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Effects of flower position on the sexual specialization within inflorescences

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"Segue o teu destino,
Rega as tuas plantas,
Ama as tuas rosas.
O resto é a sombra
De árvores alheias.

A realidade
Sempre é mais ou menos
Do que nos queremos.
Só nós somos sempre
Iguais a nós-próprios.

Suave é viver só.
Grande e nobre é sempre
Viver simplesmente.
Deixa a dor nas aras
Como ex-voto aos deuses.

Vê de longe a vida.
Nunca a interrogues.
Ela nada pode
Dizer-te. A resposta
Está além dos deuses.

Mas serenamente
Imita o Olimpo
No teu coração.
Os deuses são deuses
Porque não se pensam."

Ricardo Reis, in "Odes"
Heterónimo de Fernando Pessoa

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i. Abbreviations

BM - Brownian motion model

COI - Herbarium of the University of Coimbra

et al. – (L. *et alia*) and others

F – Fruits from female flowers

FSD - Fruit size difference

GLMM - Generalized Linear Mixed Models

GM – Gynomonoecy

H – Hermaphroditism

I – Inner fruits from bisexual flowers

i.e. – (L. *id est*) that is

Lsmeans - Least square means

M – Monoecy

O – Outer fruits from bisexual flowers

OU - Ornstein-Uhlenbeck model

Pgls - Phylogenetic generalized least squared

S - Swedish Natural History Museum Herbarium

SAS - Statistical Analysis System

SD – Standard deviation

Note: all the units used follow the SI (Système International d'Unités)

ii. Resumo

Em muitas espécies de plantas com flor, as funções sexuais são segregados em flores diferentes dentro da mesma inflorescência. Além disso, esta especialização das flores, em inflorescências, nas funções masculinas e femininas frequentemente segue um padrão posicional. Por exemplo, dentro das inflorescências da família Asteraceae, ou seja, dentro do capítulo, um padrão de posição muito conservador é observado: as flores femininas estão quase sempre localizadas nas posições mais externas, enquanto que as flores masculinas estão nas posições mais internas. A especialização sexual dentro dos capítulos das Asteraceae pode ser a consequência da diminuição do gradiente de recursos das flores proximais para as flores distais, produzido por competição por recursos entre flores e frutos e restrições arquitectónicas no desenvolvimento da inflorescência. A diminuição do gradiente de recursos parece produzir sementes com tamanhos maiores nas flores exteriores em comparação com as partes internas dos capítulos, resultando num maior sucesso reprodutor feminino nas flores mais exteriores, o que poderá ter conduzido à especialização floral em funções sexuais em diferentes posições. Tamanhos de frutos diferentes podem estar relacionados com diferentes aptidões das plantas, visto que frutos maiores podem dar origem a plantas com maior capacidade competitiva, com prováveis consequências no sucesso reprodutivo. Se as flores mais exteriores produzem frutos maiores do que as flores mais interiores, os frutos exteriores vão dar origem a plantas com uma aptidão maior. Como consequência, o aumento do sucesso reprodutor feminino irá diminuir das flores exteriores para as flores interiores, levando a uma especialização da função sexual das flores em diferentes posições. Considerando tudo isto, o objectivo principal desta Tese foi o de analisar se a especialização de flores em diferentes funções sexuais em posições diferentes dentro dos capítulos de Asteraceae está relacionado com o gradiente de disponibilidade de recursos dentro dos capítulos. Assim, testou-se se especialização sexual está relacionada com o gradiente da disponibilidade de recursos, e se o padrão na atribuição de recursos dentro de capítulos está relacionado com o desempenho da planta. Para isso, em primeiro lugar, o tamanho dos frutos e as características capítulos foram medidos em 97 espécies, em diferentes linhagens da família, com diferentes níveis de especialização sexual dentro dos capítulos. Em segundo lugar, o desempenho da planta, como uma aproximação do fitness das plantas, produzidas por frutos das posições exteriores e interiores foi avaliado através de uma experiência de jardim com a espécie anual *Anacyclus clavatus*. Os resultados obtidos revelaram que as características da

inflorescência estavam correlacionadas com o sistema sexual, apoiando que a especialização sexual pode resultar de algum processo que ocorre ao nível inflorescência. A densidade floral aumentou das espécies hermafroditas para as espécies monóicas e foi positivamente correlacionado com a diferença no tamanho dos frutos (FSD). Portanto, estes resultados suportam que um aumento da competição entre flores pode conduzir a uma maior especialização das funções sexuais em diferentes flores. Apesar de não significativas, foram observadas algumas diferenças no tamanho dos frutos entre os sistemas sexuais, o FSD foi positivo (indicando que os frutos exteriores eram geralmente maiores do que as interiores) e maior nas espécies monóicas do que em espécies hermafroditas e ginomonóicas, indicando que, neste sistema sexual a diferença no tamanho dos frutos entre posições era maior. Além disso, a experiência de jardim, apoia a hipótese de que as plantas dos frutos exteriores produzem plantas maiores. No entanto, as diferenças observadas no desempenho da planta foram influenciadas por diferenças no tempo de germinação, com os frutos exteriores a apresentar uma germinação mais precoce e melhor desempenho nas características da planta, que foram analisadas. O efeito e a importância do tempo de germinação foi comprovada, quando as plantas germinadas ao mesmo tempo, não apresentaram diferenças em nenhuma das características analisadas. Assim, este estudo está de acordo com o princípio de que a germinação precoce influencia o sucesso da planta. Os resultados obtidos nesta Tese suportam as expectativas da hipótese de que os efeitos de posição e a disponibilidade de recursos ao nível da inflorescência podem transformar-se num espaço de tempo evolutivo em efeitos de posição no género floral. Diferenças no desempenho de plantas produzidas por frutos de diferentes posições dentro de uma inflorescência podem levar a padrões diferenciais de alocação do sexo em flores de diferentes posições. No entanto, para além de outras características do tamanho do fruto, tais como tempo de germinação, o sucesso esperado das plantas produzidas a partir de frutos provenientes de diferentes posições da flor pode alterar-se. Estes resultados abrem novas linhas de investigação na especialização floral em funções sexuais e todos esses aspectos podem ser aplicados no futuro para outros grupos e outros tipos de inflorescência que seguem também um gradiente de posição, a fim de confirmar se o nível de especialização na inflorescência é uma característica evolutiva geral em várias famílias de plantas.

Palavras-chave: Asteraceae; tempo de germinação; efeito de posição; gradiente de recursos; alocação do sexo.

iii. Abstract

In many species of flowering plants, sexual functions are segregated in different flowers within the same inflorescence. Furthermore, this specialization of flowers on male and female functions within inflorescences frequently follows a positional pattern. For instance, within the inflorescences of the family Asteraceae, i.e, within the capitulum, a very conservative positional pattern is observed: female flowers are almost always located at the outermost positions, whereas male flowers are at the innermost positions. Sexual specialization within the capitula of Asteraceae might be the consequence of a decreasing resource gradient from the proximal to the distal flowers produced by both resource competition among flowers and fruits and architectural constraints in the development of the inflorescence. The decreasing gradient of resources seems to produce seeds with larger sizes in the outer flowers compared to the inner parts of the capitula resulting in a higher female fitness at the outermost flower positions, which could drive to flower specialization on sexual function at different positions. Dissimilar fruit sizes may be related with different plant fitness since larger fruits may give rise to plants with greater competitive ability, with probable consequences in the reproductive success. If outermost flowers produce bigger fruits than innermost flowers, they will give rise to plants with a higher fitness. As a consequence, female fitness gain will decline from outer to inner flowers, ultimately leading to a specialization on sexual function of flowers from different positions. Considering all this, the main objective of this Master Thesis was to explore whether the specialization of flowers on different sexual functions at different positions within the capitula of Asteraceae is related to the gradient of resource availability within the capitula. Thus, it was tested if sexual specialization is related to the gradient of resource availability and if the pattern in resource allocation within capitula was related with plant performance. For that, first, fruit size and capitula traits were measured in 97 species across several lineages of the family with different levels of sexual specialization within their capitula. Second, plant performance as a proxy of plant fitness of plants produced by fruits from the outer and innermost positions was assessed by means of a common garden experiment with the annual species *Anacyclus clavatus*. The obtained results revealed that inflorescence traits were correlated with the sexual system supporting that sexual specialization may result of some process happening at the inflorescence level. Flower density increased from hermaphroditism to monoecy and was positively correlated with the standardized fruit size difference (FSD). Therefore,

these results supports that an increase of flower competition may lead to a higher specialization of sexual functions in different flowers. Despite not significant, some differences in fruit size between sexual systems were observed, with FSD being positive (indicating that outer fruits were generally larger than the inner ones) and larger in monoecious species than in hermaphroditic and gynomonoecious species, indicating that in this sexual system the difference in fruit size among positions was bigger and that the outer fruits were larger than the inner ones. Additionally, the common garden experiment supported the hypothesis that plants from the outer fruits yield higher plants. However, the observed differences in plant performance were mediated by differences in the germination time with the outer fruits that germinated earlier presenting a high performance in the life-history traits that were analyzed. The effect and importance of germination time was corroborated, when plants germinated at the same time did not present differences in any of the analyzed traits. Thus, this study is in accordance with the premise that early emergence influences the plant success. The results provided in this Thesis supports the expectations derived from the hypothesis that positional effects on resource availability at inflorescence level might turn in evolutionary time into positional effects on floral gender. Differences in plant performance produced by fruits from different positions within an inflorescence might lead to differential patterns of sex allocation on flowers at different positions. However, other traits apart from fruit size, such as germination time might change the expected success of plants originated from fruits from different flower positions. These results open new lines of research in the flower specialization on sexual functions and all these aspects can be applied in the future to other groups and other types of inflorescences that also follow a positional gradient, in order to confirm that specialization at inflorescence level is a general evolutionary feature in several plant families.

Key words: Asteraceae; germination time; position effect; resource gradient; sex allocation.

1. Introduction

1. Introduction

1.1. Sexual specialization of floral gender

The modular nature of plants allows a distribution of gametes in different units, individuals or flowers that allows plants to show a large variety of sexual systems (Lloyd, 1979; Diggle, 2003). Most angiosperms are hermaphrodites, i.e., all their flowers have both female and male organs, being named bisexual flowers, which is also considered the ancestral condition (Bawa & Beach, 1981; Harder & Barret, 1995). However, different species across distinct lineages have unisexual flowers, i.e., flowers with either stamens or pistils (Lloyd, 1972; Barrett, 2002). Two main groups of sexual systems are described depending whether the variation on sex expression happens within or between individuals. Thus, in monomorphic sexual systems all individuals bear both male and female organs; however they can be distinguished according with the different types of flowers: i) only bisexual flowers (i.e., hermaphroditic species); ii) female unisexual and bisexual flowers (i.e., gynomonoecious species); iii) male unisexual and bisexual flowers (i.e., andromonoecious species); iv) bisexual flowers and unisexual male and female flowers (i.e., trimonoecious species) and v) male and female unisexual flowers (i.e., monoecious species). In dimorphic sexual systems, sexual organs are displayed in different individuals, such as the case of dioecious plant (i.e., individuals with male flowers and individuals with female flowers). Other dimorphic sexual systems include gynodioecy (i.e., individuals with bisexual flowers and others with only female flowers) and androdioecy (i.e., individuals with bisexual flowers and others with male flowers, only) (Harder & Barret, 1995; Barrett, 2002; Torices *et al.*, 2011).

Within monomorphic sexual systems, the specialization of flowers on male and female functions frequently follows a positional pattern within inflorescences. Thus, sexual segregation in different flowers is not equal distributed within inflorescences. Such a positional pattern has been mainly demonstrated in linear inflorescences (reviewed in Diggle 2003). Female unisexual flowers are commonly placed at the base of the racemes whilst male flowers are on the top of this type of linear inflorescences (Kudo *et al.*, 2001; Wolfe & Denton, 2001). Positional patterns on the gender of flowers are also common on other inflorescence architectures, such as capitula and umbels (Bell, 1971; Burtt, 1977). For instance, in the family Asteraceae, many species have some kind of unisexual flowers (Anderberg *et al.*, 2007; Torices *et al.*, 2011), and,

within their capitula a very conservative positional pattern can be observed: female flowers are almost always located at the outermost positions, whereas male flowers are at the innermost positions. This positional pattern is maintained for all sexual systems including different combinations of female, male and bisexual flowers (Burtt, 1977; Torices *et al.*, 2011).

1.2. Selective pressures leading to specialization in floral gender

Avoiding inbreeding seems to be a major selective pressure on the gender specialization of flowers (Bawa, 1980; Barrett, 2002). Bisexual flowers allow a strong proximity between male and female organs, leading to an interference between organs and allowing self-pollination (Harder & Barret, 1995; Harder *et al.*, 2000, 2004). Inbreeding depression produced by selfing (including geitonogamy) leads to a higher frequency of homozygosis, reducing both fecundity and the probability of long-term survival (Harder & Barret, 1995; Freeman *et al.*, 1997; Harder *et al.*, 2000; Harder & Barrett, 2006; Charlesworth, 2006). Thus, at inflorescence level, sexual segregation on different flowers might have evolved to reduce the rate of selfing (Harder & Barret, 1995). Nevertheless, in hermaphroditic plants, the ability to recognize and reject their own pollen by means of different genetically-based mechanisms, i.e. self-incompatibility, is common (Franklin-Tong & Franklin, 2003; Hiscock & Tabah, 2003). Interestingly, these self-incompatibility mechanisms are not restricted to hermaphroditic species, and unisexual flowers have evolved in lineages of self-incompatible plants (Bertin, 1993). Therefore, floral sexual specialization cannot be explained, solely, as a mechanism to avoid selfing (Bawa, 1980; Bertin, 2010).

Indeed, sexual segregation might also be the result of a division of sexual functions mediated by a differential performance on male and female functions at different flower positions within the inflorescence. Fitness in hermaphrodite flowers is composed by the sum of male (pollen dispersal) and female fertilities (fruit production). The resource allocation to both sexual functions is expected to be equal when the reproduction through both functions is also equal (Charnov, 1982). However, a biased allocation to male or female functions is expected when that function obtains a greater fitness gain, in other words, when a function obtains a higher reproductive success by unit of resources allocated (Charnov, 1982; Diggle, 2003). In some ecological contexts, the division of sexual functions implies that the unisexual flowers provide a more efficient use of resources than bisexual ones (Bawa, 1980; Bawa & Beach, 1981).

Therefore, the allocation to female or male functions should be increased in those positions where each function is more likely to have success (Harder & Barrett, 2006). Under different ecological circumstances and at specific positions within inflorescences, the unisexual flowers might have a higher reproductive success than bisexual ones, favoring the selection of those mutants that allocate the resources efficiently to those sexual functions and to those specific positions.

One example on how the ecological context can affect the reproductive success of different flower positions are those species that display protandrous flowers aggregated in racemes with a sequential blossom, and that are pollinated by hymenoptera with a predictable behavior (Brunet & Charlesworth, 1995). Many hymenoptera visit first the bottom part of the racemes, moving then up to the uppermost parts. This behavior makes it more probable that flowers at the bottom receive outcrossed pollen, whereas the uppermost and last visited flowers are more efficient dispersing pollen to other inflorescences or individuals (Harder & Barret, 1995; Harder *et al.*, 2000, 2004). In this context, flowers at the bottom have a higher female reproductive success since they receive pollen of greater quality and thus produce offspring with higher levels of outcrossing, whereas flowers at the top have a higher male reproductive success, since they are capable to disperse more pollen grains and eventually sire more offspring (Harder *et al.*, 2004). This asymmetry on expected male and female reproductive success may drive the evolution of sexual specialization by means of differential sex allocation optima at different flower positions. In summary, flowers at the bottom will have a female-biased optimum whilst, flowers at the top will have a male-biased allocation optimum (Brunet & Charlesworth, 1995).

Additionally, male and female sexual functions respond differently to the availability of resources, which can also influence the evolution of floral genders (Freeman *et al.*, 1997). In general, female reproductive success is considered to be more resource-limited than male reproductive success, which is commonly limited by the availability of ovules to mate (Brunet, 1992). As female function is usually more expensive than male function (Harder & Barrett, 2006), flowers with higher amounts of available resources increase the amount of ovules and seeds. By contrast, in resource depletion scenarios, it might be more favorable for the plant to allocate the few resources to male function, promoting pollen dispersal (Bawa, 1980; Brunet & Charlesworth, 1995; Freeman *et al.*, 1997; Bertin & Kerwin, 1998; Barrett, 2002; Harder & Barrett, 2006). For instance, resource allocation to female organs is usually

increased in larger plants (de Jong & Klinkhamer, 1994), in larger inflorescences (Torices & Méndez, 2011), and even in bigger flowers (Koelewijn & Hunscheid, 2000; Méndez & Traveset, 2003) because they usually have a higher female reproductive success than smaller ones.

