Multidimensional ecological filtering shapes a specialized yet potentially flexible pollination interaction

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Abstract

Climate change may not only disrupt plant-pollinator interactions by eliminating either partner, but also disturb the simultaneous alignment of phenology, spatial overlap, signal perception, behavior, and morphological fit necessary for successful pollen transfer. However, the ecological dimensions that make specialized pollination systems vulnerable have rarely been comprehensively assessed within the same system. Here, we combined more than a decade of field observations with floral volatile analysis, antennal electrophysiology, behavioral assays, seasonal population monitoring, quantitative morphology, and *in situ* introduction experiments to examine the rewardless alpine orchid *Cypripedium bardolphianum*. Female winter-morph *Drosophila immigrans* dominated effective pollination during the orchid's short flowering season. The orchid attracted drosophilid flies with ethyl tiglate (ET), a multifunctional volatile associated with aggregation, feeding, and oviposition, but responsiveness to this compound extended across several drosophilid lineages. Temporal and spatial overlap, seasonal phenotype, sex-specific preference, and morphological fit progressively narrowed this broad response pool to the realized pollination niche. Two species that are geographically isolated but are attracted to ET, *D. robusta* and *D. virilis*, visited flowers

and removed pollinia after *in situ* introduction, revealing compensatory potential beyond the locally realized interaction. These findings show that specialization can emerge from a narrow multidimensional interaction window even when signal-response breadth is comparatively wide. Therefore, environmental change may disrupt current partners through mismatch, while exposing latent functional compatibility through community reassembly. To predict the persistence of specialized pollination, it is necessary to measure the breadth of signal response together with the number and climate sensitivity of the filters that convert response capacity into pollen transfer.

Keywords: climate-change vulnerability; compensatory potential; deceptive pollination; ecological filtering; interaction mismatch; pollination niche; sensory bias

Introduction

Climate change is reshaping plant-pollinator interactions worldwide (Memmott et al. 2007; Schweiger et al. 2010; Trunschke et al. 2024). The interaction between plants and pollinators is particularly exposed because pollen transfer depends on coordinated biological events rather than the mere coexistence of two taxa (Burkle et al. 2013; Phillips et al. 2020; Marjakangas et al. 2025). Successful pollen transfer requires not only temporal overlap between flowering and pollinator activity and spatial encounters between the partners (Memmott et al. 2007; Schweiger et al. 2010), but also pollinator perception of floral signals and appropriate flower-visiting behavior (Chittka and Raine 2006; Schiestl and Dötterl 2012), together with morphological compatibility between the pollinator and the plant's reproductive structures (Armbruster 2014; Wang et al. 2023). Because the interacting partners often differ in their responses to temperature and other climatic drivers, phenological and spatial mismatches can arise even when both species remain locally present (Harrington et al. 1999; Schweiger et al. 2010; Trunschke et al. 2024). Thus, the climate vulnerability of pollination interactions depends not only on the persistence of the interacting species, but also on whether the multiple conditions required for effective pollination remain aligned.

This dependence on several aligned conditions should be especially consequential in specialized pollination systems. Specialized plants often rely on only a few pollinator taxa and may therefore have limited functional redundancy (Johnson and Steiner 2000; Armbruster 2014; Armbruster 2017). However, the number of pollinator taxa observed under present conditions does not reveal how specialization arises or how vulnerable the interaction is (Armbruster 2017; Phillips et al. 2020), both of which depend on the breadth of species that can respond to floral signals. Plant signals provide the first gate into pollination interactions that animals must detect and respond to them, while geographic co-occurrence, phenological overlap, flower handling, and mechanical compatibility further determine whether pollen transfer can occur

(Ollerton et al. 2007; Armbruster 2017; Wang et al. 2023; Zhang and Luo 2024; Johnson 2025). In some systems, the signal recruits only a narrow pool of responder species, potentially comprising that pollinator alone (Ayasse et al. 2000; Peakall et al. 2010). Climate change or a decline in that pollinator could therefore disrupt the interaction while leaving the plant with few immediately available, compatible replacement pollinators (Johnson and Steiner 2000; Armbruster 2017). In others, several species may detect or approach the same signal, forming a broader responder pool that is subsequently narrowed by temporal, spatial, behavioral, and morphological filters (Stökl et al. 2010; Martos et al. 2015). Community turnover, range shifts, or dispersal could then bring previously isolated responders into contact with the plant, creating opportunities to recruit new pollinators to enter the interaction and complete pollen transfer (CaraDonna et al. 2017; Domínguez-Garcia et al. 2024; Marjakangas et al. 2025). Thus, systems with similar observed pollinator richness may differ in their stability and compensatory potential under environmental change (Phillips et al. 2020; Marjakangas et al. 2025). Systems based on a narrow responder pool may be more sensitive to climatic and other environmental disturbances, whereas systems with a broader pool may have greater potential for compensation (Memmott et al. 2007; CaraDonna et al. 2017; Rodger et al. 2021).

Rewardless flowers provide a suitable system for separating signal attraction from subsequent ecological and mechanical filters because the absence of nectar or pollen removes the reward-based reinforcement of repeated visits. Initial attraction often relies on pre-existing receiver biases or sensory exploitation (Ayasse et al. 2000; Stökl et al. 2010; Zhang and Luo 2024), sometimes through non-rewarding mimicry of biologically relevant cues (Brodmann et al. 2009; Schiestl and Schlüter 2009). To examine these processes, we selected *Cypripedium bardolphianum*, a rewardless slipper orchid from the eastern Qinghai-Tibetan Plateau, for two reasons. First, the slipper orchids (genus *Cypripedium*), while most species employ deceptive pollination strategies, impose a clear mechanical filter (Cribb 1997; Li et al. 2006; BÄNziger et al. 2008; Pemberton 2013). *C. bardolphianum* possesses the smallest flowers within the genus, with a labellum diameter of only 1.0-1.5 cm, functioning as a strict morphological trap that demands precise pollinator dimensions for successful pollen transfer (Cribb 1997; Li et al. 2008). Signal attraction can therefore be distinguished from successful pollen transfer, which requires compatible body dimensions and flower-handling behavior. Second, the alpine habitat of *C. bardolphianum* provides a suitable setting for examining environmental vulnerability. The species occurs at approximately 3,000-3,500 m in an ecotonal region with steep elevational climatic gradients (Favre et al. 2015), and has a short growing season, and flowers from late May to late June. Long-term observations identified drosophilid flies as its principal visitor group. The seasonal occurrence, elevational movement, thermal tolerance, and adult phenotype of these small ectotherms can vary with temperature and resource seasonality, making them well suited for testing environmental filtering (Kimura and Beppu 1993; Kojima and Kimura 2003; Mitsui et al. 2010; Ueno et al. 2023).

In this study, we combined long-term field observations with floral chemical analysis, antennal recordings, behavioral experiments, seasonal population monitoring, thermal assays, and quantitative morphology to identify the filters that shape the realized pollination interaction. We then conducted *in situ* tests with local and non-local drosophilids to assess whether alternative responders could pass these filters and complete pollen transfer. We asked three questions: (1) Which floral signal and receiver behaviors mediate attraction and pollen transfer? (2) How do time, space, seasonal phenotype, sex-specific preference, and body size narrow the interaction within the principal pollinator? (3) How widely is the signal detected across drosophilids, and which non-local responders can pass behavioral and mechanical filters? Together, these analyses test whether specialization is established at signal recognition or emerges through multidimensional ecological filtering, and how this mechanism shapes mismatch risk and compensatory potential under environmental change.

Results

Female *D. immigrans* are the dominant pollinator for *C. bardolphianum*

We intermittently observed the pollination system of *C. bardolphianum* between 2004 and 2018 in Huanglong National Nature Reserve, Sichuan, China (103°49′ E, 32°45′ N; elevation ~3,100 m). *C. bardolphianum* is a small clonal terrestrial orchid that forms dense patches, with individual clones containing more than 20 ramets, and occurs on an open slope within dry, open shrub vegetation (Huang et al. 2008). Rhizomatous growth appears to contribute substantially to the maintenance of flowering ramets at Huanglong despite infrequent pollination. Each mature ramet of the orchid produces one flower on a 6-8 cm erect stalk. The inflated labellum is 1.0-1.5 cm long, flanked by two lateral petals and partly concealed by the dorsal sepal (Fig. 1a). The anther sacs of *C. bardolphianum* produce a discrete pollinium bearing an elongated and sticky tip. A visitor must enter through the central opening of the labellum and traverse the trap before escaping, during which a pollinium can adhere to the dorsal thorax and subsequently contact the stigma of another flower (Fig. 1b). We used pollinium carriage as evidence of pollen export and potential pollinator function. Examination of more than 5,000 flowers detected no known edible reward, consistent with most studied *Cypripedium* species (Cribb 1997) but contrasting with *C. subtropicum* (Jiang et al. 2020) and *C. wardii* (Zheng et al. 2025).

