Limited genetic differentiation between acoustically divergent populations of urban and rural silvereyes (*Zosterops lateralis*)

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Short title: Urban-rural population genetics of silvereyes

Keywords: urbanization, silvereye, population genetics, *Zosterops lateralis*

Total word count = 3484

Section Word counts: Abstract = 151; Introduction = 814; Methods = 963; Results = 404; Discussion = 973; Ethics/Acknowledgments = 129

Number of References = 48

Number of Tables = 2; Number of Figures = 3

Online Appendices = 2 (Table SI & Figure S1)

Abstract:

The bioacoustic attributes of vocalisations made by birds in urban environments often differ markedly from those of rural conspecifics. Whether such differences are result from genetic divergence between urban and rural populations, or from plasticity or cultural evolution of song remains poorly understood. Silvereyes (*Zosterops lateralis*) show evidence of acoustic adaptation to urban noise, modifying both their songs and calls in cities when compared to rural areas. We investigated whether these differences were associated with corresponding morphological and neutral genetic differences. Across six pairs of geographically separate urban and rural populations, all morphological traits measured were similar. Furthermore, genetic analyses of variation at nine microsatellite loci revealed high levels of genetic connectivity between populations, and similar levels of heterozygosity in both habitat types. Consistent directional shifts in song attributes of city birds across large geographic areas thus do not appear to be accompanied by associated morphological or neutral genetic divergence.

Introduction

Numerous recent studies have demonstrated differences in behaviour and other biological features between populations of animals living in urban and rural habitats (Partecke et al. 2004; Chace and Walsh 2006; Patricelli and Blickley 2006; Partecke and Gwinner 2007; Evans et al. 2012). In particular, changes in vocalizations of urban birds have been observed across the globe (Slabbekoorn and Ripmeester 2008). Although there is evidence that such effects are taxonomically and geographically widespread, there is currently debate about the degree to which this divergence results from plasticity (behavioural responses that are flexible across contexts, (Halfwerk and Slabbekoorn 2009), cultural evolution (non-random transmission of song types across generations; (Cardoso and Atwell 2011), or genetic change (microevolutionary change in song attributes; (Badyaev et al. 2008). Given that the ability to vocalize is directly influenced by morphological structures (Derryberry 2009), it is surprising that so few studies have explicitly examined the relationship between urban vocalizations, morphology and genetics.

Changes in selective pressures as a result of habitat differentiation can promote adaptive phenotypic change in different populations of the same species (Rice 1987; Edelaar and Benkman 2006; Blondel 2007). One profound and increasingly common source of selective pressures is global urbanization, which has produced novel habitat types with specific selective pressures for many species, especially birds. These pressures, including urban noise, can exert selection on both morphological (e.g., size) and behavioural (e.g., song) traits, which may result in urban adaptations and a genetic shift in the population

(Badyaev et al. 2008). For example, food differences between rural and urban habitats may result in modifications of bill structure, with corresponding altered song characteristics in urban birds (Badyaev et al. 2008). Furthermore, sexual selection may act on morphological and/or song features, and may result in local adaptations and preferences that can facilitate population divergence (Ripmeester et al. 2010).

Findings from studies investigating genetic or morphological differentiation between urban and rural populations have been inconsistent. Badyaev et al's (2008) study found evidence for a small but significant genetic differentiation in neutral loci between urban and rural populations of house finches (*Carpodacus mexicanus)* which paralleled bill and song differences. However, another study using both mitochondrial DNA and microsatellites found no genetic differences between urban and rural populations of the orange-tufted sunbird (*Nectarinia osea*), despite unique song dialects associated with each habitat (Leader et al. 2008). Partecke et al (2004) reported a similar trend using AFLP analysis in urban and rural European blackbirds (*Turdus merula*), which were not genetically distinct, yet demonstrated decreased genetic diversity in urban areas (Evans et al. 2009) along with differences in laying and migratory behaviour (Partecke et al. 2006; Evans et al. 2012). Thus, although differences in multiple behavioural traits between urban and rural populations have been identified, evidence for genetic divergence has been equivocal.

