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Title:
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Date:
2018-01-01

Citation:
Soanes, K., Taylor, A. C., Sunnucks, P., Vesk, P. A., Cesarini, S. & van der Ree, R. (2018). Evaluating the success of wildlife crossing structures using genetic approaches and an experimental design: Lessons from a gliding mammal. Journal of Applied Ecology, 55 (1), pp.129-138. https://doi.org/10.1111/1365-2664.12966.

Persistent Link:
https://hdl.handle.net/11343/293317

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Article type   : Standard Paper

Evaluating the success of wildlife crossing structures using genetic approaches and an experimental design: lessons from a gliding mammal

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ABSTRACT

1. Millions of dollars are spent on wildlife crossing structures intended to reduce the barrier effects of roads on wildlife. However, we know little about the degree to which these structures facilitate dispersal and gene flow.
2. Our study incorporates two elements that are rarely used in the evaluation of wildlife crossing structures: an experimental design including a before and after comparison, and the use of genetic techniques to demonstrate effects on gene flow at both population and individual levels. We evaluated the effect of wildlife crossing structures (canopy bridges and glider poles) on a gliding mammal, the squirrel glider (*Petaurus norfolcensis*). We genotyped 399 individuals at eight microsatellite markers to analyse population structure, first-generation migrants and parentage relationships.
3. We found that the freeway was not a complete genetic barrier, with a strong effect evident at only one site. We hypothesise that the presence of corridors

alongside the freeway and throughout the surrounding landscape facilitated circuitous detours for squirrel gliders.

4. Installing a crossing structure at the location with a strong barrier effect restored gene flow within just five years of mitigation.
5. *Synthesis and applications.* Our study highlights the importance of using genetic techniques not just to evaluate the success of road crossing structures for wildlife, but also to guide their placement within the landscape. Managers wishing to reduce the effects of linear infrastructure on squirrel gliders and other arboreal mammals should aim to preserve and enhance vegetation along roadsides and within centre medians, as well as mitigate large gaps by implementing wildlife crossing structures.

KEYWORDS: wildlife crossing structure, fragmentation, gliding mammals, gene flow, canopy bridge, glider pole, vegetated median, gap-crossing, road ecology, BACI

1. INTRODUCTION

The ability of animals to move across landscapes is essential for the long-term persistence of many wildlife populations (Frankham 2015; Pierson *et al.* 2015). Understanding and mitigating human-induced barriers to movement and gene flow is therefore a key goal of wildlife management. For example, millions of dollars are spent across the globe on wildlife crossing structures (underpasses and overpasses) to reduce the barrier effects of roads on wildlife. The goal is to provide connectivity across a road that would otherwise restrict animal movement, thus allowing populations to persist. However, we know surprisingly little about the degree to which such structures facilitate dispersal and gene flow (Corlatti, Hacklander & Frey-Roos 2009; Lesbarreres & Fahrig 2012). This uncertainty limits the ability of managers to decide how to best mitigate the impacts of roads on wildlife, which impacts can be successfully mitigated, and where to best allocate limited funds for conservation.

Studies evaluating wildlife crossing structures largely lack two components that would help address this gap. The first is an experimental approach, in which crossing structures are added to the landscape in a controlled way, and their effects compared to before mitigation and/or unmitigated sites. Manipulative experiments can yield great inferential power, particularly in complex landscapes, providing added confidence that the observed effect was due to mitigation and not to other factors (van der Grift *et al.* 2013; Evans, Riley & Lamberti 2015; Rytwinski *et al.* 2015).

However, implementing an experimental approach is challenging and examples in road ecology are rare (Michener 1997; Rytwinski *et al.* 2015). The second component missing from most evaluations is the use of genetic techniques (Balkenhol & Waits 2009; Simmons *et al.* 2010; Sunnucks & Balkenhol 2015). By investigating temporal and spatial patterns in genetic variation, researchers can infer changes in movement and dispersal for a large number of individuals and sites, and, critically, determine whether an animal's movement across the road results in gene flow. Despite these advantages, relatively few studies have used genetic techniques to evaluate the success of crossing structures (but see Kuehn *et al.* 2007; van Manen *et al.* 2012; Sawaya, Kalinowski & Clevenger 2014). Incorporating these two elements into road ecology research represents a next step that will allow strong inferences about the effectiveness of wildlife crossing structures.

The squirrel glider (*Petaurus norfolcensis*) is a small, nocturnal, arboreal mammal of eastern Australia. Throughout the southeast of its range, the squirrel glider is threatened with extinction due to extensive habitat loss and fragmentation. Squirrel gliders move by gliding from tree to tree, with an average glide distance of 25–40 m depending on the launch height; individuals rarely travel along the ground (van der Ree, Bennett & Gilmore 2004; Goldingay & Taylor 2009). Large, treeless gaps, such as those that occur over major roads, can restrict movements and isolate populations (Taylor *et al.* 2011; Goldingay *et al.* 2013). Over the last 10 years, transport agencies have begun implementing crossing structures such as glider poles, canopy bridges and vegetated medians (described in Soanes & van der Ree 2015) to reduce the barrier effects of roads on squirrel glider populations. However, decision-making has been hampered by a lack of information as to their effectiveness. Recognising this knowledge gap, the state transport agency of Victoria in southeast Australia (VicRoads), collaborated with researchers to experimentally test the effectiveness of crossing structures for squirrel gliders. The project involved monitoring the use of structures by wildlife, radio-tracking movements, and collecting genetic data before and after structures were installed at mitigated, unmitigated and non-freeway sites. Several associated studies (van der Ree *et al.* 2010; Soanes *et al.* 2013; Soanes, Vesk & van der Ree 2015) and other projects (e.g. Taylor & Goldingay 2012; Teixeira *et al.* 2013; Gregory *et al.* 2014) have since shown that canopy bridges, glider poles and vegetated medians are used by squirrel gliders and a range of other arboreal mammals. However, questions about the level of functional connectivity remain unanswered.

Here, we used genetic measures of connectivity within a BACI (before–after–control–impact) experimental framework to investigate the degree to which wildlife crossing structures provide connectivity across a freeway for a threatened gliding mammal. We inferred changes in connectivity over time and among site-types, based on patterns of spatial genetic structure, genetic identification of migrants between populations and evidence of cross-freeway mating.

