

Biology and Ecology of the Rare Perennial Plant Species *Salvia pratensis* (L.) and the Implications for its Conservation in England and Wales

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Industrial Placement Year Report:

NATIONAL BOTANIC GARDEN OF WALES

Start Date: 18th July 2011

Finish Date: 17th August 2012

Supervisor: **Dr Natasha de Vere**

Degree Programme: Plant Science with Industrial Experience

My Organisation: The National Botanic Garden of Wales

The National Botanic Garden of Wales (NBGW) is a young and developing botanic garden, located at a large site in Carmarthenshire, South Wales. NBGW is dedicated to the research and conservation of biodiversity, to sustainability, lifelong learning and the enjoyment of the visitor. As an organisation in a very good position to reach out and engage the public on environmental issues we work to inspire and educate through numerous methods of interpretation and engagement including: theatre, art, walks, talks, interactive exhibitions and most importantly, diverse and interesting floral displays hosting over 8000 different plant species and a 150 hectare National Nature Reserve.

During my placement I worked in the Conservation and Research department which is directed by Dr Natasha de Vere, concentrating on a number of major projects: 1) DNA Barcoding all of the native and alien flowering plants in the UK; 2) Applications of DNA barcoding including: plants for health and commercial applications and 3) Researching and conserving rare plants in Wales and the rest of the UK. The department includes five PhD and numerous Masters Students from collaborating institutes across the UK. Each year Dr de Vere also supervises 2 to 4 undergraduate placement students who work as full-time research assistants in the department for one year. The position was extremely varied and below is a breakdown of time which I spent on each project, it was only possible to present the results from one project in this report, a brief explanation of the work involved and relevant publications and articles is given (tab. i). I greatly enjoyed my time and experience at the Garden and look forward to staying in contact with my supervisor and colleagues in the future.

Table i. Breakdown of project involvement and time scales (project title in **bold** is presented here).

Project	Time and Dates of involvement	Summary of work carried out and results/publications
<i>Campanula patula</i> conservation	Full Time: 2 months, July – September 2011	Role: UK-wide survey of extremely rare species, studying its ecology and taking DNA samples for genetic analysis.
Barcode Wales	Full Time: 2 weeks, September 2011	Role: Assisted analysis and processing of large DNA Barcode database. Co-author in 2 abstracts: 1) de Vere, N. <i>et al.</i> (2011) Barcode Wales / Codbar Cymru: A complete DNA barcode dataset of a nation's native flowering plants: creation, applications and public engagement. <i>Fourth International Barcode of Life Conference, Adelaide.</i> 2) de Vere et al. (2012) DNA barcoding the flora of Wales: the creation and use of a national resource for biodiversity conservation and human health. <i>Third European Congress for the Barcode of Life (ECBOL3), Brussels.</i> - I attended this conference
Barcode UK	Part time: 4 months, January - May 2012	Role: Assisted Herbarium specimen collection of >1500 plant samples and subsequent DNA extraction, PCR, gels and sequence plating.
<u>Salvia pratensis research and conservation</u>	Time spent on results reported here: 4 months Time spent on project area: Full Time: 7 months, October – December 2011 & May – August 2012 Part time: 4 months, January - May 2012	Role: In-depth literature review, field surveys for biology and ecology, germination studies, cross pollination of <i>ex-situ</i> future reintroduction specimens. Presented HRH Prince Charles with a collection of specimens I had reared to be grown at two of his estates. Co-author: Moughan, J., Rich, T.C.G. and de Vere, N. (in prep.). Biological Flora of the British Isles: <i>Salvia pratensis</i> (L.). <i>Journal of Ecology</i> Co-author: Moughan, J. and de Vere, N. (2012). Conservation of Meadow Clary in Wales. Scientific report for Countryside Council for Wales, Cardiff. Author: Moughan, J. (2012). Saving <i>Salvia pratensis</i> . <i>Yr Ardd – National Botanic Garden of Wales Magazine</i> . 15 , 12-13. Featured in BBC online news report: Roberts, A. (2012). BBC Wales. Prince Charles urges local food strategy in Carmarthen visit.

Abstract

The impact of human activity is resulting in fragmentation and loss of plant species and ultimately depleting global biodiversity. Conservationists must apply conservation biology and restoration ecology theory and past experimental discoveries to prevent the extinction of species which are rare. In this study the biology and ecology of *Salvia pratensis* a perennial plant species, which is rare in the British Isles, is investigated. The results from ecological and biological surveys along with a seed fitness test under controlled conditions of a representative sample of 11 British populations are used to study the interaction between demographic structure, population size, population fitness, sex ratio, habitat quality and management technique.

Significant relationships were investigated using multiple regression for demographic structure, population size and habitat quality. The results were used to inform construction of a structural equation model, which investigates the effect of all variables within a system. Bivariate analysis was used for fitness variables due to reduced sample size. For sex ratio and management type comparisons of the mean of categorised groups were used.

Productivity (measured using Ellenberg bio-indicator value for nitrogen) was found to exhibit a negative effect on the population size (total plant number), the demographic structure (ratio of young/old plants) and the emergence rate of seeds. Such that populations found in sites of high productivity had reduced viability. Measures of population size, demography and fitness did not present interacting relationships with one another. Conservation implications and the requirement for knowledge of populations history in *Salvia pratensis* is discussed.

Introduction:

Destruction and fragmentation of natural habitats globally is putting tens of thousands of plant species at risk of extinction and causing worldwide biodiversity loss (Isbell et al. 2011; Butchart et al. 2010; Jackson & Kennedy 2009; Hanski & Ovaskainen 2000; Tilman et al. 1994). There are two central concepts that are the foci of biodiversity research these are: habitat space and habitat quality. These concepts are at the basis of the issues conservationists must attempt to understand in order to prevent the loss of species. The large scale destruction of natural areas by humans, for example to make way for intensive farming practises or in the process of deforestation, is the cause of the first of two threats to biodiversity - the loss of space for habitats. The depreciation of natural areas as a result of anthropogenic activity which alters their environmental characteristics and causes loss of species which are unable to adapt to the change is the second threat to biodiversity – loss of habitat quality (Ouborg et al. 2006).

Restoration Ecology and Conservation Biology

The loss of space for habitats has consequences on biotic characteristics of populations and species, which must be understood in the study of conservation biology. These biological issues are caused by loss of numbers and fragmentation of populations resulting from both the destruction and the depreciation of habitats, and put the survival prospects of species under threat (Frankham et al. 2010). Increased distance between populations incurred through fragmentation results in a number of irregularities or stochasticities in their demography, genetics and environment (Oostermeijer et al. 2003).

The concept of habitat quality is described by Ouborg et al. (2006) as the influence of abiotic characteristics in the landscape such as climate, soil composition and management type. These characteristics will dictate the species that can survive and hence the level of biodiversity found within an environment. In order to assess a population or species' ability to survive, habitat quality must be observed, with the aim of identifying the negative impacts resultant from human activity and improve them with best management techniques along with the promotion of preserving environments which are already of high quality (Saar et al. 2012; Ouborg et al. 2006). This form of conservation research and intervention is restoration ecology. Ouborg et al. (2006) describes need for research in the prevention of extinction and biodiversity loss resulting from human activity which incorporates both restoration ecology and conservation biology, in order to take actions which are well informed and will promote the best chance of maintaining species which are at risk of loss, preventing continued worldwide biodiversity reduction.

Biological aspects of conservation

The loss of habitat space, lowered numbers of individuals and isolation causes population dynamics to be altered from how they would normally operate under vital rates, this will result in numerous biological stochasticities or random variations within a population which place it at risk of being lost. The application of ecological theory is used in conservation to try and understand the biological

effects of isolation and reduced size on the populations in the study of demographics (Ouborg et al. 2006; Oostermeijer et al. 2003). The vital rates of a population such as the fecundity - ability to reproduce or survival rate will be subject to a number of stochastic effects based on the altered demographic interactions, genetic constitution and environmental variations (Boyce et al. 2006).

Demographic stochasticity is the variation in population growth rate which can be measured in things like population viability, number of reproductively active individuals or the survival rate of offspring. Demographic stochasticity will be different for every individual in a population and varies completely at random hence affecting all populations regardless of size (Simonis 2012; Kendall et al. 2011). Due to the discrete nature of each individual in a population fluctuations in demographic stochasticity will have a much more severe affect in small populations, making them more prone to extinction (Drake 2005; Fox et al. 2012; Oostermeijer et al. 2003).

Environmental factors affect populations of all sizes, when variation in the environment causes fluctuation in vital rates of populations such as growth rate and fitness this is known as environmental stochasticity. The magnitude of the effect environmental variation will have on the survival prospects of a population and in extreme cases, species, will vary based on population size, as larger populations are better able to buffer environmental stochasticity (Ouborg et al. 2006; Oostermeijer et al. 2003). A further issue a population's environment will have upon it, is the response to catastrophes, these can be natural (flooding, landslides etc.) or the result of human activity (unfavourable habitat management or habitat destruction for example to make way for building work). Catastrophic stochasticity in small populations is much more likely to lead to extinction than if there are large numbers of individuals in a population, due to the greatly increased probability that some members of larger populations will survive (Fujiwara 2009; Boyce et al. 2006; Ouborg et al. 2006; Oostermeijer et al. 2003).