Two other processes could contribute to phenotypic differences in song between urban and rural populations. Firstly, observed song differences may represent flexible responses by birds to increase transmission of a song through noise, either through frequency shifts (Halfwerk and Slabbekoorn 2009) or amplitude shifts (Nemeth and Brumm 2010).

Alternatively, songs may diverge through selective cultural transmission of particular song types or 'memes' from one generation to the next, or from modification of key attributes (e.g. minimum frequency) of those song types (Luther and Baptista 2010; Cardoso and Atwell 2011).

Cardoso and Atwell (2011) recently demonstrated that cultural modification of song types explained about 56% of the overall magnitude of population divergence in minimum frequency of songs between urban and rural populations of dark-eyed juncos (*Junco hyemalis)*. Selective cultural transmission may also arise from copying errors, for instance if parts of the source song are masked by high amplitude background noise. Furthermore, geographic (and hence genetic) isolation and low dispersal can maintain differences between populations, although these are not necessarily required for cultural evolution to occur (Yoktan et al. 2011). Cultural evolution, therefore, can act separately from, or in tandem with genetic divergence. Moreover, song differences may even promote or maintain genetic divergence between populations through assortative mating (MacDougall-Shackleton and MacDougall-Shackleton 2001; Slabbekoorn and Smith 2002b; Patten et al. 2004).

We have demonstrated elsewhere (Potvin et al. 2011) that silvereyes (*Zosterops lateralis)* in cities across Australia have both altered songs and contact calls, compared to rural counterparts. In oscine birds, songs are typically learned, whereas calls are assumed to be innate and less flexible (Marler 2004; Podos et al. 2004). One hypothesis that could account for the finding that apparently innate, as well as learned vocalizations differ in urban populations is that they have become genetically or morphologically distinct from rural populations. If so, populations in close geographic proximity but differing in habitat

type should show evidence of genetic divergence (Badyaev et al. 2008), and possibly decreased genetic diversity (Evans et al. 2009). Here, we examine morphological and neutral genetic differences between city and rural silvereye populations across eastern Australia to evaluate this possibility.

Methods

Study Species

The silvereye is an abundant, small, native sexually monomorphic Australian passerine with resident breeding populations in almost every major city. Urban silvereyes sing and call at a higher frequency and at a slower rate than those in matched rural areas across the continent (Potvin et al. 2011). Silvereyes on isolated islands around the Pacific also exhibit differing morphologies, probably as a result of local adaptations, rather than genetic drift (Clegg et al. 2002).

Field Methods

Field work was conducted between September 2009 and February 2010 during the silvereye breeding season. Sites were located in urban and rural areas, paired in the following geographic regions around Australia (Figure 1): Melbourne (latitude: -37.8, longitude: 144.9), Adelaide (-34.9, 138.6), Sydney (-33.9, 151.2), Brisbane (-27.5, 153.0), Hobart (-42.9, 147.3) and Canberra (-35.3, 149.1). Each rural site was within 150km of its corresponding urban site. Urban sites were all located within cities, surrounded by major roads and major buildings within 100m, whereas rural sites were located away from roads and all consisted of undisturbed native, local habitat (eucalypt

forest). Sound levels of sites are reported in Potvin *et al.* 2011. At each site, we caught between 15 and 20 silvereyes in mistnets over the course of two to eight days. Each captured individual was fitted with an ABBBS (Australian Bird and Bat Banding Scheme) aluminium numbered band (Sample sizes in Table 1). We weighed individuals to the nearest 0.1g using a spring balance, and measured their head-bill lengths (base of skull to bill tip), wing lengths (maximum chord), tarsus lengths (minimum tarsus) and tail lengths to the nearest 0.1mm using a caliper. A small (30µl) blood sample was taken from the brachial vein for genetic analysis before release. This work was done at the same locations concurrently with work previously described on song and call adaptations (Potvin et al. 2011).

