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ÁREA DE BIODIVERSIDAD Y CONSERVACIÓN

Alberto López Teixido

Flores grandes en un ambiente mediterráneo: costes y beneficios del despliegue floral en Cistaceae

FLORES GRANDES EN UN AMBIENTE MEDITERRÁNEO: COSTES Y BENEFICIOS DEL DESPLIEGUE FLORAL EN CISTACEAE

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TESIS DOCTORAL

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CERTIFICA:

Que los trabajos de investigación desarrollados en la memoria de tesis doctoral, **“Flores grandes en un ambiente mediterráneo: costes y beneficios del despliegue floral en Cistaceae”** son aptos para ser presentados por el Ldo. Alberto López Teixido ante el Tribunal que en su día se consigne, para aspirar al Grado de Doctor en Conservación de Recursos Naturales por la Universidad Rey Juan Carlos de Madrid.

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“Podrán cortar las flores, pero no podrán cortar la primavera”

Pablo Neruda (1904 – 1973)

Foto de portada: individuo de *Cistus ladanifer* en la localidad de Vista Real (Becerril de la Sierra), mayo 2011. Autora: Teresa Gimeno Chocarro.

Foto de contraportada: individuo albino de *Cistus albidus* en la localidad de San Agustín de Guadalix, abril 2011. Autor: Alberto López Teixido.

A Luisiño,
por su plena confianza en mí,
por su dedicación y amistad,
por su vitalidad y valores humanos.

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pero, especialmente, por ser como son,
por su apoyo incondicional,
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Índice

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RESUMEN

ANTECEDENTES

Variabilidad floral: balance entre costes y beneficios

Las plantas zoófilas (i.e. polinizadas por animales) abarcan una enorme diversidad de flores, que varían no sólo en la gama de colores, la forma y el diseño, si no también en el tamaño y el despliegue, la longevidad y el aroma floral. Históricamente, numerosos botánicos y biólogos evolutivos se han interesado en el estudio de los procesos que conducen a tal diversificación (Sprengel 1793; Darwin 1862, 1877; Stebbins 1950, 1970). En general, los científicos de ambas disciplinas acuerdan en conferir a los polinizadores un papel relevante en la evolución de la variabilidad floral observada en la naturaleza. Esta afirmación se apoya en el hecho de que las plantas zoófilas dependen de los polinizadores para reproducirse y son las flores las estructuras diseñadas para tal fin. Las flores juegan un papel crucial en la atracción a los polinizadores ofreciéndoles recompensas energéticas y nutritivas, mientras que éstos favorecen la reproducción transfiriendo el polen entre plantas dentro de las poblaciones. En conjunto, la diversidad taxonómica de polinizadores (insectos, reptiles, aves, mamíferos), que implica una gran diversidad morfológica, funcional y de comportamiento, se ajusta a la diversidad de las variantes florales (revisado en Fenster *et al*. 2004; ver también Aigner 2005; Smith *et al*. 2008). Por tanto, los polinizadores tienen un gran potencial de ejercer selección sobre los rasgos de las flores de aquellas plantas que visitan.

El despliegue floral, determinado por el número y el tamaño de las flores, es un rasgo clave en la ecología reproductiva de las plantas polinizadas por animales. Este carácter está estrechamente ligado con la atracción de polinizadores, ya que mayores despliegues son detectados más fácilmente y ofrecen menores costes en los recorridos interflorales (Bell 1985; Harder y Barrett 1996; Ohashi y Yahara 2001).

Además, las flores más grandes poseen un mayor contenido de recursos para los polinizadores, tales como polen y néctar (J Herrera 1992; Jones 2001; Arista y Ortiz 2007). El despliegue floral ha sido por tanto relacionado con incrementos en la diversidad (Andersson 1988; Aigner 2005), la frecuencia (Young y Stanton 1990; Medel *et al*. 2007), el número (Bell 1985; Johnson *et al*. 1995; Liao *et al*. 2009) y la duración de las visitas (Conner y Rush 1996; Thompson 2001; Nattero *et al*. 2011) en numerosas plantas polinizadas por insectos. Las plantas con mayor tamaño floral y en mayor número son visitadas como primera opción (Young y Stanton 1990; Brody y Mitchell 1997). Por tanto, el despliegue floral favorece la dispersión y deposición de polen incrementando así el éxito tanto masculino como femenino (Stanton *et al*. 1986; Kudoh y Whigham 1998; Harder y Johnson 2005). Como una consecuencia lógica de este hecho, muchos estudios han documentado selección fenotípica hacia mayor número o tamaño de las flores mediada por polinizador (Galen 1989; CM Herrera 1993; Conner y Rush 1997; Hodgins y Barrett 2008; Nattero *et al*. 2010a).

En última instancia, para que pueda existir evolución, los procesos de diversificación en el despliegue floral deben estar acoplados a una variación genética a nivel intraindividual. En efecto, se sabe que entre poblaciones e incluso entre individuos dentro de poblaciones, existe una gran variabilidad en el tamaño floral con una heredabilidad significativa (Anderson y Widén 1993; Galen 1996; Ashman y Majestic 2006). Pero si los polinizadores ejercen presión selectiva contra flores pequeñas y el tamaño floral es heredable, entonces las flores deberían mostrar poca variación en este rasgo (ver Ushimaru *et al*. 2006). ¿Por qué entonces se mantiene la variabilidad en el tamaño y las flores pequeñas aún persisten en las poblaciones? En un artículo seminal, Galen (1999) detalló que una visión unilateral de la evolución del tamaño floral tan sólo desde la perspectiva de la polinización es excesivamente reduccionista. La selección sobre el tamaño de las flores es más un proceso pluralístico en el que intervienen no sólo amigos (polinizadores), sino también enemigos (parásitos, predadores) así como numerosos aspectos del ambiente abiótico de la planta. En este sentido, Galen (1999) postuló las hipótesis de los "costes de recursos" y del "escape de los enemigos" para explicar la variabilidad en este rasgo y avanzar en el entendimiento de por qué flores menos vistosas pueden verse favorecidas y compensar su reducida atractividad a los polinizadores mediante una reducción de los costes y un mayor escape de los enemigos.

Las flores requieren una considerable inversión de recursos para su producción y mantenimiento, lo que se engloba dentro de los costes directos (Chapin 1989; Ashman y Schoen 1997). Agua, carbono y otros nutrientes son necesarios para aumentar la atracción y la recompensa de los polinizadores, con pétalos llamativos, pigmentos, compuestos aromáticos y néctar (Cruden y Lyon 1985; Ashman y Baker 1992; Méndez y Traveset 2003; De la Barrera y Nobel 2004a), y también para mantenerse funcionalmente activas mientras permanecen abiertas (Galen *et al*. 1993; Galen 2000, 2005; Lambers *et al*. 2008). Despliegues florales más grandes requieren por tanto mayor inversión en biomasa y agua para su construcción (Galen 1999; Caruso 2006; Halpern *et al*. 2010) y mayores costes de mantenimiento debido a las altas tasas de respiración y transpiración (Vemmos y Goldwin 1994; Galen *et al*. 1999; Galen 2000). En consecuencia, factores abióticos tales como el estrés hídrico pueden imponer límites al tamaño y número de las flores (Galen 1999), dos rasgos que, de hecho, suelen mantener una correlación negativa (Caruso 2004; Hansen y Totland 2006; Sargent *et al*. 2007).

Un principio fundamental en la economía de los recursos de las plantas es que los costes directos se traducen en costes indirectos (Chapin 1989), es decir, en efectos negativos sobre otras funciones, en este caso florales tales como el rendimiento reproductivo. Andersson (1999, 2000, 2001, 2005) cuantificó los costes indirectos de la atractividad floral en diversas especies. En general, encontró un incremento en la producción de frutos y semillas de flores cuyos periantos fueron eliminados frente a flores control no manipuladas. Sin embargo, los costes indirectos del número y tamaño de las flores desplegadas no han sido tenidos en cuenta hasta la fecha en ningún estudio, a pesar de que la evidencia en este sentido suministraría un fuerte apoyo a las predicciones de los argumentos de Galen (1999) basados en la inversión de recursos a las flores (ver además Caruso 2006 para el número de flores abiertas).

Las plantas con mayores despliegues florales tienen además que lidiar con costes adicionales impuestos por animales antagonistas, como algunos parásitos, predadores de semillas, herbívoros y ladrones que obtienen recompensas de sus visitas florales sin ofrecer beneficios a la polinización (Shykoff *et al*. 1996; Galen 1999; Irwin *et al*. 2004; Strauss y Whittall 2006). Por ejemplo, los herbívoros florales (i.e. florívoros) causan daños a flores abiertas y cualquiera de sus estructuras, ya sean pétalos, sépalos u órganos sexuales (McCall e Irwin 2006). El nivel de florivoría aumenta a medida que se incrementan los rasgos que atraen a los polinizadores, como el número de flores desplegadas o el tamaño floral (Galen 1999; Mosleh Arany *et al*. 2009). El consiguiente consumo y degradación de las flores puede reducir el éxito reproductivo de la planta por una menor producción de frutos y semillas mediante una disminución del número de flores disponibles y/o permanencia de las más pequeñas, la alteración de las propiedades de atracción a polinizadores o el consumo directo de gametos viables (Schemske y Horvitz 1988; Krupnick *et al*. 1999; McCall e Irwin 2006; Cardel y Koptur 2010).

En conclusión, el conjunto de costes que implica el despliegue floral tiene el potencial de ser un importante factor selectivo sobre este rasgo. En particular, los costes directos asociados al uso del agua y el carbono por despliegues mayores reducen tanto el número como el tamaño de las flores (Galen 2000; Elle y Hare 2002; Caruso 2006; Lambers *et al*. 2008; Halpern *et al*. 2010). Esto es probable que explique la relación entre el patrón de inversión de dichos recursos a la atractividad floral y a la descendencia (Ashman y Schoen 1996; Galen 1999, 2005), es decir, de la magnitud de los costes indirectos en términos de producción de frutos y semillas (Andersson 2005). Por otro lado, los florívoros también ejercen presiones selectivas sobre el despliegue (Galen 1999; Irwin *et al*. 2001; Irwin 2006). Por tanto, un importante aspecto en el mantenimiento de la variación en el despliegue floral es que los beneficios obtenidos por la atracción a los polinizadores pueden ser contrarrestados por un incremento de los costes de dicha atracción.

El ambiente mediterráneo: un ecosistema cálido y seco

El ecosistema mediterráneo se caracteriza por un clima con una fuerte estacionalidad y dos aspectos clave, limitantes para la vida de las plantas: (1) la asociación de un periodo de sequía con la estación en la que las temperaturas son más cálidas, y (2) la presencia de un periodo frío, más o menos intenso según las regiones, durante el invierno. La estación húmeda, generalmente con temperaturas suaves, asegura el desarrollo de las plantas, pero puede coincidir con el estrés del frío invernal (Thompson 2005). Dicho estrés puede limitar el crecimiento y la distribución de las especies, como se ha visto para algunas esclerófilas (Mitrakos

1982; Valladares *et al*. 2008) y representa por tanto una limitación adicional al estrés estival sobre la fenología y crecimiento de las plantas mediterráneas.

El calor y la sequía estival son los principales factores limitantes para la supervivencia de las plantas en el ambiente mediterráneo, por lo que las estrategias fenológicas y morfo-fisiológicas para la conservación del agua y la maximización de la eficiencia de su uso juegan un papel clave en este ecosistema (Larcher 2000; Valladares *et al*. 2000; Thompson 2005). Estas estrategias contra el estrés hídrico son diversas y heterogéneas y han sido tradicionalmente divididas en dos mecanismos: tolerancia y evitación de la sequía (Levitt 1980; Jones 1992; Larcher 2003). Especies que emplean mecanismos de tolerancia a la sequía soportan bien una conductancia estomática baja y un potencial hídrico también bajo, mientras que las especies que la evitan previenen daños mediante el cierre estomático completo (sobre todo a mediodía y en los periodos de mas aridez), una historia de vida anual o una dormancia de verano, en el caso de las perennes, y un rápido desarrollo, acortando la duración de la floración y/o aplazándola hasta después de la estación húmeda (Larcher 2000, 2003; Verdú *et al*. 2002; Thompson 2005; Aragón *et al*. 2008; ver además Valladares *et al*. 2004 y sus referencias).

Como se ha destacado con anterioridad la floración implica una gran inversión de recursos. En conjunto, la atractividad y la recompensa para promover la polinización son caros en términos de la economía de recursos de una planta. En un ambiente cálido y seco como el mediterráneo, los ya de por sí elevados costes del despliegue floral pueden verse acrecentados. Además del inherente gasto de agua para la producción floral, la exposición de las flores a altas temperaturas puede incrementar de manera significativa los costes de mantenimiento en términos de agua (transpiración) y carbono (respiración) (Galen 1999, 2005; Patiño y Grace

2002; Elle y Hare 2005). La escasez de agua puede impedir termorregulación que se obtiene en muchos tejidos biológicos mediante transpiración, por lo que, conjuntamente, el calor y la sequía pueden dañar las flores y los procesos que contribuyen a la reproducción sexual, damnificando así a la producción de frutos y semillas (Konsens *et al*. 1991; Lacey 1996; Galen 2000; Erickson y Markhart 2002; Young *et al*. 2005; Fang *et al*. 2010).

Como consecuencia, las condiciones estresantes de un ambiente cálido y seco podrían imponer límites selectivos al tamaño floral con el fin de minimizar la pérdida de agua, otorgando potencialmente ciertas ventajas a las flores pequeñas, al menos en el uso de los recursos hídricos (Galen 1999, 2000, 2005; Elle y Hare 2002). Dos estudios en el ecosistema mediterráneo mostraron que las flores fueron más grandes en las poblaciones más frescas y húmedas de *Narcissus triandrus* (Barrett *et al*. 2004) y *Rosmarinus officinalis* (J Herrera 2005). Por tanto, el cambio total en el fenotipo acompañó a un gradiente ecológico desde condiciones más cálidas y secas a otras menos estresantes. La inusual aparición de plantas con flores grandes en ambientes secos suele llevar asociadas adaptaciones para prevenir el sobrecalentamiento y una excesiva pérdida de agua. La floración nocturna es propia de algunas alcaparras (*Capparis* sp.) en zonas semiáridas (Rhizopoulou *et al*. 2006) y cactus en los desiertos (Valiente-Banuet *et al*. 1997; Fleming *et al*. 2001). Asimismo la longevidad floral, que ajusta el balance entre las tasas de dispersión y deposición de polen con los costes de mantenimiento floral, suele ser efímera (Galen 2005). Por tanto, la presencia de flores grandes y costosas contrasta con las expectativas a partir de los mecanismos de respuesta al déficit de agua y con la hipótesis de “costes de recursos” que favorecería flores pequeñas en estos ambientes (Galen 1999).

Cistaceae: una familia mediterránea con flores grandes

Cistaceae es una familia que contiene 8 géneros (*Cistus, Crocanthemum, Fumana, Halimium, Helianthemum, Hudsonia, Lechea* y *Tuberaria*) y alrededor de 200 especies de arbustos y hierbas de las zonas templadas del Hemisferio Norte y Sudamérica (Arrington y Kubitzki 2003). Su centro de diversificación es el área mediterránea, especialmente la península Ibérica (hasta 64 especies), donde aparecen ampliamente distribuidos los géneros *Cistus, Fumana, Halimium, Helianthemum* y *Tuberaria* (Muñoz-Garmendía y Navarro 1993). Los otros tres géneros sólo aparecen en el continente americano, desde el medio este de los Estados Unidos y sur de Canadá, hasta Centroamérica, especialmente México, y las zonas templadas de Sudamérica (Arrington y Kubitzki 2003). El hábitat en el que usualmente aparecen las especies de esta familia comprende áreas abiertas con suelos secos, ya sean calizos o ácidos, generalmente rocosos, formando los matorrales y estratos herbáceos de formaciones esclerófilas mixtas dominadas por *Pinus* y *Quercus* (Muñoz-Garmendía y Navarro 1993).

En el área circunmediterránea la floración ocurre casi exclusivamente en primavera, como muy pronto en febrero, y se puede extender hasta el mes de julio (Muñoz-Garmendía y Navarro 1993). Existen algunas especies anuales, pero la gran mayoría son perennes y policárpicas, floreciendo cada primavera. Las flores son generalmente casmógamas, con pétalos amarillos, blancos o rosado-purpúreos y abren cada mañana de forma sincrónica dentro de las poblaciones, tirando habitualmente sus pétalos a mediodía (J Herrera 1992). El despliegue floral es variable pero los géneros arbustivos *Cistus* y *Halimium* suelen presentar flores grandes y en elevado número. Es interesante destacar que *Cistus ladanifer*, la especie con mayores flores dentro de la familia, es también una de las especies con mayor tamaño floral de todo el ecosistema mediterráneo, con diámetros que pueden superar los 10 cm (Arrington y Kubitzki 2003). Las flores de esta familia son predominantemente auto-incompatibles y generalistas, dependiendo de un alto espectro de polinizadores para la reproducción (Bosch 1992; J Herrera 1992; Talavera *et al*. 1993, 2001). Aunque existe una correlación entre el tamaño de las flores y las tasas de visitas de polinizadores, esto no se relaciona con una tasa diferencial de producción de frutos y semillas en flores más grandes (Talavera *et al*. 2001; Arista y Ortiz 2007). El resto de géneros presenta flores de menor tamaño e incluso algunas especies cleistógamas que pueden mostrar auto-polinización (J Herrera 1992; Aragón y Escudero 2008).

En conjunto, las especies con mayor despliegue floral de la familia de las cistáceas conforman un sistema de estudio ideal para el estudio de las presiones selectivas operando sobre flores grandes en un ambiente mediterráneo. Las especies de la familia Cistaceae son, por tanto, especialmente útiles para llevar a cabo un estudio exhaustivo de los costes y beneficios que las flores grandes acarrean en un ambiente estresante como el mediterráneo, con condiciones cálidas y secas, potencialmente costosas para grandes despliegues en términos de recursos (agua y carbono) y en términos de presiones ecológicas (p.ej. florivoría).

OBJETIVOS

El objetivo general de esta tesis se centra en evaluar el balance entre costes y beneficios del despliegue floral y sus consecuencias evolutivas en un sistema representado por flores grandes en un ambiente estresante. Para ello, se han seleccionado algunas de las especies con mayor despliegue floral de la familia Cistaceae en un ecosistema mediterráneo, en condiciones cálidas y secas. A su vez, se han empleado combinaciones de diseños observacionales y experimentales, en función de las hipótesis planteadas en cada uno de los capítulos. En particular, se ha planteado la siguiente serie de objetivos específicos, desarrollados a lo largo de los cinco capítulos de esta tesis para alcanzar este objetivo general:

(1) Evaluar los beneficios del despliegue floral en términos de visitas de polinizadores. A través de un estudio espacio-temporal en *Cistus ladanifer*, una especie de gran tamaño floral con alta variabilidad en este rasgo, analizamos si la abundancia y composición de polinizadores sobre cada planta se relaciona con el despliegue floral, si un mayor tamaño o despliegue floral aumenta la producción de frutos y semillas y, en última instancia, si los polinizadores actúan como agentes selectivos sobre el despliegue floral a través de la función femenina (Capítulo 1).

(2) Cuantificar los costes de mantenimiento de las corolas en términos de agua y carbono en función de la temperatura y del tamaño floral. Para este objetivo diseñamos un estudio combinado bajo distintas temperaturas ambiente en *Cistus albidus* y *C. ladanifer* para los costes del agua, dos especies cofloreciendo en simpatría y con tamaño floral contrastado, con un estudio monoespecífico en *C. ladanifer* para los costes de carbono. Asimismo cuantificamos los costes de agua y carbono de la floración respecto a los de las hojas para así relativizar los costes de las flores en el contexto de la planta (Capítulo 2).

(3) Determinar los costes indirectos del despliegue floral en términos de producción de frutos y semillas. Específicamente cuantificamos los costes directos de producción de las corolas en términos de masa seca, nitrógeno y fósforo y, a continuación, los costes indirectos entre flores cuyos pétalos fueron eliminados y flores control no manipuladas bajo condiciones controladas de polinización, entre plantas dentro de poblaciones y entre especies simpátricas difiriendo en tamaño floral. Para ello, realizamos un estudio en dos pares de especies simpátricas: *Cistus albidus* vs *C. ladanifer* y *C. laurifolius* vs *C. ladanifer* (Capítulo 3).

(4) Analizar los efectos del tamaño de las flores y la temperatura ambiente sobre la longevidad floral. Es importante tener en cuenta que, al igual que el despliegue, la longevidad floral resulta beneficiosa para una planta ya que incrementa las tasas de dispersión y deposición de polen, pero puede incrementar los costes de mantenimiento de las flores, especialmente al aumentar su tamaño y bajo temperaturas elevadas. En concreto, medimos experimentalmente la longevidad floral en respuesta a diferencias en la temperatura ambiente, el tamaño floral y la deposición de polen en *Cistus ladanifer* (Capítulo 4).

(5) Evaluar los efectos de la atractividad floral (incluyendo despliegue y longevidad de las flores) sobre la incidencia de florívoros. En este caso llevamos a cabo un estudio observacional en cuatro poblaciones de *Cistus ladanifer* con variación en el despliegue y la longevidad floral para saber específicamente si la florivoría incrementa con mayor tamaño floral, número

de flores abiertas y longevidad de las mismas y, además, si varía entre poblaciones difiriendo en estos rasgos (Capítulo 5).

METODOLOGÍA

Área de estudio

Todas las especies y poblaciones de estudio incluidas en esta tesis están localizadas en la Comunidad de Madrid (39'53º−41'09ºN, 3'03º−4'34ºW) y fueron muestreadas durante las primaveras y principio de los veranos de 2009, 2010 y 2011. La Comunidad de Madrid se caracteriza por un clima mediterráneo con un gradiente de humedad SE-NW debido a la altitud (rango: 430−2.428 msnm). En concreto, la Comunidad presenta regiones con clima semi-árido y otras con clima hiper-húmedo, en los cuales las precipitaciones medias anuales oscilan aproximadamente entre los 425 y los 1.500 mm anuales, y las temperaturas medias anuales entre los 16 y los 7ºC, respectivamente.

Un total de 10 poblaciones fueron muestreadas a lo largo de los tres años de estudio de campo de esta tesis e incluyeron distintos gradientes de temperaturas y precipitaciones medias anuales, diferentes características geológicas y edáficas, y variación en la cubierta vegetal predominante (Tabla 1; Fig. 1). Todas las poblaciones fueron similares en orientación (sur), pendiente (0−10º) y cubierta del estrato arbóreo (0−10%). Las orientaciones al sur fueron elegidas para eliminar, en la medida de lo posible, los diferentes factores ambientales entre laderas de umbría y solana que pudieran influir en nuestro estudio. En algunos capítulos (Capítulos 1, 4 y 5), se muestrearon distintas poblaciones a lo largo de un gradiente altitudinal de temperatura y humedad, que potencialmente pudieran influir en los rasgos florales

Tabla 1. Localización y datos ecológicos de todos los sitios de estudio incluidos en esta tesis. La columna de clima muestra además la precipitación (mm) y la temperatura (ºC) media anual (Ninyerola *et al*. 2005, N = 20 años).

Capítulo	Sitio de estudio	Altitud (m)	Clima	Sustrato	Vegetación predominante
1, 4, 5	Monte del Pardo (40'34ºN 3'42ºW)	732	Seco 533 mm,14ºC	Arcilla y arena	Dehesa con *Quercus ilex* y *Pinus pinea*
1, 3, 5	Puerto de Canencia (40'50ºN 3'46ºW)	1.300	Subhúmedo 865 mm, 9ºC	Granito	Pendiente con *Quercus pyrenaica* y *Pinus sylvestris*
2, 3	S. Agustín de Gudalix (40'41ºN 3'36ºW)	740	Seco 567 mm,13ºC	Yeso y caliza	Matorral con *Quercus coccifera* y *Juniperus oxycedrus*
2, 5	Vista Real (40'44ºN 3'57ºW)	1.120	Subhúmedo 820 mm,11ºC	Granito	Roquedo con matorral y *Juniperus oxycedrus*
4	Monte del Pardo (40'33ºN 3'42ºW)	741	Seco 529 mm,14ºC	Arcilla y arena	Dehesa con *Quercus ilex* y *Pinus pinea*
4	Monte Valdelatas (40'32ºN 3'41ºW)	725	Seco 520 mm,14ºC	Arcilla y arena	Dehesa con *Quercus ilex* y *Pinus pinea*
4	Becerril de la Sierra (40'43ºN 3'58ºW)	1.163	Subhúmedo 844 mm,11ºC	Granito	Matorral con *Cistus laurifolius* y *Juniperus oxycedrus*
4	Navacerrada (40'43ºN 4'00ºW)	1.176	Subhúmedo 878 mm,11ºC	Granito	Matorral con *Cistus laurifolius* y *Juniperus oxycedrus*
4	Navacerrada (40'43ºN 4'00ºW)	1.199	Subhúmedo 878 mm,11ºC	Granito	Matorral con *Cistus laurifolius* y *Juniperus oxycedrus*
5	La Pedriza (40'44ºN 3'52ºW)	940	Subhúmedo 771 mm,12ºC	Arcilla y arena	Roquedo con matorral y *Quercus ilex*

analizados (tamaño, número y/o longevidad de las flores) y las interacciones planta-animal (polinizadores o florívoros).

Especies empleadas como modelo de estudio

En esta tesis se han utilizado un total de tres especies de *Cistus* Cistaceae con flores grandes y variabilidad interespecífica en el tamaño floral para evaluar el balance entre costes y beneficios del despliegue floral y sus consecuencias evolutivas en un ambiente mediterráneo: *Cistus albidus, C. ladanifer, C. laurifolius* (Fig. 2). A continuación se exponen las principales características ecológicas, fenológicas, morfológicas y reproductivas del género *Cistus* en general y de las tres

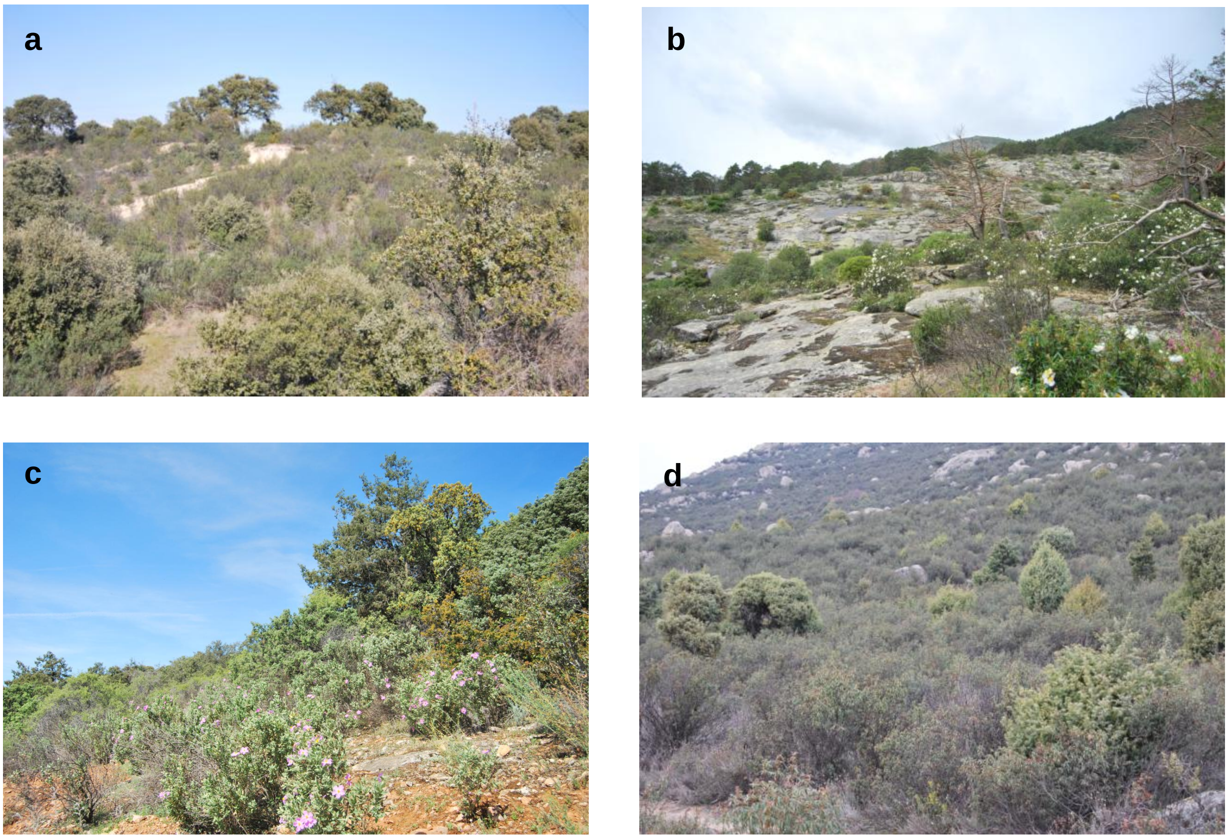


Fig. 1. Cuatro de las poblaciones utilizadas durante el estudio de esta tesis. Matorral dominado por *Cistus ladanifer* en una ladera adehesada con *Quercus ilex* en Monte del Pardo (a); individuos dispersos de *C. ladanifer* y *C. laurifolius* en simpatría en un roquedo granítico con *Pinus sylvestris* en Puerto de Canencia (b); matorral heterogéneo dominado por *Cistus albidus, Juniperus oxycedrus* y *Quercus coccifera* en los suelos calcícolas de San Agustín de Guadalix (c); roquedo granítico con *C. ladanifer* entre individuos dispersos de *Juniperus oxycedrus* en Vista Real (d). Fotos: Alberto López Teixido.

especies en particular, así como las principales similitudes y diferencias entre todas ellas.

