



Floral traits determine pollinator visitation in *Rhododendron* species across an elevation gradient in the Sikkim Himalaya

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Received: 15 January 2019 / Accepted: 30 July 2019
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Abstract

Plants growing along steep elevational gradients experience variations in abiotic conditions. The elevational gradient also affects the diversity and abundance of pollinators associated with these plants. As a result, plants may have locally adapted floral traits. However, detailed assessments of multiple floral traits along elevational gradients are often neglected despite the traits being important for plant sexual reproduction. We tested the association of floral traits with pollinators in response to elevation by identifying pollinators and measuring morphological and biochemical floral traits as well as studying the breeding systems of ten aggregated *Rhododendron* species in the Sikkim Himalaya. Corolla length, nectar volume and distance between stamen and stigma significantly decreased with elevation. In contrast, nectar concentrations were positively associated with elevation. Birds, bumblebees and flies were the three dominant pollinator groups. Bird visits showed a strong negative association with elevation while visits by bumblebees and flies increased with elevation. Species with longer corollas and higher nectar volumes showed higher rates of bird visits, while bumblebees were associated with species that had higher nectar concentrations. Fruit set following cross-pollination was high compared to self-pollination, and higher pollen limitation and auto-fertility were observed among species in higher elevations. These observed patterns in the association between floral traits and pollinator groups in response to elevation may help generate testable hypotheses on alpine plant–pollinator responses to climate warming.

Keywords Elevation · Climate change · Floral traits · Himalaya · Pollinators · *Rhododendron*

Introduction

Plant trait characteristics along elevational gradients reflect responses to temperature and other abiotic conditions which change considerably over relatively short vertical distances (Körner 2007; Junker and Larue-Kontic 2018). Differences in these conditions at different elevations favor the establishment of plant communities with certain traits (Junker

and Larue-Kontic 2018). Elevational gradients, therefore, have been considered to be the most important factor in studies related to phenotypic variations in plant species in mountains (Stöcklin and Armbruster 2016; Kiełtyk 2018). Such knowledge on how plant traits vary along elevational gradients can be valuable to predict elevation range shifts, species declines and extinction risk, especially in the wake of current climate change (Parmesan 2006). However, in comparison to vegetative traits, floral traits along elevational gradients are not well studied although they are important for the plants' sexual reproduction (Fabbro and Körner 2004). Floral traits also influence plant–pollinator interactions and, thereby, impact flower-visiting animal communities (Junker et al. 2013). Variations in floral traits within a relatively short geographical area along an elevational gradient can lead to major shifts in abundance and composition of pollinators too (Gurung et al. 2018; Lefebvre et al. 2018).

Flower color and longevity are the two commonly studied traits along elevational gradients (Shrestha et al. 2014; Trunschke and Stocklin 2017; Bergamo et al. 2018). However,

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00035-019-00225-3>) contains supplementary material, which is available to authorized users.

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we have limited information on other floral traits such as nectar, which is one of the primary rewards for birds and other pollinators (Baker et al. 1998). Nectar is known to be highly sensitive to temperature and increase in the temperature is expected to reduce the nectar secretion in plants and decrease available resources for pollinators (Takkis et al. 2015). On mountaintops, bees and flies are the most dominant pollinators and, in general, are attracted to flowers with lesser amounts of nectar and high sugar concentrations (Bergamo et al. 2018). Contrary to this, birds—the other important pollinators in mountainous areas—usually prefer flowers with high nectar volume (Martén-Rodríguez et al. 2009; Huang et al. 2017) but diluted nectar (Bolten and Feinsinger 1978). Flower size, especially corolla length, is another important morphological trait that corresponds to the size of the probing parts of major pollinators (Martén-Rodríguez et al. 2009). In general, high-elevation montane plants are known to have distinctly smaller flower sizes (Guo et al. 2010) which are selected for insect pollinators such as flies and bumblebees (Kudo 2016).

Apart from selection for certain floral traits, the harsh environments are known to influence plant reproduction (Escaravage et al. 1997) in many ways. Pollinator diversity, abundance and reproductive efficiency of plants are often low in high-elevation arctic and alpine environments due to unfavorable environmental conditions (Totland 1994), causing highly variable and often poor pollination success. In such regions where the availability of pollinators is low, the reproductive success of plants is also drastically reduced due to pollen limitation (a widespread phenomenon among angiosperms) (Larson and Barrett 2000). However, many studies have reported that plants in higher elevations adopt alternative pollination strategies and selfing becomes one of the important means by which plants reproduce due to extreme environmental conditions, short growth periods as well as reduced and unreliable pollinator services (Körner and Paulsen 2009).

Many studies across several mountain ranges have provided evidence on the variation of floral traits and pollinators along the elevation both at the community (Lefebvre et al. 2018) and species levels (Kiełtyk 2018). However, there are very few assessments of elevational variation in floral traits involving pollinators and breeding systems of flowering plant communities in the Himalayan region (see Gurung et al. 2018). The Himalayan montane ecosystem is considered as one of the major global biomes with higher alpine plant diversity than the global average (Körner 1999). Its topographical gradient represents the most extended bioclimatic elevational gradient which may enforce a strong turnover in plant taxa. Grytnes and Vetaas (2002). The Eastern Himalayan region, in particular, is one of the world's biodiversity hotspots (Myers et al. 2000) and is recognized as a critical ecosystem where the impact of climate change

is profound on flora and fauna (Telwala et al. 2013; Subba et al. 2018). This region also contains a higher presence of endemic alpine plants compared to the Western Himalaya and other adjoining regions (Behera et al. 2002).

Therefore, it is critical to understand the influence of the elevational gradient on alpine plants here, particularly with respect to floral traits and its associated pollinators which enable them to survive in the harsh environment. Furthermore, such a study will provide valuable baseline information to detect how plant–pollinator interactions in a fragile mountain ecosystem like the Himalaya will respond to climate change. In the Himalayan region, *Rhododendrons* are one of the dominant flowering plants (Singh et al. 2009); the mountain range is home to more than 90% of the world's natural populations of these plants (Kumar 2012). *Rhododendrons* are distributed across the northern temperate zone, throughout tropical southeastern Asia, and in northeastern Australia (Chamberlain et al. 1996). *Rhododendron* species in the Sikkim Himalaya are distributed along a wide altitudinal range. They vary considerably in their flowering phenology and floral morphology, while supporting a wide range of visitors including bees, butterflies and birds (Kudo 1993; Ng and Corlett 2000; Stout 2007; Georgian et al. 2015; Huang et al. 2017; Basnett et al. 2019). Their wide distribution along an elevation range and diversity in floral traits make *Rhododendrons* an ideal system to evaluate how floral traits and plant–pollinator interactions vary in response to elevational gradients. The study of the plants' breeding system will further help understand the mating strategy of species distributed along the elevational gradient. In this context, we specifically addressed three major questions: (a) Do floral traits and pollinator visits vary across *Rhododendron* species and are they influenced by elevation? (b) Do visitation frequencies of pollinators across *Rhododendron* species correspond to floral traits along the elevational gradient? (c) Do *Rhododendron* species distributed along the elevation exhibit variations in pollen limitation and auto-fertility?

