Alpine Botany (2025) 135:215–226
https://doi.org/10.1007/s00035-025-00330-6

ORIGINAL ARTICLE

The morphological fit between flowers of *Cypripedium calceolus* L. and their visitors matters but does not explain flower size variations along altitude

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Received: 21 January 2025 / Accepted: 16 April 2025 / Published online: 13 May 2025

Abstract
Studies of floral adaptations in response to divergent pollinators are important for understanding floral evolution and diversification of plants. A plant exposed to a variable pollinator climate is *Cypripedium calceolus*, a threatened lady's-slipper orchid. Insect pollinators are temporarily trapped in the pouch-like labellum and escape via one of two small posterior exit holes. In escaping, they pass the stigma and an anther, depositing and collecting pollen, respectively. Successful pollination is thought to depend on the morphological fit between pollinators and flowers, with particularly thorax and exit heights being key traits. Too small insects might neither touch the stigma nor collect pollen when exiting, and too large insects do not fit through the exit but leave the flower through the entrance hole or die inside. To discern the likelihood of floral adaptations in *C. calceolus* to varying pollinator assemblages, we investigated (a) whether floral, vegetative, and insect traits change in a concerted manner along an altitudinal gradient, and whether the morphological fit affects (b) the escape mode of a visiting insect and (c) its probability of exporting pollen. We found that floral and vegetative traits of *C. calceolus* got smaller with altitude, while insect dimensions were similar across the sites. Hymenoptera, the main visitors, were more likely to escape via the exit and to export pollen when the fit was near-exact. This shows that the morphological fit plays a critical role in the pollination of *C. calceolus* and that pollinators have the potential to drive size-related floral adaptations.

Keywords Altitudinal gradients · Morphological fit · Deceptive pollination system · Lady slipper orchids · Cypripedioideae

Introduction

Pollinators have played a major role in the diversification of angiosperms (Darwin 1862; Dodd et al.1999; Grant and Grant 1965). According to the Grant-Stebbins model (Grant and Grant 1965; Johnson 2006; Stebbins 1970), plants, particularly floral traits, adapt to their most effective pollinators at a local scale (microevolution) before they speciate (macroevolution). Therefore, understanding the underlying ecological and microevolutionary processes at the local scale is important to comprehend the role of pollinators in plant diversification (Herrera et al. 2006).

The abundance, species composition, and activity of the locally available pollinators is described by the term "pollinator climate", coined by Grant and Grant (1965). A variable pollinator climate across a plant's distribution range can exert different selective pressures on the floral phenotype and is a precondition for floral diversification via pollinator-mediated selection (Grant and Grant 1965). For example, pollinators might increase, decrease, or show no change in size with altitude (Dillon et al. 2006; Hoiss et al. 2012; Maad et al. 2013), along with a change in species composition and behaviour (Grant and Grant 1965; Maad et al. 2013; Soteras et al. 2020). Thus, floral traits dependent on pollinator sizes would be under different selective pressures along the altitudinal gradient. Variation in pollinator climate across a plant's distribution range can spring from abiotic factors, such as temperature (Dillon et al. 2006; Hoiss et al. 2012), or from biotic factors, such as vegetation structure and the availability and composition of flowering plants,

Herbert Braunschmid passed away in 2023.

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which might differ among sites (Johnson 2006). At the same time, abiotic factors also influence plant traits and can drive their diversification (Strauss and Whittall 2006), which may confound the pollinators' influence. For example, both vegetative and floral traits might get smaller with altitude as temperatures drop, the growing season becomes shorter, and solar radiation increases (Körner 2007; Körner et al. 1989). Thus, floral traits dependent on pollinator sizes could be subject to various, possibly opposing, evolutionary pressures. It is pertinent to discriminate between and study both pollinators and the abiotic environment as potential drivers for plant phenotypic divergence. Yet, few studies have so far investigated floral adaptation in response to pollinator and environmental changes simultaneously (e.g., Cosacov et al. 2014; Strauss and Whittall 2006).

The Orchidaceae, with its approximately 25000 members, is among the most species-rich plant families. Orchids are well-suited for evolutionary studies thanks to their extraordinary floral diversity and highly specialized adaptations to their pollinators. One-third of the orchids are deceptive (Cozzolino and Widmer 2005), such as the slipper orchids (Cypripedioideae). Most of them, such as *Cypripedium calceolus* (the yellow lady's slipper orchid) temporarily trap their pollinators—primarily different species of bees and, to a lesser extent, hoverflies (Braunschmid et al. 2017, 2021; Daumann 1968; Nilsson 1979; Zheng et al. 2011)—inside a modified, pouch-like labellum that originates from one of the three petals (Pemberton 2013). *Cypripedium calceolus* spans a wide altitudinal and latitudinal range, from near sea level to approximately 2500 meters above sea level, and from Northern Morocco to Scandinavia (https://www.gbif.org/species/2820517). This range exposes it to a variety of pollinator climates, making it an ideal organism for studying local adaptive divergence in response to the latter.

Cypripedium calceolus attracts insects by visual and olfactory cues (Bergström et al. 1992; Daumann 1968; Müller 1873; Przybyłowicz et al. 2012). Insects enter the labellum through a central opening (henceforth: entrance) and get temporarily trapped, as the curved, slippery inside makes escape through the entrance difficult (Pemberton 2013). Imprisonment in the labellum lasts from a few seconds to a day and can eventually lead to the death of the visitor (Braunschmid et al. 2017; Nilsson 1979). Ideally, light windows and hairs guide pollinators towards one of two narrow exit holes (henceforth: exit; Cribb 1997; Nilsson 1979). Thereby, they first pass the stigma, potentially depositing pollen from a previously visited flower, and then an anther, taking up sticky pollen mass onto their thorax before escaping. Studies investigating the plant-pollinator relationship of *C. calceolus* and other slipper orchids agree that insects too small will not touch the stigma or anthers (Li et al. 2008a; Nilsson 1979; Stoutamire 1967), and insects too large will not fit through the exit, though single observations in some species have shown that large individuals, to some extent, may be able to push the exit apart (e.g., Bänziger et al. 2005). Hence, pollination is thought to occur when a correctly sized insect enters the flower (Edens-Meier et al. 2011, 2018). This makes *C. calceolus*, like most other slipper orchids, highly selective to pollinator size (Antonelli et al. 2009; Bernhardt et al. 2014a,b; Edens-Meier et al. 2018; Erneberg and Holm 1999; Stoutamire 1967). We can expect strong stabilising selection towards increasing the accuracy and precision of this morphological fit (Argue 2012; Armbruster et al. 2009; Cosacov et al. 2014). Such pollinator-mediated selection might also have resulted in the diversification of *Cypripedium,* with some species having very small flowers and being pollinated by tiny *Drosophila* flies, and other species having much larger flowers and being pollinated by bumblebees (Bernhardt et al. 2014b).

