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Population Size Effects on Progeny Performance in *Banksia ilicifolia R. Br.* (*Proteaceae*)

BAMBANG HELIYANTO[1‡*], TIANHUA HE[2], HANS LAMBERS[1], ERIK J. VENEKLAAS[1], SIEGFRIED L. KRAUSS[1,2]

[1]School of Plant Biology, Faculty of Natural and Agricultural Sciences, The University of Western Australia,
35 Stirling Highway, Crawley, WA 6009, Australia
[2]Botanic Gardens and Parks Authority, Kings Park and Botanic Garden, West Perth, WA 6005, Australia

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A reduction in population size due to land clearing and habitat fragmentation may have negative effects on plant fitness. A relationship between population size and progeny performance for four small (n < 20 plants) and four large (n > 100 plants) populations of *Banksia ilicifolia*, a widespread but naturally fragmented species in south-western Australia, was assessed. Seeds collected from the field were germinated and a comparative growth study conducted in a greenhouse. After six months, the survival rate of seedlings from larger populations was double (36%) that of smaller populations, while germination rates and other measures of growth performance were independent of population size. The conservation and management implications of reduced fitness associated with small population size in *B. ilicifolia* are discussed.

Key words: population size effect, fragmentation, *Banksia ilicifolia*, growth study

INTRODUCTION

Ecosystem fragmentation and its consequences is a major threat to the persistence of many species (Henley *et al*. 1996; Laurance & Bieregaard 1997; Whitmore 1997; Hobbs & Yates 2003). Due to habitat destruction, many formerly widespread species have become restricted to small and fragmented populations (Fischer & Matthies 1998). Small populations are more vulnerable to environmental stochasticity (Menges 1991a,b, 1992; Boyce 1992), and may suffer from a disruption of important biotic relations, such as the frequency and behaviour of pollinators, leading to lower per capita reproductive rates (Allee effect) (Lamont *et al.* 1993; Olesen & Jain 1994; Bond 1995; Groom 1998). Mating patterns, such as the frequency of selfing, may change and lead to inbreeding and be expressed as inbreeding depression. Genetic variation may be reduced compared with that of larger populations, which may decrease plant fitness and population viability (Ellstrand & Elam 1993; Nunney & Campbell 1993; Young *et al*. 1996). Genetic drift can lead to rapid fixation of alleles, which decreases genetic variation and the evolutionary potential to adapt to environmental change (Falconer & Mackay 1996).

A comprehensive understanding of the consequences of reduced population size following fragmentation on plant fitness is crucial for designing appropriate conservation-management strategies. Despite its relevance to conservation management, the relationship between population size and fitness has been studied in very few plant species, typically annuals and in common garden experiments (Fischer & Matthies 1998). While several fitness components were found to be correlated with population size in *Gentiana pneumonanthe* (Oostermeijer *et al.* 1994) and *Ipomopsis aggregata* (Heschel & Paige 1995), no clear trend with respect to mean fitness and population size was detected in *Salvia pratensis* (Ouborg *et al*. 1991), *Scabiosa columbaria* (van Treuren *et al*. 1993) and *Lychnis flos-cuculi* (Hauser & Loeschcke 1994). These contrasting results emphasize the need for further research, particularly for poorly studied and unique landscapes such as south-western Australia, where there are numerous narrowly distributed and naturally fragmented plant species under threat (Hopper & Gioia 2004).

In this paper, we assess the effect of population size on progeny vigour in *Banksia ilicifolia* (holly-leaved banksia-), a widespread species in south-western Australia found mainly on the sandy coastal plain from Mt Lesueur to Augusta, and east to Cordingup River between Albany and Bremer Bay (Taylor & Hopper 1988). Although widespread, *B. ilicifolia* is locally restricted to swales and wetland fringes with depth of groundwater < 10 m (Groom *et al*. 2000; Groom 2004). As a consequence, populations are naturally fragmented and typically small (< 100 plants), although some large stands do exist (Taylor & Hopper 1988). While naturally fragmented, surviving populations within the Perth metropolitan area have become increasing isolated and small due to land clearing with urbanization. *Banksia ilicifolia* is self-compatible, but preferentially outcrossing, with marked inbreeding depression at seed production, seed germination, and seedling growth (Heliyanto *et al*. 2005). Plants flower intermittently in most months of the year, with an apparent peak from winter to spring. Fruit set following natural pollination is extremely low (c. 0.04-1% of flowers setting fruit; Whelan & Burbidge 1980; Lamont & Collins 1988; Heliyanto *et al*. 2005).