Methods

Study area

The study was conducted from 2013 to 2015 covering the flowering to fruiting seasons (May–October) of ten *Rhododendron* species at the Kyongnosla Alpine Sanctuary (referred to as KAS from here on) (27°N 88°E) situated in the eastern part of the Sikkim Himalaya, India. In KAS, *Rhododendron* species along with conifers form a dominant component of plant communities at every elevation. *Rhododendrons* and conifers occur as tall trees and shrubs in the sub-alpine region (ranging from 3200 to 3800 m Above Sea Level, ASL). The elevation range between 3800 to 3900 m

ASL represents the treeline and the region above the treeline gives way to the alpine plant community where *Rhododendrons* occur as bushy and stunted shrubs along with alpine herbs and dwarf conifers (Basnett et al. 2019; Fig. S1). From 1998, the Sikkim Forest Department has regulated human activities in the protected area and, therefore, pressures from cattle grazing and human settlements are controlled in the KAS. This region experiences intermittent snowfall from December to mid April. During 3 years of study from 2013 to 2015, ten IButtons were deployed along the elevational gradient at every 200 m to record atmospheric and soil temperature. During the study period, the bimonthly mean day and night air temperatures ranged from -1.87 to 10.96 °C (Fig. S2) and the total annual rainfall was 4171 mm (Basnett et al. 2019).

Floral traits across *Rhododendron* species along the elevation

To understand the floral traits of the *Rhododendron* community across the elevation, we collected quantitative data by laying three transects of 50×20 m² at every 100 m elevation starting from 3400 m to 4230 m. Totally, 27 such transects were laid across the study site. We encountered ten *Rhododendron* species inside the vegetation plots; these were then selected as the focal species. Herbarium specimens were prepared and the plants were identified to species level by consulting experts who work on *Rhododendron* taxonomy and by referring to field guides including *Rhododendrons of Sikkim* (Pradhan 2010), the *Flora of China* (Wu et al. 2005) and the *Flora of Bhutan* (Grierson and Long 1983). We measured corolla length, corolla upper width (widest part of the corolla), corolla lower width (narrowest part of the corolla), style length, closest distance between stigma and stamen as well as nectar volume and concentration in 10–12 flowers from randomly selected individual plants at every 100 m interval. For measurement, we used a calibrated digital caliper to an accuracy of 0.01 mm. To measure nectar volume and concentration, flower buds that were likely to open the next day were enclosed in mesh bags which allow air circulations but prevent use by visitors. The following day between 8:00 am and 10:00 am, the nectar volume and sugar concentration were measured using micropipette (50 µl) and a pocket refractometer. Floral trait measurements were carried out only during 2014.

Visitation rate of pollinators across *Rhododendron* species along the elevation

We randomly selected five individuals of each *Rhododendron* species to conduct studies on visitation rates of flower visitors. A flowering branch of each tree was tagged, and the number of fresh florets was counted. Each tagged branch

was observed for 10 min. The visitation study was carried out on all species occurring at every 100 m elevation for 2 years from 2014 to 2015. A minimum of 10–15 m distance was maintained between marked trees. Animal visitors were observed on flowers from a distance of 2–5 m to avoid disturbing them. When bird visitation rate was high, we used binoculars (12 × 50 mm, Eagle optic) and photos (SLR Nikon D90, Nikon) to confirm pollen load on their forehead and other parts of the body. The observation was made from 6:00 am to 12:30 pm by two observers.

All unidentified insects that visited the focus tree species and individuals were collected and pinned to an insect board and later identified in consultation with entomologists. Birds were identified using photos and videos and with the aid of the field guide *Birds of India* (Kazmierczak and Perlo 2000). The visitation study was carried out on sunny days when the frequency of visits was recorded and the visitation rate could be deduced per flower. We did not carry out any direct study to understand the efficiency of each pollinator group; however, we recorded the frequency of visits at a given time which also represents pollinator abundance (Brunet 2009). In the absence of data on a visitor's efficiency, the frequency of visits has been suggested as a surrogate for a visitor's relative importance to the plant species (Fenster et al. 2004). Insects and birds that both picked up pollen and deposited it on a receptive stigma were classified as effective pollinators, as suggested by Stout (2007). Observations were not conducted on days which were extremely foggy, cold or rainy. Total mean visitation rates were calculated for each pollinator group of *Rhododendron* species occurring at every 100 m.

Breeding system

To determine the breeding system of the target *Rhododendron* species, we carried out four pollination treatments. (1) Spontaneous self-pollination: flowers were bagged with a nylon mesh bag and left unmanipulated and this was considered as control (2) Facilitated self-pollination: Flowers were hand-pollinated with the pollen from the same flower. (3) Cross-pollination: flowers were emasculated immediately after opening and were hand-pollinated with pollen from different individuals at least 10 m apart from the recipient plants. (4) Open pollination: Unmanipulated flowers were tagged and left open for natural pollination. The breeding system of each species was investigated on 10–15 randomly selected individuals which were at a distance of 10–15 m apart. Each individual tree received all three treatments; each treatment was performed on separate inflorescences, on at least 4–7 flowers while all other flowers were removed. All treatments were performed during the peak of the flowering phase. As we required many focus trees to carry out each pollination experiment, we selected these individuals from a

specific region along the elevation where the abundance of that particular species was high. Pollen was applied on the stigma by holding the dehisced anther with forceps and rubbing the pollen on. A magnifying glass was used to confirm if pollen was deposited over the stigma and mesh bags were removed once the flowering period was over. Hand pollination experiments were carried out across species between May and July of 2014 and fruits produced by treated flowers were counted and monitored for three to 4 months. For each treatment, we calculated the fruit set as the ratio of mature fruits to the total number of flowers pollinated. This is considered as a good indicator for pollination success in species with a large number of seeds per fruit, such as *Rhododendron* (Kudo 1993).

We calculated pollen limitation index (henceforth PL) for each species to understand if there is a shortage in the supply of pollen to stigmas (Larson and Barrett 2000). The PL was calculated as $L = 1 - (P_N/P_S)$, where P_N is the number of fruit set by the natural pollination and P_S is the fruit set by the cross-pollination. $L=0$ indicates no pollen limitation in the population or species under study (Larson and Barrett 2000). To obtain measures of the capacity for autonomous selfing, we calculated the auto-fertility index (henceforth AFI) for each *Rhododendron* species. The index AFI represents the ability of flowers to self-fertilize in the absence of pollinators (Escaravage et al. 1997). AFI for each *Rhododendron* species was calculated as the ratio between the mean seed set after spontaneous selfing and cross-pollination. The AFI ranges from 0 to greater than 1 and an auto-fertility level greater than zero is a necessary prerequisite for autonomous self-fertilization (Lloyd and Schoen 1992).

Statistical analysis

We used one-way analysis of variance (ANOVA) to determine the difference in the means of floral traits and pollinator groups across *Rhododendron* species. We log transformed the data to meet the assumptions of normal distribution. The R package ‘psych’ (Revelle 2011) was used to check multicollinearity correlation coefficients among the floral traits. Variables having a Pearson’s correlation coefficient $|r| \geq 0.70$ were removed before model building, following Dormann et al. (2013). To understand the effect of elevation (fixed factors) on floral traits, pollinator visits, we build a generalized linear mixed model (GLMM) using ‘lme4’ package. Species identity was included as a random factor and the model was tested using package ‘lme4’ (Bates et al. 2011). Because of the nature of data for floral traits, we used Gamma distribution with log link function and Poisson distribution with a log function for pollinator visits.