2. MATERIALS AND METHODS

2.1 Freeway and crossing structures

We studied a 70-km section of the Hume Freeway, between the towns of Avenel (36°54'2.54"S, 145°13'59.98"E) and Benalla (36° 33'5.40"S, 145°58'54.10"E) in the state of Victoria, southeast Australia (Figure 1). The average traffic volume is approximately 10,000 vehicles per day at a maximum legal speed of 110 km/hr. The surrounding landscape is predominantly agricultural pasture and rural townships. Woodland (*Eucalyptus* spp.) supporting squirrel glider populations is mostly restricted to linear strips along roadsides (including the freeway), road reserves (land set aside for roads that have not been constructed) and waterways, forming a network of linear habitat (van der Ree 2002, Figure 1). Squirrel gliders do not appear to avoid roadside habitats and are resident within the linear woodland along the freeway (McCall *et al.* 2010; van der Ree *et al.* 2010). Therefore, the key factor expected to limit squirrel glider movement across the freeway is the width of the gap between trees on either side (hereafter referred to as the treeless gap). During the 1970s and 1980s, the Hume Freeway was widened from a two-lane highway (~20–40 m wide) to a four-lane, divided freeway, increasing the width of the treeless gap to 44–76 m depending on the width of the centre median (21–38 m) and the presence of woodland vegetation within the median and roadside. It was assumed that the freeway did not affect squirrel glider movement prior to widening, because the treeless gap across the single carriageway was within gliding range, and the species readily crosses narrow roads (van der Ree *et al.* 2010).

We studied four overhead crossing structures (two canopy bridges and two glider poles) along the Hume Freeway. These structures were installed in July 2007, approximately 30 years after the freeway was widened, as part of the experimental design described below. Bridges were approximately 70 m long in a 'rope lattice' design, providing a continuous link between roadside trees across the freeway. Glider poles were tall wooden poles (~15 m tall, ~50 cm diameter) placed in the centre median and roadsides as 'stepping stones', reducing the treeless gap to <35 m. The structures are described in Soanes *et al*. (2013, 2015) and images provided as Supporting Information (Appendix S1). The canopy bridges and glider poles were installed at sites where the treeless gap across the freeway exceeded 50 m. The roadside tree heights and expected glide ratio of squirrel gliders indicated that safe crossing of the freeway would be unlikely at these points (Soanes, Vesk & van der Ree 2015). Further, pre-mitigation radio-tracking identified that squirrel glider movement was restricted (van der Ree *et al.* 2010). The structures were intended to increase movement across the freeway, allowing habitat access, dispersal and gene flow.

2.2 Experimental design and context

We established a BACI experiment to test the success of canopy bridges and glider poles in reducing the barrier effect of the Hume Freeway on squirrel glider populations. We compared impact sites (i.e. sites at which crossing structures were added) with control sites, before and after mitigation (Figure 2). We selected three types of control sites to reflect multiple potential benchmarks for the success or failure of the crossing structures: unmitigated freeway sites (control 1), freeway sites with natural canopy connectivity or 'vegetated medians' (control 2), and non-freeway sites (control 3). At *unmitigated freeway* sites (n = 2) there were no trees in the centre median, resulting in a treeless gap across the freeway exceeding 50 m. These control sites remained unmitigated throughout the entire study, to show what would have happened if no action had been taken. At *vegetated median* sites (n = 4) tall trees (20–30 m) were present in the centre median and roadsides within 5 m of the road edge, reducing the treeless gap across the freeway to 15–20 m. We expected that this control would provide an approximation of the 'pre-widening' condition of the freeway, representing structural connectivity that was retained when the freeway was upgraded. *Non-freeway* sites (n = 3) were approximately 5 km away from the freeway, and the barrier being tested was a local, low-traffic-volume road (treeless gap <15 m, <100 vehicles per day). This control provided an approximation of squirrel glider populations residing in similar habitat without the presence of a freeway. Before mitigation, the *crossing structure* sites were in the same condition as *unmitigated* sites, in that the treeless gap exceeded 50 m. For ease of reference, we refer to impact and control sites by their more descriptive terms (e.g. mitigated/crossing structure, unmitigated, non-freeway or vegetated median).

Based on concurrent field studies of squirrel glider movement and use of the crossing structures (van der Ree *et al.* 2010; Soanes *et al.* 2013; Soanes, Vesk & van der Ree 2015), we expected that where squirrel glider populations were separated by a treeless gap across the freeway wider than 50 m (i.e. unmitigated freeway sites, or crossing structures sites prior to mitigation), connectivity would be lower than at sites where the treeless gap was within gliding range (e.g. vegetated median sites or non-freeway sites). Further, we expected that movement and gene flow would increase after crossing structures were installed.

2.3 Animal capture, DNA extraction and genotyping

We conducted trapping surveys to collect genetic material from squirrel gliders at 13 sites (four mitigated, two unmitigated, four vegetated median, and three non-freeway) before (2005–2007) and after (2008–2013) the crossing structures were installed in July 2007 (Figure 2). All sites had similar habitat configuration, consisting of a 10–20 m wide linear strip of woodland bisected either by the freeway (impact, veg median, unmitigated) or a single-lane road (non-freeway). The distance between sites varied from 1 to 11 km. Each site contained a transect of ~20–24 traps, half on the east side and half on the west side of the road barrier being investigated (Figure 2). Comparison between individuals from the east and west sampling locations was used to evaluate the degree of connectivity that occurred at each site.

Squirrel gliders were captured by setting wire-mesh cage traps (17 × 20 × 50 cm, Wiretainers Pty Ltd) in trees, following McCall *et al.* (2010). The trap effort was approximately equal across both survey periods (the average number of trap nights per site per survey was 129 before mitigation and 124 after mitigation). Captured animals were marked with an ear tattoo and PIT tag (Trovan ID100, Microchips Australia Pty Ltd) to allow identification upon recapture. Ear-tissue biopsies (2 mm diameter) were taken for genetic analysis and stored in 97% ethanol at ambient temperature until extraction. Over the eight-year study, samples were collected from 399 squirrel gliders (191 before mitigation, 208 after mitigation; Table 1).

Genomic DNA was extracted from a single tissue sample for each individual. DNA was extracted from samples collected before mitigation using the salting-out method described by Sunnucks and Hales (1996). DNA from samples collected after mitigation was extracted using the Bioline DNA extraction kit by the Australian

Genome Research Facility (Melbourne). Samples were genotyped at eight microsatellite loci: PnMs49 (Millis 2000), Pno18, Pno12, Pno7, Pno40, Pno31, Pno44 and Pno56 (Soanes, Banks & van der Ree 2014). All samples were successfully genotyped at five or more loci (average 7.5 per individual).

