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RESPONSES AND VULNERABILITY OF THE GENUS *VIOLA* TO ELEVATIONAL GRADIENTS AND ALIEN INVASIVE SPECIES IN MOUNTAIN SYSTEMS

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Doctor by the Universitat de les Illes Balears

Als meus pares i al meu germà
A n'Elena, la sal de la meva vida

Quería movimiento, no una existencia sosegada.
Quería emoción y peligro, así como la oportunidad de sacrificarme por amor.
Me sentía henchido de tanta energía que no podía
canalizarla a través de la vida tranquila que llevábamos.

León Tolstoi, Felicidad familiar

No permitas que tu trabajo sea tu forma de ganarte la vida, sino tu diversión.
Cada día deberíamos regresar al hogar de lejos, de aventuras, peligros
y descubrimientos, con experiencias nuevas y el carácter renovado.

Henry David Thoreau, Walden

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Outline and publications

This thesis is a compendium of publications, organized in four parts: Prologue, Main chapters, Epilogue, and References. The prologue presents an overview of plant ecology in alpine ecosystems, and their special vulnerability to global change, as well as of the particularities of the genus *Viola*, study model in this thesis, and finally highlights the general objectives. The second part consists of four independent chapters, each one corresponding to a different research article. One is already published, one is under review, one is resubmitted, and one is in preparation for submission. The epilogue contains a general discussion of the main findings of the thesis, the final conclusions, and ideas for future investigation in this research line. Finally, a list of references is included.

List of publications included in this thesis:

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Seguí, J., López-Darias, M., Pérez, A. J., Nogales, M., & Traveset, A. (2017). Species-environment interactions changed by introduced herbivores in an oceanic high-mountain ecosystem. *AoB Plants*. 9(1) plw091.
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Seguí, J. & Traveset, A. (2017). What explains the maintenance of outcrossing in an almost exclusively cleistogamous species? The case of the cliffhanger *Viola jaubertiana*. Resubmitted to *International Journal of Plant Sciences*.

In preparation for submission:

Seguí, J., López-Darias, M., Nogales, M., & Traveset, A (2017). Invasive rabbits change the selection that pollinators and abiotic conditions exert on floral traits of a high-mountain oceanic plant. Prepared for submission.

Resumen

Los ecosistemas de montaña son centros de biodiversidad mundial, debido a su aislamiento geográfico, a su papel como refugio de especies durante los cambios climáticos del pasado y a la gran variedad de condiciones ambientales que presentan a lo largo de sus gradientes altitudinales. Los gradientes ambientales que presentan las montañas permiten testar respuestas ecológicas de las plantas a diferentes condiciones ambientales a través de distancias cortas, por lo que pueden funcionar como laboratorios al aire libre, especialmente útiles para entender las respuestas de las plantas al cambio global. Por ejemplo, la presencia de especies invasoras en estos sistemas puede crear efectos sinérgicos con los del cambio climático, teniendo consecuencias dramáticas para especies nativas ya amenazadas, o para ecosistemas frágiles, como los presentes en la alta montaña de islas oceánicas.

El principal objetivo de esta tesis es explorar las respuestas fisiológicas, reproductivas y fenotípicas de diversas especies de plantas entomófilas de montaña del género *Viola* a las distintas condiciones bióticas y abióticas que se encuentran a lo largo de sus gradientes altitudinales de distribución.

En los capítulos 1 y 2 se estudia la eficacia biológica, la reproducción y los rasgos florales de la violeta del Teide, *Viola cheiranthifolia,* en su gradiente altitudinal de distribución en la isla de Tenerife, así como el efecto de la presencia de un herbívoro invasor (el conejo, *Oryctolagus cuniculus*) sobre estos caracteres. A pesar de que las condiciones ambientales son cada vez más adversas con la altitud, *V. cheiranthifolia* no mostró un incremento de la limitación de polen en la parte más elevada de su distribución, lo cual atribuimos a la presencia de mecanismos compensatorios, como el aumento del grado de autogamia. La presencia de conejos en el Teide reduce de forma importante el desarrollo y éxito reproductivo de este endemismo vegetal y, a su vez, modifica las relaciones rasgo-ambiente de la planta a lo largo de su gradiente altitudinal, además de alterar las presiones de selección que los polinizadores y el medio físico ejercen sobre sus rasgos florales.

El capítulo 3 indaga en cómo varían los rasgos fenotípicos de las flores y de las hojas de una violeta alto-andina (*Viola maculata*) en respuesta a un gradiente altitudinal. En dicho gradiente pueden entran en conflicto ambos fenotipos, ya que flores grandes pueden ser más atractivas para los polinizadores, pero si están asociadas positivamente a hojas grandes, podría plantearse un dilema en un escenario de sequía, ya que las hojas favorecidas serían las pequeñas. En este estudio confirmamos el gradiente inverso de estrés hídrico (es decir, mayor a menor altitud) presente en los Andes Centrales, pero no detectamos un desajuste entre la correlación de los rasgos florales y vegetativos en la población con mayor estrés hídrico, presentando los individuos con hojas pequeñas también flores pequeñas. Pero este hecho no parece comprometer la reproducción de esta planta alto-andina, debido a su alta capacidad de autofecundación.

En el capítulo 4, se analiza cómo varía la producción de flores casmógamas (CH) y cleistógamas (CL) en la especie *Viola jaubertiana* en función de las condiciones individuales de tamaño, estrés hídrico y herbivoría presentes a lo largo de su gradiente altitudinal. Además, también se evalúan los mecanismos que pueden estar favoreciendo el mantenimiento de la fecundación cruzada en una especie predominantemente cleistógama. *Viola jaubertiana* presentó una gran limitación de polen, además de un escaso éxito reproductivo de las flores CH. Por el contrario, las flores CL, más económicas fisiológicamente, fueron mucho más exitosas, produciendo 100 veces más semillas que las flores CH. A pesar de este gran desequilibrio, el mayor peso, potencial de dispersión y mejor germinación de las semillas procedentes de fecundación cruzada, juntamente con un posible efecto positivo de la heterosis, podrían explicar el mantenimiento de la casmogamia en este endemismo mallorquín.

Resum

Els ecosistemes de muntanya són centres de biodiversitat mundial, degut al seu aïllament geogràfic, al seu paper com a refugi d'espècies durant els canvis climàtics del passat, i a la gran varietat de condicions ambientals que presenten al llarg dels seus gradients altitudinals. Els gradients ambientals que presenten els sistemes alpins, permeten testar respostes ecològiques de les plantes a diferents condicions ambientals, a través de distàncies curtes, de manera que poden funcionar com a laboratoris a l'aire lliure, especialment útils per entendre les respostes de les plantes al canvi global. Per exemple, la presència d'espècies invasores en aquests sistemes pot crear efectes sinèrgics amb els del canvi climàtic, tenint conseqüències dramàtiques per a espècies natives ja amenaçades, o per ecosistemes fràgils, com els presents en l'alta muntanya d'illes oceàniques.

El principal objectiu d'aquesta tesi és explorar les respostes fisiològiques, reproductives i fenotípiques de diverses espècies de plantes entomòfiles de muntanya del gènere Viola, a les diferents condicions biòtiques i abiòtiques que es troben al llarg dels seus gradients altitudinals de distribució.

En els capítols 1 i 2 s'estudia l'eficàcia biològica, la reproducció i els trets florals de la violeta del Teide, *Viola cheiranthifolia*, en el seu gradient altitudinal de distribució a l'illa de Tenerife, així com l'efecte de la presència d'un herbívor invasor (el conill, *Oryctolagus cuniculus*) sobre aquests caràcters. Tot i que les condicions ambientals són cada vegada més adverses amb l'altitud, *V. cheiranthifolia* no va mostrar un increment de la limitació de pol·len a la part més elevada de la seva distribució, la qual cosa atribuïm a la presència de mecanismes compensatoris, com l'augment del grau d'autogàmia. La presència de conills al Teide redueix de forma important el desenvolupament i èxit reproductiu d'aquest endemisme vegetal i, al mateix temps, modifica les relacions tret-ambient de la planta al llarg del seu gradient altitudinal, a més d'alterar les pressions de selecció que els pol·linitzadors i el medi físic exerceixen sobre els seus trets florals.

El capítol 3 indaga en com varien els trets fenotípics de les flors i les fulles d'una violeta alt-andina (*Viola maculata*) en resposta a un gradient altitudinal. En aquest gradient poden entrar en conflicte ambdós fenotips, ja que flors grans poden ser més atractives per als pol·linitzadors, però si estan associades positivament a fulles grans, podria plantejar-se un dilema en un escenari de sequera, ja que les fulles afavorides serien les petites. En aquest estudi confirmem el gradient invers d'estrès hídric (és a dir, més a menys altitud) present en els Andes Centrals, però no vam detectar un desajust entre la correlació dels trets florals i vegetatius en la població amb més estrès hídric, presentant els individus amb fulles petites també flors petites. Però aquest fet sembla no comprometre la reproducció d'aquesta planta alt-andina, degut a la seva alta capacitat de autofecundació.

En el capítol 4, s'analitza com varia la producció de flors casmógames (CH) i cleistógames (CL) en l'espècie *Viola jaubertiana* en funció de les condicions individuals de mida, estrès hídric i herbivoria presents al llarg del seu gradient altitudinal. A més, també s'avaluen els mecanismes que poden estar afavorint el manteniment de la fecundació creuada en una espècie predominantment cleistógama. *Viola jaubertiana* presenta una gran limitació de pol·len, a més d'un escàs èxit reproductiu de les flors CH. Per contra, les flors CL, més econòmiques fisiològicament, van ser molt més exitoses, produint 100 vegades més llavors que les flors CH. Tot i aquest gran desequilibri, el major pes, potencial de dispersió i millor germinació de les llavors procedents de fecundació creuada, conjuntament amb un possible efecte positiu de l'heterosi, podrien explicar el manteniment de la casmogamia en aquest endemisme mallorquí.

Abstract

Mountain ecosystems are biodiversity hotspots due to their geographical isolation, their role as a species refuge during past climate changes, and the wide range of environmental conditions present along their altitudinal gradients. The environmental gradients common in alpine systems allow the ecological responses of plants to different environmental conditions to be tested over short distances. They thus function as natural laboratories, especially useful for understanding plant responses to global change. For example, the presence of invasive species in these systems can lead to synergistic effects with those of climate change, having dramatic consequences for threatened native species or fragile ecosystems, such as those found in the high mountain zones of oceanic islands.

The main objective of this thesis is to explore physiological, reproductive and phenotypic responses of entomophilous species (genus *Viola*) in mountain systems to different biotic and abiotic conditions found along their altitudinal gradients.

In Chapters 1 and 2 the performance, reproductive success and floral traits of *Viola cheiranthifolia* (Tenerife) are assessed along its altitudinal gradient, as well as the interactive effects of an invasive herbivore (rabbits) on these characters. Despite increasingly adverse environmental conditions with altitude, *V. cheiranthifolia* did not show greater pollen limitation with altitude, which we attribute to compensatory mechanisms such as increased autogamy. The rabbits in the National Park are found to greatly reduce the performance and reproductive success of *V. cheiranthifolia.* This in turn alters the species-environment interactions along its altitudinal gradient and also the selection pressures that pollinators and abiotic conditions exert on their floral traits.

Chapter 3 explores how phenotypic features of the flowers and leaves of a high Andean violet (*Viola maculata*) vary in response to an altitudinal gradient. In this gradient, these two phenotypes may conflict, as large flowers may be more attractive to pollinators. However, if they are positively associated with larger leaves, a dilemma could arise in a drought scenario, as small leaves would be selected. In this study, the inverse water stress gradient (i.e. higher to lower altitude) in the Central Andes was confirmed, but no decoupled correlation was detected between floral and vegetative traits in the population with highest water stress, plants with small leaves also having small flowers. This absence of decoupling may be due to the high self-fertilization capacity of this species, and to the strong selection pressure exerted by water stress, constraining both phenotypes.

In Chapter 4, we analyse how the production of chasmogamous (CH) and cleistogamous (CL) flowers in *Viola jaubertiana* varies according to size, water stress and herbivory per individual, and across the altitudinal gradient. In addition, we also evaluated the mechanisms that may be favouring the maintenance of outcrossing in this predominantly cleistogamous species. *Viola jaubertiana* showed a great pollen limitation, besides a very low reproductive success of the CH flowers. In contrast, the CL flowers were much more successful; they were more economical to produce and provided 100 times more seeds than CH flowers. Despite this great imbalance, the greater mass, dispersion potential and germination of seeds from outcrossing, together with a probably positive effect of heterosis, could explain the maintenance of chasmogamy in this Mallorcan endemism.

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A. PRÓLOGO

Introducción general

La ecología reproductiva de las plantas alpinas y su vulnerabilidad al cambio global.

Los ecosistemas alpinos de alrededor del mundo son núcleos muy importantes de biodiversidad (Myers *et al.* 2000; Korner & Spehn 2002; Körner 2004; Molau 2004) debido a su papel como refugio de especies durante los cambios ambientales y climáticos del pasado, a su aislamiento geográfico y a la gran cantidad de hábitats que presentan las montañas a lo largo de su gradiente altitudinal. Sin embargo, son también considerados ecosistemas frágiles y amenazados (Watson & Haeberli 2004). En las últimas dos décadas, se ha documentado una notable pérdida de biodiversidad en muchos sistemas montañosos del mundo, especialmente en las zonas de alta montaña (Foster 2001; Klein *et al.* 2004; Pauli *et al.* 2012).

La principal amenaza a la que se enfrentan en la actualidad los ecosistemas montañosos es el cambio climático que está experimentando el planeta (IPCC 2007, Gottfried *et al.* 2012; Mayor *et al.* 2017) y que implica, por ejemplo, aumentos en la temperatura, cambios en los regímenes de acumulación de nieve y posterior deshielo. De hecho, los ecosistemas de montaña son unos sistemas de alerta temprana frente al cambio global. Los distintos escenarios propuestos de cambio climático para el siglo XXI predicen, de forma consistente, una reducción de los hábitats de alta montaña, con la consecuente pérdida regional de muchas especies que habitan en estos ecosistemas (Thuiller *et al.* 2005; Engler *et al.* 2011; Dullinger *et al.* 2012; Gottfried *et al.* 2012). Obviamente, esta pérdida de especies es especialmente preocupante al tratarse de ecosistemas que albergan una gran proporción de endemismos (Spehn *et al.* 2011; Steinbauer *et al.* 2016). Por ejemplo, en las regiones montañosas de Europa, el cambio climático está teniendo un efecto incluso mayor a las predicciones iniciales. Recientemente, se ha estimado que algunas especies de plantas en estas regiones desaparecerán en el transcurso de unas pocas décadas, ya que las más adaptadas al frío verán mermadas sus poblaciones, mientras que aquellas más adaptadas a condiciones cálidas incrementarán su número (Gottfried *et al.* 2012). Por otro lado, debido al calentamiento global, los rangos de distribución de las especies adaptadas a los climas fríos de la alta montaña podrían trasladarse progresivamente a mayores altitudes (Pauli *et al.* 2012).

Además del cambio climático, los ecosistemas montañosos también afrontan otros importantes riesgos como son las especies invasoras (Alexander *et al.* 2016; Lembrechts *et al.* 2016) que resultan particularmente dañinas en zonas aisladas como las partes altas de las montañas (Irl *et al.* 2012, 2014; Krushelnycky 2014), el riesgo de deriva genética e ”inbreeding” de muchas especies alpinas debido al reducido tamaño de sus poblaciones (Young & Clarke 2000), o incluso los riesgos asociados con la rápida industrialización y crecimiento de la población que se inició en el siglo XX en todo el mundo, como son la deforestación, el sobrepastoreo por ganado, o la reciente proliferación de turistas y de deportes de montaña (Godde *et al.* 2000; Beniston 2003). Por otra parte, la disminución del manejo tradicional de la tierra, conjuntamente con los efectos del cambio climático, están provocando en muchos casos un aumento de la densidad, cubierta y biomasa de las plantas leñosas en detrimento de las plantas herbáceas (Wookey *et al.* 2009; Brandt *et al.* 2013), pudiendo producir efectos en cascada, como la alteración de las redes de polinización en las comunidades alpinas (Lara-Romero *et al.* 2016a).

La polinización en la alta montaña

Las interacciones entre organismos de distintos niveles tróficos suponen un componente importante de la funcionalidad de los ecosistemas y constituyen la base de la llamada “arquitectura de la biodiversidad” (Bascompte *et al.* 2003). Por ejemplo, la mayoría de angiospermas depende en mayor o menor medida de la actividad de los polinizadores para su reproducción (Ollerton et al. 2011). Los estudios sobre interacciones planta-polinizador en ecosistemas de alta montaña son relativamente escasos. En dichos ambientes, las bajas temperaturas, las cortas estaciones de crecimiento y los fuertes vientos son los principales factores que limitan la disponibilidad de polinizadores (Arroyo *et al.* 1982, 1985; Gómez *et al.* 1996; Torres-Díaz *et al.* 2007). La diversidad y abundancia de polinizadores en estas zonas elevadas es menor que en las zonas de altitudes inferiores (Arroyo *et al.* 1982, 1985; Kearns & Inouye 1994; Gómez & Zamora 1999), su tasa de visita es también más baja (Eriksen *et al.* 1993; Kasagi & Kudo 2003) y en general poco eficientes, sobre todo dípteros de distintas familias (ej., Muscidae, Calliphoridae, Fannidae, Mycetophyllidae, Empididae), siendo también muy comunes las hormigas (Arroyo *et al.* 1982; McCall & Primack 1992; Gómez & Zamora 1999; Dupont *et al.* 2003). Además, las extremas y cambiantes condiciones climatológicas típicas de la alta montaña (McCall & Primack 1992; Bergman *et al.* 1996) generan una gran variabilidad en la abundancia y en la identidad de los polinizadores, tanto en el espacio como en el tiempo (Totland 1994; Gómez & Zamora 1999).

Debido a la baja accesibilidad que presentan las zonas de alta montaña, la información sobre la dependencia reproductiva que tienen las plantas alpinas de los polinizadores es limitada, aunque se piensa que las plantas con flores en los ecosistemas de montaña podrían ser especialmente susceptibles a las perturbaciones que afecten a sus polinizadores, porque conllevarían a su vez, consecuencias ecológicas y evolutivas para las plantas que dependen de ellos. Por ejemplo, una menor disponibilidad de polinizadores puede conllevar un menor disponibilidad de polen, provocando una limitación en la producción de semillas (García-camacho & Totland 2009). Por otro lado, una menor diversidad y una mayor variación en la disponibilidad de polinizadores podrían también promover una menor proporción de interacciones mutualistas planta-polinizador (ej. Kearns *et al.* 1998; Losey and Vaughan 2006). Del mismo modo, la escasez de polinizadores podría promover cambios en el sistema reproductivo de las plantas; para evitar una alta dependencia de ellos, éstas podrían hacerse autocompatibles y/o aumentar sus niveles de autogamia, aunque eso conlleve una reducción en la variabilidad genética de sus poblaciones (Jarne & Charleswoth 1993).

Los gradientes altitudinales como laboratorios naturales.

Los gradientes altitudinales son “laboratorios naturales” muy útiles para testar respuestas ecológicas y evolutivas de los organismos a diferentes condiciones ambientales, como pueden ser las bajas temperaturas (Körner 2007). Se pueden diferenciar dos tipos de cambios ambientales con la altitud: (1) cambios de tendencia general y (2) cambios de tendencia regional.

Los cambios ambientales que se producen con la altitud en todos los ecosistemas montañosos del mundo son la disminución de la temperatura, de la presión atmosférica y de la presión parcial de todos los gases atmosféricos, así como el aumento de la radiación y la fracción de radiación UV-B respecto al total de ésta. Otro aspecto muy importante que varía de forma general en todos los ecosistemas montañosos es la disminución del área disponible al aumentar la altitud. Éste es un importante motor de la diversidad y evolución de las especies montañosas (MacArthur & Wilson 1967; Rosenzweig 2003). A medida que aumentamos en altitud, disminuye la disponibilidad de espacio para los organismos y aumenta el aislamiento entre sus poblaciones. Este aislamiento espacial intensifica la diferenciación de las poblaciones y, por tanto, las posibilidades para que se den procesos de especiación (Chapin & Körner 1994; Steinbauer *et al.* 2016). Por ello, el porcentaje de endemismos en las zonas alpinas aumenta de manera consistente con la altitud (Steinbauer *et al.* 2016), aunque el número global de especies disminuye a medida que aumentamos en altitud (40 angiospermas menos por cada 100 metros; Körner, 2000; Vetaas and Grytnes, 2002).

Por el contrario, los cambios de tendencia regional, como son la precipitación, la humedad del suelo, la velocidad del viento y la estacionalidad, no varían de igual forma en los diferentes sistemas montañosos del mundo (Körner 2007). Por lo tanto, cada sistema montañoso es el resultado del efecto combinado de los fenómenos generales altitudinales y de las peculiaridades regionales de cada montaña. De ahí la importancia de no generalizar al hablar de sistemas montañosos y controlar factores como la humedad del suelo en cualquier estudio que trabaje con gradientes altitudinales para testar respuestas adaptativas y fenotípicas de las plantas.

Hasta la fecha se han realizado un gran número de estudios de cómo varían las respuestas de las plantas a lo largo de un gradiente altitudinal, tanto a nivel individual como a nivel de comunidad y ecosistema (Giménez-Benavides *et al.* 2007; García-Camacho & Escudero 2009; García-Fernández *et al.* 2012; Lara-Romero *et al.* 2014b). Se ha comprobado, por ejemplo, que las comunidades vegetales varían tanto en la abundancia de especies presentes como en los rasgos intra-específicos, para ajustarse a los cambios ambientales que se dan a lo largo del gradiente (Pescador *et al.* 2015). También puede variar el tipo de interacciones (competencia, neutralidad o facilitación) entre las especies de las comunidades a lo largo del gradiente altitudinal. Así, se ha encontrado que la proporción de especies que experimenta procesos de facilitación mediada por polinizadores puede aumentar con la altitud (Tur *et al.* 2016) y se ha sugerido que, bajo condiciones desfavorables y con bajas densidades de plantas, los procesos de facilitación son más comunes (Callaway *et al.* 2002; Cavieres *et al.* 2016). En esta tesis me he centrado en los cambios a nivel individual, sin evaluar las bases genéticas de dichos cambios, y obviando los cambios a nivel de comunidad y ecosistema.

Existe una gran variedad y abundancia de estudios intra-específicos que testan las respuestas de las plantas a lo largo de su gradiente altitudinal de distribución:

(1) Estudios de respuestas fisiológicas y de caracteres funcionales. Así, con el aumento de la altitud, se espera que las condiciones ambientales más severas y la menor disponibilidad de recursos promuevan especies más tolerantes al estrés, que invierten más carbono por superficie de hoja (Körner 2012; Read *et al.* 2014). En cambio, en los hábitats alpinos de las zonas del Mediterráneo, la sequía puede limitar el rendimiento fotosintético de las plantas en las poblaciones a menor altitud (Loik & Redar, 2003; Hernández-Fuentes et al., 2015), al igual que fomentar la presencia de hojas más esclerófilas.

(2) Estudios de respuestas fenotípicas, como el desarrollado por Grassein *et al.* (2014), en el cual se ha visto que las respuestas fenotípicas (biomasa) de cinco especies de plantas a lo largo de un gradiente no siguen un patrón claro con la altitud, sino que dependen de las condiciones ambientales que ocurren localmente en cada sitio (principalmente disponibilidad de agua), y de los efectos interactivos de las especies circundantes. Otro ejemplo es el de Herrera (2005) con *Rosmarinus officinalis*, donde encontró un incremento del tamaño de las flores con la altitud que estuvo totalmente desacoplado

con la respuesta del tamaño de las hojas, argumentándose que podía ser debido a la presión selectiva ejercida por polinizadores de mayor tamaño en las zonas altas.

(3) Estudios de dinámica poblacional, como los que se han llevado a cabo con *Silene ciliata* en Guadarrama (Giménez-Benavides *et al.* 2011), o con *Argyroxyphium sandwicense* en la isla de Maui (Krushelnycky 2014). Estos estudios indican que las poblaciones a menor altitud están sufriendo unas condiciones ambientales más estresantes, limitando su supervivencia y disminuyendo su tasa de crecimiento.

(4) Estudios de dispersión y fragmentación, que predicen un efecto escalador de las poblaciones de alta montaña (Marris 2007), aumentando el aislamiento entre las poblaciones, y disminuyendo su capacidad de dispersión (Herrera and Bazaga, 2008).

(5) Estudios genéticos, donde se destaca el importante papel de los gradientes altitudinales en las plantas alpinas, dando lugar a estructuración genética con importantes implicaciones evolutivas y de conservación (Jump *et al.* 2006; Mathiasen & Premoli 2016). Así, Lara-Romero et al. (2014), encontraron que la variación fenotípica en rasgos reproductivos y fenológicos observada a lo largo del gradiente en *Armeria caespitosa* tiene una base genética, y que esa variabilidad genética asociada con la fenología de floración puede ser relevante en la especie para hacer frente al cambio climático.

(6) Estudios de éxito reproductivo. A medida que aumenta la altitud, se espera que las condiciones favorables para la polinización disminuyan, debido a las bajas temperaturas y al fuerte viento, factores que restringen notablemente la actividad de los visitantes florales (Arroyo *et al.* 1985; McCall & Primack 1992; Totland 1994). Por lo tanto, cabría esperar que eso supusiera un problema para las plantas alpinas, traducido en una importante limitación de polen, y un menor éxito reproductivo en las poblaciones más elevadas. El meta-análisis desarrollado por Camacho y Totland (2009) confirmó la presencia de limitación de polen en las especies alpinas. Sin embargo, no se corroboró que la limitación de polen aumente con la altitud, como cabría esperar. Ello se debe, en gran medida a la gran variedad de mecanismos compensatorios que se han observado en las plantas alpinas a lo largo de su distribución altitudinal. Por ejemplo, se ha detectado la presencia de polinizadores más eficientes en las zonas altas respecto a las bajas (como es el caso de la mayor abundancia de los eficientes abejorros; Arroyo et al., 1985; Galen and Stanton, 1989), una mayor longevidad de las flores en las poblaciones a mayor altitud (Pacheco *et al.* 2016), o un incremento de los niveles de autofecundación con la altitud (Medan *et al.* 2002; Arroyo *et al.* 2006).

En los cuatro capítulos que componen esta tesis, se han testado diferentes tipos de respuestas y adaptaciones de especies del género *Viola* a lo largo del gradiente altitudinal donde se desarrollan: respuestas *fenotípicas* (integración fenotípica, morfología de la flor, inversión en floración, y correlaciones entre el tamaño de las flores y las hojas), *funcionales* (área específica de la hoja; SLA), *fisiológicas* (conductancia estomática y rendimiento fotosintético) y *reproductivas* (limitación de polen, autogamia, fenología, tasa de visitas de polinizadores, fructificación, producción de semillas, viabilidad y germinación).

Efectos sinérgicos de las especies invasoras y el cambio climático en los sistemas de montaña. Especial vulnerabilidad en las islas oceánicas.

Actualmente, la proliferación de especies no nativas está considerada la segunda causa de pérdida de biodiversidad, después de la destrucción de los hábitats (UICN, lista roja de especies amenazadas). Además, los efectos de las invasiones biológicas pueden interactuar con los efectos del cambio climático (Walther *et al.* 2009; Mainka & Howard 2010), probablemente intensificando los impactos sobre los ecosistemas y aumentando la necesidad de gestionar eficazmente a las especies no nativas (Hellmann *et al.* 2008; Vorsino *et al.* 2014). En el caso de las islas, las especies invasoras son una de las mayores amenazas para su biota nativa, al tratarse a menudo de especies con mayores tasas de dispersión y reproducción, mayor plasticidad fenotípica y mayor tolerancia a los cambios climáticos que las especies endémicas que han evolucionado en condiciones de aislamiento (Harter *et al.* 2015).

Por ejemplo, se ha observado que la presión ejercida por herbívoros introducidos puede provocar efectos sinérgicos negativos en especies nativas ya amenazadas por el cambio climático (Gangoso *et al.* 2006; Krushelnycky 2014). Estos efectos sinérgicos, si ocurren, pueden tener aún peores consecuencias en islas oceánicas, donde la mayoría de las plantas nativas han perdido o nunca han desarrollado mecanismos de defensa frente a la herbivoría (Bowen & Van Vuren 1997) porque los mamíferos herbívoros estuvieron ausentes en la gran mayoría de islas hasta la llegada del hombre (Atkinson 1989). Aun así, muy pocos estudios han testado los efectos interactivos del cambio climático y la herbivoría por parte de mamíferos no nativos sobre el éxito reproductivo y la supervivencia de especies amenazadas en islas oceánicas. En la isla de La Palma (Canarias), Irl et al. (2012, 2014) encontraron que la presencia de mamíferos introducidos afectaba los patrones de dominancia de las especies de plantas y empobrecía la composición de las comunidades alpinas oceánicas, debido a la proliferación de unas pocas especies con defensas frente a la herbivoría.

Por lo tanto, a la hora de estudiar cómo responde el desarrollo y éxito reproductivo de una planta alpina a lo largo de su gradiente altitudinal de distribución, es muy importante tener en cuenta el posible efecto que pueda ejercer la presión de herbivoría a lo largo del gradiente. Herrera (2000), en un estudio con *Paeonia broteroi*, ya advirtió de los riesgos que conlleva el estudiar interacciones planta-animal, centrándose simplemente en un tipo de interacción (siendo los estudios de herbivoría o de polinización los más comunes), ya que los efectos de los distintos tipos de interacciones, con frecuencia, no son aditivos. Por ejemplo, las presiones de selección ejercidas por los polinizadores pueden ser diferentes dependiendo de la acción de los herbívoros. Utilizando cercados de exclusión de ungulados en Sierra Nevada (España), Gómez (2003) encontró que las presiones de selección ejercidas por los polinizadores sobre varios rasgos florales (ej. número de flores) y morfológicos de *Erysimum mediohispanicum* desaparecieron con la presencia de los herbívoros. Además, la herbivoría reduce notablemente el rendimiento y éxito reproductivo de las plantas, afecta a la dinámica de sus poblaciones y a su distribución en el hábitat (Gómez 2005).

En el capítulo 1, se estudia el efecto que ejercen el conejo, especie invasora en las islas Canarias, sobre la respuesta reproductiva de una planta clave en el ecosistema alpino del Teide (*Viola cheiranthifolia*), a lo largo de su amplio rango altitudinal de distribución. En el capítulo 2, se investiga el efecto interactivo de la herbivoría por conejo sobre la morfología floral y la abundancia de flores, y éstos a su vez, sobre el éxito reproductivo de la violeta del Teide.

Particularidades del género *Viola*. El porqué de su elección como modelo de estudio.

El género *Viola* comprende cerca de 600 especies distribuidas en al menos 17 secciones o linajes (Wahlert *et al.* 2014; Marcussen *et al.* 2015), la mayoría de ellas alopoliploides. Se sabe además que la hibridación y la poliploidía han jugado un papel muy importante en la historia evolutiva de este género, con una gran variación de números cromosómicos y grados de ploidía (Ballard *et al.* 1999; Marcussen *et al.* 2010). Su distribución abarca las regiones templadas del hemisferio norte, aunque sus centros de diversificación se encuentran principalmente en zonas montañosas del mundo, como son los Andes, donde según las evidencias biogeográficas, cariológicas y filogenéticas parece ser que se originó el género (Ballard *et al.* 1999; Marcussen *et al.* 2012). Son mayormente especies herbáceas perennes, aunque también se encuentran algunas especies anuales, fundamentalmente en los Andes del centro de Chile, y algunas especies arbustivas, como las presentes en Hawaii (Ballard & Sytsma 2000). Su tamaño puede ser muy variable. Por ejemplo, *Viola lilliputana*, que vive a más de 4,000 m de altura en el altiplano andino, es una de las angiospermas más pequeñas del mundo (Ballard & Iltis 2012).

En muchas de las especies del género se ha reportado la presencia de cleistogamia dimórfica, esto es, la producción, a través de diferentes vías de desarrollo (Culley & Klooster 2007), de flores casmógamas (CH), abiertas a la polinización, y flores cleistógamas (CL; que se asemejan a botones florales), de pedúnculo más corto, que no se abren y que por tanto producen semillas como resultado de la autofecundación. La producción de ambos tipos de flores de forma inherente en un mismo individuo facilita la presencia de una mezcla de autofecundación y fecundación cruzada en estas especies (Darwin 1877a). La producción de cleistogamia parece haber evolucionado de forma independiente en al menos 50 familias de plantas (Lord 1981; Culley & Klooster 2007). Además, hay muy pocos casos de pérdidas evolutivas de cleistogamia (Culley & Klooster 2007).

Las flores casmógamas presentan una estructura que suele dificultar la auto-polinización, particularmente en las especies que presentan cleistogamia, ya que estas últimas son las que aseguran la descendencia en caso de que no haya polinizadores. Aun así, se ha descrito también autogamia retardada en las cleistógamas V. *canadensis* y V. *pubescens* (Culley 2000, 2002), pero no es un proceso extendido en el género (Beattie 1969; Banasinska & Kuta 1996). La autogamia retardada ocurre en las flores CH que no han sido polinizadas, mediante la inclinación del estigma sobre el pétalo anterior, que normalmente presenta pelos para facilitar la retención de los granos de polen que han caído de las anteras. Mediante emasculaciones y observaciones de estigmas en varias especies de *Viola*, se ha visto que la transferencia de polen conespecífico y heterospecífico es frecuente, aunque depende de varios factores (Beattie 1969). La lluvia, la disposición en lugares sombríos con poca luz incidente y la coexistencia de especies de otros géneros de floración simultánea se ha visto que disminuyen el número de visitas (Beattie 1969). A pesar de la observación de gran cantidad de granos de polen depositados en los estigmas (Beattie 1969), la observación de visitas de polinizadores tiende a ser muy poco frecuentes (West 1930; Culley 2002). Entre los polinizadores observados en *Viola,* hay abejorros, mariposas, polillas, abejas solitarias y sírfidos (Darwin 1877a; Beattie 1972; Herrera 1996; Freitas & Sazima 2003). Sus polinizadores suelen llevar poco polen heterospecífico, por lo que la polinización entomófila en este género es altamente efectiva (Beattie 1972). Normalmente, las especies del género *Viola* son de floración temprana, así compiten por los polinizadores con un número menor de especies. Por tanto, al ser un género con un gran número de especies que viven en zonas de alta montaña, resultan ideales para testar respuestas y adaptaciones a lo largo de sus gradientes altitudinales de distribución. Además, puede ser un género muy útil para testar el efecto de las diferentes condiciones ambientales asociadas al gradiente altitudinal sobre la eficiencia de la polinización en las flores casmógamas, y/o la inversión en un tipo u otro de flor (autofecundación o fecundación cruzada).

Por último, otra particularidad que hace muy interesante el estudio del género *Viola* es intentar descifrar el enigma sobre las fuerzas que mantienen la fecundación cruzada en especies que presentan un sistema reproductivo mixto de cleistogamia y casmogamia. La cleistogamia es más económica en términos energéticos, y mucho más efectiva. Sin embargo, las ventajas de producir descendencia cruzada, aunque sea más incierta y costosa, deben ser mucho mayores que las de la autofecundación, en algunos casos debiendo explicar una ventaja del coste por semilla 342% superior en la cleistogamia (Oakley *et al.* 2007). La plasticidad fenotípica adaptativa (Schoen & Lloyd 1984) es la hipótesis que se plantea comúnmente para explicar al mantenimiento de este sistema de reproducción. Los individuos producirían el tipo de flor (CH o CL) que permita asegurar mayor descendencia dependiendo de las condiciones ambientales. En la mayoría de los casos (alrededor de 20) en los que se ha testado la hipótesis de Schoen and Lloyd (1984), se ha encontrado una mayor producción de flores cleistógamas en respuesta a condiciones más desfavorables para la polinización. Además, en prácticamente todos estos casos estudiados, la producción de cleistogamia es mayor que la de casmogamia con independencia del ambiente. Por otro lado, la heterosis es otra explicación propuesta al mantenimiento de un sistema reproductivo mixto (Lu 2002). La casmogamia reduce la presencia de mutaciones deletéreas que se pueden acumular con la autofecundación, ya que permite el cruzamiento (vía polinizadores) de dos líneas derivadas de la autofecundación. Por último, la presencia de ventanas temporales con una mayor producción de descendencia por casmogamia, o una mayor competitividad de la CH respecto a la CL en el posterior reclutamiento (Waller 1980; Schmitt & Ehrhardt 1987), también podría explicar que a la larga este sistema permanezca estable.

En los capítulos 3 y 4, se corrobora si la inversión en flores CL es mayor en las zonas más altas, donde las condiciones para la polinización deberían ser más desfavorables, y se evalúa qué factores (herbivoría, tamaño y estrés hídrico) promueven la producción de un tipo u otro de flor. Por otra parte, en el capítulo 4, se intentan descifrar los motivos por los cuales se mantiene este sistema mixto de reproducción en una violeta endémica de Mallorca (*Viola jaubertiana*), que presenta una elevada producción de flores casmógamas pero descritas como estériles por su descubridor (Marès & Vigineix, 1880).

Objetivos generales de la tesis

El principal objetivo de esta tesis es explorar las respuestas fisiológicas, reproductivas y fenotípicas de diversas especies de plantas entomófilas de montaña del género *Viola* a las distintas condiciones bióticas y abióticas que se encuentran a lo largo de sus respectivos gradientes altitudinales de distribución.