We used non-metric multidimensional scaling to evaluate Bray–Curtis dissimilarities (Bray and Curtis 1957) for both floral traits and pollinators of *Rhododendron* species

among different elevational bands using ‘vegan’ package (Oksanen et al. 2013). Unlike other ordination techniques, NMDS makes no assumptions about distribution of the variables (McCune and Mefford 2006) and NMDS ordines objects based on rank distances, thus preserving ordered relationships, so that similar objects are close to each other (Legendre and Legendre 1998). We built two ordinations plots. First, we used the NMDS approach to evaluate the clustering of species dissimilarity distance of morphological data across different elevational bands. Second, we used NMDS to identify the clustering of species dissimilarity distance of pollinator visitation rates across elevational bands. The observation of similar clustering in morphology traits and pollinator visits would demonstrate that the particular floral trait morphologies are associated with specific pollinator groups. We report the stress value and Shepard plot, which represent the goodness of fit of the NMDS approach. We also used non-parametric permutational multivariate analysis of variance (PERMANOVA) based on the dissimilarities matrix to evaluate the difference in floral traits and pollinator groups across *Rhododendron* species along the elevational gradient. We used 1000 permutations to compute F-statistics. All statistical analyses were performed in R version 3.2.4 (R Core Team 2014). Henceforth, species growing below the treeline are referred to as low elevation species (3400–3800) and species growing above the treeline as high-elevation species (3800–4230). Based on *Rhododendron* species composition, we divided the entire elevation bands into four groups (1) 3400–3600 m, (2) > 3600–3800, (3) > 3800–4000 and (4) > 4000–4230 (Table S1). The fruit/flower ratio for breeding experiments did not conform to normal distribution assumptions; therefore, we used generalized linear model (GLM) of binomial error with logit function to compare between control and other pollination treatments for each *Rhododendron* species. We used one-way analysis of variance (ANOVA) to determine the difference in PL and AFI across *Rhododendron* species.

Results

Variations in floral traits and pollinator visits among *Rhododendron* species

We encountered ten *Rhododendron* species inside the vegetation plots and all ten were selected as study species. *Rhododendron thomsonii*, *R. hodgsonii*, *R. cinabarinum* and *R. campylocarpum* occurred in between 3400 and 3800 m, whereas *R. campanulatum* was found from 3400 m until 3915 m. The species which occurred above 3800 m were *R. aeruginosum*, *R. setosum*, *R. lepidotum*, *R. wightii*, *R. anthopogon*. Henceforth, species growing below the treeline (3800–3900 m) are referred

to as low elevation species, and species growing above the treeline as high-elevation species. We found a strong correlation of corolla length with style length ($r^2 = 0.85$), corolla lower width ($r^2 = 0.75$) and corolla upper width ($r^2 = 0.84$; Fig. S3). Therefore, we considered only corolla length followed by nectar concentration, nectar volume and the closest distance between style and stigma for further analysis. A significant difference in corolla length (ANOVA, $F_{9, 285} = 466.4$, $P < 0.0001$), nectar concentration, (ANOVA, $F_{9, 285} = 85.44$, $P < 0.0001$), nectar volume (ANOVA, $F_{9, 285} = 168.3$, $P < 0.0001$) and distance between stamen and stigma (ANOVA, $F_{9, 285} = 21.22$, $P < 0.0001$) was observed in all ten *Rhododendron* species. Pollination visits by birds (ANOVA, $F_{9, 115} = 11.11$, $P < 0.0001$), bees (ANOVA, $F_{9, 115} = 6.33$, $P < 0.0001$) and flies (ANOVA, $F_{9, 115} = 9.633$, $P < 0.0001$) also varied significantly across the *Rhododendron* species.

Rhododendron thomsonii which occurred below the tree-line had the longest mean corolla length and highest nectar volume and was visited relatively more by birds. Other species occurring in this elevational gradient with similar floral characteristics in which birds were the dominant visitors were *R. hodgsonii* followed by *R. cinnabarinum* and *R. campanulatum*. In contrast, among species growing mainly above the treeline, *R. anthopogon*, *R. setosum* and *R. lepidotum* were visited by insects such as bumblebees and flies. These three *Rhododendron* species also had the highest concentrations of nectar, shortest corolla length and displayed a shorter distance between the stamen and stigma. *R. wightii* and *R. aeruginosum* which occurred between 3900 and 4000 m were largely visited by flies, followed by bumblebees, and were occasionally visited by birds. Their corolla

lengths were also relatively longer compared to other species occurring in the similar elevation gradient (Table 1).

We observed a significant decrease in community mean corolla length (GLMM, estimate = -0.001 ± 0.0003 , $Z = -2.522$ and $P < 0.05$) nectar volume (GLMM, estimate = -0.010 ± 0.002 , $Z = -3.757$ and $P < 0.0001$) and distance between stamen and stigma (GLMM, estimate = -0.026 ± 0.002 , $Z = -8.935$ and $P < 0.0001$) along the elevational gradient. However, in the case of nectar concentration (GLMM, estimate = 0.011 ± 0.004 , $Z = 2.458$ and $P < 0.01$), we found a significant increase along the elevational gradient (Fig. 1). The dominant pollinators visiting the flowers were grouped as flies, bees and birds. Henceforth, we will use the term ‘pollinators’ and not ‘visitors’ as flies, bees and birds came in contact with the reproductive parts of the flower and helped in pollen transfer. Visitation by other insects such as wasps, ants, small beetles, and butterflies was negligible, and many of them seldom came in contact with the reproductive parts and were thus excluded in the analysis.

The total visitation frequency of flies (GLMM, estimate = 1.09 ± 0.055 , $Z = 19.613$ and $P < 0.001$) and bumblebees (GLMM, estimate = 0.304 ± 0.066 , $Z = 4.591$ and $P < 0.001$) showed a significant positive relationship with elevation, whereas in contrast, bird visitation frequency (GLMM, estimate = -0.098 ± 0.044 , $Z = -2.204$ and $P < 0.05$) showed a significant negative relationship with elevation (Fig. 2). A detailed list of pollinators across species at every 100 m elevation is reported in Table S2. The commonly observed bird pollinators were the fire-tailed sunbird (*Aethopyga gouldiae*), Tickell’s leaf-warbler (*Phylloscopus affinis*), black-faced laughing thrush (*Trochalopteron affine*)

Table 1 Details of means floral traits, total pollinator visitation frequency (\pm SD) and elevational range of ten *Rhododendron* species in Kyongnosla Alpine Sanctuary, Sikkim