1.3. Resource availability gradient at inflorescence level

Within an inflorescence the available resources for the flowers seems to be unevenly distributed. For instance, the size of flowers and fruits is not equal, and varies according to its position in the inflorescence (Diggle, 2003; Torices & Méndez, 2010). The number of ovules, fruit size, flower size and fertility rates usually decrease from basal flower positions to apical positions on a inflorescence (Fig. 1) (Solomon, 1988; Medrano *et al.*, 2000; Diggle, 2003; Kliber & Eckert, 2004; Torices & Méndez, 2010). This positional pattern on flower and fruit traits is not restricted to raceme-like inflorescences, and other inflorescence types such as capitula of the sunflower family also display a similar pattern (Torices & Méndez, 2010). Within capitula, outer fruits are usually larger than the inner fruits (Fig. 1) (Ruiz De Clavijo, 1995; Imbert *et al.*, 1997; Imbert, 2002; El-Keblawy, 2003; Picó & Koubek, 2003). This positional variation, i.e., position effects, is mainly attributed to a combination of between-flowers competition for resources and to architectural constrains (Diggle, 2003; Kliber & Eckert, 2004; Torices & Méndez, 2010). The flowers or fruits that develop first have an earlier access to resources, and thus, the resources are limited for the flowers and / or fruits that develop later (Stephenson, 1981; Torices & Méndez, 2010). In general, basal (outer) flowers commonly start to develop its fruits before upper (inner) flowers. The first fruits will have then a temporal advantage, reducing the amount of resources available to the late and distal fruits. Furthermore, architectural constraints may restrict the size of flowers and fruits on specific flower positions. The physiological mechanism responsible for the architectural constrains remains unknown, however it seems to be related with the inflorescence development program (Alkio *et al.*, 2002; Diggle, 2003; Alkio & Grimm, 2003). Eventually, both mechanisms generate a resource gradient at inflorescence level that produces the positional gradient on flower, fruit size and fertility rates. This resource gradient from basal or outer and higher supplied flowers to upper or inner and more resource-limited flower positions might drive the evolution of flower gender specialization into female and male functions respectively.
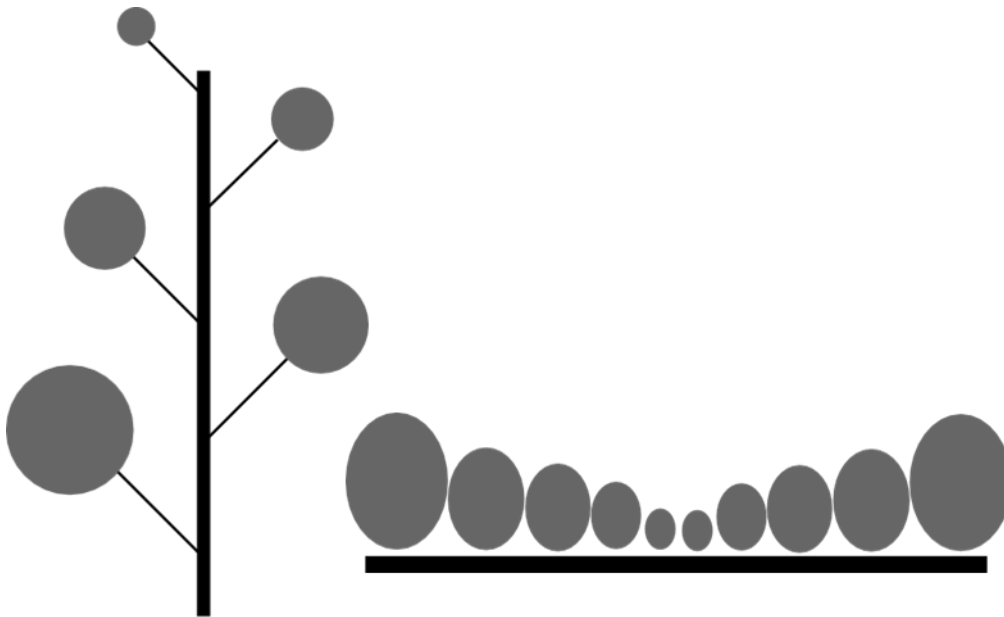
Figure 1. Position effects on fruit size in a raceme (left) and in a capitulum (right).

As referred above, sexual segregation within inflorescence is very common within capitula of the sunflower family. Torices, Méndez & Gómez (2011) hypothesized that floral gender specialization in this family might have been a consequence of differential sex allocation patterns at different floral positions mediated by the resource availability gradient. This hypothesis is connected with the centripetal flowering pattern. The outer flowers blossom firstly, having an earlier access to resources, and consequently are supplied with more resources than the inner flowers. Thus, outer flowers may give rise to larger fruits than inner ones, resulting in a continuous decline of female fitness from the outermost positions to the innermost ones within capitula (Fig. 2). Over time, the position effects in fruit size might influence the evolution of floral gender leading to specialization of the female function in outer flowers and of the male function in inner flowers.
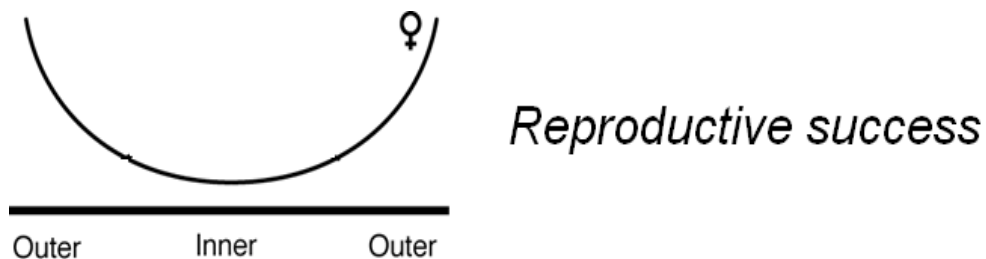

Figure 2. Female reproductive success along capitula. It decreases from outermost positions to innermost positions.

1.4. The role of fruit position on offspring´s fitness

Female fitness is expected to be higher at the outermost positions (Fig. 2) because flowers in those positions produce larger fruits compared to the innermost ones (Fig. 1). However to confirm this hypothesis it is fundamental to assess the effect that fruit position may have on the performance of its offspring. Plants resulting from outer and heavier fruits usually have a higher performance and fitness. Outer fruits may have higher germination rates, higher vigor of seedlings and survival than inner ones (Rai & Tripathi, 1987; Imbert *et al.*, 1996; Ruiz De Clavijo, 1998; Bastida & Menéndez, 2004; Dubois & Cheptou, 2012). However, this positional effect seems mediated mainly by fruit size differences (Torices & Méndez, 2010). Heavier fruits are frequently correlated with higher germination rates (Banovetz & Scheiner, 1994), higher plant growth and reproductive ability (Venable & Levin, 1985; Imbert *et al.*, 1997). Finally, variation in germination times could also affect the fitness of the offspring confounding the effects of fruit size and position. For instance, in several species larger fruits germinate early (Forsyth & Brown, 1982; Ellison, 1987; Imbert *et al.*, 1996; Espinosa-García *et al.*, 2003), have a higher survival (Venable & Levin, 1985; Rai & Tripathi, 1987; Banovetz & Scheiner, 1994; Imbert *et al.*, 1997; Dubois & Cheptou, 2012) and may be more competitive having a higher fitness (Dubois & Cheptou, 2012). Since fruit position, fruit size, and time of germination are usually correlated it is complicated to fully unravel causal links between them.

1.5. Objectives

The main objective of this Master Thesis was to explore whether the specialization of flowers on different sexual functions at different positions within the capitula of Asteraceae is related to the gradient of resource availability within the capitula. This positional pattern is particularly interesting to study in the family of Asteraceae, which is the angiosperms family with the largest number of species, and presents a worldwide distribution. In Asteraceae, a great diversity of sexual systems can be found, with 50% of the genera having a sexual system different from hermaphroditism. Also, most of species are self-incompatible or partially self-compatible and a recurrent positional effect in floral gender and fruit size can be found (Ferrer & Good-Avila, 2007; Torices *et al.*, 2011).

In Chapter 1, fruit size and several capitula traits were assessed in many species across different lineages and from all around the world. It was expected that floral

competition for resources will be positively correlated with sexual specialization, i.e., a higher floral competition for resources would lead to more positional differences within the capitulum. In addition, it was expected to find larger fruits in the outer positions than in the inner ones, and that differences between fruit sizes from different positions would be higher in gynomonoecious species than in hermaphrodite ones.

In Chapter 2, it was assessed whether the outer fruits give rise to plants with higher survival, performance and reproductive effort than those from the inner ones, using *Anacyclus clavatus*, as a study system. Besides presenting the typical positional effects in floral gender, as in this species fruit mass decreases from the outer positions to the inner ones, the effect of fruit size on plant's performance can also be evaluated. Furthermore, fruits from different positions are known to have different germination times. In *A. clavatus* outer fruits also have an early germination than inner ones (Torices *et al.*, 2013). Therefore, to fully evaluate the effect of fruit position, in one of experiments germination time was controlled constraining germination to the same time, whereas in another one, fruits were let to freely germinate. Therefore, it was possible to evaluate whether the obtained differences between plants from different fruit positions are mediated by differences in germination time or not It was expected that plants originated from fruits in the outer positions would present a higher fitness, i.e., that the position and fruit size would have an effect on the performance and fitness of the offspring. Also, it was expected that early germination would give rise to plant with a high performance.

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2. Chapter I

Sexual segregation within inflorescences: the role of floral resource competition

2.1. Introduction

Angiosperms show an extraordinary variation on the way they distribute their gametes on different units, flowers or individuals, i.e. gamete packaging strategies (Lloyd, 1979). Within the same population, individuals may differ in the relative production of male and female gametes (Lloyd, 1972; Wright & Barrett, 1999; Barrett, 2002; Méndez & Gómez, 2006), and even within the same individual, different flowers may produce different amounts of pollen grains, ovules and fruits (Solomon, 1988; Ishii & Sakai, 2002). These different strategies may have consequences on the reproductive success and therefore may lead to different adaptive responses (Lloyd, 1979). The sex allocation theory predicts that allocation to a given sexual function (female or male) should be increased in those circumstances where that function leads to a higher relative fitness (Charnov, 1982; Harder & Barrett, 2006). These adjustments in sexual allocation across individuals within a population or across flowers within an individual could ultimately lead to unisexual individuals or unisexual flowers, respectively.

In many plants from phylogenetically distant groups, the specialization of flowers on different genders shows a positional pattern (Diggle, 2003). This pattern is strikingly pervasive on the largest family of flowering plants, the sunflower family (Asteraceae) (Torices *et al.*, 2011). In this family, when unisexual flowers are present, female flowers are always placed in the outer positions within the capitulum, whereas male unisexual flowers are always placed in the innermost positions (Fig. 1). This sexual segregation within inflorescences has been hypothesized to result from other processes that are happening at the inflorescence level. For instance, Torices et al. (2011) proposed that the sexual specialization within the capitula of Asteraceae may be the consequence of unequal resource availability at different floral positions within the same inflorescence.

Not all flowers within the inflorescence are supplied with an equal amount of resources (Diggle, 2003). Within the inflorescences the proximal flowers usually start maturation before the distal ones. This phenological difference gives an advantage to the first flowers and fruits, as they have an earlier access to the resources, limiting the resources available for the last developing flowers and/or fruits. Limited resources result in smaller flowers and fruits or even abortion of these structures in distal positions. Indeed, several studies have demonstrated that when early flowers are removed, fruits from late flowers increase in size and a gradient in fruit size along the inflorescence is observed (Diggle, 1995; Ashman & Hitchens, 2000; Medrano *et al.*,

2000; Torices & Méndez, 2010). Thus, the number of flowers and ovules, fruit size and fertility rates usually decrease from proximal positions to distal positions within an inflorescence, a phenomenon called 'position effects' (Diggle, 1995, 2003; Kliber & Eckert, 2004; Medrano et al., 2000). Additionally, architectural constraints in the inflorescence development may also lead to a higher resource limitation in distal flower positions (Diggle, 2003; Kliber & Eckert, 2004; Torices & Méndez, 2010). The exact physiological mechanism responsible for these architectural constrains is yet unknown, however it has been related to a decreasing thickness of the vascular tissue along the inflorescence that limits the amount of resources distributed to distal positions (Alkio *et al.*, 2002; Alkio & Grimm, 2003). Most probably, both resource competition among flowers and fruits and architectural constraints are involved in generating a decreasing resource gradient from the proximal to the distal flowers.

The gradient of resource allocation could lead to sexual specialization in male and female flowers (Brunet & Charlesworth, 1995; Diggle, 2003; Mazer & Dawson, 2001; Torices & Méndez, 2010). Still, whether this positional effect within an inflorescence is promoting sexual specialization of flowers in different floral genders remains untested. The sunflower family may represent a suitable model to explore this hypothesis since positional patterns in both resource availability (Torices & Méndez, 2010) and gender specialization of flowers (Torices *et al.*, 2011) have been previously observed. In this family, the decreasing gradient of resources from the first opened outermost flowers to the last opened innermost flowers seems to produce seeds with larger sizes in the outer flowers than in the inner parts of the capitula (Torices & Méndez, 2010), resulting in a higher female fitness in the outermost flowers. Under this scenario, Torices *et al.* (2011) predicts that the flowers in optimal positions will allocate proportionally more resources to female organs, whereas flowers in suboptimal positions will become relatively male-biased.

The main objective of this study is, thus, to explore if the positional effects on resource availability have led to sexual specialization of flowers on different positions within the inflorescences, using Asteraceae as study system. Under this hypothesis, it is expected that i) outermost fruits will be generally higher than the innermost ones, and ii) fruit size differences between outer and inner fruits will be higher in gynomonoecious (individuals bearing female unisexual flowers at the outermost positions) species than in hermaphroditic ones. Furthermore, if the gradient on resource availability is promoting the observed floral gender specialization it is expected that inflorescence traits that

increase the resource gradient between the flowers within the inflorescence will be correlated with sexual specialization. For instance, it is expected that traits increasing the level of floral aggregation will promote floral competition, potentially leading to increased resource differences between outer and inner positions. In order to test these predictions, fruit size and capitulum traits were measured in more than one hundred species across different lineages of the sunflower family and analyses were done using a non-phylogenetic and a phylogenetic approach.

2.2. Materials and Methods

2.2.1. Study system

The sunflower family, Asteraceae, is the largest family of angiosperms and has a worldwide distribution (Funk *et al.*, 2005). This family displays an extraordinary diversity of sexual systems. While 50% of the genera is hermaphroditic, the other 50% have a different sexual system (Torices *et al.*, 2011). In addition, most of these nonhermaphroditic sexual systems are associated with floral specialization within capitula (Fig. 1), where unisexual flowers are present. Usually, female flowers are placed at the outermost positions whereas male flowers appear in the innermost ones (Fig. 1) (Torices *et al.*, 2011).
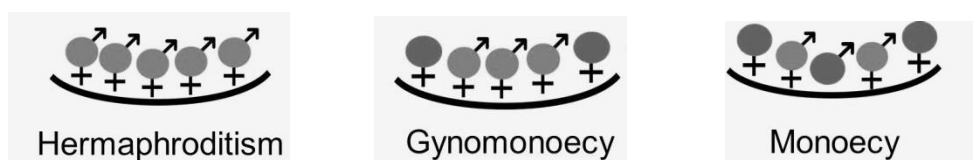
Figure 1. The position of bisexual, male and female flowers within the capitula of Asteraceae.

2.2.2. Inflorescence traits and fruit size at different positions

Inflorescence traits and fruit size were measured in herbarium material from the Asteraceae collection of the Swedish Natural History Museum Herbarium (S) and the Herbarium of the University of Coimbra (COI). First, specimens belonging to the species included in the phylogenetic supertrees published for this family (Funk *et al.*, 2005; Funk, 2009) were searched. Second, herbarium specimens with enough mature infrutescences (capitula with mature fruits) and in good conservation status were carefully selected. For each species, one specimen was selected and at least one

capitulum was sampled. Following this procedure, 100 herbarium specimens were sampled (78 from S and 22 from COI; Appendix I), comprising a total of 97 species. Sampling included 44 hermaphroditic species, and 55 non-hermaphroditic species (they bear at least some unisexual flowers), from which 30 were gynomonoecious and 23 were monoecious species. For the specimens selected in COI capitula were placed in soapy water for rehydration and easy manipulation of the material to reduce the damage to the capitulum.

All infrutescences were manually dissected to separate all fruits in their relative positions within capitula: from the outermost to the innermost positions. This separation was only possible in 70 species, due to lack of herbarium material. Infrutescences and fruits were measured using pictures taken with a tripod stabilized digital camera. The size of over 2700 fruits was measured as the two-dimensional projection of their outline using Image J 1.54s software (Abràmoff *et al.*, 2004). In addition, the inflorescence size given as the capitulum diameter was measured, and the total number of flowers in each inflorescence was counted. In some species as the flowers were not available where the number of flowers was impossible to count since flowers were not available, it was sampled another capitulum for counting them. Finally, flower density within each capitulum was calculated as the ratio between the number of flowers and the area of each capitulum. This trait was used as a measure of floral aggregation and integrates spatial constraints and resource competition between fruits within the capitulum.

