Floral Biology, Pollinator Guilds, and Male Fitness of *Aconitum spicatum* (Brühl) Stapf in Central Nepal

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DECLARATION

I, Prativa Masrangi, hereby declare that this dissertation entitled "Floral Biology, Pollinator Guilds, and Male Fitness of *Aconitum spicatum* (Brühl) Stapf in Central Nepal" is my original work, and all other sources of the information used are properly acknowledged. I have not submitted it or any of its parts to any other universities for any academic award.

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LETTER OF RECOMMENDATION

This is to certify that the dissertation work entitled **"**Floral Biology, Pollinator Guilds, and Male Fitness of *Aconitum spicatum* (Brühl) Stapf in Central Nepal**"** has been submitted by Ms. Prativa Masrangi under our supervision. This entire work was accomplished based on the candidate's original research work. The work has not been submitted to any other academic degree. It is hereby recommended for acceptance of this dissertation as part of the requirement of a Master's Degree in Botany at the Institute of Science and Technology, Tribhuvan University, Kathmandu, Nepal.

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ABSTRACT

Floral biology includes several studies on reproductive and pollination biology, such as biology of various floral parts, floral rewards (nectar and pollen), and pollination by various pollen vectors. Plant-pollinator interactions are greatly influenced by floral traits, which also determine the group of specific floral visitors of a particular plant. This study aims to document different floral traits as well as their potential floral visitors and male fitness of *Aconitum spicatum* (Brühl) Stapf. Flower (bud and mature flower) samples were collected from 3043-4197m altitude while pollinator samples were collected from 3315-4265m altitude along the Annapurna trekking route of Annapurna Conservation Area, Kaski, Central Nepal. Floral traits like tepals length, anther number, anther and stamen length, pollen and ovule number were measured in the laboratory under microscope and few floral traits were measured in the field. The result showed that all floral traits varied significantly among the populations except for the pollen-ovule ratio. The variation in floral traits of *A. spicatum* among different populations might be due to pollinator mediated selection. It was observed that, the bumblebee (*Bombus miniatus)* actively visited flower of *A. spicatum* during day time among all population while nocturnal visitors were not observed. *B. miniatus* actively visited the flower after 9 a.m. till 6 p.m. and total 1916 visits were recorded within three days for total 23 hours at an elevation of 3901m while only 5 visits were recorded at an elevation of 4265m for 2 hours of observation period when weather was extremely cold. The visitation rates vary across the different timing of the day and also among the various days. The visitation rate of *B. miniatus* was maximum when the weather was clear and sunny and least visitation rate was recorded during cloudy and windy weather. The male fitness of *A. spicatum* was found to be 14.08% and very high male fitness of *A. spicatum* linked with *B. miniatus*; the bumblebee transports 2416.65±652.85 pollen grains out of total 17169±1125 (mean ± SE) pollen grains of *A. spicatum* during a single foraging bout. These facts thus indicate that *B. miniatus* could be the prime pollinator of *A. spicatum*.

Keywords: Floral traits, potential floral visitors, Annapurna trekking route, medicinal plant

ABBREVIATIONS AND ACRONYMS

m asl	Meters above sea level
ACA	Annapurna Conservation Area
ACAP	Annapurna Conservation Area Project
NTNC	National Trust for Nature Conservation
Km^2	Kilometer square
m	Meter
P/O	Pollen-Ovule
Pos	Pollen-Ovule ratio
ml	Mili liter
µl	Micro liter
sp	species

CHAPTER 1: INTRODUCTION

1.1 Background

Angiosperm plants form a complex relationship with insects which has been studied for over a century to understand their evolution (Darwin, 1962). The first well documented study on plant-pollinator interaction was done by Aristotle (384-422BC), and scientific development of this field progressed with the work of Sprengel (1793), Darwin (1859, 1962), and Sir John Lubbock (1882). Interaction between plants and pollinators is crucial to the reproductive cycle of flowering plants as it plays an important role to achieve genetic variation (Parrish, 2004). However, depending upon the various intrinsic and extrinsic factors, this important interaction may vary temporarily and spatially. Thus, plants evolve different mating strategies to assure their reproduction. When mating choice becomes unreliable, i.e., when plants suffer from pollinator limitation, evolution to selfing is the most predominant strategy to assure the reproduction (Paudel and Li, 2020). On the other hand, in an environment of abundant pollinator availability plants usually develop outcrossing features. Although the actual directionality of pollination system transition is still a matter of dispute, a substantial proportion (~87 %) of extant angiosperms species are principally outcrossing (Ollerton et al., 2011). This fact thus suggests that evolution to outcrossing is the most favoured reproductive strategy of angiosperms.

Floral traits play the key role in determining the outcrossing features of angiosperms. In general, showy flowers can be easily detected by the visitors and thus such plants are predominately outcrossing. Presence of sufficient rewards (nectar/pollen), flowers with the corolla/labellum serving as the landing platform for its visitors, emission of floral scent favorable for the visitors, and timing of flowering (i.e., synchrony between flowering phenology and emergence time of potential pollinators) are other important outcrossing features (Faegri and van der Pijl, 1971). It is assumed that each floral parts have more or less definite role in pollination. Floral traits such as flower color, nectar, corolla tube, flower fragrance mainly used to predict effective plant pollinators, which also provide mechanistic explanation of floral diversity (Fenster et al., 2004). The evolution of such floral traits is primarily thought to have developed historically as a result of floral adaptation arising from interaction with floral visitors (Harder and Johnson, 2009). Therefore, the availability of pollinator largely determines the floral traits, and in turn plants develop floral traits in accordance to the preference of its most effective pollinator (Stebbins, 1970, Fenster et al., 2004). The flowering phenology such as timing of budding, flowering, wilting, and fruiting of any plant is an important aspect for pollination studies as it provides important information about activities of floral visitors (Kearns & Inouye, 1993). Although floral traits in angiosperms are thought to have evolved via the convergent evolution with its most effective pollinator (Stebbins, 1970), the floral traits do not necessarily predict the effective pollinators. Sometimes flower with specific floral traits that confirm to particular pollinator groups are also pollinated by generalized pollinators (Ollerton et al., 2007). For example, species of Himalayan alpine ginger (*Roscoea auriculata, R. capitata,* and *R. tumjensis*) with long tongued fly pollination syndrome are pollinated by bumblebees (Paudel et al., 2018). Moreover, the evolution of floral traits and ultimately the mating system in plants is greatly influenced by environmental variables mainly low temperature in high elevation (Tong et al., 2021). The harsh environmental condition of alpine habitats not only limits the growth and evolution of floral traits but also greatly impacts the availability/ abundance of pollinators. Therefore, study on floral biology is the fundamental requisite to understand the plant reproductive strategy and its potential variation across different environmental gradients.

The elevational gradient in the mountain regions of Nepal provides sharp environmental changes in short spatial distance and slight change in elevation can lead to rapid change in temperature, rainfall, humidity and concentration of atmospheric gaseous (Hovenden and Vander Schoor, 2004) and such environmental changes create great variation in floral traits (Jacquemyn et al., 2005). Thus, elevation gradient across the mountainous range in Nepal provides natural avenues to investigate how floral biology and plant-pollinator interaction change across different environmental gradients.

1.2 Justification

Plants and pollinators form the important component of biodiversity. Pollinators not only contribute for the reproductive success of wild plants but also impacts the productivity of many crops as approximately one third of the leading global crop species require animal vectors for pollination. Therefore, without pollinators the terrestrial ecosystem of the earth including the humans would be greatly affected.

Many previous studies suggest that the number of pollinators are declining globally due to natural as well as anthropogenic causes (Carvalheiro et al., 2013, Dirzo et al., 2014). The declining number of honeybees and local bees in many parts of the world is the evidence of "pollination crisis" and damage of plant-pollinator interaction and their biotic network. Studies indicated that there was a case of decline in apple output in the Hindu Kush-Himalayas as a result of the loss of native/wild pollinators, and consequently due to the inadequate crop pollination (Adhikari, 2003). Therefore, conservation of both plant and pollinators requires a clear understanding of plant-pollinator interaction and pollinator diversity.

The species richness, distribution, diversity, and community structure of both plant and pollinators are greatly affected by local environment (Neumüller et al., 2020). Pollinators' activities are strongly correlated with temperature, humidity (Pellissier et al., 2010) and plant species richness (Hudewenz et al., 2012). Understanding the variation in diversity of plants and their pollinator as well as interaction between plant and their pollinator under different environmental scenarios has important implications for conservation and management of important and threatened medicinal plants.

Aconitum spicatum, distributed within elevation range of 1,800-4,800m asl, the wide distribution range and diversity in floral traits make them an ideal system to study how floral traits and plant-pollinator interactions vary in response to various environmental variables. Also, complex floral morphology and abundant rewards (pollen and nectar) for pollinators of *A. spicatum* can be considered as suitable model for study of pollination ecology. *A. spicatum* is important medicinal plant and its tubers are in trade (Rana et al., 2020). Plants need to be carefully conserved because of their great commercial value or else they will be in danger. Understanding plant-pollinator interaction and pollinator diversity is the fundamental key for the conservation of both plants and pollinators. Therefore, considering the conservation need of *A. spicatum*, exploring its floral biology and pollinator guilds across different environmental gradients is of utmost need.

1.3 Objectives

The general objective of the study is to explore the floral biology, pollinator guilds, and male fitness of *Aconitum spicatum* across different environmental/elevational gradient.

The specific objectives of the study are as follows.

1. To study the spatial variation in floral biology of *A. spicatum* (Brühl) Stapf across the elevation gradient in ACA.

2. To observe the potential visitors of *A. spicatum* and to determine if the observed visitors are legitimate pollinators.

3. To explore if the pollinator guilds of *A. spicatum* vary across the elevation gradient in ACA.

4. To determine the visitation frequency and foraging behavior of prime pollinator of *A. spicatum.*

5. To estimate the prime pollinator mediated male fitness of *A. spicatum*.

1.4 Limitations

This research primarily examined the floral biology and interactions with potential pollinators, which does not provide the comprehensive details of pollination strategies and natural breeding system of *A. spicatum*. Moreover, the current finding is the results of single flowering period in different elevation bands. Therefore, current finding represents a site-specific geographic trend and thus excludes the potential spatial/ temporal variations in the floral biology and pollinator guilds of *A. spicatum* across different population throughout its entire distribution range.

CHAPTER 2: LITERATURE REVIEW

2.1 Floral Biology

Floral biology is a field of ecological studies that examined the evolutionary factors that shaped the structure, behavior, and physiological characteristics associated with flowering of plants (Sharma et al., 2022). The study of floral biology has been conducted under the topic of pollination biology, which includes overall reproductive strategies of plants and their pollen vectors. Study of floral biology is important as it seeks to understand how floral traits are related with possible pollination strategies and their pollen vectors and describes adaptive strategies of plant to pollinators with their floral traits (Eustacchio et al., 2023). Globally there has been many studies on floral biology mainly in medicinal plants as study of floral biology helps to understand reproductive biology of plants which helps in conservation of important medicinal plants. Mendes et al., (2017) studied different floral biology like length of floral axis, flower per inflorescence, size of corolla, floral longevity, time of anthesis, pollen number and pollen viability of *Mauritia flexuosa* and found that *M. flexuosa* have gender specific floral traits and male and female flowers attracted different pollen vectors. Basnet et al., (2019) also studied different floral traits like corolla length, nectar volume and distance between stamen and anther along the elevation within ten different *Rhododendron* species and found that pollinator type and their visitation rate differed along the elevation mainly due to nectar volume and nectar concentration. Also study about floral structure, stigma receptivity and time of anthesis of *Rauvolfia serpentina* found that its psychophily mode of pollination is associated with its tubular floral structure (Sharma et al., 2022). Similarly, while studying pollen and floral morphology of *Androsace brevis* it was concluded that placement of the stamen and pistil inside corolla tube and yellow to purple color of corolla mouth suggest that *A. brevis* required insect mediated pollination.

There was so many research work in floral biology around the world which studies about different floral traits, pollen vectors and overall reproductive strategies of different plant species. But only few researchers have studied about floral biology in Nepal. Adhikari (2003), Adhikari and Adhikari (2004, 2010), Subedi et al., (2011), and Paudel et al., (2015, 2016, 2017,2018, and 2019) in their research work have focused on floral biology of flowering plants in Nepal Himalaya. Paudel et al., (2015, 2016, 2017,2018, and 2019) studied different floral traits like flower per inflorescence, petal length, anther and stamen length, pollen and ovule number per flower, pollen-ovule ratio, etc. and also recorded different pollen vectors and co-evolution with their pollinators within *Roscoea* spp and their potential visitors in Himalayan region of Nepal.