Genetic stochasticity or genetic erosion can occur in small populations which have become isolated due to the reduction of genetic diversity/variation, inbreeding and mutation accumulation (Frankham et al. 2010). Population size is known to exhibit a very strong relationship to the number of different alleles which are present within the population. Reduction in population size increases the risk of alleles being lost through genetic drift (Ouborg et al. 2006; Oostermeijer et al. 2003; van Treuren et al. 1991). Species which lack the ability to perform desired breeding systems, due to reduced numbers and isolation, puts great pressure on their ability to outcross successfully. Inbreeding will occur which will increase the number homozygous alleles within the population and commonly results with reduced levels of fitness (de Vere et al. 2009). When homozygosity is high and there is reduced fitness due to population size, inbreeding depression is occurring. Inbreeding in plants is most severe in dioecious species and is much less of an issue in self-compatible species (Oostermeijer et al. 2003). The subsequent loss of genetic diversity due to both genetic erosion and inbreeding puts the populations at the mercy of their environment having a reduced ability to cope with change, making them susceptible to be lost as species altogether (Reed 2005; Luijten et al. 2002).

The prevention of biodiversity reduction has become a priority in science over the last 30 years and huge response has resulted, however many conservation research efforts focus on one or a few of the areas outlined above (Ouborg et al. 2006; Oostermeijer et al. 2003). Here we combine restoration ecology with conservation biology to try and understand the status of the rare perennial plant *Salvia pratensis* which has a fragmented distribution in the British Isles and the majority of populations are also isolated

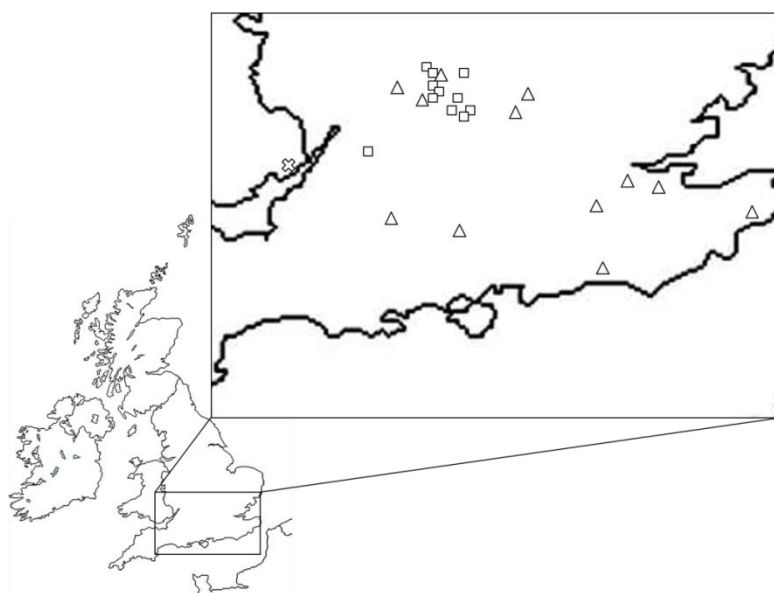


Figure 1) Extant, native distribution of the 23 *Salvia pratensis* populations in the British Isles. Square points are the 11 populations included in this report. Triangular points are the remaining 12 populations not discussed here. The cross represents a site where the species recently went extinct and is the subject of a reintroduction program.

(fig. 1) (Rich et al. 1999; Scott 1989). Using past biological and ecological findings and applying theory to study its population size, analysing its demographic constitution, floral biology, fitness and comparing this to habitat quality and the interaction of management type to try and inform how best to promote its continued survival and the application of informed efforts for its conservation in the British Isles.

Historical and biological background to Salvia pratensis (L.) and its conservation

Salvia pratensis (L.) is a Lamiaceae species found in unimproved and semi-unimproved dry, calcareous grasslands and neutral hay meadows. It is a long-lived, attractive, robust, erect perennial herb with a persistent deep tap root system and forms a basal rosette of three leaf pairs (Sell & Murrell 2009; Hedge 1972). The plant normally produces 1 – 5 flowering stems (however up to 50 have been observed) these are 30-100 cm tall, erect, quadrangular, branched and pale green. Each flowering stem will produce 2 – 50 clusters of 4 – 6 (most commonly 6) blue – violet flowers (van Treuren et al. 1993; Ouborg & van Treuren 1995; Hegland et al. 2001). As a gynodioecious species, the plants have either hermaphrodite or male-sterile flowers, distinguishable by having retracted anthers and smaller sized flowers on male-sterile inflorescences. *S. pratensis* is self-compatible, however requires pollinator foraging for seed to be produced (Ouborg & van Treuren 1994; Scott 1989).

In the British Isles *S. pratensis* is rare with a current total of 23 extant populations (fig. 1). Records for native populations occur in only 37 hectads (10km squares) - less than 1% of the overall number (Preston et al. 2002; Rich et al. 1999). This species is nationally scarce and protected under Schedule 8 of the 1981 Wildlife and Countryside Act, making it illegal to intentionally pick, uproot, destroy or sell

plants found in the wild. In the Vascular Plant Red Data List for Great Britain the species is classed as near threatened (Cheffings et al. 2005). *S. pratensis* is likely to have always been fairly uncommon in Britain however significant loss of native populations was witnessed prior to the 1950's. The decline seemingly followed intensified farming practises and altered management techniques of grassland communities. Since this time the number of populations has remained almost constant and is restricted to those sites which have been managed using more traditional, less intensive farming practises for the purpose of maintaining unimproved lowland dry pasture (Rich et al. 1999; Scott 1989; Preston et al. 2002).

Across Central Europe *S. pratensis* has a wide and often abundant distribution which means the plant is not of direct conservation concern internationally. However this species is found in a habitat that is in decline. As a result of this, at the distribution edge, where numbers were already low it is becoming increasingly rare. This has been witnessed most greatly in Britain and the Netherlands. In the British Isles the plant is a priority species in the countries Biodiversity Action Plan (Cheffings et al. 2005). Decrease in population size and extinction was also experienced in Dutch populations having diminished by over 80% between the 1960's and the 1990's. Here it also features on the countries red list as vulnerable, is legally protected and a target species for floodplain grasslands - a priority habitat for conservation in the Netherlands (van Treuren et al. 1993; Ouborg & van Treuren 1995; Hegland et al. 2001). Both British and Dutch *S. pratensis* populations are now generally only found in priority habitats which are protected under conservation efforts (Hegland et al. 2001; Ouborg & van Treuren 1995; Rich et al. 1999). The process of decline in these regions should be an example for continued management of sites in methods that promote the success of *S. pratensis* where numbers currently remain unthreatened. Using the experiences and knowledge gained through conservation research and actions that have already been performed should enable better informed practises elsewhere in the distribution range and in promotion of species rich calcareous and neutral hay meadows in Europe.

S. pratensis conservation has been studied both *in situ* and *ex situ* in the Netherlands in detail. Having a fragmented distribution and declining numbers resulted in a series of conservation genetic studies being performed to assess the influences of genetic erosion and identify if inbreeding depression was occurring. To do this, allozyme variation within and across populations was studied. Van Treuren et al. (1991) examined a total of fourteen Dutch populations, with a large variation in size from 5 to over 1500 plants with 50 plants being sampled at each site. These populations had an average nearest neighbour of 4km and with a pollination range and seed dispersal area much likely to be smaller than this, gene flow between these populations was assumed to be extremely small or nil. In twenty-nine enzyme systems, nine varied at 13 loci. Increasing population size resulted in a significant increase in both the proportion of polymorphic loci and average number of alleles observed. As well as reduced genetic variation Van Treuren also showed that smaller populations were fixed for certain alleles which could be deleterious. Van Treuren et al. (1991) concluded that genetic isolation of small populations of *S. pratensis* in the Netherlands by fragmentation has reduced genetic variation in these populations.

To complement this study phenotypic variation was studied by Ouborg et al. (1991) in 2 large populations and 3 small populations using plants grown from wild seed under standard conditions. It was found that variation in 17 phenotypic characteristics was significantly greater in the larger populations. Some of these characteristics were most likely the effect of maternal influences particularly in the juvenile and seedling stages. With these removed, regression analysis found that the significant relationship between population size and phenotypic variation was lost, however with a common trend still towards less variation in smaller populations. The results of this paper and the allozyme variation carried out by van Treuren et al. (1991) confirmed that *S. pratensis* was presenting evidence of genetic erosion in small isolated populations. It was not however possible to identify if the reduced genetic and phenotypic variation was resulting in reduced fitness or if these small populations were in fact well suited to their environment even with the inability of gene flow between populations.

In order to identify if *S. pratensis* was subject to inbreeding depression, first outcrossing rate was observed due to the gynodioecious nature of the species, to gain an understanding of the effect dioecious forms were having on the populations. Van Treuren et al. (1993) surveyed plants for sexual characteristics and reproductive output, and collected seed from four wild populations (2 large & 2 small). A controlled garden environment experiment was also performed using four populations with a known maternal plant; these were dispersed at varying levels of plant density. Plant material was used to perform multilocus estimation of outcrossing rate, by comparing maternal and progeny allelic loci of allozymes, to identify offspring which contain loci which are not represented in the maternal sample. The species was found to predominantly outcross in both wild and cultivated populations. Ranging from 38.2% (\pm 9.2%) to 95.5 (\pm 5.4%). This was found in a similar study in seed set of British *S. pratensis* by Scott (1989) where male-sterile plants set 67-74% seed. Van Treuren et al. (1993) found no relationship between the abundance of plants in a population and the outcrossing rate, with the lowest value of 38.2% occurring in the one of the largest populations (>1000 plants). It was however found that high plant density and low numbers of female-only plants promoted higher rates of outcrossing.