Cistus es uno de los géneros más representativos de la flora mediterránea como parte del estrato arbustivo perennifolio de los bosques esclerófilos. Las flores tienen cinco pétalos blancos o rosado-purpúreos, con un tamaño de entre 2 y 12 cm de diámetro, y son hermafroditas, auto-incompatibles y secretan algo de néctar

Fig. 2. Las tres especies utilizadas durante el estudio de esta tesis. *Cistus albidus* en San Agustín de Guadalix (a); *C. ladanifer* con máculas en la base de los pétalos en Puerto de Canencia (b); *C. laurifolius* en Puerto de Canencia (c); Fotos: Alberto López Teixido (a, c) y Alejandro Aparicio-Valenciano (b).

(Bosch 1992; J Herrera 1992; Muñoz-Garmendía 1993). La floración ocurre en primavera, entre febrero y junio, y las flores abren de manera sincrónica cada día dentro de las poblaciones. Los frutos son cápsulas leñosas y globulares de entre 4−15 mm de longitud, generalmente con 5 valvas (aunque el número es altamente variable en *C. ladanifer*; Narbona *et al*. 2010), y contienen numerosas semillas (rango aproximado: 100−1.000) de pequeño tamaño, entre 1−2 mm de largo (J Herrera 1992; Muñoz-Garmendía y Navarro 1993).

Cistus albidus L., es un arbusto de entre 40−100 cm de altura que habita suelos secos y calcáreos en áreas occidentales del Mediterráneo. Florece entre febrero y abril y cada planta produce numerosas flores rosado-purpúreas de entre 4−6 cm de diámetro en inflorescencias terminales y son polinizadas predominantemente por abejas y escarabajos (Bosch 1992; Muñoz-Garmendía y

Navarro 1993). Las flores contienen además, de media, más de 150 anteras y unos 70 óvulos (J Herrera 1992). Los frutos son cápsulas de entre 7−13 mm y contienen numerosas semillas de 1−1'5 mm de longitud (Muñoz-Garmendía y Navarro 1993).

Cistus ladanifer L. es un arbusto de unos 100−250 cm de alto que habita suelos secos y ácidos en áreas abiertas en el Mediterráneo occidental. El período de floración se expande de marzo a junio y las plantas producen muchas flores blancas, solitarias, de entre 7−11 cm de diámetro, a menudo exhibiendo una mácula rosado-purpúrea en la base de los pétalos. Las flores son las más grandes de la familia y tienen, de media, más de 150 anteras y 1.000 óvulos (J Herrera 1992), y son principalmente polinizadas por abejas, moscas y escarabajos (Talavera *et al*. 1993). Los frutos contienen un variable número de valvas (5−12) y semillas (rango aproximado: 300−1.200) de hasta 1 mm de diámetro (Talavera *et al*. 1993; Delgado *et al*. 2008; Narbona *et al*. 2010).

Cistus laurifolius L. es un arbusto de entre 100 y 200 cm de alto con cepa leñosa que ocupa suelos pedregosos, frescos y ácidos de las montañas alrededor del Mediterráneo, especialmente en el este, Mar Egeo y Anatolia, y el oeste, Italia, Francia, península Ibérica y norte de Marruecos (Muñoz-Garmendía y Navarro 1993). Florece entre mayo y junio, con inflorescencias cimosas de hasta 9 flores blancas, de unos 5−6 cm de diámetro. A nuestro conocimiento, no hay estudios que hayan reportado características de las estructuras reproductivas de la flor ni sus polinizadores habituales. Los frutos miden entre 9−12 mm de longitud y poseen numerosas semillas de aproximadamente 1 mm de diámetro (Muñoz-Garmendía y Navarro 1993).

Muestreos y trabajo de campo: diseños observacional y experimental

Para cumplir el objetivo general de esta tesis se ha llevado a cabo una combinación de diseños observacionales con experimentales en el campo, complementados con trabajo de laboratorio, y desarrollados en función de las preguntas específicas y las hipótesis planteadas en cada uno de los capítulos. En algunos aspectos se han utilizado diseños de muestreo y técnicas experimentales clásicos en la ecología evolutiva floral y la biología reproductiva de plantas entomófilas para responder a objetivos concretos. Sin embargo, en otros casos los diseños planteados y las técnicas utilizadas han sido novedosos con el fin de consumar aquellos objetivos nunca antes planteados en dichos campos. Durante la primavera de tres años consecutivos, comprendidos entre 2009 y 2011, se llevaron a cabo los muestreos de campo y los trabajos de manipulación floral, acompañados del trabajo de laboratorio, que consistió en el conteo de frutos y semillas y el análisis de los costes de asignación floral, durante los correspondientes otoños e inviernos.

1. Medidas del despliegue floral y su relación con las visitas de polinizadores y éxito reproductivo, costes fisiológicos y niveles de florivoría

En todas las poblaciones de estudio se escogieron y marcaron individuos adultos al azar en una superficie aproximada de 20×20 m^2. Durante el pico de floración, diariamente se marcaron números determinados de flores por individuo durante varios días consecutivos. Todas las flores marcadas abrieron durante la mañana del día correspondiente. Dependiendo del objetivo específico, se marcaron más o menos flores diarias por individuo y se registraron distintas medidas del despliegue floral. En todos los casos, se midió el tamaño floral de las flores marcadas, estimado como el diámetro en cm. Adicionalmente, el tamaño fue estimado como el área en

cm^2 para calcular las tasas de transpiración y respiración por unidad de superficie (Capítulo 2). En otros casos, se registraron además el número de flores abiertas cada día en cada planta y/o la longevidad, en días, de cada una de las flores marcadas.

Durante el primer año, 2009, y en cuatro poblaciones de *Cistus ladanifer* a lo largo de un gradiente altitudinal, los tres rasgos florales de atracción (i.e. tamaño, número y longevidad de las flores) fueron relacionados con la incidencia de florívoros a nivel individual dentro de poblaciones y, de media, a nivel interpoblacional. Dicha incidencia fue registrada como presencia/ausencia de insectos que causaran cualquier daño mecánico a flores abiertas, ya fuera daño a las brácteas, sépalos, pétalos, androceo y/o gineceo (McCall e Irwin 2006).

A lo largo de dos años consecutivos, 2010−2011, y en dos de las poblaciones de *C. ladanifer* utilizadas durante el primer año, situadas a distinta altitud y difiriendo en tamaño floral, éste y el número de flores abiertas por individuo fueron relacionados con la variación espacio-temporal en la tasa de visitas de polinizadores. Estas tasas se registraron mediante censos temporales a plantas individuales en días soleados, de modo que en todas las plantas fueron observadas la misma longitud de tiempo y la misma cantidad de flores bajo condiciones climáticas similares (Fenster *et al*. 2004). Adicionalmente, se recogieron los frutos y se cuantificaron tanto el set de frutos como el número medio de semillas por fruto para relacionar la tasa de visitas de polinizadores con el éxito reproductivo femenino de cada planta y, en última instancia, se estimaron los coeficientes de selección fenotípica sobre el tamaño y el número de flores a través del éxito femenino (Lande y Arnold 1983; Stinchcombe *et al*. 2008).

Por último, en la primavera de 2011, una población con *Cistus albidus* y *C. ladanifer* cofloreciendo en simpatría fue seleccionada para llevar a cabo el estudio de los costes fisiológicos del tamaño floral en términos de agua. Durante varios días soleados consecutivos se analizaron las diferencias de transpiración de las corolas en función del tamaño floral (tanto a nivel inter e intraespecífico, como a nivel individual, entre pares de flores de distinto tamaño sobre el mismo individuo) y en función de la temperatura del aire. Específicamente, se realizó un análisis extra para evaluar las diferencias en las tasas de transpiración de las corolas entre días soleados y nublados en *C. ladanifer*. Las tasas de transpiración fueron calculadas a partir de la conductancia estomática al vapor de agua (g_s) de las corolas, registrada a su vez mediante un porómetro portátil (SC-1 hand held porometer, Decagon, Pullman, WA, USA). Para el análisis de los costes fisiológicos del tamaño floral en términos de carbono, se seleccionó otra población de *C. ladanifer* y se evaluaron las diferencias entre las tasas de respiración de las corolas y el tamaño floral inter e intraindividual (de manera similar a cómo se realizó con las tasas de transpiración) y la temperatura del aire. Las tasas de respiración floral (R_f) fueron medidas usando un analizador infrarrojo LI−6400 (LI-COR, Lincoln, NE, USA).

2. Trabajo experimental: manipulación del tamaño y de la longevidad floral

Para cumplir los objetivos y testar las hipótesis de los Capítulos 3 y 4 llevamos a cabo diseños experimentales con distintos tratamientos. En el Capítulo 3, donde evaluamos los costes indirectos del despliegue floral y testamos si dichos costes varían entre diferentes tamaños del despliegue, manipulamos el tamaño de las flores por eliminación total de la corola y comparamos la producción de frutos y semillas con flores control no manipuladas (Anderson 1999, 2000, 2001, 2005). Cada día en cada una de las poblaciones de estudio y antes del amanecer, cuando la antesis aún no fue completa, marcamos 5 flores por individuo y eliminamos los pétalos con unas pinzas. Entonces tanto estas flores como otras 5 flores control fueron polinizadas manualmente con polen xenógamo con el uso de un pincel. Registramos a continuación el tamaño y el número de flores medio de cada individuo y cuantificamos la producción de frutos y semillas entre tratamientos, individuos y especies.

En el Capítulo 4, donde evaluamos la relación del tamaño con la longevidad floral, manipulamos el tiempo que las flores estuvieron abiertas y funcionales. Ya que algunos estudios han reportado que la longevidad floral depende de la deposición de polen en el estigma (Proctor y Harder 1995; Sargent y Roitberg 2000; Giblin 2005; Castro *et al*. 2008), llevamos a cabo tres tratamientos en cada planta: flores bajo polinización manual, envoltura de los estigmas para evitar deposición de polen y flores control expuestas a polinización natural. La polinización manual fue llevada a cabo con polen xenógamo recolectado de varios individuos alejados de la planta focal con la ayuda de un pincel. La envoltura de los estigmas se realizó antes de que la antesis fuera completa mediante cápsulas de plástico hechas manualmente a partir de pajitas de sorber y celo adaptadas sobre los estigmas. Entonces registramos la longevidad y el tamaño de cada una de las flores y de cada tratamiento y la temperatura ambiente. A continuación evaluamos las diferencias en la longevidad floral entre tratamientos y en función del tamaño floral y la temperatura.

ESTRUCTURA GENERAL

Todos los capítulos de esta tesis han sido redactados en inglés para su publicación en revistas científicas internacionales. En esta memoria, por tanto, se presentan los manuscritos originales de los artículos resultantes. En la presente sección se incluye una versión en castellano del título de cada uno de los capítulos, la lista de los coautores y un breve resumen de los mismos, incluyendo objetivos, hipótesis, principales resultados y conclusiones generales.

En el Capítulo 1 se evaluaron los beneficios del despliegue floral en términos de visitas de polinizadores y éxito reproductivo. En los capítulos sucesivos (Capítulos 2−5) se examinaron los costes, incluyendo los costes fisiológicos de las corolas en términos de agua y carbono (Capítulo 2), los costes indirectos en términos de producción de frutos y semillas (Capítulo 3), los efectos del tamaño de las flores y la temperatura en la longevidad de las mismas (Capítulo 4) y los costes ecológicos en términos de florivoría (Capítulo 5).

Capítulo 1. – *Las flores más grandes de* Cistus ladanifer *atraen a más polinizadores pero no incrementan el éxito reproductivo femenino bajo las condiciones más estresantes*. Teixido AL, Valladares F. Manuscrito inédito.

Los mayores despliegues florales suelen incrementar las visitas de polinizadores y la producción de frutos, viéndose selectivamente favorecidos en las poblaciones. Sin embargo, las condiciones ambientales estresantes pueden también modular el despliegue al imponer costes en términos de recursos y contrarrestar el efecto de los polinizadores. En este capítulo evaluamos si los despliegues mayores de *Cistus ladanifer* poseen más tasas de visitas y están bajo selección fenotípica direccional a través del éxito reproductivo femenino en un estudio espacio-temporal, con variabilidad en el despliegue y en las condiciones ambientales. En concreto, planteamos las siguientes hipótesis: i) la abundancia y composición de polinizadores está positivamente relacionada con el despliegue floral; ii) la tasa de visitas está además influenciada por diferentes condiciones ambientales (sitios y años); iii) tasas de visitas diferenciales sobre mayores despliegues incrementan el éxito reproductivo femenino sólo de manera marginal debido a la limitación impuesta por las condiciones ambientales en los hábitats más cálidos y secos; iv) esto se traduce en ausencia de selección direccional positiva sobre los rasgos del despliegue floral. Pare ello medimos el tamaño y número de flores individual en dos poblaciones durante dos años consecutivos, hicimos censos de polinizadores, registramos el número de frutos y semillas y calculamos los coeficientes de selección fenotípica. Los resultados mostraron una relación espacio-temporal positiva entre el tamaño floral y la tasa de visitas, pero esta relación no se tradujo en un éxito reproductivo femenino diferencial. En general, observamos por tanto una ausencia de selección direccional positiva, aunque ésta es dependiente del contexto ecológico. En concreto detectamos selección estabilizadora sobre el tamaño floral y selección negativa sobre el número de flores en la población con mayor despliegue y bajo condiciones más secas, probablemente debido a los costes derivados de dichas condiciones.

Capítulo 2. – *Costes desproporcionados de mantenimiento de flores grandes en ecosistemas mediterráneos en términos de agua y carbono*. Teixido AL, Valladares F. Manuscrito en revisión en *New Phytologist*.

Para confirmar si las flores más grandes en nuestro sistema de estudio acarrean mayores costes directos de mantenimiento de las corolas, en este capítulo cuantificamos dichos costes en términos de agua (transpiración) y carbono (respiración). Esperamos que las corolas más grandes y bajo temperatura ambiente más elevada aumenten significativamente los costes de agua debido a una mayor transpiración y los costes de carbono debido a una mayor respiración. Para ello realizamos estudios de campo en dos especies de *Cistus* simpátricas con distinto tamaño floral (*C. albidus* vs *C. ladanifer*) como un sistema de referencia para analizar los costes del agua. Adicionalmente, usamos la especie de flores más grandes, *C. ladanifer*, para analizar los efectos de la variación específica en el tamaño de la corola sobre la tasas de respiración floral. Las tasas de transpiración incrementaron con el tamaño de la corola y con la temperatura tanto a nivel intra (*Cistus ladanifer*) como interespecífico (*C. ladanifer* vs *C. albidus*). Además detectamos una relación alométrica entre estas tasas y el tamaño floral, por lo que las flores grandes transpiraron desproporcionadamente más por unidad de superficie. Esto supuso que las flores de mayor tamaño se calentaron menos, debido a un efecto de enfriamiento transpiracional más acusado. Las tasas de respiración también fueron mayores al incrementar la temperatura del aire, pero no existió una relación alométrica con el tamaño. En general, las corolas de *C. ladanifer* transpiraron y respiraron sobre la mitad de lo que lo hicieron las hojas sobre una base de superficie de dichos órganos. Los elevados costes de flores grandes en un ambiente mediterráneo podrían imponer límites al tamaño floral a través de una asignación diferencial de recursos entre las propiedades de atracción y la producción de frutos y semillas en las flores de mayor tamaño.

Capítulo 3. – *Flores grandes y abundantes incrementan los costes indirectos de las corolas: un estudio en especies simpátricas mediterráneas con tamaño floral contrastado*. Teixido AL, Valladares F. Manuscrito en revisión en *Oecologia*.

Hasta aquí sabemos que las flores más grandes, al menos en *Cistus ladanifer*, atraen a más polinizadores, pero conllevan además mayores costes de mantenimiento. Esto quizá explique que la relación tamaño floral-tasa de visitas no se traduzca en mayor éxito reproductivo femenino e, incluso, que exista una selección fenotípica estabilizadora sobre el tamaño floral. Por tanto, en este capítulo evaluamos los costes indirectos del tamaño floral para confirmar que los costes de mantenimiento reducen de manera indirecta la producción de frutos y semillas y que esta reducción es más acentuada en individuos y especies con mayores despliegues florales. Aquí planteamos pues las siguientes hipótesis: i) las corolas suponen costes significativos en términos de producción de frutos y semillas; ii) estos costes incrementan con mayor despliegue floral; iii) estos costes difieren entre especies simpátricas de *Cistus* con tamaño floral contrastado, aumentando en aquellas especies con mayor tamaño y/o número de flores. Para contrastar estas hipótesis llevamos a cabo un diseño experimental en dos pares de especies simpátricas de *Cistus*, cada par difiriendo en tamaño floral, y comparamos el éxito reproductivo femenino entre flores cuyos pétalos fueron eliminados y flores control no manipuladas bajo condiciones controladas de polinización. Adicionalmente, cuantificamos la pérdida relativa de frutos y semillas entre tratamientos. Demostramos que los costes de mantenimiento implican costes indirectos, principalmente en términos de producción de frutos. Dentro de especies, hubo una correlación negativa entre la pérdida relativa de frutos y el tamaño floral medio individual. En uno de los pares de especies hubo una pérdida mayor de frutos en la especie con flores más grandes. Además, en ese mismo par de especies, la relación entre pérdida relativa de frutos y el despliegue floral fue mayor en la especie con más flores abiertas y, en el otro par, en la especie con mayor tamaño floral. En general, los resultados sugieren que el ambiente mediterráneo puede limitar el despliegue floral, especialmente a través del tamaño, al reducir la producción de frutos e imponer presiones selectivas opuestas a las de los polinizadores.

Capítulo 4. – *Las flores grandes tienden a ser de corta vida en ambientes mediterráneos: un estudio experimental en* Cistus ladanifer. Teixido AL, Valladares F. Manuscrito inédito.

Después de evaluar los beneficios, los costes de mantenimiento y los costes indirectos, evaluamos como las condiciones de nuestro sistema de estudio, flores grandes en ambientes estresantes, afectan a la longevidad floral. La longevidad floral (i.e. el tiempo que las flores permaneces abiertas y funcionales) mantiene un equilibrio entre las tasas de deposición y dispersión de polen y los costes de mantenimiento de las flores, por lo que una longevidad óptima tenderá a minimizar los costes y a maximizar el fitness. En este capítulo evaluamos la longevidad floral en respuesta a diferencias en la temperatura ambiente, el tamaño floral y la deposición de polen en dos poblaciones de *Cistus ladanifer* con distinto tamaño floral y condiciones ambientales. Las hipótesis que planteamos fueron: i) la longevidad floral decrece con el aumento de la temperatura ambiente; ii) la longevidad floral decrece con el aumento del tamaño floral; y iii) la longevidad floral decrece al incrementar la deposición de polen en el estigma. Ya que se ha reportado que además del tamaño y la temperatura (y los costes asociados) la deposición de polen afecta a la longevidad floral, llevamos a cabo una manipulación experimental de la polinización en flores de distinto tamaño y bajo distintas temperaturas para determinar su efecto sobre la longevidad. Para ello utilizamos tres tratamientos: polinización manual, cobertura de los estigmas para evitar deposición de polen y flores control expuestas a polinización natural. Las flores tendieron a durar un día, aunque hubo cierta plasticidad en función de la temperatura. Corroboramos nuestras hipótesis ya que la longevidad decreció con la deposición de polen, el tamaño floral y la temperatura ambiente. Además, a partir de un umbral de temperatura (aprox. 20ºC) se limitó la longevidad a un día independientemente del tamaño de las flores y la deposición de polen. Nuestros resultados sugieren un importante efecto de la temperatura ambiente sobre la longevidad floral en flores grandes en ecosistemas mediterráneos, tal vez debido al aumento significativo de los costes de mantenimiento floral y los costes indirectos en términos reproductivos que aquéllos implican.

Capítulo 5. – *El tamaño y la longevidad floral influyen en la florivoría en el arbusto de grandes flores* Cistus ladanifer. Teixido AL, Méndez M, Valladares F. Manuscrito publicado en 2011 en *Acta Oecologica* 37: 418-421.

Algunos autores han puesto de manifiesto que además de los costes del despliegue floral en términos de recursos, existen también ciertos costes ecológicos, como interacciones con animales antagonistas. Estos animales se ven atraídos por los mismos rasgos que los polinizadores y pueden por tanto contrarrestar el efecto positivo de los mismos sobre la planta. Por ejemplo, los florívoros degradan las propiedades de atracción de las flores y afectan de manera negativa a la producción de frutos y semillas. Esto puedo imponer límites selectivos en los mayores despliegues florales. En este capítulo examinamos si tres rasgos relacionados con la atracción a polinizadores, tamaño, número y longevidad floral, incrementan también los niveles de florivoría en *Cistus ladanifer* en cuatro poblaciones difiriendo en dichos rasgos. Nuestras hipótesis fueron: i) la florivoría incrementa con mayor tamaño floral, número de flores abiertas y longevidad de las mismas; ii) el nivel de florivoría varía entre poblaciones, con mayor incidencia en aquéllas con menor atracción, debido a que altos niveles de florivoría podrían limitar el despliegue y la longevidad floral. En este estudio detectamos mayor incidencia de florivoría en flores más grandes y más longevas dentro de poblaciones, pero no detectamos efectos del número de flores ni diferencias inter-poblacionales. Aunque el número de flores dañadas fue bajo (aprox. 10%), las probabilidades de florivoría sobre flores más grandes en algunas poblaciones (rango: 18−35%) abre la posibilidad de presiones selectivas relevantes hacia flores más pequeñas. En conjunto, nuestros resultados apoyan la noción de que los rasgos de atracción floral implican costes ecológicos en términos de florivoría.

CONCLUSIONES GENERALES

En esta tesis hemos confirmado que, al menos en nuestro sistema de estudio con Cistaceae, i) las flores grandes en un ambiente mediterráneo obtienen beneficios al incrementar de manera significativa la tasa de visitas de polinizadores pero acarrean sustanciales costes de mantenimiento en términos de agua y carbono, ii) que estos costes directos se traducen en costes indirectos significativos en términos de producción de frutos y semillas, iii) que el riesgo de estos costes acorta la longevidad de las flores y, por último, iv) que existen costes ecológicos en términos de florivoría. En concreto, profundizando algo más podemos extraer las siguientes conclusiones generales:

(1) Las flores grandes obtienen recompensas en términos de tasas de visitas de polinizadores, actuando como un sistema generalista del cual forman parte una gran diversidad de insectos. A nivel específico, la variabilidad en el tamaño floral es importante en este sentido, al existir una significativa relación entre el tamaño y la atracción a polinizadores, con flores de mayor tamaño recibiendo más visitas. La consecuencia directa de este beneficio es un elevado porcentaje de producción de frutos y semillas, por lo que no parece existir una limitación de polen en este sistema. Respecto al número de flores abiertas, no encontramos ninguna relación con la tasa de visitas y, de hecho, cuando estos rasgos están positivamente correlacionados, sólo parece ser beneficioso para la planta el tamaño floral, por lo que los polinizadores parecen distinguir más tamaño que el número de flores para seleccionar las plantas que visitan.

(2) No detectamos un incremento significativo de la producción de frutos y semillas a través de la relación tamaño floral vs tasa de visitas, por lo que el éxito reproductivo femenino parece saturarse con facilidad en nuestro sistema de estudio y un número poco elevado de visitas puede ser suficiente para fertilizar todos los óvulos. En contraste, la relación tamaño floral vs tasa de visitas podría incrementar de manera significativa el éxito masculino, como ha sido detectado en otros sistemas, y las flores más grandes dispersar una

mayor cantidad de polen y conferir paternidad de un mayor número de semillas.

(3) En condiciones de limitación de polen (en poblaciones con un ambiente climático más fresco y/o con individuos con menores tamaños florales donde disminuyen las tasas de visitas de polinizadores) existe selección fenotípica direccional hacia mayores tamaños florales a través de la producción de semillas, indicando que la función femenina sí puede verse beneficiada de la atracción a los polinizadores. Sin embargo, lo más común en nuestro sistema de estudio es la ausencia de selección fenotípica sobre el despliegue floral a través del éxito reproductivo femenino. En las condiciones donde el ambiente es más estresante (condiciones climáticas más cálidas y secas) y los individuos poseen los mayores tamaños florales, la limitación de recursos derivados de los elevados costes relacionados con el mantenimiento floral acarrean la aparición de selección estabilizadora sobre el tamaño floral y/o selección direccional negativa sobre el número de flores a través de la producción de frutos. Esto indica presiones selectivas sobre el despliegue floral a través de la función femenina más allá de los polinizadores, por lo que las condiciones abióticas estresantes pueden imponer límites al tamaño y el número de las flores. Queda abierta la posibilidad de que la selección favorezca el despliegue a través de la función masculina pero, en general, detectamos que la plasticidad temporal en el tamaño floral en cada una de las poblaciones siguió los patrones impuestos por la selección fenotípica del primer año, es decir, aumentando el tamaño cuando detectamos selección direccional positiva y disminuyendo cuando encontramos selección estabilizadora. Esto pone de manifiesto la importancia que la función

femenina, la función limitada por los recursos, desempeña en la plasticidad y variabilidad temporal del despliegue en flores grandes en el mediterráneo.

(4) Los costes de mantenimiento floral en términos de agua (evapotranspiración) y carbono (respiración) son elevados en nuestro sistema de estudio, con incrementos significativos en las flores más grandes en condiciones de elevada temperatura y escasa humedad relativa en el aire. Es más, los costes del agua mantienen una relación alométrica con el tamaño de las corolas ya que incrementan de manera no linear por unidad de área, especialmente con temperaturas altas. Además, los costes de mantenimiento en términos de agua y carbono representan, aproximadamente la mitad de lo que suponen las hojas sobre la misma base de superficie de estos órganos, un porcentaje relevante más aún teniendo en cuenta la corta longevidad de las flores. En general, estos resultados sugieren que los costes de mantenimiento imponen límites importantes al éxito reproductivo femenino y pueden ser la causa de la selección estabilizadora encontrada en nuestro sistema, imponiendo en consecuencia límites a los tamaños florales más grandes.