Laboratory methods

We extracted DNA from blood samples from 204 adults using an ammonium acetate-based protocol to salt out proteins, modified from (Laitinen et al. 1994). Small samples were extracted with DNeasy blood and tissue kits (QIAGEN). We then genotyped 204 individuals at nine highly variable (5 – 10 alleles per locus) microsatellite loci: ZL12, ZL18, ZL22, ZL35, ZL38 (Degnan et al. 1999; Frentiu et al. 2003); ZL41, ZL46, ZL49, and ZL54 (Frentiu et al. 2003). Genotyping conditions were as follows: PCR was conducted in a total volume of 10 µL and included 25 mM $MgCl_2$, 2 µM dNTPs, 1 µM of forward primer, 10 µM of reverse primer, M13-Dye (D2, D3 or D4) (Sigma-Genosys), 5 U *Taq* polymerase and approximately 10ng of genomic DNA. Cycling conditions included an initial step of 3min at 94^{o}C, followed by 34 cycles of 30 s at 94^{o}C, 30 s at the annealing temperature, and 30 s at 72^{o}C, plus a final step of 4 min at 72^{o}C. Annealing

temperatures were 53^{o}C for ZL41, 55^{o}C for ZL38, ZL12, ZL18 and ZL46, 60^{o}C for ZL49 and ZL35, and 63^{o}C for ZL54. PCR products were analyzed on a Beckman-Coulter CEQ 8000. Ten percent of the samples were repeat-analyzed to ensure accuracy of the genotyping and results. All of these genotypes were identical to the originals. We also sexed individuals genetically, using the method of (Griffiths et al. 1998).

Statistical analysis

We used Bayesian linear regression in *OpenBUGS* (Meirmans and Van Tienderen 2004; Spiegelhalter 2006; McCarthy 2007) to analyse differences in morphological features between urban and rural populations. This analysis contained no prior information and therefore is equivalent to a General linear mixed model (GLMM) with two random effects. Our model used morphological features (mass, wing length, tarsus length, head-bill length) as the response variable and habitat type (urban or rural) as the explanatory variable. The models included uninformative priors to reflect an absence of prior information, and random effects for site (1-12) and geographic location (1-6). We discarded the first 100,000 samples as a burn-in and checked for convergence, then used the 200,000 samples from the posterior distribution. We calculated the mean and standard deviation of the posterior distributions of the model parameters, along with the 2.5th and 97.5th percentiles to represent the 95% credible interval around the predicted relationship. In order to test structural size in relation to body mass, we also re-ran the models to test for effect of habitat type on each structural variable (wing length, tarsus length and head-bill length) divided by mass of each individual. Finally, we ran tests to analyze the effect of sex (predictive variable) on all morphometric variables.

Population genetic analysis

We used the program GenoDive (Meirmans and Van Tienderen 2004) to calculate the inbreeding coefficient for each population after testing for linkage disequilibrium and Hardy-Weinberg equilibrium. We tested whether inbreeding and homozygosity levels differed between urban and rural populations by conducting a one-way ANOVA with a reference class in OpenBUGS using uninformative priors and the same conditions as above. We also used both GenoDive and GenePop 4.0 (Raymond and Rousset 1995; Rousset 2008) to determine F_{st} values and associated p-values for differentiation for all population pairs. We first ran a comparison between geographic locations (which included both urban and rural populations for each location, therefore comparing six geographically local populations to one another), then ran a second pairwise analysis including all twelve sampled populations. Genodive calculates standardized F_{st} values using AMOVA (Meirmans 2006) to account for within-population variability. We included the second program – GenePop - in order to subsequently perform an isolation-by-distance correlation analysis using geographic distances (from latitude and longitude coordinates). In order to test the effect of habitat type on genetic differentiation of the individual populations while controlling for geographic distance, we also performed a Mantel multivariate autocorrelation analysis on the raw genetic data from the 12 populations grouped by geographic area using the software Arlequin 3.0 (Excoffier et al. 2005).

It has recently been argued that p-values are of debatable value in assessing the magnitude of population differentiation (Jost 2009). We therefore adopted a conservative approach in evaluated differentiation, assessing the magnitude of F_{st} from the effect size

once statistical significance was established. Differentiation was considered low if F_{st} was in the range of 0 – 0.05, moderate if 0.05 – 0.15, high if 0.15 – 0.25 and very high if greater than 0.25 (Balloux and Lugon-Moulin 2002).