Across more than 200 h of direct observation in 2004-2005, 2011 and 2016-2018, we recorded eight insect taxa visiting flowers (Table S1), but only drosophilids and occasional ants entered the labellum and removed pollinia. Drosophilids accounted for 94 landings and 12 pollinium-removal events observed. Pollination is also rarely observed in sympatric bumblebee-pollinated *Cypripedium*, likely reflecting cold,

rainy flowering conditions and low visitation associated with deceptive pollination (Tremblay et al. 2005; Li et al. 2008).

Banana traps and targeted netting yielded 24 insects carrying *C. bardolphianum* pollinia: 17 *D. immigrans* (16 females and one male), four female *D. curviceps*, two ants and one *Leucophenga* sp. (Fig. 1c; Table S2). Morphology and cytochrome c oxidase subunit I barcodes confirmed fly identities; both *D. immigrans* and *D. curviceps* carried pollinia on the dorsal thorax.

The banana traps also characterized the background drosophilid assemblage. Morphological and molecular identification indicated that the local assemblage was dominated by the morphologically cryptic *D. obscura* subgroup (pooled), *D. curviceps* and *D. immigrans* (Fig. 1c). *D. immigrans* represented only 9.3% of captured flies (106/1,140) but 70.8% of recorded pollinators (17/24; 95% CI, 48.9–87.4%). Its recorded pollinator fraction exceeded that of the remaining taxa pooled (16.0% versus 0.68%; Fisher's exact test, odds ratio = 27.82, 95% CI, 10.63–81.56; $P < 0.001$). This disproportionate representation, together with the strong female bias (16/17), identifies female *D. immigrans* as the principal recorded pollinators of *C. bardolphianum*.

Natural fruit set was 13.18% in 2004 (n = 554 flowers) and 8.47% in 2005 (n = 484). To assess self-compatibility and autonomous fruit production, we removed labella from randomly selected mature buds before opening and compared unpollinated controls with flowers hand-pollinated using their own pollen (n = 18) or pollen from another plant approximately 10 m away (n = 23). No control flowers set fruit, whereas both hand-pollination treatments produced fruits, with higher fruit set following crossing than selfing. These results demonstrate self-compatibility and no autonomous fruit production under the assay conditions. Together with infrequent visitation, low natural fruit set suggests that limited pollen delivery may constrain sexual reproduction.

Ethyl tiglate is a specific but multifunctional floral attractant

We first tested whether olfaction was necessary for flower location by enclosing flowering *C. bardolphianum* clones in 40 × 40 × 60 cm field cages. Each assay released 20 artificially raised *D. immigrans* with intact antennae or surgically ablated antennae and recorded flower landings and residence time for 30 min. Antennal ablation reduced both the ability to locate flowers and the subsequent time spent on them, demonstrating that olfactory information is necessary for attraction (Fig. 2a).

We next identified the floral volatile responsible for attracting female *D. immigrans*. Among 90 field-collected scent samples, we selected 16 samples with high volatile concentrations to reduce the effect of natural heterogeneity. Eight volatile organic compounds occurred in at least three samples (Tables S3 and S4; Fig. S2). Using gas chromatography coupled with flame-ionization detection and electroantennographic

detection (GC-FID-EAD) to analyze pooled natural samples, we identified four compounds that elicited antennal responses in *D. immigrans*: ethyl butyrate, ethyl tiglate, acetoin, and isobutyl acetate (Fig. S1). To standardize testing across multiple *Drosophila* species and overcome natural sample limitations, we formulated an artificial scent mixture using reference standards from natural samples. When tested on *D. immigrans* and the model animal *D. melanogaster*, seven VOCs (ethyl tiglate, acetoin, isobutyl acetate, ethyl butyrate, 1-hexanol, 3-octanone, and 6-methyl-5-hepten-2-one) triggered similar electrophysiological responses in both species, alongside minor spikes from two isoforms of 2,3-butanediol (Fig. 2b).

Among the antennally active compounds, only a moderate concentration of ethyl tiglate (ET) significantly attracted *D. immigrans* in two-choice assays; the remaining compounds were behaviorally neutral at the tested concentrations (Fig. 2c). ET attracted females more strongly than males (Fig. 2d), matching the female bias among pollen-carrying flies in the field (Table S1). Furthermore, when presented with a synthetic mixture of all EAD-active VOCs excluding ET (MIX / ET –), female *D. immigrans* showed no attraction, whereas they strongly preferred the same mixture containing ET (MIX / ET +) (Fig. 2e). Together, these results identify ET as the key floral volatile mediating pollinator attraction in *C. bardolphianum*.

We then tested the functional contexts in which ET could be relevant to *D. immigrans*. Because *D. immigrans* has adopted generalist feeding habits during its global expansion and ET has been reported from several tropical fruits, we quantified ET emissions from fruits under different fermentation states. Large quantities of ET were detected from mangos (*Mangifera indica*), pears (*Pyrus bretschneideri* and *Pyrus sinkiangensis*), and eight additional fruit species infected with *Saccharomyces cerevisiae* or *Penicillium expansum*, but not from fresh fruit (Fig. 3a, b). This suggests that the floral scent of *C. bardolphianum* mimics chemical cues associated with fermented fruits. In feeding assays, females consumed more sucrose solution when ET was present, whereas male feeding did not increase (Fig. 3c). In two-choice oviposition assays, *D. immigrans* showed a marked oviposition preference for medium containing ET, and exposure to ET also increased the total number of oviposition (Fig. 3d). These results indicate that ET conveys nutritional and reproductive information relevant to both foraging and oviposition, thereby attracting specialized female pollinators through multiple functional pathways.

Spatiotemporal, behavioral, and morphological filters concentrate the realized niche on winter-morph female *D. immigrans*

We next examined how spatiotemporal, behavioral, and morphological filters shape the participation of individuals within *D. immigrans* in the pollination interaction. At Huanglong, *C. bardolphianum* flowers in May and June, when mean temperature is approximately 6–8 °C, whereas winter temperatures fall below freezing (Fig. 4a). To assess whether cold conditions could constrain local overwintering, we compared *D.*

immigrans survival at 23 °C and 4 °C (Fig. S3). At 23 °C, flies exhibited an approximately sigmoidal survival curve, with a median lifespan of 41 d and a maximum of approximately 80 d. At 4 °C, early mortality was high and median survival was reached on day 8; only very few individuals survived for up to 150 d. These results, together with the cold winters at Huanglong, suggested that local overwintering may be limited and that pollinators could arrive through seasonal immigration.

To investigate seasonal elevational movements, we monitored populations across the year, excluding January, along a 200-1,800 m gradient at Langshan and Shunhuangshan in the Yangtze River basin, within the species' inferred ancestral rang (Throckmorton 1975; Zhang et al. 1999; Izumitani et al. 2016). These sites permitted consistent sampling across elevations, which access restrictions and difficult terrain prevented in Huanglong Nature Reserve. Previous studies of climatic adaptation and seasonal occurrence informed this investigation (Kimura and Beppu 1993; Kojima and Kimura 2003; Mitsui et al. 2010), while temperature preference did not differ significantly among the geographic populations examined by Yamamoto (Yamamoto 1994). Trap records revealed seasonal shifts consistent with partial elevational migration: flies first appeared at low elevations in early spring, subsequently occurred at higher elevations, and peaked there in May and June (Fig. S4). Monthly ovarian assessments distinguished early-stage, mid-stage and mature females at low and high elevations. All females collected upon first detection at high elevations were reproductively mature, consistent with the arrival of mature adults (Fig. S5).

Seasonal changes also occurred in the morphology of captured flies. Representative winter-morph females collected at 800 m had larger wings than summer-morph females from the same elevation (Fig. S6). Monthly measurements, with females and males pooled within each elevation band, showed that wing length generally increased during cooler months and decreased during summer (Fig. S7). Together, these observations link seasonal elevational occurrence with variation in reproductive state and body size, providing context for the seasonal availability of different pollinator phenotypes. The low overall survival at 4 °C, together with seasonal elevational occurrence elsewhere, offer indirect support that pollinators reach Huanglong through seasonal immigration from lower elevations.

To assess the pollination-related differences between these seasonal morphs, we compared their occurrence during flowering, responses to ET, floral visitation, and mechanical compatibility with *C. bardolphianum*. Comparison of wing lengths with summer- and winter-morph populations generated by temperature-controlled rearing over three generations in the laboratory indicated that local *D.immigrans* collected during the flowering period corresponded to the winter morph (Fig. 4b). In laboratory two-choice flight-box assays, winter-morph females showed the strongest attraction to ET compared with males and summer morph individuals (Fig. 4c), indicating that seasonal migration brings the sex and phenotype with the strongest ET

response to the orchid during its short flowering period. Field-cage assays then confirmed that winter morph females exhibited the highest visitation rates to *C. bardolphianum* flowers (Fig. 4d).

