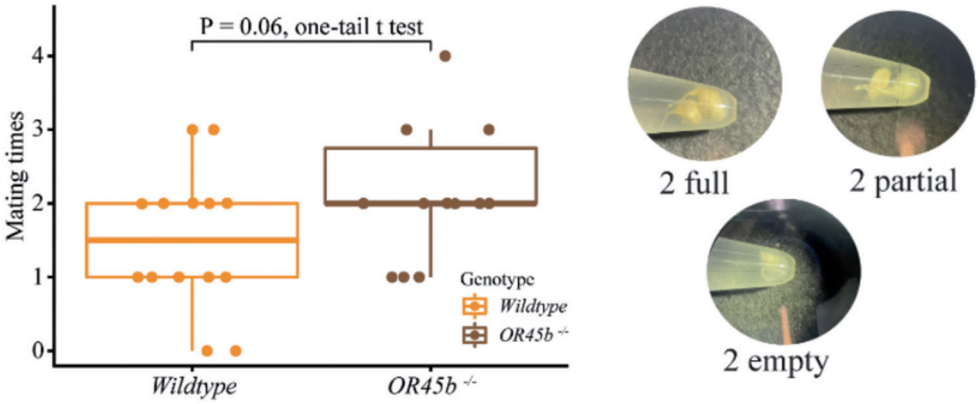



**Figure S6.8)** When allowed to mate freely over 10 days, knockout *Pieris brassicae* butterflies mated more often than wildtype butterflies and sometimes contained more than one full spermatophore at once.

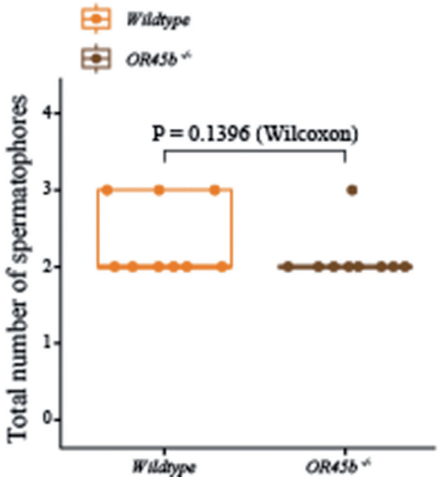



**Figure S6.9)** Similar mating rate of WT and KO *Pieris brassicae* used in the experiments where *Trichogramma* preference was tested in a static olfactometer.

**Table S6.10)** First choice of *Parus major* for virgin *Pieris brassicae* female butterflies, which were control (10 ul hexane treated) or treatment (10 ul hexane and benzyl cyanide). Results of binomial test are shown.

| | | Category | N | Observed Prop. | Test Prop. | Exact Sig. (2-tailed) |
|---|---|---|---|---|---|---|
| Choice_Trial1 | Group 1 | Control | 9 | .43 | .50 | .664 |
| | Group 2 | Treatment | 12 | .57 | | |
| | Total | | 21 | 1.00 | | |
| Choice_Trial2 | Group 1 | Control | 19 | .90 | .50 | <.001 |
| | Group 2 | Treatment | 2 | .10 | | |
| | Total | | 21 | 1.00 | | |

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First and foremost, I begin with my supervisory team: Nina, Bart, Eric, and Bas. You have worked together seamlessly over the last five years to guide my research and build my skills and confidence. I'm so grateful for the way each of you supported me at different times and in different ways. I was warned that having four supervisors might be overwhelming, but I can't imagine having succeeded any other way. You worked together to create a supportive and empowering environment that helped me grow into an independent scientist, and your input into this thesis was invaluable.

**Nina**, my inspiration in so many ways. From the first day we met, your warmth and enthusiasm drew me in. Even before we met, from reading your work, I wanted to be your student. When I told you that I felt intimidated to approach you as an MSc student, that seemed to shock and even upset you, which speaks volumes about the kind of mentor you are and how you want your students to perceive you. I couldn't have been more wrong not to try to work together with you sooner. Your kindness, approachability, and lack of judgment make you an extraordinary mentor. Your willingness to explore and defend new theories with infectious enthusiasm pulled me into the world of science and academia more deeply than ever before. You nurtured my excitement for learning and teaching in a way no one else has, and that's been integral to my growth.

