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UNIVERSIDAD DE CHILE -FACULTAD DE CIENCIAS-ESCUELA DE POSTGRADO

## **Evaluación del impacto del abejorro invasor *Bombus terrestris* en la polinización sobre la herbácea nativa *Mimulus luteus***

Tesis entregada a la Universidad de Chile en cumplimiento parcial de los requisitos para  
optar al grado de Magíster en Ciencias Biológicas

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UNIVERSIDAD DE CHILE**

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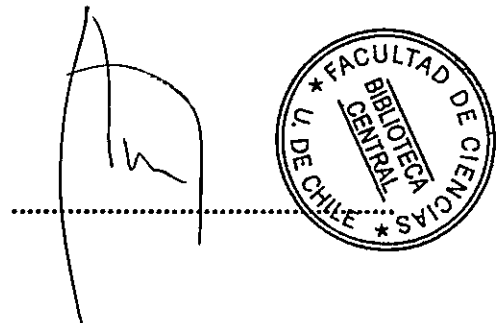
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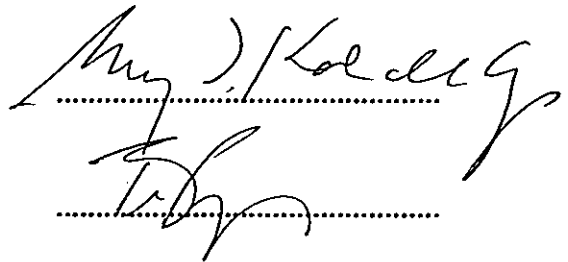
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Comisión de Evaluación de Tesis:

Dra. Mary Kalyn-Arroyo



Two handwritten signatures are written over horizontal dotted lines. The first signature is for Dra. Mary Kalyn-Arroyo and the second is for Dra. Mª Fernanda Pérez.

Dra. Mª Fernanda Pérez

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*A mi familia y amigos*

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## RESUMEN

La llegada de polinizadores exóticos a nuevos hábitats puede introducir nuevos patrones de preferencia floral y forrajeo que pueden modificar la estructura planta-polinizador de la comunidad residente. El objetivo de este trabajo es caracterizar la eficiencia y efectividad de los polinizadores nativos sobre la especie herbácea *Mimulus luteus* y examinar el impacto potencial del abejorro exótico *Bombus terrestris* en el servicio de polinización. El estudio se realizó en una localidad altoandina de Chile durante las temporadas de verano de 2010, 2011 y 2012. Se registró la tasa de visitas, el número de granos de polen transportado en el cuerpo de los polinizadores y la deposición de polen sobre los estigmas de *M. luteus*. La efectividad de la polinización (polen depositado x tasa de visita) se comparó entre las especies. Los resultados revelaron que *B. terrestris* fue un polinizador poco efectivo en comparación a las abejas nativas debido a la baja cantidad de polen depositado en el estigma y a su baja e intermitente tasa de visita a través de los años. La tasa de visita de *B. terrestris* y del abejorro nativo *Bombus dahlbomii* covarió en forma paralela a lo largo del tiempo, sugiriendo que la integración de *B. terrestris* no genera consecuencias importantes para la especie congénérica nativa. En general, *B. terrestris* contribuyó con una baja proporción a la transferencia de polen en *M. luteus*, alcanzando el 5,8% en 2010, ausencia en 2011 y solo 0,01% en 2012. Estos resultados sugieren que a pesar de ser una especie que se está propagando rápidamente en Chile, *B. terrestris* se encuentra aún en una fase inicial de invasión en el área de estudio.

## ABSTRACT

The arrival of exotic pollinators to new habitats may introduce new patterns of floral preference and foraging behavior that modify the structure of the resident plant-pollinator community. The aim of this paper is to examine the efficiency and effectiveness of native pollinators on the herb *Mimulus luteus* and the potential impact of the exotic bumblebee *Bombus terrestris* on the pollination service provided by the native pollinator assemblage. The study was performed in a high elevation locality in the Chilean Andes during the summer seasons of 2010, 2011, and 2012. We recorded visitation rate, the number of pollen grains transported on the body of *B. terrestris* and native pollinators and the pollen deposition on the stigmas of *M. luteus*. Pollinator effectiveness (pollen deposited x visitation rate) was compared among species. Results revealed that *B. terrestris* was an inefficient pollinator, due to the low amount of pollen delivered to stigmas and the low and intermittent visitation rate across years. The parallel inter-annual variation in the visitation rate of *B. terrestris* and the native bumblebee *Bombus dahlbomii* suggests that the integration of *B. terrestris* had no important consequences for the congeneric species. In general, *B. terrestris* accounted for a low proportion of the pollen transference in *M. luteus*, attaining 5.8% in 2010, absence of effect in 2011, and 0.01% in 2012. These results suggest that in spite of being a rapidly spreading species in Chile, *B. terrestris* is still in the initial phase of invasion in this area.

## INTRODUCCIÓN

La introducción de especies exóticas en los ecosistemas es una amenaza para la biodiversidad, pudiendo afectar de múltiples maneras a las especies nativas de polinizadores y plantas. Este proceso involucra, entre otros mecanismos, la usurpación de interacciones planta-polinizador, lo cual puede provocar una disminución de la conectividad entre las especies nativas, sometiéndolas a nuevas dinámicas ecológicas y evolutivas (Aizen *et al.* 2008). Por ejemplo, las especies exóticas pueden generar una serie de efectos directos e indirectos sobre los polinizadores nativos, los cuales incluyen competencia por recursos florales y sitios de anidamiento, transmisión de patógenos y riesgo de potencial hibridación con especies nativas por cruzamientos entre congéneres (Goulson 2003, Morales 2007, Stout 2009). Además de los efectos antes descritos, la introducción de polinizadores exóticos puede originar cambios en la estructura de la comunidad de plantas nativas, lo que a su vez puede impactar indirectamente sobre los polinizadores nativos (Stout 2009). Este efecto sobre la comunidad local puede ser producido tanto por la preferencia de los polinizadores introducidos por plantas no nativas (Hingston 2006), como por el hecho que los polinizadores exóticos pueden ser menos eficientes sobre plantas nativas debido a desajustes morfológicos (Goulson 2003, Montalva *et al.* 2008). La frecuencia de visitas de polinizadores nativos sobre plantas nativas puede también disminuir debido a conductas particulares de las especies invasoras en la obtención de recursos (e.g., robo de néctar, Dohzono 2010), el cual genera daños en la estructura de la flor tornándola menos atractiva a los polinizadores nativos.