We next quantified mechanical matching between flies and the trap flower. Escaping visitors must pass the stigma and anther, which form sequential mechanical checkpoints for pollen transfer (Cribb 1997). Successful transfer therefore requires thorax height to match the distances from the base of the labellum to these structures (Fig. 4e). Although the thorax heights of all tested sexes and seasonal morphs fell within the broad passage dimensions, winter-morph females matched these checkpoints more closely than the other groups (Fig. 4e). Together, spatiotemporal and behavioral ecological filters, including seasonal migration, environmentally induced phenotype, sex-specific preference, and floral morphology restrict effective pollination to the winter-morph female *D. immigrans*.

Exotic *Drosophila* species attracted by ET exhibit pollination behavior toward *C. bardolphianum*

To test whether *Drosophila* species beyond the local pollinator assemblage could enter the response pool of *C. bardolphianum*, we compared *D. immigrans* with five other drosophilid species using antennal recordings, morphological measurements, behavioral assays, and *in situ* flower-visiting tests: *D. immigrans*, *D. melanogaster*, *D. suzukii*, *D. hydei*, *D. virilis*, and *D. robusta* (Fig. 5e). These species included local taxa, phylogenetically related species, and species previously known to produce or respond to ET. This selection was based on two lines of evidence. First, ET is associated with fermenting resources and functions as a male-produced aggregation pheromone in some of the six drosophilid species (Bartelt et al. 1985; Bartelt et al. 1986; Hedlund et al. 1996; Zhang et al. 2022). Second, South American *Specklinia* orchids use ET to attract *D. hydei*, providing a direct example of its role in drosophilid-mediated orchid pollination (Karremans et al. 2015).

All six species produced antennal responses to the synthetic mixture of floral volatiles (Fig. 5a), demonstrating shared capacity to detect components of the floral blend. Morphological comparisons nevertheless revealed differences in compatibility with the floral passage. The thorax heights of *D. melanogaster* and *D. suzukii* fell below the functional range, whereas those of *D. immigrans*, *D. robusta*, *D. hydei*, and *D. virilis* overlapped this range (Fig. 5b). These comparisons identified body-size matching as a potential mechanical filter among species capable of detecting floral volatiles.

Behavioral responses to ET were also species-dependent. Species in the subgenus *Drosophila*, namely *D. immigrans*, *D. hydei*, *D. virilis* and *D. robusta*, were attracted, whereas the two species in the subgenus *Sophophora*, *D. melanogaster* and *D. suzukii*, avoided the compound (Fig. 5c). Attraction to or avoidance of ET therefore imposed a behavioral filter after sensory detection. The four ET-attracted species also

exhibited body-size compatibility with the floral passage, whereas the two ET-avoiding species lacked this match (Fig. 5b, c, e).

In subsequent in situ cage experiments, all four ET-attracted species visited *C. bardolphianum* flowers (Fig. 5e). The non-local species *D. robusta* and *D. virilis* successfully exited the flowers carrying pollinia (Fig. 5d). Although pollinium removal was not observed in *D. hydei*, floral visitation was recorded (Fig. 5e). These introduction experiments show that exotic *Drosophila* species attracted by ET can visit *C. bardolphianum*, pass its mechanical filters, and remove pollinia, demonstrating pollination behavior and the potential to form new interactions with the orchid.

Discussion

Multidimensional filtering shapes pollination specialization

Our results show that the specialized interaction of *C. bardolphianum* and winter morph female *D. immigrans* is not determined solely by signal recognition. A comparatively broad range of drosophilids can detect the floral blend, and several species are attracted to ET. However, temporal, spatial, behavioral, phenotypic, and morphological filters progressively limit the range of pollinators that participate in pollen transfer. Specialization therefore emerges through the sequential narrowing of signal-response capacity into pollinator function. This process explains how a highly specialized field interaction can arise even when other drosophilids retain the capacity to complete some of the steps required for pollination (Phillips et al. 2020; Marjakangas et al. 2025).

ET initiates this process by exploiting receiver functions that evolved outside the orchid interaction. Among eight compounds tested from the floral blend, ET alone attracted *D. immigrans*, and the removal of ET abolished the preference of *D. immigrans*. The compound occurred in microbially infected fruit, increased female feeding and stimulated oviposition, while previous work identified ET in aggregation-pheromone systems of several drosophilids (Bartelt et al. 1985; Bartelt et al. 1986; Hedlund et al. 1996; Goldman-Huertas et al. 2015; Mansourian and Stensmyr 2015; Zhang et al. 2022; He et al. 2026). These convergent behavioral contexts indicate that the orchid recruits pre-existing sensory and motivational pathways associated with resource and mate location rather than requiring an orchid-specific signal channel (Ryan 1998; Chittka and Raine 2006; Bohman et al. 2014; Martos et al. 2015; He et al. 2026). Such sensory exploitation is central to many deceptive pollination systems, in which flowers reproduce biologically salient information without providing the expected reward (Schiestl and Dötterl 2012; Zhang and Luo 2024; Suetsugu et al. 2026).

The multifunction of ET helps explain both the female bias and the breadth of the initial response pool. Feeding and oviposition are especially consequential to reproductive females, and female *D. immigrans* responded more strongly than males in behavioral assays. At the same time, all six drosophilid species detected the floral mixture and four species were attracted to ET. Floral signals can serve several ecological functions and be interpreted differently across receivers, so a single volatile need not be either universally attractive or species exclusive (Junker 2016; van der Kooi et al. 2021). Here, conserved detection was followed by divergent behavioral preference, with attraction in the subgenus *Drosophila* and avoidance in the tested *Sophophora*. This pattern has been repeatedly reported across the genus *Drosophila*: odor-detection patterns can be conserved, while the same odors elicit different behavioral responses across species (Krause Pham and Ray 2015; Depetris-Chauvin et al. 2023). Thus, the key to specialization lies not in whether pollinators detect the chemical signal, but in what happens after detection, as differences in behavioral responses among pollinator groups and subsequent ecological filters progressively narrow the pool of potential pollinators (Martos et al. 2015; Armbruster 2017).

This filtering occurred both among species and within *D. immigrans*. A candidate fly had to overlap with the orchid in space and time, respond positively to ET, and pass the behavioral and mechanical filters imposed by the floral trap (Cribb 1997; Pemberton 2013). Seasonal migration brought reproductively mature adults to higher elevations during flowering (Kimura and Beppu 1993; Mitsui et al. 2010), while winter-morph females showed the strongest attraction, highest visitation, and closest morphological fit with *C. bardolphianum*. Thus, the specialized interaction of *C. bardolphianum* is generated by sequential multi-ecology filters of a broader responder pool, showing that observed pollinator richness alone does not reveal the full range of possible interactions or the ecological conditions required for their formation (Armbruster 2017).

Multiple mismatch risks for specialized pollination systems under climate change

A multidimensional interaction filter creates several routes by which environmental change could disrupt the current pollination system (Boukal et al. 2019; Trunschke et al. 2024). In *C. bardolphianum*, clonal growth may maintain flowering ramets despite infrequent pollination, whereas sexual reproduction depends on successful pollen transfer. Although a single pollinium transfer can potentially support the production of numerous seeds (Tremblay et al. 2005), low natural fruit set suggests limited reproductive opportunities that changes in pollinator availability could further reduce. The most immediate is temporal mismatch (Memmott et al. 2007; Forrest and Thomson 2011; Inouye 2019). Flowering in *C. bardolphianum* must overlap with the elevational migration and adult female activity of *D. immigrans*. Plants and insects often differ in the magnitude and direction of their phenological responses to warming, and network models and field studies show that the phenological responses can reduce floral resources, visitation, and reproductive

success before either partner disappears (Memmott et al. 2007; Bartomeus et al. 2011; Kudo and Ida 2013). For example, a long-term analysis of a sexually deceptive orchid showed that spring warming increased phenological mismatch with its pollinating bee, reduced effective access to male pollinators, and increased the risk of complete reproductive failure (Hutchings et al. 2018). In our study, the low survival of *D. immigrans* at 4 °C and its sequential occurrence along the elevational gradient further indicate that its arrival at the orchid population is constrained by both temperature and seasonal progression. Changes in migration timing or elevational occupancy could therefore eliminate overlap during the limited flowering period (Forrest and Thomson 2011; Bauer and Hoye 2014; Muthukrishnan et al. 2025).

