

# Inbreeding avoidance in Cunningham's skinks (*Egernia cunninghami*) in natural and fragmented habitat

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## Abstract

Habitat fragmentation/alteration has been proposed as a distinct process threatening the viability of populations of many organisms. One expression of its impact may be the disruption of core population processes such as inbreeding avoidance. Using the experimental design outlined in our companion paper, we report on the impact of habitat alteration (deforestation) on inbreeding in the rock-dwelling Australian lizard *Egernia cunninghami*. Ten microsatellite loci were used to calculate relatedness coefficients of potential and actual breeding pairs, and to examine mate-choice and heterozygosity. Despite significantly less dispersal and higher within-group relatedness between potential mates in deforested than in natural habitats, this did not result in significantly more inbred matings. Average relatedness amongst breeding pairs was low, with no significant difference between natural and fragmented populations in relatedness between breeding pairs, or individual heterozygosity. Active avoidance of close kin as mates was indicated by the substantially and significantly lower relatedness in actual breeding pairs than potential ones. These facts, and heterozygote excesses in all groups of immature lizards from both habitats, show that *E. cunninghami* maintained outbreeding in the face of increased accumulation of relatives.

**Keywords:** dispersal, glass effect, habitat fragmentation, inbreeding avoidance, lizard, mate choice, microsatellite

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## Introduction

Habitat fragmentation/alteration may exert its effects by creating isolated population units that are susceptible to stochastic and deterministic threatening processes. One under-researched facet is the extent to which changes in intrapatch population processes such as mating system and kin structure contribute to population declines and thus to probability of extinction. In the companion paper published in this issue (Stow & Sunnucks 2004), we showed that the rock-dwelling skinks *Egernia cunninghami* typically breed as monogamous pairs that are stable over multiple years. Coupled with high philopatry this leads to social groups comprising multigeneration and multicohort assemblages of close relatives. This situation pertains even in rock outcrops that have been surrounded by deforested habitat for more than 70 years, despite the fact that lizards

therein exhibit significantly reduced dispersal and elevated relatedness (Stow *et al.* 2001; Stow & Sunnucks 2004).

It might then be expected that inbreeding and inbreeding depression (reviews in Frankham *et al.* 2002; Taylor 2003) will be elevated in *E. cunninghami* populations that experience deforestation (fragmentation), because the predominantly monogamous mating system may promote mating between relatives (Stow & Sunnucks 2004). The entire population biology of a species, including mating system, kin recognition and sex-biased dispersal, may be moulded by optimization of inbreeding (Tregenza & Wedell 2000; Lebas 2002). However, comparatively little work has been conducted on whether habitat fragmentation impacts on mating systems (Peacock & Smith 1997a,b). In species without strong inbreeding avoidance mechanisms, increased relatedness of surrounding individuals is likely to lead to inbred matings (Slate & Pemberton 2002). In contrast, if inbreeding avoidance is very strong, breeding with close kin may be actively avoided to such an extent that the reduced pool of acceptable mates actually limits reproductive output (Young *et al.* 2000). Inbreeding,

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inbreeding depression and mate limitation will all reduce effective population size and will have a negative impact on population fitness/viability, increasing the risk of population extinction (e.g. Saccheri *et al.* 1998; Young *et al.* 2000; Keller & Waller 2002). Thus it is important to understand what impacts habitat fragmentation has on inbreeding avoidance in a range of species.

Background on this species and experimental design is given in the companion paper (Stow & Sunnucks 2004). Here we reiterate that populations in deforested areas experience potentially inbreeding-enhancing factors (reduced dispersal and increased relatedness) superimposed on a naturally monogamous mating system that is already potentially prone to inbreeding. Thus skinks in deforested areas may have experienced substantial pressure towards inbred matings in the 70 + years since habitat alteration at the two study sites, Tarana and Bathurst.

## Materials and methods

### *Study sites and sample collection*

Samples of *Egernia cunninghami* were collected at two locations on the Central Tablelands of New South Wales, Australia. The study sites, sample collection, DNA extraction, microsatellite genotyping and characteristics of the 10 loci used are described in detail elsewhere (Stow *et al.* 2001; Stow 2002; Stow & Sunnucks 2004). Briefly, each of two replicate study sites (Bathurst and Tarana) consists of a naturally vegetated and adjacent deforested area of skink habitat. In total 125 adult and 240 immature lizards were sampled.