Debido a su amplia distribución y alta adaptabilidad a condiciones artificiales, las colonias de *Bombus terrestris* (Hymenoptera: Apidae) han sido ampliamente comercializadas como polinizadores (Dohzono 2010). Las colonias son utilizadas en invernaderos desde los cuales suelen escapar a los ambientes nativos (Inari *et al.* 2005). La especie se considera una invasora muy eficiente con una alta capacidad de establecerse y desplazar competitivamente a los polinizadores locales (Ings *et al.* 2005, 2006). Por ejemplo, la especie se ha naturalizado exitosamente en Nueva Zelanda (introducida en 1885), Israel (introducida en 1960), Japón (introducida en 1991) y Tasmania (introducida en 1992) (Inoue 2008, Dohzono 2010). En los ecosistemas donde la especie ha sido introducida ha llegado a ser un factor importante que conlleva la disminución de abejas nativas (Williams 2009). En Japón, la introducción de esta especie ha generado desplazamientos y extinción local de algunas poblaciones de abejorros nativos, siendo la competencia por los sitios de anidamiento el principal mecanismo de desplazamiento, mas que la competencia por el recurso floral (Inoue *et al.* 2008). Para Tasmania, se ha establecido que *B. terrestris* posee el potencial de generar un gran impacto en el ecosistema debido a la diversidad de plantas nativas que visita y a la amplia sobreposición de recursos de forrajeo con los polinizadores locales. Además, la especie introducida visita flores que no eran visitadas por las abejas previamente introducidas, aumentando aún mas su potencial de impacto (Hingston y McQuillan 1998). Este mismo impacto fue descrito en Israel, donde *B. terrestris* ha aumentado progresivamente y en asociación a una disminución de abejas melíferas y especies solitarias (Dafni y Shmida 1996). Como muestra de la alta capacidad de adaptación a los ecosistemas donde ha sido introducida, se ha documentado que *B. terrestris* se expande a

una velocidad de 25 km por año en un amplio rango de condiciones ambientales en Tasmania (Hingston *et al.* 2002).

En Chile se han introducido históricamente una serie de abejas con el fin de suplir las necesidades de polinización de cultivos. Algunas de ellas son *Apis mellifera*, *Megachile rotundata*, *Bombus impatiens* (de reciente introducción, Ruz 2002) y *Bombus ruderatus*, este último introducido en 1982 (Arretz 1986, Rebolledo *et al.* 2004) desde cuando se ha expandido y establecido a una alta tasa en las poblaciones naturales (Ruz 2002). El éxito de esta última especie fue tal, que en poco tiempo cruzó la cordillera de Los Andes, siendo detectada en Argentina en 1993 (Roig-Alsina y Aizen 1996). Actualmente *B. ruderatus* presenta una amplia distribución encontrándose en una gran variedad de ambientes, lo cual supone una gran plasticidad y adaptabilidad (Morales 2007), pudiendo incluso superar a los polinizadores nativos debido a su gran abundancia y frecuencias de visita (Madjidian *et al.* 2008).

La introducción del abejorro *Bombus terrestris* a Chile se produjo mediante importaciones comerciales desde Israel y Bélgica autorizadas por el Servicio Agrícola y Ganadero (SAG) en 1998 de acuerdo al “Código de conducta para la importación y liberación al medio ambiente de agentes de control biológico” (FAO). Las colonias de *B. terrestris* fueron distribuidas en diferentes localidades desde el norte a la zona central de Chile, y fueron verificados sus buenos resultados como polinizadores en distintas variedades de tomates en invernaderos en Quillota, V Región (Ruz 2002). También fueron utilizados en estudios de servicios de polinización sobre paltos en el sector de Quillota (Fried 1999). Actualmente existe una gran expansión natural de colonias de esta especie en Chile, ocurriendo desde la IV Región a la X Región y desde el nivel del mar

hasta los 3.400 m de altitud (Montalva *et al.* 2008). Las consecuencias de la introducción de *B. terrestris* sobre los abejorros nativos son aún desconocidas. Sin embargo, algunos trabajos han indicado que *Bombus terrestris* presenta una velocidad de forrajeo por flor mayor que *Bombus dahlbomii* y otras abejas nativas (Ruz y Herrera 2001), lo que sugiere que podría existir un efecto negativo de *B. terrestris* sobre las especies nativas en el caso que existiese una sobreposición en el uso de recursos limitantes. A pesar de la creciente expansión del área invadida por *B. terrestris*, la evidencia sobre su impacto ecológico todavía es escasa, fragmentada y preliminar con lo cual se hace necesario la evaluación rigurosa de su impacto sobre especies nativas de polinizadores y plantas (Morales 2007).

En este trabajo de tesis se evaluará la contribución del abejorro exótico *Bombus terrestris* en el servicio de polinización sobre *M. luteus*. Asimismo, se examinará su efecto sobre los polinizadores nativos presentes en el sitio de estudio. Más específicamente, en este trabajo se abordarán las siguientes preguntas: 1) ¿Cuál es la contribución del abejorro exótico *B. terrestris* en el servicio de polinización recibido por *M. luteus*?, 2) ¿Impacta *B. terrestris* en el servicio de polinización realizado por polinizadores nativos sobre *M. luteus*?, 3) Existe evidencia que sugiera la ocurrencia de un desplazamiento competitivo instantáneo de *B. dahlbomii* por *B. terrestris*?

## SITIO DE ESTUDIO E HISTORIA NATURAL

La toma de datos y observaciones se efectuaron sobre parches de *Mimulus luteus*, una herbácea anual o perenne autocompatible que crece en zonas húmedas a través de Chile desde el nivel del mar hasta una elevación de 3650 m y entre los 29°S y 45°S (von Bohlen 1995). Esta herbácea posee flores hermafroditas solitarias de corola amarilla de cinco pétalos lobulados y presenta una mancha roja como guía de néctar en el lóbulo inferior (Fig.1) (Medel et al. 2003, Pohl et al. 2006). En el sitio de estudio existe una amplia gama de variaciones en la forma y posición de la guía de néctar en la flor, desde una casi total ausencia hasta presencia de la mancha roja en los cinco lóbulos. Antecedentes previos indican que la corola de *M. luteus* no refleja en el espectro UV (Botto-Mahan et al. 2004).



Figura 1. Flor de *Mimulus luteus* que presenta cinco pétalos y mancha roja como guía de néctar en lóbulo inferior.

El estudio de campo fue realizado en el sector de Juncal en Portillo, V Región (32°51'S, 70°08'O, 2398 m.s.n.m.), ubicado a pocos kilómetros por debajo del complejo fronterizo Los Libertadores. El sitio corresponde a la zona altoandina con presencia de nevadas invernales que cubren completamente el sector (Fig. 2).

Otras especies de plantas presentes en el sitio de estudio incluyen a *Brassica campestris* (Brassicaceae), *Verbascum thapsus* (Scrophulariaceae), *Taraxacum officinale* (Asteraceae), *Junellia scoparia* (Verbenaceae), *Lathyrus subandinus* (Fabaceae), *Senecio glaber* (Asteraceae) y *Chuquiraga oppositifolia* (Asteraceae) como las más abundantes.



Figura 2. Sitio de estudio, sector de Juncal en Portillo, V Región (32°51'S, 70°08'O, 2398 m.s.n.m.)

## CAPÍTULO I

### **Assessing the impact of the invasive buff-tailed bumblebee (*Bombus terrestris*) on the pollination of the native Chilean herb *Mimulus luteus***

Gabriel Esterio

#### **Abstract**

The arrival of exotic pollinators to new habitats may introduce new patterns of floral preference and foraging behavior that modify the structure of the resident plant-pollinator community. The aim of this paper is to examine the potential impact of the exotic bumblebee *Bombus terrestris* on the pollination service provided by the native pollinator assemblage of the herb *Mimulus luteus*. The study was performed in a high elevation locality in the Chilean Andes during the summer seasons of 2010, 2011, and 2012. We recorded visitation rate, the number of pollen grains transported on the body of *B. terrestris* and native pollinators and the pollen deposition on the stigmas of *M. luteus*. Pollinator effectiveness (pollen deposited x visitation rate) was compared among species. Results revealed that *B. terrestris* was an inefficient pollinator, due to the low amount of pollen delivered to stigmas and the low and intermittent visitation rate across years. The parallel inter-annual variation in the visitation rate of *B. terrestris* and the native bumblebee *Bombus dahlbomii* suggests that the integration of *B. terrestris* had no important consequences for the congeneric species. In general, *B. terrestris* accounted for a low proportion of the pollen transfer in *M. luteus*, attaining 5.8% in 2010, absence of effect in 2011, and 0.01% in 2012. These results suggest that in spite of being a

rapidly spreading species in Chile, *B. terrestris* is still in the initial phase of invasion in this area.