Ouborg and van Treuren (1994) went on to perform crossing experiments in order to study if the species was subject to inbreeding depression. This was achieved through forced outcrossing, selfing and hybridising plants with known parental origins, in both a greenhouse and a field experiment for six populations of varying size and genetic diversity. Significant inbreeding depression was witnessed after one generation in all selfed populations regardless of population size. The results of the field experiment showed that survival rates were higher in outcrossed progeny than in selfed progeny, with the effect of inbreeding load increasing over the 8 months during which observations were made. Ouborg and van Treuren (1995) next investigated the effect of population size on fitness characteristics through growing wild seed under controlled conditions. However no correlation was found between the two, indicating that fitness was in no way reduced in smaller populations.

The cumulative results from all of the above experiments led to the conclusion that the small populations studied were in an initial stage of genetic erosion. The populations with fewer plants

showed reduced allozyme variation and small populations also exhibited inbreeding depression after one generation of selfing, however inbreeding depression could not be linked to population size. The negative effect of inbreeding in this species could be increased by the fact the species was found to largely reproduce by outcrossing, even in the smallest Dutch populations. This outcrossing is most likely mediated by specific pollinator-plant interactions, a factor which was not possible to identify in this investigation, however such interactions have played a significant role in the evolution of the species (ClaBen-Bockhoff et al. 2004; Walker & Sytsma 2007). Ouborg and van Treuren (1995) thus concluded that it is of paramount importance to know the history of populations of conservation concern, before making an analysis on its viability and continued survival. Some papers have commented on the lack of F₂ generation used in the above studies. Not including members which have gone to the next generation does not account for epistatic characteristics when studying inbreeding depression (Keller et al. 2000).

In Britain *S. pratensis* genetics have also been studied by Kay and John (1995) who investigated the population genetics and ecology of *S. pratensis* during a survey of rare and declining lowland grassland species of plants in the British Isles. Leaf samples from a singular Welsh population (prior to its extinction) and four English populations including one subdivided into three populations. Allozyme variation assays found 5 variable loci out of 9 discovered from six enzymatic systems. Not surprisingly the Welsh population showed the least variation, having only 3 plants, levels of variation were highest in eastern and central England. From the data collected Kay and John calculated the Nei (1972) measure of genetic distance and used these values to produce an unrooted phylogenetic tree for the populations studied. From the results the plants were all deemed native and it was concluded that the plants were of a typical edge of range distribution with the populations reducing in genetic variability with distance across the country from east to west. Differences in genetic variation witnessed between the subpopulations studied could also suggest that the British populations were at threat of genetic erosion.

Several studies have found that small populations of *S. pratensis* were subject to genetic erosion, show some evidence of inbreeding depression and that the species exhibits high but varying outcrossing rates. With this knowledge already gained genetics were not used in this study. Here we focus more deeply on the demographic structure of the populations in the UK. Demographic surveys appear less regularly in conservation research than studies which focus on genetic structure or ecology and habitat (Oostermeijer et al. 2003). A rapid and effective way of studying a species demographic performance/viability status is to compare the structure between populations (Oostermeijer et al. 1994). For perennial plants the most efficient, accurate and commonly used way of doing this is to calculate relative proportions of life stages in a population, as size and reproductive capacity are inaccurate measures of actual age in perennial plants. Assessment by the ratio of life stages have been used successfully for many perennial plant species including *Salvia pratensis*, and are effective for measuring the viability of populations and relating this to other variables such as fitness and/or management (Crone et al. 2011; Hegland et al. 2001; Oostermeijer et al. 1994).

Hegland et al. (2001) placed individuals of *S. pratensis* into six life stages and then calculated a ratio of young to old plants for 23 populations in the Netherlands. The resulting ratio for each population was grouped into three categories of demographic structure type, using K-means cluster analysis. The cluster results were used to classify the population demographic types into: 'dynamic, normal and regressive'. 'Dynamic' populations had relatively high numbers of seedlings and juveniles compared to adult plants, thus were considered highly regenerative and related structurally to 'invasive' populations described by Oostermeijer et al. (1994). 'Normal' populations had more even spread numbers of plants in all 6 observed life stages with no extremes of either young or aging plants, being regenerative and viable. 'Regressive' populations had high numbers of adult plants both vegetative and generative and no or extremely limited numbers of seedlings or juveniles and are likely to be ageing. The results of this survey were used in the current study to group British populations into one of the three categories described above.

Aim:

With fragmented and isolated populations it is feared that British *Salvia pratensis* could be aging and not regenerative. With limited outcrossing, the threat of inbreeding depression and a species known to suffer genetic erosion in small populations this leaves a very real risk of extinction for this species in Britain, without continued conservation efforts. This paper aims to study variation in *S. pratensis* populations in the UK across a representative sample by observing population size, demographic structure, fitness and sex ratio along with habitat characteristics and management types, in order to answer the following questions:

- 1) What is the current status of *Salvia pratensis* in the UK?
- 2) How does demographic structure vary at each site and is there a relationship with fitness and/or population size?
- 3) Does fitness vary across the sites?
- 4) Is sex ratio having any effect on the observed populations?
- 5) What is the effect of habitat quality on population size, demographic structure and fitness?
- 6) Is there a management type that promotes the most suitable habitat characteristics for *Salvia pratensis* viability?

The findings from the questions above will be used to define a range of habitats and management techniques in a representative sample of the native distribution of *S. pratensis* in Britain. Observing where the populations are most viable (i.e. where the population size, demographic structure and fitness are best), the variation in sexual characteristics and relating the results to the observed habitat characteristics and management types. Using these results favourable and limiting conditions for the species will be identified in order to inform conservation efforts in the British Isles. Using the theory and results from the past findings outlined above, the theorised relationship between the variables measured here can be made (fig. 2). These relationships will be tested using the results from the above questions. Finally the conclusions drawn from the results of this study will be used to evaluate

the suitability of a site where the species has recently become extinct and a reintroduction program has been proposed (fig. 1).

Materials and Methods

Study species and sites

Salvia pratensis (L.) (Lamiaceae) is a long-lived, robust perennial herb with a persistent deep tap root and three leaf pair basal rosette (Sell & Murrell 2009; Hedge 1972). The plant normally produces 1 – 5 flowering stems (sometimes up to 50). Each flowering stem will produce 2 – 50 clusters of 4 – 6 (most commonly 6) blue – violet flowers (van Treuren et al. 1993; Ouborg & van Treuren 1995; Hegland et al. 2001). As a gynodioecious species, the plants will have either hermaphrodite or male-sterile flowers, distinguishable by having retracted anthers and smaller sized flowers on male-sterile inflorescences. *S. pratensis* is self-compatible, however was found to mostly outcross in a study of Dutch populations up to a maximum of 96%, independent of population size (van Treuren et al. 1993). Each flower can produce up to 4 seeds which drop from the seed head once dry after the flower dies and falls away (Scott 1989).

The species is mainly distributed in Central and Southern Europe and reaches its north-west edge of range distribution in the British Isles, where it is rare and protected, with only twenty-three extant native populations (fig. 1). Eleven of these populations were chosen for this study that represented the variation in population size and habitat type for the species across Britain. At each site surveys were carried out in June and July 2012, when measurements and observations were made for population size, demographic structure, flower sex ratio, reproductive fitness, habitat quality and management type. A further site where the species has recently become extinct was also included in the habitat survey to assess its suitability for a future re-introduction programme.

Demographic structure

A survey of 11 populations of *S. pratensis* was carried out between 12th and 21st June 2011 (tab. 1). At each site, population area was defined by measuring the distance of plants at the extremities of a

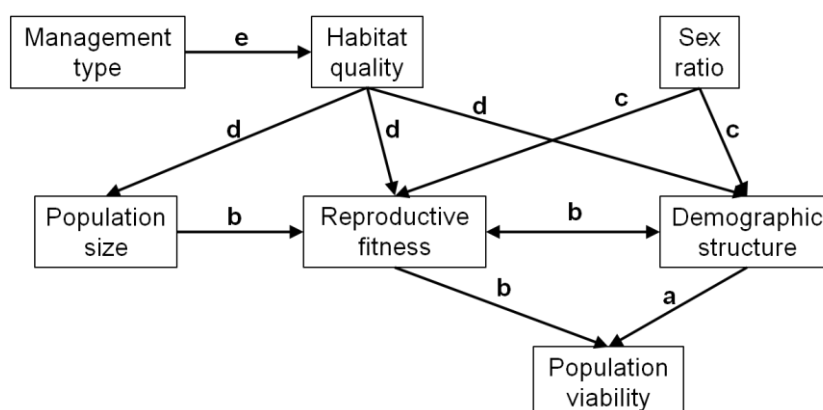


Figure 2) Theorised interactions between habitat, sex, population size, reproductive fitness, demographic structure and population viability in British *Salvia pratensis* populations. Relationships studied in this report are highlighted with letters: **a** demographic structure as a measure of population viability, **b** effect of population size and demographic ratio on fitness, **c** effect of sex ratio on fitness and demographic structure, **d** effect of habitat on population size, demographic structure and fitness and **e** effect of management on habitat.