[‡]Current address: Jalan Raya Mapanget, P.O. Box. 1004, Manado North Sulawesi, Indonesia
[*]Corresponding author. Phone: +62-431-812430, Fax: +62-341-812017, E-mail: b.heliyant@gmail.com

In this study, we extend on our earlier research identifying preferential outcrossing, inbreeding depression and heterosis with wide outcrossing in *B. ilicifolia* (Heliyanto *et al.* 2005, 2006) and ask whether the offspring from large populations outperform offspring from populations that are small and isolated as a result of land clearing and habitat fragmentation with urbanization in the Perth metropolitan area. Our results may have important implications for the viability of recently fragmented populations due to the negative genetic consequences of reduced population size.

MATERIALS AND METHODS

To assess the performance of progeny, a greenhouse experiment was carried out between May and December 2005, using seeds collected from the field. Four small, geographically isolated, populations (< 20 plants) and four large populations (> 100 plants) of *B. ilicifolia* within or near to the Perth metropolitan area were arbitrarily selected for this study (Table 1).

Mature fruits (follicles) were collected from all plants in the small populations and an arbitrary selection of up to 30 widely spaced plants (> 10 m apart) from each large population in March 2005. Closed follicles were heat-treated, by putting them onto a hot frying pan to extract seeds. Germination occurred in a growth chamber at 15 °C under dim light on two moistened Whatman filter papers in Petri dishes for three weeks. Prior to germination, seeds were treated with fungicide by soaking them in a 10% Previcur (a.i. Propamocarb hydrochloride, Bayer CorpScience, USA) solution for 15 minutes. To maintain moisture content, water was added every week. Each seedling was then transferred into a pot of 15 x 15 x 15 cm containing river sand. Pots were placed randomly on three neighboring benches in a climate-controlled greenhouse at the University of Western Australia (20/15 °C, day/night, light intensities 65% ambient), and watered as required. A slow-release fertilizer (Osmocote 17N-1.6P-18.7K, Scott), at a dose of 10 g per pot, was applied to 4-month-old seedlings. Plants were maintained for another 2 months before being harvested for growth measurements.

In an unplanned and uncontrolled element of the study during the subsequent establishment period until harvest time in the greenhouse, seedlings were affected by damping-off disease caused by a fungal pathogen. Mortality rates per population, as a result of this stress, were assessed.

At harvest, remaining seedlings (Table 1) were severed at the soil surface, and the bulk of the soil was carefully removed to count the number of cluster roots produced per plant. Measured growth traits included number of leaves, oven-dry mass (after 48 h at 70 °C) of roots and leaves and total leaf area (using a portable leaf area meter, LI-3000, Lincoln, NE, USA). Measured other parameters included germination percentage and seedling survival. Germination percentage was estimated as number of germinated seeds/total number of seeds x 100%. Seedling survival was determined on the basis of the proportion of seedlings remaining after 6 months in the greenhouse.

Statistical Analysis. Statistical analysis was performed using Statistica 6 (Statsoft Inc., Tulsa, OK, USA). Parametric tests were performed after confirming homogeneity of variance using Cochran's C test or Levene's test (Zar 1999). Effect of population size on seed germination, and seedling survival was evaluated using one-way ANOVA. Effect of population size on growth components under common environment was evaluated using a mixed model ANOVA (Zar 1999; Quinn & Keough 2002), in which populations were treated as a random factor nested within a fixed factor, population size. Since the design was strongly unbalanced (Table 1), type III MS was used and the degrees of freedom were adjusted using Satterthwaite's method (Zar 1999). To test the possible maternal influence on growth parameters, seed weight was included as a covariate in the analysis. However, as it was found to have no significant effect, it was later excluded from the analysis in order to strengthen the results. Effect of population size on the proportion of cluster rooted plants was tested using a t test.