Keywords: plant - pollinator, community impact, exotic bee, pollinator effectiveness, pollinator efficiency, invasion process, La Niña

## **Introduction**

The introduction of exotic species to natural ecosystems has been described as one of the most important threats to biodiversity (Sala et al. 2000; Courchamp et al. 2003). Exotic species may affect native plant and pollinator species through different interaction pathways, often usurping interactions and disrupting plant-animal mutualistic relationships (Traveset and Richardson 2006). The introduction of exotic pollinators may have a series of effects on native pollinators, such as competition for floral resources and nesting sites, pathogen transmission, and risk of hybridization with congeneric species (Goulson 2003; Morales 2007; Stout and Morales 2009, Goulson 2010). In addition, the introduction of exotic pollinators may modify the organization of the native pollinator assemblage (Stout and Morales 2009), which might change reproductive patterns of the native plant community (Bjerknes et al. 2007). The negative effect of exotic pollinators on the native flora may occur through at least two mechanisms. First, exotic pollinators may be less efficient than native pollinators due to morphological mismatches in the plant-pollinator interface (Goulson 2003). Second, the presence of exotic pollinators may reduce the visitation rate of native pollinators when the foraging strategies used by exotic species make flowers less attractive to native pollinators (e.g., nectar theft, Dohzono and Yokoyama 2010).

*Bombus terrestris* (Hymenoptera: Apidae) is one of the pollinator species most widely used for commercial purposes (Dozhono and Yokoyama 2010). The species often escapes from confinement, becoming naturalized in the invaded ecosystems (Inari et al. 2005). For example, the species is naturalized in New Zealand (introduced in 1885), Israel (introduced in 1960), Japan (introduced in 1991), and Tasmania (introduced in 1992) (Goulson 2003; Inoue et al. 2008; Dozhono and Yokoyama 2010). This species is an efficient invader with a high capacity to establish and displace native pollinator species, apparently through interspecific competition (Ings et al. 2005; 2006; Williams and Osborne 2009). In Japan, the invasion by *B. terrestris* has displaced native bumblebees mostly through competition for nesting sites rather than by competition for floral resources (Inoue et al. 2008). In Tasmania, where the species expands at a rate of 25 km per year in typical environmental conditions (Hingston et al. 2002), *B. terrestris* has a high potential to impact ecosystem functioning because of the high diversity of plants it visits, the ample overlap in floral resource use with native pollinators and the occurrence of visitation on flowering species not previously visited by other invasive pollinator species (Hingston and McQuillan 1998). For instance, introduced *B. terrestris* reduced the number of flowers visited and the time spent by two native megachilid bees on flowers of *Gompholobium huegelii* (Fabaceae) in Tasmania (Hingston and McQuillan 1999). In Israel, *B. terrestris* increased in abundance during the 80's and 90's and at the same time the honeybee *Apis mellifera* and solitary bee species decreased in abundance (Dafni and Schmida 1996).

In Chile, several bee species have been introduced for commercial purposes such as *Apis mellifera*, *Megachile rotundata*, *Bombus impatiens* and *Bombus ruderatus*



(Arretz and Macfarlane 1986; Ruz 2002; Rebolledo et al. 2004). The introduction of *B. terrestris* to Chile occurred in 1997-1998 (Montalva et al. 2008). Several colonies from Israel and Belgium were established in different localities of north-central Chile and their pollinator efficiency in tomato and avocado plantations was monitored (Fried 1999; Ruz 2002). The current distribution of *B. terrestris* in Chile ranges from ca. 30 - 38° S, and from the sea level to 3400 m elevation (Montalva et al. 2008). In spite of the increasing expansion of the area invaded by *B. terrestris* in southern South America, evidence on its ecological impact on native pollinators is still scarce, fragmented and preliminary. It has been suggested that the native bumblebee *Bombus dahlbomii* could be under threat due to the arrival of the exotic *B. terrestris* and *B. ruderatus*, in part because of competition for shared flower resources and nesting sites. This threat can be due to the ability of *Bombus terrestris* to visit a larger number flowers per minute in comparison to *B. dahlbomii* and other native bee species (Ruz and Herrera 2001).

In this report we present data on the pollination service of native pollinators and the exotic *B. terrestris* on the herbaceous *Mimulus luteus* (Phrymaceae) during three consecutive years. We attempt to answer the following two questions: 1) What is the contribution of the exotic *B. terrestris* to the overall pollination service received by *M. luteus*? 2) Does *B. terrestris* impact on the pollination service provided by native pollinators to *M. luteus*? To answer these questions we estimated the pollination efficiency and effectiveness of each pollinator species by recording the number of pollen grains attached to their bodies, the number of pollen grains deposited on stigmas, and visitation rates in three consecutive years in an Andean locality of central Chile.

## Materials and methods

### *Study site and natural history*

*Mimulus luteus* is a hydrophilic perennial herb that grows from 0 – 3650 m elevation and between 29° - 45°S (von Bohlen 1995). The species is hermaphroditic, slightly protogynous, and self-compatible. It presents solitary hermaphrodite flowers with yellow corollas of 721 mm<sup>2</sup> and a conspicuous red spot in the lower lobe, which acts as nectar guide (Medel et al 2003; Botto-Mahan et al 2004). Flowers are zygomorphic, 34 mm height (along the symmetry plane) and 26 mm width. Flowers remain open among 3.6 - 5.9 days, depending if pollinated or not (Medel et al 2003) and have a stigma exerted above anthers, which has been named “approach herkogamy” by Barrett (2002) (Figure 1a). Previous studies performed in different populations of *M. luteus* indicate that even in the face of complete self-compatibility, autonomous self-pollination produces low fruit set and seed production, and pollen vectors are required to assure effective pollination (Medel et al. 2003, Pohl et al. 2006, Carvallo and Medel 2010). The flowering season of *M. luteus* extends from mid December to late February with a pick of flowering during mid January - mid February. Because of its wide distribution in Chile, *M. luteus* is a useful model to examine the impact of invasive pollinator species on the composition and efficiency of native pollinator assemblages in replicated populations. For instance, current information indicates that the invasive *Bombus terrestris* is already present in four out of seven populations of *M. luteus* reported in Medel et al. (2007).

We focused on a population located in Juncal (32°51'S, 70°08'W, 2398 m elevation), a high Andean locality about 80 km northwest of Santiago. The site is a well-

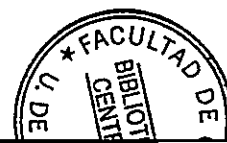
drained substrate that is entirely covered by snow during at least 3 months a year. The vegetation type corresponds to associations of the subalpine belt (Hoffmann et al 1998). The most abundant plant species at the site are *Minulus luteus* (Phrymaceae)(relative abundance from five 10 m transects: 67.8%), *Brassica campestris* (Brassicaceae) (11.7%), *Chrysanthemum parthenium* (Asteraceae) (9.5%), *Veronica anagallis-aquatica* (Scrophulariaceae) (6.4%), *Verbascum densiflorum* (Scrophulariaceae) (1.9%), *Solidago chilensis* (Asteraceae) (1.5%), and *Lactuca serriola* (Asteraceae) (1.1%). All these species co-flower with *M. luteus* but are less frequently visited by insects. As almost 70% of the plants in the patch corresponded to *M. luteus* (Figure 1b), it is unlikely that potential changes in the relative abundance of the accompanying flora had influenced the visitation rate on *M. luteus* among years.

