Spatio-Temporal Variation in Seed Production in Three *Euphorbia* Species and the Role of Animals on Seed Fate

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This study was supported by the Fundação para a Ciência e Tecnologia.
PhD Fellowship: PRAXIS XXI/BD/22785/99

Aos meus pais, Flamínia e Joaquim,
e à Carla, minha esposa, aqueles a quem muito amo.
“As estrelas mais cintilantes da minha vida”

To my parents, Flamínia e Joaquim,
and to Carla, my wife, those I love most.
“The brightest stars of my life”

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ACKNOWLEDGEMENTS

During this long journey many people have given me advice, help and most of all have shared with me very pleasant moments that I will never forget. I want to express my deepest gratitude to all of them and apologize in advance to anyone I may have forgotten to mention.

To Prof. Artur Serrano, for having accepted to support my adventure, for his help during thesis development and most of all for being my friend, for having taught me so much, for the companionship and for having changed my life one day, some time ago.

To Prof. Xavier Espadaler, my deepest gratitude for his help during planning and development of the thesis, for having shared with me knowledge on the amazing world of ants and their allies, and for the delightful conversations about science and life. It is really a pleasure to work at your side.

To Dr. Manuel João Pinto, Eng. Gomes Pedro, Dr. Adelaide Clemente, Dr. Paulo Pereira, Prof. Ana Isabel Correia, Prof. Otília Correia and Prof. Amélia Loução for advice and valuable information on the biology of Portuguese *Euphorbia.*

To Prof. Marina Zerova, Prof. Wolfgang Rabitsch, Dr. Leif Aarvik, Dr. Joaquin Baixeras and Dr. Eduardo Marabuto for help in the identification of some insect species and for providing interesting information on their biology.

To Dr. Alba Eustaquio, Prof. Crisanto Gómez and Prof. Josep Piñol for the insightful discussions on elaiosomes, myrmecochory and for making my stays at the Universitat Autònoma de Barcelona always a pleasure.

To Centro de Biologia Ambiental for the support during the development of this thesis.

To Estação Agronómica Nacional (Dep. de Protecção de Plantas, Entomologia), particularly to Belard da Fonseca, for allowing me to use its facilities in order to perform some observations and experiments, and for the bibliographic support.

To ADAPA and to my friends there – Fernando Carvalho, Susana Rosa, Silvia Albano, Ricardo Miguel and Hélder Careto – for their support, companionship and for allowing the use of their facilities to perform some experimental work.

To Paulo Marques for the interesting discussions on the fauna and flora of the Serra de Montejunto and on the conservation priorities for this protected area.

To Eugénia Alfredo, Fernando Sousa, Genage André, Leonor Florentino and Dr. Lurdes Saramago from the Departamento de Biologia Animal (Universidade de Lisboa) for their valuable help and assistance. The librarian Manuel João (Museu Laboratório do Jardim Botânico) was a great help in several occasions and was always kind with me.

Thanks to all my friends and colleagues from the Entomology Lab:

To Anabela Cardoso and Sónia Santos for the many laughs and the good moments we spent together. We jumpstarted our “adventures” almost at the same time and we shared so many wishes for the future…some of them, fortunately, have come true.

To Carlos Aguiar, the “soul” of the entomology lab, for teaching me many things in science and life, for sharing so many enjoyable moments both during fieldwork as well as in the lab and for making more pleasant our everyday life at the Entomology Lab.

To Israel Silva and Pedro Silva for being such nice pals. Thank you for the stimulating discussions on science, the interesting entomological findings made together, the very pleasant moments playing football and for introducing me to the “Stroopwaffels” world.

To Teresa Rebelo for her friendship and support during the development of this thesis. I miss the times of our community lunches (when chocolate mousse was a must) and your guidance in our incursions in Oriental gastronomy.

And to Ana Margarida Santos, Carla Barrinha, Catarina Castro, Diana Marques, Filipa Gouveia, Gabriela Juma, Paula Simões and Sofia Seabra for all the good moments shared during all these years.

I also thank all my friends from Centro de Biologia Ambiental, especially Paula Lopes, Sofia Gabriel and Rita Monarca as well as to the *Drosophila* Lab team members - Ana Duarte, Inês Fragata, Josiane Santos, Marta Santos, Pedro Simões, Raquel Gonçalves and Prof. Margarida Matos - for their companionship and support during all these years.

I appreciate the kind words and encouragement addressed by António Zuzarte, Dr. Bivar de Sousa and Prof. Luis Mendes during the performance of this project. My friend Ana Maria also provided valuable support during these years.

To Fundação para a Ciência e a Tecnologia for providing me the opportunity to develop this research (through grant PRAXIS XXI/BD/21407/99) and for the financial support for my stays in Barcelona and participation in scientific meetings.

To my parents, that always encouraged me in everything in life, including my biological endeavours, for their constant support, love and above all for always being there for me. Mom, thank you for your help during the construction of exclusion traps, for preparing delicious meals to feed and “warm” me in the field and for always having everything set before the daily departure to fieldwork, when both I and the sun were still asleep. Dad, thank you for your help, advice and company during fieldwork, for always being kind to me even when I was too anxious, sad, tired or bad-tempered, for loving my insects and “babby-sitting” my seeds, and most of all for making this PhD thesis a pleasure. With your support and love all became much easier! I love you both!!

To Carla Rego, my love, for being patient, supportive and always nice to me. Thank you also for your encouragement, for the help during fieldwork and for the advice in statistical analyses and scientific writing. You provided me the time, the tranquillity and a pleasant environment to finish this thesis … and thanks to you I made it! Thank you for being my love and for having shared with me so many enjoyable memory-lasting moments.

Thank you all for being part of my life and for being a good help during this project.

RESUMO

Os insectos e as plantas angiospérmicas constituem dois importantes grupos de organismos, ambos dominando em número de espécies os reinos em que se integram e desempenhando um papel relevante na composição, estrutura e funcionamento das comunidades terrestres. Estes dois grupos de organismos estão unidos por uma variedade de interacções bióticas que diferem no seu grau de especialização, dependência e benefício ecológico, de acordo com as espécies envolvidas. Diversos estudos têm destacado o importante papel das interacções insecto-planta, quer como importantes forças evolutivas, quer também como importantes mecanismos de modelação das comunidades e ecossistemas.

A fase que medeia entre a ântese e a germinação da semente é considerada como uma das mais importantes do ciclo de vida das plantas, uma vez que é durante ela que as plantas produzem a sua descendência sob a forma de sementes e que procuram repor o número de indivíduos localmente ou colonizar novas áreas. Esta é também considerada uma das fases de maior susceptibilidade das plantas dada a vulnerabilidade das flores, frutos e sementes à acção dos factores bióticos e abióticos. Neste período, são diversas as espécies de insectos que interagem com as plantas influenciando positiva (e.g. polinizadores, dispersores) ou negativamente (e.g. predadores, herbívoros) o seu sucesso reprodutor. Pelas razões atrás mencionadas, o estudo da biologia da reprodução das plantas tem merecido considerável atenção na literatura, particularmente quando directamente relacionado com a biologia da conservação de espécies ameaçadas.

A conservação de espécies de plantas raras constitui uma das principais prioridades das políticas de conservação nos países mais desenvolvidos e diversos investigadores têm defendido que o estudo da biologia da reprodução dessas espécies deverá constituir uma parte integrante das iniciativas de conservação. Por outro lado, é cada vez mais recomendável que a realização de estudos de conservação de espécies vegetais raras incorpore outros elementos da comunidade, preferencialmente filogeneticamente relacionados com as espécies-alvo e que sejam comuns, por forma a mais facilmente identificar e compreender as causas responsáveis pela raridade de determinada espécie. Esta abordagem tem vindo ocasionalmente a ser colocada em prática com vantagens significativas na interpretação das causas e consequências da

raridade, e consequentemente na definição de medidas de conservação mais efectivas e eficientes para a manutenção ou recuperação de populações de espécies ameaçadas.

Na presente tese foi estudada a biologia da reprodução de três espécies do género *Euphorbia*, duas das quais – *E. pedroi* Molero & Rovira e *E. welwitschii* (Boissduval & Reuter) - são endemismos nacionais localizados e a outra – *E. characias* Linnaeus - apresenta uma distribuição circum-mediterrânica. Da espécie endémica *E. pedroi* conhecem-se apenas três populações localizadas entre o Cabo Espichel e o Cabo Ares, enquanto o endemismo *E. welwitschii* apresenta uma maior área de distribuição (no centro-oeste de Portugal), mas grande parte das suas populações são constituídas por um reduzido número de indivíduos.

Os trabalhos de campo tiveram lugar em áreas onde se verificou a co-ocorrência dos pares de espécies *E. characias*/*E. welwitschii* (em Montejunto e na Tesoureira) e *E. characias*/*E. pedroi* (em Ares e na Azóia), tendo ainda sido seleccionada uma área de estudo no Risco, onde ocorre *E. welwitschii* e devido à sua proximidade de Ares. O sucesso reprodutor de cada uma das três espécies de *Euphorbia* foi monitorizado durante três anos nas cinco populações seleccionadas, desde a ântese até ao destino das sementes após a sua remoção por animais, tendo sido identificados e quantificados os principais factores responsáveis pela diminuição do seu potencial reprodutor, com especial destaque para as interacções insecto-planta.

Os principais objectivos desta tese consistiram em: 1) identificar as principais causas de perdas reprodutivas no período pré-dispersão para as três espécies de *Euphorbia* e avaliar como os seus efeitos variam no tempo, espaço e entre indivíduos e espécies; 2) estudar o impacto diferencial de insectos predadores de sementes especialistas e generalistas no sucesso reprodutor das três espécies de *Euphorbia* e avaliar como os seus efeitos se relacionam com algumas características das plantas; 3) caracterizar a composição em ácidos gordos dos elaiossomas das três espécies de *Euphorbia* e avaliar a importância dessas estruturas para a atracção das formigas; 4) identificar os principais agentes dispersores das sementes de cada uma das espécies de *Euphorbia* e analisar o destino dado às sementes por cada um deles no curto prazo.

As principais causas das perdas reprodutivas no período pré-dispersão foram idênticas para as três espécies de *Euphorbia* e consistiram na ausência ou atrofia do ovário, na expressão de factores intrínsecos/ambientais que conduziram ao aborto de flores e frutos, na predação de sementes por insectos generalistas e especialistas e na expressão de factores intrínsecos/ambientais que conduziram ao aborto de sementes. Nas espécies *E. pedroi* e *E. characias* foram também assinaladas perdas reprodutivas devido à destruição dos ciátios por lagartas da espécie *Acroclita subsequana* (Herrich-Schäffer).

A ausência/atrofia do ovário constitui uma estratégia reprodutiva das plantas, que se traduz em andromonoecia e contribui para uma redução do potencial reprodutor. Os níveis de andromonoecia foram distintos nas três espécies de *Euphorbia*, tendo sido superiores em *E. characias* (~20%) e inferiores em *E. welwitschii* (~5%). Em cada espécie, estes valores mostraram uma variação significativa no tempo e no espaço, e uma forte associação aos padrões de produção de ciátios em resultado de efeitos relacionados com a arquitectura das plantas. Os nossos resultados confirmaram a ocorrência de valores de andromonoecia mais elevados em plantas perenes e tornam evidente a necessidade de avaliar em estudos futuros a importância da filogenia e do modo de vida da planta (anual *vs.* perene herbáceo *vs.* perene lenhoso) como factores determinantes dos níveis de andromonoecia em plantas do género *Euphorbia*.

A perda de flores e frutos, devida ao aborto destas estruturas, deveu-se sobretudo a causas ambientais, relacionadas com as condições climáticas das áreas de estudo, e também à expressão de factores intrínsecos. Em duas localidades costeiras (Ares e Azóia), onde co-ocorrem *E. pedroi* e *E. characias*, verificou-se que uma percentagem dos ciátios de indivíduos de ambas as espécies apresentavam marcas evidentes de destruição devido às condições meteorológicas adversas que se fazem ali sentir, tendo culminado com o aborto desses órgãos reprodutores. Essas perdas foram mais significativas nos indivíduos da população da Azóia, uma vez que as características topográficas e o coberto vegetal mais desenvolvido em Ares funcionaram como barreira natural, protegendo o desenvolvimento de flores e frutos. De um modo geral, os indivíduos de *E. welwitschii* mostraram ser mais susceptíveis a perdas que resultaram no aborto de flores e frutos do que os seus congéneres. Na população do Risco, este tipo de perda reprodutiva foi sempre, em média, superior a 50%, parecendo traduzir os efeitos negativos de populações com reduzido número de efectivos. O reduzido número de efectivos desta população, o seu isolamento geográfico e a sistemática baixa produção de sementes sugerem que as elevadas perdas reprodutivas aqui registadas poderão estar relacionadas com fenómenos de consanguinidade, baixas taxas de polinização e/ou perda de variabilidade genética.

As perdas reprodutivas que resultaram no aborto de sementes foram significativamente mais elevadas em *E. pedroi* (com médias anuais de ~30-50%) do que nos seus congéneres. Estes resultados poderão ficar a dever-se à expressão de factores intrínsecos (i.e. depressão consanguínea, conflito progenitor-descendente, rivalidade entre sementes do mesmo fruto), como foi já relatado para uma espécie ibérica aparentada (*E. dendroides*), embora existam indícios de que os valores mais elevados registados em Azóia se possam também ficar a dever ao baixo nível de recursos existentes para a utilização pelas plantas neste local.

As traças da espécie *A. subsequana* mostraram uma clara preferência por *E. pedroi* como planta hospedeira, tendo as suas lagartas destruído uma maior percentagem de ciátios nessa espécie (~10%) do que em *E. characias* (~1%). As perdas reprodutivas devido a *A. subsequana* não se encontraram associadas a características das plantas indicativas de tamanho e fecundidade, uma vez que a selecção de plantas hospedeiras por esta traça se baseia fortemente em pistas químicas.

Dois grupos de insectos predadores de sementes, euritomídeos especialistas e hemípteros generalistas, foram responsáveis por perdas reprodutivas consideráveis nas três espécies de *Euphorbia.* De um modo geral, os hemípteros generalistas [pertencentes às espécies *Cydnus aterrimus* (Forster), *Dicranocephalus agilis* (Scopoli)*, Dolycoris baccarum* (Linnaeus) e *Spilostethus pandurus* (Scopoli)] causaram uma maior percentagem de perdas reprodutivas nas espécies endémicas de *Euphorbia* do que em *E. characias*, mas o seu impacto no sucesso reprodutor das espécies estudadas foi menor do que o verificado para os euritomídeos. Os euritomídeos foram responsáveis por perdas reprodutivas mais elevadas em *E. pedroi* (causadas por *Eurytoma fumipennis* Walker) do que nas duas outras espécies de *Euphorbia* (causadas por *E. jaltica* Zerova) e tal deveu-se sobretudo a diferenças na biologia e abundância desses predadores. As perdas causadas pelos euritomídeos especialistas mostraram estar intimamente associadas com os padrões de produção de sementes das suas plantas hospedeiras, uma vez que estes predadores se encontram delas fortemente dependentes para o desenvolvimento do seu ciclo de vida. À semelhança do que se verificou com *A. subsequana*, as perdas causadas pelos insectos especialistas e generalistas não mostraram estar associadas com características das suas plantas hospedeiras indicativas de tamanho e fecundidade. A selecção de plantas hospedeiras por ambos os grupos de insectos baseia-se fortemente na interpretação de compostos voláteis emitidos pelas plantas e numa posterior avaliação local, onde são utilizados diversos sentidos (gosto, olfacto, tacto). Os efeitos dos três grupos de insectos no sucesso reprodutor de cada uma das três espécies de *Euphorbia* foram aditivos, indicando que os diferentes grupos de insectos parecem não discriminar as plantas em função das suas perdas prévias para outros grupos de insectos e/ou que baseiam a sua selecção em diferentes características das plantas.

A produção de sementes intactas diferiu consideravelmente entre as três espécies de *Euphorbia*, tendo sido inferior em *E. welwitschii* (médias populacionais de 37-242 sementes/indivíduo/ano), intermédia em *E. pedroi* (médias populacionais de 228-399 sementes/indivíduo/ano) e mais elevada em *E. characias* (médias populacionais superiores a 595 sementes/indivíduo/ano). Estas diferenças interespecíficas significativas devem-se sobretudo às diferenças na produção de ciátios entre as três espécies de *Euphorbia*, mas também traduzem o impacto diferencial dos factores de mortalidade nas três espécies estudadas. Os nossos resultados corroboram as conclusões indicadas noutros estudos de que as espécies endémicas de distribuição localizada apresentam uma menor produção de flores e sementes do que as suas congéneres comuns.

As três espécies de *Euphorbia* estudadas apresentam sementes com elaiossomas, tendo-se verificado a existência de diferenças interespecíficas nas dimensões das sementes e dos elaiossomas, bem como na razão entre as duas variáveis. Análises cromatográficas dos elaiossomas das três espécies de *Euphorbia* também evidenciaram diferenças significativas na concentração de vários ácidos gordos, em especial nos ácidos oleico, palmítico e linoleico, cujas concentrações são cinco vezes superiores nos elaiossomas de *E. characias* e *E. welwitschii* do que nos de *E. pedroi*.

Os ensaios de oferta de sementes mostraram que as formigas mutualistas preferem as sementes de *E. characias* que possuem elaiossomas de maiores dimensões e têm uma maior concentração de ácidos gordos (especialmente ácido oleico). Por outro lado, apesar das sementes de *E. pedroi* apresentarem a maior razão entre o tamanho do elaiossoma e o da semente, foram removidas em menor percentagem que as dos seus congéneres provavelmente dada a baixa concentração de ácidos gordos (em particular de ácido oleico) nos seus elaiossomas. Constatou-se ainda que, uma vez desprovidas de elaiossoma, as sementes das três espécies de *Euphorbia* foram invariavelmente ignoradas pelas formigas mutualistas. Estes resultados destacam o importante papel desempenhado pelo elaiossoma na remoção das sementes pelas formigas mutualistas e também a importância do conhecimento da sua composição química na interpretação de diferenças nas taxas de remoção de sementes.

Os animais que interagiram com as sementes de *Euphorbia* nas cinco áreas de estudo incluíram formigas omnívoras mutualistas, formigas granívoras e vertebrados, particularmente roedores. Os resultados das experiências com armadilhas de exclusão tornaram evidente o papel preponderante das formigas na remoção das sementes de *Euphorbia*, embora uma percentagem das sementes tenha sido removida por vertebrados durante o período nocturno.

As formigas envolvidas na remoção de sementes pertenciam às espécies omnívoras *Aphaenogaster senilis* Mayr, *Formica subrufa* Roger, *Pheidole pallidula* (Nylander) e *Tapinoma nigerrimum* (Nylander) e à espécie granívora *Messor barbarus* (Linnaeus). As duas primeiras espécies removeram uma maior percentagem de sementes das três espécies de *Euphorbia* nas diversas áreas de estudo e transportaram essas sementes a maiores distâncias (em média, aproximadamente 2,5m), tendo abandonado as sementes no exterior do formigueiro uma vez retirado o elaiossoma. Estas espécies removeram a maior parte das sementes durante o período mais quente do dia, quando as outras espécies se encontravam inactivas. As características ecológicas e etológicas destas duas espécies correspondem ao padrão que alguns autores definiram como "mutualistas fundamentais" para o processo de dispersão. As outras duas espécies de formigas omnívoras, *P. pallidula* e *T. nigerrimum*, removeram uma menor percentagem de sementes nas áreas de estudo e a uma menor distância (em média, aproximadamente 0,5m para *P. pallidula* e 0,85m para *T. nigerrimum*). Verificou-se também que, a curto prazo (num período de 24h após a recolha das sementes), nenhuma das sementes recolhidas por estas espécies de formigas havia sido descartada para o exterior dos seus formigueiros. As formigas granívoras da espécie *Messor barbarus* não mostraram ter um comportamento distinto perante as sementes das diferentes espécies de *Euphorbia* e também foram indiferentes à inexistência de um elaiossoma na semente, contrariamente ao que foi registado com as formigas mutualistas. As formigas granívoras recolhem as sementes para delas se alimentarem, actividade que decorre durante as horas de menor calor, sendo a taxa de predação de virtualmente 100%.

As formigas mutualistas tiveram um papel importante na sobrevivência das sementes das três espécies de *Euphorbia* uma vez que ao removê-las rapidamente do solo reduziram a probabilidade de as mesmas serem predadas e também, ao transportá-

las a alguma distância, contribuíram para a sua redispersão e afastamento da área de influência parental.

Os resultados das nossas observações e experiências indicaram a existência de perdas reprodutivas consideráveis para os predadores de sementes na fase pós-dispersão, perdas essas que variaram entre populações e se encontraram estritamente relacionadas com a identidade das espécies de *Euphorbia*. Quando em simpatria, as espécies endémicas *E. pedroi* e *E. welwitschii* sofreram proporcionalmente perdas mais elevadas para as formigas granívoras do que a sua congénere *E. characias* e isto deveu-se sobretudo à menor atracção das formigas mutualistas pelos elaiossomas das sementes das espécies endémicas.

As três espécies de *Euphorbia* parecem pois exibir estratégias de dispersão um pouco distintas. Por um lado, *E. characias* parece investir mais na produção de sementes de maior tamanho e de elaiossomas de maior qualidade nutritiva sendo as suas sementes removidas em maior percentagem por formigas mutualistas, enquanto que as outras duas espécies produziram sementes e elaiossomas de menor tamanho e elaiossomas mais pobres em ácidos gordos (no caso de *E. pedroi*) pelo que as suas sementes foram removidas com menor probabilidade pelas formigas mutualistas. Diferenças na estratégia de dispersão foram já observadas noutras espécies diplócoras congenéricas tendo-se concluído que as mesmas resultam do facto de cada espécie procurar optimizar as vantagens conferidas por uma das duas fases de dispersão.

Os resultados obtidos na presente tese realçam a importância do estudo das interacções insecto-planta para uma melhor compreensão do sucesso reprodutor das plantas. Os insectos interagem continuamente com as plantas ao longo do ciclo reprodutor e têm um papel bastante importante no destino das flores, frutos e sementes. Desta forma, consideramos de grande importância, que os planos de conservação de espécies de plantas ameaçadas contemplem o estudo das interacções insecto-planta e que a avaliação dos factores responsáveis pela mortalidade de flores, frutos e sementes seja feita comparativamente com outros elementos da comunidade com elas relacionados.

Palavras-chave: Interacções insecto-planta; Mirmecocoria; Predação de sementes por insectos; Elaiossomas; *Euphorbia*.

ABSTRACT

Comparative studies on the reproductive biology of co-occurring related plant species have provided valuable information for the interpretation of ecological and evolutionary phenomena, with direct application in conservation management of plant populations. The aims of this thesis were to identify the causes of pre-dispersal reproductive losses in three *Euphorbia* species (the Mediterranean *E. characias* and the narrow endemics *E. pedroi* and *E. welwitschii*) and evaluate the variation of their effects in time, space and between individuals and species. Furthermore, we intended to study elaiosomes' fatty acid profiles for the three *Euphorbia* and assess the role played by the elaiosome in ant attraction. Finally, we aimed to identify the major seed dispersal agents for each *Euphorbia* species in each site and study differences in short term seed fate due to differences in ant behaviour. The results indicated that intact seed production differed significantly between the three *Euphorbia*, mostly due to differences in cyathia production. Losses to pre-dispersal seed predators were proportionately larger for the endemic species which also suffered higher losses resulting in flower, fruit (in *E. welwitschii*) and seed abortion (in *E. pedroi*). The elaiosomes of *E. pedroi* are poor in fatty acids and for this reason seeds of this species were removed in lower proportion by mutualistic dispersers than those of their congeners, being more prone to seed predation. Two larger ant species – *Aphaenogaster senilis* and *Formica subrufa* – were responsible for a larger percentage of removals with seeds being transported at larger distances and being discarded in the vicinity of their nests following elaiosome removal. Our results highlight the role of insect-plant interactions as major determinants of seed survival for the three study plants and call for the need to include more information on insect-plant interactions in plant conservation programmes.

Keywords: Insect-plant interactions; Myrmecochory; Insect seed predation; Elaiosomes; *Euphorbia*.

Chapter 1

General introduction

GENERAL INTRODUCTION

The interactions between animals and plants are diverse and omnipresent in nature. These organisms have been evolving together for millions of years, with varying types and degrees of interaction between them. In some cases, plants and animals share an extremely specialized interaction, being dependent on each other and exhibiting peculiar morphological, ethological and physiological adaptations. However, the large majority of interactions between plants and animals are not specialized and are usually facultative (Herrera & Pellmyr, 2002).

In the last decades the interest in plant-animal interactions increased dramatically and nowadays it is a firmly established discipline (Herrera & Pellmyr, 2002). The late development of this field was in part due to the historical progress of the knowledge on plants and animals in two separate lanes, only occasionally interacting with each other. It was not until the late 70s, but mostly during the 80s, that we witnessed to an explosion in the number of publications dealing with a variety of thematics on plant-animal interactions and this large amount of information allowed the development of many theories explaining ecological patterns and processes. Nowadays the study of plant-animal interactions encompasses various areas of knowledge (ecology, ethology, physiology, biochemistry, etc.) allowing a better comprehension of the phenomena under study.

Many studies have highlighted the role of plant-animal interactions as powerful evolutionary forces and as influential mechanisms in shaping the structure and composition of natural communities (Herrera & Pellmyr, 2002; Strauss & Irwin, 2004). Furthermore, the interactions involving plants and animals have important controlling influences on a variety of population, community and ecosystem-level processes.

It should also be emphasized that many findings resulting from studies on plant-animal interactions have had direct application in a wide variety of disciplines, such as agriculture, horticulture, forestry and conservation biology, translating into clear benefits for human kind (Price, 2002).

Insects and angiosperms are two major groups of living organisms, each one dominating in number of species the kingdoms to which they belong, and both playing predominant roles in the composition, structure and functioning of most terrestrial communities (Crawley, 1997; Foottit & Adler, 2009). The diversification of insects and angiosperms is still a matter of debate, but many authors consider that insect-plant interactions have played a key role as drivers of diversification in both groups (Price, 2002; Janz *et al.,* 2006; Kay & Sargent, 2009; Lengyel *et al.*, 2009; McKenna *et al.*, 2009). Insects and angiosperms are united by intricate relationships which vary in their degree of specialization, dependency and overall net benefit for the interacting species.

Among the most commonly studied insect-plant interactions we find mutualistic associations, where insects behave as pollen or seed vectors, and antagonistic associations, like herbivory and seed predation. During a single reproductive season, an individual plant may interact with tenths of insect species, including herbivores, pollinators, frugivores, seed dispersers and seed predators. This clearly illustrates the widespread nature of insect-plant interactions and how they may be crucial for the interactors.

1.1 Ovule and seed fate in vascular plants

The period that mediates from ovule formation to seed germination is a very important stage in the life cycle of plants. During this period vascular plants produce their offspring in the form of seeds and provide these propagules with structures to nurture, protect and aid in their dispersal (Fenner & Thompson, 2005).

Seeds are the result of the development of fertilized ovules, being potential new plants. Thus, seed fate has direct consequences for species survival and for the dynamics of plant populations. According to several authors (e.g. Harper, 1977; Hulme & Benkman, 2002) seeds influence plant populations in the following ways:

- seeds allow the **replacement of individuals in a population** ensuring population persistence or increase;
- seeds allow the **colonization of new areas**;
- seeds allow the **survival in unsuitable places and/or during unfavourable periods**.

From ovule formation until seed germination a variety of factors are responsible for the decrease in the potential number of new plants to be recruited. Among those factors, pollination failure, resource limitation, predation, genetic/developmental effects and agents of the physical environmental (e.g. adverse weather conditions) are the most frequently reported (Stephenson, 1981; Lee, 1988; Fenner & Thompson, 2005). These factors act sequentially or in conjunction and their effects usually show variation in space, time and between individuals. Many studies have shown how the effects of these factors may be very severe, leading to high reproductive losses, compromising plant recruitment and even threatening population survival (Hulme & Benkman, 2002; Fenner & Thompson, 2005; Knight *et al.*, 2005).

The studies on the fate of seeds have progressed in two separate lines taking in consideration a key event in the life-cycle of plants - seed dispersal. A much higher number of studies have dealt with reproductive losses during the period of seed development on the parent plant (pre-dispersal losses) while seed losses during the period following dispersal until germination (post-dispersal losses) have received less attention (Fenner & Thompson, 2005). Further, very few studies have reported both pre- and post-dispersal losses for a particular plant species (Kaye, 1999; Traveset *et al.*, 2003; Giménez-Benavides *et al.*, 2008).

A reason for the larger number of studies concerning pre-dispersal reproductive losses is the relative ease to identify and quantify the fate of ovules and seeds at the parent plant. Plants can be easily located in the field and the causes of reproductive losses can also be related with parent plant characteristics. Even so, some difficulties in evaluating accurately ovule and seed condition at this stage have been pointed out by several authors (e.g. Janzen, 1971; Andersen, 1988), who have called for caution and rigor when assessing the causes of seed loss.

After dispersal, seeds are much more difficult to follow, a reason that led several authors to argue that “seeds became nearly invisible between the time they left the parent and when the seeds germinated” (Vander Wall *et al.*, 2005). Consequently, it may prove difficult to monitor seeds during this stage in order to identify and quantify accurately the causes of seed mortality. Furthermore, in some circumstances, like for example the case of plant species whose seeds may remain dormant for many years, it may also be challenging to disentangle the causes of seed mortality (Fenner & Thompson, 2005).

The need for the integration of the information on pre- and post-dispersal losses has been stressed by many authors, in order to improve our understanding of the processes governing seed fate and consequently understand the dynamics of plant populations (Kaye, 1999; Picó & Retana, 2000; Traveset *et al.*, 2003; Giménez-Benavides *et al.*, 2008). All the factors that intervene in the reproductive stage of the life cycle should be considered since this is the only way to evaluate correctly the relative importance of each one of them in reducing the potential reproductive output of a plant species (e.g. Heithaus *et al.*, 1982). It should also be kept in mind that the relative importance of a factor is subjected to variation in space and time, so more accurate estimates of its magnitude require the replication of the study across multiple populations and over several reproductive seasons.

1.2 Seed production and pre-dispersal reproductive losses

Reproduction is an important stage in the life-cycle of plants during which a variable, but usually considerable, amount of resources are used to produce flowers, fruits, seeds and other reproductive structures. For this purpose, resources need to be diverted from other functions (e.g. vegetative growth) and may even constrain plant longevity (Crawley, 1997; Obeso, 2002). The resources channelled to plant reproduction are then used according to a strategy of resource allocation to reproduction where they are further divided between the male and female functions.

The upper limit of a plant´s reproductive output is fixed by the number of ovules produced. However, during the period of seed development (from fertilization to seed dispersal) this upper limit is further reduced due to a variety of factors. For simplicity, those factors have been categorized as extrinsic factors, when they relate to the abiotic environment and to biotic interactions, and intrinsic factors if they concern to genetic, developmental, architectural or nutritional aspects of the individual.

Among the factors known to reduce the reproductive output of plants before seed dispersal, pollination failure, genetic and developmental effects, agents of the physical environment, resource limitation and predation are the most frequently reported.

1) **Pollination failure** is considered a common phenomenon that hampers the reproductive output of plants (Knight *et al.*, 2005). The effects of pollination limitation on seed production have been thoroughly debated during the last few decades with many examples illustrating the relevance of pollen quantity and quality on fertilization success. The causes of pollination limitation may be both ecological or evolutionary in nature (Ashman *et al.*, 2004), but during the last decade, due to the large-scale of changes induced by man to most ecosystems, a great emphasis has been put on the consequences of ecological perturbations in pollination limitation. The fragmentation of habitats affects abiotic and biotic factors, which may directly or indirectly lead to changes in the abundance of plants and their pollinators. The reduced population size of both plants and their pollinators has been reported to be a major cause of pollination

failure in plants (Kearns *et al.*, 1998; Wilcock & Neiland, 2002). There is also growing evidence that the introduction of exotic species may lead to the disruption of coevolved interactions between plants and their pollinators leading to pollination limitation (Bond, 1994; Kearns *et al.*, 1998; Traveset & Richardson, 2006). Some plant reproductive strategies may also lead to pollination failure. For example, plants living in environments with stochastic pollen receipt may oversupply flowers with ovules. This bet-hedging strategy may prove to be adaptive despite the occasional high losses due to lack of pollination plants are subjected to (Burd, 1995).

2) **Genetic and developmental effects** are responsible for a reduction in the reproductive output of plants since they may lead to the abortion of ovaries, fruits and seeds. A number of articles dealing with genetic and developmental effects on plant reproduction concern the deleterious effects of inbreeding. Inbreeding depression can result from mating between genetically similar individuals, although a number of studies concern the negative effects of self-fertilization on seed production (see Fenner & Thompson, 2005 and references therein). In the last two decades, numerous studies have reported high reproductive losses due to inbreeding depression in small and isolated populations of many plant species as a consequence of the growing fragmentation of natural habitats (e.g. Byers & Waller, 1999; Keller & Waller, 2002; Severns, 2003). In particular situations, inbreeding depression has even been identified as a cause of population decline or local extinction (Ellstrand & Elam, 1993; Frankham, 2005). Genetic load and maternal effects have also been reported as causes of early embryo abortion and abnormal seed development (Wiens *et al.*, 1987; Kärkkäinen *et al.*, 1999).

3) The effects of **agents of the physical environment** have been reported for significant reproductive losses in a number of plants. For example, occasional adverse climatic conditions during the flowering and fruiting periods are known to damage the reproductive structures of plants, leading to a lower production of seeds (Green & Palmbald, 1975; Ågren, 1988; Hampe, 2005). On the other hand, plants living in harsh environments, like coastal areas, are constantly subjected to their negative effects on reproduction which usually lead to the destruction of ovaries and fruits (e.g. Rovira *et al.*, 2004). The effects of physical agents play a crucial role for many plant species since they commonly determine the reproductive success of individuals from range margin populations and consequently species distribution (Woodward, 1987; Pigott, 1992).

4) During the reproductive episode plants need to coordinate the allocation of the available resources for different tasks. This is a very complex problem due to the unpredictability associated with the number of flowers that will be successfully pollinated and the constraints they will face during seed development. Consequently, many plants face **resource limitation** and the abscission of reproductive structures (ovaries and fruits) allow plants to match their reproductive output with the available resources (Stephenson, 1981). The temporal decline in fruit set, documented in a large number of species, may be a consequence of competition between flowers and fruits for limited maternal resources (e.g. Stephenson, 1981; Lee, 1988; and references therein). Resource limitation also put in evidence the impact of architectural effects on seed set (Diggle, 1995; Espadaler & Gómez, 2001), i.e. some positions on the plant are more favourable than others on the probability of successful fruit and seed maturing.

5) **Pre-dispersal seed predation** is a frequently reported cause of seed mortality that often has a significant impact on the reproductive output of plants (Crawley, 1992; Hulme & Benkman, 2002; Kolb *et al.*, 2007). In general, seeds are very rich in nutrients, being proportionally richer in carbohydrates and proteins than most of the other plant tissues (Jordano, 1992). Due to their intrinsic and high nutritional values, seeds are often well defended physically and/or chemically from the attack of predators (Hulme & Benkman, 2002). Many plant species, from a wide variety of plant families, have dry resistant fruits that are indehiscent or dehisce only by the time seeds mature. Additionally, fruits and seeds may possess a thick coat or a cover of spines or hairs, making seed predation an insurmountable challenge for many predators (Green & Palmbald, 1975; Grubb, 1998; Bojňanský & Fargašová, 2007). Some other species have fleshy fruits, where seeds are enclosed in a protective fibrous coat that deters invertebrate seed predators and simultaneously attracts vertebrate dispersers (Mack, 2000). The physical protection of seeds constitutes the sole defence in some species, but in others it is just the first line of defence. The chemical defence of seeds is also widespread in most plant lineages and involves the accumulation of organic compounds (e.g. alkaloids, cyanogens, non-protein aminoacids, terpenoids), most of which being products of secondary metabolism. These compounds are toxic for a wide array of animals and their effects vary from feeding deterrence to poisoning (Janzen, 1969; Harborne, 1993; Tewksbury & Nabhan, 2001).

In spite of the variety of seed defences, pre-dispersal seed predators may still have a considerable impact on the reproductive output of plants (Janzen, 1971; Fenner &

Thompson, 2005). In a recent review, Kolb *et al.*, (2007) stated that "pre-dispersal seed predation rates are usually low, but sometimes high" and Hulme and Benkman (2002) found an average of 47.3% reproductive losses due to pre-dispersal seed predation from a number of selected studies. The majority of pre-dispersal seed predators are specialist insects (Janzen, 1971; Crawley, 1992; Hulme & Benkman, 2002), mostly included in the orders Coleoptera, Hymenoptera, Hemiptera and Lepidoptera. These insects have their life-cycles synchronized with the temporal patterns of seed production of their hosts and are capable to cope with their defences. Furthermore, contrary to what happens with generalist insects, there is usually a close relationship between seed production patterns and seed predation levels by insect specialists that can allow some predictability on future reproductive losses (Solbreck & Sillén-Tulberg, 1986; Sperens, 1997).

Pre-dispersal seed predators may have an important role on the dynamics of plant populations and may also play a role on the evolution of plant traits (see a review by Kolb *et al.,* 2007 and references therein). Several studies have shown that seed predators have the potential to reduce both recruitment and population growth of their host plants and may even threat population survival (Hegazy & Eesa, 1991; Kelly & Dyer, 2002; Maron *et al.*, 2002; Kolb *et al.*, 2007). For example, studies carried out on *Haplopappus squarrosus* and *Cirsium canescens* were particularly elucidative about the effects that pre-dispersal seed predators may have on determining plant distribution and abundance (Louda, 1982a, b; Louda & Potvin, 1995). On the other hand, pre-dispersal seed predators may also act as selective agents by reducing differentially the reproductive output between plants that differ in some heritable characteristic. A particular important association of pre-dispersal seed predation levels is with plant reproductive traits. In fact, the reproductive traits of many plants have evolved to increase attraction by pollinators, but those traits can also be used as cues by seed predators (Brody & Mitchell, 1997; Cariveau *et al.*, 2004). A number of studies reported how pre-dispersal seed predators may exert a significant selective pressure on a variety of plant reproductive traits (e.g. flower number and size, flowering phenology, fruit number and size, mast seeding) (Ehrlén, 1996; Colas *et al.*, 2001; Leimu *et al.*, 2002), but evolutionary change may be limited due to the occurrence of trade-offs between the effects of seed predators and those from other interactors (Brody & Mitchell, 1997; Cariveau *et al.*, 2004; Strauss & Irwin, 2004; Harder & Barrett, 2006). However, several other studies failed to detect a role of pre-dispersal seed predators in plant trait evolution and in the dynamics of plant populations (see Kolb *et al.*, 2007).