Table 1) Site name, number of quadrats surveyed, number of sample areas and the total quadrat area for 11 populations of *Salvia pratensis* in the British Isles surveyed between 12th and 21st June 2012

Site name	2m ² Quadrats surveyed	Sample areas	Population Area (m ²)
Stuart Fawkes NR - (Nature Reserve)*	32	2	294
Glyme Farm*	81	2	1066
Ardley - Middleton Stoney	10	3	20
Saltway	18	2	36
Stonesfield Common	50	1	100
Wigwell NR*	22	4	120
Cornbury Park	1	1	2
Langland Farm	20	2	40
Holly Court Farm**	60	6	6372
Abel Wood	2	2	4
Grintley Hill Bridge	18	2	36

*= Sites where estimated values were used which covered $\geq 10\%$ of the total area

**= Sites where estimated values were used which covered $\geq 1\%$ of the total area

homogeneous population. At sites with large gaps between populations sample areas of homogeneous regions were measured and only quadrats within the samples were surveyed. The population area was then divided in to 2m² quadrats using a superimposed grid as used in a previous study on British *S. pratensis* to allow comparison between previous and current results (Scott 1989). At sites where population size was too great to survey all of the plants, a random sample of quadrats were selected, which were numbered and picked using a random number generator. Where random sampling was utilised $>10\%$ of the population area was surveyed with the exception of Holly Court Bank. Here due to the extremely large size of the population just over 1% of the total area was covered (tab. 1).

In each quadrat all *S. pratensis* individuals were recorded along with their life stage. This was performed following the life stage classification system developed for a demographic structure and viability study in the Netherlands (Hegland et al. 2001). This system uses size and morphological differences to designate *S. pratensis* plants into one of six age categories: seedling, juvenile, immature, vegetative adult and two size classes of generative adult (flowering plants): small and large.

Population size

The numbers of plants counted from the demographic survey (above) were used to give two measures of population size. The total number of all individuals counted provided the total or census population. The effective population was also calculated, which is the number of breeding members of a population and hence for plants the number of flowering individuals (Frankham et al. 2010). This was measured as the total number of small and large generative adults recorded at each site. For populations where the whole area could not be surveyed (as mentioned above) estimates were used as the product of density (census or effective) per quadrat multiplied by the total number of quadrats in the population area (tab. 1).

Sex ratio

During the demographic structure and population size survey, the total number of flower stems which fell within each 2m² quadrat was recorded along with the sex of each stem. Sex of *S. pratensis* flowers can be hermaphrodite, male-sterile or intermediate. These are distinguishable by inserting a pointed object (like a pencil) into the mouth of the corolla tube to activate the floral staminal lever system (ClaBen-Bockhoff et al. 2004; ClaBen-Bockhoff et al. 2003; Scott 1989). In hermaphrodite flowers this will result in the emergence of two stamens each with two fertile thecae from the upper lip of the corolla, with a dark brown or black exterior, often bright yellow-orange pollen is visible within (though this is not vital for identification). Hermaphrodite corollas are also generally larger than male-sterile (~20mm). Male sterile plants will have aborted stamens with the base of the staminal lever often intact but with no anthers, these flowers have smaller corollas (≤ 17 mm) and are regularly observed with flowers of a lighter violet colour. Rarely intermediate flowers can be observed, where one or both anthers are formed on retracted stamens, these anthers are often small, deformed and paler brown in colour than in hermaphrodite flowers (Scott 1989). To measure the variation in sex across the sites the proportions of each sex were calculated. In order to quantify this as a single measure sex ratio of non-hermaphrodite (male sterile + intermediate) over hermaphrodite flower stems was calculated.

Reproductive fitness

Seeds were collected (where present and ripe) under licence from Natural England over a two day period between 23rd and 24th July 2012. Due to the constraints of the licence (in order to prevent any damage to the populations), the number of seed heads collected was limited to a maximum of 10% of the total number available up to a maximum of 50. The seed heads were inspected for the presence of fertilised, ripe seed before removal, and were taken from as many individual plants as possible across the entirety of the population area. Seeds were then allowed to further dry/ripen for just over a week, in airtight containers wrapped in paper bags.

Three measures of fitness were used. An initial measure of fitness was performed using a 1mm pore diameter sieve to separate and count the proportion of seeds falling into one of two size classes: those larger than 1mm (large – viable seed) and those smaller than 1mm (small – non-viable seed). This was based on findings in a study on Dutch *S. pratensis* seed viability, where only seeds which were greater than 1.25mm were found to successfully germinate (Ouborg & van Treuren 1995). Thus 1mm was a useful basic measure of the variation in fitness across the sites.

Seeds were then sown in a mix of 3:2 peat-free compost to sieved sand, in 38x24x5cm seed trays, this soil composition was previously found to promote the best growth in British *S. pratensis* samples (unpublished data) differing from 1:1 mix suggested in other papers (Ouborg & van Treuren 1995). Seeds were sown, on 31st July 2012, in individual holes ~5mm below the surface and then covered with a light sprinkling of soil. The seed trays were arranged randomly on a cold bench in a frost free glass house maintained between 8 - 13°C, in full sunlight. Emergence was monitored over 16 days, at

the same time each day, when the trays were rotated to avoid any tray being under different conditions due to its position in the glass house. Water was delivered from the base-up every fourth day when the trays were also treated with Bayer Garden® Fruit and Vegetable Disease Control Fungicide to prevent damping-off of seeds.

Percentage of total emergence after 16 days was the second measure of fitness, when the number of seeds emerging began to plateau. The final measure of fitness was the rate of emergence. Each day the total number of seedlings which had emerged were counted then at the end of the experiment the time taken for 50% of the total emergence (T_{50}) of each population was calculated using equation 1 devised by Coolbear et al. (1984) and revised by Farooq et al. (2005):

$$T_{50} = t_i + \frac{\left(\frac{N}{2} - n_i\right)(t_i - t_j)}{(n_j - n_i)} \quad (1)$$

Where N = total number of seedling emergence n_j and n_i are the cumulative number of seedlings emerged by adjacent counts at times t_j and t_i , respectively, when $n_i < N/2 < n_j$.

Habitat quality

Several variables were measured to assess the quality of habitat across the 11 populations and the reintroduction site in Wales. In each of the 2m² quadrats described above for measuring demographic structure, population size and sex ratio, the percent cover of herb layer, bryophyte layer, litter and bare soil was recorded, along with the mean height of the vegetation. The cover of herbaceous plants and the mean height of vegetation were measured as indicators of productivity. Bare soil is required for the recruitment and establishment of seed this was observed to identify the effect on ability for populations to incorporate seed effectively into the soil and allow successful growth of seedlings (Hegland et al. 2001; Scott 1989; Ouborg & van Treuren 1995).

All associated vascular plant species with *S. pratensis* in one 4m² quadrat per site were identified between 23rd and 24th July 2012. The quadrat was placed within the population area in a homogenous area of vegetation that sufficiently represented the vegetation type. Identification was performed by a taxonomic expert for vascular plants - Dr T. Rich from the National Museum Wales. The total number of species observed in the above quadrat was used as a measure of habitat quality in the form of species richness. When trying to assess environmental habitat characteristics such as soil quality, water levels and pH over a short period of time it is difficult to provide a definitive assessment for sites in one or two measurement events, with no historical records. The plant species present in a habitat give an indication of the how environment at that site fluctuates over time regardless of its condition at the time of testing (Diekmann 2003; Hill et al. 1999; Ellenberg et al. 1992; Schaffers & Sýkora 2000). Due to this survey occurring over one season bio-indicator values were used to assess variation in a number of habitat characteristics across the sites, based on the species composition of the plants in the vegetation stand type and using Ellenberg indicator values corrected for the British Isles by Hill et al. (1999) for light, moisture, reaction, nitrogen and salt tolerance. The corrected Ellenberg value for

each plant species present in the quadrat was taken, all of the values found were then averaged to give an estimated overall value measure for each site (Ellenberg et al. 1992).

Management type

The management type was designated using on site observations, conversations with land owners/managers and conservation groups. This resulted in collation into 4 management types: no management, seasonal grazing, year round low intensity grazing and high intensity grazing (table 2).

Data analysis

For the variables of population size, demographic ratio, fitness and sex ratio the total value found for each population was used in the analysis. For the variables measured for habitat quality the mean and standard deviation for each population was calculated. The remaining variables: demographic group, overall sex type and management type, were analysed using categorical methods. Tests for normality were carried out and where data deviated from normality and homogeneity of variance transformations were performed. The following variables were transformed: census population, effective population, total flower stalk number, demographic ratio and sex ratio were log-transformed and the square-root of the percent cover of bare soil was used. Some of the habitat variables were removed from the analysis at this point due to lack a variance across the 11 populations including: bryophyte cover, litter cover and the Ellenberg values for light, reaction, water and salt tolerance.

To quantify the demographic structure/viability of each population a ratio of young/aged plants was used. This was calculated as the total number of seedlings and juveniles divided by the total number generative adults (both small and large) (referred to as S+J/G ratio from here). Hegland et al. (2001) used the values of the S+J/G ratios to group populations into three categories of demographic structure type, using K-means cluster analysis. The cluster results from that paper were used to classify the population demographic types observed here as: 'dynamic' populations with S+J/G ratios of 0.67–22.0, 'normal' populations, ratios of 0.1-0.77 and 'regressive' populations had ratios of less than 0.1 (generally 0.0). Differences between the mean of S+J/G ratio (log-transformed) of each of the three demographic structure group types was tested using a one-way ANOVA with post-hoc Tukey test.