Despite already Müller (1873) being aware of size limitations in the relationship between *C. calceolus* and its pollinators, it is somewhat surprising that, 150 years later, no empirical attempt has been made to quantify when insects are too small or too large to be pollinators in any of the slipper orchids (Cypripedioideae). Many studies have investigated both floral and insect dimensions (Bernhardt et al. 2014a,b; Bänziger et al. 2005, 2008; Catling and Knerer 1980; Edens-Meier et al. 2011, 2018; Erneberg and Holm 1999; Li et al. 2008a; Li et al. 2006; Nilsson 1979; Pemberton 2013; Stoutamire 1967) and generally accepted that the insect's thorax should be of roughly the same height as that of the flower's exit (Bernhardt et al. 2014a,b; Nilsson 1979; Pemberton 2013). However, it is not known how the thorax size of a specific flower visitor stands in relation to the exit of the flower visited by that insect, and whether and how this influences the likelihood of the insect to act as a pollinator. Yet, the success (pollination) or failure (no pollination) of these individual interactions lays the foundation to the evolution of floral traits.

This study determines floral visitor, flower, and for comparative reasons also vegetative, size parameters of *C. calceolus* along an altitudinal gradient. Specifically, we measured four floral (length and height of labellum, width of entrance hole, height of exit hole) and three vegetative (plant height, length of the third leaf, stem diameter) traits of *C. calceolus* at five populations situated between 450 masl and 1450 masl. We observed flower visitors, recorded their escape mode (e.g., visitor left the flower through one of two exits or the entrance), whether they exported pollen, and measured four morphological traits of the flower visitors (length, head width, thorax width, thorax height). We test the hypotheses that (1) floral, but not vegetative traits of *C. calceolus* change in a concerted manner with those of the flower visitors along the altitudinal gradient, that (2) the plant-pollinator morphological fit, i.e., the size difference between the thorax height of an insect and the exit height of

the visited *C. calceolus* flower, influences the escape mode of a trapped insect, and (3) that this fit affects the likelihood of whether a flower visitor exports pollen or not. We also aim to establish the upper and lower limits of this mechanically dependent relationship, i.e., when is an insect too large or too small to export pollen.

Materials and methods

Plant species

Cypripedium calceolus L. is a perennial herb mainly distributed in boreal and temperate zones of Europe and Asia (Cribb 1997; Kull 1999). This pollen-limited orchid is mostly found in medium shady deciduous and coniferous forests, alpine meadows, and rubble, predominantly on calcareous soil (Kull 1999). It is one of the biggest and showiest European orchids, with stem heights of up to 70 cm (Kull 1999). Flowering shoots develop from an underground rhizome and most often produce one or two, rarely three flowers (Cribb 1997; Kull 1999). It can reproduce vegetatively through the branching rhizomes, building patches with many shoots belonging to one or several clones (Kull 1999). The flowers consist of two purple-brown petals, a purple-brown dorsal sepal, two purple-brown fused lateral sepals, and of the characteristic, pouch-shaped, yellow labellum (Cribb 1997). Medium-sized solitary *Andrena*, *Lasioglossum* and *Halictus* bees (Hymenoptera) are the most frequent pollinators (Braunschmid et al. 2017, 2021; Nilsson 1979), but hoverflies (Diptera) have also been observed to carry its pollen (Antonelli et al. 2009; Blinova 2002; Braunschmid et al. 2017, 2021). The pollen smear attached to the pollinators consists of single pollen grains contained in elastoviscin, a sticky smear similar to pollenkitt (Pacini 2000). *Cypripedium calceolus* is considered critically endangered or near-threatened in many parts of Europe, and has already gone locally extinct in parts, mostly due to collection by enthusiasts, overgrazing, or logging of the forests in which it occurs (Bilz 2011; Jakubska-Busse et al. 2021; Kull 1999).

Study sites

Investigations were conducted in 2015 and 2021, overall at five study sites with naturally occurring *C. calceolus* populations in the Austrian and German Limestone Alps nearby Salzburg. The study sites spanned an altitudinal gradient from 450 masl to 1450 masl, and we named the sites accordingly (i.e., Site450–Site1450). Flowering started, depending on altitude and year, between early May and end of June, and lasted approximately two weeks per site. Site450 is a spruce-dominated forest along a river with broad limestone gravel banks with a *C. calceolus* population of around 400 shoots. Site600 is on the shore of a mountain lake, characterized by very light spruce and willow forests with patches of marshy grassland in between; it has a population with about 2000 shoots. Site800, a forest with spruces, beeches, larches, and mountain pines along a dry, calcareous riverbed, harbours about 400 *C. calceolus* shoots. Site1300 is a dense spruce mountain slope. The steep forest is interrupted by grassy strips, a few dozen meters wide, some of which reach a few hundred meters from the summit to the valley. Up to 2000 shoots grow mainly in these clearings. At Site1450, ca. 400 shoots grow in-between dry limestone streams and mountain pines. Two of the sites were studied in both years (Site450 and Site1450), whereas Site600 and Site1300 were studied in 2015 only and Site800 in 2021 only.

Plant size measurements

Four floral and three vegetative traits of *C. calceolus* were measured. To learn about labellum traits potentially involved in the attraction of flower visitors (size properties of the labellum) as well as in the trapping and release of flower visitors, we measured, non-destructively, the length and the height of the labellum, the width of the entrance hole (Fig. 1a), and the height of the insect-chosen or a randomly chosen exit hole of the flower, i.e., the distance between the labellum wall and an anther (Argue 2012; Fig. 1b). The exit height presents the narrowest part of the escape path and the place where pollen will be taken up by a correctly sized insect (Fig. 1c).