The combination of all kind of pre-dispersal reproductive losses may lead to very low values of fruit and seed set, a situation that has often been recorded in literature (see Fenner & Thompson, 2005). There are even several examples where a reproductive season was completely lost due to the impact of factors acting before seed dispersal (Lamont *et al.*, 1993; Curran & Leighton, 2000). However, for most plant species, the magnitude of each kind of pre-dispersal reproductive loss is usually low to moderate and shows some variation between individuals and years, meaning that a variable number of intact seeds is regularly produced in each population. Some plant species have also developed strategies to maximize the successful production of offspring during episodic periods. For example, a number of species have supra-annual synchronized fruiting patterns (i.e. mast fruiting) that may lead to increased seed survival through high pollination efficiency and/or predator satiation (Kelly *et al.*, 1992; Kelly, 1994; Yasaka *et al.*, 2003; Satake *et al.*, 2004). There are also several examples of synchronized fluctuation of seed production among different co-occurring plant species (community-wide masting) that share the same seed predators (Pucek *et al.*, 1993; Shibata *et al.*, 1998; Curran & Webb, 2000).

1.3 Seed dispersal and post-dispersal seed predation

Seed dispersal is a key event in the life-cycle of plants encompassing a variety of processes that take place since a propagule separates from the parent plant until it germinates (Cousens *et al.*, 2008). Although seed dispersal ecology has for long fascinated naturalists, it remained a neglected field of research for decades probably due to the technical difficulties in monitoring seeds once they leave the parent plant together with the common belief that plants produced so many seeds making seed fate dynamics an unimportant issue. Nowadays seed dispersal is a firmly established discipline and an active field of theoretical and empirical research.

Many studies emphasize the advantages of seed dispersal for plant populations which rely on three main hypotheses (Howe & Smallwood, 1982; Howe & Miriti, 2004):

- seed dispersal avoids heavy seed and seedling mortality near the parent plant (escape hypothesis); This hypothesis followed the pioneer works of Janzen

(1970) and Connell (1971), who have found evidence for density-dependent or distance-dependent seed mortality, respectively. Many studies support this hypothesis by showing that some seed predators search for food mainly in the immediate vicinity of parent plants and seeds dispersed further away have higher probability of survival (Howe, 1993; Packer & Clay, 2000).

- **seed dispersal allows the colonization of new areas away from the parent (colonization hypothesis)**; This hypothesis was developed following studies of successional communities, where some plants were found to disseminate their seeds widely in order to find a suitable habitat where germination and establishment may occur or where seeds may remain dormant until a disturbance creates the necessary conditions for establishment (Howe & Smallwood, 1982). Some plants that have this strategy only persist for a few generations in a specific location, usually occupying ephemeral or unpredictable habitats. Others, like some forest species, depend on the dynamics of treefall gaps for successful colonization (Wenny, 2001)
- **seed dispersal permits the location of specific microhabitats suitable for establishment (directed dispersal hypothesis)**; This hypothesis states that dispersal vectors take seeds non-randomly to specific microhabitats, which are critical for plant establishment and growth (Wenny, 2001). Several examples of directed seed dispersal are known among vertebrate-dispersed plants (Wenny & Levey, 1998), but many more were reported from ant-dispersed plants (Beattie, 1985; Giladi, 2006; Rico-Gray & Oliveira, 2007).

During the process of seed dispersal, the parent plant, the propagule and the environment play major roles on seed fate. The contribution of the parent plant to dispersal may be important because both the initial position and the time of dispersal may greatly influence seed fate. Furthermore, many plants have active (launching systems) or passive (requiring abscission forces) mechanisms of seed dispersal which may also contribute decisively to the frequency distributions of dispersal distance.

The attributes of propagules also play a predominant role during the dispersal process. The structural adaptations of propagules have for long fascinated naturalists and for this reason were the subject of detailed morphological and functional studies. Furthermore, botanists and ecologists were also committed to classify the diversity of propagules according to the traits they exhibited (Ridley, 1930; Van der Pijl, 1969; Murray, 1986). Three major classes of propagules are often recognized in literature: wind-dispersal adapted (i.e. those having aerodynamic traits like plums and wings), water-dispersal adapted (i.e. those possessing hydrodynamic/buoyancy traits like air chambers and impermeable coats) and animal-dispersal adapted (i.e. fleshy and carunculate propagules, propagules with hooks and spines). There are, however, propagules that combine traits from different classes and others that seem to lack any special dispersal mechanism (Vander Wall & Longland, 2004; Fenner & Thompson, 2005).

Finally, after the propagule has left its parent plant, the environment plays a major role on dispersal success. Propagules may then interact with a variety of dispersal vectors of physical and biological nature (e.g. wind, water, animals) that will make them move in different ways, with different magnitudes. Even after reaching the ground, propagules may resume their movement away from the parent plant or even approaching it. Consequently the pathway taken by each propagule may be unique and, in general, very difficult to follow since release until its final rest.

Seed dispersal by animals has been the subject of thorough research during the last three decades and consequently the role of animals on seed fate is now much better known. In many situations, it has even been found that the dispersal of propagules by animals is critical for the functioning of the whole ecosystem (Howe & Miriti, 2004). Seeds are dispersed by a large variety of animals ranging from ants to elephants, but a great number of studies concern seed dispersal by frugivorous birds and mammals (e.g. Herrera, 2002; Dennis *et al*., 2007).

Myrmecochory, the dispersal of seeds by ants, is a widespread phenomenon with more than 3000 plant species worldwide, from eighty different families, being known to be ant-dispersed (Beattie & Hughes, 2002). Most of these species are known from Australia (~1500) and South Africa (~1300), with around 300 species reported from temperate deciduous forests of Europe and North America (Berg, 1975; Bond & Slingsby, 1983; Beattie & Hughes, 2002). However, taking in consideration recent studies where the number of ant-dispersed plant species was estimated as more than 11000 (Lengyel *et al*., 2009; 2010), those numbers clearly illustrate how poorly we still know the ecological and evolutionary relevance of myrmecochory in most terrestrial ecosystems. Ant-dispersed plants have been reported from a variety of habitats from deserts to tropical forests, but most studies were performed in temperate deciduous forests and sclerophyll scrublands (Rico-Gray & Oliveira, 2007).

Myrmecochory is a facultative mutualistic interaction between ants and plants, where seeds provided with an elaiosome (nutritious appendage attached to the seed) are carried away by ants, usually having their nests as destiny. In the nest, the elaiosome is removed from the seed and used in ant nutrition, while the seeds may rest inside the nest or may be discarded unharmed to the refuse pile outside. Thus, the elaiosome plays a critical role in ant attraction and subsequent seed removal. The elaiosomes are particularly rich in lipids, aminoacids and monosaccharides, and provide various nutrients that are essential for insect reproduction and development (Bresinski, 1963; Brew *et al.*, 1989; Fischer *et al.*, 2008). Among lipids, fatty acids are major components of elaiosomes and a few of these compounds, particularly oleic and oleyl-based glycerides, have been identified as the main responsible for elaiosome attractiveness for ants (Marshall *et al.*, 1979; Skidmore & Heithaus, 1988; Brew *et al.*, 1989; Kusmenoglu *et al.*, 1989; Lanza *et al.*, 1992). Although the key role played by oleic and oleyl-based glycerides in myrmecochory seems unquestionable, there are two different interpretations (nutrition *vs.* attraction) for their high content in elaiosomes (see Fischer *et al.*, 2008; Pfeiffer *et al.,* 2009). In some species, the elaiosome may also play additional functions besides ant attraction, such as providing the handle that allows the transportation of seeds (e.g. Gómez *et al.*, 2005) or repelling seed predators (Hanzawa *et al.*, 1985), among others (Lisci *et al.*, 1996).

Myrmecochorous plants usually possess a variety of morphological, anatomical, biochemical and phenological adaptations, besides the above mentioned specialised lipid-containing appendages (Berg, 1958; Gorb & Gorb, 2003). Some species have peduncles that bend down to the soil and the seeds are deposited directly on the soil surface, where they are easily removed by ants. Many myrmecochorous plants also have early fruiting periods, a phenological adaptation that may be explained by the higher activity of dispersal vectors during that period (Oberrath & Böhning-Gaese, 2002; Guitián & Garrido, 2006). By that time, ants face a critical period in the colony resources because there are not many alternative foods available and some of the species are engaged in brood rearing. Thus, elaiosomes seem to be crucial for ants.

The selective advantages of myrmecochory for plants have been summarized in some references (Beattie, 1985; Giladi, 2006; Rico-Gray & Oliveira, 2007) and include:

- **avoidance of predators** – after being released by their parent plants, seeds are quickly removed by ants to their nests, where they find refuge from predators. Furthermore, by being inside or near the nest, seeds may benefit from the protection by ants. A number of studies support this hypothesis by showing that seeds not removed by ants are eaten by seed predators (O'Dowd & Hay, 1980; Heithaus, 1981; Smith *et al.*, 1986; Nakanishi, 1994; Ruhren & Dudash, 1996; Pizo & Oliveira, 1998).

- **avoidance of competitors** – seeds dispersed by ants will usually rest in the ant nest or in its vicinity. There they may face low interspecific competition for germination and seedling growth from other co-occurring species, particularly from non-ant-dispersed congeners (Handel, 1976, 1978; Boyd, 1996).

- **avoidance of fire** – in dry sclerophyllous scrublands, where fire is a common event, the removal of seeds to ant nests may protect them from fire (Majer, 1982; Christian & Stanton, 2004). Furthermore, burial depth inside ant nests also allows seeds to benefit from heat to germinate, without being killed (Bond & Slingsby, 1983).

- **dispersal for distance** – by removing the seeds beyond the influence of their parent plants, ants eliminate the probability of seedling competition with established adults, thus increasing seedling survival (Pudlo *et al.*, 1980; Luond & Luond, 1981; Rice & Westoby, 1981; Boyd, 1996; Kalisz *et al.*, 1999).

- **deposition in a nutrient-rich environment** – ant nests may differ from adjacent soils both chemically and physically. Some studies have shown that ant nests are particularly rich in organic matter and in several nutrients essential to plant development and growth (Beattie & Culver, 1977, 1983; Whitford *et al.*, 2008).

On the other hand, the main advantage of myrmecochory for ant colonies stems from the nutritional value of elaiosomes. Once inside the nest, the elaiosomes are removed from the seed and are preferably used for feeding the larvae (e.g. Fischer *et al.*, 2005). The few studies where the benefits of myrmecochory to ants were assessed, reported:

- a shift in the investment on sex ratios towards females (Morales & Heithaus, 1998; Bono & Heithaus, 2002);
- an increase in production and weight of ant larvae (Gammans *et al.*, 2005);
- an increase in the number of worker pupae (Fokuhl *et al.*, 2007).

In some plant species, myrmecochory is combined with other mechanisms of seed dispersal. The combination of two-phase seed dispersal systems is named diplochory and is a common feature in various plant communities. In diplochorous systems, myrmecochory usually represents the second phase of dispersal, whereas seeds may be initially dispersed by wind (anemochory), vertebrates (endozoochory) or following the explosive dehiscence of fruits (ballistics). More complex seed dispersal systems involving myrmecochory are also known, but are extremely rare (Clifford & Monteith, 1989; Bas *et al.*, 2009). Diplochory has evolved in many plant families probably due to the advantages in combining two means of seed dispersal. It is acknowledged that phase one of the dispersal process allows seeds to escape from density-dependent mortality near the parent plant, eventually resulting in colonization at distance, while phase two usually leads to the location of specific microsites, where seed germination and seedling establishment are higher (Vander Wall & Longland, 2004).

The ants involved in myrmecochory are carnivorous or omnivorous species that generally prey upon terrestrial invertebrates (Gómez & Espadaler, 1995). These ants are attracted by the elaiosomes due to their overall chemical affinities with ant prey and also by sharing the same chemical cues that elicit removal behaviour in ants (e.g. Hughes *et al.*, 1994). They carry the seeds to their nests, but discard them unharmed soon after removing the elaiosome. There are however other ant species that carry seeds to their nests with the purpose of preying them (granivorous species). These ants, named harvester ants, collect a large number of seeds to their nests and usually destroy almost all of them (e.g. Detrain & Tasse, 2000). A number of works has highlighted the impact of harvester ants as post-dispersal seed predators and their role in shaping plant communities composition and structure (Brown & Human, 1997; MacMahon *et al.*, 2000). Furthermore, several authors have found evidence of the negative impact of harvester ants on plant recruitment, particularly in several endangered species (Duarte & Alves, 1987; White & Robertson, 2009). Nevertheless harvester ants may also contribute to seed dispersal since some foragers occasionally lose some seeds on their way back to the nest or reject some viable seeds to the refuse piles, where they may germinate (Wolff & Debussche, 1999; Detrain & Tasse, 2000; Retana *et al.*, 2004).

Harvester ants together with birds, rodents and beetles are major post-dispersal seed predators in most terrestrial ecosystems (Hulme & Benkman, 2002). Recent reviews of studies where both pre- and post-dispersal seed predation have been assessed, reported higher proportional losses due to the latter cause of seed mortality (Hulme, 2002; Hulme & Benkman, 2002). Most post-dispersal seed predators are generalist species that feed upon a spatially heterogeneous resource. Their effects on plant demography and evolution may be quite significant (see Hulme, 1998): post-dispersal seed predators may play a key role in plant regeneration, plant colonization and in determining the spatial distribution of plants, as well as in shaping seed traits and phenological strategies of seed production.

There are however specific situations where the role of post-dispersal seed predators on the dynamics of plant population may be less relevant, like for example, the cases of plants that regenerate by vegetative means, plants with a persistent seed bank and plants with microsite-limited regeneration (Hulme, 1998).

The effective conservation of plant populations depends to a great extent on the knowledge we have concerning the biology of the target species. For many plant species, information on seed fate, from fertilization to seed germination, has proved to be extremely valuable for the design of more effective conservation measures (Bernardello *et al*., 1999; Kaye, 1999; Colas *et al*., 2001; Navarro & Guitián, 2002; Burgos *et al*., 2008). This finding has led to an increase in the number of studies dealing with seed fate in threatened species and many authors now defend that these kind of studies should be "an integral feature of all conservation projects" (see Moza & Bhatnagar, 2007).

1.4 Study species and study areas

The study species are included in the genus *Euphorbia* Linnaeus, a diverse genus with a considerable number of endemics in the Iberian Peninsula. Iberian *Euphorbia* show a large variation in morphology, ecological requirements, and plant growth form, from small widespread annual species to long-lived narrow endemic perennial shrubs (Benedí *et al*., 1997). This genus was selected as a study group due to the existence of relevant information on the reproductive biology and dispersal ecology of some species (e.g. Baiges *et al*., 1992; Traveset, 1995; Espadaler & Gómez, 1996). The previous works of Xavier Espadaler and Crisanto Gómez on myrmecochory of Iberian *Euphorbia* (e.g. Gómez & Espadaler, 1994, 1997; Espadaler & Gómez, 1996; 1997) provided the stimulus to this choice and also for the development of the present work.

Three study species – *Euphorbia pedroi* Molero & Rovira, *E. characias* Linnaeus and *E. welwitschii* (Boissduval & Reuter) - were selected taking in consideration the availability of biological information concerning one of the species (*E. characias*) and the need to collect baseline information on the biology of the two Portuguese endemic species (*E. pedroi* and *E. welwitschii*). Furthermore, their affinities in phenology and the possibility to find them in sympatry were also crucial for this choice.

Euphorbia pedroi Molero & Rovira is a narrow endemic species of western Portugal restricted to three disjunct populations along the coastline, between Cabo Espichel and Cabo Ares (Fig. 1). This species, together with *E. dendroides*, are the only two members of section *Balsamis* in Iberia. *Euphorbia pedroi* is included in the *E. lamarckii* complex showing affinities with Macaronesian dendroid spurges (Molero *et al*., 2002). Individuals of this species were first discovered by Gomes Pedro, who identified them as belonging to *E. obtusifolia* (Pedro, 1983). Later, Molero and Rovira (1997) made a detailed study of specimens from the three populations and described them as belonging to a new species, honouring the discoverer of these plant populations by naming it *E. pedroi*.

Euphorbia pedroi is a caducifolious sub-succulent xerophyte that can attain about 2 m in height and more than 2 m width. There is however some spatial variability on the morphology of this species, which has led to the description of two non-co-occurring distinct forms (Pedro, 1983). Fruits are sub-spherical (4-5x6-7mm) and glabrous.

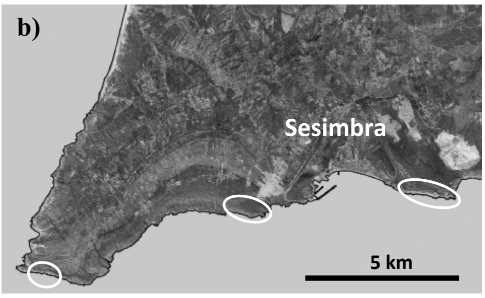

Figure 1 – A full growing adult plant of *E. pedroi* (a) and location of the three populations of this study species (b).

Seeds are subovoid, somewhat compressed laterally, reticulate grey and provided with an elaiosome. *E. pedroi* is restricted to the slopes of south-facing rocky cliffs where is subjected to regular mist, strong winds and to high levels of insolation throughout the year. *E. pedroi* makes part of a unique plant community, including several other rare or narrow endemic plant species (e.g. *Convolvulus fernandesii*, *Lavatera maritima*, *Withania frutescens*) and benefits from legal protection since its populations are included in the Arrábida Natural Park.

Euphorbia welwitschii (Boissduval & Reuter) is an herbaceous perennial endemic to western Portugal. This species is restricted to some scattered populations between Santarém and Lagoa de Melides, with most of them being located in the vicinities of Lisbon (Fig. 2). Some of the historical populations of this species failed to be located during the last decade and probably went extinct due to habitat destruction. There are, however, a number of extant populations of *E. welwitschii* that can be found within the Portuguese network of protected areas.

E. welwitschii is included in the subgenus *Esula* (section *Helioscopia*). It is a calcicolous species that occurs in Mediterranean-type ecosystems and constantly presents a small population size. The individuals of this species may attain 90cm in height and have a variable number of floral stems (1-35, usually less than 20). Fruits are spherical (4,0-4,5mm), glabrous and provided with tubercules. Seeds are shinning dark brown, spherical and have an elaiosome.

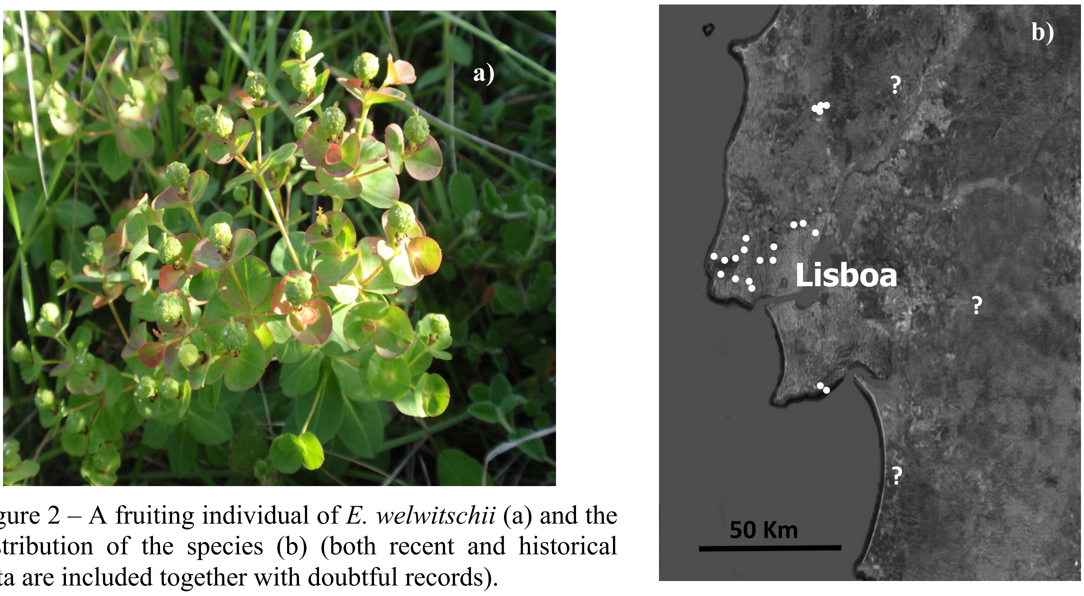

Figure 2 – A fruiting individual of *E. welwitschii* (a) and the distribution of the species (b) (both recent and historical data are included together with doubtful records).

Following a revision of the *E. squamigera* species complex, Vicens *et al.* (1996) proposed the inclusion of *E. welwitschii* as a subspecies of *E. paniculata*. However, in our opinion, taking in consideration the characteristic morphology and peculiar ecology of this taxon, *E. welwitschii* should still be considered a distinct species until scientific-based evidence resulting from comparative biology and molecular studies supports the decision of changing its taxonomical status.

Euphorbia characias Linnaeus is a woody perennial species with a wide distribution along the Mediterranean Basin, from Portugal to Greece, and has also been reported from several North African countries (Benedí *et al*., 1997) (Fig 3). In the Iberian Peninsula, *E. characias* is relatively widespread (e.g. Franco, 1971; Benedí *et al*., 1997), being commonly found in open and sunny habitats, where it usually occurs in large populations.

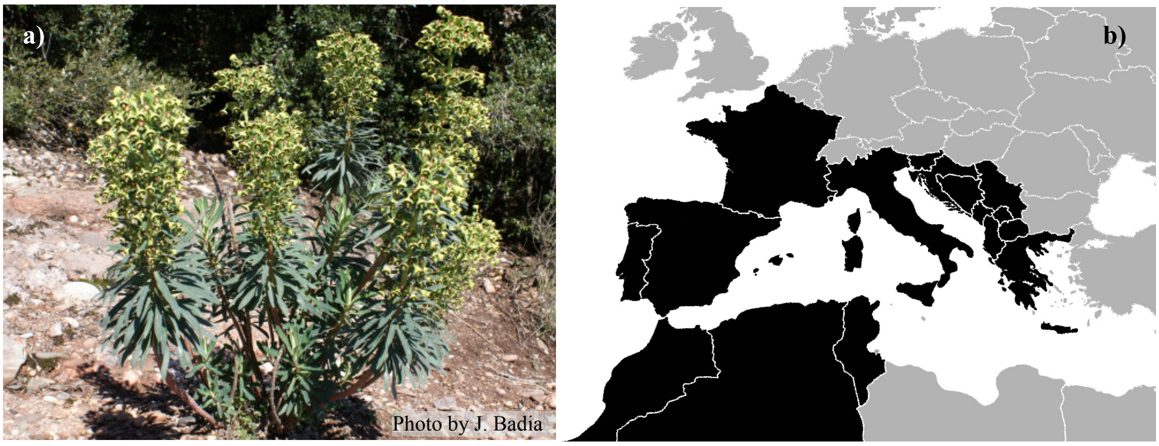

Figure 3 – A flowering individual of *E. characias* (a) and the distribution of the species (b).

E. characias is included in the subgenus *Esula* (section *Esula*). The individuals have a variable size (usually between 50-100cm) and a variable number of floral stems (1-35, but usually less than 10). Fruits are spherical (4-5mm) and pubescent. Seeds are light grey, ellipsoidal and provided with an elaiosome.

The study took place in five areas located in western Portugal, between Serra de Montejunto and Serra da Arrábida (Fig. 4). The selected sites (and their coordinates) were:

1 - **Montejunto**: at Serra de Montejunto, near the Environmental Interpretation Center (UTM: 29SMD9436; altitude 510 m)

2 - **Tesoureira**: near the village of Tesoureira (UTM: 29SMD8709; altitude 240 m)

3 - **Azóia**: near Cova da Mijona, Azóia (UTM: 29SMC8753; altitude 140 m)

4 - **Ares**: near Cabo Ares, Sesimbra (UTM: 29SMC9354; altitude 190 m)

5 - **Risco**: about 4km from Cabo Ares, near Serra da Arrábida (UTM: 29SMC9757; altitude 175 m)

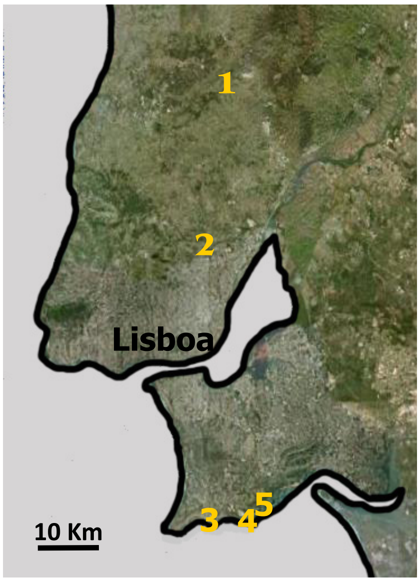

Figure 4 – Location of the study areas in Portugal (see also detailed indication of site location above).

The sites were selected taking in consideration the ease to access and perform the necessary observations and experiments and the possibility of having the co-occurrence of two of our study species. In Ares and Azóia, we can find both *E. pedroi* and *E. characias*. These sites are located in the coastline and both are included in the Arrábida Natural Park. At Risco, there is a population of *E. welwitschii*, which was selected due to its proximity to Ares. This site is also within the Arrábida Natural Park. Tesoureira and Montejunto were selected since there it is possible to find sympatric populations of *E. characias* and *E. welwitschii*. The site within Serra de Montejunto benefits from protection by being included in the Serra de Montejunto Protected Landscape, while the one located at Tesoureira lacks legal protection. All study areas are characterized by a Mediterranean-type climate and the vegetation is dominated by sclerophyllous species, particularly *Quercus coccifera*, *Pistacia lentiscus*, *Rosmarinus officinalis*, *Daphne gnidium*, *Lavandula luisieri*, and several *Cistus* species.

1.5 Objectives

With this thesis I aim to understand several aspects related with the reproductive biology of three *Euphorbia* species, particularly the role of insect-plant interactions on the plant reproductive output. The objectives I aim to address with this thesis are the following:

- 1) identify the factors responsible for reproductive losses from anthesis to seed dispersal in the three *Euphorbia* species, assess their magnitude and evaluate the variation of their effects in space, time and between individuals and species;

- 2) examine the differential impact of specialist and generalist insect seed predators in the reproductive output of the three *Euphorbia* species and evaluate how their effects relate with variables indicative of plant size and fecundity;

-3) evaluate the role of the elaiosome in ant attraction and characterize the fatty acid composition of elaiosomes from the three *Euphorbia* species;

-4) identify the seed dispersal agents of each study *Euphorbia* and evaluate seed dispersal distance and short-term seed fate by different dispersal agents.

1.6 Thesis structure

The current thesis is organized in eight chapters. The first chapter is the General Introduction, where I present an overview of the several issues addressed in the following chapters and introduce the study species, the study areas and the main objectives of the thesis. The next six chapters correspond to six scientific papers which are either published (2), submitted (2) or in preparation (2). In Chapter 2, the reproductive biology of the narrow endemic *Euphorbia pedroi* is studied from anthesis to seed dispersal and the factors responsible for reproductive losses are identified. Particular emphasis is put in insect-plant interactions and in the variation on their effects in space, time and between individuals. Chapter 3 deals with the reproductive biology of two *Euphorbia*, the rare endemic *E. welwitschii* and its widespread congener *E. characias*. The magnitude and spatiotemporal variation in the effects of factors leading to reproductive losses are analyzed comparatively between the two species to evaluate how they relate with plant rarity. In Chapter 4, it is examined the differential impact of specialist and generalist insect seed predators in the reproductive output of *E. characias* and *E. welwitschii* during the pre-dispersal phase. It is also assessed how insect predation pressure is correlated with plant traits indicative of size and fecundity and how consistent is that association in space and time. In Chapter 5 the fatty acid composition of *Euphorbia characias* elaiosomes is characterized and information on its variation within and between plants and individuals is also provided. Chapter 6 presents the fatty acid profile of two endemic *Euphorbia* species - *E. pedroi* and *E. welwitschii*. In Chapter 7 we evaluate the role of the elaiosome in seed removal and we study seed dispersal by ants and losses to post-dispersal seed predators. We also evaluate seed dispersal distance and short-term seed fate by different dispersal agents. Finally, in Chapter 8, the main findings of this work are discussed and integrated in the light of current knowledge. Further, future directions of research are suggested as well as several conservation measures for the endemic *Euphorbia*.

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PART I

PLANT FECUNDITY AND PRE-DISPERSAL REPRODUCTIVE LOSSES

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Chapter 2

Seed production and pre-dispersal reproductive losses in the narrow endemic *Euphorbia pedroi* (Euphorbiaceae)

Boieiro, M., Rego, C., Serrano, A.R.M. & X. Espadaler (submitted) Seed production and pre-dispersal reproductive losses in the narrow endemic *Euphorbia pedroi* (Euphorbiaceae). *Plant Ecology*

Seed production and pre-dispersal reproductive losses in the narrow endemic *Euphorbia pedroi* (Euphorbiaceae)

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Abstract

Euphorbia pedroi is a narrow endemic species with only three known populations located in coastal areas of western Portugal. This study focused on the reproductive biology of this species from flowering to dispersal, during a three-year period, aiming to identify the factors causing decrease in seed production potential and to assess the spatio-temporal patterns of seed production at the individual and population levels.

The abortion of reproductive structures, particularly seeds, represented a major fraction of losses in the potential seed production of *E. pedroi* and was much higher in one (Azóia) of the two studied populations. Larvae of *Acroclita subsequana*, a moth species specific to the genus *Euphorbia,* destroyed a variable proportion of cyathia in a large number of plants from the two populations, regardless of their degree of isolation. Two kinds of pre-dispersal seed predators – generalist hemipterans and specialist seed-wasps – were responsible for temporally variable seed losses unrelated with variables indicative of plant size and fecundity, and showing no consistency at the individual level. Seed losses to generalist hemipterans were mainly due to *Cydnus aterrimus* and were much higher at Azóia. *Eurytoma fumipennis*, a specialist seed-wasp, inflicted higher losses to *E. pedroi* and their impact was intimately associated with the magnitude of yearly variation in seed production. This finding highlights the role of the inter-annual variation in seed production as a key feature in this plant-seed predator system. The effect of the two groups of seed predators on the reproductive output of *E. pedroi* was additive and those insects do not seem to exert an important selective pressure on the traits measured indicative of plant size and fecundity.

The proportion of intact seeds produced by *E. pedroi* differed between locations (being higher at Ares), but not between individuals within each population, highlighting the major contribution of larger plants to the seed pool. In future, efforts should be addressed to improve our knowledge on the population biology of this narrow endemic species.

Keywords: Andromonoecy · Arrábida · Fruit and seed abortion · Insect seed predation · Reproductive success · Xerophytes

Introduction

The conservation management of a particular plant species can be much facilitated with a better understanding of its reproductive biology. Reproduction is a critical stage in the life cycle of plants, particularly for those species where regeneration occurs exclusively through seeds. In this situation, plant population dynamics is closely linked to seed survival and the design and implementation of more effective conservation measures may benefit from a better knowledge on the factors that limit seed production.

During plant reproduction, a variety of abiotic and biotic factors interfere with the processes of fruit and seed production, leading to a reduction in the reproductive potential of individual plants. Among the abiotic factors, harsh atmospheric conditions have been frequently identified as a cause of reproductive losses in plants (e.g., Stephenson, 1981). For example, coastal plants are subjected to strong winds, regular frosts and salt spray which usually damage a number of flowers and fruits and may even preclude insect pollination (Ågren 1988; Rovira et al., 2004). The destruction of flowers, fruits and seeds by animals is also a common cause of plant reproductive losses, which in some cases may be very severe and even influence the dynamics and survival of plant populations (Crawley, 2000; McCall & Irwin, 2006; Kolb et al., 2007). Seed predators belong to a variety of animal groups, but many of them are inconspicuous insects that usually feed on one or a few closely related host plant species and have their life-cycle synchronized with the phenology of their hosts (Hulme, 2002). In some circumstances, the damage inflicted by some insect seed predators may be difficult to assess. This and other reasons have led several authors to urge caution and rigor when assessing seed predation levels (e.g., Janzen, 1971; Hulme, 2002). Furthermore, the discrimination of the impact inflicted by each seed predator is paramount since it allows the identification of patterns of interaction between predators and their host plants and may even enable some predictability on subsequent reproductive losses. Nevertheless, the dynamics of the interaction between seed predators and their host plants is still largely unexplored since many of the studies on seed predation are inaccurate on the identification of predators (e.g., a variety of species unrelated phylogenetically and ecologically is, for simplicity, grouped under the same name - insects, invertebrates, etc) precluding the identification of patterns of interaction.

Understanding the reproductive success of a plant species requires not only the identification of all factors limiting seed production, but also the quantification of their impact (Heithaus et al., 1982). Furthermore, the knowledge on the spatiotemporal variation in the magnitude of each mortality factor is also crucial to a better understanding of the dynamics of plant populations (Kolb et al., 2007).

In this study we investigate seed production and the causes of pre-dispersal reproductive losses in the narrow endemic *Euphorbia pedroi*. More specifically, we: (1) identify the factors responsible for reproductive losses from anthesis to seed dispersal and (2) study the spatiotemporal variation in their magnitude. We also (3) assess how the variation in the effect of each mortality factor relates with variables indicative of plant size and fecundity. Furthermore, we (4) examine the differential impact of specialist and generalist seed predators in the reproductive output of *E. pedroi* and the consistency of their attack at the individual level.

Materials and Methods

Study plant

Euphorbia pedroi Molero & Rovira is a narrow endemic species of western Iberia restricted to three disjunct populations along the coastline of Portugal, between Cabo Espichel and Cabo Ares (Fig. 1). There, this species is restricted to the slopes of south-facing rocky cliffs being subjected to regular mist, strong winds and to high levels of insolation throughout the year. *Euphorbia pedroi* is a caducifolious sub-succulent xerophyte that can attain about 2m in height and more than 2m width. This perennial andromonoecious species is included in the *E. lamarckii* complex showing affinities with Macaronesian dendroid spurges (Molero et al., 2002) and it is possible that this species has originated following colonization from Macaronesia (Carine et al., 2004).

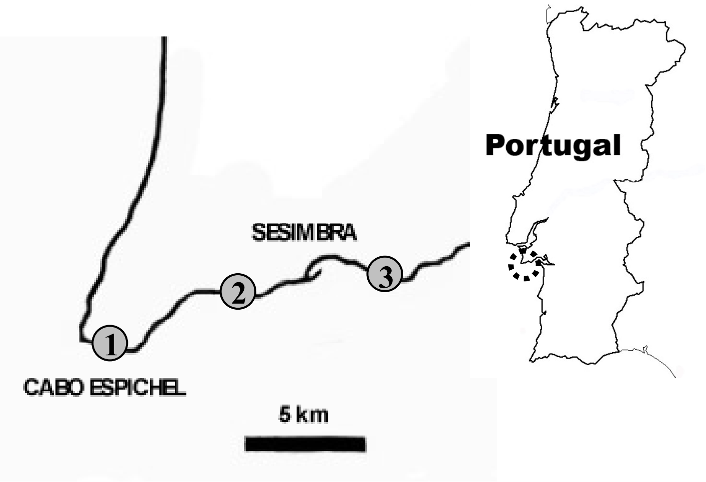

Figure 1. Map of the study area showing the location of *E. pedroi* populations (grey circles). 1 – Espichel; 2 – Azóia; 3 – Ares.

Annually, each adult plant produces a large number of cyathia, which are arranged in clusters at the top of loose pleiochasial synflorescences. Each cyathium comprises a single female flower with a three-lobed ovary surrounded by some male flowers, but some cyathia may contain solely staminate flowers (male cyathia). During the flowering period a variety of insects (wasps, bees, flies, ants and beetles) visit the inflorescences in search of nectar or pollen and unwittingly they may be engaged in pollen transfer between cyathia, benefiting the plants this way (M. Boieiro, unpublished data). Other insects, however, have a negative impact on the reproductive success of *E. pedroi*. The larvae of *Acroclita subsequana* (Herrich-Schäffer) (Tortricidae), a moth species specific to the genus *Euphorbia*, develop inside the buds and, as they grow, they join the adjacent cyathia together with the surrounding leaves, usually leading to the destruction of the whole cluster of cyathia. During fruit maturation, seed-wasps – *Eurytoma fumipennis* Walker (Eurytomidae) – are regularly observed ovipositing in full-sized fruits. This seed-wasp species is specific to a few *Euphorbia* species (Graham, 1984; Zerova & Seryogina, 1994) and depends upon their seeds to complete its life cycle. Seed-wasp larvae grow and develop inside the seeds feeding on their contents and there remain throughout the winter until spring, when the adult emerges and starts seeking for mates. Three species of hemipterans – *Cydnus aterrimus* (Forster) (Cydnidae), *Spilostethus pandurus* (Scopoli) (Lygaeidae) and *Dolycoris baccarum* (Linnaeus) (Pentatomidae) – have also been found puncturing the mature fruits of *E. pedroi*. These bugs are generalist species that feed upon different structures in a variety of plant species, but usually prefer seeds. They puncture the fruits and inject saliva into the seed leaving it irreversibly damaged. Seed dispersal in *Euphorbia pedroi* involves two different mechanisms of dispersal. In a first instance, seeds are projected ballistically some meters away from the parent plant, getting individually scattered on the soil surface. Then, several ant species may collect the seeds and transport them to their nests. Depending on the identity of the dispersal agent, seeds may then be preyed or discarded unharmed in nest galleries or in the refuse pile at the nest entrance, where germination may occur.

Study sites

This study took place in two of the three known populations of this species – Azóia and Ares (Fig. 1). We decided not to do fieldwork at Cabo Espichel due to the difficulties to access to the steep cliffs where these plants occur. The study areas are located about 6 km apart and the habitats present similar characteristics. At both sites we found cliffs and steep rockwalls, where *E. pedroi* plants grow on rocky crevices, and less inclined slopes or even balconies, where soil pockets harbour a much larger diversity of plant species, including *E. pedroi*. The vegetation is dominated by *Pistacia lentiscus*, *Quercus coccifera*, *Rhamnus oleoides* and *Juniperus turbinata*, with some *Olea europaea* interspersed (especially in Ares). The habitat-type where our study plant occurs is discontinuous along a short course of the coastline, it is extremely localised and presents unique characteristics from the biological and geological point of view. These peculiar habitats are also equivalent to ecological islands to some other rare or narrow endemic plant species (e.g., *Convolvulus fernandesii*, *Lavatera maritima*, *Withania frutescens*). For this reason, the authorities responsible for nature conservation in Portugal recognized the outstanding natural value of these areas and have included them in the Natura 2000 network (Sítio Arrábida/Espichel PTCON0010) and, more recently, in the Portuguese Reserve System – Parque Natural da Arrábida. According to the zonation defined in the management plan of this protected area, the populations of *E. pedroi* are included in "areas of partial protection – type I", where only human activities compatible with the safeguard of natural values are allowed. This legislation, together with other conservation measures that were put in practice following a LIFE project on Portuguese critically threatened plant species (ICN, 2007), are a guarantee for the safeguard of these unique habitats.

