Assessing the Long-Term Viability of the Locally Restricted Species *Calothamnus* sp. Whicher and *Hakea oldfieldii* in a Threatened Ecological Community near Busselton

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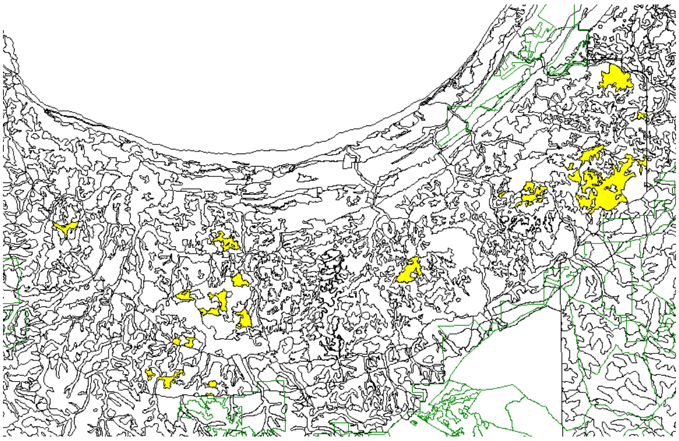

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Executive summary

Introduction

The seasonally wet ironstone plant communities of the Swan and Scott Coastal Plains are amongst the most endangered in south-west Australia and are of national and international significance (Gibson *et al*. 2000). These plant communities, although historically restricted and rare, have been extensively cleared occurring in small isolated remnants and are now under considerable threat. For plant species in these fragments, populations are typically smaller and more isolated than they were historically, the abundances of their interacting species have changed, new species have been added, and the abiotic conditions and resources necessary for growth and reproduction have been altered. For sexually reproducing plant species, changes to the pollinator community and mate availability may have a number of important consequences for pollen flow, the mating system, seed production, and offspring fitness, factors which all influence population vital rates and viability (Hobbs & Yates 2003; Yates *et al*. 2007 ab).

This report describes studies of the genetic and ecological factors affecting the viability of population fragments of two plant species, *Calothamnus* sp. Whicher and *Hakea oldfieldii*, in the Busselton ironstone community. Investigations of fruit set, seed production, seed fitness, the mating system, landscape level gene flow and demography were undertaken to determine how population size and connectivity influences the viability of plant population fragments for the two species.

Key findings – *Calothamnus* sp. Whicher

Flowering, fruit and seed production occurred in spring in each of the three years of the study. There were no significant differences in the proportion of flowers which set fruit or the number of seeds produced per fruit between years. Fruit set and the number of seeds per fruit varied between populations, but importantly there was no relationship with the number of plants in populations.

Investigations of the mating system showed that seeds arise from a mixture of inbreeding and outbreeding. Levels of inbreeding were unusually high for a bird-pollinated shrub and are most likely consequence of geitonogamous self-pollination rather than biparental inbreeding (crossing between genetically similar plants).

Rates of seed germination under controlled laboratory conditions varied between populations but there was no relationship with the number of plants in a population.

The results for *C*. sp. Whicher contrast strongly with those of a similar study for the closely related but geographically widespread species *Calothamnus quadrifidus.* This species also has a mixed mating system, but progeny arising from self-pollination are aborted and as populations become smaller and mates fewer, the number of seed produced per fruit declines. *Seed production in C. sp. Whicher is apparently robust to the effects of population fragmentation. Plants in small populations are able to go on producing substantial numbers of viable seeds despite the availability of mates declining.*

Although similar levels of seed production occurred among different sized C. sp. *Whicher* population fragments, seedlings and juveniles were only observed in the medium- and large-sized population fragments. Seedling emergence was observed in each of the three years of the study but was highest in the wettest year of the study when rainfall was above average. Seedlings emerged in greatest numbers in areas of bare ground where vegetation cover was lowest. These conditions were not present in the smallest populations where annual weeds were abundant. Seedling mortality is high, but seedlings which survive are recruited into the juvenile population of plants creating populations with mixed-age structures. *Medium and large-sized populations are currently stable or increasing in size, but small populations, because of a lack of recruitment, will decline and eventually be lost from the landscape.*

Genetic diversity in medium and large populations was higher than in small populations and was highest in the medium and large-sized populations. Genetic distances among populations are very high. A significant outcome of this is that all population fragments of C. sp. Whicher contain irreplaceable genetic diversity. *Because of the high genetic differentiation between populations the loss of small populations will result in the significant loss of genetic diversity.*

Recommendation 1: Manage weed loads in the small populations to maximize the potential for recruitment.

Recommendation 2: Conserve genetic resources of small populations by establishing *ex situ* collections and using seeds to rehabilitate suitable areas in or adjacent to medium or large populations.

Recommendation 3: Conserve genetic resources of all populations through *ex situ* collections.

Recommendation 4: Utilize seeds from all populations for replanting and restoration.

Key findings - *Hakea oldfieldii*

Flowering, fruit and seed production occurred in each of the three years of the study, but were significantly higher in the wettest year of the study when rainfall was above average. Fruit set and the number of viable seeds produced per weight of plant canopy varied between populations, but there was no clear relationship with population size, with the exception of the smallest population which produced significantly fewer seeds than other populations. Fruit were generally held on plants for no longer than two consecutive flowering seasons. *Seasonal variation in seed production may make populations susceptible to local extinction following fire.*

Investigations of seed production and population fragment size were limited by the availability of populations. However investigations of the mating system showed that seeds arise predominantly from outbreeding due to a self-incompatibility system preventing effective self-pollination. *As a* *consequence small populations of H. oldfieldii will be susceptible to mate limitation and subsequent reductions in seed production*.

Seedling emergence was observed in one population in the wettest year 2007 when rainfall was above average. The low rates of seedling emergence and predominance of plants in adult size classes indicate recruitment may be more intermittent in *H. oldfieldii* than in *C*. sp. Whicher. *Medium and large-sized populations may be relatively stable in the short term but patterns of recruitment suggest that populations will decline in the longer term.*

Genetic diversity in *H. oldfieldii* was moderate and similar among populations. Like *C.* sp. Whicher genetic distances among populations of *H. oldfieldii* are very high and all population fragments contain irreplaceable genetic diversity. *Because of the high genetic differentiation between populations the loss of populations will result in the significant loss of genetic diversity.*

Recommendation 5: Manage weed loads in the small populations to maximize potential for recruitment.

Recommendation 6: Conserve genetic resources of small populations by establishing *ex situ* collections and using seeds to rehabilitate suitable areas in or adjacent to medium or large populations.

Recommendation 7: Conserve genetic resources of all populations through ex situ collections.

Recommendation 8: Utilize seeds from all populations for replanting and restoration.

Recommendation 9: Confirm that sufficient quantities of canopy stored seed are available in populations before controlled burning is undertaken.

1.0. INTRODUCTION

The ironstone formations of the southern Swan Coastal Plain occur on massive ferricrete deposits that have formed at the foot-slopes of the Whicher Scarp over the last two million years. Skeletal soils overlying the massive ironstone become seasonally inundated and waterlogged in winter and dry out over summer. The ironstone environments support species rich shrublands containing a suite of restricted and endemic plant taxa and forming a unique plant community (Busselton ironstones, community type 10b; Gibson *et al*. 1994; Gibson *et al*. 2000; Meissner & English, 2005).

The Busselton ironstone vegetation community contains a total of 15 endemic species, 11 Declared Rare Flora and six Priority species (Gibson *et al*., 2000; Meissner & English, 2005) as well as a number of endemic 'forms' of more widespread species which are yet to have their taxonomic status resolved (Keighery 2004).

This ironstone community is under considerable threat and is restricted to small isolated remnants due to extensive clearing for agricultural activities. It is estimated that 97% of the Busselton ironstone community has been cleared for agriculture, with approximately 68ha of native vegetation remaining (Gibson *et al*. 2000). These areas are highly fragmented, and restricted to roadside verges, rail reserves and small bushland remnants. Connectivity between these fragments is generally poor as the remnants are scattered throughout the agricultural matrix. The community is threatened by the spread of plant pathogens, particularly *Phytophthora* species; weed invasion from agricultural areas; loss of diversity through altered fire regimes; salinity as a result of altered hydrology; and disturbance from the proliferation of tracks, roads and mining activities (Meissner & English, 2005).

For plant species in these fragments, populations are typically smaller and more isolated than they were historically, the abundances of their interacting species have changed, new species have been added, and the abiotic conditions and resources necessary for growth and reproduction have been altered. For sexually reproducing plant species, changes to the pollinator community and mate availability may have a number of important consequences for pollen flow, the mating system, seed production, and offspring fitness, factors all of which influence population vital rates and viability (Hobbs & Yates 2003; Yates *et al*. 2007 ab).

This report describes studies of the genetic and ecological factors affecting the viability of population fragments of two plant species, *Calothamnus* sp. Whicher and *Hakea oldfieldii,* in the Busselton ironstone community. Investigations of fruit set, seed production, seed fitness, the mating system, landscape level gene flow and demography were undertaken to determine how population size and connectivity influences the viability of plant population fragments for the two species.

The objectives of the research were to:

- Provide information on the reproductive biology and output of Busselton Ironstone species in relation to population size;
- Provide information on the population dynamics and demography of Busselton Ironstone species;
- Quantify the effects of inbreeding in relation to population size, isolation and disturbance in Busselton Ironstone species;
- Assess genetic diversity and structure in Busselton Ironstone species;
- Assess gene flow between fragmented populations of different sizes in Busselton Ironstone species.

2.0. METHODS

2.1. Study area and populations

The study was undertaken in the Busselton area in south-western Australia, approximately 200km south of Perth. The study focused on the vegetation community of the ironstone formation in the Busselton area which comprises only one of two extant areas of vegetation on ironstone of the southern Swan Coastal Plain (Gibson *et. al.,* 2000). Three species typical of the Busselton ironstones form the basis of this study. Results for two species, *Calothamnus* sp. Whicher and *Hakea oldfieldii*, are presented in this report. A third species *Banksia nivea* subsp. *uliginosa* is the subject of two BSc Honours projects being undertaken in the 2009 academic year.

Calothamnus sp. Whicher is endemic to the ironstone soils. It is closely related to *Calothamnus quadrifidus* a widespread shrub that grows from 2 to 3m high and occurs throughout south-west Western Australia. The leaves of *C.* sp. Whicher can be used to distinguish it from *C. quadrifidus.* The leaves are much more terete than *C. quadrifidus* and are densely crowded, spreading upward to form ‘brush-like’ branches (**Plate 1**). Inflorescences are a dense one sided spike (**Plate 2**). Flowers are suspected to have a similar pollination biology to the closely related *C. quadrifidus* which is protandrous and transfer of pollen between flowers by honeyeaters (Megaliphidae) or honey possums (*Tarsipes rostratus*) is necessary for seed set (Collins *et. al.,* 1984).

Hakea oldfieldii was originally thought to be endemic to the Busselton area and largely restricted to the Busselton ironstones (as *Hakea* sp(BJK & NG 226) in Gibson *et al.* 2000). More recent taxonomic work indicates that it also naturally occurs in the Porongurup – Stirling Range area. There are also old collections from the Geraldton area and central wheatbelt. Around Busselton this taxon is largely restricted to the ironstones and areas immediately surrounding them. It is a dense multi-stemmed shrub that grows from 2 to 5m high (**Plate 3**). Given its restricted distribution it is currently listed as a Priority 3 species. The species has alternate leaves that range from 10 to 70mm in length and 1mm in width. Leaves are typically linear, terete and undissected with entire margins and indumentums absent, which is a distinguishing feature. Inflorescences are an axillary raceme of 8-20 cream flowers occurring from August to October. A small proportion of flowers successfully set woody fruits which are approximately 2 cm long and 1 cm wide (**Plate 4**). These contain up to two seeds and are retained on the plant for up to two seasons. To date, no information on pollination syndrome or mating system in the species has been published. However, floral morphology suggests pollination by insects.

In the study area, both species grow in low open shrubland vegetation on relatively shallow skeletal red clay/sands over massive ironstone. Prior to clearing these shrublands would have intergraded with woodlands occurring over deeper soils. Extant populations of *H. oldfieldii* and *C* sp. Whicher vary in size and occur across a variety of landscape contexts ranging from isolated linear strips of vegetation on road verges to patches of varying size on private land and in nature reserves.

In 2006, an area approximately 50km × 50km was surveyed for *C.* sp. Whicher and *H. oldfieldii* population fragments. A number of populations of each species were chosen for study that encompassed a broad range of population sizes (**Table 1** and **Table 2**). Estimates of population size were made based on visual assessment both in the field and from aerial photography, and populations were assigned a relative population size class.

2.2. Reproductive output

In consecutive flowering seasons the numbers of flowers that produced a fruit (fruit set) from 2006 to 2007, and the numbers of seeds per fruit from 2006 to 2008 were investigated for *C.* sp. Whicher. Surveys were undertaken on a minimum of 20 plants in each of the nine populations of *C.* sp. Whicher. Where there was a surplus of reproductively mature individuals, plants spread throughout the fragment were opportunistically chosen for sampling.

On each *C.* sp. Whicher plant, three branchlets spaced equidistantly around the canopy were selected and tagged 30cm from the shoot apex. At peak flowering the number of flowers and flower buds distal to each tag were counted. When fruits had matured, the numbers of fruits distal to the tag on each branchlet were counted. Five fruits from around the canopy of each plant were also collected. Fruits were kept separate, returned to the laboratory and the number of seeds per fruit counted.

Plate 1. Growth habit of *Calothamnus* sp. Whicher. Doyle Rd, Busselton.

Plate 2. Inflorescence of *Calothamnus* sp. Whicher. Doyle Rd, Busselton.

Plate 3. Growth habit of *Hakea oldfieldii.* Gale-Jindong Rd, Busselton.

Plate 4. Fruit of *Hakea oldfieldii*. Gale-Jindong Rd, Busselton.

Table 1. Characteristics of the ten *Calothamnus* sp. Whicher populations studied.

Population	Population code	Location WGS84	Population size	Log population size	Population size class	No of plants sampled for reproductive output data			No of leaf samples collected for genetics*	No of plants from which seeds were collected for paternity analysis	Total no of progeny sampled
						2006	2007	2008			
Adams Rd	A	-33.7743 115.2299	105	2.02	Medium	20	20	20	20	-	-
Boallia Rd	B	-33.7509 115.2687	32	1.50	Small	20	20	21	20	-	-
Doyle Rd	D	-33.737 115.2896	27	1.43	Small	20	22	25	27	10	138
Hairpin Rd	H	-33.7311 115.2663	86	1.93	Small	20	20	20	86	10	147
McGibbon Track	M	-33.6908 115.4768	123	2.08	Medium	20	20	20	20	-	-
Negus Block	N	-33.6657 115.5724	13 530	4.13	Large	20	20	20	20	-	-
Oates Rd	O	-33.6804 115.5311	344	2.53	Medium	20	20	20	20	-	-
Tutanup Rd 1	T1	-33.6512 115.5246	3000	3.47	Large	20	20	20	-	-	-
Tutanup Rd 2	T2	-33.654 115.536	80	1.90	Small	-	-	-	80	10	200
Williamson Rd	W	-33.6995 115.542	3725	3.57	Large	20	20	20	-	-	-

* Only 20 samples from each population were used for the genetic diversity analysis

Table 2. Characteristics of the eleven *Hakea oldfieldii* populations studied.