#### *Field and laboratory procedures*

We recorded the visitation rate of every pollinator species on flowers of *M. luteus* during 54 h 30 min in 2010 (February 5 – February 24), 77 h 45 min in 2011 (January 22 - January 30) (Esterio 2011), and 31 h 40 min in 2012 (January 7 - January 17). A variable number of observers recorded the identity and frequency of visits during 30 min in flower patches in an area of ca. 20 m x 10 m (Figure 1a). Each observer recorded the insect activity in 222 flowers on the average (range: 110 – 420 flowers). The same patches were surveyed each year, which ensured a comparable flower density among years (ca. 137.5 flowers/m<sup>2</sup> on the average). Observations were performed on sunny days only and between 8:00 – 12:30. Visitation rate was calculated as the average of the number of visits flower<sup>-1</sup> h<sup>-1</sup> using time intervals as replicates.

To estimate the pollen grains carried by insect species, a sample of each insect species was collected in the morning, in 2010 (the sample sizes are shown in the results) and the following procedure was performed for all the species. Insects were deposited in killing jars containing a layer of paper tissue soaked in ethyl acetate to act as a rapid killing agent. The third pair of legs of each insect species was removed because pollen from these structures (corbiculae) is used by insects and not available for pollination (Inouye et al. 1994, Thorp 2000). All insects were stored in Eppendorf vials with 1ml of 70% ethanol and transported to the laboratory for pollen counting. Pollen was removed from the surface of the insect's body by shaking tubes in a vortex for 1 min. The resulting solution was stained with methylene blue and shaken again for 1 min, after which three samples of 10  $\mu$ l were taken. The pollen grains from each sample were spread on a grid set in cover slips and counted using a compound microscope. Each count was extrapolated to 1 ml to obtain an average number of pollen grains per individual (Hingston 2006). The pollen transported by a pollinator species was calculated as the average value from all the individuals measured (Esterio 2011). In addition to pollen counting, and to relate morphometric variables with pollinator efficiency we recorded the body length (mm), thorax width (mm) and head width (mm) of the six pollinator species (five specimens per species).

Pollination intensity (PI), that is, the mean number of pollen grains deposited by pollinator species (Silander and Primack 1978) was assessed by removing stigmas of virgin flowers after being visited by the first time by a pollinator. To ensure that stigmas were not previously visited, we removed the corolla of all flowers in the patch at dusk (1858 flowers on average during 8 days) leaving only flower buds ready to open next



morning. Since nocturnal pollination is absent in this species, all the flowers in anthesis on the next morning were virgin. Observations of visits began before the arrival of the diurnal pollinators. Assessment of pollination intensity was performed only in the summer of 2012. The visited stigmas were immediately deposited in Eppendorf vials with 70% ethanol. This method fixed the pollen grains on the stigma surface, aided by bilabiate stigma that closes after mechanical contact, permitted subsequent counting of pollen grains in the laboratory. Because stigmas were fixed immediately after being visited by a pollinator, no formation of pollen tubes was expected. The tissues were softened with 2 M NaOH for 14 h and washed with distilled water during other 14 h (range 12 - 16 h). We checked the solution were stigmas were immersed in the search for pollen grains potentially lost during the washing process. As no pollen grain was detected in three stigmas analyzed, we did not repeat this procedure for the remaining samples. Stigmas were treated with aniline blue 0.1% in a 0.07 M phosphate buffer ( $\text{Na}_2\text{HPO}_4$ ) solution at pH 9 for 48 h (Kearns and Inouye 1993).

To quantify the contribution of each pollinator species to pollination service we calculated two indexes: 1) Pollination efficiency (PE) (Inouye et al. 1994), calculated as  $\text{PI}/\text{TG}$ , where PI = number of pollen grains delivered on a virgin stigma after a single visit, and TG = number of pollen grains transported by the vector (i.e. total pollen load). This index was estimated from the mean PI and TG values per pollinator species; and 2) the index of pollen transfer effectiveness (PTE) (Herrera 1990), measured as PI x visitation rate. This index is species-specific and independent of population size. It permits characterization and comparison of pollinator effectiveness on the same plant species (Gross 2005). Additionally, we calculated an overall PTE per year, measured as

the addition of the PTE of each species per year.

## Results

The native pollinator assemblage for *M. luteus* at this site consists of the solitary bees *Centris chilensis* and *Centris nigerrima* (Anthophoridae), *Megachile saulcyi* and *Megachile semirufa* (Megachilidae), and the bumblebee *B. dahlbomii* (Apidae). *Bombus terrestris* (Apidae) was the only exotic pollinator species in the study site. The body size of each species is shown in Table 1. There was significant heterogeneity in the body length of species (one-way ANOVA on log-transformed data,  $F_{5,24} = 23.4$ ,  $P < 0.001$ ). Similarly, the species differed globally in thorax width ( $F_{5,24} = 17.2$ ,  $P < 0.001$ ) and head width ( $F_{5,24} = 7.3$ ,  $P < 0.001$ ). A posteriori Tukey's comparisons revealed that *B. terrestris* tend to differ mainly with *C. chilensis* and *M. saulcyi*, being largely undistinguishable from the native *B. dahlbomii* and *C. nigerrima* (Table 1). The visitation rates in 2010 differed among species (one-way ANOVA,  $F_{5,228} = 11.6$ ,  $P < 0.001$ ). *Centris nigerrima*, *B. terrestris* and *B. dahlbomii* were the species that most visited flowers of *M. luteus* (Table 2); their visitation rate was higher than that of the native *C. chilensis*, *M. saulcyi* and *M. semirufa* (Tukey's tests,  $P < 0.05$ ). A different situation was observed in 2011, when the two *Bombus* species were absent in the study site. There was significant heterogeneity in the visitation rates of the remaining species (one-way ANOVA,  $F_{3,84} = 7.0$ ,  $P < 0.001$ ). In this year, *Megachile saulcyi* had a higher visitation rate than *M. semirufa* and *C. chilensis* (Table 2). The visitation rates in 2012 differed among species (one-way ANOVA,  $F_{5,48} = 15.3$ ,  $P < 0.001$ ). *Centris chilensis* and *C. nigerrima* were the species with the highest visitation rate of the pollinator

assemblage (Table 2). In general, the visitation rate of *B. terrestris* was highly variable over years. Unlike the situation observed in 2010, when *B. terrestris* showed the highest visitation rate, this species did not visit the study site in 2011 and had the lowest visitation rate of the pollinator assemblage in 2012 (Table 2). Interestingly, the pattern of temporal change of *B. terrestris* was similar to *B. dahlbomii*, suggesting that the exotic and native bumblebees responded in a similar way to environmental variation.