Spatial mismatch provides the second pathway. Climatic responses can shift plant and pollinator ranges at different rates and restructure the local species pool (Schweiger et al. 2010; Boukal et al. 2019). For migratory pollinators, spatial overlap also depends on dispersal routes, elevational colonization, and transient local abundance, not only on the limits of the geographic range (Bauer and Hoye 2014; Gomez-Ruiz and Lacher 2019; Muthukrishnan et al. 2025). Thus, even without a decline in the overall population of *D. immigrans*, climate-driven changes could reduce the number of individuals reaching high-elevation orchid habitats or shift their seasonal abundance peak outside the flowering period (Bauer and Hoye 2014; Gomez-Ruiz and Lacher 2019). Such changes would reduce pollination without requiring local extinction (Schweiger et al. 2010; Burkle et al. 2013).

Environmental change may also alter the phenotypes that determine pollinator function (Scaven and Rafferty 2013; Boukal et al. 2019). Temperature affects developmental rate, seasonal morphology and adult body size in drosophilids (Kimura and Beppu 1993; Ueno et al. 2023). Summer and winter morphs of *D. immigrans* differed in both attraction to ET and morphological fit, showing that individuals within the same pollinator species are not functionally interchangeable (Ueno et al. 2023). If warming changes the production or relative abundance of seasonal morphs, the proportion of flies that respond strongly to the signal and successfully pass through the flower could change even when total fly abundance is stable (Boukal et al. 2019). Floral dimensions may also vary with growing conditions, creating an additional source of mechanical mismatch (Wang et al. 2023).

Finally, climate change may disrupt the chemical signal channel before physical contact occurs (Farré-Armengol et al. 2013; Gérard et al. 2023). Floral volatile emissions respond nonlinearly and in compound-specific ways to temperature, while drought and herbivory are reported to alter volatile blends and pollinator attraction (Farré-Armengol et al. 2013; Burkle and Runyon 2016). Climatic conditions may also affect volatile dispersal and the physiological state of receivers (Gérard et al. 2023; Trunschke et al. 2024). Temperature influences not only whether pollinators are present, but also how insects acquire, process, and respond to visual and olfactory information, potentially creating sensory–behavioral mismatch even when

spatial and temporal overlap is maintained (Gérard et al. 2023). Because attraction to *C. bardolphianum* depends strongly on a single component of its floral blend, changes in ET production, background blend composition, or receiver physiology could alter attraction independently of phenological and spatial overlap (Farré-Armengol et al. 2013; Burkle and Runyon 2016).

These pathways are likely to covary rather than operate independently (Zarnetske et al. 2012; Boukal et al. 2019; Trunschke et al. 2024). Temperature can simultaneously influence flowering, migration, seasonal phenotype, body size, volatile release, and sensory behavior (Farré-Armengol et al. 2013; Scaven and Rafferty 2013; Gérard et al. 2023; Ueno et al. 2023). By identifying the mechanistic dimensions along which mismatch may arise, our study provides a basis for inferring the multiple risks that climate change poses to specialized pollination systems (Schweiger et al. 2010; Trunschke et al. 2024). Interaction vulnerability therefore cannot be estimated from pollinator abundance or the continued persistence of both partners alone (Bascompte et al. 2019; Trunschke et al. 2024). Loss of overlap at any filter could further narrow an already restricted interaction window, while correlated changes across filters could amplify disruption (Burkle et al. 2013; Bascompte et al. 2019; Pauw 2019; Trunschke et al. 2024).

Balancing of vulnerability and compensatory potential

Multidimensional filtering increases mismatch risk, but the shared responses to ET provide a mechanism through which other drosophilids could enter the candidate pollinator pool (Schweiger et al. 2010; Marjakangas et al. 2025). The independent use of ET by South American *Specklinia* orchids to attract *D. hydei* shows that plants can exploit pre-existing sensory preferences without a shared geographic history, suggesting that attraction need not be limited to the current pollinator or require prolonged interaction with *C. bardolphianum* (Ryan 1998; Schiestl and Dötterl 2012; Bohman et al. 2014; Karremans et al. 2015; Suetsugu et al. 2026).

Our controlled *in situ* introductions of pollinators provide evidence for a mechanism through which non-local drosophilids could contribute to pollinator compensation and species reorganization in specialized pollination systems (Schweiger et al. 2010; CaraDonna et al. 2017). All four species attracted to ET visited *C. bardolphianum*, while the non-local *D. robusta* and *D. virilis* entered the flowers and removed pollinia. Together with their morphological compatibility, these results show that pre-existing sensory preferences can allow geographically isolated species to pass signal and behavioral filters and enter the pollen-transfer pathway (Ryan 1998; Schiestl and Dötterl 2012; Marjakangas et al. 2025). Among these species, *D. virilis* is cosmopolitan and has been sampled in China, while *D. hydei* is also widely distributed and has colonized high-latitude environments, making their arrival through human-assisted dispersal or range shifts plausible, although their ability to establish under alpine cold remains uncertain (Nunney 1990; Mirol et al. 2008;

Zhao and Begun 2017). By contrast, *D. robusta* is mainly associated with temperate forests in eastern North America; although members of the *robusta* species group occur in Yunnan, contact between *D. robusta* itself and *C. bardolphianum* would probably require rare long-distance dispersal (Watabe et al. 1990; Levitan and Etges 2005; Suwito and Watabe 2010). Thus, *D. virilis* and *D. hydei* illustrate the conditional possibility of new interactions following range shifts, whereas *D. robusta* demonstrates that geographically isolated species can retain signal and morphological compatibility (Schweiger et al. 2010; CaraDonna et al. 2017; Domínguez-Garcia et al. 2024; Marjakangas et al. 2025).

However, sensory compatibility and pollinium removal indicate only the mechanistic potential for compensation, not effective compensation under natural conditions (Marjakangas et al. 2025). Candidate species must reach the plant population, maintain sufficient abundance and visitation during flowering, tolerate the alpine environment, deposit pollinia onto conspecific stigmas, and ultimately maintain plant reproductive success (Harmon and Barton 2013; Armbruster 2017; Marjakangas et al. 2025). Newly arriving drosophilids may also reorganize local networks, and their effects need not benefit the specialized plant (Schweiger et al. 2010; CaraDonna et al. 2017; Marjakangas et al. 2025). Realized compensation will therefore depend on candidate species identity, abundance, phenology, environmental tolerance, functional traits, and behavioral plasticity (Harmon and Barton 2013; Domínguez-Garcia et al. 2024).

Together, our findings show that the narrow-specialized pollination interaction of *C. bardolphianum* can emerge when a broader pool of signal-responsive drosophilids is progressively filtered across time, space, behavior, phenotype, and morphology. This complex structure creates both mismatch risk and compensatory potential: environmental change may disrupt any filter, while shared responses to ET preserve compatibility beyond the local pollinator assemblage (Schweiger et al. 2010; Marjakangas et al. 2025). Predicting the persistence of specialized pollination should therefore move beyond species survival and geographic overlap to assess signal-response breadth, the number and climate sensitivity of ecological filters, and the availability of compatible responders capable of completing pollen transfer. This process-based framework provides a basis for identifying both where specialized interactions are likely to fail and when community reassembly could maintain pollination function under environmental change (Gérard et al. 2023; Domínguez-Garcia et al. 2024; Trunschke et al. 2024; Marjakangas et al. 2025).

Methods

Collection and survey of pollinators. We utilized banana-yeast baited traps to collect and quantify the background drosophilid community. To prepare the bait, 1 kg of crushed bananas was mixed with 10 g of yeast

and allowed to ferment at room temperature for 24 h before being dispensed into the traps. A total of 21 baited traps and seven unbaited control traps were deployed across six plots. Traps were active daily from 09:00 to 16:00. At the end of each trapping session, all captured drosophilids were collected as background samples and preserved in absolute ethanol. A small number of flies captured in the traps carrying pollinia on their dorsum were preserved separately. Because no co-flowering plant species in the study area produce similar pollinium structures, flies carrying these small pollinia were identified as pollinators of *Cypripedium bardolphianum*.

For pollinator observations, three quadrats within the plots were selected daily for 15-min observation bouts during several periods of peak insect activity. We recorded the number of insects entering the quadrats, floral visitation frequencies, visit durations, and detailed descriptions of foraging behaviors. Insects observed exiting the orchid labellum with attached pollinia were confirmed as effective pollinators, captured using a small hand net, and subsequently preserved in absolute ethanol.

Taxonomic identification of the pollinating drosophilids was conducted using DNA barcoding based on the mitochondrial cytochrome c oxidase subunit I (COI) gene. Amplification was performed using universal dipteran COI primers described by Simon et al. (Simon et al. 1994). The resulting COI sequences were queried against the NCBI database using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Pollinators were identified via these molecular alignments, while background drosophilids were manually classified and quantified based on morphology.

Fly stocks. The fly stocks of *Drosophila immigrans*, *Drosophila suzukii*, *Drosophila virilis*, *Drosophila robusta*, and *Drosophila hydei* were obtained from the Drosophila species stock center (San Diego, CA).