Study of floral biology is very important as they are associated with pollen vectors (Wang et al., 2009). In some species, inflorescences are the unit of attraction and insect visit also varies according to size of inflorescence (Thomson, 1988), many studies showed that pollinators offer longer visitation frequencies on plant with many flowers which increases the chance of more pollen to be removed from flower (Schmid-Hempel and Speiser, 1988). Petal length is most significant morphological trait that correlates with size of probing parts of pollinators (MarténRodríguez et al., 2009). The amount of pollen produced by a plant represents the sexual ability and abundance of that plant in their local habitat as well as counting pollen number becomes an important method for observing plant-pollinator interaction (Kearns and Inouye, 1993). Similarly, ovule number represents main reproductive trait among flowering plants based on which mechanism of pollen reception and seed output keeps on varying. Pollen-Ovule ratio is an important indicator of plant reproductive system (Cruden, 1977).

2.2 Pollination Syndrome

Evolutionary biologists have been enthralled by the variety of flowering plant reproductive organs for long time. Floral evolution has often been linked with differences in pollination syndromes. The term pollination syndrome describes highly correlated floral traits, that have been used to predict effective pollinators (e.g., insects, birds, mammals) of plant species and also provide a mechanistic explanation of floral diversity. Darwin (1862) and many others scientists describes the view that different floral traits promote interaction between plant and pollinator and also reflect pollinator type of specific plant species (Stebbins, 1970, Sprengel, 1996, Johnson & Steiner, 2000). When we investigated the evolutionary framework, their comparative studies suggest that different pollinators encourage selection for the variety of floral forms that produce an array of “pollination syndromes”.

Plant-pollinator interaction are greatly influenced by floral traits, which also determines floral visitor communities (Junker et al., 2013). Major shifts in abundance and composition of pollinators result from variations in floral traits even within short geographical area along an elevation gradient (Gurung et al., 2018, Lefebvre et al., 2018). Flower color is the most commonly studied floral traits along the elevational gradient (Shrestha et al., 2014) though we have limited information about other important floral traits. Flower color has been considered as an important floral trait that plays a major role to attract pollinators (Fenster et al., 2004, Shrestha et al., 2013, 2014, and 2016). Color components of pollination syndrome are usually presented in very simple way for instance flowers that are pollinated by birds are often orange or red to a human vision while blooms that are pollinated by bees are frequently violet or blue (Raven, 1972, Sutherland & Vickery, 1993) and flies favor white and yellow flowers (Lázaro et al., 2008). Nectar primarily serves as reward for birds and other pollinators, also it aids in the selection of possible pollinators. In high mountains flies and bees are the most dominant pollinators and are attracted to flower with lesser amount of nectar and higher sugar concentration but birds and other pollinators favors flower with high nectar volume (Martén-Rodríguez et al., 2009). Flower size mainly petal length is most significant morphological trait that correlates with size of probing parts of pollinators (MarténRodríguez et al., 2009). In general, high-elevation mountain plants are known to have smaller flower size (Guo et al., 2010) that is selected for insect pollinators like flies and bumblebees (Kudo, 2016).

Indeed, plants are often distinguished because of their floral traits, thus pollinator specialization has been considered critical to plant speciation and evolutionary radiation for the long time (Stebbins, 1970). According to Darwin (1859) pollinators could be the major selective agent in the evolution of floral morphology. Ever since Darwin (1862) defined pollination biology with the logic and explanatory power of evolutionary theory, there has been interest in the adaptive significance of floral traits in relation to pollinators. The most successful and frequent visitors may exert selective pressure on advertising traits such as size, color, scent, and inflorescence height and on mechanical traits such as corolla tube length (Stebbins, 1970, Anderson et al., 2014, Newman et al., 2015). Similarly, reciprocal coevolution may result from selection forces exerted by floral traits on pollinator traits (Muchhala & Thomson, 2009, Pauw et al., 2009). Darwin (1862) for the first time hypothesized co-evolution between long nectar spur Malagasy star orchid (*Angraecum sesquipedale*) and long-proboscis moth which became model for investigating co-evolution between plants and their potential pollinators (Zhang et al., 2013). The mutual relationship between long corolla tubed plants and their potential pollinators, long- tongued flies is a typical example of co-evolution, which has been reported mainly from the region of southern Africa till date. Out of Africa, in Nepal Himalaya, Paudel et al., (2015) first recorded an example of co-evolution between the long-tongued *philoliche longirostris* and the long-corolla tubed *Roscoea purpurea* (Zingiberaceae).

Along with Waser et al., (1996) other authors (Ollerton et al., 2009, Rosas-Guerrero et al., 2014) questioned the central theme of pollination syndrome/pollination specialization hypothesis that the evolution of floral traits is primarily through interactions with their specialized pollinators. Various studies show that plant with specific pollination syndromes is often pollinated by several pollinators. Even in some cases generalized pollinators contribute more than those pollinators predicted by floral syndromes (Waser et al., 1996, Fleming et al., 2001). For example, like other *Roscoea* species *R. alpina* has long-longue pollination syndrome despite this beetle (*Mylabris* sp.) and a moth (*Macroglossum nycteris*) visited its flower (Paudel et al., 2017). Also, in *R. auriculata, R. capitata* and *R. tumjensis* morphologically mismatched *Bombus haemorrhoidalis* contribute 90% pollination service to all of these species (Paudel et al., 2019).

Since Darwin started study about co-evolution, the remarkable diversity of floral traits of animal-pollinated plants has been one of the interesting phenomena studied by evolutionary biologists. Darwin's hypothesis helps us to understand how reciprocal selection can lead to trait matching or trait scalation but does not give comprehensive idea about additional trait variation. Along with pollinator-mediated selection, evolution of floral traits in Angiosperms can be exerted by antagonists, different climatic factors, and random processes. More ever in addition to pollinator mediated selection and climatic factors, there could be other possible factors such as nectar robbers, flowering time, and phylogenetic relationship of species which may be also responsible for floral trait variation.

2.3 Pollinator guilds

Plant and pollinators are the most important components of regional diversity. Animal pollinator plays an important role in the reproduction of many flowering plants also pollinator mediated selection is one of the most important evolutionary forces in flowering plant diversity (Darwin, 1862, Nilsson, 1988, Fenster et al., 2004). Floral morphology is one of the important aspects of plant pollinator interaction. Apart from this adverse climatic condition such as low temperature and precipitation influence the plant growth, reproduction system as well as pollinators behavior (Tong et al., 2021). Temperature is considered as the most important predictor of plant-pollinator interaction in mountain region (Classen et al., 2020). Numerous studies have focused on pollen vectors of different plant species, their visitation rates along the elevation as well as their interaction with plants. A study carried out in *Tibauchina pulchra* in Atlantic Rainforest, Brazil (870-1100m) showed that despite high flowering rate in higher elevation, only 5 visits were recorded by 2 species of bees but in lower elevation total 948 visits were recorded by 7 species of bees during the same period (Brito and Sazima, 2012). Also, a study carried out in Sikkim Himalayan, India (3400-4235m) in 10 *Rhododendron* species found that the major pollinators were flies, birds and bees. But in three *Rhododendron* species i.e., *R. anthopogon, R. setosum,* and *R. lepidotum* found in relatively higher elevation the birds were absent. The bird visit showed negative relationship with elevation while flies and bees increased with increasing elevation (Basnet et al., 2019). Similar study conducted in Washington State (8-728m) in *Cyticus scoparius L.* showed higher pollination rate by *Bombus* species in higher elevation but have low seed production and *Apis mellifera* was found pollinating the flower at lower elevation (Bode et al., 2020).

Only a few studies have been conducted on plant and pollinator diversity and their interaction in Nepal Himalaya. Adhikari, (2003), Adhikari and Adhikari (2004, 2010), Subedi et al., (2011), and Paudel et al., (2015, 2016, 2017, and 2019) recorded pollinators and visitation frequencies of specific plant species in Nepal Himalaya. Paudel et al., (2015, 2016, 2017, and 2019) in their research work conducted in different parts of the Nepal Himalaya within *Roscoea* species revealed that only long corolla tube *Roscoea purpurea* was pollinated by long tongue tabanid fly (*Philoliche longirostris)* as according to their pollination syndrome. Despite having long tongue pollination syndrome other *Roscoea* species were visited by morphologically mismatched pollinators for instance *R. alpina* was visited by a beetle (*Mylabris* sp.) and a moth (*Macroglossum nycteris*). Also morphologically mismatched Bumblebee (*Bombus haemorrhoidalis)* contribute more than 90 % pollination service to three species of *Roscoea* (*R. auriculata, R. capitata and R. tumjensis*), they were also visited by long tongue butterflies according to their pollination syndrome but they act as nectar robbers rather than pollinators. When it was raining, pollinators avoided visits to *Roscoea* species and they visited them less often when there was cloud cover, indicating that weather conditions could have an impact on the pollination system. Similarly, a study carried out by Adhikari and Adhikari (2010) in different parts of Kathmandu Valley in *Punica granatum* L. revealed that *Apis cerena* was the prime pollinator along with *A. mellifera* also seven other insects were found visiting the plant but they were probably the nectar robbers. The visitation rate of floral visitors was higher during the morning time with sunny weather and low when the weather was cloudy and rainy. Dyola et al., (2022) also explored the species richness and abundances of insect pollinators in four different habitats and different environmental variables in Shivapuri-Nagarjun National Park. Apart from this other similar research were carried out on other plant species in Nepal Himalaya (Adhikari, 2003, Subedi et al., 2011, Shrestha et al., 2020).

2.4 Male fitness

Male fitness is defined as the chance of pollen grains to reach the target stigma through insect body. Contact between an insect and flower in the field is the proof for plant-pollinator interaction. Pollen grains are the natural indicator of insect visits to flowers. Identification of pollen grains present on insect bodies can be an additional approach for studying plant-pollinator interaction (Manincor et al., 2019). Bisexual plants can obtain fitness through both male fitness (pollen donation) and female fitness (fruit and seed production). Unfortunately, a female-biased understanding of plant breeding systems, pollination biology, and plant ecology has developed as a result of disregarding the significance of male fitness (Sutherland and Delph, 1984). But some research papers discussed about importance of male fitness. Vivarelli et al., (2010) studied male fitness of 8 different bee species visiting flower of *Ononis masquillierii* and found that male fitness of plants was greatly affected by body size of floral visitors, visitation frequency and foraging behavior. Potascheff et al., (2020) in his study in

Qualea grandiflora found that total 60% pollen grains were removed by nocturnal visitors while 18% pollen grains were removed by diurnal visitors within 4 hours which is one third of pollen grains removed by nocturnal visitors and concluded that amount of pollen grains removed varied according to time of anthesis. Additionally, Capo et al., (2023) studied male fitness of five different rewarding and non-rewarding orchids and found that *Anacamptis coriophora*, orchid with nectar reward have maximum male fitness. Male fitness becomes less when removal of pollen grains becomes higher than seed production, which indicates that many pollen grains did not reach the stigma of flower.

2.5 *Aconitum* species

The genus *Aconitum* of the family Rannunculaceae is widely distributed in alpine sub-alpine region. The genus comprises over 300 species including some medicinal and ornamental plants (Utelli et al., 2000). Only a limited number of research work has been done in pollination system of genus *Aconitum* throughout the world while there were no such studies in Nepal which includes their floral biology, breeding system, and interaction with pollinators.

Bosch et al., (1999) in their study on *A. columbianum* found that *Bombus flavifrons* comprises 86.4% visits while, *B. appositus* constitute 14.3% visits and a single visit by *B. sylvicola and B. occidentalis* and their total visitation frequency were 2710 in 540 bouts for total 30 hours. Similarly, Utelli et al., (2000) in their research about floral biology like floral longevity and flower size, pollinators activity and breeding system of *A. lycoctonum* revealed that *Bombus hortorum* and *B. gerstaeckeri* were the major pollinators and their visitation time per flower was lower at upper elevation. Although stereotypically bees showed upward movement, 17% movements were downward in *A. lycoctonum* and plants were self-compatible.

Nautiyal et al., (2009) in his research carried out in Indian Himalayan studied about floral biology and breeding system of *A. heterophyllum* and found that their pollen grains varied from 2000-6000 per anther with an average of 80000 pollen grains per flower. Other floral characteristics, such as flower per inflorescence, floral axis length, and quantity of fruits and seeds, were also noted. Bees were seen pollinating *A. heretophyllum*, and as no seeds were seen during autonomous selfing, it is a self-incompatible plant. Additionally, similar kind of studies were carried out in *A.*

gymnandrum and found that *Bombus kashmirensis and B. sushikini* were prime pollinators. Total number of pollen grains produced per flower was $6.4\times10^5 \pm2.7\times10^4$ while mean number of ovules per flower was 126.9±7.6 and pollen-ovule ratio was found to be 5467± 357.3. And high P/O ratio might indicate *A. gymnandrum* might adapted to wind pollination (Duan et al., 2009). Zhang et al., (2006) also studied about pollination ecology of *A. gymnandrum* and found that *A. gymnandrum* were self-compatible plant and bumblebees were major pollinators and 3.9% and 2.7% of downward movements of bumblebees were recorded in two different elevations.