En los capítulos 1 y 2 se estudian la eficacia biológica, la reproducción y los rasgos florales de la violeta del Teide (*Viola cheiranthifolia*) en su gradiente altitudinal de distribución, así como el efecto de la presencia de un herbívoro invasor (el conejo, *Oryctolagus cuniculus*) sobre estos caracteres. El capítulo 3 se centra en determinar cómo varían los rasgos fenotípicos de las flores y de las hojas de una violeta alto-andina (*V. maculata*) en respuesta a un gradiente altitudinal, con un mayor estrés hídrico en las zonas bajas y un mayor estrés biológico (escasez de polinizadores) en las zonas altas. En el capítulo 4, se analiza cómo varía la producción de flores casmógamas (CH) y cleistógamas (CL) en la especie *V. jaubertiana* en función de las condiciones individuales de tamaño, estrés hídrico y herbivoría presentes a lo largo de su gradiente altitudinal. Además, también se evalúan los mecanismos que pueden estar favoreciendo el mantenimiento de la fecundación cruzada en una especie predominantemente cleistógama.

A continuación, se detallan los objetivos particulares abordados en cada uno de los capítulos:

1. (Capítulo 1) Explorar cómo varían la eficacia biológica y el éxito reproductivo de una especie de alta montaña a lo largo de su amplio gradiente altitudinal (cerca de 1,300 m), así como evaluar si esos cambios son consistentes entre años.

2. (Capítulo 1) Determinar si las poblaciones más elevadas (> 3,000 m) de la violeta del Teide presentan limitación de polen y corroborar si la autofecundación podría actuar como mecanismo compensatorio cuando ésta ocurre.

3. (Capítulo 1) Evaluar los efectos de la herbivoría por conejo sobre las respuestas observadas (de eficacia biológica y de éxito reproductivo) en *V. cheiranthifolia* a lo largo de su gradiente altitudinal de distribución.

4. (Capítulo 2) Analizar la variación de los rasgos florales en *V. cheiranthifolia* a lo largo de su gradiente altitudinal de distribución.

5. (Capítulo 2) Evaluar la influencia que ejercen las condiciones específicas de estrés hídrico y presión por herbivoría sobre los rasgos florales de *V. cheirantifolia*.

6. (Capítulo 2) Explorar cómo afectan los rasgos florales, el despliegue floral y la densidad de flores conspecíficas vecinas al éxito reproductivo de cada individuo.

7. (Capítulo 3) Estudiar la respuesta fisiológica de *V. maculata* al gradiente de estrés hídrico asociado a su distribución altitudinal; en las zonas bajas de su distribución (próximas al límite del bosque), el estrés hídrico es mayor que en las zonas altas (próximas a la cima).

8. (Capítulo 3) Determinar el tipo de estrategia reproductiva que adopta *V. maculata* para ajustarse a las condiciones más desfavorables para la polinización presentes en zonas más elevadas: (a) modificar la proporción de flores casmógamas y cleistógamas, o alternativamente: (b) incrementar la autofecundación retardada en las flores casmógamas.

9. (Capítulo 3) Determinar si la integración fenotípica y los patrones de correlación entre el área floral y el área foliar de los individuos varía a lo largo del gradiente altitudinal, de acuerdo con las condiciones bióticas y abióticas presentes en cada altitud.

10. (Capítulo 4) Estudiar la fenología de la floración de *V. jaubertiana* en diferentes poblaciones altitudinales (0-1100 m), considerando la producción de dos tipos de flores (CH and CL).

11. (Capítulo 4) Explorar cual es el coste energético de producir semillas en ambos tipos de flores (CH and CL).

12. (Capítulo 4) Identificar qué factores determinan la producción de cada tipo de flor (CH and CL): altitud, herbivoría, tamaño de la planta o estrés hídrico.

13. (Capítulo 4) Evaluar si las semillas producidas por ambos tipos de flores (CH y CL) difieren en sus rasgos (peso y producción del elaiosoma), así como en su capacidad de germinación.

B. MAIN CHAPTERS

Chapter 1

Species-environment interactions changed by introduced herbivores in an oceanic high-mountain ecosystem

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Abstract

Summit areas of oceanic islands constitute some of the most isolated ecosystems on earth, highly vulnerable to climate change and introduced species. Within the unique high-elevation communities of Tenerife (Canary Islands), reproductive success and thus long-term survival of species may depend on environmental suitability as well as threat by introduced herbivores. By experimentally modifying the endemic and vulnerable species *Viola cheiranthifolia* along its entire altitudinal occurrence range, we studied plant performance, autofertility, pollen limitation and visitation rate and the interactive effect of grazing by non-native rabbits on them. We assessed the grazing effects by recording (1) the proportion of consumed plants and flowers along the gradient, (2) comparing fitness traits of herbivore-excluded plants along the gradient, and (3) comparing fitness traits, autofertility and pollen limitation between plants excluded from herbivores with unexcluded plants at the same locality. Our results showed that *V. cheiranthifolia* performance is mainly affected by inter-annual and microhabitat variability along the gradient, especially in the lowest edge. Despite the increasingly adverse environmental conditions, the plant showed no pollen limitation with elevation, which is attributed to the increase in autofertility levels (≥ 50% of reproductive output) and decrease in competition for pollinators at higher elevations. Plant fitness is, however, extremely reduced owing to the presence of non-native rabbits in the area (consuming more than 75% of the individuals in some localities), which in turn change plant trait-environment interactions along the gradient. Taken together, these findings indicate that the elevational variation found on plant performance results from the combined action of non-native rabbits with the microhabitat variability, exerting intricate ecological influences that threaten the survival of this violet species.

Keywords: Autofertility; Canary Islands; elevational gradient; herbivory; pollen limitation; rabbits; *Viola cheiranthifolia.*

1.1 Introduction

Mountain ecosystems worldwide provide sharp elevational gradients that allow testing ecological responses of biota to different abiotic influences across short distances (Dunne *et al.* 2003; Körner 2007), and therefore, are especially useful for understanding the response of plants to global change (Körner 2007). Thus, alpine plant populations occurring at the lowest altitudinal distribution of the species should face especially harsh constraints on survival and performance mainly due to water scarcity and high temperatures, giving rise to species range shifts upward (Peñuelas & Boada 2003; Parmesan 2006). Moreover, the complex environmental heterogeneity present in alpine ecosystems may lead to phenotypic plasticity, and in some cases to genetic selection in favour of a particular reproductive behavior in a specific environment and, hence, to local adaptation in plants (Rahn 1983; Körner 2003).

Reproductive efficiency is one of the major constraints in high-mountain ecosystems, as the environmental conditions in alpine habitats often reduce the possibilities of cross-pollination, affecting several fitness traits such as pollinator visitation rate (McCall & Primack 1992; Totland 1994) and fruit and seed set (Santandreu & Lloret 1999; Lundemo & Totland 2007; Ye *et al.* 2011). However, pollen limitation does not seem to differ overall between alpine and lowland species (García-Camacho and Totland 2009), suggesting that alpine species may have compensatory mechanisms such as higher pollinator efficiency and extended flower longevity (Arroyo *et al.* 1985; Fabbro & Körner 2004) to compensate for a lower visitation frequency in terms of their reproductive success. Furthermore, alpine plants show a great variability in germination behavior, and may largely vary within a single species from one population to another (Urbanska & Schütz 1986; Giménez-Benavides *et al.* 2005).

On oceanic islands, high mountain areas represent one of the most isolated ecosystems on earth, which confers an outstanding uniqueness on their biota (Steinbauer *et al.* 2012). This biota frequently displays high endemicity (Steinbauer *et al.* 2016), usually well adapted to poor developed soils and to hydric, thermic or aeolian stresses, constituting islands within islands (MacArthur & Wilson 1967; Steinbauer *et al.* 2016). Therefore, high-mountain insular biota is particularly vulnerable to climate change and disturbance (Harter *et al.* 2015). The special vulnerability of this 'sky island' biota is mainly due to their small and isolated distributions, which prevent species migrating and escaping to a more favorable location (Fernández-Palacios *et al.* 2014). Consequently, rapid adaptation or high plasticity would be needed to cope ongoing climatic changes (Ohlemüller *et al.* 2008; Hampe & Jump 2011) Moreover, oceanic islands have relatively low species richness, simplified biotic community structures, and are characterized by having limited biotic exchange compared to mainland ecosystems (Whittaker & Fernandez-Palacios 2007;

Fordham & Brook 2010; Gilman *et al.* 2010). Hence, changes in the abundance of single members of functional groups (e.g., pollinators, herbivores) are likely to affect the structure and functional integrity of communities on oceanic islands more than in mainland areas. This is why the introduction of non-native species is one of the greatest threats to oceanic island ecosystems (Gillespie *et al.* 2008; Irl *et al.* 2014; Kueffer *et al.* 2014).

Introduced species are expected to interact with climate change (Walther *et al.* 2009; Mainka & Howard 2010), which will likely intensify impacts on island ecosystems, increasing the need to effectively manage non-native species (Hellmann *et al.* 2008; Vorsino *et al.* 2014). Antagonistic interactions, such as grazing pressure by herbivores, can cause profound synergistic effects on climatically weakened native plant species (Gangoso *et al.* 2006; Krushelnycky 2014). These synergistic effects can be even worse in oceanic islands, where most plant species have either lost or never developed defense mechanisms against herbivory. Such impacts are likely to be greater than simple additive effects of single stressors (Brook *et al.* 2008; De Sassi *et al.* 2012) and, thus, can even have more dramatic consequences on the fragile ecosystems of summit oceanic areas.

Our general objective in this study was elucidating the factors affecting performance of an oceanic high-mountain endemic plant, *Viola cheiranthifolia* Humb. & Bonpl, Specifically, we asked the following questions: 1) Does plant performance change along the pronounced elevational gradient (c. 1300 m), and are changes consistent between years? 2) Is the plant pollen-limited at high elevations, and does autofertility act as a compensatory mechanism? and 3) How does herbivory by rabbits (*Oryctolagus cuniculus* L.) influence plant performance along the elevational gradient? We expected that plant performance is limited at both edges of the altitudinal range, due to the warmer and dryer conditions (at the lowest edge) and to the harsh conditions for pollination (at the summit). We also expected to find pollen limitation at the summit due to possible lower pollinator visit rates, which could be compensated by higher selfing rates. Finally, we hypothesized that rabbits may be strongly altering the plant trait-environment interactions along the gradient, since they are known to cause pernicious effects on other endemic and threatened plant species of summit scrub systems of oceanic islands (Carqué-Álamo *et al.* 2004; Irl *et al.* 2012).

1.2 Materials and methods

Study system

Viola cheiranthifolia is a high mountain dwarf chamaephytic plant, endemic to Tenerife. It is the most dominant and structuring species within the summit vegetation of El Teide. The largest populations are found around El Teide stratovolcano at elevations from c. 2400 m to c. 3700 m at the peak of El Teide, although some small populations occur at the highest points of the caldera (Guajara; 2715 m, Pasajirón; 2531 m). The plant grows on poor soils on cinder flats amongst the volcanic rubble, mixed with pumice stones at some localities. Its height usually ranges from 3 to 6 cm, and it has oval-shaped hairy leaves. The species produces chasmogamous zygomorphic flowers mainly from late April to early July (although some flowers can be sometimes observed by mid-late winter). Flowers are found on 6-10 cm peduncles; they remain open for approximately 10 days, being white for the first 24-48 hours after anthesis and becoming purple-violet afterwards. They have a short nectar spur (9 mm long) and produce a small amount of nectar. According to the Viola dispersal syndromes described by Beattie and Lyons (1975), *V. cheiranthifolia* presents typical diplochory, with explosive ejection of seeds, and a small caruncle (shrunk elaiosome) permitting post-dispersal by ants (myrmechory). Despite this, no secondary dispersal by ants has ever been recorded.

Research site

Fieldwork was conducted during four years (2012-2015) at El Teide National Park (N.P). El Teide stratovolcano (3718 m; 28º16’15’’N 16º38’21’’O), like many tropical and sub-tropical alpine landscapes, presents xeric, arid environments (Leuschner 2000). The peak of El Teide (La Rambleta, hereafter) has a mean annual precipitation of 146 mm and a mean temperature of 4.2 º C (period 1989-1992, ICONA: in Gieger & Leuschner 2004). We set an elevational gradient along four different localities of *V. cheiranthifolia*, separated between 200 and 600 m from each other: Montaña Rajada (2400 m), Montaña Blanca (2700 m), Refugio de Altavista (3300 m), and La Rambleta (3500 m) (Fig. 1A). Temperature and edaphic (soil moisture and organic matter) data were gathered at each site to control for differential abiotic conditions along the gradient (Table 1). All study sites were located on the SE face of the stratovolcano (the only slope that covers the entire elevational distribution of the plant) in order to more accurately control for the elevational effect (Fig. 1A). Plant density varied depending on microsite availability in each site; overall, however, plant density in our study sites were rather similar, ranging from 0.01 to 0.03 individuals/m^2.

Effect of elevation on plant performance

Phenology, plant size, and flower display were studied along the main elevational gradient in 2012 and 2013 on a total of 329 and 204 individually marked plants, respectively. Since early April, we began marking reproductive individuals until we reached a minimum of 50 per locality. During 2013, aside from sprouted individuals marked in 2012, we added some new reproductive individuals to reach this number. To assess differences in flowering and fruiting phenology, each locality was visited once every 3-7 days until August. In each visit, we recorded the number of buds and flowers at different stages of anthesis, as well as the number of fruits produced. Plant size (maximum length and width of each individual) was also measured to account for its potential effect on reproductive success.

To quantify the reproductive success along the elevational gradient during 2012 and 2013, differences in fruit set (number of flowers setting fruits) and number of seeds per capsule were compared using 428 control flowers in a total of 106 marked individuals. Seed production was quantified by individually bagging 245 fruits (in a total of 97 individuals) before ripening and counting the number of seeds after fruits opened. All marked individuals from the lowest elevation (2400 m) had to be excluded from the reproductive success analyses because the number of reproductive individuals was very low (only 7 individuals in 2012) or even nil (in 2013).

To evaluate the level of autofertility (capacity to produce seeds by spontaneous self-pollination; Eckert *et al.* 2010), half of the flower buds available per individual were bagged before flower anthesis with a mesh-cloth that excluded floral visitors. Control flowers (open-pollination) from the same individuals were also marked and left un-bagged. Due to the high flower consumption by herbivores, we finally also used flowers from non-treated individuals as control. Autofertility was quantified in 2012 and 2013 in a total of 126 bagged flowers from 53 individuals from three different localities at 2700 m, 3300 and 3500 m (20, 15 and 18 individuals, respectively).

To examine whether the extent of pollen limitation differed among localities, we hand-pollinated half of the flowers available per plant, leaving the other half as controls. Pollen addition (PA) was accomplished by brushing pollen-laden anthers of about 5-10 plants from another population. This procedure was repeated two days during stigma receptivity. Pollen limitation was quantified in two consecutive years (2014 and 2015) in a total of 49 individuals from three different localities (23 individuals at 2700 meters in 68 PA flowers, 15 individuals at 3300 m in 29 PA flowers, and 11 individuals at 3500 m in 44 PA flowers).

Floral visitors were quantified and compared along the elevational gradient during 2014 and 2015. We conducted a total of 182 censuses of 15 min (32 censuses at 2400 m, 57 at 2700 m, 47 at 3300 m and 46 at 3500 m). We measured flower visitation rate as the number of visits per hour per flower. Censuses were carried out in no-windy days, mostly from 10:00 to 16:00, avoiding the beginning and the end of the day, with usually low temperatures and little insect activity in this high mountain environment. Only visitors touching the reproductive parts of the flowers were considered; these were either identified in the field or collected for later identification in the laboratory.

Seed germination was assessed along the elevational gradient in 2012. Of the 1,224 seeds collected, 44.5% were stored in the dark at room temperature and the remaining 55.5% underwent a stratification treatment (2-month below 5ºC). Seeds were buried in late February 2013, in a greenhouse located within the N.P at 2000 m. Moreover, during 2013 we evaluated the seed viability by means of the tetrazolium-chloride test in a total of 3824 control seeds.

Effect of herbivore exclusion on plant performance

The effect of herbivory upon *V. cheiranthifolia* was assessed by: 1) recording, at all localities, the proportion of individuals consumed (totally or partially) by rabbits (clipping off sprigs, stems and buds) and the number of consumed flowers per individual in 2012 and 2013 (besides rabbits, lizards and grasshoppers were occasionally observed consuming flowers); 2) comparing fitness traits (size, floral display, fruit and seed set) in protected individuals (by individual exclusion cages; Fig. 1C) during a two year period (2014 and 2015) among elevational populations; and 3) comparing plant density, fitness traits, autofertility, and pollen limitation (with the same methods described above) between 25 individuals inside and 35 outside a rabbit exclusion fence during 2013. This herbivore exclusion (20 x 20 m, located at 3500 m; Fig. 1B) was set up by the N.P. in 2009. Individuals outside the exclosure were used as controls, and were located between 5 and 90 m from the fence. The effect of herbivore exclusion on seed viability was assessed by comparing a total of 1,511 seeds from 25 individuals within the exclosure with 677 seeds from 19 individuals outside it.

Finally, we wanted to determine whether herbivore exclusion affected flower visitation rate. For this, we carried out pollinator censuses inside and outside the exclosure. A total of 49 and 46 censuses of 15 minutes were accumulated during 2014 and 2015 within and outside the exclosure, respectively; individuals selected for censuses outside were at a minimum distance of 5 m and a maximum of 100 m from the fence.

Data analyses and statistics

To test for differences in individual size among localities along the elevational gradient and between years, plant area was estimated by using maximum perpendicular diameters of the whole plant, subsequently log10-transformed to meet assumptions of normality. We used General Linear Mixed Models (GLMM; package lm4 in R) for the analyses of between-year and among-populations variations in plant size, flowering probability, floral display, fruit set, seeds per capsule, pollination rate, and herbivore rates. We included elevation, year, and the elevation x year interaction as fixed factors, plant individual as random effect, and plant size as covariate; for some variables, the interaction between plant size and elevation was also included in the model. Seed viability was compared in 2013 among elevations including plant size as covariate and individual plant as random effect. Resprouting probability (measured only in 2013) was compared among elevations using General Linear Models.

The levels of autofertility and pollen limitation in V. cheiranthifolia were compared across localities by means of GLMM, with fruit set and seed set as dependent variables, pollination treatment and elevation as fixed factors, and individual plant and year as random effects. Germination rate was compared across localities using the Kolmogorov-Smirnov test.

To test for plant responses to elevation after one and two years of individual protections we used size, floral display, seed set and fruit set as dependent variables, elevation, year and the elevation x year interaction as fixed factors, and plant individual as random effect. Finally, to test for the influence of the four-year herbivore exclusion on plant fitness, we compared those four variables between excluded and non-excluded plants.

We used error distribution and link functions that best fit the data: (1) binomial distribution for fruit set, seed viability, and probability of flowering and resprouting (2) a Gaussian distribution for plant size, (3) a Poisson distribution for floral display and seeds per capsule, and (4) a gamma distribution and inverse link function for pollination rate (visitation rate + 1, in order to avoid zeros in the response variable). When seeds per capsule showed overdispersion (this occurred when comparing elevations), we performed a negative binomial model (Zuur et al., 2009). All predictor variables showed VIF values smaller than three and were, therefore, included in the analyses (Zuur et al., 2009). We used the Akaike information criterion to select the best models with the package MuMIn 1.15.6, with ΔAIC values > 2 retained as indicators of a significantly improved model fit. All statistical analyses were performed with the R package,v.3.1.

1.3 Results

Effect of elevation on plant performance

Floral buds of *V. cheiranthifolia* showed an abundance peak between late April and mid-May both years that phenology was monitored (Fig. S1). Open flowers were available from the first week of April until early July; the highest bloom occurring by mid-end of May. The fruiting peak was by mid-end of June, although some individuals showed fruits already by mid-late April whereas others fruited during late August. Plants at the highest locality (3500 m) flowered and fruited about ten days later than plants at lower elevations at 2013, with no differences in the non-snowy 2012 (Fig. S1).

The proportion of individuals that resprouted in 2013 after wintertime was higher at low-sites (2400 m and 2700 m) than at high-sites (3300 m and 3500 m) (Z-value = -2, $P < 0.05$; Table 2A; Fig. 2A). We did not detect significant effects of plant size on resprouting probability (Table 2A). Plant size varied among elevations but not consistently between years (Table 2A): in 2012, plant size increased with elevation ($F_{3,266}$=14.58, $P < 0.001$), whereas in 2013 it was greatly reduced at the two extremes of the distribution, though mainly at 2400 m (Fig. 2C). Flowering probability (Fig. 2B), by contrast, did mainly depend on plant size, the largest plants being more likely to bloom (Table 2A); this was consistent across localities, but with different slopes. At 2700 m and 3300 m flowering probability increased more strongly with plant size (slope: 0.16 ± 0.03, 0.21 ± 0.04, $P < 0.0001$, respectively) than at the extremes of the plant distribution, at 2400 m and especially at 3500 m (slope: 0.14 ± 0.07, 0.01 ± 0.00, $P \leq 0.05$, respectively). Larger plants produced also more flowers in all localities, but this increase with size was smaller in the highest elevation (slope: 0.01 ± 0.002, $P <$ 0.0001, Table 2A); in 2013, plants produced more flowers than in 2012, except at 2400 m where no plants flowered (Fig. 2D).

Fruit set was higher in 2012 than 2013 but did not vary among elevations either of the two years (Table 2, Fig. 2E). Interestingly, while at 2700 m and 3300 m fruit set increased significantly with size (slope: 0.06 ± 0.02), this relationship was negative at 3,500 m (slope: - 0.03 ± 0.01). Number of seeds per capsule, on the other hand, only differed between years at the highest elevation, and larger plants consistently produced more seeds per fruit in all localities (Table 2A, Fig. 2F).

Seed viability varied significantly among elevations (χ^2_2 = 17.66, $P < 0.001$): seeds from 2700 m were less viable (c. 80%) than those at either 3300 m or 3500 m (≥ 90%). There was no plant size effect on seed viability (χ^2_1 = 2.6, P = 0.1).

Both fruit set and seeds per capsule were significantly higher in the open-pollination than in the autofertility treatment ($P < 0.0001$; Fig. 3A and B). Autofertility varied across elevations, being higher at 3300 m and 3500 m than at 2700 m, and this was observed both for fruit set and seeds per capsule (Table 2B; Fig. 3A and B). Fruit set was consistently over 75% in all localities during 2014 and 2015, and was only slightly pollen limited. Number of seeds per capsule, on the other hand, showed no pollen limitation at any elevation (Table 2B; Fig. 3C and D). Neither plant size nor floral display was included in any of the best models.

The number of plant species and floral visitors in the Teide's alpine community decreased along the elevational gradient during 2014 and 2015 (Lara-Romero et al., unpub. data); however, in the case of *V. cheiranthifolia* the number of interactions increased with elevation during those years (Table 3). This violet was visited by a total of 19 native insect species and the non-native *Apis mellifera* (Table 3). Of these, the most dominant species were the bee *Anthophora alluaudi* and the honeybee *Apis mellifera*. Mean visitation rate during the two years was 0.28 visits per flower per hour. Assuming a 6-h activity period for pollinators and that flower duration is c. 10 days, flowers received on average ~16.8 visits during their entire lifespan. Such visitation rate varied among elevations ($F_{3,181} = 5.14$, $P = 0.001$; Fig. 4A), tending to be higher at 3300 m than at 2700 m, and being higher in 2014 than in 2015 overall ($F_{3,181} = 4.53$, $P < 0.05$; Fig. 4A).

Germination rate was extremely low, with only 3.82% of emergence of stratified seeds and 2.92% of non-stratified seeds. Except for seeds collected at 3500 m, which showed significantly higher germination rate when stratified ($\chi^2_1 = 4.71$, $P < 0.05$), there were no differences between stratified and non-stratified seeds in other localities. Moreover, a significantly higher germination rate was encountered only when comparing seeds at 3500 m with those at 2700 m ($\chi^2_1 = 6.75$, $P < 0.01$).

Effect of herbivore exclusion on plant performance

A high fraction of the total marked individuals underwent herbivory in all localities, with a mean per locality ranging from 31 to 83% in 2012 and from 11 to 57% in 2013. Great differences were observed both in the proportion of consumed plants and flowers among elevations and between years (Table 2C). We did not observe an upward trend with elevation in both herbivory and florivory, but both were consistently low at 2700 m; Fig. 2G and H).

The density of violets was higher inside than outside the exclosure (9.05 indiv/m^2 vs. 0.15 indiv/m^2), and most reproductive parameters differed significantly between excluded and non-excluded individuals. Inside the exclosure, plants were larger ($F_{1,52}$ = 107.3, *P* < 0.001), showed greater floral display (χ^2_1 = 96.08, *P* < 0.001 with size as covariate; χ^2_1 =686, *P* < 0.001), higher fruit set (χ^2_1 = 26.69, *P* < 0.001) and seeds per capsule (χ^2_1 = 32.63, *P* < 0.001), and higher seed viability (χ^2_1= 14.1, *P* < 0.001) than controls. Seed viability was higher inside the exclosure (94 ± 0.07%) than outside it (90 ± 0.16%). Pollinator visitation rate per flower did not differ between either plants inside and outside the exclosure ($F_{1,94}$ = 0.04, *P* = 0.84; Fig. 4B) or between years ($F_{1,94}$ = 0.0001, *P* = 0.99; Fig. 4B).

Bagged flowers within the exclosure produced fewer fruits per flower –but not fewer seeds per fruit- than bagged flowers outside the exclosure (Table 2B, Fig. 3A and B). In contrast, a consistently higher fruit and seeds per capsule was found in open-pollinated flowers within the exclosure than outside it. Moreover, no pollen-limitation was detected in either excluded or non-excluded individuals (Table 2B, Fig. 3C and D).

Finally, when examining plant size and reproductive traits along the elevational gradient after either one or two years of individual rabbit exclusions, we found that plants at 3300 m were smaller, produced less flowers and had a lower fruit set than those at the other localities (Z-value > -2, *P* < 0.05, in all cases; Fig. 5A, B and C). However, we found an interaction with elevation for size and floral display (Table 2C). Seeds per capsule in excluded individuals showed no significant differences among localities (Table 2C, Fig. 5D). When plants within the exclosure were compared with those excluded either one or two years at 3500 m, all differences in plant size and reproductive fitness disappeared, except for floral display, suggesting that plants respond rapidly to herbivore exclusion (Table 2C, Fig. 5).

1.4 Discussion

The environmental variability found along the gradient showed a consistent trend only in mean temperature (Table 1), with differences in fraction of organic matter and in soil moisture depending on small-scale heterogeneity. The performance of *V. cheiranthifolia* showed to be especially constrained at the lowest elevation (2300 m), with a very low plant size and floral display, and with practically no individuals producing seeds, probably due to the warmer and dryer conditions at this site in the two dry years (2012 and 2013). Hence, it seems plausible that this endemism could be suffering an upward shift of its low edge limit under the effect of climate change (Klanderud & Birks 2003; Parmesan 2006). This, together with the strong deleterious effects of non-native rabbits observed on its fitness and reproduction reveals a very uncertain future for this unique endemic plant.

1.4 Discussion

Effect of elevation on plant performance

The annual variability in blooming suggests that it is mainly controlled by snow conditions or soil moisture, and not only by temperature and photoperiod as found in other studies (Walker *et al.* 1995; Keller, F., & Körner 2003; Giménez-Benavides *et al.* 2007). Regarding plant regrowth, we found it to be lower at higher elevations, which we attribute to the fact that the severity and frequency of freezing temperatures significantly increase with elevation (Neuner 2014). The type of soil (pumice stones, Fig. 1D) of the lower localities retains water and might thus provide safe sites for plant development (Pérez 2009) leading to higher plant regrowth there.

Plant size increased with elevation in the unusual snowless 2012, which is not concordant with the growth-limitation hypothesis (Körner 1998). In 2013, however, plant size decreased at the two extremes of the elevational distribution, especially at the lowest elevation. The small plant size observed in both dry years (2012-13; Izaña Meteorological Station; www.ogimet.com) suggests that the species might be currently contracting its distribution due to the increasing warmer and dryer conditions, as found for other species (Engler *et al.* 2011; Gottfried *et al.* 2012). During snowy years, on the other hand, plants at the lowest elevation can take advantage of longer snow-free period (Palacios *et al.* 2003) and increase their size (we did observe large flowering individuals at this site in snowy years), despite this rapid growth might imply greater weakness at maturity and lower survival (Giménez-Benavides *et al.* 2007). The presence of small snowfalls during the winter 2012-13, with snow cover maintained longer at the upper site, might explain the decrease in size observed at 3500 m in 2013. *Viola cheiranthifolia* showed a high size-dependence at several stages of the reproductive process; thus, smaller plants were less likely to flower and produced fewer flowers, as reported for other species (Ollerton & Lack 1998; Mendez & Karlsson 2004). The tiny individuals at the lowest elevation (2400 m) actually produced no flowers at all in 2013. At 3500 m, larger plants were not as likely to flower and did not produce as many flowers as expected compared to plants at lower elevations, probably due to the unfavorable environmental conditions at the summit area (snow cover). Temperature is known to indirectly affect nutrient availability at high elevation (Körner 2003), whereas water availability is known to affect it at low elevation which could thus explain, at least partly, our findings. In short, plant size, flowering probability, and floral display of *V. cheiranthifolia* seem to vary on a spatiotemporal scale, being influenced by environmental conditions at each elevation, mainly snowfall regime which determines soil moisture and temperature.

Overall, contrary to our expectations, neither fruit set nor seeds per capsule varied with elevation. In spite of the assumption of scarcity of pollinators at alpine areas, literature shows no consistent trend of plant fitness along elevational gradients, with wide interspecific differences as well as temporal variation within species (Seguí et al. meta-analysis in prep.). Fruit set of *V. cheiranthifolia* varied between years, as reported for other alpine species such as Silene ciliata (Giménez-Benavides *et al.* 2007) and *Armeria caespitosa* (García-Camacho & Escudero 2009). Number of seeds per capsule of *V. cheiranthifolia* only differed between years at 3500 m; the lower values in the snowless 2012 are possibly due to the lower water availability compared to 2013, besides also a higher herbivory the former year (Fig. 2G).

The environmental conditions in alpine habitats may reduce the possibilities of cross-pollination and are believed to represent important driving forces in the evolution of self-pollination at high elevations (Molau 1993; Totland 2001; Kalisz *et al.* 2004). *Viola cheiranthifolia* showed a high (c. 50%) autofertility level, especially at the two highest localities. This increase in autofertility level with elevation is consistent with other studies that have reported enhanced self-compatibility mechanisms at greater elevations (Medan *et al.* 2002; Arroyo *et al.* 2006). In addition, although the scarce evidence available from the literature shows that pollinator abundance is lower at high elevations (Cruden 1977; Totland 1994; Medan *et al.* 2002), we observed no increase in pollen limitation with elevation. This agrees with the findings of García-Camacho and Totland (2009) who reported that ovule fertilization is not particularly limiting in highland vs. lowland sites and attribute this to plant compensatory mechanisms to combat the scarcity of pollinators. Flower visitation rates showed to vary among localities and in time, what is often attributed to variable climatic conditions (Totland 1994; Herrera 1995), but were not found to decrease with elevation as expected. At lower elevations, *V. cheiranthifolia* coexists with a large variety of entomophilous plants whereas at 3,500 m this violet is the only plant in the pollination community; this lack of interspecific competition for pollinators at high elevations might thus lead to a greater generalization level.

The fact that seed viability was positively associated to elevation, with lower values at 2700 m than at 3300 or 3500 m, is in line with other studies (Marcora *et al.* 2008; Milla *et al.* 2009) and might be due to different reasons. First, the abundance of heterospecific pollen can be expected to be higher at lower elevations due to the presence of other flowering plants, potentially affecting seed set and viability (Morales & Traveset 2008). Second, seeds at higher elevations might be expected to ripen more slowly, owing to the lower temperatures and longer drought periods than those in the lowest localities, which might positively influence embryo viability and germination (Fenner M 2005). Despite the relatively high seed viability found, the seeds of *V. cheiranthifolia* showed a very low germination rate. This is possibly related to the ability to produce an abundant seed bank capable of dormancy that guarantees the long-term reproductive success of the species, even when climatic conditions are adverse or pollinators are scarce (Pannell & Barret 1998).

Impact of invasive rabbits on plant performance

Herbivores can have strong deleterious effects on plant growth, reproduction and even survival (Crawley 1989; Marquis 1992; Strauss & Zangrel 2002). These deleterious effects have a disproportionally greater impact on island endemic plants such *V. cheiranthifolia*, because these are expected to have lost, through the evolutionary process, the defense mechanisms and strategies from their ancestors (Burger & Gochfeld 1994; Atkinson 2001; Courchamp *et al.* 2003).

For this reason, herbivore pressure by non-native rabbits upon *V. cheiranthifolia* was huge in almost all elevational localities, affecting even more than 75% of the individuals in some of them. Pressure was variable among elevations and between years, probably due to the changes in rabbit density and population dynamics between sites and years. Very few studies have demonstrated spatial variation in the magnitude of herbivory along elevational gradients (Galen 1990; Bruelheide & Scheidel 1999), and as far as we know, this is the first study that examines so in a high-mountain oceanic ecosystem. Moreover, herbivores strongly altered many of the studied plant traits. After excluding rabbits for two years (2014-15), the effect of elevation on plant size, floral display, and fruit set changed completely from that previously observed in the non-excluded plants, highlighting that the current state that we observe in this endemism is far from what would be expected without the presence of rabbits. Consistently, both in 2014 and 2015, these three performance traits showed the lowest values at 3300 m but were similar at 2700 m and 3500 m. One reason for this may be that the population at 3300 m had the lowest soil moisture and the highest annual and daily temperature oscillation (Table 1); by contrast, soil moisture at 3500 m is maintained high after snow melt, whereas at 2700 m, the dominant substrate is pumice grits, what also maintains moisture for long periods (Fig. 1C and D).

Rabbits, in particular, are among the 100 most invasive species (Lowe *et al.* 2000), and are virtually present on all major islands of the world, where they have devastating effects (Courchamp *et al.* 2000). Although further studies are needed to quantify to what extent herbivory upon *V. cheiranthifolia* is altering population dynamics in the long-term, it is very revealing that, in all localities, plant performance is much higher when herbivores are excluded. Moreover, herbivory pressure might be affecting the reproductive ecology of the species, as we found a lower autofertility level at the rabbit exclosure. In this sense, and in the light of what is occurring within the exclosure, it is clear that rabbits considerably alter and diminish the distribution, abundance and fitness of this summit endemic violet.

Finally, herbivory can also alter mutualistic interactions, such as those between plants and their pollinators. Leaf damage by herbivores can decrease overall plant attractiveness to pollinators (Lehtilä & Strauss 1997; McCall & Irwin 2006), greatly reducing the opportunity for pollinators to select between plants (Herrera 2000; Herrera *et al.* 2002a). One reason for the increase in fruit set observed when rabbits are excluded could be individual higher floral display and therefore higher attractiveness to pollinators. No differences, however, were detected in flower visitation rate between protected and unprotected plants in any of the two years of intensive censuses (2014-15), which suggests some kind of resource limitation influencing fruit and seed set. Plants excluded from herbivores can grow larger, and possibly be less resource-limited as they may be capable of extracting more resources from the soil and accumulate rhizome reserves than small plants (in fact, we only observed rhizome reserves in plants from the exclosure; pers. obs.).

1.5 Conclusions

In short, our findings demonstrate that drivers associated with elevation are not necessarily limiting plant reproductive success, with no differences in pollen limitation and a higher self-pollination rate as compensatory mechanism in the highest localities. *V. cheiranthifolia* showed to be mainly affected by local conditions related to environmental heterogeneity, especially soil moisture (influenced by snow duration and soil type) and co-occurring plant species, at each site and year, and mainly by the interactive effect of non-native rabbits, which appear to be altering plant responses along the gradient. Rabbits are acting as ecological engineers, intensely altering this isolated, fragile, and exclusive island ecosystem, in which the violet is the most dominant species in the community above a certain elevation. We argue that only by urgently minimizing the effects of invasive herbivores by effective control campaigns can this ecosystem be restored to the most natural state possible.

1.6 Acknowledgements

The Service of Parque Nacional del Teide provided us with all kind of facilities to perform this work. We deeply thank the company Teleférico del Teide for logistic support in the summit area, Benito Pérez for assistance in the field and the laboratory, and Vicente Soler for temperature and soil data. We also thank to two anonymous reviewers for valuable comments on an earlier version of the manuscript. Our work was funded by a project of the “Organismo Autónomo de Parques Nacionales” (785/2012). Jaume Seguí was funded by a PhD grant of the Balearic Government co-financed by the European Social Fund (ESF), and Marta López-Darias by a JAE-DOC (CSIC) contract.