RESULTS

Seed Germination and Survival Rates. Seed germination rate was independent of population size ($F_{1,6} = 0.32$, P = 0.59). Inclusion of seed weight as a covariate did not change the trend. All populations showed relatively high seed germination rates, ranging from 66 to 100%, with small and large

Table 1. Population sites description, sample numbers, germination and survival of *Banksia ilicifolia* from small and large populations. The habitats of all small populations demonstrated a greater degree of disturbance than those of the large populations, as indicated by a weedy understory

Population (# plants/pop)	Longitude	Latitude	Altitude (m)	No. of follicles collected	No. of seeds extracted	No. of seeds germinated (% germination)	No. of seeds survived (survival %)
Small:							
Beechboro/Reid (17)	31°51'48"	115°55'32"	75	64	54	52 (96)	8 (15)
Caporn St. (17)	31°43'52"	115°48'35"	47	50	43	30 (70)	8 (27)
Chiquita Park (12)	31° 45'27"	115°49'36"	72	42	38	38 (100)	4 (11)
Kensington Bushland (18)	31°51'48"	115°49'00"	50	30	37	37 (100)	6 (16)
Average						(91.5)	(17.3)
Large:							
Harry Waring (> 100)	32°10'48"	115°50'32"	50	133	101	75 (74)	27 (36)
Pinjar (> 1000)	31°40'24"	115°48'49"	27	25	15	10 (66)	5 (50)
Lancelin/Mimegara (> 1000)	31°01'05"	115°32'07"	60	20	20	20 (100)	11 (55)
Rowe/Hopeland (>300)	32°20'96"	115°54'51"	28	30	14	14 (100)	4 (29)
Average						(86)	(36)

populations averaging 92% (± 7.2) and 86% (± 17.6), respectively.

Following naturally infection, seedling mortality per population ranged from 45 to 89% (Table 1). Seedling survival was strongly affected by population size ($F_{1,6} = 11.20$, $P < 0.005$), and the mean percentage for large populations (36% ± 6) was double that of small populations (17% ± 4.1).

Growth. The mean number of leaves per seedling, leaf area per seedling, and root and shoot dry mass per seedling were independent of population size (Table 2; Figures 1a-d). For large and small populations respectively, mean (± SE) number of leaves per seedling was 14.62 (0.91) and 12.96 (1.0), leaf area per seedling was 70.6 (5) and 69.45 (7) and root dry mass per seedling was 0.3 g (0.02) and 0.35 g (0.03) and shoot dry mass per seedling was 1.4 (0.5) and 1.5 (0.7). Inclusion of seed weight as a covariate did not alter the trend.

The Caporn street population produced a higher proportion of seedlings with cluster-roots (88%) than all other populations. Fifty percent of seedlings from the Chiquita Park population produced cluster roots, and 33% of Kensington

Tabel 2. Mixed model ANOVA results for different growth parameters of *Banksia ilicifolia* progeny grown for 6 months in river sand in a greenhouse. DF error was computed using the Satterthwaite method (Zar 1999)

Source of variation	Effect	D.F.effect	M.S.effect	D.F.error	M.S.error	F	P
Number of leaves							
Population size	Fixed	1	104.68	7.90	49.13	2.13	0.18
Population (PS)	Random	6	52.49	66.00	34.32	1.53	0.18
Leaf area							
Population size	Fixed	1	289.40	11.42	677.48	0.43	0.53
Population (PS)	Random	6	598.32	66.00	1026.29	0.58	0.74
Root dry mass							
Population size	Fixed	1	0.06	8.25	0.03	2.39	0.16
Population (PS)	Random	6	0.03	66.00	0.02	1.30	0.27
Shoot dry mass							
Population size	Fixed	1	0.13	11.16	0.27	0.47	0.51
Population (PS)	Random	6	0.24	66.00	0.40	0.61	0.72