2.6 Research Gap

The majority of earlier studies on plant communities have focused on how abiotic factors (soil moisture and mineral composition) or direct interactions (competition for nutrients and space) affect plant establishment, while studies incorporating plant-pollinator interaction as a major driver of community composition are meagre. Only, a few studies have focused on the detailed pollination and reproductive biology of flowering plant in the Nepal Himalaya. Adhikari (2003), Adhikari and Adhikari (2004, 2010), Subedi et al., (2011), and Paudel et al., (2015, 2016, 2017, 2018, and 2019) made rigorous study on floral biology, pollinator interaction as well as breeding system of particular plant species. Paudel and Li, (2020) in their comprehensive study on Himalayan *Roscoea* spp. suggest that how variation in floral trait among the congeners drives for the evolution of different mating system. Even though more research on the reproductive biology of several significant medicinal plants found in the Nepal Himalaya is still required, such studies are yet meagre.

Chapagain et al., (2019) in their research on life history traits of *Aconitum spicatum* studied some floral traits like flowers per plant, fruits per plant, and reproductive parts per plant. Although Chapagain et al., (2019) made a comprehensive study on the vegetative and floral structures of *A. spicatum* studies incorporating floral trait variation in relation to pollinator guilds are entirely lacking. Therefore, this research that includes study of major floral traits of *A. spicatum* such as petal length, anther number, anther and stamen length, pollen and ovule number, and P/O ratio, as well as the exploration of spatial variation in potential visitors and legitimate pollinators across the different elevation gradient, and the contribution of legitimate pollinator in male fitness of *A. spicatum*, provides clear insight into their reproductive strategies.

CHAPTER 3: MATERIALS AND METHODS

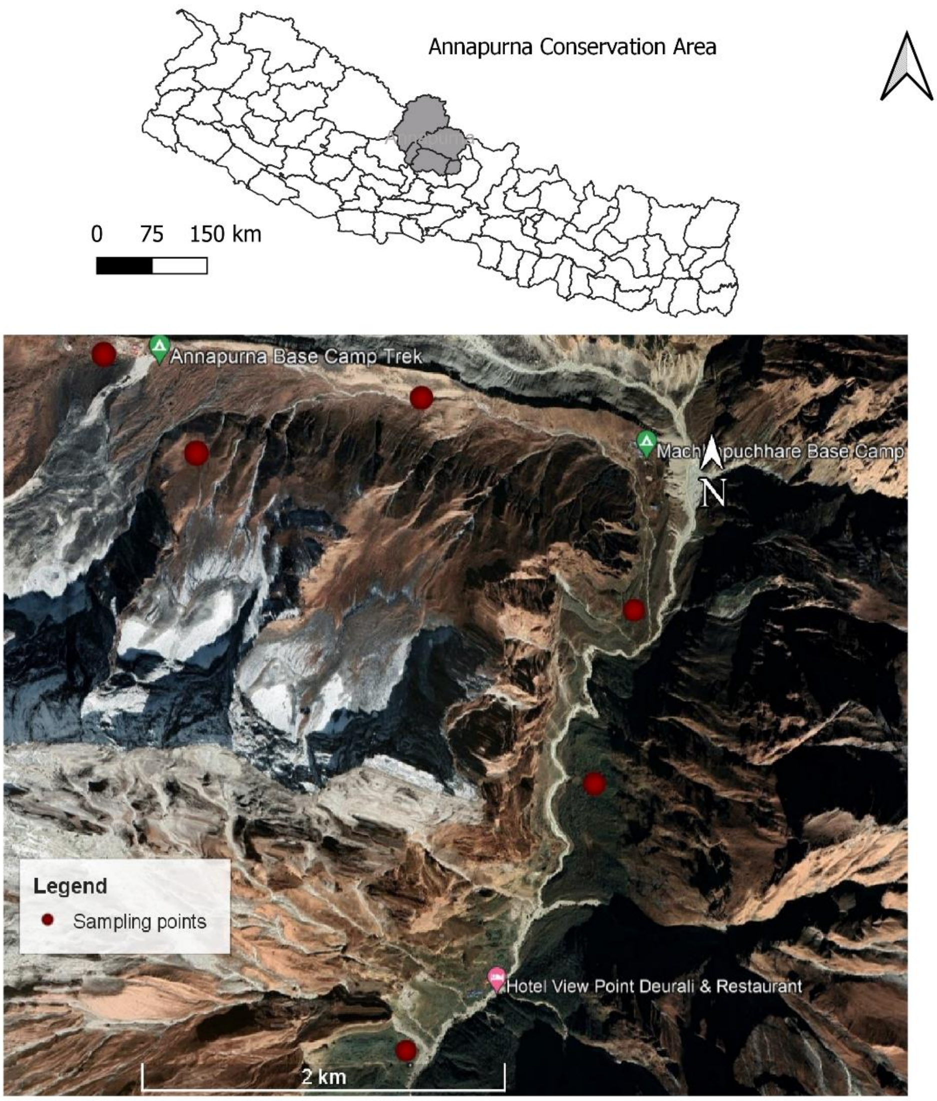
3.1 Study area

The study area lies in Annapurna Conservation Area (ACA) between 28°12′48″ - 29°19′48″N and 83°28′48″ - 84°26′ 24″E with an elevation range of 790m to 8091m asl, central part of Nepal (figure 1). Annapurna Conservation Area Project (ACAP) was established in 1986, mainly for biodiversity conservation and tourism promotion. It is the first conservation area and the largest protected area of Nepal managed by the National Trust for Nature Conservation (NTNC) with a total area of 7,629 km^2. ACA occupies 5.18% of the total area of Nepal and 35.10% of the total area of Gandaki Province (Baral et al., 2019). ACA encompasses Annapurna Himalayan range and known for several trekking routes including Annapurna trekking route. The conservation area extended in five districts (Manang, Mustang, Kaski, Myagdi, and Lamjung) (Baral et al.,2019).

Within ACA, the study was carried out at six sites in Annapurna trekking route, Annapurna rural municipality, Kaski district. The sampling locations were determined along the trekking route of Annapurna Base Camp starting from Deurali to Annapurna Base Camp with an elevation range of 3043m to 4265m. The geographical details of the specific study sites are tabulated below.

Table 1: Study sites

Study sites	Latitude	Longitude	Altitude(m)
Below Deurali	28.49408N	83.89353E	3043
Above Deurali	28.50935N	83.90481E	3315
Between Deurali and Machhapuchhre Base Camp	28.51847N	83.90775E	3504
Between Machhapuchhre Base Camp and Annapurna Base Camp	28.52880N	83.89422E	3901
Annapurna Base Camp	28.53037N	83.87641E	4197
Above Annapurna Base Camp	28.52534N	83.88187E	4265



Source: Google Earth

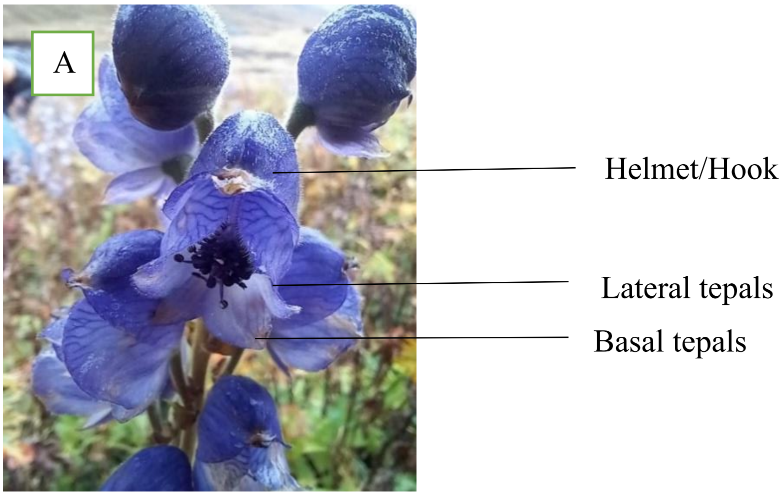
Figure 1: Map of study area

3.2 Vegetation

The study area is characterized by temperate and alpine climate, which contains a variety of ecosystems, including trans-Himalayan cold deserts, subalpine meadows, and subtropical Sal forests. The vegetation type of ACA at lower elevation (>3,000-3,500m) includes upper temperate coniferous and mixed broad-leaved forests. The dominant species of this region are *Abies spectabilis* at subalpine mixed-forests, *Betula utilis* at and below(>3,500-4,000m), dwarf bushes of *Rhododendron* spp. at lower alpine region and above tree line (ca. 4,000m), and alpine meadows and grasslands above 4,000m respectively (Chapagain et al., 2019).

3.3 Study Species

Genus *Aconitum,* belonging to the family Ranunculaceae comprises over 300 species including some ornamental and medicinal plants (Utelli et al., 2000). It is commonly known as bikh in Nepali and Monk's hood or Aconite in English, they are distributed in subalpine and alpine regions. Total 28 *Aconitum* species are reported from the Nepal (Shrestha et al., 2022). The study species *Aconitum spicatum* is one of the important medicinal plant species which is restricted to Himalayas of Nepal, India, Bhutan and China at an elevation range of 1,800-4,800 m asl (Ghimire et al., 2008, Shrestha et al., 2022). The plant is usually perennial or biennial herb with erect stem reaching up to two meter in height. Leaves are mostly cauline, lobed, and rarely divided and dentate. Flowers are simple or branched racemes bearing 1-70 dark blue, violet or white zygomorphic flowers with numerous stamens, and 1-110 fruits per plant with average of 41 seeds per fruit (Chapagain et al., 2019). Flowers has three different tepals, the uppermost sepal form a helmet-shaped hood and known as helmet or hood which covers sparsely pubescent petaline claw bearing nectar. The central pair of tepals is known as lateral tepals which brackets the stamens and ovary. And the lowermost tepals known as basal tepals and that lies under the lateral tepals. Each fruit is an aggregate of five to six follicles. The plant's flowering time is from July to September and fruiting time is from August to November (Lama et al., 2001). The aerial parts are annual but its tubers are perennial. The plant is highly poisonous and tubers contain alkaloids (C-19 norditerpenoid, C-19 diterpenoid alkaloids) (Shyaula et al.,2012, 2016). It is one of the highly valued medicinal plant used against disease like headache, infections in lungs, intestine and to heal cuts and wounds (Lama et al., 2001) and its tubers are in trade (Rana et al., 2020).



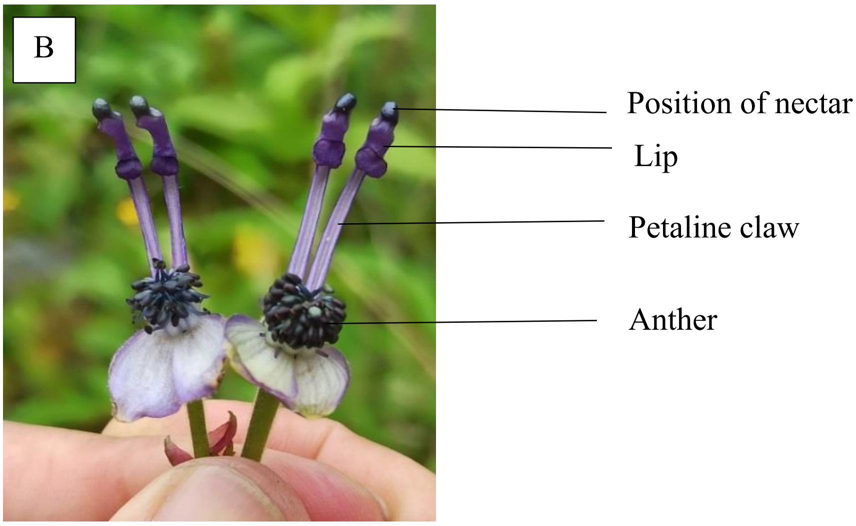


Figure 2: (A) Inflorescence of *A. spicatum* showing basal tepals, lateral tepals and helmet/hook (B) Petaline claws and anthers of *A. spicatum*

3.4 Floral Biology

Plots with dense flowering plants were selected at each sampling site, and a plot of 5m × 5m was laid down for the study of floral biology of *Aconitum spicatum*. The sampling points for each plot were determined by purposive sampling method. For estimating various floral traits of *A. spicatum*, at each study site, 10 mature buds of plants lying within the sampling plots were randomly selected and collected from four populations with an elevation range from 3043m to 3901m. The buds were collected in vials with 70% ethanol and vials were well labeled with identification code. Also 20 healthy flowers were randomly selected and collected from three populations with an elevation range from 3315m to 4197m. Then the flowers were pressed in herbarium sheet.

