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# Science

## POPULATION GENETICS, DEMOGRAPHY AND POPULATION VIABILITY OF LITTLE PENGUINS (*EUDYPTULA MINOR*) IN AUSTRALIA

A thesis submitted for the degree of

Doctor of Philosophy

By

Sandra Vardeh

School of Biological, Earth and Environmental Sciences,  
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Evolution and Ecology Research Centre

April 2015

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## Title: Population genetics, demography and population viability of little penguins (*Eudyptula minor*) in Australia

**Abstract 350 words maximum: (PLEASE TYPE)**

One of Australia's most iconic native bird species, the little penguin (*Eudyptula minor*), is in decline at numerous locations due to manifold anthropogenic impacts including predation by introduced predators, habitat destruction and modification, effects of fishing activity and climate change. The fundamental aim of this thesis was to combine genetic and demographic data about the penguins to forecast likely population changes under a range of possible scenarios, including evaluation of anthropogenic threats. To target this aim, four separate studies were undertaken. The first collated demographic data on population size and survival rates in New South Wales (NSW), using data from existing databases and new surveys. Non-invasive techniques were trialled to estimate population size and survival rates, with the latter resulting in estimates consistent with mark-recapture based approaches. The second study investigated size and connectivity of little penguin populations in Australia using traditional measures of genetic structure and diversity. Smaller colonies were genetically not less diverse than larger ones and differentiation was limited among colonies in NSW. Isolation by distance was not observed within NSW and weak at longer distances between different states. Complex interactions between the influence of oceanic currents and demography were furthermore suggested by contrasting dispersal patterns at different scales and using different genetic markers. The third study was based on an adaptive genetic marker of the immune system (Major Histocompatibility Complex, MHC) to complement the aforementioned population genetic study that was based on neutral genes. Genetic diversity at the MHC locus largely mirrors diversity at neutral loci, indicating a strong effect of stochasticity. Nevertheless, selective pressures are acting on the MHC. The lowest rates of parasite infestation were suspected for individuals of intermediate MHC diversity, and penguins showed differential mortality or inbreeding avoidance at the MHC locus. Results from the first two studies were incorporated into a population viability analysis model to evaluate outcomes of different scenarios and management strategies in the fourth and last study conducted as part of this thesis. All models based on current estimates predicted severe population declines in NSW and Western Australia, and forecasts were particularly sensitive to changes of mortality estimates.

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## Table of Contents

<b>List of Figures .....</b>	VII
<b>List of Tables .....</b>	XI
<b>Acknowledgements .....</b>	XVII
<b>Thesis abstract .....</b>	1
<b>Chapter 1: Introduction / Review of the status of the Little Penguin (<i>Eudyptula minor</i>).....</b>	3
1.1. Biology of the little penguin .....	6
1.2. Penguin distribution and population trends in New South Wales .....	7
1.3. Connectivity and dispersal.....	11
1.4. Rationale of the study.....	12
1.5. Thesis overview.....	13
References.....	17
Appendix 1 .....	20
<b>Chapter 2: Identifying a decline of flightless, burrow-nesting seabirds using non-invasive methods to replace mark-recapture approaches for estimating population size and survival rates .....</b>	35
Introduction .....	39
Methods .....	48
Results .....	65
Discussion.....	77
Acknowledgements.....	82
References.....	83
Appendix 2 .....	88
<b>Chapter 3: Population genetics of Little Penguins (<i>Eudyptula minor</i>) in Australia ...</b>	89
Abstract .....	91
Introduction .....	93
Materials and Methods.....	98
Results .....	107
Discussion.....	120
Acknowledgements.....	124
Conflict of Interest .....	124
References.....	125
Appendix 3 .....	131

<b>Chapter 4:</b> Patterns of Major Histocompatibility Complex diversity, parasitism and mate choice in Little Penguins ( <i>Eudyptula minor</i> ) .....	153
Introduction .....	157
Materials and methods .....	162
Results .....	173
Discussion.....	184
Acknowledgements.....	189
References.....	190
Appendix 4 .....	196
<b>Chapter 5:</b> Viability of little penguin ( <i>Eudyptula minor</i> ) populations at their northern range edge .....	201
Summary .....	205
Introduction .....	205
Method.....	210
Results .....	224
Discussion.....	242
Acknowledgements.....	245
References.....	247
Appendix 5 .....	252
<b>Chapter 6:</b> Discussion / Final conclusions and recommendations for research and management of little penguins ( <i>Eudyptula minor</i> ).....	267
Main Findings .....	269
Management recommendations .....	273
Further research.....	276
References.....	278

