Floral development and pollination of *Centropogon granulosus* (Campanulaceae) in an Andean cloud forest

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**Submission Type**: Note

**Abstract**: (200 max for Botany)

**Main Body**: (4200 max for Botany)

**Figures**: # Figures

**Keywords**: (3-6 for Botany)

#### Acknowledgements

Servicio Nacional Forestal y de Fauna Silvestre (SERFOR) of Peru. Herbarium faculty and staff at the Universidad Nacional de San Antonio Abad del Cusco (UNSAAC). Staff of the Gallieto de las Rocas Lodge. B. Freeman, M.N. Scholer, and E. Llacta-Cuno

#### Introduction

The Andes comprise the highest mountains in the western hemisphere and are one of the most biodiverse regions on Earth (Myers et al., [2000](#ref-myers_2000); Hoorn et al., [2018](#ref-hoorn_2018)). With a mean elevation of 4000 m, the Andes encompass a wide environmental gradient ranging from warm Amazonian rainforests, to dry, montane grasslands. During Andean orogeny, numerous plant clades diversified as they migrated and adapted to novel montane environments (Gentry, [1982](#ref-gentry_1982)), e.g. *Lupinus* L. (Fabaceae, Hughes and Eastwood, [2006](#ref-hughes_2006)), centropogonids (Lobelioideae, Lagomarsino et al., [2016](#ref-lagomarsino_2016)), epiphytic orchids (Epidendroideae, Pérez-Escobar et al., [2017](#ref-perez_2017)). At present, ~10% of global angiosperm diversity is found in the Andes eco-region (Joppa et al., [2011](#ref-joppa_2011)).

   Plant-pollinator co-evolution offers a powerful model explaining the rapid diversification of the Andean flora (e.g. Kay et al., [2005](#ref-kay_2005); Lagomarsino et al., [2017](#ref-lagomarsino_2017); Serrano-Serrano et al., [2017](#ref-serrano_2017)). As a plant species expands into a novel montane environment, floral traits may drift, evolve via pleiotropy, or adapt to a pre-existing pollinator community (Harder and Johnson, [2009](#ref-harder_2009); Smith, [2016](#ref-smith_2016)). Subsequently, divergence in floral phenotype can be reinforced when reciprocal adaptation between the plant and pollinator promotes floral isolation (Grant, [1949](#ref-grant_1949); Thompson, [2005](#ref-thompson_2005)). Co-evolution acting as a source of divergent selection has been framed as a special case of ecological speciation (Althoff et al., [2014](#ref-althoff_2014)).

   In many cases, there is compelling phenotypic and phylogenetic evidence for co-evolutionary diversification, but direct observations of plant-pollinator interactions are missing (although this gap is generally closing, Dellinger, [2020](#ref-dellinger_2020)). Linking these patterns to process is a major goal in testing the generality of co-evolutionary diversification (Althoff et al., [2014](#ref-althoff_2014)). In this study, we recorded pollinator visits and performed a pollinator exclusion experiment to test the theory of hummingbird-mediated diversification in the Andean bellflowers (Campanulaceae).

   Here, we focus on *Centropogon granulosus* C.Presl and buff-tailed sicklebill, *Eutoxeres condamini* (Phaethornithinae). The Andean bellflowers (*Centropogon* C. Presl), along with *Siphocampylus* Pohl and *Burmeistera* H.Karst & Triana form a South American clade of Campanulaceae known as the ‘centropogonids’ (Lagomarsino et al., [2014](#ref-lagomarsino_2014)). The centropogonids arose in the last 5 million years and have diversified into over 550 species. This rapid radiation has been attributed to increased speciation rates driven by the Andean uplift, simultaneous with decreased extinction rates during periods of global cooling (Lagomarsino et al., [2016](#ref-lagomarsino_2016)). The Andean clade *Centropogon* section *Centropogon* (McVaugh 1949) or the ‘eucentropogonids’ (38 spp., Lagomarsino et al 2014) are distinct because of their elongated, hook-shaped flower tube, and the presence of a scale made of fused hairs that serves as a lever to deposit pollen. Both traits are likely adaptations to facilitate pollination by sicklebill hummingbirds (Stein, [1987](#ref-stein_1987)). Generally, the elevational distribution of the eucentropogonids is mid- to high-montane (Figure 1).