2.2.3. Statistical analyses

a)   Positional variation on fruit size

In order to assess whether a general pattern on fruit size variation from outer and larger fruits to inner and smaller ones is observed, the fruit size and the position of the fruits within the capitulum of each sampled specimen were compared using two complementary analytical approaches.

First, a nonparametric test, Mann-Whitney U-test, was used to test whether outer fruits were larger than inner ones in each species. This conservative approach was used because data between fruit positions and across species was very heterogeneous regarding sample size and variance. Despite of the use of a nonparametric test, the presence of outliers, homogeneity of variances and normality of residuals were explored for each group of data.

Second, the meta-analytical effect size was used to get a standardized measure of the magnitude of the difference among the size of the outer and inner fruits (fruit size difference, hereafter FSD).This procedure allows comparing the differences between species because it takes into account measures of error and sample size (Gurevitch et al., 2001). The meta-analytical effect was originally designed to summarize statistical differences between different published studies (Gurevitch *et al.*, 2001), however it use is not restricted to such an approach. For instance, Hegland and Totland (2008) used effect size to study the magnitude of pollen limitation in a plant community. Thus, Hedges'd (Gurevitch & Hedges, 1999; Gurevitch *et al.*, 2001) was used as the measure of effect size. In particular, a random-effects meta-analysis was used. In this type of analysis the effect size is weighed by the inverse of their sampling variances to provide unbiased estimates with minimum variance (Gurevitch *et al.*, 2001). Effect sizes were calculated using the META package for R (R Development Core Team, 2008).

It was expected that the FSD increases with the sexual system specialization, that is, from hermaphroditism to gynomonoecy and monoecy. In order to evaluate if the FSD was correlated with sexual specialization, a phylogenetic comparative model was used. This is most appropriate approach as the sampling units, i.e., the species, are not independent. In other words, species are correlated within evolutionary time and, thus, some species are closer in time than others. When we have non-independent data and when phylogenetic information is ignored, statistical errors, such as, correlations between two variables that are not, in fact, correlated in their evolutionary history, or the opposite, such as missing patterns and correlations that in fact exist but get undetected. Considering all this, it is fundamental to introduce a phylogenetic control (Nunn, 2011). For that, the effect of sexual specialization on FSD was tested by means of phylogenetic generalized least squared (pgls) models (Freckleton *et al.*, 2002; Paradis, 2006). A phylogenetic supertree published for the family (Funk *et al.*, 2005), with a modification on its branch lengths (Torices, 2010) was used. All species without information were removed from the tree. Pgls were fitted using the "ape" and "geiger" packages for R (R Development Core Team, 2008). All models were evaluated under both an adaptive model (OU, Ornstein-Uhlenbeck model) (Butler & King, 2004) and a neutral model of evolution (BM, Brownian motion model) (Felsenstein, 2004). The fittest model for each combination of variables was selected using the Akaike Information Criterion and a likelihood ratio test comparing BM and OU models (Burnham & Anderson, 2002). For all fitted models the OU model had a higher

goodness of fit than the BM model (see Appendix II). Therefore, the results provided in this study originated from OU models. All analyses were fitted using R (R Development Core Team, 2008). Specific comparisons between hermaphroditism, gynomonoecy and monoecy were explored using least square means, which are marginal means (the group means after having controlled for covariates) using the 'lsmeans' package for R, which can be defined as a linear combination (sum) of the estimated effects from a linear model (R Development Core Team, 2008).

b)   Correlated evolutionary change between inflorescence traits and sexual specialization on floral gender

To explore whether floral competition by resources between flowers within capitula were associated with sexual specialization, it was assessed whether those inflorescence traits that may influence the intensity of floral competition were correlated with sexual specialization. Thus, it was expected that a higher floral density would be correlated with a higher sexual specialization. This means that hermaphroditic species would have lower floral density than gynomonoecious and monoecious species. Furthermore, it was explored whether FSD was correlated with inflorescence traits. For instance, it is expected that i) larger capitula also have higher FSD, ii) FSD should be positively correlated with floral competition. As above, these effects were tested by means of phylogenetic generalized least squared (pgls) models. All models were evaluated under both an adaptive model (OU, Ornstein-Uhlenbeck model) and a neutral model of evolution (BM, Brownian motion model) (see above for details).

Inflorescence trait differences between sexual systems were explored with (i) the nonparametric Kruskal-Wallis' H test, and (ii) a pgls approach (see above for details). Specific comparisons between hermaphroditism, gynomonoecy and monoecy were explored using least square means (see above for details).

2.3. Results

2.3.1. Positional variation on fruit size and sexual systems

Twenty-four out of 70 species had outermost fruits significantly different than innermost ones (Table I). In 13 species (6 hermaphroditic, 5 gynomonoecious, and 2 monoecious) the outer fruits were larger than inner ones whereas in the other 11 species

(6 hermaphroditic and 5 gynomoecious) inner fruits were significantly larger than outer ones (Table I).

No statistical differences between sexual systems in the size of outer and inner fruits were found using both a phylogenetic corrected test and a non-phylogenetic test (Table II). However, a different pattern can be envisaged from both approaches. When the phylogenetic relationships were not considered, and the arithmetic means were calculated, monoecious species had the largest outer fruits and the smallest inner fruits (Table II). The standardized fruit size difference (FSD), was higher in monoecious species and only in this sexual system was positive. Thus, a high difference among outer and inner fruit size was found in monoecious and outer fruits were larger than inner fruits. Nevertheless, this was not statically different (Table II).

When phylogenetic relationships were considered, outer fruits were larger than inner fruits in all sexual systems, however in gynomonoecy the size of the outer and inner fruits were almost the same. Fruit size in both outer and inner positions decreased from hermaphroditism, to gynomonoecy, and monoecy. However these differences were not statistically significant (Fig. 2A and B). Regarding the FSD, it was only positive for monoecious species, indicating that outer fruits were in general larger than the inner fruits (Fig. 2C). Hermaphroditic and gynomonoecious species had a negative value, indicating that inner fruits were larger than outer ones (Fig. 2C). Nevertheless, the FSD values for all sexual systems were neither statistically different from zero nor between sexual systems (Table II).

However, with exception of the number of flowers (Fig. 3B), the FSD was significantly affected by other inflorescence traits. Capitulum diameter showed a negative correlation with FSD (Fig. 3A), implying that capitula with higher diameters presented larger inner fruits than outer ones. By contrast, FSD and flower density showed a positively significant correlation (Fig. 3C), revealing that an increase of flower density was associated what a higher difference between the size of outer and inner fruits, with the outer fruits being larger.

Table I. Outer and inner fruit mean sizes (± SD) of hermaphrotidic, gynomonoecious and monoecious species. Diff. Difference between outer and inner fruits (+ indicates outer fruits were larger than inner ones; - shows the opposite; and 0 indicates no difference). Z (normal deviate) values for U Mann-Whitney statistic and standardized fruit size difference (FSD) are shown. ¹Sexual system: H – Hermaphroditism; GM – Gynomonoecy; M – Monoecy.

Species	Sexual System[1]	Diff.	Outermost fruits		Innermost fruits		P value	Z value	FSD ± SE
			n	Mean ± SD	n	Mean ± SD			
Ageratina calaminthaefolia	H	-	7	1.14±0.18	3	1.33±0.31	0.153	1.026	-0.781±0.726
Amellus strigosus	GM	+	33	**2.27±0.16**	15	**2.14±0.20**	**0.013**	**-2.224**	**0.738±0.321**
Ammobium alatum	H	+	53	**1.25±0.24**	88	**1.08±0.27**	**0.000**	**-3.652**	**0.652±0.178**
Arnica lanceolata	GM	-	43	**3.20±0.49**	7	**4.51±0.48**	**0.000**	**3.900**	**-2.638±0.492**
Baccharoides adoensis	H	-	29	4.47±0.55	18	4.57±0.59	0.384	0.295	-0.174±0.301
Baileya pleniradiata	GM	+	100	**1.49±0.18**	25	**1.37±0.14**	**0.012**	**-2.256**	**0.690±0.228**
Barnadesia spinosa	H	-	12	3.89±0.59	3	5.17±1.78	0.156	1.010	-1.362±0.708
Blumea riparia	GM	+	172	**0.39±0.05**	44	**0.34±0.05**	**0.000**	**4.524**	**0.207±0.166**
Brickellia chlorolepis	H	+	13	2.10±0.26	9	1.88±0.45	0.055	-1.603	0.607±0.445
Calendula arvensis	M	+	6	**17.47±5.02**	8	**8.98±0.47**	**0.001**	**3.098**	**2.438±0.766**
Calotis erinaceae	GM	+	38	3.24±0.51	12	3.14±0.43	0.289	-0.557	0.200±0.332
Chaptalia nutans	GM	-	32	**2.57±0.37**	19	**2.90±0.26**	**0.000**	**3.292**	**-0.973±0.307**
Chromolaena odorata	H	-	17	1.36±0.18	13	1.40±0.21	0.353	0.377	-0.201±0.369
Cyanthillium cinereum	H	-	13	0.70±0.05	6	0.76±0.17	0.396	-0.263	-0.566±0.504
Dasyphyllum diacanthoides	GM	+	14	2.56±0.38	4	2.25±0.58	0.144	-1.062	0.695±0.582
Dasyphyllum ferox	GM	-	7	4.10±0.38	5	4.35±0.49	0.146	1.056	-0.540±0.601
Dicoma anomala	H	-	9	2.37±0.33	4	2.75±0.43	0.061	1.543	-0.982±0.644
Doniophiton anomalon	GM	+	31	**12.41±1.63**	15	**13.99±1.47**	**0.002**	**2.835**	**-0.982±0.332**
Dubautia laxa	H	-	8	**1.24±0.21**	2	**1.79±0.10**	**0.018**	**2.089**	**-2.489±1.066**

Species	Sexual System[1]	Diff.	Outermost fruits		Innermost fruits		P value	Z value	FSD ± SE
			n	Mean ± SD	n	Mean ± SD			
Epaltes cunninghamii	M	+	16	0.43±0.19	11	0.42±0.08	0.394	0.888	0.062±0.392
Ethulia conyzoides	H	+	15	0.88±0.14	7	0.85±0.03	0.376	-0.317	0.244±0.460
Florestina pedata	**H**	**+**	**6**	**2.25±0.28**	**4**	**1.85±0.19**	**0.017**	**-2.132**	**1.445±0.767**
Gnaphalium microcephalum	GM	0	27	0.08±0.02	10	0.08±0.02	0.286	-0.564	0.000±0.370
Grindelia arenicola	**GM**	**-**	**54**	**3.36±0.45**	**25**	**3.86±0.56**	**0.000**	**3.826**	**-1.017±0.256**
Gymnarrhena micrantha	M	-	13	1.31±0.40	7	1.60±0.24	0.052	-1.624	-0.783±0.489
Hirpicium echinus	H	+	11	2.28±0.44	5	2.24±0.39	0.433	0.170	0.089±0.540
Inula oculus-christi	GM	-	26	0.88±0.14	75	0.97±0.11	3.076	0.002	-0.755±0.234
Inula peacockiana	H	+	16	5.63±0.49	4	5.52±0.43	0.378	0.705	0.219±0.560
Jungia paniculata	H	+	19	0.69±0.12	8	0.62±0.12	0.106	-1.248	0.566±0.430
Kleinia longiflora	H	-	8	6.74±1.19	3	7.46±0.28	0.110	1.225	-0.622±0.697
Layia platyglossa	GM	-	20	1.93±0.17	15	1.98±0.17	0.184	-0.900	-0.287±0.344
Liabum bourgeaui	**GM**	**-**	**52**	**0.15±0.03**	**17**	**0.17±0.03**	**0.045**	**1.692**	**-0.659±0.285**
Liatris aspera	**H**	**-**	**17**	**4.15±0.37**	**8**	**4.49±0.36**	**0.016**	**2.155**	**-0.896±0.450**
Ligularia fischeri	GM	-	11	4.96±0.50	7	4.97±0.65	0.482	0.045	-0.017±0.484
Marshallia graminifolia	**H**	**-**	**26**	**3.12±0.32**	**29**	**3.61±0.35**	**0.000**	**4.628**	**-1.437±0.305**
Microseris douglasii	**H**	**+**	**25**	**2.33±0.14**	**15**	**2.19±0.19**	**0.009**	**-2.375**	**0.856±0.342**
Millotia myosotidifolia	**H**	**-**	**44**	**0.84±0.18**	**21**	**1.07±0.23**	**0.000**	**3.584**	**-1.152±0.285**
Monolopia lanceolata	GM	+	22	1.05±0.15	10	0.99±0.17	0.186	-0.894	0.374±0.385
Onoseris alata	**GM**	**+**	**16**	**4.42±0.90**	**9**	**3.14±0.46**	**0.001**	**-3.199**	**1.596±0.484**
Onoseris odorata	**GM**	**-**	**20**	**3.41±0.35**	**10**	**3.78±0.50**	**0.024**	**1.980**	**-0.890±0.406**
Oxypappus scaber	GM	+	22	0.17±0.03	8	0.16±0.02	0.279	-0.586	0.350±0.416
Palafoxia arida	H	+	12	7.95±1.31	5	7.64±1.01	0.337	-0.422	0.238±0.534
Perezia multiflora	H	+	22	3.78±0.48	24	3.73±0.61	0.383	-0.297	0.089±0.295
Perityle emoryi	GM	+	44	1.63±0.14	23	1.59±0.24	0.424	-0.191	0.219±0.258
Philoglossa peruviana	GM	+	15	1.10±0.08	4	1.08±0.07	0.382	-0.300	0.244±0.564