Results

Morphology

Effect sizes and 95% credible intervals (all encompassing zero) for the Bayesian analyses indicated that all morphological features including mass, wing length, tarsus length and head-bill length, were similar between urban and rural individuals regardless of geographic location (Fig. 1; model coefficients are reported in the supporting material Table S1, graphs showing results for each geographic area in Figure S1). Results from structural size comparisons were also similar between habitats when controlling for body mass (Table S1). Sex was not an important predictor of any morphological variable (effect sizes were very close to zero and 95% credible intervals all encompassed zero).

Microsatellite variation

GenoDive revealed no significant deviation from Hardy-Weinberg equilibrium in loci, nor linkage disequilibrium among loci (all $p > 0.05$, consistent with the published descriptions of these loci; (Degnan et al. 1999; Frentiu et al. 2003). Observed heterozygosities were significantly lower than expected heterozygosities for all populations, however, whether grouped by location or by location and habitat type (Table 1). These calculated figures differed negligibly between the two programs. Comparing inbreeding coefficients between urban and rural populations revealed no differences

between the two habitat types (mean difference in G_{is} = 0.01926, 95% CI = -0.04638, 0.08524; Fig. 2).

F_{st} values calculated by both GenoDive and GenePop revealed generally low levels of differentiation (Genodive results in Table 2). The only geographic area showing a significant, high level of differentiation from any other area was Adelaide (Table 2a): with the Adelaide rural population being the most highly differentiated from all others (Table 2b). All of the F_{st} values for the urban-rural pairs of populations were low and most were also non-significant (Table 2b). GenePop revealed a very small positive correlation of isolation by distance (ρ = 0.0077, CI = 0.003, 0.015; Fig. 3). Autocorrelation analysis of raw genotype data in Arlequin revealed that the genetic distance between two populations was unassociated with habitat type when accounting for geographic distance (r = - 0.001, p = 0.73).

Discussion

We found little evidence that consistent directional shifts in songs and call attributes of urban silvereye populations (Potvin et al. 2011) are associated with systematic morphological or neutral genetic differentiation between nearby rural and urban populations. Given that we sampled only neutral loci, and a fraction of the genome, we cannot rule out the possibility that habitat-specific selection has affected other, unsampled coding regions of the genome. Nevertheless, our data are not consistent with an expectation of predictable genetic differentiation between populations in relation to habitat type. Coupled with recent findings that song shifts in some species result from flexible adaptive responses (e.g. frequency or amplitude shifts - (Halfwerk and

Slabbekoorn 2009) (Nemeth and Brumm 2010) or cultural transmission of particular song types or 'memes' from one generation to the next (Luther and Baptista 2010; Cardoso and Atwell 2011) our results suggest that directional song changes in city habitats may not be associated with corresponding microevolutionary changes.

Morphology

Urban and rural silvereyes around Australia were morphologically similar. In particular, despite differences in song structure (Potvin et al. 2011) we found no differences in head-bill length between city and rural birds. Differences in other, dimensions of bill morphology (e.g. bill depth or width) seem unlikely because silvereyes are mainly frugivorous (Higgins 2006), and food sources tend to be consistent between rural and urban habitats in similar geographic areas, so diet-related selection on bill morphology is unlikely to vary. In the absence of obvious differences in bill size, song differences in silvereyes may be due to other structural changes, for instance to beak gape (Podos et al. 2004), song apparatus (Riede et al. 2006), physiological changes (Cynx et al. 2005), or they may be completely unrelated to morphological structure, as in dialect formation (Slabbekoorn and Smith 2002a).

Genetics

Urban and rural populations showed similar inbreeding coefficients, and in all populations, observed heterozygosities were lower than expected. Silvereye flocks in the breeding season often contain a high percentage of young (first-year) related, non-nesting individuals and dispersal between flocks is generally low, regardless of habitat type (Catterall et al. 1982; Kikkawa and Wilson 1983; Kikkawa 1987, pers. obs.), and this

may have deflated our estimates of H_0. Contrary to house sparrows (Vangestel et al 2011), urban habitats also did not seem to promote higher inbreeding or lower dispersal rates in silvereyes. Silvereyes are partial migrants, and have shown a strong ability to move across various landscapes (Chan and Kikkawa 1997). It therefore seems implausible that dispersal of such a mobile species would be restricted by habitat type, though differentiation of song and call repertoires could limit gene flow between populations.