As a strong female scientist and a loving mom, you've shown me what's possible both professionally and personally. You always prioritized my well-being, which made my success possible. You didn't just supervise me, but you mentored me in life and career. Working with you on my thesis has truly been a dream come true. I hope to supervise students in your style throughout my own career. I'm endlessly grateful for your support and belief in me. Your observations of butterflies and egg parasitoids inspired my life's work, and your way of being inspires the person I want to be. Thank you so much for everything.

**Bart**, thank you for being such a generous and thoughtful supervisor. You were always available when I needed you and consistently went above and beyond to support both my work and my personal life. I've always felt at ease speaking with you about anything,

from questions about parasitoid genetics to chats about how to deal with the current state of the world, your door was always open.

Your steady guidance helped me through challenging times, whether I needed advice on relaxing, handling life's chaos, or navigating DNA barcoding. You were always there with better advice than I could've hoped for. Your vocal faith in me helped me believe in myself as a researcher. Thank you for that incredibly important gift.

**Eric**, I'm here because of you. Genomics was the last course of my master's, which I took on a whim to broaden my horizons. Despite the online format during the COVID lockdown, your passion for genomics made me excited about evolution and how to investigate in a brand new way. I've always loved evolutionary theory but didn't know how to merge it with my love of ecology, until you came along and showed me how genomics and phylogenetics could bridge that gap. I had been intimidated by genetics, but with you, everything clicked. Now I think in phylogenetic trees. You noticed me, too, a fellow American interested in insect research and pursuing a PhD in Wageningen, and connected me with Nina right away, a gift that changed everything.

Throughout my PhD, you challenged me to clarify my ideas and connect with broader research in our Biosystematics group. As chair of this group, you've fostered a dynamic and welcoming environment, where fundamental sciences flourishes, topics are (bio) diverse, and exciting new ideas are encouraged. Your way of thinking is always exciting and inspiring, and I've loved following along in your thought process. Your belief in my potential to be part of this biosystematics group gave me the path and the confidence I needed to succeed. Thank you.

**Bas**, your support as my promotor gave me the foundation for this thesis and the rest of my career. From our first conversation, united by our shared love of butterflies, ideas flowed naturally. You have a gift for extracting value from my messy thoughts and helping me refine them. Every question you asked pushed me to think harder, and your insights greatly improved both my experiments and my writing. You made time for in your very busy workload, and I always felt welcome to schedule a meeting or chat in the hallway. I've truly enjoyed every minute of those discussions.

Your belief in my work gave me the courage to challenge norms and defend my ideas. Thanks to your encouragement, I feel ready to do my own science. I also deeply admire your vocal stance on ethics in academia. You've inspired me to fight not only for good science, but for fair science. Thank you for your guidance and integrity.

## Earlier Mentors and the Laboratory of Entomology

Before beginning my PhD, I was fortunate to have supervisors who nurtured my love for research and encouraged me to pursue this path.

First, there was **Chris Frost**, my mentor during my bachelor's at New College, who opened my eyes to insect–plant interactions. He gave me incredible opportunities to explore IPI with field work in the Amazon rainforest in Peru, to my first scientific job in his lab in Louisville, and, perhaps most importantly, to connect with the IPI network in Wageningen. When he heard I wanted to do an internship abroad, he knew exactly where I should go. He contacted Marcel Dicke, recommended me as a student, and in doing so, set the next phase of my career and life in motion.

**Marcel** warmly welcomed me into the Entomology department at WUR, first for two summer internships during my bachelor's, and later for two MSc theses when I returned as a master's student. He and **Joop van Loon** challenged me in my course work and later as my thesis examiners. Throughout these interactions, they helped shape the way I think about entomology and academia.