Plant fecundity and the production of fruits

In 2002, at the beginning of the flowering period, we tagged 25 randomly chosen individual plants of *Euphorbia pedroi* in each population. During the flowering and fruiting periods we visited regularly the study areas to record the total number of cyathia produced, the number of cyathia that lacked ovary, the number of ovaries and fruits aborted and the number of cyathia damaged by moth larvae in all marked plants. This procedure was repeated on the same plants in the following years (2003 and 2004). Occasionally, at Ares, we had some difficulties in assessing plant fecundity and the cyathia condition in several individuals of larger dimensions. In this case, we simultaneously sampled a fixed number of cyathia clusters (at least 80) and counted the whole number of cyathia clusters of the selected plant. Then, we extrapolated this result to the whole individual by multiplying the two variables. Male cyathia were easily distinguished from the hermaphrodite ones. In male cyathia the pistillate flower was generally absent, although a vestigial non-functional pistillate flower was also occasionally found. The data on male cyathia levels were analyzed as a form of reduction of the potential seed production, but taking in consideration that these results translate the reproductive allocation strategy of plants we discuss these findings separately. Individual seed production was estimated by multiplying the number of intact fruits by 3, the usual number of seeds enclosed in each trilocular capsule (Berg, 1990; Traveset, 1995). For each individual plant, we recorded plant height, crown width and stem diameter (at 10cm from the trunk base) as attributes indicative of plant size, together with the distance to the nearest conspecific, in order to evaluate if there was an influence of these variables on seed production and pre-dispersal seed predation levels.

Seed production and losses to seed predators

Mature fruit collection from the selected individuals was regularly performed during the fruiting season with an increased sampling effort during the peak of fructification (in May-June). The fruits were brought to the lab, and seeds were obtained by either dissecting the fruits or by exposing them to sunlight, which led to xerochastic dehiscence. A random sample of 100 seeds/individual was then dissected under a stereomicroscope to determine which were preyed, aborted or intact. This stage posed no difficulties since seeds present conspicuous non-overlapping features which are indicative of their condition. Aborted seeds were readily recognized due to their whitish or yellowish colour, light weight, wizened appearance and the presence of an undeveloped elaiosome while the distinction between preyed and intact seeds was only possible after inspecting the seed content. Seeds preyed by generalist hemipterans were left empty. These bugs inject saliva into the seed, where extra-oral digestion takes place, and then they suck back the resulting liquids leaving the seed deprived of its contents. On the other hand, the presence of a larva inside the seed was indicative of seed-wasp predation by *E. fumipennis*. Seed-wasps oviposit in the seeds, where their larvae grow and develop at the expense of the nutritive tissue within the seed locule. In intact seeds the seed locule is filled with an unharmed embryo surrounded by white endosperm and the seed coat shows no signs of puncturing by seed predators.

Data analysis

The effects of population and year on cyathia, fruit and seed production, fruit set, abortion levels and losses to insects were analyzed using repeated-measures ANOVA, with year as the within subject (repeated) factor. The effects of the interaction between factors (population and year) on the studied variables were also assessed, but only the significant results are presented. We tested the association between variables indicative of plant size (height, crown width and stem diameter), plant fecundity (cyathia and fruit production), plant isolation and insect predation levels by Spearman Rank correlation analysis. The association analyses were performed separately for each population and study year. Interpopulation comparisons on traits indicative of plant size were made by means of Student's *t*-tests. The temporal dynamics of seed predation by generalist and specialist seed predators was examined by plotting together the average annual seed production (only considering non-aborted seeds) and the average seed predation levels by the two insect groups. All analyses were performed using Statistica (StatSoft, 2008).

Results

Spatiotemporal variation in cyathia and fruit production

There was temporal variation in cyathia ($F_{2,96} = 58.81$; $p < 0.0001$) and fruit ($F_{2,96} = 30.49$; $p < 0.0001$) production in the two study populations, with the average number of cyathia produced in Ares showing higher fluctuations during the study period than it was recorded by the same time at Azóia (Table 1). Nevertheless, no significant differences were found in cyathia and fruit production between the two study populations (both $p > 0.05$).

Table 1. Cyathia and fruit production per plant and proportion of losses due to the different factors that reduced fruit set in the study populations of *Euphorbia pedroi* from 2002 to 2004. The number of individuals sampled was 25. Results are presented as mean±S.D..

Location	Variable	2002	2003	2004
Ares	N of cyathia	628.1±408.9	266.8±142.0	447.8±226.6
	% Male cyathia	11.4±2.7	17.1±2.4	13.5±2.9
	% Ovary and fruit abortion	5.9±2.7	6.8±2.6	9.5±4.9
	% Cyathia infested by larvae	9.6±6.1	10.9±6.6	7.3±7.0
	N of fruits	471.6±337.0	179.3±103.2	319.8±173.9
Azóia	N of cyathia	542.4±304.9	459.9±272.2	335.3±201.3
	% Male cyathia	14.5±2.4	13.9±2.4	16.3±2.8
	% Ovary and fruit abortion	17.0±9.4	11.6±7.6	12.8±7.2
	% Cyathia infested by larvae	14.1±8.9	10.3±10.4	17.0±10.4
	N of fruits	321.6±219.5	312.9±218.3	202.0±149.0

In both study populations, the individuals with a larger crown diameter produced a high number of cyathia and a high number of fruits (all $r > 0.72$ and $p < 0.001$). The other variables indicative of plant size (plant height and trunk diameter) were less consistently related with cyathia and fruit production. Plant size differed among the two populations with the individuals from Ares being taller (mean±S.D.: 137.2±37.1 *vs.* 98.2±22.7 cm; $t = 4.86$, $p < 0.0001$) and having a larger crown diameter (mean±S.D.: 162.2±38.4 *vs.* 133.6±33.4 cm; $t = 2.35$, $p < 0.05$) than those in Azóia. During the three-year period, there was a strong consistency in cyathia production at the individual level (all $r > 0.84$; $p < 0.0001$), meaning that, in general, the most and less productive plants were the same throughout the years.

The proportion of cyathia that set fruit was higher in Ares than at Azóia ($F_{1,48} = 18.16$; $p < 0.0001$), but no significant variation was observed during the three-year study in each population ($F_{2,96} = 0.89$; $p > 0.05$). In general, fruit-set was not correlated with any of the variables indicative of plant size ($|r| < 30.0$; $p > 0.05$) and there was no consistency of fruit set at the individual level (all $p > 0.05$, except a significant result for the years 2002 and 2004 in Azóia).

Magnitude and variability in losses due to the factors that affect fruit-set

The factors responsible for reduction in fruit-set were the lack of a functional pistillate flower in the cyathia, inflorescence destruction by moth larvae and the abortion of ovaries and fruits (Table 1). Together, these factors accounted for considerable reproductive losses and their combined effect showed high variation between individuals (Ares, mean±S.D.: 30.7±9.6 %; range = 13.6-67.2 %; Azóia, mean±S.D.: 42.5±16.8 %; range = 18.9-88.4 %).

Male cyathia levels were similar between the two study areas ($F_{1,48} = 1.79$; $p > 0.05$), but showed significant temporal variations throughout the study ($F_{2,96} = 34.03$; $p < 0.0001$). The absolute number of male cyathia was positively correlated with the number of cyathia produced (all $r > 0.92$; $p < 0.0001$) and with the variables indicative of plant size, particularly crown diameter (where all $r > 0.71$; $p < 0.001$).

The destruction of cyathia by moth larvae was evident during the study. A large proportion of plants in the two populations was attacked by moth larvae (Ares, mean±S.D.: 88.0±4.0 % infested plants; Azóia, mean±S.D.: 96.0±6.9 % infested plants) and the average proportion of cyathia destroyed per individual was moderate (Table 1). The losses due to moth larvae showed significant differences between the study areas ($F_{1,48} = 8.15$; $p < 0.01$), but not between years ($F_{2,96} = 0.53$; $p > 0.05$). The relative number of cyathia damaged by moth larvae was not correlated with any of the variables indicative of plant size ($|r| < 0.24$; $p > 0.05$), not even with the distance between individual plants (all $|r| < 0.35$; $p > 0.05$). Cyathia production also did not influence infestation levels by moth larvae, with a single exception recorded at Azóia in 2004 ($r = -0.59$; $p < 0.01$). No consistency was found in the proportion of cyathia damaged by moth larvae at the individual level in the two study populations (all $r < 0.30$ and $p > 0.05$).

The proportion of aborted ovaries and fruits differed among the populations of *E. pedroi* ($F_{1,48} = 25.71$; $p < 0.0001$), being lower in Ares and higher at Azóia. Among-year variation in the proportion of aborted ovaries and fruits was not significant ($F_{2,96} = 2.34$; $p > 0.05$). Occasionally, ovary and fruit abortion levels were found to be negatively correlated with the number of cyathia produced and with plant size (both plant height and crown diameter), but this pattern failed to be consistent throughout the study. Annual variation in losses due to ovary and fruit abortion were also not consistent at the individual level (all $r < 0.32$ and $p > 0.05$) with a single exception recorded at Azóia for the years 2002 and 2004 (where $r = 0.64$; $p < 0.01$).

Seed production, abortion and the impact of specialist and generalist insect predators

The proportion of viable, aborted and preyed seeds is shown in Table 2. Heavy losses due to seed abortion were recorded in the two study populations and there were also significant spatial ($F_{1,48} = 259.52$; $p < 0.0001$) and temporal variations ($F_{2,96} = 11.38$; $p < 0.0001$) in seed abortion levels. No associations were found between seed abortion levels and any of the variables indicative of plant size or fecundity (all $p > 0.05$). Seed predation levels were relatively high and variable throughout the study. Two groups of insect seed predators were responsible for seed losses: specialist seed-wasps and generalist hemipterans.

Table 2. Proportion of intact, aborted and preyed seeds in the study populations of *Euphorbia pedroi* from 2002 to 2004. The number of plants sampled was 25. Results are presented as mean±S.D.

Location	Variable	2002	2003	2004
Ares	% Seed predation	18.2±12.0	49.2±10.0	33.2±11.3
	% Seed abortion	31.7±4.6	34.8±5.4	29.9±4.5
	% Viable seeds	50.2±13.4	16.0±8.3	36.9±11.0
Azóia	% Seed predation	28.3±11.4	22.9±10.7	36.6±9.1
	% Seed abortion	41.8±4.5	47.7±5.1	51.0±7.0
	% Viable seeds	30.0±12.9	29.4±11.4	12.4±10.2

The two insect groups had a different impact on the reproductive output of *E. pedroi* and they also showed distinct temporal patterns of interaction with this host species. On average, hemipterans attacked a lower proportion of individuals than did seed-wasps (mean±S.D.: 76.0±18.4 *vs.* 99.3±1.6 %) and they also inflicted minor losses to the attacked plants (Fig. 2). There were spatial ($F_{1,48} = 89.37$; $p < 0.0001$) and temporal ($F_{2,96} = 4.68$; $p < 0.05$) variations in the losses due to hemipterans. The damage inflicted by hemipterans was not related with plant traits related to size or fecundity (all $p > 0.05$) and only once their effect was negatively correlated with the losses inflicted by seed-wasps (in Azóia during 2004, $r = -0.54$; $p < 0.01$). Seed-wasps inflicted heavy damage to *E. pedroi* plants in both populations. Seed predation levels by seed-wasps showed a wide temporal variation ($F_{2,96} = 34.82$; $p < 0.0001$), particularly in Ares (Fig. 2). Higher losses to seed-wasps were recorded during years of low seed production while lower losses occurred in high productive years. Thus, the impact of the specialist seed-wasps seems to be intimately associated with the seed production patterns of *E. pedroi* (Fig. 2). The losses inflicted by seed-wasps were not related with variables indicative of plant size, fecundity or isolation (all $p > 0.05$). In both populations, during 2002, seed predation levels by seed-wasps were negatively correlated with the number of cyathia damaged by moth larvae (both $p < 0.05$), but this trend was not detected in the two following years. We found no consistency of seed predation levels on individual plants throughout the study for both specialist and generalist seed predators (all $p > 0.05$).

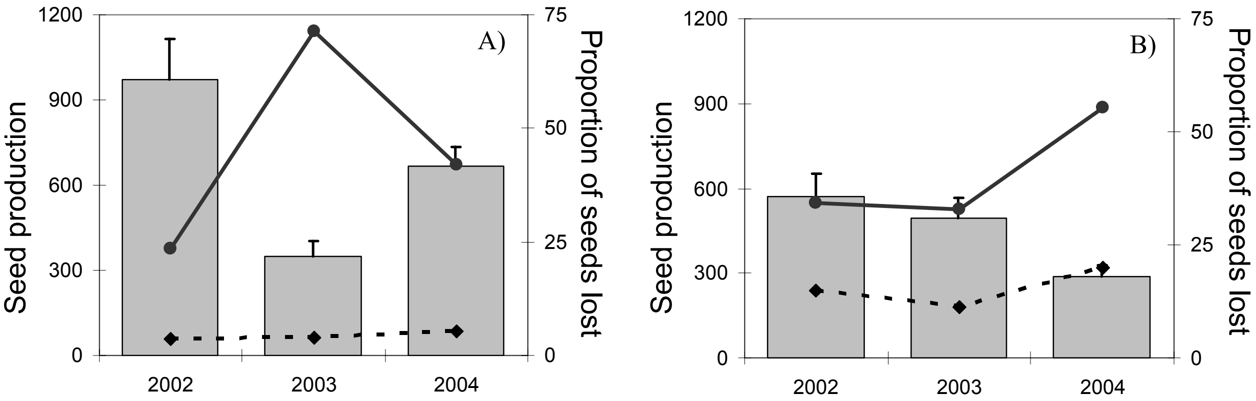

Figure 2. Dynamics of seed production and pre-dispersal seed predation by seed-wasps (solid line) and hemipterans (dashed line) in the study populations of *E. pedroi* from 2002–2004. Annual average (+ SE) seed production per plant (bars) is plotted together with the mean proportion of losses due to the two different kinds of seed predators. A) Ares; B) Azóia.

The average number (mean±S.D.) of intact seeds produced by individual during the study was 399.0±455.5 at Ares and 228.3±251.9 at Azóia. Intact seed production levels showed wide temporal fluctuations throughout the study ($F_{2,96} = 39.03$; $p < 0.0001$) and were unrelated with the traits measured (all $p > 0.05$). No consistency in intact seed production levels was detected among the study individuals.

Discussion

The effective conservation of plant populations depends to a great extent on the knowledge we have concerning the biology of the target species. For many plant species, information on the reproductive biology has proved to be extremely valuable for the design of more effective conservation measures (Bernardello et al., 1999; Colas et al., 2001; Navarro & Guitián 2002; Burgos et al., 2008). This has led several authors to defend that reproductive biology studies should be “an integral feature of all conservation projects” (see Moza & Bhatnagar, 2007).

Euphorbia pedroi, despite being a narrow endemic with only three known populations that have been recently included in the Portuguese protected areas system, is still a poorly studied species. During this study we found that cyathia production in *E. pedroi* was variable throughout the 3-year period and showed no significant differences between Ares and Azóia, despite the differences on traits indicative of plant size recorded among the two populations. However, within each population, cyathia production was positively related with crown diameter and showed a strong consistency at the individual level, with the most and least productive plants being, in general, the same during the study period. Many other studies have also found a high positive correlation between flower production and traits indicative of plant size (e.g., De Steven 1981; Traveset, 1995; McIntosh 2002), leading several authors to consider that this association appears to hold universally among plants (e.g., Herrera, 1991 and references therein).

Euphorbia pedroi individuals produce both hermaphrodite and male cyathia, being the latter restricted to the lowest level of the inflorescence. Male cyathia levels were relatively constant with annual averages usually around 15%. These values differ from the ones reported to other Iberian perennial *Euphorbia* (Narbona et al., 2002; Boieiro et al., 2010a), probably due to the fact that those species are phylogenetically unrelated with *E. pedroi* and have a striking different morphology. Male cyathia were also recorded on the related *E. dendroides* (Traveset, 1995), but since their proportion was indicated combined with the proportion of reproductive losses due to ovary abortion no comparisons can be made. Even so, from the joint values reported to one population during 1993 and 1994, we realize that male cyathia levels in *E. dendroides* may achieve low values, being less than 3-5% of total cyathia (Traveset, 1995). The prevalence of male cyathia in perennial *Euphorbia* has been interpreted as a mechanism to promote outcrossing in long-lived plants by enhancing pollen receipt of pistillate flowers (Narbona et al., 2002, 2005). In these species, protogyny (together with pollen transfer probabilities) may have favored variation in sex allocation leading to a higher presence of male cyathia in early-blooming flowers (Brunet & Charlesworth, 1995; Narbona et al., 2005), which occur on the lowest levels of the inflorescence.

The destruction of cyathia by *Acroclita subsequana* larvae affected most of the individuals in both populations with higher reproductive losses being reported in Azóia. Individual variation on the magnitude of reproductive losses was unrelated with the traits measured indicative of plant size and fecundity, suggesting that this moth species may use other cues during host selection. Similar findings were reported from two other *Euphorbia* species where *A. subsequana* larvae inflicted disparate reproductive losses, but, in general, no association between damage levels and plant traits indicative of size and fecundity was found (Traveset, 1995; Boieiro et al. 2010a). Moths use a variety of sensory cues during the stages of host finding and acceptance, including information on plant morphology and fecundity, but plant chemistry is probably the most important source of information to elicit oviposition (Renwick & Chew, 1994). This finding is supported by a number of experimental studies made on other tortricid moth species, mostly of economic importance, where the key role played by a variety of plant volatiles in host finding and oviposition eliciting has been highlighted (e.g., Suckling et al., 1996; Yan et al., 1999; Hern & Dorn, 2004; Masante-Roca et al., 2007). Moths may have also used chemical cues to evaluate plant infestation levels by conspecifics, as it seems to be suggested by the high proportion of plants moderately infested in the two populations. Distance between plants did not seem to affect the degree of infestation by this moth, given that even more isolated plants suffered infestation levels similar to those recorded in individuals at the population core. This is probably due to the fact that moths can easily travel between plants separated by tenths of meters in search of a suitable host for oviposition.

Euphorbia pedroi was also susceptible to reproductive losses that resulted in the abortion of reproductive structures. Ovary and fruit abortion was higher at Azóia and in both populations the individuals were affected inconsistently throughout the study period. The harsh environmental conditions found in these coastal populations were responsible for some of the reproductive losses that resulted in ovary and fruit abortion. The strong winds, frosts and regular salt spray during the flowering and fruiting periods damaged both ovaries and early fruits in some individuals leading to the abortion of these reproductive structures. Furthermore, the unfavorable atmospheric conditions may have also played an indirect role in ovary abortion by precluding insect pollination during the period of stigma receptivity. Flying and non-flying insects are important pollinators of *E. pedroi* (M. Boieiro, unpublished data) and the activity of both groups is strongly influenced by weather conditions. Other studies dealing with the reproductive biology of coastal plants have shown a decrease in pollen transfer by insects as a consequence of unfavorable weather (e.g., Kevan & Baker, 1983; Rovira et al., 2004). A number of aborted ovaries also corresponded to late flowering cyathia which have failed to develop probably due to resource limitation. By the time of the development of these ovaries, most of the fruits were maturing and a few others had already dehisced. So, maternal resources are probably very limited at this stage and are mostly channeled to maturing fruits. The temporal decline in fruit set has been documented in a large number of species and experimental work has provided evidence that this is mostly due to competition of flowers and fruits for limited maternal resources (e.g., Stephenson, 1981; Lee, 1988; and references therein). Many studies have shown that fruits produce growth inhibitors and/or growth stimulating phytohormones allowing them to use the resources that otherwise would be made available to the development of late-flowers, thus leading to high levels of late-flower abortion (see Lee, 1988).

Seed abortion was a major form of reproductive loss in the two populations, with mortality levels averaging from a third to half the number of the seeds produced. The significantly higher reproductive losses due to seed abortion recorded in Azóia comparatively to Ares were also found in a co-occurring congener (Boieiro et al., 2010a) and may be due to a lower availability of resources in this area. In Azóia, the individuals of *E. pedroi* are shorter than their conspecifics at Ares and grow predominantly on rocky crevices or inclined coastal slopes, where the availability of nutrients is usually low. Nevertheless, specific experimental work is needed to clearly assess the causes of high seed abortion in the two populations of *E. pedroi*.

Two groups of pre-dispersal seed predators (specialist seed-wasps and generalist hemipterans) were responsible for a major fraction of the reproductive losses recorded in *E. pedroi.* The losses due to generalist seed predators were mainly due to *Cydnus aterrimus*, the most common of the three hemipteran species detected in the two study populations. Mostly adults, but also nymphs, of this bug species were found puncturing the fruits of *E. pedroi* to feed upon the intact seeds enclosed inside. Traveset (1995) clearly misinterpreted the feeding biology of *C. aterrimus* by considering that this species punctured the fruits of *E. dendroides* to feed exclusively upon the seed-wasp larvae developing inside infested seeds. *C. aterrimus* is a true insect seed predator that feeds upon the seeds of a variety of plant species, but also on other plant material (Stichel, 1955/1962; Schorr, 1957; W. Rabitsch, pers. comm.). The seed losses inflicted by hemipterans were not related with variables indicative of plant size and fecundity. This may be due to the fact that these generalist species use a combination of visual and chemical cues to locate their hosts, but after contact with the plant surface, mechanoreception, gustation and olfaction are the main senses used to decide upon feeding (Bernays & Chapman, 1994). In two other *Euphorbia* species, reproductive losses due to hemipterans were also found to be unrelated with attributes related to plant size and fecundity (Boieiro et al., 2010b). The generalist feeding habits of many hemipteran species together with the aggregation behaviour observed in some of those species hinders the prediction of the impact of these insects on the reproductive output of their hosts.

Seed-wasps were responsible for high and variable seed losses in the two populations of *E. pedroi*. The damage caused by seed-wasps to some individuals was extremely high (above 85%), but no consistency of seed predation levels among individuals was found during the study. The variation in seed predation levels by seed-wasps among individuals could not be attributed to any of the traits measured indicative of plant size or fecundity. This finding was also reported in other studies on seed-wasp predation in *Euphorbia* (Traveset, 1995; Boieiro *et al.*, 2010b) and probably reflects the use of other cues by seed-wasps during host-plant selection. Kouloussis and Katsoyannos (1994) found that *Eurytoma amygdali* females were attracted by active compounds present in the surface of its host and the same components also stimulated oviposition. Furthermore, these authors discovered that *E. amygdali* females produce an oviposition-marking pheromone following egg-laying to avert additional ovipositions by conspecifics, thus avoiding superparasitation of the host (Kouloussis & Katsoyannos

(1991, 1993). Fruits marked with the pheromone can be perceived by seed-wasp females even before landing (Kouloussis & Katsoyannos, 1991), usually leading to fruit rejection. Infochemicals may also play a key role on host-plant selection in our study system. In fact, *E. fumipennis* seed-wasp females were regularly observed dragging the tip of the abdomen on the fruits of *E. pedroi* following oviposition, a characteristic behaviour of pheromone host-marking (Kouloussis & Katsoyannos, 1991, 1993). The predominant use of chemical senses by seed-wasps to select their hosts may then explain the lack of association between the measured variables indicative of plant size and fecundity and the individual variation in seed predation levels. The temporal variation in seed predation levels due to seed-wasps was high, particularly at Ares, and was intimately associated with the magnitude of yearly variation in seed production in *E. pedroi*. Seed-wasps are specialist seed predators that depend upon the availability of intact seeds to complete their life-cycle and consequently their population dynamics is mostly determined by the availability of resources for oviposition and larvae development during the previous season. For this reason, seed-wasps show a delay in tracking the variation in their food resources leading to the observed mismatch between seed predation levels and seed production patterns. According to several authors (e.g., De Steven, 1983; Solbreck & Sillén-Tulberg, 1986) the inter-annual variation in seed production is considered a key feature in these plant-seed predator systems since it allows the regulation of seed predators´ populations providing cyclical opportunities of seed escape from predation. Some other studies have reported similar patterns of interaction between specialist seed predators and their host plants (Sperens, 1997; Poncet et al., 2009 and references therein), including some recent findings on the genus *Euphorbia* (Boieiro et al., 2010b), and there is also some evidence that an equivalent system can be found in *E. dendroides* (Traveset, 1995), a close relative of *E. pedroi*.

Although the studied insect-plant interactions have contributed to a considerable reduction in seed-set, the damage inflicted by each insect group does not seem to be associated with each other. The effect of both groups of pre-dispersal seed predators on the reproductive success of *E. pedroi* was, in general, additive and the proportion of seed losses inflicted by those two groups of insects was also unrelated with the damage due to moth larvae. These findings suggest that the two groups of seed predators do not seem to discriminate between plants with different levels of reproductive losses due to insects and/or probably use different plant traits to select their hosts for feeding or oviposition. Furthermore, the lack of association between the proportion of reproductive losses to insects (to each insect group and overall) and the measured plant traits suggest that these insects do not exert an important selective pressure on these traits.

By the time xerochastic dehiscence occurs, the proportion of intact seeds dispersed is just a fraction of the potential seed production (24% at Ares and less than 14% at Azóia). The total percentage loss of reproductive potential did not differ among larger and smaller plants, meaning that, due to their large crop size, larger individuals contribute with a much higher number of seeds to the seed pool.

Seed production is crucial not just only for providing propagules to the replacement of individuals, but also for the colonization of new sites. Some of the areas occupied by *E. pedroi* are prone to rock slides which usually lead to the mortality of some individuals. Nevertheless, these events are important for the population dynamics of *E. pedroi* since they provide an opportunity for the establishment of individuals in areas free of competitors. Although *E. pedroi* is not in immediate danger of extinction, it is confined to just three populations making it very vulnerable to habitat destruction. Efforts should be addressed to devise a conservation plan for this species, including the collection of basic information on the biology of the species (e.g., demography, population dynamics and the identification of the limiting life history stages and the factors affecting them), the regular monitoring of populations and the exploration of suitable habitats in the areas near the known populations. Furthermore, simple and efficient measures, like seed deposition on gene banks, *ex-situ* study and conservation of individuals in botanical gardens and the *in-situ* experimental seed plantation into clefts of rocks and cleared areas (free of competitors), should also be put in practice.

Acknowledgements

We dedicate this paper to the botanist José Gomes Pedro for the discovery of interesting plant taxa, including *Euphorbia pedroi*, but also for his long-lasting commitment to the study and safeguard of the flora of Arrábida. We thank J. Boieiro for his valuable help in the field. M. Zerova, W. Rabitsch, and E. Marabuto helped with the identification of insect species and discussed with us several aspects on seed predator biology. M.J. Pinto provided valuable help on the biology and distribution of *Euphorbia pedroi*. Support was provided by Fundação para a Ciência e Tecnologia through grants PRAXIS XXI/BD/21407/99 to MB and SFRH/BPD/66934/2009 to CR.

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Chapter 3

Plant fecundity and pre-dispersal reproductive losses in a common and a rare *Euphorbia* species (Euphorbiaceae)

Boieiro, M., Serrano, A.R.M., Rego, C. & X. Espadaler (2010) Plant fecundity and pre-dispersal reproductive losses in a common and a rare *Euphorbia* species (Euphorbiaceae). *Ecological Research* 25: 447-456
DOI: :10.1007/s11284-009-0674-6

Ecol Res (2010) 25: 447–456
DOI 10.1007/s11284-009-0674-6

ORIGINAL ARTICLE

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Plant fecundity and pre-dispersal reproductive losses in a common and a rare *Euphorbia* species (Euphorbiaceae)

Received: 18 September 2009 / Accepted: 6 November 2009 / Published online: 5 January 2010

Abstract Comparative studies on the reproductive biology of closely related plant species have provided valuable information to understand the causes and consequences of common-rare differences with possible applications for the management of threatened populations. The magnitude and spatiotemporal variability of seed production and pre-dispersal reproductive losses were studied for 3 years in the rare endemic *Euphorbia welwitschii* and in its widespread congener *E. characias*. The factors responsible for a decrease in potential seed production in these species were the lack of a functional ovary in the cyathium, ovary and fruit abortion, seed predation by insects and seed abortion. In *E. characias*, the moth *Acroclita subsequana* was also responsible for minor reproductive losses. The proportion of male cyathia varied significantly between seasons, populations and species, being consistently higher in *E. characias* than in *E. welwitschii*. Reproductive losses that resulted in ovary, fruit and seed abortion affected mostly the endemic species and were heavier in the populations located near the sea due to local adverse climatic conditions. Seed predators inflicted higher reproductive losses to the endemic species than to its widespread congener and their impact was particularly heavy at Risco. The two *Euphorbia* species differed markedly in cyathia production, with *E. welwitschii* producing systematically a lower number of cyathia than its widespread congener and this, together with higher levels of ovary, fruit and seed abortion, seemed to be the main reasons for the low reproductive output of this rare species.

Keywords Andromonoecy · Fruit abortion · Plant rarity · Reproductive success · Seed predation

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Introduction

Reproduction is a critical stage in the life cycle of plants. During this period, plants experience a variety of interactions with other plants and animals and are subjected to particular environmental conditions that determine that only a fraction of the ovules produced will develop into viable seeds. Numerous studies have emphasized how plant–animal interactions may greatly affect the reproductive success of a plant species (e.g., Herrera and Pellmyr 2002). Adverse climatic conditions during the flowering and fruiting periods may also damage significantly the reproductive organs of plants, leading to a lower production of seeds (Ågren 1988; Hampe 2005). When several types of interactions influence the reproductive output of a plant species it is important to assess their relative contribution before assigning importance to any of them. The factors affecting the reproductive output of a plant species have the selective potential to shape phenological characteristics and to favor particular plant traits (Wright and Meagher 2003; Strauss and Whittall 2006). These factors may act as important selective forces by discriminating between phenotypes, thus promoting variation in seed set at the individual level. However, the magnitude of the reproductive losses caused by each mortality factor usually varies between individuals, populations, and years (Ågren 1988; Traveset 1995; Simon and Hay 2003). So, only by studying how consistent in time and space the effect of a particular mortality factor is in promoting variation in plant reproductive success can we understand its role on the evolution of plant traits and more clearly evaluate its overall effect on plant reproduction and plant population dynamics.

In this study we investigate seed production and the causes of pre-dispersal reproductive losses in two *Euphorbia* species, a common and a rare species. *Euphorbia welwitschii* (Boiss. & Reut.), an endemic species of western Portugal, is restricted to scattered populations near Lisbon and its biology is still poorly known. On the other hand, *Euphorbia characias* (Linnaeus) is a relatively well studied common species (Espadaler and Gómez 1996, 2001) that can be found in sympatry with *E. welwitschii*. The use of comparisons within groups of co-occurring close relatives has for long been recognized as a powerful method for studying patterns and processes in evolutionary ecology. This procedure is now being increasingly applied since the analysis of plant reproductive success in groups of common–rare species may lead to a clearer understanding of the underlying causes of rarity, eventually providing valuable information for the management of endangered species (Simon and Hay 2003; Scott and Gross 2004; Lavergne et al. 2005). Here, we (1) identify the factors responsible for reproductive losses from anthesis to seed dispersal in *Euphorbia characias* and *E. welwitschii* and (2) examine the spatiotemporal variation in the magnitude of the reproductive losses caused by each of those mortality factors. We also (3) assess how the variation in the effect of mortality factors is related with plant size and reproductive plant traits and evaluate the consistency of that association in space and time.

Materials and methods

Plant species and study sites

Iberian *Euphorbia* are diverse and show a large variation in morphology, ecological requirements, and plant growth form, from small widespread annual species to long-lived narrow endemic perennial shrubs (Benedí et al. 1997). The two study plants were selected due to their affinities in phylogeny, phenology, and the possibility to find them in sympatry. Both *Euphorbia* are calcicolous perennials that occur in Mediterranean-type ecosystems and can be found in sympatry in some areas of western Portugal. Although these species are not direct sister species, they are both included in the subgenus *Esula*. *Euphorbia welwitschii* is an herbaceous perennial endemic to western Portugal. This species is restricted to scattered populations around Lisbon (Franco 1971) and constantly presents a small population size. During the last century, *E. welwitschii* suffered a considerable decline as a result of habitat destruction, which is still considered the major threat to its existence. *Euphorbia characias* is a woody perennial species with a wide distribution along the Mediterranean Basin, from Portugal to Greece, and has also been reported from several North African countries (Benedí et al. 1997). In the Iberian Peninsula, *E. characias* is relatively widespread, being commonly found in open and sunny habitats, where it usually occurs in large populations. Both species are andromonoecious, produce a variable number of floral stems, and flower between February and early May. In each floral stem there is a variable number of cyathia arranged in pleiochasia or dichasia. Each cyathium has a single female flower with a three-lobed ovary surrounded by some male flowers, but male cyathia are relatively common in the lowest levels of the floral stem (Narbona et al. 2002). In male cyathia, the pistillate flower is generally absent or is vestigial and non-functional while the staminate flowers may remain unaffected or the male function may be altered (Narbona et al. 2005). During the flowering period, a variety of insects visit the inflorescences in search of nectar/pollen and may simultaneously participate in pollen transfer within and between plants. Cryptic larvae of the microlepidopteran *Acroclita subsequana* (Herrich-Schäffer) (Tortricidae) can also be observed within the complex inflorescence architecture of *Euphorbia characias*. These larvae can negatively affect fruit production because they join adjacent cyathia together, precluding the development of the ovaries. In case the ovaries have already developed, the damage inflicted by moth larvae will usually lead to their abortion. In both study species, the fruit is a trilocular green capsule enclosing one smooth carunculate seed per loculum. The fruits of the two species are similar in size, but differ in pubescence: *E. welwitschii* fruits are glabrous while the ones from *E. characias* are pubescent. During fruit maturation, which takes place in May–June, insect seed predators (hemipterans and seed-wasps) are regularly observed puncturing or ovipositing in full-sized fruits. Specialist seed-wasps—*Eurytoma jaltica* Zerova (Eurytomidae)—oviposit singly in seeds, where their larvae develop feeding on the nutritious seed content. The generalist hemipterans—*Cydnus aterrimus* (Forster) (Cydnidae) and *Dicranocephalus agilis* (Scopoli) (Stenocephalidae)—puncture the fruits to inject saliva inside the seeds, and then they suck the resulting nutritive fluids, leaving the seed empty. Both study plants are diplochorous species. Seeds are first dispersed ballistically to a distance several meters away from the parent plant. Then, secondary seed dispersal is promoted by a variety of ant species that carry the seeds to their nests (Espadaler and Gómez 1996; Gómez and Espadaler 1998) where they may germinate. The study was carried out from 2002 to 2004 at five sites in western Portugal. In two of these sites, Montejunto (UTM: 29SMD9436; altitude 510 m) and Tesoureira (UTM: 29SMD8709; altitude 240 m), both plants co-occur. The populations of Montejunto are located on the northern border of the distribution area of *E. welwitschii* on the north-facing slope of a limestone mountain. These populations are included in the Paisagem Protegida da Serra de Montejunto protected area. At Tesoureira, the study populations are situated in a plateau slightly facing south. Despite the lack of legal protection of this study area, no human disturbance was detected during our study. Two other populations of *Euphorbia characias* were selected in coastal areas of the Península de Setúbal (Azóia—UTM: 29SMC8753; altitude 140 m and Ares—UTM: 29SMC9354; altitude 190 m) together with the nearest known population of the rare *E. welwitschii* (at Risco—UTM: 29SMC9757; altitude 175 m), which is located about 4 km from Ares. Due to their proximity to the sea, these populations are regularly subjected to salty winds and harsh atmospheric conditions. These three study areas are included in the Arrábida Natural Park. All study areas are characterized by a Mediterranean-type climate and the vegetation is dominated by sclerophyllous species, particularly *Quercus coccifera*, *Pistacia lentiscus*, *Rosmarinus officinalis*, *Daphne gnidium*, *Lavandula luisieri*, and several *Cistus* species. During 2004 we were unable to collect data at Montejunto because a severe fire profoundly affected our study site.

Cyathia and fruit production

Each year, at the beginning of the flowering period, we randomly selected and marked 25 individual plants of *E. welwitschii* in each study area. In Risco, where this species is very rare, only 20 plants were monitored in 2002. During the flowering and fruiting periods, we regularly visited the study areas at 5 to 15-day intervals to record the number of hermaphrodite and male cyathia, the number of ovaries and fruits aborted, the total number of mature fruits, and the total number of floral stems per plant. Then, by the end of the fruiting period, we counted the total number of mature fruits present in each selected individual. A similar methodology was adopted in the study of fruit production in *E. characias*, but due to its larger size, a single floral stem was sampled per individual. In each population, 25 individual plants of *Euphorbia characias* were randomly selected and the same variables indicated for *E. welwitschii* were monitored together with the damage inflicted by moth larvae throughout the flowering and fruiting periods. Following a previous study on the reproductive biology of *Euphorbia* (Traveset 1995), we analyzed the data on the proportion of male cyathia as a form of reduction of the potential seed production. However, we are aware that andromonoecy levels are the result of a reproductive strategy allocation and for this reason we comment these findings in a separate section in the discussion. For both *Euphorbia* species we measured individual plant height and the distance to the nearest reproductive conspecific to assess if these factors affected seed production and pre-dispersal reproductive losses.

Seed production, abortion, and predation by insects

During the fruiting season we collected the mature fruits from each marked plant of both study species prior to dehiscence. Once in the laboratory, the fruits were opened and a random sample of 60 seeds/plant was carefully dissected to distinguish between predated, aborted, and intact seeds. Aborted seeds are easily recognized due to their whitish or yellowish color, light weight, wizened appearance, and the presence of an undeveloped elaiosome. Seed predation was evaluated by examining the seed content. Both the presence of a larva inside the seed or the finding of a seed depleted of its contents is indicative of insect predation. Intact seeds were recognized by verifying the presence of an embryo surrounded by white endosperm within the seed locule.

Statistical analysis

Spatial and temporal variations for the studied variables were analyzed through analysis of variance. Proportions were arcsine square-root transformed and absolute values were $\log_{10}$-transformed prior to analysis in order to improve normality and meet the criterion of equal variances within groups. Since we were unable to collect data at Montejunto during 2004, for each studied variable we assessed the significance of the interaction terms (year × location) by performing a pair of two-way ANOVA on subsets of the data (Underwood 1997). Spatial variations in plant fecundity and in the relative contribution of factors affecting reproductive potential of *Euphorbia* were assessed through one-way ANOVA followed by multiple comparison tests (Tukey HSD post hoc test) to evaluate which treatments were significantly different. For both *Euphorbia* species we tested the association between variables indicative of plant size and reproductive plant traits, and causes of reproductive failure by Spearman Rank correlation analysis. Interspecific comparisons were performed by means of Student's *t*-tests. All analyses were performed using Statistica (StatSoft Inc. 2008).