All the insect species carried a higher proportion of pollen of *M. luteus* (70-90%) on their bodies than pollen from the accompanying flora. There was significant heterogeneity in the amount of pollen transported by pollinator species (one-way ANOVA,  $F_{5,93} = 3.43$ ,  $P = 0.007$ ). The introduced *B. terrestris* was the species with the greatest number of pollen grains on the average (Table 2). *Bombus terrestris* and *C. nigerrima* transported more pollen than *M. semirufa* (Tukey's test,  $P < 0.05$ ). Albeit not significant, the exotic *B. terrestris* transported a two-fold greater amount of pollen than its congeneric native bumblebee *B. dahlbomii* and almost 25% more than *C. nigerrima*. These differences were not paralleled by the number of pollen grains deposited on stigmas per visit (pollination intensity). Even though pollination intensity differed among species (Kruskal-Wallis test,  $X^2 = 25.0$ ,  $df = 5$ ,  $P < 0.001$ ), the two *Bombus* species tended to deposit a lower number of pollen grains than *Centris* and *Megachile* species (Table 2). Notwithstanding, the only significant effects at  $P < 0.05$  were observed in comparisons of the native species: *B. dahlbomii* - *M. saulcyi*, and *B. dahlbomii* - *C. nigerrima*. It is likely that the absence of significance in the contrast of *B. terrestris* with the native species results from the low sample size for this species, reflecting its low relative abundance in the study site. However, even if not significant,

the mean number of pollen grains deposited on stigmas by *B. terrestris* is sufficiently low to assume this result has biological significance.

The pollination efficiency of *B. terrestris*, that is, the number of pollen grains delivered onto stigmas relative to the amount of pollen on the body was the lowest of the pollinator assemblage, only comparable to the native bumblebee *B. dahlbomii* (Table 2). The overall effectiveness of the pollinator assemblage, corresponding to the sum of the PTE-values per year, varied substantially across years (2.70 in 2010, 0.64 in 2011, and 6.98 in 2012), indicating that the overall contribution of the pollinator assemblage to the reproductive success of *M. luteus* is variable among years. The solitary bee *C. nigerrima* was the species that consistently contributed most to pollen transfer, reaching 82.9% in 2010, 45.9% in 2011, and 88.8% in 2012 (Figure 2). Regarding *B. terrestris*, PTE-values indicate that this species had an overall low importance, reaching 5.8% in 2010, absence in 2011, and 0.01% in 2012 (Figure 2).

## Discussion

The aim of this study was to evaluate the potential impact of the exotic bumblebee *B. terrestris* on the pollination service received by the Andean monkeyflower, *M. luteus*. Our results indicate that the native bee *C. nigerrima* provided consistently most pollination service (Figure 2). Even though *B. terrestris* was the species with the greatest amount of pollen of *M. luteus* on the body, the low number of pollen grains deposited on stigmas and the medium-low visitation rate exhibited by the exotic pollinator in comparison to native pollinators in 2010 and 2012 make this species one of low effectiveness (Table 2, Figure 2). Why did *B. terrestris* have no impact on the pollination



service of *M. luteus*? We suggest two non-mutually exclusive explanations. First, *B. terrestris* is one of the largest bee species currently inhabiting Chile, only equivalent to the exotic *B. ruderatus* and the native *B. dahlbomii* in size. As such, it does not match the flower phenotype of most native flowering plants, leading to pollen removal and subsequent delivery in wrong places, especially in species with narrow and small floral tubes. Kenta et al. (2007) suggested that limitation in accessibility to nectar make the short-tongued *B. terrestris* more prone to nectar robbing than native pollinators. We did not observe nectar robbing of *B. terrestris* on *M. luteus*, which allows us to reject this pathway of detrimental impact. Second, it is likely that the importance of *B. terrestris* in pollen transfer depends on the plant species involved. *Mimulus luteus* has the peculiarity of having a mobile stigma with lobes that close quickly (10 s on the average) after mechanical contact and remain closed for 13 min on the average (Carvallo and Medel 2010) (Figure 1a). As *B. terrestris* and *B. dahlbomii* have a large size relative to *Centris* and *Megachile* species (Table 1) they have a high chance of accidentally touch and close stigmas while manipulating flowers. This phenomenon may be increased by the foraging behavior of *B. terrestris*. Unlike *Centris* and *Megachile* bees that access easily to the nectar concealed at the end of the corolla tube, *B. terrestris* often bend downward and manipulate extensively the flowers of *M. luteus* increasing the chance of accidental contact with bilabiate stigmas.

The low abundance of *B. terrestris* and *B. dahlbomii* in 2010 and 2012 and their absence in 2011 is intriguing and may relate to several factors. On the one hand, the occurrence of bumblebees may be restricted only to years with favorable environmental conditions, especially as the study site is located at the edge of their elevation range in

the Andes, which may render bumblebees more sensitive to environmental change (see also Goulson et al. 2005). El Niño/La Niña events are the most important factors determining inter-annual variation in climate conditions in central Chile, especially in the Andean zone comprised between 30° - 35° S (Escobar and Aceituno 1988). It is known that many ecological processes in central Chile are strongly influenced by these large-scale climate processes (see reviews in Jaksic 1998, 2001). Winters with extremely low temperatures and extensive rainfall and snow in central Chile characteristic of El Niño events probably limit the successful emergence of queens from hibernation reducing colony initiation. On the contrary, La Niña events associated with hot and dry years may influence bumblebee colony development through environmental conditions that exceed the thermal and humidity range needed for colony homeostasis (e.g., Vogt 1986, Yoon et al. 2002). It is likely that the absence of *B. terrestris* and *B. dahlbomii* in the summer season of 2011 occurred as a consequence of the strong La Niña episode that occurred from autumn 2010 - summer 2011 in central Chile. In spite of the importance of linking population-level pollination processes with El Niño/La Niña events few studies have analyzed the consequences of large-scale climate processes on natural populations (e.g., Inouye et al. 2002).

Interestingly, the congeneric native bumblebee, *B. dahlbomii*, showed a parallel pattern of occurrence with *B. terrestris* over years (Table 2) suggesting that similar thermal niche constraints account in part for this pattern in bumblebees as a whole (see also Goulson 2003; Wiggam and Ferguson 2005; Dohzono et al. 2008). Alternatively, bumblebees and other species may all be responding in the same way to inter-annual variation in floral density and nectar availability in the area rather than climate variation

(e.g., Bowers 1985). Even though we cannot rule out this explanation at present, ecological reasons make this possibility unlikely. *Mimulus luteus* occurs in dense patches of ca. 137 flowers/m<sup>2</sup> (Figure 1b) that do not change substantially across years, ensuring nectar availability for the limited demand imposed by pollinators as revealed by their extremely low visitation rates (see also Medel et al. 2003; Carvallo and Medel 2010).

In summary, we did not detect an important impact of the exotic bumblebee *B. terrestris* on the pollinator assemblage of *M. luteus*. Even though information of the time of arrival of this species to the study site is lacking, judging from the time of introduction to Chile, it should not exceed 15 years. It is known that as the population size of invasive species changes with environmental variation, their impact in new habitats does not necessarily occur at the beginning of the invasion process (Strayer et al. 2006). In general, the invasion process occurs through a series of events that include: a) transport, b) introduction, c) establishment, d) propagation, and e) impact (Lockwood et al. 2007). The observation that *B. terrestris* has not yet become the dominant species in the area but occurred intermittently in the study site suggests that the invasion process is still in the establishment stage. It is likely that the impact of *B. terrestris* on the native pollinator assemblage occurs in more developed stages of the invasion process, once colonies have established definitively. In spite of being a quickly spreading species in Chile, *B. terrestris* currently has a low impact on the pollination service of the Andean monkeyflower *M. luteus*.