### *Examining kin-bias in breeding pairs*

Patterns of dispersal and spatial organization of relatives may influence mating between close kin. To assess the effect of deforestation on relatedness structure, the relationship of pairwise  $R$  (Goodnight *et al.* 1998) between adult opposite-sex pairs and geographical distance between them was examined in deforested and naturally vegetated habitats.

Microsatellite analysis identified 56 parents, including 24 breeding pairs, for 201 offspring at the two sites (Stow & Sunnucks 2004). In addition to the 24 progeny groups for which both parents were sampled, there were five (each consisting of at least five offspring) for which only one parent was sampled. In each of these cases, a single parental genotype that could explain all offspring genotypes was deduced (= 'reconstructed') and included in the calculation of pairwise relatedness ( $R$ ) (see below). Their addition increased not only the number of parental genotypes but also the proportion of sampled groups from which relatedness data on breeding pairs were available. For the calculation of pairwise  $R$ -values, reconstructed genotypes were used only from progeny arrays consisting of five or more

offspring because this number was sufficient to allow reliable reconstruction of a missing parental genotype (e.g. the probability of a falsely deduced homozygous parental genotype from five offspring is approximately  $0.5^5$  (0.031).

Because of the stable group structure in *E. cunninghami*, the influence of relatedness on mate-choice can be assessed by comparing the relatedness in breeding pairs with that in pairs within the same group that might have mated but were not detected to have done so (as used for *Egernia stokesii* in Gardner *et al.* 2001). Three males in the 29 breeding pairs analysed here were detected breeding in two separate groups (Stow & Sunnucks 2004). For these individuals, separate relatedness comparisons were made with potential mates in both groups. Unsampled parents whose genotypes were reconstructed (see above) were considered members of the group occupied by their offspring and breeding partner. These lizards had their genotypes inferred from offspring taken from at least two age cohorts.

Relatedness between breeding pairs was further assessed by examining the inbreeding coefficient  $F_{IS}$  (details below) in offspring. However, even if kin avoidance results in heterozygote excesses within offspring at the group level, local isolation could still result in inbreeding through genetic drift. This was examined via individual multilocus heterozygosity (Slate & Pemberton 2002).

### *Statistical approaches*

Pairwise relatedness estimates were calculated from 10 independently segregating microsatellites, based on site- and habitat-specific allele frequencies using KINSHIP version 1.2 (Goodnight *et al.* 1998). Thus comparisons were based on distributions of pairwise  $R$  relative to the mean level of relatedness ( $R = 0$ ) in each site  $\times$  habitat block (rationale in Stow *et al.* 2001). Comparable KINSHIP simulations were used to determine the  $R$ -values expected among 1000 of each of: full siblings, half siblings and unrelated individuals. The distribution of relatedness between simulated full sibling pairs (mean  $\pm$  SD;  $0.496 \pm 0.145$ ) was consistent with the distribution of relatedness obtained between full siblings identified from parentage analysis ( $N = 651$ ,  $0.509 \pm 0.150$ ) (Stow & Sunnucks 2004).

The spatial distribution of pairwise  $R$  was assessed with the 'mantelize it' module in FSTAT version 2.9.3 (Goudet 2001). The significance of differences in means was determined using randomization (RT version 2.1, Manly 1997). All reported probability values are two-tailed.

Inbreeding coefficients ( $F_{IS}$ ) were determined for groups of immature lizards. Inbreeding coefficients (Weir & Cockerham 1984) were calculated using FSTAT version 2.9.3. Tests for deviations from Hardy-Weinberg equilibrium were carried out for each group of lizards inhabiting a rock outcrop using a randomization procedure and adjusted for multiple tests at  $\alpha = 0.05$  (Rice 1989; Goudet

2001), also checking for particular loci or groups that are over-represented in uncorrected significant results. Average  $F_{IS}$  for all groups takes into account sample size differences, with 95% confidence intervals calculated using a bootstrapping routine (Goudet 2001).