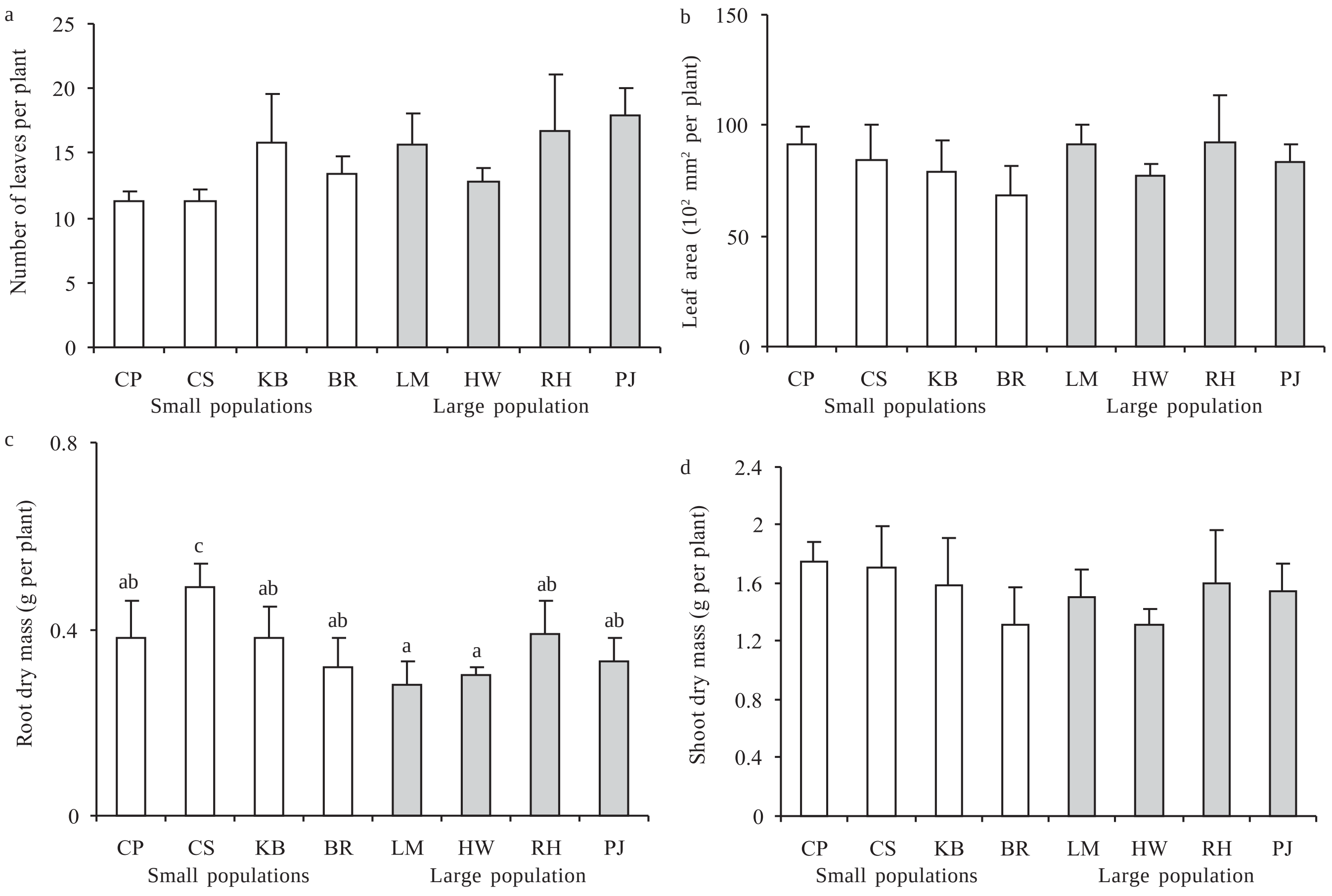

Figure 1. Growth performance of *Banksia ilicifolia* progeny across four small and four large populations grown for six months on river sand. CP: Chiquita Park; CS: Caporn Street; KB: Kensington Bushland; BR: Beechboro Reid; LM: Lancelin Mimegara; HW: Harry Waring; RH: Rowe Hopeland; PJ: Pinjar. Error bars represent standard errors. a. Number of leaves per plant; b. Leaf area per plant; c. Shoot dry mass per plant; d. Root dry mass per plant.