Is necessary to note some limitations in this paper. First, the different dates used each year, this monthly variation may confound the annual variation in visitation rates, so that the low numbers of *Bombus* in 2011 and 2012 could reflect their lower numbers



in January than in February. This may be possibly, however, even though the sampling in 2010 was performed on February, there was plenty of activity of *Bombus terrestris* in January 2010. In general, the summer season of 2010 was favorable as most pollinator species showed a high activity in the study site. Observations in February 2011 and 2012 do not show an increase in activity of *Bombus* in the study site. Second, we were unable to sample a higher number of stigmas visits by *B. terrestris* and *B. dahlbomii* due to the low abundance of *Bombus* in 2012 in the study site.

More research into the effects of *B. terrestris* on pollination of other species of native plants seems necessary to extract legitimate conclusions of its impact on Chilean plant-pollinator communities.

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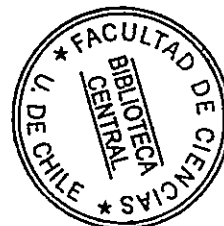
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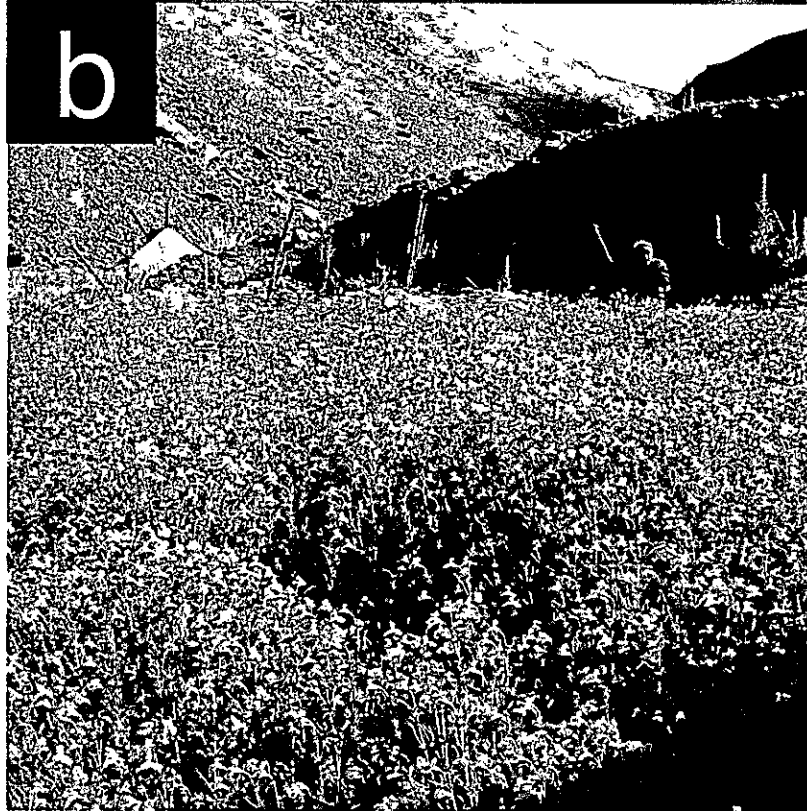
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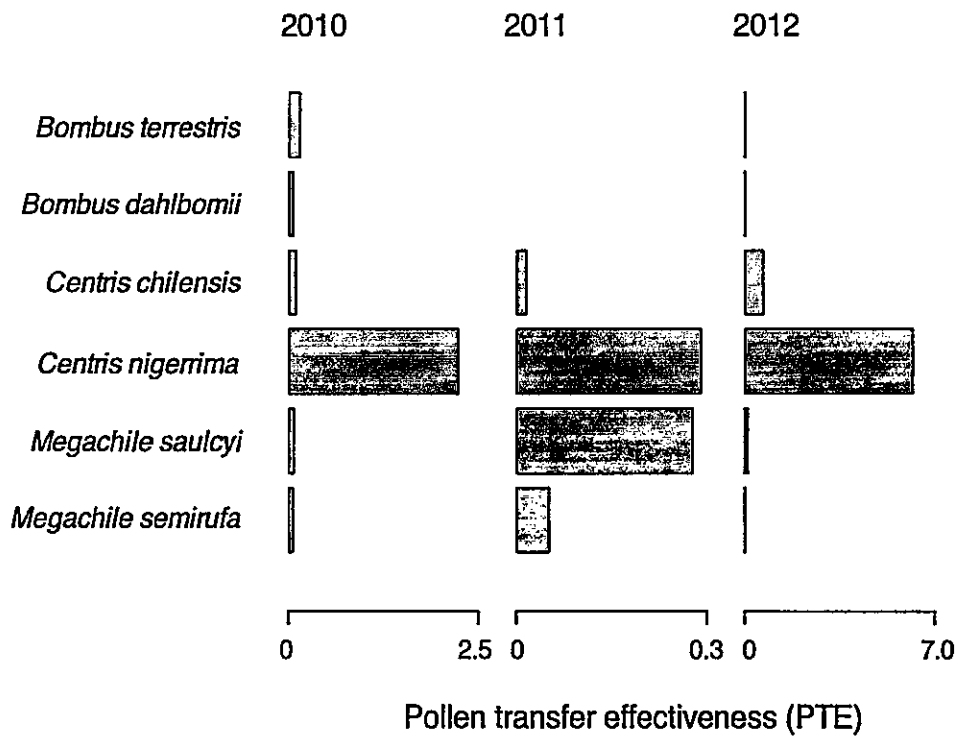
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## Caption for Figures

Figure 1. a) *Centris nigerrima* (Apidae) in a flower of *Mimulus luteus*. Arrow indicates the bilabiate stigma that closes after mechanical contact. b) Patch of *Mimulus luteus* in the study site. Patches are relatively discrete units of thousands of flowers located in well-drained areas of the Chilean Andes.

Figure 2. Pollen transfer effectiveness (PTE) per year and pollinator species. PTE values were estimated from the product between visitation rate x pollination intensity (see Materials and Methods section). Note that PTE is not in the same scale among years.





Species	Body length (mm)	Thorax width (mm)	Head width (mm)
<i>Bombus terrestris</i>	16.78 ± 1.08 <sup>ab</sup>	7.31 ± 0.33 <sup>a</sup>	5.12 ± 0.41 <sup>a</sup>
<i>Bombus dahlbomii</i>	18.35 ± 1.46 <sup>a</sup>	7.06 ± 0.64 <sup>a</sup>	5.13 ± 0.40 <sup>a</sup>
<i>Centris chilensis</i>	14.10 ± 0.55 <sup>cd</sup>	5.63 ± 0.35 <sup>bc</sup>	5.13 ± 0.09 <sup>a</sup>
<i>Centris nigerrima</i>	15.26 ± 0.73 <sup>bc</sup>	6.43 ± 0.33 <sup>ab</sup>	5.16 ± 0.22 <sup>a</sup>
<i>Megachile saulcyi</i>	12.71 ± 0.99 <sup>d</sup>	5.00 ± 0.65 <sup>c</sup>	4.29 ± 0.32 <sup>b</sup>
<i>Megachile semirufa</i>	14.94 ± 0.45 <sup>bc</sup>	5.79 ± 0.32 <sup>bc</sup>	5.05 ± 0.14 <sup>a</sup>

Table 1. Size descriptors of the insect pollinators visiting *Mimulus luteus* in the study site. Figures are mean ± SE. Within columns, values that do not share letters are significantly different ( $P < 0.05$ , one-way ANOVA followed by Tukey's hsd test). All measurements were performed on five individuals per species.