## **List of Figures**

### **Chapter 1**

Fig. 1: Map of known penguin colonies in Australia .....	8
--	---

### **Chapter 2**

Fig. 1: Map of all known penguin colonies in Australia .....	48
Fig. 2: Map of Lion Island. ....	50
Fig. 3: Map of Bowen Island. ....	50
Fig. 4: Beach counts of penguins arriving on Penguin Beach, Bowen Island.....	66
Fig. 5: Beach counts of penguins arriving on the main beach, Lion Island .....	67
Fig. 6: Estimates of the size of the population using Penguin Beach, Bowen Island.....	69
Fig. 7: Estimates of the size of the population using Lion Island's main beach.....	71

### **Chapter 3**

Fig. 1: Map of all known penguin colonies in Australia with sampling locations. ....	98
Fig. 2: STRUCTURE bar plot of microsatellite genotypes .....	111
Fig. 3: Isolation by distance at microsatellite loci, NSW only .....	112
Fig. 4: Isolation by distance at microsatellite loci, all locations.....	112
Fig. 5: Haplotype network for mitochondrial control region sequences.....	113
Fig. 6: Isolation by distance of mitochondrial haplotypes, NSW only .....	115
Fig. 7: Isolation by distance of mitochondrial haplotypes, all locations.....	116

### *Appendix 3*

Figure 1A: Haplotype network for mitochondrial control region.....	151
--	-----

### **Chapter 4**

Fig. 1: Map of all known penguin colonies in Australia with sampling locations. ....	162
Fig. 2: Number of MHC alleles and average individual parasite loads .....	180
Fig. 3: Average MHC allele sharing value D.....	182

## **Chapter 5**

Fig. 1: Map of known penguin colonies in Australia .....	209
Fig. 2: Forecast of population size, baseline scenario for NSW .....	224
Fig. 3: Forecast of population size, stratification 1 for two populations in WA .....	225
Fig. 4: Forecast of population size, stratification 2 for three populations in WA.....	226
Fig. 5: Sensitivity analysis - reproductive rates for northern NSW .....	227
Fig. 6: Sensitivity analysis - reproductive rates for southern NSW .....	228
Fig. 7: Sensitivity analysis - reproductive rates for WA metapop stratification 1 .....	229
Fig. 8: Sensitivity analysis - reproductive rates for WA metapop stratification 2 .....	230
Fig. 9: Sensitivity analysis - mortality rates for northern NSW .....	231
Fig. 10: Sensitivity analysis - mortality rates for southern NSW.....	232
Fig. 11: Sensitivity analysis - mortality rates for WA metapop stratification 1 .....	234
Fig. 12: Sensitivity analysis - mortality rates for WA metapop stratification 2 .....	236
Fig. 13: Sensitivity analysis - strength of La Niña for NSW metapop .....	237
Fig. 14: Sensitivity analysis - strength of La Niña for WA metapop stratification 2 .....	238
Fig. 15: Sensitivity analysis - La Niña frequencies for NSW metapop.....	239
Fig. 16: Sensitivity analysis - La Niña frequencies for WA metapop stratification 1 ....	240

## *Appendix 5*

Fig. 1A: Sensitivity analysis - reproductive rates for NSW metapop .....	256
Fig. 2A: Sensitivity analysis - reproductive rates for WA pops in stratification 1 .....	257
Fig. 3A: Sensitivity analysis - reproductive rates for WA pops in stratification 2 .....	259
Fig. 4A: Sensitivity analysis - mortality rates for NSW metapopulation .....	259
Fig. 5A: Sensitivity analysis - mortality rates for WA pops under stratification 1 .....	260
Fig. 6A: Sensitivity analysis - mortality rates for WA pops under stratification 2 .....	262
Fig. 7A: Sensitivity analysis - La Niña frequencies for two NSW populations.....	263
Fig. 8A: Sensitivity analysis - La Niña frequencies for WA pops (stratification 1).....	264
Fig. 9A: Sensitivity analysis - La Niña frequencies for WA pops (stratification 2).....	266