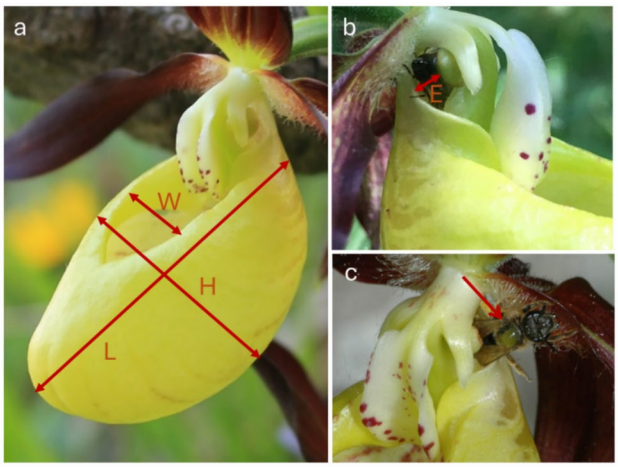

Fig. 1 **a** The labellum of a *Cypripedium calceolus* flower with the three traits measured: length (L) and height (H) of the labellum, and width (W) of the entrance hole. **b** We also measured the height of one of the exit holes of a flower (E), i.e., the smallest distance between the labellum wall and an anther; here, a flower visitor is on its way of passing the exit. **c** A *Lasioglossum* bee passed the exit already with its head, thorax, and part of the abdomen, with pollen smear thereby deposited on its thorax (arrow)

We did not measure the distance between the labellum wall and the stigma of a flower, where previously collected *C. calceolus* pollen will be deposited by a pollinator on its way to the exit, because this would have required damaging the flower. For the same reason, we did not assess whether pollen had been deposited or not. As vegetative traits, we measured the height of the plant, the length of the third leaf (from the bottom), and the stem diameter (3 cm above ground). Flower measurements were performed with a digital slide gauge to the nearest 0.01 cm and vegetative traits with a measuring tape to the nearest 0.1 cm. Per year, all measurements were performed by the same person, but that person changed from 2015 to 2021.

Traits of overall 376 *C. calceolus* flowers (one per shoot) were measured at the five study sites: 90 at Site450, 71 at Site600, 66 at Site800, 45 at Site1300 and 104 at Site1450 (for more details refer to Table SI1). Of those, 176 flowers (some of which were visited more than once) were selected by insects, and these were used to test for an effect of the morphological fit (see below) on escape mode and pollen export. Another 200 shoots were randomly chosen to have a set of measurements that is not influenced by potential pollinator preferences. These measurements were used to determine altitude-related changes in the size of plant traits. We randomly selected patches of *C. calceolus*, of which we measured one flower per one, two, or three shoots (depending on whether a patch consisted of less than eight, between eight and 15, or more than 15 flowering shoots, respectively). Given *C. calceolus'* ability to reproduce vegetatively, it was unknown whether different shoots within the same patch were from the same or a different clone (see also Brzosko 2002). For our analyses, the up to three flowers measured per patch were treated as being independent measures.

Flower visitor observations and measurements

On warm and sunny days, when putative pollinators of *C. calceolus* are most active (Nilsson 1979), we repeatedly went from patch to patch to look for insects trapped in flowers. If an insect was found inside a labellum, a perforated plastic bag was put over the terminal part of the shoot that contained the flower, and the escape mode of the insect was observed. The plastic bag, which did not affect the insects while visiting the flower, assured that they did not escape after having left the flower but were available for measurements. Five different escape modes we discriminated: (1) "exit", if the insect left through the exit hole; (2) "exit^{D}", if the insect got stuck and died in the exit hole; (3) "entrance", if the insect left through the entrance; (4) "labellum^{D}" if the insect died inside the labellum and was found dead on the bottom of the labellum; (5) "undefined" if the escape mode could not be observed. Exiting insects captured in the bag were immediately examined for fresh pollen smear of *C. calceolus* on their back (see Fig. 1c).

Insects were subdivided into different categories, similar as proposed by Antonelli et al. (2009): "flower visitors" were all insects that had been inside the labellum, including those whose escape mode was not observed; "pollen vectors" were the subset of insects that were observed to leave through the exit, thereby got pollen deposited on their back, and were considered able to fly afterwards. This last point we considered critical as the wings of some Diptera were glued together by pollen smear, so that they were unlikely to continue flying or pollinating (see Fig. SI1 for an example). These were then considered "non-pollen vectors," alongside those that left the flower without pollen.

Following the examination for pollen, insects were either collected for later measurements and identification in the laboratory (data collected 2015; insects were the same as reported in Braunschmid et al. 2017), or they were briefly anesthetized with CO_2, measured, photographed for later identification to the order level and released (data collected 2021). Using a digital sliding gauge, we measured the length of the insect (head to abdomen), head width (maximum lateral extent), thorax width (maximum lateral extent), and thorax height (maximum vertical extent) to the nearest 0.01 cm each. The insect and the respective flower received the same ID code to be able to match up plant and insect dimensions during the statistical analyses (see below). In two cases, we observed that the same insect visited a specific flower twice. In these cases, we only considered the first visit.

In total, we collected data on 299 insects. Of those, 86 were collected from *C. calceolus* flowers without measuring the flowers and 213 were associated with flower measurements Thirty-one flowers were visited more than once (26 flowers were visited twice, four flowers three times, and one flower four times), so that, overall, 213 unique insects visited 176 specific flowers. For a more detailed overview of the insects' sample size per year, site, and the escape mode, see Table SI2.