3.4.1 Measurement of floral traits

Different floral traits like length of tetals, length of anther and stamen, length of petaline claw, lip distance, number of anthers per flower, number of pollen grains per flower, numbers of ovules per ovary and P/O ratio were measured from preserved flower and bud samples at the laboratory of Central Department of Botany, Tribhuvan University. *Aconitum spicatum* has three different tetals, named as basal tepals, lateral tepals, and helmet and their lengths were measured using ruler. Petaline claw was separated from the flower then its length and lip distance were measured under microscope (BEL, ITALY, STMLAB-T LED), using wave image software.

To measure anther and stamen length, ten filaments with intact anthers were selected and measured under stereomicroscope (BEL, ITALY, STMLAB-T LED), using wave image software. Total anther number were counted from preserved bud samples.

3.4.2. Pollen count

The preserved bud samples were used to estimate pollen number in Central Department of Botany, Tribhuvan University laboratory. Pollen grains were counted using haemocytometer (Neubauer improved Haemocytometer, MARIENFELD, Germany, Tiefe-depth profundeur 0.100 mm) under the light microscope (BEL, ITALY, BIO2T-LED), with magnification of 100x following the standard methods of (Kearns & Inouye, 1993). The vials of suitable size and 70% ethanol were used to prepare 1ml of homogenized suspension and a glass rod of appropriate size was used to crush the anthers. A micropipette of 20-100 µl capacity and a micropipette lip were used to transfer pollen grains to a haemocytometer.

First of all, total anthers of a bud were separated from the stamen with the help of blade and kept in the vial and 1ml of 70% ethanol was added. The anthers were crushed finely with the help of glass rod to release pollen grains from the pollen sac and 1ml of homogenized suspension were prepared. The homogenized suspension was well shaken and transferred to the haemocytometer with the help of micropipette then covered with cover slip. All the pollen grains present on the grids of haemocytometer were observed under the microscope with 100x magnification and pollen grains were counted. Same process was repeated for three times for each sample. Total number of pollen grains in the suspension were calculated following formula by Dafni et al., 1992.

Total pollen grain in a bud = $\frac{\text{No.of pollen grains observed under microscope}}{\text{Volume of suspension pipetted out}} \times$ final volume of suspension

3.4.3 Ovule count

Preserved bud samples were used to count the total ovule number per ovary. First carpels were separated from the bud and placed on a slide. Ovary was carefully dissected vertically with the help of blade and ovules were counted under the stereomicroscope (BEL, ITALY, STMLAB-T LED) following the standard methods of (Kearns & Inouye, 1993). Total ovules in all five follicles were counted and total ovules per ovary were calculated as:

Total ovules in a bud=Total sum of ovules present in all follicles.

3.4.4 Pollen-ovule ratio (Pos)

Both pollen and ovule number of plant helps to determine the efficiency of plant in pollination (Dafni, 1992). Pollen-ovule ratio is the ration of total numbers of pollen grains to total numbers of ovules per flower. The pollen-ovule ratios were calculated using following formula by Dafni et al., 1992.

$$\text{Pollen-ovule ratio} = \frac{\text{total pollen grain per flower}}{\text{total ovules per flower}}$$

3.4.5 Daily display area

Daily display area and number of flowers per inflorescence were recorded in the field. For daily display area total number of flowers at different stages i.e., bud, flower, and wilt were counted. Daily display area was calculated by following formula by Harder and Barrett, 1995.

$$\text{Daily display area} = \frac{\text{flower}}{\text{bud+flower+wilted}} \times 100\%$$

For total number of flowers per inflorescence, ten healthy plants were randomly selected from the plot and total number of flowers at different stage i.e., buds, flowers, and fruits per inflorescence were counted.

3.5 Observation of floral visitors

To observe the floral visitors of *Aconitum spicatum,* four observation plots of 5m × 5m were prepared with an elevation range 3315m to 4265m where plants were densely flowering. At middle elevation (3901m), for diurnal visitor's observations were made from 9 am to 6 pm with hour-based observation periods (9am to 10am, 10am to 11am, 11am to 12am, and so on) per day. For nocturnal visitors observations were made from 7pm to 9pm. Diurnal visitors were observed for a total of 23 hours for three days whereas nocturnal visitors were observed for a total of 2 hours in one day. Due to logistic and other constraints, at other three populations, only diurnal floral visitors were observed for only 2 hours while nocturnal visitors were not observed.

Foraging behavior of floral visitors i.e., floral visitors were legitimate or illegitimate were recorded from direct observation. A visit was considered legitimate if the visitors' body came in contact with female reproductive part of flower and ultimately transferred pollen grains to the stigma of flower.

To estimate visitation frequency, total number of plants and total numbers of visits per hours within observation plots (observation time was 23 hours at an elevation of 3901m while only 2 hours at an elevation of 4265m) were counted. Visitation frequency of floral visitors were calculated as:

Visitation frequency = total number of visits per flower per hour

During observation, different weather events (rainy, cloudy, windy, and sunny) were also recorded.

The observed floral visitors from three populations with an elevation range from 3315m to 4265m were captured with the help of insect net and killed using a killing jar (a jar with ethyl acetate). Then the specimens were collected in vials with 70% ethanol for specimen identification. Specimens were collected in individual vial to calculate male fitness. The specimens of collected visitors were identified in consultation with an entomologist.

3.6 Male fitness of *Aconitum spicatum*

Male fitness is defined as chance of pollen grains to reach the target stigma through insect body. For this, floral visitors were collected in individual vial with 1 ml of 70% ethanol. The vial was well shaken then floral visitor was removed from the vial. The suspension was well shaken and transferred to the haemocytometer with the help of micropipette then covered with cover slip. All the pollen grains (viable and non-viable) present on the grids of haemocytometer were observed under the microscope (BEL, ITALY, BIO2T- LED), with 100x magnification and pollen grains were counted. Same process was repeated for three times for each sample. Total number of pollen grains in the suspension were calculated by following formula by Dafni et al., 1992.

Total pollen grain in a floral visitor's body=

$$\frac{\text{No. of pollen grains observed under microscope} \times \text{final volume of suspension}}{\text{Volume of suspension pipetted out}}$$

Later male fitness was calculated by following formula by Sutherland and Delph, 1984.

$$\text{Male Fitness (M.F)} = \frac{\text{Pollen grains present in bumblebees body}}{\text{Total pollen grains/flower}} \times 100\%$$

3.7 Statistical analysis

Statistical analysis was done using Microsoft excel and IBM SPSS Statistics 25 (IBM Corp. 2017). All the data were firstly tested for normality and homogeneity of variance before running parametric statistical analysis. The number of flowers per inflorescence, petals length (basal tepals, lateral tepals, and helmet), anther number, anther's length, stamen length, and P/O ratio were log transformed to make the data normally distributed; however other data did not require any transformation.

Later, one-way analysis of variance (ANOVA) was conducted to determine the collective interaction effect of different populations (elevation) on different floral traits. This model incorporates different populations as independent variables and floral traits as dependent variables. The mean values of floral traits were compared among different populations using Tukey's post-hoc test

CHAPTER 4: RESULTS

4.1 Floral Biology

All the studied floral traits of *Aconitum spicatum* except pollen-ovule ratio were differed significantly among the populations. The flower number per inflorescence was significantly higher in population 1 (elevation of 3315m) as compared to two other populations (P=0.00). On average, the plant possesses 10-195 flowers (69±9.99) per inflorescence. The daily display area of plant was significantly higher at elevation 4197m and 4265m as compared to elevation 3901m (P=0.003). Plant has three different types of tepals namely basal tepals, lateral tepals and helmet with mean value of 1.14±0.018 cm, 1.60±0.018 cm, and 2.17±0.025 cm respectively. Basal petals and helmet length were significantly higher in population 3 (4197m) and lateral tepals length was significantly higher in population 2 (3901m) and population 3 (3901m) as compared to population 1(3315m) (P=0.00). Flowers (helmet) have a pair of petaline claw of length 25.04±0.19 mm with lip distance/nectar accessible length of 6.54±0.11 mm which were significantly higher in population 2 (3901m) and population 3 (3901m) as compared to population 1(3315) (P=0.00).

A flower produced 33-44 anthers per flower (38.74±0.46) with an average of 0.84±0.0083 mm anther length and 7.21±0.06 mm stamen length. Both anther and stamen length were significantly higher in population 2 (3901m) than in other two populations (P=0.00). Each flower produced 8163-23172 pollen grains (14024±679) and 53-100 ovules (70.78±1.82) with an average 201±10.95 pollen-ovule ratio. Population 4 (3901m) had significantly higher number of ovules per flower(P=0.001) while pollen numbers per flower (P=0.002) and pollen-ovule ratio(P=0.087) did not differ significantly within populations.

Table 2: Floral traits (Mean ± SE) of *Aconitum spicatum* among different population studied in Annapurna trekking route, Kaski, Central Nepal (different letters indicate significance value among populations at p <=0.05).

Floral traits	Sample no. (No)	Mean (±SE) value of floral traits				F-value	P-value
		Population1 (3315 m)	Population2 (3901 m)	Population3 (4197 m)	Population4 (4265 m)		
Daily Display Area	340		61.37%[a]	79.76% [bc]	73.37%[ac]	5.85	**0.003**
No. of flowers/inflorescence	30	125.7±18.15[a]	36.60±8.61[b]	46.10±5.39[b]		11.11	**0.00**
Basal tepals length (cm)	60	1.05±0.20[a]	1.08±0.023[a]	1.27±0.024[b]		22.99	**0.00**
Lateral tepals length (cm)	60	1.5±0.02[a]	1.63±0.03[b]	1.68±0.03[b]		12.11	**0.00**
Helmet/Hook length (cm)	60	2.02±0.03[a]	2.14±0.03[b]	2.34±0.03[c]		24.26	**0.00**
Petaline claw length (mm)	60	23.92±0.25[a]	25.29±0.29[b]	25.90±0.28[b]		13.74	**0.00**
Lip distance (mm)	60	5.83±0.11[a]	7.03±0.21[b]	6.75±0.12[b]		17.17	**0.00**
Anther length (mm)	300	0.733±0.0079[a]	0.96±0.014[b]	0.84±0.011[c]		108.51	**0.00**
Stamen length (mm)	300	6.32±0.05[a]	8.1334±0.09[b]	7.16±0.07[c]		167.78	**0.00**

Floral traits	Sample no. (No)	Mean (±SE) value of floral traits				F-value	P-value
		Pooulation1 (3043 m)	Population2 (3315 m)	Population3 (3504 m)	Population4 (3901 m)		
Anther number/flower	32	39.25±0.53[a]	39.80±0.36[ab]	41.20±0.61[b]	34.80±0.25[c]	42.28	**0.00**
Pollen number/flower	32	13250±979[a]	11238±646[ab]	14678±2407[a]	17169±1125[ac]	6.22	**0.002**
Ovule number/flower	32	66±1.31[a]	68.40±1.87[a]	62.75±3.75[a]	80.20±3.87[b]	6.72	**0.001**
P/O ratio	32	224±18[a]	167±12[a]	243±56[a]	218±17[a]	2.42	**0.087**

4.2 Pollination guilds

During the field observation, there were no spatial variation in the pollinator composition of *A. spicatum*. Out of five days of field observation, within total four plots during the peak blooming period of plant across their respective distribution site along Annapurna trekking route, we found that the bumblebee (*Bombus miniatus*) was the single visitor species that actively visited the flower of *A. spicatum* during day time. However, we did not observe any nocturnal visitors to forage the flowers of *A. spicatum*. While observing foraging behavior *B. miniatus* in the field, *B. miniatus* placed their legs in basal tepals and their abdominal part rest in the anther and stamen which were covered by lateral tepals and extend their proboscis into lips of petaline claw to collect the nectar. While doing so, their abdominal part touched reproductive part of plant i.e., pollen grains were observed in the stigma of *A. spicatum* following the visit of *B. miniatus.* Based on that, it was concluded that *B. miniatus* were the legitimate visitors (pollinators) of *A. spicatum.*

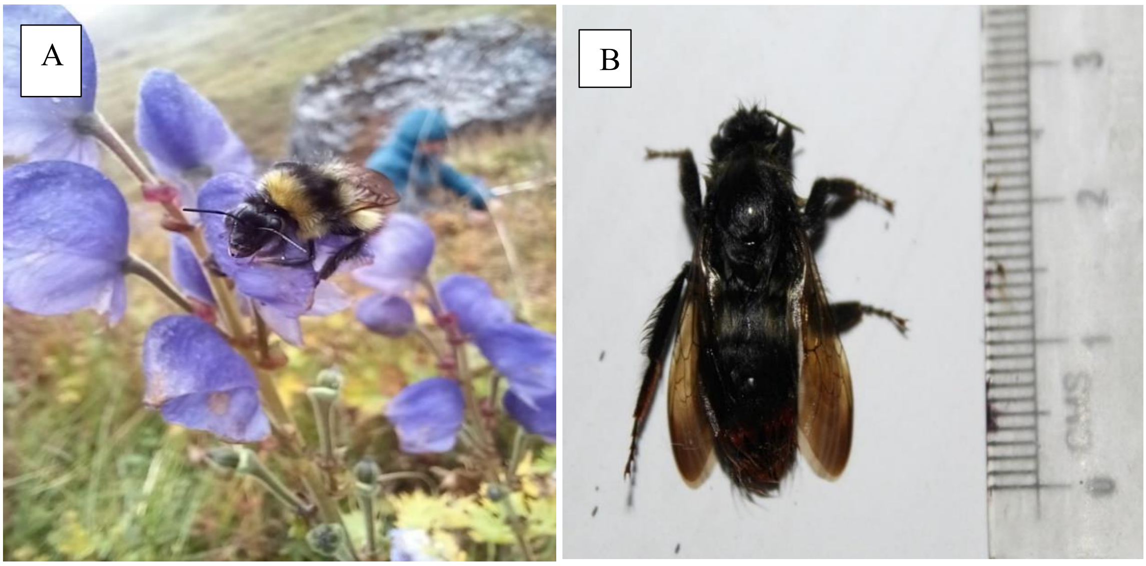


Figure 3: (A) *Bombus miniatus* visiting the flower of *Aconitum spicatum* in the field. (B) Image of *B. miniatus* taken in the laboratory.