Results

Spatiotemporal variation in cyathia and fruit production

The average number of cyathia and fruits produced per plant are shown in Tables 1 and 2, respectively, to *Euphorbia characias* and *E. welwitschii*. Both study species exhibited spatiotemporal variation in cyathia and fruit production, but *Euphorbia characias* was always more fecund and produced a larger number of fruits per plant (Tables 1, 2).

In *Euphorbia characias*, the production of fruits varied considerably among the study populations ($F_{3,271} = 11.77$; $p < 0.0001$) and within populations during the 3-year study ($F_{2,272} = 8.60$; $p < 0.001$). The differences in fruit production were also unequal during the sampling period in the study areas as testified by a significant

Table 1 Cyathia and fruit production per plant and proportion of the different factors that reduced fruit set in the study populations of *Euphorbia characias* from 2002 to 2004

Location	Variable	2002	2003	2004
Ares	*N* of cyathia	1142.6 ± 967.1	516.6 ± 438.6	1084.8 ± 1209.5
	Male cyathia (%)	19.0 ± 3.2	24.1 ± 6.8	20.6 ± 4.2
	Ovary and fruit abortion (%)	18.2 ± 7.2	17.9 ± 5.1	22.9 ± 8.5
	Cyathia infested by larvae (%)	0.5 ± 1.2	1.5 ± 3.2	0.4 ± 1.0
	N of fruits	739.4 ± 640.8	308.9 ± 306.1	653.1 ± 772.2
Azóia	*N* of cyathia	988.7 ± 674.6	773.6 ± 535.5	371.4 ± 234.7
	Male cyathia (%)	18.2 ± 3.7	21.0 ± 5.0	21.8 ± 3.9
	Ovary and fruit abortion (%)	34.5 ± 8.9	26.7 ± 8.2	30.3 ± 9.7
	Cyathia infested by larvae (%)	0.2 ± 0.6	0.4 ± 1.2	0.1 ± 0.5
	N of fruits	458.5 ± 325.4	401.4 ± 283.4	184.4 ± 139.0
Montejunto	*N* of cyathia	1197.2 ± 872.0	946.0 ± 575.6	–
	Male cyathia (%)	17.3 ± 4.1	20.9 ± 4.5	–
	Ovary and fruit abortion (%)	10.6 ± 4.0	14.4 ± 4.0	–
	Cyathia infested by larvae (%)	0.1 ± 0.4	0.3 ± 0.8	–
	N of fruits	895.5 ± 672.4	631.6 ± 412.5	–
Tesoureira	*N* of cyathia	502.5 ± 485.5	724.7 ± 359.4	350.5 ± 183.7
	Male cyathia (%)	25.4 ± 5.9	19.6 ± 3.2	27.6 ± 4.3
	Ovary and fruit abortion (%)	16.9 ± 5.2	21.4 ± 9.4	23.6 ± 7.5
	Cyathia infested by larvae (%)	1.2 ± 3.6	0.1 ± 0.6	1.6 ± 3.5
	N of fruits	308.9 ± 323.7	437.7 ± 257.4	171.0 ± 121.4

The number of individuals sampled was 25. Number of cyathia and fruits per plant were estimated by multiplying the data collected in a single floral stem by the number of floral stems per plant. Results are presented as means ± SD

Table 2 Cyathia and fruit production per plant and proportion of the different factors that reduced fruit set in the study populations of *Euphorbia welwitschii* from 2002 to 2004

Location	Variable	2002	2003	2004
Montejunto	*N* of cyathia	224.2 ± 124.1	185.0 ± 102.2	–
	Male cyathia (%)	4.4 ± 1.6	3.7 ± 2.0	–
	Ovary and fruit abortion (%)	24.0 ± 5.9	27.7 ± 6.9	–
	N of fruits	164.0 ± 95.0	123.9 ± 65.3	–
Risco	*N* of cyathia	105.1 ± 93.6	120.2 ± 102.7	77.4 ± 65.2
	Male cyathia (%)	5.3 ± 5.8	14.7 ± 20.3	4.3 ± 5.6
	Ovary and fruit abortion (%)	52.1 ± 19.8	69.4 ± 18.9	56.3 ± 24.2
	N of fruits	52.9 ± 56.2	23.9 ± 27.6	40.8 ± 44.0
Tesoureira	*N* of cyathia	100.7 ± 81.6	157.2 ± 120.0	86.6 ± 90.3
	Male cyathia (%)	6.8 ± 5.6	7.7 ± 4.1	10.0 ± 7.7
	Ovary and fruit abortion (%)	27.4 ± 18.1	20.7 ± 13.2	32.6 ± 14.1
	N of fruits	71.1 ± 59.4	117.7 ± 88.4	54.7 ± 59.7

The number of plants sampled was 25, except for the data collected at Risco during 2002 (where $n = 20$). Results are presented as means ± SD

interaction between the two factors ($F_{4,216} = 8.00$; $p < 0.0001$). A multiple comparison test indicated that the significant difference in fruit production among populations was mainly due to the highest productivity at Montejunto (Tukey's test, $p < 0.05$). Fruit production per plant was significantly correlated with the number of cyathia per plant ($r = 79.8$; $p < 0.001$) and with variables indicative of plant morphology, namely plant size ($r = 43.0$; $p < 0.001$) and the number of floral stems per plant ($r = 74.5$; $p < 0.001$). Plant size differed among populations ($F_{3,271} = 42.75$; $p = 0.0001$), being larger in Montejunto than in the other study areas (Tukey's test, $p < 0.05$). No significant differences were found in the number of floral stems among populations ($F_{3,271} = 2.53$; $p = 0.06$).

There was also a large variation in fruit production among populations of *Euphorbia welwitschii* ($F_{2,192} = 37.24$; $p < 0.0001$) and within populations during the 3-year study ($F_{2,192} = 5.41$; $p < 0.01$). The interaction between location and year was also significant ($p < 0.01$). The wide spatial variation in fruit production was due to considerable differences in productivity between the three study areas (Tukey's test, $p < 0.05$) with the individuals from Montejunto consistently producing a larger mean number of fruits while those at Risco always produced the lowest. Fruit production was significantly correlated with the number of cyathia ($r = 72.0$; $p < 0.001$) as well as with the number of floral stems per plant ($r = 74.6$; $p < 0.001$), but not with plant size ($r = 2.1$; $p > 0.05$). There was a significant difference between populations in plant size ($F_{2,192} = 5.79$; $p < 0.01$) and in the number of floral stems per plant ($F_{2,192} = 7.85$; $p < 0.001$).

The two species showed a different strategy of resource allocation to reproduction since *E. characias* produced taller inflorescences (mean ± SD: 71.6 ± 19.8 vs. 37.2 ± 14.8) while *E. welwitschii* produced a higher number of floral stems per individual (mean ± SD: 9.2 ± 7.1 vs. 3.6 ± 2.6).

Magnitude and variability in losses due to factors that affect fruit-set

The main factors that reduced fruit set in *Euphorbia characias* were the lack of a functional pistillate flower in the cyathia and ovary/fruit abortion (Table 1). Together, these factors accounted for losses that, on average, almost reached half of a plant's reproductive potential (mean ± SD: 43.0 ± 11.3%; range: 20.6–72.9%). There were considerable spatial and temporal variations in the proportion of male cyathia in the study populations (respectively $F_{3,271} = 20.09$; $p < 0.0001$ and $F_{2,272} = 10.77$; $p < 0.0001$) and the effect of location × year interaction was highly significant ($F_{4,216} = 13.16$; $p < 0.0001$). During the study period, the population at Tesoureira differed considerably from the other populations by having the highest proportion of male cyathia (Tukey's test, $p < 0.05$). The absolute number of male cyathia was positively correlated with the number of cyathia produced ($r = 87.2$; $p < 0.0001$) while the relative number showed an opposite trend ($r = -52.2$; $p < 0.0001$). These distinct patterns are the result of an architectural effect since male cyathia are strictly associated to the lower levels of the inflorescence and virtually absent from the more productive higher levels. No significant correlations were found between the proportion of male cyathia with plant height and with the number of floral stems per plant. Ovary and fruit abortion varied significantly among populations ($F_{3,271} = 78.18$; $p < 0.0001$) and years ($F_{2,272} = 11.54$; $p < 0.0001$). The location × year interaction was also statistically significant ($F_{4,216} = 4.76$; $p < 0.0001$). The proportion of aborted ovaries and fruits differed among populations, being lower in Montejunto and higher at Azóia (Tukey's test, $p < 0.05$). Despite circumstantial correlations, no consistent association was found between the levels of ovary/fruit abortion with variables indicative of plant size and fecundity. Inflorescence infestation by moth larvae of *Acroclita subsequana* accounted for minor losses in the reproductive potential of *E. characias* in all of the populations (Table 1). Only a low proportion of plants was attacked by larvae (mean ± SD: 13.5 ± 7.2% infested plants/population) and the proportion of cyathia destroyed per floral stem was also low (mean ± SD: 4.4 ± 3.6% cyathia; range: 1.0–14.0%). Our estimates on the proportion of attacked plants are conservative since they were based on the analysis of individual floral stems. Cyathia damage by moth larvae was not correlated with plant size ($r = 2.2$, $p > 0.05$) neither with the number of floral stems per plant ($r = 5.7$, $p > 0.05$), nor even with distance between plants ($r = -9.0$, $p > 0.05$). The proportion of moth-damaged cyathia was also not related with any of the plant attributes associated with plant fecundity (all $p > 0.05$).

Ovary and fruit abortion was the kind of mortality factor that most affected *E. welwitschii* (Table 2), accounting on average for losses of 38.4 ± 23.2% (mean ± SD) (range: 5.0–66.7%). The proportion of aborted ovaries and fruits differed considerably between study areas ($F_{2,192} = 75.96$; $p < 0.0001$) and was particularly high at Risco (Tukey's test, $p < 0.05$). Among-year variation in the proportion of aborted ovaries and fruits was marginally significant ($p < 0.05$). The number of aborted ovaries and fruits was correlated with the number of floral stems per plant ($r = 71.4$; $p < 0.001$) and with the number of cyathia produced ($r = 73.5$; $p < 0.001$). The proportion of male cyathia recorded in *E. welwitschii* was low, around 5%. Spatial and temporal variations in the proportion of male cyathia were significant (both $p < 0.05$) as well as the interaction location × year ($p < 0.01$). The relative number of male cyathia was not correlated with any variable indicative of plant size or fecundity. During this study no microlepidopteran larvae were found on *E. welwitschii* and we were also unable to detect signs of moth damage within the inflorescences. Interspecific comparisons showed that male cyathia and ovary and fruit abortion affected differently the two *Euphorbia* (all $p < 0.001$), both considering all populations and only sympatric populations.

Seed abortion, insect seed predation, and production of intact seeds

The proportion of viable, aborted, and preyed seeds is shown in Tables 3 and 4, respectively, to *Euphorbia characias* and *E. welwitschii*. Both *Euphorbia* species had a relatively large proportion of aborted seeds, but, on average, losses were higher for *E. welwitschii* ($t = -9.14$; $p < 0.0001$). Inter-plant variation in the levels of seed abortion was high for both plant species since some individuals had minor losses (3–5%) while others showed a considerable reduction in seed production due to seed abortion (up to 67%). The proportion of aborted seeds of the two *Euphorbia* species varied significantly between areas (in both cases $p < 0.0001$), but not among the 3 years (both $p > 0.05$). The most severe losses resulting in seed abortion were recorded at Azóia (for *E. characias*) and Risco (for *E. welwitschii*) and were consistently high during the study period. Seed abortion was not associated with traits related to plant size or fecundity (all $p > 0.05$), but significant positive correlations (Montejunto 2003 for *E. characias*; Montejunto 2002 and Tesoureira 2004 for *E. welwitschii*) were found with

Table 3 Proportion of intact, aborted, and preyed seeds in the study populations of *Euphorbia characias* from 2002 to 2004

Location	Variable	2002	2003	2004
Ares	Seed predation (%)	5.3 ± 4.9	12.3 ± 8.1	10.2 ± 7.3
	Seed abortion (%)	21.8 ± 4.5	23.1 ± 4.7	14.5 ± 5.0
	Viable seeds (%)	72.9 ± 5.9	64.6 ± 9.1	75.3 ± 8.8
Azóia	Seed predation (%)	3.1 ± 3.9	7.3 ± 5.3	13.5 ± 7.7
	Seed abortion (%)	29.1 ± 6.4	23.6 ± 4.5	29.7 ± 6.1
	Viable seeds (%)	67.7 ± 7.3	69.1 ± 7.4	56.8 ± 9.8
Montejunto	Seed predation (%)	19.0 ± 6.9	16.0 ± 9.0	–
	Seed abortion (%)	19.9 ± 5.0	16.0 ± 6.0	–
	Viable seeds (%)	61.1 ± 6.3	68.0 ± 7.8	–
Tesoureira	Seed predation (%)	14.1 ± 6.9	8.2 ± 4.6	25.8 ± 10.0
	Seed abortion (%)	19.6 ± 5.8	23.5 ± 6.6	16.9 ± 4.8
	Viable seeds (%)	66.3 ± 6.2	68.3 ± 8.8	57.4 ± 12.2

The number of plants sampled was 25. Results are presented as means ± SD

Table 4 Proportion of intact, aborted, and preyed seeds in the study populations of *Euphorbia welwitschii* from 2002 to 2004

Location	Variable	2002	2003	2004
Montejunto	Seed predation (%)	16.2 ± 8.4	21.5 ± 10.7	–
	Seed abortion (%)	23.3 ± 9.0	29.9 ± 6.6	–
	Viable seeds (%)	60.5 ± 14.7	48.6 ± 13.2	–
Risco	Seed predation (%)	34.4 ± 14.0 (20)	35.4 ± 12.6 (20)	29.2 ± 9.2 (23)
	Seed abortion (%)	41.2 ± 9.0 (20)	38.0 ± 9.7 (20)	31.4 ± 8.3 (23)
	Viable seeds (%)	24.4 ± 14.4 (20)	26.5 ± 7.9 (20)	39.4 ± 10.2 (23)
Tesoureira	Seed predation (%)	21.9 ± 11.0 (24)	11.0 ± 6.8	27.7 ± 9.6
	Seed abortion (%)	29.7 ± 7.1 (24)	24.0 ± 10.3	21.1 ± 10.4
	Viable seeds (%)	48.4 ± 14.0 (24)	65.0 ± 10.6	51.3 ± 16.0

The number of plants sampled was 25, except when given in parentheses (*n*). Results are presented as means ± SD

ovary and fruit abortion ($p < 0.05$), suggesting that the individuals most susceptible to ovary and fruit abortion also suffered higher losses by seed abortion. Nevertheless, this association was not consistent in space and time. Seed predation was responsible for the reduction in the reproductive potential of both *Euphorbia*, but the proportion of preyed seeds was higher in *Euphorbia welwitschii* than in *E. characias* ($t = -11.39$; $p < 0.0001$). However, if considering only sympatric populations, this difference is much less pronounced ($t = -2.34$; $p < 0.05$). Spatial and temporal variations in seed predation were highly significant for *E. characias* (both $p < 0.0001$) and the percentage of preyed seeds of *E. welwitschii* also varied considerably between the study areas ($p < 0.0001$). In general, the losses inflicted by pre-dispersal seed predators were unrelated with plant isolation and with morphological and reproductive plant traits for both study plants. A significant correlation between reproductive losses to seed predators and the proportion of aborted fruits and ovaries was however detected in *Euphorbia welwitschii* ($r = 46.9$; $p < 0.001$). It was also noticed that plants infested by moth larvae were not more susceptible to seed predator attack than undamaged plants. The proportion of intact seeds was high in *Euphorbia characias* (mean ± SD: 66.1 ± 9.9%), but there was a wide variability among plants (range: 32.2–86.7%). Spatial and temporal variations in the proportion of viable seeds were significant (both $p < 0.01$) as well as the location × year interaction ($F_{4,216} = 14.02$; $p < 0.0001$). *Euphorbia welwitschii* produced a lower proportion of intact seeds than its congener, and also presented a high among plant variation for this trait (mean ± SD: 46.6 ± 18.4%; range: 0–91.7%). Intact seed production varied among study sites ($p < 0.0001$), but not between years ($p > 0.05$) and the location × year interaction was highly significant ($F_{2,114} = 7.42$; $p < 0.001$). No association was found between intact seed production and variables indicative of plant fecundity or morphology, for either *Euphorbia* species.

Discussion

Magnitude and variation in cyathia production

The two study plant species differed markedly and consistently in the number of cyathia produced per individual due to their differences in plant size and plant growth form. Herbaceous perennial species, like *Euphorbia welwitschii*, have in general a lower capacity for storing resources than do woody perennials, and every year they face the need to allocate resources simultaneously for vegetative growth and reproduction from an overwintering subterranean stem. Instead, woody perennials, like *E. characias*, may concentrate a higher proportion of resources in reproduction at the expenses of stored reserves or through a more flexible strategy of resource allocation. In some woody perennials, the overwintering leaves may also act as a storage organ, particularly for carbohydrates, which can be rapidly allocated to reproduction in the following season (Jonasson 1995). Furthermore, in the open habitats where this study took place, early photosynthetic production by overwintering leaves may allow a larger investment in reproduction, conferring a competitive advantage to woody perennials over other co-occurring plants.

The temporal patterns of cyathia production were similar in the populations where both *Euphorbia* co-occur suggesting that local environmental factors (e.g., climatic conditions) govern plant fecundity. Furthermore, a constant large difference in cyathia production was observed between those two study areas for both *Euphorbia*, which can be, at least in part, assigned to differences in soil nutrient levels. At Montejunto most of the soil is covered with grass species, an indication of higher nutrient content (Heil and Diemont 1983). Thus, more nutrients are possibly available to allocate for reproduction in Montejunto comparatively to what happen in Tesoureira. Within each species there was also a high variation in cyathia production among individuals due to differences in plant morphology. In general, taller plants with a higher number of floral stems produced a larger number of cyathia whilst shorter plants were less fecund. Other studies have also reported significant positive correlations between traits related to plant morphology and flower production, emphasizing the major contribution of larger individuals to the overall seed-set within a population (e.g., McIntosh 2002). Nevertheless, seed production is not always positively associated with flower production since the overall effect of pre-dispersal hazards may affect individuals differently (Traveset 1995).

Reproductive strategies in the two *Euphorbia*

Sex expression in andromonoecious *Euphorbia* can be quite variable between individuals and populations (Narbona et al. 2002) and this was also reported in this study for both *E. characias* and *E. welwitschii*. This labile sex expression is probably the result of the spatiotemporal variability in the interplay between genetic and environmental factors that, under ontogenic contingencies, govern the patterns of reproductive allocation as it has been shown in other andromonoecious plant species (Diggle 1994). Despite some expected differences due to the spatiotemporal variability in sex expression, our results on the proportion of male cyathia for *Euphorbia characias* also differed from the ones previously reported (Narbona et al. 2002) because we analyzed the whole inflorescence and we recorded a higher number of inflorescence levels in our sample individuals. The number of inflorescence levels in some *Euphorbia* species is apparently a variable trait as it has recently been shown for *E. boetica* and *E. nicaeensis*, where variability was detected both at the individual and population levels (Al-Samman et al. 2005; Narbona et al. 2005). In their study on andromonoecy in *Euphorbia*, Narbona et al. (2002) found that the proportion of male cyathia varied according to plant life form, with perennial species producing a significantly higher proportion of male cyathia than annuals. The larger production of male cyathia by perennial *Euphorbia* species, like *E. characias*, was then interpreted as a mechanism to promote outcrossing in long-lived plants (Narbona et al. 2002 and references therein). However, these authors have also identified two exceptions to this pattern among the study species (*E. hirsuta* and *E. caecorum*). In this study we found a consistent difference in the proportion of male cyathia between the two species: *Euphorbia welwitschii*, an herbaceous perennial plant species, is weakly andromonoecious and always had a much lower proportion of male cyathia than its relative woody perennial species, *E. characias*. Since both *E. hirsuta* and *E. welwitschii* belong to the same section (Helioscopia) of the subgenus *Esula,* in future it would be convenient to evaluate the role of phylogenetic relatedness on male cyathia production. The evolution and maintenance of andromonoecy in the genus *Euphorbia* is probably the result of a strategy to enhance pollen receipt of pistillate flowers, thus improving seed production (Narbona et al. 2002). Similar findings have also been reported for other plant genera (Podolsky 1992; Elle and Meagher 2000). Nevertheless, alternative hypothesis have been presented to explain the prevalence of andromonoecy in plants suggesting that further experimental work is needed (for a review see Vallejo-Marín and Rauscher 2007).

Spatiotemporal variation on the impact of factors that constrain seed production

The spatiotemporal variability in the proportion of aborted ovaries and fruits found in this study can be partially explained by population-specific environmental conditions. The populations near the sea suffered significant reproductive losses due to their exposition to high winds carrying harsh salty air and the regular heavy mist. These atmospheric conditions imposed permanent physical damage to the floral parts, recognized by the brownish-grey tinge of ovaries and fruits, which lead to the abortion of these reproductive structures. Reproductive losses were, however, quite distinct in the two populations of *Euphorbia characias* at the seaside since the individuals at Ares benefited of protection from the adverse weather by particular topographic conditions and a more developed vegetation cover. In certain circumstances, plant cover may act as a natural barrier attenuating the effect of harsh weather and providing particular microenvironmental conditions suitable for the development of ovaries and fruits of ground layer plants (Ågren 1988). The heavy reproductive losses of *E. welwitschii* plants at Risco were also, in part, due to the effect of adverse climatic conditions since a fraction of the aborted ovaries and fruits exhibited signs of physical damage. Nevertheless part of the reproductive losses were aborted ovaries without signs of physical damage suggesting that other factors may be also limiting seed production. The low number of individuals of *E. welwitschii* at Risco, their geographical isolation from other suitable habitats and the low individual seed production recorded here suggest that this population is facing the negative effects of small population size. Many other studies, mostly dealing with rare endemic plants, have shown that small isolated populations are frequently prone to increased inbreeding, low pollination rates and to the loss of genetic variation, which often result in low individual seed production and eventually population decline (e.g., Severns 2003). In the study areas where the two *Euphorbia* species co-occur, it was found that the proportion of aborted ovaries and fruits was on average invariably larger for *E. welwitschii*. This finding may be due to reproductive interference (see Kuno 1992; Takakura et al. 2009) since the widespread *E. characias* presents a showier flowering display attracting a higher number and diversity of pollinators than its congener, thus having proportionately fewer losses due to lack of pollination (M. Boieiro, unpubl. data). Larger plants with showier floral traits generally benefit from a higher number of visits from pollinators and may indirectly decrease both visit quantity and quality of the other sympatric co-flowering plants (Williams et al. 2001). However, only by conducting specific experimental work in order to evaluate the roles of pollen and resource limitation in limiting seed production can we assess the causes of ovary abortion in these study plants. The array of pre-dispersal predators was identical for both study plants with the exception of *Acroclita subsequana*, a microlepidopteran not recorded in *E. welwitschii*. *A. subsequana* damaged a low proportion of cyathia in *E. characias* in all study populations. There was, however, some variation among plants within a population in the proportion of reproductive losses to larvae, but such variation was not associated with traits related with plant morphology or fecundity, neither with the distance to the nearest conspecific. Most interplant distances were small (usually less than 2.0 m) and, within a population, only a few plants were relatively isolated. Thus, the adult of *A. subsequana* may easily travel between different plants in search of a suitable host for oviposition. In other *Euphorbia* species, where this moth species inflicted high reproductive losses, the variation on infestation levels was also, in general, unrelated with morphological and reproductive traits (Traveset 1995). Insect seed predators imposed larger reproductive losses to both *Euphorbia* species during the pre-dispersal phase. In sympatry, both study plants suffered losses of the same magnitude, probably due to their identical fruiting phenology and affinities in fruit and seed characteristics. Fruits are similar in size and seeds have identical weight, size and toughness, thus both *Euphorbia* present similar conditions for insect landing, fruit and seed puncturing and larvae development. The two plants differ, however, in fruit pubescence, but, unlike to what was reported by Green and Palmbad (1975) from two *Astragalus* species, this aspect did not interfere with the attack of seed predators. In allopatry, *E. welwitschii* suffered proportionately larger losses to seed predators than its widespread congener. However, this finding is most likely due to differences in the abundance of insect seed predators between study areas, a key aspect on the dynamics of seed predation, like has recently been shown for another plant species (Honek and Martinkova 2005). The effect of moths and insect seed predators on the reproductive output of *Euphorbia characias* was additive. During oviposition and fruit puncturing, insect seed predators do not appear to discriminate between plants with different levels of inflorescence infestation by moths and consequently the losses they inflict are unrelated to moth damage. The lack of discrimination by seed predators may be due to the low impact that moths have on limiting their host plant's reproductive potential. However, other explanations may be possible since in *Euphorbia dendroides*, despite the high losses attributed to moths, the same kind of seed predators attacked plants irrespectively to their infestation levels (Traveset 1995). In general, the associations between reproductive losses due to insects and the measured plant traits related to size and fecundity in both *Euphorbia* were low and inconsistent throughout the study. This finding suggests that the selective pressures exerted by insects on the evolution of those traits may be rather weak, i.e. insufficient to cause changes in plant trait–fitness relationships.

Comparison of the cumulative effect of reproductive losses in two *Euphorbia* species

During the 3-year study the common *Euphorbia characias* produced, on average, about four times more hermaphrodite cyathia per plant than its endemic congener *E. welwitschii*. Comparative studies on the biology of congeneric co-occurring plant species have shown that rare species have, in general, lower flower production than their widespread relatives (Fiedler 1987; Murray et al. 2002). Recently, Lavergne et al. (2004) reinforced this idea after comparing various traits in 20 congeneric pairs of narrow endemic and widespread plant species from the Western Mediterranean. These authors argued that the low investment in reproduction by endemics may be an evolutionary consequence of population confinement and persistence in isolated habitats. This situation may also apply to *E. welwitschii* since both historical and current data on species distribution turn evident the isolation of most of its populations.

Overall, the cumulative proportional reduction in potential seed production was relatively high for both

Euphorbia species, but affected mostly the endemic *E. welwitschii*. The factors responsible for reproductive losses during the pre-dispersal phase showed a significant interspecific variation in their values and invariably were responsible for higher losses in the endemic species, which was particularly susceptible to reproductive losses that resulted in ovary, fruit and seed abortion. These differences between the two study plants reflect their different morphology and life form, but are also related with the consequences of rarity. Rare species characterized by several small populations, like *E. welwitschii*, suffer the effects of small-population size on plant reproduction, including pollinator limitation and inbreeding depression, which frequently lead to the abortion of reproductive structures. These effects of small population size on plant reproductive output have been reported to be the main cause of low seed set in a variety of rare species (Severns 2003; Leimu et al. 2006) and are currently an issue of major concern when driving conservation plans for critically endangered species. The array of pre-dispersal seed predators was identical for both *Euphorbia* and apparently there was no feeding preference for a particular plant species, despite a somewhat larger fraction of preyed seeds in *E. welwitschii*. Nevertheless, this low difference, but statistically significant, in proportional seed predation increased the already wide disparity in potential seed production between the rare and the common *Euphorbia*. According to several studies, the impact of seed predators may represent a mechanism that might help explain plant rarity (e.g., Hegazy and Eesa 1991), but the results of this study do not seem to support those findings. Instead, they confirm the opinion of other authors who defend that seed predation levels *per se* do not appear sufficient to represent a mechanism that might help to explain rarity (Madeira and Fernandes 1999; Simon and Hay 2003; Lavergne et al. 2004). Recently, it has been shown that even equal rates of seed predation in co-occurring congeners may lead to very different outcomes on plant population dynamics (Münzbergová 2005). So, only by performing studies encompassing the whole life cycle of study plants can we unambiguously assess the determinants of common rare differences (Münzbergová 2005). At the time of ballistic seed dispersal, the common *E. characias* produced a much higher number of intact seeds per individual than its congener *E. welwitschii*. This difference in seed production was not only due to the high differences in cyathia production between the two *Euphorbia*, but also to the higher levels of pre-dispersal reproductive losses affecting the endemic species.

Acknowledgements We thank J. Boieiro for his valuable help in the field, E. Marabuto for help with the identification of microlepidopteran specimens and discussions on the biology of tortricid moths, and M. J. Pinto for discussions on plant rarity and Iberian *Euphorbia* taxonomy and distribution. Support was provided by Fundação para a Ciência e Tecnologia through grants PRAXIS XXI/BD/21407/99 to MB and SFRH/BPD/39998/2007 to CR.

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Chapter 4

The impact of specialist and generalist pre-dispersal seed predators on the reproductive output of a common and a rare *Euphorbia* species

Boieiro, M., Rego, C., Serrano, A.R.M. & X. Espadaler (2010) The impact of specialist and generalist pre-dispersal seed predators on the reproductive output of a common and a rare *Euphorbia* species. *Acta Oecologica* 36: 227-233
DOI: 10.1016/j.actao.2010.01.001

Acta Oecologica 36 (2010) 227–233

Contents lists available at ScienceDirect

Acta Oecologica

journal homepage: www.elsevier.com/locate/actoec

Original article

The impact of specialist and generalist pre-dispersal seed predators on the reproductive output of a common and a rare *Euphorbia* species

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ARTICLE INFO

Article history:
Received 31 August 2009
Accepted 8 January 2010
Published online 4 February 2010

Keywords:
Seed predation
Seed-feeding insects
Congeneric species
Plant rarity

ABSTRACT

Pre-dispersal seed predators can have a severe impact on the reproductive output of their hosts, which can translate into negative effects on population dynamics. Here we compared the losses due to specialist and generalist insect seed predators in two *Euphorbia* species, a rare (*Euphorbia welwitschii*) and a common one (*Euphorbia characias*). Pre-dispersal losses to specialist seed-wasps (*Eurytoma jaltica*) and generalist hemipterans (*Cydnus aterrimus* and *Dicranocephalus agilis*) were on average higher for the rare *E. welwitschii* than for its widespread congener. In both *Euphorbia* species, the variation in losses to specialist and generalist seed predators was not related with traits indicative of plant size, fecundity, or isolation. Nevertheless, the temporal variation in losses to seed-wasps seemed to be intimately associated with the magnitude of yearly variation in fruit production. The impact of seed-wasps and hemipterans on the reproductive output of both *Euphorbia* species was additive, though there was evidence for infochemical-mediated interference at the fruit level. The moderate levels of seed predation in *E. welwitschii*, together with the results from the comparative analysis with its widespread congener, suggest that insect seed predation is not a causal effect of plant rarity.

1. Introduction

From anthesis until seed germination the reproductive potential of a plant species diminishes progressively as a consequence of the losses imposed by a variety of factors acting in conjunction or sequentially. Despite the plurality of causes governing the reproductive success of plant species, seed predation has frequently been reported as a major form of seed mortality (Janzen, 1971; Crawley, 2000) with seeds being consumed both before and after the dispersal phase. Although either form of seed predation may impose considerable reproductive losses (Hulme, 2002), pre-dispersal seed predation has also the potential to negatively influence seed dispersal mechanisms. For example, by damaging fruits pre-dispersal seed predators may render them unattractive to frugivorous dispersers and even contribute to a lower number of plant–disperser interactions (e.g., Jordano, 1987; Sallabanks and Courtney, 1992; Izhaki, 1998; Bas et al., 2005). Pre-dispersal seed predators belong to a variety of animal groups, but many of them are inconspicuous specialized insects that attack a particular plant species or a few closely related species (Hulme, 2002). These insects usually feed directly upon the seeds or use them as oviposition sites, ensuring a rich food resource for the development of their progeny. Many studies have reported that pre-dispersal seed predation by insects could be extremely severe, frequently leading to losses greater than 50% of the total seed crop (Crawley, 2000; Fenner and Thompson, 2005; Kolb et al., 2007). Even so, several authors (e.g., Janzen, 1971; Andersen, 1988) claim that estimations of insect pre-dispersal seed predation are still conservative or even misleading due to the inadequacy of the sampling methodology. Therefore, the loss of seeds as a result of predation may be a potential threat for plant population growth or maintenance. Various examples have highlighted how insect seed predation has clearly the potential to affect the population dynamics of its host species by limiting plant recruitment (Louda, 1982a,b; Louda and Potvin, 1995; Kelly and Dyer, 2002; Münzbergová, 2005), which may ultimately lead to population decline. This was the case stated by Hegazy and Eesa (1991) where extraordinarily heavy seed predation by bruchid beetles was considered a major threat to the existence of the narrow endemic *Ebenus armitagei*. According to some authors, the impact of seed predators on the reproductive output of endemic plants may represent a mechanism that might help explain plant rarity (e.g., Lavergne et al., 2005; Münzbergová, 2005). Other studies, however, identified other factors that correlate with common–rare differences and found no evidence for

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doi:10.1016/j.actao.2010.01.001

a key role of seed predation in determining plant rarity (Fiedler, 1987; Simon and Hay, 2003; Lavergne et al., 2004).

Insect seed predators, particularly the host-specific ones, can also act as important selective forces by exerting consistent selection on particular plant traits (Zimmerman, 1980; Brody and Mitchell, 1997; Caruso, 2001; Cariveau et al., 2004). Pre-dispersal seed predators may, together with pollinators and other flower visitors, drive flowering synchrony, flowering phenology and influence inflorescence characteristics, flower size and flower longevity (Brody, 1997; Fenner et al., 2002; Wright and Meagher, 2003; Strauss and Whittall, 2006).

Some of the few studies comparing pre-dispersal seed predation in co-occurring closely related plant species revealed how particular plant traits may correlate with levels of insect damage. Green and Palmbad (1975) stated that differential predation on two *Astragalus* species may be due to differences in fruit morphology and biochemistry, fruit internal temperature, and seed energy contents. They found that lower levels of seed predation were correlated with fruit and seed characteristics that impose severe constraints on insect oviposition and larvae development. In another study, Greig (1993) evaluated the impact of pre-dispersal seed predators in a set of *Piper* species that exhibited alternative reproductive strategies (partitioning between seed size and seed number). She recorded the highest levels of insect damage in the less fecund species, which were also the ones that produced the larger seeds. These species are attractive to a wider array of predators, particularly weevils that can develop on large-sized seeds, but are unable to do so in the small-sized seeds of their congeners. This kind of comparison among groups of congeneric co-occurring species is considered a powerful method for studying patterns and processes in evolutionary ecology. This procedure is now being increasingly applied in plant conservation biology since the analysis of the main causes of mortality in pairs of common-rare species may lead to a clearer understanding of the underlying causes of rarity and provide valuable information for the management of endangered species (e.g., Bevill and Louda, 1999; Simon and Hay, 2003; Scott and Gross, 2004; Lavergne et al., 2005).

The aim of this study is to investigate the magnitude and spatio-temporal variation in seed predation levels in two sympatric *Euphorbia* species, a common (*Euphorbia characias*) and a rare one (*Euphorbia welwitschii*). We examine the differential impact of specialist and generalist seed predators in the reproductive output of both species and evaluate how those effects relate with plant rarity. Furthermore, we assess how insect predation pressure is correlated with plant traits indicative of size and fecundity and how consistent is that association in space and time.

2. Materials and methods

2.1. Plant species and their pre-dispersal seed predators

E. characias Linnaeus and *E. welwitschii* Boissduval & Reuter are perennial species associated with calcareous soils and both can be found in Mediterranean-type ecosystems. The two study plants were selected due to their affinities in phylogeny and phenology, the possibility to found them in sympatry and due to their differences in abundance and distribution. *E. characias* is widely distributed along the Mediterranean Basin, from Portugal to Greece and has also been reported from several North African countries (Benedí et al., 1997). In the Iberian Peninsula, *E. characias* is relatively widespread being commonly found in open and sunny habitats, where it usually occurs in large populations. On the other hand, *E. welwitschii* is endemic to Western Portugal, being restricted to some populations scattered around the vicinities of Lisbon (Franco, 1971). Some of those populations have a low number of individuals (less than 20 individuals) and the observation of recruitment failure during consecutive years in several of them may be indicative of their susceptibility to the negative effects of small population size (M. Boieiro, unpubl. data). During the last century, *E. welwitschii* suffered a considerable decline as a result of habitat destruction, which is still considered the major threat to its existence.

In both *Euphorbia* species, the fruit is a trilocular green capsule enclosing one smooth caruncIulate seed per loculum. The fruits differ in external morphology since the capsules of *E. characias* are pubescent while those of *E. welwitschii* are glabrous and somewhat thicker. During fruit maturation seed predators are regularly observed puncturing or ovipositing in full-sized fruits. Two kinds of insect predators are responsible for reproductive losses in both *Euphorbia* species: a specialist seed-wasp, *Eurytoma jaltica* Zerova (Eurytomidae) and generalist hemipterans, *Cydnus aterrimus* (Forster) (Cydnidae) and *Dicranocephalus agilis* (Scopoli) (Stenocephalidae). The seed-wasp *E. jaltica* can be found mating and ovipositing in the fruits of *Euphorbia* species in April and May. The larvae of this species develop inside the seeds feeding on their contents. They overwinter inside the seeds and the adults emerge in the following spring, when fruits are again available for oviposition. In Iberia, *E. characias* and *E. welwitschii* are the only known hosts of *E. jaltica* (M. Boieiro, unpubl. data). The hemipterans *C. aterrimus* and *D. agilis* are generalist species that feed upon a variety of plant species from different genera (Stichel, 1955/1962; Moulet, 1995). These insects are frequently seen on the fruits of *Euphorbia* species puncturing the seeds during the fruiting period. They inject saliva into the seed where extra-oral digestion takes place and then suck back the resulting liquids leaving the seed deprived of its contents.

2.2. Study sites

The study was carried out from 2002 to 2004 at five sites in western Portugal. In two of these sites, Montejunto (39°10′N, 9°03′W) and Tesoureira (38°56′N, 9°08′W), both plant species co-occur. Two other populations of *E. characias* were selected in coastal areas of the Peninsula de Setúbal (Azóia – 38°25′N, 9°08′W and Ares – 38°26′N, 9°04′W) altogether with the nearest known *E. welwitschii* population (Risco – 38°27′N, 9°01′W), located about 4 km from Ares. All study areas, except Tesoureira, are included in natural protected areas of the Portuguese Reserve System, namely the Área de Paisagem Protegida da Serra de Montejunto (Montejunto site) and the Parque Natural da Arrábida (Azóia, Ares and Risco sites). The five study sites are characterized by a Mediterranean-type climate and the vegetation is dominated by sclerophyllous species, particularly *Quercus coccifera*, *Pistacia lentiscus*, *Rosmarinus officinalis*, *Daphne gnidium*, *Lavandula luisieri* and several *Cistus* species.