CHAPTER I

| Species	Sexual System[1]	Diff.	Outermost fruits		Innermost fruits		P value	Z value	FSD ± SE
			n	Mean ± SD	n	Mean ± SD			
Pluchea dentex	M	0	57	0.18±0.04	81	0.18±0.03	1.271	0.204	0.000±0.173
Porophyllum scoparium	**H**	-	**39**	**1.56±0.23**	**24**	**1.82±0.21**	**0.000**	**3.892**	**-1.153±0.280**
Roldana mexicana	H	+	14	1.42±0.24	5	1.37±0.26	0.391	-0.278	0.195±0.522
Rosenia hulilis	GM	+	10	2.45±0.32	8	2.23±0.23	0.153	-1.022	0.737±0.494
Rudbeckia fulgida	H	-	51	1.15±0.14	32	1.16±0.10	0.379	0.309	-0.079±0.226
Senecio inornatus	GM	-	22	0.66±0.14	7	0.74±0.05	0.063	1.529	-0.619±0.443
Senecio subsessilis	GM	-	20	3.16±0.35	8	3.38±0.45	0.143	1.068	-0.563±0.426
Sinclairia polyantha	GM	-	14	0.43±0.07	4	0.49±0.11	0.050	1.646	-0.723±0.583
Soliva pterosperma	**M**	+	**10**	**1.55±0.25**	**5**	**1.02±0.21**	**0.003**	**2.756**	**2.092±0.706**
Streptoglossa liatroides	GM	-	34	1.79±0.21	27	1.86±0.18	1.038	0.299	-0.350±0.260
Trixis antimenorrhoea	H	+	7	1.23±0.04	2	1.21±0.14	0.500	0.000	0.275±0.806
Uropappus lindleyi	**H**	-	**23**	**4.54±0.13**	**9**	**4.81±0.13**	**0.000**	**3.919**	**-2.025±0.477**
Vernonanthura patens	H	+	9	0.90±0.09	3	0.82±0.14	0.230	-0.740	0.724±0.691
Vernonia alamanii	H	-	46	4.10±0.69	25	4.43±0.76	0.064	1.523	-0.456±0.252
Vernonia amygdalina	H	+	7	1.74±0.29	5	1.57±0.19	0.232	-0.731	0.616±0.605
Vernonia angustifolia	H	0	13	1.69±0.31	5	1.69±0.23	0.215	0.789	0.000±0.526
Vernonia anisochaetoides	H	-	15	1.04±0.14	7	1.06±0.18	0.472	-0.070	-0.126±0.458
Vernonia cinerascens	H	+	10	1.22±0.14	5	1.08±0.22	0.099	-1.286	0.781±0.572
Vernonia fastigiata	**H**	+	**36**	**2.26±0.55**	**19**	**1.66±0.31**	**0.000**	**-3.894**	**1.227±0.308**
Vernonia galamensis	H	-	22	3.62±0.28	14	3.73±0.42	0.118	1.184	-0.316±0.344
Vernonia glabra	H	+	8	4.58±0.61	2	4.36±0.19	0.217	-0.783	0.346±0.797
Vernonia lasiopus	**H**	+	**10**	**1.38±0.28**	**5**	**0.93±0.24**	**0.004**	**-2.694**	**1.578±0.642**
Vernonia poskeana	H	-	30	1.90±0.22	13	1.91±0.36	0.210	0.807	-0.037±0.332
Vernonia tortuosa	**H**	+	**22**	**1.40±0.32**	**11**	**1.12±0.31**	**0.026**	**-1.948**	**0.862±0.386**
Warionia saharae	H	-	21	15.31±2.69	19	15.51±2.49	0.414	0.217	-0.075±0.317
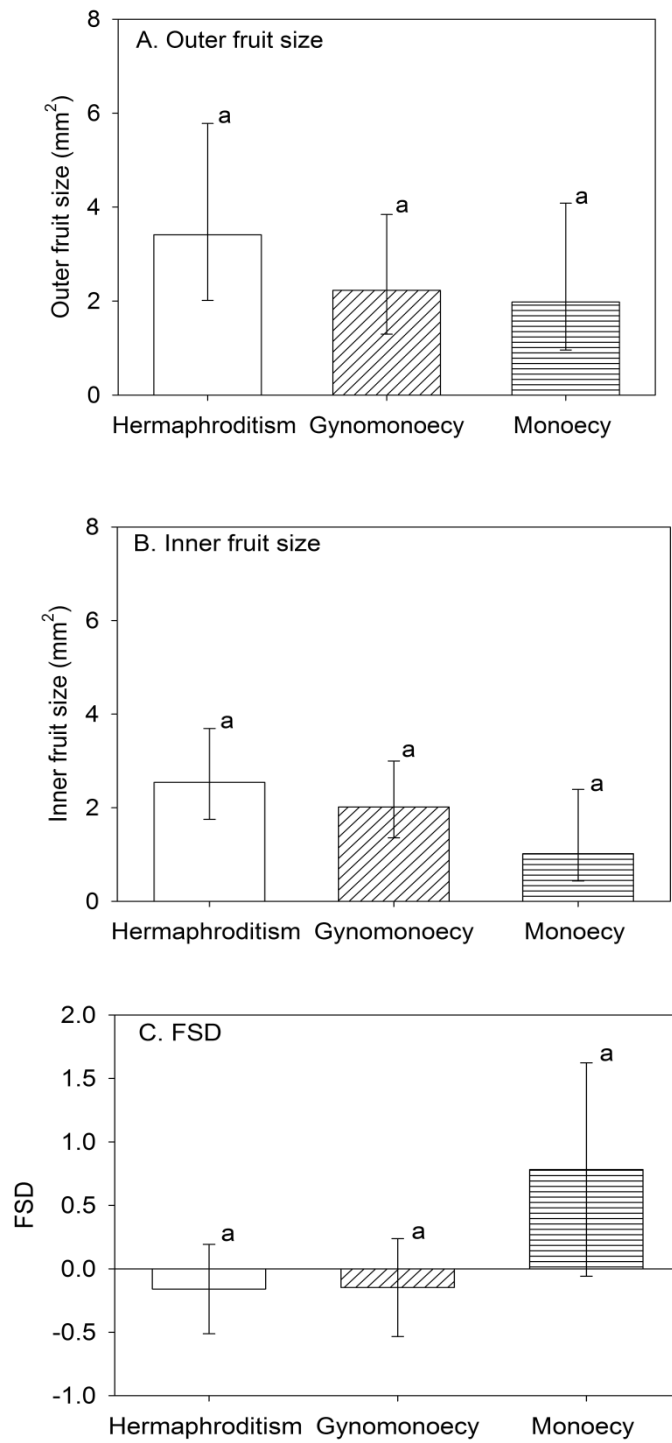
Figure 2. Least squares means (± Confidence Interval) of outer fruits size (A), inner fruits size (B) and FSD (C) for different sexual systems. Values sharing a superscript were not significantly different at the $P < 0.05$ level.
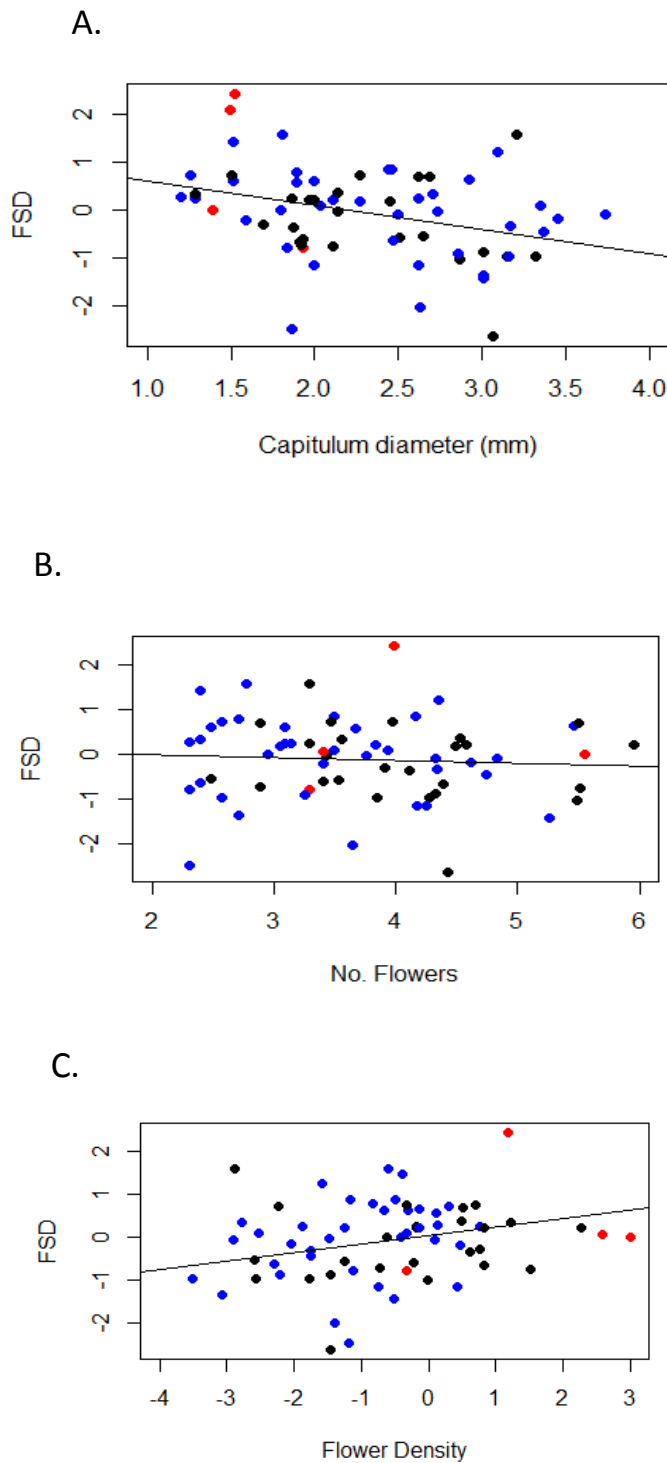
A.

B.

C.

Figure. 3. Phylogenetic regression between FSD and (A) capitulum diameter ($b \pm$ SE= -0.50 ± 0.16; $t = -3.112$; $P = 0.003$); (B) number of flowers ($b \pm$ SE= -0.06 ± 0.12; $t = -0.497$; $P = 0.621$) and (C) flower density ($b \pm$ SE= 0.20 ± 0.08; $t = 2.451$; $P = 0.02$), among sexual systems (blue dots: hermaphroditic species; black dots: gynomonoecious species and red dots: monoecious species).

Table II. Mean ± SD for inflorescence traits of Asteraceae species with different sexual systems. For the non-phylogenetic test a Kruskal-Wallis statistic was used and the H and P values are presented. For the phylogenetic test (phylogenetic generalized least squared - pgls), the F and P values, as well as the degrees of freedom (d.f.), are showed. n – sample size. For fruit size, the total number of species used is shown in parentheses. FSD – fruit size difference measured as meta-analytical effect size. For each inflorescence trait, means sharing the same superscript letter were not significantly different at the $P < 0.05$ level.

Inflorescence traits		Sexual system			Non-phylogenetic test		Phylogenetic test		
		Hermaphroditism	Gynomonoecy	Monoecy	H	P	F	d.f.	P
Fruit size (mm^2)	Outer	2.95 ± 2.63 (42)[a]	2.68 ± 2.84 (29)[a]	3.11 ± 4.19(15)[a]	0.71	0.700	1.29	2, 77	0.281
	Inner	2.91 ± 2.75 (40)[a]	2.73 ± 2.91 (28)[a]	2.44 ± 3.70 (5)[a]	1.90	0.390	2.01	2, 66	0.142
FSD		-0.09 ± 0.89 (39)[a]	-0.19 ± 0.82 (26)[a]	0.76 ± 1.27 (5)[a]	1.51	0.470	2.30	2, 64	0.109
Capitulum diameter (mm)		12.34 ± 8.91[a]	11.11 ± 6.73[a]	5.04 ± 2.18[b]	19.99	0.000	11.92	2, 85	<0.001
No. Flowers		42.93 ± 48.44[ab]	76.43 ± 88.74[a]	32.13 ± 50.81[b]	9.66	0.008	3.72	2, 84	0.028
Flower density (no. flowers/mm^2)		0.59 ± 0.56[a]	1.53 ± 2.10[ab]	2.57 ± 4.72[b]	11.85	0.003	6.65	2, 84	0.002
n		44	30	23					

2.3.2. Inflorescence traits and sexual specialization

Capitulum diameter, number of flowers and flower density were significantly different between sexual systems in both, the non-parametric test (Table II) and in the pgls in which the phylogenetic relationships were taken into account (Fig. 4). Hermaphroditic and gynomonoecious species had higher capitulum diameters than monoecious species (Table II; Fig. 4A). The hermaphroditic and monoecious species had the lower number of flowers (Fig. 4B), but there were only significant differences between monoecious and gynomonoecious species when the phylogenetic relationships were not taken into account (Table II). The density of flowers, measured as the ratio between the number of flowers and the capitulum area, was significanly correlated with the level of sexual specialization. Monoecious species presented the highest values of flower density, being this value significantly different from the one obtained for hermaphroditic species (Table II, Fig. 4C).
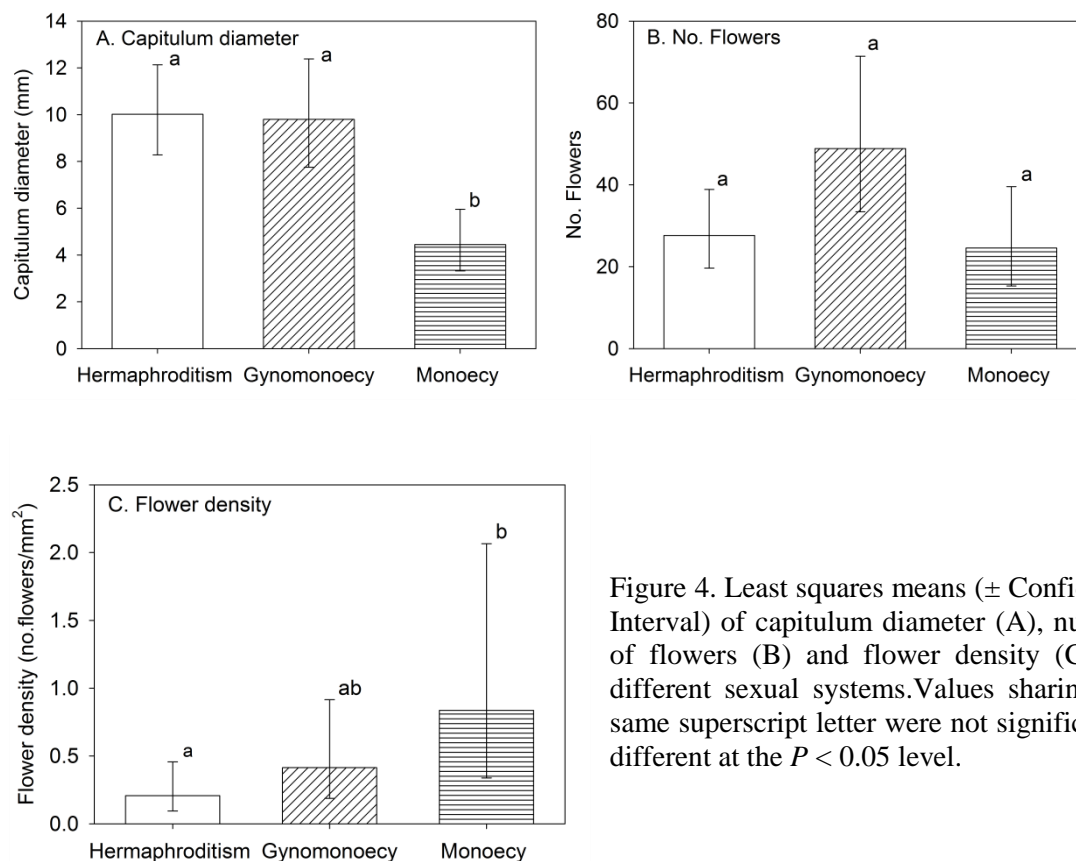

Figure 4. Least squares means (± Confidence Interval) of capitulum diameter (A), number of flowers (B) and flower density (C) for different sexual systems. Values sharing the same superscript letter were not significantly different at the $P < 0.05$ level.

2.4. Discussion

It has been hypothesized that floral sexual specialization within Asteraceae inflorescences may be mediated by a resource gradient within capitula that is in part a

consequence of a centripetal anthesis of the flowers within the inflorescence (Torices *et al.*, 2011). This study provides the first test of this hypothesis. Although, not all species presented larger fruits at the outer positions, there are other evidences that support it. For instance, the level of floral aggregation, which is related with the level of resources available for flowers and fruits, was correlated with the specialization of flowers on different sex functions suggesting that sexual specialization may be a consequence of resource partitioning between competing functions within inflorescences.

According with the resource gradient hypothesis, fruits from earlier and outer flowers should be larger because they have an earlier access to resources and consequently more resources than inner fruits which will be smaller. Such pattern has been already observed in previous studies in different Asteraceae species (Tanowitz *et al.*, 1987; Diggle, 1995, 2003; Imbert, 1999; Gibson, 2001; Ruiz De Clavijo, 2001; El-Keblawy, 2003; Torices & Méndez, 2010). Still, in this case, most of the analyzed species did not show a decreasing pattern on fruit size from the outer to the inner fruits within capitula.

Despite no significant differences in fruit size between sexual systems were observed, FSD was positive and higher in monoecious species than in other sexual systems, indicating that in this sexual system the difference in fruit size among positions is high and that the outer fruits were larger than the inner ones. In the other sexual systems the negative FSD indicates that inner fruits were larger, despite the value is very close to zero, indicating that there are almost no differences in fruit size between both positions. Therefore, only for monoecious species the results are in agreement with the resource gradient hypothesis. As, monoecy is the more specialized sexual system, it was already expected that the differences in resource allocation and consequently in fruit size would be more evident in the species with such a system.

The absence of an evident pattern in this data set, which contrasts with previous studies, may be due to the different methodological approaches that were followed. In many previous papers, weight was used as a proxy of the investment on fruit size, (Eriksson, 1999; Gibson, 2001; Ruiz De Clavijo, 2001; Picó *et al.*, 2003; Mölken *et al.*, 2005; Torices & Méndez, 2010), while in this case fruit size was actually measured by means of fruit area, which might not eventually describe accurately the magnitude of the position effect on fruit size. Another factor that may have influenced the results was the criteria used to divide the fruits in outer and inner fruits. According with the resource gradient hypothesis the more remarkable differences in fruit size are found in the

extremes of the capitula. Despite this methodology was followed in most species, in some cases due to material limitation this was not possible.

Inflorescence traits were correlated with the sexual system supporting that sexual specialization may result of some process happening at the inflorescence level. Flower density (the ratio between the numbers of flowers per capitulum area) increased from hermaphroditism to monoecy and was positively correlated with FSD. Therefore, these results agree with the positive correlation between floral density and level of sexual specialization, evolving from hermaphroditism to gynomonoecy and monoecy. (Bawa, 1980; Bawa & Beach, 1981; Torices, 2009; Torices & Méndez, 2010). Thus, the evolution of these sexual systems was correlated with a gradient of flower density.

Overall, monoecious species may be the result of flower competition. Monoecious species had small capitula but with many flowers producing a high competition for resources between them and leading to a high difference in fruit size within capitula. These small capitula are also associated with larger outer fruits compared to inner ones. In the past, Torices and Anderberg (2009) also demonstrated that gynomonoecious and hermaphroditic species of the tribe Inuleae frequently display solitary capitulum or small groups of few capitula, whereas monoecious species bear agglomerates of many small capitula. They suggest that evolution of capitulum size, number of flowers and flower density can be shaped by other factors (Torices & Anderberg, 2009) such as pollinators (Celedón-Neghme *et al.*, 2007; Andersson, 2008) or predators (Fenner *et al.*, 2002; Bertin, 2010) driving afterwards the specialization of flowers on different sexual functions.