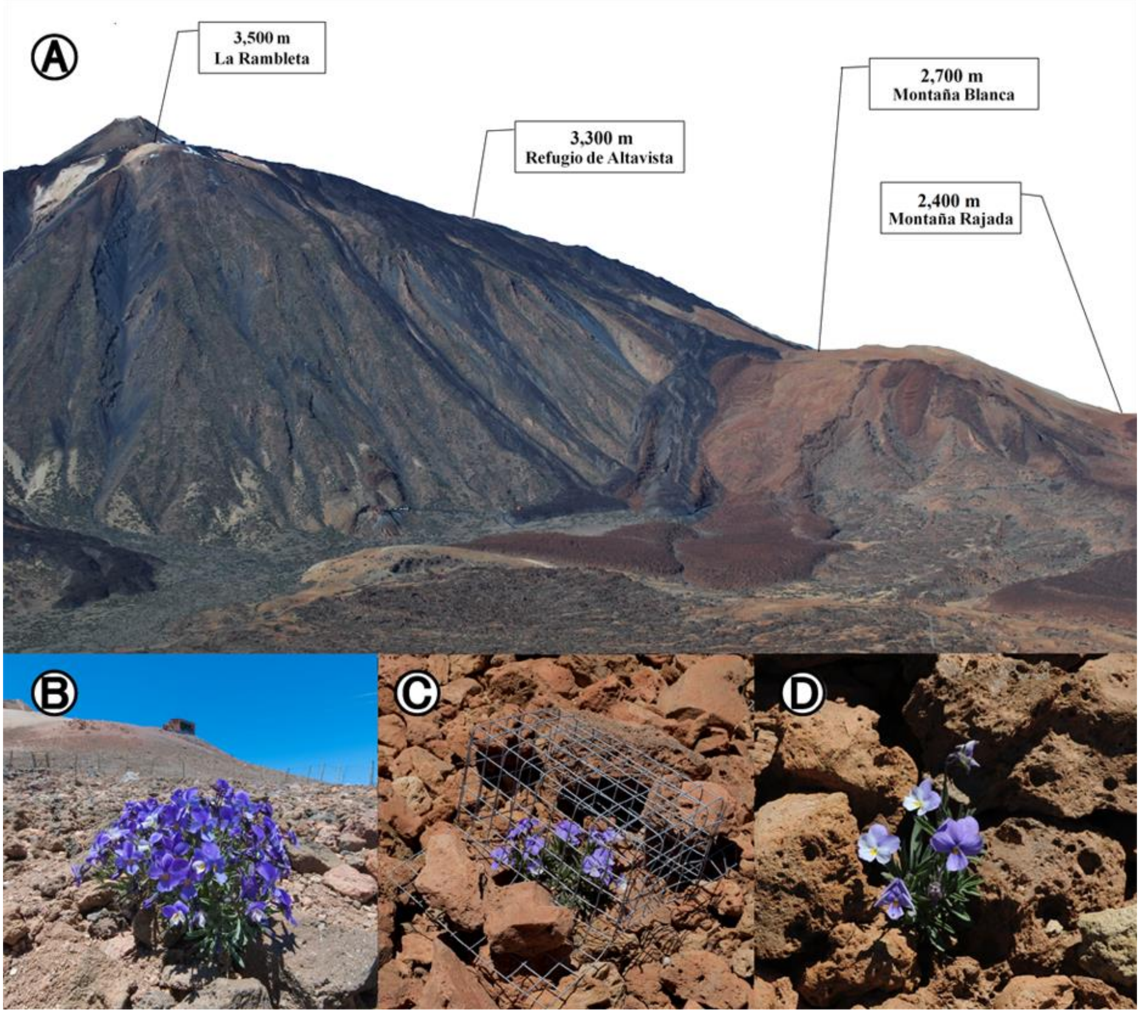

Figure 1. 1A; study sites selected on the SE slope of the Teide stratovolcano. 1B; Plant located inside the herbivore fence exclusion at 3,500 m. 1C; Plant with the individual exclusion cage. 1D; Image of a whole non-protected plant, with presence of pumice, which protects individuals and maintains soil moisture.

1.7 Figures

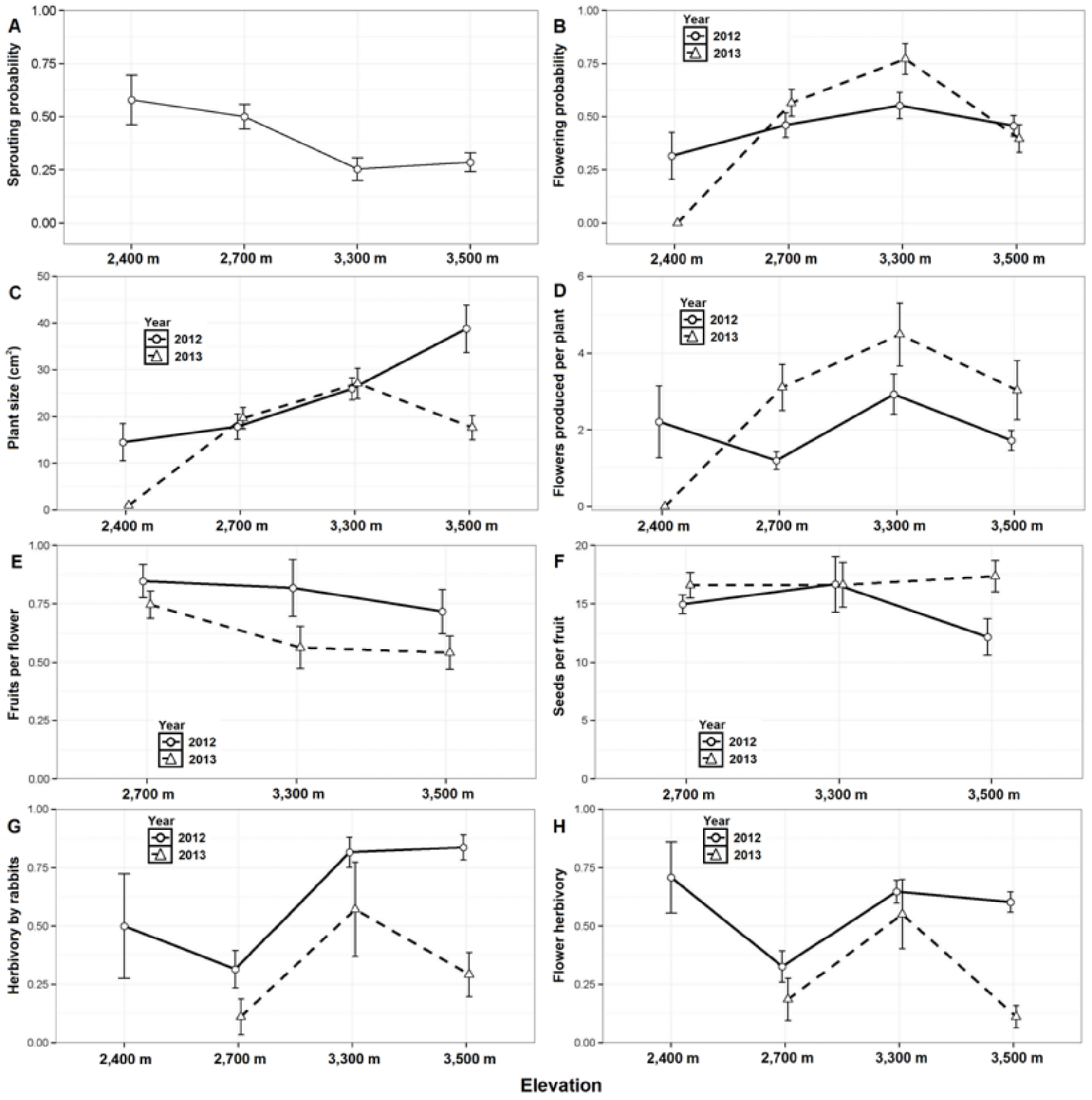

Figure 2. Plant traits studied along the elevational gradient during 2012 and 2013. 2A; Sprouting probability represents the percentage of individuals marked in 2012 that sprouted in 2013. Points represent means and vertical bars represent standard errors.

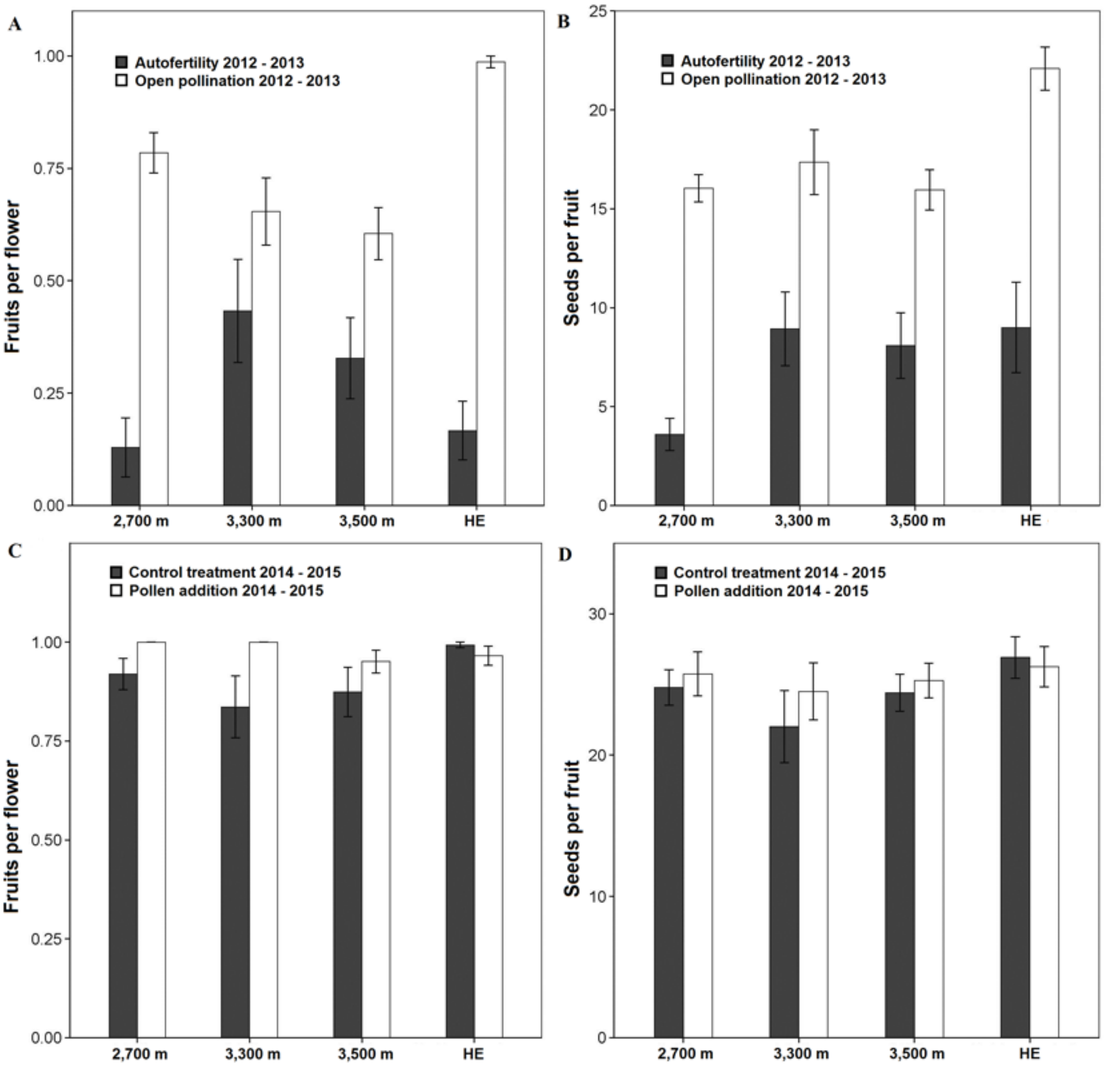

Figure 3. Autofertility and pollen addition treatments accounting for fruit set and seed set. Treatments were performed in different pairs of years (2012-13 and 2014-15) due to the low flower display per plant. 3AB; Autofertility treatment in the herbivore fence exclusion was performed only during 2013. Bars represent means and vertical bars represent standard errors. HE indicates herbivore exclusion.

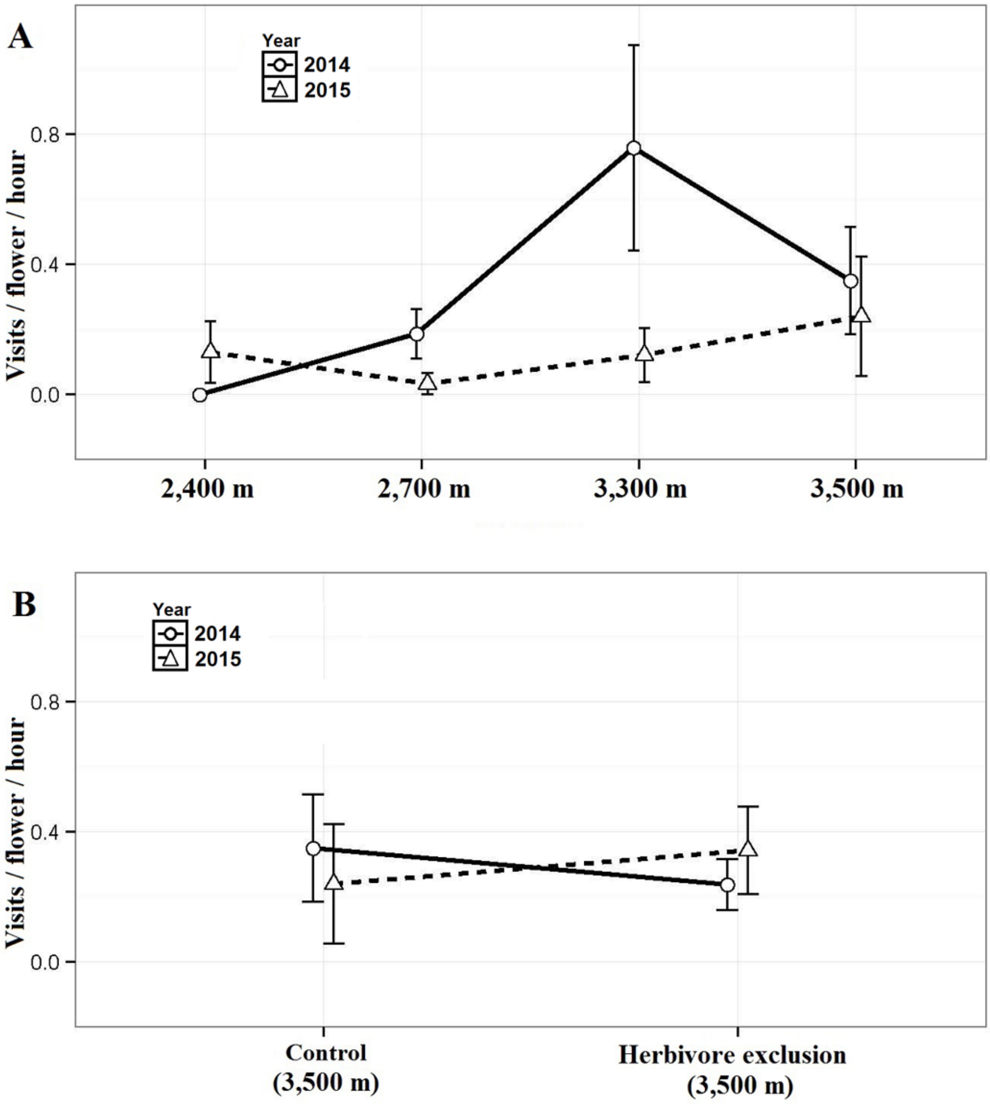

Figure 4. Flower visitation rate among elevational localities and between rabbit-excluded and non-excluded plants (2014-2015). Points represent means and vertical bars represent standard errors.

1.7 Figures

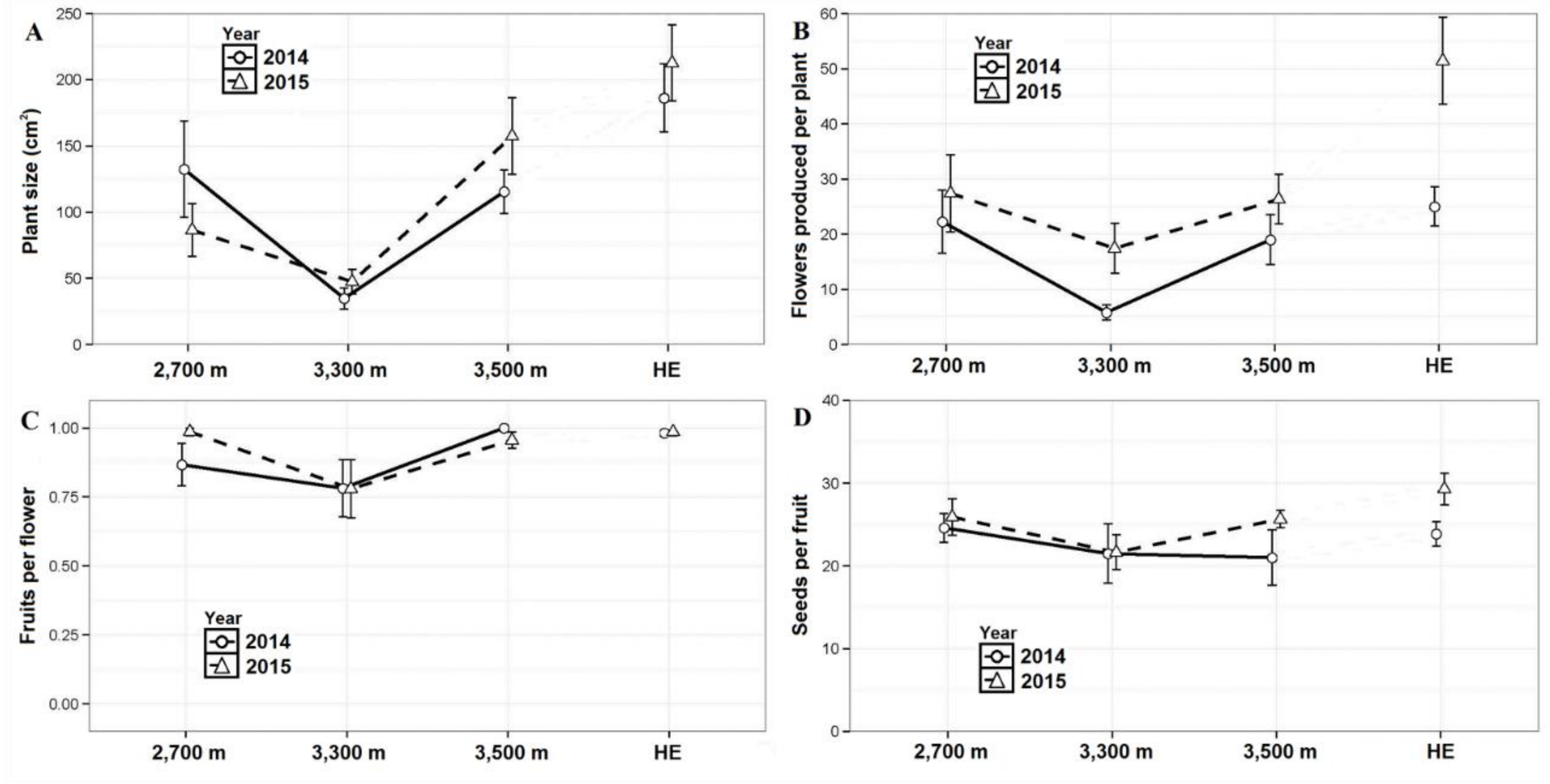

Figure 5. Plant traits of rabbit-excluded individuals along the elevational gradient and in the 3500 m fence exclusion during 2014 and 2015. Points represent means and vertical bars represent standard errors. HE indicates herbivore exclusion.

Table 1. Temperature and edaphic data at each site. Temperature (T) was measured with a temperature data logger during a whole year (2014-15). Soil moisture was measured with a soil moisture sensor with 7-11 replications per site. We did not get climatic data from the lowest site (2400 m), instead we present data of a data logger located at 2153 m. O.M. (Organic matter).

Site	Coordinates	Altitude	T. Max. (ºC)	T. Min. (ºC)	Mean (ºC)	Max. Diurnal oscillation (ºC)	O.M. (%)	Soil moisture (%)
Cañadas del Teide	28 13.455 N 16 37455 W	2153 m	30.78	-5.87	11.69	22.25	(-)	(-)
Montaña Blanca	28 16.195 N 16 36.938 W	2732 m	34.05	-6.86	10.85	29.88	1.76	11.63
Refugio de Altavista	28 16.469 N 16 37.789 W	3296 m	33.24	-10.91	8.34	35.11	0.02	9.53
La Rambleta	28 16.490 N 16 38.768 W	3518 m	24.96	-13.03	5.02	23.19	0.03	12.98

1.8 Tables

Table 2. Results of GLMM for (A) response variables measured in non-protected individuals (2012-2013), (B) autofertility (2012-13) and pollen addition (2014-15) treatments and (C) response variables measured in caged plants along the altitudinal gradient (2014-15). Rows: factors included in each model. Columns: the response variables. We provide the sign of the effect for the continuous predictors, the chi-squared value and the significance level: non-significant (n.s.) *P* ≥ 0.05, **P* < 0.05, ***P* < 0.01, ****P* > 0.001.

A) **Factors**	**Resprouting**	**Flowering**	**Plant size**	**Floral display**	**Fruit set**	**Seed set**	**Herbivory**	**Florivory**
Altitude	14.36**	(-)	(-)	(-)	1.88 n.s.	0.03 n.s.	33.53***	54.71***
Year	(-)	(-)	(-)	11.1***	9.62**	2.06 n.s.	23.7***	81.03***
Altitude x Year	(-)	7.03 n.s.	12.08**	(-)	(-)	(-)	(-)	(-)
Size	(-)	(-)	(-)	(-)	(-)	+10.79**	(-)	(-)
Altitude x Size	(-)	141.5***	(-)	201.34***	19.98***	(-)	(-)	(-)

B) **Factors**	**Fruit set**	**Seed set**		**Fruit set**	**Seed set**
Altitude	(-)	(-)	Altitude	6.39 n.s.	0.71 n.s.
Autofertility	(-)	(-)	Pollen addition (PA)	6.20*	0.97 n.s.
Altitude x Autofertility	11.17**	15.51**	Altitude x PA	(-)	(-)
Herbivore exclusion (HE)	(-)	30.03***	Herbivore exclusion (HE)	4.31*	1.55 n.s.
Autofertility	(-)	99.64***	Pollen addition (PA)	0.08 n.s.	0 n.s.
HE x Autofertility	21.02***	(-)	HE x PA	(-)	(-)

C) **Factors**	**Fruit set**	**Seed set**	**Fruit set**	**Seed set**
Altitude	(-)	(-)	8.63*	3.06 n.s.
Year	(-)	(-)	(-)	1.31 n.s.
Altitude x Year	6.42*	7.77*	(-)	(-)
Cages – Fence Exclusion	3.26 n.s.	5.01*	0.75 n.s.	2.1 n.s.
Year	3.36 n.s.	129.16 n.s.	(-)	8.17**

1.8 Tables

Table 3. Floral visitors of *V. cheiranthifolia* observed at each altitudinal site during 2014-15.

Order	**Species**	**2400 m**	**2700 m**	**3300 m**	**3500 m**
Hymenoptera					
Anthophoridae	*Amegilla quadrifasciata*		X		
Anthophoridae	*Antophora alluaudi*	X	X		X
Apidae	*Apis mellifera*		X	X	X
Encyrtidae	*Homalotylus sp.*				X
Eulophidae	*Elasmus sp.*				X
Megachilidae	*Megachile canariensis*				X
Pteromalidae	*Systasis sp.*				X
Lepidoptera					
Lycaenidae	*Cyclyrius webbianus*			X	X
Pieridae	*Pieris rapae*		X		
Pieridae	*Euchloe belemia*		X		
Sphingidae	*Macroglossum stellatarum*				X
Diptera					
Phoridae	*Phoridae sp.*			X	
Sirfidae	*Scaeva albomaculata*			X	X
Tachinidae	*Linnaemya soror*			X	
Tachinidae	*Pseudogonia fasciata*		X		
Coleoptera					
Melyridae	*Melyrosoma hirtum*			X	X
Nitidulidae	*Meligethes varicollis*			X	
Buprestidae	*Acmaeodera cisti*		X		
Hemiptera					
Lygaeidae	*Nysius cymoides*			X	
Anthocoridae	*Orius laevigatus*			X	
TOTAL		**1**	**7**	**9**	**10**

1.9 Supplementary material

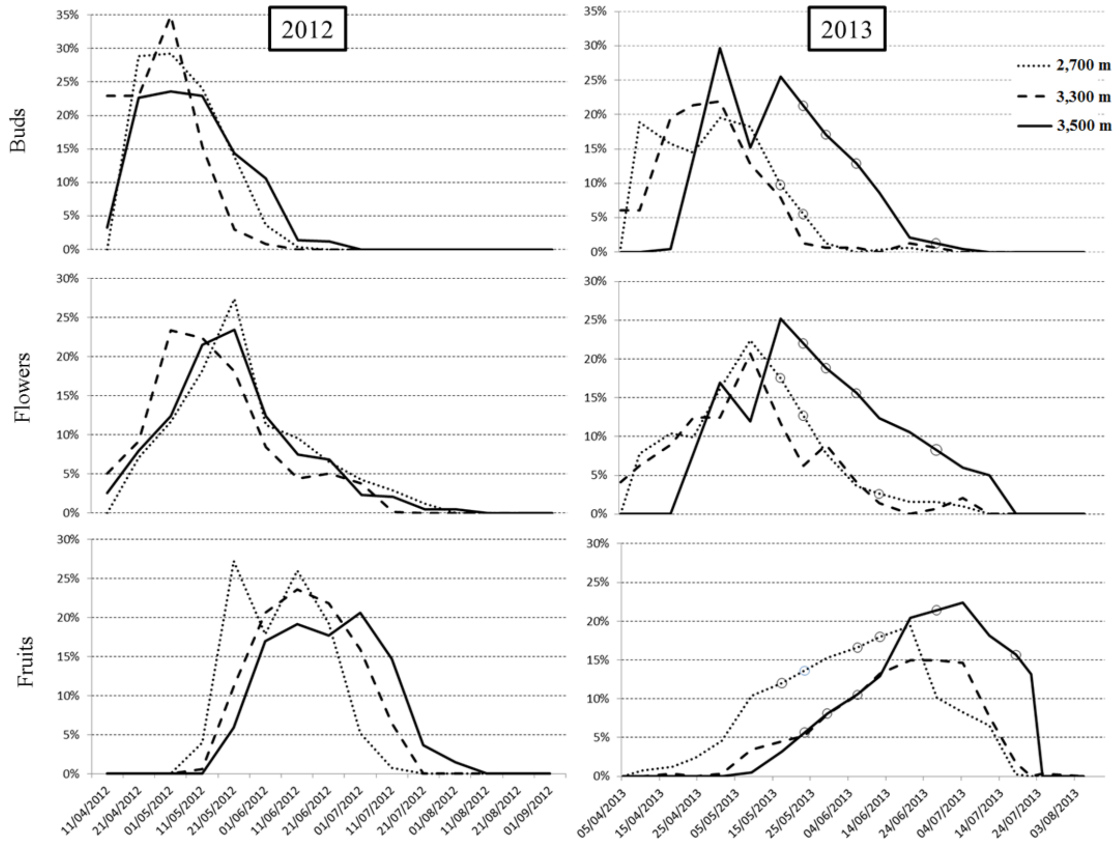

Figure S1. Percentage of buds, flowers and fruits produced during the flowering season of *Viola cheiranthifolia* during 2012 and 2013 at each elevation. Round unfilled marks reflect inferred values obtained by averaging adjacent dates, due to the impossibility of visiting that particular locality on that date.

Chapter 2

Invasive rabbits change the selection that pollinators and abiotic conditions exert on floral traits of a high-mountain oceanic plant

Seguí, J., López-Darias, M., Nogales, M., & Traveset, A. Invasive rabbits change the selection that pollinators and abiotic conditions exert on floral traits of a high-mountain oceanic plant. Prepared for submission.

Abstract

Floral trait variation across the geographic range of a plant species is usually interpreted as the result of different species composition and abundance, with functional groups of pollinators exerting contrasting selective pressures. However, much evidence suggests that selection acts on flower morphology in a more pluralistic way across any particular habitat, involving not only pollinators but also enemies and abiotic agents. For this reason, we assessed the variation in flower traits along a wide elevational gradient (2700 m – 3500 m) and between herbivore-excluded and non-excluded plants of the high-mountain endemic *Viola cheiranthifolia*, which is strongly affected by invasive rabbits. Secondly, we tested whether water shortage (indirectly estimated by specific leaf area) influences floral traits, and finally, whether and how floral traits, floral display and density of conspecific flowers explain differences in its reproductive success. Significant variation was found in most of the floral traits of the violet along its elevational distribution and between herbivore-excluded and non-excluded plants. Water stress conditions affected the morphology of the flowers, accompanied by an interactive effect with herbivory. Spur width constrained the reproductive success of the individuals developing at the highest site, but this constraint disappeared in herbivore-excluded individuals. Plant floral display and density of conspecific flowers were other crucial factors limiting plant reproductive success. In short, herbivores seem to be altering both flower morphology and plant floral display and abundance, thus changing the selection that either pollinators or abiotic factors may normally exert on such plant traits.

Keywords: elevational gradient, flower morphology, herbivory by rabbits, specific leaf area, plant-pollinator interactions, *Viola cheiranthifolia*

2.1 Introduction

The great variety of animal-pollinated flower shapes and sizes observed in nature has fascinated ecologists for a long time (Darwin 1877b; Van Der Pijl & Dodson 1966). Much of this flower diversity is thought to result from their interaction with pollinators, as these mediate pollen transfer between plants, and in turn these interactions are crucial for plant reproduction (Ollerton *et al.* 2011). Thus, the variation observed in floral morphology across the geographic range of a plant species is usually interpreted as the result of the different species composition of pollinator assemblages, as well as different species abundances and efficiencies, creating mosaics of contrasting selection (Silva-Montellano & Eguiarte 2003; Medel *et al.* 2007; Pérez-Barrales *et al.* 2007). However, much evidence suggests that selection acts on flower form and size through more pluralistic processes, involving not only pollinators but also enemies and abiotic agents (Lehtilä & Strauss 1997; Galen 1999; Herrera 2005b; Zhao & Wang 2015). Accordingly, geographic variation in flower traits may be due to multiple conflicting selection pressures, difficult to predict. Herbivores have the potential to select for changes in plant traits by the direct effects of folivory on seed production through decreased resource availability (Mothershead & Marquis 2000). Similarly, herbivores can indirectly affect plant fitness by delaying flowering time (Frazee & Marquis 1994; Juenger & Bergelson 1997), modifying flower size (Frazee & Marquis 1994; Strauss 1997) and morphology (Cunningham 1995; Lohman *et al.* 1996). This influences the probability of pollination, and reduces the quality or quantity of pollinator reward (Quesada *et al.* 1995; Strauss 1997).

In addition, geographic variation in flower traits can be associated with abiotic conditions, which may conflict with the adaptive responses of flower size to pollinator-mediated selection. Some studies have suggested that vegetative plasticity may affect the expression of floral traits in certain environments, and that there may be environment-specific constraints on the evolution of both floral and vegetative traits (Brock & Weinig 2007; Lambrecht & Dawson 2007). Furthermore, plant density (neighborhood conditions) can also result in either competitive interactions (for nutrient resources and pollinator visits; e.g., Steven et al., 2003) or facilitative processes (for pollination attraction; e.g., Hegland and Boeke, 2006) as well as in different herbivore-plant interactions (e.g., Colling and Matthies, 2004), which can also indirectly affect plant traits and success.

Despite the increasing evidence of the importance of focusing not only on single interactors (e.g. pollinators, herbivores), very few studies have examined the combined influence of both mutualist and antagonistic interactors and abiotic conditions on floral traits variation and evolution. Studying *Paeonia broteroi* and *Helleborus foetidus* more than a decade ago, Herrera et al. (2000; 2002) found that plants possessing a combination of ''traits'' that simultaneously enhanced pollination and facilitated escape from herbivores enjoyed a disproportionate fitness advantage over others. These authors showed that such a combination favored the adaptive correlated evolution of these mutualism- and antagonism-related plant traits. Other studies have shown that smaller corollas provide an advantage to some plant species that live under prevailingly stressful conditions (Galen 2000; Carroll *et al.* 2001; Herrera 2005b).

Elevational gradients show changes in abiotic conditions, in abundance and diversity of pollinators, in plant species composition and density, and even in herbivore predation pressures. Therefore, they are ideal natural laboratories to explore adaptations and phenotypic variance in plants over very short distances. This study focuses on an alpine plant species, *Viola cheiranthifolia* Humb. & Bonpl*.,* which lives within a wide elevational gradient exposed to different biotic and abiotic environments. We first assessed the variation in flower traits along its altitudinal distribution and whether and how this could be influenced by herbivores (non-native rabbits), by comparing herbivore-excluded plants with controls (non-excluded). Secondly, we aimed to evaluate the relative importance of specific environmental conditions on the floral traits observed at each site, testing whether drought stress (indirectly estimated by measuring specific leaf area) influences floral traits. Finally, we explored whether and how flower traits, floral display and density of conspecific flowers explain individual differences in plant reproductive success, and tested for an interactive effect of elevation and herbivory on such a relationship between floral traits and reproductive success.

2.2 Material and methods

Study species

Viola cheiranthifolia is a high-mountain dwarf plant, endemic to Teide stratovolcano (Tenerife, Canary Islands). It is the only entomophilous species within the summit vegetation of Teide, while at lower elevations it shares habitat with other species from which the most abundant are *Echium auberianum* Webb & Berthel., *Descuraina bourgaeana* (E.Fourn.) O.E.Schulz or *Erysimum scoparium* (Brouss. ex Willd.) Wettst. The largest populations of *V. cheiranthifolia* are found from c. 2400 m to c. 3700 m a.s.l. along El Teide stratovolcano, although currently there are also two small populations at the highest southern points of the Las Cañadas caldera (Guajara; 2715 m, Pasajirón; 2531 m). The plant grows on poor soils (low organic matter, nitrogen and phosphorus) on cinders amongst the volcanic rubble, mixed with pumice at some localities. The species is self-compatible, with an increase in selfing level with elevation (Seguí *et al.* 2017); the number of ovules per flower has also been found to be higher at 3500 m than at 2700 m (38.03 ± 1.54 *vs*. 32.9 ± 1.48, respectively, *n* = 63, χ^2_1 = 11.65, *P* < 0.001). Despite the wide elevational gradient where it lives, pollen limitation does not appear to increase with elevation, but is present at all elevations (Seguí *et al.* 2017). It produces bears chasmogamous zygomorphic flowers, mainly from late April to early July. Flowers have a lifespan of 10 days and are purple-violet when mature (being white for the first 24-48 hours after anthesis), with a short nectar spur (9 mm long), and a 6-10 cm peduncle. They are pollinated by more than 20 different pollinator species, mainly bees, with *Anthophora alluaudi* and the honeybee *Apis mellifera* as the most common pollinators, but also butterflies (such as *Macroglossum stellatarum* and *Cyclyrius webbianus*), flies (*Scaeva albomaculata*), beetles (such as *Melyrosoma hirtum*) and several hemipteran species, although a low visitation rate was encountered (Seguí et al. 2017). Plant fitness and abundance are being decimated owing to the presence of non-native herbivores, mainly rabbits, in the National Park (Seguí et al., 2017; Fig. 6A, below).

Study site

This study was carried out during 2013 on El Teide stratovolcano (28º16’15’’N 16º38’21’’O); at 3718 m it is one of the highest island volcanos in the world, located on Tenerife (Canary Islands). It offers a subtropical high-elevation arid environment (Leuschner 2000; Gieger & Leuschner 2004). For the study, we selected four elevational sites where *V. cheiranthifolia* is present, covering most of its distribution from 2700 m to 3500 m (Fig. 6B), and providing contrasting combinations of temperature, moisture and nutrients (Table 4). The areas encompassing each study site varied among the sites, ranging from 4,000 to 40,000 m^2, depending on plant density and population structure (Table 4). At the highest site (3500 m), we marked and monitored 25 individuals inside an herbivore fence exclusion (20 x 20 m) set up by the National Park in 2009 (Fig.6B, below), with a plant density of 9.05 indiv/m^2. Temperature, organic matter, soil moisture and nitrogen availability decreased along the elevational gradient, whilst phosphorus availability was low at all sites (Table 4). We found no differences in either soil moisture or nutrient availability between inside and outside of the herbivore exclosure (Table 4).

Plant trait measurements and reproductive success

At the end of April, 2013, we marked and mapped (using a GPS; Garmin, GPSMAP 76CS) all reproductive individuals found at each study site (Table 4). Such data were used to account for the density of *V. cheiranthifolia* at each, and the potential scale-dependent effects on plant success. Distance-dependent effects on plant success are easily recognizable via the spatial range and strength of spatial correlation in plant success (Lara-Romero et al., 2017).