## **Chapter 6**

Fig. 1: Map of known penguin colonies in Australia.....	268
---	-----



## **List of Tables**

### **Chapter 1**

Table 1: Historically known breeding places of Little Penguins in New South Wales .....9

#### *Appendix 1*

Table A1: Current information on Australian little penguin breeding colonies..... 20

### **Chapter 2**

Table 1: Timeline of beach counts at Bowen and Lion Islands .....51

Table 2: Demographic methods used to estimate population size and survival rates...52

Table 3: Sampling dates in 2012 to 2014 at Bowen and Lion Islands .....53

Table 4: Mark-recapture/mark-resight population size estimates and methods .....57

Table 5: Model parameters for Cormack-Jolly-Seber Models .....60

Table 6: Model parameters for Robust Design Models .....62

Table 7: Model results for the best six mark-resight models – Bowen Island.....68

Table 8: Estimates of number of penguins using Penguin Beach on Bowen Island .....68

Table 9: Model results for the best six mark-resight models – Lion Island .....70

Table 10: Estimates of number of penguins using the main beach on Lion Island. ....70

Table 11: Estimate of apparent survival based on burrow occupancy.....71

Table 12: Model results for CJS models – Bowen Island .....72

Table 13: Model results for CJS models – Lion Island .....73

Table 14: CJS estimates of apparent survival.....74

Table 15: Model results for robust design models .....75

Table 16: Survival estimates for Bowen and Lion Islands .....76

#### *Appendix 2*

Table 1A: Summary of burrow checks on Lion Island .....88



## **Chapter 3**

Table 1: Genetic diversity of ten Little Penguin populations in Eastern Australia .....	108
Table 2: AMOVA results for microsatellite genotypes.....	108
Table 3: $\Phi_{PT}$ genetic differentiation at eleven microsatellite loci .....	109
Table 4: AMOVA results for mitochondrial control region sequences .....	114
Table 5: $\Phi_{PT}$ genetic differentiation at the mitochondrial control region .....	115
Table 6: MIGRATE estimates of the number of immigrants per generation .....	118
Table 7: MIGRATE estimates of mutation-scaled population size. ....	119
Table 8: Estimates of the effective population size $N_e$ based on $\Theta$ .....	119

## **Appendix 3**

Table 1A: Sampling locations in Eastern Australia.....	131
Table 2A: Details of microsatellites used for genotyping .....	131
Table 3A: $F_{ST}$ genetic differentiation at 11 microsatellite loci .....	132
Table 4A: Shannon's mutual information index at 11 microsatellite loci.....	132
Table 5A: MIGRATE estimates of the number of immigrants per generation .....	133
Table 6A: MIGRATE estimates of mutation-scaled population size $\Theta$ .....	134
Table 7A: Likelihood plots of xNm into Bowen or Lion Island - microsats - Bayesian ..	135
Table 8A: Likelihood plots of xNm into Bowen or Lion Island - mtDNA - Bayesian.....	136
Table 9A: Likelihood plots of xNm into NSW or SA - microsats - Bayesian.....	137
Table 10A: Likelihood plots of xNm into NSW or SA - mtDNA - Bayesian .....	138
Table 11A: Likelihood plots of $\Theta$ , Bowen and Lion Island - microsats - Bayesian.....	139
Table 12A: Likelihood plots of $\Theta$ , Bowen and Lion Island - mtDNA - Bayesian .....	140
Table 13A: Likelihood plots of $\Theta$ , NSW and SA - microsats - Bayesian .....	141
Table 14A: Likelihood plots of $\Theta$ , NSW and SA - mtDNA - Bayesian .....	142
Table 15A: Likelihood plots of xNm into Bowen or Lion Island - microsats - ML .....	143
Table 16A: Likelihood plots of xNm into Bowen or Lion Island - mtDNA - ML .....	144
Table 17A: Likelihood plots of xNm into NSW or SA - microsats - ML.....	145
Table 18A: Likelihood plots of xNm into NSW or SA - mtDNA - ML.....	146
Table 19A: Likelihood plots of $\Theta$ , Bowen and Lion Island - microsats - ML .....	147
Table 20A: Likelihood plots of $\Theta$ , Bowen and Lion Island; - mtDNA - ML.....	148
Table 21A: Likelihood plots $\Theta$ , NSW and SA - microsats - ML.....	149
Table 22A: Likelihood plots of $\Theta$ , NSW and SA - mtDNA - ML .....	150