***In situ* flight cage assays.** During the flowering season of *C. bardolphianum*, we conducted *in situ* flight cage assays (40 × 40 × 60 cm) across five selected plots within the natural population in the Huanglong Valley. Assays were performed daily between 10:00 and 16:00. Each flight cage was placed to enclose a single orchid clone bearing 4–5 flowers at the early blooming stage. For each trial, we introduced 20 *D. immigrans* individuals either as intact controls or with their antennae surgically ablated (antennectomized) into the cage. Over a 30-minute observation period, we quantified two behavioral metrics: the number of floral landings (visitation frequency, n = 15) and the floral residence time. Residence time was meticulously tracked for every fly that visited a flower within the 30-min window, measured from the exact moment of landing until departure.

To evaluate the floral visitation and pollination capacity of different *Drosophila* species, individual *C. bardolphianum* clones bearing 4–5 early-blooming flowers were enclosed in flight cages (40 × 40 × 60 cm). For each trial, 10 male and 10 female flies of the respective tested species were introduced into the cage and allowed to interact with the flowers for 24 h. Following this period, we observed and recorded whether the flies had successfully removed pollinia.

To assess the behavioral responses of different seasonal ecotypes and sexes of *D. immigrans*, another set of flight cage assays was conducted. Each cage enclosed a single early-blooming orchid clone and six *D. immigrans*

individuals. We established four experimental groups: winter-morph females, winter-morph males, summer-morph females, and summer-morph males, with 20 replicates (n = 20) per group. All flies utilized in these assays were on the second day of their oviposition period (with males age-matched to this reproductive stage). During a 2-h observation period for each trial, we quantified the floral visitation frequencies and the residence time of each visit.

Headspace odor extraction. Floral scents were collected from *C. bardolphianum* and ambient air (the control group) in Huanglong Valley (3300 m), Sichuan Province, during July 2012, 2016, and 2017. The volatile samples were obtained using dynamic headspace sorption techniques(Gaskett et al. 2005). Entire inflorescences and other organisms were carefully enclosed in polyester oven bags (Top-its, Germany), and volatile compounds were trapped in an adsorbent tube containing a thin layer of activated charcoal (CLSA, 1.5 mg, Granicher and Quartero) with a pump adjusted to a flow rate of 1000 ml/min for approximately 5 hr. The inflowing airstream was cleaned by a charcoal filter (activated charcoal, Supelco, Orbo 32 large). The trapped volatiles in the adsorbent tube were eluted with 50 µl of dichloromethane (GC299-4HC, Honeywell, U.S.). The dichloromethane with the headspace sample was stored at -20 ℃. After each sampling session, the sorbent tubes were cleaned three times with ethanol, dichloromethane, and hexane (39199, Alfa Aesar, U.S.).

GC-MS and GC-EAD analyses. The floral odor extracts were analyzed by gas chromatographic-electroantennographic detection (GC-EAD) using the antenna of fruit flies(Salzmann et al. 2007). GC-EAD was performed using an Agilent Technologies 7890A GC system equipped with an HP-5MS UI column (30 m × 0.25 mm × 0.25 µm; Agilent, USA). Helium served as the carrier gas at a flow rate of 1 ml/min. For the on-column injection, 2 µl of the headspace sample was injected into a front inlet at 280 ℃ with the splitness mode. The column oven temperature program was held at 40 ℃ for 2 min, then programmed to 300 ℃ at a rate of 12 ℃ per min and held for 3 mins. The MS source was operated at 230 ℃ and the MS quadrupole at 150℃ in the electron impact ionization mode at 70 eV (S.A.= sigma-Aldrich).

Structure elucidation gas chromatographic analyses with mass spectrometry (GC-MS) of the extracts were performed on the same GC setup(Mondello et al. 2008). Quantitative gas chromatographic analyses were conducted using the same GC parameters. A systematic investigation on the mass spectrometric fragmentation patterns of the putative active compound(s) was confirmed by checking the NIST 2011b MS Library SW Kit (Agilent Technologies) and mass spectra with co-injected authentic standards. Commercial standard products (> 95% purity, Sigma Co., U.S.) of the putative compounds were used to compare retention time and diagnostic ions of the headspace volatile compounds. The retention index of each compound based on the GC analysis of C7–C30 saturated alkanes mixture (49451-U, Supelco-Sigma Aldrich, U.S.) was calculated by the program with the same column and oven(Mondello et al. 2008). The CAS number and other information of the standard compound are shown in the table below.

A fixed outlet splitter (1:1 split ratio; SGE) was used with the EAD outlet placed in a purified and humidified airstream after passing through the GC oven wall via a Syntech heater (Syntech, Kirchzarten, Germany). This flow was directed over a prepared female fly antenna. The electrode holding the base of the antenna was connected to a grounded Ag-AgCl wire. The distal end of the antenna was connected in the same way via an interface box to a Syntech signal acquisition interface board for signal transfer to a PC. Signals obtained by EAD and flame ionization detection (FID) signals were simultaneously recorded using Syntech GC-EAD software.

Dilution preparation. The commercial products of the VOC compounds and solvent used were acetoin (A1,795-1, Sigma-Aldrich, U.S.); 2,3-butanediol (B84904, Sigma-Aldrich, U.S.); isobutyl acetate (C10016100, Dr. Ehrenstorfer GmbH, Germany); ethyl butyrate (E15701, Sigma-Aldrich, U.S.); 1-hexanol (73117, Fluka, U.S.); ethyl tiglate (W246018, Sigma-Aldrich, U.S.); 3-octanone (13,691-3, Sigma-Aldrich, U.S.); 6-methyl-5-hepten-2-one (S52972-429, Sigma-Aldrich, U.S.) and n-hexane (39199, Alfa Aesar, U.S.). Besides, formulas for the mixed orchid floral scents were listed in Table S2.

The diluent was prepared in concentrations varying from 0 (solvent (0.1% Triton X100 solution) as the control), 10-6 (0.01 μl compound in 10 ml 0.1% Triton X100 solution), 10-5, and 10-4.

T-maze odor preference test. Flies were loaded into a T-maze and allowed to choose between a specific VOC odor and air for 2 mins. A performance index (PI) was calculated from the distribution of flies trapped in the two arms of the T-maze(Tully and Quinn 1985). To balance the space bias, the preference test of an odor was comprised of two reciprocal groups of flies, in which one group received the odor in the left arm of the T-maze, while the other received the odor in the right arm. The final PI was the average of the PIs from the two groups.

PI = [(number of flies in the test odor arm of the T-maze) − (number of flies in the air arm of the T-maze)] / total number of flies

Analysis of volatile compounds in the fruits of different conditions. Fresh Fruits were acquired from a local market and cultivated with either *Saccharomyces cerevisiae* or *Penicillium expansum* at 28℃ for 72 hours before inoculation. The fungi-inoculated mangos were fermented at 28℃ for 7-10 days before analysis. As a control group, 1 g yeast power (*S. cerevisiae*) was dissolved in sterile water and cultivated at 28℃ for four days. The volatile compounds of fresh fruits, fermented fruits (with *S. cerevisiae* or *P. expansum*), and yeast culture (*S. cerevisiae*) were analyzed by SPME-GC-MS.

SPME-GC-MS Analysis: In Solid Phase Micro-extraction (SPME), the fruits or a yeast culture were placed into a 100 ml glass beaker flask covered with sealing film. The 85μm CAR/PDMS SPME Fiber (57334-U, Supelco-Sigma Aldrich, U.S.) was used to extract volatiles from the samples, and the equilibrium of each sample lasted for 30 minutes when the flask was incubated in the 30℃-water bath. After extraction, the fiber was transferred to the injection port of GC inlets with a manual injection for 15 s at 280 ℃ with a 1:10 split mode. The column and oven temperature programs were consistent with the GC-MS analysis.

Food intake assay. The quantity of artificial sucrose liquid was evaluated by the food intake of an adult fly in a fly's body. Blue dye (Erioglaucine disodium salt, #S0158, Ameresco LLC, U.S.) was added to the sucrose liquid

for 4-hour food-intake trials (Fig. 3c, shown by the diagram in the top panel), and the quantity of sucrose liquid was given by an Abs (absorptivity at 630 nm) of the supernatant segregated from fly homogenate.

Two-choice egg-laying assay. Three to 5 days after eclosion, female flies were transferred to a small food tube for adequate copulation with males at 23℃ for three days. Then, female flies were separated into a group of twenty flies and maintained on a food bottle with abundant yeast power for three hours to induce the activation for spawning before the assay. During the test, each fly group was loaded into a 1% agarose plate chamber for egg-laying under conditions of 25 °C, 60% humidity, and darkness. After 1 hour, we removed the flies and counted the egg numbers on each semi-plate. The oviposition performance index of flies affected by an odor was evaluated by the egg numbers laid on the semi-plate covered by the odor, with the whole plain plate as the negative control and the plain-yeast plate as the positive control (Fig. 3d, illuminated by the diagram in the top panel).