For each marked individual, we recorded during May: plant size (maximum length and width), number of stems and leaves, number of flowers produced, number of flowers consumed by herbivores, and the area and dry weight of the three largest leaves. By means of a digital caliper (Mitutoyo, with a precision of 0·01 mm), we measured the length and width of the anterior (AP), lateral (LP) and upper petals (UP), as well as the length (SL), width (SW) and curvature (SC) of the spur in each opened "intact" flower (with no symptoms of flower herbivory) (Fig.6C; selected measures). These traits were recorded for a total of 185 flowers (Table 4). Due to the strong florivory suffered by plants at the Refugio de Altavista site (3,300 m) (≥ 50% of the flowers in 2012 and 2013; Seguí et al., 2017), it was excluded from the floral traits analyses. The area and dry weight of the leaves provided the widely used *Specific Leaf Area* (SLA) per plant, applied here to indirectly describe the drought-stress conditions (Westoby & Wright 2006; Poorter *et al.* 2009) in which each individual plant has developed. It indicates a trade-off between an investment in leaf surface area to capture light for photosynthesis or in constructing more protected tissues to avoid dehydration and herbivory (Westoby 1998; Westoby *et al.* 2002). Plant SLA was obtained from the ratio between the mean of the areas of the three largest leaves of each plant and the mean of its respective dry weight. The area of recently collected leaves was calculated with the image analysis software ImageJ (ImageJ ver. 1.6). Once measured, leaves were dried in an oven for four days at 60 ºC and subsequently weighed in a balance to the nearest 0.01 mg.

Individual reproductive success was evaluated by measuring the number of viable seeds produced per flower (hereafter, viable seed set). We first recorded all the flowers produced per individual. From each individual, we marked flowers and monitored their fruit production (fruit set). To quantify the number of seeds per fruit (seed set), we bagged fruits before ripening and counted the number of seeds after fruits opened. Finally, we quantified seed viability with the TTC test, consisting of exposing seeds to a positive red staining test of viable embryos (+) during a 24-hour dark period in a 0.1% triphenyl tetrazolium chloride (TTC) solution (Cottrell 1947). A total of 3063 seeds were tested (774, 228, 494, 584 seeds at 2700, 3100, 3300 and 3500 m, respectively, and 983 seeds from the rabbit exclosure).

Data analyses and statistics

First, given that multicollinearity among explanatory variables can hamper the identification of the most causal variables (Mac Nally 2000), the number of explanatory variables was reduced using variance inflation factor (VIF) analysis. Plant size and number of stems and leaves per plant were strongly correlated with floral display, the width of the petals with their length, and the lateral petal length with the upper petal length. Accordingly, only *floral display*, *petal length* and *upper petal length* were included in the analyses. The VIF values of all remaining variables were smaller than five, indicating there was no strong collinearity between them (Zuur *et al.* 2009). Differences among mean floral traits at sites were tested using MANOVA, followed by separate one-way ANOVAs on averaged individual and floral traits. We used post hoc Tukey tests (library *multcomp*) to perform *a posteriori* contrasts between levels of significant factors.

In order to test for SLA effects on floral traits, different generalized linear mixed models (GLMM; package lme4 in R) examined *floral traits* as response variables, *SLA* and its *interaction with site* as predictors, and *individual plant* as random effect. To evaluate how the most important floral traits were related to plant success, GLMMs were used with *viable seed set* as response variable, all floral traits with their interaction with site as predictors, and *individual plant* as random effect. In the models, elevational sites were analyzed separately from the herbivore-excluded and non-excluded sites at 3,500 m. Before analyses, all traits were log-transformed to reach normality.

To test if conspecific flower density around each plant affects individual reproductive success, we used separate linear models with reproductive success: *fruit set, seed set and seed viability* as response variables, *density of conspecific flowers* around each plant as predictor variables, and *number of flowers per plant* as covariate. We assumed binomial distribution and log-link functions for fruit set and seed viability. Density of conspecific flowers was estimated using a Gaussian kernel with a standard deviation equal to the mean distance to the nearest neighbor in each study population (following Lara-Romero et al., 2016). The Akaike information criterion (AIC) was used to select the best models. Final models were assessed for goodness-of-fit to the data using the conditional R^2 for GLMMs, described by Nakagawa and Schielzeth (2013). All statistical analyses were performed with the R package, v. 3.

2.3 Results

Changes in floral traits with elevation

Considering all floral traits together in the MANOVA, significant differences were found across sites (*Wilks* = 0.389, *df* = 2, $P < 0.0001$). The subsequent separate ANOVAs showed that all floral traits differed from one site to another, except spur length ($F_{2,106} = 0.969$, $P = 0.38$; Fig. 7C). Both anterior ($F_{2,108} = 4.80$, $P < 0.05$) and upper petals ($F_{2,110} = 37.87$, $P < 0.001$) were smaller at the highest site (Fig. 7A and B), while spurs were wider ($F_{2,108} = 12.47$, $P < 0.001$; Fig. 7D) and more curved there ($F_{2,108} = 7.61$, $P < 0.001$), compared to the lower sites. Plants had similar floral display ($F_{2,68} = 1.74$, $P = 0.18$), leaf area ($F_{2,62} = 0.15$, $P = 0.86$) and SLA ($F_{2,62} = 0.68$, $P = 0.51$) according to elevation, but plants from the intermediate site (3100 m) showed a lower number of viable seeds per flower compared to the highest and lowest sites ($F_{2,44} = 7.94$, $P < 0.001$).

The length of the upper petals and their spurs ($\chi^2_1 = 4.01$, and $\chi^2_1 = 4.34$, respectively, $n = 99$, $P < 0.05$; Fig. 8A and B) were significantly associated with SLA, consistently across the sites, which suggests that the abiotic conditions influence flower morphology. The effect of floral traits on individual reproductive success varied with elevation, with a significant spur width x site interaction ($\chi^2_2 = 7.33$, $n = 69$, $P < 0.05$). This interaction effect was due to a greater increase in the viable seed set with spur width at the highest site, while no correlation occurred at the lower sites (Fig. 9A).

The effect of density of conspecific flowers around each plant on its reproductive success depended on the parameter used in the model. For fruit set, the best model (Table S1) included plant floral display ($\chi^2_1 = 31.09$, $n = 75$, $P < 0.001$; Fig. 10A) but not flower neighborhood density. Thus, plants with greater floral displays, but regardless of flowering plant densities, showed a higher fraction of flowers setting fruit. For seed set, none of the selected models fit well with the data (Table S1, $R^2 < 0.08$), thus indicating that neither floral display nor flower neighborhood density affected consistently to seed set. Lastly, the best model for seed viability (Table S1) included both conspecific flower neighborhood density ($\chi^2_1 = 43.4$, $n = 53$, $P < 0.001$; Fig. 10A) and floral display ($\chi^2_1 = 8.37$, $n = 53$, $P < 0.01$; Fig. 10B); that is, the proportion of viable seeds produced per individual increased with density of conspecific flowering individuals and plant floral display.

Effect of herbivore exclusion on floral traits

Significant differences between excluded and non-excluded plants were observed when considering all floral traits in the MANOVA (*Wilks* = 0.555, *df* = 1, $P < 0.0001$). The separate ANOVAs showed that herbivore-exclusion influenced all floral traits except spur curvature ($F_{1,116} = 414$, $P = 0.5$). Individuals inside the herbivore exclosure had longer anterior and upper petals ($F_{1,118} = 29.87$ and $F_{1,120} = 39.43$, $P < 0.001$, respectively; Figure 7A and B) and also longer and thinner spurs ($F_{1,118} = 38.19$ and $F_{1,118} = 11.93$, respectively, $P < 0.001$; Fig. 7C and D) than those outside it. At 3,500 m, herbivore-excluded individuals produced more flowers ($F_{1,50} = 80.79$, $P < 0.001$) and showed a greater viable seed set ($F_{1,39} = 17.29$, $P < 0.001$) than the non-excluded. Likewise, individuals protected from herbivores bore larger leaves ($F_{1,47} = 4.72$, $P < 0.05$), though such leaves had only slightly greater SLA ($F_{1,47} = 3.08$, $P = 0.08$).

Models comparing the effect of SLA on floral traits between excluded and non-excluded individuals showed a significant herbivore exclusion x upper petal length interaction ($\chi^2_1 = 5.91$, $n = 112$, $P < 0.05$; Fig. 11) as well as a significant positive effect on floral display (χ^2_1 = 6.59, *n* = 112, *P* < 0.01), anterior petal length (χ^2_1 = 3.89, *n*= 112, *P* < 0.05) and spur length (χ^2_1 = 4.52, *n* = 112, *P* < 0.05). By contrast, its effect was negative on spur width (χ^2_1 = 5.12, *n* = 112, *P* < 0.05). That is, individuals with greater SLA at 3,500 m had larger upper petals (especially at the herbivore-exclusion), longer but narrower spurs, and produced a higher number of flowers.

Finally, the analysis to test how floral traits affect plant success showed a significant spur width x herbivore exclusion interaction (χ^2_1 = 15.98*,* *n* = 99, *P* < 0.001; Fig. 9B), reflecting the positive relationship between plant success and spur width on individuals exposed to herbivores but not on the excluded ones. In other words, individuals bearing flowers with wider spurs produced more viable seeds per flower than those with narrow spurs, but this association was found only outside the herbivore exclosure.

2.4 Discussion

The flowers of *V. cheiranthifolia* showed a smaller size and wider spurs along the elevational gradient. This is actually the opposite pattern to that usually reported, with flower size increasing with elevation despite a decrease in plant size (Scobell & Scott 2002; Herrera 2005b). Such elevational variation in flower size has often been explained as a response to local pollinator assemblages or visitation frequencies (Galen 1987; Kudo & Molau 1999). Thus, for instance, the larger size of pollinators at higher elevations -due to the advantages of a large body size in ectotherms living in cold environments may select for larger flowers (Malo & Baonza 2002; Maad *et al.* 2013). Moreover, flower size is also known to be associated with the degree of resource limitation and time to complete the life-cycle (Snell & Aarssen 2005), and thus the more resource-constrained conditions at higher elevations might select for smaller flowers. In the extreme conditions on Teide stratovolcano, resource allocation to floral display is probably costly in terms of growth and survival, as found in other species (Galen 1999; Totland 2001).

Our previous findings on the breeding system and pollinators of *V. cheiranthifolia* do not support the hypothesis that pollinators are an important selective force for flower size in this species (Seguí *et al.* 2017). Flower visitation rates showed variations among sites depending on the year, which is often attributed to variable climatic conditions (Totland 1994; Herrera 1995). However, they were not found to decrease with elevation, nor did pollen limitation vary across sites. Such pollen limitation is a requirement for the selection acting on floral traits engaged in pollinator attraction, and selection is expected to be most intense in populations with greatest pollen limitation (Johnston, 1991; Totland, 2001). Nevertheless, we did find that pollinator richness increased with elevation (Seguí *et al.* 2017). Such a high number of species visiting *V. cheiranthifolia* at the highest elevation might be associated with the wider flower spurs found there, since wider spurs would allow a greater number of pollinators to accede to the nectar. Indeed, Stang et al. (2006) found that the number of flower visitor species increased with nectar holder width, especially in species with short spurs, as is the case of *V. cheiranthifolia*. As far as we know, no previous studies have documented an increase in spur width with elevation. We hypothesize that this might be a mechanism to increase flower visitation by greater richness of pollinators.

Furthermore, we found that the length of the petals and spurs was positively associated with SLA (and with leaf area). The SLA is considered a good indicator of water stress in plants, since it has been found to be correlated with relative growth rate (RGR) under stress conditions (Poorter *et al.* 2009), except in woody plants (Galmés *et al.* 2005). Accordingly, the consequent correlation between SLA and flower size suggests that pollinators do not exert a strong selection for a specific flower shape, independently of environmental conditions. In such a case, the flower-leaf correlation is not decoupled in this species (Berg 1960). Moreover, it suggests that environmental conditions may be responsible for the altitudinal differences in petal size, but not for those observed in spur width.

Plants protected from herbivores had larger flowers with longer and thinner spurs than plants exposed to them. In addition, in the herbivore-excluded individuals, SLA was even more strongly associated with flower size, as well as with floral display, and spur length and width. This indicates an important interactive effect of herbivores shaping floral traits, even greater than that of the abiotic conditions. Herbivores further have an indirect effect on plant survival on comparing the years. The Teide violet is a rhizomatous species, but we detected no reserves in its rhizome under herbivory pressure. By contrast, individuals inside the exclosure presented thick rhizomes, as well as larger leaves. This is a long-lived species, as are other perennial violets (Culley 2002; Herrera & Bazaga 2010) and after five years monitoring it, the evidence shows that rhizome reserves accumulate when herbivores are absent. This probably allows it to produce larger flower sizes and a greater number of flowers. By contrast, plants under herbivore pressure do not have the possibility of accumulating such reserves and thus produce fewer and smaller flowers.

On the other hand, plants exposed to herbivores showed a spur width positively correlated to their reproductive success, though only at the highest site. In other words, flowers with wider spurs produced more viable seeds, which may indicate that pollinators select for wider spurs at the highest elevation. However, when herbivores were absent, selection acting on wider spurs completely disappeared, since there was no correlation between spur width and seed set. Hence, herbivory effects can alter the pollinator-mediated selection acting on flower traits. Plants within the exclosure showed no pollen limitation (Seguí *et al.* 2017), supporting the hypothesis that selection of floral traits is less intense in plants that are not pollen-limited (Totland 2001). Very few studies have tested the effects of vertebrates changing the strength of pollinator-mediated selection on the shape and size of flowers. When studying *Erysimum mediohispanicum*, Gómez (2003) suggested that pollinator-mediated selection on floral traits could be disrupted by conflicting effects of plant enemies during or subsequent to pollination, with potential consequences for plant specialization.

Therefore, the greatest reproductive success (viable seeds per flower) found in the herbivore-excluded individuals may be due to distinct factors related to the pollinator-mediated selection on floral traits and display. On the one hand, the longer and thinner spurs when herbivores are absent probably determine the pollinator species that will visit the flowers, allowing only long-tongued pollinators access to the nectar. Longer corollas normally conceal larger volumes of nectar (Kaczorowski *et al.* 2005; Gómez *et al.* 2008; Martins & Johnson 2013), and this is normally associated with increased pollination effectiveness because they increase the frequency (Thomson *et al.* 1989; Real & Rathcke 1991) and/or duration of pollinator visits (Cresswell 1999). This hypothesis of narrower plant specialization in herbivore-excluded plants might also explain the decrease in autonomous selfing and the higher seed viability observed inside the exclosure (Seguí *et al.* 2017). Long spurs as nectar barriers may be favoring long-tongue pollinators such as *Anthophora alluaudi* and *Macroglossum stellatarum*, rather than small insects and short-tongued pollinators (such as the small beetles *Melyrosoma hirtum* and *Meligethes varicollis*), which may favor selfing in the species. Furthermore, the higher density of conspecific flowering individuals and the greater production of flowers per plant inside the herbivore-exclusion may be another important factor explaining the greater reproductive success there. We found that plants with more flowers produced more fruits, and that plants with more flowers and more conspecific flowering plants in their vicinity increased the viability of their seeds. The number of flowers is a trait that indirectly affects the fitness of many plant species by increasing the visitation rate of many pollinators (Conner & Rush 1996; Thompson 2001). However, our censuses did not show significant differences in flower visitation rates between plants excluded from and exposed to herbivores (Seguí *et al.* 2017), at least when comparing plants with many flowers. It is also plausible that plants inside the exclosure were less resource-limited in producing more flowers and fruits and setting more viable seeds, although we did not detect significant differences in our analyses regarding the main nutrients and water availability.

In short, the floral traits currently observed in *V. cheiranthifolia* may be quite different from those one would expect in the absence of herbivores like the invasive rabbits. These introduced vertebrate herbivores seem to be altering both flower morphology and plant floral display and abundance, thus changing the selection that either pollinators or abiotic factors may be exerting on such plant traits. Besides the strong negative impact of invasive herbivores on this iconic endemic species on Teide, our findings show the importance of simultaneously assessing the multiple factors that influence plant floral traits.

2.5 Acknowledgments

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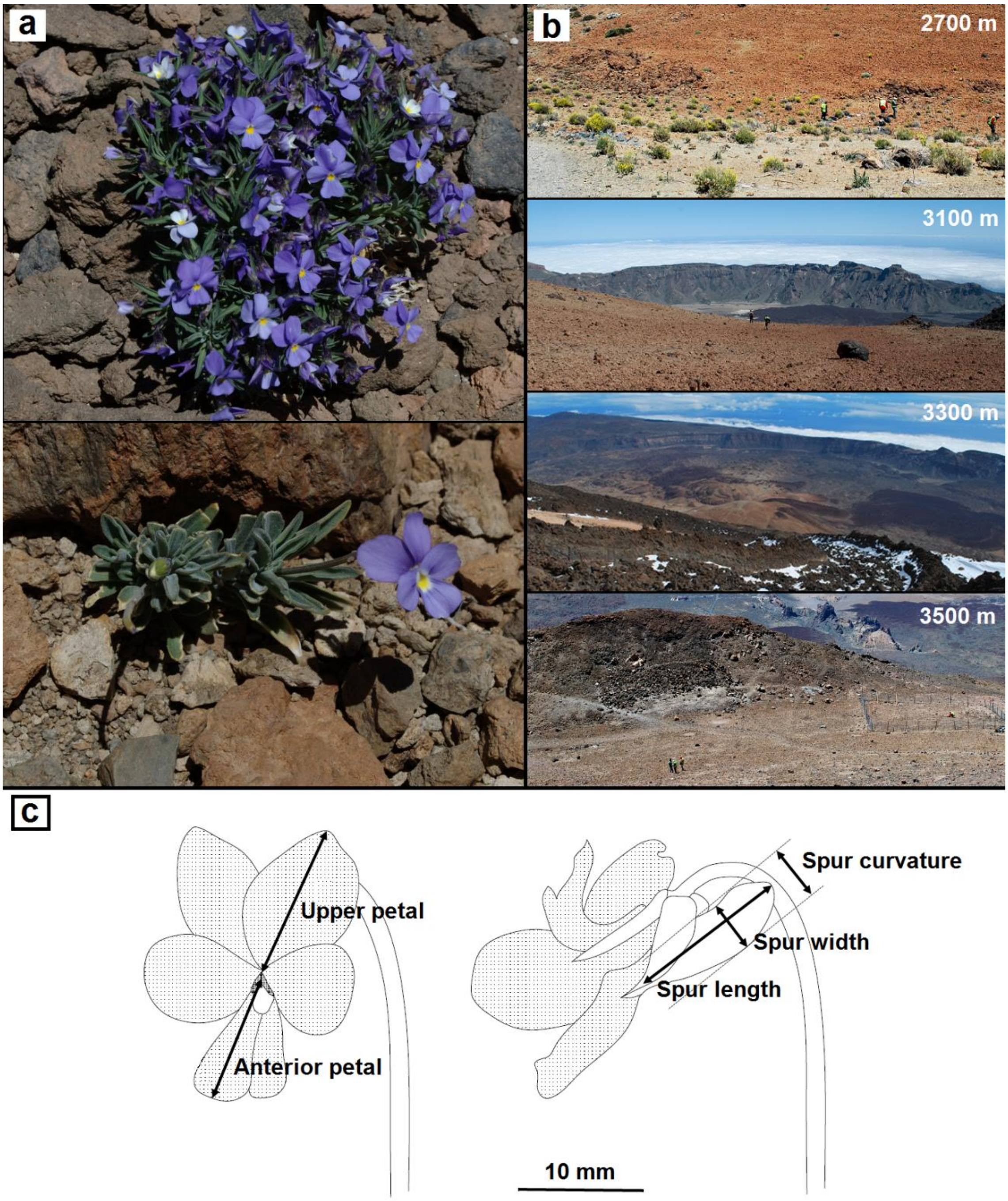

Figure 6. Images of (A) plants rabbit-excluded and non-excluded, (B) the four elevational study sites, and (C) floral traits measured.

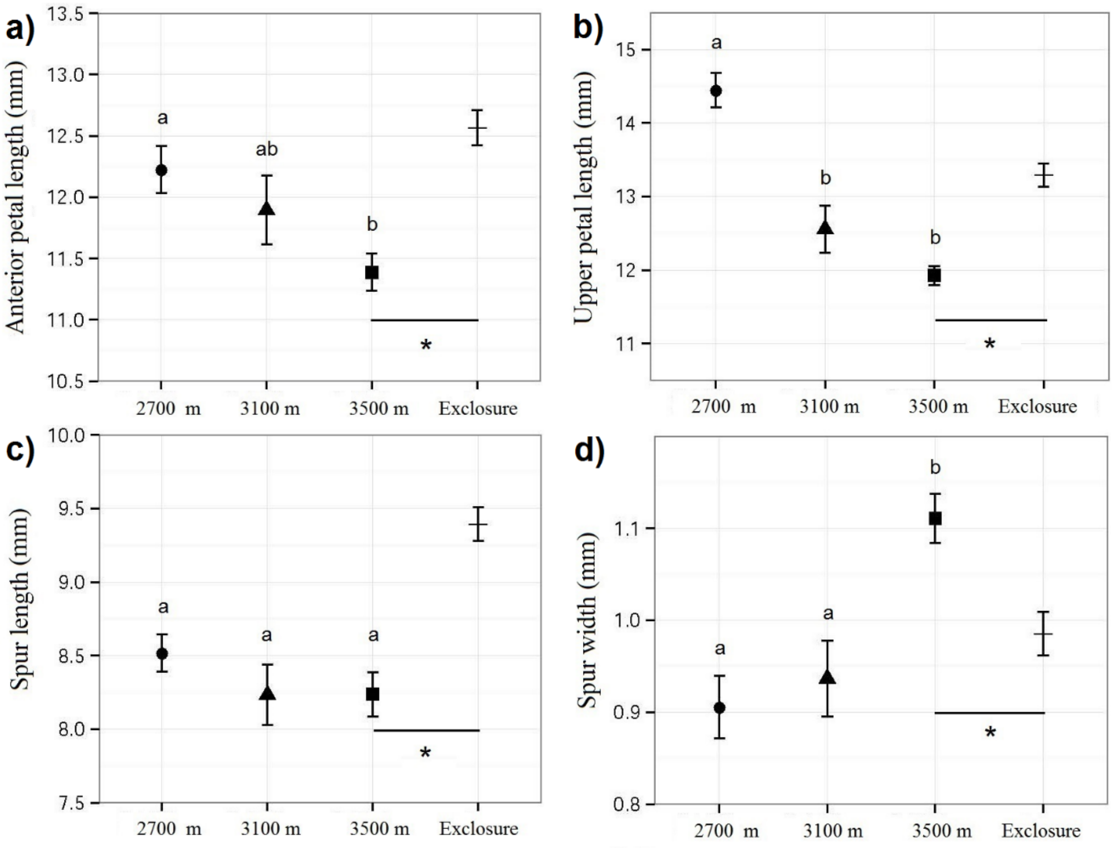

Figure 7. Mean and standard deviation of floral traits measured in *V. cheiranthifolia* across study sites. Different letters and * indicate significant differences within sites.

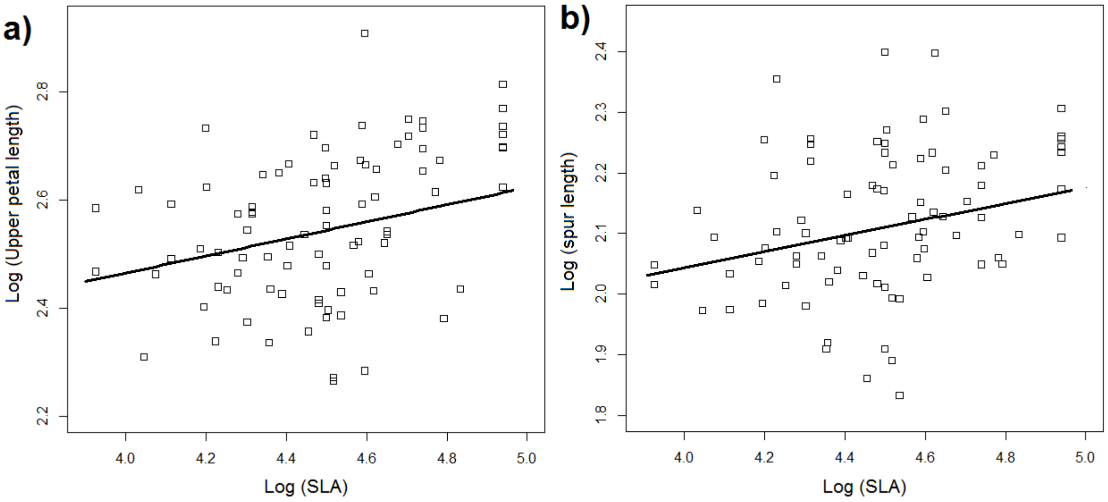

Figure 8. Upper petal and spur length (open-squares) in response to specific leaf area in *V. cheiranthifolia*. Lines represent the estimates of the models.

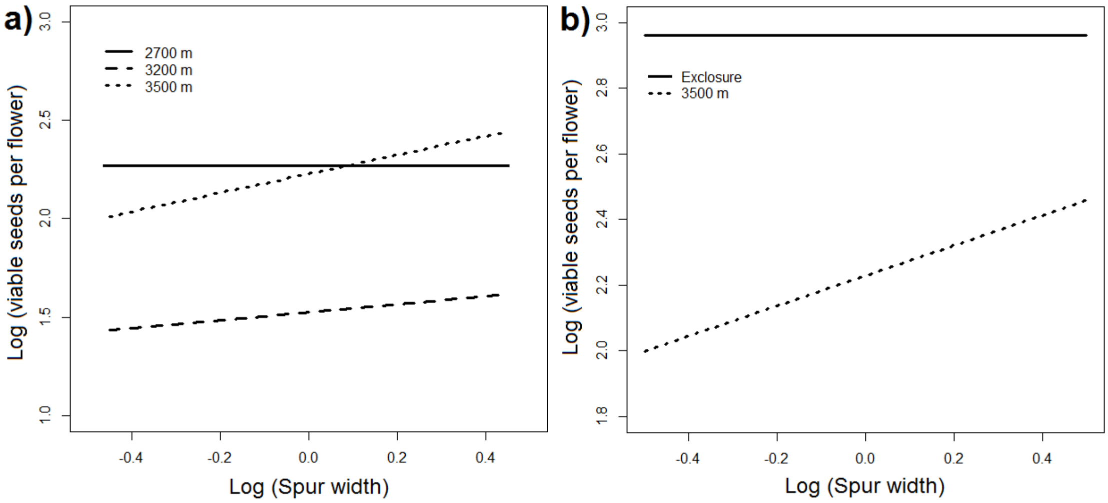

Figure 9. Plant reproductive success (viable seeds per flower) in response to spur width across the elevational sites, and between excluded and non-excluded pants. The lines represent the best estimates for each study site.

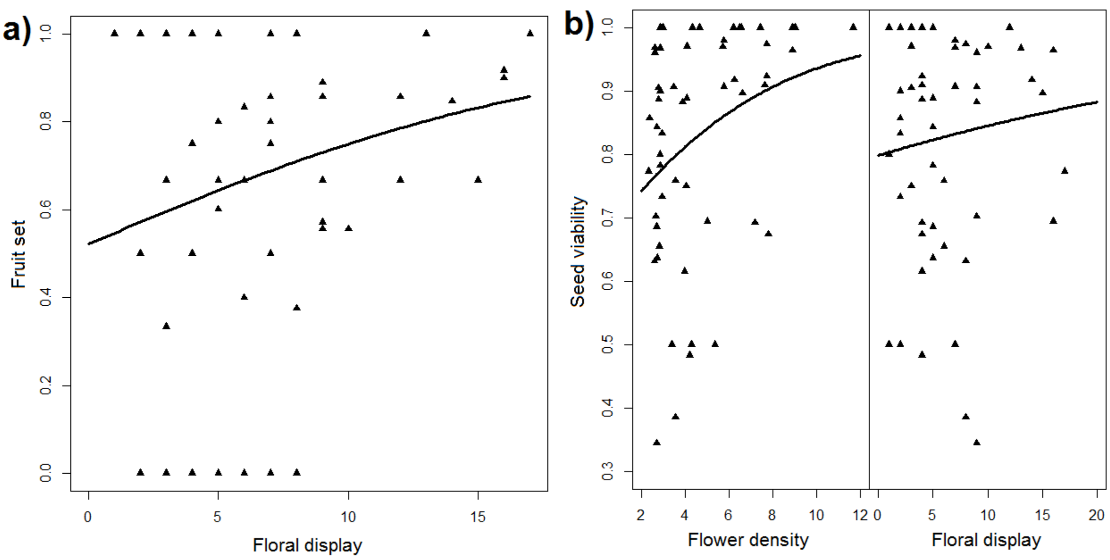

Figure 10. (A) Fruit set (filled triangles) in response to floral display, and (B) seed viability (filled triangles) in response to floral display and conspecific flower density. Lines represent the estimates of the model.

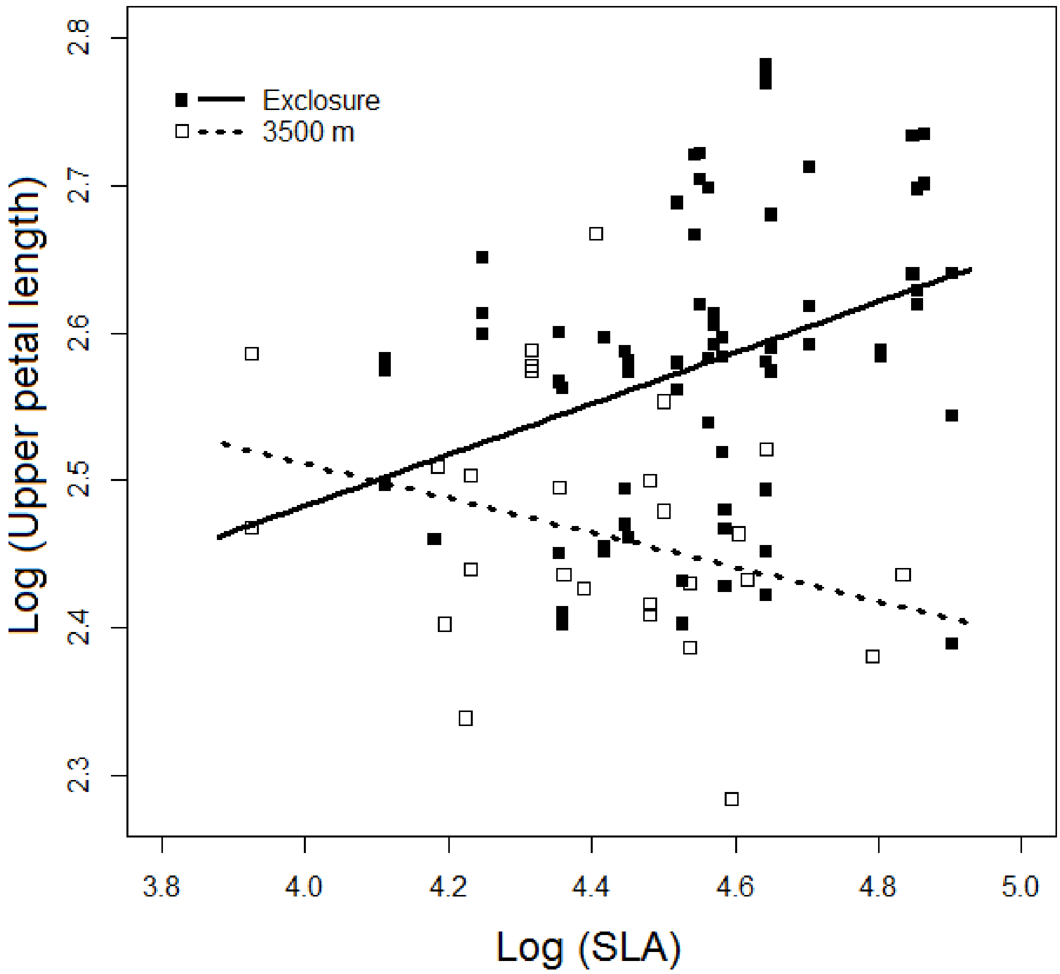

Figure 11. Interactive effect of rabbit excluded (filled squares) and non-excluded (open squares) on the response of spur width to specific leaf area in *V. cheiranthifolia*. Lines represent the best estimates for each study site.

Table 4. Microclimatic and soil data at each study site.

Site	Coordinates	Elevation	Reproductive individuals	Individuals per hectare	Flowers measured	Mean (ºC)	O.M. (%)	Moisture (%)	P_2O_5 (ppm)	Nitrogen (g/Kg)
Montaña Blanca	28 16.195 N 16 36.938 W	2732 m	22	19.96	32	10.85	1.76	1.70	0.00	1.4
Pico Viejo	28 15.696 N 16 39.407 W	3050 m	20	7	30	9.17	0.25	0.90	0.00	0.25
Refugio de Altavista	28 16.469 N 16 37.789 W	3296 m	17	19.63	-	8.34	0.02	0.30	0.00	0.25
La Rambleta	28 16.490 N 16 38.768 W	3518 m	24	49.26	51	5.02	0.03	0.90	0.00	0.17
Fenced exclosure	28 16.111 N 16 38.338W	3518 m	25	-	71	5.02	0.08	0.90	0.00	0.14

Notes: Temperature was measured with a temperature data logger at 1 m height above ground at each site through a whole year (2014-15).

2.8 Supplemental material

Table S1. Set of best-ranked linear models (*i.e.,* models with ΔAIC < 2) fitted for reproductive performance of *V. cheiranthifolia*. Log(L), maximised log-likelihood function.

	Log(L)	**AICc**	**Akaike weight**	**R^2**
FS ~ Floral display	-118.624	241.4	1	0.33230
SS ~ Floral display + Flower density	-162.096	332.9	0.291	0.07
SS ~ Floral display	-163.319	333	0.267	0.03
SS ~ 1	-164.477	333.1	0.252	0.00
SS ~ Flower density	-163.655	333.7	0.191	0.02
SV ~ Flower density + Floral display	-249.196	504.9	1	0.6544

Chapter 3

Phenotypic and reproductive responses of an Andean violet to environmental variation across an elevation gradient

Seguí, J., Lázaro, A., Traveset, A., Salgado-Luarte, C., & Gianoli, E. (2017). Phenotypic and reproductive responses of an Andean violet to environmental variation across an elevation gradient. Under review in *Alpine Botany*.

Abstract

Environmental gradients in alpine systems may lead to differences in both abiotic conditions and species interactions in very short distances. This may lead to physiological, reproductive and phenotypic changes in plants to enhance fitness in each environment. In this study, we explored how the Central Andean *Viola maculata* responds to the elevation gradient where it is distributed, with an expected increase in water availability and a decrease in pollinator availability with elevation. We hypothesized that: (1) plants would be more water-stressed at low elevations, (2) investment in and success of cleistogamous flowers (closed, self-pollinated) would increase with elevation, and (3) correlation patterns between floral and vegetative traits would vary along the gradient according to changes in biotic/abiotic selection pressures across sites. We partially confirmed the inverse gradient of water stress with elevation, with *Viola maculata* populations in the lowest site experiencing lower soil moisture and showing thicker leaves and lower stomatal conductance. Cleistogamy was more prevalent and successful at the highest site, thus confirming the hypothesis of maintenance of a mixed mating system as reproductive assurance. Correlation patterns between flower and leaf size differed across sites, with stronger vegetative-floral correlation at the lower sites and a weak correlation at the highest site. This finding disagrees with the notion of pollinators as drivers of correlation between floral and vegetative traits. Our study shows how a narrow gradient in an alpine system may affect not only reproductive and physiological responses in plants, but also floral and vegetative covariance.

Keywords: cleistogamy, correlation pleiades, drought, floral-vegetative correlation, phenotypic integration, *Viola maculata*

3.1 Introduction

High-mountain (alpine) ecosystems are highly heterogeneous, with environmental factors changing across short distances (Körner 2003), and therefore are useful systems to study the patterns and processes underlying adaptive phenotypic plasticity (von Wettberg *et al.* 2005; Gianoli & Valladares 2012; Hamann *et al.* 2017) and local adaptation in plants (Leimu & Fischer 2008); which are both important mechanisms enabling individual lineages and plant populations to maximize fitness in response to environmental heterogeneity. Small-scale differences in abiotic (e.g., nutrients, light, water) and biotic (e.g., pollinators and seed dispersers) resources for plants may lead to different ecological strategies, which would reflect distinct patterns of variation in physiological and morphological traits and biomass allocation. Such high heterogeneity at small spatial scales makes mountains large natural laboratories in which plant responses to different abiotic and biotic factors can be tested.

In the case of elevational gradients, as elevation increases, temperature and atmospheric pressure decrease steadily, while solar radiation increases (Körner 2003). Mountain ranges around the world, however, exhibit different trends in moisture, growing season length and cloudiness with elevation (Körner 2007). For example, in alpine habitats of Mediterranean-climate areas, drought may limit plant photosynthetic performance at low elevation (Loik & Redar 2003; Hernández-Fuentes *et al.* 2015), and low temperature together with high solar radiation early in the morning may cause photoinhibition, consequently reducing photosynthetic efficiency (Germino & Smith 2000). Low water environments select for sclerophyllous leaves with low SLA (specific leaf area, $cm^2 mg^{-1}$) and tend to reduce stomatal conductance to control for water loss (Lázaro-Nogal *et al.* 2015). Although stomatal density generally increases with aridity (e.g., Carlson *et al.* 2016), it may vary idiosyncratically along environmental gradients because of the simultaneous influence of several factors (e.g., soil moisture and wind exposure); hence elevation-related patterns in stomatal density are difficult to predict (Wang et al. 2014).