Park seedlings produced cluster roots. For all other populations, percentages ranged from 9 to 25%. However, the proportion of seedlings with cluster-roots was independent of population size (t_6 = -1.71, P = 0.14).

DISCUSSION

Small populations tend to be subject to an increased probability of stochastic extinction over large populations, due to demographic, environmental and/or genetic factors (Shaffer 1981; Ellstrand & Elam 1993; Reed 2005). In the present study, we found a rate of mortality for seedlings sourced from small populations that was twice as high as that for seedlings sourced from relatively large populations of *B. ilicifolia* when subjected to stressful conditions. Seedlings from relatively large populations possessed on average a higher resistance to a fungal pathogen than seedlings from relatively smaller populations. Our results suggest that these small populations of *B. ilicifolia* are suffering from the negative genetic effects associated with a reduction in size, and increased isolation of populations.

Although the pathogen was introduced as an unplanned part of the experiment, presumably as a result of over-watering, the seedlings were equally exposed to the possibility of infection, as they were randomly allocated to location on glasshouse benches, and the genetic vigor of seedlings apparently determined resistance. In support to this, we found some seedlings bearing fungus infection at their roots but still survived until the harvest time. Another possible source of pathogen infection is through the seeds (seed borne disease), but this is unlikely as we had sterilized the seeds prior to germination. Upon infection, the seedlings either died or survived until the harvest time.

Differences in seedling mortality with population size may be due to genetic and/or non-genetic maternal effects, such as seed size (Roach & Wulf 1987; Outborg & van Treuren 1995). Our inclusion of seed size as a covariate did not affect the ANOVA results, suggesting a negligible maternal seed size effect and indicated that the lower survival rate of seedlings from small populations probably has a genetic basis. Theory predicts that inbreeding and genetic drift is more likely to occur in small populations, and that these processes will lead to increased homozygosity and the random loss of alleles. The loss of genetic diversity in small populations can be expressed as inbreeding depression through, for example, an increased susceptibility to diseases (O’Brien *et al*. 1983, 1985; O’Brien & Evermann 1988; Ferguson & Drahuschak 1990). We (Heliyanto *et al*. 2005) have previously shown that more inbred offspring (selfs) of *B. ilicifolia* show lower resistance to, and higher mortality from, root fungal pathogens, than more outbred offspring (outcrossed). In an attempt to study the potential role of pathogens as selective agents in plant populations, Schmid (1994) also found a positive correlation between genetic diversity and plant resistance, following natural infestations by the mildew *Erysiphe cichoracearum* in experimental stands of *Solidago altissima*.

We suggest that the small populations of *B. ilicifolia* produced on average relatively more inbred offspring that demonstrate lower resistance to a pathogen induced stress than seedlings from large populations. This conclusion is supported by our earlier results that showed high and marked coefficients of inbreeding depression for selfed versus outcrossed progeny and heterosis for locally outcrossed versus long-distance outcrossed progeny (Heliyanto *et al*. 2006). Whilst we do not have data on outcrossing rates for these populations, much lower levels of seed set following hand self-pollination compared to pollination with outcross pollen suggests that *B. ilicifolia* is preferentially outcrossing and outcrossing rates are expected to be high in large populations (Heliyanto *et al*. 2005). Lower levels of outcrossing and higher levels of correlated paternity are found in smaller populations of the closely related *B. cuneata* compared to larger populations (Coates & Sokolowski 1992), suggesting that similar changes to mating patterns might be expected in small isolated populations of *B. ilicifolia*.