   Sicklebill hummingbirds (*Eutoxeres*) constitute two species of large hermits (Phaethornithinae) that are defined by their dramatically curved bill. Their unique bill shape is likely an adaptation to specialize on those *Centropogon* and *Heliconia* L. (Zingiberales) with similarly curved flowers. The hermits diverged early in the evolutionary history of hummingbirds (~26 Ma, McGuire et al., [2014](#ref-mcguire_2014)), and represent one of two major ecotypes of hummingbirds: the trapliners. There are currently 36 extant hermit species (McGuire et al., [2014](#ref-mcguire_2014)). *Eutoxeres* diverged 21.45 Ma, and like other hermits, probably co-adapted to *Heliconia* before major uplifts of the Andes (Abrahamczyk et al., [2017](#ref-abrahamczyk_2017)). At present, *Eutoxeres* has the largest range of any hermit hummingbird — it can be found in its ancestral range, the Amazonian lowlands, as well as the foothills and mid-montane habitats of the Andes up to 2800 m (Schulenberg et al., [2010](#ref-schulenberg_2010)).

   Because *Heliconia* and the hermit hummingbirds co-existed before the emergence of the eucentropogonids, it is likely that sicklebills and *Heliconia* subgenus *Stenochlamys* and/or *Heliconia* subgenus *Griggsia* co-evolved extreme bill and flower curvature in lowland Amazonia, before the uplift of the Andes (Stein, [1987](#ref-stein_1987); Abrahamczyk et al., [2017](#ref-abrahamczyk_2017)). During a recent period of major Andean uplift (~5 Ma) *Centropogon* colonized and radiated into the novel montane habitat, with some species adapting to sicklebill pollination. This meditated the elevational expansion of sicklebills, and explains why they are able to exist outside of the range of *Heliconia*. However, the physiological pre-adaptations that allowed sicklebills to expand, and not other hermits, remains unknown (Stiles, [2004](#ref-stiles_2004)). This scenario presents the possibility of a plant-hummingbird interaction cline, with *Centropogon* and *Eutoxeres* co-evolving in the absence of *Heliconia* at medium to high elevations (Figure 1), and *vice versa* in the lowlands. The distribution of putatively sicklebill-pollinated *Centropogon* and *Heliconia* suggests that above ~1500 m, there are few Heliconia-Sicklebill interactions (Figure 2).

   While trait-matching and anecdotal reports strongly suggest that sicklebills are pollinators of *Centropogon*, empirical evidence remains absent. While the pollination syndrome concept has proven useful in inferring pollinators from floral phenotypes (Dellinger, [2020](#ref-dellinger_2020)), a complete theory of pollinator-mediated plant diversification ultimately requires *in situ* observations of visitation, and some evidence of pollination. Moreover, without a dedicated focus on the organisms’ natural history, it cannot be ruled out that yet-unknown floral visitors directly or indirectly interact with the pollinator. All said, we aim to provide some missing fundamental information on this fascinating and increasingly well-studied pollination system.

#### Materials and Methods

*Field Site*

   We based our field work at the Gallieto de las Rocas Lodge, in the Kosñipata Valley, Peru (-13.055, -71.548 DD). Research Permit No. 0441-2017 was administered by the Servicio Nacional Forestal y de Fauna Silvestre (SERFOR). In this study we focused on the pollination biology of *Centropogon granulosus*. While other eucentropogonid species were present at the study site (Figure 1), *C. granulosus* was the most widespread and accessible for study. We located and recorded the locations of *C. granulosus* individuals in a preliminary survey (Boehm et al., [2018](#ref-boehm_2018_a)).