2.4 Data analysis

Squirrel gliders can live for up to seven years (van der Ree, Harper & Crane 2006), with an estimated generation time of four years; therefore some generational overlap was expected between samples collected before and after mitigation. We used the approximate age at time of capture (assessed as per van der Ree 2002) to categorise individuals as belonging to the generation born before or after mitigation, as this more accurately reflects the landscape context under which their genotypes originated. Each animal was included in only one category. We found that 67 individuals born before mitigation were still alive after mitigation (~17% of genotypes). These were all placed in the before-mitigation group.

Geographic distance and landscape features such as towns were likely to swamp fine-scale genetic patterns caused by the freeway and mitigation. To combat this and maintain focus on our primary question, we used the individual-based Bayesian clustering approach implemented by the program STRUCTURE 2.3.4 (Pritchard, Stephens & Donnelly 2000) to identify appropriate groupings within which to conduct fine-scale analysis. STRUCTURE defined three genetic groups corresponding with northern, central and southern regions of our study area (Figure 1, Supporting Information, Appendix S2). Analyses in GenAlEx 6.5 (Peakall & Smouse 2012) revealed no significant deviations from Hardy–Weinberg equilibrium or evidence of linkage disequilibrium within any region at any locus or pairs of loci respectively, and measures of genetic variation were approximately equal across all regions in both periods (average heterozygosity, H_E 0.68–0.70; average number of alleles, N_a 6.4–8.0; full details in the Supporting Information, Appendix S3). All further analyses investigated genetic substructure across the freeway within each region.

We tested the hypothesis that the freeway created two genetic clusters at each site, one on each side (Blair *et al.* 2012). We used STRUCTURE to calculate the proportion of each individual's genotype (represented as a Q-value) that belonged to each of two genetic clusters (i.e. $K = 2$). If individuals sampled to the east of the freeway showed membership to one cluster (high Q-values) and individuals to the west did not (low *Q*-values), then this would suggest a barrier effect of the freeway. Alternatively, if the freeway was not a 'barrier', we expected individuals on opposite sides to show strong membership to the same cluster, or show similar membership (*Q* ~0.5) to both clusters. The genotypes were run at $K = 2$ (admixture allowed, allele frequencies correlated) for 500,000 iterations after a burn-in of 500,000. We used ObStruct (Gayevskiy *et al.* 2014) to generate coefficients of determination (R^2) to measure the strength and statistical significance of the relationship between the inferred genetic structure and the spatial location of samples (east and west of the freeway). A high R^2-value suggests a strong relationship between the observed genetic structure and the presence of a linear barrier within a site.

We used GENECLASS 2.0 (Piry *et al.* 2004) to identify first-generation migrants that crossed the freeway. We used the Bayesian criteria developed by Rannala and Mountain (1997) and the Monte Carlo resampling method described by Paetkau *et al*. (2004) to calculate assignment probabilities for each squirrel glider based on the genotype frequencies of 10,000 simulated individuals. Migrants were classified based on the ratio *Lhome/Lmax* at a threshold of 0.01.

We used CERVUS (Kalinowski, Taper & Marshall 2007) to identify the likely father and mother of each individual simultaneously – that is, the most statistically likely 'parent pair' for each individual based on our sample. For a given offspring, if the accepted mother and father occur on opposite sides of the freeway, this provides strong evidence that movement across the road resulted in successful reproduction and therefore gene flow. We accepted parentage assignments with strict (>95%) and relaxed (80%) assignment probabilities, and allelic mismatch at one or no loci. The simulation was based on 10,000 offspring and the parameters were estimated from field data (Supporting Information, Appendix S5).

3. RESULTS

3.1 Spatial genetic structure

Over the whole sampled landscape, there was little evidence that the freeway affected the spatial genetic structure of squirrel glider populations at any site type, before or after mitigation. The influence of the freeway on spatial genetic structure at unmitigated and vegetated median sites was similar to the influence of narrow, low-traffic volume roads present at non-freeway sites, with individuals on either side of belonging to the same genetic cluster (*Q*) or showing mixed membership to both clusters (Table 2). Surprisingly, given the very low rate of road-crossing shown by radio-tracking (van der Ree *et al*. 2010), the absence of vegetated medians at a site rarely resulted in spatial genetic structuring between individuals on opposite sides of the freeway. The unmitigated sites RR 169 and RR 182 showed no evidence of genetic structuring due to the freeway, nor did the crossing structure sites Warrenbayne or Violet Town prior to their mitigation ($R^2 < 0.44$, P = NS, Table 2). Only one site, Longwood, showed evidence of significant structuring before mitigation ($R^2 = 0.83$, $P < 0.001$). After a canopy bridge was installed at this site, genetic structure was significantly reduced and was comparable to vegetated median and non-freeway sites ($R^2 = 0.02$, P =NS).

While the R^2-values at some other sites appeared high (Table 2), these were not significant and the distribution of Q-values did not suggest that squirrel gliders sampled on opposite sides of the freeway showed strong proportional membership to opposing clusters.

3.2 Evidence of migration and gene flow across the freeway before and after mitigation

To investigate current and recent gene flow across the freeway, we identified individuals captured at freeway sites that were first-generation migrants from the opposite side of the freeway (i.e. *cross-freeway migrants*) and individuals whose mother and father were on opposite sides of the freeway (i.e. *cross-freeway parent-pairs*). We further distinguished between *direct* crossings, in which an individual (or parent) was assigned to the sampling location directly opposite the freeway, and *detour* crossings, in which an individual (or parent) was assigned to a site on the opposite side of the freeway that was not directly across it (inset, Figure 3). Analysis of first-generation migrants revealed 33 individuals that were born at a site other than the one in which they were detected, 19 of which had crossed the freeway (details provided as Supplementary Information, Appendix S4). We identified the most likely mother and father for 81 individuals (16 at 95% confidence, 65 at 80% confidence), 16 of which resulted from cross-freeway pairings (2 at 95% confidence, 14 at 80% confidence). As the small sample size precluded statistical analysis, we provide descriptive information.

The number of cross-freeway parent-pairs increased at mitigated sites after crossing structures were installed (from one to five). This included successful reproduction evident at the mitigated site Longwood, which showed a significant reduction in genetic structure after a crossing structure was installed. While there was no difference in the number of cross-freeway migrants after mitigation, the apparent path taken to cross the freeway changed from *detour* to *direct* (Figure 3). That is, after crossing structures were added to mitigated sites, all first-generation migrants crossing the freeway were classed as direct crossings (Figure 3). Direct crossings were most common at sites where vegetated medians or crossing structures were present. Cross-freeway migrants and parent-pairs were detected at unmitigated sites in both study periods; however all were classed as detour crossings.