Statistical analysis

To investigate whether floral and vegetative plant traits and flower visitor traits differed between years and among sites (altitude) (including their interaction), 2-factorial PERMANOVA analyses (10000 permutations), based on Euclidean distances calculated on the normalised (mean of 0, standard deviation of 1) plant and insect traits, were run using PRIMER 7.0.23 (PRIMER-E Ltd, http://primer-e.com). Where significant, we ran post-hoc tests (not performed between 600 masl and 800 masl and between 800 masl and 1300 masl as for these pairs of sites data were available for different years only). In R, version 4.2.3 (R Core Team 2023), Principal Component Analyses (PCAs)

were performed with the vegan library (Oksanen et al. 2022) to visualize similarities and differences of floral, vegetative, flower visitor, and Hymenoptera sizes across the samples of the different altitudes. All plant/insect traits as well as altitude were normalised (as before). We used the envfit function of the R vegan library, which calculates correlations with the PCA axes by fitting environmental variables (here: altitude) onto the ordination using linear regression. The function uses permutation (n = 999) tests to test for significance. We only plotted the altitude vectors when $p < 0.05$. We also plotted morphological traits to visualize how much of their variability is captured by the PCA axes and to illustrate their relationship with altitude. Ellipses showing the 95% confidence interval around the centroids of each year/site combination were drawn, using the R ggpubr library (Kassambara 2023a).

We further assessed interpopulation and year differences of each plant/insect trait separately, using Kruskal-Wallis rank sum tests (KW-tests) of the rstatix R library (Kassambara 2023b). Where KW-tests were significant, we used Dunn's post-hoc tests with Bonferroni correction (of the same library) to further assess these differences. We chose non-parametric approaches because not all traits followed a normal distribution according to Shapiro-Wilk tests. Because we found differences in some plant traits between randomly and insect-selected flowers (e.g., plant height at 1450 masl in 2015: Dunn's $z_{1,59} = 2.99$, $p = 0.003$; exit height at Site1450 in 2021: Dunn's $z_{1,47} = 2.17$, $p = 0.03$), we based all the analyses on plant traits (floral and vegetative) on the random subset of plants. For insects, we separately analysed all flower visitors and the subset of pollen vectors only.

We calculated the morphological fit by subtracting the insect's thorax height from the flower's exit height of the flower visited by that insect. The non-parametric data were analysed using KW-tests followed by Dunn's post-hoc tests with Bonferroni correction. First, we tested whether morphological fit differed between altitudes (450 masl, 600 masl, 800 masl, 1300 masl, 1450 masl) or years (2015, 2021). Although there were no differences ($p > 0.05$), we decided against pooling the data by year as some traits—specifically exit height—differed between years, likely due to an observer bias (Fig. SI2). Introducing year as a random effect resulted in singularity for Diptera, thus, in tests that included either exit height or fit, we considered the measurements of the different years as different groups. Second, to test whether the morphological fit of Diptera and Hymenoptera (not Coleoptera, as they all exited via the exit hole) differed between escape modes (exit, death in exit, entrance, death in labellum), KW-tests followed by Dunn's post-hoc tests were used. We calculated the medians and the median absolute deviations (MAD) of morphological fit per insect subset.

To assess whether the morphological fit affected the likelihood that a flower visitor was also a pollen vector, we first ran permutation tests (10000 permutations) on their density distributions using the sm.density.compare function of the R sm library (Bowman and Azzalini 2021). Secondly, we built logistic regression models using the absolute values (moduli) of the morphological fit to assess whether the absolute mismatch (distance from zero) affected whether a flower insect was a pollen vector (value = 1) or a non-pollen vector (value = 0). Other R packages used for loading, manipulating, and analysing data were biostat (Gegzna 2020), ggpmisc (Aphalo 2023), patchwork (Pedersen 2024), plyr (Wickham 2011), PMCMRplus (Pohlert 2023), tidyverse (Wickham et al. 2019), and viridis (Garnier et al. 2024). Inkscape 1.3.2 (Inkscape's developers, http://inkscape.org) was used to develop figures.

Results

Plant traits

An increase in altitude led to an overall decrease in vegetative (Pseudo-$F_{4,191} = 33.92$, $p < 0.001$) and floral (Pseudo-$F_{4,192} = 26.62$, $p < 0.001$) traits. Also, year affected both vegetative (Pseudo-$F_{1,191} = 4.06$, $p = 0.03$) and floral traits (Pseudo-$F_{1,192} = 36.54$, $p < 0.001$), but less consistently (Fig. SI2). The interaction of year and altitude affected floral (Pseudo-$F_{1,192} = 5.55$, p = 0.001) but not vegetative (Pseudo-$F_{1,191} = 0.15$, $p = 0.88$) traits. As shown by post-hoc analyses, both floral and vegetative traits differed among all altitudes ($p < 0.05$ each) except for floral traits between 450 masl and 600 masl ($p = 0.15$) and for vegetative traits between 1300 masl and 1450 masl ($p = 0.05$).

Flower traits were particularly small at the highest altitude site when compared to the other sites (Figs. 2a, SI2), while most of the vegetative traits continuously decreased with increasing altitude (Figs. 2b, SI2). In the floral PCA, 57% of variance were explained by PC1, mostly due to its correlation with mouth width, labellum length, and labellum height. Exit height, the only floral trait not correlated with PC1 or with altitude, strongly correlated with PC2, which explained 23% of the variance (Fig. 2a). Year significantly affected exit height (Kruskal-Wallis H = 112, df = 6, $p < 0.001$, n = 200), with larger values recorded in 2015 (Fig. 3a). Other plant traits, with the exception of mouth width at 450 masl, were similar across years (Fig. SI2). Vegetative traits correlated (moderately) strongly with PC1, which explained 82% of the variance (Fig. 2b). Altitude correlated weakly with PC2, which explained 13% of the variance (Fig. 2b).