(5) Esta hipótesis es verificada a través de los costes indirectos del despliegue floral en términos de frutos y semillas. Bajo condiciones controladas de polinización y manipulando experimentalmente el tamaño floral, detectamos costes indirectos de las corolas a través de la función femenina, disminuyendo la producción de frutos y semillas en flores cuyos pétalos fueron eliminados y, en adición, manteniendo una relación negativa entre pérdida relativa de frutos y el tamaño floral entre individuos dentro de cada especie y entre especies simpátricas con tamaño floral contrastado. Por tanto,

esto implica que los costes de mantenimiento de las corolas más grandes limitan la función femenina al disminuir la producción de frutos y semillas lo que, en última instancia, se traduce en presiones selectivas abióticas que imponen limitaciones a un elevado despliegue floral, especialmente a través del tamaño de las flores.

(6) La deposición de polen en los estigmas, el tamaño floral y, sobre todo, la temperatura del aire, reducen la longevidad floral en nuestro sistema de estudio, limitándose a exclusivamente un día. Esto sugiere que cuando la deposición de polen es suficiente para consumar la polinización y fertilización de todos los óvulos, la atractividad floral cesa y los pétalos caen, probablemente para no incrementar los costes de mantenimiento de las corolas cuando la función femenina se ha desempeñado. Esto es particularmente importante en las flores más grandes y bajo temperaturas altas, ya que en flores de menor tamaño y bajo condiciones climáticas más suaves y frescas, la deposición de polen no necesariamente implica una reducida longevidad floral, posiblemente para incrementar el éxito reproductivo masculino debido a una menor tasa de visitas de polinizadores. No obstante, el tamaño floral y, especialmente, las altas temperaturas, pueden limitar la longevidad floral a un día independientemente de la polinización, lo que corrobora que los costes de mantenimiento pueden verse acrecentados e imponer ciertas limitaciones a la producción de frutos y semillas en el conjunto de la planta.

(7) Además de los costes de mantenimiento del despliegue floral, nuestro sistema de estudio también acarrea costes ecológicos en términos de florivoría. Las

flores más grandes y más longevas suponen un mayor riesgo de visita por parte de distintos florívoros, lo que supone costes indirectos adicionales en términos de producción de frutos y semillas al decrecer la atractividad a los polinizadores por daños mecánicos a los pétalos y/o disminuir la deposición y dispersión de polen por consumo directo de los gametos (estambres y carpelos). Nuestros resultados apoyan la noción de que los florívoros se ven atraídos por los mismos rasgos empleados por los polinizadores para detectar las plantas que visitan. Desde una perspectiva evolutiva, esto podría tener consecuencias negativas sobre el despliegue floral, de tal manera que la florivoría, independientemente de los polinizadores, tiene el potencial de ejercer presiones selectivas relevantes, al menos en el tamaño de las flores, contrarrestando los efectos positivos de las visitas de los polinizadores e imponiendo límites al tamaño floral.

(8) En conjunto, nuestros resultados permiten concluir que, a pesar de los beneficios que pueden reportar las flores grandes, el estrés ambiental del ecosistema mediterráneo implica costes significativos de un despliegue floral numeroso y de flores grandes reduciendo el éxito reproductivo femenino e imponiendo ciertos límites a los rasgos de atracción floral. Por tanto, la función femenina, debido a que es la función estrechamente ligada a la limitación de recursos, juega un papel relevante en la variabilidad de estos rasgos y posee el potencial de amortiguar y modular el tamaño floral en nuestro sistema de estudio.

SÍNTESIS

La presente tesis doctoral se enmarcar dentro de varios proyectos de investigación más amplios sobre procesos ecofisiológicos, plasticidad fenotípica y ecología evolutiva de especies leñosas mediterráneas que se vienen desarrollando desde hace algunos años por los equipos de la Universidad Rey Juan Carlos y el Instituto de Recursos naturales del Centro de Ciencias Medioambientales (actualmente integrado en el MNCN) del CSIC, en colaboración con investigadores de otros grupos, áreas y nacionalidades.

El objetivo principal de esta tesis es contribuir al conocimiento de las consecuencias ecológicas y evolutivas que acarrea tener flores grandes en plantas mediterráneas, un rasgo anómalo en este ambiente. Existen abundantes evidencias sobre las limitaciones de crecimiento y supervivencia a las que se ven sometidas las plantas mediterráneas. Por un lado, es bien sabido que factores abióticos como temperaturas extremas, déficit hídrico y ausencia de recursos en general, factores todos ellos característicos de los sistemas mediterráneos, imponen importantes presiones selectivas sobre las plantas. Por otro, se conocen estrategias conservativas en el uso de los recursos y se han analizado en bastante detalle los mecanismos morfo-fisiológicos subyacentes de tolerancia y evitación de las condiciones estresantes. Adicionalmente, se han documentado algunos de los costes que dichos mecanismos suponen para el crecimiento y supervivencia de las plantas en estos ecosistemas, así como lo que implica en estas medidas de rendimiento y eficacia biológicasentido los costes de reproducción. Sin embargo, poco se sabe sobre los costes de la floración y los efectos del tamaño floral, un coste adicional potencialmente gravoso para las plantas mediterráneas. Ante esta laguna de conocimiento, hemos abordado en esta tesis doctoral esta cuestión combinando muestreo de campo con experimentos y empleando las especies de la familia Cistaceae, muchas de ellas con flores grandes, para alcanzar una visión lo más general posible de los costes y limitaciones funcionales de flores y despliegues florales grandes en estos ambientes.

En el **capítulo 1** evaluamos los beneficios del tamaño floral a través de su relación con las tasas de visitas de polinizadores y el éxito reproductivo femenino en un estudio espacio-temporal. Las flores más grandes atrajeron más polinizadores pero esto no aumentó la producción de frutos y semillas y, de hecho, hubo una tendencia estabilizadora en la selección sobre este fenotipo en las condiciones ambientales más estresantes y donde el tamaño floral fue mayor. Esto nos lleva a discutir las hipótesis que postulan que las flores más grandes en los hábitats más cálidos y secos son más costosas de mantener y permiten asignar menos recursos a la propia producción de frutos.

En el **capítulo 2** analizamos si las flores más grandes bajo condiciones cálidas y secas incrementan significativamente sus costes de mantenimiento en términos de agua y carbono. Para ello diseñamos un estudio de campo sobre dos especies simpátricas con tamaño floral contrastado y con variación en la temperatura ambiente para evaluar la transpiración floral acoplado a un estudio sobre los efectos de la variabilidad intraespecífica del tamaño de las corolas en las tasas de respiración floral. Detectamos que el área de la corola y la temperatura ambiente incrementaron significativamente los costes de mantenimiento y que, además, los costes del agua incrementaron de manera no linear con el tamaño, ya que las corolas mayores transpiraron más por unidad de superficie. Como lógica implicación de este hecho detectamos que las corolas más grandes se calentaron menos, por un efecto de enfriamiento transpiracional más acentuado. Con estos resultados confirmamos la hipótesis de que las flores más grandes en ambientes estresantes son más costosas y nos lleva a plantear si, efectivamente, acarrean grandes costes indirectos, en concreto disminuyendo el éxito femenino.

El **capítulo 3** confirma que las flores más grandes y abundantes dan lugar a una menor producción de frutos y semillas. Empleando técnicas experimentales de reducción de las corolas y métodos de polinización controlada sobre poblaciones con especies simpátricas de distinto tamaño floral, detectamos costes indirectos de las corolas en términos de producción de frutos, una correlación entre la pérdida relativa de frutos y el tamaño floral medio individual y una pérdida mayor de frutos en la especies con flores más grandes o más abundantes. Esto nos conduce a sugerir que el ambiente mediterráneo limita el tamaño floral, al reducir la producción de frutos por los mayores costes de mantenimiento de flores más grandes. Esto impondría presiones selectivas opuestas a las de los polinizadores, y permitiría comprender los resultados que encontramos en el capítulo 1.

En el **capítulo 4** evaluamos los efectos de la temperatura ambiente, el tamaño floral y la deposición de polen sobre la longevidad floral. Este rasgo resulta del equilibrio entre las tasas de deposición y dispersión del polen y los costes del mantenimiento floral, por lo que una longevidad óptima tenderá a minimizar los costes y a maximizar el fitness. En este capítulo planteamos que la polinización reducirá el tiempo que las flores permanecen abiertas y que el tamaño floral y la temperatura limitarán la longevidad debido al incremento de los costes de mantenimiento. Mediante manipulación experimental de la polinización sobre flores de distinto tamaño en ambientes con temperatura contrastada, medimos la longevidad floral y detectamos que tendió a ser corta (aprox. 1 día) y que decreció con la deposición de polen, el tamaño floral y la temperatura ambiente. Nuestros resultados sugieren que los costes de mantenimiento de las flores más grandes en un ambiente mediterráneo limitan la longevidad floral para maximizar el éxito reproductivo femenino.

Por último, en el **capítulo 5** examinamos si tres rasgos relacionados con la atracción a polinizadores, tamaño, número de flores abiertas y longevidad floral, incrementan también los costes ecológicos en términos de florivoría. Mediante un estudio en cuatro poblaciones de *Cistus ladanifer* difiriendo en dichos rasgos detectamos mayor incidencia de florivoría en flores más grandes y más longevas dentro de las poblaciones, pero no detectamos diferencias significativas entre poblaciones. La incidencia significativa de la florivoría sobre flores de mayor tamaño podría ejercer presiones selectivas relevantes hacia flores más pequeñas. En conjunto, nuestros resultados reafirman el hecho de que los rasgos de atracción floral implican, además de costes de mantenimiento, costes ecológicos en términos de florivoría, lo que lleva en última instancia a costes indirectos en términos de producción de frutos y semillas viables, lo que podría conducir a una reducción del tamaño floral en nuestro sistema de estudio.

CHAPTER 1

Larger flowers of *Cistus ladanifer* attract more pollinators but do not increase female fitness under the most stressful conditions

Teixido AL, Valladares F.

Manuscrito inédito

Abstract

Larger and numerous flowers increase pollinator visit rates and female fitness so they usually are under pollinator-mediated selection. However, stressful environmental conditions can also modulate these traits by means of resource costs counteracting the effect of pollinators. We evaluate whether larger floral displays in the large-flowered Mediterranean shrub *Cistus ladanifer* attract more pollinators and are under directional phenotypic selection through female fitness in two populations along two years with contrasting flower size and climatic conditions. We measured individual mean flower size and number and related them with pollinator visit rates and, subsequently, recorded fruit and seed production and estimated the coefficients of phenotypic selection. We detected a positive relationship between flower size and visit rates, but this did not increase female fitness. We also detected stabilizing selection on flower size and negative selection on flower number in the drier population. However, we found positive directional selection on flower size the first year in the wetter population. Overall, our results suggest that in natural populations of *C. ladanifer* pollinator attraction alone does not always drive selection on floral display through female fitness, but rather it is context dependent due to pollen and/or resource limitation. Hence, evolution of this trait could be constrained by conflicting selective pressures acting on female function associated to floral costs of flower size in this large-flowered Mediterranean species.

Keywords: drier site, floral maintenance costs, flower size, fruit and seed production, number of flowers, pollinator visit rates, wetter site

Introduction

Pollinators are considered to be a major factor driving evolution of floral phenotypes (Darwin 1862; Stebbins 1970; Fenster *et al*. 2004). Because flower size and number are involved in pollinator attraction, these floral traits have often been described to evolve under pollinator-mediated selection (e.g. Galen 1989; CM Herrera 1993; Conner and Rush 1997; Hodgins and Barrett 2008; Harder and Johnson 2009). However, large and numerous flowers are associated with greater requirements of biomass, energy and water for floral development (Galen 1999; Halpern *et al*. 2010), as well as with larger maintenance costs due to high respiration and transpiration rates (Vemmos and Goldwin 1994; Galen 2000). Accordingly, several studies have documented that simultaneous constraints from pollen availability and other environmental factors influencing female's reproductive output can affect attraction floral traits (Galen 1999, 2000; Totland 2001; Caruso 2006; see also review by Strauss and Whittall 2006).

Species composition and the visit rates of pollinator assemblage that determine the amount of pollen availability, together with constraints from abiotic environmental factors are likely to show spatial and temporal variation. It follows that the strength and direction of selection may differ in space and time, which complicates our understanding on the factors underlying the evolution of floral traits. Geographic and temporal variation in abundance and composition of pollinators has been broadly reported (e.g. CM Herrera 1988, 1996; Fenster and Dudash 2001; Aigner 2005; Minckley and Roulston 2006). Such variation has mostly explained the existence of generalized pollination systems, which thus promote efficient animal-mediated cross-pollination (reviewed in Gómez and Zamora 2006). Few studies have analysed spatio-temporal variation in phenotypic selection on floral traits involved in attractiveness to pollinators (Caruso *et al*. 2003; Maad and Alexandersson 2004; Wright and Meagher 2004), but they are essential to identify reliable estimates of selection (Conner 2006; see also CM Herrera 2009). Despite the extensive literature on geographic and temporal variation in pollination environment there have been no investigations on effects of such variation in the direction and magnitude of pollinator-mediated selection acting on intraspecific spatio-temporal floral diversity (but see Aigner 2005).

From an evolutionary perspective, the generalization to pollinators due to the high spatio-temporal variability in pollination service may reduce pollen limitation and, consequently, lead to stabilizing selection on flower size through female fitness (Aigner 2001; Gómez and Zamora 2006; Sahli and Conner 2011). In addition, selection on flower size and number through fruit and seed production may be relaxed because of other mechanisms, such as nonpollinator agents of selection or resource allocation among reproductive traits (reviewed in Strauss and Whittall 2006). For example, a large pollen deposition due to a high attractiveness to pollinators may not involve a differential fruit and seed production due to resource limitation (Ashman and Morgan 2004). However, despite some evidence that stabilizing selection on floral traits engaged in pollinator attraction may be relatively common in plant populations (Cresswell 1998; Kingsolver and Diamond 2011), this form of selection has rarely been measured in natural populations visited by a high and variable pollinator assemblage.

Here, we explicitly test whether pollinators were acting as agents of selection on flower size and number through female function in natural populations of *Cistus ladanifer*, a large-flowered, generalized and outcrossing hermaphroditic shrub native to the Mediterranean area. High temperatures and water shortage in Mediterranean ecosystems strongly limit plant reproduction (Larcher 2000; Thompson 2005; Aragón *et al*. 2008). Under such stressful conditions, a large-flowered species in a generalist pollination environment is a good system to determine pollinator-mediated selection on floral traits. We conducted our study in two populations along two years to examine spatio-temporal variation in the evolution of floral traits involved in pollinator attraction. Geographical variation in seed number and in flower size as well as high variability in flower number within-populations has been documented in natural populations of *C. ladanifer* (Narbona *et al*. 2010; Teixido *et al*. 2011). Specifically, our study addressed the following hypotheses: (1) pollinator visit rate is affected by flower size, with larger flowers attracting more visitors; (2) pollinator visit rate is influenced by different environmental conditions (sites and years) further modulating the relationship between flower size and abundance and composition of pollinators; (3) increased pollinator visit rates enhance female fitness only marginally due to the limiting environmental conditions where *C. ladanifer* dwell, and this positive influence can only be seen in the most favourable sites and years; (4) direction and magnitude of pollinator-mediated selection is consistent with the relationship between visit rates and female fitness and, therefore, dependent of environmental conditions.

Materials and methods

Study species

Cistus ladanifer L. (Cistaceae) is a shrub 100-250 cm tall that inhabits acid and dry soils in warm open areas on western Mediterranean. Flowering period spans from March to June and each plant produces many white flowers of approximately 7-11 cm in diameter, often exhibiting dark coloured spots at their bases. Flowers are the largest in the family and have on average more than 150 anthers and 1000 ovules (J

Herrera 1992). Flowers are solitary, hermaphroditic, self-incompatible and secrete some nectar (J Herrera 1992). Flower opening occurs synchronously each day within the populations and flowers last only several hours when pollinated and/or under warm temperatures (AL Teixido and F. Valladares, unpub. data). Fruits are globular woody capsules with a variable number of valves (5-12) and seeds (approx. range: 300-1200) 0.8 x 0.6 mm in size (Talavera *et al*. 1993; Delgado *et al*. 2008; Narbona *et al*. 2010).

Study sites

We recorded pollinator visit rates and measured phenotypic selection on floral display (i.e. flower size and number) of *C. ladanifer* at two populations differing in flower size and climatic conditions during two consecutive years (2010 and 2011) in Madrid province, central Spain (39.53º-41.09º N 3.03º-4.34º W). Our survey at two populations along two years was intended to include spatio-temporal variations in floral display, pollinator environment and phenotypic selection. Both populations had similar orientation (south), slope (0-10º) and tree canopy cover (0-10%). First population was located in Tres Cantos (732 m; 40.34ºN 3.42ºW; hereafter drier site) where individuals bloom between April and May. Substrate is predominantly clay and sand and vegetation is dehesa-like with *Quercus ilex* and *Pinus pinea* interspersed in a shrub matrix. Second population was located in Puerto de Canencia (1300 m; 40.50ºN 3.46ºW; hereafter wetter site) where individuals bloom from late May to late June. Substrate is granite and vegetation is sparsely wooded with *Quercus pyrenaica* and *Pinus sylvestris.* In the drier site climate is dry with annual average temperature of 12ºC and annual average rainfall of 533 mm, whereas in the wetter site climate is subhumid with annual average temperature of 9ºC and annual average rainfall of 865 mm (Ninyerola et al. 2005).

Floral display and female fitness

During the flowering peak (when all individuals bloomed more than 5 flowers per day) 39 plants in the drier site and 34 in the wetter site were selected and tagged in a plot of 25 × 25 m. We recorded two plant traits related with floral display (flower size and number) in order to evaluate their effects on female fitness. Under mild weather conditions, suitable for pollinator activity, the number of open flowers was noted and 4-6 flowers at random were tagged daily at each plant. Overall, 18-53 flowers were tagged per plant, for a total sample size of 1765 and 1170 flowers in the drier site and 1029 and 1003 in the wetter site, in 2010 and 2011, respectively (total N = 4967). Diameter (cm) of 18-30 tagged flowers per plant was measured and then flower size and number of flowers at each plant averaged. Both mean flower size and mean number of flowers per plant were utilised for averaging these traits at each population and year.

In July, all ripe fruits were picked before seed dispersal to evaluate female fitness, recorded as fruit set and mean seed number per fruit. Fruit set estimates pollination intensity as a proportion of pollinated flowers whereas mean seed number per fruit (hereafter seed number) assesses the quality of mating. Fruit set per plant was obtained by dividing the number of mature fruits set between all flowers tagged per plant. To determine seed number, 8-15 mature fruits were randomly selected per plant. In the lab, all seeds of each fruit were collected and compiled in an envelope per plant. Then all seeds of each envelope (i.e. of each plant) were carefully scattered on a white sheet and photographed to be counted by means of image processing in ImageJ v1.43 (ImageJ, US National Institutes of Health, Bethesda, MD, USA 1997-2011, http://imagej.nih.gov/ij/). Lastly, recorded seed number was averaged at each plant by dividing by the number of fruits utilised.

Pollinators

We evaluated the relationship between number and identity of flower visitors with floral display and subsequent effects on female fitness. The insect observations were conducted on 4 warm sunny days with little wind during the flowering peak at each population and year. 14 plants per population and year utilised for female fitness measures and differing in mean flower size were chosen. On each day, we tagged and observed 5 flowers on each of 14 plants during 5-min periods between 1200 and 1700 hours in the drier site and 0900 and 1400 hours in the wetter site. Overall, we observed 20 flowers per plant during 50 min. All plants were observed every day in order to avoid possible differences in individual visit rates due to climatic variability. We observed every plant at each of the five hours twice on two different days, to give a total of 10 observation periods per plant and 140 periods during 700 min (approx. 12 h) at each population and year. The order of observation of plants was random and we never observed the same plant two consecutive times or more than three times per day. Every day we recorded flower size of every observed flowers as well as the number of displayed flowers per plant. Then both traits were averaged at each plant.

During each observation period we noted the number and identity of visitors to flowers and number of visits per each visitor. A visit was defined to have occurred when the visitor's body contacted the stigma of the flower. At each plant, we calculated visit rate as total number of visits per 50 min. We categorised each visitor into seven pollinator functional groups or clusters of pollinator species that behave in a similar way in the flowers (Fenster et al. 2004). The functional groups were: bumblebees (*Bombus* spp.), solitary bees (Andrenidae, Colletidae and Halictidae), honeybees (*Apis mellifera*), wasps (Icneunonidae), hover flies (Syrphidae), muscoid flies (Muscidae and Anthomyiidae) and beetles (Coleoptera). Then we recorded the frequency of visits of each pollinator functional group to each plant. In the absence of data on a visitor's efficiency, the frequency of visits can be used as a surrogate of their relative potential importance for the plant species (Fenster *et al*. 2004). Lastly, we evaluated female fitness as pointed above (see "Floral display and female fitness").

Data analysis

1. Floral display and female fitness

We tested for differences in floral display and in female fitness components between populations and years (fixed factors), plant nested within population (random factor) and the interaction between population and year, by fitting Generalized Linear Mixed Models (GLMM) for every response variable, i.e., flower number, flower size, fruit set and seed number. In all cases, but fruit set, we assumed a normal error distribution with an identity link function. For fruit set we assumed a binomial error distribution with a logit link function. For all models we used the restricted maximum likelihood (REML). All the computations were performed using the GLIMMIX Macro of SAS (SAS Statistical Package, 1990; SAS Institute, Cary, NC, USA).

2. Pollinators

To test spatio-temporal differences in the frequency of visits of each pollinator functional group to each plant we used PerMANOVA. PerMANOVA is a permutation-based version of the multivariate analysis of variance (Anderson 2001). It uses the distances between samples to partition variance and randomizations or permutations of the data to produce the p-value for the hypothesis test. It is non-parametric (or semi-parametric for multi-factor models) and, therefore, robust to the assumption of multivariate normality making it less prone to Type I errors. Count data of visits of each pollinator functional group were square root transformed to improve normality. Bray-Curtis similarity index was calculated before performing the analysis (Anderson 2001). All PerMANOVA analyses were performed in Primer 6.0 (Clarke and Gorley 2006).

We analysed for relationship between visit rates and floral display and whether that relationship differed between sites and years. We conducted an ANCOVA including population and year (fixed factors), flower number and flower size (covariates) and all the possible interactions. Significant interactions show spatio-temporal differences in pollinator visit rates and their correlation with flower number and/or flower size. We subsequently analysed whether any spatio-temporal variation in visit rates had effects on female fitness in terms of fruit and seed production. We conducted two new ANCOVAs, where the dependent variables were fruit set and seed number, respectively. Fruit set was arcsin transformed to achieve normality. We included population and year (fixed factor), visit rates (covariate) and all the possible interactions.

3. *Phenotypic selection*

We estimated selection through female function in both populations and both years using standardized linear selection differentials (*s*). Selection differentials are the univariate regression coefficient between relative fitness (individual fitness/population mean fitness, *w*) and each of the standardized traits (Lande and Arnold 1983). We conducted all analyses on traits standardized with a mean of 0 and a variance of 1 for each population and each year. This facilitates comparisons of selection acting on traits measured in different units and across different populations and/or years (Lande and Arnold 1983). We used fruit set and seed number produced per plant, divided by the mean of each population to provide a measure of relative female fitness.

Selection differentials suffer from the fact that selection acting on the trait of interest cannot be disentangled from selection acting indirectly on correlated characters. Therefore, to reduce the confounding effects of indirect selection, we also calculated selection gradients (*β*) using a standard multivariate regression analysis, where phenotypic selection is estimated as the regression coefficients of relative fitness (*w*) on standardized measures of multiple traits and their pairwise combinations (Lande and Arnold 1983). Additionally, we calculated nonlinear selection gradients (*γ*) to estimate stabilizing/disruptive selection, by obtaining quadratic deviations from the mean for both single and quadratic terms of characters (Lande and Arnold 1983). Therefore, we used flower number and flower size as well as the quadratic components flower number^2 and flower size^2 in the regression model. Quadratic regression coefficients were doubled to estimate properly stabilizing/disruptive selection gradients (Lande and Arnold 1983; Stinchcombe *et al*. 2008). All the regression models, both univariate and multivariate, were performed using the GLM procedure of SAS (SAS Statistical Package 1990; SAS Institute, Cary, NC, USA).

Results

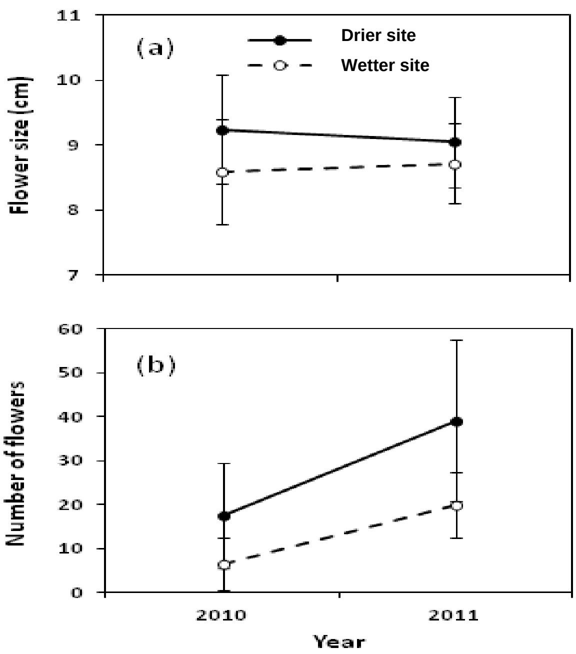
Floral display and female fitness

Flower size and number showed spatio-temporal variability and differed between populations and plants within populations (Table 1). Correlation between both floral traits was positively significant in the wetter site in 2010 ($r = 0.45$, $p < 0.01$, $n = 34$). Individual mean flower size ranged 7.2 ±0.4 to 10.5 ± 0.5 cm and, overall, was significantly larger in the drier site both in 2010 and in 2011 (Fig. 1a). Over time, variation in flower size was opposite-signed between populations (Population × Year significant, Table 1), decreasing up to a 2% in the drier site and increasing a 1.5% in the wetter site (Fig. 1a). Individual mean flower number showed a large variation (range: 5.8 ± 2.0 – 100.3 ± 36.4) and, overall, was also significantly higher in the drier site during the two study years (Fig. 1b). Over time, flower number significantly

Table 1. Generalized linear mixed models for differences in flower size and number of flowers of *C. ladanifer* between populations, years, plants within populations and the interaction population × year. Plant (Population), as random factor, was tested with Wald Z-test, and fixed factors with Type III F-tests. Significant *P*-values are marked in bold.