Plants that cope with varying ecological factors associated with altitude may show reproductive mechanisms that enhance fitness in each environment. For example, high levels of autogamy (Arroyo *et al.* 2006) or a mixed-mating system strategy, such as the production of both cleistogamous (CL) and chasmogamous (CH) flowers, may provide reproductive assurance when pollinators are scarce (Culley 2002; Albert *et al.* 2011). The production of CL flowers (closed, self-pollinated) might compensate for decreased fruit set due to unsuccessful fertilization of many CH flowers (showy, supposedly outcross-pollinated), thus conferring plants reproductive assurance (Schoen & Lloyd 1984; Redbo-Torstensson & Berg 1995). Schoen and Lloyd (1984) proposed a model where the CH/CL system would be favoured in a heterogeneous parental environment if individuals could assess and respond appropriately to environmental variation (mainly in terms of pollinator or seed predator abundance).

Plant traits are often correlated and selection should favour an integrated phenotype (Pigliucci 2003). Phenotypic integration, the pattern and magnitude of covariation among traits in a given organism (Gianoli & Palacio-López 2009), often increases with environmental stress and should be particularly observed among certain groups of characters that have been subjected to joint selection or are functionally related (Schilichting 1989; Waitt & Levin 1993; Gianoli 2004). Thus, plants with specialized pollination should exhibit floral characters that strongly covary with one another but not with vegetative traits, a pattern known as *correlation pleiades* (Berg 1960). However, studies analysing phenotypic variation and covariation between floral and vegetative traits in species with specialized pollination found inconsistent evidence for Berg's hypothesis (Waitt & Levin 1993; Conner & Sterling 1996). Pélabon et al. (2013) found that the decoupling of phenotypic covariation between vegetative and floral traits in an outcrossing species can be environment-dependent. Brock and Weinig (2007) further suggested that vegetative plasticity may affect the expression of floral traits in some environments, and that there may be environment-specific constraints on the evolution of floral and vegetative traits.

The Central Chilean Andes are under the influence of a Mediterranean-type climate (Di Castri & Hajek 1976). Consequently, during the dry summer plants at lower elevations are exposed to high temperatures and water stress (Cavieres *et al.* 2006), leading to a low photosynthetic performance (Hernández-Fuentes et al. 2015). By contrast, at higher elevations water is not a limiting factor and the increase in temperature may have a positive effect on photosynthetic performance (Hernández-Fuentes et al. 2015). Furthermore, ecological studies in this region have reported that pollinator abundance (Arroyo *et al.* 1985; Arroyo & Squeo 1990), community-level flower visitation rate (Arroyo *et al.* 1985) and pollination-networks' organization (Ramos-Jiliberto *et al.* 2010) decline with elevation. Given that reduced water availability leads to smaller flowers (Carroll *et al.* 2001; Caruso 2006) and pollinators usually prefer larger flowers (Suárez *et al.* 2009; Lázaro & Totland 2014) for such greater pollinator abundance at lower elevations to translate into effective selective pressures there should be a decoupling of vegetative and floral traits.

In the present study, we aimed at understanding the physiological, phenotypic and reproductive responses of an Andean violet to environmental variation across an elevation gradient, where both water stress and pollinator abundance should decrease with elevation. Specifically, the goals of this study were: (1) to evaluate how does this violet species respond physiologically to the environmental conditions along the gradient, (2) to assess whether this species adjusts its strategy to particular environments by modifying the proportion of flower types produced (CH/CL) and/or by delaying selfing in CH flowers to assure reproduction, (3) to evaluate whether the relation between floral and leaf traits change along the elevation gradient, according to changes in abiotic and biotic conditions, and (4) to evaluate how phenotypic integration changes with environmental stress. Our hypotheses were: (1) plants would be more water-stressed at low elevations and therefore would show increased water-saving physiological responses, (2) to enhance reproductive output at each environment, cleistogamy would be favoured at higher elevations and chasmogamy at lower elevations, 3) a decoupling between vegetative and floral traits at low elevation, where a higher pollinator selection pressure is expected (this assumes selection forces of opposite sign acting on vegetative and floral traits and pollinator-mediated selection on floral size), and (4) since phenotypic integration often increases with environmental stress, we expected a higher phenotypic integration in the site where plants showed decreased physiological performance.

3.2 Materials and methods

Study species and study sites

Viola maculata Cav. (Violaceae) is an alpine perennial rhizomatous herb, distributed along the southern part of the Andes (33ºS-55ºS) at elevations from 1600 m to 2500 m. This species grows in grasslands, steppes and stony places, often under the protection of other herbs or rocks. Leaves are alternate, ovate to lanceolate petiolate. *Viola maculata* produces chasmogamous zygomorphic yellow flowers from December to February, on 6-12 cm long peduncles. At the end of the chasmogamous flowering period, it starts producing cleistogamous flowers on 2 cm peduncles (J. Seguí, personal observation; first time recorded in the species). This plant species has clonal (vegetative) growth, with the production of ramets through horizontal growth (339 ± 30 cm^2; n = 34); for this reason, for all the data collected in this study, we selected ramets with a minimum separation between them of 1 meter, so we could ensure their independence. According to Lovett Doust (1981), it presents “phalanx” growth, whereby ramets are packed close to each other and the plant stays rooted in the same place on a year-to-year basis.

We conducted this study on three sites located along an altitudinal gradient in Central Chilean Andes, in Altos de Lircay National Reserve (35°36'07.0"S 70°56'49.0"W), spanning the whole altitudinal range of the species in the Reserve, ranging from 1950 m to 2350 m (Fig. S2). The low-alpine site was located at 1950 m, close to the timberline of *Nothofagus antartica* and *N. obliqua*, and accompanied by *Chusquea coleou* Desv. and *Festuca pallescens* (St.-Yves) Parodi. The mid-alpine site, located at 2200 m, was accompanied by *Mulinum echinus* DC and *Berberis empetrifolia* Lam. Finally, the highest site, at 2350 m, was a sub-nival site close to the summit of "El Cerro Peine", and accompanied by *B. empetrifolia* Lam. and *F. pallescens* (St.-Yves). *Viola maculata* phenology at the lowest site was advanced about two weeks with respect to the two highest sites (Fig. S3). The vegetation cover decreased with elevation (Fig. S2).

Soil moisture and physiological traits

We measured soil moisture at the study sites by means of a moisture sensor (Irrometer Company, Riverside, California), which measures soil water tension in centibars (cb), i.e, the effort required by root systems to extract water from the soil. We placed the tensiometer in a hole 20 cm deep, in the root zone of a *Viola maculata* ramet, and waited between 30-60 min to take the measure. A high reading indicated the dry end of the scale whereas a low reading indicated the wet one. We took two measures for each elevation site, one at the end of December 2014 and another one at the end of January 2015. Measures across elevation sites were taken on the same day within the shortest time possible (≤ 4 h).

During the first week of February 2015, in 20 ramets per population, we measured the maximum photochemical efficiency of PSII (Fv/Fm), an estimate of photosynthetic performance (Maxwell & Johnson 2000), by means of a modulated fluorometer PAM-2000 and leaf clip holder 2030-B. In the same ramets we also measured stomatal conductance (mmol.m^{-2}. s^{-1}), a parameter that decreases with water stress (e.g., Lázaro-Nogal et al. 2015) using a leaf porometer (model SC-1, Decagon Devices, Inc., Pullman, WA, USA). We measured stomatal conductance and Fv/Fm between 14:30 h and 17:30 h. The minimum and maximum dark-adapted fluorescence Fv/Fm were obtained after dark adaptation of the leaves for at least 20–25 min. During measurements, photosynthetically active radiation (PAR) was ~1900 (µmol/m^2 per second) at the lowest site, and ~2100 (µmol/m^2 per second) at the two upper sites. We also estimated the specific leaf area (SLA) from the ratio between leaf area and leaf dry weight (see *flower and leaf traits* section). SLA provides information on local water availability, decreasing with water shortage. For example, Ramírez-Valiente et al (2010) studied 13 cork oak populations in the Iberian Peninsula and reported that sclerophyllous leaves (low SLA) provide fitness benefits under water-limited environments. This because sclerophylly minimizes water loss while maintaining photosynthetic rates, thus allowing a conservative water use.

We also recorded the density of stomata and trichomes per mm^2 in the same 20 ramets per population. Stomata density reflects adaptive responses of plants to the environment at larger scales (Hetherington and Woodward 2003; Wang et al. 2014).

We took a piece of epithelium of approximately 25 mm^2 from one leaf per stem and let it soak in 1.5% NaClO at 36°C for 30 minutes, and then stained for 10 min in a solution of safranin 750µg/ml. Finally, we photographed the sample using an optical PCE-MM200 Microscope, and digital images were used to count the number of stomata and trichomes in a 1 mm^2 grid, with the ImageJ software (ImageJ ver. 1.6).

Reproductive traits

To study the reproductive ecology of the species, we marked 39 ramets at the beginning of December 2014 (12 ramets at 1950 m; 14 ramets at 2200 m; 13 ramets at 2350 m) and estimated floral display, reproductive success, selfing levels and visitation rates. In these marked ramets we estimated the floral display by recording the total number of cleistogamous (CL) and chasmogamous (CH) flowers produced per ramet (flowers per ramet: 32.83 ± 4.74 at 1950 m, 15.53 ± 2.14 at 2200 m and 35.84 ± 8.11 at 2350 m), as well as their reproductive output (from a total of 472 CL flowers and 586 CH flowers). The reproductive success of CH and CL flowers was compared by measuring fruit set (proportion of flowers setting fruits) and seeds per capsule across sites. To avoid seed loss in the field, we individually bagged fruits before ripening. Seed production was quantified in the lab by counting the number of seeds after fruits opened.

To evaluate selfing level (capacity to produce seeds by spontaneous self-pollination), in a total of 38 ramets nearly half of the CH flower buds available per ramet were bagged before anthesis with a mesh-cloth that excluded floral visitors. Control flowers (open-pollination) from the same ramet were also marked and left unbagged. To quantify the reproductive success of each treatment we estimated the number of seeds produced per flower of the bagged and unbagged flowers.

We conducted a number of 30-min censuses in different ramets of *Viola maculata* distributed along the gradient: total 450 min at 1950 m, total 300 min at 2200 m, and total 150 min at 2350 m. During each 30-min observation period, the researcher remained in front of a single ramet, recording all the insects that visited the flowers and the number of open CH flowers available per ramet. Only visitors touching the reproductive parts of the flowers were considered. We measured flower visitation rate as the number of visits per hour per flower. Censuses were carried out during December 2013 and January 2014, mostly from 12:00 h to 17:00 h (low temperatures preclude insect activity before and after this time), avoiding windy days. Due to the high frequency of windy days and the time needed to arrive at the upper site, we censused visitors during fewer hours at this site.

Floral and leaf traits and their relation

To characterize phenotypic variation in floral and leaf traits, we selected a total of 121 independent ramets in the altitudinal gradient (45, 40 and 36 ramets at 1950 m, 2200 m and 2350 m, respectively) in January 2015. Within these ramets, we randomly chose a stem, counted the number of leaves and flowers, and selected a ripe leaf with a CH flower associated to its base (Fig. S4) to measure: (1) leaf area and dry weight; (2) flower peduncle length (cm; measured with a steel ruler); (3) area and dry weight of petals (measuring separately the upper, lateral and anterior petals); and (4) area and dry weight of the five sepals, separately. We scanned each leaf (measured within a week from collection) and flower (preserved in alcohol) and calculated the area with the image analysis software ImageJ (ImageJ ver. 1.6). We weighed flowers and leaves after oven-drying (4 days at 60 ºC). We obtained the floral and leaf biomass by multiplying the weight of the leaf and flower traits (weight of the 5 petals plus the 5 sepals) by the number of leaves and flowers in each stem, respectively. Finally, we obtained the coefficient of variation (standard deviation / mean) of each leaf and flower trait to estimate phenotypic variation within each site.

Data analyses and statistics

All the analyses were conducted in R (R 12.2, R Development Core Team, 2008), except otherwise indicated. We assessed differences among sites in mean values of physiological traits (stomatal conductance, Fv/Fm, stomata and trichome density, SLA) and morphological traits (table 1) by using linear models, with log-transformed variables to meet the assumptions of normality. To compare reproductive success among sites and its interaction with treatment (selfing) and flower type (CH and CL), we used generalized linear mixed models (GLMM), including ramets as the random factor. To compare flower visitation rate (visits / 30 min census), we used a generalized linear model including site as predictor variable, and number of flowers as covariate. The coefficient of variation (standard deviation /mean) of floral and leaf areas was obtained using the *cv* function of the *raster* package (Hijmans & Van Etten 2012) in R. For generalized and mixed models, we used error distribution and link functions that best fit the data: (1) binomial distribution for fruit set and CH/CL relative proportion of total flowers, (2) a Poisson distribution for visitation rate and seeds per capsule and (3) a Gamma distribution for seeds per flower. After all the models, we performed *a posteriori* tests based on least-squares means comparisons among levels of significant factors, by using the package *lsmeans* (Lenth 2016) in R.

To study how reproductive and vegetative traits were related at each elevation, we used three GLM models in which reproductive traits (petal area, flower peduncle length and floral biomass) were the response variables, whereas vegetative traits (leaf area and leaf biomass) and their interaction with site were included as predictors. We fitted GLM model with all variables log-transformed to achieve normally distributed residuals. Floral and leaf traits showed VIF (Variation Inflation Factor) values < 3, indicating no collinearity problems, and therefore could be included together in the analysis (Zuur *et al.* 2009).

We estimated phenotypic integration of floral and leaf characters using Wagner (1984) index of phenotypic integration (INT), which measures the variance among the eigenvalues of a phenotypic or genetic correlation matrix (Lázaro & Santamaria 2016). Each eigenvalue represents the amount of variation explained by a given principal component. A high variance among eigenvalues indicates high integration because most of the phenotypic variation is accounted for by the first principal components and hence traits are strongly associated. We calculated phenotypic integration indices per population. Whenever we had several measurements for one character (i.e., the two lateral petals) we averaged them to obtain a single value per individual. The expected eigenvalue variance under the hypothesis of random covariation among traits is Exp (INT) = (number of traits -1) / number of ramets. As the number of ramets measured varied among populations, Exp (INT) also did. Therefore, in order to compare populations, we calculated corrected integration indices (INTc) by subtracting from each value of INT its expected value Exp (INT) (Wagner 1984; Cheverud 1988). Bootstrapping was used to calculate the standard deviations and 95% confidence intervals of INTc in R. The INTc was considered to indicate significant floral integration when its 95% confidence interval did not include zero, and INTc was expressed as the percentage of the maximum possible value (the number of traits considered; Herrera *et al.* 2002a).

Finally, we also studied the differences in the floral and leaf covariance matrix structure across sites following the ‘jump-up’ approach of Phillips and Arnold (1999). In this approach, based on Flury's (1988) hierarchical method, a model assuming heterogeneity of covariance matrices is sequentially compared, by means of likelihood ratio tests, with models that specify different relationships among the populations’ covariance matrices (common principal components, partial principal components, proportionality, and equality). These tests were performed using ‘The Common Principal Component Analysis’ software by Patrick Phillips (pages.uoregon.edu). Correlation matrices were calculated in R with the package Hmisc (Harrell 2017).

3.3 Results

Soil moisture and physiological traits

Soil water tension, which is inversely proportional to soil moisture, was higher at the lowest site compared to the two other sites at the end of December (1950 m: 32 cb;

2200 m and 2350 m: 26 cb). However, at the end of January soil water tension at the lowest site (64 cb) was only slightly higher than that at the upper site (62 cb), while it was lowest at the mid-elevation site (48 cb).

There were no differences in photosynthetic efficiency of PSII (Fv/Fm) among sites ($F_{2,59}$ = 1.85, *P* = 0.16; Fig. 12A). Stomatal conductance increased with elevation ($F_{2,59}$ = 15.56, *P* < 0.001; Fig. 12B) and specific leaf area (SLA, cm^2/g) was higher at the upper site ($F_{2,122}$ = 9.91, *P* < 0.0001: Fig. 12C). Stomatal density was lower in plants at the upper site (Fig. 12D), while leaf trichome density did not differ among sites ($F_{2,58}$ = 0.17, *P* = 0.84).

Reproductive traits

The GLMM showed that the proportion of CH and CL flowers varied across sites (χ^2 = 134.3, df = 2, *P* < 0.0001; R^2 = 0.32). Ramets at the intermediate site (2200 m) produced the highest proportion of CH flowers (estimate: 1.87 ± 0.2, *P* < 0.001), while those at the highest site produced the lowest proportion of CH flowers (estimate: -0.30 ± 0.09). When we compared reproductive success between CH and CL flowers as estimated by fruit set, we found a significant interaction between site and flower type (χ^2= 83.12, df = 2, *P* < 0.0001; R^2 = 0.32). This was because fruit set of CL flowers was higher than fruit set of CH flowers at the upper and intermediate site, whereas at the lowest site the fruit set of different flower types did not significantly differ (Fig. 13A). We also found a significant interaction between the type of fruit (CH or CL fruits) and site on the number of seeds per capsule (χ^2 = 6.66, df = 2, *P* = 0.03; R^2 = 0.41), because there were no differences in seed number across sites in CL fruits, while CH fruits had more seeds at the lowest site compared to the upper ones (Fig. 13B). In addition, ramets with a higher proportion of chasmogamous flowers were less successful –in terms of fruit set– at the highest site (r = -0.80, *P* < 0.001), whereas at the other sites CH:CL ratios in each ramet were not correlated with such measure of success (Fig. S5).

GLMM showed that bagged CH flowers produced fewer seeds per flower than unbagged ones (χ^2 = 38.7, df = 1, *P* < 0.001, R^2 = 0.54), a pattern consistent across sites (selfing x site; χ^2 = 3.05, df = 2, *P* = 0.22). Autonomous selfing accounted for only 11.5% (0.81 ± 0.3 seeds per flower) of reproductive output of CH flowers (6.97 ± 1.5 seeds per flower). We observed very low visitation rates overall (0.4 visits per hour and flower), with most of the flower visits corresponding to ant species (8 interactions; 80% of the total visits). At 1950 m we observed two interactions by a butterfly (*Vanessa* sp.), and two visits by ant species, at 2200 m six visits by ant species, and at 2350 m one visit by an ant. The overall very low visitation rates precluded performing the statistical analysis planned.

Floral and leaf traits and their correlations

Flowers were larger and heavier at the intermediate site (Table 5). By contrast, flower peduncles were longer at the lowest site (Table 5). Leaf area and dry biomass per stem did not vary among study sites. The size of leaves and flowers was more constant at the lowest elevation (Table 5). Flower area increased with leaf area, but not consistently across sites (site x leaf area: $F_{2,120} = 3.38$, $P < 0.05$). Flower area increased with leaf area in the two lower sites, whereas there was no relationship between these two variables at the upper site (Fig. 14A). Flower peduncle length was correlated with leaf area, but this association tended to be stronger as elevation decreased (site x leaf area: $F_{2,120} = 2.69$, $P = 0.07$). By contrast, flower biomass increased with leaf biomass consistently in all sites ($F_{1,120} = 28.08$, $P < 0.0001$).

Significant phenotypic integration was found at the three elevations, as 95% confidence intervals of INTc did not include zero. However, such phenotypic integration did not differ significantly among populations (INTc showed overlapping confidence intervals), with the intermediate site showing slightly higher INTc (36.5%) than the low-elevation (30.66%) and the high-elevation site (25%). Although the magnitude of phenotypic integration (INTc) did not differ among populations, its pattern did. Phillips and Arnold's (1999) analysis showed that the matrices for the study populations differed in principal components ($\chi^2 = 78.74$, df = 30, $P < 0.001$), correlation structure ($\chi^2 = 137.95$, df = 42, $P < 0.0001$) and proportionality ($\chi^2_2 = 109.83$, df = 40, $P < 0.001$). Significant correlations between floral and leaf traits were always positive in all three populations (Fig. 14B), indicating the absence of trade-offs between floral traits and leaf area. As expected, the size of upper, lateral and anterior petals covaried in all sites. However, other trait correlations differed among sites. Leaf area was correlated with sepal area and petal area at the two lower sites, especially at the intermediate site, but not at the highest one. By contrast, flower peduncle length was highly correlated with petal area only at the highest elevation (Figure 14B).

3.4 Discussion

The hypotheses of lower pollinator visitation rates and reduced water stress with elevation were partially supported by our results. Physiological data and soil moisture measurements partially show lower water stress at higher elevations. We did not find clear differences among sites in visitation rates (we would need more years of censuses). Nonetheless, reproductive traits indicate that cross-pollination is favoured at low elevation (CH flowers were more abundant and successful than CL flowers) and intermediate elevation (it showed the largest ratio of CH:CL flowers per ramet, and the largest flowers), while self-pollination (cleistogamy) is favoured at high elevation.

3.4. Discussion

Soil moisture and physiological traits

The Andes of central Chile present complex altitudinal gradients where temperature and soil water availability vary inversely with elevation: temperature decreases and water availability increases with elevation (Cavieres *et al.* 2006). Our soil moisture measurements agree with this pattern, with drier soils at the lowest site, although it seems that at the end of the growing season the upper site also became water stressed, probably because of the end of the influence of snow melting.

We expected higher photoinhibition at the upper elevation due to lower temperatures and higher irradiance, but we found no differences across elevations in Fv/Fm. This may be related to the greater (drought-driven) stomatal closure at the lower sites, which also impairs photosynthetic performance by reduction of CO_2 uptake. Studies in elevation gradients show that SLA generally decreases with elevation (Körner 2003; Milla *et al.* 2009), mainly as a response to decreasing temperature and increasing light and nutrient stress. Our results found the opposite direction, with lowest SLA at the lowest site, because of the greater water stress in this site. We found that stomatal conductance increased with elevation, which indicates that water stress in *V. maculata* decreased with elevation, a result that agrees with soil moisture data. The pattern of lower stomatal density at the upper site is also consistent with the general relationship between stomatal density and water availability (Carlson et al. 2016). It also suggests that this violet species is able to develop differential responses to the environment across relatively short distances, either by phenotypic plasticity or by genetically-based adaptations.

Reproductive traits

Our results supported the reproductive assurance hypothesis, as we found a high autonomous selfing capacity in the study species (although selfing level did not differ across sites), and different proportion and success of CH and CL flowers at each elevational site, but similar total reproductive output among sites. The pattern found of increased investment and fitness (fruit set) of CH and CL flowers at the lowest site and the upper sites, respectively, suggests the occurrence of reproductive assurance adaptation (most likely genetically-based).

Several studies have reported that pollinator abundance and diversity tend to decrease above the treeline (Arroyo et al., 1985; Totland, 1997) because of lower temperatures and increased cloudiness. In the Andes of central Chile this decline in flower visitation rates has been also confirmed from the lower to the upper alpine zones (134 plant species included, Arroyo et al. 1985). We found a very low visitation rate and fruit set (≤ 50%) in the CH flowers in all sites, which could indicate the presence of pollen limitation in this alpine species. These results are consistent with the low pollinator/plant ratios and relatively high prevalence of specialized interactions described in Central Chile (Carvallo & Medel 2010; Carvallo *et al.* 2013) due to the depauperate species assemblages caused by geographical barriers in Mediterranean Andes. However, limited resource availability cannot be discarded as explanation (Totland and Eide, 1999; Galen, 2000; Muñoz et al., 2005), particularly in view of the especially dry summer of 2014-15 in the Central Chilean Andes.

Floral and leaf traits and their correlations

Contrary to expected we found no differences in leaf size across sites. We did find larger and heavier flowers at the intermediate site, where we also found the highest proportion of CH flowers per ramet and the higher floral integration (although INT was not significant across sites). Taken together, these results suggest that cross-pollination might be favoured at this intermediate site, probably due to a higher pollinator abundance that we did not detect during the year studied. We would expect also larger flowers at the lowest elevation, but the co-occurrence of water stress and the growing season probably constrains flower size at this site, as has been shown in other systems (Galen 2000; Strauss and Whittall 2006).

Most studies addressing variation in floral traits across different environments, have focused on how pollinators promote this variation (Herrera 2005; Lázaro and Totland 2014), but other factors may also contribute to floral size variation, such as environmental factors influencing resource availability (Strauss and Whittall 2006). Few studies have determined to what extent foliar responses to the environment are correlated with flower size (Armbruster *et al.* 1999; Pélabon *et al.* 2013), particularly in species with predominantly selfing breeding or mixed breeding system (Brock & Weinig 2007). Both GLMs and matrix correlation analysis showed the same pattern, with flower traits (petal and sepal size, and flower peduncle length) and leaf size strongly correlated at the two lower sites, whereas they were weakly correlated at the highest site. These findings disagree with Berg's hypothesis (1960), since floral and vegetative traits are expected to be decoupled when they depend more on pollinators, enabling vegetative traits to respond plastically to environmental heterogeneity without disrupting the reproductive function of CH flowers. As the reproductive success of CH flowers was higher at the lowest elevation, and CH flower size and investment peaked at the intermediate elevation, we expected decoupling between vegetative and floral traits at these two sites, and not at the highest one.

Several studies have tested the *correlation pleiades hypothesis* between floral and leaf traits, but some have already reported results inconsistent with the hypothesis. Armbruster et al. (1999) found that four species with specialized pollination had floral traits decoupled from variation in vegetative traits; however, the same trend was also observed in three of four species with unspecialized pollination. Therefore, the correlation pattern cannot be predicted from the reproductive ecology of the plant. Lambrecht and Dawson (2007) found that the effect of moisture availability on leaf traits may mediate an indirect effect on floral size, with stronger correlations between leaf and floral size at wettest sites. Contrariwise, we found the stronger correlation at the driest site, which indicates that increased soil moisture does not always determine such correlation pattern. A study with the predominantly selfing *Arabidopsis thaliana* suggested that vegetative plasticity may affect the expression of floral traits under some environments, finding a decoupled correlation between floral and leaf traits under conditions resembling sunlit settings and a strong correlation under foliar shade treatment (Brock and Weinig 2007). Our results along the elevation gradient might be interpreted to agree with those reported by Brock and Weinig (2007). Although we did not quantify specifically light intensity in the microsites where *V. maculata* was found, it was evident that in the lowest (and driest) site neighbouring vegetation was denser (see Fig. S2) than in the upper sites. This could have triggered a shade-avoidance syndrome (Smith 2000; Schmitt et al. 2003), with elongation of the floral organs in response to increased plant density. The simultaneous influence of shading on both floral and vegetative structures might have contributed to the observed pattern of correlations between these functions at low elevation. Nonetheless, we cannot discern which environmental factor is driving the correlation patterns, being habitat heterogeneity and temperature other drivers to consider. Furthermore, despite strong vegetative-floral correlations could result in increased variation in floral morphology, which is likely to disrupt the reproductive function of flowers (Conner & Via 1993), reproduction would be assured by the production of CL flowers in the case of *Viola maculata*. Finally, in terms of floral integration and correlations patterns among all traits, our results are consistent with our hypothesis of increasing phenotypic integration and correlation among traits with environmental stress, which result in a more “tight” phenotype at the lowest and intermediate sites (see. Fig. 14B).

3.5. Conclusions

We confirmed partially the inverse gradient of water stress with elevation in Chilean Central Andes, with *Viola maculata* populations in the lowest site experiencing lower soil moisture and showing thicker leaves, lower stomatal conductance and higher stomatal density. Reproductive results support the hypothesis of maintenance of a mixed mating system, with different proportion and success of each flower type depending on the site. Phenotypic integration did not differ across sites, but correlation patterns between flower and leaf size did, with stronger vegetative-floral correlation at the lower sites and a weak correlation at the highest site. The nonreplicated nature of our study prevents drawing general conclusions about the study species or high-mountain violets; for instance, we cannot discern which environmental factor is influencing the different correlations found between floral and leaf traits along the gradient. Nevertheless, it seems that in this violet species with mixed-mating system pollinators are not the main force decoupling floral-vegetative traits. Our study shows how a relatively narrow gradient in an alpine system may affect not only reproductive and physiological responses in plants, but also floral and vegetative covariance and phenotypic integration, with a tighter phenotype under more stressful conditions.

3.6 Acknowledgements

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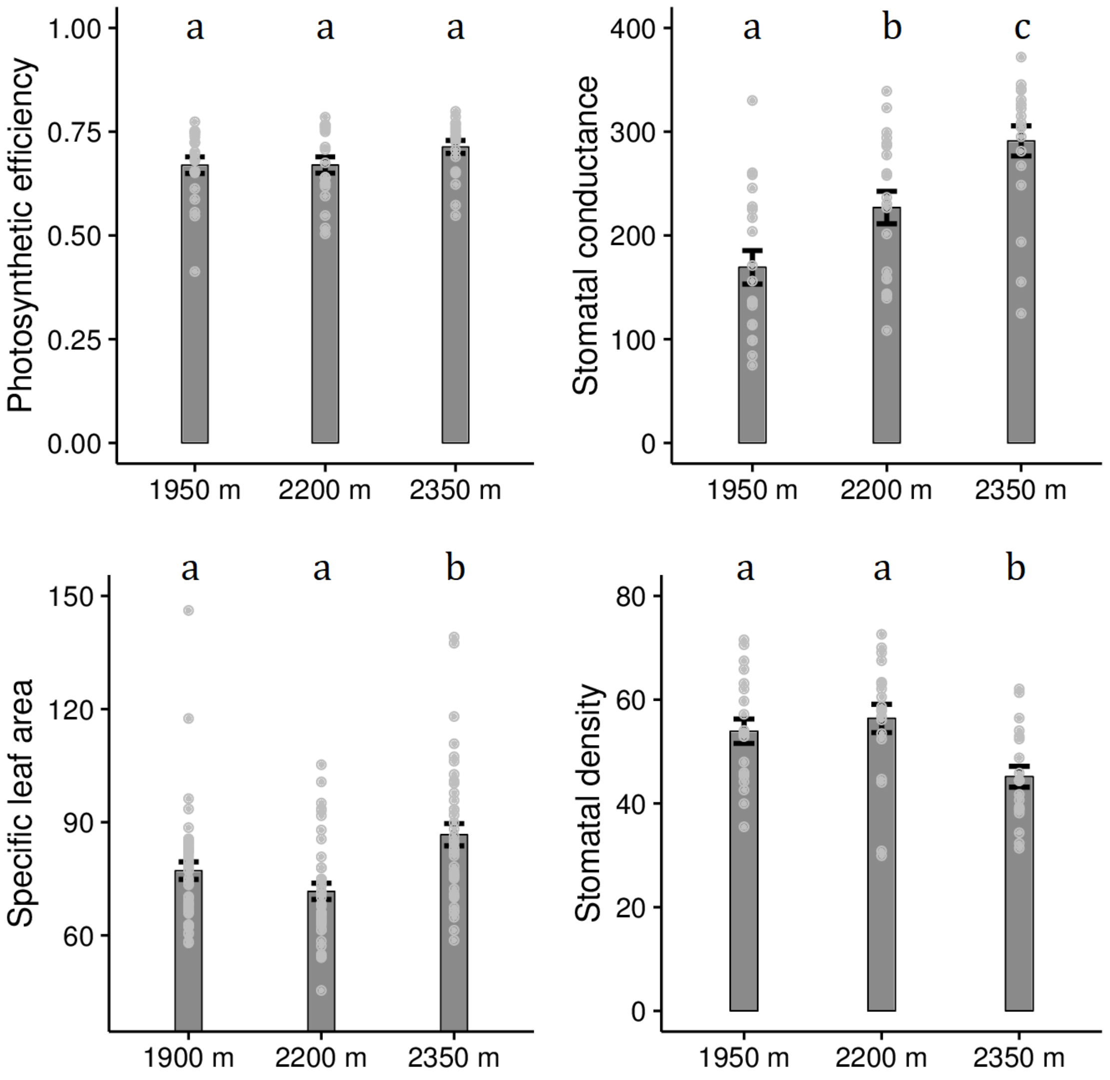

Figure 12 Bar plots of the physiological variables measured in *Viola maculata* at each site. Different letters indicate significant differences among sites within each model. 12A: Photosynthetic efficiency (Fv/Fm), 12B: Stomatal conductance (mol H_2O/m^2 per seg), 12C: Specific leaf area (mm^2/mg), 12D: Stomatal density (stomata per mm^2).

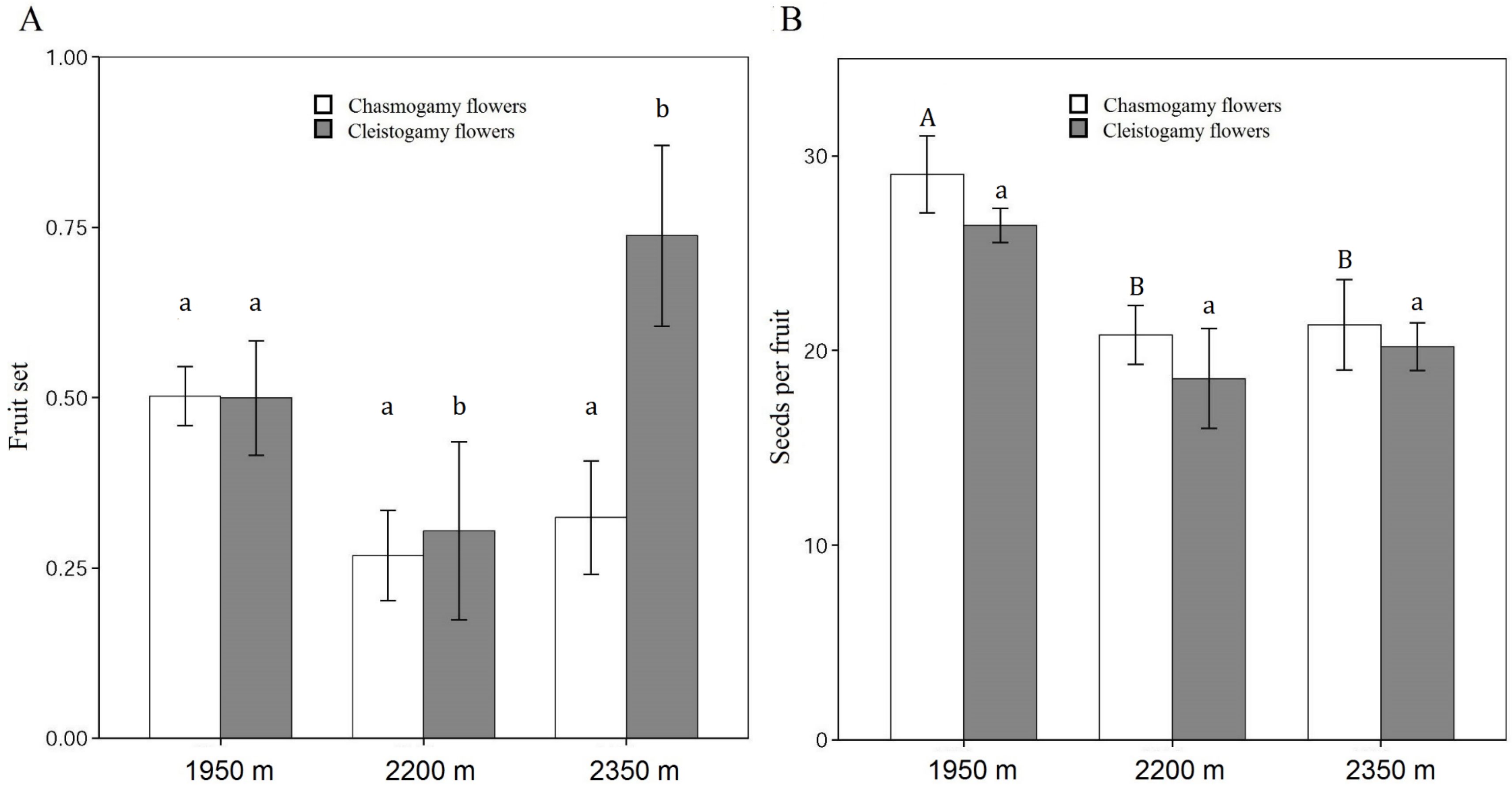

Figure 13 (**13A**) Mean ± SE fruit set (percentage of flowers setting fruits). Different letters indicate significant differences within sites. (**13B**) Seeds per fruit in chasmogamous and cleistogamous flowers. Different capital letters indicate significant differences across sites for chasmogamy flowers, and different letters indicate significant differences across sites for cleistogamy flower

3.7 Figures

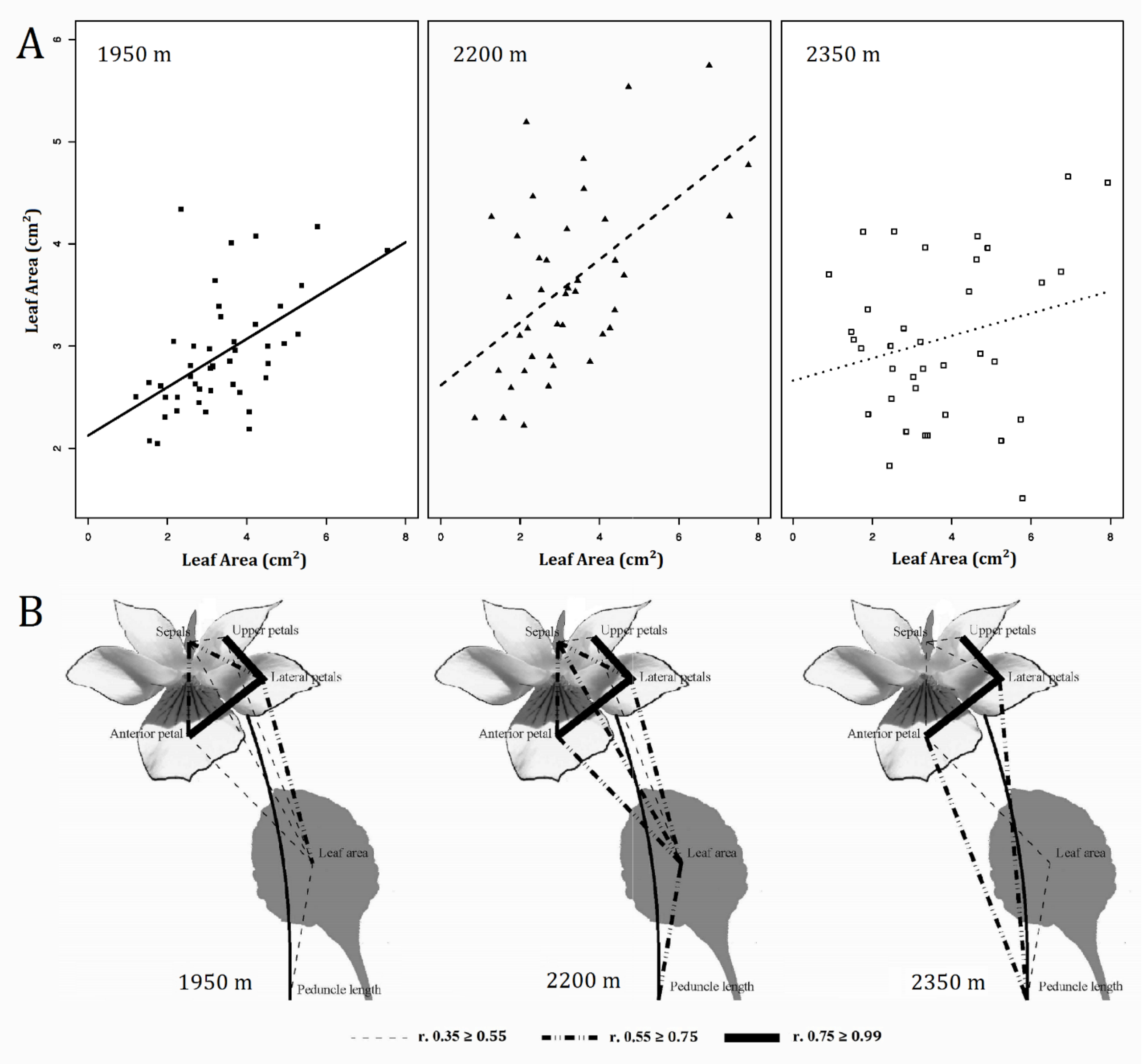

Figure 14 (**14A**) Partial residual plots showing the relationships between flower area and leaf area at each elevational site. The lines represent the best estimates for each study site while the symbols represent the partial residuals of the model. (**14B**) Patterns of correlations among plant traits in the three study populations of Viola maculata. Lines indicate significant positive correlations among floral and leaf traits

3.8. Tables

Table 5: Statistics and mean and standard errors of the floral and leaf traits measured at each elevation. The coefficient of variance is given in brackets. Different letters indicate significant differences within each elevation.