2.3. Seed production in a common and a rare Euphorbia species

At the beginning of the flowering period, in each study site, we randomly selected 25 reproductive individuals from each plant species, to quantify fruit and seed production. The only exception occurred at Risco, during 2002, when we were unable to locate more than 20 reproductive individuals of the rare *E. welwitschii*. Each year, by the end of the fruiting period, which usually takes place in May–June, we counted all mature fruits produced in each selected individual of *E. welwitschii*. A different methodological approach was adopted in the study of fruit and seed production in *E. characias* due to the larger size and fecundity of this species. Subsamples were collected by counting the fruits from a single randomly selected floral stem of each individual and simultaneously recording the number of floral stems per individual. Fruit production per individual was subsequently estimated by multiplying the two variables.

For both study species, individual seed production was estimated by multiplying the total number of mature fruits by 3 since this is the usual number of seeds enclosed in each trilocular capsule (Berg, 1990; Traveset, 1995). In 2004 we were unable to collect data at Montejunto because the study site was devastated by a fire which destroyed almost 70% of the plant cover of this protected area.

2.4. Pre-dispersal seed predation by seed-wasps and hemipterans

During the fruiting season we collected all mature fruits from each marked plant of both study species. Fruit collection was performed regularly at 4–10 days intervals since the studied *Euphorbia* species present sequential fruiting from the lower to the upper levels of the infructescence. Once in the laboratory, the fruits were opened and a random sample of 60 seeds per plant was carefully dissected using a microscope to distinguish between predated, aborted and intact seeds. Aborted seeds are easily recognized due to their whitish or yellowish colour, light weight, wizened appearance and the presence of an undeveloped elaiosome. Seed predation was evaluated by examining the seed content. Precise differentiation of seed destruction by hemipterans versus seed-wasps was easy, because these seed predators use the seeds for different purposes leaving specific evidences in the seeds. The presence of a larva inside the seed is indicative of seed-wasp predation by *E. jaltica*. On the other hand, the seeds predated by hemipterans are left empty as a result of the action of salivary secretions inserted by these insects in the seeds and the subsequent uptake of the nutritive fluids. Precise discrimination of the feeding damage by the two hemipteran species was however not possible. Intact seeds were recognized by verifying the presence of an embryo surrounded by white endosperm within the seed locule. Seed predation levels were subsequently calculated as the fraction of seeds destroyed from the pool of seeds available to predators (non-aborted seeds). During 2003, we haphazardly chose and dissected 500 fruits of *E. characias* and 200 of *E. welwitschii* from the study populations where both species co-occur (Montejunto and Tesoureira) to study seed predator segregation at the fruit level. In order to evaluate the role of plant morphology, fruiting display and relative spatial isolation on pre-dispersal seed predation levels we took the following measures from each individual: plant height, distance to the nearest conspecific, number of mature fruits and proportion of aborted fruits.

2.5. Statistical analysis

The differences in the proportion of predated seeds by generalist and specialist insect predators among populations and years were assessed separately for each study plant using ANOVAs on arcsine square-root transformed proportions. Significant spatial variations in seed predation levels were subsequently assessed by multiple comparisons of means using the Tukey HSD *post hoc* test with a 95% confidence level. Differences between the two *Euphorbia* species in losses due to specialist and generalist seed predators were assessed by means of *t*-tests.

The temporal dynamics of seed predation by seed-wasps were examined by plotting the average proportion of predated seeds as a function of year-to-year difference in seed production. The year-to-year variation in seed production was calculated as the difference between the average seed production in year t1 and year t0 divided by the range in year t1. Data from the study populations were pooled and presented graphically for each plant species. For both *Euphorbia* species we tested the association between morphological traits, fruiting display, spatial isolation and pre-dispersal seed predation levels by Spearman rank correlation analysis. Data from the study populations were pooled for a general analysis after performing single population-based analyses for each year. All statistical analyses were performed using the Statistica software (StatSoft, 2008).

3. Results

3.1. Spatio-temporal variation in seed production and seed predation levels by specialist and generalist insects

Most individuals of both study plants were attacked by seed predators, but losses were on average low. Hemipterans inflicted seed losses to the majority of individuals of both *Euphorbia* species in all study populations (mean ± SD: 77.8 ± 17.7% for *E. characias* and 82.5 ± 11.2% for *E. welwitschii*). The proportion of seed losses by plant due to hemipterans was higher for *E. welwitschii* (14.3 ± 14.6%, range 0.0–100.0%, $n = 187$) than for *E. characias* (7.6 ± 7.9%, range 0.0–35.7%, $n = 275$) ($t = -5.53$, $P < 0.0001$). Seed-wasps also attacked a large proportion of individuals of both study plants (75.6 ± 26.1% for *E. characias* and 90.6 ± 6.2% for *E. welwitschii*) and inflicted higher seed losses to *E. welwitschii* (21.1 ± 17.2%, range 0.0–100.0%, $n = 187$) than to *E. characias* (7.9 ± 8.3%, range 0.0–41.2%, $n = 275$) ($t = -10.75$, $P < 0.0001$).

The impact of pre-dispersal seed predators on *E. characias* and *E. welwitschii* varied in space and time, but distinct patterns of seed predation dynamics were identified for seed-wasps and hemipterans (Fig. 1). Seed predation by seed-wasps appears to be negatively associated with seed production patterns in both *Euphorbia* species, with higher losses reported in periods of low seed production and minor losses in years of high seed production. On the other hand, seed predation by hemipterans seems more unpredictable since fluctuations in seed production do not always elicit direct changes in predation intensity.

In the widespread *E. characias*, the seed losses inflicted by seed-wasps varied considerably among the study populations ($F_{3,271} = 26.11$, $P < 0.0001$) and within populations during the three-year study ($F_{2,272} = 23.80$, $P < 0.0001$) with a more severe impact on the individuals at Tesoureira (Tukey's test, $P < 0.05$). Losses due to hemipterans differed between populations ($F_{3,271} = 16.08$, $P < 0.0001$), but not between years ($F_{2,272} = 1.67$, $P = 0.19$), with higher seed losses reported from Montejunto (Tukey's test, $P < 0.05$). On the other hand, the losses inflicted by seed-wasps and hemipterans on the endemic *E. welwitschii* differed between populations (respectively $F_{2,157} = 10.75$, $P < 0.0001$ and $F_{2,157} = 11.95$, $P < 0.0001$) but not between years (for seed-wasps $P = 0.48$ and for hemipterans $P = 0.18$). The individuals from Risco were more affected because both kinds of predator inflicted heavier damages in this study area (Tukey's test, $P < 0.05$). In general, when in sympatry, the rare *E. welwitschii* had a higher proportion of losses to both kinds of seed predator than its widespread congener (Table 1). However, although the proportion of seeds destroyed by predators was higher in *E. welwitschii*, they caused by far more losses to *E. characias* due to the larger fecundity of this species.

3.2. Temporal dynamics of seed predation by specialist insects

For both study plants, the proportion of seeds preyed upon by seed-wasps showed an inverse pattern from the one recorded for seed production suggesting that seed predation levels may depend on the inter-annual variation in seed production (Fig. 1). Annual increases in seed production were, in general, accompanied by a reduction in the proportion of attacked seeds and when seed production was low compared to the previous year, higher levels of

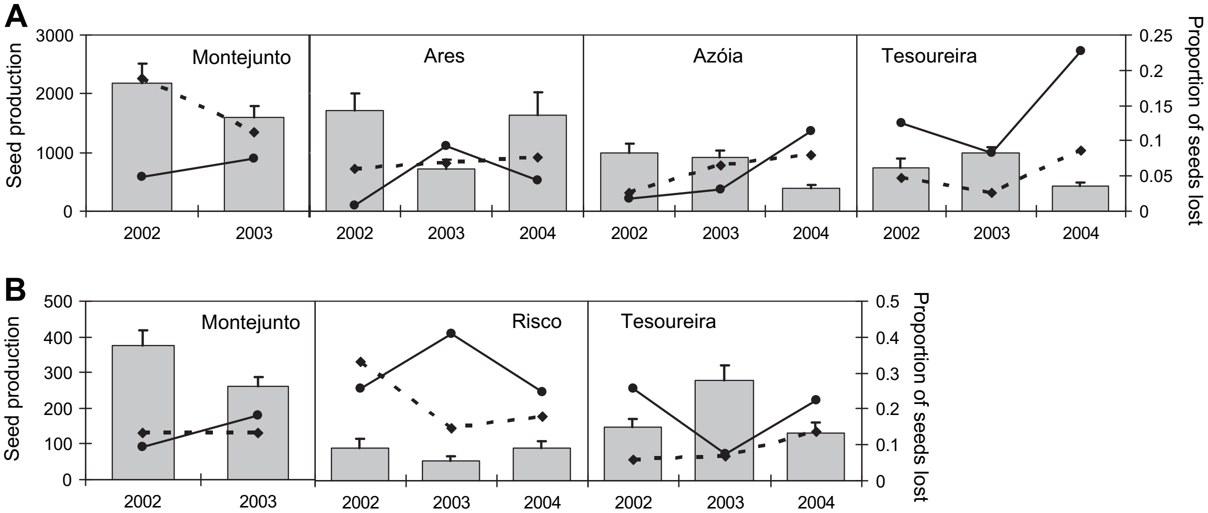

Fig. 1. Dynamics of seed production and pre-dispersal seed predation by seed-wasps (solid line) and hemipterans (dashed line) in the study populations from 2002 to 2004. Annual average (+SE) seed production per plant (bars) is plotted together with the mean proportion of losses due to the two different kinds of seed predator. A) *E. characias*. B) *E. welwitschii*.

seed predation by seed-wasps were then recorded. This trend was found for both *Euphorbia* species in all the study populations (Fig. 2). However, the low number of points of each time series and the large variation associated with each data point demand caution in drawing conclusions from these findings.

3.3. Correlates of seed predation levels in two Euphorbia species

Overall, the proportions of losses to specialist and generalist seed predators appear to be only weakly or not directly related with variables indicative of size, fecundity and ovary/fruit abortion in both study plants (Table 2). This lack of association between the measured variables and pre-dispersal seed predation levels resulted from a wide spatio-temporal variation in the correlation values, since occasional significant correlations were found. For example, the losses inflicted by hemipterans to *E. welwitschii* in Tesoureira during 2004 seemed to be associated with the percentage of damaged ovaries and fruits ($r = 0.61$, $P < 0.01$). However, in the preceding years no association was found between these two variables (in 2002, $r = 0.23$, $P = 0.32$ and in 2003, $r = 0.07$, $P = 0.75$) and distinct findings were recorded for its co-occurring congener at the same time ($r = 0.17$, $P = 0.41$). The significant positive association between the proportion of losses due to hemipterans and plant size found in Ares during 2002 for *E. characias* ($r = 0.42$, $P < 0.05$) was not detected in the following years (in 2003, $r = 0.01$, $P = 0.95$ and in 2004, $r = 0.02$, $P = 0.93$). The spatial dispersion of individuals of both study species does not seem to influence the levels of pre-dispersal seed predation by specialist and generalist predators (Table 2) as most correlation values recorded in each study population were low and non-significant.

The impact of specialist and generalist pre-dispersal seed predators on the reproductive output of the two *Euphorbia* species was additive. In *E. characias*, the damage inflicted by specialist seed predators was unrelated with the losses to generalist predators ($r = 0.07$, $P = 0.22$). Identical findings were recorded for *E. welwitschii* ($r = -0.06$, $P = 0.40$), notwithstanding the fact that a significant result was found in one population ($r = -0.56$, $P < 0.001$, at Risco). At the individual fruit level, generalist seed predators showed a higher tendency in feeding on more seeds in a same fruit than specialists for both study plants (Fig. 3). We also

Table 1
Differences in the proportion of pre-dispersal losses to specialist (seed-wasps) and generalist (hemipterans) seed predators in sympatric populations of *E. welwitschii* and *E. characias*. The range of seed losses is shown in parentheses. Data from 2 years (Montejunto) and 3 years (Tesoureira) were pooled. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{\text{n.s.}}$ = not significant.

Site	Seed predator	*E. welwitschii*	*E. characias*	*P* value
Montejunto	Seed-wasps	13.6 (0.0–45.2)	6.1 (0.0–18.6)	***
	Hemipterans	13.1 (0.0–37.0)	15.0 (0.0–35.7)	n.s.
Tesoureira	Seed-wasps	18.5 (0.0–61.8)	14.4 (0.0–41.2)	*
	Hemipterans	8.9 (0.0–47.1)	5.2 (0.0–22.7)	**

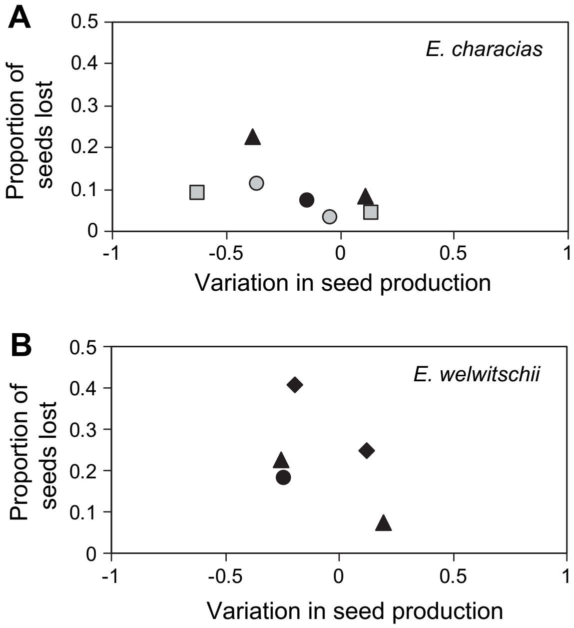

Fig. 2. Mean proportion of losses to seed-wasps as a function of year-to-year variation in seed production. Data from the study populations were pooled and presented as: Ares (light square), Azóia (light circle), Montejunto (black circle), Risco (black diamond) and Tesoureira (black triangle). A) *E. characias*. B) *E. welwitschii*.

Table 2
Correlations between traits related with plant morphology and fecundity and the proportion of losses to specialist and generalist seed predators for *E. characias* and *E. welwitschii*. Data from the study populations were pooled (four populations for *E. characias*, three populations for *E. welwitschii)*. (pAbortion – proportion of ovary and fruit abortion, pSeed-wasps – proportion of losses to seed-wasps, pHemipt – proportion of losses to hemipterans). $^{*}P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$, $^{\text{n.s.}}$ = not significant.

	E. characias		*E. welwitschii*	
	pSeed-wasps	pHemipt	pSeed-wasps	pHemipt
Plant size	$0.11^{\text{n.s.}}$	0.24^{***}	$0.01^{\text{n.s.}}$	$0.05^{\text{n.s.}}$
Plant spacing	$-0.08^{\text{n.s.}}$	$0.05^{\text{n.s.}}$	$0.07^{\text{n.s.}}$	$-0.09^{\text{n.s.}}$
Plant fecundity	-0.17^{**}	$0.05^{\text{n.s.}}$	-0.17^{*}	-0.18^{*}
pAbortion	$-0.09^{\text{n.s.}}$	-0.20^{***}	0.21^{**}	0.32^{***}

found evidence of a segregation mechanism since the two insect groups rarely attacked the same fruit.

4. Discussion

4.1. Magnitude, variation and correlates of pre-dispersal seed predation in two Euphorbia species

The impact of pre-dispersal seed predators on the reproductive output of both study plants was moderate and, in general, varied within and between habitats within each species. In areas of sympatry, the two plants suffered seed losses of the same magnitude to generalist and specialist predators and the patterns of temporal variation in seed predation were also similar for both *Euphorbia* species. This finding may be due to the identical fruiting phenology and affinities in fruit and seed characteristics of both plants. Fruits are similar in size and seeds have identical weight, size and toughness; thus both *Euphorbia* species present similar conditions for insect landing, fruit and seed puncturing and larval development. The two plants differ, however, in fruit pubescence, but, unlike what was reported by Green and Palmbad (1975) from two *Astragalus* species, this aspect did not appear to interfere with the attack of seed predators. Additionally, the short interplant distances recorded in all populations for both study species may also have eased the location and movement of insect seed predators between individuals. A long-term study on seed production in four co-occurring *Carpinus* species with similar seed size and flowering phenology (Shibata et al., 1998) also showed that the magnitude and the temporal pattern of variation in pre-dispersal losses to insect predators may be quite similar between species for several years. Nevertheless, examples can be found where congeneric co-occurring plants may exhibit disparate losses to pre-dispersal seed predators due to differences in fruiting phenology, fruit and seed characteristics (e.g., size, toughness and chemistry), plant morphology and fecundity or a combination of these factors (Green and Palmbad, 1975; Siemens et al., 1992; Greig, 1993; Simon and Hay, 2003; Nakagawa et al., 2005; Espelta et al., 2009).

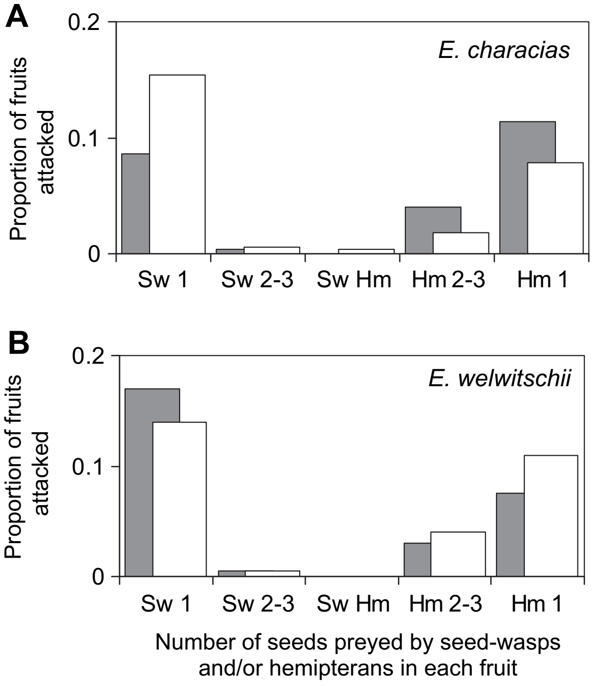

Fig. 3. Segregation of attack by specialist and generalist pre-dispersal seed predators within a fruit in two sympatric populations of *E. characias* and *E. welwitschii* (Montejunto – grey bars and Tesoureira – white bars). The proportions of fruit losses are indicated as follows: Sw1 – fruits with one seed lost to seed-wasps, Sw 2–3 – fruits with two or three seeds lost to seed-wasps, Sw Hm– fruits with at least one seed lost to each predator group, Hm 2–3 – fruits with two or three seeds lost to hemipterans, Hm1 – fruits with one seed lost to hemipterans. A) *E. characias*, $n = 500$/population. B) *E. welwitschii*, $n = 200$/population.

In the study populations near the sea, where allopatric populations of the two species were located, *E. welwitschii* suffered proportionately larger losses to seed predators. At Risco, *E. welwitschii* seems to face the effects of small population size expressed in low fecundity and large reproductive losses in the form of ovary, fruit and seed abortion (Boieiro et al., in press). Thus, the larger impact of pre-dispersal seed predators in this population may be due to low resource availability (low seed production by *E. welwitschii* and the absence of alternative hosts), although other site-specific factors associated with the abundance and dynamics of insect populations may also be implicated. For example, Honek and Martinkova (2005) showed how pre-dispersal seed predation levels in *Taraxacum officinale* were related with the local abundance of consumer species. Other authors have also stressed that the occurrence of interacting species from a higher trophic level may lead to spatial variation in seed predation levels (e.g., Guimarães et al., 2006; von Zeipel et al., 2006).

Individual differences in plant size, fecundity and seed crop quality do not seem to influence host plant selection by specialist and generalist seed predators, probably because these insects use other cues to assess potential hosts. It has been shown that chemical cues play a major role in host plant selection by seed-wasps. Seed-wasp females are attracted from a distance by odours from fruits of their host plants and the same substances also proved to play a key role in eliciting oviposition (Kouloussis and Katsoyannos, 1994). On the other hand, host location by phytophagous hemipterans seems to be driven by a combination of visual and chemical cues, but after contact with the plant surface multiple sensory modalities (including mechanoreception, gustation and olfaction) govern the decision of host acceptance (Bernays and Chapman, 1994). For these reasons, the lack of temporal consistency in the association between losses to seed predators and the measured plant characteristics found here, suggest that the selective pressures exerted by the insects on those traits are rather weak.

4.2. The differential impact of pre-dispersal seed predators on seed output

Seed losses inflicted by hemipterans were moderate, although highly variable, and unrelated with plant traits associated with morphology and fecundity. This variability in the proportion of predated seeds is, in part, due to the generalist feeding habits of these insects. They usually prey upon a variety of other species besides *Euphorbia* spp. and frequently feed on various parts of the host plant, not exclusively upon seeds. For example, *C. aterrimus* has been reported on host plants from different plant families and although it prefers feeding upon the seeds of *Euphorbia* species, may also puncture the stem, the roots or the leaves (Stichel, 1955/1962). The dynamics of seed predation by these insects is thus extremely complex, involving interactions with several host plants and capability to cope with wide fluctuations in a variety of potential resources, which leads to unpredictable reproductive losses in some of their host plants. Several hemipteran species, including *D. agilis*, may aggregate in particular plant species as a result of attraction between conspecifics. In some plants, we found up to 40 individuals of this species feeding, resting, mating and hiding rapidly within the inflorescence when disturbed. The aggregation of individuals as a response to attraction between conspecifics contributes to disproportional losses between individual host plants and consequently may mask the selectivity by herbivores, thus explaining, in part, the lack of association between the proportion of seed losses to hemipterans and plant characteristics.

Seed-wasps were also responsible for highly variable reproductive losses in both *Euphorbia* species, but the levels of seed predation could be neither attributed to traits related with plant morphology or fecundity, nor with the distance between conspecific host plants. The temporal variation in the proportion of losses to seed-wasps appears to be intimately associated with the magnitude of yearly variation in fruit production (Fig. 2). A large increase in fruit production from one year to another swamped seed-wasps with sites for oviposition and resources for larvae development, leading to a lower proportion of attacked seeds. On the other hand, a large decrease in fruit production led to a higher proportion of reproductive losses to host plants. This negative association between the variation in fruit production and seed-wasp predation intensity was found in all populations of the two *Euphorbia* species. Due to their host specificity, seed-wasps are highly susceptible to fluctuations on the availability of fruits upon which to complete their life cycle. Nevertheless, these seed predators show a delay in tracking the variation in resources since the insect population is, in part, determined by the availability of resources for oviposition and larvae development during the previous season. Similar patterns of interaction between a specialist pre-dispersal seed predator and its host plant have been reported in a few other studies (De Steven, 1983; Solbreck and Sillén-Tulberg, 1986; Sperens, 1997; Poncet et al., 2009). In those systems, the inter-annual variation in fruit production is considered a key feature since it appears to regulate the populations of specialist seed predators and may provide a mechanism of predator satiation, allowing more seeds to escape from predation during episodic good fruiting years (De Steven, 1983; Poncet et al., 2009).

The effect of pre-dispersal seed predators on the reproductive success of the two *Euphorbia* species appears to be additive since the proportion of seed losses imposed by hemipterans to a plant was unrelated with the magnitude of reproductive losses due to seed-wasps and vice versa. This lack of association suggests that, at the plant level, both groups of predators do not seem to discriminate between plants with different levels of attack, probably due to the moderate levels of predation recorded in most study areas. This finding is reinforced by the fact that at Risco, where lower fruit production and higher levels of seed predation were recorded, the impact of specialist and generalist seed predators was negatively correlated. At the fruit level, the two groups of insects exhibited a distinct behavior since they seemed to avoid fruits that were already predated. *Eurytoma* females are known to apply an oviposition-marking pheromone on fruits following egg-laying (Kouloussis and Katsoyannos, 1991, 1993) which enables the discrimination of its condition by conspecifics, preventing additional ovipositions in the same fruit (Fig. 3). As a consequence, the resulting pattern of egg distribution will tend to be uniform while the infestation levels are still low. Hemipterans may be able to detect the presence of the seed-wasp oviposition-marking pheromone explaining, in part, the lack of co-predation at the fruit level. Interspecific recognition of oviposition-marking pheromones has been reported for various insect species, even when included in different taxonomical orders and trophic levels (Stelinski et al., 2009). On the other hand, seed-wasps also seem to perceive the condition of fruits when assessing their quality for oviposition. Further experimental studies on the complex infochemical interactions between plants and their associated insects are needed to help us understand more clearly the dynamics of the host plant selection process by seed-feeding insects.

4.3. Pre-dispersal seed predation in a rare and a widespread Euphorbia species

Direct evidence showed that herbivores, including seed predators, can have a significant effect on plant population dynamics, recruitment and survival and may even limit the geographic range of plant species (Kolb et al., 2007). In our study, where a pair of closely related co-occurring *Euphorbia* species was monitored for three years, significant differences were detected in seed predation levels between the rare and the widespread species. Data from allopatric populations of the two study plants also revealed a larger percentage of seed loss to predators in *E. welwitschii*, but this finding may also, at least in part, reflect differences in abiotic and biotic factors between those study sites. Some other studies also reported a larger percentage of pre-dispersal seed predation in endemic compared to widespread congeners (see references in Kolb et al., 2007) and, in a few of them, seed predation has even been identified as a causal factor of plant rarity (e.g., Münzbergová, 2005). Despite the somewhat larger fraction of losses to seed predators in the endemic *E. welwitschii*, our study does not enable us to reach the same conclusion since the overall impact of seed predators did not change substantially the already large difference in potential seed production between the two species. Our findings agree with other comparative studies on pairs of common–rare species that argue that seed predation rates *per se* appear to be insufficient as a causal effect of plant rarity (Fiedler, 1987; Madeira and Fernandes, 1999; Simon and Hay, 2003; Lavergne et al., 2004).

Acknowledgements

We thank J. Boieiro for the valuable help in the field, M. Zerova and W. Rabitsch for help with the identification of insect species and discussions on seed predator biology, and M.J. Pinto for discussions on plant rarity and Iberian *Euphorbia* species taxonomy and distribution. Support was provided by Fundação para a Ciência e a Tecnologia through grants PRAXIS XXI/BD/21407/99 to MB and SFRH/BPD/39998/2007 to CR.

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PART II

MYRMECOCHORY AND POST-DISPERSAL SEED PREDATION


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Chapter 5

Spatial variation in the fatty acid composition of elaiosomes in an ant-dispersed plant: differences within and between individuals and populations

Boieiro, M., Espadaler, X., Gómez, C. & A. Eustaquio (submitted) Spatial variation in the fatty acid composition of elaiosomes in an ant-dispersed plant: differences within and between individuals and populations. *Journal of Chemical Ecology*

Spatial Variation in the Fatty Acid Composition of Elaiosomes in an Ant-Dispersed Plant: Differences Within and Between Individuals and Populations

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Abstract

Here we investigate the variation in elaiosomes' fatty acid composition in the Mediterranean *Euphorbia characias* at three hierarchical levels (subindividual, individual and population). We found that plant architecture effects do not seem to influence the fatty acid composition of elaiosomes, providing to each propagule an equal chance of being dispersed. However, significant differences between individuals and populations in elaiosome fatty acid composition were found for most of the compounds identified. Oleic acid, a key mediator in the ant-seed interaction, showed a wide difference between populations, probably reflecting geographic variations in co-adaptation between plants and their dispersers. The finding that the fatty acid composition of *E. characias* elaiosomes is distinct from the seed itself, but very similar to elaiosomes from unrelated species, reinforces the idea of convergent evolution in the chemical composition of these structures.

Keywords Architectural effects · Elaiosome · *Euphorbia characias* · Fatty acids · Myrmecochory · Oleic · Seeds

Introduction

Seeds are biological structures subjected to strong natural selection by both animal dispersers and predators (Harper et al. 1970; Howe and Smallwood 1982; Fenner and Thompson 2005). In myrmecochorous plants (plants whose seeds are mainly dispersed by ants) the seeds have a specialized external appendage named elaiosome. The elaiosome is a lipid rich structure that functions by luring ants to retrieve the seeds to their nests (Robertson 1897; Sernander 1906; Beattie 1985). This ant-plant relationship has been labelled as a facultative mutualism, with plants benefiting from having their seeds dispersed away from the parent plant, having lower losses to post-dispersal seed predators and eventually having the possibility to reach better microsites for germination and establishment (Beattie 1985; Giladi 2006), and ants getting benefits by feeding on the nutrient rich elaiosomes (e.g., Morales and Heithaus 1998; Bono and Heithaus 2002; Gammans et al. 2005; Fischer et al. 2008). The elaiosomes are particularly rich in lipids, aminoacids and monosaccharides, and provide various nutrients that are essential for insect reproduction and development (Bresinski 1963; Brew et al. 1989; Fischer et al. 2008). Among lipids, fatty acids are major components of elaiosomes and a few of these compounds (e.g., oleic or oleyl-based glycerides) have been identified as main responsible for elaiosome attractiveness to ants (Marshall et al. 1979; Skidmore and Heithaus 1988; Brew et al. 1989). Consequently, some studies have focused in assessing the fatty acid composition of elaiosomes of plants, occasionally drawing interspecific comparisons on fatty acid profiles (Soukup and Holman 1987; Lanza et al. 1992; Mackay and Whalen 1998), but only a few of them have assessed the effects of natural variation in the fatty acid profile (Boulay et al. 2006, 2007). Biological characteristics are subjected to variation, which can be wide even within the same spatial and temporal scale. By incorporating natural variation in ecological studies, we may achieve a better interpretation of the phenomena under study and provide more accurate predictions. This was specifically shown in our study species in a previous work. Seed set, seed quality and seedling survival was shown to be intimately related with variation due to plant architectural effects (Espadaler and Gómez 2001) - distal fruits produced more and bigger seeds than basal ones. Moreover, seedlings issued from distal seeds have enhanced emergence and survival than seedlings from basal fruits. The question then arises if elaiosomes of distal fruits have a different quality from those of basal fruits and how large is that variation between individuals and populations. The aim of this study is to identify the fatty acid profile of *Euphorbia characias* elaiosomes and assess the variation in their composition across three hierarchical levels (subindividual, individual and population). We specifically investigate if a) fruit position affects elaiosome fatty acid composition and if b) elaiosome composition shows variation between populations in the Iberian Peninsula. Additionally, we were able to explore if c) elaiosome composition shows variation between individuals within a population. In the biology of the system *E. characias*-ants, this information is pertinent to the understanding of post-dispersal processes determining seed fate.

Methods and Materials

Plant and Study Sites

Euphorbia characias is a common woody perennial spurge with a Western Mediterranean distribution and presents distinctive morphological features making its discrimination from related congeners relatively easy (Benedí et al. 1997). This species has a variable number of floral stems where flowers are arranged in inflorescences. The floral stems have a long leaf-free basal part, a leafy medial zone, and a distal elongated inflorescence somewhat cylindrical in shape with a terminal umbrella-like structure on top (Fig. 1). Flowers can be found in the upper part of the floral stem, where they occur sparsely distributed around the stem or more aggregated in the terminal umbrella-like structure (Fig. 1). The flowering period takes place between January and April and fruit maturation lasts until early June. The fruit is a green capsule enclosing three seeds. Seeds are light grey, ellipsoidal in form and have a yellowish elaiosome. *Euphorbia characias* has a diplochorous dispersal system where seeds are first ballistically ejected at short distances (up to 4 m; unpublished data) getting scattered on soil. Then, some ant species (e.g., *Aphaenogaster senilis*, *Tapinoma nigerrimum*, *Pheidole pallidula*) retrieve the seeds to their nests where they consume the elaiosome, leaving the intact seeds inside the galleries or discarding them outside, where they may germinate (Espadaler and Gómez 1996). The samples for analysis were collected from four populations of the Iberian Peninsula: 1) Can Llevallol (41°24'N 2°05'E; elevation 315 m), within the Collserola Natural Park, a preserved area close to Barcelona (eastern Spain), 2) Montejunto (39º10'N, 9º03'W; elevation 510 m) included in the Serra de Montejunto protected area (western Portugal), 3) Tesoureira (38º56'N, 9º08'W; elevation 240 m) near Lisbon (western Portugal), and 4) Ares (38º26'N, 9º04W; elevation 190 m) in the western coast of Portugal, within the Arrábida Natural Park. Seed samples of each population are held in reference collections in the Faculty of Sciences, University of Lisbon and in CREAF, Autonomous University of Barcelona.

Fig. 1. A floral stem of *Euphorbia characias* showing in detail the distal part of the inflorescence (drawing by J. Conca).

Elaiosome Extraction and Chemical Analysis

In each population, a single randomly selected mature floral stem was collected from different individuals (seven at Can Llevallol and five from the other populations) and then transported to the laboratory. There, the floral stems were cut and the distal (the terminal umbrella-like structure) and basal parts of the inflorescence were kept separated inside carton boxes, until seeds were ejected from the drying capsules. All elaiosomes from each individual were mixed and a subsample was used for analysis. Elaiosomes were carefully detached from the seeds with the aid of forceps and their semi-dry weight was measured to the nearest 0.1 mg. For chemical analysis, each sample contained 8-13 elaiosomes (mean±S.D.=10.34±1.86), totalling a mean of 5.86±0.80 mg/sample. The fatty acid profile of elaiosomes was obtained via gas-liquid chromatographic analyses of the fatty acids methyl esters (FAME). The FAME were prepared by trans-methylation using the following procedure: 6 mg of elaiosomes were weighted and transferred to a flask, then adding 500 µl 20 % BF3/MeOH (for synthesis, Merck) and 2 ml 25 ppm nonadecanoic acid (internal standard) (99 %, Sigma Aldrich) in Methanol. The trans-methylation was carried out by heating to reflux at 95 ºC for 15 min. 5 ml of 200 g/L NaCl (pro analysis, Merck) and 1 ml hexane were added to the reaction mixture and the FAME were extracted for 2 minutes. The mixture was centrifuged at 3300 rpm for 3 minutes and the hexane layer was transferred to a vial to be analyzed. Analyses were performed employing a 6890 Series II Agilent GC equipped with FID as detector and an HP-23 cis/trans FAME column (60 m x 250 µm x 0.25 µm, Agilent). Operating conditions: injector temperature 280 ºC, detector temperature 275 ºC, splitless during 0.5 minute, carrier gas helium at a flow of 1.8 ml/min; oven temperature was initially set at 130 ºC for 1 minute with programmed increases (130-160 ºC at 1.5 ºC/min, 160-170 ºC at 0.5 ºC/min, 170-230 ºC at 7 ºC/min and held for 12 minutes). Two microliters of the hexane solution were injected onto the column. The FAME were identified by retention time comparation to a FAME standard mixture (37 FAME mix, Supelco) and a vaccenic methyl ester solution in hexane, obtained by applying the same experimental procedure to vaccenic acid (99%, Sigma Aldrich). Quantitative results were obtained using nonadecanoic acid as internal standard, which was added at the beginning of the experimental procedure. Two injections onto the column were performed from each sample.

Statistical Analysis

For any given sample, the area of each of the 9 peaks was divided by the total area of all peaks, thus assessing the relative composition of each compound to allow direct comparisons across samples. However, due to the constraint of these compositional data to sum unity, log contrasts need to be calculated before the performance of exploratory multivariate analyses (Aitchison 1986). Log contrasts were calculated by dividing all relative amounts by an arbitrarily chosen one (in this case, the peak corresponding to vaccenic acid) and taking the log of the 8 remaining new variables (see Neems and

Butlin 1995; Simmons et al. 2003, for similar procedures). A multivariate approach using principal component analysis was then run to reveal patterns in the data. We used univariate ANOVAs, with population and fruit position in the inflorescence as fixed factors, to test for differences in the relative fatty acid composition of elaiosomes (µg compound/mg elaiosome). Significant interpopulation differences in fatty acids' composition were subsequently assessed by multiple comparison tests (Unequal N HSD *post hoc* test) to identify which treatments were significantly different. Among plant variation in elaiosome composition was assessed in one study population (Collserola) by conducting a one-way ANOVA on the relative composition of each compound (µg compound/mg elaiosome). All analyses were run under Statistica (StatSoft 2008).

Results

A representative profile of the fatty acid composition of elaisomes from the basal and distal parts of the inflorescence of *Euphorbia characias* is presented in figure 2. The chromatogram produced nine peaks corresponding to the following fatty acids: miristic (14:0), palmitic (16:0), palmitoleic (16:1), estearic (18:0), oleic (18:1, 9c), vaccenic (18:1, 11c), linoleic (18:2), linolenic (18:3) and an unidentified component. The relative amount of each compound is shown in Table 1.

Table 1 An example of the fatty acid composition of elaiosomes from an individual of Ares.

Fatty acids	14:0 miristic	16:0 palmitic	16:1 palmitoleic	18:0 estearic	18:1,9c oleic	18:1,11c vaccenic	18:2 linoleic	18:3 linolenic	Unident.
Rel. Amount (%)	5.9	17.2	1.0	4.2	65.6	0.6	3.7	1.1	0.6

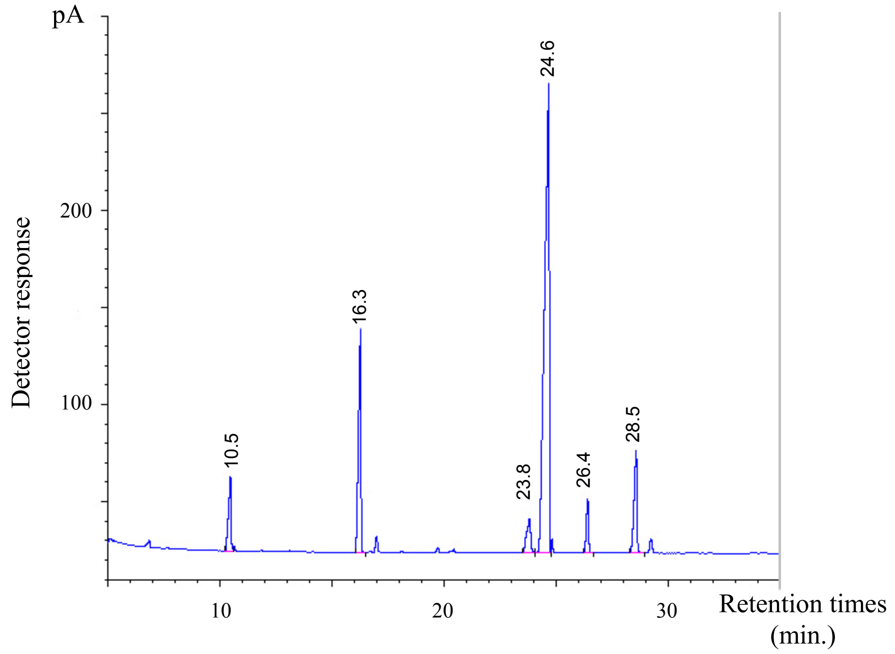

Fig. 2. Typical gas chromatographs of FAME from *Euphorbia characias* elaiosomes. The retention times are indicated in each peak. The sample was collected from an individual in Ares.

The major component is clearly oleic acid followed by palmitic. The other fatty acids occur in a lower proportion, usually around 5% or less. The PCA plot of the data on the fatty acid profiles of the individuals from the four study populations is shown in figure 3.