Species	TG (SE, N)	PI (SE, N)	Eff <sub>1</sub>	Eff <sub>2</sub> (SE, N)	Visitation rate (SE, N)		
					2010	2011	2012
<i>Bombus terrestris</i>	22076 (3394, 22) <sup>a</sup>	8.5 (6.5, 2) <sup>ab</sup>	0.39	2.42 (1.20, 22) <sup>a</sup>	1.833 (0.537, 273) <sup>a</sup>	0	0.009 (0.009, 3) <sup>a</sup>
<i>Bombus dahibomii</i>	9502 (2049, 30) <sup>ab</sup>	4.0 (3.0, 2) <sup>b</sup>	0.42	4.51 (1.91, 30) <sup>a</sup>	1.657 (0.290, 216) <sup>a</sup>	0	0.048 (0.045, 10) <sup>a</sup>
<i>Centris chilensis</i>	15887 (6523, 5) <sup>ab</sup>	28.1 (8.3, 22) <sup>ab</sup>	1.77	3.07 (0.98, 5) <sup>ab</sup>	0.350 (0.107, 48) <sup>b</sup>	0.055 (0.024, 13) <sup>c</sup>	2.395 (0.627, 699) <sup>b</sup>
<i>Centris nigerrima</i>	17165 (3627, 24) <sup>a</sup>	117.1 (15.1, 92) <sup>a</sup>	6.82	30.22 (8.18, 24) <sup>b</sup>	1.908 (0.546, 228) <sup>a</sup>	0.249 (0.065, 99) <sup>ab</sup>	5.295 (2.385, 1703) <sup>b</sup>
<i>Megachile saulcyi</i>	13133 (5773, 7) <sup>ab</sup>	83.6 (8.5, 137) <sup>a</sup>	6.37	78.20 (57.14, 7) <sup>b</sup>	0.090 (0.048, 11) <sup>b</sup>	0.333 (0.057, 145) <sup>a</sup>	0.111 (0.060, 33) <sup>a</sup>
<i>Megachile semirufa</i>	8062 (3463, 11) <sup>b</sup>	37.6 (6.8, 57) <sup>ab</sup>	4.66	132.76 (132.76, 11) <sup>b</sup>	0.174 (0.057, 22) <sup>b</sup>	0.137 (0.035, 60) <sup>bc</sup>	0.025 (0.013, 7) <sup>a</sup>

Table 2. Summary of results of pollination variables per insect species. TG = number of pollen grains transported by the vector, PI = number of pollen grains deposited on virgin stigmas after a single visit, Eff = Efficiency. Efficiency<sub>1</sub> was calculated as the ratio of means PI/TG x 1000; Efficiency<sub>2</sub> was calculated from the mean of ratios PI/TG x 1000 considering TG-values from every individual as replicates. Visitation rate are expressed as visits flower<sup>-1</sup> h<sup>-1</sup>. Within columns, efficiency<sub>2</sub>, TG, and Visitation rate-values with no letters in common indicate significant difference (one-way ANOVA followed by significant Tukey's hsd test,  $P < 0.05$ ). Non parametric *a posteriori* contrasts (Zar 1999) for PI-values were performed in R (R Core Team, 2013). Parentheses in Efficiency<sub>2</sub>, TG, and PI indicate standard error (SE) and sample size (N). Parentheses in visitation rate indicate standard error (SE) and total number of visits during the observation period (N).



## CONCLUSIONES

De este trabajo se desprenden algunas conclusiones importantes. La primera de ellas es que la mayor cantidad de visitas y polinización por insectos sobre el parche de flores de *Mimulus luteus* ocurre por parte de un reducido grupo de abejas sociales (2 especies) y solitarias (4 especies) encontradas durante los tres años de observación: *Bombus terrestris*, *Bombus dahlbomii*, *Centris nigerrima*, *Centris chilensis*, *Megachile semirufa* y *Megachile saulcyi*. Con excepción de *B. terrestris*, todas las especies del ensamble de polinizadores son nativas. La composición del ensamble de polinizadores varía entre un año y otro, presentando fenómenos de presencia/ausencia de visitantes dependiendo del año. Cuatro de las seis especies antes descritas están presentes los tres años consecutivos, *C. nigerrima*, *C. chilensis*, *M. semirufa* y *M. saulcyi*. Sin embargo, las especies de *Bombus* presentaron actividad intermitente, probablemente como consecuencia de fluctuaciones abióticas en el sistema de estudio.

La intensidad de la polinización (cantidad de granos de polen depositados sobre los estigmas de *M. luteus*) difirió entre las especies, siendo máxima en *C. nigerrima*. A pesar que *B. terrestris* fue la especie que transportó la mayor cantidad de granos, depositó muy pocos de ellos en el estigma de *M. luteus*. Este hecho, conjuntamente con los bajos valores de eficiencia obtenidos por dos métricas ( $Eff_1$  y  $Eff_2$ , ver Tabla 2), sugiere que *B. terrestris* es una de las especies menos eficientes del ensamble de polinizadores, comparable solamente a *B. dahlbomii* y *C. chilensis*.

Los resultados revelaron diferencias significativas en la tasa de visita de las especies para cada año estudiado, *C. nigerrima* es la que presenta una mayor tasa de

visita durante el año 2010 y 2012. Solo el año 2010 *B. terrestris* actuó como un importante visitante de *M. luteus* relativo a las otras especies. *Bombus terrestris* presentó una alta variación entre años en su aparición en el sitio de estudio, desde una alta tasa de visita el año 2010, ausencia total el año 2011 y una muy baja actividad el 2012. Contrario a lo esperado, ninguna de las especies residentes disminuyó en presencia de *B. terrestris*. Por el contrario, algunas especies incluso aumentaron en actividad. Respecto a una potencial interferencia entre *B. terrestris* y *B. dahlbomii*, se observó una variación paralela entre los años, por lo que no habría evidencia suficiente para apoyar la idea de un desplazamiento competitivo instantáneo de la especie nativa por la alóctona. Sin embargo, estudios experimentales son necesarios para confirmar inequívocamente esta afirmación.

Al analizar la contribución del ensamble de polinizadores sobre el total de polinización recibida por *M. luteus* de acuerdo a la efectividad de transferencia de polen (PTE), se observó una amplia variación entre los años de estudio. En particular, se observó un bajo impacto del abejorro exótico *B. terrestris* y un alto y consistente impacto de *C. nigerrima*. Esta última especie mostró la máxima efectividad debido a su alta tasa de visitas y al alto número de granos de polen depositado en los estigmas de *M. luteus*. De acuerdo a lo anteriormente expuesto, la presencia de *B. terrestris* en la comunidad estudiada no afectaría de manera importante el servicio de polinización en *M. luteus*.