## **Chapter 4**

Table 1: Sampling locations with samples collected and methods used.....	164
Table 2: Haplotype diversity at an 80 bp sequence of the MHC gene .....	174
Table 3: Allelic diversity of MHC genotypes according to MiSeq analyses.....	175
Table 4: Differences in allele pools among WA populations (a) and colonies (b) .....	177
Table 5: Average pN/pS ratio by state .....	178
Table 6: Neutrality test results by state .....	178
Table 7: Families with MHC genotype information .....	181
Table 8: Inbreeding coefficients O and I' (Sherwin, pers. comm.).....	183

## **Appendix 4**

Table A1: Sampling locations in eastern Australia and Western Australia.....	196
Table A2: ANOSIM results with different numbers of permutations .....	197
Table A3: Genetic differentiation of Australian penguin populations at the MHC .....	198
Table A4: Genetic differentiation of Australian penguins at MHC, by state of origin..	198
Table A5: Inbreeding coefficient $F_{IS}$ for known penguin families from Perth .....	199

## **Chapter 5**

Table 1: Simulation model parameters for PVA .....	211
Table 2: Population-specific parameters for NSW PVA .....	214
Table 3: Population-specific parameters for WA PVAs.....	215
Table 4: Dispersal rates based on mitochondrial DNA .....	216
Table 5: Probabilities of extinction for NSW under different reproductive scenarios .	228
Table 6: Probabilities of extinction for WA under different reproductive scenarios ...	229
Table 7: Probabilities of extinction for NSW under different mortality scenarios .....	233
Table 8: Probabilities of extinction for WA under different mortality scenarios .....	234
Table 9: Probabilities of extinction for all La Niña scenarios .....	241

## **Appendix 5**

Table 1A: Population size estimates and location of known NSW penguin colonies ...	252
Table 2A: Population size estimates and location of known WA penguin colonies .....	254



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3

#### **4 Thesis abstract:** 350 words

5 One of Australia's most iconic native bird species, the little penguin (*Eudyptula minor*),  
6 is in decline at numerous locations due to manifold anthropogenic impacts including  
7 predation by introduced predators, habitat destruction and modification, effects of  
8 fishing activity and climate change. The fundamental aim of this thesis was to combine  
9 genetic and demographic data about the penguins to forecast likely population  
10 changes under a range of possible scenarios, including evaluation of anthropogenic  
11 threats. To target this aim, four separate studies were undertaken. The first collated  
12 demographic data on population size and survival rates in New South Wales (NSW),  
13 using data from existing databases and new surveys. Non-invasive techniques were  
14 trialled to estimate population size and survival rates, with the latter resulting in  
15 estimates consistent with mark-recapture based approaches. The second study  
16 investigated size and connectivity of little penguin populations in Australia using  
17 traditional measures of genetic structure and diversity. Smaller colonies were  
18 genetically not less diverse than larger ones and differentiation was limited among  
19 colonies in NSW. Isolation by distance was not observed within NSW and weak at  
20 longer distances between different states. Complex interactions between the influence  
21 of oceanic currents and demography were furthermore suggested by contrasting  
22 dispersal patterns at different scales and using different genetic markers. The third  
23 study was based on an adaptive genetic marker of the immune system (Major

24 Histocompatibility Complex, MHC) to complement the aforementioned population  
25 genetic study that was based on neutral genes. Genetic diversity at the MHC locus  
26 largely mirrors diversity at neutral loci, indicating a strong effect of stochasticity.  
27 Nevertheless, selective pressures are acting on the MHC. The lowest rates of parasite  
28 infestation were suspected for individuals of intermediate MHC diversity, and penguins  
29 showed differential mortality or inbreeding avoidance at the MHC locus. Results from  
30 the first two studies were incorporated into a population viability analysis model to  
31 evaluate outcomes of different scenarios and management strategies in the fourth and  
32 last study conducted as part of this thesis. All models based on current estimates  
33 predicted severe population declines in NSW and Western Australia, and forecasts  
34 were particularly sensitive to changes of mortality estimates.