Effect	Flower size				Number of flowers			
	d.f.	Estimate ± SD	Test value	*P*-value	d.f.	Estimate ± SD	Test value	*P*-value
Random								
Plant (Population)	-	0.564 ± 0.072	3.72	**<0.001**	-	0.501 ± 0.044	4.51	**<0.001**
Fixed								
Population	1, 71.5	1.075 ± 0.381	19.82	**<0.001**	1, 71.5	1.425 ± 0.350	30.64	**<0.001**
Year	1, 2825	0.194 ± 0.032	0.15	0.703	1, 824	1.001 ± 0.119	35.61	**<0.001**
Population × Year	1, 2825	1.405 ± 0.041	35.74	**<0.001**	1, 824	1.202 ± 0.110	19.12	**<0.001**

Figure 1. (a) Flower size ± SD (cm) and (b) number of flowers ± SD at drier and wetter sites for each year of study in *C. ladanifer*. Interactions Population × Year were significant for both traits (Table 1)



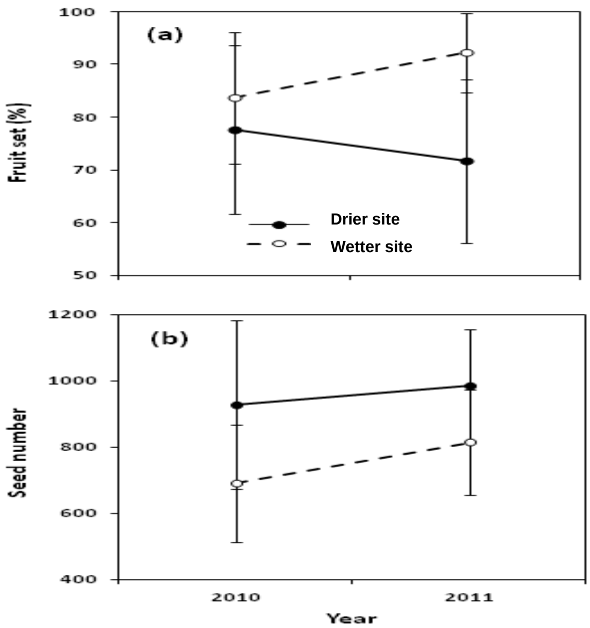
increased, but this increase differed between populations (more than two-fold in The drier site vs more than three-fold in The wetter site; Population × Year significant, Table 1; see also Fig. 1b).

Fruit set and seed number also showed patterns of spatio-temporal variation. Overall, fruit set averaged around 80% (range: 13.5 – 100%), significantly differed among populations and plants within populations and remained invariable over time (Table 2). Fruit set was constantly higher in the wetter site than in the drier site and this difference significantly increased over time (Population × Year significant, Table 2). Regarding 2010, in 2011 the plants set a 10% more of fruits in the wetter site and a 7.6% less in the drier site (Fig. 2a). Seed number was variable (range: 362 – 1513) and averaged 855±233. Mean seed number significantly differed among populations and plants within populations (Table 2). Contrary to fruit set, mean seed number was constantly higher in the drier site than in the wetter site (Fig. 2b). Over time, mean seed number increased up to a 5.8% in the drier site and a 15.1% en the wetter site, but this increase was similar (Population × Year not significant, Table 2; see also Fig. 2b).

Table 2. Generalized linear mixed models for differences in fruit set and seed number of *C. ladanifer* between populations, years, plants within populations and the interaction population × year. Plant (Population), as random factor, was tested with Wald Z-test, and fixed factors with Type III F-tests. Significant *P*-values are marked in bold.

Effect	Fruit set				Seed number			
	d.f.	Estimate ± SD	Test value	*P*-value	d.f.	Estimate ± SD	Test value	*P*-value
Random								
Plant (Population)	-	0.418 ± 0.091	4.57	**<0.001**	-	1.229 ± 0.244	4.51	**<0.001**
Fixed								
Population	1, 71.5	1.358 ± 0.203	24.22	**<0.001**	1, 71.5	1.425 ± 0.350	9.26	**0.003**
Year	1, 4798	0.647 ± 0.143	3.31	0.089	1, 824	1.001 ± 0.119	0.56	0.458
Population × Year	1, 4798	0.988 ± 0.169	34.18	**<0.001**	1, 824	1.202 ± 0.110	3.58	0.063

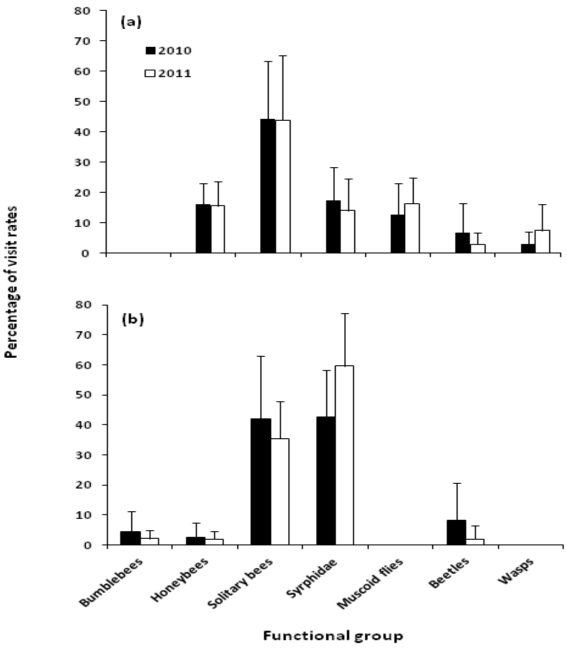
Figure 2. (a) Fruit set ± SD (%) and (b) seed number ± SD at drier and wetter sites for each year of study in *C. ladanifer*. Interactions Population × Year was only significant for fruit set (Table 2)



Pollinators

Six and five functional groups of pollinators were identified in the drier and in the wetter site, respectively (Fig. 3). In the drier site, solitary bees were dominant and accounted for about 45% of the visits over time. Overall, honeybees and flies

Figure 3. Frequency ± SD (%) of each pollinator functional group in *C. ladanifer* over time in (a) the drier site and (b) the wetter site



(Syrphidae, Muscidae and Anthomyiidae) approximately spanned the same frequency of visits than solitary bees, whereas beetles and wasps included about the remaining 10% of visits. In the wetter site, solitary bees were also abundant, but hover flies were the dominant group. Taking together, both groups accounted for about 85-95% of visits, in 2010 and 2011, respectively. Muscoid flies and wasps were absent and bumblebees were present, though in a low frequency (<5%). Differences in composition of pollinators were significant between populations and years (Pseudo-$F_{1,52}$ = 47.39, $p < 0.001$; Pseudo-$F_{1,52}$ = 5.27, $p = 0.001$, respectively), but they uniformly varied among populations over time (Population × Year non significant; Pseudo-$F_{1,52}$ = 2.09, $p = 0.102$).

Visit rates showed spatio-temporal variation (range: 0.26 ± 0.08 – 0.43 ± 0.15) and were significantly and positively correlated with flower size (Table 3). However, visit rates were kept constant independently of flower number. Overall, visit rates were similar between sites, but significantly increased about a 19% over time (Fig. 4). This difference was mainly due to the high increase (>38%) in the wetter site from

Table 3. ANCOVA for differences in pollinator visit rates of *C. ladanifer* between populations, years, flower size, number of flowers and all the interactions. Significant *P*-values are marked in bold.

Effect	df	MS	F	p
Population	1	0.004	0.327	0.570
Year	1	0.089	8.287	**0.006**
Flower size	1	0.172	17.401	**<0.001**
Number of flowers	1	0.008	0.813	0.372
Population × Year	1	0.079	7.298	**0.010**
Population × Flower size	1	0.074	7.433	**0.009**
Population × Number of flowers	1	0.002	0.249	0.620
Year × Flower size	1	0.010	1.054	0.311
Year × Number of flowers	1	0.001	0.058	0.811
Population × Year × Flower size	1	0.001	0.143	0.707
Population × Year × Number of flowers	1	0.000	0.014	0.908
Error	44	0.010	-	-

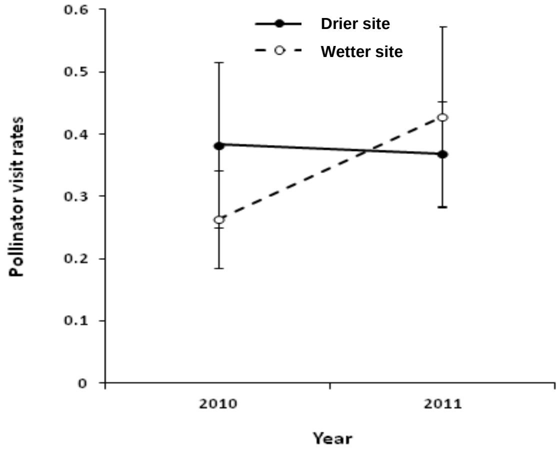


Figure 4. Interaction between population and year in pollinator visit rates in *C. ladanifer*.

2010 to 2011. Consequently, the interaction Population × Year was significant (Table 3). Likewise, visit rates showed a higher positive correlation with flower size in the wetter site over the study period (Population × Flower size significant, Table 3; see also Fig. 5). Pollinator visit rates did not affect fruit set or seed number (Table 4), indicating that the positive correlation between visit rates and flower size does not entail an increase in female fitness within or between populations. Overall, fruit set of the observed plants for pollinators followed the same pattern that for the populations

Figure 5. Interaction between population and flower size in pollinator visit rates in *C. ladanifer*.

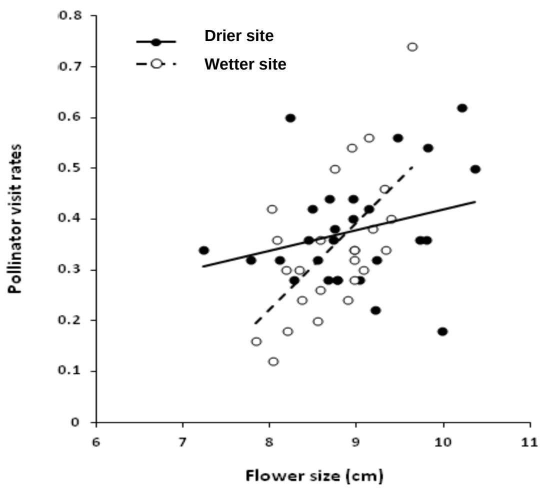


Table 4. ANCOVAs for differences in fruit set and seed number of *C. ladanifer* between populations, years, visit rates and all the interactions. Significant *P*-values are marked in bold.

Effect	Fruit set				Seed number			
	df	MS	F	p	df	MS	F	p
Population	1	0.090	4.213	**0.046**	1	3.46×10^5	14.080	**0.009**
Year	1	0.003	0.140	0.710	1	5.70×10^4	3.210	0.082
Visit rates	1	0.069	2.721	0.105	1	3.33×10^4	2.678	0.110
Population × Year	1	0.032	1.477	0.231	1	2.74×10^4	1.543	0.223
Population × Visit rates	1	0.021	0.960	0.333	1	1.80×10^4	1.013	0.321
Year × Visit rates	1	0.000	0.015	0.905	1	4.08×10^4	2.298	0.139
Population × Year × Visit rates	1	0.024	1.115	0.297	1	1.66×10^4	0.935	0.341
Error	48	0.021	-	-	48	1.78×10^4	-	-

as a whole and was significantly higher in the wetter site than in the drier site (90.2 ± 6.6 vs 81.5 ± 11.3, respectively). Seed number also followed that same pattern and was significantly higher in the drier site than in the wetter one (1024.7 ± 153.7 vs 798.0 ± 98.0, respectively).

Phenotypic selection

Selection differentials for floral display through female fitness were variable between populations and over time (Table 5). The results revealed total selection on flower size through seed number and marginally significant total selection on flower number through fruit set in the wetter site during 2010. However, total selection in the drier site was detected during 2011, but for decreased flower number through fruit set. When analysing direct selection (i.e., selection gradients) on floral display, the results were relatively similar to those of total selection but we also detected non-linear selection (Table 6). There was positive direct selection on flower size through seed number but, conversely to total selection, not on flower number in the wetter site during 2010. This indicates that total selection on flower number was indirectly detected. There also was direct negative selection on the combination of floral display traits (Flower size × Number of flowers significant, Table 6), indicating contrasting selection. Over time, plasticity in flower size in the wetter site was related with direct selection, since a positive directional selection through seed number involved an increase in flower size in 2011. In the drier site, there was consistent directional selection to decrease flower number through fruit set and seed number during 2011. In addition, we detected constant stabilizing selection on flower size

Table 5. Standardized directional selection differentials ±SD for flower size and number of *C. ladanifer* on both female fitness measures (fruit set and seed number) at each population for each year of study. Significant *P*-values are marked in bold. * p = 0.059; **p = 0.001; ***p < 0.001.

Year Trait	Drier site		Wetter site	
	Fruit set	Seed number	Fruit set	Seed number
2010				
Flower size	-0.022±0.034	-0.012±0.057	0.028±0.025	**0.159±0.042*** **
Number of flowers	-0.044±0.034	-0.073±0.070	**0.047±0.025***	0.026±0.076
2011				
Flower size	-0.003±0.039	0.067±0.035	-0.007±0.014	0.039±0.056
Number of flowers	**-0.130±0.029*****	0.026±0.068	0.005±0.014	-0.025±0.039

Table 6. Standardized selection gradients for flower size, flower number and their correlation in *C. ladanifer* on both female fitness measures at each population for each year of study. Linear (β') and quadratic (γ') coefficients ± SD are shown. Significant *P*-values are marked in bold. *p < 0.05; **p < 0.01; ***p = 0.001.

Year / Trait	Drier site				Wetter site			
	Fruit set		Seed number		Fruit set		Seed number	
	β'	γ'	β'	γ'	β'	γ'	β'	γ'
2010								
Flower size	-0.025 ±0.033	**-0.124 ±0.058***	-0.013 ±0.012	-0.086 ±0.090	0.010 ±0.028	0.076 ±0.058	**0.123 ±0.042****	0.002 ±0.004
Number of flowers	-0.048 ±0.033	0.070 ±0.050	-0.051 ±0.047	0.082 ±0.168	0.045 ±0.028	-0.034 ±0.060	0.017 ±0.058	0.028 ±0.130
Flower size × Number of flowers	0.057 ±0.061	–	0.066 ±0.091	–	0.017 ±0.057	–	**-0.186 ±0.077***	–
2011								
Flower size	-0.018 ±0.032	**-0.094 ±0.040***	0.070 ±0.108	0.076 ±0.138	-0.003 ±0.016	0.034 ±0.024	0.051 ±0.060	-0.066 ±0.114
Number of flowers	**-0.134 ±0.035*****	-0.022 ±0.038	**-0.158 ±0.130***	0.094 ±0.137	-0.003 ±0.019	0.002 ±0.016	0.008 ±0.057	0.048 ±0.048
Flower size × Number of flowers	0.000 ±0.009	–	-0.055 ±0.033	–	0.019 ±0.019	–	-0.077 ±0.058	–

through fruit set (Table 6). This was related with plasticity in flower size over time, since flower size decreased from 2010 to 2011. Overall, the results for phenotypic selection on floral display through female fitness are reliable with those of the effect of visit rates on fitness.

Discussion

Our study relates spatio-temporal responses in patterns of pollinator visits to analysis of phenotypic selection on traits involved in floral attraction. We found evidence of a spatio-temporal positive relation between flower size and pollinator visit rates in natural populations of *C. ladanifer*. We also found that this relation was not translated into a differential female reproductive success. According to this we did not generally detect a significant positive directional phenotypic selection on flower size through female fitness. Specifically, we found evidence for stabilizing selection over time through fruit set in the drier site. However, we did detect significant directional selection through seed number for increased flower size in the wetter site in 2010 indicating that context dependence (i.e., variation in pollination environment and/or resources among populations) might have potential to affect patterns of sex-specific selection. We also detected significant negative directional selection on flower number through fruit set in the drier site in 2011. Overall, our study indicates that in populations of the large-flowered Mediterranean shrub *C. ladanifer* selection for increasing flower size and number is not always to be pollinator-mediated through female function.

Effects of floral display on visit rates and female fitness

The interactions between *C. ladanifer* and pollinating agents recorded in our study were generalist in nature; the species was visited by an array of pollinators that in fact varied both in space and in time. Variety of plant-pollinator systems seems to be a rule in large-flowered *Cistus* species, in which the relative proportions of bees, beetles and flies are highly variable among species and sites (Bosch 1992; Talavera *et al*. 1993, 2001). Specifically, our results contrast with those of Talavera *et al*. (1993) who recorded a high proportion of flies (79%) and a very low one of bees (4%) in pollinator spectra of a population of *C. ladanifer* in SW Spain. Hence, generalization is common in this species and the high variability in pollination environment among populations might entail some adaptation in floral display to local pollinating conditions.

Our results show the importance of evaluating flower size and number together since may have contrasted or additive effects on visit rates (Conner and Rush 1996; Thompson 2001). Flower size is a common determinant of attractiveness to pollinators with larger flowered individuals receiving higher visit rates (Bell 1985; Young and Stanton 1990; Kudoh and Whigham 1998; Medel *et al*. 2007). Larger flowers are disproportionally visited by pollinators because of a greater content of resources such as nectar and pollen (reviewed in Jones 2001). In particular, flower size is related with greater amounts of nectar and pollen in *Cistus* (J Herrera 1992; Arista and Ortiz 2007) which may act as a key factor for differential visit rates on larger flowers in our study species. This could explain the higher relationship between flower size and visit rates in the wetter site, where flowers were smaller. Other studies have evaluated the influence of flower number on pollinator visit rates. Results of these studies have mostly reported negative or absence of any relationship (Bell 1985; Brody and Mitchell 1997; Thompson 2001; Liao *et al*. 2009; but see Mitchell 1994; Grindeland *et al*. 2005). Despite that experimental manipulation of number of open flowers would improve the effect of this trait on pollinator visit rates (Aigner 2005), our data suggest that number of open flowers do not entail significant changes in visit rates in *C. ladanifer*. Thus, it appears that many-flowered plants did not have a higher percentage of their flowers visited.

As reported here, increased pollinator visit rates did not enhance fruit or seed production in other species (Young and Stanton 1990; Kudoh and Whigham 1998; Talavera *et al*. 2001; but see Johnson *et al*. 1995). The absence of this relation in our populations is likely due to the generally low levels of pollen limitation experienced in *Cistus* (Bosch 1992; J Herrera 1992; Talavera *et al*. 1993, 2001; Arista and Ortiz 2007). Studies have reported high fruit set for *C. ladanifer* (95%; Talavera *et al*. 1993) as well as for *C. libanotis* (70–88%; Talavera *et al*. 2001) and *C. salviifolius* (78–91%; Arista and Ortiz 2007). Relative to seeds, Narbona *et al*. (2010) reported that the mean number per fruit was highly variable in natural populations of *C.*

ladanifer (range: 318.0 ± 55.8 – 1364.3 ± 63.1). In addition, we reported 73–86% of fruit set and 622.9 ± 127.3 – 946.0 ± 188.1 seeds per fruit in hand-pollinated flowers of *C. ladanifer* in our study populations (A.L. Teixido and F. Valladares unpub. data). Spatio-temporal ranges of fruit set (72–92%) and seed number (690.6 ± 177.7 – 985.1 ± 170.3) reported in the present work fit in the ranges recorded in the studies above.

Assessment of pollinator-mediated phenotypic selection

We detected positive directional selection in the wetter site in 2010 for flower size through seed number, indicating that there can be an effect of pollinators on female fitness in *C. ladanifer* in our study populations. This effect could be a result of a certain pollen limitation in the wetter site in 2010, where we recorded both the smallest flower size and the lowest pollinator visit rates of our study. A high number of studies has reported selection on flower size through seed number associated with pollen limitation (e.g. Totland 2001; van Kleunen and Ritland 2004; Gómez *et al*. 2006; Hodgins and Barrett 2008; Nattero *et al*. 2010a).

On the other hand, we detected negative directional selection for flower number through both fruit set and seed number in the drier site in 2011 and contrasting selection between larger and less abundant flower in the wetter site in 2010, which suggests costs of having many open flowers together with a balance between flower size and number. To our knowledge, this is the first study detecting negative selection on flower number in natural populations. Other researchers have identified selection for increased flower number (Conner and Rush 1997; Caruso *et al*. 2003; Sletvold *et al*. 2010), no selection (Nattero *et al*. 2010a) or stabilizing selection (Parachnowitsch and Kessler 2010). Contrasting selection towards larger but less flowers suggests that attraction to pollinators is modulated through flower size more than number of flowers in *C. ladanifer*. A plausible explanation of the patterns found could be that higher number of flowers generally involves higher geitonogamous rates, thus decreasing fruit and seed set (Brys *et al*. 2004; Liao *et al*. 2009; Brys and Jacquemyn 2010). However, since we found no compelling evidence of any effect of flower number on visit rates and a subsequent differential fruit production, this pattern is more difficult to explain. Alternatively, high flower number in *C. ladanifer* in the drier site in 2011 could be related to significant costs of floral allocation translated into negative effects on reproductive output. Relative to this, we found that the species with higher number of open flowers showed a higher correlation with indirect costs in terms of fruit production between coflowering sympatric *Cistus* species in our study area (AL Teixido and F Valladares unpub. results).

Apart from these exceptions we identified absence of selection through both female functions on floral display in the wetter site and through seed number in the drier site. No selection through female fitness on traits involved in floral attraction has also been reported in other plant species, particularly under resource-limited conditions (reviewed in Ashman and Morgan 2004) and, specifically, in the sister species *C. salviifolius* (Arista and Ortiz 2007). Our finding supports the prediction based on a common lack of pollen limitation in natural populations of *C. ladanifer*. More interestingly, we identified stabilizing selection for flower size through fruit set in the drier site over time. Several studies of female selection gradients on flower size have found evidence for this form of selection (CM Herrera 1993; Wright and Meagler 2004; Nattero *et al*. 2010b; Sahli and Conner 2011). Stabilizing selection on flower size through fruit set is not possible to explain based on pollinator attraction, because increased visit rates on larger flowers in *C. ladanifer* did not entail increased pollen deposition and maternal success. Cresswell (1998) suggested several possible mechanisms that might underlie stabilizing selection on floral traits, including the structural match between pollinators and flowers and resource allocation among fitness-related sinks.

From an evolutionary perspective, generalist plants to a multiple-pollinator environment may have less capacity to respond to directional selection on floral traits (Johnson and Steiner 2000; Gómez and Zamora 2006). In fact, these plants may be under stabilizing selection since medium-sized flowers may result the optimal values in their pollination environment it is an optimal under such conditions (reviewed in Kingsolver and Diamond 2011). In a recent study, Sahli and Conner (2011) identified floral adaptation to multiple pollinators by reporting strong selection on flower size through female fitness with an only pollinator but stabilizing selection with all pollinators combined in *Raphanus raphanistrum*. Therefore, flower size of *C. ladanifer* in the drier site may be near local fitness peaks being an optimal for the effectiveness to a multiple-pollinator environment (see Aigner 2001 for details).

Other alternative mechanism influencing in this form of selection could be based on resource limitation and floral costs. Larger flowers require higher investment in biomass and water for their construction and entail larger maintenance costs due to high respiration and transpiration rates (Galen 1999, 2000). From a resource economy perspective, allocation to floral structures entails indirect costs (Chapin 1989), i.e., negative effects on other floral functions such as reproductive output. Therefore increased allocation to flower size may divert resources away from fruits and reduce fitness on larger flowers (Cresswell 1998). This process could be far more relevant under Mediterranean hot and dry conditions, under which larger flowers are expected to entail increased maintenance costs (Galen 2000, 2005; Elle and Hare 2002). We know that floral transpiration significantly increases both with high temperatures and with flower size in *C. ladanifer* in our study area (AL Teixido and F Valladares, unpub. data). Likewise, corollas imply indirect costs by reducing fruit production a 24% relative to flowers with their petals removed and these costs increased with increasing individual mean flower size (AL Teixido and F Valladares, unpub. data). This fact could also explain the lower fruit set in the drier site since flowers were larger and conditions hotter and drier than in the wetter site.

Additionally, the presence of floral herbivores (i.e., florivores) could help to explain the existence of stabilizing selection on fruit set in our study populations. The incidence of florivory was significantly influenced by flower size in *C. ladanifer* and was higher than 30% on largest flowers in populations near the drier site (Teixido *et al*. 2011). Florivores may reduce fruit production by degrading the attractive properties of flowers for pollinator service or by direct consumption of viable gametes (Schemske and Horvitz 1988; Krupnick *et al*. 1999; Cardel and Koptur 2010). In this way, florivores can exert negative selective pressures on the same floral traits positively selected for pollinators (Galen 1999; Irwin 2006).

Overall, selection on largest flowers seems to be mostly limited in *C. ladanifer* through female fitness under the most stressful conditions, but selection for increasing flower size is feasible, keeping large flowers in this Mediterranean species. Selection on this trait could occur through male fitness and the correlation between flower size and pollinator visit rates to enhance pollen dispersal and siring success. Sex allocation theory suggests that selection on flower size should be stronger through male fitness whereas female fitness is resource-limited, especially in no pollen-limited species (Bateman 1948; Bell 1985; Ashman and Morgan 2004).

Despite the growing body of literature questioning the generality of the "male function" hypothesis (Wilson *et al*. 1994; Morgan and Conner 2001; Wright and Meagher 2004; Ashman and Morgan 2004; Hodgins and Barrett 2008), this seems to be the pattern for another *Cistus* species in the Mediterranean (Talavera *et al*. 2001; Arista and Ortiz 2007). However the directional selection detected in the wetter site in 2010 through seed number supports the idea that the relative strength of selection through female function in our study is context-dependent (Ashman and Morgan 2004). More interestingly, flower size showed temporal plasticity at each population in response to phenotypic selection detected the first year, indicating that female function is likely to be stronger than male function in modulating flower size. Therefore, total selection exerted on flower size in *C. ladanifer* may strongly depend on whether plants are more resource or pollen limited in their female reproduction.

Conclusions

The occurrence of stabilizing selection and the different patterns of selection on flower size and number through fruit and seed production demonstrate the importance of assessing nonlinear selection gradients and quantifying selection on different traits related to pollinator attractiveness through different female functions. We identified a positive relation between flower size and pollinator visit rates which was not translated into a differential female fitness and, consequently, into a directional selection for larger flowers. However, we detected positive selection on flower size through seed number in one population during the first study year. Additionally, we found evidence for negative selection on flower number through fruit set in the other population during the second study year. Therefore, in natural populations of *C. ladanifer* pollinator attraction alone does not appear to always drive selection on floral display through female fitness, but rather it varies with ecological context due to pollen and/or resource limitation. In fact, evidence of stabilizing selection on flower size through fruit set suggests that the evolution of this trait could be constrained by conflicting selective pressures acting on female function associated to resource limitation in this large-flowered Mediterranean species.

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CHAPTER 2

Disproportionate carbon and water maintenance costs of large corollas in hot Mediterranean ecosystems

Teixido AL, Valladares F.

Una versión de este manuscrito está en revisión en *New Phytologist*

Abstract

Larger corollas increase the reproductive success of entomophilous plants, but are also associated with increased carbon and water costs, especially under hot and dry conditions. We quantify maintenance costs of corollas (water and carbon) in large-flowered rockroses (*Cistus* spp.) in a Mediterranean ecosystem. We performed field studies of two coflowering sympatric *Cistus* species of contrasting corolla size to analyze water costs. Additionally, we used the larger-flowered species (*C. ladanifer*) to analyze the effects of intraspecific variation in corolla size on floral respiration and transpiration rates. Temperature and corolla area increased water maintenance costs, following an allometric relationship where transpiration increased non- linearly with corolla area. Larger flowers tended to heat less under strong irradiance than smaller ones in both species, especially in *C. ladanifer*, demonstrating a stronger transpirational cooling effect on larger flowers. In terms of carbon, temperature significantly affected floral respiration rates, which were not affected by corolla size. Daily water and carbon expenses of corolla were ca. 50% of those of leaves on an organ surface area basis. Our results suggest that water and carbon maintenance costs of large flowers in the Mediterranean impose significant constrains to corolla size, ecophysiologically favoring smaller-flowered individuals in these ecosystems.