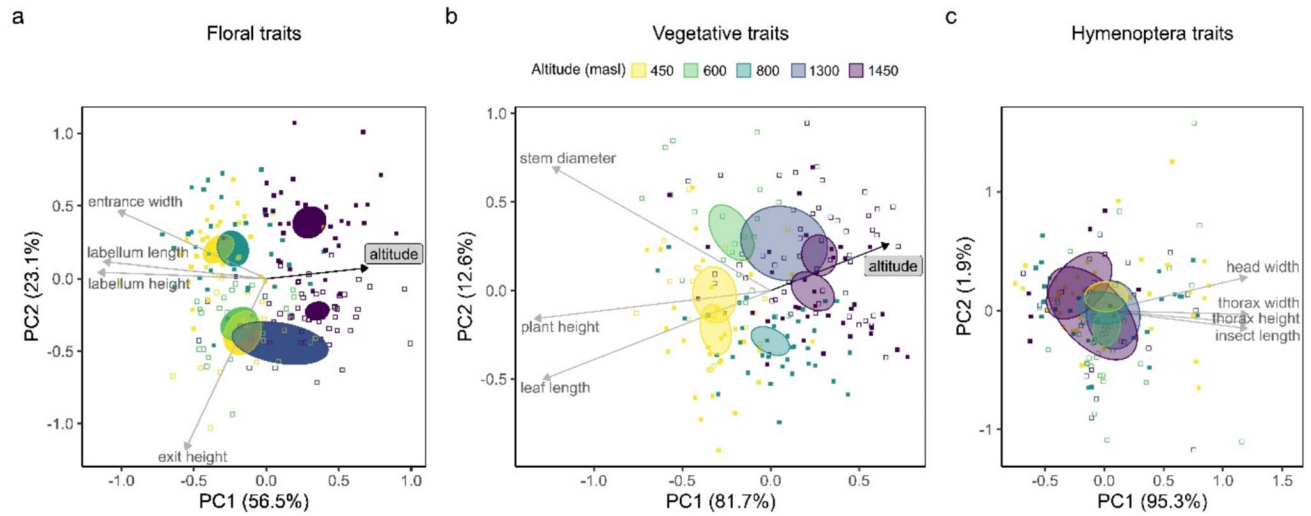

Fig. 2 Principal component analyses of floral (**a**) and vegetative (**b**) traits of *Cypripedium calceolus* flowers, and of morphological traits of hymenopteran floral visitors (**c**) measured at five different altitudes during two flowering seasons. The length of the arrows indicates the amount of variation explained by a variable and the angle of arrows to each other indicate the strength of correlation (more acute angles are stronger correlations). Grey arrows are response variables (morphological traits), and black arrows are predictors (shown when $p < 0.05$). Ellipses show the 95% confidence interval of the centroid of each group. For visualisation purposes, all vectors are reduced to 0.5 times their original length

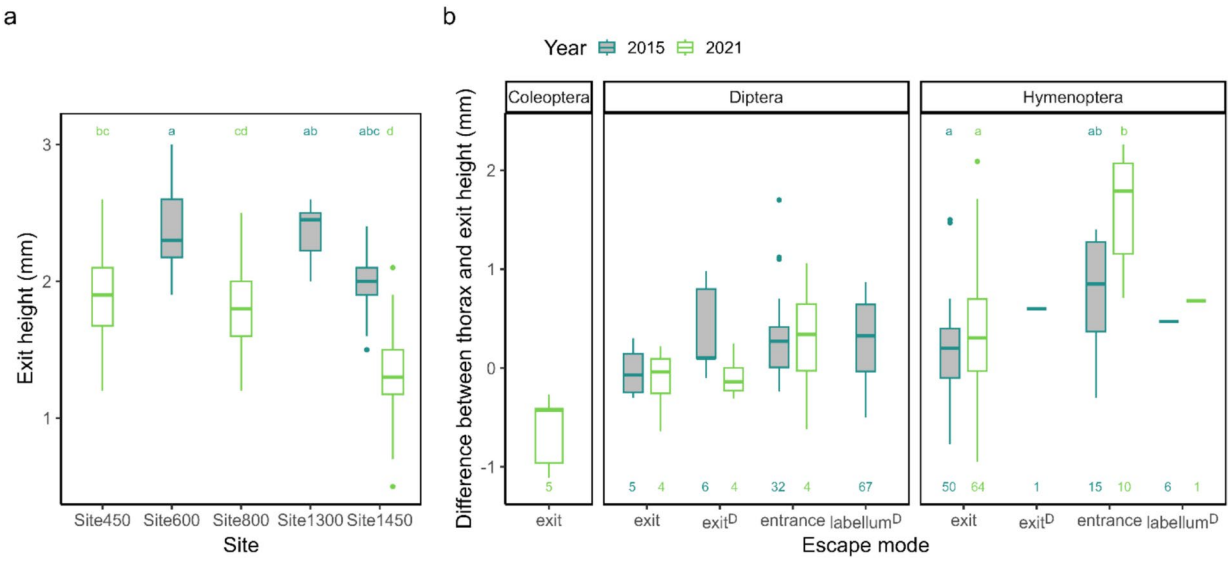

Fig. 3 Differences in the exit height (mm) of randomly chosen *Cypripedium calceolus* flowers across study sites and years (**a**), and the relationship between the escape mode and the difference in thorax height and exit height (morphological fit) of Coleoptera, Diptera, and Hymenoptera visiting *C. calceolus* flowers (**b**)**.** The bars present the median, the boxes the interquartile range (first and third quartile), the whiskers show the data that fall within 1.5 times the interquartile range (minimum and maximum), and the dots represent outliers. Numbers above the x-axes give the number of observed interactions (n), and letters (only given where significant differences were detected) above indicate significant differences in fit among escape modes according to Dunn's post hoc tests

Pollinator climate and traits

Most flower visitors were bees (Hymenoptera; 53.8%, 160 individuals; see Table SI2 for details), followed by hoverflies (Diptera; 44.5%, 133 individuals), and these two groups differed markedly in their effectiveness of pollen removal. While 57% (91 individuals) of the Hymenoptera that entered the labellum exited the flower via the exit and exported pollen, this was true for only 5% (13 individuals) of Diptera. Instead, most (59%, 78 individuals) of Diptera trapped in

the labellum did not manage to exit and died (partly because they get stuck in the exit). In contrast, just 5% (8 individuals) of Hymenoptera died in the flower (one individual in the exit). We also recorded five Coleoptera (1.7% of the total insect count), four of which were pollen vectors (Table SI2, Fig. SI3). Generally, pollen was deposited on dorsal parts of the insect bodies, mainly the thorax, though in some Diptera and Coleoptera also on the wings (Figs. SI1, SI3). Only in three cases insects passed the exit upside-down, with pollen deposited on their ventral sides: one *Lasioglossum* and two *Andrena* individuals. Hymenoptera often began buzzing while passing through the exit (see video in SII).