The hypothesis and results discussed here for Asteraceae, can also have implications in the evolution of unisexual flowers of other plant families. In Liliaceae, Myrtaceae and Solanaceae flower position and traits within the inflorescence also follow a pattern (Primack & Lloyd, 1980; Solomon, 1988; Spalik, 1991; Emms, 1993; Diggle, 2003; Miller & Diggle, 2003). Bisexual flowers are heavier than male flowers, and all flower structures are lighter in male flowers (Emms, 1993). The number of ovules per ovary and the size of the ovaries, as well as the mass and seed number decreased significantly along the inflorescence (from basal to distal positions) (Solomon, 1988). Plants with larger inflorescences have more male flowers and the percentage of hermaphrodite flowers on a plant increased with resource availability (Primack & Lloyd, 1980), suggesting a resource dependent sex allocation. Therefore, the differential resource avaiability ate different flowers positions, may also affect the

evolution of unisexual flowers on distant lineages of Angiosperms, and deserve further research.

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3. Chapter II

The effect of achene position on plant performance in *Anacyclus clavatus*

3.1. Introduction

Many Asteraceae present variation in the size of the fruits depending on their position within the capitulum, with the inner fruits being typically smaller than outer fruits (Tanowitz *et al.*, 1987; Diggle, 1995, 2003; Imbert, 1999; Gibson, 2001; Ruiz De Clavijo, 2001; El-Keblawy, 2003; Torices & Méndez, 2010). It has been argued that the decline in fruit size when heading into the interior of the capitulum may mainly result from two mechanisms (Torices & Méndez, 2010): resource competition between flowers and fruits where the outer flowers have earlier access to the resources, and thus limiting them to inner ones (Stephenson, 1981), and architectural constraints in the organ development (Diggle, 2003). The combination of both mechanisms generates a gradient in the availability of resources from the outer to the inner positions within a capitulum leading to the common pattern of variation of achene size, i.e., outer fruits are larger than inner ones.

This positional variation of fruit traits can have consequences on post-dispersal life history traits, such germination time and probability of survival, influencing the offspring in space and time. Dissimilar fruit sizes may be related with different plant fitness since larger fruits may give rise to plants with greater competitive ability, with probable consequences in the reproductive success. Larger fruits typically have a higher percentage of viability, germination and survival (Rai & Tripathi, 1987; Banovetz & Scheiner, 1994; Imbert *et al.*, 1997). Torices and Méndez (2010) also showed that fruit size affects seedling survival and growth. The heavier fruits were normally correlated with increased growth rates and reproductive ability. As a consequence of such differences, plants originated from larger fruits are described to be more stress tolerant than plants from smaller fruits (Venable & Levin, 1985a; Imbert, 2002). In addition, when growing under competition, heavier fruits were shown to be more competitive and to have high reproductive outputs. Facing all this, it is clear that the initial size of the fruits could influence plant development (Imbert *et al.*, 1997). Also, fruits with different sizes and from different positions within an inflorescence may have different germination times, with fruits germinating earlier having an advantage over the others (Rai & Tripathi, 1987; Imbert *et al.*, 1997; Imbert, 2002; Donohue *et al.*, 2010).

Furthermore, variation in plant performance due to differences in diaspore traits may have deep implications on other important biological processes of the plant's life cycle, such as, dispersal strategies (Imbert, 2002) and/or floral gender specialization

(Torices *et al.*, 2011). Within the inflorescences of an Asteraceae, besides the differences in fruit size, the outer flowers are commonly specialized on female function whereas the inner ones are either bisexual or male. This matching between positional patterns: fruit size and floral gender specialization within capitula is used to propose an hypothesis to explain the evolution of floral gender specialization in this family (Torices *et al.*, 2011). If outermost flowers produce larger fruits than innermost flowers, they will give rise to plants with a higher fitness. As a consequence, female fitness gain will decline from outer to inner flowers, ultimately leading to a specialization on sexual function of flowers from different positions. Because the cost of producing the female function (fruits) is more expensive than the male function (pollen), when under resource limitation, the female function will be detrimentally affected and resources will mostly be allocated to male function. Thus, in outer positions of capitula where the resources are higher producing larger fruits, the female fitness of those flowers should be also higher, whereas in inner positions where the resources are more limited producing smaller fruits, the female fitness should be smaller. Over time, a permanent change to the female function in outer positions and for male function in inner positions can occur. Still, to our knowledge no study has tested if fruits from different positions yield plants with different fitness, more specifically, if plants from outer fruits have a higher fitness when compared to plants from inner ones.

Therefore, the objective of this study was to evaluate if the position of the fruit (hereafter achene) within the inflorescence affects plant fitness, using the annual *Anacyclus clavatus* (Asteraceae) as a study species. This species is an appropriate model because previous studies by Torices *et al.* (2013) have shown that achene mass significantly decreases from the outer positions to the inner ones. Additionally, the achenes of *A. clavatus* have different germination rates, with the outer achenes germinating earlier than the inner achenes (Torices *et al.*, 2013). In annual species, this early germination may result in a competitive advantage (Imbert *et al.*, 1997; Donohue *et al.*, 2010; Dubois & Cheptou, 2012), therefore to evaluate the effect of achene position per se in plant developmental traits, it is important to consider the effects of germination time. For that, two different common garden experiments were set up, one where achenes were sowed at the same time in pots, and another where they first germinated in Petri dishes, and only after germination they were transferred into pots. This experimental design will be able to answer the following specific questions: i) do plants from the outer achenes have a higher survival rate?; ii) do the outer achenes

produce larger plants and with higher below- and above-ground biomass?; iii) do outer achenes produce plants with a higher reproductive effort?; iv) do the earliest germinating achenes have a higher plant performance?; and finally, v) are the differences between plants originated from different achene positions mediated by differences in germination time?

3.2. Materials and Methods

3.2.1. Study system

The effect of fruit position, fruit size and time of germination on plant performance was studied in *Anacyclus clavatus* (Desf.) Pers. This species occurs in the Western Mediterranean Peninsula and is an appropriate model because: (i) fruit size has a position pattern; (ii) their capitula are gynomonoecious, bearing female and bisexual flowers, and (iii) it is a winter annual species, and therefore it enables to explore the effect of fruit size and position on the later stages of its life history such as plant size, flowering traits and total reproductive effort. Female flowers are always placed at the outermost positions of the capitula whilst bisexual flowers are placed at inner positions. This trait allows to test whether fruits from flowers specialized on female function may produce offspring of higher quality than bisexual flowers.

3.2.2. Fruit material, experiment and grow conditions

Capitula were sampled from 37 different mother plants in a population in the south of Spain (36°41'49"N; 3°27'33"W, 13 m a.s.l., Carchuna, Spain). For each capitulum, the achenes were separated in the following categories depending on its position: F - achenes from female flowers, which were in the outermost position; O - the outermost achenes from bisexual flowers; and I - the innermost achenes from bisexual flowers, which were also the innermost achenes of the whole capitulum. All achenes were weighed until the nearest 0.1 mg before sowing. They were weighed in groups, due to their reduced weight. Achenes from female flowers were weighed in groups of two and achenes from the other two positions in groups of 10.

To explore the different effects of position and time of germination, two different experiments were performed. In the first experiment, achenes were sowed directly in pots at the same time. In this way, achenes germinated at different times, allowing testing the effect of germination time in plant traits, such as, plant survival, and plant performance. Achenes from each of the three positions (F, O, and I) of 30

distinct capitula, representing 30 different genetic families were sowed in pots of 8.0 x 8.0 cm and 9.5 cm height filled with a mixture of gardening substrate and sand (1:2), and kept in a greenhouse (Fig. 1). Two achenes of each of the positions were placed to ensure the germination of at least one. Later, when both seeds germinated, one of the seedlings was removed, so that each pot presented only one achene.

As previous studies have shown that outermost achenes (F and O) can germinate within 1-2 days after watering and approximately 10 days before than the innermost ones (Torices *et al.*, 2013), in the second experiment, the germination time was manipulated to remove the effect of germination time and observe the effect of achene position *per se*. Thus, first achenes from the inner positions belonging to 37 different capitula were placed to germinate in Petri dishes with sand (Fig. 2). The germination was controlled every day and when the first achenes of a given capitulum germinated (achenes were considered germinated after radicle emergence), the F and O achenes of that capitulum were immediately placed to germinate at the same conditions. Those seedlings germinated within the same day or in the day after and thus seedlings from each position were transplanted at the same time to pots. Using this procedure, for 29 out of the 37 families it was possible to obtain seedlings from F, O and I positions that germinated approximately at the same time. In this experiment, only one achene from each of the three different positions was transplanted to the pots under the same conditions described above.

Both experiments began in October and run until June 2013. Pots were monitored weekly to record plant survival and flowering traits. The first and last day of flowering of each capitulum were recorded, and capitulum and disk diameter were measured. After dying, plants were harvested and in the laboratory, flowering heads, stems, leaves and roots were separated into paper bags, dried at 68 ˚C for 48h and weighed in an analytical scale up to the nearest 0.1 mg. Plant performance was measured as total biomass including, above- and below-ground biomass. In addition, the reproductive effort of each plant was estimated as the number and size of capitula, the biomass allocated to capitula and the flowering duration (how many days each plant was flowering). Percentage of above- and below-ground and reproductive biomass was assessed to study the proportion of resources allocated to each of these parts in respect to the total biomass of each individual, and thus, evaluate if different fruit positions lead to different patterns of resource allocation.

3.3.3. Statistical analyzes

To evaluate the effect of fruit position, germination time and genetic family on plant performance, Generalized Linear Mixed Models (GLMM) (Bolker *et al.*, 2009) were used. Using GLMM the distinction in random and fixed factors could be made. First, the effect of fruit position (fixed factor) was analyzed, using genetic family as random factor and probability of germination and germination time as response variables (only for the first experiment, i.e., when achenes were placed to germinate directly into the pots). In the same analysis, for both experiments, i) placed directly into the pots and ii) with controlled germination time the response variables were total, above- and below-ground biomass, reproductive biomass and their proportions, capitulum and disk diameter and duration of flowering. The number of capitula and probability of flowering were not evaluated because in both cases similar values among all individuals were recorded, and therefore there was no variation to model (results not shown). The probability of germination and survival were modeled with a binary distribution; whereas germination time and flowering duration were adjusted to a Poisson distribution and total, above- and below-ground biomass and reproductive biomass and capitulum and disk diameter were fitted to a Gaussian distribution. Finally, the biomass proportions were modeled with gamma distribution. Differences between the positions of the achenes were analyzed using least square means (LSmeans). These were marginal means, in other words, the group means after having controlled for covariates.

In addition, the effect of achene position on plant performance was also analyzed using germination time as covariate, but only for the experiment where the achenes were placed directly into the pots and therefore they germinated at different times. The same GLMM approach was employed. For this model, achene position and germination time were the fixed factors, whereas again, family was considered as a random factor. Plant performance was evaluated throughout several traits: survival, total plant biomass, above-ground biomass, below-ground biomass and reproductive biomass and their proportions (see above for details), capitulum and disk diameter and flowering duration. Error distributions and link functions were set as above. Statistical differences between the different positions of achenes were analyzed using least square means (LSmeans) (see above for details). All models were fitted using the GLIMMIX procedure for SAS, with LSMEANS option (SAS Institute Inc., Cary, USA).

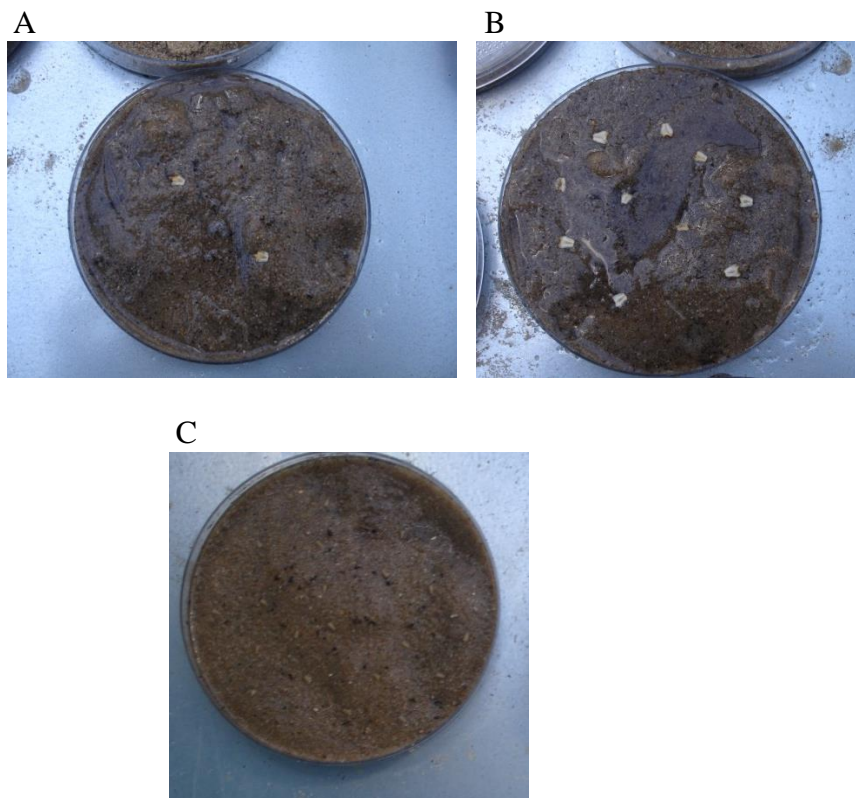Figure 1. Pots with seedlings from achenes of different positions.

Figure 2. Different types of achenes in *Anacyclus clavatus.* Achenes from female flowers - F (A), outer achenes from bisexual flowers – O (B) and inner achenes from bisexual flowers – I (C).

3.3. Results

3.3.1. Effect of achene position on plant performance under different germination times

Achene position affected significantly all the analyzed post-dispersal life history traits (Fig. 3), except survival rates (Table I, Fig. 3C). The outermost achenes, F and O showed a statistically significant higher probability of germination than I (Table I, Fig. 3A). These achenes also germinated significantly earlier than I (Table I, Fig. 3B). This trend was also observed for total, above-ground and below-ground biomass, with outer achenes presenting higher values than inner achenes (Table I, Fig. 3D, E and F). The proportional resource allocation pattern, i.e., the proportion of biomass allocated to one plant part with respect to the total biomass was also significantly affected by achene position. When compared with O and I achenes, F achenes produced plants that allocated proportionally less biomass to above-ground organs than to below-ground organs (Table I, Fig. 3G and H). The probability of survival was not different between achenes (Fig. 3C).

The reproductive effort also was partially affected by achene position (Table I). The results from reproductive biomass and its proportion, measured as the ratio between reproductive biomass and total biomass, were surprising. Statistically significant differences for both reproductive traits were only observed for the I achenes, but whereas for reproductive biomass, I achenes presented significantly lower values than F and O, for the proportion of reproductive biomass the opposite was observed (Table I, Fig. 3I and J). Capitulum and disk diameter was also affected and in both traits, with I achenes presenting the smallest sizes, whereas O achenes presented the largest sizes (Table I, Fig. 3K and L). Finally, flowering duration was also affected by achene position, with plants from O achenes presenting a significantly shorter flowering period (Table I, Fig. 3 M). The genetic family appeared to have a greater effect on flowering duration (Table 1).