*Pollinator Observations*

   In this study, we deployed five camera traps (Reconyx Hyperfire HC600) near *Centropogon granulosus* vines located in a previous survey of the area (Boehm et al., [2018](#ref-boehm_2018_a)). Camera traps were checked for new captures every 24 hours. If no floral visitors were recorded within three days, the camera traps were moved to different *Centropogon* individuals. Where floral visitors were recorded, we attempted targeted (manual) video recording to better document visitation behaviour. Camera traps were active continuously from August 17 to Septermber 20, 2017. Monitored locations are marked on Figure S2.

*Pollinator Exclusion Experiment*

   Following the methods and designs of Sun et al. ([2017](#ref-sun_2017)), we constructed and deployed wire cages covering one inflorescence each from six *Centropogon* individuals. Wire cages prevent hummingbirds from contacting the flowers while allowing invertebrates to move freely. An additional ten inflorenscences were monitored as controls. Each inflorescence produced on average 11 and 13 flowers, respectively. Using the control flowers, we defined five discrete stages of floral development (Table 1). These stages were used to quantify and compare the developmental trajectories of the control and pollinator-excluded flowers. Monitoring for a sample stopped when 1) berry development completed (stage E), 2) the flower died prematurely, or 3) the study period ended. Flowers that did not reach berry development were treated as right-censored (Allison, [2014](#ref-allison_2014)). Daily observations were recorded between August 17 and September 20, 2017. Flower development data was inputted to R v3.6.3 (R Core Team, [2017](#ref-R_2017)) and organized using here v0.1 (Müller, [2017](#ref-muller_2017)) and tidyverse v1.3.0 (Wickham et al., [2019](#ref-wickham_2019))

   Flowering stage data was staggered because each flower developed independently. The data was also fragmented because some samples abscissed prematurely due to herbivory or weather. The flower development data was therefore a ‘censored’ dataset i.e. some subjects left the study before reaching maturity (Allison, [2014](#ref-allison_2014)). Although manually aligning the stage data is possible, for convenience we used a multiple sequence alignment protocol to automate the process. To do this, we ran the stage data through ClustalW implemented in the R package msa v.3.9 (Bodenhofer et al., [2015](#ref-bodenhofer_2015)) with a neutral (identity) substitution matrix. Aligned developmental sequence data was then used to compute a consensus (mean) sequence for each treatment — this was done using the ConsensusSequence() function in DECIPHER v2.14.0 (Wright, [2015](#ref-wright_2015)).

   We then conducted an event history analysis to compare the probability of post-anthesis survival between pollinator-excluded and untreated flowers. To do this, we counted the number of days elapsed from anthesis (Stage D) through berry development (Stage E). Daily survival probability (from anthesis) was calculated using the survival package v3.1 (Therneau, [2015](#ref-therneau_2015)).

#### Results

*Pollinator Exclusion and Floral Development*

   We identified and described five discrete stages of floral development in *C. granulosus* (Table 1). These were used to compare differences in development between control and pollinator-excluded flowers. From the floral phenology data we estimated consensus developmental sequences for each treatment (Figure 2). While the the developmental rates are identical from stages A to D, the control flowers spend an additional 24 days developing berries, while hummingbird-excluded flowers did not produce berries. Event history analysis showed that, following anthesis, survival probability decreased daily at similar rates. However, at 13 days post-anthesis, hummingbird-excluded flowers abscice, while untreated plants continued to produce berries (Figure XXX). Overall, the survival curves differed significantly ( = 4.5, = 0.0336).

*Floral Visitors*

Numerous invertibrates occupy or visit the flowers of *C. granulosus*. As noted in previous studies, we observed ants (Stein, [1992](#ref-stein_1992)), mites (Naskrecki et al., [1998](#ref-naskrecki_1998)), and dipterids (Weiss, [1996](#ref-weiss_1996)) in or on the flowers of this species. Though *Anelosimus* spiders (Araneae) are known to build webs scaffolded by *Centropogon coccineus* (Hook.) Regel ex B.D.Jacks. (Nentwig and Christenson, [1986](#ref-nentwig_1986)), we observed unidentified Aranids *inside* of the floral tubes. We also recorded an unidentified larval leptidoptran inhabitating a flower (Figure S1). Two vertebrate visitors were recorded by camera traps and later observed *in situ*: the nectar robbing, wedge-billed hummingbird (*Schistes geoffroyi*), and the likely pollinator of *C. granulosus*, buff-tailed sicklebill (*Eutoxeres condamini*).