Hymenoptera sizes were similar across the altitudinal gradient (Pseudo-$F_{4,145}$ = 0.82, p = 0.52; Fig. 2c) and across years (Pseudo-$F_{1,145}$ = 0.35, p = 0.58). Approximately 97% of their variance was explained by the two PCA-axes (Fig. 2c). Also, the traits of pollen vectors were similar across altitude (all insect orders; altitude: Pseudo-$F_{4,95}$ = 1.01, p = 0.40) and years (Pseudo-$F_{1,95}$ = 0.25, p = 0.67; Fig. SI4). In contrast, flower visitor sizes changed with altitude (Pseudo-F4,270 = 2.65, p = 0.03) but not between years (Pseudo-$F_{1,270}$ = 0.75, p = 0.39; Fig. SI5). Post-hoc tests showed that sizes at 1300 masl, where Diptera were more abundant (Table SI2), differed from those at 600 masl (p = 0.04) and 1450 masl (p < 0.001; see also Fig. SI4). With regards to the single flower visitor traits, they were largest at 1300 masl (Fig. SI4).

Effects of the morphological fit on escape mode and pollen export

Coleoptera, which all left through the exit, were all smaller than the exit, with a median and MAD of − 0.43 ± 0.16 mm. In Diptera, the morphological fit did not affect the escape modes in 2015 (Kruskal-Wallis H = 2.85, df = 3, p = 0.42, n = 37) and 2021 (Kruskal-Wallis H = 1.42, df = 2, p = 0.49, n = 12; Fig. 3b), with medians and MADs ranging from 0.04 ± 0.17 mm for an escape through the exit to 0.34 ± 0.44 mm for an escape through the entrance (also see Table SI3). Diptera leaving through the exit were maximally 0.3 mm larger and 0.6 mm smaller than the exit, those dying in the exit were between 1.0 mm larger and 0.3 mm smaller than the exit, and those escaping or dying in the labellum were up to 1.7 mm larger and 0.6 mm smaller than the exit (Fig. 3b). Also, possibly because sample sizes were limited, we did not find that the morphological fit affected the likelihood that a visit resulted in pollen export in neither 2015 nor 2021, although both density distributions and logistic regressions appeared markedly different (p > 0.05; Fig. 4a, b). The median morphological fit for pollen and non-pollen vectors was – 0.2 mm and 0.3 mm in 2015 and 0.1 mm and − 0.1 mm in 2021, respectively.

In Hymenoptera, the morphological fit differed between the escape modes exit and entrance in 2021 (Dunn's Z = 4.38, p < 0.001, n = 64 [exit] and 10 [entrance]), with a

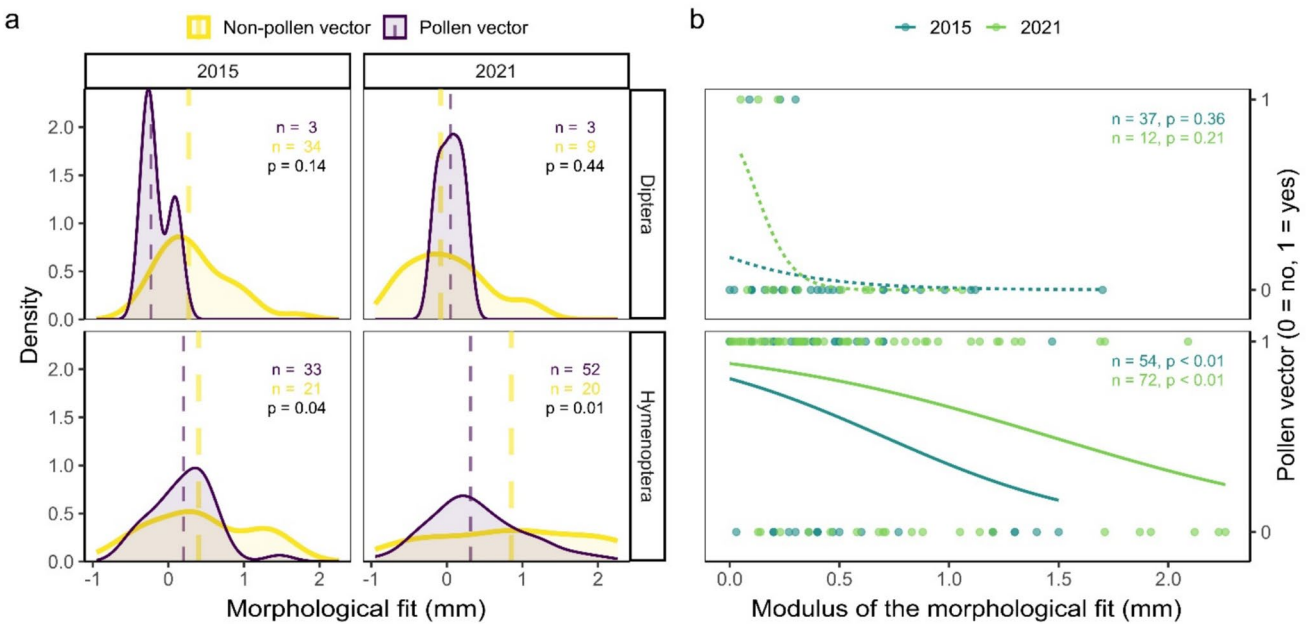

Fig. 4 The influence of the morphological fit (the difference between thorax height and exit height) on the likelihood that Diptera and Hymenoptera, the most abundant flower visitors, exported pollen from *Cypripedium calceolus* flowers in 2015 and 2021. **a** Density distributions of the morphological fit for cases where an insect visit did (pink) or did not (yellow) result in pollen export and (**b**) binomial logistic regressions of the modulus of the morphological fit, where pollen vectors received the value 1 and non-pollen vectors the value 0. n = sample sizes

median and MAD of 0.2 ± 0.3 mm and 1.8 ± 0.5 mm, respectively (Fig. 3b; also see Table SI3). The smallest hymenopteran escaping through the exit was 1.0 mm smaller than the exit, and the largest was 2.1 mm larger than the exit. Only single ones were found dying in the exit or the labellum, with fit being average in all these few cases. The average morphological fit was smaller and closer to zero in pollen vectors in both 2015 ($p = 0.04$, median = 0.2 mm) and 2021 ($p = 0.01$; median = 0.3 mm) compared to non-pollen vectors (medians of 0.4 mm and 0.9 mm, respectively; Fig. 4a). A higher density towards a more accurate fit in Hymenoptera is also confirmed by logistic regressions for both study years. They evidenced that the likelihood that a visit resulted in pollen export is increased when the morphological fit approaches zero (Fig. 4a, b).