Population	Population code	Location WGS84	Population size	Population size class	No of plants sampled for reproductive output			No of leaf samples collected for genetics*	No of plants from which seed were collected for paternity analysis	Total no of progeny sampled
					2006	2007	2008			
Adams Rd	A	-33.7743 115.2199	>100	Medium	10	0	0	20	-	-
Gale Rd	G	-33.7946 115.2113	>1000	Large	10	10	10	102	10	199
Hairpin Rd	H	-33.7311 115.2663		Small	3	3	3	-	-	-
Ironstone Gully	IG	-33.8135 115.2218	>2000	Large	10	10	10	20	-	-
Kolhagen Rd	K	-33.808 115.241	29	Medium	10	10	10	29	11	210
McGibbon Track	M	-33.6908 115.4768		Small	0	0	0	-	-	-
Oates Rd	O	-33.6859 115.5171	3	Small	-	-	-	3	2	34
Payne Rd	P	-33.7509 115.2015	20	Medium	10	10	10	20	-	-
Price Rd	PR	-33.7961 115.2447	21	Medium	-	-	-	21	10	180
Tutanup Rd 3	T3	-33.6512 115.5246	>1000	Large	10	10	10	20	-	-
Williamson Rd	W	-33.6995 115.542	>300	Large	-	-	-	20	-	-

* Only 20 samples from each population were used for the genetic diversity analysis

In consecutive seasons when fruits were mature, the numbers of seeds produced per branch weight were investigated for *H. oldfieldii* between 2006 and 2008. Collections were made from a minimum of ten plants per population. Where there was less than ten reproductively mature individuals, all individuals from the population were sampled. On each *H. oldfieldii* plant, the branch with the greatest number of fruit was harvested and the four closest neighboring branches. Fruits were harvested from the branches and kept separate, returned to the laboratory and the fruits were counted under the categories of green fruit, closed fruit and open fruit. Branches were cross referenced to the fruits, kept separate and returned to the laboratory to obtain dry-weight values. Both fruit category and number of viable seeds were expressed as products of dry branch weight.

The effect of population on fruit set and seeds per fruit (*C.* sp. Whicher) and seeds produced per branch weight (*H. oldfieldii*) were examined. Population estimates of seed production for *C.* sp. Whicher and *H. oldfieldii* were made by calculating the mean number of fruit set and seeds per fruit/branch weight for each plant in each population in each year. For each population the mean numbers of fruits set and seeds per fruit/branch weight were calculated from the means of each plant. The population means from each year were used to calculate a single mean estimation of the number of fruits set and seeds per fruit/branch weight for each population.

Prior to performing statistical analysis, data were tested for normality and homogeneity of variances using Levene's test. As the data was normally distributed, the effects of population were examined using generalised linear models with randomized complete block designs. Population was treated as a fixed factor and year as a random blocking factor. The effects of population size were also examined using generalized linear models with population size treated as a fixed factor and year as a random blocking factor. All statistical analysis of the reproductive output was undertaken using the software Statistica v.6.1. (Statsoft).

2.3. Demography and population dynamics

For *H. oldfieldii* and *C.* sp. Whicher, permanent monitoring belt transects were established in the populations outlined in **Tables 1** and **2** in order to monitor demographic parameters. In 2006 belt transects were searched to ensure the target species were found and tagged. For each plant height (and canopy width for *H. oldfieldii*) was measured. Life stage was also recorded (seedling, 1 year old seedling, juvenile and reproductive adult). The following year (2007) the demographic survey was repeated, measuring the survival and growth of all tagged plants. Belt transects were thoroughly searched for seedlings that had emerged since the last survey, when seedlings were found they were tagged and height was recorded. Tagged plants that had died since the last survey were replaced with 'new census plants' to ensure sample sizes remained relatively constant. In 2008 the demographic survey was continued on the *C.* sp. Whicher populations only.

Data was entered into an Access database and for the 2006-2007 and 2007-2008 periods rates of seedling recruitment, fecundity and mortality were calculated.

2.4. Habitat preferences

For both *C.* sp. Whicher and *H. oldfieldii,* nine environmental variables typifying the habitat in the immediate vicinity were measured for seedlings, juveniles and eleven randomly selected points within the boundaries of each population. The environmental variables included measures of cover from canopy to ground cover so that the effects of differences in cover at differing heights could be established for seedlings and juveniles. Distance to the nearest reproductive individual was recorded as this is important in terms of habitat preferences and distribution of seedlings. Dominant vegetation type and soil depth were also recorded as physical identifiers of habitat preference. All the environmental variables are outlined in **Table 3** and **Appendix A**.

The habitat preferences of seedlings, juveniles and random points were compared with multivariate ordination of the nine measured variables. Patterns of similarity among the environmental variables for seedlings, juveniles and random points were assessed using Principal Components Analysis (PCA). Prior to analysis a draftsman plot was used to ascertain the necessity of and the type of transformation required (**Table 3**). Each variable was then normalized (mean subtracted and then divided by the standard deviation) so that the variables would be on the same dimensionless scale. Euclidean distance was the measure of dissimilarity utilized. The significance of dissimilarities in the environmental variable composition between seedlings, juveniles and random points was determined using a permutation test applied to the dissimilarity matrix (Analysis of Similarity (ANOSIM), Primer v6.0; Clarke and Gorley, 2006). In using ANOSIM, the significance is tested by permuntation of the data and indicates the chance of obtaining the an value equal to or larger than the R values by chance alone (Clarke and Gorley, 2006).

The environmental variables responsible and the degree of their influence on the differences observed between groups in the ANOSIM procedure can be determined using the SIMPER procedure (PRIMER v6.0; Clarke and Gorley, 2006). SIMPER investigates the percentage contribution of variables to the separation between points seen in the PCA ordination.

SIMPER analysis was also used to determine the percentage contribution of the dominant life-forms to separation between seedling, juveniles and no plant points seen in the PCA ordination.

Table 3. Environmental variables used to determine habitat preferences.

Variable	Type	Description	Transformation
Distance to reproductive	Continuous	cm	Log(0.1+v)
Overhead cover	Categorical	Projected cover above plant. None, low or heavy.	None
Overhead cover height	Continuous	cm	Log(0.1+v)
Bareground cover	Categorical	Proportion of bareground in 25×25cm quadrat surrounding plant. <5%, 6-24%, 25-49%, 50-74%, >75%	None
Litter cover	Categorical	Proportion of litter cover in 25×25cm quadrat surrounding plant. <5%, 6-24%, 25-49%, 50-74%, >75%	None
Vegetation cover	Categorical	Projected vegetation cover in 25×25cm quadrat surrounding plant. <5%, 6-24%, 25-49%, 50-74%, >75%	None
Vegetation height	Continuous	Height of surrounding dominant vegetation. cm	Log(0.1+v)
Soil depth	Continuous	cm	None
Dominant life form	Categorical	Dominant life form immediately surrounding plant. See **Appendix A** for dominant life form codes.	None

2.5. Genetic analysis

Collections of leaf material were made from adult plants of *C.* sp. Whicher and *H. oldfieldii* at locations listed in **Tables 1** and **2** respectively. Seed capsules were collected from selected plants in three small and medium sized populations of *H. oldfieldii* and three small populations of *C.* sp Whicher. Seeds were extracted and germinated for DNA extraction.

DNA was extracted from seedlings of *C.* sp. Whicher and *H. oldfieldii* and adult leaf tissue of *H. oldfieldii* using a scaled-down version of the CTAB method of Doyle and Doyle (1990). DNA was extracted from adult leaf tissue of *C.* sp. Whicher using Qiagen DNeasy^{TM} plant mini extraction kits. Adult DNA samples were screened for nine DNA microsatellite loci in *C.* sp. Whicher using primers developed by Elliot and Byrne (2005) and 13 microsatellite loci in *H. oldfieldii* using primers developed by Byrne and Hankinson (2009). *Calothamnus* sp. Whicher and *H. oldfieldii* seedlings were assayed for six and seven of these loci, respectively, for mating system and gene (pollen) dispersal analyses.

Genotypes were scored from the raw data using GENEMAPPER v4.0 (Applied Biosystems) and statistical analyses undertaken using POWERMARKER V3.25, MEGA4, GenAlEx (v.6), MLTR V3.2, NEWPAT V5, GENEPOP 4.0 and BOTTLENECK 1.2.02.

2.6. Germination

Germination trials were undertaken for *C.* sp. Whicher for seed collected in 2006, 2007, 2008. The seed utilized was that collected for the reproductive output study in accordance with the methods previously outlined in section 2.2. Ten plants were utilized from each of the nine populations and a mean number of germinants at 28 days was used for the analysis. Seeds were plated onto water agar plates and placed in incubators running at 15℃ on a 12 hour diurnal light and dark cycle. Plates were scored every five days for the number of germinants until the cumulative number of germinants at 28 days was obtained.

The effects of population and year of seed collection were examined using ANOVA. All statistical analysis of the reproductive output was undertaken using the software Statistica v.6.1. (Statsoft).

3.0. RESULTS

3.1. Climate

Long-term rainfall patterns are particularly important for reproduction, seedling recruitment, plant growth and survival for *H. oldfieldii* and *C.* sp. Whicher, growing on the skeletal soils of the Busselton ironstones. Long term rainfall trends at Busselton show that annual rainfall is quite variable (**Figure 1**). There also appears to be a trend of wetting and drying cycles with higher and lower rainfall operating over 20 year periods.

Rainfall patterns indicate a trend of decreasing rainfall, particularly over the last ten years (**Figure 1**). Average rainfall in the last ten years is similar to that observed 100 years ago and may be part of a much longer term cycle.

Monthly rainfall patterns indicate that the winter months are the periods of highest rainfall (**Figure 2**). Spring and autumn rainfall is also quite high although is more variable between years. Summer has consistently low rainfall, as a result of the high pressure systems that dominate the area during these months.

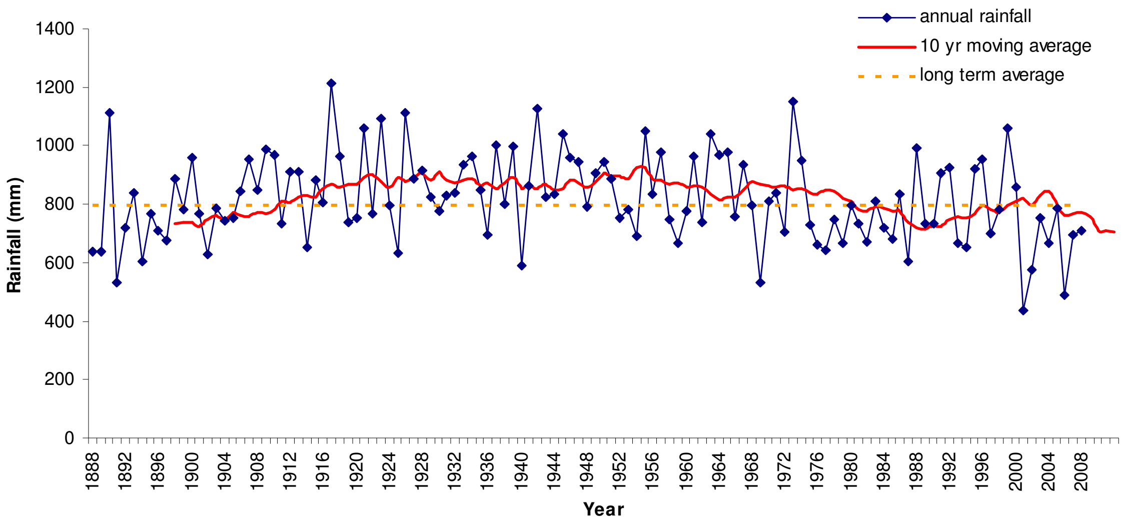


Figure 1. Long term annual rainfall for Busselton (1888-2008) with long term average. Bureau of Meteorology (2008)

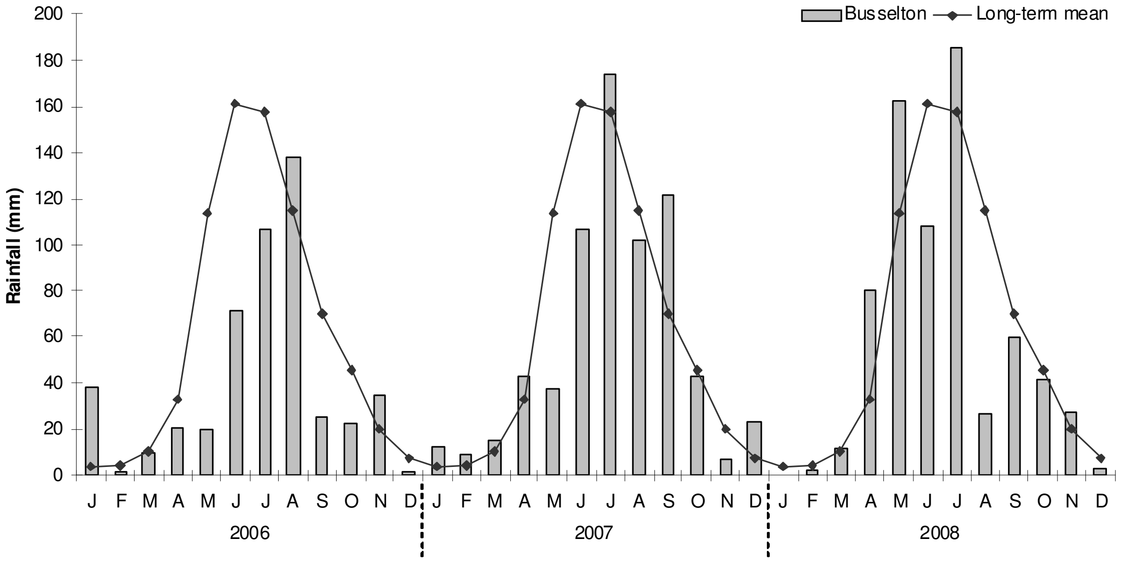


Figure 2. Recorded monthly rainfall and long term monthly average for Busselton (2006-2008). Bureau of Meteorology (2008)

3.2. Reproductive output

3.2.1. Fruit set in *Calothamnus* sp. Whicher

There was a significant difference in fruit set between years ($F_{1,8}$=8.04, p=0.004) (**Figure 3**). There were significant differences among populations in the proportion of flowers that set fruit (F1,8=39.06, p<0.001). Mean fruit set varied between 0.22 (± 0.02) and 0.67 (± 0.02) (**Figure 4**). Mean fruit set was significantly higher for population at Boallia Rd (B) compared to all other populations. Populations at Oates Rd (O) and McGibbon Track (M) had significantly lower fruit set than all other populations apart from the population Doyle Rd (D) (**Figure 4**).

There was no significant linear relationship between fruit set and log population size in either 2007 ($F_{1,7}$=0.06, p=0.80, r^2=0.009) or 2008 ($F_{1,7}$=0.01, p=0.90, r^2=0.002) (**Figure 5 A, B**).

3.2.2. Number of seeds per fruit in *Calothamnus* sp. Whicher

There was no significant difference in the mean numbers of seeds per fruit between years ($F_{1,16}$=3.83, p=0.15) (**Figure 6**). There were significant differences in the mean number of seeds per fruit between populations ($F_{1,16}$=3.83, p<0.05). Mean seed set (±SE) between populations at Adams Rd (A) and Hairpin Rd (H) varied between 14.15 ± 0.70 and 10.52 ± 0.52 seeds per fruit (**Figure 7**).

There was no significant linear relationship between mean numbers of seeds per fruit and log population size in any year; 2006 ($F_{1,7}$=0.15, p=0.70, r^2=0.02), 2007 ($F_{1,7}$=0.31, p=0.59, r^2=0.04), 2008 ($F_{1,7}$=0.00, p=0.98, r^2=0.00) (**Figure 8 A-C**).

Mean seed set between the populations of *C.* sp. Whicher did not show as much variation as that in mean fruit set values. While the general trends for relative mean seed set follow that of mean fruit set, populations such as B (Boallia) showed significant differences with all other populations in terms of fruit production (**Figure 4**). Mean seed production did not reflect fruit production in this instance (**Figure 7**).