   Unlike its relatives (e.g. *Phaethornis*), the sicklebills we encoutered were wary of human prescence and activity. Of the few *in situ* observations, and one video recording (link to recording), we noted the following. Unlike previous accounts of sicklebill visitation (Stein, [1987](#ref-stein_1987)), we did not observe any individuals using the lignfied inflorescence of *C. granulosus* as a perch. As with other hummingbirds, they are adept fliers and appear to probe flowers with ease, even while hovering. Reviewing frames from the video recording reveals an interesting feeding problem posed by the sharply curved flowers of *C. granulosus*. The hook-shape forces the hummingbird to raise its head so that it is facing skyward in order to insert the tip of its bill into the flower apeture (sicklebill5.png), the rest of its bill is further inserted by tilting the head down back to resting position. Due to the system’s unique morphology, this maneuveur is unlikely to be performed by any other hummingbird during nectar probing.

*Other Observations*

   In studying the development and pollination of *C. granulosus* flowers, we noted some natural history traits yet published elsewhere. -fruit being eaten - up to 68 flowers/peduncle scars on one inflorescence

* Figure 2. Developmental stages of Centropogon.
* Figure 3. Pollination by Sicklebills

#### Discussion

While the pollination syndrome concept is generally held as robust, empirical observations remain foundational to understanding the dynamics of plants-pollinator evolution (Dellinger, [2020](#ref-dellinger_2020)). >>maybe insert the big Qs in this field?

Here, we photo-documented sicklebill visitation to *C. granulosus*, and demonstrated that visitation is required for berry development. Presumably the inability of hummingbird-exluded flowers to produce berries is indicative of a lack of pollination. While *Centropogon* pollen from *Eutoxeres* has been identified in several studies [laura dyck chan, maglianesi], species-level identification for this clade remains difficult, and confirmation of *C. granulosus* pollen would help to bolster the evidence presented here.

In some ways, confirming sicklebill visitation in *C. granulosus* creates many more questions than have been answered. For example, given that the eucentropgonid clade is so diverse and widespread in Manu (Figure 1), is *Eutoxeres* the pollinator of all of these species? Furthermore, what mechanisms, if any, prevent interspecific pollen transfer within this clade? Through the effort of making pollinator observations, we were surprised to learn that another hummingbird (*Schistes geoffroyi*) is a nectar robber of *C. granulosus* (described in Boehm, [2018](#ref-boehm_2018_b)). An entire literature on the competitive interactions for nectar and effects on the focal plant offer many avenues for future research [XXX]. For example,

At the heart of this study, and the many excellent investigations into this system, is the question “why and what are the mechanims that select for and maintain extreme curvature in these plant and hummingbird clades?” and “what is the role (if any) of curvature in the rapid diversification of the eucentropogonids?”

Two additional vertebrates, a rat and bat were recorded near the inflorescences, but not observed to interact with the plant directly. This is worth mentioning because it is yet unknown how the berries are disperesed.

#### Concluding Remarks

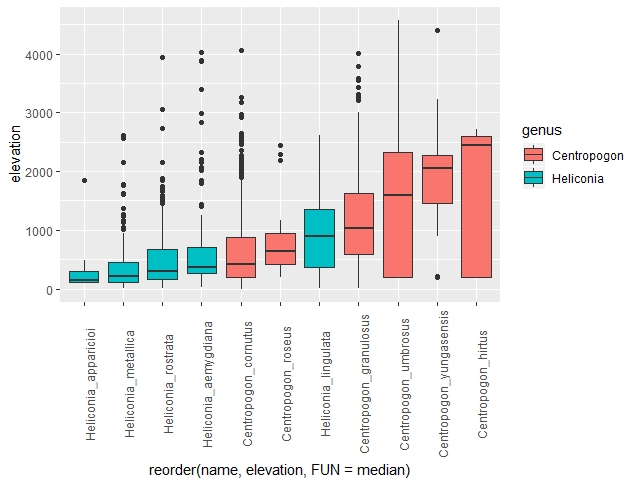


Figure 1. Distribution of Centropogon and Heliconia in Manu.