Species <i>N</i> =10 <i>n</i> =30	Elevation (m)	Total observa- tion (h)	Corolla length (mm)	Nectar vol (μ l)	Nectar con (%)	Distance (mm)	Bird visit Frequency	Bee visit	Fly visit
<i>R. hodgsonii</i>	3500–3745	114	37.36 \pm 4.20	13.48 \pm 5.05	4.84 \pm 1.32	7.62 \pm 3.00	46.42 \pm 12.52	2.26 \pm 4.75	2.42 \pm 1.80
<i>R. thomsonii</i>	3450–3805	102	51.17 \pm 4.42	46.94 \pm 8.30	2.97 \pm 0.97	5.82 \pm 1.93	36.87 \pm 10.23	4.43 \pm 5.94	1.31 \pm 1.85
<i>R. campanu- latum</i>	3400–3915	120	46.09 \pm 2.99	10.92 \pm 4.22	10.35 \pm 3.79	6.76 \pm 3.05	23.65 \pm 10.12	4.95 \pm 3.33	19.95 \pm 12.33
<i>R. campylo- carpum</i>	3602–3787	48	38.56 \pm 3.76	4.64 \pm 2.39	10.35 \pm 3.79	5.74 \pm 2.26	17.75 \pm 6.34	18 \pm 6.98	32.5 \pm 10.54
<i>R. cinna- barinum</i>	3430–3581	48	40.9 \pm 3.93	17.06 \pm 7.01	5.22 \pm 1.74	5.14 \pm 1.47	42.5 \pm 10.23	17.37 \pm 8.38	5.62 \pm 3.30
<i>R. aerugino- sum</i>	3973–4230	86	35.26 \pm 1.82	4.35 \pm 2.22	3.00 \pm 1.71	5.50 \pm 2.64	10.71 \pm 8.80	11.78 \pm 11.1	48 \pm 10.23
<i>R. wightii</i>	4040–4223	54	45.00 \pm 3.97	5.11 \pm 3.55	9.73 \pm 2.76	6.14 \pm 2.97	7.22 \pm 7.10	19 \pm 7.84	30.11 \pm 9.96
<i>R. anthopo- gon</i>	3830–4230	84	18.19 \pm 2.63	0.86 \pm 0.57	12.33 \pm 5.10	1.12 \pm 0.51	0.00	16.42 \pm 6.89	1.35 \pm 1.94
<i>R. setosum</i>	3990–4235	44	18.96 \pm 1.27	1.31 \pm 0.69	13.67 \pm 5.07	5.10 \pm 1.75	0.00	25.11 \pm 8.97	10.88 \pm 3.35
<i>R. lepidotum</i>	3920–4230	54	13.80 \pm 1.44	8.29 \pm 3.20	28.03 \pm 6.0	3.07 \pm 0.79	0.00	12.33 \pm 9.06	18.88 \pm 4.4

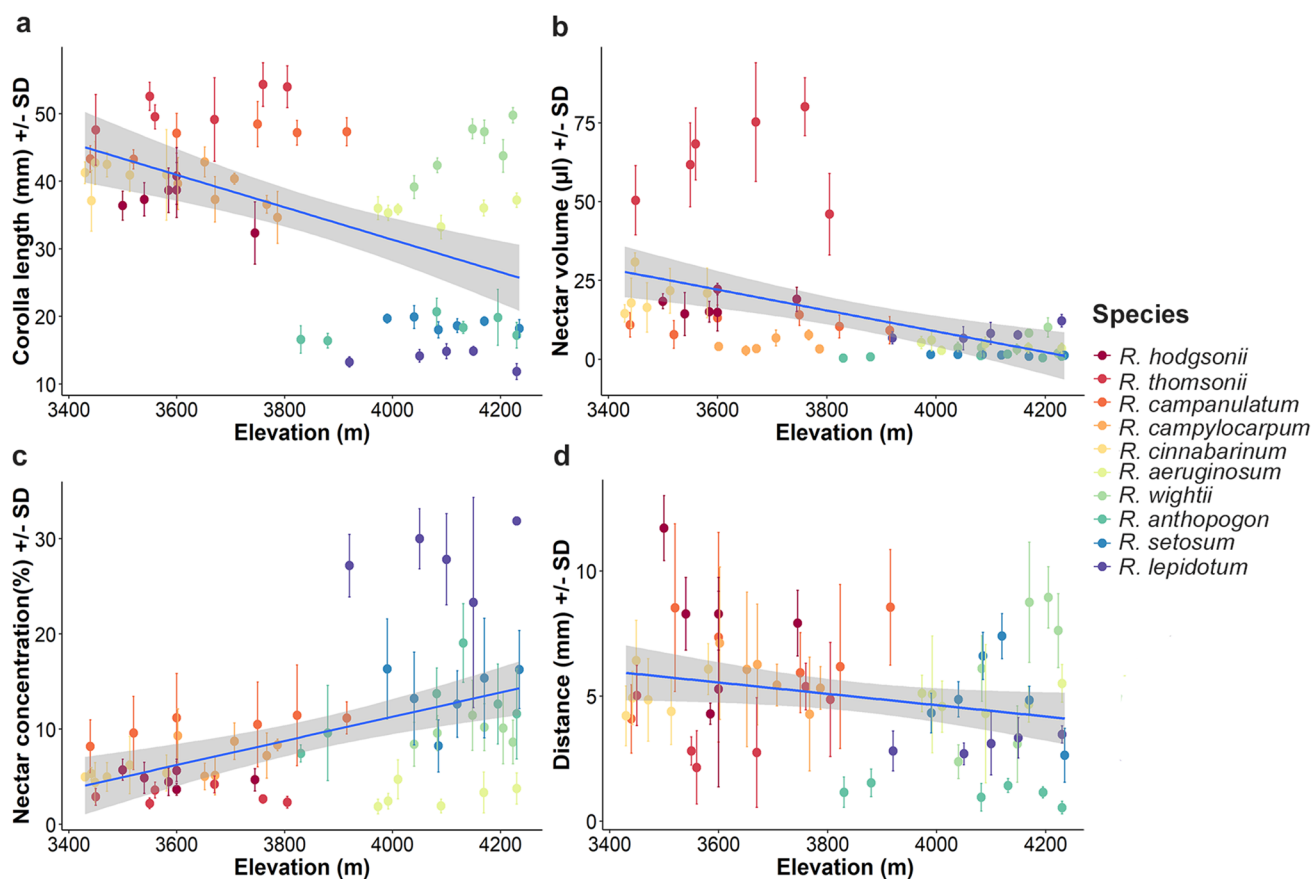


Fig. 1 Relationship of floral traits with elevation. The point represents the species mean for each elevation with \pm SD and blue solid line with gray loess curves shows trend in floral traits with elevation measured using linear regression

and Himalayan white-browed roset finch (*Carpodacus thura*). We encountered four bumblebee species *Bombus tunicatus*, *Bombus pyrosoma*, *Bombus rufofaciatus* and *Bombus festivus*. Most of the pollinating flies belonged to the families Muscidae and Syrphidae (Fig. 3). At the lower elevation, between 3400 and 3500 m, bumblebees were often seen robbing nectar from holes at the base of the flower which were side probed by birds such as the gold-naped finch (*Pyrrhopterus epaulette*), rufous-vented yuhina (*Yuhina occipitalis*) and rufous-vented tit (*Parus rubidiventris*).