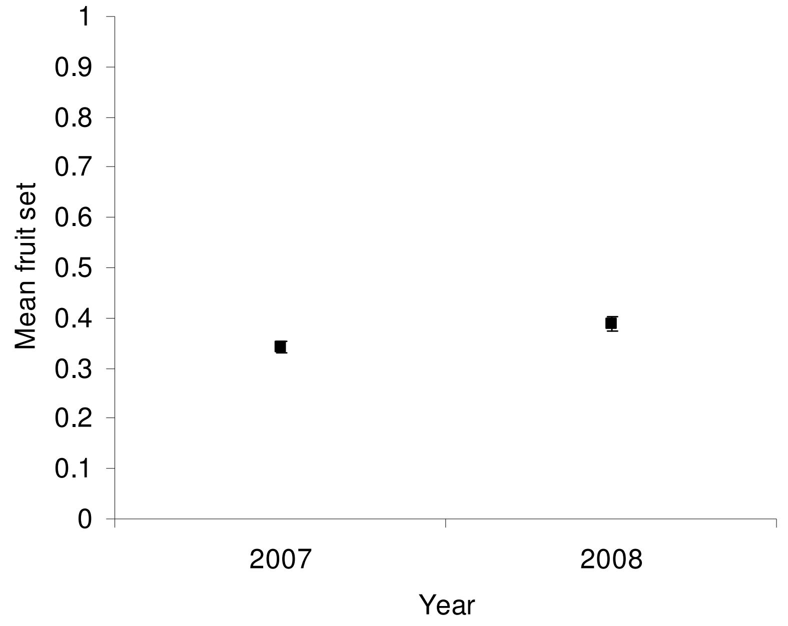


Figure 3. Mean fruit set per year for all populations of *Calothamnus* sp. Whicher. Values are means (± SE) estimated for fruit set for nine populations (2007-2008).

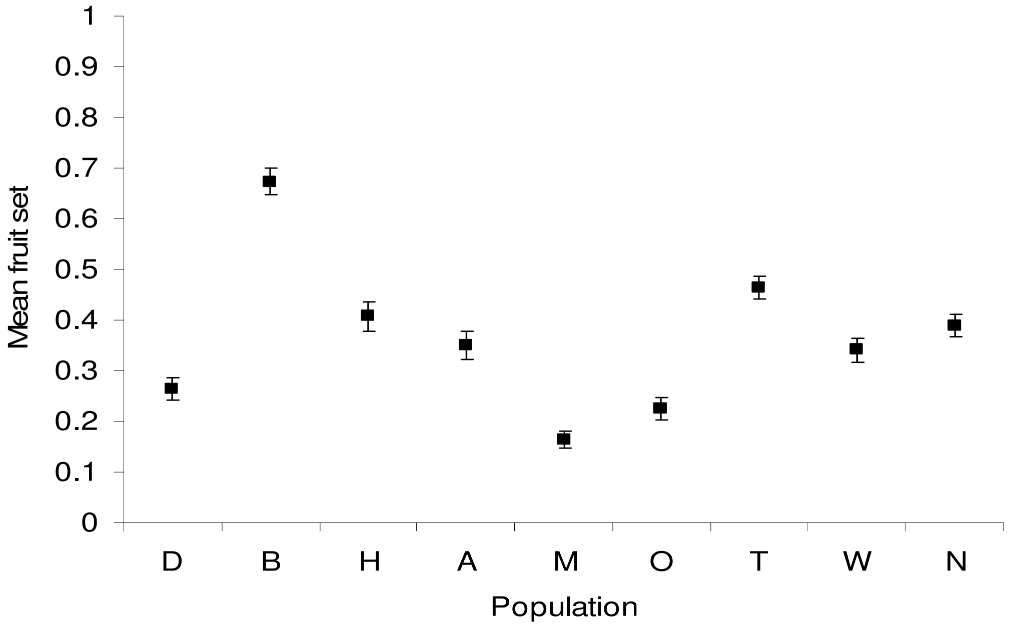


Figure 4. Mean fruit set (proportion of flowers that set fruit) for the nine *Calothamnus* sp Whicher populations. Populations are arranged in order of increasing size (number of reproductive individuals). T = Tutanup 1 population. Values are means (± SE) estimated from population estimates of fruit set for two consecutive years (2007-2008).

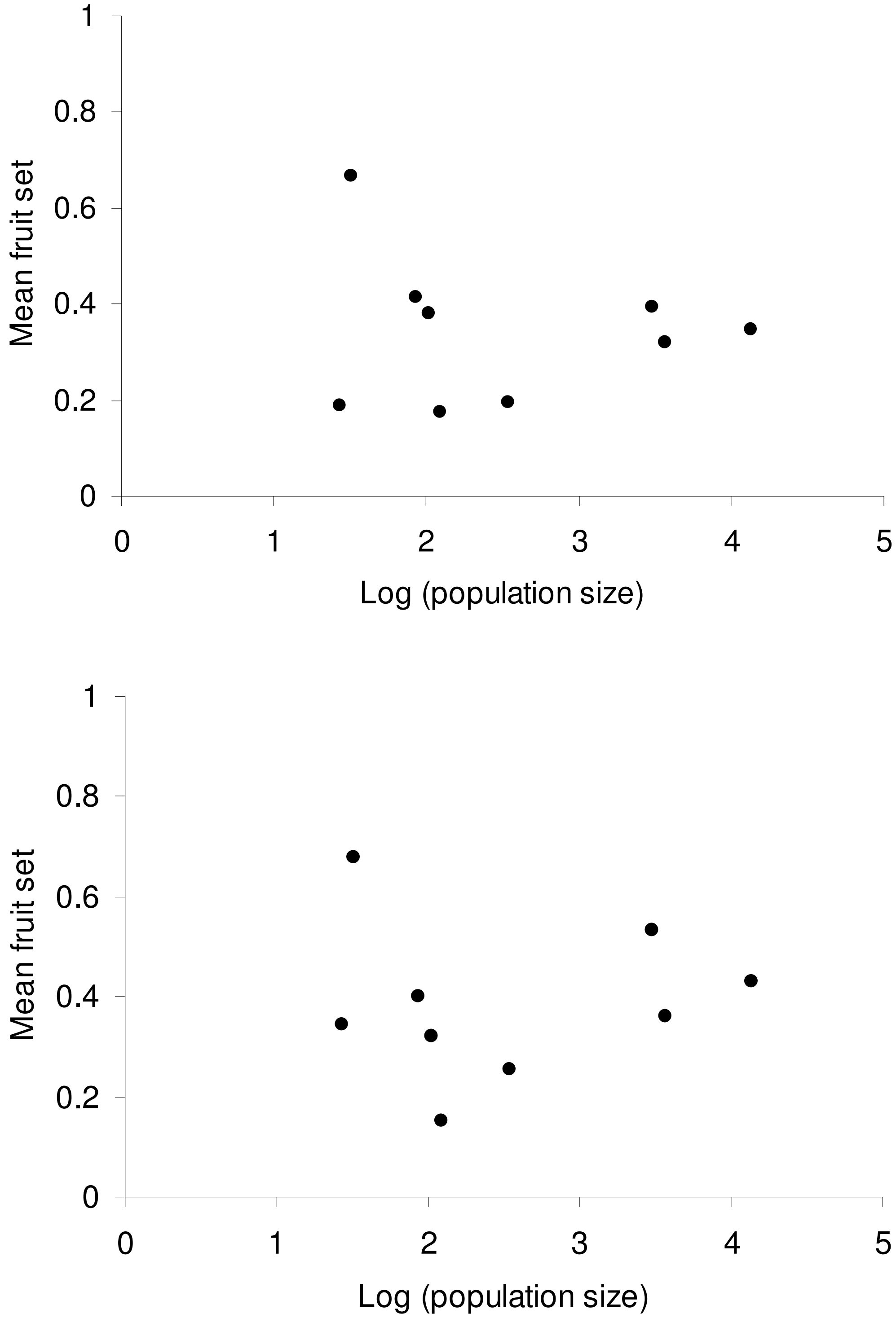


Figure 5 A, B. The relationship between mean fruit set and log population size for the nine *Calothamnus* sp. Whicher populations in 2007 (A), and 2008 (B). Values are means estimated from counts of seed from each population.

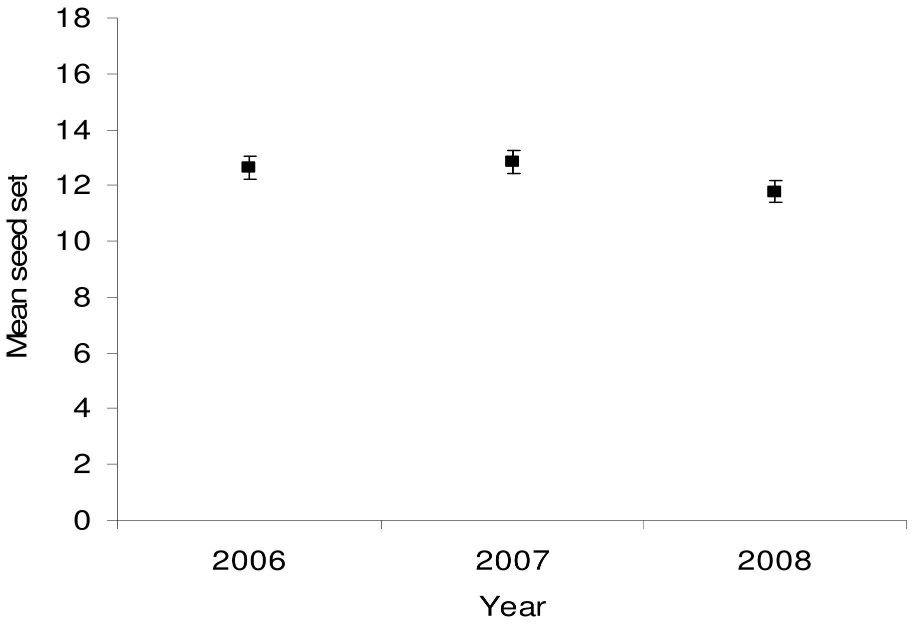


Figure 6. Mean seed set per year for all *Calothamnus* sp. Whicher populations. Values are means (± SE) estimated from seed set for nine populations.

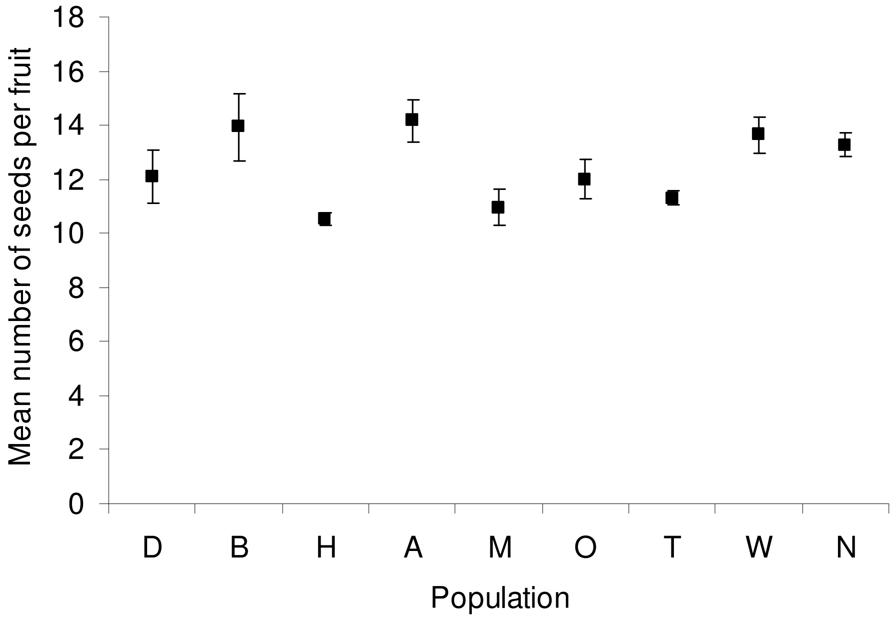


Figure 7. Mean seed set (number of seeds per fruit) for the nine *Calothamnus* sp Whicher populations. Populations are arranged in order of increasing size (number of reproductive individuals). T = Tutanup 1 population. Values are means (± SE) estimated from population estimates of seed set for three consecutive years (2006-2008).

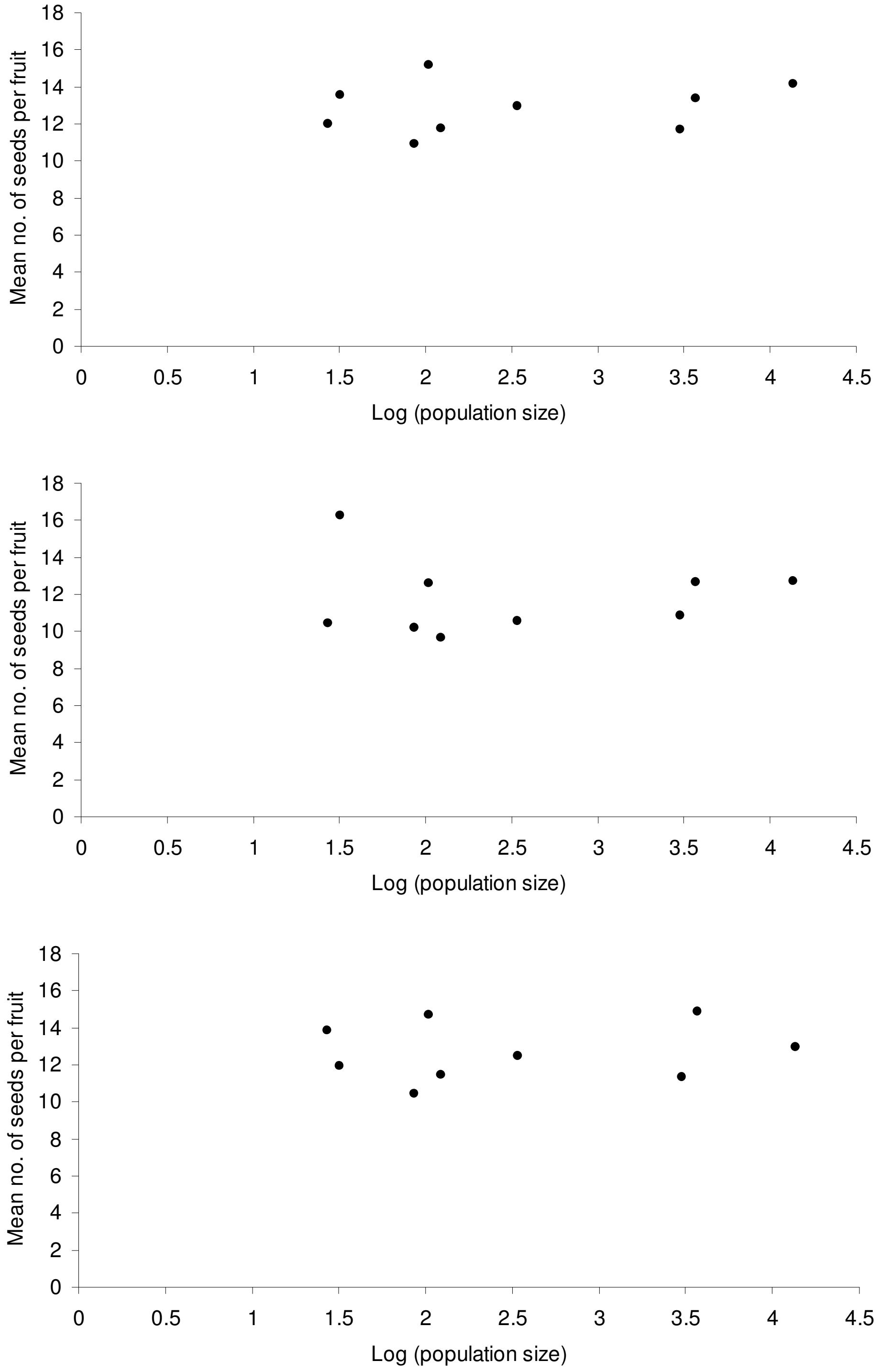


Figure 8 A-C. The relationship between the mean number of seeds per fruit and log population size for the nine *Calothamnus* sp. Whicher populations in 2006 (A), 2007 (B), and 2008 (C). Values are means estimated from 10 plants per population.