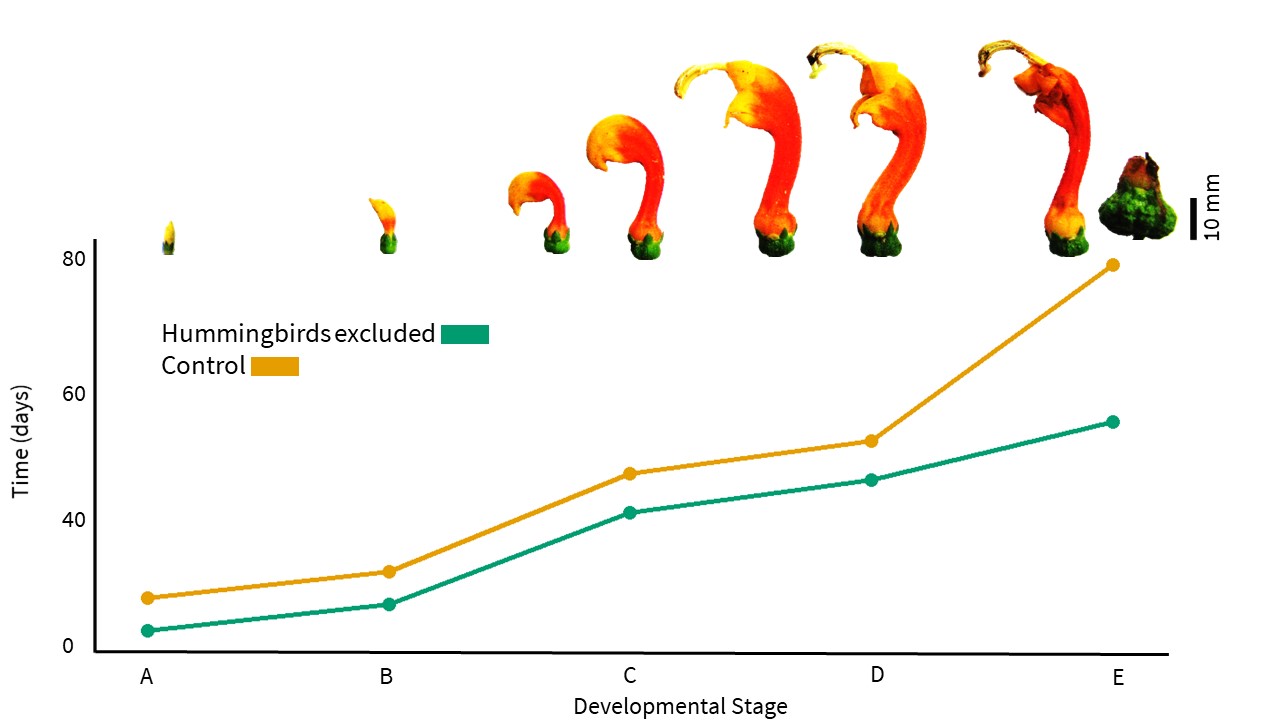


Figure 2. Effects of pollinator exclusion on floral development.