Relationship of pollinator visits and floral traits among *Rhododendron* species along the elevation

Pollinator visits in *Rhododendron* species across four elevation bands differed significantly (PERMANOVA: $F = 3.432$, $df = 8$, $P = 0.002$). A two-dimensional representation of the NMDS analysis based on the distance between the pollinator visits across the four elevation bands in all *Rhododendron* species revealed clear groupings (Stress = 0.02; Figs. 4a; S4a). We found three separate clusters with some overlapping. The first cluster above the zero value of Dimension 2

represented by *R. thomsonii* located at two low elevation bands (3400–3600 to 3600–3800) were associated with bird visits. The second cluster represented by high-elevation species (3800–4000 to 4000–4230) *R. anthopogon* and *R. lepidotum* and *R. setosum* were associated with bumblebees. Whereas the third cluster represented by both high and low elevation species such as *R. cinnabarinum*, *R. aeruginosum*, *R. hodgsonii*, *R. campylocarpum* and *R. wightii* at the zero value of Dimension 1 were associated with flies as well as birds (Fig. 4a).

The floral traits of *Rhododendron* species across the different elevation bands also differed significantly (PERMANOVA: $F = 2.243$, $df = 8$, $P = 0.02$). Ordinations plot based on the floral traits among four elevation bands across *Rhododendron* species also revealed similar clustering (Stress = 0.07; Figs. 4b; S4b). The first cluster represented mostly by *R. thomsonii*, followed by *R. hodgsonii* and *R. cinnabarinum*, was closely associated with nectar volume and then to corolla length. The second cluster represented by *R. anthopogon* and *R. lepidotum* and *R. setosum* was associated with nectar concentration whereas the third cluster represented by rest of the *Rhododendron* species was associated

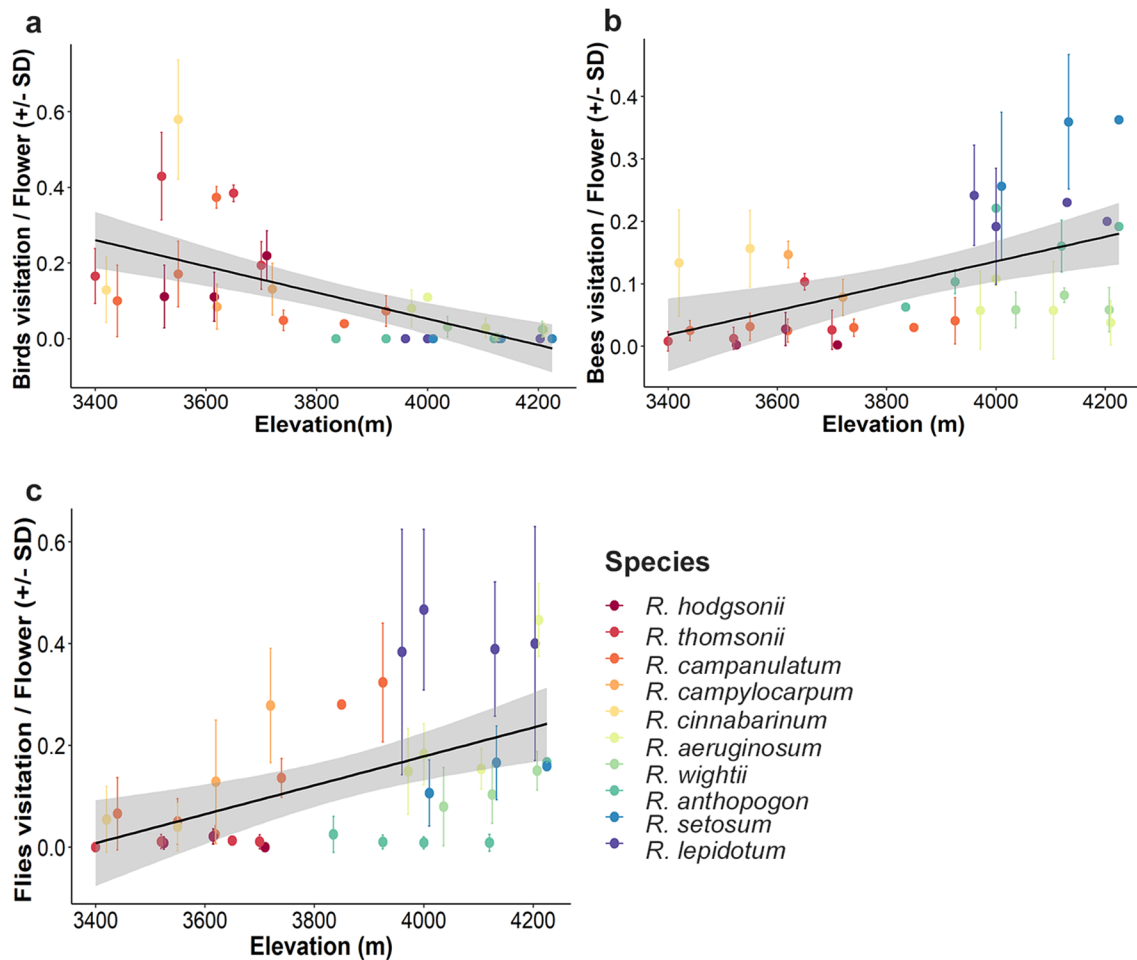


Fig. 2 Relationship of pollinator's visitation rate with elevation. The point represents the species mean for each elevation with \pm SD and blue solid line with gray loess curves shows trend in pollinator's visits with elevation measured using linear regression

with distance between stigma and stamen (Fig. 4b). Overall, the results of both ordination plots revealed that low elevation species such as *R. thomsonii* followed by *R. cinnabarinum*—which had high nectar volumes and longer corolla lengths—were preferred by birds. In contrast, high-elevation species such as *R. anthopogon*, *R. setosum* and *R. lepidotum* with their short corolla lengths and high nectar concentrations were preferred by bees. The third cluster consisting of both high and low elevation species including *R. aeruginosum*, *R. hodgsonii*, *R. campylocarpum* and *R. wightii*, which contained medium levels of nectar volume and concentration, were preferred mostly by flies followed by birds.

Breeding systems of *Rhododendron* species

There was a significant difference in fruit/flower ratio between control and cross-pollination of *R. hodgsonii* (estimate = 0.601 ± 0.295 , Z value = 2.034, $P = 0.04$), *R. thomsonii* (estimate = 1.512 ± 0.371 , Z value = 4.073, $P = 0.0001$), *R. campanulatum* (estimate = 0.696 ± 0.320 , Z value = 2.174,

$P = 0.029$), and *R. wightii* (estimate = 1.917 ± 0.565 , Z value = 3.393, $P = 0.0001$). In the case of *R. cinnabarinum* (estimate = 0.890 ± 0.448 , Z value = 1.986, $P = 0.047$), *R. aeruginosum* (estimate = 0.891 ± 0.449 , Z value = 1.987, $P = 0.047$) and *R. wightii* (estimate = 1.325 ± 0.448 , Z value = 2.716, $P = 0.001$), a significant difference in fruit/flower ratio between control and facilitated self-pollination was observed. Except in *R. cinnabarinum* (estimate = -0.989 ± 0.324 , Z value = -3.053 , $P = 0.002$), we did not find a significant difference in fruit set between control and self-pollination treatment in all other species (Table S3). The bagged plants of all species also set fruit, and except for *R. campanulatum* and *R. anthopogon*, lower fruit set were observed for open-pollinated flowers compared to cross-pollinated flowers (Fig. 5). A significant variation was observed in AFI among *Rhododendron* species (ANOVA; $F = 2.69$, $P < 0.006$, Fig. 6a). The across-species average AFI ranged between 0.60 and 1 and species occurring at higher elevation showed relatively higher AFI compared to lower elevation species. The average PL across