3.2.3. Fruit production in *Hakea oldfieldii*

Mean fruit set as a product of branch weight was significantly different between years (($F_{1,10}$=14.93, $p<0.05$). Mean fruit set was significantly higher in 2007, there was no significant difference in fruit set between 2006 and 2008. Mean seed set varied between years from 0.14 ± 0.03 in 2008 to 1.47 ± 0.25 in 2007 (**Figure 9**).

Mean fruit set by branch weight showed significant differences between populations ($F_{1,10}$=3.27, $p<0.05$). Mean fruit set (±SE) varied between 0.08 ± 0.02 for the Hairpin Rd population (H) and 1.22 ± 0.35 for the Tutanup Rd population (T) (**Figure 10**).

3.2.4. Viable seeds as a product of dry branch weight of *Hakea oldfieldii*

There were significant differences in the mean numbers of seeds by branch weight between years ($F_{1,10}$=17.35, $p<0.05$). Mean seed set was significantly higher in 2007 than in either 2006 or 2008. Mean seed set (±SE) between years varied between 0.11 ± 0.01 and 0.44 ± 0.04 (**Figure 11**).

There were significant differences in the mean number of seeds by branch weight between populations ($F_{1,10}$=4.14, $p<0.05$). Mean seed set (±SE) varied between 0.15 ± 0.04 at Tutanup Rd (T) and 0.03 ± 0.01 at Hairpin Rd (H) (**Figure 12**).

Mean seed set amongst populations generally followed the trends in mean fruit set amongst populations (**Figure 10** and **12**). Low mean fruit set was reflected in low mean seed set for corresponding populations (ie. the population at Hairpin Rd - H). However, there were a greater number of populations recording a significant difference between populations for mean seed set in comparison with mean fruit set.

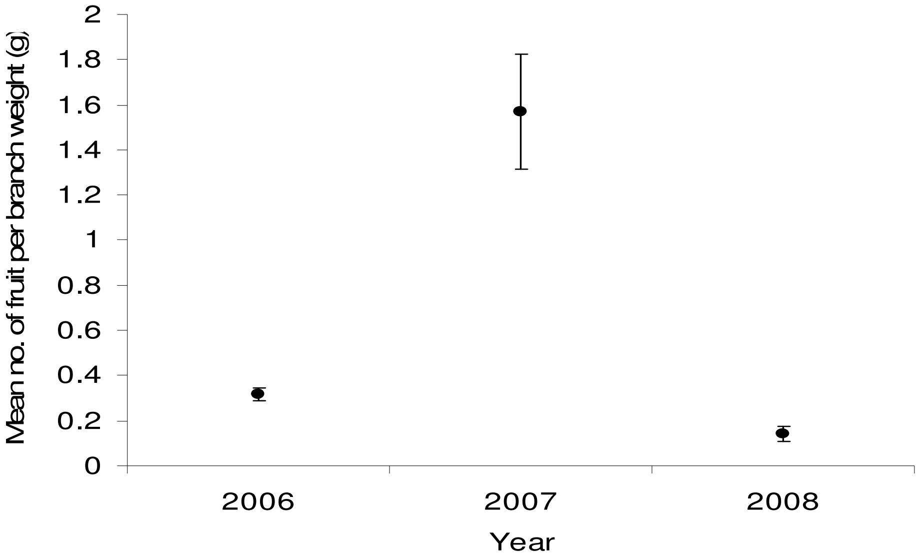


Figure 9. Mean fruit set as a product of branch weight per year for all populations of *Hakea oldfieldii*. Values are means (± SE) estimated for fruit set for three consecutive years (2006-2008).

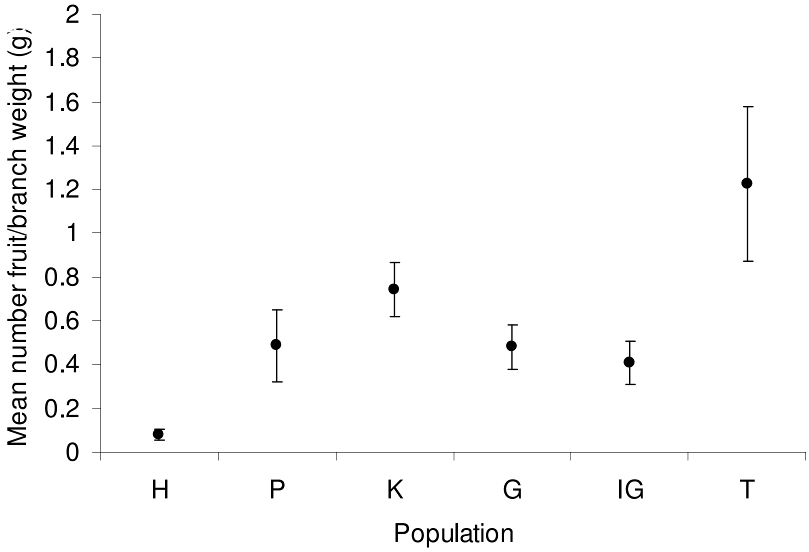


Figure 10. Mean fruit set as a product of branch weight for the six *Hakea oldfieldii* populations. Populations are arranged in order of increasing size (number of reproductive individuals). T = Tutanup 3 population. Values are means (± SE) estimated from population estimates of seed set for three consecutive years (2006-2008).

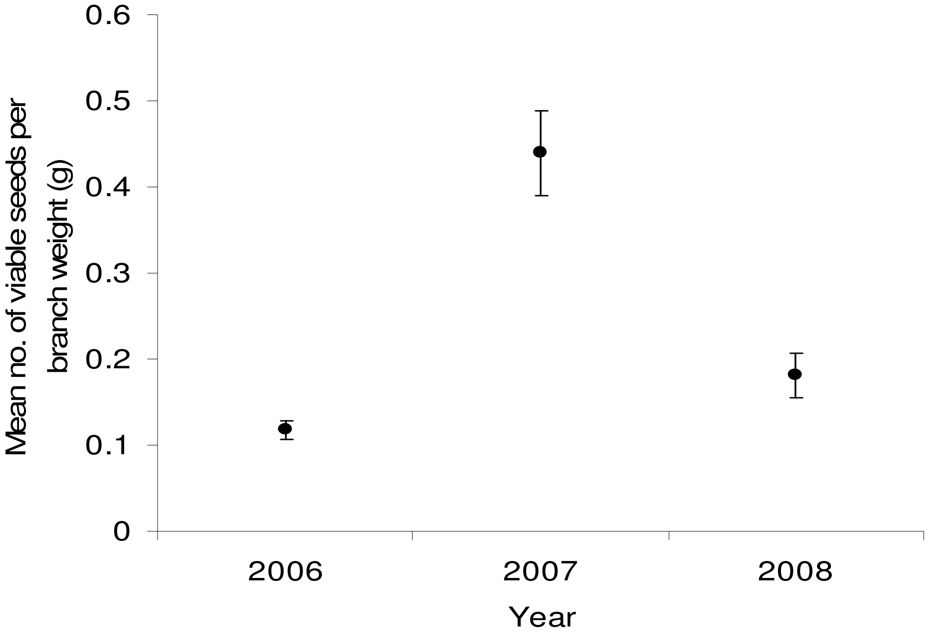


Figure 11. Mean seed set (number of viable seeds as a product of branch weight) per year for all *Hakea oldfieldii* populations. Values are means (± SE) estimated from seed set for six populations (2006-2008).

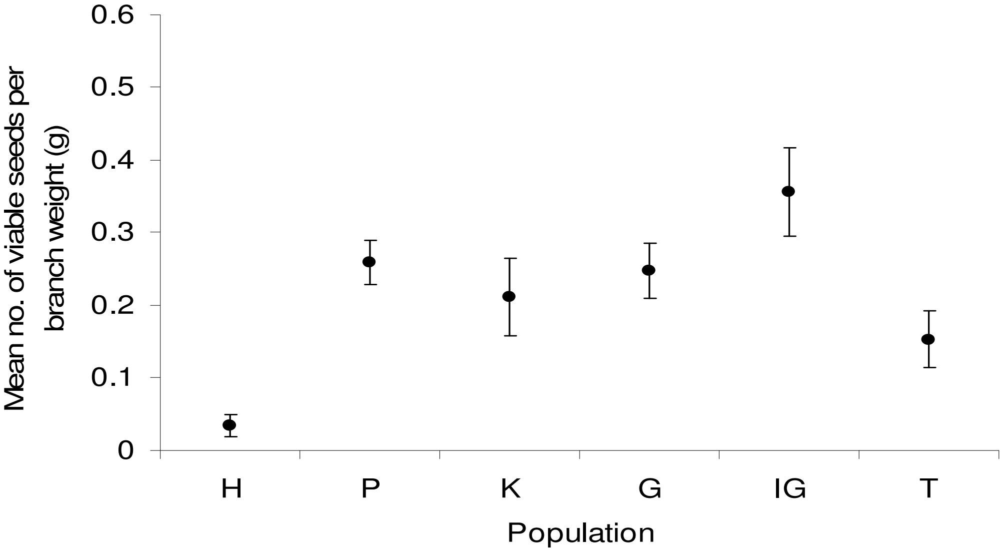


Figure 12. Mean seed set (number of viable seeds as a product of branch weight) for the six *Hakea oldfieldii* populations. Populations are arranged in order of increasing size (number of reproductive individuals). T = Tutanup 3 population. Values are means (± SE) estimated from population estimates of seed set for three consecutive years (2006-2008).

3.3. Habitat Preferences

3.3.1. Habitat preferences of *Calothamnus* sp. Whicher

The PCA of the environmental variables of seedlings, juveniles and no plant points found that the first two axes explained 52% of the variance of the multidimensional data. This indicates that the two dimensional PCA ordination presented (**Figure 13**) adequately represents the relationship among samples. Loading heavily on the PC1 axis from right to left is increasing distance to reproductive individuals (DR) and increasing areas of bare-ground. Decreasing vegetation height and soil depth also influence the PC1 axis from right to left.

Variables loading heavily on the PC2 axis include decreasing vegetation cover from top to bottom. Overhead cover and height of the overhead vegetation is increasing along the PC2 axis from top to bottom.

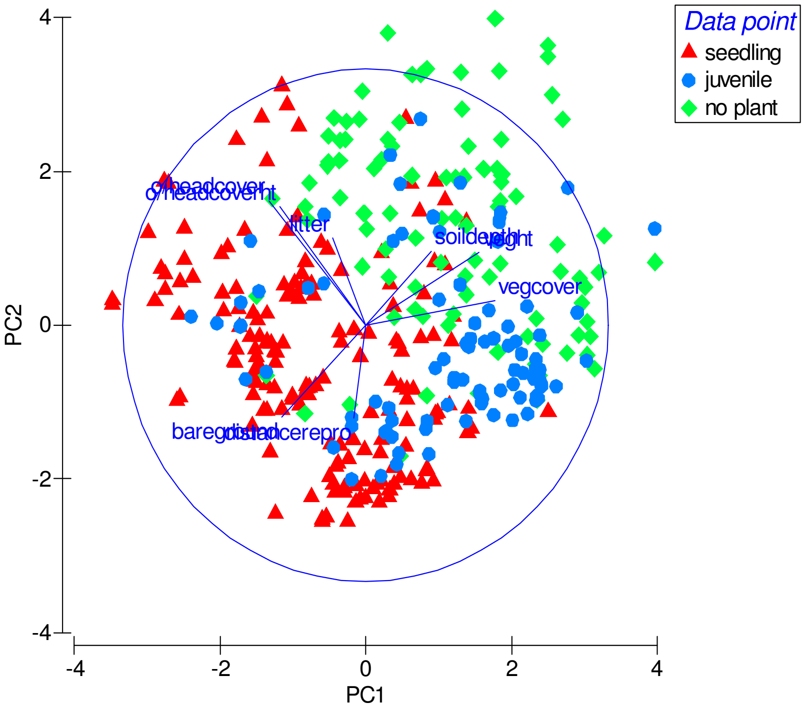


Figure 13. Principal Component Analysis (PCA) of the environmental variables for seedlings, juveniles and no plant points of *Calothamnus* sp. Whicher. The proximity of each point indicates the degree of similarity in the measured environmental variables. The blue lines and circle is the vector plot which represents the importance of an environmental variable's contribution to the PCA axes displayed.

There is a clear differentiation between seedlings and no plant points, both groups are clearly defined in the PCA ordination with few sample points overlapping in multivariate space (**Figure 13**). Juveniles appear to share environmental variables with both seedlings and no plant points. The majority of juvenile samples form a tighter group associated with deeper soils, closer proximity to reproductive individuals and greater vegetation cover than seedlings. These environmental differences, or habitat preferences seen in the PCA are confirmed with ANOSIM (Global R=0.39, p<0.001, **Table 4**). Pairwise comparisons show a significant difference between all combinations of seedlings, juveniles and no plant points.

Table 4. ANOSIM R statistic and probability of differences between environmental variables for seedlings, juveniles and no plant points of *Calothamnus* sp. Whicher.

	R statistic	**p value**
Global	0.39	<0.001
Seedling v no plant	0.53	<0.001
Seedling v juvenile	0.29	<0.001
No plant v juvenile	0.19	<0.001

3.3.1.1 Influence of individual environmental variables

The influence of individual environmental variables on the separation of data points can be further investigated with the SIMPER procedure (Primer v6.0; Clarke and Gorley, 2006) which looks at the percentage contribution of environmental variables to the separation between points seen in the PCA ordination (**Table 5**). Soil depth contributes 23% to the separation of seedlings and no plant points. Soil depth also contributes 25% to the separation of juvenile and no plant points. Vegetation cover contributes 19% to the separation of seedlings and juveniles.

Differences between the data points were also examined using Kruskall-Wallis one-way ANOVAs (**Table 6**). The ANOVA results support the findings of the multivariate analyses PCA ordination, SIMPER and ANOSIM procedures. Seedlings grow in areas with significantly greater distances to reproductive individuals than either no plant points or juveniles (**Table 6**). Seedlings grow in areas of moderate height of overhead cover, which is significantly higher than the height attained by the overhead cover immediately surrounding juveniles. The height of overhead cover was also one of the environmental variables that contributed most to the separation of seedlings and juveniles (**Table 5**). Seedlings show a preference for areas of bare-ground with the mean value of bare-ground cover for seedlings being significantly higher than either no plant points or juveniles (**Table 5**). Significantly lower values for vegetation cover and vegetation height were recorded for seedlings, as well as significantly shallower soils as compared to both no plant points and juveniles (**Table 5**).

Juveniles grow in areas of significantly lower overhead cover and overhead cover heights than both seedlings and no plant points (**Table 5**). This supports the findings of the PCA ordination where juveniles are shown grouping away from high overhead cover values and heights (**Figure 13**). Bare-ground and litter cover values are significantly lower than seedlings and significantly higher than no plant points, showing a transition between areas of new recruitment and unoccupied areas. This transition between areas of recruitment and unoccupied areas is also reflected in the vegetation cover and soil depth values, where juveniles show a preference for deeper soils, greater vegetation cover and greater heights of the surrounding vegetation than seedlings (**Table 5**).

Significant differences in the dominant life-form of the surrounding vegetation is shown in the ANOSIM (Global R=0.29, p<0.001, **Table 7**). Pairwise comparisons between seedlings, juveniles and no plant points show significant differences in the surrounding dominant life-forms for all combinations (**Table 7**). SIMPER analysis revealed that native annual sedges were the most abundant life-form found in close proximity to seedlings, contributing 87% of the similarity within the seedling group (**Table 8**). Native annual sedges also contributed to the separation (32%) of the seedling group from the juveniles and no plant points. Weedy annual grasses were the most abundant life-form found in close proximity to both no plant points and juveniles, contributing 88% and 83% of the similarity within groups respectively (**Table 8**).

Table 5. SIMPER results showing the environmental variables which contribute the most to the separation between seedlings, juveniles and no plant groups of *Calothamnus* sp. Whicher seen in the PCA.