4. DISCUSSION

Extensive radio-tracking and trapping had previously shown that squirrel glider movement across the freeway was strongly inhibited where the treeless gap created by the freeway exceeded 50 m (i.e. unmitigated sites). However, the present study showed that this restricted mobility did not cause strong genetic differentiation at the majority of unmitigated sites (including crossing structure sites prior to mitigation). Before mitigation, the level of genetic structure between squirrel gliders on either side of the freeway at vegetated median and unmitigated sites was similar to non-freeway sites at which the only impediment was a narrow, low traffic-volume road. Further, the analysis of first-generation migrants and parentage indicated current gene flow across the freeway at all site types. One site was an exception. A crossing structure site, before it was mitigated, showed significant differentiation and low gene flow. After mitigation was installed, gene flow at this site increased, and genetic differentiation significantly declined. Thus, we did not find evidence that the Hume Freeway restricted genetic connectivity for squirrel gliders overall, but did detect site-level effects that were successfully mitigated following the addition of wildlife crossing structures.

Many sections of the Hume Freeway present a large physical barrier to squirrel glider movement, yet this effect was rarely observed at the genetic level. Studies on other species have found only limited impacts of roads on gene flow (e.g. Prunier *et al.* 2014; Grilo *et al.* 2016), with strong genetic effects usually associated with large, fenced roads (e.g. Kuehn *et al.* 2007; Hepenstrick *et al.* 2012), or species with very limited mobility (e.g. a flightless beetle and a salamander: Keller, Nentwig & Largiadér 2004; Marsh *et al.* 2008). It appears that in many cases, habitat connectivity throughout the surrounding landscape can temper the effect of linear infrastructure on wildlife. For example, the use of scattered trees and roadside corridors as ‘stepping stones’ was thought to reduce the genetic impacts of habitat fragmentation and infrastructure on wildlife in agricultural and urban landscapes (Munshi-South 2012; Goldingay *et al.* 2013; Amos *et al.* 2014), while forest birds and arboreal mammals take circuitous detours to avoid inhospitable terrain (Belisle & Desrochers 2002; Bakker & Van Vuren 2004). The combined evidence from previous radio-tracking and the current genetic analysis suggest that this is also true for squirrel gliders in our study. Squirrel gliders only crossed the freeway directly, that is, between sampling locations on the east and west, at sites where vegetated medians or crossing structures were present. The landscape surrounding the Hume Freeway comprises a network of linear woodland strips as well as sparse or isolated trees. Further, at the site where a strong genetic effect was evident, there were no tall trees in the centre median or verges for more than 1 km in each direction. The detection of a genetic effect at one site (and restoration following mitigation) suggests that our findings are not due to a 'time-lag', but rather that patterns of genetic connectivity are driven by the effect of structural connectivity on species mobility. Therefore we infer that when a direct route across the freeway was not available (e.g. at an unmitigated site) animals took circuitous detours to reach an alternative crossing point.

'Detour' movements are difficult to observe directly, and can be easily missed in field-based studies of connectivity. It is therefore imperative that genetic studies be used not just to evaluate the success of crossing structures, but also to guide their placement within the landscape where disruptions to gene flow are a key concern. Future studies should aim to conduct genetic sampling within a broader, landscape-genetics framework, to provide a more comprehensive understanding of the location and relative importance of features that impede and facilitate movement (e.g. Delaney, Riley & Fisher 2010; Munshi-South 2012), allowing mitigation to be better targeted. Such applications are tractable and affordable via outsourcing of genome-wide screens of thousands of genetic markers for almost any wildlife species (Sunnucks & Balkenhol 2015).

At the single site where we observed genetic structuring and restricted gene flow for squirrel gliders, i.e. Longwood, the installation of a canopy bridge restored gene flow across the freeway. After the canopy bridge was installed there was no evidence of genetic differentiation between the east and west sides of the freeway. The change was rapid, detectable within just five years. Further, this was associated with changes in both animal movement and gene flow. Some studies have raised concerns that crossing structures may not result in gene flow if social barriers reduce the reproductive success of crossing individuals (Riley *et al.* 2006). However, this was not the case here. The analysis of first-generation migrants and parentage, combined with data from field studies (Soanes *et al.* 2013; Soanes, Vesk & van der Ree 2015) provides clear evidence that the individuals that used crossing structures and vegetated medians to cross the freeway contributed to gene flow. Similar results were reported in a study of black (*Ursus americanus)* and grizzly (*U. arctos*) bears using crossing structures to cross a major highway in Canada (Sawaya, Kalinowski & Clevenger 2014). Taken together, these studies provide encouraging examples of gene flow being restored through the retrofitting of crossing structures to existing linear infrastructure.

While the current study suggests that many of the crossing structures installed were not necessary to increase genetic connectivity across the freeway on the timescale of observation here, it is important to recognise that gene flow is rarely the only goal of mitigation. Managers must often consider multiple potential goals, both when selecting where crossing structures should be installed, and when evaluating their success. Ultimately this depends on understanding the mechanisms by which roads are expected to affect the wildlife population prior to mitigation, usually revealed through rigorous research (van der Grift *et al.* 2013; Rytwinski *et al.* 2015). In our case, previous research clearly demonstrated that the home-range movements of squirrel gliders were disrupted by the Hume Freeway at unmitigated sites (van der Ree *et al.* 2010), and that the installation of crossing structures re-established regular cross-freeway movements for individuals living alongside the freeway (Soanes *et al.* 2013; Soanes, Vesk & van der Ree 2015). In this way, the crossing structures were a benefit to local populations. Providing safe access to resources that occur on both sides of freeway could be critical to population persistence if it sufficiently improved demographic connectivity (Lowe & Allendorf 2010). Managers wishing to reduce the effects of linear infrastructure on squirrel glider populations in heavily-modified landscapes such as ours should aim both to 1) install crossing structures across treeless gaps exceeding 50 m to enable direct cross-road movements, and 2) preserve and enhance vegetation corridors that extend along the roadsides and centre medians to prevent long, treeless stretches that may result in a local genetic discontinuity (e.g. such as the ~2km stretch observed at the Longwood site).