Keywords: air temperature, allometric relationship, *Cistus albidus*, *Cistus ladanifer*, corolla area, floral respiration rates, floral temperature, floral transpiration rates

Introduction

Flower size is closely related to pollinator attraction because this trait plays a decisive role in the reproductive ecology of entomophilous plants. Larger corollas increase pollen deposition and removal and, as a result, increase both female and male fitness (Stanton *et al*. 1986; Young and Stanton 1990; Kudoh and Whigham 1998; Arista and Ortiz 2007; Nattero *et al*. 2010a). Accordingly, many studies have documented pollinator-mediated phenotypic selection towards larger corollas (e.g. Galen 1989; Campbell *et al.* 1991; Conner and Rush 1997; Totland 2001; Nattero *et al*. 2010a). However, since yet small-flowered plants persist in populations, Galen (1999) pointed out that a unilateral view of the evolution of flower size from a pollinator perspective was probably oversimplistic. Advantages associated with pollinator attraction can be offset by increased resource costs. For example, larger corollas are associated with greater requirements of biomass and water for floral development (Galen 1999; Carroll *et al*. 2001; Elle and Hare 2002), as well as with larger demographic costs due to water use under dry conditions (Galen 2000).

Carbon use during flowering plays a key role in floral attraction and respiratory demands of corollas may consequently be high and even exceed the daily production of photosynthates (Galen *et al*. 1993; Vemmos and Goldwin 1994; Lambers *et al*. 2008). Likewise, water is a limiting essential resource that is needed for the maintenance of corolla turgor and temperature (Galen 2005). Temperature regulation is especially important in flowers, as thermal optima for processes contributing to sexual reproduction are narrower than optima for growth functions (Lacey 1996; Erickson and Markhart 2002; Young *et al*. 2004). Work on floral temperature has usually focused on thermogenic flowers (reviewed by Seymour 2010) and flowers in alpine and subarctic climates where an increase of floral temperature has a positive effect on the development (Kevan 1975; Stanton and Galen 1989; Kudo 1995; Tsukaya *et al*. 2002; Galen 2006). However, floral overheating in hot climates can be damaging so transpirational cooling becomes crucial to minimize it (Galen 2005). However, water shortage in dry environments can lead to an efficient transpirational cooling. Heat and drought, acting together, can disrupt the normal performance of flowers, affecting both fruit and seed production (Konsens *et al*. 1991; Galen 2000; Erickson and Markhart 2002; Fang *et al*. 2010).

Mediterranean climates involve high temperatures and water and carbon shortages, imposing constraints in plant reproduction by speeding up development, shortening flowering duration (Larcher 2000; Thompson 2005a; Aragón *et al*. 2008) and, occasionally, delaying the initiation of flowering till the rainy season to maximize water use efficiency (Blionis *et al*. 2001; Verdú *et al*. 2002). Minimizing floral water loss by reducing corolla size should be potentially advantageous for plants living in these environments (Galen 2000, 2005; Elle and Hare 2002; J Herrera 2005). However, many common Mediterranean plants such as rockroses (*Cistus* spp.) do exhibit large corollas. Large flowers in these hot and dry environments may suffer increased respiration and excessive evaporative demand. While there can be stomata on corollas, evidence suggests that there are too few to regulate water loss (Galen *et al*. 1993; Patiño and Grace 2002; Nobel 2009). Some large-flowered plants in these and other hot and dry ecosystems show adaptations to prevent overheating and excessive water loss. Nocturnal flowering and pollination are features of several caperbushes (*Capparis* sp.) of semi-arid areas (Rhizopoulou *et al*. 2006), and most desert large-flowered cacti (Valiente-Banuet *et al*. 1997; Fleming *et al*. 2001). Floral cooling mechanisms appeared to be critical for the reproductive success of convolvulaceous large flowers in hot tropical environments (Patiño and Grace 2002).

In this study we quantify maintenance costs of corollas in terms of water and carbon in large-flowered rockroses (*Cistus* spp.) in a Mediterranean ecosystem. We refer here as carbon costs of corollas the carbon loss from photosynthates due to respiration in petals (as in Galen *et al*. 1993), while in other studies carbon costs of corollas were indirect costs taken as decreased photosynthetic rates of leaves associated to large corollas (Galen *et al*. 1999; Lambrecht and Dawson 2007). We expect that in dry Mediterranean ecosystems large corolla size coupled with a high ambient temperature involve significant costs in terms of carbon and water supplies. We hypothesize that large and numerous flowers are highly costly, not only regarding water use, but also, since corollas barely contribute to photosynthesis (Galen *et al*. 1993; Vemmos and Goldwin 1994), regarding carbon for the maintenance of respiring tissues.

We performed field studies of two *Cistus* species (Cistaceae) to test our hypotheses. Species of *Cistus* are iteroparous evergreen shrubs and their disc-shaped five-petaled flowers are hermaphrodite and depend on multiple insect pollinators to set fruits (Bosch 1992; J Herrera 1992; Talavera *et al*. 1993, 2001). Corolla size is positively related to intraspecific variation in pollinator visit rates, but this is not translated into a differential female fitness in the study species (Talavera *et al*. 2001; Arista and Ortiz 2007; A. L. Teixido and F. Valladares, unpublished). Specifically, we chose two coflowering sympatric species of contrasting corolla size, *C. albidus* (smaller) and *C. ladanifer* (larger), as a reference system to analyze water costs. *C. ladanifer*'s flowers are one of the largest in the Mediterranean (Arrington and Kubitzki 2003), reaching up to approx. 11 cm in diameter (Teixido *et al*., 2011). Differences in corolla size in sympatric species contribute to differential pollinator visit rates and pollen transfer (Aigner 2005; Ortigosa and Gómez 2010; see also Bosch

1992 for *Cistus*), reducing reproductive success of the smaller-flowered species (reviewed by Jones 2001). Additionally, we used *C. ladanifer* to analyze the effects of specific variation in corolla size on floral transpiration and respiration rates. High within-individual variation in corolla size in this species (48%, A. L. Teixido, pers. obs.) creates an opportunity to test whether the physiological costs of large flowers vary not only among species but also among individual flowers within a population and even within an individual plant.

Materials and methods

Study system

C. albidus and *C. ladanifer* are related species with contrasting flower size (mean: 5.30 ± 0.49 vs 9.19 ± 0.77 cm, respectively, n = 42). *C. albidus* is a shrub 40-100 cm tall that inhabits calcareous and dry soils. Flowering phenology spans from February to June and each plant produces purplish-pink flowers in terminal heads pollinated by beetles and bees (Bosch 1992; Muñoz-Garmendía and Navarro 1993). *C. ladanifer* is a shrub 100-250 cm tall that inhabits acid and dry soils. Flowering phenology spans from March to June and each plant produces solitary white flowers often exhibiting dark coloured spots at their bases (Muñoz-Garmendía and Navarro 1993). Flowers are mainly pollinated by bees, beetles and flies (Talavera *et al* 1993). Both *C. albidus* and *C. ladanifer* are self-incompatible, but self-pollinated flowers of the former species can set some seeds (Bosch 1992; J Herrera 1992). Flowers open synchronously each morning within populations and lose their petals in the afternoon.

The study was conducted between April and May of 2011 in the Madrid province, central Spain (39º53−41º09N 3º03−4º34W). Two different sites were utilised to study floral maintenance costs in *C. albidus* and *C. ladanifer*. Both differences in floral transpiration rates between these coflowering sympatric species and between sunny and cloudy days in *C. ladanifer* were monitored in San Agustín de Guadalix (740 m; 40º41N 3º36W; hereafter lower site) between April and May. The area is on a limestone and gypsum soil with granitic outcrops and is covered by an open scrubland vegetation on a south facing hilly slope. Climate is dry, with an annual mean precipitation of 567 mm and an annual mean temperature of 13ºC (Ninyerola *et al*. 2005). Floral respiration rates in *C. ladanifer* were monitored in Becerril de la Sierra (1120 m; 40º44N 3º57W; hereafter higher site) in May. This area is on a granite soil covered by a sparsely wood on a south facing slope. Climate is subhumid, with an annual mean precipitation of 820 mm and an annual mean temperature of 11ºC (Ninyerola *et al*. 2005).

Microclimate was recorded during the study period at each population to relate it to floral maintenance costs. Microclimatic variables measured were air temperature (ºC), air relative humidity (%), soil moisture (%) and solar irradiance (µmol PAR m^{-2} s^{-1}). Sensors were used for air temperature and relative humidity (Hobo H08-032-08, Onset, Pocasset, MA, USA) located 1 m above the ground. Readings of each sensor were recorded every 15 min with a Hobo H08-006-04 data-logger. Soil moisture was recorded at 3-5 points below the canopy in the understorey of each plant with sensors based on integrated TDR technology (ECH_2O EC-20, Decagon Devices, Pullman, WA, USA). Solar irradiance was recorded every 30 min with a quantum sensor (Apogee quantum sensor QSO-SUN, Logan, UT, USA). At each site, all microclimatic variables were significantly correlated each other (Table S1). Air temperature was positively correlated with solar irradiance and negatively correlated to air relative humidity and soil moisture. As a consequence, we only used air temperature as a predictor variable to explain floral maintenance costs.

Additionally, we recorded floral temperature of *C. albidus* and *C. ladanifer* in the lower site to evaluate whether that temperature is higher or lower than air temperature and whether it follows a predictable pattern regarding corolla size. Since floral temperature is likely to be influenced by both air temperature and corolla size we corrected this matter by comparing floral temperature with temperature of a reference surface. We used a wet white filter paper to evaluate differences between floral temperature and a filter paper temperature as a standard surface exposed to evaporation. The effect of corolla size on floral temperature is affected by transpiration, which in turn is affected by boundary layer resistance. A positive relationship indicates the importance of the boundary layer of large flowers to increasing floral transpiration rates. A negative relation would indicate a higher floral transpirational cooling in large flowers due a higher water uptake. To address all these assumptions filter paper was wet early in the morning before measuring floral temperature and transpiration rates. Then we recorded filter paper temperature every 30 min and floral temperature of every flower using an infrared thermal Camera (FLIR B2 IR Thermal Imager, 2006; FLIR Systems, Inc, Willsonville, OR, USA) right before measuring corolla size and transpiration rates. All images were subsequently analysed in ThermaCam QuickView v1.3 (FLIR Systems, Inc, 2005; Willsonville, OR, USA).

Effects of temperature and corolla size on floral transpiration rates and temperature

We carried out two different observational designs to evaluate the effects of temperature and corolla size on floral transpiration rates. First, we examined differences in floral transpiration between the coflowering sympatric *C. albidus* and *C. ladanifer*. During the coflowering peak in mid-April, we selected 21 similar adult plants per species. At each plant, we selected two mature, healthy, fully-expanded flowers of contrasting size on the same terminal branch. Since the flowers of these species lack solar tracking (i.e. heliotropism), all flowers were carefully selected to be sun-oriented. All plants and their paired-flowers were measured only once. We measured three plants per species per day between 08:00 and 14:00 h with the same number of plants every hour during seven consecutive days. Between those hours all flowers were completely open and after 14:00 h petals started falling off. On a petal of every flower we recorded stomatal conductance to water vapor, g_s (mmoles m^{-2} s^{-1}), with a portable steady-state porometer (Model SC-1, Decagon Devices, Pullman, WA, USA). Air and floral temperature and the other microclimatic variable data were recorded as described above (see "Study system"). Then floral transpiration rate on a surface area basis, E_f (g H_2O m^{-2} h^{-1}), was calculated from air and floral temperature, relative humidity and stomatal conductance to water vapor (g_s) data (see the Supporting Information presented in Methods for further details).

As fully-expanded organs in plants mainly lose water through their outer epidermis, corolla surface area (cm^2) was used to test for relationships between corolla size and water use after flower growth has ceased. To carry out this, we recorded corolla diameter (cm) after transpiration rates were taken on each paired-flowers. On the first 20 flowers measured per species we also excised one petal per flower. Excised petals were carefully unfolded and photographed on a black sheet to assess their surface area by means of image processing in ImageJ v1.43 (ImageJ, 2010; US National Institutes of Health, Bethesda, MD, USA, http,//imagej.nih.gov/ij/). Then we multiplied each value by five to estimate corolla surface area, since flowers have five petals. Corolla surface area and corolla diameter were closely correlated in both species (r_P = 0.912, P < 0.001, n = 20 for *C. albidus*; r_P = 0.837, P < 0.001, n =

20 for *C. ladanifer*). Then we indirectly estimated corolla surface area from corolla diameter for the remaining flowers by means of each given regression equation.

Second, we examined differences in floral transpiration rates depending on among and within-individual variation in corolla size and temperature variation between sunny and cloudy days in *C. ladanifer*. During the flowering peak between mid-April and early May we selected 35 similar adult individuals. Data of the 21 plants used in the differences in floral transpiration rates with *C. albidus* were added, so we selected the 14 remaining plants. Overall, 18 plants were measured in sunny days and 17 in cloudy ones. At each plant, we selected a pair of mature, healthy, fully-expanded flowers of contrasting size on the same terminal branch. Sun orientation followed the same criteria as described above even for cloudy days. All plants and their paired-flowers were measured only once between 10:00 and 13:00 h when the flowers were fully-expanded. All measurements including stomatal conductance to water vapor, floral temperature, microclimatic variables, transpiration rates and corolla size were recorded as described above. Corolla size significantly differed between paired-flowers within-individuals (mean ± SD: 58.86 ± 8.60 vs 52.29 ± 8.16 cm^2, respectively; Paired $t\text{-test}_{1,34} = 11.03$, $P < 0.001$), but did not between sunny and cloudy days (mean ± SD: 56.78 ± 9.90 vs 54.50 ± 7.98 cm^2, respectively; Student $t\text{-test}_{1,68} = 1.07$, $P = 0.290$). In cloudy days, air temperature was significantly lower (mean ± SD: 20.99 ± 1.86 vs 26.60 ± 1.28 ºC respectively; Student $t\text{-test}_{1,68} = -15.02$, $P < 0.001$) and air relative humidity significantly higher (mean ± SD: 42.24 ± 4.12 vs 28.58 ± 3.81%, respectively; Student $t\text{-test}_{1,68} = 17.00$, $P < 0.001$).

Effects of temperature and corolla size on floral respiration rates

During the flowering peak in late May in the higher site, we selected 20 similar adult individuals of *C. ladanifer*. Criteria of paired-flower selection and sun orientation were followed as described above. During six study days all plants and their paired-flowers were measured only one time between 10:00 and 13:00 h. Corolla size significantly differed between paired-flowers within-individuals (mean ± SD: 56.50 ± 9.26 vs 49.28 ± 9.03 cm^2, respectively; Paired t-$test_{1,19}$ = 6.35, $P < 0.001$). On a petal of every flower we recorded petal light respiration, R_f (µmoles CO_2 m^{-2} s^{-1}), using a LI-6400 infrared gas analyser. R_f was measured at constant and ambient CO_2 concentration (400 µmol CO_2 mol^{-1}) using the built-in LI-6400 CO_2 controller, and saturating light intensity (1500 µmol PAR m^{-2} s^{-1}) provided by the built-in LI-6400 blue-red light source. Monitored microclimatic variable (air temperature and relative humidity) were kept constant and close to ambient conditions each day. All remaining measurements including floral temperature, microclimatic variables and corolla size were recorded as described above.

Water and carbon maintenance costs of flowering

Comparisons of daily floral transpiration rates between *C. albidus* and *C. ladanifer* were made per flower and plant. At each species, we assessed the mean corolla size (m^2 ± SD) and the mean E_f (g H_2O m^{-2} h^{-1} ± SD). Then we recorded the mean daily E_f per flower (g H_2O $flower^{-1}$ d^{-1} ± SD) by considering the 6 hours (08:00 – 14:00 h) that flowers were open and functional. To calculate the mean daily E_f per plant (g H_2O $plant^{-1}$ d^{-1} ± SD) we recorded the mean number of open flowers at each species.

We also assessed the mean daily percentage of plant water and carbon consumed by corollas by comparing with that of leaves at the time of flowering in *C.*

ladanifer. To evaluate floral costs in the context of the plant, data of leaf transpiration, E_l (moles H_2O m^{-2} d^{-1}), leaf respiration, R_l (µmoles CO_2 m^{-2} s^{-1}), and leaf area index, LAI (m^2 m^{-2}), were taken from a previous study on *C. ladanifer* in our study populations during the spring of 1998 using the same equipment and sampling scheme (J. Navarro and F. Valladares, unpublished).

To evaluate daily water cost experienced by flowering in *C. ladanifer*, floral transpiration rates were compared to leaf transpiration rates. We measured the area (m^2) –as the area of an ellipse (π a b) – of all 21 individuals used in the study of floral transpiration between *C. albidus* and *C. ladanifer*. Then we recorded the total leaf area per plant (m^2 $plant^{-1}$) by multiplying the projected area of the crown by the mean leaf area index (LAI). Subsequently, from mean E_l (g H_2O m^{-2} h^{-1} ± SD) we recorded mean daily E_l per plant (g H_2O $plant^{-1}$ d^{-1} ± SD). We then compared mean daily E_f (as calculated above) with mean daily E_l and assessed the percentage of daily water expenditure by flowers. Furthermore, we evaluated the role of flowers in plant water status by considering how important daily water cost of flowering is relative to the area of corollas versus that of leaves. We recorded the mean floral display area (m^2 ± SD) by multiplying mean corolla size by mean number of open flowers and next we compared the daily water cost of corollas relative to their overall area.

To evaluate daily carbon cost involved in *C. ladanifer* flowering, floral respiration rates were compared to leaf respiration rates. In this case, we could not compare daily respiration rates since we only recorded measurements between 10:00 and 13:00 h, so we compared values during the hours of highest floral activity. We measured both the area (m^2) and the leaf area per plant (m^2 $plant^{-1}$) of all 20 individuals in the higher site as described above. Subsequently, from mean R_l (µmoles CO_2 m^{-2} s^{-1} ± SD) we recorded the mean R_l per plant (µmoles CO_2 $plant^{-1}$ s^{-1}

± SD). Then we calculated the mean R_f per plant (μmoles CO_2 $plant^{-1}$ s^{-1} ± SD) by considering the mean R_f per flower (μmoles CO_2 $flower^{-1}$ s^{-1} ± SD) and the mean number of open flowers. Thus, we compared both the mean R_f and the mean R_l per plant and recorded the percentage of carbon cost of flowering. We also evaluated the impact of flowers in plant carbon balance by considering how important carbon cost of flowering is relative to the area that corollas cover against leaf area. This step was carried out as described above for daily water cost of corollas, but we compared carbon cost of corollas in s^{-1} instead of d^{-1}.

Statistical analysis

We tested for differences in floral transpiration rates between coflowering species differing in flower size and between sunny and cloudy days in *C. ladanifer* by conducting two different three-way ANCOVAs. The assumptions of normality and homogeneity of variance were tested using Shapiro-Wilk's and Levene's tests, respectively. Then floral transpiration rates were log-transformed before statistical analysis to improve normality. In the first ANCOVA we included species (fixed factor), air temperature (covariate) and the interaction species × air temperature in the analysis. In the second one, we included day (fixed factor), corolla size (covariate) and the interaction day × corolla size. Within-individual variation in floral transpiration rates between paired-flowers with contrasting size in *C. ladanifer* was analysed by means of a paired *t*-test.

To test differences in floral temperature between species, air temperature and corolla size we used the difference between floral temperature and filter paper temperature as our response variable to correct the likely effect of the relation between air temperature and corolla size within-species in floral temperature. The assumptions of normality and homogeneity of variance were tested using Shapiro-Wilk's and Levene's tests, respectively. Then we first conducted a three-way ANCOVA with species (fixed factor), air temperature (covariate) and their interaction to test these effects on floral temperature. Second, we evaluated the effects of corolla size on floral temperature by conducting two different lineal regressions, one for each species.

We used regression analysis to test for significant effects of corolla size and air temperature on floral respiration rates in *C. ladanifer*. Since both predictive variables were not correlated with each other (r = 0.192, P = 0.235, n = 40), they were included together in a multivariate regression model. The assumptions of normality and homogeneity of variance to meet assumptions of regression were tested using Shapiro-Wilk's and Levene's tests, respectively. Within-individual variation in floral respiration rates between paired-flowers with contrasting size was analysed by means of a paired *t*-test.

ANCOVAs, paired *t*-test and regression analysis were performed using the R statistical package version 2.12.1 (R Development Core Team 2010).

Results

Effects of air temperature and corolla size on floral transpiration rates and temperature

Larger corollas of *C. ladanifer* versus *C. albidus* translated into significantly higher transpiration rates (mean ± SD: 64.1 ± 45.6 and 17.6 ± 6.4 g H_2O m^{-2} h^{-1}, respectively; Table 1). The influence of corolla size on floral transpiration was allometric since transpiration did not scale linearly with corolla area. Air temperature significantly affected floral transpiration rates but this effect differed depending on the species (Species × Air temperature significant, Table 1). The higher the temperature the higher the transpiration in both species (*r* = 0.721, *P* < 0.001, *n* = 42 for *C. albidus*; *r* = 0.876, *P* < 0.001, *n* = 42 for *C. ladanifer*), with similar floral transpiration rates at lower temperatures in the two species, but with increasing rates in the larger-flowered species *C. ladanifer* at higher temperature (Fig. 1).

Floral temperature averaged 26.9 ± 4.2 ºC for *C. albidus* and 26.5 ± 5.4 ºC for *C. ladanifer*. Air temperature averaged 23.4 ± 3.4 ºC and filter paper temperature averaged 15.4 ± 2.2 ºC. Flowers were significantly hotter than air (Paired t-test$_{1,79}$ = 7.30, *P* < 0.001) and, on average, 11.0 ± 3.6 ºC hotter than filter paper. Difference between floral temperature and filter paper temperature used in our analysis did not significantly differ between species (Table 2), so interspecific differences in corolla

Table 1. ANCOVA testing for the effects of species, air temperature and the interaction between species and air temperature on corolla transpiration rates of *C. albidus* and *C. ladanifer*. Significant P values are marked in bold.

Effect	d.f.	MS	*F*	*P*-value
Species	1	3372.1	61.0	<**0.001**
Air temperature	1	6774.4	122.5	<**0.001**
Species × Air temperature	1	4993.5	90.3	<**0.001**
Error	80	55.3		

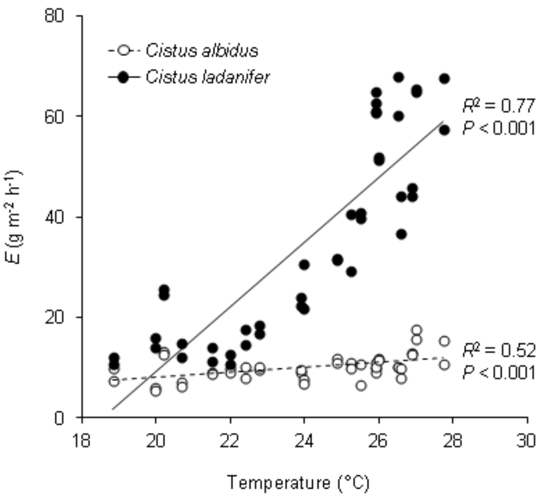


Fig. 1. Corolla transpiration rates (*E*) versus air temperature in the two coflowering sympatric rockroses *Cistus albidus* and *C. ladanifer*. Each point represents an independent measurement on a different flower, taking two flowers on each individual plant

size did not affect floral temperature. The only effect on floral temperature was air temperature, which similarly affected both species (Species × Air temperature not significant, Table 2). Intraspecific variation in corolla size did not explain differences in floral temperature among-individuals either, but larger flowers tended to heat less than smaller ones in both species, especially in *C. ladanifer* (R^2 = 0.07, β = -0.11±0.07, $F_{1,39}$ = 2.14, P = 0.105 for *C. albidus*; R^2 = 0.12, β = -0.09±0.05, $F_{1,39}$ = 3.57, P = 0.068 for *C. ladanifer*), demonstrating a stronger transpirational cooling effect on larger flowers, matching the allometry between corolla size and floral transpiration rates.

The intraspecific study in *C. ladanifer* showed significant differences in floral transpiration rates between sunny and cloudy days (Table 3). On average, in sunny days water loss from corollas was 108.7 ± 6.4 g H_2O m^{-2} h^{-1}, about five-fold than in cloudy days, when corollas transpired 24.6 ± 16.8 g H_2O m^{-2} h^{-1}. Both the effect of corolla size and its interaction with the type of day on floral transpiration rates were marginally significant (Table 3). Larger-flowered individuals tended to spend more water in sunny days (Fig. 2). Within-individual variation in corolla size also significantly affected floral transpiration rates (Paired t-test$_{1,34}$ = 4.52, P < 0.001). Between paired-flowers differing in corolla size larger flowers transpired, on average, 72.4 ± 54.8 g H_2O m^{-2} h^{-1} whereas smaller flowers transpired 63.3 ± 47.2 g H_2O m^{-2} h^{-1}.

Table 2. ANCOVA testing for the effects of species, air temperature and the interaction between species and air temperature on differences between floral temperature of *C. albidus* and *C. ladanifer* and filter paper temperature

Effect	d.f.	MS	*F*	*P*-value
Species	1	0.9	0.3	0.725
Air temperature	1	62.3	8.2	**0.006**
Species × Air temperature	1	1.0	0.4	0.718
Error	80	7.6		

Effects of air temperature and corolla size on floral respiration rates

Respiration rate of corollas of *C. ladanifer* averaged -0.9 ± 0.4 µmol CO_2 m^{-2} s^{-1}. Overall, air temperature and corolla size explained 15% of variation in respiration (R^2 = 0.15, $F_{1,39}$ = 3.14, P = 0.049). This was especially due to air temperature, which showed a marginal significance in the whole model (β = -0.152 ± 0.084, P = 0.055). Air temperature was positively related to floral respiration rates (Fig. 3), but coefficient β was negative because CO_2 exchange was negative respiration. Corolla size itself was not significant (β = -0.008 ± 0.006, P = 0.187), indicating that there is not an allometric relationship with floral respiration rates. Within-individual variation in respiration did not differ between paired-flowers with contrasting size (mean ± SD: -1.0 ± 0.4 vs -0.9 ± 0.4 µmol CO_2 m^{-2} s^{-1}, for large and small flowers, respectively;

Table 3. ANCOVA testing for the effects of the type of day (sunny vs cloudy), corolla size and the interaction between day and corolla size on corolla transpiration rates of *C. ladanifer*

Effect	d.f.	MS	*F*	*P*-value
Day	1	1990.4	14.1	**<0.001**
Corolla size	1	180.2	3.9	0.053
Day × Corolla size	1	511.0	3.6	0.062
Error	66	0.3		

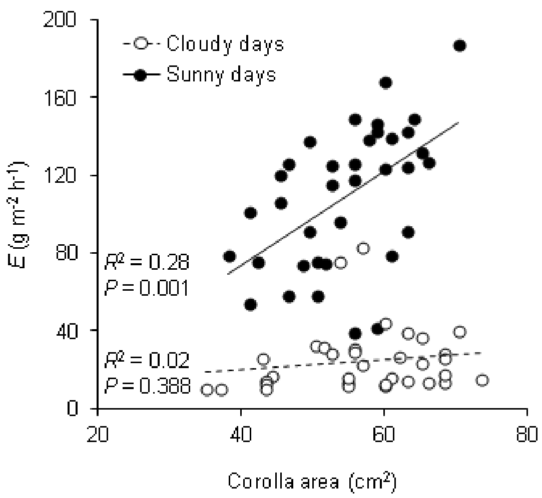


Fig. 2. Interaction between type of day (cloudy vs. sunny) and corolla area in corolla transpiration rates in *Cistus ladanifer*. Each point represents an independent measurement on a different flower, taking two flowers on each individual plant

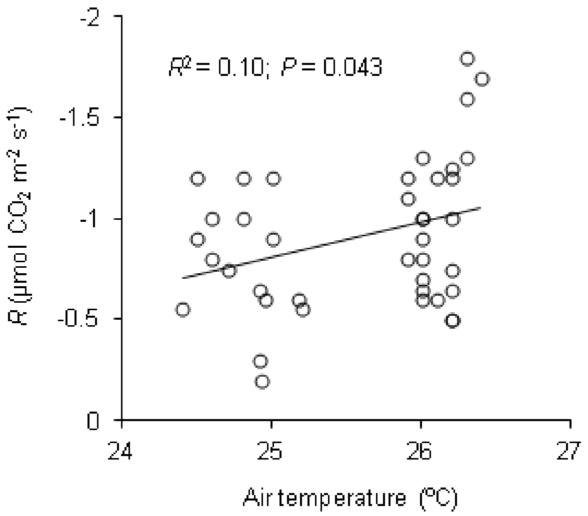
Paired *t*-test$_{1,19}$ = -1.829, P = 0.083). However, larger-flowered individuals involved increased carbon costs per flower (µmol CO_2 flower^{-1} h^{-1}; R^2 = 0.33, $F_{1,39}$ = 18.47, P < 0.001).