Seasonal population dynamics and altitudinal migration investigation of *Drosophila immigrans*. To investigate the seasonal immigration dynamics of *D. immigrans*, we established altitudinal gradient transect within its presumed ancestral range in Hunan, China: Langshan (290–810 m, 100-m intervals) and Shunhuangshan (200–1,880 m, 200-m intervals), with three replicate sites per elevation. From July 2020 to July 2021 (excluding the coldest January), we deployed banana-yeast baited traps. Collections were conducted over three consecutive days each month, with traps emptied every 24 h or 48 h during cold months.

Captured flies were quantified and identified via morphological characteristics and DNA barcoding. To evaluate the relationship between population dynamics and reproductive status, we dissected the ovaries of up to 50 randomly selected *D. immigrans* females monthly from both high- and low-elevation sites. Ovarian development was examined under a stereomicroscope and categorized into early and mature phases based on standard ovariole and oocyte developmental stages (Stages 1-14). This facilitated the spatiotemporal quantification of reproductive states across the altitudinal gradient.

Morphological measurements of the pollination channel and pollinators. To quantify the mechanical fit between the flowers and their pollinators, we measured the floral traits and pollination channel dimensions of *C. bardolphianum*. A total of 20 individual plants were randomly selected across five study plots. Using a precision digital caliper (0.02 mm accuracy), we recorded three key parameters defining the restrictive pollination channel: the exit length (EL), the vertical height from the base of the labellum to the stigma (SL), and the vertical height from the base of the labellum to the pollinium (AL). We quantified the morphology of potential pollinators using thorax height (TH), a key trait determining their ability to pass through the orchid's floral passage. Measurements were taken from five to 20 freshly collected specimens per *Drosophila* species. TH was strictly defined and measured as the vertical distance from the distal end of the foreleg femur to the dorsal surface of the thorax.

Statistical analysis and reproducibility

Student's *t* tests were used for the comparisons explicitly identified in Figs. 2, 3, and 5, and one-way analysis of variance (ANOVA) was used for the multi-group comparisons indicated in Figs. 4 and 5. Results are shown as means ± s.e.m. where stated, and significance symbols follow the thresholds defined in the figure legends. Multi-group comparisons represented by letter groupings were interpreted as different at $P < 0.05$ when letters did not overlap. Statistical analyses and figure preparation were performed using GraphPad Prism 10.1.2 and R version 4.6.1 in RStudio 2026.07.1 (Wickham 2016; Team 2026). Microsoft Excel and PowerPoint were used for data organization and figure assembly.

Supplementary information

Supplementary Materials accompany this manuscript and contain extended experimental procedures, insect-visitor records (Table S1), species and sex of pollinium-carrying insects (Table S2), information on identified floral volatiles (Table S3), floral volatile quantities across three sampling years (Table S4), natural-scent GC-FID-EAD traces and mass spectra (Figs. S1 and S2), and supplementary data on *D. immigrans*: survival at 4 and 23 °C (Fig. S3), seasonal abundance across elevations (Fig. S4), ovarian development (Fig. S5), representative seasonal-morph morphology (Fig. S6), seasonal wing-length variation (Fig. S7), and seasonal-morph proportions (Fig. S8).

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Competing interests

The authors declare no competing interests.

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Figures and legends

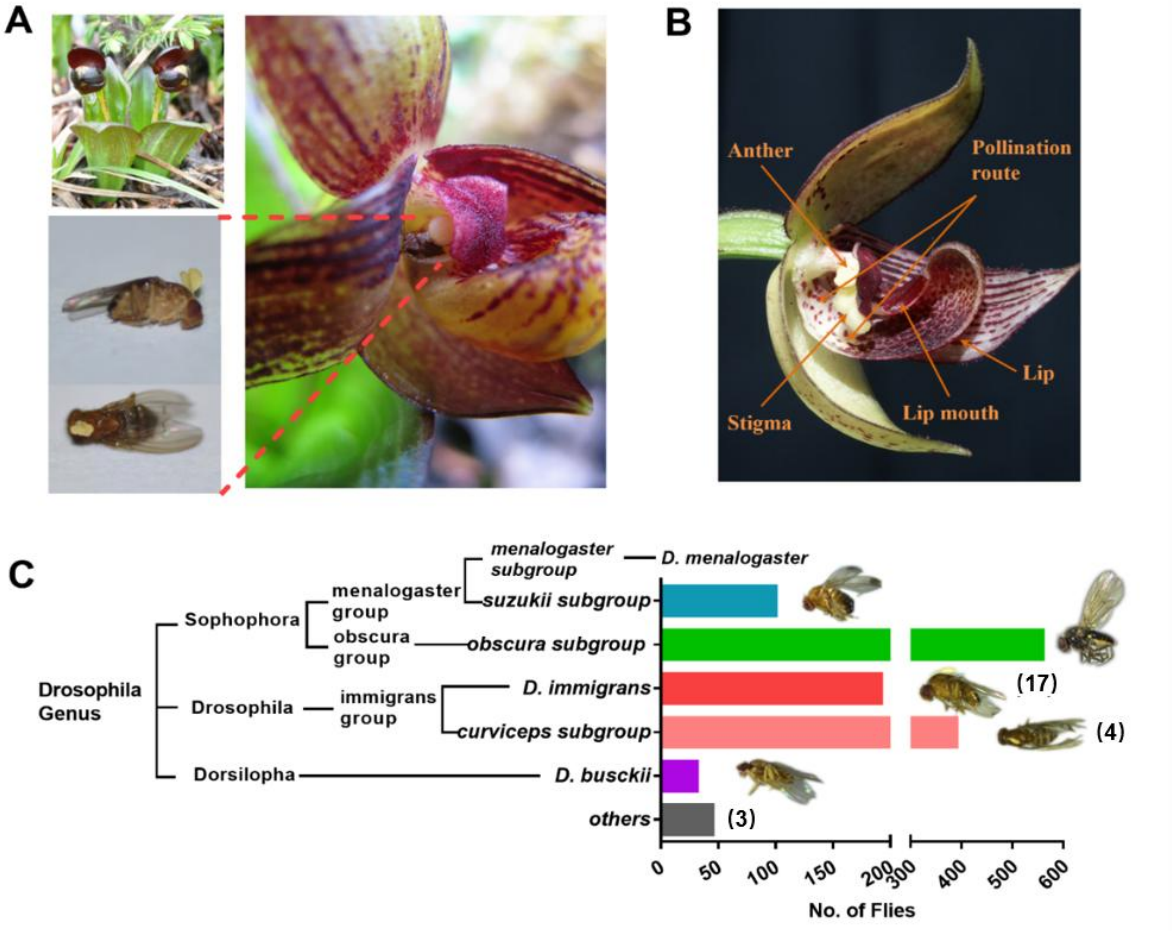


Figure 1 | The pollination system of *Cypripedium bardolphianum* and its principal pollinator, *D. immigrans*.

(a) Flowering *C. bardolphianum* and views of a pollinator. A fly leaving beneath the anther carries a pollinium on its dorsal thorax; frontal and posterior views show the attachment position. (b) Cross-section of the flower showing the trap, reproductive organs and pollination route. Orange arrows indicate key floral positions and movement through the flower. (c) Phylogenetic placement of drosophilids captured in banana-yeast traps in Huanglong Valley. Bars show the number captured; numbers in parentheses indicate pollen-carrying flies.