F_{st} analyses revealed that urban populations are not subject to higher rates of genetic population divergence from nearby rural populations than would be expected based on the distance between them. F_{st} values were all low between city and urban population pairs, indicating little genetic differentiation (Table 2). The relative values of F_{st} between urban-population pairs were much lower than the average values for all pairs, and for most populations, the F_{st} value corresponding to the paired population was lower than that for any other population. There were two exceptions to this: both Canberra and Hobart rural populations were more closely related to populations other than their urban counterparts. For instance, the Hobart rural population was more closely related to the Melbourne populations (urban and rural) than the corresponding Hobart urban population. There are several possible explanations for this pattern. Silvereyes are partially migratory (Chan and Kikkawa 1997), with some individuals breeding in Tasmania and flying northward during winter. Although we sampled breeding populations, urban Tasmanian birds may be more sedentary than rural birds, as has been found in urban European blackbirds (Partecke and Gwinner 2007; Evans et al. 2012). If so, gene flow between rural Hobart populations and mainland populations may be greater

than between urban and rural Hobart populations. Alternatively, this pattern could result from genetic drift, especially if effective population sizes are small and heterozygosity is low. Finally, apparent genetic similarity between some population pairs could be due to size homoplasy among microsatellite alleles, rather than indicating gene flow.

Nevertheless, in all population pairs, F_{st} suggested relatively high gene flow between urban and rural areas. In contrast to a recent study on blackbirds (Evans et al. 2009), we found that urban populations were not consistently more highly differentiated from each other than from rural populations, suggesting high levels of gene flow between all populations in this species. In addition, F_{st} was only weakly correlated with geographic distance. Since rural and urban paired sites were less than 150km apart, distance was not likely to have been an important factor in population differentiation. Although two rural populations (Canberra and Hobart) appeared to be slightly more connected with populations other than their urban counterparts, the lack of consistency of this finding indicates that urban habitat is not a reliable predictor of neutral genetic divergence. Further supporting evidence for this conclusion was the autocorrelation analysis controlling for geographic distance, which importantly, also showed no effect of habitat type on genetic divergence within groups.

Our results indicate that song differences in urban silvereyes are not associated with basic morphological changes or consistent shifts in neutral genetic markers. However, if urban adaptation is a recent phenomenon, genetic differentiation between city and rural populations may take more time to manifest, and detecting functional changes using microsatellite (neutral) markers may be difficult (DiBattista 2008). Nevertheless, it seems more likely that differences in vocalisations result from vocal plasticity, cultural

evolution or physiological effects of noise on song development, rather than genetic isolation. This is due to the absence of important genetic or morphological differences between matched urban and rural populations over a large geographic area, and the finding that urbanization does not seem to affect natural levels of gene flow in this species.

Ethical note

Procedures were undertaken with the approval of the following agencies: Animal Ethics Committee at the University of Melbourne, Director-General's Animal Care and Ethics Committee at the NSW Department of Primary Industries, and Wildlife Ethics Committee at the SA Department for Environment and Heritage.

Acknowledgments:

We thank three anonymous reviewers for helpful comments improving the manuscript. We thank J. Kruckel for field assistance, G. Fry, A. Leishman, D. Paton, D. Williams, E. Woehler, R. Fuller, and A. Fletcher for assistance in locating and banding, the Australian National Botanic Gardens (ACT), Namadgi National Park (ACT), Glenorchy City Council (Tas), Brisbane City Council (Qld), Munghorn Gap Nature Reserve (NSW), Kogarah City Council (NSW), Darebin City Council (Vic) and Darebin Parklands Association (Vic) for permission to conduct work on their lands.

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Table 1. Observed and expected heterozygosities and inbreeding coefficients (G_{is}) as calculated by Genodive for all locations, first including both rural and urban populations together, then each urban and rural population separately. Sample sizes for each location are shown in parentheses.