Large-scale ecological experiments are often called for in ecology and conservation, but are notoriously difficult to implement and often heavily constrained in the real world (Michener 1997; Roedenbeck *et al.* 2007; van der Ree *et al.* 2015). Our study was no exception. While it represents a significant step forward in its approach, it is still a far cry from the 'ideal' ecological experiment. We lacked pre-road data, had limited replication, spatially clustered treatments, and small sample sizes. All of these issues were beyond our control – we could not increase the number of structures that the road agency installed, nor move the location of pre-existing vegetated medians. However, important management questions should not go untested simply because the ideal study cannot be performed, and we used the strongest study design and combination of methods possible within the constraints of the project. In the field of road ecology, the quest for well-controlled studies will depend upon establishing strong relationships between researchers and road agencies early in the planning process (Rytwinski *et al.* 2015; van der Ree *et al.* 2015). Following repeated calls-to-action over the last decade for improved inferential strength in road ecology research (Roedenbeck *et al.* 2007; Balkenhol & Waits 2009; Rytwinski *et al.* 2015) we are certain that others are working to adopt similar approaches and look forward to seeing the resulting publications in the years to come.

ACKNOWLEDGEMENTS

We thank the Baker Foundation, The Australian Research Centre for Urban Ecology, the Australian Research Council (grant LP0560443), The Australian Research Council Centre of Excellence for Environmental Decisions, the Holsworth Wildlife Research Endowment (ANZ Trustees Foundation), Wildlife Preservation Society Australia, RSPCA Alan White Scholarship, VicRoads and the New South Wales Roads and Maritime Services for support. Animals were trapped under the University of Melbourne Animal Ethics Committee permits (0810924.3 and 1112269.1) and the Department of Sustainability and Environment research permits (10004763 and 1006094). W. Sowersby, S. Harvey, E. Hynes, R. Soanes, P. Zambrano, M. Carmody Lobo, B. Mitchell, S. McCall, N. Gulle and R. Bull provided invaluable help with field work. We are grateful to the anonymous reviewers on this and previous versions of the manuscript for their constructive comments and guiding insights, which unarguably improved the paper.

AUTHORS' CONTRIBUTIONS

RvdR, AT, and PS conceived the initial ideas and methodology, with significant contributions from CS, KS and PV at later stages; KS, RvdR and CS collected the data; KS analysed the data; KS, AT, PS, PV and RvdR interpreted the analysis; KS led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

DATA ACCESSIBILITY

Data are available via Figshare https://doi.org/10.4225/49/5940d4fb22c26 (Soanes et, al. 2017)

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SUPPORTING INFORMATION

Additional supporting information is available with the online version of this article.

Table 1. The number of squirrel gliders captured at each location before and after mitigation. Site code abbreviations in parentheses.

Site	Location	Before	After
Impact: Crossing structure (*n*=4 sites)			
Longwood (LON)	East	10	8
	West	5	6
Balmattum (BAL)	East	2	8
	West	0	6
Violet Town (VTN)	East	9	4
	West	9	8
Warrenbayne (WRB)	East	3	5
	West	6	6
Control 1: Unmitigated freeway (*n*=2 sites)			
RR 169 (169)	East	20	18
	West	11	7
RR 182 (182)	East	5	3
	West	5	6
Control 2: Vegetated median (*n*=4 sites)			
RR 121 (121)	East	15	8
	West	3	10
RR 122 (122)	East	10	6
	West	6	5
Alexandersons (ALX)	East	40	12
	West	21	6

RR 142 (142)	East	10	4
	West	8	5
Control 3: Non-freeway (*n*=3 sites)			
Geodetic (GEO)	East	7	5
	West	12	11
Angle-Pranjip (A-P)	East	4	1
	West	1	7
Moglonemby Hall (MOH)	East	6	3
	West	3	0

Table 2. Spatial genetic structure due to the freeway within each site and treatment type. *Q*-values indicate the proportional membership of individuals within each sampling location to one of two genetic clusters (analysed at K=2). R^2–values indicate the strength of the effect of the freeway on spatial genetic structure. Bold = P-value <0.0001.

			Before		**After**	
	Site sampling location	Code	Q	R^2	Q	R^2
Impact: Crossing structures	Longwood East	LON	0.07	**0.83**	0.82	0.02
	Longwood West		0.81		0.75	
	Balmattum East	BAL	0.57	NA	0.60	0.22
	Balmattum West		-		0.91	
	Violet Town East	VTN	0.07	0.62	0.12	0.37
	Violet Town West		0.65		0.73	
	Warrenbayne East	WRB	0.06	0.12	0.41	0.11
	Warrenbayne West		0.25		0.61	
Control 1: Unmitigated	RR 169 East	169	0.64	0.11	0.65	0.04
	RR 169 West		0.36		0.72	
	RR 182 East	182	0.06	0.44	0.14	0.10
	RR 182 West		0.10		0.07	
Control 2: Vegetated medians	RR 121 East	121	0.52	0.10	0.53	0.00
	RR 121 West		0.55		0.54	
	RR 122 East	122	0.52	0.31	0.54	0.73
	RR 122 West		0.46		0.42	
	Alexandersons East	ALX	0.48	0.04	0.51	0.14
	Alexandersons West		0.50		0.47	
	RR 142 East	142	0.22	0.16	0.09	0.43

	RR 142 West		0.50		0.50	
Control 3: Non-freeway	Geodetic East	GEO	0.71	0.09	0.52	0.01
	Geodetic West		0.88		0.62	
	Moglonemby Hall East	MOH	0.16	0.16	0.11	NA
	Moglonemby Hall West		0.09		-	