3.3.2. The effect of achene position on plant performance under the same germination times

When the germination time was controlled, achene position did not affect any post-dispersal life-history trait (Table I, Fig. 4), suggesting that all differences between

achene positions detected above may be mediated by the differences on germination timing.
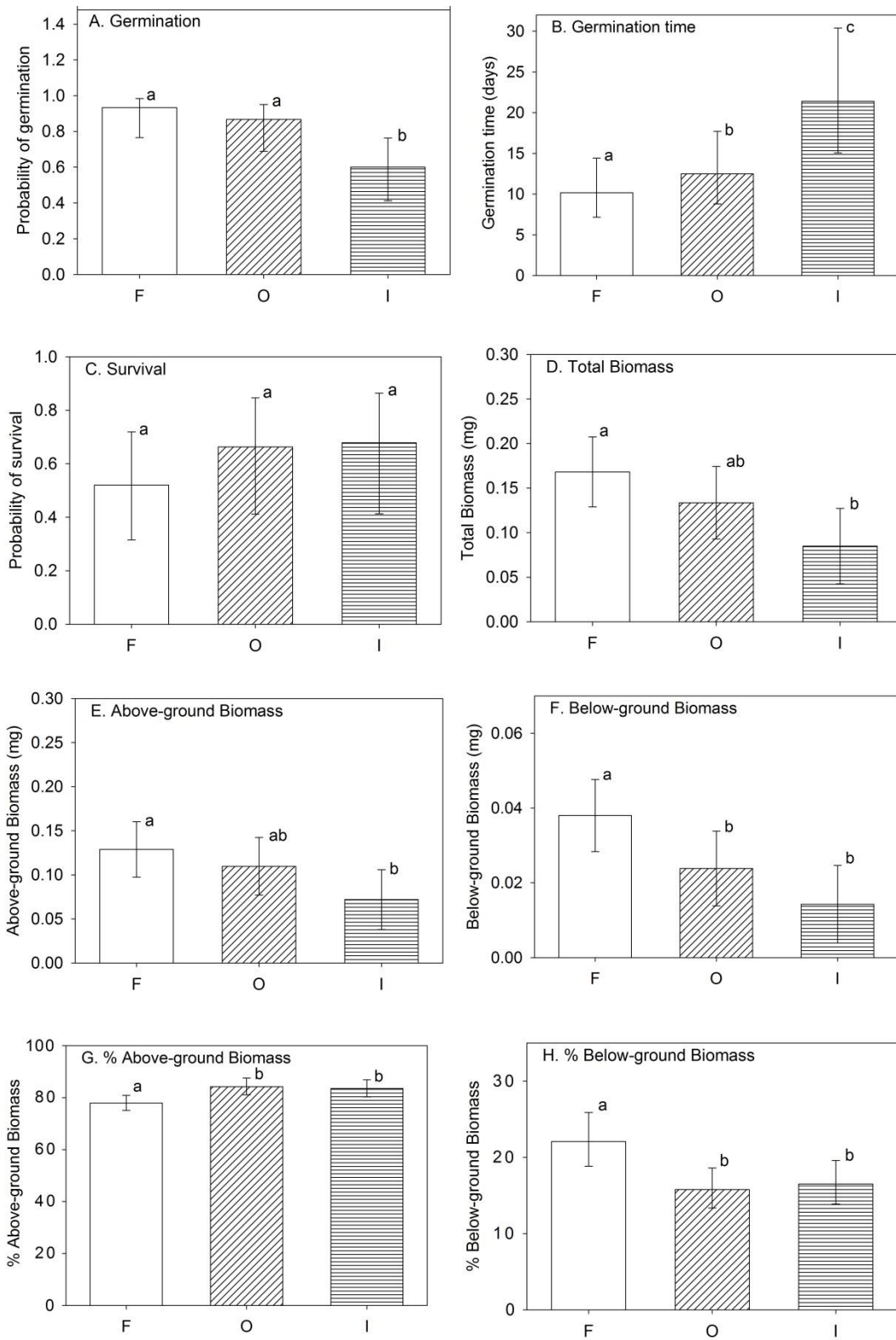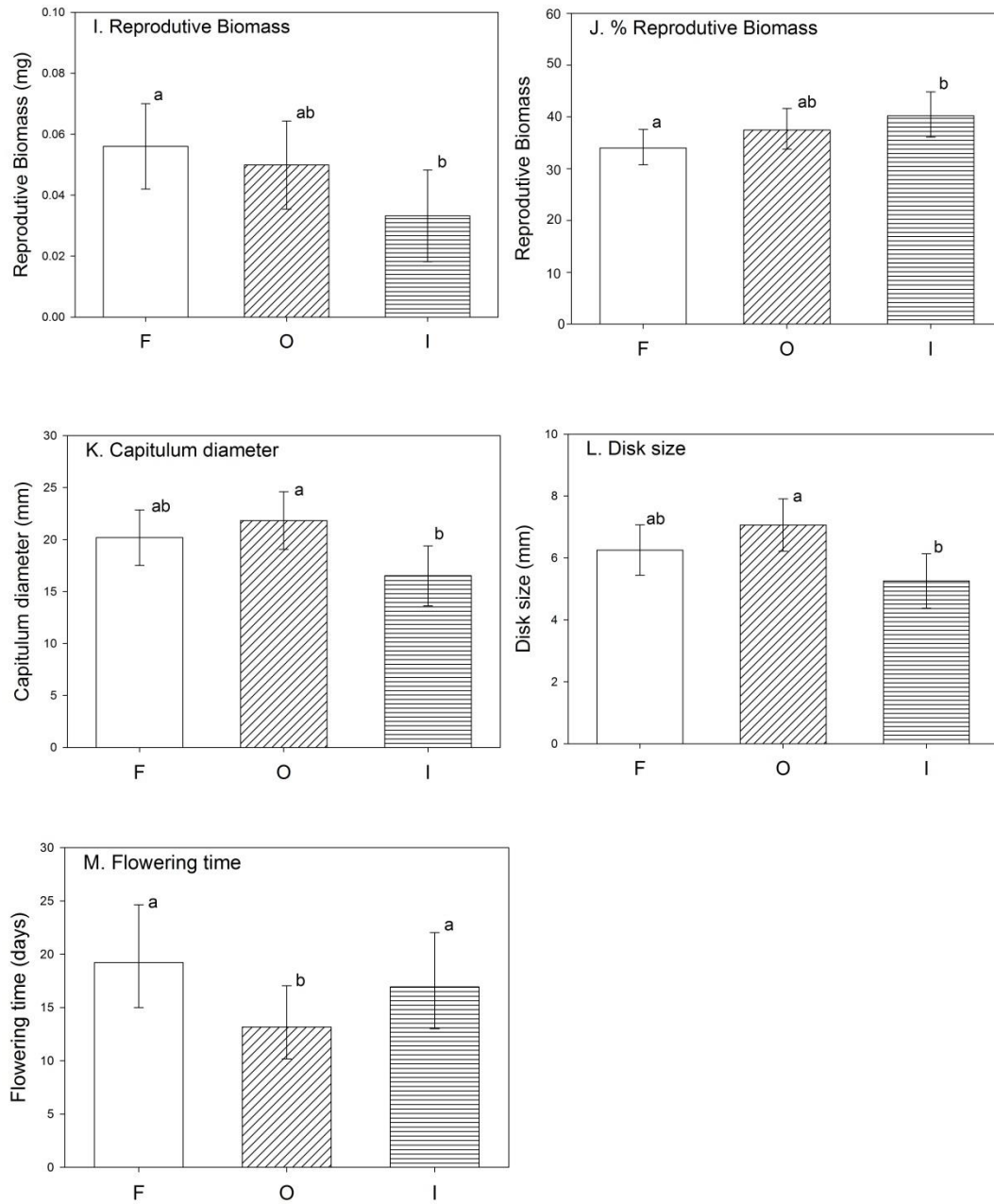
Figure 3. Least squares means (± confidence interval) of probability of germination (A), germination time (B), probability of survival (C), plant total biomass (D), above-ground biomass (E), below-ground biomass (F), % above-ground biomass (G), % below-ground biomass (H), reproductive biomass (I), % reproductive biomass (J), capitulum diameter (K), disk diameter (L), flowering duration (M), of plants from different achene positions germinated under different times (F = achenes from female flowers; O = outer achenes from bisexual flowers; I = inner achenes from bisexual flowers). Values sharing the same superscript letter were not significantly different at $P < 0.05$.

Table I. Effects of achene position on post dispersal life-history traits. GLMM from achenes with same germination times and achenes germinated directly in the pots (different germination times) with achene position as a fixed factor and generic family as a random factor. F-statistic with degrees of freedom is showed for the fixed factor. Covariate estimation ± SE is showed for the random factor. The samples size (n) is also provided.

| Variables | Time of germination | Fixed factor | | | Random factor | | Sample size |
		Achene position			Genetic family		
		df	F	*P*	Estimate	SE	n
Germination traits							
Probability of germination	Different	2, 87.00	4.98	0.0089	0.2159	0.6690	90
Germination time	Different	2, 69.00	56.56	<.0001	0.7919	0.2166	90
Probability of survival	Different	2, 62.00	0.69	0.5051	0.7840	0.7630	65
	Same	2, 79.00	0.21	0.8137	1.4740	0.8946	82
Size traits							
Total biomass	Different	2, 24.73	4.91	0.0161	0.0017	0.0015	39
	Same	2, 19.87	1.94	0.1694	0.0041	0.0018	41
Above-ground Biomass	Different	2, 25.38	3.59	0.0424	0.0010	0.0009	39
	Same	2, 20.36	1.78	0.1945	0.0027	0.0012	41
Below-ground biomass	Different	2, 21.01	6.85	0.0051	0.0001	0.0001	39
	Same	2, 20.51	1.31	0.2927	0.0001	0.0001	41
% of above-ground biomass	Different	2, 36.00	5.36	0.0092	0.0000	0.0000	39
	Same	2, 29.80	1.47	0.2460	0.0001	0.0008	41
% of below-ground biomass	Different	2, 36.00	5.20	0.0104	0.0000	0.0000	39
	Same	2, 29.21	1.55	0.2299	0.0019	0.0171	41
Reproductive traits							
Reproductive biomass	Different	2, 23.62	3.19	0.0592	0.0003	0.0002	39
	Same	2, 21.49	1.05	0.3675	0.0005	0.0002	40
% of reproductive biomass	Different	2, 23.74	2.80	0.0811	0.0033	0.0117	39
	Same	2, 27.22	0.00	0.9988	0.0084	0.0070	40
Capitulum diameter	Different	2, 28.59	3.92	0.0313	1.7244	5.9589	39
	Same	2, 19.39	0.06	0.9435	15.4160	9.8327	40
Disk diameter	Different	2, 27.40	4.95	0.0146	0.4339	0.5720	39
	Same	2, 21.71	0.16	0.8495	1.3250	0.7047	40
Flowering duration	Different	2, 36.00	6.31	0.0045	0.2310	0.0833	39
	Same	2, 37.00	0.65	0.5262	0.1789	0.0667	40
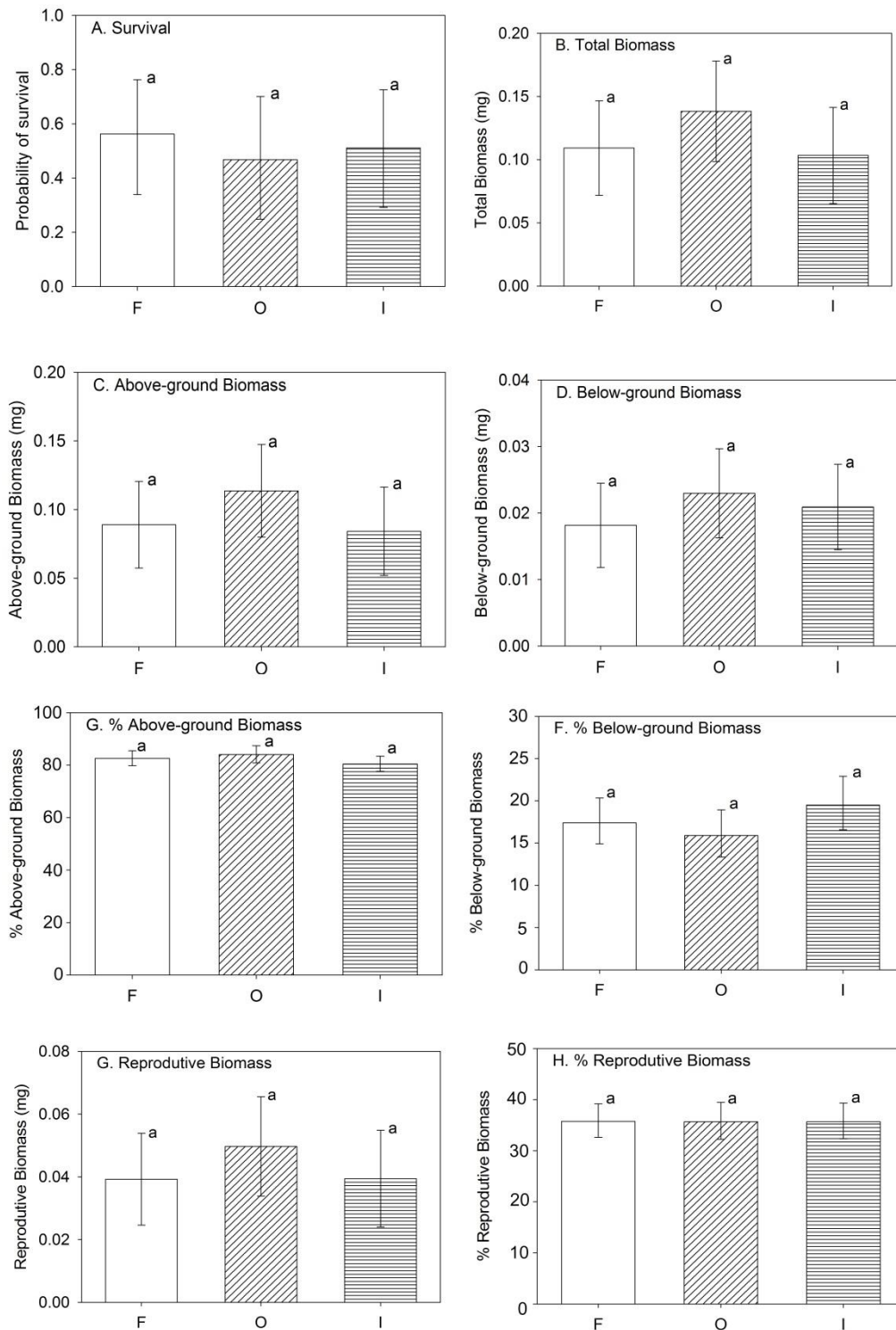
Figure 4. Least squares means (± confidence interval) of survival probability (A), plant total biomass (B), above-ground biomass (C), below-ground biomass (D), % above-ground biomass (E), % below-ground biomass (F), reproductive biomass (G), % reproductive biomass (H), capitulum diameter (I), disk diameter (J), flowering duration (K), of plants from different achene positions germinated under the same times (F = achenes from female flowers; O = outer achenes from bisexual flowers; I = inner achenes from bisexual flowers). Values sharing the same superscript letter were not significantly different at $P < 0.05$.
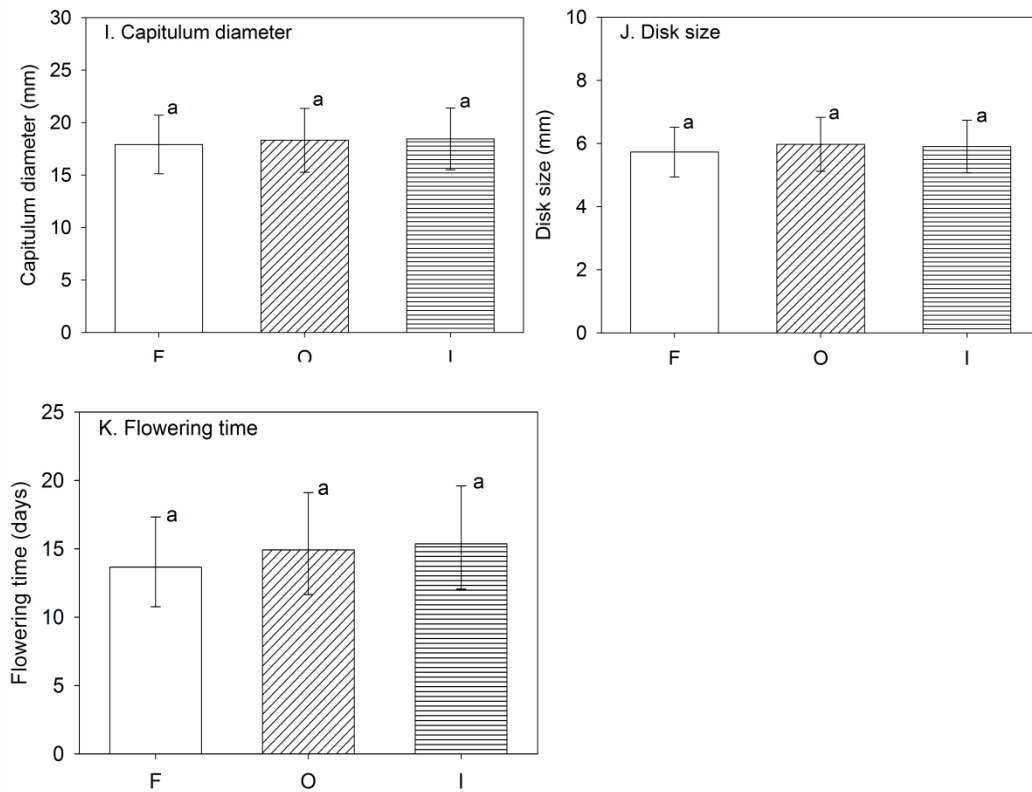
Figure 4. (Continued)

3.3.3. The effect of germination time on plant performance

When the time of germination was included in the models as a covariate (only for the achenes that germination was controlled), most of significant effects of achene position disappeared and germination time was the only significant factor (Table II). Thus, the time of germination affected significantly all traits except the proportional patterns of allocation (Table II, Fig. 5). Germination time affected positively probability of survival, then those achenes that germinated early had a lower probability of survival compared to those achenes that germinated later (Table II; Fig. 6A). Nevertheless, O achenes, which germinated earlier than I ones, had a higher probability of survival but not statistically different (Fig. 5A and 6A). By contrast, germination time affected negatively total biomass, above-, below-ground biomass, reproductive biomass, capitulum and disk diameter and flowering duration (Table II). Achenes that germinated earlier (F and O) had a higher plant biomass, either above- or below-ground, (Table II, Fig. 5B, C and D, and Fig. 6B) and showed a higher reproductive effort, as measured through biomass allocated to capitula, through capitulum and disk diameter, than those achenes that germinated later (Table II, Fig. 5G, H, I and J; and Fig. 6C). F achenes,

which germinate earlier, produced plants that allocated proportionally less biomass to above-ground organs and more biomass to below-ground organs than O and I (Table II, Fig. 5E, F) and O achenes had a lower duration of flowering (Table II, Fig. 5K and Fig. 6D). Achene position still affected significantly, plant biomass (only marginally significant), plant below-ground biomass, and the proportional allocation to both above- and below-ground parts and the capitulum and disk diameter and flowering duration (Table II, Fig. 5). The random factor family appeared to have a greater effect on flowering duration (Table II).