Water and carbon maintenance costs of flowering

On average, one flower of *C. albidus* transpired 0.2 ± 0.1 g H_2O d^{-1} whereas one flower of *C. ladanifer* transpired 1.9 ± 1.4 g H_2O d^{-1}. Thus, water costs of floral transpiration increased ten-fold in the larger-flowered species *C. ladanifer*. When considering plant daily flowering this relationship slightly decreased since, on average, *C. albidus* displayed more flowers than *C. ladanifer* (mean ± SD: 39.6 ± 37.7 vs 29.2 ± 16.7 daily open flowers, respectively). Overall, daily flowering involved 7.6 ± 2.4 g H_2O plant^{-1} d^{-1} in *C. albidus* versus 56.7 ± 41.7 g H_2O plant^{-1} d^{-1} in *C. ladanifer*.

Water and carbon maintenance costs of corollas of *C. ladanifer* relative that of the foliage of the plant are shown in Table 4. Instantaneous transpiration rates of corollas were more than two times higher than those of leaves on a surface area basis of the organ. In terms of carbon, respiration rates of corollas reached up to

Fig. 3. Corolla respiration rates versus air temperature in *Cistus ladanifer*. Each point represents an independent measurement on a different flower, taking two flowers on each individual plant



64% of leaf respiration rates per unit of organ area and time. Considering the total floral display and leaf surface area of the plant, transpiration and respiration of corollas accounted for 5.7% and 3.2%, respectively, of the whole water and carbon spent daily for the maintenance of the aboveground part of the plant. On average, corollas transpire and respire ca. 50% than leaves on an organ surface area basis. This value is noticeable because corollas remain open and functional only about 6 h.

Discussion

Our study demonstrates the importance of corolla size and air temperature on floral maintenance costs in large-flowered plants in a Mediterranean environment. We found significant species differences on transpiration rates of corollas in natural populations of the coflowering sympatric *Cistus albidus* and *C. ladanifer* differing in flower size. We also found that these transpiration rates strongly increased with air temperature in the larger-flowered species *C. ladanifer*. In agreement with this we found evidence for an allometric relationship between size and transpiration of corollas. Furthermore, we detected a reduced overheating in larger corollas with increasing air temperature indicating that corolla size affects transpirational cooling (i.e., water used as a thermoregulator mechanism). However, transpiration in corollas did not counteract the effect of radiation since floral temperature was always higher than air temperature. We also found a significant effect of air temperature, but not of corolla size, on floral respiration rates on corolla surface area basis in *C. ladanifer*. Overall, we estimated that 1 m^2 of corollas used about half of the water and carbon spent by 1 m^2 of foliage of a plant in our study populations. Our results indicate that both water and carbon losses by functional corollas are elevated, particularly in large-flowered Mediterranean species.

Table 4. Water and carbon maintenance costs of flowering in comparison with that of leaves in *C. ladanifer*. LAI and mean plant area of each population are provided. Mean transpiration rates (*E*) were only estimated in the lower site and mean respiration rates (*R*) were only estimated in the higher site

	Lower site				Higher site			
	LAI (m^2 leaf·m^{-2})	Mean area (m^2·$plant^{-1}$)	Mean *E* (g·m^{-2}·h^{-1})	Mean *E*/plant/d (g·$plant^{-1}$·d^{-1})	LAI (m^2 leaf·m^{-2})	Mean area (m^2·$plant^{-1}$)	Mean *R* (µmoles·m^{-2}·s^{-1})	Mean *R*/plant/d (µmoles·$plant^{-1}$·s^{-1})
Corollas	–	0.17 ± 0.02	64.13 ± 45.58	56.67 ± 41.73	–	0.15 ± 0.03	-0.91 ± 0.24	-0.14 ± 0.07
Leaves	0.94	1.43 ± 0.96	29.26 ± 16.25	1003.54 ± 671.36	1.50	2.89 ± 0.91	-1.42 ± 0.47	-4.33 ± 1.37

Water costs of corollas

Previous works have revealed that there is an increase in transpiration associated with flowering, thus making flowering particularly expensive in the use of water (Nobel 1977a; Whiley *et al*. 1988; Galen *et al*. 1999; Galen 2006). The relevance of water spent by transpiration in corollas is dependent, among other factors, on their size and on the environmental conditions. Galen *et al*. (1999) reported a positive relationship between corolla area and rates of water loss by fully-expanded flowers in the alpine wildflower *Polemonium viscosum* (mean: 0.024 g H_2O flower^{-1} h^{-1}). Other studies have reported higher rates of water loss in corollas of large-flowered trees in tropical dry forests flowering during the dry season (mean rates: 0.05 – 0.115 g H_2O flower^{-1} h^{-1}; Chapotin *et al*. 2003) and of large-flowered cacti in California (mean rates: 0.07 – 0.46 g H_2O flower^{-1} h^{-1}; Nobel and De la Barrera 2000; De la Barrera and Nobel 2004b; E. De la Barrera, pers. comm.). Mean floral transpiration rate in *C. albidus* found here (0.03 g H_2O flower^{-1} h^{-1}) was within the lower range of previous reports, while that of *C. ladanifer* (0.32 g H_2O flower^{-1} h^{-1}) was within the highest range reported previously.

The high rates of water loss by corollas of *C. ladanifer* indicate the high potential for transpirational cooling of these large corollas in the Mediterranean. In agreement with the allometric relationship reported in our study, Galen (2006) found that *Ranunculus adoneus* reduced excess temperature in solar tracking flowers, but not in non-tracking flowers at a water cost (transpiration). We did not find any effect of the species on floral temperature between *C. albidus* and *C. ladanifer* despite their contrasting flower size, suggesting that transpirational cooling was more effective in *C. ladanifer*. In contrast, Patiño and Grace (2002) reported that the species with larger corollas developed higher excess temperatures than those of medium and small-sized corollas among tropical convolvulaceous flowers.

Despite that large corollas appear to be expensive in terms of water, our reference system (filter paper) revealed that their transpiration rates were not maximal, suggesting constrains in floral transpiration. This limited transpiration can influence two essential aspects of the reproductive ecology of these plants. First, regulation of floral transpiration improves the water economy of the whole plant, especially important in a Mediterranean environment (Thompson 2005a). Second, temperature increase may provide a better microclimate for visiting insects (Dyer *et al*. 2006; Sapir *et al*. 2006; Rands and Whitney 2008), although the primary benefit of increased temperatures for the plant would be speeding up pollen tube growth, viability of fertilized ovules and, ultimately, seed and fruit set (Kjellberg *et al*. 1982; Erickson and Markhart 2002).

From an evolutionary perspective, the high water costs of corollas in hot and dry environments can influence flower size. Corolla tube length of long-flowered gilly, *Ipomopsis longiflora*, was affected by both high temperature and water stress (Villarreal and Freeman 1990). Drought conditions favor selection towards small-flowered plants through female fitness in the alpine wildflower *Polemonium viscosum* (Galen 2000) and plants producing fewer flowers with shorter corollas in the short-lived perennial *Lobelia siphilitica* (Caruso 2006). Likewise, non-irrigated plants produced shorted corollas in the long and large-flowered perennial *Datura wrightii* (Elle and Hare 2002) and smaller flowers with less nectar in the Indian tobacco, *Nicotiana quadrivalvis* (Halpern *et al*. 2010). In the Mediterranean, a pattern of increasing corolla size with milder conditions was reported both in the drought-tolerant shrub *Rosmarinus officinalis* in southern Spain (J Herrera 2005) and in the daffodil *Narcissus triandrus* along an ecological gradient (Barrett *et al*. 2004). Between coflowering sympatric species differing in flower size, the smaller-flowered species could benefit from lower floral costs and reallocate greater resources to reproductive output. Under experimental conditions we detected lower indirect costs of corollas in terms of fruit production in *C. albidus* than in *C. ladanifer*, counteracting attractiveness to pollinators (A. L. Teixido and F. Valladares, unpublished).

Carbon costs of corollas

Corollas account for the higher respiration rates in the perianth as a whole but these rates are low relative to those of carpels and stamens (Vemmos and Goldwin 1994; Seymour and Schultze-Motel 1998; Seymour and Matthews 2006; Seymour *et al*. 2010). For fully-expanded corollas, mean net carbon assimilation rates approached zero in the alpine snow-buttercup *Ranunculus adoneus* (-0.25 µmol CO_2 m^{-2} s^{-1}; Galen *et al*. 1993). Therefore, mean respiration rate recorded in corollas of *C. ladanifer* (-0.91 µmol CO_2 m^{-2} s^{-1}) suggests high respiratory demands in this large-flowered Mediterranean species. Higher floral respiration rates have been reported in thermogenic flowers by modulating the rate of heat production to remain much warmer than ambient temperature (Seymour 2010). Explanations for these high rates include enhancement of scent production, protection from freezing and a thermal reward to pollinators (Seymour 2001). Species with thermogenic flowers were the only group where the effects of ambient temperature and flower size on corolla respiration rates were studied. Logically but contrary to our results, Seymour (2001) reported that an increase in ambient temperature decreases the rate of carbon degradation in those species, since respiration was coupled to heat production. We did not found evidence of any allometric effect on respiration rates of corolla size. In contrast with our results, Seymour (2010) found that heat production (i.e., respiration rate) decreased with flower size to the -0.42 power in 15 species of Araceae.

Carbon costs in relation to corolla size were indirectly measured in water use studies of flowers (Galen *et al*. 1999; Galen 2000). Galen and colleagues reported that leaf photosynthetic rate at the time of flowering significantly declined with increasing corolla size in *Polemonium viscosum* under dry conditions. Therefore, water use by large corollas influenced leaf stomata closure, constraining carbon gain (see also Lambrecht and Dawson 2007). This connection between water economy and carbon balance could have evolutionary consequences on flower size in our populations of *C. ladanifer*. As this large-flowered species under hot and dry conditions increases carbon and water losses at increasing corolla sizes, reduced leaf photosynthesis translates into additional carbon costs by missed opportunity for carbon gain and eventually giving rise to dysfunctional corollas and lack of photosynthates to set fruits (Galen 2000; Lambers *et al*. 2008).

Whole plant water and carbon impacts at flowering

Large flowers of *C. ladanifer* use significant amounts of water and carbon per unit area through their corollas relative to those of leaves (47 and 62% of the water and carbon spent by leaves, respectively and on an organ surface area basis). Hence, maintenance costs by flowers along the flowering period represent a significant effort. This effort was actually underestimated because we did not consider construction costs, nectar costs, nor transpiration and respiration by calyx, stamens and carpels, which also use high amounts of water and energy (Blanke and Lovatt 1993; Galen *et al*. 1993, 1999; Patiño and Grace 2002; De la Barrera and Nobel 2004a). This whole plant analysis of flower costs is particularly scant in the literature. Some studies had also observed that water loss from flowers can exceed that of leaves in hot and dry environments in *Persea americana* (Whiley *et al*. 1988; Blanke and Lovatt 1993) and in *Agave deserti* (Nobel 1977a). Additionally, Nobel (1977b) reported that flowers of the CAM barrel cactus *Ferocactus acanthodes* use 300 g of water along the flowering, about a 2% of the annual transpiration rate of the plant as a whole.

In the Mediterranean, water and carbon shortage are among the main factors limiting plant survival so conservative resource use strategies seem to be adaptative for evergreen woody plants in these ecosystems (Valladares *et al*. 2000; Thompson 2005a). Large and costly flowers not only contrast with these strategies, but also with resource-cost hypotheses on floral evolution that postulate that reduced corollas are advantageous under stressful conditions (Galen 1999, 2005). Water and carbon costs of flowering in *C. ladanifer* are, however, consistent with its low efficiency in photosynthetic water use. This drought-avoiding schlerophyllous shrub fully exploits the brief favorable conditions of spring to maximize carbon assimilation at the expense of high rates of leaf transpiration (Valladares *et al*. 2004). Large corolla size in this species fits well with this water spending strategy of the plant that can be afforded only during a relatively brief spring period.

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Supporting Information

Methods

Floral transpiration rate on a surface area basis, E_f (g H_2O m^{-2} h^{-1}), was calculated from energy balance models using air and floral temperature, relative humidity and stomatal conductance to water vapor (g_s) data by means of the formula,

$$E_f\ (\text{g H}_2\text{O m}^{-2}\ \text{h}^{-1}) = \frac{[\text{water vapor concentration in flower (g m}^{-3}) - \text{water vapor concentration in air (g m}^{-3})]}{[\text{stomatal resistance (s m}^{-1}) + \text{boundary layer resistance (s m}^{-1})]} \times 3600\ \text{s}$$

where water vapor concentration in flower is,

saturation vapor pressure at flower temperature × 216.68 (Reynolds constant, Re) / floral temperature (ºC) + 273;

saturation vapor pressure at flower temperature was calculated using the formula,

$6.1078^{[17.269 \times \text{floral temperature (ºC)}] / [237.3 + \text{floral temperature (ºC)}]}$;

water vapor concentration in air is,

relative humidity × $6.1078^{[17.269 \times \text{air temperature (ºC)}] / [237.3 + \text{air temperature (ºC)}]}$;

stomatal resistance is,

100 / [stomatal conductance to water vapor, g_s (moles m^{-2} s^{-1}) / (0.446 × {273 / [floral temperature (ºC) + 273]})];

and boundary layer resistance for water vapor is,

1 / [(1 × Laminar flow) + (1 / Free convenction)], where laminar flow is 55.83 (s m^{-1}) and free convenction is 263.97 (s m^{-1}) using a characteristic dimension of 3.5 cm.

Table S1. Spearman's rank correlation coefficients among all microclimatic variables both in lower (first values in every cell) and higher site (second values in every cell). *P < 0.1; **P < 0.01; ***P < 0.001; n = 35 for all correlations

	Air relative humidity	Solar irradiance	Soil moisture
Air temperature	-0.769*** -0.412*	0.466** 0.562***	-0.401* -0.623***
Air relative humidity		-0.419* -0.619***	-0.532** -0.819***
Solar irradiance			-0.491* -0.616***

CHAPTER 3

Large and abundant flowers increase indirect costs of corollas: a study in coflowering sympatric Mediterranean species with contrasting flower size

Teixido AL, Valladares F.

Una versión de este manuscrito está en revisión en *Oecologia*

Abstract

Large floral displays receive more pollinator visits but they also involve higher construction and maintenance direct costs. From a plant resource economy large displays also translate into indirect costs, i.e., negative effects on functions such as reproductive output. Two pairs of coflowering sympatric Mediterranean *Cistus* with contrasting flower size were used to test the following hypotheses, (1) corollas entail significant indirect costs in terms of fruit set and seed production, (2) these costs increase with increasing floral display, and (3) these costs increase in larger-flowered species between sympatric paired-species. We compared fruit and seed production among petal-removed flowers (R flowers) and unmanipulated, control flowers (C flowers). Additionally, we evaluated the influence of mean floral display on the relative loss of fruits and seeds between C and R flowers. Fruit and seed production were significantly higher in R flowers of all study species. Within-species, there was a negative correlation between relative loss of fruits and individual mean flower size. In one pair of coexisting species, there was a higher loss of fruits in the large-flowered species, which also exhibited a higher loss of fruits to mean number of open flowers ratio. In the other species pair, the relation between loss of fruits and mean flower size was higher in the larger-flowered species. These results indicate that Mediterranean environments impose constrains on flower size opposing selective pressures by pollinators. This is a complex evolutionary scenario for coflowering sympatric generalist species with contrasting flower size, challenging attractiveness by the magnitude of indirect costs.

Keywords: *Cistus albidus, Cistus ladanifer*, floral display, fruit production, petal removal, relative loss of fruits

Introduction

Attractiveness to pollinators plays a decisive role in the reproductive ecology of entomophilous plants. Floral display (i.e., number and size of open flowers) has been broadly associated with higher pollinator attraction and, as a result, an increase in both male and female fitness (Stanton *et al*. 1986; Young and Stanton 1990; Kudoh and Whigham 1998; Thompson 2001; Harder and Johnson 2005; Arista and Ortiz 2007). Accordingly, many studies have documented pollinator-mediated phenotypic selection towards larger floral displays (Galen 1989; CM Herrera 1993; Conner and Rush 1997; Hodgins and Barrett 2008; Nattero *et al*. 2010a). However, larger displays also require higher investment in biomass and water for their construction (Galen 1999; Halpern *et al*. 2010), and entail larger maintenance costs due to high respiration and transpiration rates (Vemmos and Goldwin 1994; Galen *et al*. 1999; Galen 2000). Abiotic factors such as water stress may set further limits to the number and size of flowers (Galen 1999; Caruso 2006).

From a resource economy perspective, carbon, nutrients, water and other resources invested in floral structures can be considered as direct costs (Chapin 1989; Ashman and Schoen 1997). A fundamental tenet of plant resource economy is that direct costs translate into indirect costs (Chapin 1989), i.e., negative effects on other floral functions such as reproductive output. Indirect costs have been empirically shown for nectar production (Pyke 1991) and floral longevity (Ashman and Schoen 1997; Castro *et al*. 2008). Andersson (1999, 2000, 2001, 2005) has pioneered the assessment of indirect costs of floral attractiveness. He generally reported an increase in fruit and seed production from perianth/ray removal treatments compared to non-manipulated control flowers, under controlled, garden conditions. A next logical, but underexplored, step is to ascertain whether those indirect costs are dependent on floral display. Overall, evidence in this sense would provide support to the predictions of dry mass and water investment-based arguments (Galen 1999; Caruso 2006).

The objective of this study is to evaluate the indirect costs of floral display in two pairs of coflowering sympatric *Cistus* species differing in flower size; *Cistus* is a large-flowered genus widespread in Mediterranean ecosystems (Arrington and Kubitzki 2003). We chose this study system due to potential interspecific differences both in fitness and floral physiological costs between coflowering sympatric species with contrasting flower size. Differences in flower size in sympatric species contribute to differential pollinator visit rates and pollen transfer (Aigner 2005; Vespirini and Pacini 2010; see also Bosch 1992 for *Cistus*), potentially reducing reproductive success of the smaller-flowered species (reviewed by Jones 2001). However, smaller-flowered species entail lower floral costs (AL Teixido and F Valladares, unpublished) and, consequently, could reallocate greater resources to reproductive output. In Mediterranean ecosystems, high temperatures and water shortage constrain plant reproduction (Larcher 2000; Thompson 2005a; Aragón *et al*. 2008). In such stressful conditions, large flowers are expected to entail increased maintenance costs (Galen 2000, 2005; Elle and Hare 2002). Specifically, we evaluate here the direct costs of production of corollas and, next, the indirect costs to three different levels, between flowers whose petals were removed and non-manipulated flowers, between plants within-populations differing in floral display, and between coflowering sympatric species of contrasting flower size.

The specific goals of this study were (1) to analyse allocation of resources to corollas in terms of dry mass, N and P; (2) to verify if corollas entail sizable indirect costs in terms of fruit set and seed production; (3) to determine whether those indirect costs increase with increasing flower size; (4) to determine whether those indirect costs increase with increasing number of open flowers; and (5) to evaluate whether those indirect costs differ between each of the coflowering sympatric pairs of species with contrasting flower size.

Materials and methods

Species and study area

Cistus (Cistaceae) is one of the most characteristic genera of the Mediterranean flora as part of the shrub layer of schlerophyllous forests. Flowers have five pink or white petals, range 2-12 cm in diameter, and are hermaphrodite, self-incompatible and last one day (Bosch 1992; J Herrera 1992; Muñoz-Garmendía and Navarro 1993). Flowering opening occurs synchronously each day within the populations. Fruits are globular woody capsules 4-15 mm in size containing numerous (approx. range, 100-1000) seeds 1-2 mm (Muñoz-Garmendía and Navarro 1993). We chose two pairs of coflowering sympatric *Cistus* species differing in flower size at two different ecological ranges in Madrid region, central Spain (40º27′40″N, 3º27′10″W). The first pair of species was composed by *C. albidus* and *C. ladanifer* (hereafter, Calb-Clad; mean flower size: 5.30 ± 0.49 vs 9.19 ± 0.77 cm, respectively, n = 250). This pair of species was monitored in San Agustín de Guadalix (740 m; 40º41′10″N, 3º36′00″W) in April 2011. The area is on a limestone and gypsum soil and is covered by an open scrubland vegetation on a south facing hilly slope. Climate is dry, with an annual mean precipitation of 567 mm and an annual mean temperature of 13ºC (Ninyerola *et al*. 2005; n = 20 years). The second pair of species was composed by *C. laurifolius* and *C. ladanifer* (hereafter, Clau-Clad; mean flower size: 5.95 ± 0.46 vs 9.15 ± 0.71 cm, respectively, n = 250). This pair of species was monitored in Puerto de Canencia (1,300 m; 40º49′20″N, 3º45′50″W) in June 2011. This area is on a granite soil covered by a sparsely wood on a south facing slope. Climate is subhumid, with an annual mean precipitation of 867 mm and an annual mean temperature of 9ºC (Ninyerola *et al*. 2005; n = 20 years).

Resource allocation to corollas

At each site and during the flowering peak of every species, 15 similar plants were haphazardly selected. *C. ladanifer* was only monitored in San Agustín de Guadalix. At each plant, two flower buds were randomly harvested and kept in alcohol 70%. In the lab, each flower was divided into pedicel, sepals, petals, carpels and stamens. All the portions were subsequently oven-dried for 2 days at 60ºC and weighed to the nearest 0.1 mg with a microbalance (MX5, Mettler-Toledo International, Greifensee, Switzerland). For N and P content analysis, floral structures were pooled per species if necessary, just to weigh ≥ 2.5 mg, and then digested in sulphuric acid and analysed for total N and P by means of a SKALAR San++ Analyzer (Skalar, Breda, The Netherlands).

Experimental design

At each site and during the coflowering peak, other 10 similar plants per species were randomly selected and tagged. On each plant, we manipulated flowers by removing all petals (R flowers) and left other unmanipulated flowers as control (C flowers). We selected 5 R and C flowers every day per plant and species. Overall, 25 R and C flowers were randomly selected and tagged at every plant. Petal removal was conducted at predawn on flower buds about to open, with a pair of tweezers. All R and C flowers were hand-pollinated to avoid differences in pollen receipt between treatments. Hand-pollination was carried out with a fresh pollen mixture collected from 5 different individuals within a 5 m radius of the recipient flower and then sufficient outcross pollen was deposited on the stigma with a paintbrush three hours after anthesis in order to ensure the stigmatic receptivity in these species (J Herrera 1992). On every control flower, we noted flower diameter (cm) and, only in coflowering sympatric species, we noted the total number of open flowers every day at each plant. In early July, all ripe fruits were picked before seed dispersal and fruit set scored for R and C flowers and per plant. Ten fruits both R and C flowers per plant were randomly selected to record the mean number of seeds per fruit.

Statistical analysis

For each pair of species, we tested the effects of species (fixed factor), plant (random factor nested to species) and petal removal (fixed factor), along with the interaction species × petal removal, on fruit set and mean seed number. The interaction, if significant, indicates variation in production of fruits and/or seeds between coflowering species. Effects on fruit set were tested by fitting Generalized Linear Mixed Models (GLMMs). We assumed a binomial error distribution with a logit link function. We used the restricted maximum likelihood (REML). Effects on mean seed number were tested by means of ANOVAs. The GLMMs were performed using the GLIMMIX Macro of SAS (SAS Statistical Package 1990; SAS Institute, Cary, NC, USA) and the ANOVAs were performed with R (R Development Core Team 2010).

For every plant in each species we calculated the relative loss (RL_i) of fruits and seeds between R and C flowers using the formula,

$RL_i = (C_i – R_i) / R_i$

where C_i and R_i are the fruit set or the mean seed number of plant i in C and R flowers, respectively. For each pair of species, we assessed the influence of species and floral display on the relative loss of fruits or seeds by conducting four five-ways ANOVAs, two at each pair of species, where the dependent variables were relative loss of fruits and relative loss of seeds, respectively. We included species (fixed factor), flower size and number of open flowers (covariates), and the interactions species × flower size and species × number of open flowers. Significant interactions show different correlations between relative loss of fruits and/or seeds with mean individual flower size and/or number of open flowers between coflowering species. If there is a higher correlation in the larger-flowered species and/or with higher number of open flowers indicates that, with larger floral displays, small increases in these traits entail large increases in relative loss of fruits and/or seeds.

Results

Resource allocation to corollas

Allocation of resources to corollas differed among the study species (Table 1). Production of large showy corollas of *C. ladanifer* involves a high investment of resources so floral attractiveness to pollinators of this species is more costly in terms of C, N and P. These corollas allocate up to five-fold the amount of resources in their production than the other *Cistus* species. However, corollas of this species have lower concentrations of N and P than *C. albidus* and *C. laurifolius*. Overall, the study species invest in production of corollas about 27% of floral dry mass, 23% of N and 24% of P.

Effects of petal removal

Fruit set significantly differed between C and R flowers and between plants in both pairs of species (Table 2). In all cases, fruit set was significantly higher in R,

Table 1. Mean ± SD diameter (n = 250 flowers) and allocation of resources to corollas (%) in the three species of *Cistus* of our study in terms of dry mass, nitrogen and phosphorous (n = 15 flowers). Percentage (%) of allocation is in brackets. Concentrations are given in mmol/g dry mass

Species	Diameter (cm)	Dry mass (mg)	Nitrogen (µg)	Phosphorous (µg)	[Nitrogen]	[Phosphorous]
C. albidus	5.30 ± 0.49	14.9 ± 2.8 (20.9)	242.7 ± 4.6 (18.4)	14.5 ± 1.0 (19.7)	116.3 ± 2.2	3.1 ± 0.2
C. ladanifer	9.19 ± 0.77	65.1 ± 8.0 (35.3)	896.0 ± 391.3 (27.8)	57.3 ± 35.2 (28.6)	98.3 ± 42.9	2.8 ± 1.7
C. laurifolius	5.95 ± 0.46	13.2 ± 2.3 (27.0)	213.1 ± 50.2 (22.8)	11.9 ± 3.4 (25.2)	115.3 ± 27.2	2.9 ± 0.8

compared to C, flowers (Fig. 1a, b). Comparing coflowering species, larger-flowered ones tended to decrease fruit set between C and R flowers (15 vs 22% for Calb-Clad and 9 vs 12% for Clau-Clad), but these differences were only significant in the first species pair (Species × Petal removal marginally significant, Table 2). Seed production significantly increased in large-flowered species and differed between plants within species (Fig. 1c, d; Table 3). All species quantitatively increased seed production in R flowers, but differences between C and R were only significant for the

Table 2. Generalized linear mixed model for fruit set (250 C flowers and 250 R flowers from 10 plants per species). Plant (Species), as random factor, was tested with Wald Z-test, and fixed factors with Type III F-tests. Significant P values are marked in bold

Effect	Calb-Clad				Clau-Clad			
	d.f.	Estimate ± SD	*Test value*	*P*-value	d.f.	Estimate ± SD	*Test value*	*P*-value
Random								
Plant (Species)	-	0.589 ± 0.234	2.33	**0.010**	-	0.502 ± 0.240	2.09	**0.018**
Fixed								
Species	1, 18	0.136 ± 0.412	0.14	0.731	1, 18	0.056 ± 0.404	0.12	0.756
Petal removal	1, 978	1.248 ± 0.231	33.82	**<0.001**	1, 978	1.425 ± 0.350	27.79	**<0.001**
Species × Petal removal	1, 978	-0.738 ± 0.265	3.48	**0.051**	1, 978	-0.408 ± 0.463	0.78	0.379

Table 3. ANOVAs testing for the effects of species, plant and petal removal on mean seed number in each pair of sympatric species. Species and petal removal are fixed factors and plant is a random factor. N = 200 (100 C flowers and 100 R flowers). Significant P values are marked in bold

Effect	Calb-Clad				Clau-Clad			
	d.f.	MS	*F*	*P*-value	d.f.	MS	*F*	*P*-value
Species	1, 19	4.42×10^{7}	296.05	**<0.001**	1, 19	1.95×10^{7}	222.36	**<0.001**
Plant (Species)	19, 341	1.73×10^{5}	17.94	**<0.001**	9, 349	9.38×10^{4}	12.80	**<0.001**
Petal removal	1, 341	9.31×10^{2}	0.10	0.755	1, 349	4.71×10^{4}	6.42	**0.012**
Species × Petal removal	1, 341	5.23×10^{3}	0.55	0.461	9, 349	2.16×10^{4}	1.95	0.107

pair Clau-Clad (Table 3). In this case, the increase in seed production in R flowers was similar (Species × Petal removal non significant, Table 3).