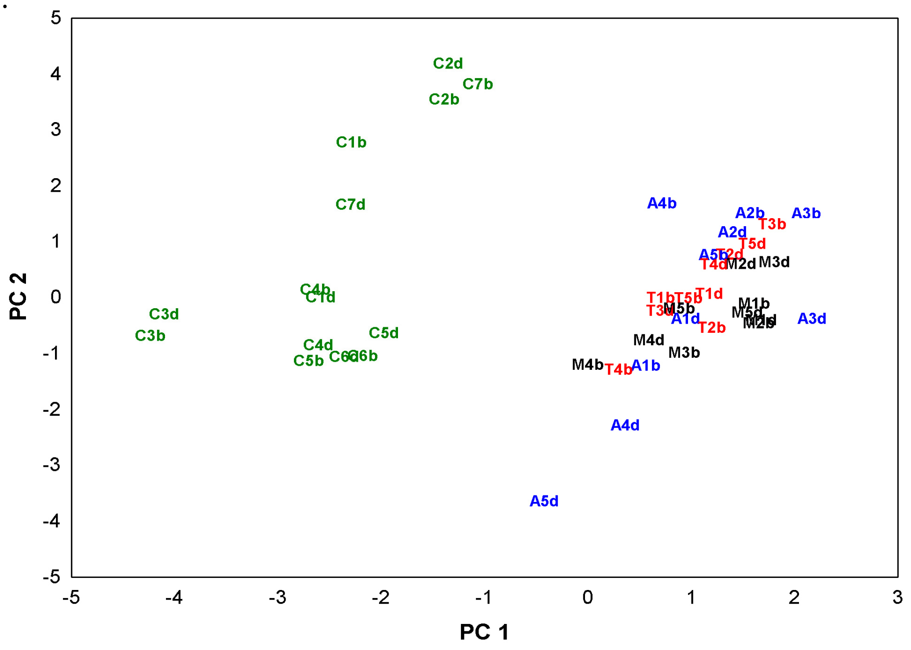

Fig. 3. Fatty acid profile similarity of elaiosomes samples based on the first two principal components (PCs) extracted from the PCA. Each sample has a three-letter code meaning: population (A - Ares, C – Collserola, M – Montejunto, T – Tesoureira), individual (from 1 to 7 for Collserola, from 1 to 5 for the other populations) and position on plant (b – basal, d - distal). Samples from different populations are signalled with different colours.

The two first principal components explain 41.3% and 28.6% of the variance; the third component adds 13.3%. The three components have eigenvalues greater than one (PC1: 3.30; PC2: 2.29; PC3: 1.07) and explain over 80% of the total variance. Some ordination of data points is apparent as shown by their segregation along the x-axis (PC1). The Spanish samples from Collserola are well separated from the Portuguese samples, with Ares, Montejunto and Tesoureira grouping together. In general, the two data points (basal and distal) for each plant are found close to each other suggesting similarity in elaiosome's fatty acid composition at the intra-plant level. Some inter-individual variation within populations can also be depicted from figure 3. A multivariate analysis of variance confirmed that the population factor had a significant effect on the fatty acid profile of elaiosomes ($F = 10.22$, $P < 0.0001$), while no differences due to plant architectural effects (fruit position) were detected ($F = 0.30$, $P = 0.96$). For most of the compounds there were significant differences between levels of the population factor, mostly due to the differences in elaiosome composition between Collserola and the other study populations (Table 2, Figure 4).

Table 2 Differences in the relative amount of fatty acids between populations (Pop), according to fruit position (Pos) and considering the interaction of the two factors (Pos x Pop). *F*-values are presented followed by their significance level and the results of *post hoc* tests are also shown (Populations: A – Ares; C – Collserola; M – Montejunto; T – Tesoureira).

	Pos	Pos x Pop	Pop	*Post hoc test*
Miristic	1.07^{ns}	0.80^{ns}	10.59^{***}	C ≤ A ≤ T ≤ M
Palmitic	1.45^{ns}	1.16^{ns}	51.23^{***}	C < A ≤ M ≤ T
Palmitoleic	0.74^{ns}	0.77^{ns}	40.50^{***}	C < AMT
Estearic	0.78^{ns}	1.08^{ns}	21.02^{***}	C < AMT
Oleic	0.72^{ns}	0.80^{ns}	19.40^{***}	C ≤ A ≤ M ≤ T
Vaccenic	0.49^{ns}	1.10^{ns}	20.71^{***}	C < A ≤ M ≤ T
Linoleic	0.46^{ns}	0.49^{ns}	4.36^{*}	C ≤ MAT
Linolenic	0.70^{ns}	0.85^{ns}	0.27^{ns}	-

$^{\text{ns}}$ $P > 0.05$; * $0.01 < P \leq 0.05$; ** $0.001 < P \leq 0.01$; *** $P \leq 0.001$.

On the other hand, the position of the fruit on the stem (basal vs. distal) did not influence the chemical composition of elaiosomes for any of the compounds (Figure 4), neither did the interaction between the two main factors (Population x Fruit Position) (Table 2).

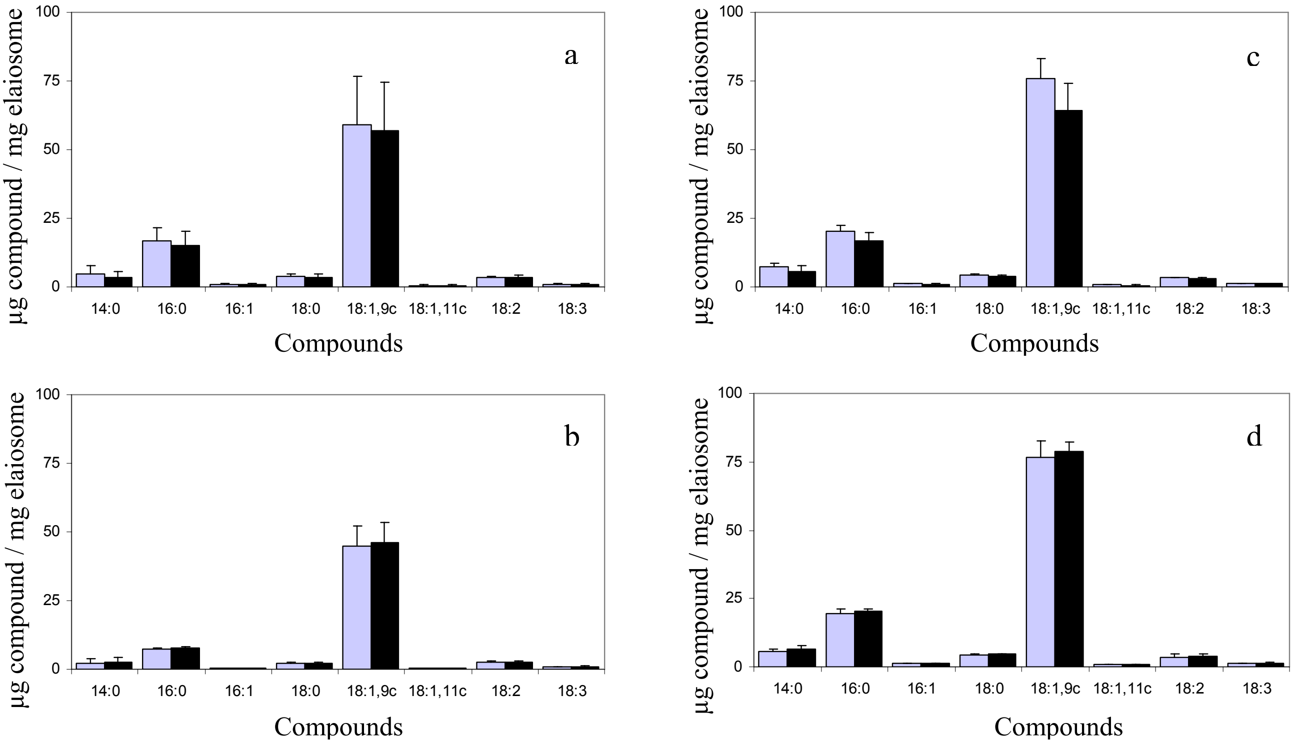

Fig. 4. Fatty acids profiles of distal (grey bars) and basal (black bars) elaiosomes from individuals of each of the four study populations. Results are presented as mean ± S.D.. **a**) Ares; **b**) Collserola; **c**) Montejunto; **d**) Tesoureira.

For Collserola, we found significant differences among plants in the fatty acid profile of elaiosomes. All components except linolenic acid (*F* = 2.19, *P* = 0.07), showed significant variation in their relative amount in elaiosomes at the individual level and in most cases that variation was highly significant (*P* < 0.0001 for miristic, palmitic, palmitoleic, estearic, oleic and vaccenic acids).

Discussion

The chemical composition of elaiosomes in *Euphorbia* had previously been studied by Bresinsky (1963), who detected various nutrients, including lipids, in several species of this genus. The occurrence of lipids in elaiosomes from other genera of Euphorbiaceae was reported in several other studies, but their constituents have only occasionally been identified (Berg 1975; Mackay and Whalen 1998; Pizo and Oliveira 2001; Peternelli et al. 2008). We identified nine fatty acids in the elaiosomes of *E. characias*, with oleic being clearly the dominant fraction, followed by palmitic (Table 1). These findings agree with other studies on the fatty acid composition of elaiosomes from other plant families, where oleic acid has been identified as one of their major components (Soukup and Holman 1987; Skidmore and Heithaus 1988; Kusmenoglu et al. 1989; Lanza et al. 1992; Hughes et al. 1994; Mackay and Whalen 1998; Bebawi and Campbell 2005; Fischer et al. 2008). Oleic acid, either in its free form or as a glyceride (e.g., 1,2-diolein, triolein), has been shown to be a major elicitor of carrying behaviour in ants, playing a key role in myrmecochory (Marshall et al. 1979; Skidmore and Heithaus 1988; Brew et al. 1989; Kusmenoglu et al. 1989; Lanza et al. 1992). These oleyl-based compounds are also major components of insect haemolymph (e.g., Beenakkers et al. 1985), a reason that led Hughes et al. (1994) to defend the evolutionary convergence of elaiosomes' chemical composition with insect prey. The extremely short retention time of seeds on soil surface following ballistic dispersal (Espadaler & Gómez 1996, 1997) makes highly unlikely that ant attraction towards seeds may also be due to the degradation products (i.e., shorter chain fatty acids) of the main fatty acid components.

Previous studies showed experimentally the role played by the elaiosome of *E. characias* in ant attraction and effective seed dispersal (Gómez et al. 2005). While both the elaiosome and the whole diaspore are avidly collected by mutualistic ants, the seed alone presents no interest for these insects (Baiges et al. 1992). In fact, the fatty acid composition of *E. characias* seeds is different from the one reported for the elaiosomes and this is probably the main reason for the observed differences in removal rates. In seeds, linoleic is the dominant component and estearic occurs in a much higher proportion than in the elaiosomes (Carriere et al. 1992). None of these fatty acids seems to play a key role in elaiosome attractiveness to ants for most of the plant species studied to date, though it has been suggested that in *Trillium* linoleic may, together with oleic, act as a chemical cue for ants (Gunther and Lanza 1989; Lanza et al. 1992). These findings suggest some segregation of specific compounds into the elaiosomes making the diaspores more attractive to ants, thus enhancing the probability of being dispersed. An alternative non-conflicting hypothesis suggests that the elaiosome chemical composition differs from the seed itself, as a result of an evolutionary process that has been tuned to provide a rich and easily accessible source of nutrients to ants (see Fischer et al. 2008 and references therein). Fischer et al. (2008) showed that elaiosomes are particularly rich in aminoacids and monossacharides, nutrients that are essential for larval development. In fact, elaiosomes are preferentially fed to larvae and colonies supplemented with elaiosomes produced a higher number and heavier progeny or female-biased progeny (Morales and Heithaus 1998; Gammans et al. 2005; Fokuhl et al. 2007).

To our knowledge this is the first study where the variation in elaiosome fatty acid composition was studied at three different hierarchical levels (subindividual, individual, population). In ecological literature, there is still a lack of studies dealing with the variation in chemical composition of plant traits across various levels of organization and, proportionally, little attention has been devoted to study variation in plant traits within individuals. Although, significant differences due to architectural effects were found in seed production, seed size, seedling emergence and survival in *E. characias* (Espadaler and Gómez 2001), elaiosome chemical composition seems not to be influenced by the position of the fruit on the stem (basal vs. distal). This finding suggests that despite the differential probability of survival between basal and distal seeds, each diaspore has, from the point of view of its chemical composition, an equal chance of being collected by seed-dispersing ants. In this case, other characteristics, such as elaiosome and seed size, may then play a key role in selection of seeds by ants (Mark and Olesen 1996; Bas et al. 2009).

For the Collserola population, interplant variation in the relative concentration of elaiosomes' fatty acids was in general significant for most of the compounds. Oleic acid showed the largest variation in relative concentration within populations, but this finding also reflects the fact that it is by far the main component in elaiosomes. Nevertheless, variation in the relative concentration of fatty acids between individuals may have important ecological consequences, like has recently been proposed for *Helleborus foetidus*. In that plant species, Boulay et al. (2007) found that the frequency of visits to plants by ant dispersers (and consequently seed removal) was highly correlated with interindividual differences in oleic acid content of elaiosomes. Thus, the chemical composition of elaiosomes seems to be under strong selection by ants and may indirectly play an important role in determining local plant demography. Geographic variation in the fatty acid composition of elaiosomes has seldom been studied, but the few studies on this subject provide evidence for significant variations along distance gradients (Soukup and Holman 1987; Mackay and Whalen 1998; Boulay et al. 2006). In our study, the Spanish population presented significant differences in elaiosome fatty acid composition from the Portuguese populations (located 1000 km apart), for most of the constituents. Although the differences were more evident between the Spanish and the Portuguese populations, within the latter it was also possible to detect significant geographic differences for some compounds. This finding reflects the effect of local selective pressures, and genetic and environmental constrains in shaping the chemical composition of elaiosomes. The role played by ant seed dispersers as selective forces on elaiosome chemical composition was highlighted by Boulay et al. (2006) after studying ant-seed interactions in two separated localities. Other studies have also suggested that other interactors (e.g., predators) may also drive the evolution of elaiosome chemical composition (Hanzawa et al. 1985; Gammans et al. 2006). However, it is debatable if the statistically significant differences here detected are biologically relevant and translate into differences in seed dispersal success. Field bioassays on the probability of seed removal by ants according to elaiosome chemical composition will be an interesting extension to the results presented in this study.

Our knowledge on the chemical composition of elaisomes and their implications in ant attraction and nutrition is still scarce. Only recently, some work has been made to evaluate the spatial variation in elaiosome chemical composition and how quantitative differences in key compounds may lead to different seed fates. Further studies are needed to elucidate the chemical basis of ant-seed interactions and their consequences for seed survival, seedling establishment and plant demography.

Acknowledgements

We thank J. Boieiro for the valuable help during fieldwork and C. Rego for comments on an early draft of the manuscript. Partial funding was provided by grants from MCYT-FEDER, Spain (CGL2004-05240-C02) and Fundação para a Ciência e a Tecnologia, Portugal (PRAXIS XXI/BD/21407/99).

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Chapter 6

Fatty acids from the elaiosomes of two endemic *Euphorbia* Linnaeus of Portugal

Boieiro, M., Espadaler, X., Eustáquio, A. & A.R.M. Serrano (in preparation) Fatty acids from the elaiosomes of two endemic *Euphorbia* Linnaeus of Portugal.

Fatty acids from the elaiosomes of two endemic *Euphorbia* Linnaeus of Portugal

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Abstract

In myrmecochorous plant species the elaiosome plays a crucial role in the dispersal process. Most elaiosomes are particularly rich in lipids and evidence suggests that fatty acids are involved in ant attraction. Despite the large number of myrmecochores worldwide, there is still a tremendous lack of information on the fatty acid composition of elaiosomes. In this study we identify and quantify the fatty acids present in the elaiosomes of two Portuguese endemic *Euphorbia* species (*E. pedroi* and *E. welwitschii*). The fatty acid profiles of the two study species show some similarities, with a number of compounds in common and the dominant fatty acids being the same for both *Euphorbia* species. However, the elaiosomes of *E. welwitschii* have a much higher content in fatty acids than those of *E. pedroi*. There is a wide difference in oleic acid content between the two *Euphorbia* species which is probably indicative of different dispersal strategies.

Keywords: Elaiosome • *Euphorbia* • Fatty acids · Myrmecochory · Oleic · Seeds

Introduction

The dispersal of fruits and seeds is a key process during the life-cycle of plants since enables them to escape from higher predation and competition near the parent plant and even reach microsites suitable for germination and establishment (e.g., Wenny, 2001). This way, dispersal is crucial for the maintenance or increase of local population size as well as for the colonization of new areas at a distance from the parent population. The diaspores (fruits and seeds) of many plants have specialized traits to enhance their dispersal by abiotic agents (water, wind) or by a variety of animals (e.g., ants, birds, mammals, reptiles) (Ridley, 1930; van der Pijl, 1969; Murray, 1986; Cousens *et al.,* 2008). In one kind of plants, usually named myrmecochores, the seeds are mainly dispersed by ants due to a specialized external appendage attached to the seed - the elaiosome. The elaiosome is a nutrient-rich structure that functions by luring ants to retrieve the seeds to their nests (Beattie, 1985; Handel & Beattie, 1990). In some species, the elaiosome may also play other functions, such as providing the handle that allows the transportation of seeds (e.g., Gómez *et al.*, 2005) or repelling seed predators (Hanzawa *et al.*, 1985). The dispersal of seeds by ants (myrmecochory) is considered a facultative mutualism with plants benefiting from having their seeds dispersed away from the parent plant and ants getting benefits from feeding on the nutrient rich elaiosomes (Beattie, 1985; Gorb & Gorb, 2003; Fischer *et al.*, 2008 and references therein).

The elaiosomes are particularly rich in lipids, aminoacids and monosaccharides, and provide various nutrients that are essential for insect reproduction and development (Bresinski, 1963; Brew *et al.*, 1989; Fischer *et al.*, 2008). Among lipids, fatty acids are major components of elaiosomes and a few of these compounds (e.g., oleic or oleyl-based glycerides) have been identified as main responsible for elaiosome attractiveness to ants (Marshall *et al.*, 1979; Skidmore & Heithaus, 1988; Brew *et al.*, 1989).

Despite the large number of myrmecochores described worldwide (Beattie & Hughes, 2002; Rico-Gray & Oliveira, 2007) there is still a lack of information on the chemical composition of elaiosomes for most of the species. Here, we report the fatty acid composition of the elaiosomes of two endemic *Euphorbia* as part of our work on ant-plant interactions in this genus.

Materials and Methods

Plant species and sampling sites

The two study species are Portuguese endemics that can only be found in the western part of this country. *Euphorbia pedroi* Molero & Rovira is a narrow endemic species restricted to three disjunct populations between Cabo Espichel and Cabo Ares. These populations are restricted to south-facing cliffs, where individuals can be found growing in soil pockets as well as directly from the rock fissures. This species is a perennial spurge of large dimensions (up to 2 m in height and more than 2 m width) showing phylogenetic affinities with the Macaronesian *Euphorbia* of the complex *E. lamarckii* (Molero *et al.*, 2002).

Euphorbia welwitschii (Boissduval & Reuter) is also endemic to western Portugal and has been recorded from some sparse locations, most of them situated around Lisbon (Franco, 1971). The consistent low number of individuals found in many populations of this species, together with the difficulty in locating some historical populations, is a matter of concern for the conservation of this species. *E. welwitschii* is an herbaceous perennial that may attain 1 m in height and occur on calcareous soils in scrublands and woodland margins. Both *Euphorbia* species have a diplochorous dispersal system with seeds being dispersed by ants following ballistic dispersal. The seeds of both species are very similar in size, but they can be easily separated according to form and colour.

The samples were collected from a single population of each species. Fruits of *E. pedroi* were collected from Ares (UTM: 29SMC9354) while those of *E. welwitschii* were sampled in Montejunto (UTM: 29SMD9436).

Elaiosome extraction and chemical analysis

In each of the two sampling sites, five individuals were randomly selected and their fruits collected and transported to the laboratory. There, the fruits of the same individual were placed together in a plastic box and left exposed to sunlight allowing xerochastic dehiscence. Seeds were then collected and the elaiosomes were carefully detached with the aid of forceps. Prior to the chemical analysis, the semi-dry weight of elaiosomes was measured to the nearest 0.1 mg.

The fatty acids methyl esters (FAME) were prepared according to the following procedure: 6 mg of elaiosomes were combined with 500 µl BF3/MeOH (20 %, for synthesis, Merck) and with 2 ml nonadecanoic acid (25 ppm, internal standard) (99 %, Sigma Aldrich) in Methanol. Trans-methylation was then carried out by heating to reflux at 95 ºC for 15 min. FAMEs were extracted for 2 minutes following the addition of 5 ml of NaCl (200 g/L, pro analysis, Merck) and 1 ml hexane to the reaction mixture. Then, the mixture was centrifuged during 3 minutes at 3300 rpm and the hexane layer was isolated for chromatographic analysis.

Chromatographic analyses were carried out using a 6890 Series II Agilent GC equipped with dual flame ionisation detection (FID) system and a HP-23 cis/trans FAME column (60 m x 0.25 mm x 0.25 µm, Agilent). The oven temperature was programmed from 130 to 230 °C. After 1 minute set at 130 °C, the increases progressed as follows: 130-160 ºC at 1.5 ºC/min, 160-170 ºC at 0.5 ºC/min, 170-230 ºC at 7 ºC/min and then held isothermally at 230 °C for 12 minutes. The injector and detector temperatures were maintained at 280 °C and 275 °C, respectively. The samples were injected (0.2 µL of pure oil) in the split mode (1:50) and helium was employed as carrier gas (1 mL/min). Two injections of the hexane solution (2 µL each) onto the column were performed from each sample.

Retention indices (RI) of compounds were determined relative to the retention times of a FAME standard mixture (37 FAME mix, Supelco) and a vaccenic methyl ester solution in hexane. Quantitative analyses were performed using nonadecanoic acid as internal standard, which was added at the beginning of the experimental procedure.

Results and discussion

Representative profiles of the fatty acid composition of the elaisomes of *Euphorbia pedroi* and *E. welwitschii* are presented in figures 1a and 1b, respectively.

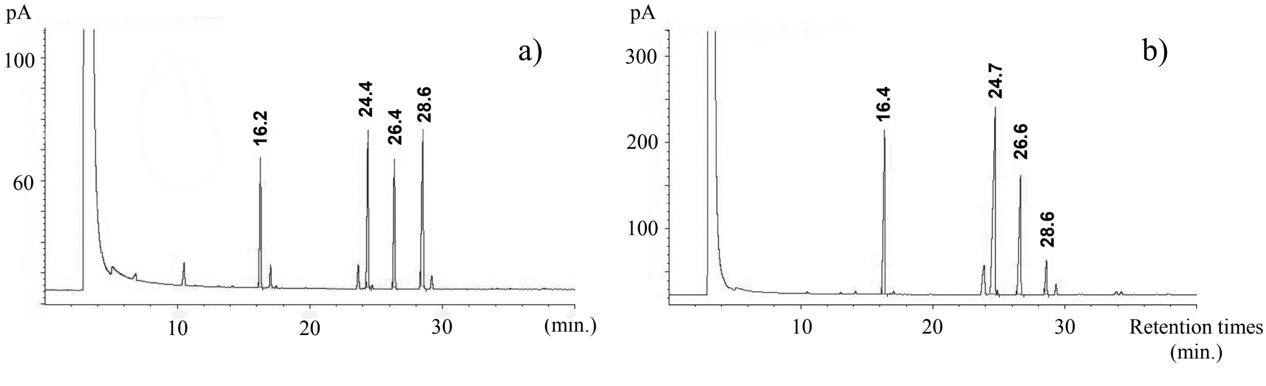

Figure 1. Typical gas chromatographs of FAME from *Euphorbia pedroi* (a) and *Euphorbia welwitschii* (b) elaiosomes. The retention times are indicated in each peak.

For comparative purposes, the fatty acid composition is presented jointly for both study plants (Fig. 2) with indication of interindividual variation levels.

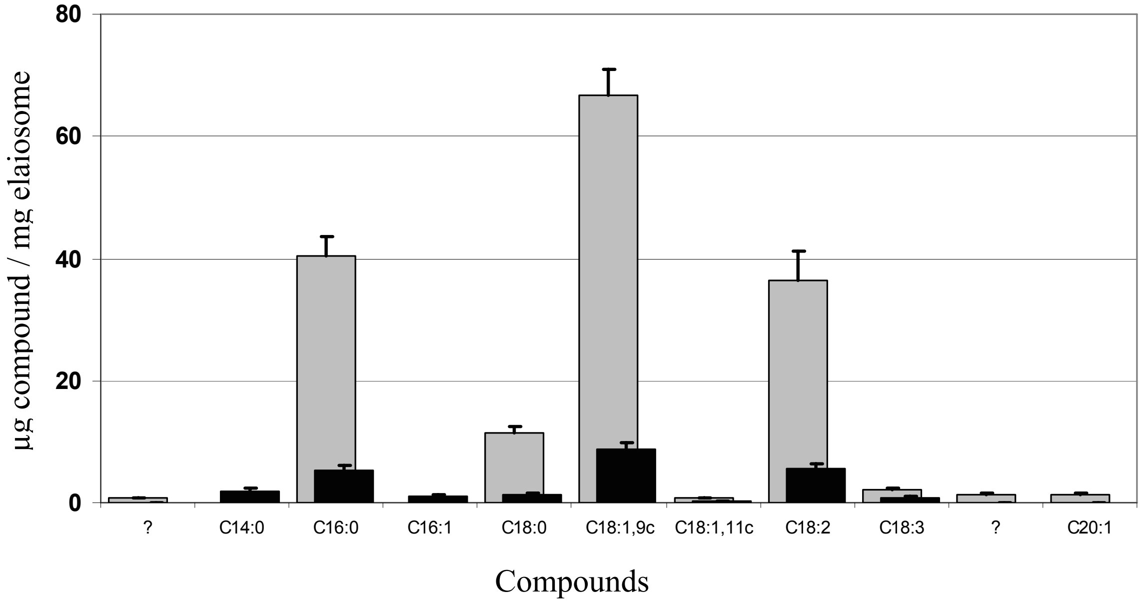

Figure 2. Fatty acid profiles of *Euphorbia pedroi* (black bars) and *Euphorbia welwitschii* (grey bars). Results are presented as mean ± S.D..

The chromatogram of *Euphorbia pedroi* shows eight peaks corresponding to the following fatty acids: miristic (14:0), palmitic (16:0), palmitoleic (16:1), estearic (18:0), oleic (18:1,9c), vaccenic (18:1,11c), linoleic (18:2), linolenic (18:3). The chromatogram of *E. welwitschii* identifies the same fatty acids found in the elaiosomes of *E. pedroi*, except miristic and palmitoleic acids. Additionally, eicosanoic acid (20:1) and two unidentified components were found in very low quantities in the elaiosomes of *E. welwitschii*. The major component in both *Euphorbia* species is oleic acid followed by palmitic and linoleic. All the other fatty acids occur in a much lower proportion (below 8%).

In spite of the marked differences in phylogeny and ecology between the two study species, these results highlight the similarities in their fatty acid profile. The few other studies on the chemical composition of elaiosomes from other plants of the family

Euphorbiaceae (Mackay & Whalen, 1998; Pizo & Oliveira, 2001; Peternelli *et al.*, 2008) also showed some interspecific affinities in the fatty acid profile.

The dominant fatty acid in the elaiosomes of both *Euphorbia* species – oleic acid – is most frequently identified as the major fatty acid component in the elaiosomes of other myrmecochores (Soukup & Holman, 1987; Skidmore & Heithaus, 1988; Kusmenoglu *et al.*, 1989; Lanza *et al.*, 1992; Hughes *et al.*, 1994; Mackay & Whalen, 1998; Bebawi & Campbell, 2005; Fischer *et al.*, 2008). Oleic acid, either in its free form or as a glyceride, has been identified as the chemical cue responsible for triggering a sequence of behaviours in ants which may culminate in seed carrying (Marshall *et al.*, 1979; Skidmore & Heithaus, 1988; Brew *et al.*, 1989; Kusmenoglu *et al.*, 1989; Lanza *et al.*, 1992). Early authors (e.g., Carroll & Janzen, 1973) provided an explanation for the ant removal of elaiosome-bearing seeds by considering that, from the ant's point of view, "the elaiosome is simply a dead insect analogue". However, two decades passed until Hughes and colleagues (1994) finally tested this hypothesis showing that the chemical composition of the elaiosomes have converged with the insect prey of ants. These authors showed experimentally that oleyl-based compounds, major components of both insect haemolymph (e.g., Beenakkers *et al.*, 1985) and elaiosomes, also play a key role as behaviour releasers in ants.

The differences in the amount of fatty acids in the elaiosomes of the two *Euphorbia* species may be related with their differential investment on seed dispersal by ants. Field observations and experiments showed that ants remove *E. welwitschii* seeds at higher rates than those of *E. pedroi* (Boieiro *et al.*, in prep). A positive correlation between oleic acid content in the elaiosomes and removal by ants has been suggested by some authors. For example, Boulay and colleagues (2007) found higher frequency of visits by dispersers to *Helleborus foetidus* plants with seeds bearing elaiosomes richer in oleic acid. Other authors have also stressed how interspecific differences in elaiosome chemical composition may translate in differences in removal rates by ants (Gunther & Lanza, 1989; Lanza *et al*., 1992; Pizo & Oliveira, 2001).

Despite the large number of myrmecochores identified worldwide (Beattie & Hughes 2002; Rico-Gray & Oliveira, 2007), the chemical composition of elaiosomes of most of the species remains unknown. In future, efforts should be addressed to improve our knowledge on the ecological chemistry of elaiosomes from different species and populations and to identify the sources of variation of elaiosome chemical composition and their consequences for myrmecochory, plant demography and community structure.

Acknowledgements

We thank J. Boieiro for help in collecting fruit samples. The Fundação para a Ciência e a Tecnologia, Portugal provided finantial support through grant PRAXIS XXI/BD/21407/99 to MB.

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Chapter 7

Seed dispersal by ants and short-term seed fate in three *Euphorbia* species

Boieiro, M., Serrano, A.R.M., Rego, C. & X. Espadaler (in preparation) Seed dispersal by ants and short-term seed fate in three *Euphorbia* species.

Seed dispersal by ants and short-term seed fate in three *Euphorbia* species

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Abstract

Myrmecochory is a widespread dispersal mechanism in plants and despite its prevalence in the Mediterranean region, it still remains poorly understood. Here we assessed the short-term fate of seeds from three *Euphorbia* species – the Portuguese endemics *E. pedroi* and *E. welwitschii* and the Mediterranean *E. characias* – which are known to be secondarily dispersed by ants. The three *Euphorbia* species showed significant differences in seed and elaiosome size, with *E. characias* having the largest seeds and elaiosomes. These differences in elaiosome size explained, in part, the differential attraction of ants for the seeds of the three *Euphorbia* species.

Observations on seed removal and exclusion experiments showed that ants are the most important seed dispersers in the five study areas with vertebrates (mostly rodents) being responsible for a minor fraction of seed removal. The major dispersers were two larger ant species (*Aphaenogaster senilis* and *Formica subrufa*) that rapidly collected the seeds and transported them for larger distances (on average around 2.5m). These two species were the only ants involved in seed removal during the hottest period of the day. After removing the elaiosome, both *A. senilis* and *F. subrufa* discarded most of the seeds unharmed outside the nest. Two smaller ant species (*Pheidole pallidula* and *Tapinoma nigerrimum*) and a granivorous one (*Messor barbarus*) removed a considerable percentage of seeds, mostly in the morning and afternoon. Seed dispersal distance for the two smaller ants was much shorter (on average around 0.75m) and once carried to the nest, seeds were soon after abandoned in a gallery following elaiosome removal.

The three *Euphorbia* showed different dispersal strategies and a direct consequence of the less attractiveness of seeds to seed-dispersing mutualistic ants was the higher average frequency of seed removal performed by granivorous *Messor barbarus*.

Keywords Ant-seed interactions · Dispersal distance · Elaiosome · Myrmecochory · Seed predation · Seed removal

Introduction

Seed dispersal is a key event in the life-cycle of plants encompassing a variety of processes that take place since a propagule separates from the parent plant until it comes to a final rest and germinates (Cousens *et al.*, 2008). Myrmecochory, the dispersal of seeds by ants, has been identified in at least 11,000 species from about a fifth of all plant families and seems to be a prevalent seed dispersal mechanism in particular environments (Beattie & Culver, 1981; Rice & Westoby, 1981; Bond & Slingsby, 1983; Beattie & Hughes, 2002; Rico-Gray & Oliveira, 2007). Furthermore, recent studies have highlighted the role of myrmecochory in angiosperm diversification and have clearly illustrated how poorly we still know the ecological and evolutionary relevance of myrmecochory in most terrestrial ecosystems (Lengyel *et al.*, 2009; 2010).

Myrmecochorous plants have seeds adapted for ant dispersal by having a specialized nutrient-rich appendage, the elaiosome. Mutualistic seed-dispersing ants usually carry the seeds to their nests, where the elaiosomes are removed and used for colony nutrition. The seeds are then left unharmed inside the nest or are discarded in the vicinities of the nest entrance.

The removal of seeds by ants may confer various benefits to the plant (see Beattie, 1985; Giladi, 2006; Rico-Gray & Oliveira, 2007), one of them being the reduction in seed losses to seed-eating animals. Rodents, birds and harvester ants are major post-dispersal seed predators in many terrestrial ecosystems and they may have a significant impact on the dynamics of plant populations (Hulme, 1998; Crawley, 2000; Hulme & Benkman, 2002). By removing the seeds away from the parent plant, mutualistic ant species allow them to escape predation and simultaneously relocate them in a microhabitat free from predation or where seed predators forage less intensively (*e.g.*, O'Dowd & Hay, 1980; Heithaus, 1981; Ruhren & Dudash, 1996).

The characteristics of the diaspore (the dispersal unit in plants) play a crucial role in ant attraction and in the success of transport by ants. Gorb and Gorb (2003) indicated what diaspore characteristics play a major role during the various stages (discovery and recognition, identification, removal and transport) leading to a successful transport by ants. In general, diaspore size, weight and shape together with elaiosome size and its chemical composition are key factors determining the probability of removal by ants (Beattie *et al.*, 1979; Gunther & Lanza, 1989; Oostermeijer, 1989). For example, Hughes and Westoby (1992a) performed some experiments on the effects of diaspore characteristics on removal by ants and concluded that two ant species responded positively to increasing elaiosome/diaspore ratios, but not all seed-dispersing ant species behaved this way.

The identity of the dispersal agent is fundamental for the comprehensive understanding of seed fate. In a particular habitat, there are usually a number of ant species involved in seed removal that differ in size and feeding ecology. Granivorous ants remove the seeds with the purpose of preying upon them and their negative effects on the reproductive output of plants may be quite significant (White & Robertson, 2009). Nevertheless there are some examples of successful seed dispersal provided by granivorous ants, resulting from seed dropping during transport to the nest (Retana *et al.*, 2004; Martínez-Duro *et al.*, 2010) or the rejection of viable seeds to the ant midden (O'Dowd & Hay, 1980). Mutualistic seed-dispersing ants are omnivorous species which carry elaiosome-bearing seeds to their nests and leave the seeds undamaged after elaiosome removal. Following a world review of myrmecochorous dispersal distances, Gómez and Espadaler (1998a) found that ants dispersed the seeds mostly at short distances (between 0 and 2m) with a maximum of 77m. More recently, Whitney (2002) reported a new record of ant seed dispersal distance, at

180m. The differences in seed dispersal distance by ants are usually related with ant size, with the larger species being responsible for removal at larger distances (Pudlo *et al.*, 1980; Gómez & Espadaler, 1998b; Ness *et al.*, 2004). In stable environments, where safe sites are rare, dispersal distance *per se* may be a potential benefit for plants since the generated seed dispersal curves, with a narrow peak and a long tail, are assumed to be optimal (Andersen, 1988a; Whitney, 2002).

Here we study the dispersal of seeds by ants in three *Euphorbia*, a common and relatively well studied species (*E. characias*) and two endemic species whose biology is still poorly known (*E. pedroi* and *E. welwitschii*). The aims of our work were: (1) assess the differences in diaspore size between the three *Euphorbia* species and evaluate the role of the elaiosome in seed removal, (2) identify the ant species involved in seed removal and assess their role as dispersal agents of the three *Euphorbia* in five study areas, and (3) evaluate seed dispersal distance and short-term seed fate by different dispersal agents.

Materials and Methods

Study species and sites

The three study species belong to genus *Euphorbia* Linnaeus, a diverse genus with a considerable number of endemics in the Iberian Peninsula (Benedí *et al.*, 1997):

- *Euphorbia pedroi* Molero & Rovira is a narrow endemic restricted to three populations along the western coast of Portugal, between Cabo Espichel and Cabo Ares. This perennial species is a sub-succulent xerophyte that may attain 2m in height and shows phylogenetic affinities with Macaronesian dendroid spurges (Molero *et al.*, 2002).

- *Euphorbia welwitschii* (Boissduval & Reuter) is also a Portuguese endemic, being known from scattered populations, most of them located near Lisbon. Some of these populations have a low number of individuals, often fail to recruit and face various threats from human activities. Furthermore, several historical populations of this species have not been relocated in recent years. This herbaceous perennial species may reach 90cm in height and is restricted to calcareous soils.

- *Euphorbia characias* Linnaeus presents a wide distribution along the Mediterranean Basin, from Portugal to Greece, and has also been reported from several North African countries. The species is commonly found in open and sunny habitats, where it usually occurs in large populations. This woody perennial species shows a variable size (usually between 50-100cm) with several individuals reaching about 150cm.

The fruits of all the three *Euphorbia* species are three-locular capsules, enclosing a seed per loculum. Fruit size, pubescence and ornamentation differ between the three species (Benedí *et al.*, 1997). In these species, as well as in other Iberian *Euphorbia*, primary seed dispersal results from explosive dehiscence of the fruit, with seeds being projected from some centimetres to a few meters away from the parent plant (Baiges *et al.*, 1991; Narbona *et al.*, 2005). The three study species have seeds with particular morphological characteristics and are all provided with an elaiosome.

The study took place in five sites where it is possible to find sympatric populations of two of the three study species, except for one of them (Risco). Risco was chosen for being the closest population of *E. welwitschii* to Ares, where the two other *Euphorbia* species co-occur. Site name, location (UTM coordinates) and study species present were as follows (see also Fig. 1):

- Montejunto (UTM: 29SMD9436), presence of *E. characias* and *E. welwitschii*.
- Tesoureira (UTM: 29SMD8709), presence of *E. characias* and *E. welwitschii*.