Table II. Effects of achene position, germination time and family. GLMM from achenes germinated directly in pots with achene position and germination time as fixed factors and genetic family as a random factor. F-statistic with degrees of freedom is showed for the fixed factor. Standard error (SE) and estimation is showed for random factor. When the differences were statistically significant, a sign that indicates the direction of the germination time effect was added. Covariate estimation ± SE is showed for the random factor. The sample size (n) is also provided.

|	Fixed factors						Random factor		
Variables	**Achene position**			**Germination time**			**Genetic family**		**Sample size**
	df	F	*P*	df	F	*P*	Estimate	SE	n
Germination traits									
Survival	2, 61	0.29	0.7516	1,61	+4.49	0.0383	0.6441	0.7667	65
Size traits									
Total biomass	2, 24.7	2.97	0.0697	1,24.58	-16.27	0.0005	0.0004	0.0012	39
Above Biomass	2, 26.19	2.00	0.1554	1,23.92	-14.93	0.0007	0.0001	0.0007	39
Below biomass	2, 20.35	5.05	0.0166	1,27.95	-12.85	0.0013	0	0	39
% Above biomass	2, 26.36	4.48	0.0211	1, 24.67	+1.42	0.2449	0.0003	0.0014	39
% Below biomass	2, 28.91	4.30	0.0232	1, 26.17	-1.58	0.2192	0.0034	0.0211	39
Reprodutive traits									
Reproductive biomass	2, 23.55	1.78	0.1899	1,27.19	-12.59	0.0014	0.0001	0.0001	39
Reproductive biomass (%)	1, 25.64	3.44	0.1684	2, 25.67	+1.91	0.0754	0.0037	0.0096	39
Capitulum diameter	2, 35	3.57	0.0387	1,35	-4.76	0.0359	0	0	39
Disk diameter	2, 35	4.81	0.0142	1,35	-6.30	0.0169	0	0	39
Flowering duration	2, 35	5.35	0.0094	1,35	-7.82	0.0083	0.0759	0.0759	39
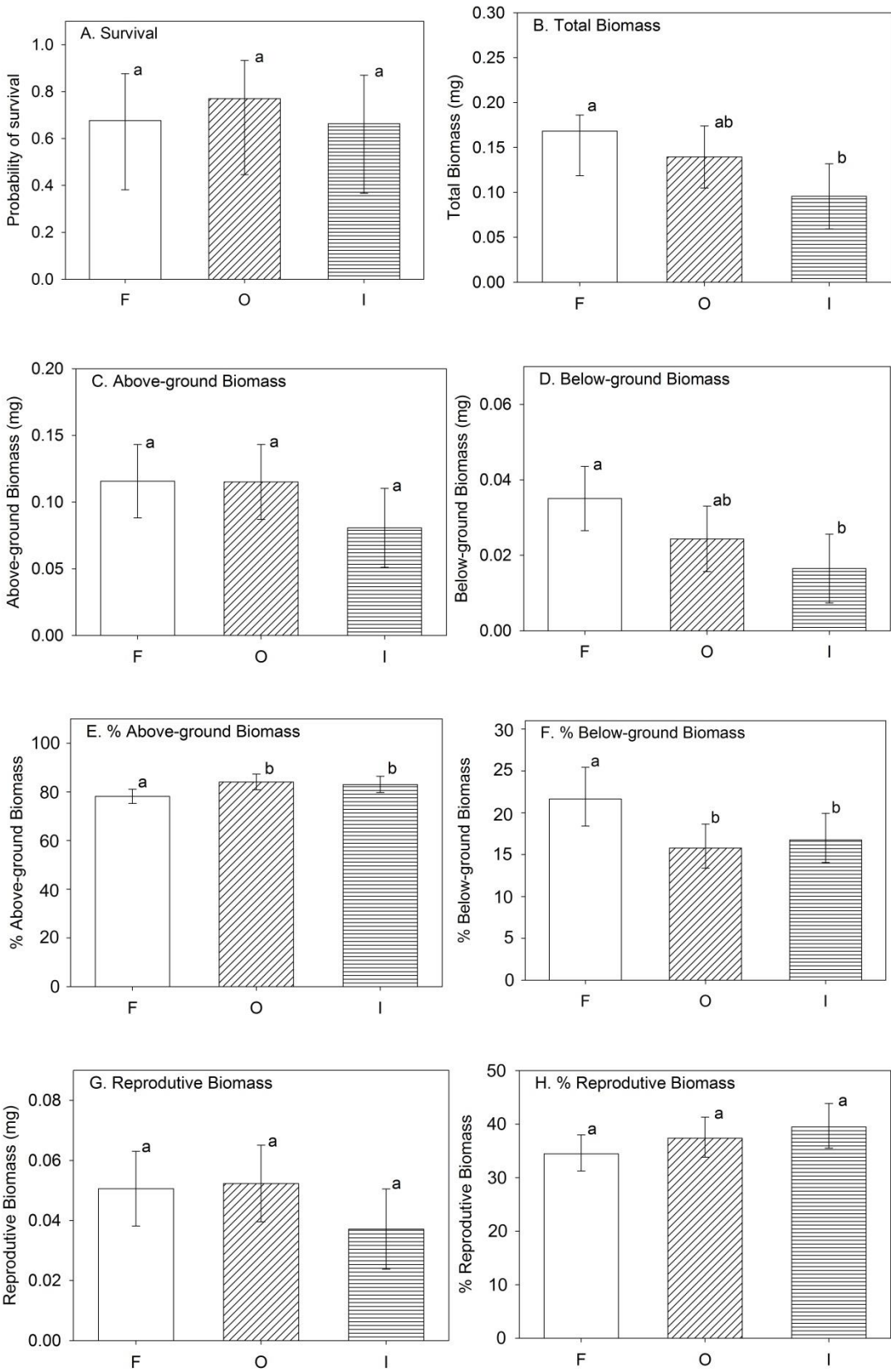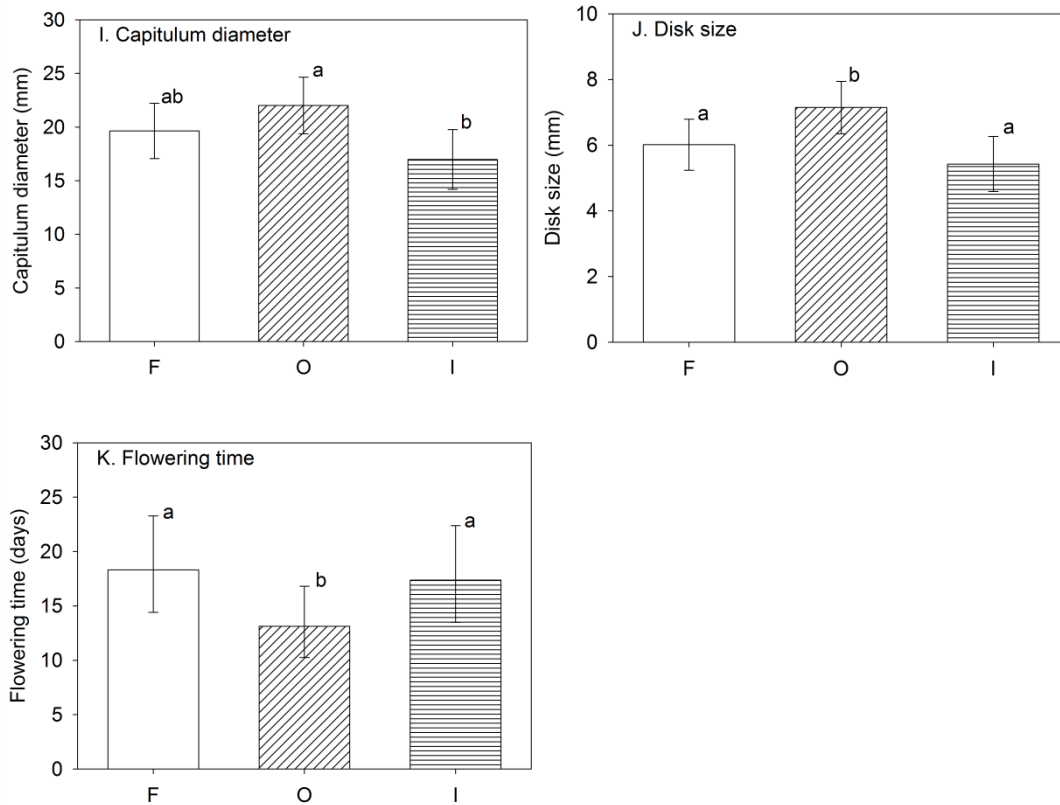
Figure 5. Least squares means (± confidence interval) of survival probability (A), total biomass (B), above-ground biomass (C), below-ground biomass (D), % above-ground biomass (E), % below-ground biomass (F), reproductive biomass (G), % reproductive biomass (H), capitulum diameter (I), disk diameter (J), flowering duration (K), of plants from different achene positions germinated under different times (F = achenes from female flowers; O = outer achenes from bisexual flowers; I = inner achenes from bisexual flowers) and using germination time as a covariate. Values sharing a superscript were not significantly different at the $P < 0.05$ level.
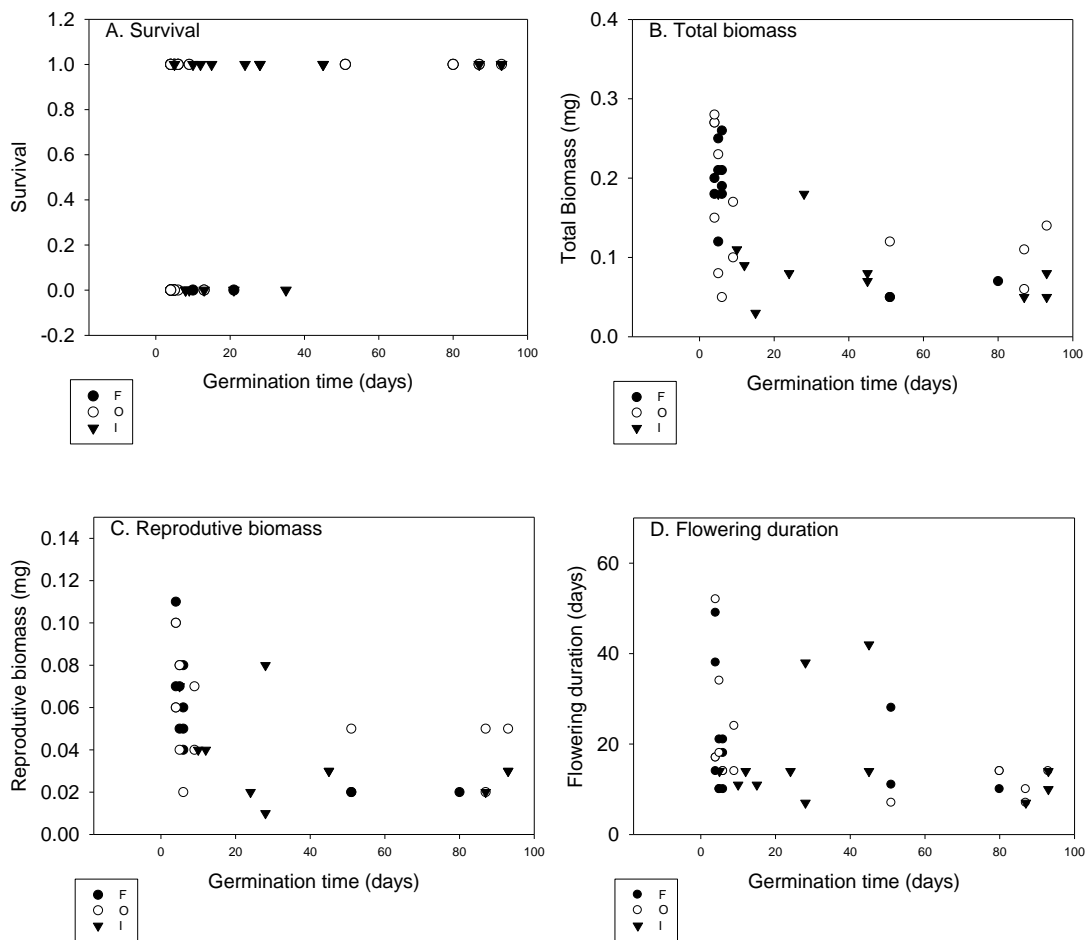
Figure 6. Effects of germination time for achenes non-pre-germinated. In Survival (A), 1 is for yes and o for no. In total biomass (B), reproductive biomass (C) and flowering duration (D).

3.4. Discussion

The results obtained in this study revealed a significant effect of germination time in several life-history traits, which considering that outer achenes are the ones that germinate faster, pointed for an effect of the achene position that was not evident when the germination time was controlled. The fact that outer achenes present a higher percentage of germination and faster germination rates than inner ones has been previous documented (Imbert *et al.*, 1996; Bastida & Menéndez, 2004; Brändel, 2004; Torices *et al.*, 2013). Still, this is not always the case. In other species the inner achenes germinate rapidly (Imbert et al., 1996; Bastida and Menéndez, 2004; Brändel, 2004), being the slower germination rates of outer achenes related with their dispersal ability (Venable & Levin, 1985b; Tanowitz *et al.*, 1987; Gibson, 2001; Ruiz De Clavijo, 2001).

The common garden experiment supports the hypothesis that plants from outer achenes yield higher plants. Previous studies have demonstrated that outer achenes produce seedlings with higher vigor and biomass and also plants with higher biomass (Imbert *et al.*, 1996; Ruiz De Clavijo, 1998; Benard & Toft, 2007; Dubois & Cheptou, 2012). Allocation for the growth above-ground was higher in outer achenes from female flowers, whereas the growth below-ground was lower. This is similar to what was observed by Imbert et al. (1997) in *Crepis sancta* (Imbert *et al.*, 1997). In the same study, it was demonstrated that outer and larger achenes had higher reproductive biomass, but only in competition. In this case survival did not differ between positions, but in some previous studies it has already been documented that plants from outer achenes presented a higher survival than those from inner positions (Rai & Tripathi, 1987; Espinosa-García *et al.*, 2003; Dubois & Cheptou, 2012).

Regarding germination time, regardless of the achene position, those achenes with a delayed germination showed a high survival probability. This is in contrast with other studies that showed that early germination leads to higher survival (Forsyth & Brown, 1982; Venable & Levin, 1985b; Rai & Tripathi, 1987; Mercer *et al.*, 2011; Dubois & Cheptou, 2012). When germination time decreased, as in F and O, total, above- and below-ground biomass and also reproductive effort increased. Thus, this study is in accordance with the premise that early emergence influences the final plant, by producing plants with higher performance (Rai & Tripathi, 1987; Imbert *et al.*, 1997; Imbert, 2002; Donohue *et al.*, 2010; Mercer *et al.*, 2011). Germination time was related with achene position, but, in *Anacyclus clavatus* it appears that germination is the main factor affecting plant performance, as plants germinated at the same time did not present differences in any of the analyzed traits. Flowering duration appears to have been influenced by the genetic family, most probably by the genetic basis of this plant trait.

The different types of achenes and consequently different times in germination are linked to achene size. As outer achenes were larger than inner ones, the probability of germination increased and germination time decreased. There are species where larger achenes germinate earlier (Forsyth & Brown, 1982; Ellison, 1987; Imbert *et al.*, 1996; Espinosa-García *et al.*, 2003), however, in other species the opposite trend was observed, i.e., the smaller achenes germinate faster (Susko & Lovett-Doust, 2000; Gibson, 2001; Ruiz De Clavijo, 2001). Curiously, achene size affected positively survival rates (results not shown). This is in accordance with several studies where large

achenes presented a higher percentage of viability, survival and germination (Rai & Tripathi, 1987; Banovetz & Scheiner, 1994; Imbert *et al.*, 1997). All this data indicates that correlation between achene size and germination time might exist. Still, in some species it has been shown that germination time and germination probability were not affected by seed size (Eriksson, 1999), and that differences in germination rate were due to the structure of pericarp (Tanowitz *et al.*, 1987; Ruiz De Clavijo, 2001). Also, when achene size increased the biomass and reproductive effort also increased (results not shown). Previous studies have demonstrated that heavier achenes produced seedlings with higher vigor and biomass, as well, as higher growth and reproductive ability (Venable & Levin, 1985b; Ellison, 1987; Bretagnolle *et al.*, 1995; Imbert *et al.*, 1996, 1997; He *et al.*, 2007).