Location	**H_o**	**H_e**	**G_{is}**	**Habitat**	**H_o**	**H_e**	**G_{is}**
Canberra	0.555	0.747	0.258	Urban (16)	0.569	0.736	0.227
				Rural (17)	0.573	0.785	0.270
Sydney	0.589	0.695	0.153	Urban (16)	0.570	0.675	0.155
				Rural (15)	0.615	0.733	0.160
Brisbane	0.560	0.692	0.191	Urban (16)	0.635	0.701	0.094
				Rural (15)	0.658	0.771	0.146
Adelaide	0.506	0.599	0.155	Urban (20)	0.501	0.584	0.143
				Rural (20)	0.520	0.672	0.225
Hobart	0.647	0.791	0.181	Urban (19)	0.509	0.641	0.207
				Rural (17)	0.693	0.842	0.177
Melbourne	0.586	0.708	0.173	Urban (18)	0.582	0.746	0.220
				Rural (15)	0.577	0.707	0.183

Table 2. F_{st} values for pairwise analysis of population differentiation for all populations sampled as defined by a) location (urban and rural populations are combined for each of six locations) including p-values and b) site (12 urban and rural populations sampled separately). Shaded cells indicate differentiation values between urban-rural population pairs in the same geographic location. Asterisked F_{st} values (*) are significantly different from zero at $p < 0.05$.

a)

Population	Canberra	Sydney	Brisbane	Adelaide	Hobart
Sydney	0.004				
Brisbane	0.003	0.03*			
Adelaide	0.13*	0.17*	0.16*		
Hobart	0.03*	0.048*	0.06*	0.101*	
Melbourne	0.039*	0.064*	0.06*	0.098*	0.025*

b)

Pop	Can U	Can R	Syd U	Syd R	Bri U	Bri R	Ade U	Ade R	Hob U	Hob R	Mel U
Can R	0.043*										
Syd U	0.022*	0.034*									
Syd R	0.029*	0.022	0.0003								
Bri U	0.040*	0.053*	0.032*	0.044*							
Bri R	0.026*	0.012*	0.018*	0.010*	0.021						
Ade U	0.134*	0.081*	0.166*	0.135*	0.155*	0.159*					
Ade R	0.163	0.121*	0.199*	0.165	0.183	0.183	0.006				

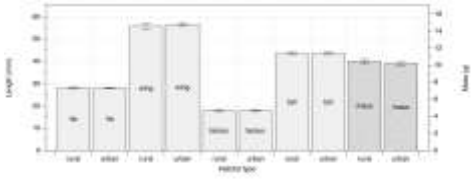
R	*			*	*	*	*				
HobU	0.071*	0.041*	0.083*	0.054*	0.091*	0.087*	0.092*	0.115*			
HobR	0.056*	0.025*	0.059*	0.037*	0.066*	0.049*	0.064*	0.098*	0.042*		
MelU	0.046*	0.029*	0.063*	0.037*	0.068*	0.065*	0.068*	0.092*	0.050*	0.010	
Mel R	0.048*	0.032*	0.059*	0.044*	0.072*	0.068*	0.065*	0.106*	0.055*	0.003	0.009