Crucially, Marcel paired me with **Dani** as my supervisor during these internships. Dani taught me everything from volatile collection and analysis to designing common garden experiments and writing results clearly. Her passion for entomology was contagious, and through her, I met three women who would deeply influence both my career and personal life: **Lucille**, **Kay**, and **Kathe**, my supervisors and dear friends. Each of these women inspired me with their high-quality work, tenacity, organization, and ability to turn great ideas into practical experiments. I was lucky that each of them invited me into the world of publishing as a coauthor, and they have remained a constant source of support in both my professional and personal life. Lucille and Kathe even traveled to Florida for my wedding, and I cherish our friendship across the globe—wherever life takes us. The two of you inspire me with your ability to balance work and life, to always keep time for fun and travel, and helped me learn to prioritize this, too. Other dear friends from my early Wageningen Entomology days who became my ento family here are **Julia**, **Daniel**, **Bram**, **Alessia**, **Max**, **Giuddi**, **Daan**, **Mitchel**, and **Filippo**. These friends made Wageningen a home away from home, always welcoming me to have fun and keep my head focused on the joy of life and not just my work.. Though we've started to scatter across the Netherlands and the globe, every time we reunite, it's as if no time has passed. I hope we'll continue to be there for one another in life's important moments.

## During my PhD

From the Laboratory of Entomology, my co-author **Qi** became a wonderful collaborator. His work ethic was inspiring and allowed him to carry out excellent experiments, some of

which I was lucky enough to join. I loved our conversations, always full of creative ideas and new connections. I hope we'll have more chances to work together in the future. Qi's supervisor, **Alexander Haverkamp**, has also been an invaluable advisor to me since the beginning of my PhD. Always happy to talk butterflies and chemical ecology, he dedicated his time and knowledge to teaching me the tools of electrophysiology. He was the one who connected me with Qi when he saw overlap in our results, and since then he has been an essential source of input into my work. I especially enjoyed our long train ride to the chemical ecology conference in Prague, where I came to appreciate your ideas even more. Thank you, Alex, for always being an enthusiastic, kind, and patient partner in my work.

I am deeply grateful to **Astrid Groot**, who helped shape key ideas in this thesis and taught me the skills I needed to collect and analyze pheromone data. Her sharp critiques and insightful feedback strengthened my work tremendously, and I was consistently inspired by her talks and publications. Thank you for welcoming me into your lab, where your students and technicians made me feel at home and supported some of the most exciting parts of my PhD.

During my fieldwork in Spain, **Constanti Stefanescu** was an incredible guide to the butterflies of the region. His deep passion and readiness to support researchers made my work possible. I am also grateful to **Estrella Hernández-Suárez**, who hosted my student in Tenerife, welcomed her warmly, and enabled her to explore *Trichogramma* and *Pieridae* there. Similarly, **Karl Gotthard** and **Krister Wiklund** offered essential guidance and support that made my research in Sweden possible. Krister, who we remember with deep fondness and miss dearly, left a lasting impact on me. His thoughtful conversations, enthusiasm for science, and vast body of work on *Pieris* butterflies and their pheromones were integral to this thesis. I'm so grateful for the communications we shared while he was still here.

In Gamboa, Panama, **Owen McMillan** welcomed me and my students to STRI with open arms. Thanks to his incredible research group and facilities, we were able to work with the fascinating *Heliconius* butterflies. His passion for new ideas and projects was contagious, and his support invaluable. Thank you also to **Remi**, for the fun times, the warm welcome, and for rearing so many beautiful butterflies.

**Kees van Oers** introduced me to the exciting world of bird behavior. His generous support, including access to rearings and his thoughtful input, made some of the most exciting parts of my work possible. I am very grateful for his guidance.

**Graziella**, I'm so glad you came to work with Nina. Thank you for teaching me all about egg pores! It was a joy to learn about your work and then to collaborate on our manuscript with Teun.