Fig. 3 Pollinator interaction of *Rhododendron* species studied in Kyongnosla Alpine sanctuary. **a** Flies (Calliphoridae) visiting broadly campanulate flowers of *R. aeruginosum*. **b** Female Fire-tailed sunbird (*Aethopyga gouldiae*) visiting tubular-campanulate flowers of *R. hodgsonii* **c** Fly and **d** *Bombus* sp visiting campanulate flowers of *R. campylocarpum*. **e** Male Fire-tailed sunbird (*Aethopyga gouldiae*)

visiting campanulate flowers of *R. thomsonii*. **f** Laughing thrush (*Trochalopteron affine*) visiting tubular-campanulate of *R. hodgsonii*. **g** *Bombus* sp visiting flowers of **g** widely funnel shaped *R. setsoum*. **h** *Bombus* sp visiting flowers of broadly campanulate *R. aeruginosum*. **i** *Bombus* sp visiting flowers of narrowly tubular *R. anthopogon*

Rhododendrons ranged between 0.71 and 1.11 but we did not observe a significant variation (ANOVA: $F = 1.61$, $P > 0.05$; Fig. 6b) across species. However, PL was relatively high among high-elevation *Rhododendron* species than in low elevation species (Table S4).

Discussion

To our knowledge, this is one of the first studies from the Eastern Himalaya to examine the variations in floral traits, pollinator groups and the association between floral traits and pollinator groups of *Rhododendron* species distributed along an elevational gradient. We observed a significant variation in floral traits and pollinator visitation rate across *Rhododendron* species and also along elevation. The proportion of bird visits in *Rhododendron* species in lower elevations was high compared to species at higher elevations which

were mostly visited by flies and bumblebees. Low elevation species such as *R. thomsonii*, with a longer corolla length and higher nectar volume, were more associated with bird visits. In contrast, higher elevation species such as *R. lepidotum*, *R. anthopogon*, and *R. setosum*, with their shorter corolla lengths and higher nectar concentrations, were associated with bees followed by flies. Apart from being cross-pollinated, higher elevation species also showed higher rates of selfing and pollen limitation.

Variation in proportion of pollinator visits along the elevation

The diverse floral traits across *Rhododendron* species make this genus highly attractive to different pollinator groups. For some *Rhododendron* species, birds are reported as important pollinators. For example, *R. floccigerum* which displays red flowers and occurs between 2400 and 2600 m is

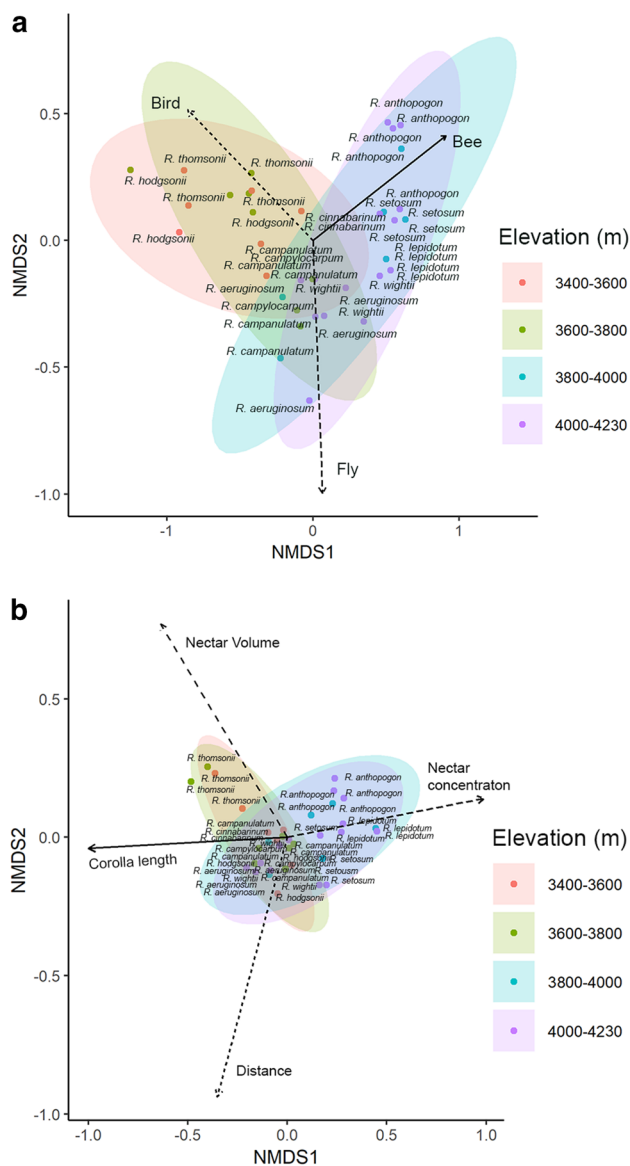


Fig. 4 **a** NMDS ordinations using Bray–Curtis dissimilarities distance on abundance data of pollinator groups of 10 *Rhododendron* species across four elevation bands. Species which are closer to each other are visited by similar pollinator groups. Vectors visualize the direction of the effect of pollinator groups in positioning the *Rhododendron* species within the two-dimensional plot. **b** NMDS ordinations on Bray–Curtis dissimilarities on floral traits groups data of 10 *Rhododendron* species across four elevation bands. Species which are closer to each other are share similar floral traits. Vectors visualize the direction of the effect of floral traits in positioning the *Rhododendron* species within the two-dimensional plot

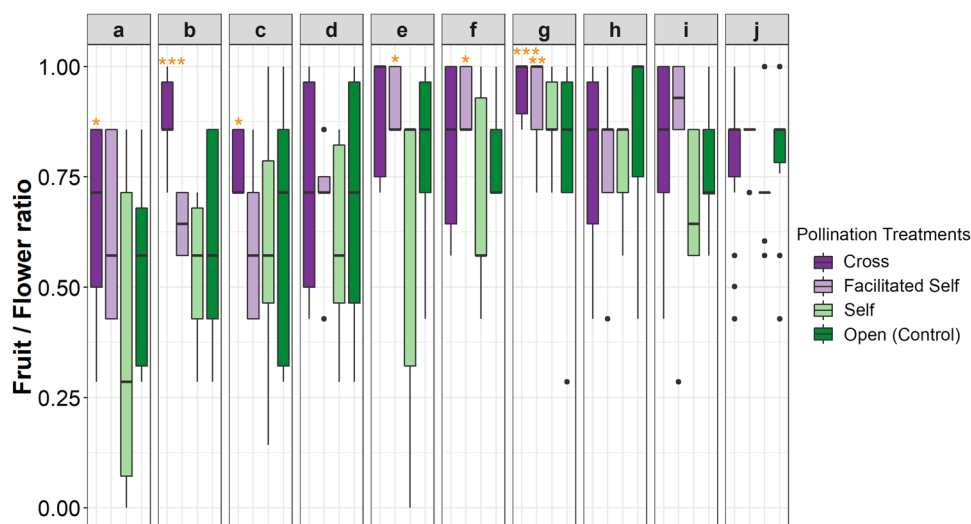
pollinated by birds (Georgian et al. 2015). Similarly, a study on 12 *Rhododendron* species along an elevational gradient from 1800 to 4200 m also reports a higher rate of bird visits in these species (Huang et al. 2017). Several studies have reported bumblebees to be the major pollinator for many *Rhododendron* species such as *R. ponticum* (Stout 2007)