Visitation frequencies of *B. miniatus* were recorded at an elevation of 3901 m where plants were densely populated. Within 23 hours of observation for three days, a total of 810, 609, and 497 visits of *B. miniatus* were recorded for day 1, day 2, and day 3 respectively in a plot with total 284 flowering plant. The visitation frequencies of *B.*

miniatus were not consistent in different weather condition and timing of the day. The visitation frequencies of *B. miniatus* were at peak after 9am to 3 pm then gradually decreases after sunset. Moreover, the visitation frequencies were fluctuated according to different whether events (sunny, cloudy, rainy, and windy). Maximum number of visits were recorded when whether were clear or sunny and minimum visits were recorded when whether were extremely cloudy and windy (figure 4, 5, and 6). The hourly variation in visitation frequency of *B. miniatus* in different timing of the day and different weather conditions were as shown in the figures (Figure 4, 5 and 6).

Visitation frequencies of *B. miniatus* were also recorded at an elevation of 4265m within total 41 flowering plants for total 2 hours (1:15pm to 3:15pm) and only 5 visits were recorded as weather was extremely cloudy and windy there.

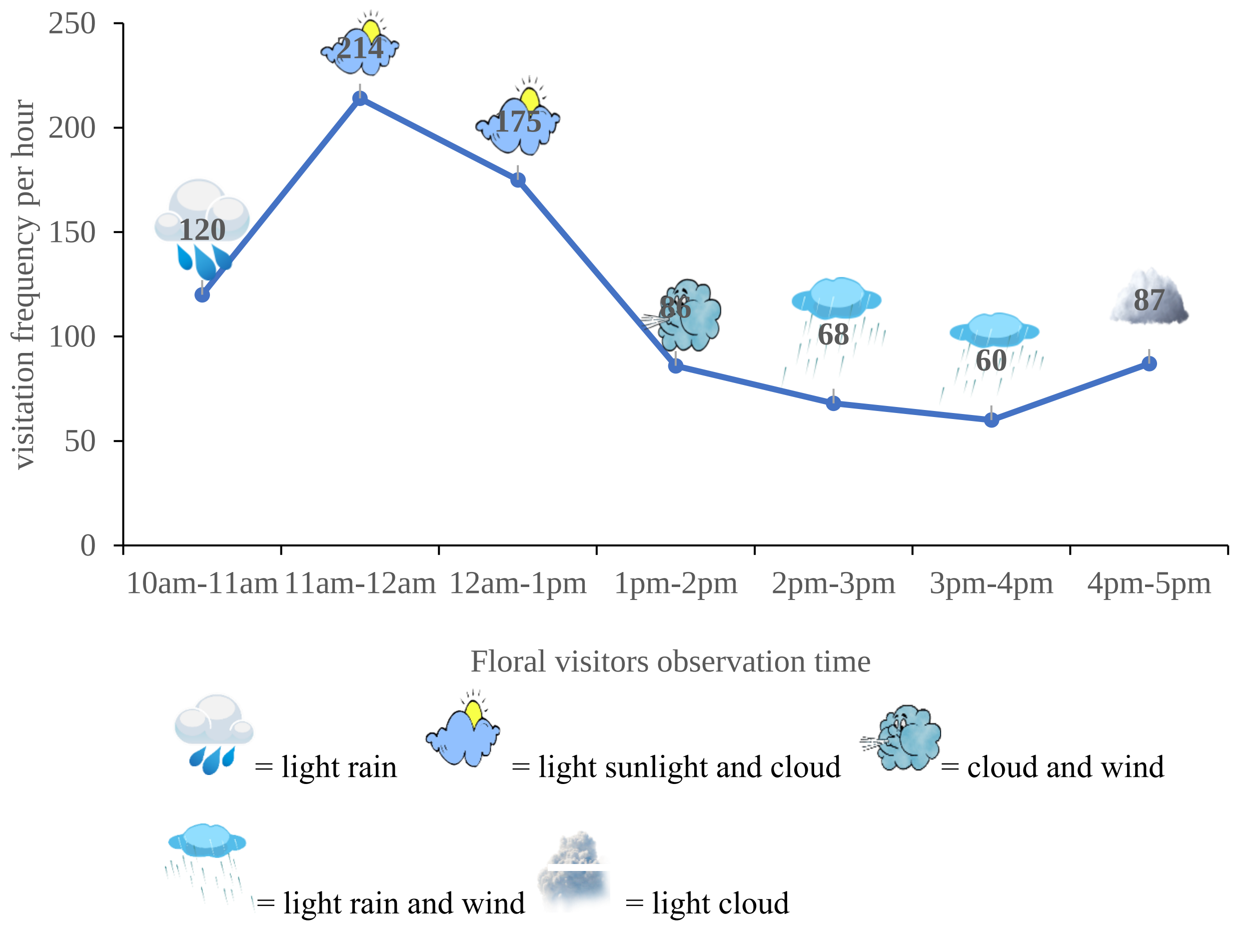


Figure 4: Visitation frequencies of *Bombus miniatus* showing different weather conditions.

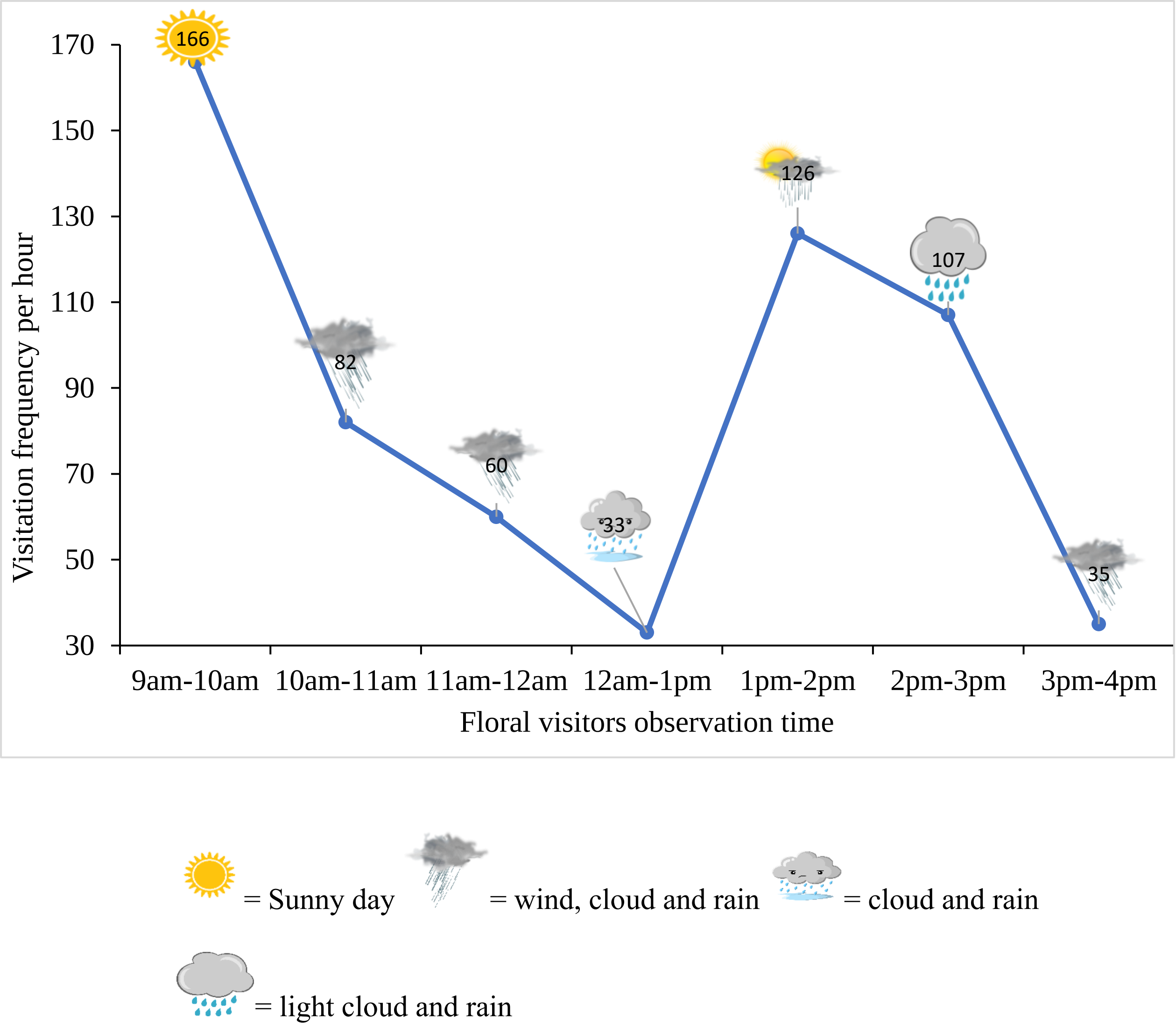


Figure 5: Visitation frequencies of *Bombus miniatus* showing different weather conditions.

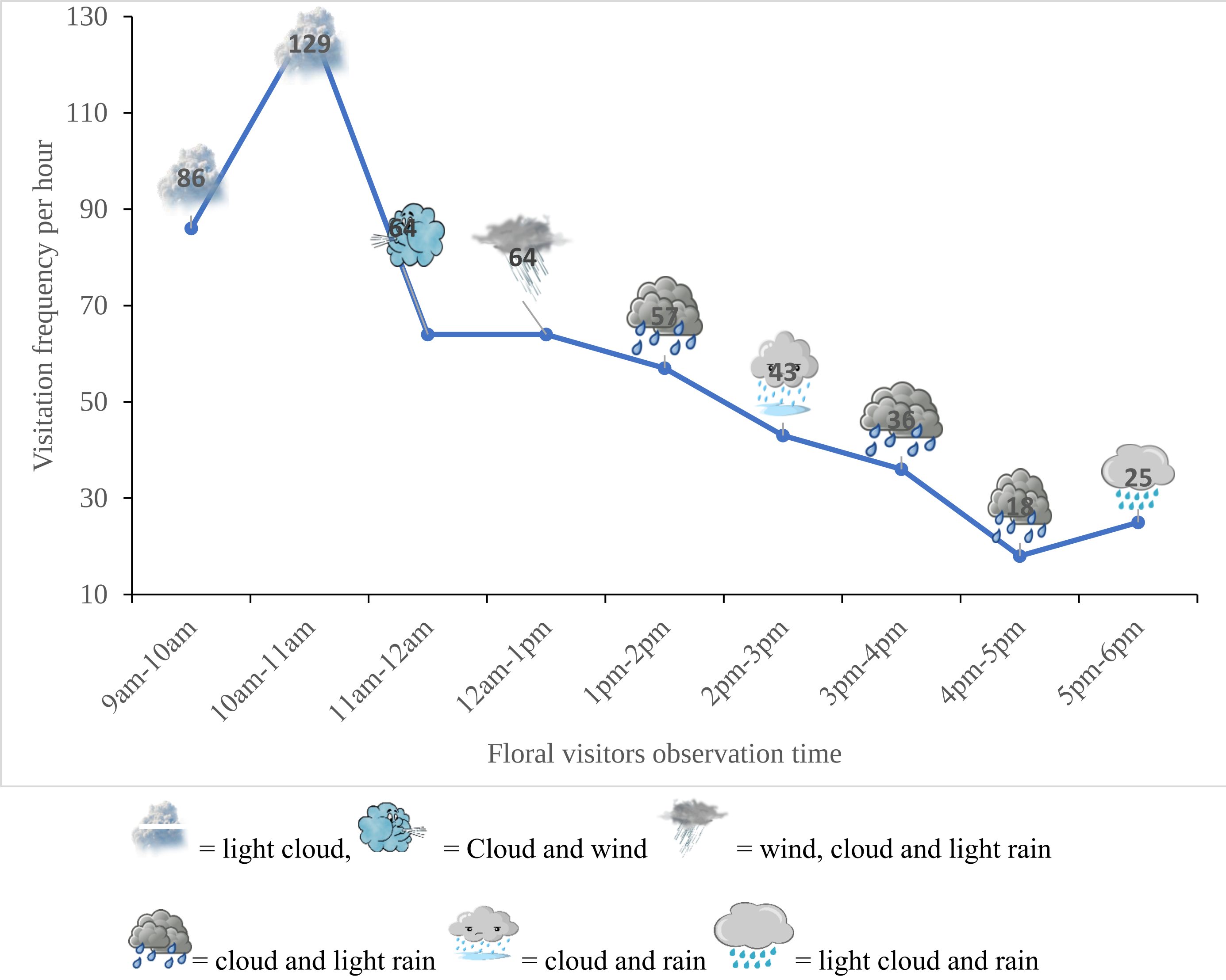


Figure 6: Visitation frequencies of *Bombus miniatus* showing different weather conditions.