At the Biosystematics and Genetics groups where I spent most of my time, I was surrounded by talented and friendly colleagues who made my PhD experience so much richer. First and foremost, I want to sincerely thank the technicians and helpers whose expertise and tireless, often thankless work makes every PhD thesis possible. **Patrick**, **Jordy**, and **Violet** dedicated countless hours to rearing butterflies I collected for endless student projects. This work is demanding, sometimes even requiring weekends, and yet they made so much of our research possible through their care and dedication. Patrick was essential to my lab work, patiently teaching me DNA barcoding in my very first week and setting me on a path of countless barcoding adventures. He was patient with me, even though I was far more comfortable in the field than the lab, and he gave me the skills I needed to succeed (and to not destroy the lab). In the field, Jordy was a great partner, and I loved our time catching butterflies and searching for parasitized eggs, even if it meant listening to country music while doing it. Thank you also for facilitating so many student projects, managing last-minute requests, and being patient through all of it. Violet, thank you for being my friend. I always really enjoy our time together!

During my PhD in Biosystemtcis and Genetics I was lucky to gain not just colleagues but friends. **Cloé**, **Ale**, **June**, **Eva**, **Wouter**, **Corné**, **Kelley, Sophie, Michiel, Pamela,** and **Lisa,** working and growing with you during our time at BIS has been such a wonderful aspect of my experience here. I wish we had spent more time together outside the office, because I truly loved every minute. Your support brought me peace of mind and comfort and a break from my stress, and I'll always be grateful for that. And Cloé, thank you for being an artistic inspiration. You did such an incredible job bringing my cover ideas to life for this thesis! Thank you for this beautiful illustration that brings all of these chapters together. And I am grateful for you always inspiring me to draw. I cherished our time at the chalk board.

**Wilma,** thank you for all you did to keep things running smoothly for me and all of biosystematics. I love talking with you, and especially hearing your strong opinions! You always bring a new and important perspective, and we'd be lost without you.

To the many students I had the honor of supervising, many of whom also became co-authors, I cannot thank you enough for what you've contributed to this thesis. Your excellent work forms the backbone of many chapters, but more importantly, you gave me the greatest joy of this PhD: mentoring you. Watching you grow, struggle, overcome, and

become excited by insects brought me deep energy and happiness. I have been incredibly lucky to work with one inspiring student after another.

It all began with **Djan** and **Niek**. From day one, you made it clear how much I would love supervising students. Your projects were so much fun, and I loved catching butterflies together, visiting the Passiflorahoeve, and seeing your enthusiasm for the work. Djan has moved on to study the oceans, which we will allow and I wish her all the best.

Niek, you've always been by my side. You've become not just a colleague but a dear friend. We've shared project after project at BIS, and watching you grow into the scientist you are today has been a gift. Seeing you now with your own gaggle of students warms my heart. In Panama, I got to see you thrive, running lab work, supervising others, and it was so clear how far you've come from BSc student I first met (who was already so capable). You've had the mind and curiosity of a great scientist from the very beginning, and I can't wait to keep watching your career unfold. You're also just a lot of fun, and exactly the right person to have around when things go wrong. Your positive attitude always makes everything feel more manageable. I'm so glad you're now my paranymph, one last time I get to ask for your help, and as always, you came through.
That same summer, we had the joy of working with **Florenske**, whose memory still tugs at my heart. She was a super sharp student, bringing her much-needed statistical skills to our survey, and I would have loved to work with her again. She was a pleasure to have in our little team. Her passing left a hole in her community and made a lasting impact on me as a supervisor, a reminder of the importance of caring for our students far beyond academic boundaries.

I also had the pleasure of supervising **Alex** through both his BSc and MSc theses. The growth in your work and writing was incredible. You were such a pleasure to work with, easygoing, thoughtful, and dedicated. Your excellent projects helped me immensely. You dove into genomics with almost no background, and the way you taught yourself and solved problems was truly impressive. With that kind of tenacity, I honestly believe there's nothing you can't do.