Effects of floral display

All coflowering sympatric species showed a mean relative loss in fruit set for C flowers (range: -0.09 ± 0.07 to -0.24 ± 0.16). The relative loss index (RL_i) of fruits was always significantly negatively related with mean flower size (Table 4). Comparing coflowering species, the effect of species was significant in the pair Calb-Clad since the larger-flowered species increased the relative loss of fruits up to a 71%. In the pair Clau-Clad, the larger-flowered species had a higher slope in the relationship

Fig. 1. Differences in (a, b) fruit set and (c, d) mean seed number between C and R flowers and coflowering species. Squares denote the means and bars the standard errors.

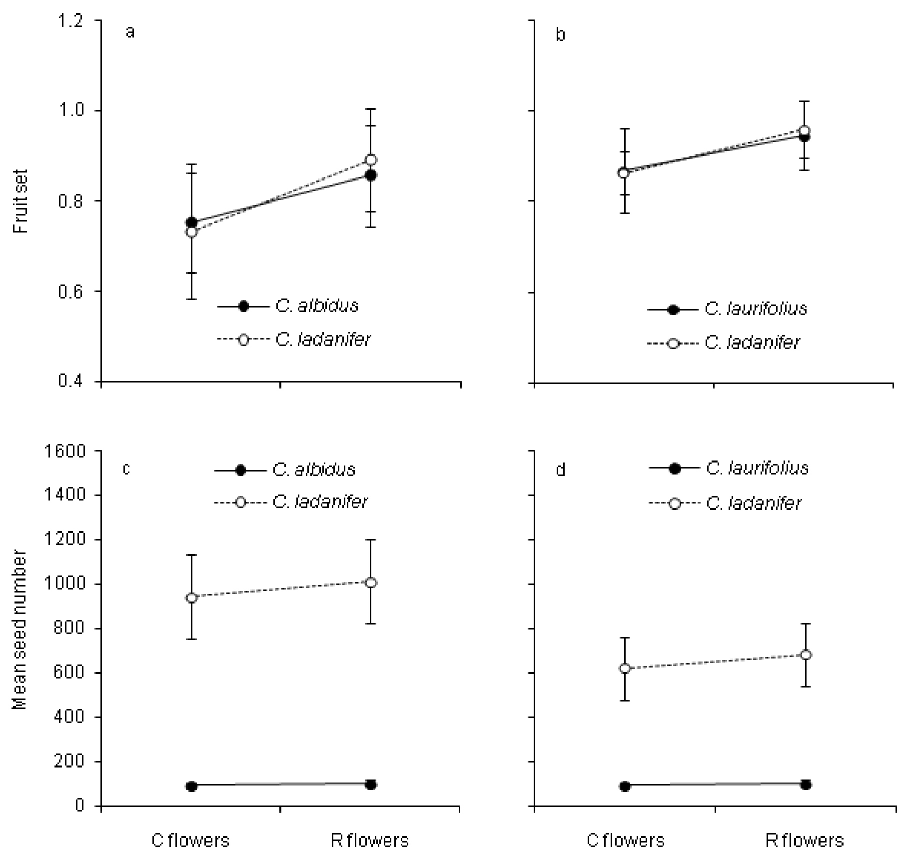
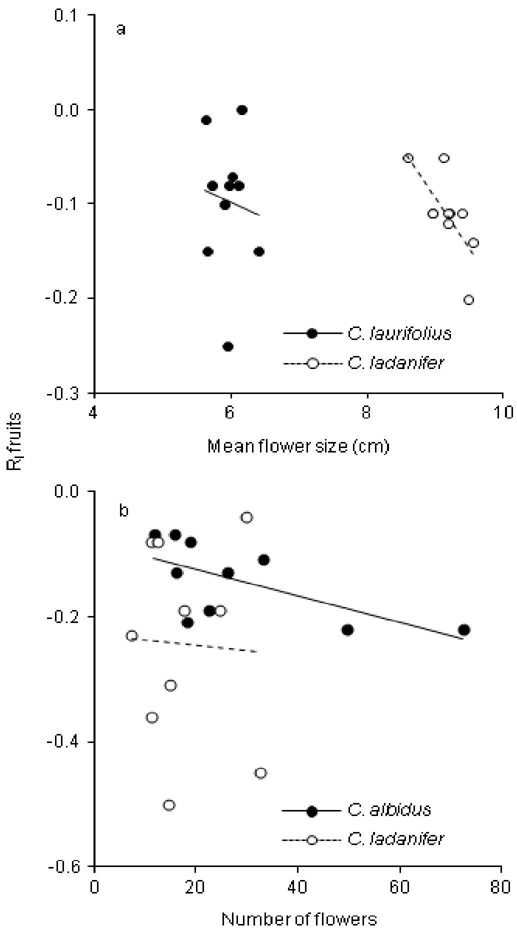


Table 4. ANCOVAs testing for the effects of species, flower size, number of flowers and the interactions between species with flower size and number of flowers on relative loss of fruits and seeds in each pair of sympatric species. Species is a fixed factor and flower size and number of flowers are covariates. Significant P values are marked in bold

Effect	RL_i fruits								RL_i seeds							
	Calb-Clad				Clau-Clad				Calb-Clad				Clau-Clad			
	d.f.	MS	*F*	*P*-value	d.f.	MS	*F*	*P*-value	d.f.	MS	*F*	*P*-value	d.f.	MS	*F*	*P*-value
Species	1, 19	0.05	4.44	**0.046**	1, 19	0.00	0.28	0.606	1, 19	0.01	1.05	0.319	1, 19	0.02	1.67	0.221
Flower size	1, 19	0.06	6.35	**0.016**	1, 19	0.02	6.07	**0.020**	1, 19	0.04	3.16	0.089	1, 19	0.03	3.32	0.081
No of flowers	1, 19	0.02	1.44	0.161	1, 19	0.00	0.03	0.865	1, 19	0.00	0.00	0.967	1, 19	0.03	3.37	0.082
Species × Flower size	1, 19	0.02	1.36	0.205	1, 19	0.01	4.41	**0.046**	1, 19	0.01	2.12	0.149	1, 19	0.01	1.43	0.266
Species × No of flowers	1, 19	0.02	4.68	**0.043**	1, 19	0.00	0.01	0.911	1, 19	0.00	0.04	0.850	1, 19	0.03	3.26	0.087

between relative loss and flower size (Species × Flower size significant, Table 4; Fig. 2a). Contrary to flower size, number of open flowers did not affect to relative loss of fruits in any case. However, in the pair Calb-Clad, the species with higher number of open flowers showed a higher correlation with relative loss of fruits (Species × No of flowers significant, Table 4; Fig. 2b). When considering seeds, all study species also showed a mean relative loss for C flowers (range: -0.09 ± 0.05 to -0.12 ± 0.11). Nevertheless, there were no significant trends and coflowering species showed similar relative loss of seeds.

Fig. 2. Significant differences (see Table 2) between coflowering species for the correlations between relative loss of fruits between C and R flowers and (a) individual mean flower size and (b) individual mean number of open flowers



Discussion

This study reported differences in direct costs of corollas among all the three *Cistus* species differing in flower size. The largest-flowered species, *C. ladanifer*, allocated the highest amount of resources to corollas in terms of dry mass, N and P. Likewise, we confirmed sizeable indirect costs of floral display, at least in terms of fruit production, at three different levels. First, we recorded indirect costs of corollas in all the three species. Second, we also verified that indirect costs increased with increasing flower size within-populations. Third, and more interestingly, we confirmed that those indirect costs differed between species pairs of contrasting flower size. In one of the pairs, indirect costs in terms of fruit production increased in the large-flowered species. Additionally, there was a higher correlation between relative loss of fruits and number of flowers in the species displaying more flowers. For the other pair, the species with larger flowers had higher slope of the relationship between flower size and relative loss of fruits.

Cruden and Lyon (1985) reported that dry mass of corollas of several xenogamous species was higher than those of autogamous species, ranged 0.31-17.91 mg and, on average, represented about 41% of all floral dry mass, thus being the heaviest structure. Likewise, corollas entailed 61% of floral dry mass in the large-flowered self-incompatible tropical tree *Ipomoea wolcottiana* (Parra-Tabla and Bullock 2000). In a study focused on the large-flowered Mediterranean herbaceous perennial *Paeonia cambessedesii*, Méndez and Traveset (2003) found that corollas represented on average 153 mg of dry mass (31%), about 2000 µg of N (19%) and about 317 µg of P (21%). Overall, our study species invested smaller amounts of resources to corollas relative to the studies above, although data of *C. ladanifer* fit relatively well with those of other large-flowered Mediterranean species.

The relevance of indirect costs, estimated as reduced fruit and seed production, is dependent on their magnitude in terms of offspring loss. Previous studies have reported from a high significant relative loss of fruit set (-28% in Andersson 1999) to a non significant loss (Andersson 2000, 2001). In our study, significant relative loss of fruits (range: -9 to -24%) and seeds (range: -8 to -12%) fit in the lower part of the range recorded in Andersson's studies. However, *C. ladanifer*, the largest-flowered species, fits in the higher part of the range. Overall, these results are remarkable considering the short floral longevity in the study species.

Several mechanisms might be involved in determining the indirect costs of floral display, the most important ones related with resource availability and allocation. For example, in *Nigella sativa* plants in which their perianth was experimentally removed, a high proportion of biomass and nutrients was reallocated to seed production (Andersson 2005). Corollas of the species used in this study entail remarkable differences of direct costs in terms of floral dry mass (range: 21-35%, Table 1). In addition, petals differ in their content of floral N (range: 18-28%) and floral P (range: 20-29%). However, we removed petals once produced so reallocation of biomass and nutrients was equally prevented in all species.

Indirect costs of corolla can also derive from the resources needed to maintenance, such as water. Andersson (2000, 2001) did not find effects of watering level on the relative loss of fruits and seeds. However, this process could be far more relevant under Mediterranean hot and dry conditions, where these stressful conditions strongly limit reproduction (Thompson 2005a; Aragón *et al*. 2008), as well as fruit and seed production (Konsens *et al*. 1991; Galen 2000; Fang *et al*. 2010). Although we lack information for the four species, we do know that high temperatures increase water costs by transpiration in corollas in *C. albidus* and *C. ladanifer* in our study area (AL Teixido and F Valladares, unpublished).

Indirect costs for fruit set, but not for number of seeds, increased with increasing flower size in all three species of *Cistus* studied. More interestingly, indirect costs in terms of fruit production increased in the larger-flowered species between a coflowering species pair. Moreover, the relationship between relative loss of fruits and flower size was higher in the larger-flowered species in the other coflowering pair. Contrary to our expectations, we did not find any effect of number of flowers on indirect costs. This is surprising, because number of flowers has been previously associated with reduction in fruit production by resource depletion (Montalvo and Ackerman 1987). However, in one of the species pairs there was a higher relationship between relative loss of fruits and number of open flowers in the species displaying more flowers. Overall, although relationships between indirect costs and floral display are expected if resource allocation or transpiration underlay indirect costs of corollas, these experimental results are, to our knowledge, novel. In the coflowering pair Calb-Clad, transpiration increased with increasing flower size and transpiration reached up to a three-fold increase in the larger-flowered *C. ladanifer* (AL Teixido and F Valladares, unpublished). Increases of indirect costs in larger displays, and their underlying mechanisms, deserve, thus, further study.

The pattern of resource investment between attractiveness and offspring could be an important selective factor on plant reproductive function (Ashman and Schoen 1996; Galen 1999, 2005). In particular, the magnitude of fruit and seed loss depending on investment in attractiveness, could determine selective pressures on floral display. In the species of *Cistus* studied, a higher relative loss of fruits in larger-flowered individuals may entail relevant selective pressures towards smaller flowers, at least in our study populations. Likewise, a higher correlation between relative loss of fruits and floral display between coflowering *Cistus* species with contrasting flower size could counteract the differential pollinator attractiveness. However, we did not detect a significant relative seed loss with increasing floral display. This may be due to a positive correlation between flower size and ovule number in the Cistaceae (J Herrera 1992). A potential complication in assessing fitness effects of indirect costs is compensatory resource reallocation to the remaining seeds. Although Andersson (2005) found indirect costs of floral attraction structures in terms of mean seed mass and germination rates, these estimates of seed mass and viability would be useful additions to future studies.

In summary, our results support that corollas imply relevant direct costs for their production and sizeable indirect costs in terms of fruit and seed production in *Cistus*. Additionally, indirect costs increased with increasing mean flower size between individuals within populations. More interestingly, we reported that indirect costs in terms of fruits increased in larger-flowered individuals between coflowering sympatric congeneric species differing in flower size. In fact, our results go further in reporting a higher correlation between relative loss of fruits and floral display. These results suggest that heat and drought in Mediterranean environments may limit floral display, especially through flower size, opposing the selective pressures of pollinators. This may be relevant in coflowering, sympatric, generalist species with contrasting flower size, counteracting attractiveness to pollinators with the magnitude of indirect costs.

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CHAPTER 4

Large flowers tend to be short-lived in Mediterranean environments: an experimental study in *Cistus ladanifer*

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Manuscrito inédito

Abstract

Both larger and longer-lived flowers receive more pollinator visits, but may also involve high maintenance costs associated with water use, especially in hot and dry ecosystems. In this context, smaller or short-lived flowers could play an important role to buffering such costs. We experimentally surveyed floral longevity in *Cistus ladanifer*, a large-flowered Mediterranean shrub, in response to differences in temperature, flower size and pollen deposition at two different altitudes differing in climatic conditions. We pollinated and capped flowers to compare their longevity with that of flowers exposed to natural pollination. We included air temperature and flower size as additional predictors to statistically control their potential effect on floral longevity. Flowers of *C. ladanifer* increased in size with altitude and tended to be short-lived, though our study reported variability in floral longevity. We detected that floral longevity depended on pollen receipt. Relative to treatment effects, capped flowers lasted longer (1.56±0.55 days) that control (1.25±0.36 days) and hand-pollinated (1.04±0.11 days) flowers. On the other hand, both larger flowers and higher temperatures had a differential effect significantly shortening floral longevity independently of pollination. Our experimental survey suggests that low floral longevity was not phylogenetically constrained in *C. ladanifer* but changed depending on temperature, flower size and pollen receipt. More importantly, larger flowers under high temperatures tend to be short-lived even in the absence of pollination. Together, these findings suggest an important effect of temperature in increasing maintenance costs and thus limiting flower size and longevity in large-flowered Mediterranean plants.

Keywords: floral maintenance costs, floral longevity, flower size, low vs. high altitude, pollen receipt, temperature

Introduction

It has been frequently reported that larger flowers increase pollinator visit rates and, as a result, increase both female and male fitness (Stanton *et al*. 1986; Young and Stanton 1990; Campbell *et al*. 1991; Kudoh and Whigham 1998; Arista and Ortiz 2007). Accordingly, many studies have documented pollinator-mediated phenotypic selection towards larger corollas (e.g. Galen 1989; Campbell *et al.* 1991; Conner and Rush 1997; Medel *et al*. 2003; see also CM Herrera 2009 and references therein). However, Galen (1999) pointed out that a unilateral view of the evolution of flower size from a pollinator perspective was probably oversimplistic. For example, large flowers are associated with greater requirements of biomass and water for floral development (Galen 1999; Carroll *et al*. 2001; Elle and Hare 2002), as well as with larger maintenance costs due to high respiration and transpiration rates (Vemmos and Goldwin 1994; Galen 2000).

Higher floral longevity, the length of time that flowers remain open and functional, leads to a higher amount and quality of pollen removal from and pollen deposition on the flower (Primack 1985; Ashman and Schoen 1994, 1996; Harder and Johnson 2005). Hence, flowers lasting longer increase the opportunity for reproductive success. Nevertheless, floral longevity also entails costs (Ashman and Schoen 1997; Castro *et al*. 2008). From an evolutionary perspective, floral longevity must balance the rates of pollen deposition and removal against floral maintenance costs, so that an optimal floral longevity will tend to minimize costs and to maximize the fitness rates (Ashman and Schoen 1994). Many factors of the environment have been suggested as selective factors on floral longevity (reviewed by Primack 1985; Sargent and Roitberg 2000; Rathcke 2003). For example, several studies have reported longer flower periods in pollinator absence, but floral senescence induced by pollen deposition on stigma (Proctor and Harder 1995; Sargent and Roitberg 2000; Giblin 2005).

The exposure of flowers to high temperatures causes a significant increase of maintenance costs to keep an optimal physiological temperature (Galen 2005). Water shortage can prevent this homeostasis by means of an inefficient transpirational cooling, particularly in hot and dry ecosystems. Acting together, heat and drought can disrupt the normal performance of flowers, affecting fruit and seed production (Konsens *et al*. 1991; Galen 2000; Erickson and Markhart 2002; Aragón *et al*. 2008; Fang *et al*. 2010). As a consequence, the stressful conditions of hot and dry ecosystems may constrain flower size and longevity, making small, short-lived flowers, potentially advantageous for plants living in these environments (Galen 2000, 2005; Elle and Hare 2002). The pattern of covariation between both floral traits should be apparent once variation in temperature is accounted for. Within species, smaller flowers can occur in more xeric environments, and phenotypic plasticity in flower size commonly takes reduced forms in hotter and drier sites (Barrett *et al*. 2004; J Herrera 2005; Lambrecht and Dawson 2007). Therefore, because floral longevity and flower size are both predicted to decrease with increasing temperature, these conditions would tend to correlate both traits negatively due to limitation in water allocation,

Mediterranean climate includes high temperatures and water shortage, constraining plant reproduction by speeding up development, shortening flowering duration (Larcher 2000; Thompson 2005; Aragón *et al*. 2008), and, occasionally, delaying the initiation of flowering till the rainy season to maximize water use efficiency (Blionis *et al*. 2001; Verdú *et al*. 2002). Some large-flowered plants in these and other hot and dry ecosystems show adaptations to prevent overheating and excessive water loss. Nocturnal flowering and pollination are features of several caperbushes (*Capparis* sp.) of semi-arid areas (Rhizopoulou *et al*. 2006), and most desert large-flowered cacti (Valiente-Banuet *et al*. 1997; Fleming *et al*. 2001). Floral cooling mechanisms appeared to be critical for the reproductive success of convolvulaceous large flowers in tropical environments (Patiño and Grace 2002).

In this paper, we utilise *Cistus ladanifer* (L.), a large-flowered common shrub occurring in Mediterranean ecosystems with generalist and self-incompatible flowers, as a model system to evaluate the response of floral longevity to flower size, temperature and pollen receipt. Specifically, we conducted an experimental manipulation of floral longevity by means different pollination treatments at two different altitudes differing in climatic conditions. Given the potential relationship between flower size, floral longevity and temperature, and also floral longevity and pollen receipt, we hypothesize: (1) flower size to be smaller in drier conditions, i.e., at the lower altitude, (2) floral longevity to decrease with increasing temperature, tending to be shorter at the lower altitude and in hotter days within-populations, (3) floral longevity to decrease with increasing flower size at each altitude and (4) floral longevity decreases with increasing pollen receipt.

Material and methods

Species and study area

Cistus ladanifer (Cistaceae) is a shrub 100-250 cm tall that inhabits acid and dry soils in warm open areas on western Mediterranean. Flowering phenology spans from March to June and each plant produces white flowers often exhibiting dark coloured spots at their bases. Flowers are the largest in the family, and are homogamous, polliniferous, self-incompatible and secrete nectar (J Herrera 1992; Talavera *et al*.

1993). Flower opening occurs synchronously each day within the populations. Pollinators are mainly bees, beetles and flies (Talavera *et al*. 1993). The study was conducted from April to June of 2009 at two different altitudes in the Madrid province, central Spain (39.53º-41.09º N 3.03º-4.34º W). These altitudes were suitable to reproduce a gradient of temperature and humidity. Three populations per altitude approx. 1 km apart were selected to study floral longevity of *C. ladanifer* (Table 1). All sites had similar orientation (south), slope (0-10º) and tree canopy cover (0-10%). Sunny and southern slopes were chosen to remove as much as possible environmental effects.

Response of floral longevity to pollination treatments

At each population, 10 similar plants > 1 m high were randomly selected and tagged during the flowering peak in a plot of 20 x 20 m. For each plant, total size –as the volume of an ellipsoid (4/3 πabc)– was recorded. Plant size averaged 1.04 ± 0.39 m^3 and was similar among populations (Kruskal-Wallis test, χ^2 = 1.801, *P* = 0.566, N = 60). Air temperature (ºC) in each population were also recorded every 15 min during the study period with data loggers (Pro H8032, Onset Hobo, MA, USA) located 1 m above the ground. We evaluated the response of floral longevity to pollination by conducting three different treatments, each of them on twelve randomly selected individual flowers per plant: (POL) hand-pollinated flowers with xenogamous pollen; (CAP) capping of stigmas to avoid pollen deposition; (CON) control flowers exposed to natural pollination.

Capping was carried out before anthesis to prevent natural pollination with plastic capsules made of straw and sellotape and fitted to the stigma. Hand-pollination was carried out with a fresh pollen mixture collected from 10 different

Table 1. Location and ecological data of the experimental study sites of *Cistus ladanifer*. Column of climate shows the annual mean rainfall (mm) and the annual mean temperature (ºC) (Ninyerola, Pons & Roure 2005, N = 20 years).

Study sites	Altitude (m)	Climate	Substrate	Vegetation cover
Low altitude				
Monte del Pardo (40.34ºN 3.42ºW)	732	Dry 533 mm,14ºC	Clay and sand	Dehesa with *Q. ilex* and *P. pinea* interspersed in a shrub matrix
Monte del Pardo (40.33ºN 3.42ºW)	741	Dry 529 mm,14ºC	Clay and sand	Dehesa with *Q. ilex* and *P. pinea* interspersed in a shrub matrix
Monte Valdelatas (40.32ºN 3.41ºW)	725	Dry 520 mm,14ºC	Clay and sand	Dehesa with *Q. ilex* and *P. pinea* interspersed in a shrub matrix
High altitude				
Becerril de la Sierra (40.43ºN 3.58ºW)	1163	Subhumid 844 mm,11ºC	Granite	Patchy scrubland with *Cistus laurifolius* and *J. oxycedrus*
Navacerrada (40.43ºN 4.00ºW)	1176	Subhumid 878 mm,11ºC	Granite	Patchy scrubland with *Cistus laurifolius* and *J. oxycedrus*
Navacerrada (40.43ºN 4.00ºW)	1199	Subhumid 878 mm,11ºC	Granite	Patchy scrubland with *Cistus laurifolius* and *J. oxycedrus*

individuals within a 10 m radius of the recipient flower (Giblin 2005; Castro *et al*. 2008) and then sufficient outcross pollen was deposited on the stigma with a paintbrush two hours after anthesis to ensure stigmatic receptivity (J Herrera 1992). These treatments were conducted between 20 April and 3 May at low altitude and between 26 May and 8 June at high altitude. Treatments were applied between 07:00 and 11:00 am. Flowers were tagged and flower diameter (cm) was noted to determine the effects of flower size on floral longevity. Flowers were monitored every day after treatment application and floral longevity (d) recorded. This sampling protocol, surveying flowers once a day, did not allow us proper estimations of longevity for flowers lasting <1 d.

Statistical analysis

We performed two different GLMMs to model the variability of flower size as a function of altitude (fixed factor) and the variability of floral longevity as a function of altitude and treatment (fixed factors). In the first GLMM, modeling the variability of flower size, we included population within altitude and plant within population as random factors. In the second one, modeling the variability of floral longevity, we included the interaction altitude × treatment, with population within altitude and plant within population as random factors and flower size and temperature as covariates. We assumed a normal error distribution with an identity link function for flower size and a Poisson error distribution with a logit link function for floral longevity. To assess significant differences among treatments at each altitude for floral longevity, their means were compared using *post hoc* HSD tests.

In addition, we specifically examined how flower size and temperature influenced floral longevity. Using our experimental data, for each treatment and altitude we correlated mean longevity of flowers measured every day, with their mean flower size, and mean longevity of flowers measured every day with mean air temperature in the days those flowers remained open by means of liner regressions.. These analyses were performed using GraphPad Prism (GraphPad Prism 5.0, 2007; GraphPad Software, Inc., San Diego, CA, USA).

Results

Flower size in *C. ladanifer* ranged between 5.9 and 10.8 cm and differed significantly between altitudes, populations within altitudes and plants within populations (Table 2). Mean flower size (± SD) increased about 12% with altitude (7.3 ± 1.3 cm at the lower altitude vs. 8.2 ± 1.1 cm at the higher altitude). Longevity of individual flowers ranged from 1 to 3 d, though longevities of 3 d only occurred in CAP and CON flowers. Floral longevity significantly differed between altitudes and treatments, decreasing at high altitude, and also decreased with increasing flower size and

Table 2. Generalized linear mixed model for flower size (2160 flowers from 60 plants in 6 populations at two altitudes). Analyses were based on GLMM with normal errors with identity link using REML estimation. Random effects were tested with Wald Z-tests and the fixed effect with Type III F-tests.

Effect	d.f.	Estimate ± SD	Test value	*P*-value
Random				
Population (Altitude)	−	1.27 ± 0.24	5.21	0.006
Plant (Population)	−	9.36 ± 1.17	20.31	<0.001
Fixed				
Altitude	1, 4	1.54 ± 0.09	99.32	<0.001

temperature (Table 3). Mean temperature (± SD) averaged 14.5 ± 1.7 vs. 17.4 ± 3.3º C at the lower and at the higher altitude, respectively, and this difference was significant ($F_{1,\ 23}$ = 7.788, *P*-value = 0.011). Overall, flowers at high altitude lasted shorter than at low altitude. At both altitudes, POL flowers were the shortest-lived ones and CAP flowers the longest-lived ones (Fig. 1). CAP flowers lasted 13% longer and POL flowers did 14% shorter than CON flowers at high altitude, whereas, at low altitude, CAP flowers lasted 31.5% longer and POL flowers did 24% shorter that CON ones (Fig. 1).

Specific analysis of flower size and temperature did not report any effect on floral longevity at low altitude (Table 4). However, the effects of flower size and temperature on floral longevity reported shorter-lived flowers at larger flower sizes and at higher temperatures in all three treatments at high altitude (Table 4).