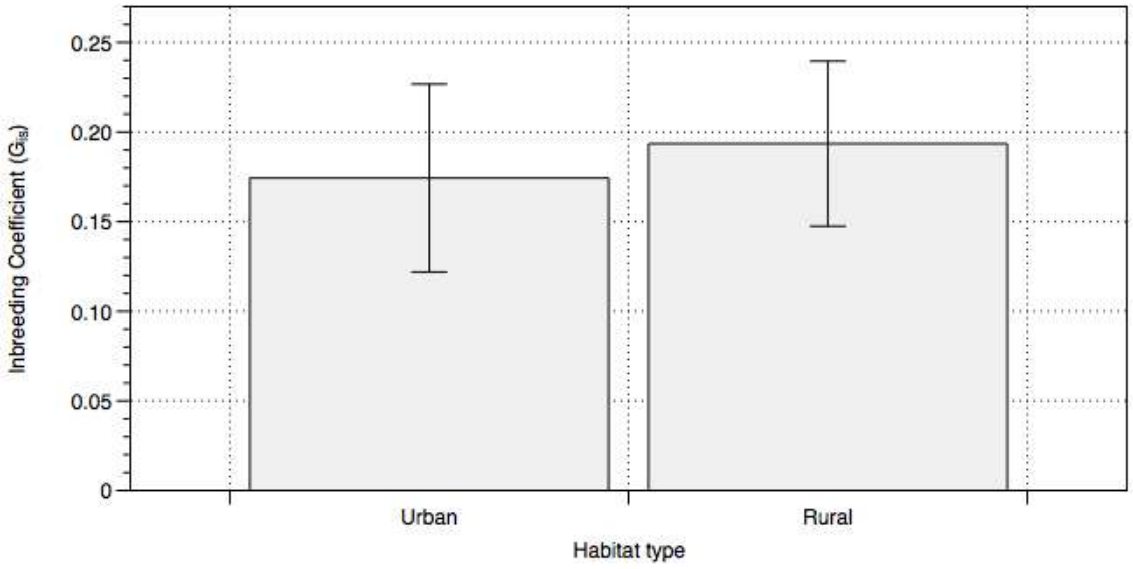
b)

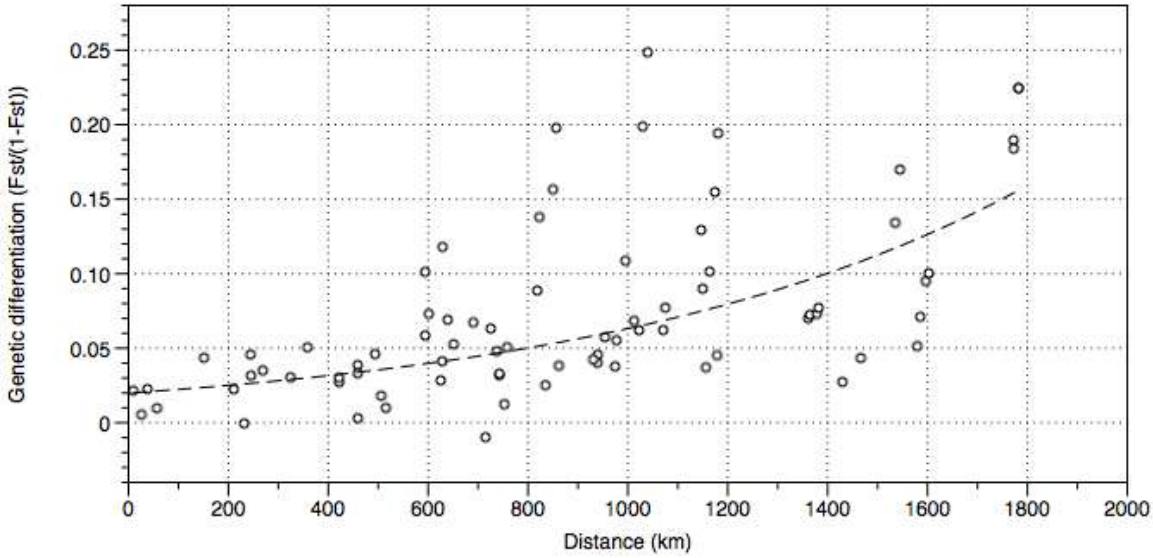
Figure 1. Mean measurements of head-bill length, wing length, tarsus length, tail length and mass of rural and urban silvereyes. Error bars represent standard deviation.

Figure 2. Mean inbreeding coefficients (G_{is}) of urban and rural populations of silvereyes. Bars represent standard deviations.

Figure 3. Genetic differentiation ($F_{st}/(1-F_{st})$ between all pairs of populations (twelve populations compared with each of the other eleven populations) in the study plotted against distance between sites in km.




Inbreeding Coefficient (G_{is})
0.25
0.20
0.15
0.10
0.05
0
Urban
Rural
Habitat type


Genetic differentiation (Fst/(1-Fst))
0.25
0.20
0.15
0.10
0.05
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Distance (km)