	% contribution		**% contribution**
Seedling v no plant		**No plant v juvenile**	
Soil depth	22.5	Soil depth	24.6
Vegetation height	14.4	Overhead cover height	14.2
Distance to reproductive individual	12.1	Distance to reproductive individual	13.2
Seedling v juvenile			
Vegetation cover	18.8		
Overhead cover height	14.1		
Vegetation height	13.6		

Table 6. Results of Kruskall Wallis one-way ANOVAs testing for differences between data points in environmental variables for *Calothamnus* sp. Whicher. Means with the same letter are not significantly different (Mann-Whitney U test, post hoc).

	Seedling	**Juvenile**	**No plant**	**p**
Distance to reproductive individual (cm)	189.3a	150.7b	151.9b	<0.001
Overhead cover (%)	1.1a	0.4b	1.1a	<0.001
Overhead cover height (cm)	40.3a	36.6b	83.7c	<0.001
Bareground cover (%)	3.2a	2.2b	1.5c	<0.001
Litter cover (%)	2.5a	1.9b	1.5a	<0.001
Vegetation cover (%)	1.6a	3.3b	3.1b	<0.001
Vegetation height (cm)	8.8a	25.4b	39.9c	<0.001
Soil depth (cm)	14.8a	20.0b	40.0c	<0.001

Table 7. ANOSIM R statistic and probability of differences between dominant life form classes for seedlings, juveniles and no plant points of *Calothamnus* sp. Whicher.

	R statistic	**p value**
Global	0.29	<0.001
Seedling v no plant	0.33	<0.001
Seedling v juvenile	0.32	<0.001
No plant v juvenile	0.00	<0.001

Table 8. SIMPER results showing the dominant life-forms which contribute the most to the similarity within seedlings, juveniles and no plant groups of *Calothamnus* sp. Whicher.

	% contribution		**% contribution**
Seedling		**Juvenile**	
Native annual sedges	87.0	Weedy annual grasses	83.2
Weedy annual herbs	10.5	Native annual herbs	6.0
No plant		Native perennial shrubs	5.1
Weedy annual grasses	88.3		
Native perennial shrubs	3.5		

3.3.1.2. Habitat preferences of *Hakea oldfieldii*

The PCA ordination of the environmental variables for *H. oldfieldii* seedlings, juveniles and no plant points found that 47.9% of the variance of the multidimensional data could be explained by the first two PC axes. The two dimensional PCA ordination presented in **Figure 14** adequately represents the relationship among samples. There were too few seedlings to make comparisons between seedlings and other groups. Loading on the PC1 axis from left to right is increasing distance from reproductive individuals, vegetation cover, and soil depth.

Variables loading onto the PC2 axis include a decreasing bare-ground cover from top to bottom and increasing litter cover from bottom to top (**Figure 14**). Visual interpretation of the loose grouping of juvenile points interspersed with no plant points would suggest very weak associations with environmental variables. Analysis by the ANOSIM procedure revealed there were no statistically significant differences between seedlings, juveniles and no plant points based on environmental variables (Global R=-0.09, p>0.05). Pairwise comparisons and further analysis were not undertaken based on these results.

No significant differences were found using ANOSIM between seedlings, juveniles and no plant points based on surrounding dominant life-forms (Global R=0.01, p>0.05). No further analysis was undertaken.

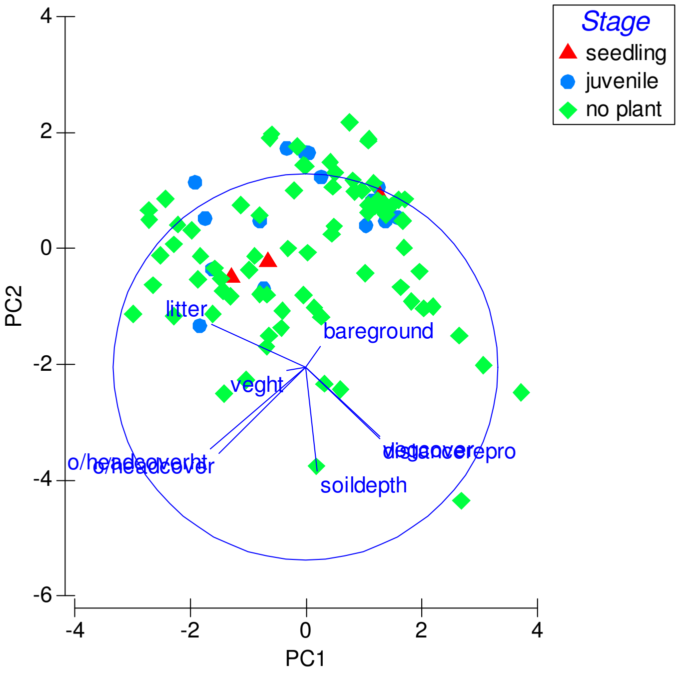


Figure 14. Principal Component Analysis (PCA) of the environmental variables for seedlings, juveniles and no plant points for *Hakea oldfieldii*. The proximity of each point indicates the degree of similarity in the measured environmental variables. The blue lines and circle is the vector plot which represents the importance of an environmental variable's contribution to the PCA axes displayed.

3.4. Demography and Population Dynamics

3.4.1. Demography and Population Dynamics of *Calothamnus* sp. Whicher

3.4.1.1 Population structure

Newly emerged seedlings, juveniles and reproductive adults were observed in populations of *C.* sp. Whicher (**Figure 15**). The majority of the plants were reproductive adults, with a large number of seedlings tagged in 2007. Subsequently there was a significant decrease in the number of seedlings in 2008 following widespread mortality of the cohort. The frequency of juveniles in stages 1 to 3 (stages being related to plant size) shows a decrease over the survey years. Generally, the frequency of reproductive adults in all size classes increased over the term of the study (**Figure 15**).

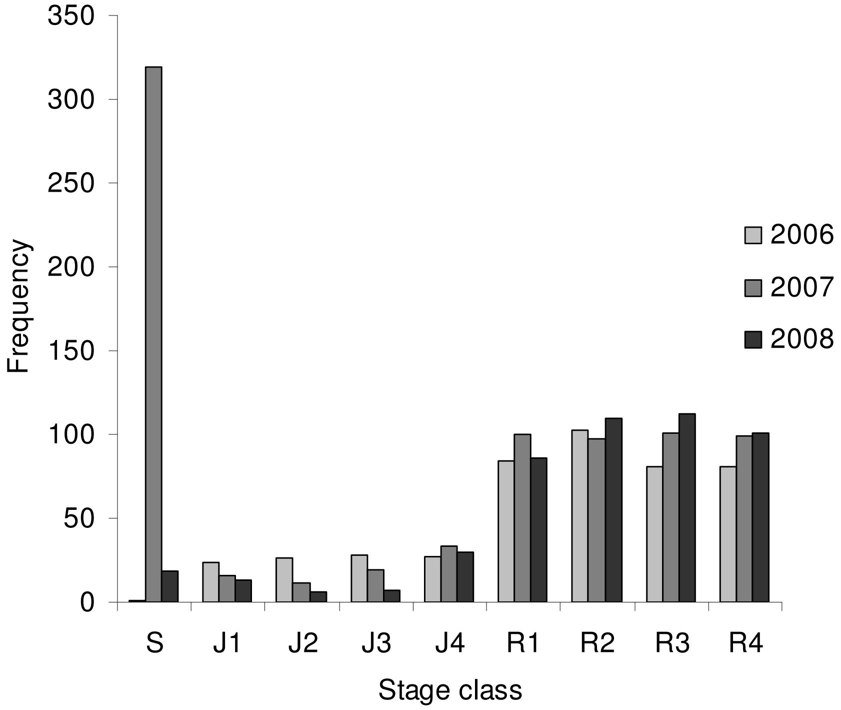


Figure 15. The frequency distribution of tagged *Calothamnus* sp. Whicher among stage classes in each of the consecutive years for all populations. Stages related to plant size.

Medium populations had an anomalous recording of high seedling frequency, and this was due to a high number of seedlings found at one population (Adams Rd). Medium populations had representatives from all stage classes (**Figure 16**). Small sized populations had very few seedlings or juveniles recorded in any year. The majority of plants recorded were reproductive adults, suggesting that small sized populations may be ageing populations with no evidence of recruitment taking place. Large sized populations also had representatives in each stage class, although seedling and juvenile stage classes were less frequently recorded than in the medium sized populations (**Figure 16**). The majority of plants recorded were reproductive adults between stages 1 and 3. These results would suggest that there is a larger ageing cohort, with minimal recruitment taking place.

3.4.1.2. Seedling recruitment

Medium populations had the greatest proportions of seedlings and large populations had the lowest (**Figure 16, Table 9**). Seedling emergence was highest in 2007 for the small and medium populations and in 2008 for the large populations.

3.4.1.3. Mortality

Annual mortality was highest and most variable in the seedling and juveniles stages and lowest and least variable in the reproductive stages (**Figure 17**). Annual mortality occurred through all stage classes in the medium populations (**Figure 17**). Annual mortality was highest for small populations overall in the time step 2007-2008. Boallia Rd had the highest mortality of all the small populations. Annual mortality was also highest in 2007-2008 in the small and medium sized populations (**Figure 17**). Annual mortality was highest in the time step 2006-2007 in the large populations (**Figure 17**).

Figure 16. The frequency distribution of tagged *Calothamnus* sp. Whicher among stage classes in each of the consecutive years for small, medium and large populations

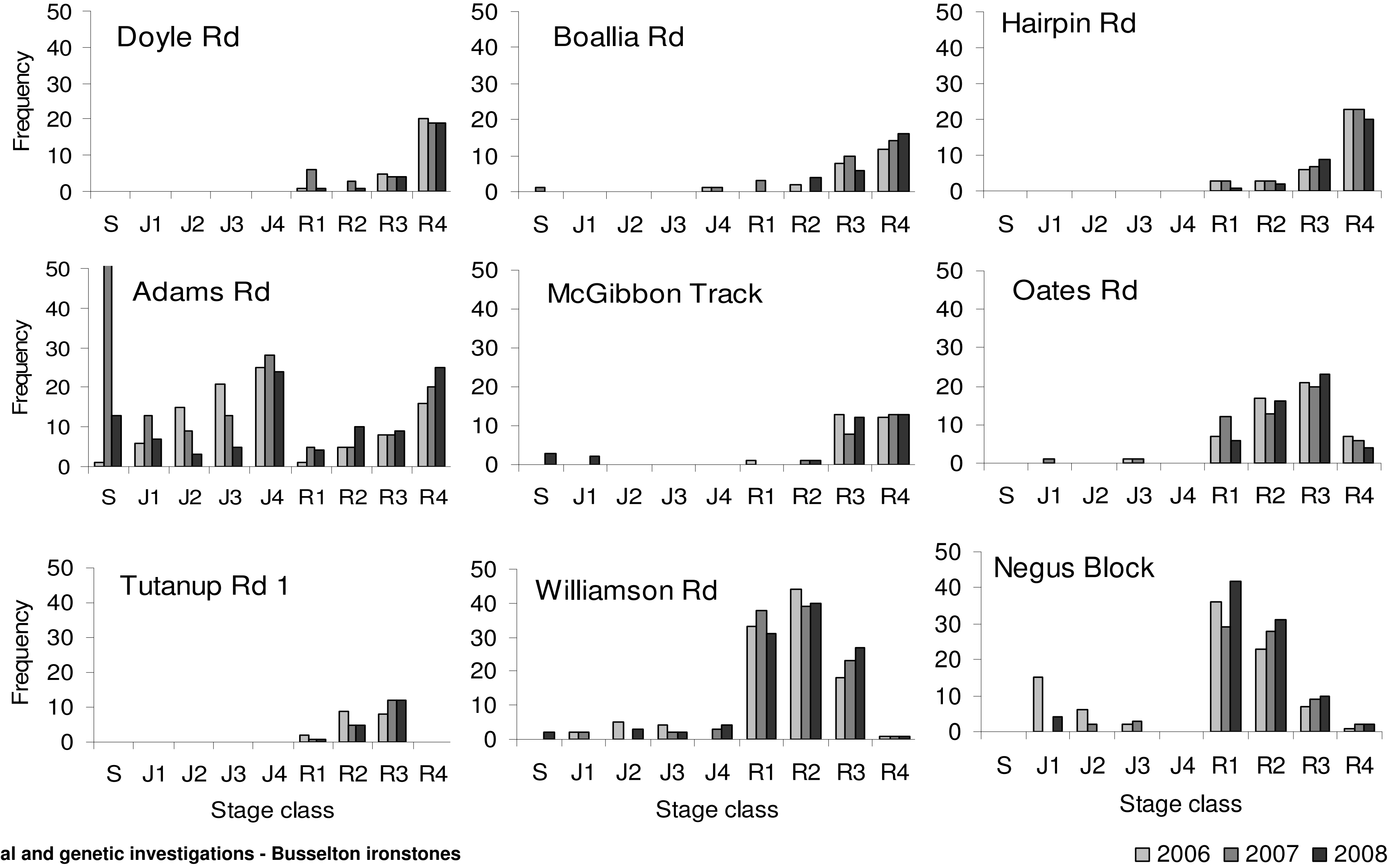
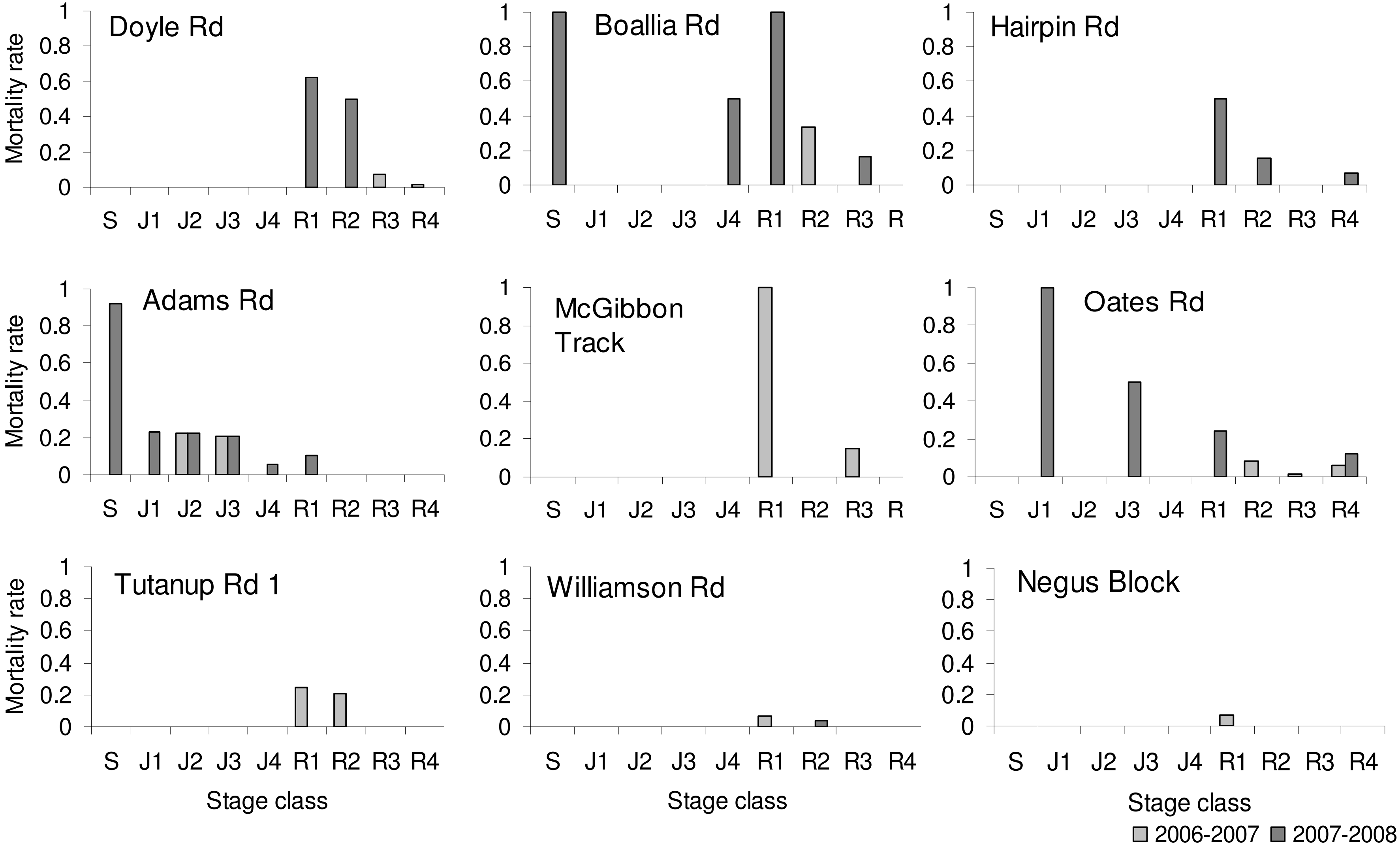


Table 9. The number of *Calothamnus* sp. Whicher seedlings, reproductive adults and their ratio in the nine populations in 2006, 2007 and 2008.