Next came **Sander** and **Dorette**, two excellent students who couldn't have been more different, and who taught me one of my most important lessons: how to tailor supervision to each unique person.Sander, you were so much fun to be around, and you found so many *Pieris mannii* eggs, including parasitized ones, making a big discovery in the process! Dorette, you were officially Nina's student, not mine, but I ended up spending a lot of time on your project anyway, just because we had such a nice time together. And now, you're my dear friend. And my co-author. I loved working together to turn your MSc thesis (and all its beautiful photos) into a publication. I'm so grateful for your friendship

(and Bram's!), the BBQs, and the gossiping. Your hard work and your friendship have been invaluable throughout this journey, and seeing you begin your own PhD has been exciting. I'm so glad I've been here to see the start of it and I'm certain you'll succeed. And now, with your keen eye, you helped me edit this thesis. You spent so much time being your meticulous self to help me catch many details I missed, and I'm incredibly grateful. Besides being an amazing academic partner, I'm certain you and I will remain great friends. You are loyal and caring in a way that few are, and I cherish you.

**Severin** and **Esther**—it all started with a *Capita Selecta*. Thank you both for the excellent hitchhiker survey data (still the best data sheet I've ever received from a student!). Esther, I loved working with you on your thesis. Your careful data collection was so valuable, and your dedication to your work throughout your pregnancy was incredibly inspiring. Watching you both go from students to friends, to partners, to parents has been one of the great joys of my time here. Your wedding was a true highlight, and seeing you now with Ella and Simmie is profoundly moving. Thank you for always sharing their joy with me.

When it came to fieldwork, so many students played an essential role.

**Jonne B.** was so meticulous and independent during her fieldwork in Sweden, and careful and percise in collecting chemical data at UVA. You were always a joy to work with, and added so much to this project. In Spain, **Tjomme** was the perfect partner: adept at insect ID, totally at ease in the field, and just a great person to have along for this first big trip. We had so much fun, and I'm so glad you've now become a friend. **Frank** joined us at the end of that field trip, bringing his sunny disposition and a contagious love of bees and orchids. Even though the trip didn't go quite to plan, your resilience and cheerful presence made a big difference. Seeing you continue to thrive in research at the Genetics and Biosystematics groups makes me so happy. You're a talented scientist who doesn't get knocked down easily.
The following summer brought another fantastic field crew: **Samu**, **Jade**, and **Jonne V.** Samu, thank you for all the driving, the meals, the chats. You were a wonderful field assistant and such a kind presence. Jade, I'm so glad you joined us! You were a huge help in the field, and your wonderful animation of our research is an absolute treasure. It remains one of my favorite outputs of the entire project.Jonne, your insect ID skills are always impressive and incredibly helpful in the field. You were the perfect person for the NIOO experiments with Kees, and you carried them out so capably in my absence. I never had to worry, and you are always a pleasure to work with.

**Esther T.**, thank you for collecting such valuable data in Tenerife! You taught yourself how to work with these butterflies and parasitoids on the fly, and your independence and re-

sourcefulness were impressive. I only wish you hadn't been so far away for the internship so that we could've spent more time together. But your report made me feel like I was there. But now, I love our coffee chats and watching your career as an ecologist take off!

**Jip**, I don't know where to place you chronologically because, lucky for us, you keep coming back! And every time, we're better for it. Your energy is contagious, and your data and reports are always top-notch. Most of all, you're just a joy to be around. Talking with you is always exciting, your thoughts come so fast they finally make me feel like I'm operating at the right tempo. You have a real mind for research, and if you choose to continue, I know you'll go far.

**Jasmijn** and **Teun**, you two are both so special, and I can hardly believe you were raised by the same parents, you're so different! I'm so glad you both joined me at BIS. You were total superstars in Gamboa, making me a very proud supervisor. I kept hearing how helpful and smart and wonderful you were that they almost didn't want to let you leave. Jasmijn, your no-nonsense attitude and your organization were a dream for someone like me. The way you structured your data and integrated earlier students' work was super impressive, especially in a topic far from your usual field. You're clearly going far in your new job. Teun, you were such a capable assistant, both independent and communicative, and when you took over the behavioral assays, I knew we were in good hands. Working on our manuscript together, you really proved your scientific strength. You had so much responsibility and handled it all while starting your own PhD project, sometimes emailing from the jungle, always with a great attitude. As long as you don't pick up something super poisonous and die, I'm sure you're going to go very far in science!