Factors	**ANOVA results**	**1950 m**	**2200 m**	**2350 m**
Leaf area (cm^2)	$F_{2,118} = 0.88$, $P = 0.41$	3.36 ± 0.19^{a} (37.95)	3.18 ± 0.24^{a} (47.91)	3.69 ± 0.27^{a}(45.66)
Petal area (cm^2)	$F_{2,118} = 8.73$, $P < 0.001$	2.92 ± 0.08^{a} (19.22)	3.59 ± 0.13^{b} (24.20)	3.06 ± 0.13^{a} (25.87)
Sepal area (cm^2)	$F_{2,118} = 1.02$, $P = 0.36$	0.54 ± 0.02^{a} (21.82)	0.52 ± 0.03^{a} (45.58)	0.53 ± 0.02^{a} (28.95)
Flower peduncle length (cm)	$F_{2,118} = 4.52$, $P < 0.05$	10.21 ± 0.33^{a} (22.19)	8.96 ± 0.31^{b} (21.99)	9.18 ± 0.35^{b} (22.54)
Number of leaves per stem	$F_{2,118} = 4.29$, $P < 0.05$	7.24 ± 0.48^{a} (45. 13)	6.4 ± 0.23^{ab} (23.08)	5.6 ± 0.18^{b} (19.65)
Number of flowers per stem	$F_{2,118} = 1.81$, $P = 0.16$	1.53 ± 0.14^{a} (59.96)	1.82 ± 0.15^{a} (53.92)	1.5 ± 0.12^{a} (49.12)
Leaf dry biomass (g)	$F_{2,118} = 1.9$, $P = 0.15$	0.31 ± 0.02^{a} (50.37)	0.30 ± 0.06^{a} (64.72)	0.25 ± 0.02^{a} (53.78)
Flowers dry biomass (g)	$F_{2,118} = 3.7$, $P < 0.05$	0.011 ± 0.001^{a} (67.99)	0.016 ± 0.002^{b} (95.55)	0.010 ± 0.001^{a} (61.40)

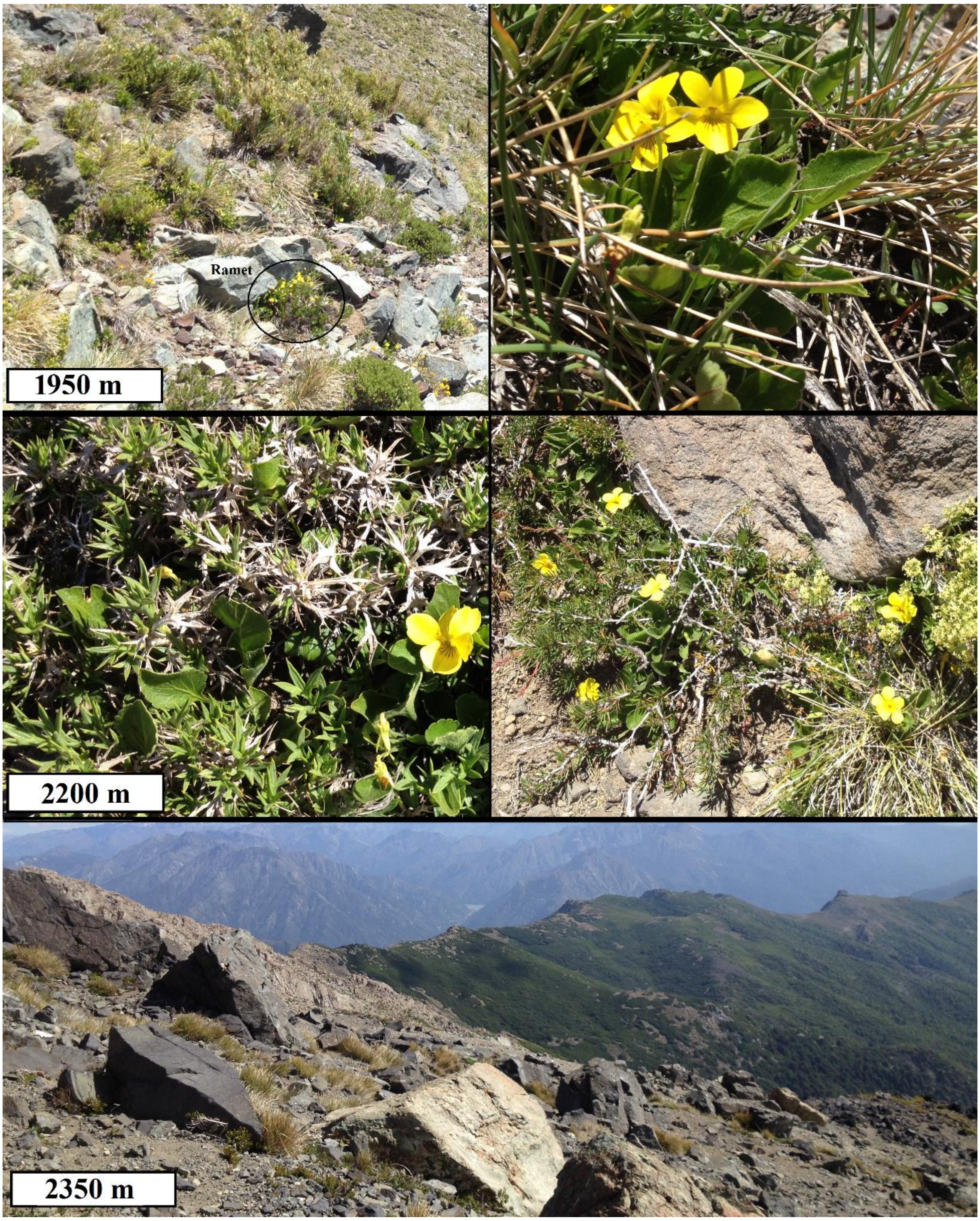

Figure S2: Images of the habitat and co-occurring plant species at each site. *Viola maculata* co-occurs mainly by *Chusquea coleou* and *Festuca pallescens at 1950 m*, with *Mulinum echinus* and *Berberis empetrifolia* at 2200 m, and with *Festuca pallescens* and *Berberis empetrifolia* at 2350 m.

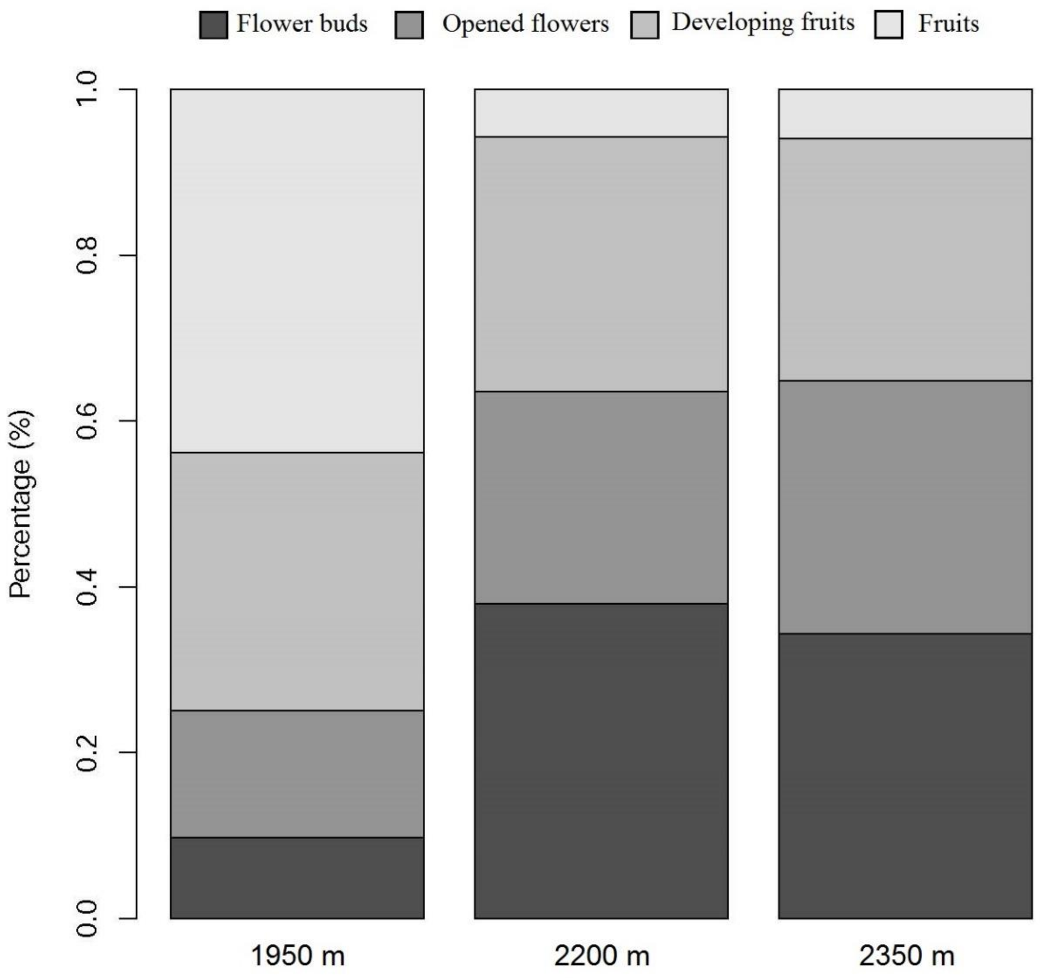

Figure S3. Phenology recorded between 27th and 29th December 2014. Bars represent the percentage of floral buds, opened flowers, developing fruits and fruits present in each population on those dates.

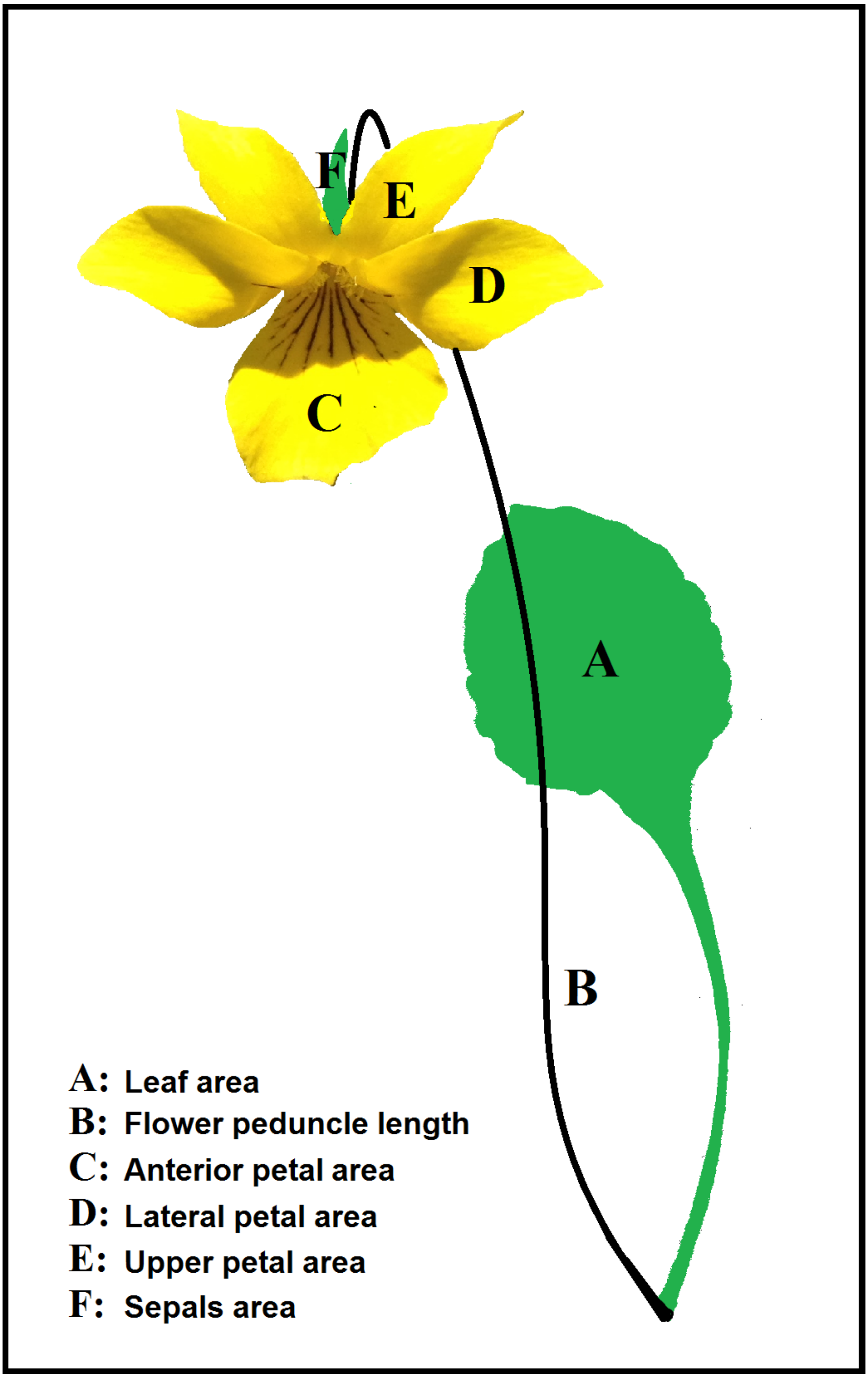

Figure S4: Schematic drawing of a *V.maculata* leaf and associated CH flower, illustrating the different plant traits examined in this study.

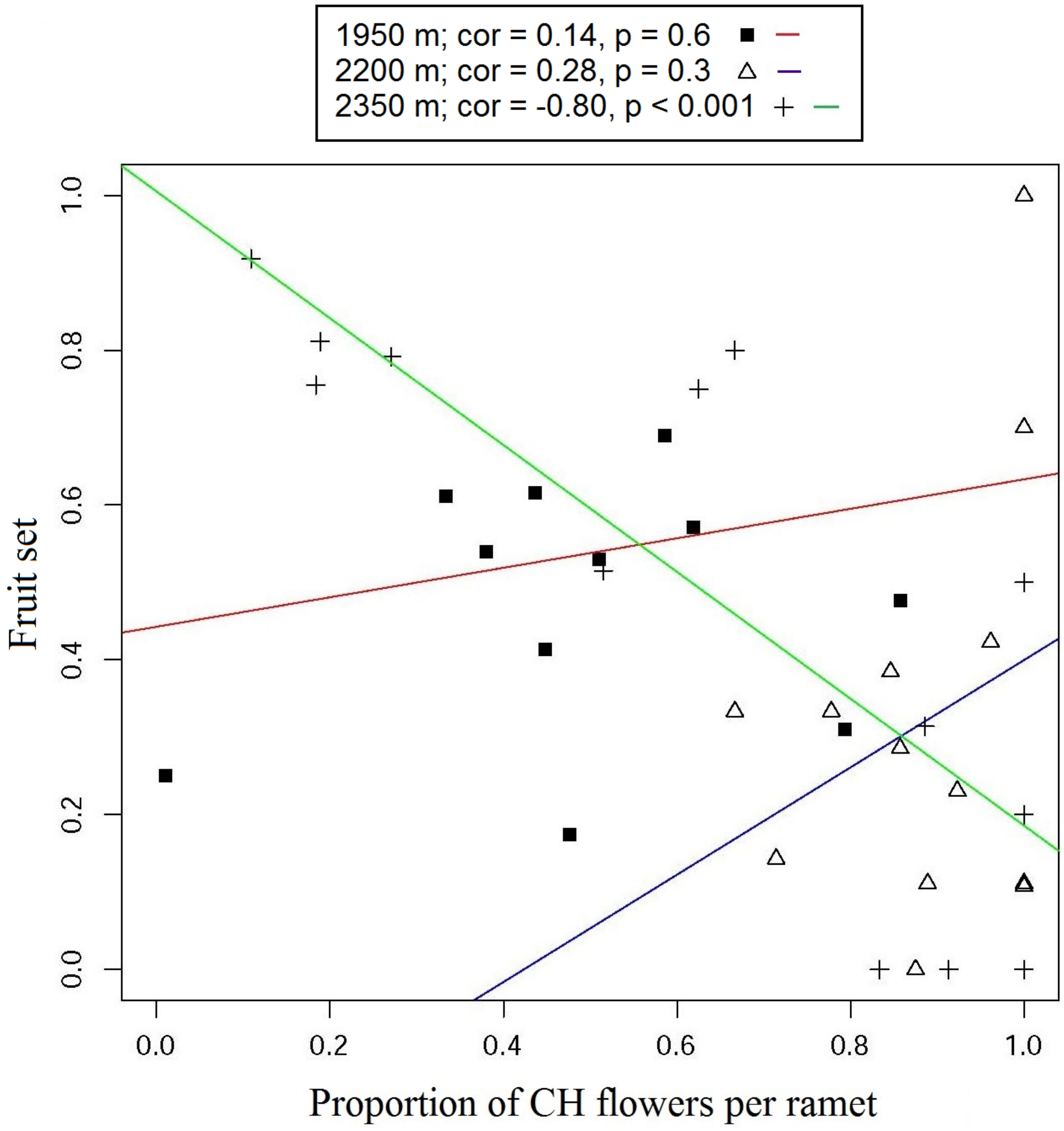

Figure S5: Relationship between fruit set and proportion of chasmogamous flowers per ramet at each study site.

Chapter 4

What explains the maintenance of outcrossing in an almost exclusively cleistogamous species? The case of the cliffhanger *Viola jaubertiana*

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Abstract

Cleistogamous species constitute an interesting evolutionary study system to resolve the longstanding question of what selective forces maintain outcrossing given that seed production is assured through selfing. In this study, we use the rupicolous endemic *Viola jaubertiana,* which produces self-pollinated cleistogamous flowers (CL) and large chasmogamous flowers (CH), to assess how this mixed mating system can be maintained. We found that CH flowers are open in winter, during unfavorable conditions for pollination, showing a very high pollen limitation and no-delayed selfing, and thus produce a very low amount of seeds. Conversely, CL flowers are produced in early spring, are physiologically cheaper to produce (i.e. their dry weight is 3.4 times lower than that of CH flowers), and yield c. 100 times more seeds than CH flowers. However, we detected that outcrossed seeds have a higher mass, produce a larger elaiosome (which makes them suitable for ant seed dispersal), and show higher seed germination rates compared to selfed (cleistogamous) seeds. We suggest that the combination of inbreeding depression, heterosis, phenotypic plasticity and infrequent favorable conditions for pollination ("ecological windows") in the species are probably sufficient forces to maintain chasmogamy in this long-lived species, ensuring a certain genetic diversity and reducing deleterious fixed mutations in the selfed lines.

Keywords: altitudinal gradient, Balearic Islands, inbreeding/outbreeding depression, pollen limitation, rupicolous.

4.1 Introduction

A plant mixed mating system in which both obligatory selfing cleistogamous flowers (CL) and potentially outcrossed chasmogamous flowers (CH) are produced, is a relatively widespread condition occurring in around 50 angiosperm families (Lord 1981; Culley & Klooster 2007). This implies that the strategy of producing these two types of flowers may be selected under certain circumstances, such as in heterogeneous and pollinator-unpredictable environments (Culley 2002; Albert *et al.* 2011). In fact, the effect of a heterogeneous environment on floral production has been studied in several dimorphic cleistogamous species, and factors such as light, moisture availability, herbivory and plant size have been found to affect the relative number of CH and CL flowers produced (Waller 1980; Berg & Redbo-Torstensson 1999; Forrest & Thomson 2008).

This peculiar reproductive strategy has also puzzled biologists for a long time as it goes against the prediction that mixed mating is not evolutionary stable (Goodwillie *et al.* 2005). Several models have addressed the conditions favoring the maintenance of both flower types in cleistogamous species. According to Schoen and Lloyd (1984), cleistogamy would be favored in a heterogeneous environment if individuals could assess and respond appropriately to this variation by producing the most appropriate flower type depending on pollinator availability (adaptive plasticity hypothesis). On the other hand, Zeide (1978) and Waller (1980, 1984) suggested that there may be periods of time during which CH are favored (although CL flowers are cheaper to produce and more reliable in terms of fruit set), or CH progeny have higher fitness than CL when grown in competition (Schmitt & Ehrhardt 1987). In these cases, producing both flower types could reduce the long-term variance in fitness and recruitment (variance discounting hypothesis). Moreover, Lu (2002) argued that despite CL flowers provide a purging of inbreeding depression, complete CL could lead to the fixation of deleterious mutations within selfing lineages. Therefore, crossing between selfed lines mediated by pollinators on CH flowers might reduce these fixed mutations through recombination (heterosis hypothesis). More recently, the review performed by Oakley et al. (2007) on the maintenance of outcrossing in cleistogamous species suggested that adaptive plasticity is unlikely to explain the evolutionary stability of this mixed mating system. Given that CL flowers do not require pollinators to set seeds, are energetically cheaper to produce, have higher fruit set, and little inbreeding depression, it is difficult to envision why CL flowers should not be produced in all environments. Although variance discounting (Waller 1984; Schmitt & Ehrhardt 1987) and heterosis (Lu 2002) might contribute to the maintenance of outcrossing in CL species, Oakley et al. (2007) concluded that additional forces, might also be relevant to explain the up to 342% higher fitness cost of CH compared to CL in some species. Altogether, it seems that the combination of features of cleistogamous species (such as different floral investment, seed performance, dispersal capacity and phenology between CH and CL flowers), the heterosis phenomenon, and the effects of high pollinator availability during "ecological windows" (Ehrlen 1991), make this mixed mating system a stable and successful strategy in some particular environments and species.

Viola jaubertiana, an endemic species from Mallorca (Balearic Islands), was originally described by Marès and Vigineix (1880) as a species producing sterile chasmogamous flowers and fertile cleistogamous flowers, but our preliminary observations suggest that some chasmogamous flowers are fertile. Our main aim in this study was to confirm and study such expected mixed mating strategy and assess how outcrossing it is maintained. Specifically, we asked the following questions: 1) Does flowering phenology of *V. jaubertiana* differ between CL and CH flowers? and do CH flowers occur during the favorable season for pollination? 2) What is the relative cost and contribution to seed production of each flower type? 3) Do biotic (herbivory) and abiotic factors (water stress conditions) influence the production of CL and CH flowers? 4) Do flower types differ in traits of the seeds they produce and in their germinability? and 5) is fitness of *V. jaubertiana* affected by pollen limitation, inbreeding and outbreeding depression?

4.2 Material and methods

Study species

Viola jaubertiana Marès & Vigin. is a narrow endemic violet, restricted to small and fragmented populations in north-central of the Sierra de Tramuntana, in Mallorca Island (Balearic Islands). It lives from 0 m to 1,100 m a.s.l. and is strictly rupicolous (unique among the European violets), inhabiting inaccessible shady rocky places, in fissures and overhangs of calcareous vertical cliffs (Figure 15A). It is a perennial herb (20-30 cm), with woody rhizome and long radicant stolons, and coriaceous, glabrous leaves. Personal observations during five years, and using estimates of other perennial violets lifespans (Solbrig 1981; Batista & Sosa 2002; Culley 2002; Herrera & Bazaga 2010), suggest that *V. jaubertiana* may live at least 10 years. It produces odorless CH flowers (Figure 15B and C), with long peduncles (6-15 cm), light blue-violet petals and a short spur (2-3 mm). CL flowers appear after CH flowers, resembling small buds at axils of the leaves, with short peduncles (2-5 cm; although occasionally they can reach 9 cm), at ground level and hidden by leaves (Fig. 15D). Rarely, *V. jaubertiana* produces some transitional flowers (morphologically intermediate between CH and CL flowers). Both flower types produce capsules (similar in size and shape, but those from CH flowers present longer sepals, surpassing more than half the length of the capsule) with seeds inside that are not ballistically dispersed but have elaiosomes that attract ants and facilitate seed dispersal to overhangs and fissures (J. Seguí, pers. obs.). The reproductive biology of this species has remained unexplored, though Conesa et al. (2008) confirmed the presence of a hybrid between *V. alba* subsp. *dehnhardtii* and *V. jaubertiana* (*V.* x *balearica*), and suggested the endemic *V. jaubertiana* as the unique ovule donor (calling into question the sterility of CH flowers). *Viola jaubertiana* was described as a relict and isolated lineage among European violets (Marcussen & Borgen 2000), but phylogenetic analysis with ITS sequences (Conesa *et al.* 2008) refuted this view, forming a well-supported clade with the sympatric *V. odorata* and *V. alba* subsp. *dehnhardtii*, and therefore, suggesting a recent origin. Life-history traits of the species suggest that it originated from one of these two sympatric species when colonizing a new rupicolous habitat, out of the grazing effects of *Myotragus balearicus*, the extinct largest mammal inhabiting Mallorca until human arrival (Borba Nunes *et al.* 2017).

Fieldwork was conducted during a 2-year period (2014-2015) at three populations of *V. jaubertiana*, at different elevations: Torrent de Pareis (0 m a.s.l.; torrent calcareous cliff), Pla de'n Gumbal (700 m a.s.l.; vertical fissures of north-facing rocks) and Massanella (1100 m a.s.l; karst fissures at the pinnacle of the mountain). Additionally, in 2015 and 2017, hand-pollination experiments were conducted at the Sóller Botanic Garden, due to the difficulty of accessing and manipulating plants in the field. Temperature and relative humidity were gathered with a data logger located a meter distance to an individual plant during an entire year at each site, to control for differential abiotic conditions along the elevational gradient (Fig. S6).

Flowering phenology, herbivory and specific leaf area

In January 2014, we marked all the accessible individuals we found in each population. The number of leaves, as well as the number of flowers of each type, were counted on each individual every 1-2 weeks during 2014-15. We also evaluated plant damage due to herbivory, expressing it as the proportion of damaged leaves per plant (Brown et al. 1987). We then obtained an herbivory index considering the product of the proportion of damaged leaves on a plant and the mean damage to each leaf. The latter was recorded on a 1-5 scale, 1 indicating < 20% leaf area removed and 5 indicating total leaf removal (only petiole remaining). From each marked plant, we also obtained specific leaf area (thereafter SLA) as the ratio between leaf area and dry weight. SLA indirectly describes the plant drought stress conditions, so that low values of this variable indicate higher water stress than high values. We took the two largest leaves of each plant, scanned them while fresh, and calculated the area with the image analysis software ImageJ (ImageJ ver. 1.6). We then weighed those leaves after oven-drying them (4 days at 60 ºC). We monitored a total of 90 individuals during 2014, and 89 individuals during 2015.

Relative success and cost of CH and CL flowers

Quantifying the relative contribution of each flower type to parental fitness depends on the energetic cost of seeds and the fitness of offspring produced by each flower type. During the two years (2014-15), we recorded floral display (total number of flowers produced) in all marked reproductive individuals (77 and 64 in 2014 and 2015, respectively) and evaluated reproductive success of each flower type by measuring fruit set (number of flowers setting fruits) and seed set (number of seeds per capsule). To avoid seed loss in the field, we individually bagged fruits before ripening. We evaluated the selfing level (i.e. the capacity to produce seeds by spontaneous self-pollination) of CH flowers in a total of 26 and 22 individuals during 2014 and 2015, respectively. Nearly half of the CH flower buds available per individual were bagged before anthesis with a mesh-cloth that excluded floral visitors, and were compared with unbagged flowers (controls) of the same individual to also test for differences in performance between selfing and outbreeding CH seeds. We further estimated the energetic cost of CL flowers relative to that CH flowers. For this, we weighed 15 CH and 13 CL flowers from five individuals after oven-drying them (2 days at 60 ºC) and obtained the relative weight of each flower type.

We evaluated the pollen:ovule ratio (P/O ratio, hereafter) of CH flowers by dividing the number of pollen grains by the number of carpels of the same flower, following Dafni et al. (2005). We measured a total of 94 flowers of five different natural populations of *V. jaubertiana* (each flower came from a different individual). We sampled the five anthers of each flower, placing them into a microcentrifuge tube containing 500 µl of an isotonic water solution, disintegrated with a pestle and agitated for 5 min using a vortex mixer for homogenization. The pollen grains from each aliquot were counted with an optical microscope (100x) using double Fuchs-Rosenthal slides, with a volume of 3.2 mm^3 per aliquot. The number of pollen grains was then extrapolated to the original solution (500 µl), and the average was obtained.

We also conducted pollinator censuses of 15 min intervals in different individuals and elevation sites of *V. jaubertiana* (1110 min at 0 m; 1575 min at 700 m; 1080 min at 1100 m) measuring flower visitation rate as the number of visits per hour per flower. Censuses were carried out during February-April of 2014-15, mostly from 11:00 h to 16:00 h, avoiding windy days.

Hand-pollination of CH flowers

In the Sóller Botanic Garden, we worked on two plots located 60 m from each other, with c. 30 individuals per plot, though coming from the same locality (Gorg Blau; 39º48’42.87N 02º49’28.74’’E). Plants were haphazardly selected for each of the following four treatments: (1) Xenogamy I; 30 emasculated flowers in a plot were hand-pollinated with pollen from ~10 individuals of the other plot, (2) Xenogamy II; 30 emasculated flowers were hand-pollinated with pollen from ~10 individuals of a natural population located 4 km away from the original population of the garden individuals, (3) Hybridization; 30 emasculated flowers of a plot were hand-pollinated with pollen from ~10 flowers of *V. alba* subsp. *dehnhardtii,* and (4) 30 control flowers for each of the above treatments were left open to pollination. Treatments (1) and (3) were performed in 2015 whereas treatment (2) was performed in 2017 (as no flowers were produced during 2016). This was due to the limited number of CH flowers per plant in a given year (usually 1-3). We calculated a pollen limitation index for each individual as PL=1-C/XI, where C and X represent fruit/seed set of the control and xenogamy I, respectively (following Tamura and Kudo 2000).

Performance differences in CL and CH treatments

To test for differences in reproductive success between both floral types and to assess the effect of inbreeding and outbreeding depression, we collected in the field the mature capsules from all reproductive individuals in 2015, as well as from the autonomous selfing treatment, and further gathered those from the hand-pollinated individuals at the botanical garden. In the laboratory, we then measured the following traits: seed number per capsule, seed mass, elaiosome mass and seed germinability. We weighed seeds with a balance to the nearest 0.01 mg, and then extracted the elaiosome from the seed and weighted it separately. To test for seed germinability, we performed a pre-sowing treatment, keeping seeds during four weeks at 4ºC (resembling the winter conditions in natural populations) in a chamber at dark conditions. Seeds were placed in Petri dishes filled with 1% agar and 250 ml/L of gibberellic acid and, after scarifying them, they were incubated in a germination chamber at 10ºC/20ºC, and 12h/12h photoperiod (dark/light), respectively. Each Petri dish contained 25 seeds and the number of dishes depended on seed availability (Table 6). Inbreeding depression was estimated as $RP_i = (W_o-W_s)/W$ maximum, where W_o and W_s are the mean performance of xenogamy I progeny and selfed progeny, respectively, and W maximum = W_o when $W_o \geq W_s$ (Ågren & Schemske 1993). The index ranges from -1 to 1, with positive values indicating inbreeding depression. Similarly, we estimated outbreeding depression as $RP_o = (W_o-W_{op})/ W$ maximum, where W_{op} is the mean performance of xenogamy II and W maximum = W_o when $W_{op} \geq W_o$. As for RP_i, positive values of the index indicate outbreeding depression. Due to the very low selfing of CH flowers in 2015 (only one fruit; Table 6), we calculated inbreeding depression (RP_i) from CL instead of from selfed-CH progeny. By using this method we are not able to control for the floral type effects on fitness differences (such as ovule number), as (Culley 2000) warns, although she found no differences in fitness (seeds per capsule and emergence rates) between CL and selfed-CH progeny in *V. canadensis*. We estimated the inbreeding and the outbreeding effects on: (1) seed set, (2) seed mass, (3) elaiosome mass and (4) germination rate. We further calculated a multiplicative estimate of inbreeding and outbreeding depression as $RP_{i}\mathrm{Total} = (W_{o}1*2*3*4-W_{s}1*2*3*4)/ W$ maximum, and $RP_{o}\mathrm{Total} = (W_{o}1*2*3-W_{op}1*2*3)/ W$ maximum (following Affre and Thompson 1997).

Data analyses and statistics

To explore the factors that influenced reproductive variables, we used general linear models (GLM) with fruit set and seed set (of both CH and CL flowers) as response variables, and population, year, CH:CL ratio, herbivory index, florivory (for CH analysis) and number of leaves (a proxy of plant size) as predictor variables. To test for the factors determining the proportion of CH and CL flowers per plant, we used a model with population, mean SLA per plant, herbivory index and number of leaves as predictor variables. Differences among treatments in seed set, seed mass and elaiosome mass were tested by means of general linear mixed models (GLMM) with treatment as predictor variable, and including individual plant as random effect. We used error distribution and link functions that best fit the data: (1) binomial distribution for fruit set and degree of CH flowers, (2) a Gaussian distribution for seed set, seed size and seed mass, and (3) a gamma distribution and inverse link function for pollination rate (visitation rate+1, to avoid zeros in the response variable). We used the Akaike information criterion to select the best models with the package MuMIn 1.15.6, with AIC values > 2 retained as indicators of a significantly improved model fit. All statistical analyses were performed with the R package, v. 3.1

4.3. Results

Flowering phenology, herbivory and specific leaf area

Consistently at all sites, CH flowers appeared first, by mid-end of the winter, overlapping partly in time with CL flowers which appeared in early spring (Fig. 16). Three clear patterns were found with elevation: (1) a delay in flowering onset; the peak of CH flowering was during February at the lowest site and during April at the intermediate and highest sites, (2) a decrease of CH flowering production compared to CL ($\chi^2_2 = 27.89$, $P < 0.0001$; Table 6), and (3) a reduction of CL flowering during autumn (October-November), i.e. practically no CL flowers were produced at the highest elevation during this season compared to the lower sites (Fig. 16). Fruit ripening was observed to be much slower in CH flowers (three months) than in CL flowers (one month).

The herbivory index varied among populations and between years, being especially high in the highest population in 2015 (Population x Year; $\chi^2_2 = 6.91$, $P < 0.05$); by contrast, it was rather similar in the two lower populations both years of the study (Table 6). Florivory of CH flowers showed much greater figures, especially in 2014 at the lowest site (90%; Table 6); there was also an interactive effect between population and year (Population x Year: $\chi^2_2 = 6.33$, $P < 0.05$). Leaves were damaged by endemic snails (*Allognathus graellsianus* and *Iberellicus balearicus*) and slugs (*Limax majoricensis*; dark morph), and by an unidentified caterpillar.