and *R. aureum* (Kudo et al. 2011). In the Sikkim Himalayan region, however, we did not observe an association of specific *Rhododendron* species with a specific pollinator groups. Instead, our study highlights the variation in proportions of visitation rate of three pollinator groups—birds, bumblebees, and flies—among *Rhododendron* species, as well as along the elevational gradient. We observed that birds in general were active pollinators only in the lower elevation while with the increase in elevation especially from 3800 to 4200 m, bumblebees and flies were more prominent pollinators of *Rhododendron* species. The treeline which lies between 3800 and 3900 m acted as a point above which bird visits especially that of sunbirds, fell drastically. We noticed that sunbirds, which are more specialized nectarivores, were common on *Rhododendrons* below 3800 m; however, above this elevation, birds such as warblers, laughing thrushes and finches were more common. Johnson and Nicolson (2008) have distinguished between the nectar traits of specialized nectarivores such as sunbirds and non-specialized ones such as bulbuls, weaverbirds and orioles. Specialized nectarivores species are characterized by associations with flowers that contain high volumes of nectar (approx. 40–100 μ l) that is extremely diluted (approx. 8–12%), which is similar to the range in flowers visited by sunbirds in the Sikkim Himalaya. Birds such as laughing thrushes, finches and warblers were more often seen feeding on insects inside flowers than on nectar itself. This might contribute only sporadic pollination whenever they make contact with essential parts and carry pollen loads on their bodies. Hence, they may only fall into the category of ‘facultative’ or opportunistic nectarivores occupying the other end of the spectrum, where insects comprise their primary diet with a minor intake of nectar. These bird species that are opportunistic nectar feeders were recorded on flowers all along the elevational gradient. They could be tracking the shift of insect emergence along the elevation rather than the flowering phenology.

In general, insect pollination is known to be predominant in the temperate alpine areas (Kudo 2016) and alpine plants are known to be totally or partially dependent on them for seed set (Peeters and Totland 1999). One such important pollinator of temperate and alpine habitats is the bumblebee and our results also indicate that bumblebees were one of the prominent pollinators of high-elevation *Rhododendron* species. A study on bumblebees from the Nepal Himalaya also reported the increase in richness and abundance of bumblebees along an elevational gradient, up to 4000 m (Williams et al. 2010). Their dominance especially in the higher elevations might be favored by their ability to forage at lower temperatures (Kudo 1993) due to their strong endothermic abilities and well-insulated furry bodies (Willmer 2011).

Dipterans in general are reported to be more abundant with increasing elevation while hymenopterans are reported to be found in mid-elevations (Lefebvre et al.

Fig. 5 Fruit/flower ratio under four different treatments in the pollination experiment for **a** *R. hodgsonii*, **b** *R. thomsonii*, **c** *R. campanulatum*, **d** *R. campylocarpum*, **e** *R. cinnabarinum*, **f** *R. aeruginosum*, **g** *R. wightii*, **h** *R. anthopogon*, **i** *R. setosum*, **j** *R. lepidotum*. The asterisk (*) sign shows significant variation between pollination treatment and the control treatment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$



2018). Through our pollinator visitation study, we found that bumblebees and flies were the prominent pollinators of *Rhododendron* species occurring between 3800 and 4230 m. However, with rise in elevation, the overall proportion of fly

visits was higher compared to bumblebees. Flies were not highly prominent at lower elevations where birds dominate, but gradually increased in the region just below the treeline where bumble bees are dominant. Finally, flies became more prominent pollinators in the high elevations. We encountered flies belonging to various families such as Muscidae, Syrphidae, Scathophagidae and Bibionidae. We observed that Muscidae serves as a dominant pollinator with the rise in elevation and this result is consistent with earlier work which also highlights flies (Muscidae) as important pollinators above the timberline (Orford et al. 2015). However, though most of the other dipteran families have not been reported as pollinators, they are understudied. Some existing work does suggest that the larvae of flies develop in moist and wet soils, so the cold temperature and high moisture in high-elevation areas might create such environments (Skidmore 1985). Flies' roles as pollinators are highly unexplored especially in the Himalaya despite their potential to contribute to plant reproduction and stability in the face of environmental change (Tiusanen et al. 2016).

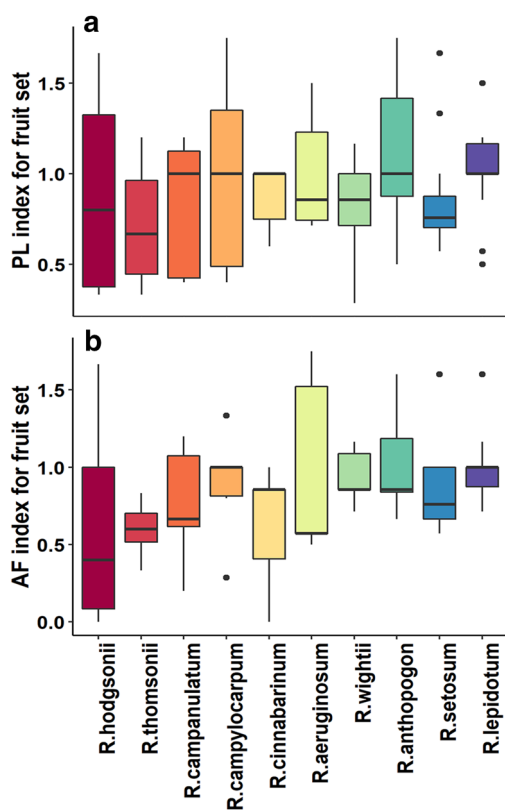


Fig. 6 **a** Pollen Limitation (PL) and **b** Auto-fertility (AF) index for fruit set of ten *Rhododendron* species. Bottom and top limits of each box plot are the lower and upper quartiles, respectively. The horizontal black lines across boxes are medians. Error bars represent the 95% confidence interval of the median

Association between pollinator visits and floral traits

We did not notice any strong association of *Rhododendron* species with any single pollinator group. However, a few species located at lower elevations, such as *R. thomsonii* which has red flowers and the crimson-orange-flowered *R. cinnabarinum* with their longer corolla lengths, lower nectar concentrations and higher nectar volume, were more associated with birds. In general, *Rhododendrons* below 3800 m had large, showy flowers with high volumes of nectar which are pollinated mainly by birds followed by flies and bees. The higher nectar volume may be related to the energy requirement of larger body sizes of obligatory

and facultative nectarivores (Johnson and Nicolson 2008). Lower nectar concentration in plants is also considered as an adaptation to avoid nectar robbing by bees (Bolten and Feinsinger 1978). Besides pollinators, *Rhododendrons* also have a high rate of visits by nectar robbers which may also play an important role in floral evolution (Georgian et al. 2015). Our field observations also show that bumblebees were the dominant nectar robbers in some of the *Rhododendron* species at lower elevations, whereas birds such as rosefinch were the prominent nectar robbers in higher elevation species such as *R. setosum* and *R. lepidotum*.