**Linz**, one of the last students of my PhD, I'm so glad you found Bart and that I got to work with you through him. Your project on *Trichogramma* haplotypes was excellent, and your diligent work continues to benefit our group. And then you stayed! Now working with Nina on your own PhD in a completely different topic, I've loved seeing your rapid growth. You've also become my friend, someone I can always count on for a hug and a good chat about keeping our minds calm. You also give the most thoughtful and beautiful gifts, which I truly cherish. Thank you for now being my paranymph, too!
And finally, **Veerle**, though not officially my student, thank you for including me in your excellent *Trichogramma* research at Naturalis. Your results were exciting and added depth to my discussion. I'm so glad to see your own passion for these amazing little creatures taking shape!

## My Family

**Mom and Dad**, I've missed you so much while living abroad, but you always made me feel like I was exactly where I needed to be, doing exactly what I should be doing. I'm so

lucky that you noticed how much I loved all things green, creepy, and crawly when I was little.

Mom, letting me raise caterpillars and tadpoles in the house, with your kitchen supplies, was the foundation for all of this. Taking me to Butterfly World whenever I asked, sending me off to SeaCamp, encouraging every spark of curiosity- you made me the scientist I am today. You've always nurtured this part of me and helped it thrive. I can't wait to come home to you again soon. Thank you for all of your love and support throughout this journey.

**Rebecca, Maya**, thank you for your love and your visits throughout these years. Our phone calls ground me and help me reconnect with my core in a way no one else can. I love you all so much. Thank you for always being there for me.

**Gabe**, this accomplishment is as much yours as it is mine. Here, words truly fail me. You are my best friend, the love of my life, and my biggest supporter, every step of the way since I met you. You moved across the world for me, not knowing what you would do next, only that we'd do it together. The ways you care for me are endless: every hug, every meal, every drive, every time you take care of our pets, every time you listen to me vent about experiments or anxieties, every time you call just to say you love me, you're just always there with exactly what I need. I don't know how you give so much. But it's because of everything you give that I was able to throw myself completely into this research without worrying about the rest. You've kept me happy, healthy, grounded, and sane. Now that it's done, I can finally find some balance and take care of *you*, and our little one, too. Your love is the most valuable thing in my life, and you are, and always will be, my favorite person. I could not have done this without you. We did this together.

**Juniper** - It's hard to believe I wrote this all before you were here. And now, you are my everything. Thank you for being the best inspiration and motivator possible. And thank you for being your perfect, sweet, silly self. Thank you for teaching me about motherhood from the human perspective. Because of you, I know I can do anything I need to. And for you, I want to do everything.

# About the Author



Liana Owitz Greenberg was born on December 26, 1994, in Boca Raton, Florida. Growing up amidst the stunning nature of the Everglades sparked an early passion for all things green and a deep love for butterflies. Raising butterflies through their metamorphosis has been both a hobby and a source of joy for as long as she can remember. A very happy realization came when she discovered that her love of learning and her love of butterflies could merge into a career as an entomologist.

This dream began to take shape when she attended New College of Florida, once a thriving liberal arts school, where she studied environmental studies. There she also met her beloved husband and partner in both life and science, Gabe. Together, they studied entomology, botany, and eventually plant–insect interactions, which led Liana to pursue an internship in the renowned Wageningen University.

She stayed on at Wageningen to complete an MSc in Plant Sciences specializing in entomology. Taking courses and conducting research about her passion was extremely motivating, and she graduated *cum laude* for doing what she loved.

Pursuing a PhD was the natural next step, as her love of learning only grew with each degree. Being a researcher, getting paid to ask questions and seek answers, was a dream come true. She discovered that supervising students and guiding them to do the same was the most rewarding part. The results of her own research, combined with those of the students she mentored, come together in this thesis. Liana hopes to continue working with her great passions, butterflies and students, for the rest of her life.

# List of publications

de Jong, T*, **Greenberg, L.O**.*, Fatouros N.E., and Iossa, G. (2025). Butterfly eggs and their plasticity in physiologically and reproductively relevant traits: Adaptation to climate? Submitted.