The results of the fitness survey were tested with bivariate correlation to see if the seed size, total emergence or emergence rate were influencing one another. Sex ratio presented two groups those with hermaphrodite only plants and those with both hermaphrodite and male sterile. Hence the two groups were tested for differences in fitness, demographic structure and habitat variables against one

Table 2) Management types in 12 study sites for British *Salvia pratensis* (11 extant & 1 extinct).

Type	Description
1	No or limited management – Often wild animal grazing e.g. rabbits along with some small scale conservation efforts such as scrub clearance and scarification
2	Seasonal livestock grazing - Exclusion of livestock during April to August (to allow flowering and seed set)
3	Year-round low-intensity livestock grazing - Some sites requiring scrub clearance
4	Year-round high-intensity livestock grazing

another using a two sample T-test.

To examine the effect of habitat on population size and demographic structure multivariate analysis was used. Due to the smaller sample size of 9 for fitness variables multivariate analysis was not possible, so bivariate correlation was analysed instead. Prior to multiple regressions, bivariate correlation was performed to test for multi co-linearity (correlations of 0.7 or higher) in groups with several variables: population size and habitat quality (Field 2009; Pallant 2007). The log-transformed values for census and effective population size correlated highly ($r = 0.987$). The census population was used as a single measure of population size in regressions, due to the fact it represented a larger number of individuals. The habitat variable percent herb cover correlated highly with the square root of percent bare soil ($r = -0.985$) and the Ellenberg nitrogen value ($r = -0.765$). Lastly the percent bare soil cover correlated the Ellenberg nitrogen value ($r = 0.673$). Hence the bare soil and herb cover percentages were removed from the study.

With the other habitat variables removed due to lack of variance (mentioned above) the following were the final independent variables used in the multiple regressions analysed: average height of vegetation, species richness and nitrogen Ellenberg indicator value. The two dependent variables were census population and S+J/G ratio in the two separate multiple regressions performed. Investigation of the relationship amongst all interacting variables was performed using structural equation modelling. Only those variables which were suitable for multivariate analysis identified in the steps above could be tested. Once again variables which were subject to multi co-linearity were removed to avoid the influences on one another affecting the path coefficients. Fitness variables were not used due to the sample size being smaller than 10.

The model was constructed by including all variables which had shown interactions in bivariate correlation and multiple regression analysis. Tests were performed where all theorised relationship pathways between variables were observed and also where any biological relationships could be possible. Models which did not fit the data as it was not possible to reject the null hypothesis that the model covariance matrix will be the same as the matrix inferred by the model, had path coefficients removed to analyse their affect, until a final model of best fit was found. The final model fitted with a non-significant maximum likelihood chi-square value, meaning that it was possible to reject the null hypothesis outlined above having a good fit with the data used. An additional measure of comparative fit index produced by Bentler was also used to test model fit of which a figure greater than 0.9 suggests a good fit between the model and the dataset (Laughlin et al. 2009; de Vere et al. 2009; Grace 2006; Byrne 2001).

To identify if there were relationships between fitness variables and the population size, demographic ratio and habitat quality variables bivariate correlations were performed. Where bivariate correlation analysis of the dataset was used with multiple tests performed, assumptions based on the mean of the dataset could produce relationships amongst the data when they were not actually present, in the form of type I errors (Field 2009). For this reason Bonferroni adjustment to the α -level of significance was used to check for this occurrence.

Finally the effect on management method and habitat variables was tested for differences using a one way ANOVA with a post hoc Tukey test. All statistical tests were performed using the IBM statistics program SPSS 19.0 software with the exception of structural equation modelling for which IBM AMOS 19.0 software was utilised.

Results

Current status of Salvia pratensis in the UK

Across the 11 sites studied the population area ranged from 2m² to roughly 6372m² (tab. 1). The number of plants varied on a large scale across the 11 sites surveyed with the census population ranging from 2 plants up to an approximate value of over 16,000 (tab. 3). Of the census number observed, the effective population constituted between 25.0% and 88.9% varying in total number from 1 flowering plant to approximately 7146. A range of demographic structures was observed in the different populations showing variance in S+J/G ratio, which allowed classification of populations into 3 population structure types. Of the 11 populations sampled 2 were categorised as dynamic, 4 as normal and the remaining 5 sites where all classified as regressive.

Table 3) Population size, demographic structure, fitness and sex type variable results for 11 British *Salvia pratensis* populations.

Site Name	Population size		Demographic structure		Fitness			Sex
	Census (n)	Effective (n)	S+J/G Ratio [‡]	Type [†]	Seeds >1mm (%)	Seedling emergence (%)	T ₅₀ emergence time (days)	Sex type [¥]
Stuart Fawkes NR	183*	148*	0.007	R	80.0	21.0	11.33	M
Glyme Farm	459*	239*	0.163	N	90.0	17.5	10.17	M
Ardley - Middleton Stoney	12	7	0.000	R	-	-	-	H
Saltway	47	35	0.000	R	70.2	14.3	12.57	H
Stonesfield Common	124	73	0.014	R	89.5	39.5	10.58	M
Wigwell NR	94*	32*	1.906	D	86.3	22.5	9.94	H
Cornbury Park	12	3	2.667	D	59.4	21.7	10.54	H
Langland Farm	58	29	0.103	N	85.0	14.5	12.13	M
Holly Court Farm	16668*	7146*	0.347	N	82.86	40.5	9.41	H
Abel Wood	2	1	0.000	R	-	-	-	-
Grintley Hill Bridge	203	56	0.589	N	88.0	11.5	11.58	H

‡ = Total Number of Seedlings + Juveniles / Generative adults

† = R = Regressive, N = Normal and D = Dynamic

¥ = H = Hermaphrodite only flowers, M = Mixed hermaphrodite and male sterile flowers

* = Estimated figure based on density of measure multiplied by population area, all others are total counts or 'true' values

Fitness was measured by three factors presented in table 3, and demonstrated clear variation across the 9 populations where seed was successfully collected. The percentage of large viable seed (those greater than 1mm) varied from a minimum of 59.4% to a maximum of 90.0%. Variation in the percentage of seedling emergence for these seeds was observed, ranging from a minimum of 11.5% to a maximum of 40.5% of the total seeds sown. The T_{50} emergence rate also varied across the populations with the time taken for 50% of the total emergence ranging from 9.4 - 12.6 days. The sex of inflorescences was found to be hermaphrodite only, at six sites, four sites presented between 44.5% and 96.2% hermaphrodite flower spikes and at one site it was not possible to distinguish sex. Where male sterile flowers were present the number was only greater than 5.8% at two sites where values of 29.4% and 53.7% were seen. Intermediate flowers were very uncommon. Ratio of non-hermaphrodite to hermaphrodite plants for most sites was 0 and ranged from 0.04 to 1.25 at the 4 sites where sex varied

Demographic structure of British populations

The populations studied were successfully grouped into the three demographic types as specified by Hegland et al. (2001) with 2 dynamic populations, 4 normal and the remaining 5 being regressive (fig. 3). Results from the one way ANOVA showed that the S+J/G ratio for the three groups were significantly different from each other (MS (Mean square) = 12.19, F = 18.98, df = 10, P <0.005). A post-hoc Tukey test found that the means were all significantly different with the exception of the dynamic and normal, this maybe the influence of the small sample size of the dynamic group of only 2 (fig. 3).

Dynamic populations presented a high proportion of both seedlings and juveniles; however this group also contained high numbers of large adult generative plants. With low numbers of small adult generative plants resulted in an overall high S+J/G ratio. Figure 3 shows the proportion of each life stage in each population individually. The dynamic populations had S+J/G ratios of 1.91 and 2.67. The two sites in this category had both been significantly impacted by small scale conservation efforts by local people, such as clearance directly around mature plants and intentional spread of seed on these areas. These sites were also suspected of having young plants grown in captivity and unofficially reintroduced at the sites. The populations categorised as normal contained an overall lower proportion of seedlings and juveniles, along with increased proportions of immature, vegetative and small generative plants. Proportion of large generative adults decreased compared to the pooled dynamic populations. In figure 3 the 4 sites which fell into the normal category and had S+J/G ratios ranging from 0.103 to 0.347. The 5 regressive populations were composed almost entirely of vegetative adults, small generative adults and large generative adults. These sites had no or limited proportions of seedlings and juveniles observed respectively and total counted number in the pooled data of seedlings being 0 and juveniles being 2. Of the 11 sites just under half were classified as regressive having a ratio of 0 with the highest ratio being 0.014 (fig. 3). It should be noted that at Abel Wood a total of only 2 plants were recorded, one vegetative and one large generative adult, this site could still however be confidently classified as regressive.