Es pertinente en este punto hacer notar tres observaciones. Primero, la estimación de intensidad de polinización para las dos especies de *Bombus* fueron efectuadas sobre la base de dos estigmas en cada caso. Un aumento en el tamaño muestral, probablemente

reduzca el error estándar en torno a la intensidad, tornando aún más significativas las diferencias con el resto del ensamble. Segundo, todas las mediciones de cargas de polen fueron efectuadas en el año 2010, repitiéndose aquel valor en los años 2011 y 2012. Se desconoce si las cargas de polen varían entre los años. Sin embargo, usualmente el polen depositado en el cuerpo de las abejas es reflejo de propiedades de ajuste morfológico polinizador-flor más que de variación ecológica. De hecho, las cargas fueron altamente variables entre los ejemplares de una misma especie en el mismo año. Por consiguiente, si bien es deseable caracterizar cargas polínicas en cada año por separado, es poco probable que existan diferencias interanuales en esta variable. Tercero, todas las mediciones de *B. terrestris* fueron efectuadas considerando individuos reinas, las únicas visitantes en el sitio de estudio durante los años de estudio. Sin embargo, recientes observaciones han revelado la presencia marginal de obreras de *B. terrestris* de menor tamaño, las que podrían ingresar más fácilmente a las flores y ser más eficientes en la transferencia de polen.

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## ANEXOS

### ANEXO 1: Obtención de datos en terreno

Con el fin de estimar la variación de visitas de polinizadores entre los años se cuantificó su tasa de visita sobre parches de *M. luteus* en el mismo sector para el 2010, 2011 y 2012.

La toma de datos correspondiente a las visitas del año 2010 se extendió durante 54 horas y 30 minutos (3270 min) con periodos de 30 min de manera continua principalmente entre las 8:30 hrs y las 12:30 hrs. Para el año 2011 se acumularon 77 horas y 45 minutos de observación, principalmente entre las 8:00 hrs y las 12:30 hrs (Esterio 2011), y para el año 2012 la toma de datos se extendió durante 31 horas y 40 minutos en similar horario del año 2011, período correspondiente a las horas de mayor actividad de polinizadores.

Tabla 1. Resumen de períodos de observación en el sitio de estudio.

Año	Observaciones			
	Fecha inicio	Fecha término	Tiempo	Horario
2010	5 - febrero	24 - febrero	54 h 30 min	08:30-12:30 hrs
2011	22 - enero	30 - enero	77 h 45 min	08:00-12:30 hrs
2012	7 - enero	17 - enero	31 h 40 min	08:00-12:30 hrs

## ANEXO 2: Cargas de polen sobre polinizadores

Las cargas de polen mostradas representan la cantidad promedio de granos de polen transportados por cada especie recolectada y analizada para el año 2010. De acuerdo a los resultados, todas las especies transportan una gran cantidad de polen de *M. luteus* sobre sus cuerpos, siendo el abejorro introducido *B. terrestris* la especie que transporta mayor cantidad de granos en promedio, seguido por *C. nigerrima*, *C. chilensis*, *M. saulcyi*. Las especies menos importantes en el transporte de polen son el abejorro nativo *B. dahlbomii* y *M. semirufa* (Esterio 2011).

Tabla 2. Promedio de cargas de polen de *M. luteus* por especie. Se indica el error estándar (EE). N ejemplares es el número de individuos analizados.

Especie	Polen (EE)	N ejemplares
<i>Bombus dahlbomii</i>	9502,41 (2084,69)	30
<i>Bombus terrestris</i>	22076,26 (3394,35)	22
<i>Centris chilensis</i>	15886,67 (6522,6)	5
<i>Centris nigerrima</i>	17165,16 (3627,14)	24
<i>Megachile saulcyi</i>	13133,33 (5772,87)	7
<i>Megachile semirufa</i>	8062,12 (3462,99)	11

### ANEXO 3: Polen depositado en estigmas

Los resultados mostrados representan la cantidad promedio de granos de polen de *Mimulus luteus* depositados en el estigma virgen de su flor luego de la visita de los polinizadores. Con los datos obtenidos se determina que la especie que deposita mayor cantidad de granos de polen promedio sobre el estigma de *M. luteus* es *C. nigerrima*, seguida de *M. saulcyi*, además es posible observar que las especies de mayor tamaño como *B. terrestris* y *B. dahlbomii* son las que depositan menor cantidad de polen sobre el estigma.

Tabla 3. Promedio de polen depositado en estigmas vírgenes de *Mimulus luteus* por cada especie visitante. Se indica el error estándar (EE).

Especie	Polen depositado en estigmas (EE)	N
<i>Bombus dahlbomii</i>	4 (3)	2
<i>Bombus terrestris</i>	8,50 (6,5)	2
<i>Centris chilensis</i>	28,10 (8,33)	22
<i>Centris nigerrima</i>	117,07 (15,09)	92
<i>Megachile saulcyi</i>	83,60 (8,46)	137
<i>Megachile semirufa</i>	37,65 (6,78)	57

## **ANEXO 4: Protocolo de tinción de tubos polínicos**

### **I. Reactivos**

1. Buffer fosfato de sodio 0,07 M pH = 9

Se necesitan dos reactivos:  $\text{KH}_2\text{PO}_4$  (12,460 g ·  $1\text{L}^{-1}$   $\text{H}_2\text{O}$ , compuesto A) y

$\text{Na}_2\text{HPO}_4$  (0,966 g ·  $100\text{ mL}^{-1}$   $\text{H}_2\text{O}$ , compuesto B). Para preparar 1L de

buffer 400 mL A + 600 mL B, disolver B sobre A. Se regula el pH con una solución

de NaOH 0.1M

2. Azul de anilina 0,1%

Disolver 0,1 g de sal anilina en 100 mL de buffer.

### **II. Procedimiento de tinción**

1. Guardar la flor o estigma en etanol 70°.

2. Ablandar el tejido en NaOH 2M por 14 horas.

3. Lavar con agua destilada por 12-16 horas.

4. Teñir con azul de anilina al 0,1% por 48 horas.

5. Lavar pasando por agua destilada para sacar el excedente de colorante .

6. Montar en portaobjeto, aplastar, sellar y observar.



## ANEXO 5: Flora acompañante

La flora que acompaña *Mimulus luteus* en el sector de Juncal se compone principalmente de las siguientes especies: *Brassica campestris* (Brassicaceae), *Verbascum densiflorum* (Scrophulariaceae), *Taraxacum officinale* (Asteraceae), *Junellia scoparia* (Verbenaceae), *Lathyrus subandinus* (Fabaceae), *Senecio glaber* (Asteraceae), *Chrysanthemum parthenium* (Asteraceae), *Verónica anagallis-aquatica* (Scrophulariaceae), *Solidago chilensis* (Asteraceae) y *Chuquiraga oppositifolia* (Asteraceae).

A partir de la delimitación del sitio específico de estudio, que corresponde a un área de 165 m<sup>2</sup>, es posible determinar el número de especies acompañantes y estimar su densidad dentro de ese sector.

Tabla 4. Estimación de densidad (N° plantas/m<sup>2</sup>) de especies vegetales acompañantes presentes dentro del sitio delimitado de estudio de 165 m<sup>2</sup>.

Especie	N° de plantas	Densidad
<i>Brassica campestris</i>	11	0,07
<i>Chrysanthemum parthenium</i>	27	0,16
<i>Senecio glaber</i>	5	0,03
<i>Solidago chilensis</i>	2	0,01
<i>Verbascum densiflorum</i>	5	0,03
<i>Verónica anagallis-aquatica</i>	4	0,02