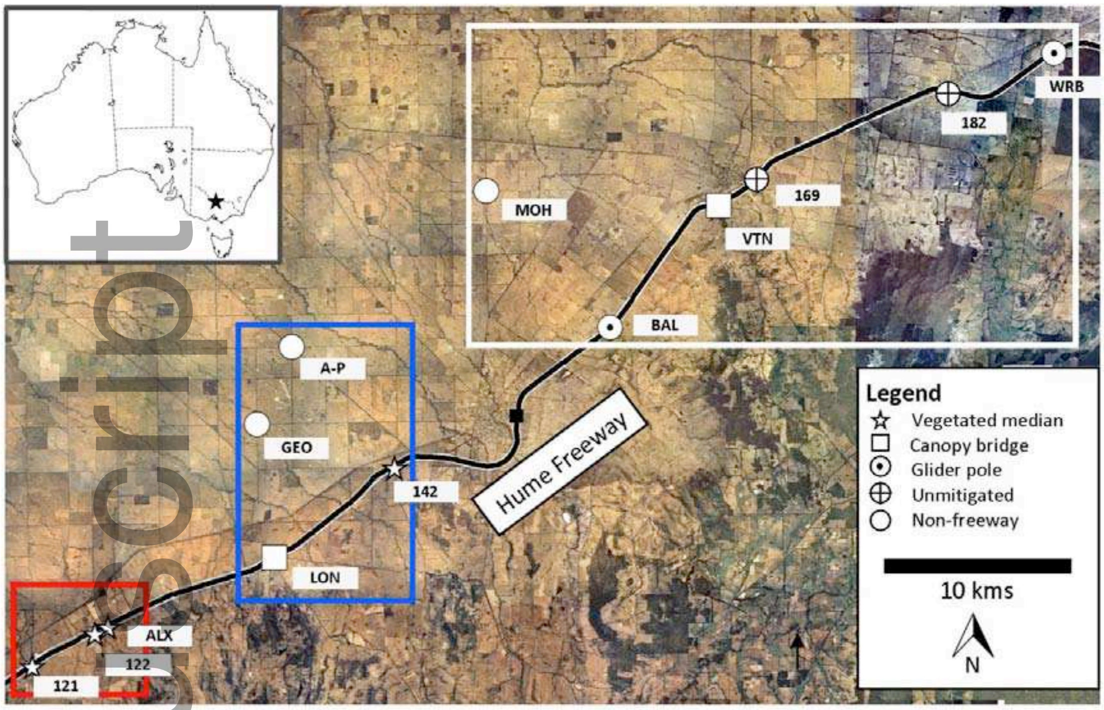


Figure 1. Map of study sites along the Hume Freeway, southeast Australia. The black square indicates the town of Euroa. Sites were grouped into the Southern (red), Central (blue) and Northern (white) geographic regions for sub-analysis. Site codes are provided in Table 1. (Source of satellite image: DSE, 2011).

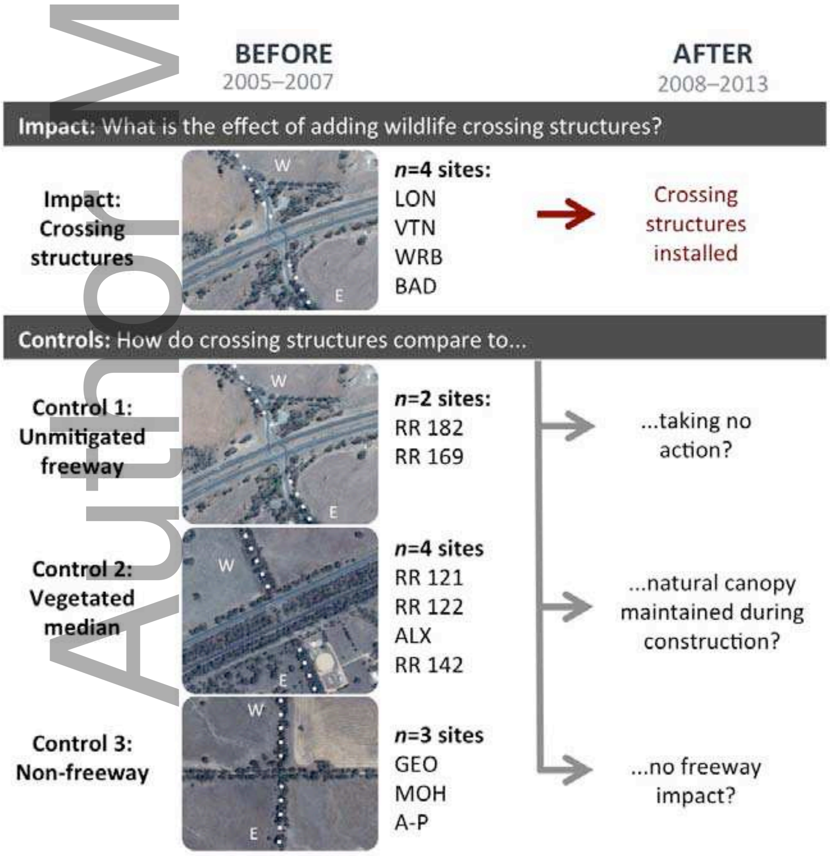


Figure 2. Experimental design used to evaluate the effectiveness of wildlife crossing structures

installed across the Hume Freeway. The layout of survey transects (dotted lines) including east (E) and west (W) sampling locations is shown as an example within each treatment type. Site codes are provided in Table 1.