- Risco (UTM: 29SMC9757), presence of *E. welwitschii*.
- Azóia (UTM: 29SMC8753), presence of *E. characias* and *E. pedroi*
- Ares (UTM: 29SMC9354), presence of *E. characias* and *E. pedroi*.

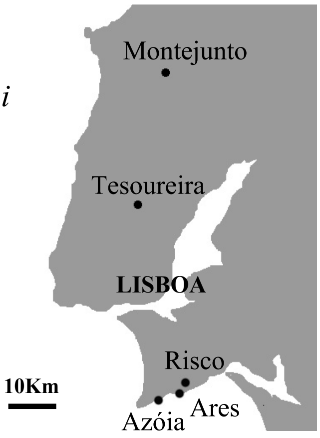

Figure 1 – Location of the study sites in western Portugal.

These sites were also selected since all of them present sparse vegetation cover allowing the performance of observations and experiments on ant-seed interactions.

All study areas are characterized by a Mediterranean-type climate and the vegetation is dominated by sclerophyllous species, particularly *Quercus coccifera*, *Pistacia lentiscus*, *Rosmarinus officinalis*, *Daphne gnidium*, *Lavandula luisieri*, and several *Cistus* species.

Seed characteristics

Differences in seed and elaiosome morphology between the three *Euphorbia* species were assessed by interspecific comparisons of seed size, seed weight and elaiosome size. Samples of 60 diaspores of *E. pedroi* and *E. welwitschii* and 120 diaspores of *E. characias*, collected from two populations (Ares and Montejunto), were used for the comparisons. Seed size and elaiosome size were measured with a stereoscopic microscope (Olympus SZX7) provided with an ocular micrometer at 25X magnification. Two measures (maximum length and maximum width) were taken from both seeds and elaiosomes. Seed mass was measured using a precision balance Precisa 205A SuperBal - series (±0.1mg).

Experiments on seed removal

The removal of seeds by ants was compared with removal by vertebrates (rodents and birds) by setting exclusion experiments which prevented the access to seeds of specific groups of animals. Four treatments were applied:

- Ant exclosure: single seeds were placed inside a small Petri dish (Ø=5.5cm, h=1.5cm) together with some soil to prevent seed rolling. The external border of the dish was covered with a sticky barrier made of polybutene-based glue to prevent access by ants. Vertebrates had access to the seeds.
- Vertebrate exclosure: single seeds were placed on the ground and covered by a wire cage (12x12x7cm) with mesh size of 1cm to prevent access by vertebrates. The cages were fixed to soil using wires. Ants could move freely inside the cage.
- Ant and vertebrate exclosure: a combination of the preceding treatments. Single seeds were placed with some soil inside a Petri dish, which had the external border coated with polybutene-based glue. The Petri dish was then covered with a wire cage.
- Free access: single seeds were placed on the ground in the open.

Twenty five sets, with the four treatments each, were set randomly in each of four study areas (Ares, Azóia, Montejunto and Tesoureira) to study seed removal of *E. characias*. The same design was adopted for the study of seed removal of *E. pedroi* in only two study areas (Ares and Azóia). Seed removal of the rare *E. welwitschii* was studied in Montejunto and Tesoureira, using only fifteen sets per study area. In each set the treatments were arranged at random with a minimum distance of half a meter between them. The experiment lasted for three-consecutive day periods with seed removal being monitored at 12h-periods (8h00-20h00 and 20h00-8h00). During monitoring, seed replacement was performed whenever necessary. Experiments were carried out during May-June 2002 and again in May-June 2003, encompassing the peak of explosive dispersal in each population.

Ant species, ant-seed interactions and short-term seed fate

The ant species present in each study area were identified following pitfall sampling as well as occasional direct collection and sampling with the use of tuna baits. Pitfall traps were set in continuum from April to July during two consecutive years (2002 and 2003) and the samples were collected after two-week periods. Four groups of five pitfall traps (Ø=2cm, h=10.5cm) were set randomly in each study area, with the traps lined and spaced by five meters in each group. Ethylene glycol (5%) was used as preservative together with some drops of detergent to break the surface tension. During the driest periods, the pitfall traps occasionally needed to be refilled with preservative due to evaporation. The ant specimens were identified to species level whenever possible. Voucher specimens of all ant species are held in a reference collection in the Faculty of Sciences, University of Lisbon.

Seeds from the three *Euphorbia* species were collected from mature fruits and used for the study of behavioural interactions in the same sites of collection. The field observations were performed during May-July in 2002 and 2003, when natural primary seed dispersal takes place. Single seeds were offered to ant foragers and their behaviour towards the seed was recorded together with ant species identity. A seed was considered to have been removed when was transported for at least 5cm (Hughes & Westoby, 1992b). Only the species actively involved in seed removal were further investigated. For these ant species we evaluated the role of elaiosome on seed removal by presenting separately entire diaspores, seeds deprived of elaiosome and elaiosomes of the three study *Euphorbia* and recording ant species behaviour towards those items.

The role of each ant species as a seed dispersal agent of *Euphorbia* was assessed by placing four conspecific seeds, spaced by approximately 10cm, at the soil surface and waiting for an ant-seed contact. The use of a set with four seeds was merely to improve the probability of ant-seed interactions since after the first successful removal the location of the observations was again selected at random. When a seed was taken by an ant, the individual was then identified to species level. These observations were made between 8h00-20h00, at hourly intervals, during the peak of fruit dehiscence in each study area. Fruit dehiscence in *E. characias* is known to be relatively homogeneous throughout the day (Espadaler & Gómez, 1996). The observations on ant-seed interactions and seed removal by ants were made by direct observation and with the aid of a digital camera JVC GR-DVL 355EG.

The distances of dispersal by ants were assessed by applying the same observational design described above. When a seed was taken by an ant, the individual was identified to species level and followed until it reached its nest, dropped the seed on its way or was robbed by other ant species. Seed dispersal distance and destination were then recorded.

To evaluate short-term fate of seeds once carried to ant nests, we fed four nests of four ant species (*Aphaenogaster senilis*, *Formica subrufa*, *Pheidole pallidula* and *Tapinoma nigerrimum*) with 40 marked seeds of *E. characias.* Nest entrances were isolated with collared carton cages (Ø=45cm, h=15cm) having the superior inner border coated with polybutene-based glue to prevent ants taking seeds away from the enclosure. Seed rejection from nests was monitored following a 24h-period. These observations were performed at Montejunto, Tesoureira and Azóia by the end of the primary dispersal period in *E. characias*. We decided not to include *Messor barbarus* in these observations since this species may use several nest entrances during foraging activities posing some technical difficulties to set the observational design. Nevertheless, we know that this ant species behaves as a post-dispersal seed predator destroying nearly all *E. characias* seeds they collect (Gómez & Espadaler, 1997; M. Boieiro, unpubl. data).

Data analysis

The differences on seed and elaiosome size between the three study plants were assessed by one-way ANOVA on log-transformed data. The data were previously transformed in order to meet the criteria of normality and variances homogeneity. Where ANOVAs were significant, individual means were compared using Tukey *post hoc* tests with the significance level set at 0.05. The same procedure was adopted to assess the differences on seed weight between the three *Euphorbia* (in this case the data were square-root-transformed). The association between variables measuring seed and elaiosome size for each *Euphorbia* species were analyzed by Spearman Rank correlation analysis. Data on removal rates by different animal groups were analyzed by three-way ANOVA. The analyses were performed separately for each plant species on seed removal frequencies by daily period (dependent variable). The independent variables were population, day period and treatment (free, ant exclusion, vertebrate exclusion and, ant and vertebrate exclusion). Significant differences between means were subsequently assessed by Tukey's multiple comparison tests. The distribution of the data on seed dispersal distances originated by each ant species was highly skewed to the right. For this reason, data transformations did not help to improve normality. The dispersal distances provided by dispersal agents were thus compared using the Kruskal-Wallis test. All analyses were performed using Statistica (StatSoft, 2008).

Results

Seed characteristics

The diaspores of the three study *Euphorbia* showed interspecific differences in seed ($F_{2,237}$=63.1, P<0.0001) and elaiosome size ($F_{2,237}$=632.6, P<0.0001). Both, seed and elaiosome size were larger in *E. characias* than in the two other congeners (Fig. 2)(Tukey's test, P<0.05). In general, there was a low positive correlation between seed size and elaiosome size for each study species (most $r_s \leq 0.29$), although higher correlation values were found between elaiosome width and seed height (r_s = 54.4) and width (r_s = 68.2) in *E. welwitschii*. Elaiosome/seed size ratios differed between the three species being higher in *E. pedroi* (0.45), intermediate in *E. characias* (0.39) and lower in *E. welwitschii* (0.35). Seed weight also differed between the three *Euphorbia* species ($F_{2,237}$=358.7, P<0.0001). Mean±SD: *E. characias* = 6.7±0.7mg; *E. welwitschii* 4.6±0.6mg; *E. pedroi* 7.6±0.7mg.

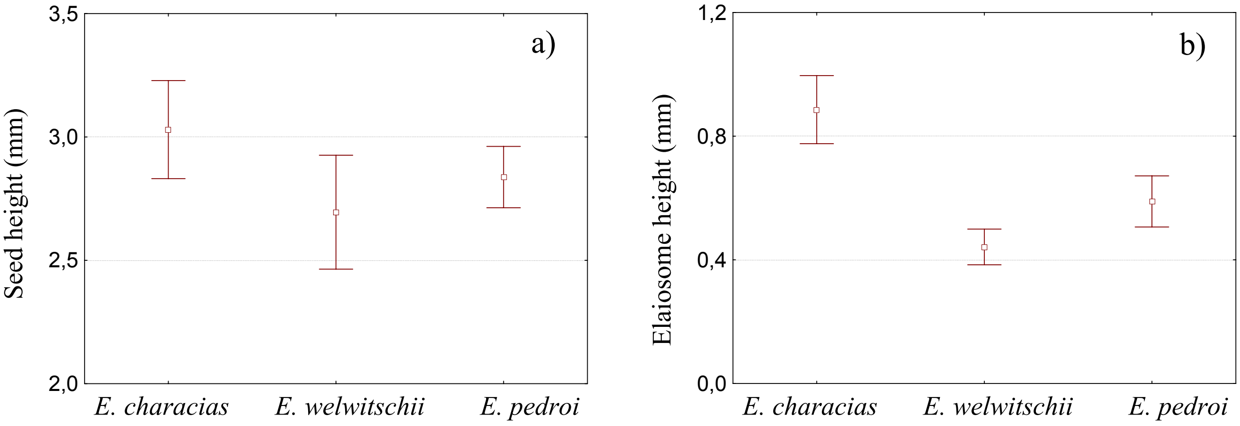

Figure 2 – Seed (a) and elaiosome (b) height of the three *Euphorbia*. Results are presented as means±SD.

Experiments on seed removal

There were marked differences on seed removal between the four treatments for all the three *Euphorbia* (Table 1; *E. characias*: $F_{3,184}$=1774.8, $P<0.0001$; *E. welwitschii*: $F_{3,90}$=698.8, $P<0.0001$; *E. pedroi*: $F_{3,90}$=352.6, $P<0.0001$). Ants were by far the major group involved in seed removal with the values reported from vertebrate exclosures (accessible to ants) being similar to those recorded from freely available seeds (Table 1). Seed removal by ants took place both during day and night. Vertebrates were responsible for a low percentage of seed removal, which occurred mainly during the night period (all $P<0.01$), an indication that rodents were probably responsible for seed removal. Nocturnal vertebrate seed removal levels were somewhat related with ground vegetation cover since the habitats with poor herbaceous cover (Ares and Azóia) had the lowest removal rates.

Table 1 – Seed removal of three *Euphorbia* by ants and vertebrates (in percentage) during two periods of the day (D=8h-20h, N=20h-8h). Data from 2002 and 2003 were combined. The treatments are vertebrate exclusion (VertExc), ant exclusion (AntExc), vertebrate and ant exclusion (ExcAll) and no exclusion (Free).

Plant Species	Site	Period	Free	VertExc	AntExc	ExcAll
E. pedroi	Azóia	D	39.3	46.0	2.7	0.0
		N	56.7	53.3	6.7	0.0
	Ares	D	34.0	32.7	0.0	0.0
		N	48.0	40.7	2.0	0.0
E. welwitschii	Montejunto	D	74.4	81.1	0.0	0.0
		N	85.6	77.8	8.9	0.0
	Tesoureira	D	74.4	76.7	2.2	0.0
		N	78.9	85.6	18.9	0.0
E. characias	Montejunto	D	86.7	79.3	4.0	0.0
		N	74.7	77.3	12.7	0.0
	Tesoureira	D	83.3	72.7	5.3	0.0
		N	81.3	78.7	12.0	0.7
	Azóia	D	64.7	72.7	0.0	0.0
		N	72.0	69.3	3.3	0.0
	Ares	D	64.7	56.0	1.3	0.0
		N	59.3	64.0	0.7	0.0

In two of the treatments (freely available seeds and vertebrate exclosures), some seeds were found to have been handled, but not removed from the spot, and most of them lacked part or the whole elaiosome. This was due to small ants (e.g., *Crematogaster* spp., *Temnothorax* spp. and *Tetramorium* spp.), which are not able or have difficulty in carrying the seeds and thus feed on the elaiosome *in situ*, leaving the seed unharmed.

Ants, ant-seed interactions and short-term seed fate

Forty six ant species were recorded from the five study areas (see Appendix 1). Most of those species were Myrmicinae (28), some were Formicinae (15) and a few were Dolichoderinae (3). Species richness varied between study areas from 19 to 25 with the highest value being recorded at Risco. Eight species were common to all five study areas, many (22 species) were exclusive to a single study area and a high number of them were recorded with low abundance. Most of the species recorded are nectarivorous/omnivorous with only seven species using seeds as the main food resource (genera *Messor*, *Goniomma* and *Oxyopomyrmex*). Furthermore, nectarivorous/omnivorous ant species were dominant in all study sites while all granivorous species, but *Messor barbarus,* were uncommon or rare.

Only a number of species in each study area were assessed to evaluate their role on the dispersal of *Euphorbia* seeds. Those that were *a priori* excluded from the assays exhibited specific morphological (low ant size, low mandible gap), ecological (nectar-feeding, aphid-tending) and populational (rare species, no nests located) features indicative of their unimportant or minor role as seed dispersers. Six ant species – *Aphaenogaster senilis*, *Messor barbarus*, *M. structor*, *Pheidole pallidula*, *Tapinoma nigerrimum* and *Formica subrufa* – removed a considerable percentage of the seeds they contact with (Table 2).

Table 2 – Percentage of seed removal of three *Euphorbia* species by different ant species (data were pooled for ant species occurring in more than one population).

	E. pedroi		***E. welwitschii***		***E. characias***	
Ant species	**% removal**	**N**	**% removal**	**n**	**% removal**	**n**
Aphaenogaster senilis	29.3	75	58.3	120	75.5	225
Crematogaster auberti	0.0	25	0.0	50	2.0	50
C. scutellaris	0.0	25	0.0	25	0.0	25
Goniomma kugleri	0.0	25	-	-	0.0	25
Messor barbarus	85.3	150	92.0	50	90.5	190
M. structor	87.1	62	-	-	94.4	54
Pheidole pallidula	52.7	150	80.7	150	86.2	225
Tetramorium semilaeve	11.7	60	14.6	48	23.8	80
Tapinoma nigerrimum	22.0	50	36.0	25	55.0	80
Camponotus cruentatus	-	-	0.0	25	8.0	25
C. sylvaticus	0.0	25	-	-	0.0	25
Formica subrufa	41.7	60	-	-	68.3	60
Lasius grandis	-	-	4.0	25	4.0	25

The other ant species showed no interest on the seeds, were not capable to transport them despite the attraction shown for the elaiosome or made removal of seeds sporadically. The ant species involved in seed removal (except *Messor* spp.) showed different attraction for the seeds of the three *Euphorbia*, since almost invariably *E. characias* seeds were removed in high percentage followed by those of *E. welwitschii* and *E. pedroi* (Table 2).

The observations on the removal of diaspores, elaiosomes and seeds deprived of elaiosomes highlighted the differences in the behaviour of the dispersal agents. *Messor barbarus*, a granivorous species, showed no preference removing equally the three items while all the other ant species showed little interest for the seeds deprived of elaiosomes (Table 3). Among the latter species, removal of elaiosomes was frequently higher than removal of the whole diaspore. The percentage of diaspore and elaiosome removal by ants was, in general, higher for both *E. characias* and *E. welwitschii* than for *E. pedroi* (Table 3).

Table 3 – Ant behaviour in the presence of seeds (S), elaiosomes (E) and seeds deprived of elaiosomes (SE). The observations were carried at Ares, Tesoureira and Azóia (just for *F. subrufa*). (results on removal probability are in percentages, data were pooled for ant species occurring in Ares and Tesoureira, all n=50).

	E. pedroi			***E. welwitschii***			***E. characias***		
Ant species	**S**	**E**	**SE**	**S**	**E**	**SE**	**S**	**E**	**SE**
Aphaenogaster senilis	38	46	0	64	90	2	68	92	6
Messor barbarus	92	96	88	98	94	94	96	88	100
Pheidole pallidula	54	76	4	72	100	2	90	98	4
Tapinoma nigerrimum	32	36	0	22	70	0	48	68	0
Formica subrufa	32	38	2	-	-	-	62	74	0

There was spatial variation in the role of each ant species as dispersal agent of *Euphorbia* seeds, with omnivorous ants being responsible for a larger fraction of seed removals (Appendix 2). *A. senilis* and *F. subrufa* were the major seed dispersal agents of the three *Euphorbia* species, with *P. pallidula* having also an important role as seed disperser at Risco and Azóia. Granivorous ants (*M. barbarus*) removed a reasonable proportion of seeds of the three *Euphorbia* species in all study areas (between 7-29%). In each study area, there was also variation in the percentage of seed removal by each ant species according to the specific identity of seeds (Appendix 2). In general, granivorous ants removed a larger percentage of seeds of *E. welwitschii* and *E. pedroi* than from their co-occurring congener *E. characias*. The seeds of this latter species were removed predominantly by the larger omnivorous ants (*A. senilis*, *F. subrufa*). The differences in seed removal by larger omnivorous ants at Ares for the two *Euphorbia* species were due to different microhabitat preferences since *F. subrufa* foraged preferentially near the stands of *E. pedroi*, where drier conditions prevail. During the day, the role of each ant species as a seed disperser varied accordingly to its daily activity and the competition from other ant species. *F. subrufa* and *A. senilis* were responsible for the seed removal during the hottest period of the day since these were the only dispersal agents active at this time (Appendix 2). During the morning and evening a larger number of ant species was involved in seed removal and occasionally ants of different species became involved in disputes over the seeds.

The average and maximum distances that seeds were carried differed between ant species (Table 4)(Kruskal-Wallis test: $H_{4,828}$=428.9, $P<0.0001$). *A. senilis* and *F. subrufa* removed the seeds to larger distances, on average near 2.5m from the point of collection and occasionally those distances were higher than five meters. On the other hand, *T. nigerrimum* and *P. pallidula* displaced the seeds at shorter distances, on average less than a meter and rarely exceeding two meters.

Table 4 – Average distance (±SD) of seed transport by five ant species and the percentage of seeds carried to the nest of the dispersal agent. Data from different seed species and years were grouped. Distance data are in centimetres.

Ant species	Distance (X±SD)	Amplitude	Nest (%)
A. senilis	242.2± 127.7	17-664	97.7, n=221
M. barbarus	123.6±65.6	16-405	93.7, n=190
P. pallidula	51.1±41.5	9-239	87.0, n=154
T. nigerrimum	84.6±37.7	9-214	76.6, n=171
F. subrufa	274.7±140.4	43-906	98.9, n=92

For the five ant species, the destination of the seeds was almost invariably the ant nest (Table 4). However, the two smaller species – *T. nigerrimum* and *P. pallidula* – were occasionally subjected to cleptoparasitism by larger species, particularly at Ares. These two species together with *M. barbarus* also lost a fraction of seeds during transport to the nest. Once carried to the nests, the fate of seeds usually depends on the dispersal agent identity (Kruskal-Wallis test: $H_{3,24}$=19.9, $P<0.001$). *Messor barbarus* destroys the seeds and discards the remains outside the nest while the four omnivorous species remove the elaiosome from the seed, leaving it undamaged (Gómez & Espadaler, 1997; M. Boieiro, unpubl. data). During a 24h-period following seed transport to the nest, the large omnivorous species (*A. senilis* and *F. subrufa*) discarded most of the seeds outside the nest

(Table 5), all of them lacking the elaiosome. On the other hand, the two smaller ant species made no rejection of seeds from their nests.

Table 5 – Short-term fate of *E. characias* seeds following a 24h-period after being fed to ant nests. Four nests per species were monitored in each site. Forty seeds were fed to each nest.

	Montejunto		**Tesoureira**		**Azóia**	
Ant species	Rejected (%)	Inside nest (%)	Rejected (%)	Inside nest (%)	Rejected (%)	Inside nest (%)
Aphaenogaster senilis	81.9	18.1	85.6	14.4	-	-
Pheidole pallidula	0.0	100.0	-	-	0.0	100.0
Tapinoma nigerrimum	-	-	0.0	100.0	-	-
Formica subrufa	-	-	-	-	89.4	10.6

Discussion

The three *Euphorbia* species showed differences in seed weight and on traits indicative of seed and elaiosome size and those differences may, at least in part, be associated with the seed dispersal strategy of each species. *Euphorbia characias*, the species with larger seeds and elaiosomes, was invariably the one whose seeds were collected in higher frequency by ants. Seed and elaiosome size have been reported as determinant traits for the interpretation of removal success by both predators and mutualistic dispersers in a variety of ecosystems (Janzen, 1971; Hulme & Benkman, 2002; Gorb & Gorb, 2003; Edwards *et al.*, 2006). For example, Hughes and Westoby (1992a) showed experimentally that both diaspore size and the relative size of the elaiosome and seed had significant effects on removal by ants in Australian open woodlands. However, they noticed that different ant species based their selection on different seed cues: for small granivorous *Pheidole* diaspore size was the most important influence on removal while two large omnivorous species responded positively to increasing elaiosome/seed size ratios. Other observations on seed removal rates by ants, from different areas of the world, also pointed to a role of elaiosome/seed ratios and elaiosome size on removal rates by ants (*e.g.*, Oostermeijer, 1989; Gorb & Gorb, 1995; Gorb, 1998; Edwards *et al.*, 2006). There is however a number of exceptions to this rule and some authors have hypothesized that ants may respond, not just only to characteristics related with size, but to a complex of diaspore characters (Gunther & Lanza, 1989; Lanza *et al.*, 1992; Midgley & Bond, 1995). Our results on seed removal rates can also not be fully understood solely considering seed and elaiosome size characteristics. In fact, *E. pedroi*, despite having the largest elaiosome/seed size ratios was the species whose seeds were removed with the lowest frequency. In two companion manuscripts, we showed that the chemical composition of the elaiosomes of *E. pedroi* is different from those of the two other species, particularly in what concerns the concentration of components (*e.g.*, oleyl-based compounds) that are known to be responsible for the attractiveness of elaiosomes to ants and to stimulate seed carrying behaviour (Boieiro *et al.*, subm.; Boieiro *et al.*, in prep.). Gómez and Espadaler (1995) had also found that the low removal rates of *Euphorbia helioscopia* seeds by *Pheidole pallidula* ants were probably due to the lack or low concentration of lipids on its elaiosomes (see also Bresinsky, 1963). Thus, the chemical composition of elaiosomes seems to be a critical parameter that should be considered if we are to understand differences in diaspore attractiveness to ants.

The elaiosome is undoubtedly the key element of myrmecochory like has been shown in a large number of studies that followed the monumental work of Sernander (1906). Elaiosomes do vary in size, shape and chemical composition, but most of all is their presence or absence that strongly determines seed fate. It has been shown that seeds with tiny elaiosomes or with elaiosomes scraped off still remain very attractive to ants (Sernander, 1906). However, we found that once deprived of the elaiosome, seed removal rates of the three study species decreased dramatically since seeds were ignored by most ant species. Similar results were found in many other plant genera from various places around the world (Sernander, 1906; Oostermeijer, 1989; Hughes & Westoby, 1992a; Garrido *et al.*, 2002; Leal *et al.*, 2007; Servigne & Detrain, 2008; Bas *et al.*, 2009). When deprived of the elaiosome, undispersed seeds become more vulnerable to seed predator attack (*e.g.*, harvester ants, rodents and birds) and, in general, strongly dependent on abiotic means of seed dispersal (*e.g.*, wind, water) to reach favourable microsites. On the contrary, ant dispersal often moves seeds to discrete and predictable microsites, where the probabilities of seed germination and seedling establishment are generally much higher (Beattie, 1985; Giladi, 2006).

Our results show that ants were the major animal group involved in the removal of *Euphorbia* seeds. This conclusion is based on evidence from both direct observations and exclosure experiments which showed that seeds were removed by ants at high rate, during both day and night, in all study sites. Other co-occurring terrestrial arthropods (*e.g.*, ground beetles, seed bugs) were also assessed for their attraction towards the seeds and to evaluate their role as seed removers, but they showed no interest on seeds or seed removal was negligible (M. Boieiro, unpubl. data). Vertebrates were responsible for a minor fraction of seed removal and their effects were more evident during the night period, an indication that rodents were probably the agents of dispersal. Rodents are known to prey upon elaiosome-bearing seeds in various habitats and their impact on seed removal may occasionally be high (Heithaus, 1981; Bond & Breytenbach, 1985; Smith *et al.*, 1986; Hughes & Westoby, 1990; Gibson, 1993; Espadaler & Gómez, 1996; Auld & Denham, 1999; Manzaneda *et al.*, 2005). Rodents have often been considered to have a dual role on seed fate since they may prey the major fraction of seeds they collect, but some seeds may be forgotten in seed caches and eventually germinate (*e.g.*, Herrera, 2002). In the present study, rodents acted as true seed predators because remains of the testa were found on the ant exclosures indicating feeding *in situ* by nocturnal vertebrates. Furthermore, seed dispersal in the study *Euphorbia* takes place mostly during May-early June, a period when rodents are actively in search of food and not involved in food hoarding. Birds may have also been involved in post-dispersal predation of *Euphorbia* seeds since low levels of seed removal were detected from ant exclosures during the day period. In a study on the dispersal biology of *Euphorbia balsamifera*, Berg (1990) has shown that seed removal by pigeons (*Columba livia canariensis*) was relatively high (near 21%) and made some comments on the probability of those dry dispersal units passing unharmed the digestive tract of the bird (a termed he coined sclerendochory). Pigeons were quite rare in our study sites and we suppose that seed removal by vertebrates during the 8h00-20h00 period was due to granivorous Passeriformes and/or to the activity of rodents early in the morning or in the evening. The frequencies of seed removal by vertebrates recorded in this study may be underestimates due to avoidance behaviour in face of the exclosures. Nevertheless, similar experimental designs have been applied frequently since the early 80s to assess seed removal rates by vertebrates comparatively to ants in a large number of ecosystems worldwide and their results are assumed to be reliable estimates (O´Dowd & Hay, 1980; Heithaus, 1981; Bond & Breytenbach, 1985; Smith *et al.*, 1986; Hughes & Westoby, 1990; Gibson, 1993; Espadaler & Gómez, 1996; Auld & Denham, 1999).

Ants were the main seed dispersal agent for the three *Euphorbia* species, but only a number of ant species in each site were actively engaged in seed removal. Despite the attractiveness of the elaiosomes for most of the ant species, some of them were unable or had difficulties in carrying the seeds due to their small size and short mandible gap (e.g., *Crematogaster* spp., *Temnothorax* spp. and *Tetramorium* spp.). These smaller ant species frequently chew the elaiosomes, remove small pieces of it and may also be involved in whole elaiosome feeding/removal *in situ*. The consequences of this behaviour on seed fate have rarely been studied, but these ant species are often coined as parasites of the mutualistic seed dispersal system since by removing the elaiosome they leave seeds unlikely to be removed by their legitimate dispersers and thus more susceptible to predation.

The ant species involved in seed removal were, in general, the same for the three *Euphorbia* species. This is in accordance with previous studies that have shown that the interaction between ants and myrmecochorous diaspores is characterized by a lack of specificity with local ants removing seeds from a variety of syntopic plant species. Furthermore, there were no considerable differences in the number and identity of the seed dispersal agents between sites, with two species being common to all five study areas, probably due to the proximity of the five study areas (the largest distance between them being 85km). Nevertheless, several authors defend that there is some predictability on the association between myrmecochores and the number and identity of their seed dispersal agents at different spatial scales. For example, Espadaler and Gómez (1996), who have studied myrmecochory in *Euphorbia characias,* found that the four ant genera involved in seed removal at Collserola (Barcelona) also co-occur in a large number of Mediterranean countries, covering the distribution of that plant species. In fact, the same four ant species are also the major seed dispersers of *E. characias* in this study. So, in some dispersal systems, there seems to be some taxonomic consistency on the guild of dispersal agents at both the interpopulational and regional scales. Other studies however failed to find such a relation. A recent study on the geographic variation in the ant assemblages engaged on seed dispersal of *Helleborus foetidus*, performed along a latitudinal transect in the Iberian Peninsula, reported significant spatial differences on the composition of ant guilds (Manzaneda *et al.*, 2007; Rey & Manzaneda, 2007). The most frequent visitors of plants in the different study areas were, in general, ants from different genera, most of them formicines (*e.g.*, *Camponotus*, *Formica* and *Lasius* species). In our study system and at each site, the ant assemblage involved in seed removal comprehended both granivorous species (*M. barbarus*) that collected the seeds and virtually fed on all of them and omnivorous mutualistic seed-dispersing species (*A. senilis*, *F. subrufa*, *P. pallidula* and *T. nigerrimum*). The latter group of species showed significant differences in seed removal between the three *Euphorbia* species (Table 2) as a response to differences in diaspore traits. On the other hand, granivorous *Messor* removed equally seeds of the three *Euphorbia* species (Table 2). These harvester ants feed upon a large number of seed species and usually rapidly collect any edible vegetal material they find during their foraging trips (Detrain & Pasteels, 2000; Azcárate *et al.*, 2005), which is later subjected to a more careful selection inside the nest.

There was some spatial variation at the population level in the average frequency of seed removal by each ant species. *A. senilis* and *F. subrufa* were co-dominant as the most frequent seed dispersal agents – they were the most frequent seed dispersers when in allopatry and when they co-occur their seed removal frequency values reflected their foraging preferences at the microhabitat level. Both genera have been reported as major seed dispersers in other habitats and continents highlighting their role as keystone mutualists (Gorb & Gorb, 2003; Manzaneda & Rey, 2009; Ness *et al.*, 2009). Species of these genera are medium to large size ants that intensively search for food, move rapidly and are capable to transport heavier loads at larger distances than co-occurring smaller species. In the present study, *A. senilis* and *F. subrufa* matched those characteristics and quickly removed seeds at larger distances (on average around 2.5m) than any of the other species. Several authors defend that ants of these genera may provide simultaneously several benefits to myrmecochores since rapid removal reduces the probability of seeds being lost to predators and larger distances of transport minimize the negative effects of distance- and density-dependent factors (Gorb & Gorb, 2003; Ness *et al.*, 2009).

Furthermore, seeds may be relocated to more favourable microsites where seed germination and seedling growth may be enhanced (*e.g.*, Beattie & Culver, 1983; Andersen, 1988b; McGinley *et al.*, 1994). We found that, after feeding on the elaiosome inside the nest, both *A. senilis* and *F. subrufa* abandoned most of the seeds unharmed outside the nest following a short period of time. The seeds deprived of the elaiosome are dropped at a short distance from the nest entrance (8-43cm) and may benefit from the specific properties of the soil that surround ant nests which have occasionally been considered to be richer on several nutrients important for seedling growth and establishment (*e.g.*, Beattie & Culver, 1983; Oostermeijer, 1989). The prominent role of *A. senilis* and *F. subrufa* as seed dispersal agents of the three *Euphorbia* species was also due to the fact that these species were the only dispersers during the hottest hours of the day, when the other species are inactive. These heat-tolerant ant species forage near their critical thermal limits and it has been found that they have better performances at high temperatures despite the high mortality risk they incur (Cerdá *et al.*, 1997; 1998). Cerdá and colleagues (1998) found that heat-tolerant species of Mediterranean communities occupy a low position in the dominance hierarchy of the community, *i.e.* they are frequently victims of aggression and have to escape from other species during interspecific encounters. The subordinate behaviour of these ants is also a determinant trait to explain their important role as mutualistic seed dispersers (Ness *et al.*, 2009; see also Gove *et al.*, 2007 and Lubertazzi *et al.*, 2010 on *Rhytidoponera*). Subordinate species rapidly remove seeds following their discovery and do not feed *in situ* to avoid interference competition from dominant species. Furthermore, the colonies of subordinate species are usually well represented in ant communities since those species are tolerated by dominant ones and their foraging areas overlap with those of conspecific and heterospecific colonies.

The other ant species (*e.g., P. pallidula* and *M. barbarus*) also played an important role on the removal of *Euphorbia* seeds since they were frequently involved in seed removal during the morning and afternoon. These species also removed *Euphorbia* seeds during night (from 20h00 to 8h00), but it was not possible to evaluate the frequency of removal made by each ant species during that period. *Pheidole pallidula* and *T. nigerrimum* carried the seeds at short distances and due to their difficulties in handling the seeds (mostly *T. nigerrimum*) they occasionally dropped some seeds during transport or were robbed by larger, fast-moving species. Even so, the major fraction of seeds was successfully carried to their nests. Gómez and Espadaler (1997) have studied in detail what happens to *E. characias* seeds inside the nests of these two species and found that once removed the elaiosome, ants lose their interest on seeds and abandon them in nest galleries. This behaviour is a consequence of the difficulty of these small ants, with a low mandible gap, to grab the seeds and transport them outside the nest once the elaiosome has been removed (Gómez *et al.*, 2005). In many plant species, the elaiosome provides the handle that allows ants to hold the seeds and transport them to their nests (*e.g.*, Oostermeijer, 1989; Gómez *et al.*, 2005). However, once removed the elaiosome, the rejection of larger seeds and seeds with a smooth surface outside the nest turns a hard challenge (occasionally insurmountable) for smaller ant species. Both minor and major workers of the harvester ant *Messor barbarus* were engaged in the removal of *Euphorbia* seeds. Several studies highlighted the dual role of harvester ants as seed predators and dispersers in a number of plant species (Danin & Yom-Tov, 1990; Pacini, 1990; Boyd, 2001; Retana *et al.*, 2004; Martínez-Duro *et al.*, 2010), but that has not been found in this ant-plant system since virtually all seeds collected were destroyed and used to feed the colony (Gómez & Espadaler, 1997; M. Boieiro, unpubl. data). Furthermore, the intense movement of workers along the trails makes unlikely that the low proportion of seeds dropped during transport to the nest may escape from seed predation. Only in one occasion we observed four seedlings of *E. characias* in a mound of *M. barbarus* at Ares and all failed to establish probably as a consequence of predation by the ants.

From our observations and experiments with seeds from three *Euphorbia* species we conclude that both elaiosome size and chemistry are determinant to explain the differential probability of removal by seed-dispersing mutualistic ant species. *Euphorbia characias* has the largest seeds and elaiosomes and was the species that mostly attracted the dispersal agents. On the other hand, the two other *Euphorbia* species invested less on seed and elaiosome size and were less attractive to ants, particularly *E. pedroi* whose elaiosomes have a low concentration of some important fatty acids on their composition. A consequence of the less attractiveness of *E. pedroi* seeds to mutualistic ants is the higher average frequency of seed removal performed by granivorous *Messor* compared to what was recorded in the other *Euphorbia,* meaning that a high percentage of seeds are lost to predation. The assemblage of ants involved in seed removal showed some similarities between the five study areas and the most frequent dispersal agents were two larger ant species that matched the profile of a keystone seed disperser. Seed-dispersing mutualistic ants rapidly removed the seeds from the surface near the parent plant and this may confer several benefits to the plants, including seed predator avoidance. In this study, we found that both vertebrates and harvester ants removed a considerable fraction of seeds in all study areas and, in the short-term, their negative effect on seed survival was prevented from being much higher by the activity of mutualistic disperser ants. Studies on the long-term fate of seeds inside the nests of *Pheidole* and *Tapinoma* and on those discarded out of the nest by *Aphaenogaster* and *Formica* are needed to ascertain the true biological benefits of this seed-dispersal mutualism.

Acknowledgements

We greatly acknowledge J. Boieiro for his valuable help both during field work preparation as well as during the performance of observations and experiments in the field. M.J. Pinto discussed with us important aspects related with the taxonomy, ecology and distribution of Portuguese *Euphorbia*. Support was provided by Fundação para a Ciência e a Tecnologia through grants PRAXIS XXI/BD/21407/99 to MB and SFRH/BPD/66934/2009 to CR.

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Appendix 1 – Ant species recorded in the five study areas during May-July in the years 2002 and 2003. Feeding habits are indicated as follows: O - omnivorous; G - granivorous; N - nectarivorous and/or aphid tending.