	Year								
	2006			2007			2008		
Population	Seedlings	Adults	Ratio	Seedlings	Adults	Ratio	Seedlings	Adults	Ratio
Doyle Rd	0	26	0	0	32	0	0	25	0
Boallia Rd	0	22	0	1	27	0.03	0	26	0
Hairpin Rd	0	35	0	0	36	0	0	32	0
Adams Rd	1	30	0.03	318	38	8.36	13	48	0.27
McGibbon Track	0	26	0	0	22	0	3	26	0.11
Oates Rd	0	52	0	0	51	0	0	49	0
Tutanup Rd	0	19	0	0	18	0	0	18	0
Williamson Rd	0	96	0	0	101	0	2	99	0.02
Negus Block	0	67	0	0	68	0	0	85	0
Mean			0			0.93			0.04

Figure 17. Annual mortality in *Calothamnus* sp. Whicher plant stage classes for the two 1 year time steps between 2006 and 2008 in small, medium, and large populations.



3.4.2. Demography and Population Dynamics of *Hakea oldfieldii*

3.4.2.1. Population structure

Representatives from all seedling, juvenile and reproductive stage classes were observed in *H. oldfieldii* populations (**Figure 18**). The majority of plants were reproductive adults, with few juveniles and seedlings observed. In 2006 the number of reproductive adults increased from stages 2 to 4. There was a corresponding decrease observed through these stage classes in 2007, largely resulting from the partial clearance due to road works at one population (Adams Road). There was very little variance between years for the juvenile stages and seedlings were only recorded in 2007.

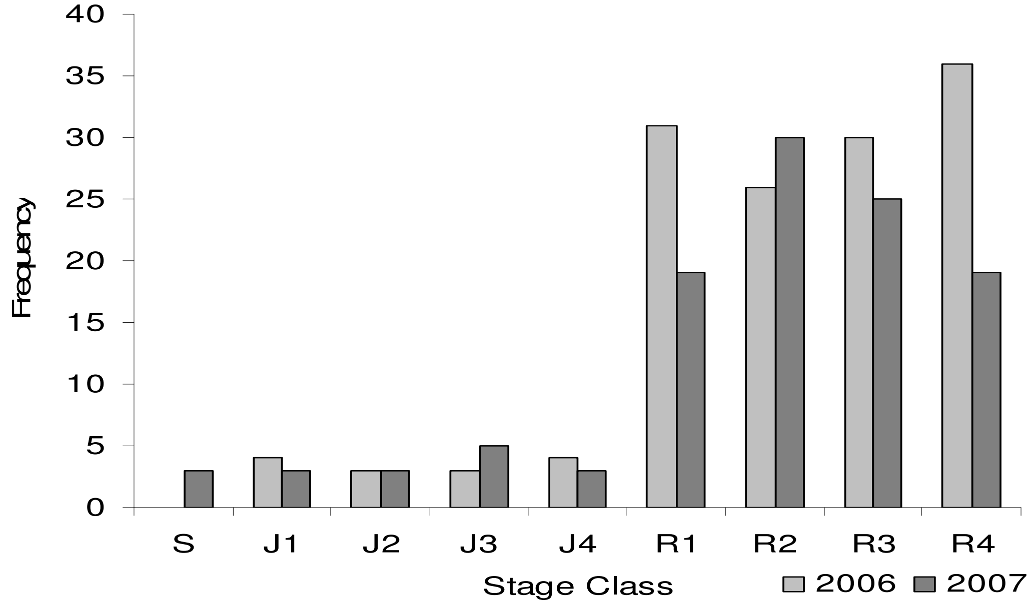


Figure 18. The frequency distribution of tagged *Hakea oldfieldii* among stage classes in each of the consecutive years for all populations.

All individuals recorded in small populations were reproductive adults between stages 2 and 4. Recruitment within small populations was not recorded (**Figure 19**). Medium sized populations were the only populations to record recruitment with newly emerging seedlings. Increasing numbers of reproductive adults in stage classes 1 to 4 in 2006 suggest an ageing population. Plants in large populations were spread across all stage classes except seedlings (**Figure 19**).

3.4.2.2. Seedling recruitment

Seedlings were recorded in the medium sized populations only (**Figure 19** and **Table 10**). Seedling emergence was recorded in 2007 only in very low numbers as compared to the adult population (ratio of 0.27) at Payne Rd.

3.4.2.3. Mortality

Mortality data was recorded for one time step in *H. oldfieldii* between 2006 and 2007. Annual mortality was highest in the reproductive stages with mortality recorded in all the population size classes (**Figure 20**). The high level of mortality in the reproductive plants was largely due to road works at Adams Rd where extensive pruning to increase visibility resulted in the loss of all tagged plants and serious impact on a high proportion of other plants in this population.

Small populations recorded mortality in the reproductive stage class (3) only. The impact of the road works at Adams Rd is largely responsible for the highest mortality in the reproductive stages in the medium sized populations (**Figure 19**). Large populations had the highest rate of mortality in the juvenile stage classes (stage 1), reflecting the death of the entire cohort in 2007 (**Figure 20**). Mortality rates in the reproductive stage classes in large populations are comparatively low. This result is reflected in the low variance of the frequency of reproductive individuals between 2006 and 2007 in large populations (**Figure 19**).

Figure 19. The frequency distribution of tagged *Hakea oldfieldii* among stage classes for small, medium and large populations in 2006 and 2007. All tagged plants removed by road works at Adams Road in 2007.

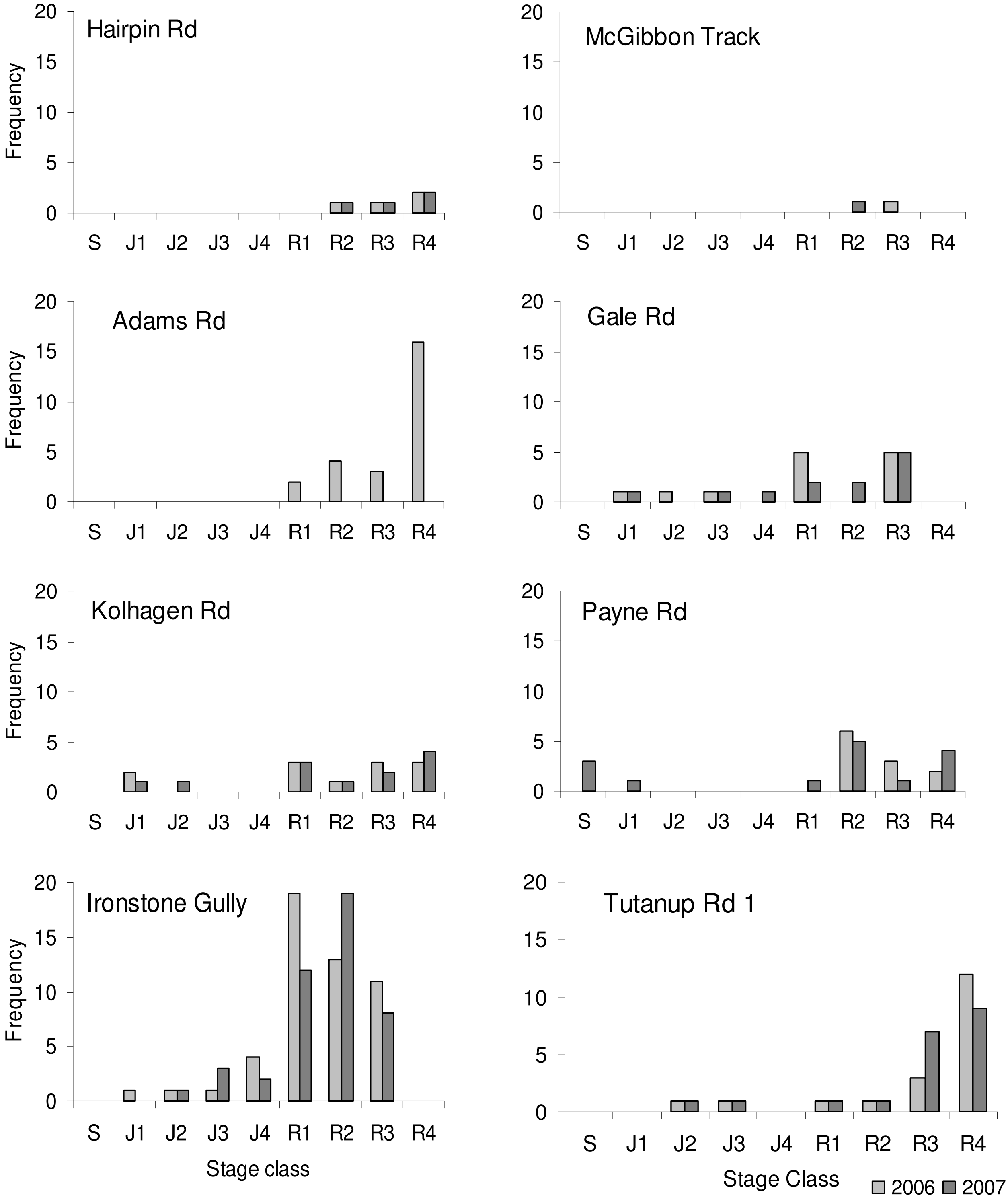


Table 10. The number of *Hakea oldfieldii* seedlings, reproductive adults and their ratio in all six populations in 2006 and 2007. Adult plants at Adams Rd removed by road works in 2007.

	Year					
	2006			2007		
Population	Seedlings	Adults	Ratio	Seedlings	Adults	Ratio
Hairpin Rd	0	4	0	0	4	0
McGibbon Track	0	1	0	0	1	0
Adams Rd	0	25	0	0	0	0
Gale Rd	0	10	0	0	9	0
Kolhagen Rd	0	10	0	0	10	0
Payne Rd	0	11	0	3	11	0.27
Ironstone Gully	0	43	0	0	39	0
Tutanup Rd	0	17	0	0	18	0
Mean			0			0.03

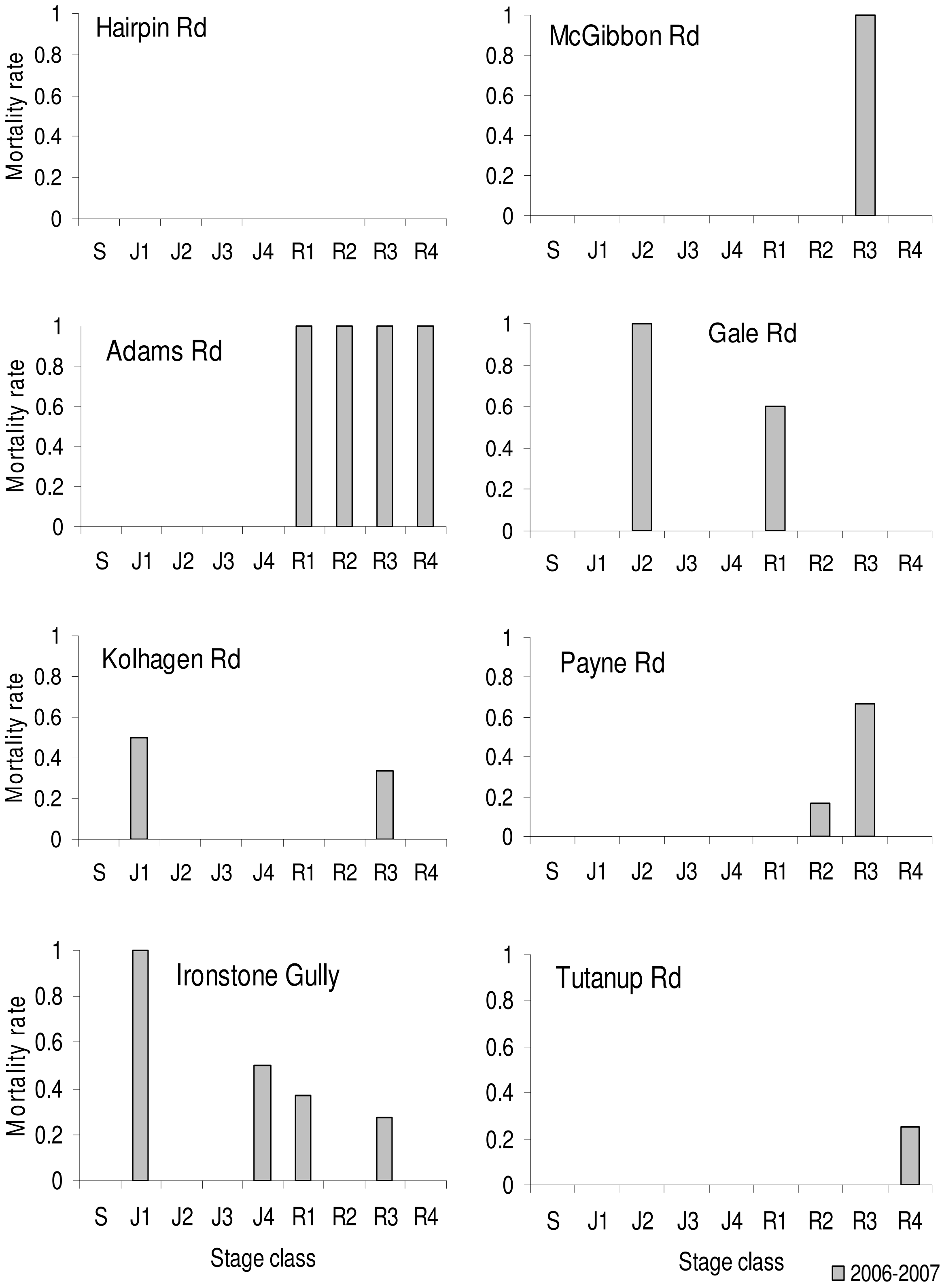


Figure 20. Annual mortality in *Hakea oldfieldii* plant stage classes for the single 1 year time step between 2006 and 2007 in small, medium, and large populations.

3.5. Genetic analysis

3.5.1. Levels and patterns of genetic diversity in *Calothamnus* sp Whicher

Calothamnus sp. Whicher was moderately diverse (D = 0.694) over all populations with 102 alleles identified from nine loci (**Table 11**). The mean number of alleles per locus was 4.2 and mean heterozygosity was 0.428. Measures of genetic diversity were similar in the medium and larger populations but were lower in the two smaller populations, Doyle Rd and Boallia. Most loci were polymorphic in populations although six populations had 1 to 3 monomorphic loci. Fixation indices were significantly positive in all populations except Adams Rd, McGibbon Track and Tutanup Rd 2. Wilcoxon's signed rank tests indicated that the Boallia Rd and Hairpin Rd populations have been through recent bottlenecks in size which have affected diversity within the populations (P = 0.008 and P=0.014 respectively; **Table 11**).