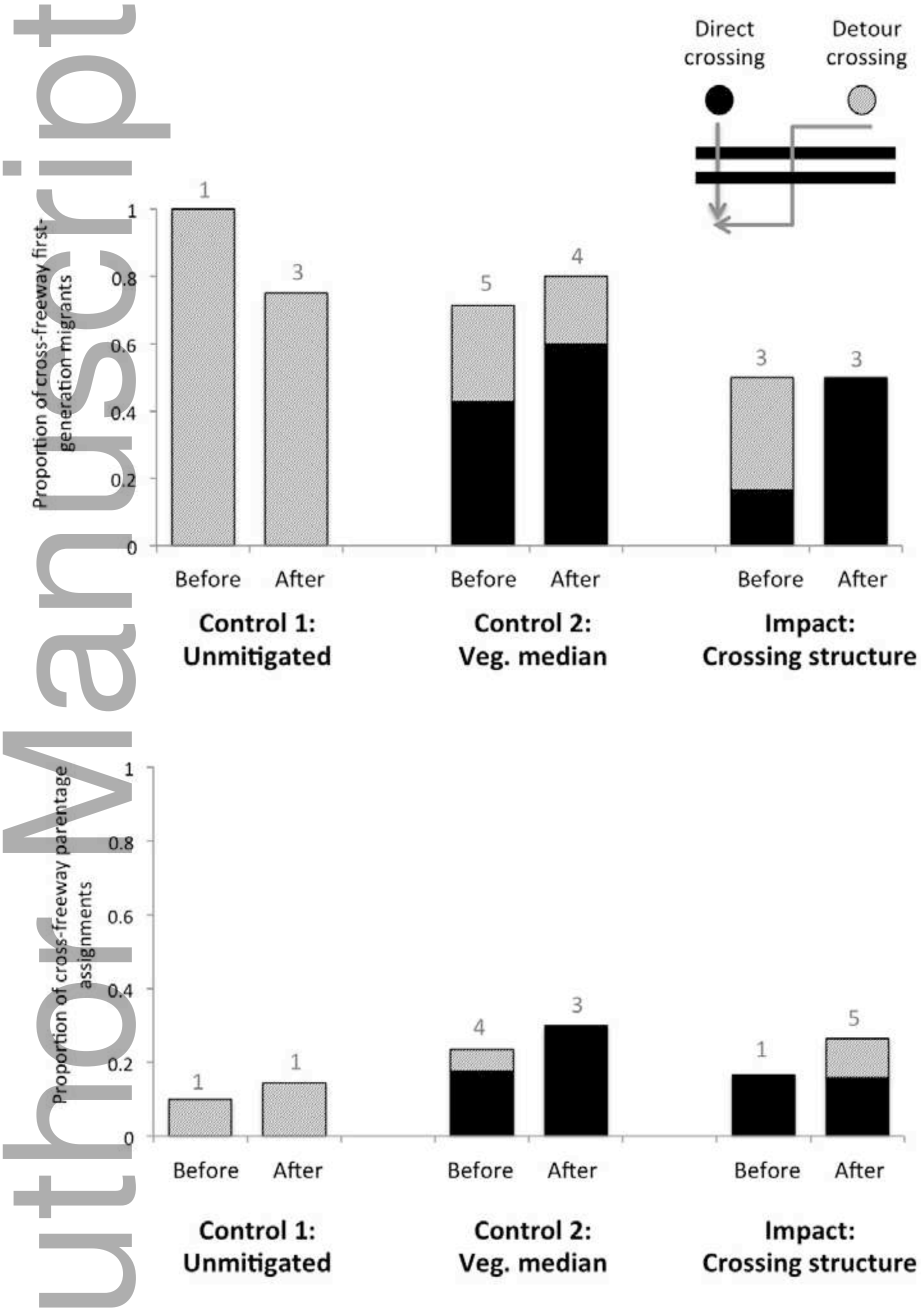
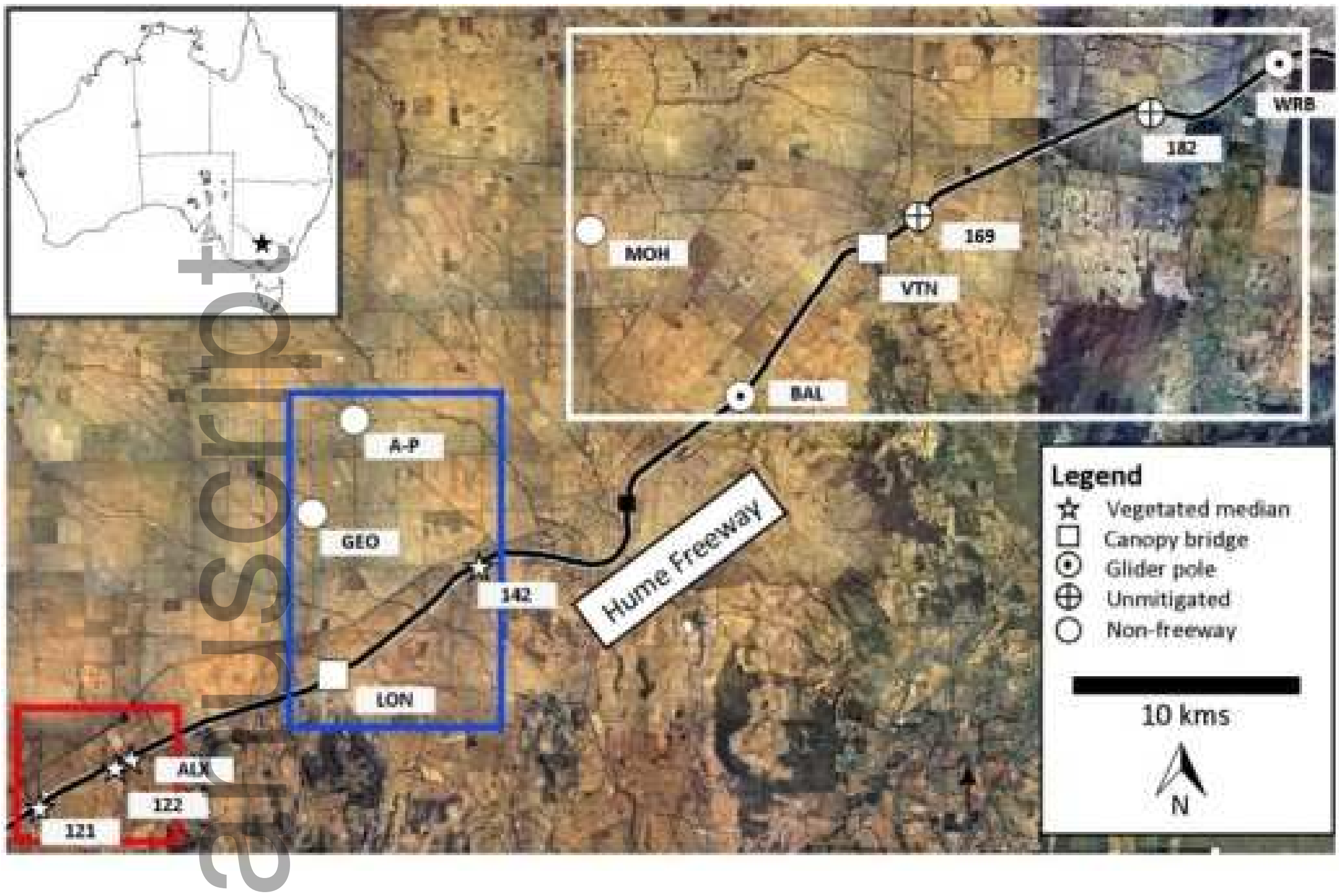


Figure 3. The proportion of first-generation migrants (top) and cross-freeway parentage assignments (bottom) that originated directly from the opposite side of the freeway (black) or via a detour from another site on the opposite side of the freeway (hashed), before and after mitigation at

each site type. Numbers above bars indicate the total number of cross-freeway individuals detected for each treatment and period.