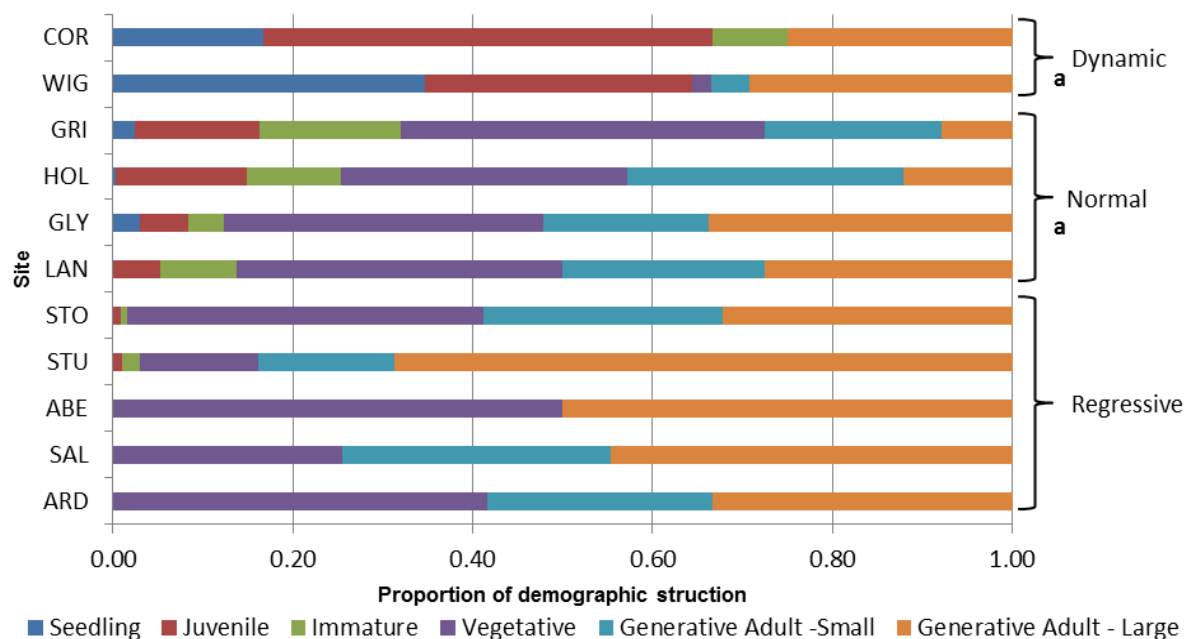


Figure 3) Demographic structure of *Salvia pratensis* by proportion of total number in 11 different British populations. Sites are displayed in ascending order of Seedling to Juvenile/Generative Adult ratio (S+J/G ratio). Site abbreviations: ARD (Ardley-Middleton Stoney); ABE (Abel Wood); COR (Cornbury Park); GLY (Glyme Farm); GRI (Grintley Hill Bridge); HOL (Holly Court); LAN (Langland Farm); SAL (Saltway); STO (Stonesfield Common), STU (Stuart Fawkes NR) & WIG (Wigwell NR). Sites were grouped for demographic structure using the S+J/G ratio results from Hegland et al. (2001). The mean ratio for each group was tested for differences with a one-way ANOVA = MS (Mean square) = 12.19, $F = 18.98$, $df = 10$, $P < 0.005$. Results of *post-hoc* Tukey test are shown: demographic groups which share a letter are not significantly different.

Study of fitness across the sites

Seed was collected from 9 of the 11 sites. It was not possible to collect from Abel Wood due to heavy predation of flower stalks and at Ardley – Middleton Stoney as there were limited numbers of seed and where present they were not ripe enough for collection. 200 seeds were used in the emergence test with the exception of Saltway and Cornbury Park the low number of seed heads available required less seed to be removed ($\leq 10\%$ of total) resulting in 84 and 180 seeds respectively. Across the 9 populations fitness variables varied quite substantially (table 3). Cornbury Park had the smallest percentage of large size seed (those seeds $> 1\text{mm}$) was 59.4% the second smallest being Saltway with 70.2% with the rest of the sites ranged between 80.0 and 90.0%. Emergence success ranged from 11.5% to 40.5% after 16 days. Lastly the T_{50} emergence had a range of over 3 days varying from 9.4 to 12.6 days.

Relationships between fitness variables were tested for using bivariate correlation with Pearson's coefficient. The percent seedling emergence had a negative correlation with seed emergence rate T_{50} ($r = -0.675$, $n = 9$, $P < 0.05$), indicating that sites which had the most successful seed also had the fastest emerging.

Sex ratio

The sex ratio survey found that 6 sites of the 11 had hermaphrodite inflorescences only and 4 populations presented between 44.5% and 96.2% hermaphrodite flower spikes. Of these 4 sites the proportion of male sterile spikes was only greater than 5.8% at two sites where values of 29.4% and 53.7% were witnessed. Intermediate flowers were recorded in very small numbers at two sites only constituting 1.7% and 5.8%. These flowers were always found to be incapable of producing pollen, forming a detracted or deformed stamen. At Abel Wood the sex of the single adult generative plant was indistinguishable due to predation of flower stalks and subsequent production of secondary shoots which had not yet flowered at the time of the survey.

Table 4) Habitat characteristics for 11 British *Salvia pratensis* populations and a test site for a reintroduction program.

Variable	Mean (SD)	Minimum	Maximum	Rectory Meadow
Bare soil (%) ^a	10 (9)	1	26	0
Herb cover (%) ^a	88 (11)	66	99	100
Bryophyte cover (%) ^b	1 (2)	0	7	0
Litter cover (%) ^b	0.05 (0.1)	0	0.5	0
Height of vegetation (m)	0.16 (0.05)	0.075	0.26	0.05
Species richness	23 (6)	15	31	17
EIV - Light ^b	7.1 (0.25)	6.5	7.4	7.1
EIV - Moisture ^b	4.6 (0.27)	4.2	5.0	5.1
EIV - Reaction ^b	6.8 (0.17)	6.4	7.0	6.4
EIV - Nitrogen	4.6 (0.69)	3.5	5.8	5.3
EIV - Salt Tolerance ^b	0.2 (0.08)	0	0.3	0.2

Mean (over all 11 populations) with the standard deviation (SD), accompanied by the minimum and maximum recorded value from these populations and the observed value at Rectory Meadow reintroduction site.

EIV = Ellenberg Indicator Value

^a = Removed from multiple regressions to avoid multi-collinearity

^b = Limited variation across sites

The sex of each population was put into one of two groups, either hermaphrodite only or mixed hermaphrodite and male sterile (tab.3). To investigate if there were any differences in mean values of the two groups habitat characteristics, population size, demographic or fitness the groups were tested with a two sample T-test, which produced no significant difference in any of the variables measured.

Interaction between habitat quality, population size, demographic structure and fitness

The results of habitat variables measured are summarised in table 4. Bryophyte and litter cover were extremely low over the 11 sites surveyed for habitat, whilst the herb cover and average bare soil showed substantial variation ranging from 66 – 99% and 1 – 26% respectively. Average height of the herb layer varied from 0.075m to 0.26m and a range of species richness were observed from a minimum of 15 species to a maximum of 31 species counted in one 4m² quadrat.

Of the bio-indicator values calculated for the sites only nitrogen presented a notable amount of variation (tables 4 & 5). The values for light, moisture, reaction and salt tolerance showed variances of less than 1 between all of the sites meaning that these characteristics were consistent across all of the populations. These values correspond to the Ellenberg explanations described in table 5.

Table 5 Average Ellenberg indicator values for 11 *Salvia pratensis* populations in the British Isles for light, moisture, reaction and salt tolerance with an explanation of the result based on Ellenberg et al. (1992) and corrections for the British Isles by Hill et al. (1999).

Indicator Value	Range Measured	Explanation
Light	7	Generally well lit places, but also occurring in partial shade.
Moisture	4 - 5	On the dry end of moist sites mainly on fresh soils of average dampness.
Reaction	6 - 7	Weakly acid to weakly basic, with a range reaching towards 6 means the sites were more likely a weak acid or neutral. Rarely acidic.
Salt Tolerance	0	Absent from saline soils.

The average indicator value for nitrogen varied across the sites more substantially ranging from 3.5 to 5.8, spanning 3 three habitat types described by Ellenberg et al. (1992) and Hill et al. (1999) as sites which range from more or less infertile (unproductive) to sites which are reaching levels of rich fertility (productive).

To investigate the interaction of habitat, population size, demographic structure and fitness, multivariate analysis was performed using multiple regression and structural equation modelling. The small sample size of 9 would not be powerful enough to allow the fitness variables measured to be

used in multivariate analysis thus they were removed and analysed using bivariate analysis. Bivariate correlation between fitness variables and population size showed no significant relationships. This was also the case between demographic structure and the fitness variables of percent large viable seeds and percentage of seedling emergence. However a negative correlation relationship with near significance was found between the rate of emergence (T_{50}) and the demographic ratio ($r = -0.627$, $n = 9$, $P = 0.07$). Suggesting the populations which had larger numbers of young plants produced seeds which had a mild trend of faster emergence time than those produced from regressive populations (table 3).

The fitness variables were test for relationships with habitat quality variables. Positive correlation was found between Ellenberg nitrogen value and the T_{50} seedling emergence rate ($r = 0.727$, $n = 9$, $P = 0.0264$), meaning a low nitrogen value at a site results in a shorter seedling emergence time. However after Bonferroni adjustment a significance level of $P < 0.0167$ was required due to the fact multiple tests were performed. Hence this relationship was not significant under the corrected α -level.

In the multivariate analysis multiple regression was initially tested using the variables remaining after removal of data which exhibited multi co-linearity or lack of variance. A significant explanation of variance was found for population size with a negative effect of nitrogen value on the site, species richness and vegetation height had limited effect on the variance in population size (tab. 6). Demographic structure produced a non-significant regression model with habitat quality, however when tested with bivariate correlation was found to have a negative relationship with Ellenberg value for nitrogen ($r = -0.666$, $n = 11$, $P < 0.05$).