Genetic distances among populations were very high (mean D_{Nei} = 0.468) and populations were genetically very different from each other (Wright's F_{ST} = 0.284, SE = 0.018) but there was no significant relationship between geographical and genetic distance between populations (**Figure 21**). Analyses based on private alleles suggested very low historical gene flow (N_{m} = 0.319) between populations sampled.

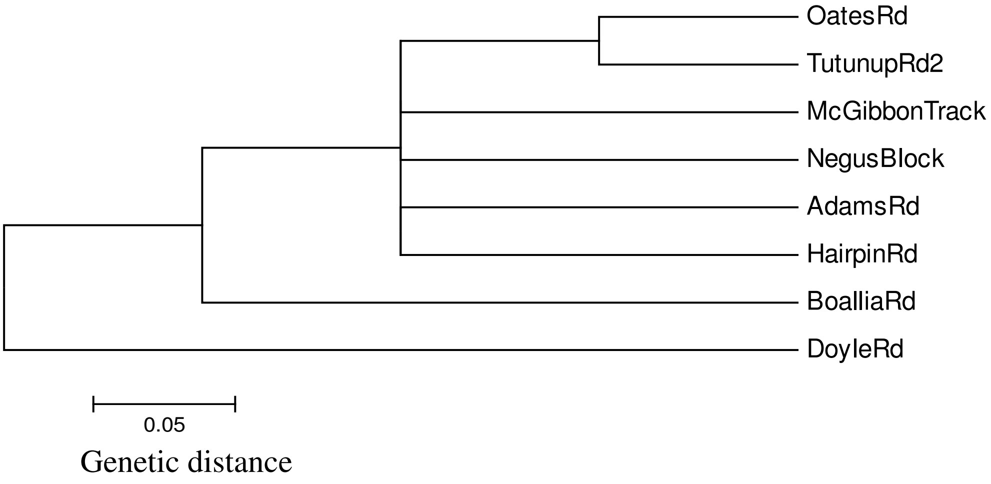


Figure 21. The relationships between populations of *Calothamnus* sp. Whicher based on genetic distance.

Table 11. Genetic diversity of sampled *Calothamnus* sp. Whicher adults.

Population	**Mean number of alleles per locus, *A***	**Percentage of loci polymorphic, *P***	**Gene diversity, *D***	**Observed heterozygosity, H_O**	**Inbreeding coefficient, *f***	**Probability of heterozygote excess^A**
Doyle Rd	2.667	88.89	0.407	0.297	0.294*	0.422
Boallia Rd	1.778	66.67	0.331	0.258	0.247*	0.008
Hairpin Rd	4.444	88.89	0.606	0.481	0.231*	0.014
Adams Rd	4.778	88.89	0.571	0.556	0.052	0.578
McGibbon Track	4.778	100.00	0.567	0.477	0.185	0.875
Oates Rd	5.333	88.89	0.509	0.433	0.175*	0.902
Tutanup Rd 2	4.333	88.89	0.463	0.450	0.054	0.990
Negus Block	5.556	100.00	0.553	0.473	0.168*	0.976
Mean	4.2	88.89	0.501	0.428		

^{A}, Wilcoxon's signed rank test for a bottleneck; * Significantly different from zero

3.5.2. Levels and patterns of genetic diversity in *Hakea oldfieldii*

Genetic diversity in *H. oldfieldii* was moderate with 198 alleles found at 13 loci over all populations. Mean gene diversity over all loci (*D*) of 0.759 with mean number of alleles per locus of 5.499 and mean heterozygosity of 0.532. The level of diversity among populations was moderate (*D* = 0.401 - 0.658) and similar among populations, except for lower diversity in Ironstone Gully (a large sized population) and Price Road (a small population). All loci were polymorphic in all populations except Ironstone Gully and Price Rd where 1 or 2 loci were monomorphic (**Table 12**). Inbreeding coefficients were low in all populations except Adams Rd and Gale Rd where a significantly positive value indicated a deficiency of heterozygotes in the sample. At Gale Rd this may have been an artefact of a large sample taken over several breeding neighbourhoods. There was no evidence of size bottlenecks in any population, including the small populations.

Wright's F_{ST} indicated significant differentiation of populations (F_{ST} = 0.23, SE = 0.023) and this pattern was reflected in high genetic distances between populations (mean D_{Nei} = 0.482). There was no significant relationship between geographical and genetic distance (**Figure 22**). Estimates of historical levels of gene flow based on private alleles were at a level at which differentiation of populations would be expected to occur (N_m = 1.087) and therefore consistent with the observed pattern.

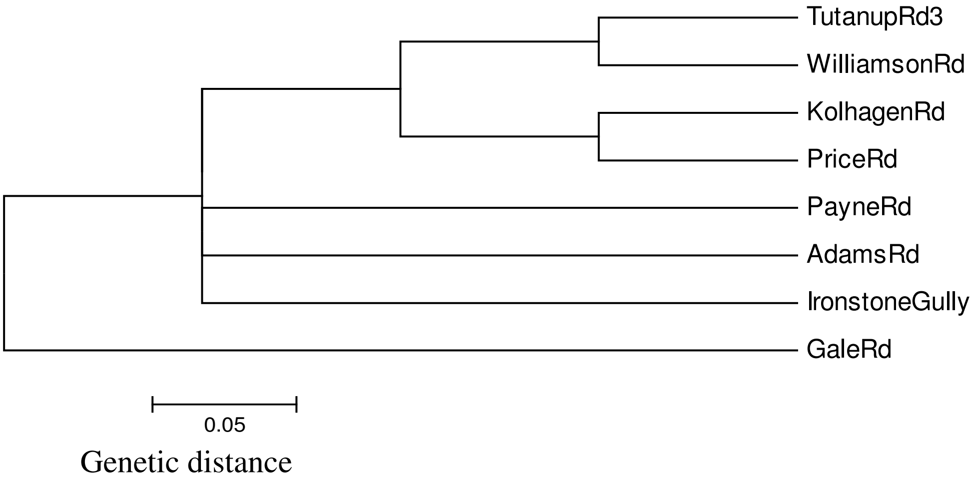


Figure 22. The relationships between populations of *Hakea oldfieldii* based on genetic distance

Table 12. Genetic diversity of sampled *Hakea oldfieldii* adults

Population	**Mean number of alleles per locus, *A***	**Percentage of loci polymorphic, *P***	**Gene diversity, *D***	**Observed heterozygosity, H_O**	**Inbreding coefficient, *f***	**Probability of heterozygote excess^A**
Adams Rd	5.461	100.00	0.605	0.529	0.181*	0.207
Gale Rd	6.461	100.00	0.608	0.504	0.221*	0.997
Ironstone Gully	3.769	84.62	0.452	0.443	0.073	0.850
Kolhagen Rd	4.231	100.00	0.577	0.557	0.086	0.500
Price Rd	3.846	92.31	0.401	0.406	0.038	0.995
Payne Rd	5.769	100.00	0.636	0.611	0.091	0.393
Negus Block	6.615	100.00	0.607	0.568	0.119	0.983
Williamson Rd	7.846	100.00	0.658	0.641	0.081	0.966
Mean	5.499	97.11	0.568	0.532		

^{A}, Wilcoxon's signed rank test for a bottleneck; * Significantly different from zero

3.5.3. Mating patterns and gene dispersal in *Calothamnus* sp. Whicher

The level of outcrossing in all three populations of C. sp. Whicher assayed was low indicating that the mating system includes both inbreeding and outbreeding (*t* = 0.417 – 0.540; **Table 13**). There were significant differences between plants in outcrossing rate (range of 7 -100%) and analyses suggested that inbreeding was from self-pollination rather than biparental inbreeding (crossing between genetically similar plants). Pollen dispersal was high within the large, open Tutanup Rd 2 population and the breeding neighbourhood of plants were large (11.63 plants) representing a substantial proportion of the population. In contrast, pollen dispersal within the small dense populations, Hairpin Road and Doyle Road, was more restricted with most pollination resulting from a pollen parent located within 10m of the mother plant and generally with nearest neighbours. The average number of fathers per seed crop was 3.1 to 4.6.

Estimates of contemporary gene dispersal between populations estimated as apparent pollen immigration ranged from low in the small, western populations Hairpin Rd and Doyle Rd (2.7% and 4.0% respectively) to moderate in the larger, eastern Tutanup Rd East population (15%).

Table 13. Estimates of mating system parameters for progeny of *Calothamnus* sp. Whicher

Parameter	Tutanup Rd 2	Hairpin Rd	Doyle Rd
Number and percentage of adult plants in the population genotyped	80 (100%)	88 (100%)	44 (100%)
N	10	10	10
n	191	147	138
t_m	0.418		
t_m - t_s	0.081		
r_p	0.086		
t_{pat}	0.665	0.54	0.42
Mean number of outcross pollen parents identified for each mother plant from mating system analyses	11.63		
Mean number of outcross pollen parents identified from within the population for each mother plant from paternity exclusion	6.9	4.6	3.1
Percentage of seedlings for which no male parent was found within the population	15	2.7	4.0

N, number of adult plants for which seedlings were analysed; n, total number of seedlings analysed; t_m, multilocus population outcrossing rate; t_m - t_s, biparantal inbreeding; r_p, correlated paternity; t_{pat}, outcrossing rate determined from paternity assignment or non-maternal alleles

3.5.4. Mating patterns and gene dispersal in *H. oldfieldii*

Hakea oldfieldii was predominantly outcrossed in all populations studied regardless of size with outcrossing rates of 0.84 to 0.96 (**Table 14**). Small amounts of effective inbreeding from different sources were found in the smaller populations with self-pollination (15.4%) occurring at Price Rd and crossing between genetically similar plants (11.9%) at Kolhagen Rd. The outcrossing rates of individual plants were similar in the larger Gale Rd population but there were significant differences between plants in the Kolhagen Rd and Price Rd populations. In the Oats Rd population, where only two of the three plants produced seed, almost all seeds were full siblings with each reproductive plant being the father of almost all the seeds of the other plant. Paternal diversity was also lower in the smaller populations with the size of the breeding neighbourhoods at Price Rd and Kolhagen Rd (5.4 and 5.2 respectively) being less than half of that of Gale Rd (11.5).

Estimates of pollen dispersal were very high (41.7- 48.2%) for all populations except the isolated Oats Road population into which effective dispersal of pollen was very low (3.1%).

Table 14. Estimates of mating system parameters for progeny of *Hakea oldfieldii*

Parameter	Price Rd	Kolhagen Rd	Gale Rd	Oats Rd
Number and percentage of adult plants in the population genotyped	21 (100%)	29 (100%)	102 (< 10%)	2 (100%)
N	10	11	10	2
n	160	211	199	32
t_m	0.846	0.962	0.965	nc
t_m - t_s	0.061	0.119	0.035	nc
r_p	0.185	0.191	0.087	nc
t_{pat}	0.826	0.905	0.974	0.938
Mean number of outcross pollen parents identified for each mother plant from mating system analyses	5.4	5.2	11.5	
Mean number of outcross pollen parents identified from within the population for each mother plant from paternity exclusion	5.2	7.0	6.6	1, 2*
Percentage of seedlings for which no male parent was found within the population	43.7	41.7	48.2	3.1

N, number of adult plants for which seedlings were analysed; n, total number of seedlings analysed; t_m, multilocus population outcrossing rate; t_m - t_s, biparantal inbreeding; r_p, correlated paternity; t_{pat}, outcrossing rate determined from paternity assignment or non-maternal alleles; *, actual number for each plant; nc, not calculated for small data set.

3.6. Germination

Germination of *C.* sp. Whicher was comparable across years with no significant differences between years ($F_{2,243}$=2.14, p>0.05) (**Figure 23**). Within years there were significant differences amongst populations.

There were significant differences in the numbers of germinants from seed collected in 2006 ($F_{1,7}$=4.42, p<0.05). There was significantly lower germination recorded at Oates Rd as compared to any other site. Adams Rd had significantly higher germination than both Oates Rd and Negus block (**Figure 24A**).

Seed collected from Oates Rd in 2007 also recorded significantly lower germination than any other population ($F_{1,7}$=2.73, $p<0.05$). Germination of seed collected from Hairpin Rd and Adams Rd was significantly higher than Oates, Tutanup Rd and Negus block in 2007 (**Figure 24B**).

The trend of the Oates Rd population having significantly lower germinants at 28 days, is continued in the germination results for seed collected in 2008 (**Figure 24C**). Germination of seed from Oates Rd is significantly lower than Adams Rd and Doyle Rd ($F_{1,7}$=3.27, $p<0.05$). Germination results from all other populations were not significantly different.

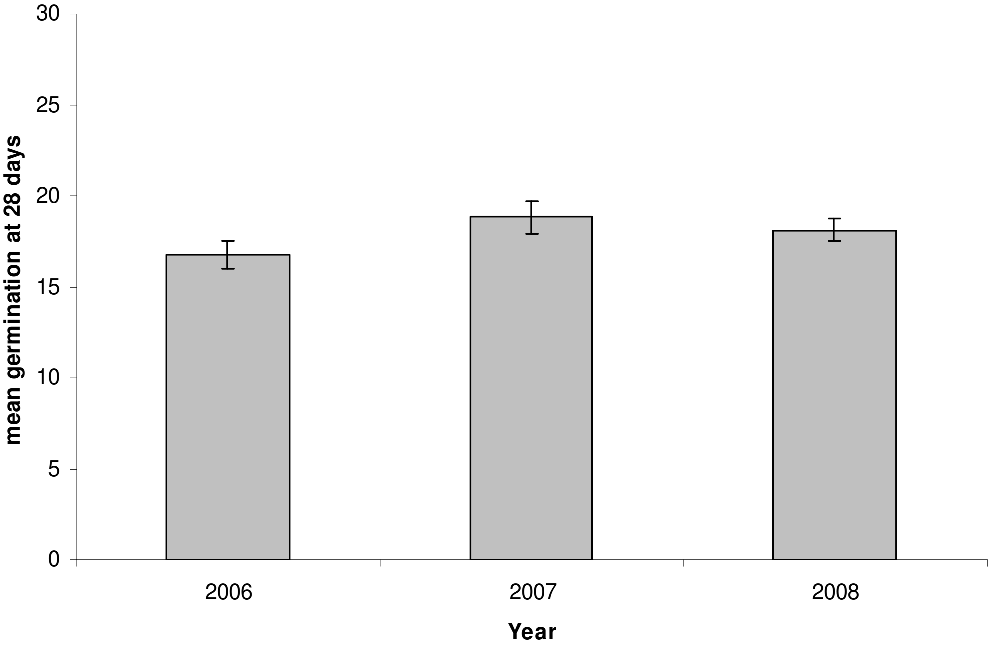


Figure 23. Germination (mean cumulative germination at 28 days) of seed lots from three consecutive years for *Calothamnus* sp. Whicher.