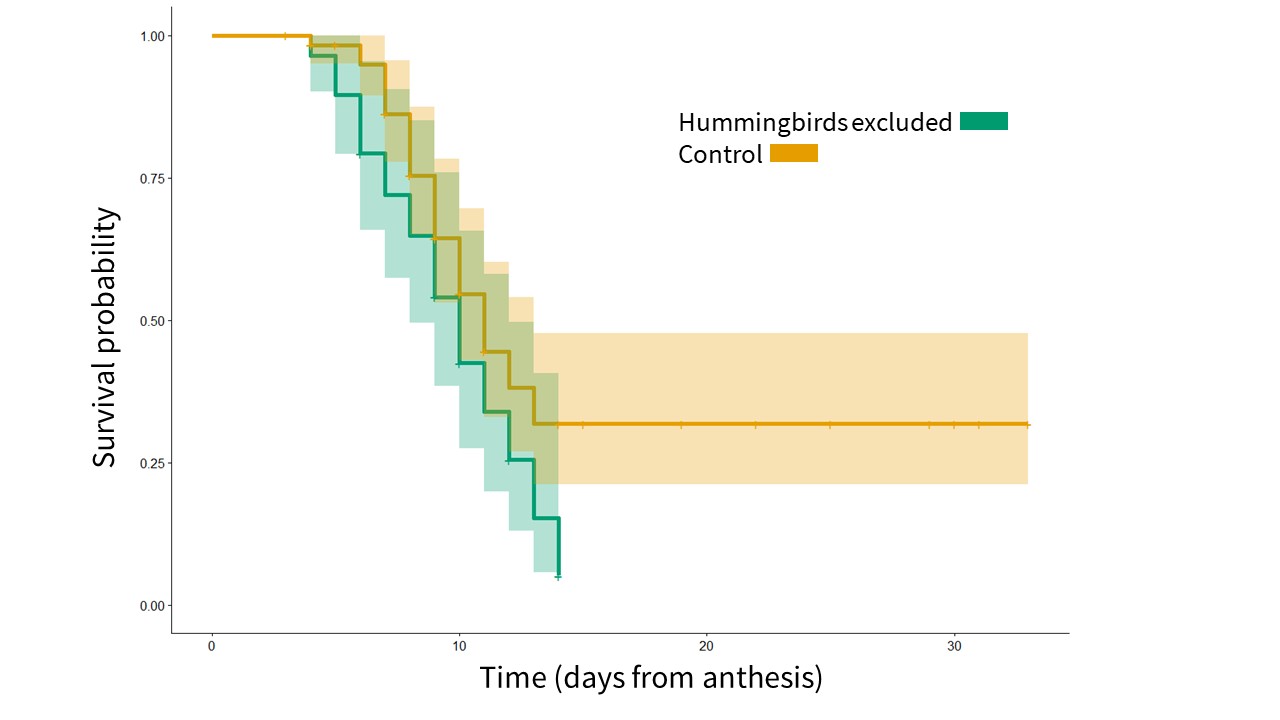


Figure 3. Effects of pollinator exclusion on survival probability.

Table 1: Stages of floral development in *Centropogon granulosus*.

|  |  |  |
| --- | --- | --- |
| Stage | Description | Duration (days) |
| 1 | Flower primordia appears above bracts. No curvature and red-orange pigmentation is not continuous around the base. Flower up to 9 mm tall (as measured from the top of the bracts). Basal diameter up to 4 mm. | NA |
| 2 | Initiation of curvature, creating 90 degree angle. Red-orange pigmentation is continuous around the base. Flower up to 10 mm tall. Basal diameter up to 6 mm. | NA |
| 3 | Growth phase. 180 degree angle formed. Red-orange pigmentation outweighs yellow. Flower 14 to 20 mm tall. Basal diameter 6 â€“ 8 mm. | NA |
| 4 | Pre-anthesis. >180 degree angle formed. Flower 30 mm tall. Basal diameter 7 â€“ 8 mm. | NA |
| 5 | Anthesis. Mature male-phase flower. Flower 34 -36 mm tall. Basal diameter 7 â€“ 8 mm. | NA |
| 6 | Female-phase flower. Pistil grows overtop of the pollen trap. Flower up to 36 mm tall. Basal diameter up to 10 mm. | NA |
| 7 | Senescing flower. Petals wilting but retained. Basal diameter 8 â€“ 10 mm. | NA |
| 8 | Berry development. Petals senesced and lost. Basal diameter grows from 11 â€“ 17 mm. | NA |

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