Subfamily/Species		Montejunto	Tesoureira	Azóia	Ares	Risco
Myrmicinae	**Feeding habits**					
Aphaenogaster dulcinea	**N**	X		X	X	
A. gibbosa	**O**	X	X	X	X	X
A. iberica	**O**				X	
A. senilis	**O**	X	X		X	X
Crematogaster auberti	**O,N**	X	X	X	X	X
C. scutellaris	**O,N**				X	X
C. sordidula	**N**	X	X	X	X	X
Goniomma hispanicum	**G**					X
G. kugleri	**G**			X		
Messor barbarus	**G**	X	X	X	X	X
M. bouvieri	**G**	X		X	X	
M. capitatus	**G**			X	X	
M. structor	**G**			X	X	
Myrmecina graminicola	**O**	X				
Myrmica aloba	**O,N**	X				
Oxyopomyrmex saulcyi	**G**					X
Pheidole pallidula	**O**	X	X	X	X	X
Solenopsis sp.	**O**	X	X	X	X	X
Temnothorax pardoi	**N**			X	X	X
T. parvulus	**N**					X
T. rabaudi	**N**					X
T. racovitzae	**N**				X	
T recedens	**N**		X		X	X
T. specularis	**N**	X	X			X
T. unifasciatus	**N**	X				
Tetramorium caespitum	**O**	X				
T. hispanicum	**O**		X			
T. semilaeve	**O**	X	X	X	X	X
Dolichoderinae						
Linepithema humile	**O**				X	
Tapinoma madeirense	**N**	X				
T. nigerrimum	**N**		X		X	X
Formicinae						
Camponotus cruentatus	**N,O**		X			
C. fallax	**N**		X			
C. figaro	**N**	X	X			X
C. lateralis	**N**					X
C. micans	**N**			X	X	
C. pilicornis	**N**		X	X		X
C. sylvaticus	**N**		X	X	X	X
C. vagus	**N**	X				
Cataglyphis ibericus	**O**					X
Formica cunicularia	**O**	X				
F. fusca	**O**	X				
F. subrufa	**O**		X	X	X	X
Lasius grandis	**N,O**	X				
Plagiolepis pygmaea	**N**	X	X	X	X	X
P. schmitzii	**N**			X	X	X
Species richness		**22**	**19**	**19**	**24**	**25**

Appendix 2 – The role of ant species as seed removal agents of each *Euphorbia* species in the five study areas. For each study plant, it is shown what were the major seed removers in each area (data in average percentages) together with the daily variation (from 08h00 to 20h00) in the removal performed by each ant species. Ballistic dispersal was assumed to be homogeneous throughout the day (Espadaler & Gómez, 1996). A) *Euphorbia pedroi*; B) *E. welwitschii*; C) *E. characias.*

A) *E. pedroi*

Ant species	Azóia	Ares
Aphaenogaster senilis	-	0.165
Messor barbarus	0.297	0.270
Pheidole pallidula	0.261	0.231
Tapinoma nigerrimum	-	0.009
Formica subrufa	0.441	0.325

Azóia

	08h-09h	09h-10h	10h-11h	11h-12h	12h-13h	13h-14h	14h-15h	15h-16h	16h-17h	17h-18h	18h-19h	19h-20h
M. barbarus	0.560	0.567	0.414	0.303	0.258	0.000	0.000	0.000	0.143	0.375	0.563	0.387
P. pallidula	0.360	0.333	0.379	0.424	0.161	0.000	0.000	0.000	0.179	0.375	0.313	0.613
F. subrufa	0.080	0.100	0.207	0.273	0.581	1.000	1.000	1.000	0.679	0.250	0.125	0.000
n	25	30	29	33	31	25	25	25	28	32	32	31

Ares

	08h-09h	09h-10h	10h-11h	11h-12h	12h-13h	13h-14h	14h-15h	15h-16h	16h-17h	17h-18h	18h-19h	19h-20h
A. senilis	0.000	0.080	0.067	0.133	0.179	0.360	0.440	0.407	0.120	0.115	0.077	0.000
M. barbarus	0.320	0.360	0.400	0.333	0.143	0.000	0.000	0.000	0.200	0.346	0.500	0.640
P. pallidula	0.560	0.440	0.300	0.300	0.071	0.000	0.000	0.000	0.240	0.308	0.269	0.280
T. nigerrimum	0.000	0.000	0.033	0.000	0.036	0.000	0.000	0.000	0.000	0.038	0.000	0.000
F. subrufa	0.120	0.120	0.200	0.233	0.571	0.640	0.560	0.593	0.440	0.192	0.154	0.080
n	25	25	30	30	28	25	25	27	25	26	26	25

Appendix 2 (cont.) – The role of ant species as seed removal agents of each *Euphorbia* species in the five study areas. For each study plant, it is shown what were the major seed removers in each area (data in average percentages) together with the daily variation (from 08h00 to 20h00) in the removal performed by each ant species. Ballistic dispersal was assumed to be homogeneous throughout the day (Espadaler & Gómez, 1996). A) *Euphorbia pedroi*; B) *E. welwitschii*; C) *E. characias.*

B) *E. welwitschii*

Ant species	Montejunto	Tesoureira	Risco
Aphaenogaster senilis	0.462	0.425	0.347
Messor barbarus	0.299	0.252	0.218
Pheidole pallidula	0.239	0.254	0.368
Tapinoma nigerrimum	-	0.068	0.067

Montejunto

	08h-09h	09h-10h	10h-11h	11h-12h	12h-13h	13h-14h	14h-15h	15h-16h	16h-17h	17h-18h	18h-19h	19h-20h
A. senilis	0.136	0.160	0.241	0.600	1.000	1.000	1.000	0.706	0.273	0.250	0.179	0.000
M. barbarus	0.500	0.360	0.448	0.200	0.000	0.000	0.000	0.000	0.364	0.500	0.607	0.611
P. pallidula	0.364	0.480	0.310	0.200	0.000	0.000	0.000	0.294	0.364	0.250	0.214	0.389
n	22	25	29	15	15	15	16	17	22	28	28	18

Tesoureira

	08h-09h	09h-10h	10h-11h	11h-12h	12h-13h	13h-14h	14h-15h	15h-16h	16h-17h	17h-18h	18h-19h	19h-20h
A. senilis	0.000	0.000	0.185	0.174	0.750	1.000	1.000	1.000	0.647	0.167	0.182	0.000
M. barbarus	0.353	0.471	0.259	0.391	0.188	0.000	0.000	0.000	0.059	0.333	0.500	0.476
P. pallidula	0.529	0.471	0.481	0.304	0.000	0.000	0.000	0.000	0.235	0.375	0.273	0.381
T. nigerrimum	0.118	0.059	0.074	0.130	0.063	0.000	0.000	0.000	0.059	0.125	0.045	0.143
n	17	17	27	23	16	15	15	15	17	24	22	21

Risco

	08h-09h	09h-10h	10h-11h	11h-12h	12h-13h	13h-14h	14h-15h	15h-16h	16h-17h	17h-18h	18h-19h	19h-20h
A. senilis	0.000	0.048	0.190	0.083	0.467	1.000	1.000	0.867	0.267	0.056	0.190	0.000
M. barbarus	0.235	0.286	0.381	0.292	0.200	0.000	0.000	0.000	0.200	0.333	0.190	0.500
P. pallidula	0.706	0.524	0.333	0.542	0.267	0.000	0.000	0.133	0.467	0.556	0.476	0.409
T. nigerrimum	0.059	0.143	0.095	0.083	0.067	0.000	0.000	0.000	0.067	0.056	0.143	0.091
n	17	21	21	24	15	15	15	15	15	18	21	22

Appendix 2 (cont.) – The role of ant species as seed removal agents of each *Euphorbia* species in the five study areas. For each study plant, it is shown what were the major seed removers in each area (data in average percentages) together with the daily variation (from 08h00 to 20h00) in the removal performed by each ant species. Ballistic dispersal was assumed to be homogeneous throughout the day (Espadaler & Gómez, 1996). A) *Euphorbia pedroi*; B) *E. welwitschii*; C) *E. characias.*

C) *E. characias*

Ant species	**Montejunto**	**Tesoureira**	**Azóia**	**Ares**
Aphaenogaster senilis	0.559	0.460	-	0.484
Messor barbarus	0.212	0.161	0.068	0.132
Pheidole pallidula	0.230	0.289	0.356	0.231
Tapinoma nigerrimum	-	0.090	-	0.143
Formica subrufa	-	-	0.576	0.010

Montejunto

	08h-09h	09h-10h	10h-11h	11h-12h	12h-13h	13h-14h	14h-15h	15h-16h	16h-17h	17h-18h	18h-19h	19h-20h
A. senilis	0.214	0.235	0.267	0.524	1.000	1.000	1.000	1.000	0.700	0.423	0.207	0.133
M. barbarus	0.357	0.265	0.433	0.190	0.000	0.000	0.000	0.000	0.200	0.346	0.414	0.333
P. pallidula	0.429	0.500	0.300	0.286	0.000	0.000	0.000	0.000	0.100	0.231	0.379	0.533
n	28	34	30	21	22	19	26	19	20	26	29	30

Tesoureira

	08h-09h	09h-10h	10h-11h	11h-12h	12h-13h	13h-14h	14h-15h	15h-16h	16h-17h	17h-18h	18h-19h	19h-20h
A. senilis	0.000	0.061	0.241	0.267	0.893	1.000	1.000	1.000	0.480	0.222	0.280	0.074
M. barbarus	0.400	0.212	0.138	0.200	0.000	0.000	0.000	0.000	0.080	0.296	0.200	0.407
P. pallidula	0.560	0.485	0.483	0.367	0.071	0.000	0.000	0.000	0.320	0.333	0.440	0.407
T. nigerrimum	0.040	0.242	0.138	0.167	0.036	0.000	0.000	0.000	0.120	0.148	0.080	0.111
n	25	33	29	30	28	25	25	25	25	27	25	27

Azóia

	08h-09h	09h-10h	10h-11h	11h-12h	12h-13h	13h-14h	14h-15h	15h-16h	16h-17h	17h-18h	18h-19h	19h-20h
M. barbarus	0.120	0.179	0.077	0.036	0.000	0.000	0.000	0.000	0.000	0.074	0.182	0.148
P. pallidula	0.760	0.679	0.538	0.571	0.240	0.000	0.000	0.000	0.000	0.296	0.485	0.704
F. subrufa	0.120	0.143	0.385	0.393	0.760	1.000	1.000	1.000	1.000	0.630	0.333	0.148
n	25	28	26	28	25	25	25	25	25	27	33	27

Ares

	08h-09h	09h-10h	10h-11h	11h-12h	12h-13h	13h-14h	14h-15h	15h-16h	16h-17h	17h-18h	18h-19h	19h-20h
A. senilis	0.120	0.185	0.286	0.440	0.692	0.840	0.960	0.960	0.704	0.393	0.154	0.077
M. barbarus	0.240	0.296	0.143	0.040	0.038	0.000	0.000	0.000	0.074	0.179	0.231	0.346
P. pallidula	0.400	0.333	0.286	0.240	0.077	0.000	0.000	0.000	0.148	0.250	0.500	0.538
T. nigerrimum	0.240	0.185	0.286	0.280	0.154	0.160	0.000	0.000	0.074	0.179	0.115	0.038
F. subrufa	0.000	0.000	0.000	0.000	0.038	0.000	0.040	0.040	0.000	0.000	0.000	0.000
n	25	27	28	25	26	25	25	25	27	28	26	26

Chapter 8

General discussion

GENERAL DISCUSSION

The work here presented contributes to a better understanding of some of the processes that intervene in the reproductive biology of three *Euphorbia* species, from anthesis to short-term seed fate following dispersal by ants.

Some of the *Euphorbia* species investigated here can be found in sympatry (the species pairs *E. characias*/*E. pedroi* and *E. characias*/*E. welwitschii*), have similar flowering seasons and share similar suites of herbivores, seed predators and seed dispersers, thus providing a good model system to comparatively study the impact of factors responsible for reproductive losses. This issue is of the outmost importance since two study species are Portuguese endemics whose biology is still poorly known and both of them have narrow distribution ranges and face several conservation threats. Thus, this comparative approach may provide insights on the determinants of common/rare differences and make available valuable information on species ecology to be taken in consideration for management strategies addressed to these singular plant *taxa*.

Some of the studies presented in this thesis focus on subtle and less frequently studied biotic interactions having the insects as major protagonists. Insect-plant interactions were frequent during the various stages of the plants' reproductive cycle and their effects on plant reproductive success were, in general, significant, although variable in time, space and related with the identity of the interacting insect species.

Many studies throughout the world have highlighted the key role of insect-plant interactions on plant reproductive success and consequently as determinants of plant population dynamics, demography and evolution (see reviews in Herrera & Pellmyr, 2002; Ashman *et al.*, 2004; Fenner & Thompson, 2005; Knight *et al.*, 2005; Harder & Barrett, 2006; Kolb *et al.*, 2007; Rico-Gray & Oliveira, 2007; Kay & Sargent, 2009; Lengyel *et al.*, 2009). In the last few decades those findings have been valued in applied ecology and conservation and proved to be of great help when designing more effective management strategies for endangered plant species (e.g. Louda & Bevill, 2000; Colas *et al.*, 2001; Rovira *et al.*, 2004; Hegazi *et al.*, 2010).

In this chapter I bring together the main findings of the preceding chapters and discuss how they link together and how do they relate with the extant knowledge in this scientific field. The main results of the thesis are discussed in two more specific sections, corresponding to two well-defined areas of scientific research, followed by a more general section (on the role of insect-plant interactions on seed fate), where I also outline some recommendations for the conservation management of the two endemic *Euphorbia*.

8.1 Seed production and pre-dispersal reproductive losses in *Euphorbia*

The production of seeds is a key event of plant life cycle allowing the reposition of individuals in populations, the possibility to incorporate novel genetic combinations in the species' gene pool and consequently ensuring species maintenance. During the pre-dispersal stage the potential seed production of plants diminishes progressively as a consequence of reproductive losses due to a variety of intrinsic and extrinsic factors (Stephenson, 1981; Lee, 1988; Wilcock & Neiland, 2002; Fenner & Thompson, 2005; Knight *et al.*, 2005; Kolb *et al.*, 2007).

The factors responsible for a decrease in the reproductive potential in our study plants were, in general, the same, but their magnitude and variation differed between the three *Euphorbia* species (see chapters 2, 3 and 4). Furthermore, significant spatial and temporal variations in the average effect of each mortality factor on the reproductive output of each *Euphorbia* species were also recorded during this study.

Andromonoecy levels

The three study species presented different levels of andromonoecy, ranging from around 5% in *E. welwitschii* to 20% in *E. characias*. Narbona and colleagues (2002) after studying a set of *Euphorbia* species concluded that andromonoecy levels were intimately associated with plant life form, with perennial species producing a much higher proportion of male cyathia than annuals. Higher andromonoecy levels were then considered a mechanism to promote outcrossing in long-lived plants (Narbona *et al.*, 2002), a conclusion also drawn by other authors when studying other plant genera (Podolsky, 1992; Elle & Meagher, 2000). Our results reinforce their interpretation of andromonoecy levels and provide evidence for the occurrence of significant differences also between herbaceous and woody perennials (chapter 3). Furthermore, our findings on the proportion of male cyathia in *E. pedroi* and *E. welwitschii* highlight the need to evaluate the role of phylogeny and inflorescence architecture in andromonoecy levels in this plant genus (chapters 2 and 3). Sex expression in our study plants was quite variable between populations, years and individuals translating the interplay between genetic and environmental factors that, under developmental constrains, govern the patterns of reproductive allocation (Diggle, 1994; Narbona *et al.*, 2005a). In the three study species, male cyathia were restricted to the lowest levels of the inflorescence, thus andromonoecy levels were intimately associated with cyathia production patterns and highly dependent upon plant fecundity determinants.

Flower, fruit and seed abortion

The three study plants were particularly susceptible to losses resulting in the abortion of reproductive organs. The rare endemic *E. welwitschii* systematically had a high percentage of flowers and fruits aborted, especially at Risco, where more than half of the reproductive potential was lost each year (chapter 3). Those results clearly suggest that this population is facing the negative effects of small population size on plant reproduction. Small isolated populations of many rare species are known to be prone to increased inbreeding, loss of genetic variability and low pollination rates which lead to high levels of flower and fruit abortion (Severns, 2003; Leimu *et al.*, 2006). The other two *Euphorbia* also suffered losses resulting in flower and fruit abortion, particularly in the coastal population of Azóia, where the physical damage due to adverse weather conditions was evident in ovaries and early fruits. Seed abortion levels also differed between species and were much high in the narrow endemic *E. pedroi* (chapter 2). In a study on the pre-dispersal reproductive losses of *E. dendroides*, a close relative of *E. pedroi*, Traveset (1995) reported high seed abortion levels and concluded that those losses were mostly due to intrinsic factors. Intrinsic factors (i.e. inbreeding depression, parent-offspring conflict, intra-fruit sibling rivalry) are frequently reported as major causes of reproductive losses in plants (Stephenson, 1981; Lee, 1988 and references therein) and seem to have played a role as determinants of seed abortion levels in *E. pedroi*. Nevertheless, evidence suggests that the high levels of seed abortion recorded at Azóia for both *E. pedroi* and *E. characias* are, in part, due to a low availability of resources in this study area.

Reproductive losses due to insects

Acroclita subsequana, a polyvoltine moth species specific to the genus *Euphorbia*, clearly showed a preference for *E. pedroi* as a host plant for oviposition and larval development and this was probably due to aspects related with plant architecture and flowering phenology duration. Some other *Euphorbia* species are known to be host plants of *A. subsequana* in Iberia (Brown *et al.*, 2008), but heavier reproductive losses have been reported only from the larger species of the *Balsamis* section (Traveset, 1995; chapter 2) since these species represent a more economic foraging effort from the insect point of view (Fenner *et al.*, 2002). Host plant selection by tortricid moths like *A. subsequana* is mostly based on chemical cues as has been highlighted by experimental studies on several economically important species (e.g. Yan *et al.*, 1999; Hern & Dorn, 2004; Masante-Roca *et al.*, 2007) and this was the main reason for the lack of association between the magnitude of reproductive losses due to *A. subsequana* and the measured traits indicative of plant size and fecundity.

Two groups of pre-dispersal seed predators – specialist seed-wasps and generalist hemipterans – were responsible for considerable reproductive losses in all three study plants (chapters 2 and 4).

Generalist hemipterans inflicted losses of the same magnitude to co-occurring *Euphorbia* species (although somewhat higher in *E. pedroi* at Azóia) and this was in part due to the fact that the fruits and seeds of the three study plants are somewhat similar in size, form and toughness, thus providing similar conditions for generalist insect feeding. Similar findings were reported from four co-occurring *Carpinus* species with similar sized seeds and fruiting phenologies (Shibata *et al.*, 1998). However, in many other study systems, differences in plant morphology, fruiting phenology and fruit and seed characteristics were found to be responsible for disparate losses to pre-dispersal seed predators between co-occurring congeners (Green & Palmbald, 1975; Siemens *et al.*, 1992; Greig, 1993; Simon & Hay, 2003; Nakagawa *et al.*, 2005; Espelta *et al.*, 2009).

Specialist seed-wasps were responsible for much higher seed losses in *E. pedroi* than in the two other study plants. However, interspecific comparisons on losses due to seed-wasps have to be made with caution since the identity of the insect seed predator was not the same in the three study systems: *Eurytoma fumipennis* seed-wasps attacked the seeds of *E. pedroi* while the seeds of the two other *Euphorbia* species were preyed upon by *Eurytoma jaltica*. Thus, we believe that differences in biology, local abundance and population dynamics between the two seed-wasp species can be determinant to interpret the observed differences in seed predation levels (M. Boieiro, unpublished data). The *Eurytoma-Euphorbia* seed predation system is quite unique since the specialist seed predators are highly dependent of their host plants for food, shelter and larval development. Consequently, the population dynamics of seed-wasps is intimately associated with the temporal fluctuations in *Euphorbia* seed production patterns. Only a few other studies have reported a similar pattern of interaction between a specialist pre-dispersal seed predator and its host plant (De Steven, 1983; Solbreck & Sillén-Tullberg, 1986; Sperens, 1997; Poncet *et al.*, 2009). In those systems, the inter-annual variation in fruit production is considered a key feature since it appears to regulate the populations of specialist seed predators and may provide a mechanism of predator satiation, allowing more seeds to escape from predation during episodic good fruiting years (De Steven, 1983; Poncet *et al.*, 2009).

The lack of association between losses due to both groups of insect seed predators and the measured traits indicative of plant size and fecundity suggests that host plant selection by insects is based on other cues. In fact, chemical senses seem to play a major role in host plant selection by both seed-wasps and hemipterans. Seed-wasps are attracted by plant volatiles and the same odours also elicit oviposition in these insects (Kouloussis & Katsoyannos, 1994). Furthermore, seed-wasps are known to apply a host-marking pheromone on fruits following egg-laying to prevent additional ovipositions by conspecifics and consequently impede host superparasitism (Kouloussis & Katsoyannos, 1991, 1993). On the other hand, generalist hemipterans, which prey upon a variety of plant species and may feed on various plant tissues, use mostly chemical-based multiple sensory modalities to decide upon host acceptance (Bernays & Chapman, 1994).

The effects of the different insect groups on the reproductive success of the three study plants were, in general, additive suggesting that they do not seem to discriminate between plants with different levels of reproductive losses due to heterospecific insects and/or probably use different plant traits to select their hosts for feeding or oviposition. A similar conclusion was drawn from a study on pre-dispersal reproductive losses in another *Euphorbia* species, where the same insect groups were also responsible for considerable losses (Traveset, 1995). There are however many examples from literature where the patterns of host plant use by one species were altered by other interactors acting synergistically or antagonistically (Evans *et al.*, 1989; Strauss & Irwin, 2004).

Intact seed production

Intact seed production differed significantly between the three study species. The average seed production per individual was lower in *E. welwitschii* (ranging from 37 seeds/individual at Risco to 242 seeds/individual in Montejunto), intermediate in *E.*

pedroi (with 228 seeds/individual in Azóia and 399 seeds/individual in Ares) and higher in *E. characias* (where more than 595 seeds/individual were recorded in all study populations). These interspecific differences in average intact seed production per individual were mostly due to the large differences in cyathia production between the three *Euphorbia* species, although significant interspecific differences in the effects of pre-dispersal mortality factors have also been detected. The two narrow endemics studied here had a lower cyathia production when compared both with *E. characias* and other widespread congeners (Berg, 1990; Traveset, 1995; Espadaler & Gómez, 1996; Chapters 2 and 3).

Comparative studies on the determinants of plant rarity between co-occurring congeners concluded that, in general, rare species have lower flower production than their widespread relatives (e.g. Fiedler, 1987; Murray *et al.*, 2002). This finding was reinforced in a recent study, where a large number of ecological characteristics and biological traits were compared in 20 congeneric pairs of narrow endemic and widespread plant species from the Western Mediterranean (Lavergne *et al.*, 2004). These authors found that narrow endemics produce fewer flowers and seeds than their widespread congeners, even after accounting for differences in plant size and seed weight. The low investment in reproduction by narrow endemics was then interpreted as an evolutionary consequence of population confinement and persistence in isolated habitats (see also Byers & Meagher, 1997; Orians, 1997).

The effects of isolation on natural populations have been the subject of thorough research for long, but during the last decades interest on this issue raised considerably due to our need to understand an increasingly fragmented natural world and the effects of isolation on a growing number of small populations. During this study we found evidence that one population (Risco) of the endemic *E. welwitschii* is facing the negative effects of small population size on plant reproduction. There, reproductive losses leading to ovary, fruit and seed abortion were quite high and consequently intact seed production was low. On the other hand, no such evidence was found in the two populations of the extremely localized *E. pedroi*, where insects were responsible for high pre-dispersal reproductive losses.

8.2 Myrmecochory and post-dispersal reproductive losses in *Euphorbia*

Seed dispersal is a fundamental stage of plant life-cycle linking the end of the reproductive cycle of adult plants with the establishment of their offspring (Fenner & Thompson, 2005; Dennis *et al.*, 2007; Cousens *et al.*, 2008). During this stage, several complex processes, involving one or more seed dispersal agents, may take place in discrete and sequential phases. Diplochory, the dispersal of seeds in a sequence of two or more steps, each involving a different dispersal agent, is a common strategy among plants (Vander Wall & Longland, 2004) and a frequent dispersal mode within the genus *Euphorbia* (Baiges *et al.,* 1991; Espadaler & Gómez, 1996). In this plant genus, primary seed dispersal consists in the explosive dispersal of seeds that scatter them around the parent plant from distances of a few meters to more than 20m in the larger species (Berg, 1990; Narbona *et al.*, 2005b). Explosive seed dispersal contributes to low density-dependent seed mortality near the parent plant, but seems to play a minor role in directing seeds to favourable microsites (Vander Wall & Longland, 2004). The secondary dispersal of *Euphorbia* seeds may be carried by a variety of dispersal agents which transport the seeds farther away and may also reduce losses to seed predators (Blockstein *et al.*, 1987; Baiges *et al.,* 1991; Berg, 1990; Espadaler & Gómez, 1996; Olson *et al.*, 1997; Wald *et al.*, 2005). Furthermore, many *Euphorbia* species are provided with a specialized seed appendage – the elaiosome – which attract ants and elicit the transport of seeds to their nests. This directed dispersal is considered an advantage of secondary dispersal by animals since seeds can be relocated to situations where germination and seedling establishment may be considerably higher (Wenny, 2001; Vander Wall & Longland, 2004).

The elaiosome – characteristics and the role played in seed dispersal

All three study plants have seeds provided with an elaiosome. Both elaiosome and seed size differed between the three *Euphorbia* species, with *E. characias* having the larger seeds and elaiosomes. Differences in seed and elaiosome physical characteristics are known to be responsible for different outcomes in interactions with dispersers and predators (Gorb & Gorb, 2003; Edwards *et al.*, 2006). Observations and experiments on the effects of seed and elaiosome size on the probability of seed removal by ants have shown that both variables (seed and elaiosome size), together with a combination of them (elaiosome/seed size ratio) are determinant traits to understand seed removal success (Oostermeijer, 1989; Hughes & Westoby, 1992; Gorb & Gorb, 1995; Gorb, 1998; Peters *et al.*, 2003; Edwards *et al.*, 2006). Furthermore, it was also found that not all ant species behaved the same way with differences in seed selection being intimately related with their feeding ecology: granivorous ants responded positively to seeds with larger size while omnivorous privileged higher elaiosome/seed size ratios (e.g. Hughes & Westoby, 1992). A number of studies questioned the major role played by physical characteristics on seed selection by ants after providing evidence that, at least in myrmecochorous plant species, chemical cues are also determinant for seed removal success (Marshall *et al.*, 1979; Skidmore & Heithaus, 1988; Brew *et al.*, 1989; Gunther & Lanza, 1989). Differences in the chemical composition of elaiosomes, particularly the concentration of specific fatty acids (mostly oleyl-based compounds), have been shown to lead to significant differences in seed attraction and removal by ants (Marshall *et al.*, 1979; Skidmore & Heithaus, 1988; Brew *et al.*, 1989; Boulay *et al.*, 2006, 2007).

Our results on seed removal in *Euphorbia* can only be fully understood by considering both physical and chemical characteristics of the diaspores. *Euphorbia characias*, the species with larger seed and elaiosome size and high content in oleic acid, was removed with higher probability by mutualistic ants than its congeners (chapter 7). On the other hand, in spite of having the largest elaiosome/seed size ratio, *E. pedroi* was removed with the lowest probability. This is probably associated with the poor concentration of fatty acids in the elaiosomes of this species, particularly the low quantities of oleic acid (with concentrations 5-fold less than those of the two other *Euphorbia*) (chapters 5 and 6). A direct consequence of the low attractiveness of *E. pedroi* seeds to mutualistic ants is a longer exposition at soil surface leading to a high probability of those seeds being destroyed by local seed predators, particularly harvester ants and vertebrates (chapter 7).

Seeds of myrmecochorous species once deprived of the elaiosome also get ignored by mutualistic ants and face a high probability of being preyed as was shown in this study. Independently of the study species, the lack of elaiosome on the seeds led to a large decrease in the probability of removal by mutualistic ants while no such effects were detected on their attractiveness towards granivorous ants (chapter 7). These results clearly illustrate the major role played by the elaiosome on the rapid seed removal and successful relocation of seeds away from the point of collection. Many other studies on myrmecochory in different plant genera and on different continents are unanimous in highlighting the crucial role played by the elaiosome on the removal of seeds by mutualistic seed-dispersing ants (Sernander, 1906; Berg, 1959; Oostermeijer, 1989; Hughes & Westoby, 1992; Garrido *et al.*, 2002; Leal *et al.*, 2007; Servigne & Detrain, 2008; Bas *et al.*, 2009).

Seed dispersal by ants and losses to post-dispersal seed predators

The array of animals that interacted with the seeds of the study plants following ballistic dispersal included mutualistic seed-dispersing ants, granivorous ants and vertebrates (mostly rodents). The results from observations and experiments indicated that reproductive losses to post-dispersal seed predators were significant, variable in space and related with the identity of the study plant. When in sympatry, both *E. pedroi* and *E. welwitschii* suffered proportionally larger losses to the granivorous *Messor barbarus* than their congener *E. characias* and this was mostly due to the low attractiveness of the former species to mutualistic seed-dispersing species.

The rapid removal of seeds from the soil surface by mutualistic ants contributes to reduce the losses to seed predators being one of the main advantages of myrmecochory (Beattie, 1985; Giladi, 2006; Rico-Gray & Oliveira, 2007). Espadaler and Gómez (1996, 1997) showed how efficient and effective are ant species in searching the soil surface in Mediterranean habitats during primary seed dispersal in *E. characias:* ant activity was intense and an area equivalent to the whole study site was scanned in just 43 minutes! A number of studies on myrmecochory have shown that potential high losses to post-dispersal seed predators were considerably lowered due to ant activity (Heithaus, 1981; Bond & Breytenbach, 1985; Smith *et al.*, 1986; Nakanishi, 1994; Ruhren & Dudash, 1996; Pizo & Oliveira, 1998). For example, Heithaus (1981) reported heavy seed losses to rodents in the absence of ants (around 70-84%) for three plant species, but those values changed dramatically (to 13-43%) when ants were allowed to interact and transport the seeds. Similar findings were reported by Bond and Breytenbach (1985), who have concluded that the cause of recruitment failure in Proteaceae was seed predation by small mammals and the major benefit of myrmecochory was precisely seed escape from those seed predators.

In general, the myrmecofauna of a particular area only contains some ant species that are actively involved in seed dispersal. The other species usually include ants too small to carry the seeds or with a specific feeding ecology (e.g. nectar-feeding, aphid-tending). Nevertheless, several of those species may feel attracted by the elaiosome and be capable of removing small pieces of it or even feed *in situ* on the whole elaiosome (e.g. Manzaneda *et al.*, 2007; Castro *et al.*, 2010). This behaviour may interfere with myrmecochory by rendering seeds unattractive to mutualistic seed-dispersing ant species, but to my knowledge no detailed study has specifically addressed this issue hitherto.

In spite of the large number of ant species identified in this study, only five of them were actively engaged in seed removal. Two larger omnivorous species matching the characteristics of keystone seed dispersal mutualists (see Ness *et al.*, 2009) were the main seed dispersers in the five study areas. These species – *Aphaenogaster senilis* and *Formica subrufa* - were common in the study sites, removed rapidly most of the seeds they contact with and transported them at larger distances. The submissive behaviour of these species led dominants to tolerate them and consequently their colonies were widespread, providing a good coverage of the soil surface in each study area. Ants of these genera have been reported to play a major role in myrmecochory in several habitats of Europe and North America (e.g. Gorb & Gorb, 2003; Manzaneda & Rey, 2009; Ness *et al.*, 2009). In Australia, another ant genus – *Rhytidoponera* - has also been identified as a keystone mutualist due to its predominat role as dispersal agent at several spatial scales of analysis (Gove *et al.*, 2007; Lubertazzi *et al.*, 2010). The other three species involved in the dispersal of *Euphorbia* seeds included the granivorous *Messor barbarus* (that virtually destroys all seeds collected) and two smaller omnivorous species (*Pheidole pallidula* and *Tapinoma nigerrimum*) which removed a lower fraction of seeds and dispersed them at shorter distances than co-occurring larger species.

Overall, most of the seeds were removed by mutualistic ants at distances ranging from 9cm to 9m and, in the short term, seed fate was the ant nest or its vicinities. Thus, the four mutualistic seed-dispersing species were important to rearrange the seed shadow and to transport the seeds farther away from the parent plant, thus minimising the effects of distance- and density-dependent mortality factors. In other plant species, the rearrangement of seed spatial distribution by ants was considered to be advantageous for plants since it allowed the exploitation of heterogeneous spatial and temporal conditions for seed germination and seedling establishment (Ohkawara & Higashi, 1994; López-Vila & García-Fayos, 2005; Beaumont *et al.*, 2009). According to several authors seed deposition inside ant nests or outside in its vicinities is one of the main advantages of myrmecochory since those microsites provide favourable conditions for seed germination and seedling establishment and, once buried, seeds may easily escape from seed predator attack (Beattie, 1985; Giladi, 2006; Rico-Gray & Oliveira, 2007). A number of studies have shown that ant nests may differ chemically, physically and biologically from the surrounding soil, being richer in several nutrients which are known to enhance the probability of seedling establishment (e.g. Beattie & Culver, 1983; Andersen, 1988; Oostermeijer, 1989; see also a review by Giladi, 2006 and references therein). Other studies, however, failed to find such differences (Rice & Westoby, 1986; Bond & Stock, 1989). The literature strongly suggests that the way ants handle seeds inside the nest may also be beneficial to plants, e.g. elaiosome removal may estimulate seed germination (Beattie & Lyons, 1975; Horvitz, 1981; Pacini, 1990; Pizo & Oliveira, 1998; Passos & Oliveira, 2002; Ohkawara, 2005), seed cleaning prevents fungal infections improving seed survivorship (Ohkawara & Akino, 2005; Rico-Gray & Oliveira, 2007) and seed burial by ants is often at an adequate depth for seed germination and seedling emergence (Majer, 1982; Christian & Stanton, 2004.; Lubertazzi *et al.*, 2010; Renard *et al.*, 2010).

The literature on myrmecochory provides various examples where benefits to plants were reported in the form of higher seed survival or germination, or increased seedling emergence or establishment (see Beattie, 1985; Gorb & Gorb, 2003; Giladi, 2006; Rico-Gray & Oliveira, 2007 and references therein). However, there is still a lack of solid information on how, and if, those advantages translate in terms of plant-fitness components and on their influence in plant population dynamics (e.g. Hanzawa *et al.*, 1988; Gorb *et al.*, 2000). Even so, evidence suggests that myrmecochory influences the abundance and structure of plant communities (Handel *et al.*, 1981; Bond & Slingsby, 1984; Christian, 2001; Rico-Gray & Oliveira, 2007) and may play a role in ecological restoration (Andersen *et al.*, 1998; Wanless, 2003; Thompson & McLachlan, 2007).

The three *Euphorbia* species exhibited different dispersal strategies: *E. characias* invested more on elaiosome size and quality and was the species that most attracted mutualistic seed-dispersing ants. On the other hand, the two other *Euphorbia* species invested less on elaiosome size and quality and were much less attractive to ants, particularly *E. pedroi* whose elaiosomes have a low concentration of fatty acids important for ant attraction. Comparative studies on the dispersal biology of congeners using both explosive and ant seed dispersal have found that plants tend to optimise the investment in one of the two dispersal phases (Ohkawara & Higashi, 1994; Narbona *et al.*, 2005b). For example, Narbona and colleagues (2005b) reported that in two Mediterranean *Euphorbia,* the species with shorter and loose elaiosomes and lighter seeds seems to maximize the investment in explosive dispersal while the species with larger elaiosomes, reach lower distances through ballistic dispersal and seems to favor myrmecochory. In our study system, the lack of fatty acids in the elaiosomes of *E. pedroi* together with the larger distances achieved through explosive dispersal (Berg, 1990) clearly indicates that this species, contrary to its congeners, privileges primary dispersal.

8.3 The role of insect-plant interactions on *Euphorbia* seed fate and management recommendations for the two Portuguese endemics

Insect-plant interactions played a major role in determining the reproductive success of the three study species and were omnipresent during flowering, fruiting and seed dispersal. The array of insects that interacted with plants included both antagonists (pre- and post-dispersal seed predators) and mutualists (seed dispersers), with quite unique associations being found (*Eurytoma/Euphorbia* system). Most of these interactions are rather inconspicuous but their effects on seed survival are too important and should not be neglected. The magnitude of the effects of insect-plant interactions on plant reproductive output was considerable and can potentially influence plant population dynamics as has been shown in other study systems (Hanzawa *et al.*, 1988; Gorb *et al.*, 2000; Hulme, 2002; Kolb *et al.*, 2007). Furthermore, some of those effects showed significant variation in space, time and between individuals. Differences in interactions among populations provide the raw material upon which evolutionary change is made (Thompson, 1999, 2002). Insect-plant interactions may show selection mosaics, where different traits and outcomes are favoured by natural selection in different populations, and understanding their dynamics is one of the most fundamental problems in ecology and evolutionary biology (Thompson, 2002).

Management recommendations for Euphorbia pedroi and E. welwitschii

These two Portuguese endemic species should be the focus of further research in order to clarify the conservation status of their populations and the identification of the major threats they face. Basic and crucial information on the biology of both species is still lacking and efforts should be addressed to fill this serious knowledge gap.

The geographic distribution of *E. welwitschii* should be updated and comprehensive studies on the population ecology of both *Euphorbia* should be carried on. These studies should encompass the whole life cycle to identify the most critical transitions and the major causes of mortality in these species. The genetic diversity of populations should be assessed taking in consideration that both species are narrow endemics and gene flow between populations seems to be very low. This kind of studies is critical for *E. welwitschii* since most populations of this species have a small number of individuals being prone to the negative effects of small population size on fitness components, as it seems to be suggested from the data collected at Risco.

Efforts should also be addressed to develop a program for conservation *ex situ* aiming to improve the knowledge on the biology of both species (e.g. seed germination, seedling establishment) and, particularly in the case of *E. welwitschii*, to evaluate the possibility of reinforcement of the effectives in the populations most threatened by the negative effects of small population size (i.e. inbreeding depression, accumulation of deleterious mutations, low genetic variability, reduced genetic variability and its consequences). *In situ* field experiments of cross-pollination between populations of *E. welwitschii* to enhance within-population genetic diversity would also be on order.

Conservation and management of *E. welwitschii* is challenging due to the dispersion of its populations, their small size and location mostly outside protected areas. On the other hand, the three populations of *E. pedroi* are already included in a protected area and may benefit from further protection from recently proposed conservation measures at the habitat level following a LIFE project on critically threatened plants (ICN, 2007). However, this does not preclude the monitoring of its populations as a conservation measure, in fact, both Portuguese endemics should be regularly monitored and the viability of their populations should also be assessed. Further, local communities should be involved in the conservation strategies for these endemic species in order to increase their chances of success, namely in reducing disturbance in their natural habitats.

8.4 Future research

The development of this research project raised several questions that warrant further research. Insect-plant interactions have a strong chemical component whose study has only been grasped in this thesis when the fatty acid composition of elaiosomes was determined. We found evidence that plant volatiles play a major role in host plant selection at least in seed-wasps and those chemical cues are crucial for the interpretation of variation in seed predation levels. The chemical basis of host plant selection and avoidance behaviour of parasitized fruits should be clarified in the future.

Experimental work should be carried out to determine the causes of flower and fruit abortion in the three study species. Physical damage of flower and fruits due to adverse weather was easily identified, but the contributions of pollen and resource limitations to abortion levels were not assessed. Furthermore, research should also be conducted to identify the determinants of andromonoecy levels in the three *Euphorbia* species and to disentangle the contributions of plant life form and phylogeny to andromonoecy in this genus.

More complete analyses on the chemical composition of elaiosomes should be taken in future, particularly to elucidate the arrangement of fatty acids in glycerides and the occurrence of other nutrients that may be essential to ant nutrition. The assessment of benefits of myrmecochory to ants is also an interesting field for future research.

Finally, it will be important to assess the advantages of myrmecochory to *Euphorbia* species in the long term and its consequences in terms of plant fitness. This research will demand the performance of experimental work on seed germination, seedling emergence and recruitment as well as monitoring the various stages of plant development until adult plant first reproduction.

8.5 References

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See the white light, the light within.
Be your own disciple, fan the sparks of will.
For all of us waiting, Your kingdom will come !

Manowar, 1990