A structural equation model was constructed to analyse the overall interaction between population size, demographic structure and habitat quality. The findings from both the multi and bivariate analysis were used to feed into the model construction, however all coefficient pathways for variables

suitable for multivariate analysis were tested until the model of best fit was found. Ellenberg nitrogen value was the only major significant effecting habitat variable having a negative influence on both population size and demographic structure. This gave the structural equation model which produced the best fit shown in figure 4 and illustrated by a likelihood chi-square value of 2.29, $df = 1$, $P = 0.130$, CFI = 0.95. The Ellenberg indicator value for nitrogen was shown to be having a highly significant negative relationship with population size and a significant negative effect the demographic structure. Therefore for the 11 populations surveyed when the Ellenberg nitrogen value or productivity at the site increases the population size decreases and the demographic structure becomes more regressive.

Table 6) Multiple regression results for the effect of habitat quality on population size in 11 *Salvia pratensis* populations in the British Isles.

Dependant Variable: Census population ($R^2 = 0.819$, $P = 0.001$)			
Independent Variables:	β	t	P
Ellenberg nitrogen value (productivity)	-0.966	-5.140	0.002
Species richness	0.189	1.352	0.219
Average height of vegetation	-0.204	-1.371	0.213

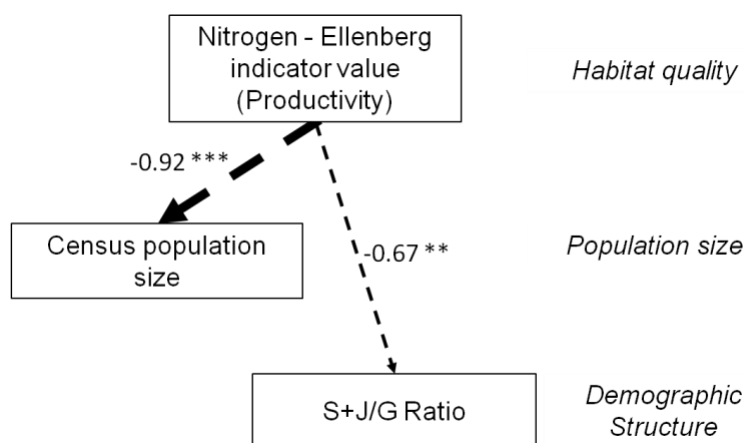


Figure 4) Structural equation model showing the relationship between habitat quality, population size and demographic structure for 11 *Salvia pratensis* populations in Britain. Single head arrows represent directional relationships amongst variables. There were no significant correlations between the variables included. Line width is proportional to the standardised path coefficient with the value given. The dash represents the negativity of the relationships. ** $P < 0.01$ and *** $P < 0.001$. Chi-square = 2.29, $df = 1$, $P = 0.130$, CFI = 0.95.

Furthermore the impact of nitrogen value on a population negatively affected the number of plants and the demographic structure independently as these two variables did not correlate with one another. When a correlation path coefficient for census population and demographic ratio was included it rendered the model non-significant. Hence demographic ratio could not be attributed to variation in population size for the sites observed.

Management type and habitat characteristics

Four management types were observed (table 2), 5 sites were classified in management type 1, 3 sites per management type 2 and 3 under type 3. Finally only the Rectory Meadow (reintroduction) site was found to be under type 4. Some variation in mean habitat variables of bare soil and vegetation height across the management types can be seen in figure 5; however none of these variances were significantly different when tested by one-way ANOVA. Productivity and species richness did not present notable variation across the sites. It is therefore not possible from the results of this study to draw any definitive conclusions on which management type is promoting the best

habitat conditions. Variables which did not show variation across the sites were again not included in this part of the study (tab. 4).

Reintroduction site – Rectory meadow habitat quality and management type comparison

The habitat values for Rectory meadow are presented in table 4, where these values significantly differ from the mean of the 11 extant sites is a good indication of which variables are limiting the ability for *Salvia pratensis* survival at this site. Four of the observed values fell outside or at the vary edge of the standard deviation of the mean these were percent bare soil, mean vegetation height, species richness and Ellenberg nitrogen value (productivity). The bare soil cover, vegetation height and species richness were notably lower and the productivity for the site was notably higher than the 11 other sites. Rectory Meadow did not fit into any of the management types observed being labelled in its own category of type 4, year round high intensity livestock grazing (tab. 2).

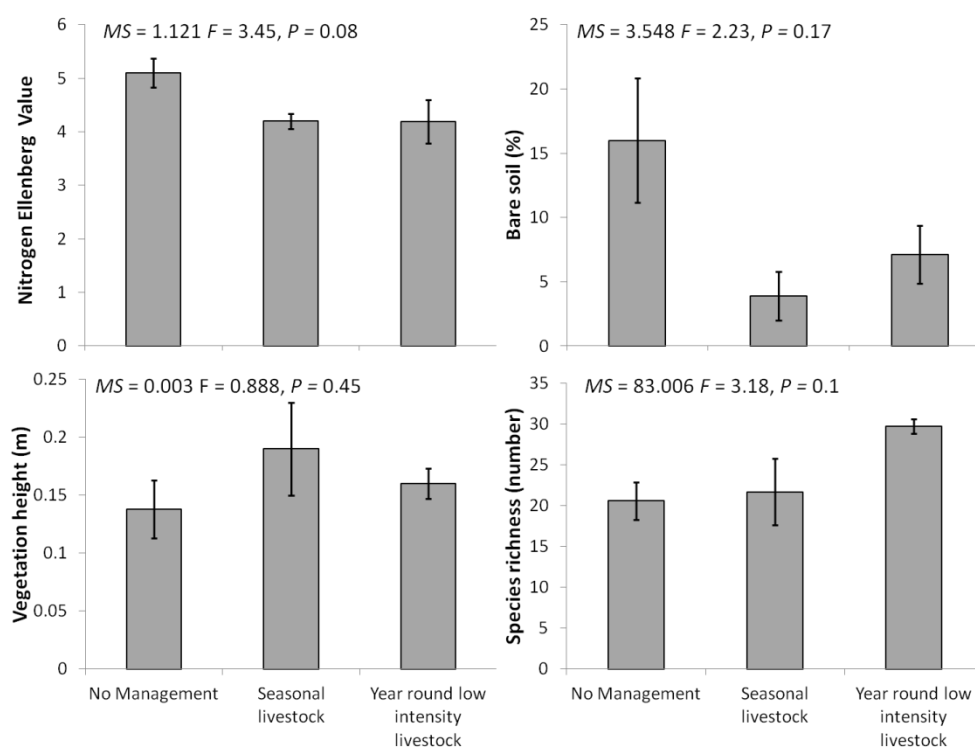


Figure 5) Means \pm SE for habitat variables measured at 11 *Salvia pratensis* sites in the UK, under three observed management types: No management, seasonal livestock grazing or year round low intensity livestock grazing (more detail table 2). The findings from one-way ANOVA are presented, MS = Mean squares, $df = 2$. No management type presented significantly different means; hence *post hoc* results are not shown.

Discussion

Demographic structure of British Salvia pratensis

All 11 populations were classified using Hegland et al. (2001) life stage classification system, which transferred well for use comparing British *Salvia pratensis* populations. The populations observed were most predominantly regressive (5/11), with four normal and two dynamic. The distribution of life stage structure of the populations is concerning as it is quite different from the findings in Hegland et al. (2001) where a greater proportion of Dutch populations surveyed were dynamic or normal. The findings are more similar to Oostermeijer et al. (1994) in *Gentiana pneumonanthe* where populations were predominantly regressive. Hegland et al. (2001) concluded that the populations surveyed in the Netherlands were mostly under the correct management, this would suggest that in the UK the management of many of the sites may need to be reviewed. In this study the two dynamic populations were suspected to have been influenced by conservation efforts and unauthorised reintroductions, and both had obvious signs of clearance around the plants and one had purposeful distribution of seed heads on bare soil. It is difficult to establish the true viability of the plants at these two sites due to human intervention, however this greatly exemplifies the need of conservation efforts to enable the populations to be regenerative or invasive in this species in the British Isles.

As was hypothesised in the theorised relationship model (figure 2), the demographic ratio did not present a relationship with population size in the structural equation model. Notably one normal population had only 58 plants and a 'regressive' population had ~183 plants. This was similarly found by Hegland et al. (2001) where normal populations ranged from 6 to ~700 and regressive ranged from 2 to ~700. This suggests that regardless of population size those which are regressive could be in the early stages of being lost. Some issues with the survey could be limited young plant numbers at the time of surveying, as *S. pratensis* sets seed later in the season and is not thought to incorporate seed into the seed bank (Ouborg & van Treuren 1995; Hegland et al. 2001; Scott 1989). However the fact that individuals in young life stage categories were recorded at several sites reduces the likelihood of this issue. The classification system could also act as a good measure of the survival rate of the plants, as out of the seedling and juvenile categories mostly juveniles were recorded. This suggests that the populations with higher S+J/G ratios are producing offspring with greater longevity as more young plants survive to be recorded in the next flowering season.

Effect of demographic structure and population size on fitness

Fitness in *S. pratensis* seeds has been studied in a number of publications including the effect of seed size and germination rate. Ouborg and van Treuren (1995) found that seed size significantly affects viability using sieved size classes. A minimum size of 1.25mm was necessary for germination to occur and below this all seeds were non-viable. This was used as the basis for measuring the proportion of large viable seed here. In the four Dutch populations surveyed the proportion of non-viable seeds (\pm SE) varied from 27.0 \pm 3.0% to 45.0 \pm 4.0% this variation was not related to population size. A similar level of variation was seen here, ranging from 10.0 to 40.6% non-viable seed, with a slightly smaller

minimum size of 1mm to place seeds into the large viable class measured in this investigation, thus the presence of non-viable seed was likely slightly higher than the value observed, however as this was not tested here it is difficult to conclude the effect of seed size on germination/emergence success.