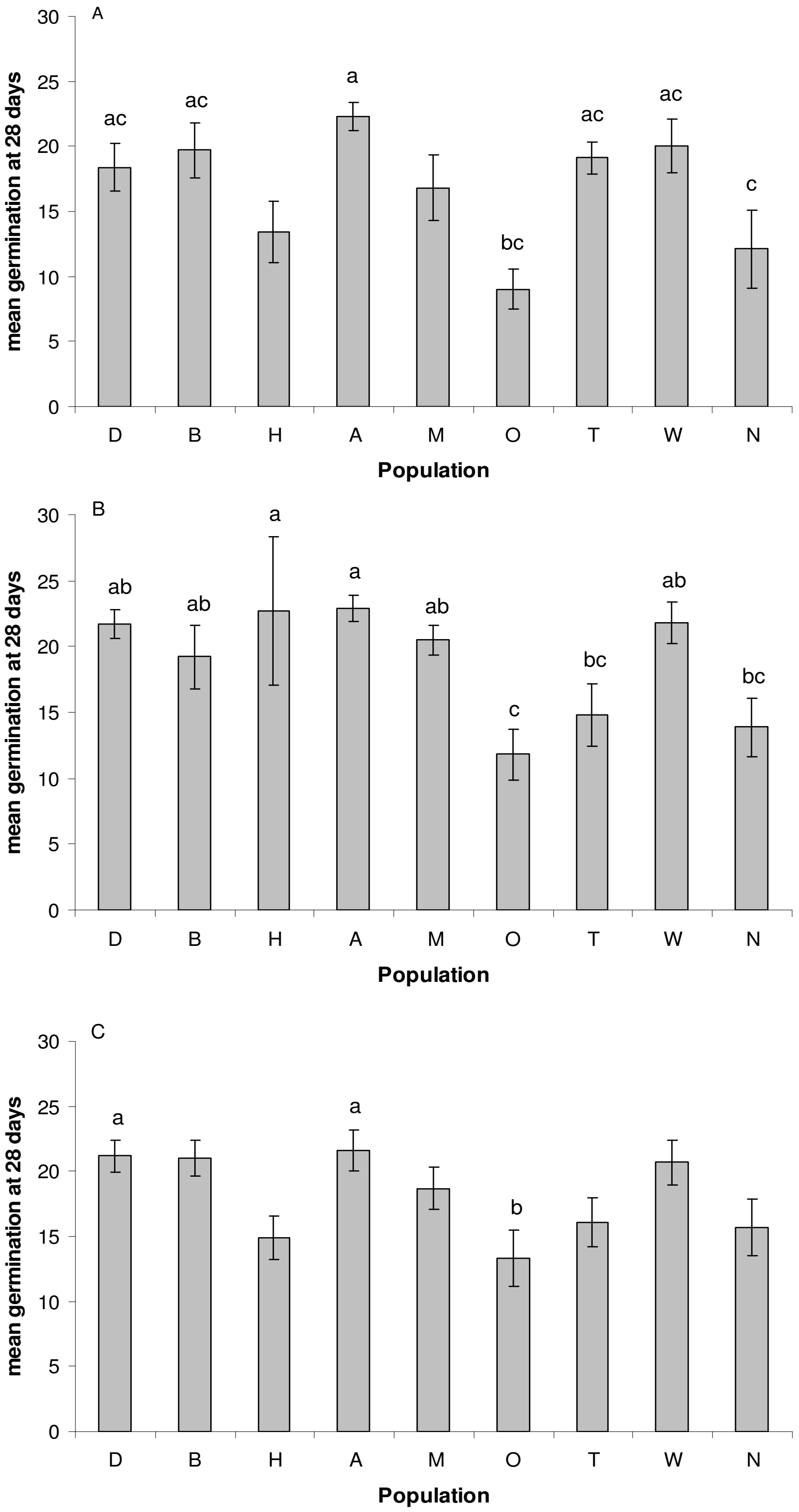


Figure 24 A-C. Germination (mean cumulative germination at 28 days) for *Calothamnus* sp. Whicher using seed collected in 2006 (A), 2007 (B) and 2008 (C). Populations are arranged in increasing order of size (left to right). Values are means (± SE) estimated for germination for three consecutive years (2006-2008).

4.0. DISCUSSION

4.1. Reproductive Output

4.1.1. *Calothamnus* sp. Whicher

Fruit and seed production occurred annually in spring in *C.* sp. Whicher. Significant differences in fruit and seed production occurred between populations but were not related to population size. Fruit set was highly variable amongst populations with low variability within a population. Variability in seed set was much higher within populations but relatively low between populations. There were no significant differences in fruit set or seed set between years despite variable rainfall patterns over the course of the study.

The results for *C.* sp. Whicher contrast with a similar study on the more widely distributed and closely related species, *Calothamnus quadrifidus* for which decreases in seed production occurred with decreasing population fragment size (Yates *et al.*, 2007b).

High rates of self-pollination have been observed in *C.* sp. Whicher, much higher than those observed in *C. quadrifidus* (Stankowski *et al.*, unpublished) and population size and the availability of mates is therefore likely to have less of an impact on fecundity in *C.* sp Whicher than *C. quadrifidus*.

4.1.2. *Hakea oldfieldii*

There were significant differences between populations in fruit and seed production in *H. oldfieldii,* but there were no clear relationships with population size with the exception of the smallest population which had lower fruit and seed set than the others. Evidence from genetic studies suggests that although pollinators may visit very small populations (two plants) less frequently than larger populations, lower seed and fruit set in smaller populations is probably due to a lack of genetically compatible mates rather than a lack of pollinators.

Fruit and seed production was significantly higher in 2007 than in either 2006 or 2008. Rainfall in 2007 from July to October was higher than the long term average (**Figure 2**). In combination with consistent rainfall over the 2006-2007 summer, this rainfall is likely to have provided optimal conditions during flowering and seed formation.

4.2. Habitat preferences

4.2.1*. Calothamnus* sp. Whicher

Seedlings, juveniles and no plant points formed clearly defined habitat groups following multivariate analysis. Seedlings exhibited a preference for relatively open, sparsely vegetated areas with moderate over head cover and low surrounding vegetation. Seedlings favour areas of bare-ground with high litter cover and preferentially grow in shallow soils. Vegetation cover provided the strongest separation between seedling habitats and juvenile habitats, with seedlings showing a distinct preference for sites with greater distance to the canopy layer. The data indicates that seedling recruitment is most successful in open areas with a high canopy layer allowing a greater degree of light to reach the ground. High litter cover is likely to encourage growth by holding moisture in the root growing zone of the plants. This is particularly important as the drought-prone skeletal soils over ironstone become dry in the summer months (Poot and Lambers, 2008).

Juveniles in contrast show preferences for areas that have a greater degree of canopy cover and higher surrounding vegetation typical of an established area of ironstone vegetation. These results indicate that *C.* sp. Whicher seedlings recruit in recently cleared areas where competition for resources with other plants is reduced ie. fire scars or tree fall areas. Juveniles show a strong preference for deeper soils and less bareground than seedlings, where established vegetation cover provides organic matter necessary to build a deeper soil profile.

The distinction in preferred habitats for seedlings and juveniles of *C.* sp. Whicher extends to the dominant life-form of the surrounding vegetation. Seedlings occurred almost exclusively in vegetation dominated by native annual sedges. Native annual sedges are low lying, short lived plants that provide adequate light penetration to the ground. Native annual sedges may not provide any competition during establishment providing an optimal environment for recruitment.

Juveniles and no plant points were most often associated with weedy annual grasses. However, the presence of native annual herbs in juvenile habitats differentiated the juvenile and no plant point environments. Unlike seedlings, juveniles have established root systems, survived their first summer and so are better able to withstand competition from other plants. The presence of juvenile shrubs may also change the soil chemistry or light availability to make the ironstone soils a more hospitable place for native annual herbs to establish.

4.3. Population dynamics and demography

4.3.1. *Calothamnus* sp. Whicher

Reproductive adults were the most numerous stage-class category in all populations. Representatives from the majority of stage classes were observed in medium and large populations (**Figure 17**). However, only very low numbers of seedlings were found in large and small sized populations, with a small amount of recruitment observed in 2008. Recruitment was observed in all years in medium sized populations with an anomalous recruitment event in 2007 (Adams Rd) followed by widespread mortality of the cohort (**Figure 18**). The mortality of the entire seedling cohort is likely to be related to the lack of summer

(January) rainfall in 2008, whereby the seedlings were not able to survive the dry conditions and become established.

In general, mortality was highest in the 2007-2008 time-step for small and medium populations and in the 2006-2007 time-step for large populations. The juvenile stage class category suffered the greatest mortality in the 2006-2007 time-step in large populations. In 2007-2008 seedlings, juveniles and adults all suffered mortality in medium and small populations. It is unclear from the data collected the reasons for the differences in mortality between populations and between years.

4.3.2. *Hakea oldfieldii*

Plants in small populations of *H. oldfieldii* restricted to reproductive stage class categories. No recruitment of seedlings was observed in small populations. Genetic analyses indicate that most *H. oldfieldii* seeds are outbred, even in very small populations, probably due to a gametophytic lethal or self-incompatability system so it seems unlikely low recruitment is due to lack of fitness in seeds. Fecundity was low in the smallest populations because of the elimination of inbred seeds and therefore recruitment may be limited by a lack of sufficient seeds.

An ageing cohort of adults was observed in medium sized populations due to significant loss of mature plants at one population due to road works. Only one of the nine populations of *Hakea* recorded seedling recruitment over the study period and then of only a few individuals.

All stage classes apart from seedlings were represented in large populations. Large populations were also home to the greatest numbers of plants in each stage class and overall. Mortality of adults in large populations is relatively low compared to the other population sizes, suggesting a healthy adult population is present. The highest mortality was recorded in the juveniles (Stage J1), reflected in the mortality of the entire cohort in the 2006-2007 time-step. Mortality within the other stage classes was not nearly as high and is unlikely to dramatically affect the population structure. Despite the seeming robustness of the population structure of large populations, if the lack of recruitment observed over the 2006-2007 time-step is a long term trend, large populations can not be considered sustainable.

4.4. Genetic analysis

4.4.1. *Calothamnus* sp Whicher

Genetic analysis of *C.* sp. Whicher showed a species with moderate diversity and high differentiation among populations. The diversity showed little genetic structure among populations and they did not cluster into groups concordant with the western and eastern geographic areas. The species showed a mixed mating system with relatively high levels of inbreeding for a bird pollinated woody shrub. The observed pattern of genetic diversity in *C.* sp Whicher may reflect a history of inbreeding and low gene flow that has resulted in a divergent group of populations through genetic drift.

Mating system analysis indicates a large number of males pollinate each plant suggesting that outcrossing is not limited because of an absence of pollinators or compatible mates. Rather, it seems the genetic load (expression of negative genetic effects) has been purged sufficiently to permit a high level of inbreeding and the survival of a large proportion of inbred seeds leading to reduced diversity within populations.

Historically, gene flow seems to have been very low in *C*. sp. Whicher presumably because pollinators either did not move between populations over the range studied here during the flowering season or did not carry significant pollen loads. Contemporary pollen dispersal was also very low in the western populations but was high in the eastern population. This eastern population occurs in continuous vegetation along the Tutanup rail/road reserve, compared to the more isolated populations of Hairpin Road and Doyle Road in the western region. The greater pollen dispersal in the Tutanup population may reflect an increase in pollen dispersal more recently. Such a change might arise from changes in the suite and/or behaviour of pollinators following fragmentation. An increase in gene flow might be expected to reduce the divergence of populations in the long term and form a source of diversity for populations. The likelihood of local extinction would depend on the balance between increased loss of diversity in small fragments and increased diversity from pollen dispersal following changes in pollinator behaviour.

4.4.2 ***Hakea oldfieldii***

Genetic analysis of *Hakea oldfieldii* showed moderate diversity and high differentiation among populations. There was some geographic structure to the diversity but no major partitioning of diversity according to western and eastern areas. *Hakea oldfieldii* has a breeding system dominated by outbreeding. The very low level of selfing in a population of two reproductive plants and the correlation of fecundity with population size suggests the operation of a gametophytic lethal or self-incompatibility system, as found in other species of the Proteaceae family.

Although predominantly outbred, mating patterns did vary in populations of different sizes in *H. oldfieldii*, with paternal diversity higher in the larger population presumably because of the availability of compatible mates and/or more pollinators. Inbreeding in smaller populations arose from both self-pollination and inbreeding between similar plants. The smaller mating neighbourhoods of smaller populations would result from fewer compatible males being available. The lower fecundity of smaller populations would also be expected as an expression of genetic load resulting from this increased inbreeding.

The pattern of genetic diversity in extant *H. oldfieldii* populations indicates that gene flow was low in the past and populations were affected by drift. Within populations, high diversity is likely to be maintained by a gametophytic lethal or self-incompatibility system that limited or prevented self-pollination. Inbreeding, caused by crossing between fewer compatible plants, crossing between relatives and also, some self-pollination, would erode diversity within populations limiting evolutionary potential. Estimates of contemporary gene dispersal between post-fragmentation populations indicate that there have been substantial increases in pollen dispersal recently. Pollen dispersal would retard genetic drift and reduce divergence if genes become established in the populations. *Hakea oldfieldii* might be expected to have reduced genetic divergence in the future if gene flow overrides the impact of any increased inbreeding following reduction in population size.

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REFERENCES

Bureau of Meteorology (2008). *Climate Statistics for Australian Locations; Busselton Shire*. Available from: http://www.bom.gov.au/climate/averages/tables/cw_009515.shtml

Byrne, M. and Hankinson, M. (2009). Characterization of polymorphic microsatellite DNA markers in *Hakea oldfieldii* Benth. (Proteaceae). *Molecular Ecology Resources,* **9**, 1214–1216.

Clark, K., and Gorley, R. (2006). *PRIMER v6: User Manual/Tutorial,* PRIMER-E: Plymouth.

Collins, B., Newland, C., and Briffa, P. (1984). Nectar utilization and pollination by Australian honeyeaters and insects visiting *Calothamnus quadrifidus* (Myrtaceae). *Australian Journal of Ecology*, **9**, 353-365.

Doyle, J.J. and Doyle, J.L. (1990). Isolation of DNA from fresh tissue. *Focus*, **12**, 13-15.

Elliott, C. P. and Byrne, M. (2005). Isolation and characterization of microsatellites in the woody shrub *Calothamnus quadrifidus* (Myrtaceae). *Molecular Ecology Notes,* **5**, 757–759.

Hobbs R.J. and Yates C.J. (2003). Turner Review No. 7. Impacts of ecosystem fragmentation on plant populations generalizing the idiosyncratic. *Australian Journal of Botany*, **51**, 471-488.

Keighery, G. (2004). *Ironstone Communities of South-Western Australia*. Presented at the Busselton Ironstone Workshop, November 2004.

Gibson, N., Keighery, G., and Keighery, B. (2000). Threatened plant communities of Western Australia. 1. The ironstone communities of the Swan and Scott Coastal Plains. *Journal of the Royal Society of Western Australia*, **83**, 1-11.

Gibson, N., Keighery, B., Keighery, G., Burbidge, A., and Lyons, M. (1994). *A Floristic Survey of the Swan Coastal Plain.* Unpublished Report. Department of Conservation and Land Management and the Conservation Council of Western Australia.

Meissner, R., and English, V. (2005). *Interim Recovery Plan No. 215; Shrubland Association on Southern Swan Coastal Plain Ironstone (Busselton Area) (Souther Ironstone Association) Interim Recovery Plan 2005-2010.* Department of Conservation and Land Management; Perth, Western Australia.

Poot, P., and Lambers, H. (2008). Shallow-soil endemics: adaptive advantages and constraints of a specialized root-system morphology. *New Phytologist*, **178**, 371-381.

Stankowski, S., Byrne, M., and Bennett, I. (unpublished). Extremely low levels of pollen dispersal between isolated populations of a rare, bird pollinated shrub: A consequence of human disturbance or consistent with historical levels?

Yates, C., Coates, D.J, Byrne, M., Elliott, C. (2007a). Composition of the pollinator community, pollination and the mating system for a shrub in fragments of species rich kwongan in south-west Western Australia. *Biodiversity and Conservation,* **16**, 1379-1395.

Yates, C., Elliott, C., Byrne, M., Coates, D., and Fairman, R. (2007b). Seed production, germinability and seedling growth for a bird-pollinated shrub in fragments of kwongan in south-west Australia. *Biological Conservation,* **136**, 306-314.

Appendix A.

Dominant Life-form Codes

Definitions of dominant life form codes.

Code	Description	Code	Description
MOS	Moss	NPR	Native perennial restionaceae
NAG	Native annual grass	NPS	Native perennial sedge
NAH	Native annual herb	NPSH	Native perennial shrub
NAM	Native arborescent monocot	WAG	Weedy annual grass
NAS	Native annual sedge	WAGE	Weedy annual geophyte
NPG	Native perennial grass	WAH	Weedy annual herb
NPGE	Native perennial geophyte	WAS	Weedy annual sedge
NPH	Native perennial herb	WPG	Weedy perennial grass