## Results

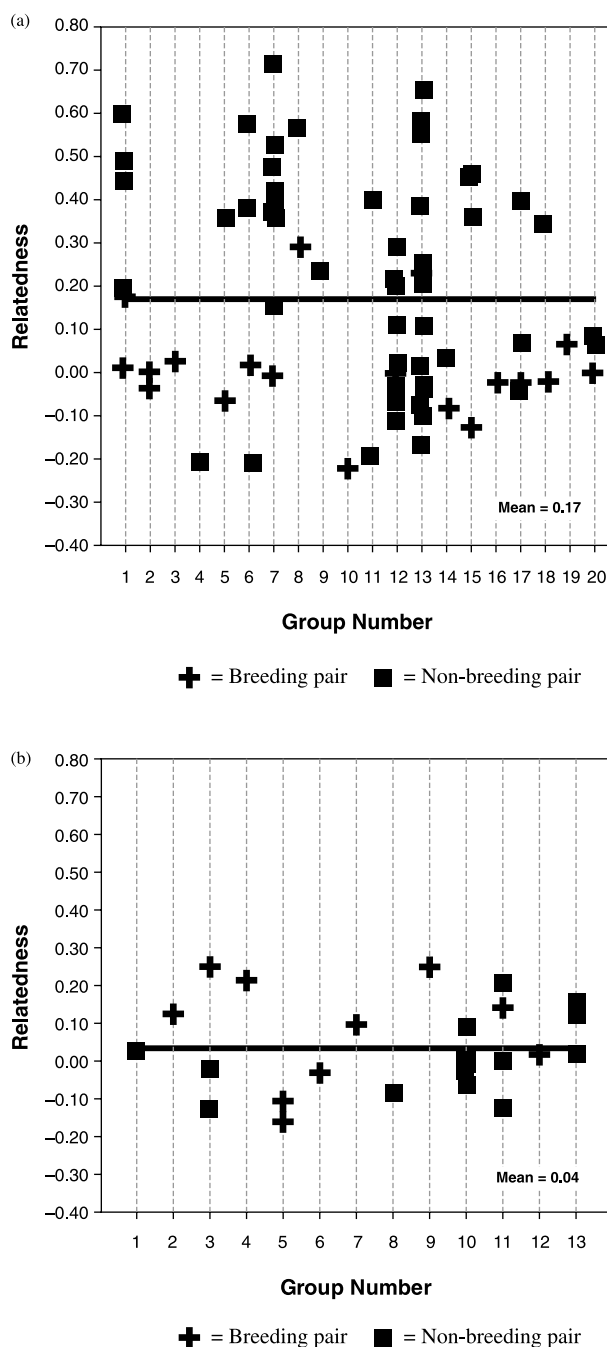
### *Deforestation was associated with increased relatedness amongst potential mates*

Opposite-sex adults showed relatively higher levels of within-group pairwise  $R$  in the deforested than the naturally vegetated habitats, significantly so at Bathurst ( $P = 0.02$ ) and overall ( $P = 0.009$ ; Fig. 1a,b). Additionally a significant decline of pairwise relatedness with increasing geographical distance was found in deforested but not natural habitats at each site and overall (Fig. 2a,b). Increased relatedness among potential mates relative to that in naturally vegetated habitats was seen for the majority of pairs of skins in the majority of deforested outcrops (Fig. 1a,b).

### *Egernia cunninghami avoids breeding with close relatives*

Our data indicate that *E. cunninghami* preferentially breed with less related individuals (Fig. 1). The observed range of  $R$  between estimated breeding pairs was 0.223–0.284: no known breeding pair had pairwise  $R$  close to the mean value predicted for first-degree relatives (mean  $\pm$  SD;  $0.496 \pm 0.145$ ). Indeed the 29 identified breeding pairs include only five (two in deforested habitats, three in naturally vegetated habitats) that were even close to the value of  $R$  expected for half siblings ( $0.248 \pm 0.131$ ) (Fig. 2a,b). The low  $R$  in breeding pairs may reflect active avoidance because members of breeding pairs were significantly less related to each other than they were to other potential mates available in the same rock outcrops (Bathurst  $P < 0.001$ , Tarana  $P = 0.029$ , overall  $P < 0.001$ ). The magnitude of the effect is impressive – successful breeding pairs have on average approximately an order of magnitude lower  $R$  than pairs without detected offspring (Table 1).

The greatly elevated levels of within-group relatedness between opposite-sex pairs in the deforested habitats (above, Fig. 1a,b and 2a,b) did not translate into greatly elevated levels of inbreeding. There was no significant difference ( $P = 0.185$ ) in relatedness between breeding pairs in naturally vegetated ( $R = 0.079 \pm 0.145$ ;  $N = 10$ ) and deforested ( $R = 0.011 \pm 0.120$ ;  $N = 19$ ) treatments. Remarkably, even absolute  $R$  (i.e. calculated from site-specific allele frequencies pooled over habitat types) of breeding pairs at each site were not significantly different (deforested, mean  $0.028 \pm 0.115$ ; natural,  $0.077 \pm 0.135$ ;  $P = 0.325$ ). Furthermore, mean individual multilocus heterozygosities for all sampled lizards in deforested habitats ( $0.860 \pm 0.095$ ) and naturally vegetated