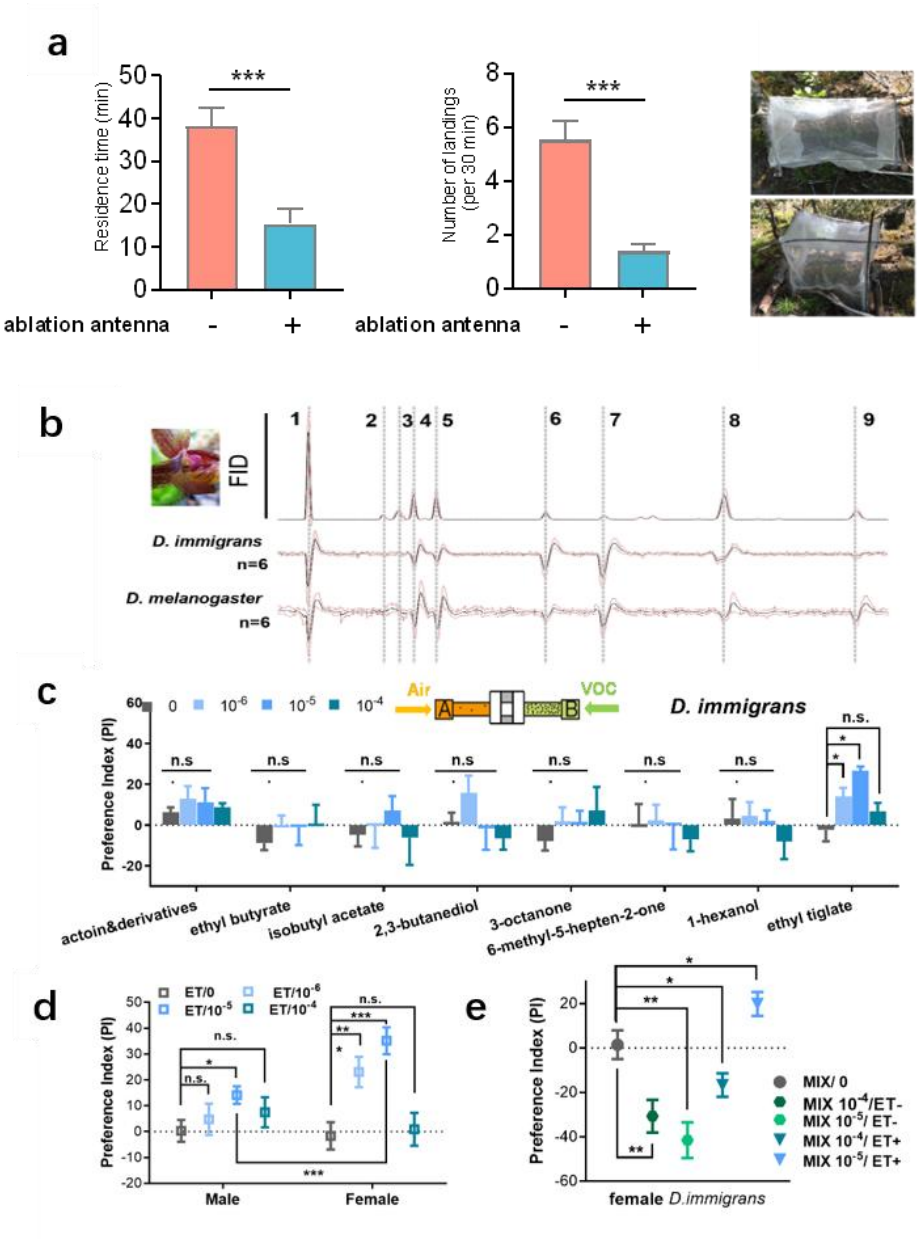


Figure 2 | Ethyl tiglate is the key floral attractant of *Cypripedium bardolphianum*.

(a) Surgical removal of the antennae reduced flower-visitation frequency and residence time of female *D. immigrans* (n = 12-15). (b) Gas chromatography with flame-ionization detection and electroantennographic detection of synthetic *C. bardolphianum* headspace by female *D. melanogaster* and *D. immigrans* (n = 6). (c) Behavioral responses of *D. immigrans* to dilutions of eight antennally active floral volatile organic compounds, from solvent control to 10^-4 (1 µL compound in 10 mL solvent; n = 5 per group). (d) ET attracted female *D. immigrans* more strongly than males (n = 8). (e) Removing ET abolished attraction to the synthetic mixture, whereas the complete mixture remained attractive (n = 8-12). Data are means ± s.e.m. Student's *t* tests: n.s., $P > 0.05$; , $P < 0.05$; , $P < 0.01$; , $P < 0.001$.

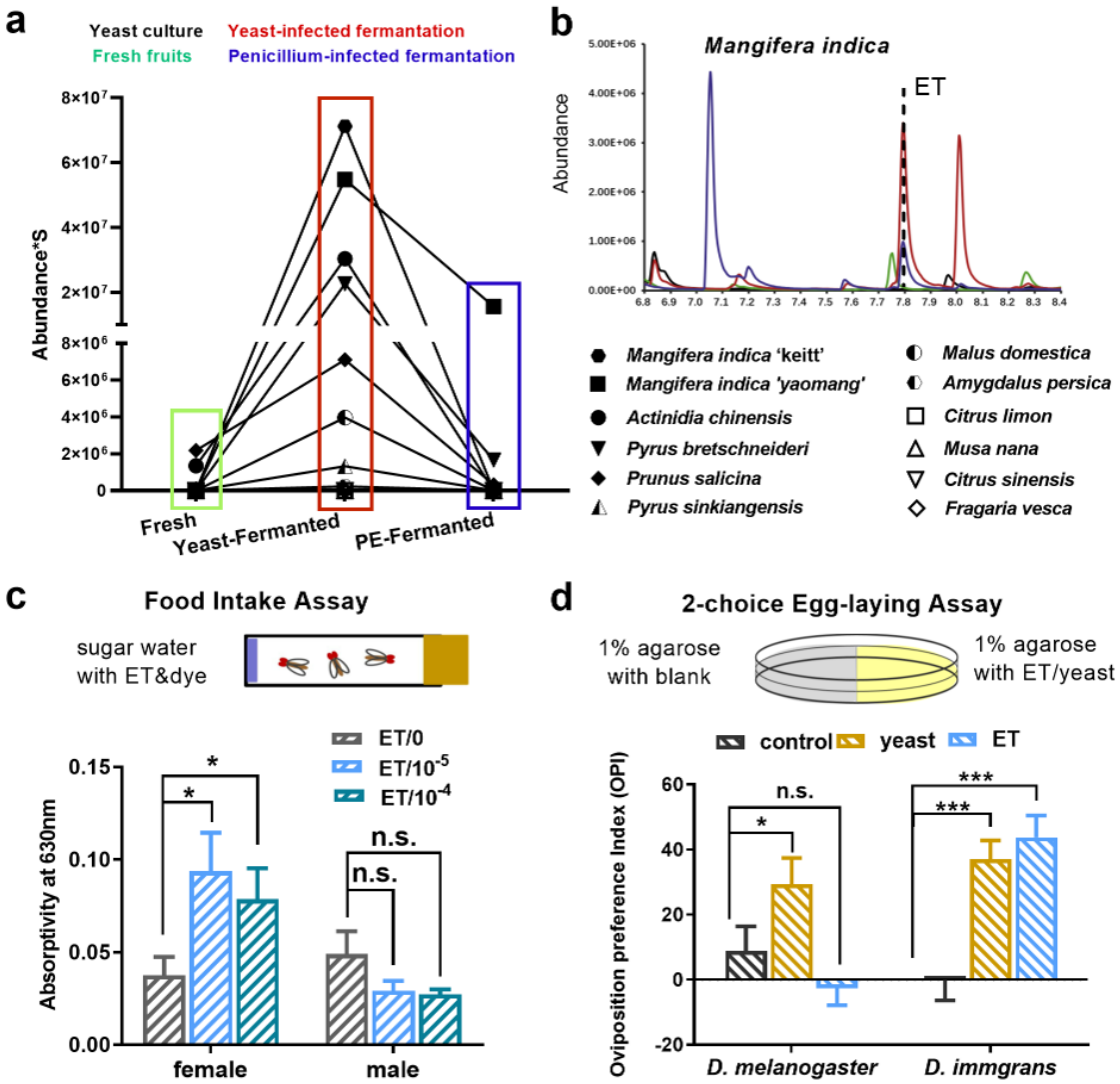
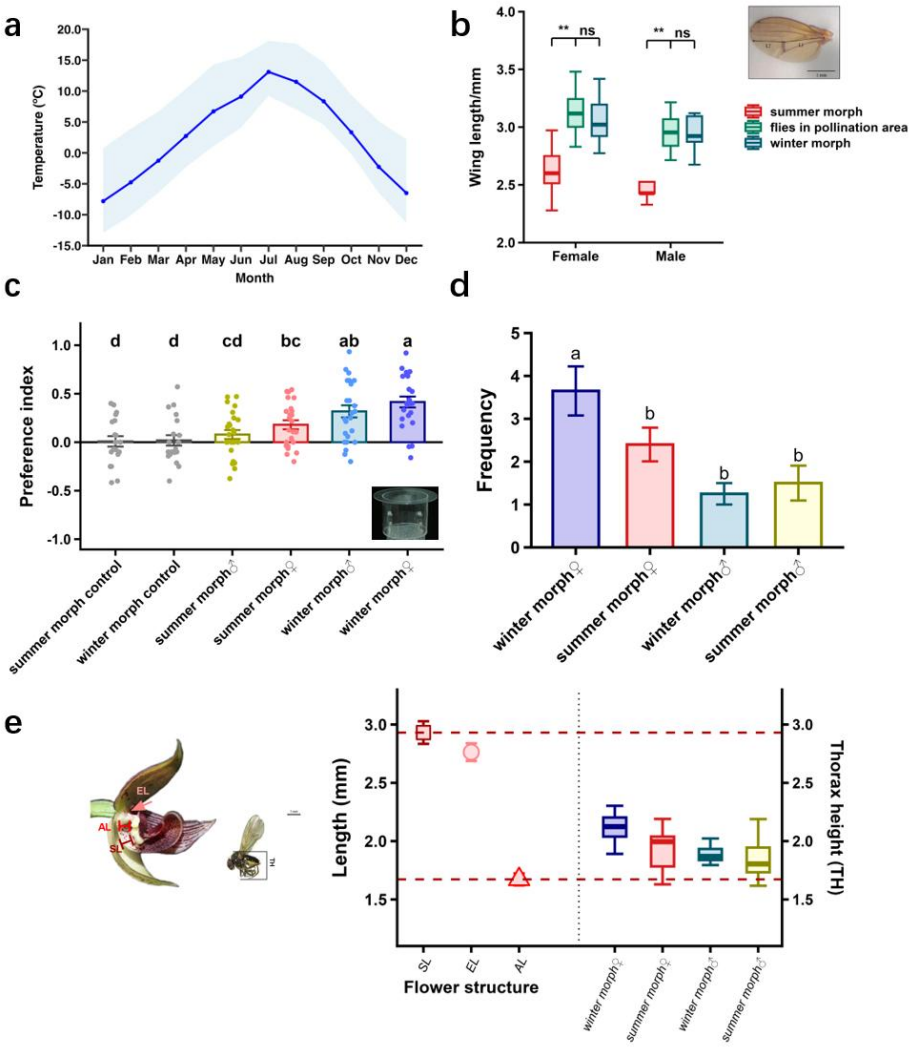