Discussion

Our experimental survey in *C. ladanifer* showed a short floral longevity. This finding contrasts with floral longevities of several days in Mediterranean ecosystems (Petanidou *et al*. 1995; Blionis *et al*. 2001; Berjano *et al*. 2009). Over 60% of the species in a phryganic system had average floral longevities higher than 2 days

Table 3. Generalized linear mixed model for floral longevity (2160 flowers from 60 plants in 6 populations at two altitudes). Analyses were based on GLMM with Poisson errors with log link using REML estimation. Random effects were tested with Wald Z-tests and fixed effects with Type III F-tests. Significant *P*-values are marked in bold.

Effect	d.f.	Estimate ± SD	Test value	*P*-value
Random				
Population (Altitude)	-	0.01 ± 0.00	0.98	0.163
Plant (Population)	-	0.01 ± 0.00	1.01	0.159
Fixed				
Altitude	1, 4	-0.28 ± 0.04	16.58	**0.015**
Treatment	2, 2094	-0.27 ± 0.02	344.55	**<0.001**
Altitude × Treatment	2, 2094	-0.24 ± 0.03	34.96	**<0.001**
Flower size	1, 2094	-0.14 ± 0.01	96.32	**<0.001**
Temperature	1, 2094	-0.03 ± 0.00	209.71	**<0.001**

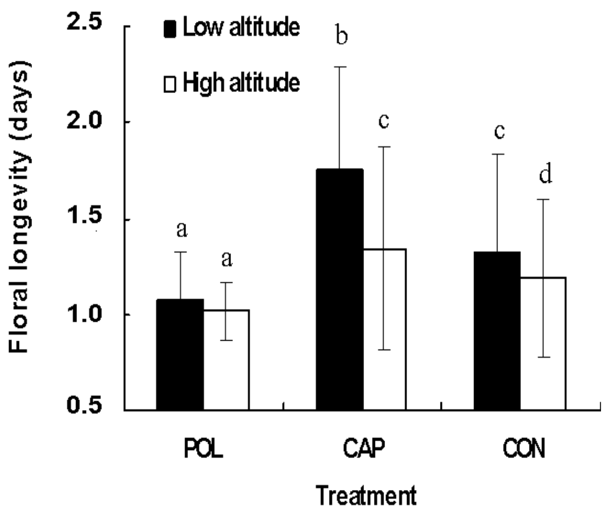

Fig. 1. Differences in floral longevity of *Cistus ladanifer* in the interaction altitude × treatment. Columns denote the means and bars the standard errors. Different letters indicate significant differences among treatments by unequal HSD *post hoc* test ($P < 0.05$).

(Petanidou *et al*. 1995). Nevertheless, floral longevities widely differed between families and Cistaceae in that study also showed floral longevities of 1.0 days, with the exception of *Fumana amarisia* (Petanidou *et al*. 1995). Phylogenetic constraints or phylogenetic conservatism could account for this pattern. Several families typically show one-day flowers (e.g. Commelinaceae, Convolvulaceae, Pontederiaceae: Primack 1985; Acanthaceae: Endler 1994; Turneraceae: Arbo 2007). Alternatively,

Table 4. Linear regressions of every treatment at each altitude for mean floral longevity (days) against mean temperature (ºC) and flower size (cm). Significant *P*-values are marked in bold. N = 12 data for all regressions.

Effect	Treatment	Low altitude						High altitude					
		R^2	$\beta_0 \pm SD$	$\beta_1 \pm SD$	d.f.	*F*	*P*-value	R^2	$\beta_0 \pm SD$	$\beta_1 \pm SD$	d.f.	*F*	*P*-value
Temperature	POL	0.01	1.12 ± 0.21	-0.00 ± 0.02	1,11	0.070	0.797	0.56	1.21 ± 0.05	-0.01 ± 0.00	1,11	12.885	**0.005**
	CAP	0.06	2.20 ± 0.55	-0.03 ± 0.04	1,11	0.581	0.463	0.67	2.93 ± 0.36	-0.09 ± 0.02	1,11	19.991	**0.001**
	CON	0.02	1.53 ± 0.47	-0.01 ± 0.03	1,11	0.182	0.679	0.60	2.50 ± 0.35	-0.08 ± 0.02	1,11	14.697	**0.003**
Flower size	POL	0.01	1.50 ± 1.26	-0.06 ± 0.17	1,11	0.118	0.739	0.49	2.65 ± 0.53	-0.20 ± 0.07	1,11	9.453	**0.012**
	CAP	0.03	3.66 ± 3.71	-0.25 ± 0.50	1,11	0.257	0.623	0.39	9.87 ± 4.01	-1.22 ± 0.49	1,11	6.253	**0.031**
	CON	0.02	2.63 ± 2.86	-0.18 ± 0.39	1,11	0.205	0.660	0.41	9.46 ± 3.55	-1.13 ± 0.43	1,11	6.827	**0.026**

low floral longevity found for *C. ladanifer* in our study may be due to climatic conditions, such as high temperature, because our populations were located at south-facing slopes. High temperatures could limit floral longevity by increasing the physiological costs of floral maintenance (Galen 2005). Alternative plausible explanations could explain the patterns reported in our study. For example, an association between air temperature and flower longevity can be interpreted alternatively via the role of correlated factors (e.g., time-related changes in temperature and plant resource status conditioning flower longevity). In addition, a high activity of pollinators (mediated by high temperature or not) could lead to a rapid and effective pollination (van Doorn 1997; Ishii and Sakai 2000). Which of these mechanisms is responsible for our results is difficult to disentangle from observational evidence alone. Nevertheless, our experimental study provided evidence against the phylogenetic constraint and supported a combined action of an efficient pollinator service and environmental (temperature) influences.

We found that floral longevity in *C. ladanifer* decreased along an altitudinal gradient. In general, floral longevity has been found to increase with increases in altitude (Blionis *et al*. 2001; Giblin 2005). In our study, the altitudinal gradient was intended to reflect a gradient in temperature and, thus, higher altitude was expected to be related to lower temperature and longer floral longevity. Although we found exactly the opposite pattern, the expected relationship between floral longevity and temperature still held.. However, increased floral longevity at higher altitude has been related to low pollinator activity (Blionis *et al*. 2001). An indirect effect of temperature on pollinator activity cannot be excluded from these data alone. Nevertheless, our experimental approach allowed to disentangle both effects.

Our experimental data showed a negative relationship of floral longevity with both pollen receipt and increased temperature. The former result has been found in many other studies; pollination of flowers decreases longevity (Clayton and Aizen 1996; Ishii and Sakai 2000; Arathi *et al*. 2002; Gibblin 2005; Harder and Johnson 2005) while lack of pollinator visits leads to extended floral longevity (Ishii and Sakai 2000; Blionis and Vokou 2001; Harder and Johnson 2005; Steinacher and Wagner 2010). An inverse relationship between temperature and floral longevity has also been reported in the field (Sargent and Roitberg 2000; Rathcke 2003; Giblin 2005) and under controlled conditions to avoid confounding effects of pollinator activity (Michaud 1990; Yasaka *et al*. 1998). Interestingly, we detected short floral longevity with increasing temperature even for unpollinated flowers. Our finding suggests that under high temperatures, floral maintenance costs may be excessive in this large-flowered species and the theoretical expectation of a balance between maintenance costs and fitness accrual rates (Ashman and Schoen 1994) simply breaks down. The negative correlation between flower size and longevity at higher altitude, where flower were larger, also points out to elevated costs of maintenance.

Further work is required to disentangle the proximate mechanisms of the patterns reported here, but temperature-mediated increases in transpiration rates are likely involved. For example, decreasing water availability reduced floral longevity in *Mimulus* (Arathi *et al*. 2002). In addition, transpiration rates may significantly increase in large flowers, especially for species living under dry and hot conditions (Galen 1999, 2005) and a negative correlation between flower size and time to abscission has been found for cultivars of rose (van Doorn and Schröder 1995). We also know that flower size and, especially, temperature increased carbon and water maintenance costs in corollas, involving an allometric relationship in terms of water since transpiration did not scale linearly with corolla area (AL Teixido and F Valladares, unpub. data).

From an evolutionary perspective, the negative phenotypic correlation between flower size and floral longevity found in our study suggests that larger flowers seem to need to be short-lived. In addition, flower size in *C. ladanifer* increased with altitude and also varied among plants. Though temperatures were higher at high altitude in the time of flowering, annual hotter and drier conditions at low altitude are expected to decrease plant size and organs (Thompson 2005). Flowers are significantly larger in the colder parts of the range of some Mediterranean species (*Narcissus triandrus*: Barrett *et al*. 2004; *Rosmarinus officinalis*: J Herrera 2005). Thus, this variation could, in principle, be adjusted to the thermic and pollination environments faced by the plants. However, it is not disentangled whether this pattern is something besides simple scaling effects and is not due to other evolutionary drivers of flower size. Although the heritability of flower size in this species is unknown, it is realistic to think that variability among plants generates potential of phenotypic selection on this trait (Ashman and Majestic 2006). Our study points out that maintenance costs are a likely selection pressure in addition to pollinators. In conclusion, our survey of *C. ladanifer* showed an altitudinal variation in flower size and a low but not phylogenetically constrained floral longevity, which changed depending on temperature, flower size and pollen receipt. The main found patterns are that pollen receipt reduces floral longevity and that both larger flowers and high temperatures also significantly reduce floral longevity even in the absence of pollination. Together, these findings suggest an important effect of temperature in increasing maintenance costs and thus limiting flower size and longevity in large-flowered Mediterranean plants.

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CHAPTER 5

Flower size and longevity increase florivory in the large-flowered shrub *Cistus ladanifer*

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Abstract

Plants with larger and longer-lived flowers receive more pollinator visits and increase reproductive success, though may also suffer more from antagonistic interactions with animals. Florivores can reduce fruit and seed production, so selection on flower size, floral longevity and/or number of flowers may thus be determined by the relative effects of both pollinators and florivores. In this study, flowers of *Cistus ladanifer*, a large-flowered Mediterranean shrub, were monitored to evaluate the effects of flower size, floral longevity and number of flowers on levels of florivory in four populations. Number of flowers was variable but did not differ among populations. Both flower size and floral longevity of *C. ladanifer* showed broad variation and significantly differed among populations. Overall, 7% of flowers suffered attack by florivores, which were mainly ants picking the stamens and beetles consuming petals and pollen. Within-populations, larger and longer-lived flowers tended to be affected by florivores more frequently. The low overall incidence of florivores and its lack of between-population variation suggest that florivory may not influence intraspecific variation of these floral traits. However, moderate florivory levels on the largest and longest-lived flowers open the possibility of exerting selection towards smaller and shorter-lived flowers in some of the populations studied.

Keywords: Floral longevity, florivores, flower size, number of flowers, variation within-populations

Introduction

Attractiveness to pollinators plays a decisive role in the reproductive ecology of entomophilous plants. The importance of the number of displayed flowers for pollinator visitation rates and fruit production has been broadly reported (Brody and Mitchell 1997; Thompson 2001; Harder and Johnson 2005). Larger flowers have also been associated with higher pollinator attraction and, as a result, an increase in cross-pollination and reproductive success (Galen 1989; Kudoh and Whigham 1998; Arista and Ortiz 2007). In the same way, floral longevity (the length of time that flowers remain open and functional) involves both a greater amount of pollen removal, and higher amount and quality of pollen deposition, on the flower (Primack 1985; Ashman and Schoen 1994, 1996). As a consequence, longer-lived flowers may also increase reproductive success.

Despite its benefits, floral attractiveness can also be related with greater plant-animal antagonist interactions. For example, floral herbivores (i.e. florivores) cause damage to open flowers, including damage to bracts, sepals, petals, androecium and/or gynoecium (McCall and Irwin 2006). Thus, florivores may reduce fruit and seed production by degrading the attractive properties of flowers for pollinator service or by direct consumption of viable gametes (Schemske and Horvitz 1988; Krupnick *et al*. 1999; Irwin 2006; Cardel and Koptur 2010). In this way, florivores can exert negative selective pressures on the same floral traits positively selected for pollinators (Galen 1999; Irwin *et al*. 2001; Irwin 2006). There is evidence that florivory increases with increasing components of plant attractiveness to pollinators such as the number of flowers displayed and flower size (Galen 1999; Mosleh Arany *et al*. 2009). Longer floral longevity should also increase the risk of florivory, as documented for other antagonistic interactions (e.g., fungal infection: Shykoff *et al*.

1996, Kaltz and Shykoff 2001). However, the effects of floral longevity on the incidence of florivory seem to have been only scarcely studied and are not even mentioned in reviews of non-pollinator influences on floral traits (Strauss and Whittall 2006).

The strength of agents of selection can vary geographically and lead to contrasting selective pressures at different locations (Thompson 1982, 2005b). Several studies have reported that individuals in those populations with a higher incidence of florivores display fewer, smaller flowers (Galen 1999; Mosleh Arany *et al*. 2009). Thus, documenting spatial variation in incidence of florivory is important to understand differences in floral display related traits among populations.

In this study, we evaluate the effect of three floral attractiveness-related traits (flower size, floral longevity and number of flowers displayed) on incidence of florivory in four populations of *Cistus ladanifer*. We address the following specific questions: (1) Does florivory increase with flower size, floral longevity and number of flowers? (2) Does florivory vary among populations? Given the potential relationship between floral attractiveness and florivory incidence, we expect florivory to be higher both on larger and on longer-lived flowers within-populations, as well as on flowers of showier plants that produce more flowers.

Materials and methods

Species and study area

Cistus ladanifer (Cistaceae) is a shrub 100-250 cm tall that inhabits acid and dry soils in warm open areas of the western Mediterranean. The flowering period spans March to June and each plant produces white flowers of approximately 7-10 cm in diameter, often exhibiting dark coloured spots at their bases. The flowers are the largest in the family with on average more than 150 anthers and 1000 ovules, are self-incompatible and secrete some nectar (J Herrera 1992). The pollinators are mainly bees, beetles and flies (Talavera *et al*. 1993). A predispersal seed predator, the larva of the moth *Cleonymia yvanii* (Noctuidae), attacks very young fruits, where it spends part or all its pre-imaginal development (Serrano *et al*. 2001; Delgado *et al*. 2007). Flowers last at least 1 day, with individual plants showing some plasticity for this trait (AL Teixido, M Méndez and F Valladares, unpublished).

The study was conducted from March to June of 2009 in a south-north altitudinal gradient from 720 to 1300 m a.s.l. in Madrid province, central Spain (39.53º-41.09º N 3.03º-4.34º W). A total of four populations were chosen to study florivory (Table 1). All populations had similar orientation (south), slope (0-10º) and tree canopy cover (0-10%).

Floral traits and florivory incidence

At each population, 10 similarly-sized plants (1.03 ± 0.37 m^3, Kruskal-Wallis test, χ^2 = 1.432, *P* = 0.698, N = 40) were randomly selected and tagged before the beginning of the flowering in a plot of 20 x 20 m. For each plant, presence or absence of a spot on its flowers was recorded. In other species, dark petal spots have been shown to act as visual signals for insect pollinators (Johnson and Midgley 1997; van Kleunen *et al*. 2007; Thomas *et al*. 2009). Hence, spots might also be visual signals to florivores. During the flowering peak (when all individuals produced more than 10 flowers per day), 63-74 flowers were haphazardly selected and tagged per plant, for a total sample size of 645 flowers in Monte Valdelatas, 701 in La Pedriza, 706 in Vista Real and 671 in Puerto de Canencia (total N = 2723). On each plant, the number of open flowers was counted each day. In addition, 5-10 randomly selected flowers were monitored daily to assess floral longevity (number of days open). Flower

Table 1 - Location and ecological data of study populations of *C. ladanifer*. Column of climate shows the annual mean rainfall (mm) and the annual mean temperature (ºC) (Ninyerola *et al.* 2005, N = 20 yr)

Study sites	Altitude (m)	Climate	Substrate	Vegetation cover
Monte Valdelatas (40.32ºN 3.41ºW)	720	Dry 520 mm, 14ºC	Clay and sand	Dehesa with *Quercus ilex* and *Pinus pinea* interspersed in a shrub matrix
La Pedriza (40.44ºN 3.52ºW)	940	Subhumid 771 mm, 12ºC	Clay and sand	Patchy scrubland with *Q. ilex* among boulders and rocks
Vista Real (40.44ºN 3.57ºW)	1120	Subhumid 820 mm, 11ºC	Granite and sand	Patchy scrubland with *Juniperus oxycedrus* among boulders and rocks
Puerto de Canencia (40.50ºN 3.46ºW)	1300	Subhumid 865 mm, 9ºC	Granite and sand	Dispersed wooded slope with *Pinus sylvestris* and *Quercus pyrenaica*

diameter (cm) was also measured every day that they remained open and then averaged.

Florivory was considered as any type of damage to open flowers (see Introduction). Hence, we included animals that consumed pollen and/or picked the stamens, as well as full flower loss by eating the pedicel. Thus, these animal-flower interactions did not allow effective pollination (Inouye 1980; McCall and Irwin 2006). We limited our study of florivory to open flowers, so floral bud attack was not considered. Florivory was scored shortly before floral senescence, usually in the afternoon or the evening.

Statistical analysis

We tested the effects of population (fixed factor), plant within population (random factor), plant size, presence of spot and floral longevity (fixed factors) on presence of florivores by fitting Generalized Linear Mixed Models (GLMMs). Both flower size and number of flowers were also included as covariates. We assumed a binomial error distribution with a logit canonical link function. We used the restricted maximum likelihood (REML) and, because our data were unbalanced, we used Satterthwaite's method to determine the approximate denominator degrees of freedom. Due to the complexity of the model structure and the relatively large number of potential explanatory variables, we only considered the interaction between floral longevity and population, flower size and population and number of flowers and population. All the computations were performed using the GLIMMIX Macro of SAS (SAS Statistical Package, 1990; SAS Institute, Cary, NC, USA).

Due to the limitations of GLIMMIX to carry out *a posteriori* tests, two additional analyses were carried out to further explore the exact relationship of both flower size and floral longevity with florivory. Firstly, we correlated flower size with florivory by means of logistic regressions for each population. Secondly, we used contingency tables to test whether florivores disproportionately occurred on flowers of greater longevity at each population. Contingency tables were analysed using the G-test (Sokal and Rohlf 1995). For population 3, flowers lasting 2 and 3 days were pooled in order to avoid empty cells (Sokal and Rohlf 1995). These two analyses were performed with SPSS 15.0 (SPSS Inc., Chicago, Illinois, USA).

Results

Flower size, floral longevity and number of flowers

Flower size ranged between 4.2 and 10.8 cm and significantly differed among populations ($F_{3, 2720}$ = 385.21, P < 0.001). Longevity of individual flowers ranged from 1 to 5 days, but mean floral longevity (± SD) of the species was 1.44 ± 0.62 d and significantly differed among populations (Wald Z-test, Z = 2.01, P = 0.022). Number of flowers ranged between 10 and 63 flowers per plant and did not significantly differ among populations ($F_{1, 3}$ = 0.25, P = 0.859), but did differ among plants within-populations ($F_{3, 35}$ = 41.89, P < 0.001). Table 2 shows mean flower size, mean floral longevity and mean number of flowers in each population.

Table 2 - Mean ± SD flower size (cm), floral longevity (d) and number of flowers of *C. ladanifer*, and florivory incidence (%) on flowers at each population

Population	Flower size	Floral longevity	Number of flowers	Florivory incidence
Monte Valdelatas	7.00 ± 1.13	1.38 ± 0.56	17.31 ± 8.26	9.77
La Pedriza	8.02 ± 0.74	1.92 ± 0.70	19.93 ± 9.08	9.70
Vista Real	8.12 ± 0.81	1.21 ± 0.41	18.57 ± 11.30	3.68
Puerto de Canencia	8.60 ± 0.79	1.23 ± 0.48	19.40 ± 8.71	4.77
Total	7.95 ± 0.86	1.44 ± 0.62	18.82 ± 10.08	6.94

Table 3 - Generalized linear mixed model for presence of florivores on *C. ladanifer* (2723 flowers from 40 plants in 4 populations). Analyses were based on one GLMM with binomial error with logit canonical link function using REML estimation. Random effects were tested with Wald Z-tests and fixed effects with Type III F-tests. Significant *P*-values are marked in bold

Effect	d.f.	Estimate ± SD	*Test value*	*P*-value
Random				
Plant (Population)	-	0.27 ± 0.14	2.00	0.116
Fixed				
Population	1, 3	-3.62 ± 2.05	1.80	0.146
Plant size	1, 28.4	-0.25 ± 0.21	1.44	0.239
Spot	1, 29.4	0.29 ± 0.27	1.16	0.290
Floral longevity	1, 2681	0.99 ± 0.27	45.16	**<0.001**
Flower size	1, 1045	0.12 ± 0.21	13.83	**<0.001**
Number of flowers	1, 1663	-0.07 ± 0.04	0.64	0.422
Floral longevity × population	3, 952	-0.39 ± 0.34	0.87	0.458
Flower size × population	3, 433	0.57 ± 0.26	1.12	0.183
Number of flowers × population	3, 1222	0.13 ± 0.05	2.05	0.101

Florivory incidence

Overall, 189 flowers (ca. 7%) had some type of florivory (Table 2). The main florivores were several ant species picking stamens and beetles consuming petals and pollen. The incidence of florivory on *C. ladanifer* flowers did not differ among populations (Table 3). The incidence of florivory was significantly influenced by flower size and floral longevity (Table 3). Effects of flower size on florivory were significantly positive in three populations (Table 4) and were higher than 30% in Monte Valdelatas (Fig. 1). In addition, there was a significant positive effect of floral longevity on the proportion of damaged flowers, ranging from approximately 3% in 1 day flowers to 67% in 5 day flowers (Table 5).

Discussion

Both flower size and longevity positively influenced florivory incidence in *Cistus ladanifer*. The effect of flower size on the probability of damage by florivores is relatively well documented (Galen 1999; Galen and Cuba 2001; Lara and Ornelas 2001). In contrast, our finding of increased florivory on longer-lived flowers is novel. Previously, higher floral longevity has been related only to increased risk of anther smut infection (Shykoff *et al*. 1996; Kaltz and Shykoff 2001). Thus, floral longevity seems to have been understudied in relation to plant-animal antagonistic interactions and deserves further research.

Contrary to our expectations, number of flowers did not affect florivory. This is surprising, because florivore incidence has been previously associated with reduction in the number of flowers displayed (Krupnick *et al*. 1999; Mosleh Arany *et al*. 2009; Penet *et al*. 2009), as well as with other plant-animal antagonistic interactions, such as higher incidence of predispersal seed predators (Hainsworth *et al*. 1984; English-Loeb and Karban 1992; Kudoh and Whigham 1998) and herbivores (Ehrlén 1997;

Table 4 - Logistic regressions for the effects of flower size on presence of florivores for each of populations of *C. ladanifer*. Significant *P*-values are marked in bold

Population	R^2	$\beta \pm SD$	d.f.	N	*P*-value
Monte Valdelatas	0.26	0.56 ± 0.12	1	645	**<0.001**
La Pedriza	0.15	0.48 ± 0.18	1	701	**0.013**
Vista Real	0.18	0.65 ± 0.27	1	706	**0.016**
Puerto de Canencia	0.01	0.16 ± 0.23	1	671	0.477

Strauss and Whittall 2006; Sandring *et al*. 2007). In our case, the dominant florivores (ants and beetles) may use cues other than number of flowers to locate their food plants.

The relevance of florivores as agents of natural selection is dependent, among other factors, on their overall incidence. Studies on florivory have reported moderate to high incidences (e.g., 75% in Galen 1999; see however Breadmore and Kirk 1998; Malo *et al*. 2001). Maximum values of damaged flowers reported in the present work

Fig. 1 - Logistic adjustments showing the increased probability of florivory with increases in flower size, in the three populations of *C. ladanifer* where the relationship between flower size and florivory were significant (Table 4).

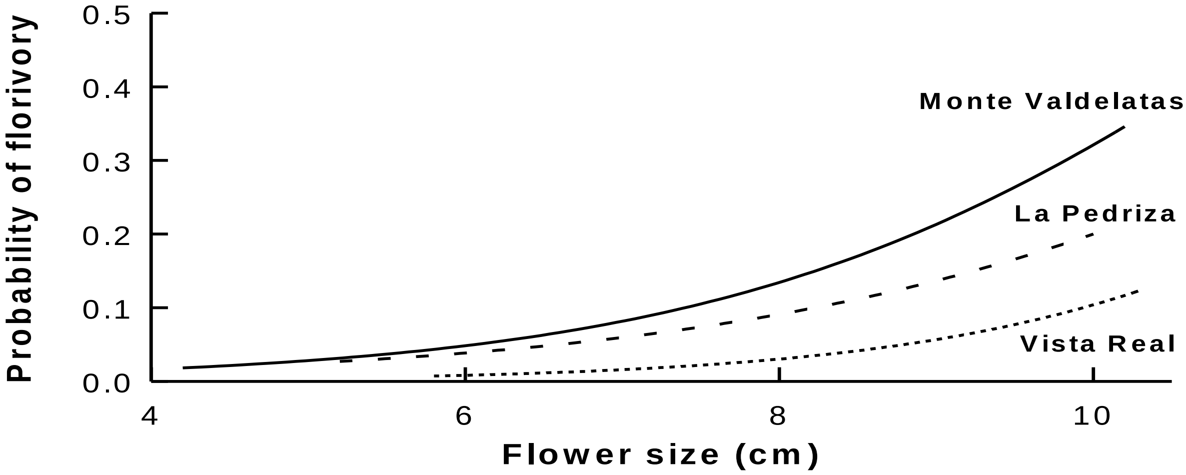

Table 5 - Proportions of damaged flowers by florivores for each floral longevity of each of populations of *C. ladanifer*. Sample size is in brackets. Likelihood ratio is the value of the G-statistic for testing that flowers with florivores are distributed randomly with respect to floral longevity. Significant *P*-values are marked in bold

Population	Floral longevity (d)					d.f.	Likelihood ratio (G)	*P*-value
	1	2	3	4	5			
Monte Valdelatas	0.075 (425)	0.108 (195)	0.308 (26)	-	-	2	11.561	**0.003**
La Pedriza	0.033 (182)	0.075 (414)	0.250 (84)	0.429 (14)	0.666 (3)	4	46.210	**<0.001**
Vista Real	0.027 (561)	0.069 (145)	-	-	-	1	5.119	**0.024**
Puerto de Canencia	0.038 (532)	0.074 (121)	0.111 (18)	-	-	2	4.061	0.131

(approximately 10%) along with the absence of significant differences of florivory incidence among populations would suggest at best a mild selective influence of florivores on *C. ladanifer*. As a comparison, the incidence of predispersal seed predation of *C. ladanifer* in the same area was greater than 40% (Delgado *et al*. 2007). However, temporal variation should be considered since annual variation in the influence of florivores has occasionally been documented (Galen and Cuba 2001; Kawagoe and Kudoh 2010).

Another important factor in determining the selective relevance of florivores is the extent to which damage is greater on larger or longer-lived flowers. For flower size, florivory probabilities of approximately 18% to 35% on largest flowers (Fig. 1) open the possibility of relevant selective pressures towards smaller flowers in Monte Valdelatas and La Pedriza. For floral longevity, florivory incidences of 25% to -67% on flowers lasting three or more days may also open these same possibilities towards short-lived flowers at those two populations (Table 5). Nevertheless, formal phenotypic selection analysis, and verification that flower longevity is a genetically controlled trait, would be needed to confirm these possibilities.

In conclusion, our results support the notion that some floral traits associated with attractiveness to pollinators are also attractive to different types of florivores. Both larger and longer-lived flowers suffered higher incidence of florivory within populations of *C. ladanifer*. The low overall incidence of florivores and its lack of between-population variation suggest that these florivory patterns may not influence intraspecific variation of these floral traits. However, moderate florivory levels on the largest and longest-lived flowers open the possibility of exerting a relevant selective pressure towards smaller and shorter-lived flowers in some of our study populations.

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