In contrast to the lower elevation *Rhododendrons*, higher elevation *Rhododendron* species such as *R. anthopogon*, *R. lepidotum*, and *R. setosum* with their highly concentrated nectar, short tubular corollas with narrow openings and wheel-shaped flowers were mostly associated with bumblebees. Overall, nectar concentration was highest in the elevations dominated by bumblebees and flies. Bees in general prefer to forage on floral types associated with more concentrated nectar (Hill et al. 2001) to guarantee energetically profitable foraging (Bolten and Feinsinger 1978). Nectar is the most important reward offered to pollinators (Baker and Baker 1990) and our work also highlights how the variations in nectar concentration and volume affect the visitation rate of birds and insects along elevation. Nectar secretion is known to reduce with the increase in temperature (Takis et al. 2015) and this might have a negative impact on plant–pollinator interaction of high nectar content bird-pollinated *Rhododendron* species such as *R. thomsonii* and *R. cinnabrinum*. As suggested by Hegland and Totland 2005, flowers visited by flies showed a strong association with species showing campanulate open-mouthed flowers such as *R. campylocarpum*, *R. hodgsonii*, *R. aeruginosum* and *R. wightii*. However, unlike in the case of birds and bumblebees, we did not observe clear associations of flies with certain floral traits. Unlike other groups, flies were also found all across the elevational gradient and were among the most common pollinators of all *Rhododendron* species. But their proportions of visits increased along the elevational gradient where *Rhododendron* species had more concentrated nectar. Flies that were encountered in lower elevations were of a smaller body size compared to those in higher elevations. We also observed that the high-elevation species visited mostly by flies and bumbles also flowered later in the season (Table S5). Their flowers were of smaller size and set smaller fruits compared to *Rhododendrons* in lower elevations. Plants in higher elevations are known to show such unique adaptations due to their short growing season. Despite flowering late, they complete their fruit maturation and seed dehiscence within a short period due to their small fruit size (Basnett et al. 2019).

Pollen limitation and auto-fertility in *Rhododendron* species

For most *Rhododendron* species, cross-pollination fruit set was higher compared to open pollination fruit set where the flowers were naturally exposed to flower visitors. This suggests that fruit set was pollen limited in the studied system. The lower availability of pollinators has been reported in many other mountain ranges around the world (Bingham and Orthner 1998). Our results are consistent with earlier findings that pollen limitation is usually higher in the alpine region (Larson and Barrett 2000). Overall, PL was more than 0.7 across all *Rhododendron* species and it was relatively higher for high-elevation species compared to species at the lower elevation. During the flowering months, the bimonthly average atmospheric temperature of KAS ranged between 3 and 10 °C and the average temperature significantly decreased with rise in elevation (Basnett et al. 2019). Apart from low temperatures, this region also experiences very dense fogs especially during this time. Therefore, weather may be one of the factors contributing to lower pollinator visitation rate (0.1–0.6 visits per flower). In addition, pollinators such as bumblebees do frequently visit flowers in higher elevations too but they are known to deposit low pollen thread tangles compared to pollinators such as birds (Song et al. 2019). This may also have contributed to high pollen limitation among higher elevation *Rhododendron* species such as *R. anthopogon*, *R. setosum* and *R. Lepidotum*.

All ten *Rhododendron* species were auto-fertile and showed high auto-fertility indices, indicating that there is no barrier to autonomous self-pollination (Lloyd and Schoen 1992). However, it is beyond the scope of this study to explain the selfing ability and the fitness of the fruit set obtained from the breeding experiment. But earlier studies on *Rhododendrons* have highlighted the higher abortion rate of self-pollinated seeds caused by strong inbreeding depression (Kudo et al. 2011). The high level of inbreeding in alpine/arctic taxa has evolved because strong PL causes selection to favor selfing as a reproductive assurance mechanism (Crawford 1989).

Most studies have shown that Ericaceae, the family to which *Rhododendrons* belong, are mostly self-compatible (Escaravage et al. 1997) with one exception in Hong Kong where six species of *Rhododendron* found along elevation of the 350–650 m gradient were self-sterile (Ng and Corlett 2000). These species occur at a lower elevation compared to other self-compatible *Rhododendron* species (Stout 2007; Kudo et al. 2011). Also, since Hong Kong is an island, different selection processes may be operating here compared to within continental populations. The foraging behavior of insects might also favor selfing. Due to the large display of flowers, bumblebees were often seen foraging in adjacent flowers minimizing inter-flower travels across different

individuals which could contribute to selfing through geitonogamous pollen transfer (Stout 2007). Similarly, flies were also noticed making less movement across different individuals. In contrast in lower elevations, birds might initiate more cross-pollination as they can travel across longer distances. Therefore, selfing might be a more reliable, faster and cheaper alternative breeding strategy in harsh environmental conditions when crossing is unreliable (Lloyd and Schoen 1992) and species get only a short window to complete its reproductive cycle (Hart et al. 2016).

Several considerations that could influence our results have to be taken into account. Our inferences are mainly based on three different pollinator groups, but a family- and species-level pollinator study will provide more insight into pollinator behaviors and their interactions with each *Rhododendron* species. We did not measure the pollination effectiveness of each pollination group, which restricts us from deriving a conclusion on a specific pollinator's efficacy. Our sampling took place only during the day which limits us from understanding the role of nocturnal pollinators.

Conclusions

The ten *Rhododendron* species studied in the Sikkim Himalaya suggest that species growing in increasingly limiting environments show adaptations in corolla length as well as nectar concentration and volume. Moreover, in addition to climatic factors and pollinator selection pressures, there could be other possible factors such as nectar robbers, flowering timing and phylogenetic relationship of species which may be also responsible for the observed pattern of elevation variations in floral traits. Also, earlier studies have rarely looked at both morphological and biochemical floral traits across multiple species along the elevation. Therefore, it is difficult to determine if the trend observed in *Rhododendron* species represents a wider pattern in alpine plants. Studies which span multiple genera and inter- and intra-specific variations in floral traits are necessary to be able to comment on the generality of these observed elevational patterns in alpine species. For the first time, this work also highlights flies as dominant pollinators of the high-elevation Himalayan *Rhododendrons*. As highlighted by earlier studies, our results also support the finding that at highly stochastic pollination environments, selection might favor generalist plants open to different pollinators and plants might adopt selfing as an alternative strategy for reproduction.

Acknowledgements This research was funded by the Department of Biotechnology, Government of India (Grant No. BT/01/NE/PS/NCBS/09) and National Mission on Himalayan Studies, and implemented by the Ministry of Environment, Forest & Climate Change of India (Grant No. GBPI/NMHS/HF/RA/2015-2016). We thank the Sikkim State Department of Forests and Wildlife, Sikkim Police

Department and Indian Army for research permits and logistical support. We thank Saini Mallikat, Aarti Pannure, Yeshwanth H, Vasuki Belavadi, Kumar Ghorpade and Seena Narayanan for insect identifications. Rakesh Basnett helped with some part of the field work. Sonam Bhutia provided assistance for the data collection throughout the field work. We thank Gaku Kudo and Shoko Shakai for their valuable comments on the analysis. We are very thankful to three anonymous reviewers whose comments have vastly improved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Declaration of authorship SB and SD conceived and designed the research. SB carried out the field work, analyzed the data and wrote the manuscript. All authors contributed to writing and approved the manuscript.

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