The proportion of seeds which germinate varies quite vastly between populations and by year in *S. pratensis* (Scott 1989). Ouborg and van Treuren (1995) found differing germination proportions (\pm SE) between $11.0\pm 4.0\%$ to $86.0\pm 4.0\%$ in samples of 'optimum' size seed (between 1.4-1.6mm). A similar amount of variation was witnessed when measuring the seedling emergence rate in this investigation ranging from 11.0% to 40.5%. The effect of inbreeding on *S. pratensis* seeds was investigated in another study by Ouborg and van Treuren (1994). Seeds resulting from self-treatment had a lower germination proportion, averaging 29% less which suggested some impact of inbreeding depression in the species. In this study fitness level across the populations showed considerable variation particularly percentage emergence and the rate of emergence, which also correlated with one another. Thus populations with 'fitter' seed produced higher proportion of effective emergence at the fastest rate. With previous findings of seeds which had been forced to inbreed showing lower germination rate this could indicate that the sites with low emergence rate here are being forced to inbreed.

The fitness of populations was theorised to be effected by demographic structure and population size (fig. 2), however none of the variables measured correlated with seed size, emergence success or rate. Fitness was notably best in the extremely large population at Holly Court Bank having both highest percentage and the fastest emerging seedlings. However this did not follow with small populations having reduced fitness and hence no relationship could be identified with population size and fitness in this study, an observation which is commonly found to have positive correlation (de Vere et al. 2009; Oostermeijer et al. 2003). Ouborg and van Treuren (1994) found a similar lack of correlation in Dutch populations. The small number of populations used here for fitness of 9 and the even smaller number used in the aforementioned study make it statistically difficult to study relationships in the data, however based on the findings from both investigations it can be said that population size is not related to fitness in this species.

Interestingly a mild negative (non-significant) trend was witnessed between demographic ratio and emergence rate. This trend suggested that the populations with higher proportion of young individuals also had faster emerging seed, which could be aiding survival of these plants making them able to cope with competition of other grassland species. However this observation would need to be further tested with larger number of populations included in the analysis, as the sample size was particularly small (9) making in un-statistically powerful. This is a common problem in conservation research of rare species, as small population numbers make statistical strength in the analysis difficult to draw definitive results (Ouborg & van Treuren 1995; Hegland et al. 2001). It was not possible to collect data for two of the eleven populations as seeds were not available. Both of these were extremely small populations (census populations of 2 and 12) that were both regressive (table 3). The lack of seed,

small population size and aging demographic of these two populations, Abel Wood and Ardley, places them under extremely high potential of extinction.

Effect of sex ratio on fitness and demographic structure

There were only two sites with major differences in their sexual composition Langland Farm and Stuart Fawkes nature reserve which had 29.4% and 44.9% male sterile flower stems respectively. It is non-conclusive if this is having an impact on the populations. No significant differences were seen between grouped variable means for hermaphrodite only and mix sex populations. Langland Farm had a 'normal' demographic structure, meaning this population had good fecundity; however fitness was low with an emergence success of only 14.5% and a slow rate of 12.1 days. It is notable that the number of male sterile plants at the Stuart Fawkes site was very high considering that the majority of populations had no male sterile plants. This site was regressive and yet had the 4th largest population size. This could be the impact of the dioecious nature of male sterile plants causing the effects of inbreeding to be felt more intensely in these two populations. Van Treuren et al. (1993) found in both natural and experimental populations having a higher number of hermaphrodite plants promoted outcrossing. It would be interesting to study this further to investigate if the increased proportion of different sex plants is resulting in inbreeding depression in these populations. The sex ratio and viability of both should continue to be monitored closely.

*Effect of habitat quality on *Salvia pratensis* populations*

The greatest impact on the populations of the habitat variables was the Ellenberg indicator value for nitrogen or productivity (Hill et al. 1999; Ellenberg et al. 1992). This gave the final structural equation model which found that an increase in productivity was having a highly negative impact on population size and a negative influence on demographic ratio. This negative effect was also witnessed in the seedling emergence time, where sites which had higher nitrogen values produced seed with the longest emergence times, though after Bonferroni adjustment this value was non-significant. The relevance of Bonferroni α -level correction in biological relationships is sometimes doubted and therefore it is likely this correlation value is still of importance (Curtin & Schulz 1998; Field 2009).

Ellenberg indicator values are sometimes criticised due to the fact that the values for species are generally based on observational data making them subjective and/or in data sets which can be influenced by circularity caused by the collection or calibration method (Zelený & Schaffers 2012; Diekmann 2003). However indicator values have objective meaning in biology based on calibrations and statistical significance in the results of the authors who produced them, the corrections for the British Isles alone encompassed data from over 14 thousand quadrats (Diekmann 2003; Hill et al. 1999; Ellenberg et al. 1992). The large amount of data they use makes them an extremely useful resource for giving an indicated value of habitat quality characteristics using a method which allows for temporal fluctuations at sites, provides an insight into characteristics which cannot be measured easily with equipment over short periods and requires little cost and no technical equipment.

Diekmann (2003) reviews the use of indicator values and findings that the value measured often correspond to a number of varying factors in the environment. Whilst the Ellenberg value for nitrogen is a good indicator for the total amount of nitrogen (or soil mineralisation rate) it is an even stronger indication of the amount of biomass in that environment. The value integrates the bioavailability of nutrients in the soil and the site conditions which effect how productive the plants at the location will be. Hence the indicator value for nitrogen is best described as a measure of productivity (Diekmann 2003; Ellenberg et al. 1992). Here the productivity was found to be the greatest influencing factor on *S. pratensis* on population size, demographic ratio and on fitness. This is furthered as there was no seed from the two sites with the highest Ellenberg nitrogen values. It is justified in saying that *S. pratensis* needs non-productive or unimproved conditions, which is the case for many threatened hay meadow species emphasising the need for reduced fertiliser application and havens where no fertiliser is applied commonly described in the literature (Stevens et al. 2010; Hegland et al. 2001; Ouborg & van Treuren 1995; Scott 1989). This investigation also highlights the variation in productivity level in which *S. pratensis* will be able to survive in for the British Isles. It is likely that a habitat with an Ellenberg value for nitrogen of 6 or greater will result in the loss of the species and low values of around 3 will promote the best demographic structure, population sizes and fitness.

For the other habitat variables measured even though they were not found to have a significant impact on the viability of populations, these represent the habitat type which the species will survive in, where they varied and also where they did not. Where the habitat variables did not vary this could be indicative of habitat traits for which the species has a narrow range of tolerance. *S. pratensis* could be more fixed for these values, such that it can only survive in sites with such characteristics and if they were to begin to change this could have a large impact on the plants survival prospects. For the other habitat variables, which did show notable variation, the standard deviation provides the ideal range in which these values should fall to allow survival of the species. Therefore the mean values for habitat observations presented in table 4 and the Ellenberg indicator values presented in table 5 are a good resource for surveying or restoring and hence are guideline values for *S. pratensis* habitat in the UK.

Suggestions for the improvement of the reintroduction site - Rectory Meadow.

At the Rectory Meadow site the habitat variables which differed most significantly were productivity, bare soil, vegetation height and species richness. As the productivity was found to be the most heavily influencing factor this would be of high priority to restore before any plants are reintroduced. The Ellenberg nitrogen value was high at 5.3 it would be recommended that the productivity of this site was lowered before any plants are re-introduced, having a direct negative effect on both the number of individuals and the demographic viability of *Salvia pratensis*. A major difference at this site compared to the 11 extant populations was observed in its management type of year round high intensity livestock grazing, this is mirrored by the fact the vegetation height was so low. Low intensity or seasonal live stock would be encouraged at this site as an ideal management type and it is likely that this will promote a much lower level of productivity and enable the growth of species which are

not able to survive with fast growing species in these kinds of habitats, this would also improve the species richness at the site (Hegland et al. 2001).

Conservation implications for the British Isles

A large amount of variation was witnessed across the 11 populations of *Salvia pratensis* examined, in terms of total plant numbers, demographic ratio, fitness and sex ratio. From the results of the analysis several interesting and noteworthy points have arisen. The size of a population, the fitness level or population viability are not necessarily indicative of one another in the populations studied. Hence having a small population size in several cases does not represent a non-viable population, similarly for large populations which can be unfit and regressive. With this in mind as previously suggested by Ouborg and van Treuren (1995) a good knowledge of the structure, status and history of the site should be known before any intervention on the population is carried out on this species. With some of the sites at real concern of loss, a review of how they are managed is needed most urgently at the five sites classified as regressive.

S. pratensis is long lived and has been known to survive for over 30 years, thus small populations can continue to survive for long periods of time even though they may be in decline (Rich et al. 1999; Hegland et al. 2001; van Treuren et al. 1991). It is likely that a few successful reproductive events in this species can prolong this survival for even longer and hence it has many long standing small population records. Management of the sites will play a large role in prevention of loss. None of the management types here presented significant differences in their habitat structure. However it has been previously shown that low-intensity grazing or late hay cut are the most successful management types (Hegland et al. 2001). The most influential factor on populations from the results presented here is the productivity of the sites. Thus efforts should be maintained to prevent productivity from increasing at the sites by seasonal or low intensity grazing and/or regular clearing of scrub and no fertiliser application, whilst working to preserve the sites in state which falls within the habitat characteristics described above.

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