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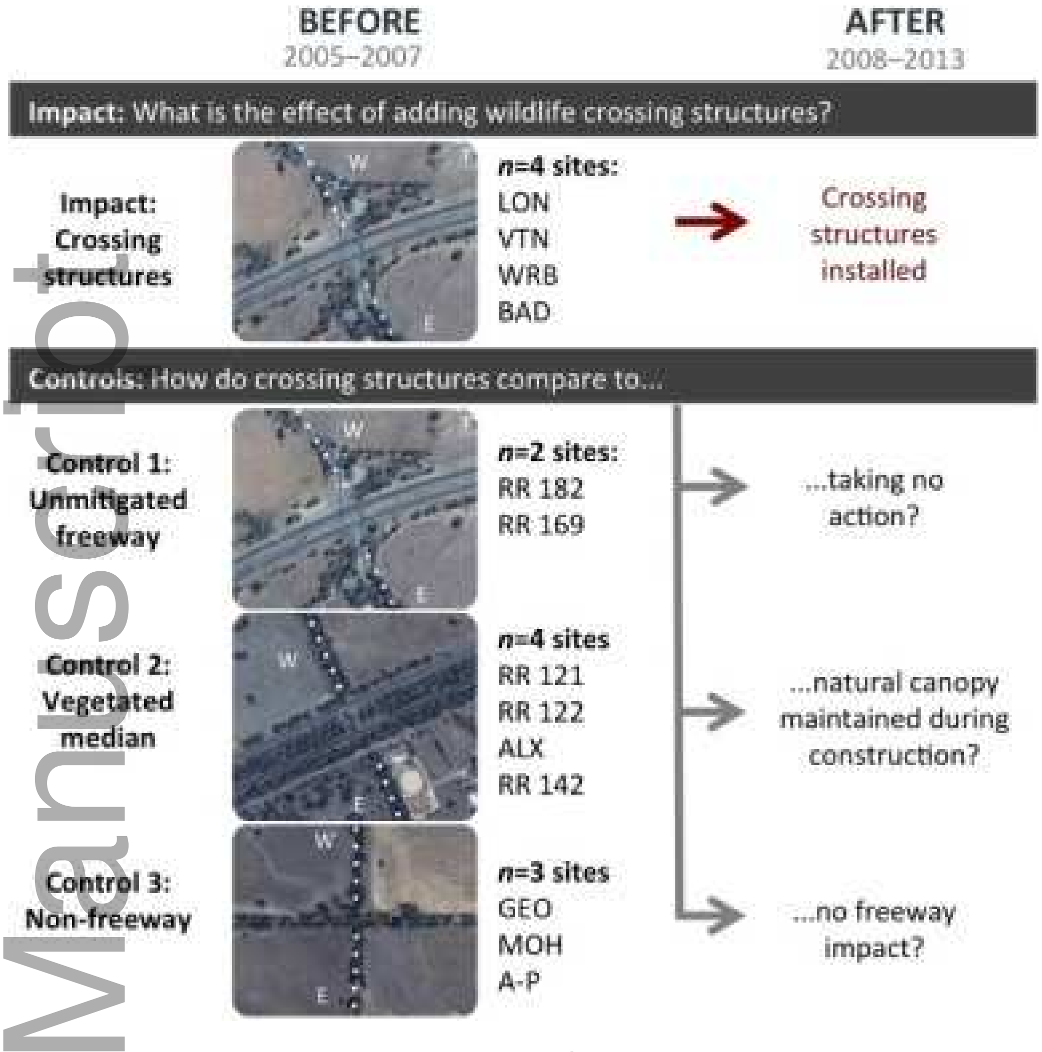

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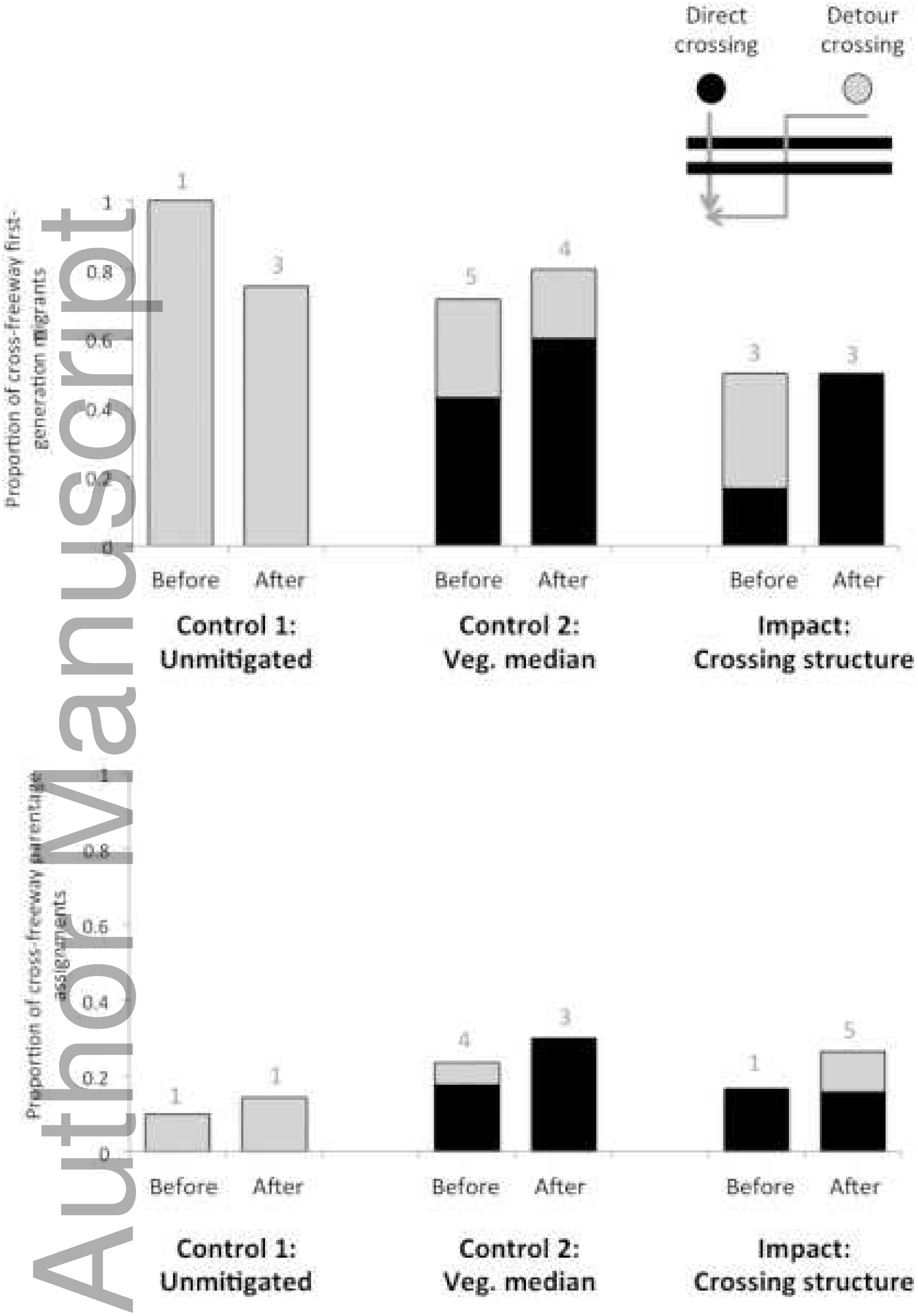

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