Figure 3 | ET is a multifunctional signal for *D. immigrans*.

(a) ET released by fresh fruit and fruit fermented by yeast or infected with *Penicillium expansum*. (b) Representative chromatographic detection of ET from infected fruit. (c) Exposure to ET increased food intake by female but not male *D. immigrans* (n = 10-14). (d) *Drosophila immigrans* laid more eggs on medium containing ET than on control medium (n = 12-14). Data are means ± s.e.m. Student's *t* tests: n.s., $P > 0.05$; , P < *0.05;* , P < *0.01;* , $P < 0.001$.



793

794 **Figure 4 | Spatiotemporal, behavioral, and morphological filters concentrate pollination on winter-morph female *D.***
795 ***immigrans*.**

796 (a) Monthly temperature profile at the *C. bardolphianum* site in Huanglong Valley. The blue line shows monthly temperature and
797 the shaded band indicates variation across observations. (b) Wing lengths of female and male *D. immigrans* representing laboratory-
798 induced summer and winter morphs, together with flies collected from the orchid pollination area. *$P < 0.01$; ns, not significant.
799 (c) Preference indices of summer- and winter-morph females and males in laboratory two-choice assays with ethyl tiglate. Controls
800 received solvent only. Points represent individual replicates; boxplots show medians and interquartile ranges. Groups with different
801 letters differed at $P < 0.05$. (d) Visitation frequency of the four sex-by-seasonal-morph groups in field-cage assays with *C.*
802 *bardolphianum*. Bars show means ± s.e.m.; different letters indicate $P < 0.05$. (e) Mechanical correspondence between floral exit
803 dimensions and fly thorax height (TH). Stigma length (SL), exit length (EL), and anther length (AL) are shown on the left; thorax
804 heights of the four *D. immigrans* groups are shown on the right. Red dashed lines indicate the floral dimensions defining the
805 functional passage.

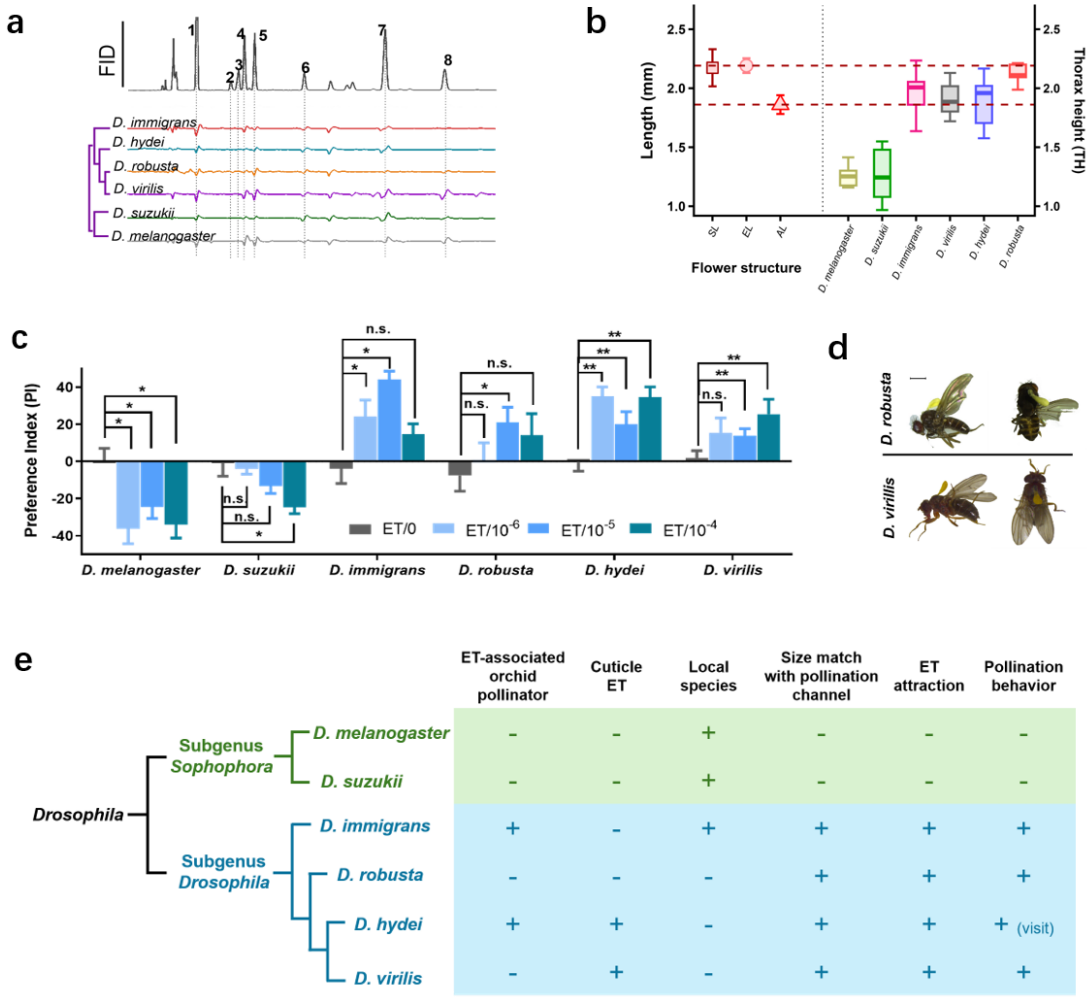


Figure 5 | Exotic *Drosophila* species attracted by ET exhibit pollination behavior toward *C. bardolphianum*.

(a) Gas chromatography with flame-ionization detection (FID; upper trace) and electroantennographic detection (EAD; lower traces) of the synthetic floral volatile mixture by the six drosophilid species. Numbers indicate 1, acetoin; 2 and 3, two stereoisomers of 2,3-butanediol; 4, isobutyl acetate; 5, ethyl butyrate; 6, 1-hexanol; 7, ethyl tiglate; and 8, 3-octanone and 6-methyl-5-hepten-2-one. (b) Mechanical correspondence between floral exit dimensions and thorax height (TH) of the six tested drosophilid species. Stigma length (SL), exit length (EL), and anther length (AL) are shown on the left; red dashed lines mark the stigma and anther positions that bound the functional floral passage. (c) Behavioral responses to ethyl tiglate at three concentrations relative to the solvent control (ET/0) in two-choice assays ($n = 5$ per treatment). Bars show means ± s.e.m.; asterisks indicate the comparisons shown ($P < 0.05$; $*P < 0.01$; n.s., not significant). (d) Non-local *D. robusta* and *D. virilis* carrying *C. bardolphianum* pollinia on the dorsal thorax after *in situ* cage assays. (e) Schematic relationships among the six drosophilid species and a summary of interspecific filters and pollination-related traits. Columns indicate documented pollination of ET-associated orchids, reported ethyl tiglate (ET) in cuticular extracts, occurrence in Huanglong Valley, body-size compatibility with the floral passage of *C. bardolphianum*, attraction to ET, and observed pollination-related behavior. Symbols indicate positive (+) or negative (−) status based on available records or assays. In the final column, + denotes pollinium removal, whereas + (visit) denotes floral visitation without observed pollinium removal in *D. hydei*.