Mean SLA (cm^2/g) per individual varied among populations ($F_{2,57} = 15.14$, $P < 0.001$), being significantly lower at the intermediate population compared to the others (Table 6). SLA tended to be larger at the lowest population than at the highest one (Z-value = 2.20, $P = 0.07$).

Relative success and cost of CH and CL flowers

There were consistently more CL than CH flowers at all sites, although the ratio between the two flower types varied among populations and years (Population x Year: $\chi^2_2 = 35.08$, $P < 0.0001$; Table 6). However, both years, that ratio was consistently higher at the lowest population compared to the higher elevation sites (Table 6). Two models were selected to predict the ratio CH:CL flowers in response to SLA, herbivory and number of leaves. The first model included the first two predictors, which showed a negative effect on the response variable ($\chi^2_1 = 14.37$, $P < 0.001$ and $\chi^2_1 = 4.62$, $P < 0.01$, respectively; Fig. 17), whereas the second model included number of leaves, which also was negatively associated with CH:CL ratio ($\chi^2_1 = 2.77$, $P < 0.09$), i.e. the higher the number of leaves in an individual the lower was the proportion of CH flowers it produced.

Fruit set was extremely low on average (0.06 ± 0.02 fruits/flower) in CH flowers, but varied across populations ($\chi^2_1 = 6.96$, $P < 0.05$), reaching consistently the minimum value at the lowest site (Table 6). Bagged CH flowers produced no or very few fruits (0.05 ± 0.03; Table 6), indicating a lack or irrelevant autonomous selfing. Open flowers showed a slightly higher fruit set although this did not differ significantly from that of bagged flowers ($\chi^2_1 = 1.38$, $P = 0.24$). By contrast, fruit set of CL flowers was high (0.86 ± 0.02) although highly variable between years and populations (Population x Year; $\chi^2_1 = 33.30$, $P < 0.001$; Table 6). Fruit set did not show to be affected in either case by any of the predictor variables (herbivory, number of leaves and number of CH flowers), as they were not selected in any of the best models. Capsules produced a mean of 6.21 ± 1.10 seeds (n = 14); unfortunately, seed set of CH capsules could not be analyzed due to the very small number of fruits developed. Seed set of CL capsules did not vary among populations, but increased with the ratio CH:CL flowers ($F_{1,99} = 7.78$, $P < 0.01$; Fig. 18) and was marginally higher in 2015 than in 2014 ($F_{1,99} = 3.91$, $P = 0.05$; Table 6). Both CH and CL seeds showed a significantly higher germination rate at the highest population compared to the lower ones ($\chi^2_1 = 14.20$, $P < 0.001$; Table 6). The mean P/O ratio was found to be 1816.57 ± 129.79, indicative of a xenogamous species, and it increased significantly with elevation (χ^2_1 =15.72, $P < 0.01$; Table 6).

Few flower visitors were observed (0.088 visits/flower/hour) at either site (χ^2_2 = 0.9, $P = 0.6$) and this was consistent the two years of the study (χ^2_1 = 3.19, $P = 0.07$). However, insect visits to plants increased with the number of flowers (χ^2_1 = 7.70, $P < 0.01$). These insects included two butterflies (*Macroglossum stellatarum* and *Lycaena phlaeas*) and two bees (*Anthophora plumipes* and *Bombus terrestris*).

CH flowers were greater than three times heavier as CL flowers (Table 7), but had an extremely lower reproductive success and higher florivory than them (Table 7). Overall, a plant produced c. 100 CL seeds for each CH seed set (Table 7).

Pollen limitation and inbreeding and outbreeding depression

The experiments performed at the Sóller Botanical Garden showed a very strong pollen limitation in this species (Fig. 19). The Xenogamy I treatment produced 40% of fruit set (n = 30; Fig. 19) and a mean of 8.8 ± 0.91 seeds per capsule (Table 8), whereas none of the 90 flowers used as control (open pollination) produced any fruit at all (data of 2015 and 2017 pooled). The hand-pollination using pollen from another population (Xenogamy II) increased fruit set to 54% (n = 22; Fig. 19) and seed set to 11.57 ± 0.72 seeds per capsule. The addition of pollen of the co-flowering *V. alba* on *V. jaubertiana* stigmas produced a low fruit set (13%) and seed set (2.75 seeds per fruit), but confirmed the potential hybridization between these two closely related species in nature (Fig. 19).

Seed mass differed strongly among pollen-addition treatments (χ^2_1 = 199.28, $P < 0.001$), being higher in Xenogamy II than in Xenogamy I (Z-value = 4.42, $P < 0.001$; Table 8). CH seeds were also heavier than CL seeds (Z-value = 11.92, $P < 0.001$; Table 8). The mass of the elaiosome in each seed also differed among treatments (χ^2_1 = 89.78, $P < 0.001$), but only between seeds coming from the outcrossing treatments (Z-value = 9.47, $P < 0.001$); seeds from the xenogamy II treatment showed heavier elaiosomes than those form the xenogamy I treatment (Table 8). CH seeds showed higher germination rates than CL seeds (χ^2_1 = 19.79, $P < 0.001$). Likewise, seeds from the xenogamy I treatment germinated better than control CH seeds (Table 8).

Inbreeding depression was detected for both seed mass and germination rate (0.29 and 0.55, respectively; Table 8). That is, seeds coming from outcrossing were heavier and germinated more readily than those following selfing (CL). By contrast, inbreeding depression was negative for seed set, due to the higher seed set in CL flowers (Table 8). Overall, the multiplicative inbreeding depression showed an important depression in the species (Table 8). We found no outbreeding depression for any of the variables studied, and multiplicative outbreeding depression either (Table 8); by contrast, outbreeding between distant populations increased the number of seeds produced, seed weight and the elaiosome mass produced per seed.

4.4 Discussion

As many *Viola* species, *V. jaubertiana* displays dimorphic cleistogamy (Culley & Klooster 2007), first producing CH flowers and CL flowers later in the flowering season (Redbo-Torstensson & Berg 1995; Culley 2002). Such delayed reproductive assurance has been shown in many species that face uncertain pollination environments (Kalisz *et al.* 2012), as is the case of *V. jaubertiana*. In other species of *Viola*, CH flowers are predicted to appear under favorable pollination conditions whereas CL under unfavorable ones (Culley 2000, 2002). For example, in *V. pubescens* and *V. canadiensis,* CH flowers appear under high light levels in early spring when pollinators are most active, whilst CL flowers are produced under low light levels after the overstory trees have leafed out and pollinators disappear (Culley 2000, 2002). In some species, like *V. pubescens*, CH flowers are capable of delayed selfing if left unvisited by insect pollinators. Conversely, in *V. jaubertiana*, CH flowers are produced in the coolest season in Mallorca (end of winter), and in a shady habitat in which pollinators are very scarce, and with no capacity of delayed selfing. CL flowers in this violet are produced during mid-spring, when pollinators are expected to be abundant. These observations run counter to the prediction to be a plastic strategy to adapt the phenology of CH flowers to the favorable conditions for pollination. This suggests that producing first CH flowers in the season than CL flowers is a genetically conserved trait in the genus, to adapt CH blooming before the canopy has been fully developed and light availability has strongly decreased. For example, in the sympatric and phylogenetically close *V. odorata* (Mayers & Lord 1983), CH flowers were seen to be formed in response to 11 hr or less of daylight and CL flowers in response to 14 hr or more of daylight.

Our results are consistent with the adaptive phenotypic plasticity model of Schoen and Lloyd's (1984). Showing a decreasing CH:CL ratio with elevation probably due to the reduced possibilities for cross-pollination at high altitudinal conditions (Totland 2001). All recent studies that have examined variation in the CH:CL ratio actually report evidence for phenotypic plasticity (Oakley *et al.* 2007). Furthermore, we found that the production of CH flowers decreased with plant SLA, i.e. that more CH flowers were produced at sunnier-drier sites (with low SLA). This might also be an adaptive strategy to increase CH production at warmer microsites environments where pollinator activity and visitation rate are expected to be higher. In fact, the few CH fruits and pollinator visits observed in *V. jaubertiana* were on few individuals with high CH flower production and located on visible and lightly faces, and rarely in cracks or narrow rocky entrances. On the other hand, the reduction of the proportion of CH flowers with herbivory agrees with other studies that have shown that CH flowers and fruits are more susceptible than CL structures to suffer herbivory (Trapp & Hendrix 1988; Redbo-Torstensson & Berg 1995), which has been shown to be an important selection force towards self-fertilization (Eckert *et al.* 2006). We also found a tendency to decrease relative allocation to CH reproduction with increasing plant size, perhaps because of an increased likelihood of geitonogamous self-pollination and pollen discounting (Harder & Barrett 1995), and/or herbivore attraction (Gómez 2003; Sandring *et al.* 2007) with increasing CH floral display.

A high P/O ratio, and almost nonexistent autonomous selfing found in *V. jaubertiana* CH flowers, show that these flowers depend entirely on pollinators to produce seeds. Therefore, the low success rate of CH flowers at all sites, together with the high pollen limitation, seem to reflect the scarcity of suitable pollinators in wild populations of *V. jaubertiana*. Actually, in his studies on the pollination ecology of *Viola*, Beattie (1969) found that pollination decreases in sheltered and north-facing habitats due to the small amount of sunlight incident upon them. However, the reduction in CH progeny expenditure may result in much improved efficiency of CL self-pollination, as shown in *V. jaubertiana* by the high fruit set (86%) of CL flowers. A very low fruit set has also been observed in many species, including other *Viola* species (Kerner & Oliver 1902; Knuth 1908; West 1930), and several functional advantages of nonfruiting have been proposed. The reserve-ovary model developed by Ehrlen (1991) predicts that more flowers may be produced than there are resources necessary for them to develop to fruits, even if the only function of the surplus flowers is to serve as reserve ovaries. Therefore, the high production of CH flowers in some individuals of *V. jaubertiana* may be an adaptive mechanism to ensure a few costly CH seeds in an unpredictable environment, with very low pollinator activity but high flower damage (up to 90% in some cases). Furthermore, a continuous production of surplus flowers may enable plants to exploit conditions extremely favorable, in terms of resources, pollinator availability, or relaxed predation pressure, that occur in an unpredictable way in time and space (Aker 1982; Holtsford 1985). To confirm this, however, it would be necessary to follow fruit production in CH flowers in the long term. Other alternative explanations for the surplus flowers are (1) to increase pollinator attraction (i.e. Schemske 1980) and (2) to serve as pollen donors (i.e. Sutherland 1986). However, we found no support for these hypotheses as we did not detect an increase of CH fruits/seeds with the increase in CH flower display, despite the increase in visitation rate. Seed set of CL flowers decreased as the proportion of CL flowers per plant increase, probably due to resource limitation in individuals predominantly cleistogamous, in which individuals may reach a point where they cannot produce more seeds in a such a resource-poor habitat (Larson & Barrett 2000).

The energetic cost per seed was threefold higher for CH than for CL flowers. However, the species produces one CH seed per plant for each 100 CL seeds. In a review of all studies testing the relative cost of reproduction via CH and CL flowers, Oakley et al. (2007) suggested a consistent trend for CL to be less costly to produce and to have a greater probability to set fruits, but with a mean of CL fertility 3.06 times higher than CH fertility. So far, only in the cleistogamous *Scutellaria indica* (Sun 1999) a similar relative cost per seed (between CH and CL) to *V. jaubertiana* has been found. In *V. jaubertiana*, as in *S. indica*, spatial separation of anthers and stigmas of CH flowers seems to be preventing spontaneous self-pollination, which renders the fertilization of a CH flower entirely pollinator-dependent.

At this point, the question raised by our data is what type of forces or advantages can be important enough to maintain the production of CH flowers, given that CL flowers are energetically cheaper and 100% more productive than CH flowers. Inbreeding depression has been used to explain the maintenance of mating systems than enhance cross-fertilization (Lande & Schemske 1985; Charlesworth & Charlesworth 1987). Nevertheless, selfing is expected to purge recessive deleterious alleles from populations, so that inbreeding depression is expected to be reduced in highly selfing species compared with species that normally outbred (Charlesworth & Charlesworth 1987; Husband & Schemske 1996), although selfing species tend to exhibit greater inbreeding depression in later life-history stages (Husband & Schemske 1996). A low inbreeding depression was reported, for instance, in the cleistogamous species *V. canadensis* (Culley 2000). Despite we had no information on the fitness of self-pollinated CH flowers in the field, our data gathered at the botanical garden are indicative of an also important inbreeding depression in *V. jaubertiana*, with a greater fitness of outcrossed compared to selfed progeny (CL), especially at the level of seed mass and seed germination. Such high inbreeding depression could partly explain the maintenance of the mixed mating system. The increase in mass, elaiosome production and germination of the outcrossed seeds suggests that, despite the much lower seed production of CH compared to CL, successful and recruitment of both flower types may be compensated by a greater fitness, dispersal capacity to cliff fissures and germination of CH progeny. Furthermore, in a long-lived species such as *V. jaubertiana* (probably living ≥ 25 years), producing even a small number of outcrossed seeds each year may be sufficient to avoid the detrimental effects of inbreeding depression (Barrett & Kohn 1991; Lande 1994). Moreover, living in a climatically very stable habitat, inaccessible to large herbivores, may also contribute to the stability of this mixed mating system.

To further identify forces or advantages that maintain the production of CH flowers in *V. jaubertiana*, the next step would be to test the Lu’s heterosis model (Lu 2002) by crosses among inbred small populations (previously determining how much genetic load accumulates each selfing lineage), since empirical evidences support a potentially large advantage to maintaining the potential for periodic outcrossing between inbred lines (Paland & Schmid 2003; Weller *et al.* 2005). In fact, having found no outbreeding depression in *V. jaubertiana* suggests that heterosis is another plausible explanation for the maintenance of outcrossing in the species. A second step might be to compare dispersal and predation rates between seeds from both flower types, and the resulting contribution of each flower type to recruitment in the vertical cliffs. Our preliminary observations suggest that *V. jaubertiana* is purely myrmecochorous, which might explain its limited distribution, as reported for other *Viola* species (Beattie & Lyons 1975). It seems reasonable to think that the heavier CH seeds, with greater elaiosome mass, may be more attractive to ants, besides germinating more than lighter seeds. A third and last step to fully understand the unusual reproductive ecology of this endemic cliffhanger, would be to assess the role of its hybridization with *V. alba*, a closely related species that coexists with it in the same habitat. What seems clear is that *V. jaubertiana* has been able to adapt to a low nutrient availability, and to an unpredictable environment for pollination, by producing a continuous amount of CL seeds to ensure reproduction, and a low amount of more costly but greater performance CH seeds, to avoid the detrimental effects of inbreeding depression. Furthermore, its ability to live under such conditions grants *V. jaubertiana* to live in an unusual very stable habitat, with low interspecific competitiveness, low mortality by climatic extremes, and low predation by abundant vertebrate herbivores.

4.5 Acknowledgments

We deeply thank the owners of *Bini Gran* and *Finca de Comafreda* for permissions to access to study localities, and the Government of the Balearic Islands (*Conselleria d'Agricultura, Medi Ambient i Territori*) for authorizations to collect material during 2014 and 2015. We are also very grateful to Sóller Botanical Garden and its staff, which provided us with all kinds of aid and facilities in carrying out the experiments, and to A. Lázaro and M. Nogales for their useful comments and reviews on the manuscript. Jaume Seguí was supported by the Graduate Fellowship Program co-funded by the European Social Fund (ESF) and the Government of the Balearic Islands (*Conselleria d'Educació i Universitat*). This work is framed within a project of the "Organismo Autónomo de Parques Nacionales" (785/2012).

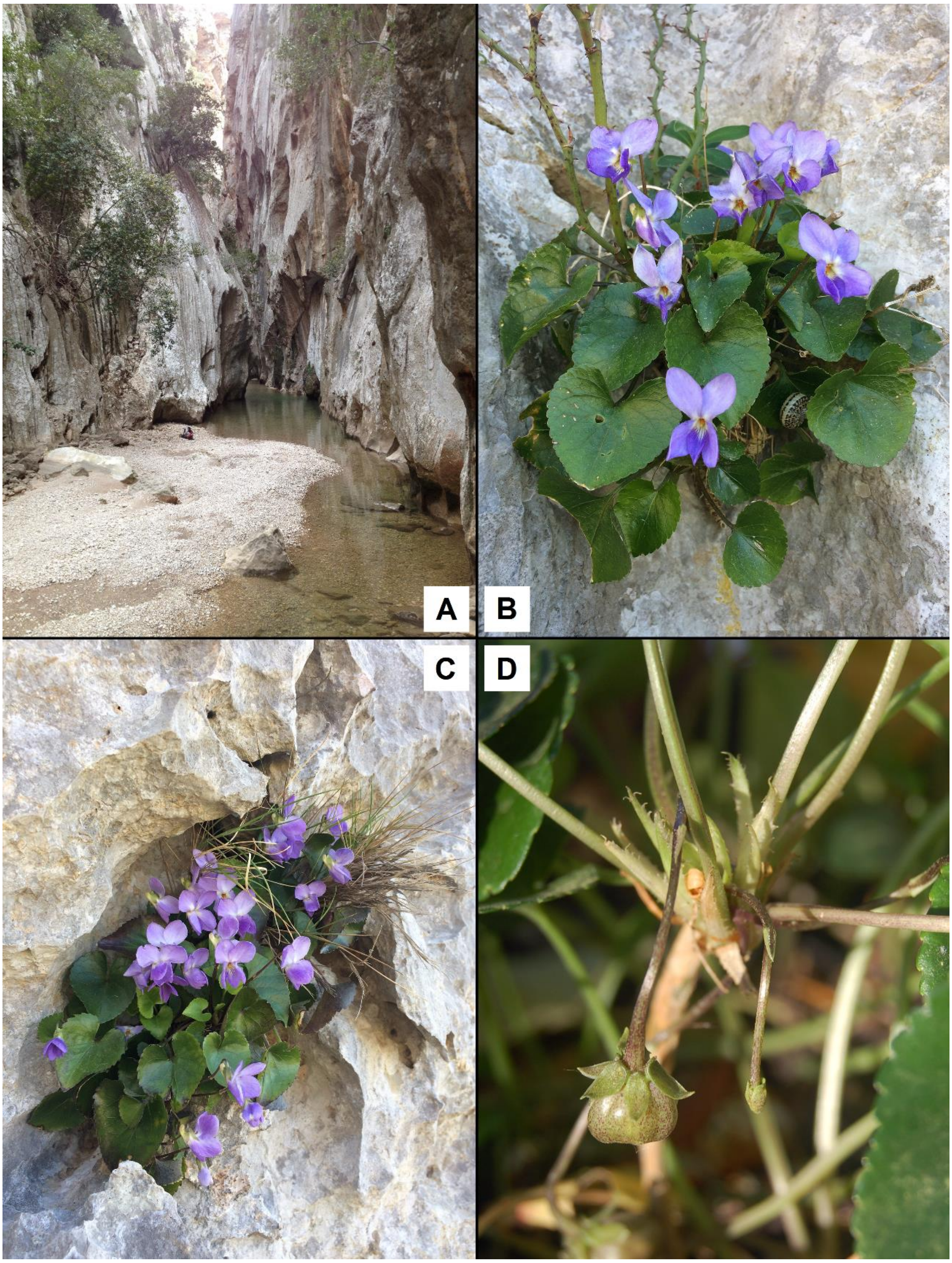

Figure 15. Examples of (A) a vertical cliff habitat type of *Viola jaubertiana*, (B)(C) two chasmogamous flowering individuals, showing an endemic snail (*Iberellicus balearicus*) with some damaged leaves, and (D) details of a cleistogamous flower and fruit.

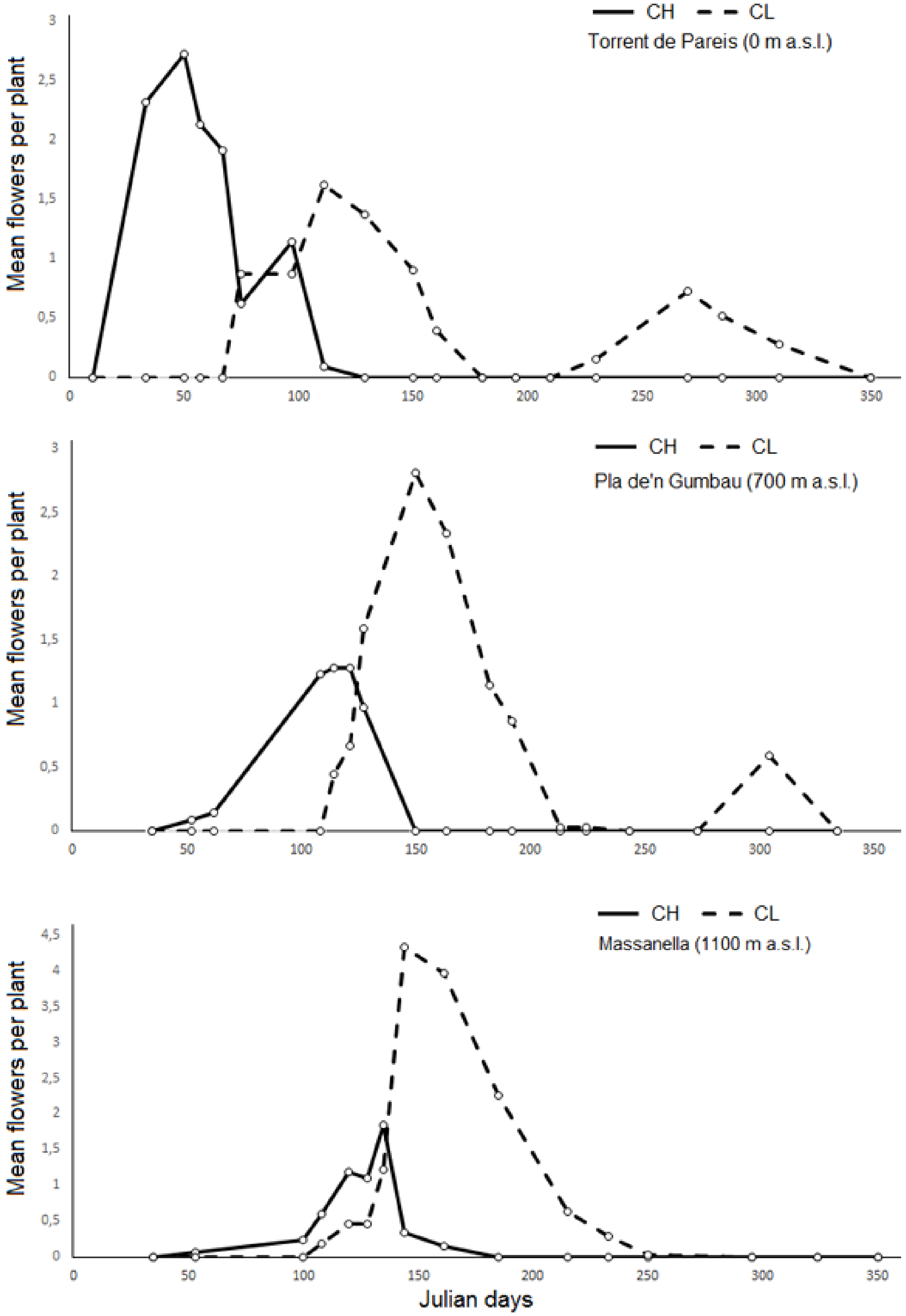

Figure. 16. Flowering phenology of *V. jaubertiana* presented as the mean number of flowers (CH and CL) per plant observed at each census and elevational site during 2014. All round unfilled marks indicate dates of observation.

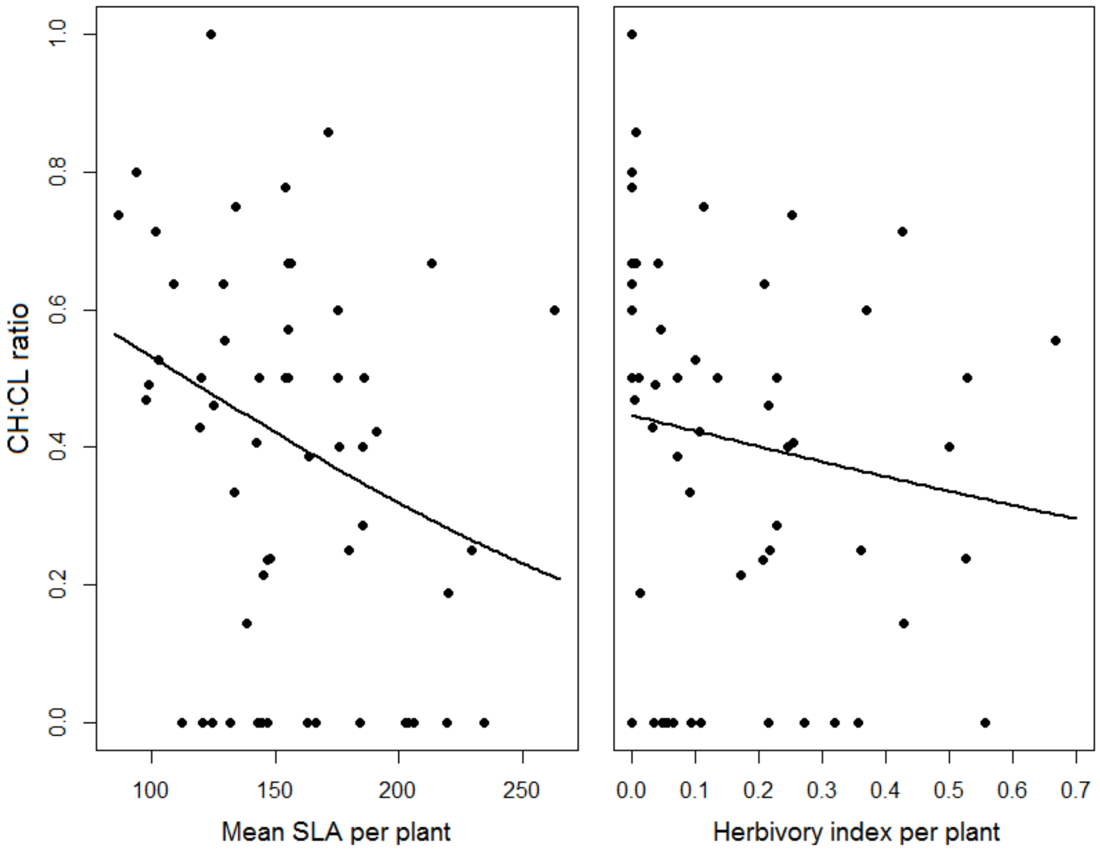

Figure. 17. CH:CL flower ratio in response to mean *specific leaf area* and *herbivory index*. The lines represent the estimates of each variable in the model whereas points are the empirical data.

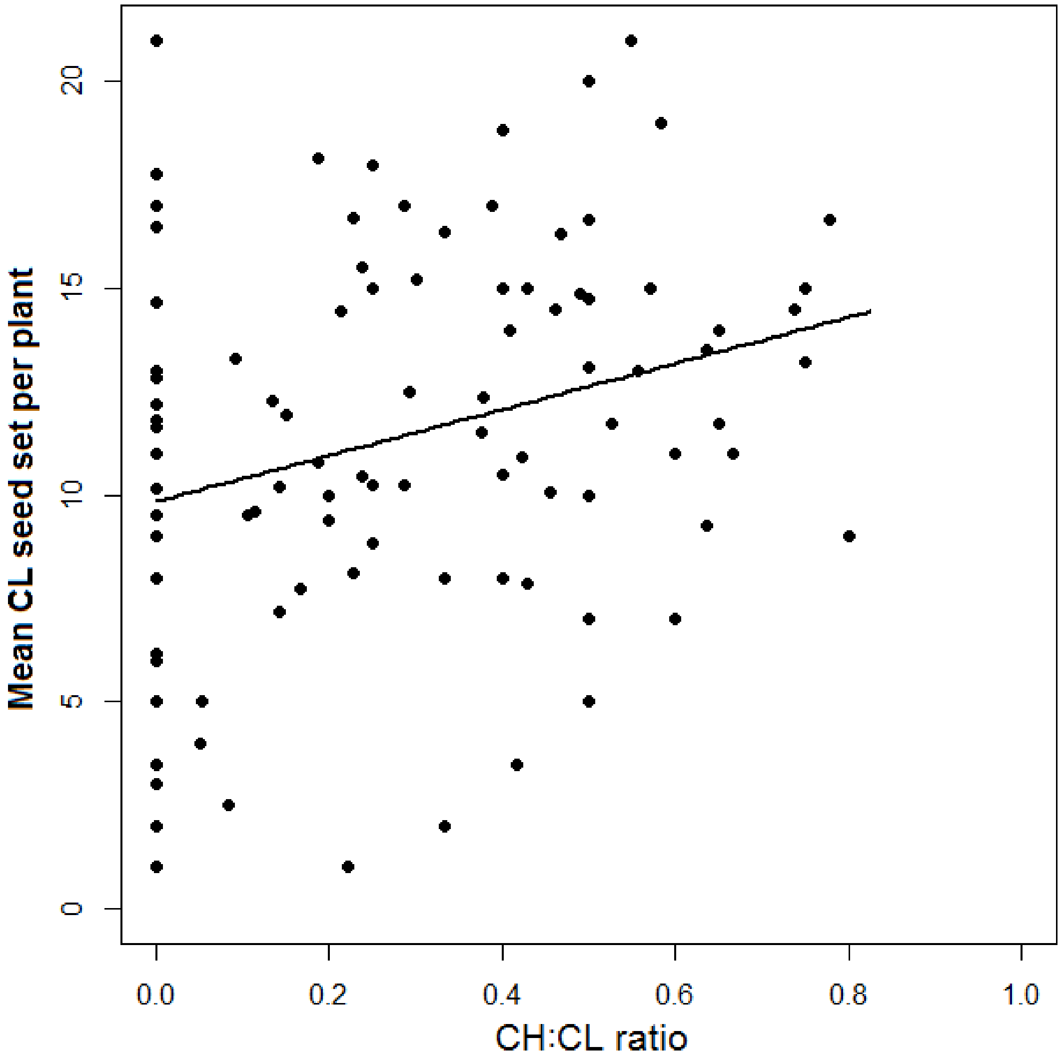

Figure 18. Mean CL seed set per plant as a function of its CH:CL flower ratio. Line represents the estimate of the model, whereas points are the empirical data.

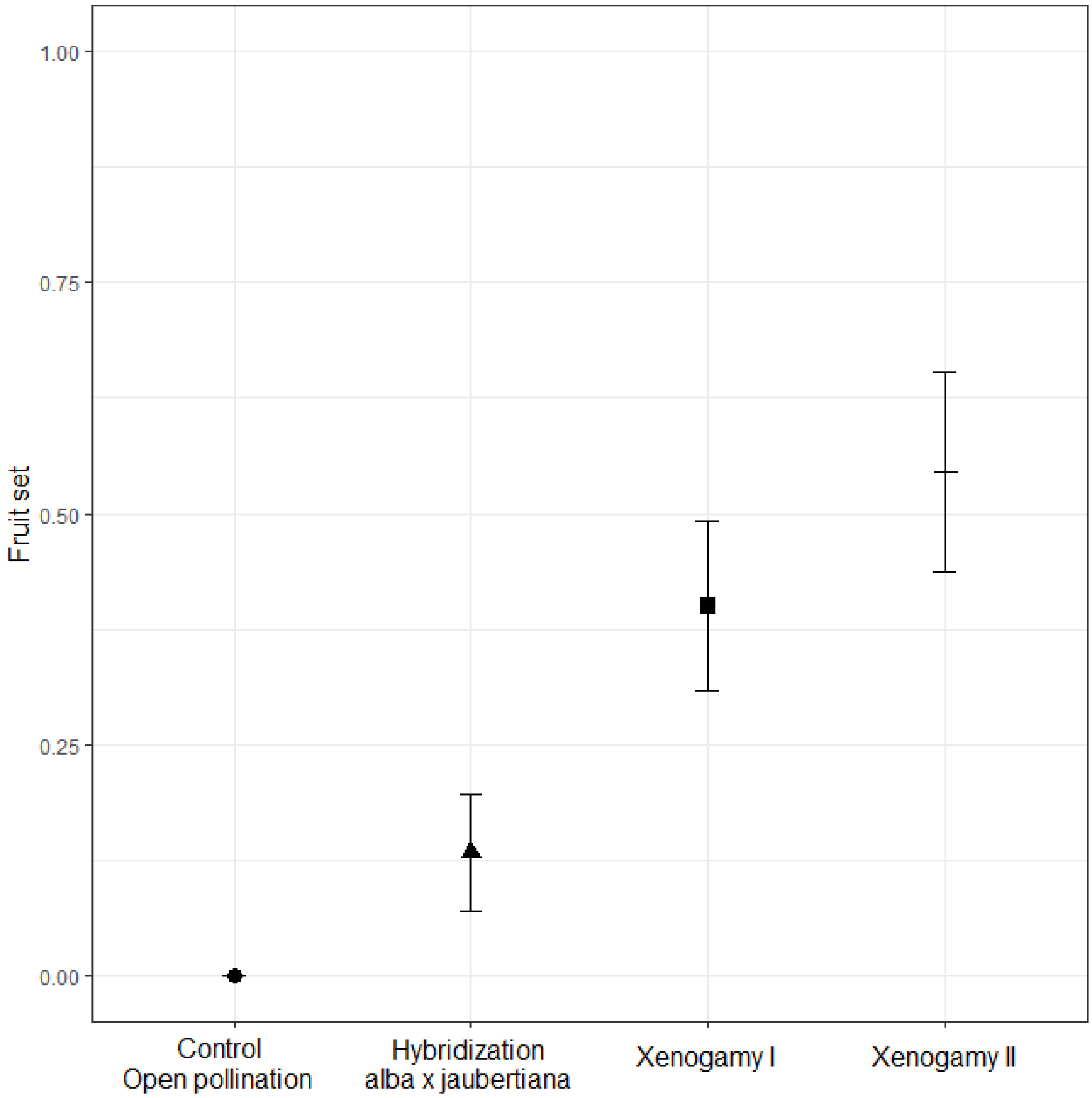

Figure. 19. Mean and standard deviation of fruit set (proportion of flowers setting fruits) obtained in each treatment and control. Experiments were carried out at Sóller Botanical Garden.

4.7 Tables

Table 6. Mean and standard errors of the traits measured in *Viola jaubertiana* during 2014 and 2015 at the three altitudinal sites.

		Torrent de Pareis 0 m	Pla de'n Gumbal 700 m	Massanella 1100 m
Herbivory index				
	2014	0.14 ± 0.02 (32)	0.18 ± 0.04 (30)	0.16 ± 0.03 (28)
	2015	0.14 ± 0.04 (31)	0.18 ± 0.04 (33)	0.24 ± 0.04 (25)
Florivory CH flowers				
	2014	0.90 ± 0.05 (20)	0.46 ± 0.11 (16)	0.54 ± 0.10 (22)
	2015	0.14 ± 0.07 (15)	0.07 ± 0.04 (17)	0.25 ± 0.12 (11)
Specific leaf area (SLA)				
	2015	192.8 ± 9.08 (23)	144.55 ± 9.3 (30)	161.82 ± 7 (19)
CH:CL ratio				
	2014	0.46 ± 0.06 (25)	0.20 ± 0.05 (24)	0.28 ± 0.05 (28)
	2015	0.45 ± 0.06 (19)	0.38 ± 0.06 (25)	0.19 ± 0.04 (20)
Fruit set CH flowers				
	2014	0 ± 0 (4)	0.16 ± 0.11 (10)	0.01 ± 0.01 (11)
	2015	0.01 ± 0.01 (14)	0.06 ± 0.02 (16)	0.17 ± 0.07 (8)
Fruit set bagged CH flowers				
	2014	0.00 ± 0.00 (7)	0.11 ± 0.11 (9)	0.05 ± 0.05 (10)
	2015	0.07 ± 0.07 (14)	0.00 ± 0.00 (6)	0.00 ± 0.00 (2)
Fruit set CL flowers				
	2014	0.70 ± 0.09 (21)	0.96 ± 0.02 (23)	0.85 ± 0.04 (26)
	2015	0.79 ± 0.08 (19)	0.85 ± 0.07 (23)	1 ± 0.00 (18)
Seed set CL flowers				
	2014	12.42 ± 1.19 (15)	8.54 ± 0.98 (19)	10.32 ± 1.47 (19)
	2015	14.28 ± 1.2 (13)	12.21 ± 0.87 (19)	12.03 ± 0.9 (15)
Germination rate (%)				
	CL	27 (100)	29 (100)	47 (75)
	CH	45.4 (11)	57.1 (25)	77.5 (50)
Pollen ovule ratio				
	2015	1102.8 ± 385 (14)	1537.1 ± 283 (8)	2207.2 ± 220 (24)

Note. Sample sizes (number of seeds in the case of germination rate and number of individuals for the rest of variables) are given in parentheses

4.7 Tables

Table 7. Estimates of the relative flower cost, and relative reproductive success of both CH and CL flowers.

	Chasmogamy	Cleistogamy	Ratio CH:CL
Flower cost (mg)	26.15 ± 1.57	7.65 ± 1.15	3.42
Florivory per plant	0.43 ± 0.04	0.00 ± 0.00	-
Floral display per plant	4.36 ± 0.54	8.55 ± 0.93	0.51
Fruit set (fruits/flowers)	0.06 ± 0.02	0.86 ± 0.02	0.07
Seed set (seeds/capsule)	6.21 ± 1.10	12.97 ± 0.40	0.47
Mean seeds per plant	1.47 ± 0.42	110.7 ± 13.4	0.01

Note. Flower cost was obtained of the mean dry weight per flower from a total of 15 (CH) and 13 (CL) flowers.