**Fig. 1** The distribution of pairwise  $R$  in opposite-sex pairs (distinguished as breeding or nonbreeding) within individual rock outcrop groups for (a) deforested and (b) naturally vegetated habitats. The allele frequencies used to calculate  $R$  were site  $\times$  habitat-specific. For each habitat a horizontal line represents mean within-group  $R$ . Within-group relatedness (mean  $\pm$  SD) between opposite-sex pairs in deforested and naturally vegetated habitats and  $P$ -values ( $P$ ) for habitat comparisons at each site and overall ( $N$  = number of relatedness values). Bathurst: deforested area  $N = 30$ ,  $0.234 \pm 0.267$ ; natural area  $N = 44$ ,  $0.035 \pm 0.148$ ;  $P = 0.020$ . Tarana: deforested area  $N = 44$ ,  $0.125 \pm 0.220$ ; natural area  $N = 14$ ,  $0.038 \pm 0.093$ ;  $P = 0.145$ . Overall: deforested area  $N = 74$ ,  $0.170 \pm 0.244$ ; natural area  $N = 26$ ,  $0.037 \pm 0.119$ ;  $P = 0.009$ .

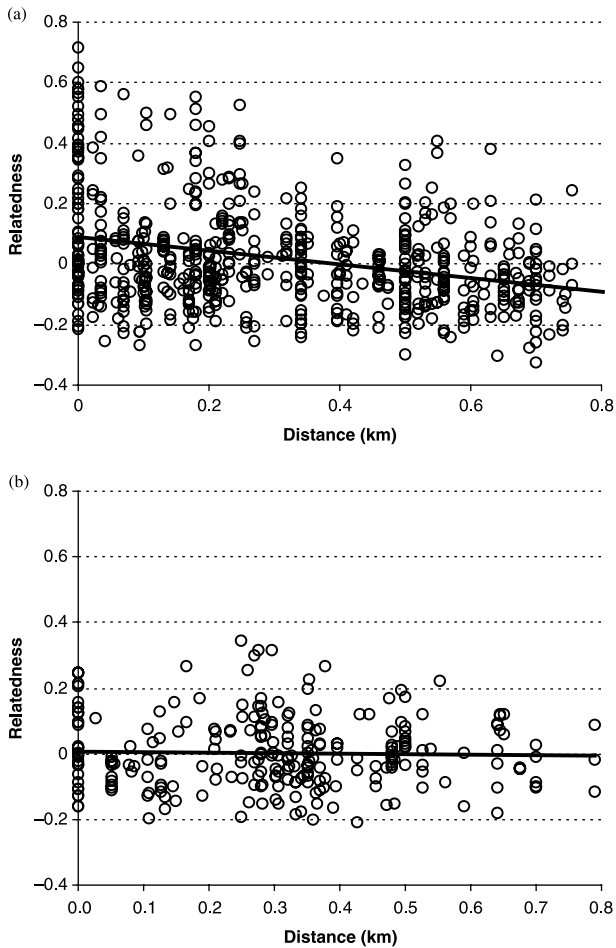


Fig. 2 Pairwise  $R$  in opposite-sex adults against geographical distance for (a) deforested habitats and (b) naturally vegetated habitats. The allele frequencies used to calculate  $R$  were site  $\times$  habitat-specific. The number of pairwise comparisons ( $N$ ), and probability of an association ( $P$ ) in each habitat at each site, and overall. Bathurst: deforested area  $N = 127$ ,  $P = 0.0002$ ; natural area  $N = 155$ ,  $P = 0.670$ . Tarana: deforested area  $N = 486$ ,  $P = 0.0001$ ; natural area  $N = 90$ ,  $P = 0.733$ . Overall: deforested area  $N = 613$ ,  $P = 0.0001$ ; natural area  $N = 245$ ,  $P = 0.673$ .

habitats ( $0.852 \pm 0.116$ ) were not significantly different (Mann–Whitney  $U$ ,  $Z = -0.222$ ,  $P = 0.824$ ). The shapes of these distributions in each habitat were not significantly different for adult lizards (Kolmogorov–Smirnov  $Z$ -test;  $Z = 0.500$ ,  $P = 0.964$ ) or for immature lizards alone ( $Z = 0.638$ ,  $P = 0.638$ ).