4.3 Male fitness

We only tracked male fitness at one population (3901) due to some logistic constraints. *Aconitum spicatum* flowers were found to produce 17169±1125 (mean ± SE) pollen grains among which 15949±1115 were viable (mean ± SE) and 80.20±3.87 (mean ± SE) ovules per flower, and bumblebee bodies had a total of 2417±653 (mean ± SE) pollen grains with total 1549±490 (mean ± SE) viable pollen grains. And it was discovered that male fitness was 14.08%. If sufficient pollen grains will reach the stigma of *A. spicatum* to fertilize the total ovules, *A. spicatum* might achieve 100% pollination success.

CHAPTER 5: DISCUSSION

5.1 *Bombus miniatus* as the potent prime pollinator

The genus *Aconitum* are thought to be adapted for pollination by bumblebee species, due to its unique floral traits which was first described in *A. nepellus* by Darwin (1876). The dependence of genus *Aconitum* on bumblebees may be associated with floral traits such as distinct spurs and zygomorphic sepals also body size and tongue length of bumblebees corresponds well with bilaterally symmetrical sepals and specialized helmet of *Aconitum* flower. During the field *Bombus miniatus* was observed as the exclusive visitors of *A. spicatum*. Several previous findings also found that bumblebees were the major pollinators of *Aconitum* species for example, *A. columbianum* was visited by 86.4% *Bombus flavifrons*, 14.3% by *B. appositus* and single visit by *B. sylvicola* and *B. occidentalis* and their total visitation frequency were 2710 in 540 bouts for total 30 hours (Bosch et al., 1999), Likewise *Bombus hortorum and B. gerstaeckeri* were the major pollinators of *A. lycoctonum* and their visitation time per flower was lower at upper elevation (Utelli et al., 2000). Similarly, *Bombus kashmirensis and B. sushikini* were prime pollinators of *A. gymnandrum* (Duan et al., 2009). The current finding suggests that *B. miniatus* visited the flowers of *A. spicatum* with relatively higher frequency with almost all the legitimate visits. Similar foraging behavior of *Bombus* spp was also recorded in *A. lycoctonum* (Utelli et al., 2000) and *A. japonicum var. montanum* (Fakuda et al., 2001). The current finding reveals the consistent foraging behavior of *B. miniatus* to the flowers of *A. spicatum*, however the visitation rates vary across the different timing of the day and also among the different days. During the field observation, the maximum number of visits were recorded when weather were clear or sunny and minimum visits were recorded when weather was extremely cloudy and windy. Previous findings on other plant species suggest that pollinators' visitation frequencies are affected not only by plant floral morphology, mainly floral display area but also by environmental variables. Adhikari, (2003), Adhikari and Adhikari (2004, 2010) and Paudel et al., (2017) also recorded maximum number of visits during sunny day and minimum number of visits during cloudy and rainy day. Therefore, the hourly/daily variation in visitation frequency of *B. miniatus* to the flowers of *A. spicatum* is more likely to be associated with weather pattern. Thus, it suggests that the visit of *B. miniatus* to the flowers of *A. spicatum* is not a fortuitous visit. The very high

(19 times higher viable pollen grains than ovule number of *A. spicatum*) male fitness of *A. spicatum* linked with *B. miniatus*; the bumblebee transports 2416.65±652.85 (N=4) pollen grains during a single foraging bout among which 1549±490 are viable which would be sufficient enough to fertilize all the ovule (average ovule number 80.20±3.87; N= 32) if only a fraction of transported pollen grains gets deposited onto the stigma during flower foraging by the bumblebee which suggests that this bumblebee can efficiently pollinate the flowers of *A. spicatum* even in a single foraging bout. These facts thus indicate that *B. miniatus* could be the prime pollinator of *A. spicatum*, at least across the current study sites. However, the current limited data do not have strong evidence in this favor. Therefore, further study including a detailed and manipulated pollination experiments along with estimation of pollination efficiency of *B. miniatus* would be required to ascertain that this bumblebee is the prime pollinator of *A. spicatum*.

5.2 Pollinator mediated selection as the potential driver of floral trait evolution

Flowers of *A. spicatum* bear 33-44 anthers per flower and flower contains 8163-23172 pollen grains and 53-100 ovules with an average 201±10.95 pollen-ovule ratio. The number of pollen grains produced by a plant indicate sexual ability of plant and counting pollen number becomes an important method for observing plant-pollinator interaction (Kearns and Inouye, 1993). Similarly, ovule number represents main reproductive trait among flowering plants based on which mechanism of pollen reception and seed output keeps on varying. Cruden (1977) provided evidence that insect pollinated plants have higher number of pollen grains and pollen-ovule ratio than self-compatible plants i.e., pollen-ovule ratio is associated with breeding system of plant and it is better indicator of breeding system than other floral morphology. Pollen-ovule ratio also reflect pollination efficiency i.e., likelihood of pollen grains reaching to the stigma (Cruden, 1977, 2000). The higher pollen production in *A. spicatum* thus suggests this plant species adopts insect-mediated pollination mechanism.

The suites of floral traits in *A. spicatum* such as dark blue or violet zygomorphic flowers with wide modified petaloid sepals namely basal tepals and lateral tepals serving as the landing platform for its visitors, stigma hidden within anthers which is exposed only when visitors probes the flower, nectar source situated away from the reproductive organs and protected by a cover of helmet etc suggest the insect pollination syndromes, particularly bumblebee pollination syndrome. Consistent with the floral syndrome, the current study reveals *B. mimiatus* as the exclusive visitors of *A. spicatum* across all the study sites. Likewise, the flower colour shows a uniform pattern (dark blue) across the different studied populations. Several previous findings (Raven, 1972, Sutherland & Vickery, 1993) also reveal that blue flowers show strong preference for pollination by bumblebees. Therefore, the uniform floral colour (for human vision) of *A. spicatum* across the different studied populations and the visitation by the same pollinators suggest that flower colour in *A. spicatum* is under the direct selection pressure of its exclusive visitor *B. mimiatus*. However, flower number per inflorescence and daily display area do not show the signature of pollinator mediated selection because despite the presence of same pollinator across all the study populations, these traits vary significantly among populations. The variation in these traits across the populations may be associated with variation in visitation frequency but this study does not test this potential hypothesis.

Current study reveals a variation in the length of all petaloid sepals and petaline claw among different populations. A study, suggests that petal length among Gesneriaceae spp is most significant morphological trait that correlates with size of probing parts of pollinators (MarténRodríguez et al., 2009). Darwin (1862) proposed that variation in floral diversity is the result of pollinator mediated selection. The findings of Paudel et al., (2015, 2016) suggest the linear correlation between corolla tube length of *Roscoea purpurea* (Zingiberaceae) and proboscis length of its obligate pollinator, a long-tongued fly *(Philoliche longirostris*). Moreover, Paudel and Li, (2020) suggest that the variation in staminal appendages among the different *Roscoea* species is directly linked with pollinator types/visitation rates. Therefore, it is likely that these floral traits in *A. spicatum* are selected by pollinators.

CHAPTER 6: CONCLUSIONS & RECOMMENDATIONS

6.1 Conclusions

In this research work, altogether 10 floral traits of *Aconitum spicatum* including daily display area, flowers per inflorescence, petaloid sepal length, petaline claw length and lip distance, anther number, anther stamen length, pollen and ovule number and P/O ratio were studied among different population within 3043- 4265m elevation range of ACA. All floral traits varied significantly among the populations except for the pollen-ovule ratio. The variation in floral traits of *A. spicatum* among different populations might be due pollinator mediated selection. Mainly, floral structure and flower colour of *A. spicatum* as well as pollinators foraging behavior suggests that *A. spicatum* have insect pollination syndrome. During this field work, *Bombus miniatus* actively visited flower of *A. spicatum* during daytime among all population while nocturnal visitors were absent. *B. miniatus* actively visited the flower after 9 a.m. till 6 p.m. and total 1916 visits were recorded within three days for total 23 hours at an elevation of 3901m while only 5 visits were recorded at an elevation of 4265 m for 2 hours. The visitation rates vary across the different timing of the day and also among the various days. The visitation rate of *B. miniatus* were maximum when weather is clear and sunny and least visitation rate were recorded when weather was cloudy and windy. Thus, variation in visitation frequency of *B. miniatus* was more likely to associated with weather events. The male fitness of *A. spicatum* was found to be 14.08% and very high male fitness of *A. spicatum* linked with *B. miniatus*; the bumblebee transports 2416.65±652.85 pollen grains out of total 17169±1125 (mean ± SE) pollen grains of *A. spicatum* during a single foraging bout which would be sufficient enough to fertilize all the ovule (average ovule number 80.20±3.87) if only a fraction of transported pollen grains gets deposited onto the stigma during flower foraging by the bumblebee; which suggests that this bumblebee can efficiently pollinate the flowers of *A. spicatum* even in a single foraging bout. These facts thus indicate that *B. miniatus* could be the prime pollinator of *A. spicatum*, at least across the current study sites of this species and variation in floral traits might due to pollinator mediated selection.

6.2 Recommendations

- The current study explores only the floral biology and potential pollinator guilds of *A. spicatum*. Therefore, further study including manipulated pollination experiments would be required which sheds light on the natural breeding system of this important medicinal plant.
- The floral traits of *Aconitum spicatum*, with the exception of the pollen ovule ratio (Pos), varied significantly across populations. While the results of this study do not indicate whether the variation in those characteristics are caused by pollinator-mediated selection or by geographic variation, this finding can be used as baseline for future research to study pollinator mediated selection in floral traits.

REFERENCES

Adhikari, S. (2003). Floral Phenology and Pollination Ecology of *Pedicularis dendrothauma* R. R. Mill and D J Allard in Sikles (ACAP) Kaski, Nepal. M.Sc. Dissertation. Central Department of Botany, Tribhuvan University Kathmandu, Nepal.

Adhikari, S., and Adhikari, M, K. (2010). Floral Phenology and Pollination Ecology of *Punica granatum* L. in Kathmandu, Nepal. *Nepal Journal of Science and Technology* 11, 115-124.

Adhikari, S., and Adhikari, M. K. (2004). Bumblebees and their Relationship with Flower Morphology of *Pedicularis* species in Nepal. In: Proceedings of IV National Conference on Science and Technology, Kathmandu. *Nepal Academy of Science and Technology*. 997-1004.

Anderson, B., Ros, P., Wiese, T. J., Ellis, A. G. (2014). Intraspecific divergence and convergence of floral tube length in specialized pollination interactions. Proceedings of the Royal Society B: *Biological Sciences* 281, 20141420. http://dx.doi.org/10.1098/rspb.2014.1420

Baral, R., Subedi, A., and Yadav, S.K. (2019). Wild Mammals of Conservation Area.