**Greenberg, L.O.***, Wang, Q.*, Bonnet, J., Te Lindert-Blommert, E.L., van Buul, J., Fernhout, T., Friman, J., Palmen, N., Rozing, A., van Tatenhove, E., Veldboom, J., Groot, A.T., van Oers, K, Schranz, M.E., Zwaan, B.J., Haverkamp, A.^, Pannebakker, B.A. ^,. Fatouros, N.E.^. (2025). Enemies, not sex, as a driving force of butterfly post-mating odor evolution. *In prep.*

**Greenberg, L.O**., Fatouros, N.E. Agroecology: It's about time for oviposition-induced cue use. (2025) Current Biology, Volume 35, Issue 13, R651 - R654. https://doi.org/10.1016/j.cub.2025.04.069

Wang Q., Yufei, J, Smid H.M., Weldegergis B.T., **Greenberg L.O**, Jongsma, M., Dicke, M., Haverkamp, A. (2025). Loss of olfaction reduces caterpillar performance and increases susceptibility to a natural enemy eLife. 14:RP105585 https://doi.org/10.7554/eLife.105585.2

**Greenberg, L. O**., Huigens, M. E., Groot, A. T., Cusumano, A., & Fatouros, N. E. (2023). Finding an egg in a haystack: variation in chemical cue use by egg parasitoids of herbivorous insects. *Current Opinion in Insect Science*, *55*, 101002. https://doi.org/10.1016/j.cois.2022.101002

Peters, D. H.*, **Greenberg, L. O**.*, & Fatouros, N. E. (2024). Oviposition strategies in Pieridae butterflies and the role of an egg-killing plant trait therein. Ecology and Evolution, 14(7), e11697. https://doi.org/10.1002/ece3.11697.

Afentoulis, D. G., Cusumano, A., **Greenberg, L. O**., Caarls, L., & Fatouros, N. E. (2021). Attraction of Trichogramma wasps to butterfly oviposition-induced plant volatiles depends on *Brassica* species, wasp strain and leaf necrosis. *Frontiers in Ecology and Evolution*, *9*, 703134. https://doi.org/10.3389/fevo.2021.703134

Barragán-Fonseca, K. Y., **Greenberg, L. O**., Gort, G., Dicke, M., & Van Loon, J. J. (2023). Amending soil with insect exuviae improves herbivore tolerance, pollinator attraction and seed yield of *Brassica nigra* plants. *Agriculture, Ecosystems & Environment*, *342*, 108219. https://doi.org/10.1016/j.agee.2022.108219

Chrétien, L. T., van Der Heide, H., **Greenberg, L. O**., Giron, D., Dicke, M., & Lucas-Barbosa, D. (2021). Multiple attack to inflorescences of an annual plant does not interfere with the attraction of parasitoids and pollinators. *Journal of Chemical Ecology*, *47*(2), 175-191. 10.1007/s10886-020-01239-6

Moisan, K., Lucas-Barbosa, D., Villela, A., **Greenberg, L. O**., Cordovez, V., Raaijmakers, J. M., & Dicke, M. (2020). No evidence of modulation of indirect plant resistance of *Brassica rapa* plants by volatiles from soil-borne fungi. *Ecological Entomology*, *45*(5), 1200-1211. https://doi.org/10.1111/een.12906

*Both authors contributed equally to this study.
^These authors contributed equally to this study and share senior authorship

# PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 30 ECTS (= 20 weeks of activities)

**Review/project proposal (6 ECTS)**

- Review: Variation in chemical cue use by egg parasitoids of herbivorous insects
- Proposal: Natural selection on butterfly sex scents by egg parasitoids and implications for biological control -NWO ENW M1

**Post-graduate courses (3.3 ECTS)**

- Genome assembly, PE&RC (2023)
- ICE International Chemical Ecology Course, MPI Jena (2025)

**Deficiency, refresh, brush-up courses (6 ECTS)**

- Comparative biology and systematics, WUR BIS (2021)

**Laboratory training and working visits (2 ECTS)**

- Chemical analysis of pheromones, UvA (2022)
- Heliconius ecology and genomics, Smithsonian Tropical Research Institute (2023)