Discussion

We observed the behaviour and measured size traits of nearly 300 flower visitors of *Cypripedium calceolus*, often in parallel with size traits of the flowers visited by these insects and of vegetative traits, at five different altitudes. In this highly size-dependent pollination relationship, we unexpectedly found a decoupling of floral and insect dimensions: increasing altitude caused a decrease in vegetative and floral dimensions of *C. calceolus,* but flower visitors were largest at the second highest site, which was characterised by a high abundance of hoverflies (Diptera), the second most frequent visitors but inefficient pollen vectors. In bees (Hymenoptera), the most frequent flower visitors and most efficient pollinators, no change in the dimensions were found at all along the altitudinal gradient. Coleoptera, unknown as pollinators of this orchid before our study, were infrequent but rather efficient at exporting pollen. The morphological fit, i.e., the similarity of the thorax height of a visitor and the exit height of a flower influenced whether visiting Hymenoptera escaped the labellum through the entrance hole or the exit, and whether they exported pollen or not. In Diptera, we did not find that the morphological fit affected the escape mode or the likelihood of pollen export, though this may be a result of low sample sizes. Overall, an insect was more likely to export pollen from a flower when its thorax was close in size to the exit, although Hymenoptera up to 2 mm larger were observed exporting pollen.

Pollinator climate and effect of the morphological fit on escape mode and pollen export

Our results confirm previous findings that primarily Hymenoptera and rarely Diptera export pollen and hence are potential pollinators of *C. calceolus* (Antonelli et al. 2009; Blinova 2002; Braunschmid et al. 2017, 2021; Erneberg and Holm 1999; Nilsson 1979). Hymenoptera were highly efficient: most of them were able to squeeze through the exit and collected pollen, few escaped through the entrance hole, and even fewer died in the labellum or the exit (Fig. 3). The vibrations caused by the bees when exiting possibly decreases friction momentarily, facilitating their escape (Bernhardt et al. 2014a; Bänziger et al. 2005; Li et al. 2008a). Diptera, on the other hand, were rather inefficient: only a few managed to escape through the exit and then only rarely exported pollen. Many got stuck inside the exit and died, or they came out with wings glued together by pollen smear (Fig. SI1). Also, a large proportion died inside the labellum (Fig. 3). The low effectiveness of Diptera in the pollination of some *Cypripedium* spp. was noted in a number of earlier studies (Antonelli et al. 2009; Bänziger et al. 2005; Edens-Meier et al. 2011; Li et al. 2008a,b), though in some *Cypripedium* species they are the main pollinators (e.g., Jiang et al. 2020; also summarised in Bernhardt et al. 2014b).

By coupling plant and insect traits, i.e., determining the morphology of flower visitors together with the morphology of the flower actually visited by that visitor, and analysing the outcome of a flower visit, we were able to quantify the boundaries of the morphological fit. It shows that individuals that have a thorax height similar to the exit height are most effective at exporting pollen (Fig. 4). However, our study also shows that the system is more flexible than previously thought in *Cypripedium* (but see Antonelli et al. 2009; Bänziger et al. 2005, 2008; Jiang et al. 2020; Li et al. 2006), at least when it comes to Hymenoptera, the main pollen vectors: they were up to 2 mm larger and down to 1.3 mm smaller than the exit, showing that *C. calceolus* flowers possess a ca. 3 mm tolerance of insect sizes (thorax height) fitting through its exit and exporting pollen. In Diptera, however, the few pollen-exporting individuals all had a thorax height very similar to the exit height (± 0.3mm) (Figs. 3, 4). In a study on *C. flavum*, Zheng et al. (2011) found that Diptera successfully emerging from the exit were, on average, smaller than Hymenoptera. Diptera, thus, seem to be inferior to Hymenoptera in strength, and less capable of pushing the anther and labellum wall—which determines the size of the exit height—apart. This suggests that it is not only size that matters in the pollination of *C. calceolus* but that also other visitor traits are of importance.

How large is too large? Prior studies proposed that insects can deflect the flexible anthers of certain *Cypripedium spp.* by more than 0.5 mm (Bänziger et al. 2005; Case and Bradford 2009; Nilsson 1979). We observed three large Hymenoptera that—though visibly struggling—managed to push the exit apart by up to 2 mm. In total, nine Hymenoptera (but no Diptera or Coleoptera) were more than 1 mm larger (Figs. 3, 4). Although these Hymenoptera may have been exceptionally strong, it is also a possibility that the tissue of

these flowers was quite soft, which facilitated the passage. Nilsson (1979) noted that the escape time in ageing flowers was lower than in young flowers, possibly because the tissue gets softer while ageing. The more elastic floral tissue of older flowers decreases the force needed to squeeze through the opening. Thus, older flowers may simplify the escape for flower visitors and allow much larger Hymenoptera to escape via the exit, effectively increasing the upper limit of the morphological fit during anthesis. Assuming that old flowers are still receptive (see Argue 2012), the flowers of *C. calceolus* would become more generalized during anthesis when it comes to pollinator sizes.