Although population size strongly influenced seedling survival under biotic-stress conditions, there was no significant effect on germination rate and growth performance of the surviving offspring. These observations suggest that under relatively benign conditions of the glasshouse and in the absence of the potentially lethal pathogen, inbreeding depression effects are masked in *B. ilicifolia*. In a review of 34 studies of inbreeding depression, Armbruster and Reed (2005) found that inbreeding depression increases under stress in 76% of cases, with a 69% increase in inbreeding depression in stressful versus benign environments. *Banksia ilicifolia* is preferentially outcrossing (Heliyanto *et al*. 2005), which maintains individual heterozygosity and masks a significant genetic load. However, changes in population size and breeding behaviour, such as increased inbreeding, increases individual homozygosity in offspring and exposes deleterious recessive alleles to selection. This appears to be occurring in the small populations sampled, with significantly higher mortality of seedlings under biotic-stress conditions compared to larger populations. Our results suggest that these small populations have not purged recessive deleterious alleles (Barrett & Charlesworth 1991; Byers & Waller 1999), as might be expected for these long-lived resprouters in populations that have probably recruited rarely, if at all, since fragmentation.

A genetic study using allozymes in the *Banksia* subgenus *Isostylis* (*B. ilicifolia*, *B. cuneata*, and *B. oliganta*) has indicated relatively low but similar levels of genetic diversity within these species (Broadhurst & Coates 2004). Although Broadhurst and Coates (2004) concluded that there was no relation between genetic diversity and population size, their conclusion was based on a comparison of the levels of genetic variability amongst the widespread *B. ilicifolia* (all sampled populations > 300 plants) and the two geographically restricted taxa *B. oligantha* (population size 15-250 plants) and *B. cuneata* (population size 13-97 plants). Moreover, the geographically restricted species may have been part of much larger populations prior to land-clearing-induced fragmentation over the past 60 years (Broadhurst & Coates 2004). Further, allozyme variation is known to be selectively neutral (Ouborg & van Treuren 1995) and as a consequence it might not represent characters (and thus genes) under selection, such as those affecting growth and disease resistance/susceptibility.

Increased mortality of offspring originating from small populations was also found by Fischer and Matthies (1998). However, we did not find any association between population size and seedling growth beyond seedling survival. The inconsistent association between population size and plant fitness is not uncommon (David 1998; Slate *et al.* 2004) and can be attributed to the presence or absence of linkages between polygenic characters (fitness) and the measured genetic diversity markers (Ouborg & van Treuren 1995). Similarly, weak association between seedling mortality/ resistance, following disease incidence, and fitness has also been reported in plant species and was, in some cases, attributable to the absence and presence of a pleiotropy gene effect (Parker 1990).

An extreme example of the relationship between population size and an effect on fitness in *Banksia* is for *B. goodie*; Lamont *et al*. (1993) found complete reproductive failure (no seed set) in small clonal populations which was attributed to an absence of pollinators. This is an example of the Allee effect (Allee 1931), where low population size leads to low per capita reproductive output. This effect is most pronounced in plants that rely on animal pollinators, such as banksias. While we did not directly measure fruit set, there appeared to be little difference between small and large populations. However, the number of seeds that were predated by insects was generally higher in the larger populations, and probably related to fewer natural enemies and greater quantity of natural resources (food plants as suggested by Lee *et al.* 2002).

Our results imply that genetic effects (inbreeding depression), rather than non-genetic effects (e.g. pollinator service), are potentially compromising the viability of small *B. ilicifolia* populations. However, while reasonable seed set indicates that pollinators remain present and are servicing the pollen dispersal needs of these plants, the nature and frequency of pollinator movements in small populations may be different to large populations. More limited cross-pollination would affect the "genetic quality" of seeds in small populations. For populations recently fragmented and potentially isolated by, for example, urbanisation, a genetic rescue program through wide-outcrossing may be required to restore lost genetic variation and viability. We have demonstrated earlier that outcrossed products of mating between individuals from *B. ilicifolia* populations 30 km apart showed a marked heterosis effect (Heliyanto *et al*. 2006).

Our observation that mortality under stress was higher for seedlings originating from small populations than for those from large populations is of concern for the conservation and management of previously widespread but recently fragmented species, especially with threats like climate change (Epps *et al*. 2004) and surrounding land use impacts on remnants, that may increase stress levels in remaining populations. A greater understanding of how these effects impact on long-term population viability for naturally fragmented species is urgently required for better conservation and management of our unique flora.

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