The generality of outbreeding is shown by the inbreeding coefficients ( $F_{IS}$ ) calculated for 22 groups of immature lizards. Group size ranged from 4 to 23 (mean  $9.55 \pm 4.21$ ) with no significant difference of group size between habitats (Mann–Whitney  $U$ ,  $Z = -0.172$ ,  $P = 0.868$ ). All groups of immature lizards showed strong heterozygote excesses with no significant difference in average  $F_{IS}$  observed between sites and habitats (95% confidence intervals overlap). In total, 15 of 22 groups still show significant hetero-

**Table 1** Relatedness between breeding pairs and potential mates for each breeding partner within groups

Site	Breeding pairs		Potential mates	
	$N$	$R \pm SD$	$N$	$R \pm SD$
Bathurst	18	$0.046 \pm 0.146$	10	$0.343 \pm 0.278$
Tarana	11	$0.014 \pm 0.104$	16	$0.156 \pm 0.179$
Overall	29	$0.034 \pm 0.131$	26	$0.245 \pm 0.237$

$N$  = number of pairwise comparisons.

zygote excess, even after Bonferroni correction. There was no significant difference between natural and deforested habitat in the proportion of groups with significant heterozygote excesses (Fisher's exact  $P = 0.724$ ).

## Discussion

Higher levels of within-group relatedness in *Egernia cunninghami* in deforested habitats were not associated with elevated relatedness amongst successful breeding pairs and had little impact on heterozygosity. We infer that this was achieved by avoidance of breeding with close kin. The alternative possibility, that inbred matings were not rare but resultant offspring were removed by inbreeding depression, is unlikely for two main reasons. First, inbreeding depression would have to be almost invariably fatal above a modest inbreeding threshold, and such a pattern has not been observed previously (Frankham *et al.* 2002). Second, matings would have to occur (and fail) outside the normal mating structure of a single pair of adults dominating the breeding in a rock outcrop (Stow & Sunnucks 2004). If, as we propose, active inbreeding avoidance is employed, it has been effective for 70+ years in buffering populations in deforested habitat from inbreeding: successful breeding pairs under these conditions have approximately an order of magnitude lower  $R$  than if they mated at random. In two species closely related to *E. cunninghami*, *Tiliqua rugosa* and *Egernia stokesii*, relatedness between breeding pairs was also shown to be lower than that of other nearby potential mates (Bull & Cooper 1999; Gardner *et al.* 2001). As with *E. cunninghami*, these species show long-term monogamy (references in Stow & Sunnucks 2004). Because of the large reproductive investment with their partners (including long-term fidelity and retreat-sharing), individuals may be expected to be choosy about their mates. Our data indicate that low genetic relatedness is an important determinant or correlate of that choosiness.

Because the majority of rock outcrop groups consist of a long-term breeding pair and their offspring, avoidance of breeding with first-degree relatives and to a large extent second-degree ones points to the importance of dispersal in finding a mate. Preferential choice of migrant mates could account for consistently low  $R$  between breeding

pairs in an environment dominated by close relatives, and consistently negative  $F_{IS}$  in offspring. Inhibited adult dispersal and increased relatedness among potential mates may account for the higher proportion of nonbreeding adult males per rock outcrop in fragmented habitats (Stow & Sunnucks 2004). Demographic consequences of mate limitation, however, may not be expected until females are affected, and presently there is little evidence that fragmentation has had an impact on recruitment (Stow unpublished data). Constraints on mate availability for male lizards may promote 'frustrated' dispersal for breeding purposes, giving rise to the stronger male-biased dispersal seen in fragmented habitats (Stow *et al.* 2001).

While *E. cunninghami* in our system have been able to avoid breeding with close relatives, the longer-term outcomes of increasing relatedness are not likely to be favourable. On one hand, extreme inbreeding avoidance alongside increasing relatedness can lead to loss of fertility through mate limitation (Young *et al.* 2000), i.e. the 'glass effect' (Tainaka & Itoh 1996). On the other hand, incomplete inbreeding avoidance under restricted dispersal will lead inevitably over time to the accumulation of inbreeding and inbreeding depression (Frankham *et al.* 2002). Despite these possible future deleterious outcomes, the natural biology of *E. cunninghami* seems to have provided it with at least a temporary buffer against inbreeding. Currently we see little evidence of demographic impacts of the 'glass effect', although the elevated proportion of nonbreeding males and significant sex asymmetry in dispersal may be an early signal of it.

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This work is part of the program outlined in Stow *et al.* (2001) and in the companion paper published in this issue, Stow & Sunnucks (2004). It forms part of Adam Stow's PhD research programme. Adam has interests in behavioural ecology, especially of reptiles, and in the consequences of habitat fragmentation on population genetics and social interactions. Paul Sunnucks' research focuses on the application of molecular markers to mobility and gene flow of animals in natural and human-impacted habitats.

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