**Invited review of journal manuscripts (6 ECTS)**

- Journal of Chemical Ecology, Effect of leaf maturity on host habitat location by the egg- larval parasitoid (2021)
- Frontiers in Plant Science, Cycad-weevil pollination symbiosis is characterized by rapidly evolving and highly specific plant-insect chemical communication (2021)
- Oikos, Periodical cicadas increase defenses in North American forest trees: Before, during, and after a mass emergence (2021)
- Annals of the Entomological Society of America, Trophic host-parasitoid interactions of two Neotropical butterfly species (Lepidoptera: Papilionoidea) in southeastern Mexico (2021)
- Ecology and evolution, What attracts the allies of Aristolochia contora? (2024)
- Ecological entomology, Making a Healthy Choice: Tactical Host Selection Behavior of Parasitoid Wasp , Bracon brevicornis (2024)

**Competence, skills and career-oriented activities (3.8 ECTS)**
- Start to supervise BSc & MSc thesis students, ESC (2021)
- Supervising BSc & MSc thesis students, ESC (2021)
- Stress Identification and Management, WGS (2021)
- Project and Time Management, WGS (2022)
- Mindful Scientific Writing, BIS (2024)
- Introduction to LaTeX, PE&RC (2025)

**Scientific Integrity/Ethics in science activities (0.3 ECTS)**
- Ethics in Plant and Environmental Sciences, WGS (2024)

**PE&RC Annual meetings, seminars and PE&RC weekend/retreat (1.2 ECTS)**
- PE&RC Day (2021, 2025)
- PE&RC Midterm retreat (2023)

**National scientific meetings, local seminars, and discussion groups (4.6 ECTS)**
- Behavior discussion group, STRI (presentation) (2021)
- NLSEB PhD / postdoc day and annual meeting (2021)
- Entomologendag, NEV (presentation x3) (2020-2025)
- Future of Butterfly and Moths symposium, Vlinderstichting (2025)
- Co-evolution insect-plant interactions, Gordon conference, California (2025)

**International symposia, workshops and conferences (4.5 ECTS)**
- DGAAE entomologentagun (presentation), Bolzano (2023)
- Chemical ecology society annual meeting (presentation), Prague (2024)

**Societally relevant exposure (1.2 ECTS)**
- Current Biology dispatch (2025)
- BSc course Biodiversity of the Netherlands, WUR (guest lecture x 4) (2024–2025)

**Committee work (4 ECTS)**
- WEES ecology & evolution seminars and workshops (organizing committee) (2021-2024)

**Lecturing/Supervision of practicals/tutorials (3 ECTS)**
- Genomics (2021-2022)
- Comparative biology and systematics (2020, 2021)
- Molecular ecology and evolution (external expert) (2025)

**BSc/MSc thesis supervision (3 ECTS)**

- Research topic 1: BSc thesis: Phoresy survey in Pieridae
- Research topic 2: BSc thesis: Parasitoid control at the Passiflorahoeve
- Research topic 3: BSc thesis: Parasitism rates and haplotypes across habitats
- Research topic 4: BSc thesis: Influence of habitat structure on phoresy
- Research topic 5: BSc thesis: Predators respond to Pieris post-mating odors
- Research topic 6: BSc thesis: Parisitism rates across a latitudinal cline
- Research topic 7: BSC thesis: Trichogramma adaptations for phoresy
- Research topic 8: MSc thesis: Have egg parasitoids already found P. *mannii*?
- Research topic 9: MSc thesis: Population genetics of P. napi along cline
- Research topic 10: MSc thesis: Can eDNA detect phoretic Trichogramma?
- Research topic 11: MSc thesis: Wasp-plant-Heliconius cues, networks in Panama
- Research topic 12: MSc thesis: Population variation in post-mating odors
- Research topic 13: MSc thesis: T. evanescens haplotype ID for biocontrol
- Research topic 14: Research practice: Do P. napi odors attract Trichogramma?
- Research topic 15: Internship: Pieris pheromones and parasitoids in Tenerife
- Research topic 16: Internship: Phoresy and cue-used survey in Panama

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