Basnet, S., Ganesan R., and Devy S, M. (2019). Floral traits determine pollinator visitation in *Rhododendron* species across an elevation gradient in the Sikkim Himalaya. *Alpine Botany* https://doi.org/10.1007/s00035-019-00225-3

Bosch, M., and Waser, N. M. (1999). Effects of local density on pollination and reproduction in *Delphinium nuttallianum* and *Aconitum columbianum* (Ranunculaceae). *Reproductive Biology* 86(6),871-879. https://doi.org/10.2307/2656707

Bode, R. F., Linhart, R. D., and Dufresne, C. (2020). Variation in the Pollinator community visiting invasive *Cytisus scoparius* L. Link (Fabaceae) along an elevation gradient. *Anthropod-Plant Interactions* https://doi.org/10.1007/s11829-020-09755-8

Brito, V. L. G., and Sazima, M. (2012). *Tibouchina pulchra* (Melastomataceae): Reproductive biology of a tree species at two sites of an elevational gradient in the Atlantic rainforest in Brazil. 123 Plant *Syst Evol* 298, 1271–1279. DOI 10.1007/s00606-012-0633-5

Capo, M., Borras, J., Perello-suau, S., Rita, J., and Cursach, J. (2023). Phenotype–fitness relationships and pollen-transfer efficiency of five orchid species with different pollination strategies. *American Journaal of Botany* 110(6), e16198. https://doi.org/10.1002/ajb2.16198

Carvalheiro, L. G., Kunin, W. E., Keil, P., Aguirre-Gutiérrez, J., Ellis, W. N., Fox, R., Groom, Q., Hennekens, S., Van Landuyt, W., Maes, D., & Van de Meutter, F. (2013). Species richness declines and biotic homogenisation have slowed down for NW-European pollinators and plants. *Ecology Letters*,16(7),870-878. https://doi.org/10.1111/ele.12121

Chapagain, D, J., Meilby, H., and Ghimire, S. K. (2019). Plant density and life history traits of *Aconitum spicatum* in North central Nepal: effects of elevation and anthropogenic disturbances. *PeerJ 2019*(9), 1–22. http://doi.org/10.7717/peerj.7574

Classen, A., Eardley, C. D., Hemp, A., Peters, M.K., Peters, R. S., Ssymank, A., Steffan-Dewenter, I. (2020). Specialization of plant–pollinator interactions increases with temperature at Mt. Kilimanjaro. *Ecology and Evolution* 10, 2182– 2195. https://doi.org/10.1002/ece3.6056

Cruden, R.W. (1977). Pollen-Ovule Ratios: A Conservative Indicator of Breeding Systems in Flowering Plants. *Evolution*. 31, 32-46.

Cruden, R.W. (2000). Pollen grains: why so many? Plant systematics and evolution. 222,143-165.

Dafni, A. (1992). Pollination ecology: a practical approach. Oxford Univ. Press, Oxford, U.K

Darwin, C. (1859). *The effect of Cross and Self- fertilization in the Vegetable Kingdom.* John Murray, London, UK.

Darwin, C. (1862). *On the various contrivances by which British and foreign orchids are fertilized by insects*. John Murray, London, U.K.

Darwin, C. (1876). The Effects of Cross and Self Fertilization in the Vegetable Kingdom. John Murray, London.

Dirzo, R., Young, H. S., Galetti, M., Ceballos, G., Isaac, N. J., & Collen, B. (2014). Deforestation in the Anthropocene. S*cience,* 345(6195), 401-406. https:/doi.org/10.1126/science.125181

Duan, Y-W., Zhang, T-F., He, Y-P., and Liu, J-Q. (2009). Insect and wind pollination of an alpine biennial *Aconitum gymnandrum* (Ranunculaceae). Plant Biology 11(6),796-802. https://doi.org/10.1111/j.1438-8677.2009.00195.x

Dyola, U., Baniya, C. B., Acharya, P. R., Subedi, P., Pandey, A., and Sapkota, K. (2022). Community structure of pollinating insects and its driving factors in different habitats of Shivapuri-Nagarjun National Park, Nepal. *Ecology and Evolution* 12(3),e8653. doi: 10.1002/ece3.8653

Eustacchio, E., Bonelli, M., Beretta, obbi, M., Casartelli, M., and Caccianiga, M. (2023). Pollen and floral morphology of *Androsace brevis* (Hegetschw.) Ces. (Primulaceae), a vulnerable narrow endemic plant of the Southern European Alps. *Flora* 301, 152256 DOI: 10.1016/j.flora.2023.152256

Faegri, K., and Van der Pijl, L. (1971). *The Principles of Pollination Biology*. Pergamon Press, New York.281.

Fenster, C. B., Armbruster, W.S., Wilson, P., Dudash, M.R., and Thomson, J. D. (2004). pollination syndromes and floral specialization. *Annu. Rev. Ecol. Syst*.35:375-403.

Fleming, T., Sahley, C., Holland, J., Nason, J., and Hamrick, J. (2001). Sonoran Desert columnar cacti and the evolution of generalised pollination systems. *Ecological Monographs*71,511530. https://doi.org/10.1890/00129615(2001)071[0511:SDCCAT]2.0.CO;2

Fukuda, Y., Suzuki, S., and Murata, J. (2001). The function of each sepal in pollinator behavior and effective pollination in *Aconitum japonicum var. montanum*. *Plant Species Biology* 16, 151–157. https://doi.org/10.1046/j.1442-1984.2001.00059.x

Ghimire, S. K., Sapkota, I. B., Oli, B. R., and Parajuli-Rai, R. (2008). Non-Timber Forest Products of Nepal Himalaya: Database of Some Important Species Found in the Mountain Protected Areas and Surrounding Regions. WWF Nepal, Kathmandu, Nepal.

Guo, H., Mazer, S.J., Du, G. (2010). Geographic variation in seed mass within and among nine species of *Pedicularis* (Orobanchaceae): effects of elevation, plant size and seed number per fruit. *Journal of Ecology* 98,1232–1242. https://doi.org/10.1111/j.1365-2745.2010.01688.x

Gurung, P. D., Ratnam, J., & Ramakrishnan, U. (2018). Facilitative interactions among co-flowering *Primula* species mediated by pollinator sharing. *Plant Ecol* 219, 1159–1168. https://doi.org/10.1007/s11258-018-0868-5

Harder, L., Barrett, S. (1995). Mating cost of large floral displays in hermaphrodite plants. *Nature* 373, 512–515. https://doi.org/10.1038/373512a0

Harder, L. D., and Johnson S. D. (2009). Darwin's beautiful contrivances: evolutionary and functional evidence for floral adaptation. New Phytologist 183 (3), 530-545. https://doi.org/10.1111/j.1469-8137.2009.02914.x

Hovenden, M. J., and Vander Schoor, J. K. (2004). Nature vs nurture in the leaf morphology of Southern beech, *Nothofagus cunninghamii* (Nothofagaceae). *New Phytologist* 161,585–594. DOI 10.1046/j.1469-8137.2003.00931.x

Hudewenz, A., Klein, A. M., Scherber, C., Stanke, L., Tscharntke, T., Vogel, A., Weigelt, A., Weisser, W. W., & Ebeling, A. (2012). Herbivore and pollinator responses to grassland management intensity along experimental changes in plant species richness. *Biological Conservation*,150(1),42-52. https://doi.org/10.1016/j.biocon.2012.02.024

Jacquemyn, H., Micheneau, C., Roberts, D. L., and Pailler, T. (2005). Elevational gradients of species diversity, breeding system and floral traits of orchid species on Re´union Island. *Journal of Biogeography* 32, 1751–1761. https://doi.org/10.1111/j.1365-2699.2005.01307.x

Johnson, S. D., Steiner, K. E. (2000). Generalization vs. specialization in plant pollination systems. *Trends in Ecology and Evolution*. 15,140–143. https://doi.org/10.1016/S0169-5347(99)01811-X

Junker, R. R., Blüthgen, N., Brehm, T., Binkenstein, J., Paulus, J., Schaefer, H. M., Stang, M. (2013). Specialization on traits as basis for the niche-breadth of flower visitors and as structuring mechanism of ecological networks. *Functional Ecology* 27,329–341. https://doi. org/10.1111/1365-2435.12005

Kearns, C.A., Inouye, D.W. (1993). Techniques for pollination biologists. University Press of Colorado, Niwot, CO, USA.

Kudo, G. (2016). Landscape Structure of Flowering Phenology in Alpine Ecosystems: Significance of Plant-Pollinator Interactions and Evolutionary Aspects. In: Kudo G (ed) Structure and Function of Mountain Ecosystems in Japan. Springer, Tokyo ttps://doi.org/10.1007/978-4-431-55954-2_3

Lama, Y. C., Ghimire, S. K., Aumeeruddy-Thomas, Y. (2001). Medicinal plants of Dolpo: Amchis' knowledge and conservation. Kathmandu: WWF Nepal.

Lázaro, A., Hegland, S.J., Totland, Ø. (2008). The relationships between floral traits and specificity of pollination systems in three Scandinavian plant communities. *Oecologia* 157, 249–257. https://doi.org/10.1007/s00442-008-1066-2

Lefebvre, V., Villemant, C., Fontaine, C., Daugeron, C. (2018). Altitudinal, temporal and trophic partitioning of fower-visitors in Alpine communities. *Sci Rep* 8,1–12. https://doi.org/10.1038/s4159 8-018-23210-y

Manincor, N. D., Hautekèete, N., Mazoyer, C., Moreau, P., Piquot, Y., Schatz, B., Schmitt, E., Zélazny, M., and Massol, F. (2019). How biased is our perception of plant-pollinator networks? A comparison of visit- and pollen-based representations of the same networks. *Acta Oecologica* 146-609X https://doi.org/10.1016/j.actao.2020.103551

Martén-Rodríguez, S., Almarales-Castro, A., Fenster, C. B. (2009). Evaluation of pollination syndromes in Antillean Gesneriaceae: evidence for bat, hummingbird and generalized fowers. *J Ecol* 97,348–359. https://doi.org/10.1111/j.1365-2745.2008.01465.x

Mendes, F.N., Valente, R. d. M., Rêgo, M.M.C., and Esposito, M. C. (2017). The floral biology and reproductive system of *Mauritia flexuosa* (Arecaceae) in a restinga environment in northeastern Brazil. *Brittonia* 69, 11–25. https://doi.org/10.1007/s12228-016-9444-2

Muchhala, N., and Thomson, J. D. (2009). Going to great lengths: Selection for long corolla tubes in an extremely specialized bat-flower mutualism. *Proceedings of the Royal Society B. Biological Science* 276(1665),2147-1152. DOI:10.1098/rspb.2009.0102

Nautiyal, B. P., Nautiyal, M. C., Khanduri, V. P., and Rawat, N. (2009). Floral Biology of *Aconitum heterophyllum* Wall.: A Critically Endangered Alpine Medicinal Plant of Himalaya, India. *Turk J Bot* 33, 13-20. doi:10.3906/bot-0805-8

Neumüller, U., Burger, H., Krausch, S., Blüthgen, N., & Ayasse, M. (2020). Interactions of local habitat type, landscape composition and flower availability moderate wild bee communities. *Landscape Ecology*, 35(10), 2209– 2224. https://doi.org/10.1007/s10980-020-01096-4

Newman, E., Manning, J., Anderson, B. (2015). Local adaptation: Mechanical fit between floral ecotypes of *Nerine humilis* (Amaryllidaceae) and pollinator communities. *South African Journal of Botany* 98(9),194-195. DOI:10.1016/j.sajb.2015.03.099

Nilsson, L. (1988). The evolution of flowers with deep corolla tubes. *Nature* 334,147-149.

Ollerton, J., Alarcon, R., Waser, N. M., Price, M. V., Watts, S., Cranmer, L., Hingston, A., Peter, C. I., and Rotenberry, J. (2009). A global test of the pollination syndrome hypothesis. *Annals of Botany* 103,1471– 1480. https://doi.org/10.1093/aob/mcp031

Ollerton, J., Killick, A., Lamborn, E., Watts, S. & Whiston, M. (2007). Multiple meanings and modes: on the many ways to be a generalist flower. *Taxon* 56, 717–728.

Ollerton, J., Winfree, R., and Tarrant, S. (2011). How many flowering plants are pollinated by animals? *OIKOS* 120(3),321-326. https://doi.org/10.1111/j.1600-0706.2010 .18644.x

Parrish, J. (2004). Pollination Ecology: Field Studies of insect Visitation and Pollen Transfer Rates. *Teaching Issues and Experiments in Ecology* 2 http://tiee.ecoed.net/vol/v2/experiments/pollinate/abstract.html

Paudel, B. R., and Li, Q-J. (2020). Functional implications of the specialized staminal appendages in alpine ginger (*Roscoea* spp.: Zingiberaceae). *Journal of Evolutionary Biology* 33(9),1265-1275. https://doi.org/10.1111/jeb.13670

Paudel, B. R., Burd, M., Shrestha, M., Dyer, A. G., and Li, Q. J. (2018). Reproductive isolation in alpine gingers: How do coexisting *Roscoea (R. purpurea and R. tumjensis)* conserve species integrity? *Evolution,* 72,1840-1850. https://doi.org/10.5061/dryad.3vn8p2m

Paudel, B. R., Kessler, A., Shrestha, M., Zhao, J.L., and Li, Q-J. (2019). Geographic isolation, pollination syndromes, and pollinator generalization in Himalayan *Roscoea* spp. (Zingiberaceae). *Ecosphere* 10(11), e02943.10.1002/ecs2.2949.

Paudel, B. R., Shrestha, M., Burd, M., & Adhikari, S., Sun, Y-S., and Li, Q-J. (2016). Coevolutionary elaboration of pollination-related traits in an alpine ginger *(Roscoea purpurea)* and a tabanid fly in the Nepalese Himalayas. *New Phytologist* 211,1402–1411. doi: 10.1111/nph.13974.