Therefore, it is very difficult to disentangle the direct effects of achene position from those produced by different achene sizes or different germination times. The results showed that achene position and size were linked, and consequently both were linked to germination time. However, it seems that germination time was the main factor influencing the plant fitness because when germination was controlled there were no difference in survival or plant performance and reproductive effort. Such significant influence of germination time was already observed in *Crepis sancta* by Dubois and Cheptou (2012). In that species, germination time affected germination rates, survival and final plant biomass, but the position of achene alone had a little effect. Also in Imbert *et al.* (1997), germination appeared to be the main factor, surpassing the possible effect of achene size. Previous studies have suggested that the effect of germination time appears to be stronger in controlled conditions than in the field, due the unlimited availability of nutrients and water resources (Verdú & Traveset, 2005). In the current study, despite the experiment occurred in controlled conditions, the resources were very limited, and both nutrients and irrigation were scarce. For instance, in the experiments performed in this study, plant size and capitula number were smaller compared to plants growing in the field. Dubois and Cheptou (2012) suggest that germination time results from an adaptive process and is linked to dispersal ability and competition. It is also suggested that the early germination of outer achenes lead to a greater plant performance, as they grow quickly and near to the mother plant, whereas the inner achenes due to the lower germination rates have the opportunity to disperse further and have a higher survival rate allocating more resources to reproductive biomass. Thus, these differences in performance and fitness between plants from different achenes due

to achene position, size and germination time could be a strategy to survive in different environments.

The differences in plant performance produced by achenes from different flower positions was proposed to explain floral sexual specialization within Asteraceae capitula (Torices *et al.*, 2011). These authors forecast that the outermost achenes should give rise to plants with higher fitness than the innermost ones, and that, these differences in plant performance might lead to differential patterns of sex allocation on flowers at different positions. The results presented in this study support the first expectation since outer and larger achenes with an early germination in *A. clavatus* produced plants with high performance and reproductive effort. Further, there were also differences between outer achenes from female flowers (F) and from bisexual flowers (O). F achenes produced plants with higher performance. These two types of achenes were closely placed within capitula and therefore the positional differences were reduced suggesting that the differences observed might be due to the sexual specialization on female function of F flowers compared to O ones. Therefore, female fitness of flowers within capitula seems to decline from the outermost positions to the innermost ones.

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4. Conclusion

4. Conclusion

The sexual specialization observed between flowers within capitula has been recently hypothesized to be due to positional effects within the capitula. Among the factors influencing floral gender, the resource gradient may play one of the important roles. Positional effects may also affect plant performance and fitness, although these effects may be mediated by other factors such as germination time.

The findings provided in this Thesis contribute with further data to support these hypotheses. Several findings support the hypothesis that the resource gradient within inflorescence might lead to a specialization of flowers on different sexual functions at specific flower positions. Inflorescence traits, such as capitulum size, number of flowers and flower density, were correlated with the sexual system, supporting that sexual specialization may result of some process occurring at the inflorescence level (Chapter I). Furthermore, sexual segregation was higher in those inflorescences with higher floral aggregation indicating that a higher specialization may be the result of intense competition between flowers.

Nevertheless, no support was given to the hypothesis that outer fruits are usually larger than inner ones within capitula (Tanowitz *et al.*, 1987; Imbert, 1999; Gibson, 2001; Ruiz De Clavijo, 2001; El-Keblawy, 2003; Torices & Méndez, 2010). This disagreement may be the result of the use of different methodological approaches. Commonly, fruit size variation has been assessed in terms of mass (Eriksson, 1999; Gibson, 2001; Ruiz De Clavijo, 2001; Picó *et al.*, 2003; Mölken *et al.*, 2005; Torices & Méndez, 2010). In this thesis, due to the impossibility of obtaining accurate estimates of mass for fruits from herbarium material, fruit size was estimated as the area in a digital photograph. Fruit area and fruit weight may not be directly proportional, thus an increase of fruit area may not be proportional to an increase in fruit weight. In addition, as fruit area seems to vary gradually from the outermost to the innermost positions (Maxwell *et al.*, 1994; Torices & Méndez, 2010), statistical differences between outer and inner fruits might be only detected when only the outermost and the innermost fruits are compared. Such approach is difficult in species with very small capitula, as was the case of some species included in this study, and only the sampling of more capitula would allow achieving enough statistical power.

The resource gradient at inflorescence level seems to influence differentially female fitness of flowers at different positions. In this Thesis, the outer flowers of a

capitulum had a higher female fitness than the inner ones since fruits from outer positions produced plants with a higher probability of germination, size and reproductive effort (Chapter II). The expectation was that plants from outer fruits had a higher success due to the higher size of the outer fruits compared to the inner ones (Imbert *et al.*, 1997; Dubois & Cheptou, 2012). However, the early germination of outer achenes was the main factor affecting the performance of plants.

The results of this Thesis also highlight the importance that other traits rather than fruit size might have on the fitness achieved from each flower at different positions. Actually, fruit size is not the only trait that varies from outer to inner positions. For instance, dispersal ability usually varies from outer to inner fruits leading to mixed strategies on dispersal or heterocarpy (Venable & Levin, 1985; Tanowitz *et al.*, 1987; Imbert *et al.*, 1996; Gibson, 2001; Ruiz De Clavijo, 2001, 2005). The presence of heterocarpy might change the expectation of success of a seedling from achenes at different positions to germinate and establish within a population. Thus, dispersal ability could correlate with survival, fitness and competition ability (Meyer & Carlson, 2001; Mazer & Lowry, 2003; Brändel, 2004, 2007). Indeed different dispersal abilities and germination times allowed the spread of the offspring in space and time, reducing sibling competition (Bastida & Menéndez, 2004). Therefore, all these traits should be jointly analyzed to obtain accurate estimates on the fitness of plants produced by different achenes and with different dispersal structures and germination behavior.

Summarizing, the positional effects on resource availability at the inflorescence level might lead, in evolutionary time, to positional effects on floral gender. Differences in plant performance produced by different fruits within an inflorescence might lead to differential patterns of sex allocation on flowers at those different positions. These results open up new lines of research in the flower specialization on sexual functions. All these aspects could be applied to other groups and other types of inflorescences. For instance, in Apiaceae, Liliaceae, Myrtaceae and Solanaceae sexual traits within the inflorescences also follow a positional pattern (Bell, 1971; Primack & Lloyd, 1980; Solomon, 1988; Spalik, 1991; Emms, 1993; Diggle, 2003; Miller & Diggle, 2003), suggesting that sexual specialization at inflorescence level might follow a common line at different families.

4.1. Literature cited

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5. Appendix

Appendix I. List of studied specimens.
Herbarium[1]: Swedish Museum of Natural History (S); Herbarium of University of Coimbra (COI).
Sexual system[2]: H: Hermaphroditism; GM: Gynomonoecy; M: Monoecy.

Species	Herbarium[1]	Herbarium voucher	Sexual system[2]
Ageratina calaminthaefolia	S	Robert Merrill King & Paul M. Peterson, no. 9957	H
Ambrosia elatior	COI	J. Vivant, no. 9146-4471	M
Amellus strigosus	S	E. Wall, no. 137	GM
Ammobium alatum	S	A. Anderberg & A.L. Anderberg, no. 7148	H
Anaxeton arborescens	COI	A. Meelbold, no.13494	M
Anaxeton laeve	S	A.&B. Strid, no. 37217	M
Anaxeton laeve	S	A.&B. Strid, no. 37218	M
Anisocarpus madioides	COI	H.N. Bolander, no. 9253	M
Anisocarpus scabridus	S	M.S. Baker, no 10658	GM
*Arnica lanceolata**	S	Galen Smith, no. 2049	GM
Artemisia crithmifolia	COI	Aarão F. de Lacerda, no. 751-780	M
Baccharoides adoensis	S	M Reekmans, no. 9172	H
*Baileya pleniradiata**	S	J. Laubert, no 113	GM
Barnadesia spinosa	S	H. Humbert, no. 26923	H
Blennosperma bakeri	S	John Thomas Howell, no. 25303	M
Blennosperma californicum	COI	Lewis S. Rose, no. 9308-33008	M
Blepharispermum spinulosa	COI	Cyossmeiler, no. 8059	M
Blumea riparia	S	Chieng-Chang Hsu, no. 5201	GM
Brickellia chlorolepis	S	Robert Merrill King & Paul M. Peterson, no.9836	H
Calendula arvensis	COI	J. Nogueirs, no. 757-10962	M
Calendula arvensis	COI	M. Queirós, no. 757-5492	M
Calotis erinaceae	S	E.N.S. Jackson, no. 5948	GM
Calotis hispidula	COI	K. Stove, no. 88875-671	GM
Chaptalia nutans	S	E. Wall, no. 729	GM
Chromolaena odorata	S	Erik Wall, no.72	H
Critoniopsis leiocarpa	S	Ynes Mexia, No. 9119	H
Cyanthillium cinereum	S	Dick Hummel, s.n	H
Dasyphyllum diacanthoides	S	Mleyer, no. 8161	GM
Dasyphyllum ferox	S	C. Hammarlund, no. 534	GM
Dicoma anomala subsp. gerrardii	S	H. & HE. Wanntorp, no. 464	H
Dimorphotheca simata	COI	Sange Kloof, no. 8598	M
Doniophiton anomalon	S	F. Barkley & O. Paci, s.n.	GM
Dubautia laxa	S	L.M. Cranwell, no. 3417	H
Epaltes cunninghamii	S	B. Nordenstam & A. Anderberg, no. 972	M
Eriocephalus umbellatus	COI	Heron, s.n.	M
Eriochephalus aspalathoides	COI	R. Seydel, no. 3608	M
Ethulia conyzoides	S	H.J. Venter & A. Venter, no. 9677	H
Florestina pedata	S	Maury, no.24	H

Species	Herbarium[1]	Herbarium voucher	Sexual system[2]
Gnaphalium microcephalum	S	Lewis S. Rose, no. 51179	GM
Grindelia arenicola	S	E. K. Balls, no. 10161	GM
Gymnarrhena micrantha	COI	A. Grizi, no. 8970-383	M
Hemizonia fasciculata	COI	S B e W F Parish, no. 9254	M
Hippia fruticosa	COI	Lason, no. 10686	M
Hirpicium echinus	S	Lars Erik Kers, no. 2179	H
Holozonia filipes	COI	S B e W F Parish, no. 9257-486	M
Hoplophyllum spinosoum	S	P. Goldblatt, no. 4325	H
Inula oculus-christi	S	I. Segelberg, no. 13761/5	GM
Inula peacockiana	S	K. H. Rechinger, no. 49051	H
Jungia paniculata	S	S.G. Saunders, no. 1244	H
Kleinia longiflora	S	E. Wall, no. 622	H
Layia platyglossa	COI	William H. Beble, no. 9258	GM
Liabum bourgeaui	S	Robert Merrill King & Victor Castro, no. 9997	GM
Liatris aspera	S	D.S. Correll & H. B. Correll, no. 36587	H
Ligularia fischeri	S	M Mizushim, no. 13766	GM
Madia anomala	S	David. D. Keck, no. 2313	GM
Marshallia graminifolia	S	C. Ritchie Bell, no. 15744	H
Melampodium leucanthemum	COI	W.P. Cottam, no. 9129-10231	M
Melampodium leucanthemum	S	B. H. Warnock, no. 46217	M
Microseris douglasii	S	Lewis S. Rose, no. 66037B	H
Millotia myosotidifolia	S	F.J. Badman, no. 8397	H
Monolopia lanceolata	S	E.K. Balls, no. 8547	GM
Onoseris alata	S	J. Olea, s.n.	GM
Onoseris odorata	S	Francis W Pennell, no 14468	GM
Osteospermum hispidum	COI	Elands, no. 9755	M
Othonna coronopifolia	COI	Iaron, no. 7885	M
Oxypappus scaber	S	Mexia, no. 1367	GM
Ozothamnus diosmifolius	S	A Anderberg & A.-L Anderberg, no. 7043	H
Palafoxia arida	S	K. Bremer, no. 2479	H
Perezia multiflora	S	Kjell von Sneiden, no. A333	H
Perityle emoryi	S	M.O. Dillon & D.O. Dillon, no. 4850	GM
Philoglossa peruviana	S	E. Asplund, no. 13735	GM
Plazia argentea	S	E. Carrette, s.n.	H
Pluchea dentex	S	B. Nordenstam & A. Anderberg, no. 325	M
Plecostachys serpyllifolia	S	R. D. A. BAYLISS, no. 8375	GM
Polymnia canadensis L.	COI	Grady L. & Barbara D. Webter, no. 9122-7088	M
Porophyllum scoparium	S	K. Bremer, no. 2379	H
Pteronia incana	S	A. & B. Strid, no. 37701	H
Roldana mexicana	S	Geo. B. Hinton, no 8745	H
Rosenia hulilis	S	Kare Bremer, no. 164	GM
Rudbeckia fulgida	S	F.T. McFarland, no. 347	H
Senecio inornatus	S	DM Hilliard & B.L. Burtt, no. 7492	GM

Species	Herbarium[1]	Herbarium voucher	Sexual system[2]
Senecio subsessilis	S	J.A. Mlangwa , P.B. Phillipson, H. van Vlaenderen & W. Kindeketa, no. 305	GM
Sinclairia polyantha	S	C. L. Lundell & Elias Contreras, no 20619	GM
Soliva pterosperma	COI	J. Matos; A Matos & A. Marques, no. 750-4806	M
Streptoglossa liatroides	S	A. Strid, no. 4269	GM
Trixis antimenorrhoea	S	F.J. Breteler, no. 3502	H
Uropappus lindleyi	S	L.S. Rose, no. 63059	H
Vernonanthura patens	S	E. Wall, no. 9301	H
Vernonia alamanii	S	H. Fröderström & E. Hultén, no. 321	H
Vernonia amygdalina	S	Fernandez Casas, no. 11433	H
Vernonia angustifolia	S	Ted Bradley, no. 3502	H
Vernonia anisochaetoides	S	J. Stewart, no. 1798	H
Vernonia cinerascens	S	Lars Erik Kers, no. 593	H
Vernonia fastigiata	S	O.H. Volk, no. 00367	H
Vernonia galamensis	S	T. Eriksson, V. Kalema & G. Leliyo, no. TE 533	H
Vernonia glabra	S	E. Lawrence, no. 112	H
Vernonia lasiopus	S	T. Erikson, V. Kalerna & G. Leliyo, no. TE 546	H
Vernonia poskeana	S	E.S. Pooley, no. 477	H
Vernonia tortuosa	S	Llewelyn Willians, s.n.	H
Warionia saharae	S	E.K. Balls, no. 2530	H

Appendix II. Model selection between the BM (Brownian motion) and the OU (Ornstein-Uhlenbeck) model for all phylogenetic generalized least squares models fitted. For each model, the Akaike Information Criterion (AIC), Log-likelihood (Loglik), and Likelihood ratio test comparing both models (LTR) are showed.

Response variable – Predictor variable	Model	AIC	Loglik	LTR	*P*
No. Flowers – Sexual System	BM	132.10	-62.05	-------	------
	OU	111.22	-50.61	-------	------
	BM vs OU	-------	-------	22.87	<.0001
Capitulum diameter – Sexual System	BM	70.37	-31.19	-------	------
	OU	38.92	-14.46	-------	------
	BM vs OU	-------	-------	33.45	<.0001
Flowers density – Sexual System	BM	154.12	-73.06	-------	------
	OU	143.89	-66.94	-------	------
	BM vs OU	-------	-------	12.24	0.0004
Outer fruits – Sexual System	BM	143.10	-67.55	-------	------
	OU	131.93	-60.96	-------	------
	BM vs OU	-------	-------	13.17	0.0003
Inner fruits – Sexual System	BM	100.36	-46.18	-------	------
	OU	85.99	-38.00	-------	------
	BM vs OU	-------	-------	16.37	0.0001
Sexual System – FSD	BM	210.60	-101.30	-------	------
	OU	189.85	-89.92	-------	------
	BM vs OU	-------	-------	22.76	<.0001
No. Flowers – FSD	BM	207.76	-100.90	-------	------
	OU	186.62	-89.31	-------	------
	BM vs OU	-------	-------	23.14	<.0001
Capitulum diameter – FSD	BM	201.15	-97.57	-------	------
	OU	184.69	-88.34	-------	------
	BM vs OU	-------	-------	18.46	<.0001
Flower density – FSD	BM	199.76	-96.88	-------	------
	OU	182.31	-87.15	-------	------
	BM vs OU	-------	-------	19.46	<.0001