Table 8. Performance seed traits of each flower type in the field, and in each treatment performed in the botanical garden. Inbreeding and outbreeding depression estimates are presented.

	Field		Botanical garden			
	Cleistogamy	Chasmogamy	Xenogamy I	Xenogamy II	RP_i	RP_o
Seed set	12.97 ± 0.40 (169)	6.21 ± 1.10 (14)	8.8 ± 0.90 (5)	11.57 ± 0.72 (7)	-0.32	-0.24
Seed mass	44.68 ± 0.39 (517)	55.74 ± 1.4 (86)	63.33 ± 1.15 (42)	84.16 ± 1.39 (68)	0.29	-0.25
Elaiosome mass	3.46 ± 0.07 (517)	3.91 ± 0.30 (86)	3.71 ± 0.46 (42)	16.41 ± 0.52 (68)	0.07	-0.77
Germination rate (%)	34.3% (275)	60% (86)	76.9% (15)	NA	0.55	NA
Total RP					0.56	-0.87

Note. **RP_i**: Inbreeding depression: (Xenogamy I – Cleistogamy) / Xenogamy I. **RP_o:** Outbreeding depression: (Xenogamy I – Xenogamy II) / Xenogamy II. Sample sizes (number of fruits in the case of seed set and number of seeds for the rest of variables) are given in parentheses.

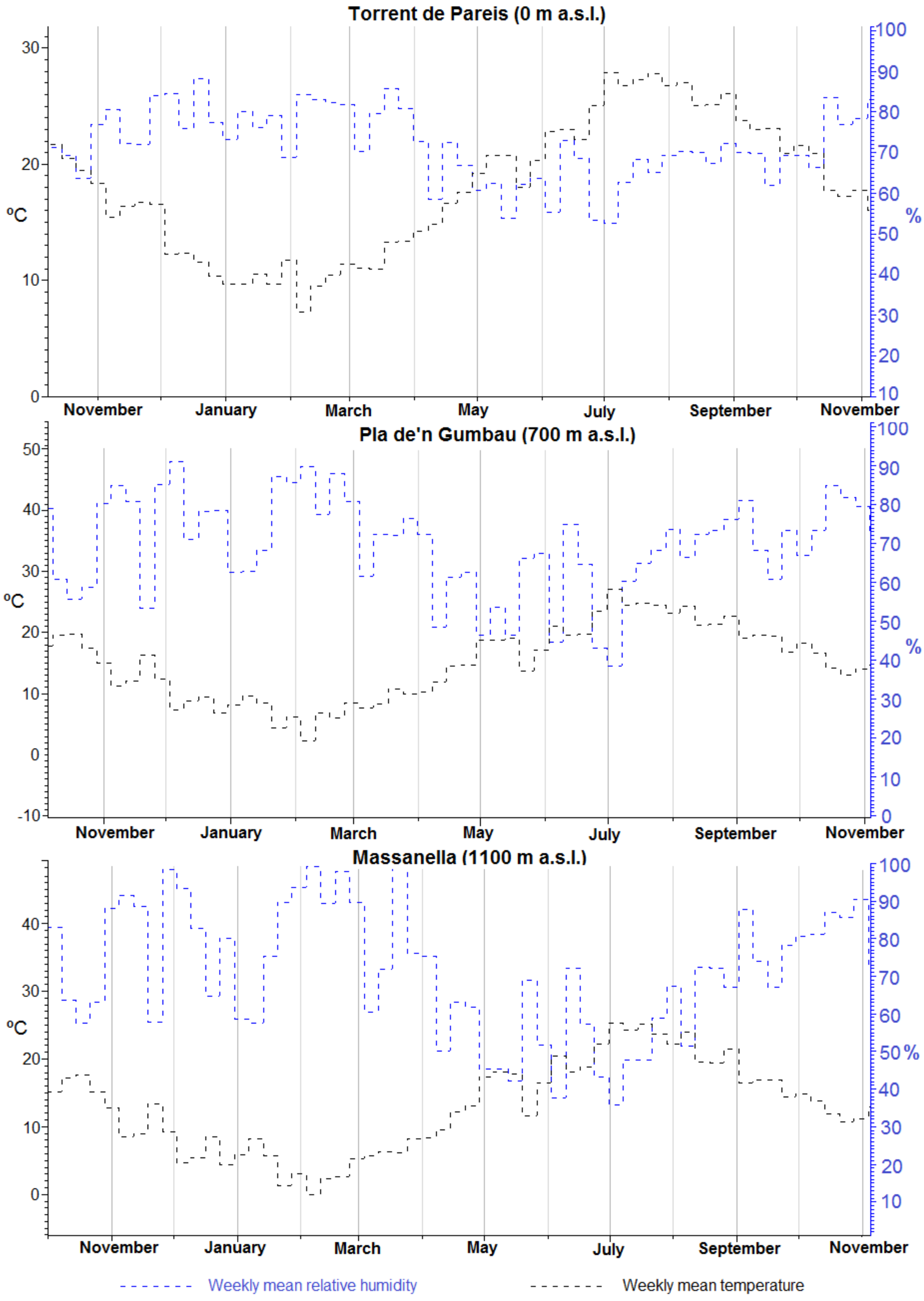

Figure S6. Temperature (ºC) and relative humidity data (%) at each site during a whole year (November 2014-November 15).

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C. EPÍLOGO

Discusión general

Las montañas, gracias a su efecto "isla ecológica" y a los gradientes ambientales que presentan, son puntos calientes de biodiversidad (Steinbauer *et al.* 2012, 2016). Desde que empezaron las expediciones de Alexander von Humboldt (1799 –1804) y Charles Darwin (1831–1836), reportando la gran variación de hábitats y especies presentes a lo largo de los gradientes altitudinales de las montañas, estos sistemas han captado el interés de un gran número de científicos.

Hoy en día, los principales parámetros del clima (la temperatura y la precipitación), están cambiando rápidamente (IPCC 2014), con drásticas variaciones en los ecosistemas; cambios en los rangos de distribución de las especies, desajustes fenológicos, cambios en la composición de especies, así como en las interacciones entre ellas, entre otros (Walther *et al.* 2002; Parmesan 2006). Los gradientes altitudinales son especialmente adecuados para estudiar estos efectos, debido a las cortas distancias entre diferentes condiciones ambientales. Dichos gradientes presentan retos importantes y recurrentes para la adaptación exitosa de las plantas en muchas partes del mundo. Aunque algunas especies vegetales pueden crecer en un amplio gradiente altitudinal, adaptándose a las diferentes condiciones ambientales a través de la plasticidad fenotípica o la adaptación local (Cordell *et al.* 1998; Körner 2003; Byars *et al.* 2007), la mayoría de las especies están restringidas en su distribución a rangos de altitud más estrechos. Los estudios reportados en esta tesis engloban diferentes especies, cada una distribuida en diferentes islas y continentes, y en distintos gradientes altitudinales. Por lo tanto, los patrones observados en cada una de ellas son altamente dependientes de las condiciones particulares de cada zona de estudio y de sus características biológicas intrínsecas.

La información obtenida en los diferentes gradientes altitudinales estudiados indica que las especies (violetas) objeto de estudio tienen capacidad para vivir bajo condiciones ambientales muy variadas, con una gran plasticidad fenotípica y una gran variedad de estrategias reproductivas de seguro reproductivo. Estas características sugieren que dichas especies tienen potencial para mantener poblaciones viables bajo las nuevas condiciones de cambio climático actual. En el caso de *V. cheiranthifolia*, no obstante, la viabilidad de la especie a corto y medio plazo está seriamente amenazada debido al efecto de los conejos, los cuales están reduciendo drásticamente su abundancia y eficacia biológica y alterando sus interacciones con el medio abiótico y biótico. Además, el efecto combinado del cambio climático - el cual muestra reducir la viabilidad de las poblaciones inferiores- con el de las especies invasoras, probablemente intensificará aún más los efectos negativos observados en *V. cheiranthifolia* de cara al futuro. Por tanto, se hace necesario actuar en el Parque

Nacional del Teide, donde ya hay claras evidencias de que la vegetación actual es el resultado de los efectos que están produciendo los lagomorfos introducidos (Kyncl *et al.* 2006).

En esta tesis, se destaca el efecto de una gran variedad de factores que interactúan con los de tendencia global con la altitud (principalmente la temperatura), lo cual dificulta la detección de patrones generales en las diferentes respuestas observadas a lo largo de los gradientes. Un motor muy importante de cambio en estos sistemas de montaña es la variabilidad interanual (Totland 1994; Herrera 1995), afectando especialmente a rasgos fenotípicos, como el tamaño de los individuos o la intensidad de la floración, y a la abundancia de polinizadores. De todas formas, no deberíamos hablar de años favorables o desfavorables, sino de condiciones climáticas que son más o menos favorables dependiendo de la localidad. Por ejemplo, en un año con importantes y persistentes nevadas, las poblaciones inferiores suelen responder con una alta tasa de crecimiento y floración (Palacios *et al.* 2003), mientras que las poblaciones elevadas tienen que hacer frente a un corto período de actividad vegetativa, con poco tiempo para el crecimiento y la reproducción. Por el contrario, un año con pocas nevadas merma especialmente las poblaciones inferiores, las cuales se enfrentan a un importante estrés hídrico, reduciendo de forma considerable el tamaño de los individuos, y consecuentemente sus probabilidades de floración (Giménez-Benavides *et al.* 2011).

Los factores más importantes que han mostrado tener un efecto interactivo sobre las respuestas observadas han sido:

1. *La humedad del suelo*: variable entre años y altitudes, dependiendo en gran medida de la abundancia y duración de las nevadas, así como del tipo de sustrato e inclinación en cada localidad. El aumento de las temperaturas y la disminución de la intensidad y duración de las nevadas que se prevé para el Teide por efecto del cambio climático, probablemente afectará de manera especial la viabilidad de las poblaciones a menor altitud, donde ya se observa una baja densidad de individuos, con tamaños muy pequeños, y poca floración.

2. *La competencia o facilitación intra- e inter-específica*: se ha observado que factores cómo la disponibilidad de flores de un individuo y la abundancia de flores conspecíficas vecinas pueden tener un efecto positivo sobre el éxito reproductivo y la viabilidad de las semillas de ese individuo. Por otro lado, la riqueza de especies vegetales acompañantes en la comunidad también puede tener un efecto sobre la tasa y la riqueza de visitas de polinizadores en las violetas. Por ejemplo, *V. cheiranthifolia* tiende a volverse más generalista a medida que asciende en altitud, a pesar de la disminución en abundancia y

riqueza de insectos y en el número de interacciones en la comunidad (Lara-Romero et al. in prep). Este efecto puede deberse a la menor competencia inter-específica en las zonas más elevadas, donde la violeta del Teide llega a ser la única especie entomófila en la comunidad y, por tanto, la única fuente de recursos de los pocos insectos que llegan a estas altitudes, ya sea de manera voluntaria o accidental.

3. *La herbivoría*: el principal resultado de los capítulos 1 y 2 ha sido precisamente el importante efecto interactivo del ramoneo por conejo sobre las respuestas observadas en *V. cheiranthifolia* a lo largo del gradiente. Así, en el capítulo 1 se ha visto que el patrón de variación de los rasgos de la planta a lo largo del gradiente cambia completamente cuando los individuos son protegidos del conejo. En el capítulo 2, además, se ha visto que la presión de herbivoría también influye sobre la morfología y el tamaño de las flores de *V. cheiranthifolia*, modificando la selección que los polinizadores y las condiciones abióticas ejercen sobre los rasgos florales. Además, la reducción del despliegue floral de las violetas por parte de los conejos, haciéndolas menos atractivas a los polinizadores, probablemente sea la causa de la limitación de polen observada en la especie en el capítulo 1. Por lo tanto, los patrones fenotípicos y reproductivos que observamos hoy en día en *V. cheiranthifolia*, probablemente nada tengan que ver con los patrones altitudinales que se observarían en esta planta antes de la llegada de los conejos por la mano del hombre.

En las tres especies del genero *Viola* estudiadas, se ha observado la presencia de estrategias reproductivas que favorecen el seguro reproductivo bajo condiciones desfavorables para la polinización, como son las presentes en las cimas de los respectivos gradientes altitudinales. Así, en *V. cheiranthifolia* se ha observado un incremento de la autofecundación retardada con la altitud, llegando a suponer la mitad de la producción total de semillas por flor en la población más elevada (Seguí *et al.* 2017). Por otro lado, en *V. maculata* y *V. jaubertiana* se ha detectado por primera vez un incremento del grado y/o éxito reproductivo de la cleistogamia respecto a la casmogamia a lo largo de un gradiente altitudinal. Estos resultados apoyan el modelo de Schoen y Lloyd (1984) sobre la cleistogamia como mecanismo de plasticidad fenotípica adaptada, al haberse observado una mayor producción o éxito reproductivo de flores cleistógamas en las zonas más desfavorables para la polinización. De todas formas, en el caso de *V. jaubertiana*, se ha visto que la inversión en un tipo u otro de flor se ve influenciada no sólo por la altitud, sino también por la presión de herbivoría, el tamaño y las condiciones particulares de estrés hídrico de cada individuo. Por lo tanto, la producción relativa de cada tipo de flor que vemos a lo largo del gradiente es el resultado de todo un conjunto de factores que actúan en dicha plasticidad. En términos generales, podemos decir que las tres especies de *Viola* estudiadas en esta tesis presentan mecanismos aseguradores de la reproducción, ya sea por medio de la autogamia retardada (observada en *V. cheiranthifolia* y *V. maculata*) o por medio de la cleistogamia (*V. maculata* y *V. jaubertiana*). Esta alta persistencia de la autofecundación en dichas especies parece que no les impide mantener una importante plasticidad fenotípica a lo largo del gradiente y, al menos en el caso de *V. cheiranthifolia*, también una importante variabilidad genética, ya que se ha observado un importante flujo génico entre las poblaciones altitudinales del estratovolcán y una baja diferenciación inter-poblacional (Rodríguez et al. In prep). En otra especie, *Silene ciliata,* también se ha encontrado un importante flujo génico entre las poblaciones a lo largo de un gradiente altitudinal en Sierra de Guadarrama, y una baja diferenciación genética entre poblaciones altitudinales, a pesar de mostrar adaptación local en las poblaciones del límite inferior de distribución (García-Gernández et al. 2012a; 2012b); estos autores han sugerido que los movimientos pasados de ascensión y descenso en las montañas, asociados a procesos glaciares-interglaciares, pueden estar detrás de la ausencia de patrones altitudinales en la diversidad genética. Posiblemente, en las especies del género *Viola* abordadas en esta tesis, las semillas producidas por autofecundación aseguran la reproducción en estos ambientes impredecibles, permitiendo mantener los procesos de adaptación local y, a su vez, un cierto grado de fecundación cruzada permite evitar fenómenos de deriva genética, además de mantener la variabilidad genética.

La eficiencia reproductiva se ha descrito como uno de los factores más limitantes en los ecosistemas de alta montaña (Arroyo *et al.* 1985; Totland 1994; Medan *et al.* 2002; Muñoz & Arroyo 2006). En la violeta del Teide, se esperaba encontrar mayor limitación de polen a mayor altitud, al igual que una menor tasa de visitas de polinizadores, debido a las condiciones desfavorables para la polinización en dichas elevaciones (temperatura media de 5 ^{0}C y fuertes vientos). No obstante, no se observaron diferencias en el éxito reproductivo ni en la limitación de polen entre altitudes, ni tampoco una reducción del número de visitantes florales con la altitud. Ello posiblemente se deba a los mecanismos compensatorios observados, como son el aumento del grado de autogamia, el mayor número de óvulos por flor con la altitud, y/o el presentar espolones más anchos y más cortos a elevaciones altas, para poder aprovechar los pocos polinizadores disponibles.

Mediante la realización de un metaanálisis (Seguí et al., en prep.) en el que se ha incluido un total de 122 especies de plantas, y en el que se han utilizado como variables respuesta el cuajado de frutos, la producción de semillas y las tasas de visitas de polinizadores, se ha observado que la altitud no limita de manera consistente el éxito reproductivo de las plantas (Fig. 20A). En un gran número de los estudios, el efecto fue prácticamente nulo, probablemente debido a la presencia de mecanismos compensatorios en dichas especies (Fig. 20A). Existe, además, un número similar de estudios donde el efecto es o bien positivo o bien negativo (Fig. 20ª). Ello es posiblemente debido al gran número de variables locales que pueden influir en el éxito reproductivo de las plantas, además de a las particularidades de cada sistema montañoso; por ejemplo, la tendencia general es a aumentar el estrés hídrico en la mayoría de sistemas montañosos del mundo, pero en los mediterráneos, la tendencia es a la inversa, con mayor estrés hídrico en las poblaciones inferiores.

En gran parte de la bibliografía consultada, relativa a gradientes altitudinales alrededor del mundo (Fig. 20B), el foco de atención normalmente está en las particularidades climáticas de cada altitud, considerándose la temperatura como la principal. Sin embargo, esto es una aproximación demasiado generalista. Como se ha visto, una gran variedad de factores ecológicos varía con la altitud. Por lo tanto, correlacionar los patrones de variación de las especies a lo largo del gradiente con muchos de los parámetros que pueden afectar a dicha especie es una labor muy importante para identificar cuáles son los factores ecológicos realmente importantes para dichas especies. Hasta la fecha, pocos estudios tienen en cuenta los diferentes tipos de interacción que ocurren en los gradientes, ya que necesitan de grupos interdisciplinares que puedan abarcar estudios fisiológicos, genéticos, ecológicos y a nivel de comunidad (como el conjunto de trabajos desarrollados con *Silene ciliata* y *Armeria caespitosa* en Guadarrama; Giménez-Benavides 2006; García-Camacho 2009; García-Fernandez 2011; Lara-Romero 2014). De hecho, la gran mayoría de estudios sobre interacciones en gradientes altitudinales progresan en líneas separadas (ej. herbivoría, polinización, dispersión, genética), lo que podría llevar a la simplificación excesiva de la ecología y evolución de estos sistemas complejos (Herrera et al. 2002). Otro problema de estos estudios es el aspecto metodológico, ya que normalmente en estos sistemas de estudio no se puede disponer del número de réplicas deseable, y a menudo dichas especies están concentradas en una sola montaña, o incluso en una sola vertiente de la montaña (Fischer *et al.* 2011). Las montañas, como los gradientes altitudinales de distribución, funcionan como islas ecológicas, con una particular y única composición de especies. Por lo tanto, estos estudios se suelen centrar muchas veces en un solo gradiente de una misma montaña, limitándose así a casos de estudio locales. A pesar de ello, los resultados obtenidos en este tipo de estudios son de gran importancia para entender los cambios, adaptaciones y procesos ecológicos que ocurren en las especies en tiempo real, debido a los cambios bióticos y abióticos que ocurren en sus gradientes de distribución. Además, pueden ser especialmente útiles para estudiar el efecto combinado de diferentes motores del cambio global, como son la interacción de cambios de temperatura y precipitación con el efecto de las especies invasoras. Por lo tanto, los gradientes también pueden ser de gran utilidad para desarrollar estrategias de conservación en especies amenazadas por el cambio global.

A continuación, proponemos las siguientes directrices para futuros estudios en gradientes altitudinales:

1. El uso de modelos espaciales puede resultar muy útil para controlar los posibles efectos de competencia y facilitación de los individuos vecinos, ya sean inter- o conspecíficos, sobre el éxito reproductivo o el vigor de las plantas a lo largo del gradiente.

2. Aparte de registrar las condiciones micro-climáticas en cada altitud, resulta de gran interés la toma de rasgos funcionales en los individuos, como el área foliar específica (SLA), la tasa fotosintética o la conductancia estomática, los cuales dan una medida indirecta de las condiciones específicas en las cuales se encuentra cada individuo y de los efectos de las condiciones abióticas sobre los rasgos observados a nivel individual.

3. El uso de exclusiones o adiciones, tanto de antagonistas como de facilitadores, a lo largo de los gradientes altitudinales puede resultar una herramienta muy útil para predecir efectos interactivos entre especies a lo largo de diferentes condiciones ambientales. Por ejemplo, el uso de exclusiones individuales de herbívoros, combinados con parcelas excluidas en cada una de las altitudes, puede ser una buena herramienta para testar el efecto interactivo de los mamíferos herbívoros, tanto a nivel de éxito reproductivo como a nivel de reclutamiento y desarrollo de las plántulas.

4. Un seguimiento a largo plazo es esencial para determinar el alcance y dirección de los cambios observados en las especies. Puede haber años inusualmente secos o húmedos que pueden cambiar totalmente el patrón observado a lo largo del gradiente.

5. En especies especialistas, con una baja tasa de visitas de polinizadores, como suele ser el caso del género *Viola*, la combinación de métodos directos de observación de visitantes florales, como los desarrollados en esta tesis, con el uso de métodos indirectos, como la deposición de polen en estigmas de flores, podría resultar útil para detectar procesos de facilitación o competencia (Tur et al. 2016), cambios en las tasas de deposición de polen entre altitudes, o incluso distancias de dispersión de polen (Schulke & Waser 2001).

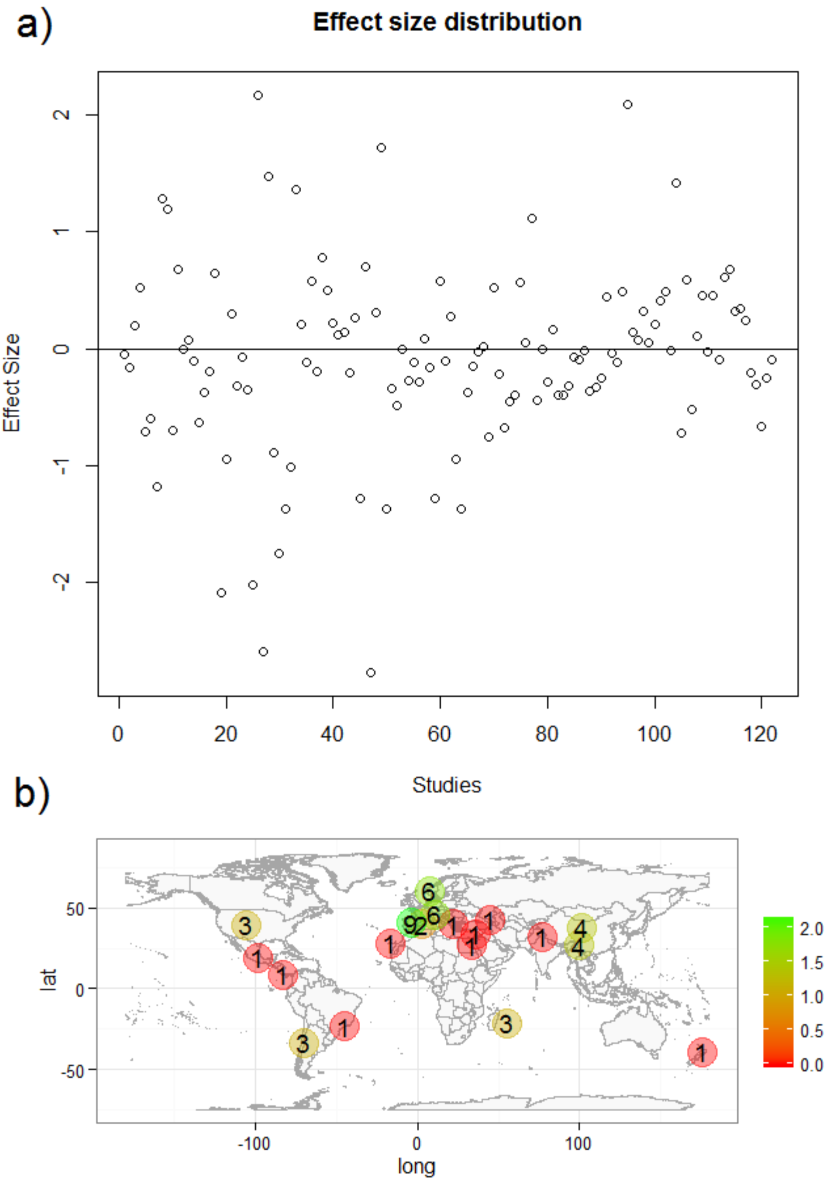

Figura 20. Resultados del metaanálisis realizado para analizar el efecto de la altitud sobre el éxito reproductivo de 122 especies de plantas en 49 gradientes altitudinales estudiados. (A) *Effect size* medido como el coeficiente de regresión basado en las estimas de las diferentes altitudes a lo largo del gradiente altitudinal, o bien calculado a partir del coeficiente *Cohen's d*, cuando se trató de comparar valores medios entre una población a altitud alta y otra a baja. (B) Ubicación de los gradientes estudiados; los números indican el número de gradientes altitudinales estudiados en cada zona geográfica.

Conclusiones generales

1. La eficiencia biológica de la violeta del Teide (*Viola cheiranthifolia*) a lo largo del gradiente altitudinal está básicamente afectada por las variaciones interanuales en el régimen de acumulación de nieve y de su posterior deshielo, además de por las condiciones particulares de cada microhábitat (abundancia de herbívoros, tipo de sustrato e inclinación, mantenimiento de la humedad del suelo, temperatura ambiental) en cada altitud, especialmente en su borde inferior de distribución durante los años secos.

2. A pesar de las condiciones ambientales cada vez más adversas para los polinizadores (como son bajas temperaturas, poca disponibilidad de recursos y fuertes vientos), *V. cheiranthifolia* no mostró un incremento en altitud en limitación de polen, lo cual se atribuye al aumento de los niveles de autofecundación en cotas elevadas y a la disminución de la competencia por los polinizadores en las poblaciones más altas, dado que la violeta es la única especie entomófila de la comunidad.

3. La presencia de conejos (*Oryctolagus cuniculus*) en el Parque Nacional del Teide reduce extremadamente el desarrollo, y éxito reproductivo de *V. cheiranthifolia*, consumiendo más del 75% de los individuos en algunas localidades. Este herbívoro invasor, además, modifica las relaciones rasgo-ambiente de la planta a lo largo de su gradiente altitudinal. Por lo tanto, y juntamente con evidencias de otras especies de este ecosistema, los conejos están alterando profundamente este exclusivo, frágil y aislado ecosistema insular, en el cual la violeta es la especie más abundante en la comunidad a partir de una cierta altitud.

4. Los rasgos florales de *V. cheiranthifolia* mostraron una gran variación a lo largo del gradiente altitudinal, con flores más grandes y espolones más largos y estrechos en la población de menor altitud, y flores más pequeñas con espolones cortos y anchos en la de mayor altitud. La exclusión de herbivoría mostró que los conejos están modificando la morfología de las flores, reduciendo también su despliegue floral y abundancia. Además, estos herbívoros alteran el patrón de selección que ejercen tanto los polinizadores como los factores abióticos sobre los rasgos florales, por lo que a su vez podrían estar indirectamente variando la comunidad de polinizadores y su grado de autogamia.

5. La tasa de visita de polinizadores en *V. cheiranthifolia* no difirió de forma consistente entre altitudes, pero se observó una mayor diversidad de visitantes florales a mayor altitud, lo cual concuerda con la presencia de espolones más anchos en esta localidad. Esto puede ser debido a que los individuos con espolones más anchos permiten que un rango más amplio de visitantes florales acceda al polen. Por otra parte, tanto el número de flores por individuo como el de flores conspecíficas vecinas fueron también factores

determinantes del éxito reproductivo de *V. cheiranthifolia*, al incrementar el cuajado de frutos y la viabilidad de las semillas. Todo parece indicar que la florivoría ejercida por el conejo, reduciendo de forma importante el despliegue floral de los individuos, es el causante de la limitación de polen observada en la especie.

6. La integración fenotípica en *V. maculata* no difirió entre las poblaciones altitudinales, pero los patrones de correlación entre el tamaño de la flor y la hoja cambiaron, con una correlación vegetativo-floral más fuerte en los sitios más bajos y una correlación débil en el sitio más alto. Este hallazgo no concuerda con la *hipótesis de Berg*, según la cual los polinizadores deben desacoplar la correlación entre los fenotipos florales y vegetativos, en plantas con sistemas de polinización especializados. Factores como el alto grado de autopolinización observado en la especie, y las diferencias observadas de estrés hídrico y competencia por la luz a lo largo del gradiente, podrían estar detrás del patrón de correlación encontrado.

7. Los resultados tanto de *V. maculata* como de *V. jaubertiana* apoyan la hipótesis del mantenimiento del sistema mixto de reproducción (con producción de flores CH y CL), como un mecanismo plástico adaptativo de garantía reproductiva frente a condiciones desfavorables para la polinización en las zonas más elevadas.

8. *Viola jaubertiana* produce flores casmógamas durante el invierno, justo cuando las visitas de polinizadores son muy escasas, por lo que se observa una gran limitación de polen, así como un escaso éxito reproductivo, ya que dichas flores tampoco disponen de autogamia retardada. Por el contrario, las flores CL, más baratas fisiológicamente, fueron mucho más exitosas, produciendo 100 veces más semillas que las flores CH. A pesar de este gran desequilibrio, el mayor peso, potencial de dispersión y mejor germinación de las semillas procedentes de fecundación cruzada, conjuntamente con el posible efecto de la heterosis, podrían explicar el mantenimiento de la casmogamia en este endemismo rupícola mallorquín.

9. En general, las tres especies del género *Viola* mostraron una importante plasticidad fenotípica y reproductiva a lo largo de sus gradientes ambientales, sugiriendo que tienen potencial para mantener poblaciones viables bajo las condiciones de cambio climático actual, con la presencia de mecanismos para hacer frente a la falta de polinizadores, como la autopolinización retardada o la producción de flores cleistógamas. A pesar de ello, la presencia de especies invasoras, la alta prevalencia de autogamia en las especies, y su distribución fragmentada y en pequeñas poblaciones, podrían poner en peligro dichas especies bajo importantes cambios climáticos de cara al futuro. El aumento de las temperaturas y la disminución del régimen de nevadas que se prevé para el Teide por efecto del cambio climático, probablemente afectará de manera especial la viabilidad de

las poblaciones a menor altitud, donde ya se observa una baja densidad de individuos, con tamaños muy pequeños, y poca floración.

Ideas para investigaciones futuras

Después de este largo y atractivo inicio a la investigación, siento ganas de aplicar todo lo aprendido en futuros estudios, y sobre todo de poder responder a todas las preguntas e inquietudes que me han ido surgiendo durante estos 4 años, fruto de los resultados obtenidos y de todas las horas de observación acumuladas en el campo. A continuación, destaco algunas ideas para futuras investigaciones:

1. Profundizar en las consecuencias ecológicas y evolutivas de la alta presión de herbivoría por conejo sobre *Viola cheiranthifolia,* a raíz de los cambios observados tanto sobre sus rasgos florales como en su despliegue floral. Además, sería muy interesante comprobar si los espolones más largos y delgados (bajo condiciones "sin" herbivoría) fuerzan a la planta hacia una mayor especialización en la polinización y a una menor autofecundación. Esta tendencia condicionaría a las flores de las plantas a ser visitadas casi exclusivamente por visitantes florales de trompa larga. El Parque Nacional, a petición nuestra, y como resultado de esta tesis, ya está instalando cercados de exclusión en las tres poblaciones altitudinales principales, lo cual permitirá futuros estudios sobre el efecto interactivo del conejo a lo largo del gradiente altitudinal.

2. Analizar la posible competencia entre especies coexistentes durante la época de floración a lo largo del gradiente altitudinal. Para ello habría que determinar la tasa de deposición de polen, tanto mono-específico como hetero-específico, en los estigmas de flores emasculadas en las 4 poblaciones altitudinales de la violeta del Teide.

3. Hacer un seguimiento prolongado (> 10 años) de la fecundidad de las flores casmógamas en *V. jaubertiana*, para comprobar si efectivamente existen esas *ventanas temporales* o años favorables, en los cuales el éxito reproductivo de las flores CH se ve incrementado notablemente.

4. Comprobar el fenómeno de la heterosis en *V. jaubertiana*, ya que se espera que los individuos procedentes de cruzamiento tengan más eficacia biológica, y mayor proporción de flores casmógamas. Mediante experimentos en invernadero, se podrían generar plantas procedentes de cruzamiento, y otras procedentes de autofecundación (de cleistogamia y autopolinización de flores CH), y comparar su desarrollo, vigor y producción de flores (CH y CL), y el fitness de su descendencia (Fig. 21).

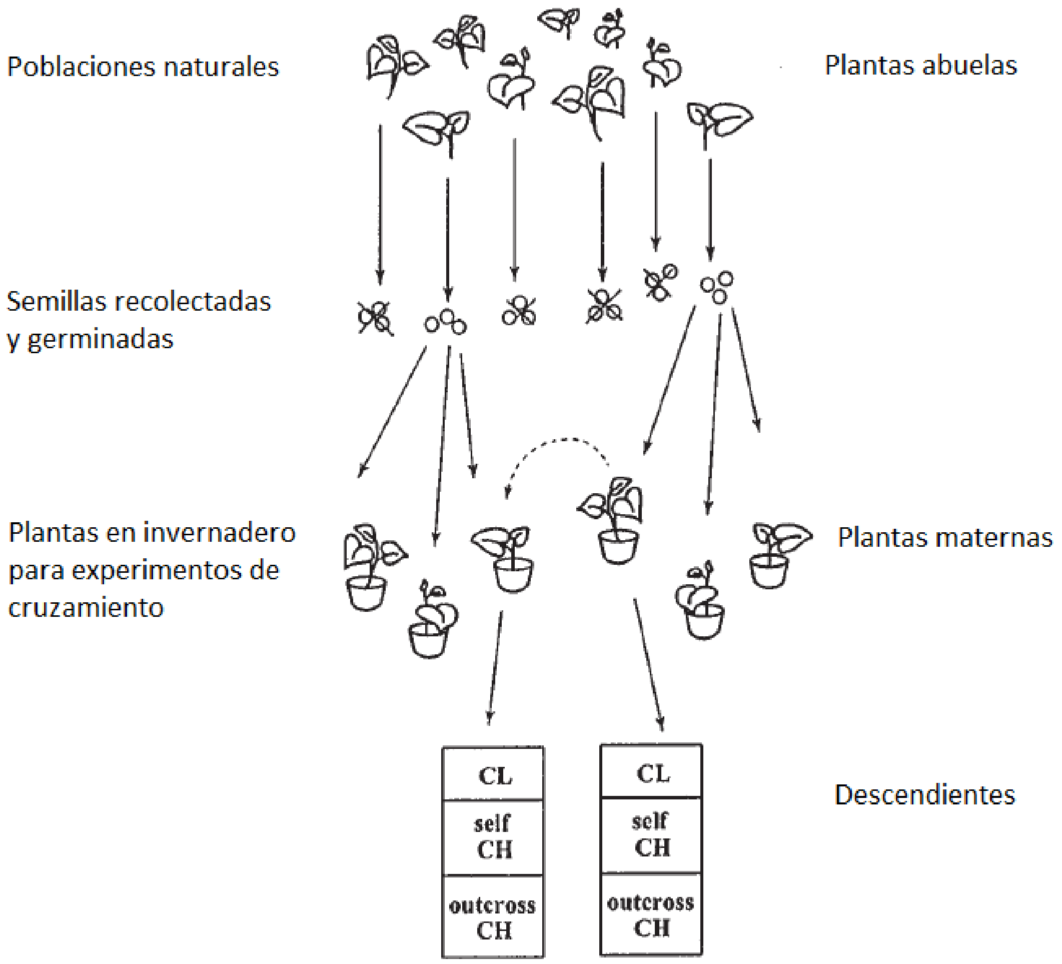

Figura 21. Diagrama de los diseños de cruzamiento para producir descendencia procedente de autofecundación y descendencia procedente de cruzamientos (Culley, 2000).

5. Al coincidir la dispersión de semillas provenientes de las flores CH y CL en el tiempo, sería interesante investigar las diferencias en el éxito de dispersión (por hormigas), reclutamiento y supervivencia en el medio natural entre ambos tipos de semillas.

6. Fruto de los 3 meses de campo en los Andes chilenos, han quedado datos sin analizar de otra especie de violeta alto-andina (*Viola cotyledon*). Se trata de una especie que vive entre los 1600 m y los 2200 m, y que presenta polimorfismo floral, con individuos con flores albinas, individuos con flores violetas, e individuos con flores rosadas. Lo interesante de la especie, bajo mi punto de vista, es que se encuentra distribuida a lo largo de un importante gradiente latitudinal en los Andes chilenos, de más de 500 km, abarcando la zona mediterránea de Chile, y la templada. A lo largo de este gradiente, la distribución relativa de los diferentes morfos de color en la especie varía, habiéndose observado que los morfos claros son más comunes en latitudes más

cálidas, y los oscuros en latitudes más frías. De momento hemos tomado datos de cómo varía la distribución relativa de los diferentes morfos a lo largo del gradiente latitudinal, del éxito reproductivo de los diferentes morfos (en cuanto a cuajado de frutos y producción de semillas), de su nivel de autofecundación, y de la tasa y diversidad de visitas de polinizadores. Sería interesante seguir tomando datos al respecto, para testar las consecuencias reproductivas del polimorfismo floral en la especie (Fig. 22).

Figura 22. Polimorfismo floral en *Viola cotyledon*; morfo violeta y morfo blanco.

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