Paudel, B. R., Shrestha, M., Dyer, A. G., ZHU, X., Abdusalam, A., and Li, Q-J. (2015). Out of Africa: evidence of the obligate mutualism between long corolla tubed plant and long-tongued fly in the Himalayas. *Ecology And Evolution* 5,5240±5241. https://doi.org/10.1002/ece3.1784

Paudel, B.R., Shrestha, M., Dyer, A.G., and Li, Q-J. (2017). Ginger and the beetle: Evidence of primitive pollination system in a Himalayan endemic alpine ginger (*Roscozea alpina*, Zingiberaceae). *PLoS ONE* 12(7), e0180460 https://doi.org/10.1371/journal.pone.0180460

Pauw, A., Stofberg, J., Waterman, R. J. (2009). Flies and flowers in Darwin's race. *Evolution*63(1),268–279. https://doi.org/10.1111/j.1558-5646.2008.00547.x

Pellissier, L., Pottier, J., Vittoz, P., Dubuis, A., & Guisan, A. (2010). Spatial pattern of floral morphology: Possible insight into the effects of pollinators on plant distributions. *Oikos*, 119(11), 1805– 1813. https://doi.org/10.1111/j.1600-0706.2010.18560.x

Potascheff, C. M., de Brito, V.L.G., Galetto, L., Stebbenn A. M., and Oliveira, P. (2020). Nectar features, diurnal and nocturnal pollinators, and male fitness in *Qualea grandiflora* (Vochysiaceae). *Plant Systematics and Evolution*306,3 https://doi.org/10.1007/s00606-020-01646-y

Rana, S. K., Rana, H. K., Ranjitkar, S., Ghimire, S. K., Gurmachhan, C. M., O'Neill, A. R., & Sun, H. (2020). Climate-change threats to distribution, habitats, sustainability and conservation of highly traded medicinal and aromatic plants in Nepal. *Ecological Indicators* 115, 106435. https://doi.org/10.1016/j.ecolind.2020.106435

Raven, P. H. (1972). Why are bird-visited flowers predominantly red? *Evolution* 26: 674.

Rosas-Guerrero, V., Aguilar, R., Marten-Rodrıguez, S., Ashworth, L., Lopezaraiza-Mikel, M., Bastida, J. M., and Quesada, M. (2014). A quantitative review of pollination syndromes: Do floral traits predict effective pollinators? *Ecology Letters* 17,388–400. https://doi.org/10.1111/ele.12224

Schmid-Hempel, P., and Speiser, B. (1988). Effects of inflorescence size on pollination in *Epilobium angustifolium*. Oi*kos* 53, 98–104. https://doi.org/10.2307/3565669

Sharma, P., Roy, M., and Roy, B. (2022). A review on influence of floral biology, pollination efficiency and conservation strategies of endangered medicinal plant, *Rauvolfia serpentina* (L.) Benth. ex Kurz. *Annals of Phytomedicine* 11(1), 86-98. DOI: http://dx.doi.org/10.54085/ap.2022.11.1.9

Shrestha, B. R., Timsina, B., Münzbergová, Z., Dostálek, T., Gaudel, P., Basnet, T. B., & Rokaya, M. B. (2020). Butterfly-plant interactions and body size patterns along an elevational gradient in the Manang region of central Nepal. *Journal of Mountain Science.* 17,1115-1127. doi:10.1007/s11629-019-5381-3

Shrestha, K. K., Bhandari, P., and Bhattarai, S. (2022). Plants of Nepal *(Gymnosperms and Angiosperms).* Heritage Publishers and Distributors Pvt. Ltd., Kathmandu.

Shrestha, M., Dyer, A. G., Bhattarai, P., and Burd, M. (2014). Flower colour and phylogeny along an altitudinal gradient in the Himalayas of Nepal. *Journal of Ecology* 102,126-135. https://doi.org/10.1111/1365-2745.12185

Shrestha, M., Dyer, A. G., Boyd-Gerny, S., Wong, B. B. M., and Burd, M. (2013). Shades of red: bird-pollinated flowers target the specific colour discrimination abilities of avian vision. *New phytology* 198,301-310. https://doi.org/10.1111/nph.12135

Shrestha, M., Lunau, K., Dorin, A., Schulze, B., Bischoff, M., Burd, M., and Dyer, A. G. (2016). Floral colours in a world without birds and bees: the plants of Macquarie Island. *Plant Biology* 18,842-850. https://doi.org/10.1111/plb.12456

Shrestha, T. B. (2001). Forest Diversity of Nepal's Protected Areas in the Mountains. *The Himalayan Review* 32, 25-28.

Shyaula, S. L., Ngakushi, A. B., Maharjan, B. L., and Manandhar, M. D. (2012). Estimation of Alkaloids and Antibacterial Activity of *Aconitum spicatum* Bruhl Stapf from Manaslu Conservation Area. *Nepal Journal of Science and Technology*.13(1), 67-71.

Shyaula, S. L., Tamang, T., Ghouri, N., Adhikari, A., Marasini, S., Bajracharya, G. B., Manandhar, M. D., & Choudhary, M. I. (2016). Antileishmanial diterpenoid alkaloids from *Aconitum spicatum* (Bruhl) Stapf. *Natural Product Research*, *30*(22), 2590–2593. https://doi.org/10.1080/14786419.2015.1114941

Sir John, L. (1882). *Ants, Bees, and Wasps: A Record of Observation of the Habitats of the Social Hymenoptera*. D. Appleton & company, New York, USA.

Sprengel, C. K. (1793). *Das entdeckte Geheimnis der Naturi m bau und in der Befruchtung der Blumen*. Friedrich Vieweg Berlin.

Sprengel, C. K. (1996). Discovery of the secret of nature in the structure and fertilization of flowers. In Floral Biology: Studies on Floral Evolution in Animal-Pollinated Plants, ed. D. G. Lloyd, and S.C. H. Barrett, pp. 3–43. Transl. https://doi.org/10.1007/978-1-4613-1165-2_1

Stebbins, G, L. (1970). Adaptive Radiation of Reproductive Characteristics in Angiosperms, I: Pollination Mechanisms. *Annual Review of Ecology and Systematics* 1(1),307-326. DOI:10.1146/annurev.es.01.110170.001515

Subedi, A., Chaudhary, R. P., Van, A.C., Heijerman, T., Lens, F., Van Dooren, T. J., and Gravendeel, B. (2011). Pollination and protection against herbivory of Nepalese *Coelogyninae* (Orchidaceae). *American Journal of Botany*. 987,1095-1103. https://doi.org/10.3732/ajb.1000306

Sutherland, S. D., Vickery, R. K. (1993). On the relative importance of floral color, shape, and nectar rewards in attracting pollinators to *Mimulus*. *Great Basin Naturalist* 53,107–117. https://www.jstor.org/stable/41712764

Sutherland, S., and Delph, L. F. (1984). On the importance of male fitness in plants: patterns of fruit-set. *Ecology* 65(4): 1093-1104. https://doi.org/10.2307/1938317

Thomson, J. D. (1988). Effects of variation in inflorescence size and floral rewards on the visitation rates of trap lining pollinators of *Aralia hispida*. *Evol Ecol* 2, 65–76. https://doi.org/10.1007/BF02071589

Tong, Z., Yun Wu, L., Quan Huang, S. (2021). Reproductive strategies of animal-pollinated plants on high mountains: A review of studies from the "Third Pole". *Journal of Systematics and Evolution* 59(6),1159–1169. https://doi.org/10.1111/jse.12680

Utelli, A-B., and Roy, B. A. (2000). Pollinator abundance and behavior on *Aconitum lycoctonum* (Ranunculaceae): an analysis of the quantity and quality components of pollination. *OIKOS* 89, 461–470. https://doi.org/10.1034/j.1600-0706.2000.890305.x

Wang, H., Yu, W. B., Chen, J. Q., Blackmore, S. (2009). Pollen morphology in relation to floral types and pollination syndromes in *Pedicularis* (Orobanchaceae*). Plant Syst Evolution* 277,153–162. DOI 10.1007/s00606-008-0112-1

Waser, N. M., Chittka, L., Price, M. V., Williams, N. M., and Ollerton, J. (1996). Generalization in pollination systems, and why it matters. *Ecology* 77(4), 1043-1060. https://doi.org/10.2307/2265575

Vivarelli, D., Petanidou, T., Nielsen, A., and Cristofolini, G. (2011). Small size bees reduce male fitness of the flowers of *Ononis masquillierii* (Fabaceae), a rare endemic plant in the northern Apennines. *Botanical Journal of Linnean Society* 165,267–277. https://doi.org/10.111 1/j.1095-8339.2010.01105.x

Zhang, F., Hui, C., and Pauw, A. (2013). Adaptive divergence in Darwin's race: how coevolution can generate trait diversity in a pollination system. *Evolution* 67,548-560. doi:10.1111/j.1558-5646.201

Zhang, T-F., Duan, Y-W., and Liu, J-Q. (2006). Pollination ecology of *Aconitum gymnandrum* (Ranunculaceae) at two sites with different altitudes. *Journal of Systematics and Evolution* 44(4),362-370. DOI: 10.1360/aps050146

PHOTOPLATES

Photo plate 1: Field work

Diurnal and nocturnal floral visitors' observation

Recording visitation frequency of floral visitors

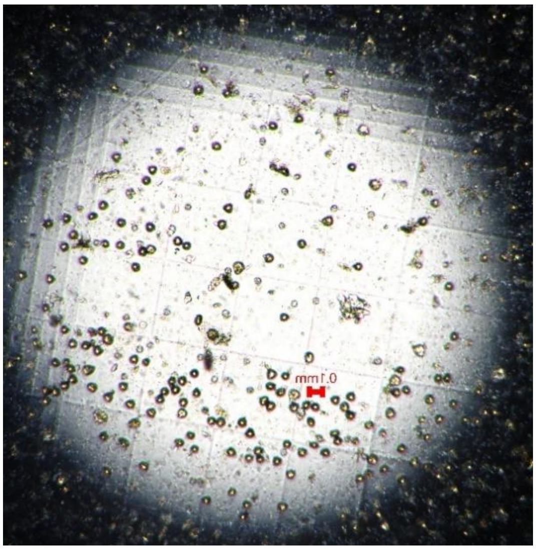
Photo plate 2: Sample collection

Bud collection

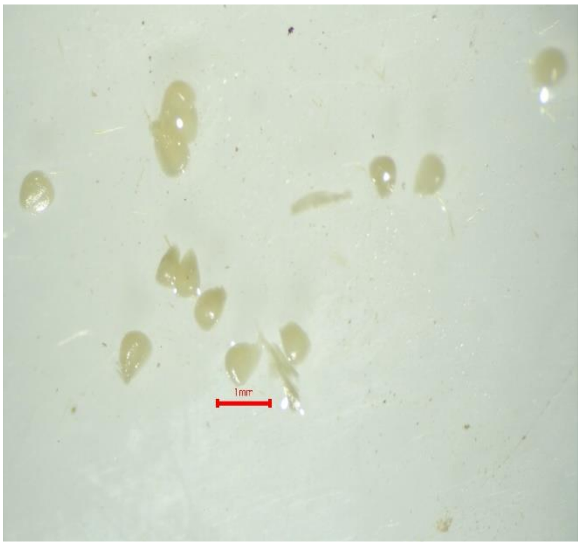
Bumblebee Collection

Flower pressed in herbarium

Photo plate 3: Laboratory work

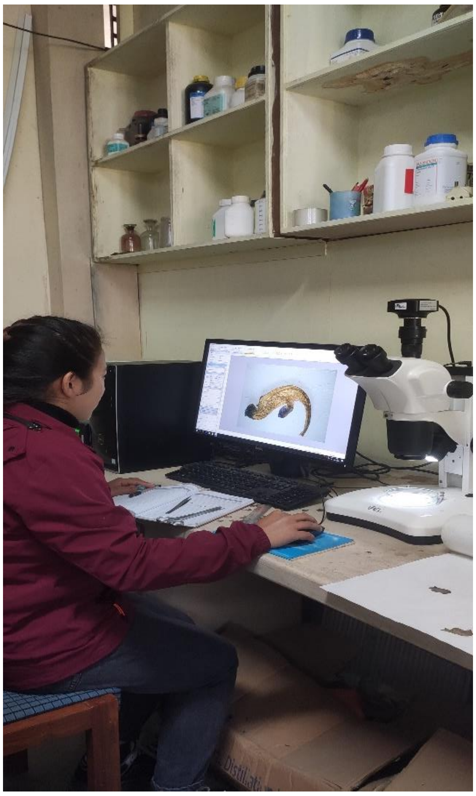


Pollen grains count



Ovule count

Anther and stamen length measurement



Petaline claw length measurement