How small is too small? Researchers have often assumed that a thorax smaller than the exit of *C. calceolus* and other *Cypripedium* species would not get in contact with the stigma or anthers (Antonelli et al. 2009; Bänziger et al. 2005, 2008; Li et al. 2006). However, Wright (1975, cited by Argue 2012) observed a Hymenoptera considerably smaller than the exit leaving it with pollen, yet did not consider it an effective pollinator. Our findings emphasise that pollen vectors smaller than the exit are not unusual: three Diptera, and more than ten Hymenoptera exported pollen despite being smaller (thorax height) than the exit. They likely contacted the anthers because their body, including the thorax, was lifted by their legs. Thus, in small insects, not only the height of the thorax needs to be considered, but also to which extent the body is lifted while walking. The same may hold for thorax hair, which can increase contact with the pollen smear. Three times we observed an upside-down escape, in which the insect's anterior or ventral side faced the stigma or anthers. Thus, depending on how exactly an insect escapes, pollen smear may be deposited on its head, abdomen or on the legs, as has been observed in other studies (Antonelli et al. 2009). The fact that insects can escape by crawling over the anther itself raises the question whether there is a lower limit at all. Manipulative experiments, placing tiny insects into the labellum and watching their possible escape through the exit, could help illuminate this question. In such experiments it should also be tested if insects that have collected pollen on other parts than their dorsal site are capable of transferring pollen to the stigma of the flowers. Anyhow, given the fact that insects only very rarely pass the exit upside-down, such insects contribute, if at all, only marginally to the reproductive success of *C. calceolus*.

Pollinator-mediated natural selection versus divergence of floral size traits in response to other factors than pollinators

In agreement with previous studies on *Cypripedium* or other plants (e.g., Argue 2012; Armbruster et al. 2009; Cosacov et al. 2014), our results show that pollinators of *C. calceolus* should have the potential to exert size-related selection pressures, given that only correctly sized insects can export pollen. However, our findings do not suggest that differences in size traits along altitude are a result of differential selection pressures exerted by variable pollinator climates that differ in size among populations. This is because only floral sizes—but not Hymenoptera (main pollinators) sizes and not consistently sizes of all flower visitors pooled (including ineffective Diptera pollinators)—changed with altitude. The finding that hymenopteran sizes do not differ along the altitudinal gradient is in line with a previous bee survey in the same study region. When excluding bumblebees, which did not occur as flower visitors in our study, Hoiss et al. (2012) found that Hymenoptera sizes are independent of altitude. Depending on the study, body sizes of Diptera increase, decrease, or are constant along an altitudinal gradient (de Groot and Vrezec 2019; McCabe et al. 2019).

Despite similar insect sizes along altitude in our study, there still might be different selection pressures at different sites, as the relative abundance of Hymenoptera and Diptera differed along altitude and the two insect groups have different properties (e.g., strength; see before). Weaker Diptera may select for larger exits at sites where they are abundant pollinators. At our montane sites (Site1300 and Site1450), Diptera outnumbered Hymenoptera at least in 2015 (Table SI1, Braunschmid et al. 2017), suggesting that at least in some seasons Diptera are more abundant flower visitors of *C. calceolus* at higher altitudes. This agrees with other studies showing that the abundance of Diptera, in contrast to that of Hymenoptera, often increases towards higher altitudes. In the Dolomitian Alps, Diptera abundance peaks at around 1400 m (Sommaggio et al. 2022). However, due to their ineffectiveness as pollen vectors, and in spite of their (relatively) high abundance at high-altitude populations of *C. calceolus*, they probably exert only weak selection pressures.

It is very likely that the phenotypic divergence of flower traits recorded in our study arises from abiotic factors related to altitudinal changes. They may mask any effects pollinators have on floral morphology (Strauss and Whittall 2006). Decreasing temperatures and shortened growing seasons decrease plant productivity higher up, resulting in lower resource allocation to growth (e.g., Chen and Zhang 2023; Körner et al. 1989). In *C. calceolus*, many of the floral and vegetative traits were particularly small at Site1450. This may be related to the specific local conditions at this site. Being a limestone scree, disturbances by rockslides, extreme frost events, or desiccation provide harsh and stressful conditions for the immobile vegetation (Deil et al. 2008). We assume that *C. calceolus* is severely affected by these conditions, also regarding its reproductive success: if flowers, including the exit height, become smaller, but insect sizes are unaffected, a mismatch in the fit might arise. The lower reproductive success might then

become exacerbated by the anyway low insect visitation rate at Site1450 (pers. observation).

Conclusion: does size matter?

We for the first time determined the size (thorax height) visiting insects need to have in relation to the *C. calceolus* flower (exit height) that they visit to act as pollen vectors. We found that insects whose thorax heights were within 1 mm smaller and 2 mm larger than the exit height were more likely to leave the flower through this exit and to export pollen. The size was more restrictive in Diptera than in Hymenoptera, which suggests that the insects' physical properties may also play a role in pollination: Diptera may lack the strength to push through a relatively small exit hole. This might drive the selection for larger exit holes where Diptera are abundant pollen vectors. Size was less restrictive in Hymenoptera, where we found that pollen vectors are more variable in size than previously assumed. Overall, our findings show that flower and pollinator sizes matter in the pollination of *C. calceolus*. However, differences in flower sizes along the studied altitudinal gradient cannot be explained by changes in flower visitor sizes: they are likely due to variable abiotic parameters that change with altitude.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00035-025-00330-6.

Acknowledgements We are grateful to the National Park Berchtesgaden, especially to Doris Huber and Fritz Eder, for supporting this study and the district government of Upper Bavaria (55.1-8645-2-2013 and ROB-55.1-8646.NAT_02-8-75-3) as well as the government of Salzburg (205-05RI/551/112-2014; 205-05RI/551/135/14-2019) for the collection permits. We thank Jonas Eberle for confirming beetle identities and are deeply grateful to Ansuela Braunschmid for her invaluable support. Øystein H. Opedal and João C. F. Cardoso provided valuable comments to an earlier version of the manuscript.

Author contribution Marie Hoensbroech: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. Stefan Dötterl: Writing – review & editing, Methodology, Formal analysis, Conceptualization, Funding acquisition. Herbert Braunschmid: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization, Funding acquisition.

Funding Open access funding provided by Paris Lodron University of Salzburg. This research was funded in whole or in part by the Austrian Science Fund (FWF; [10.55776/P32142]. For open access purposes, the author has applied a CC BY public copyright license to any author-accepted manuscript version arising from this submission.

Data availability The data generated and analysed during the current study will be made available in the Supplementary Information.

Declarations

Competing interest The authors declare no competing interests.

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