35   **Chapter 1: Introduction / Review of the status of the Little Penguin**  
36   **(*Eudyptula minor*)**

37   Australia is one of the most biodiverse nations in the world, with most of its non-fish  
38   vertebrate species (mammals, reptiles, birds and amphibians) being endemic: of 2009  
39   non-fish vertebrate species, 1489, or 74 %, are found only in Australia (Commonwealth  
40   of Australia 1994; Strahan 1995; Cogger 1996). This high rate of endemism makes  
41   Australian species particularly vulnerable to extinction, and indeed, Australia is also the  
42   country with the highest mammal extinction rate in the world (Woinarski et al. 2015).  
43   Additionally, more than one in seven Australian bird species are currently listed as  
44   threatened (Australian Government 1999 - EPBC Act - List of Threatened Fauna).

45   One of Australia's most iconic native bird species is the little penguin (*Eudyptula*  
46   *minor*), populations of which are declining in numerous locations (e.g. Dann et al.  
47   2000; Wiebkin 2011). Common threats to which declines are attributed include  
48   introduced predators, habitat destruction and modification, as well as direct and  
49   indirect effects of fishing activity (e.g. Croxall & Butchart 2012; Cannell et al. 2011;  
50   Wiebkin 2011). At the same time, some other penguin populations are increasing,  
51   most notably at Phillip Island, where a decline in penguin numbers during the 1970s  
52   and 1980s was followed by an increase from 12,000 breeding birds to 32,000 breeding  
53   birds (Sutherland & Dann 2014). Other locations where increases in penguin numbers  
54   were reported include islands off Wilsons Promontory, in northern-central Bass Strait  
55   (Schumann et al. 2013) and several other sites in Australia and New Zealand (Dann  
56   2013). These increases in estimates of penguin numbers likely balance the declines  
57   reported elsewhere, but the establishment of new colonies has rarely been recorded.

58 St Kilda pier, where penguins were first recorded breeding in 1974, is a notable  
59 exception (Eades 1975; Parks Victoria 1998). Consequently, the overall number of little  
60 penguins in Australia and New Zealand may not have declined, but the number of sites  
61 has. This reduction in the number of penguin colonies might lead to a reduction or  
62 fragmentation of the little penguin's breeding range, which might lead to reduced  
63 gene flow among colonies due to reduced connectivity. Furthermore, declining  
64 colonies might rely on immigrants from stable or increasing populations, which would  
65 act as source populations.

66 Changes in climate put further stress on little penguins, with higher sea surface  
67 temperatures being linked to poorer breeding success in Western Australia (WA,  
68 Cannell et al. 2012), whereas the opposite trend was observed for penguin colonies in  
69 Northern Bass Strait (Cullen et al. 2009). It has been projected that warming of land  
70 surface temperatures would have a negative impact on breeding and moult in little  
71 penguins (Dann & Chambers 2013). Burrow temperatures above 35 °C lead to  
72 increased heat stress, which could be partly balanced by improved breeding success  
73 with increased sea-surface temperature in South-Eastern Australia, but not WA (Dann  
74 & Chambers 2013; Cannell et al. 2012).

75 Furthermore, rising sea levels will affect availability of nesting habitat for little  
76 penguins (Schumann et al. 2013), possibly eradicating existing colonies but opening up  
77 new habitat through formation of new, potentially predator-free islands. Additionally,  
78 the likelihood of disease outbreaks and parasite infestations might be altered (Cannell  
79 et al. 2013; Burge et al. 2014). It is therefore essential to investigate the current status

80 and vulnerability of little penguin populations to anthropogenic impacts to ensure  
81 their long-term viability.

82   **1.1. Biology of the little penguin**

83

84   The little penguin (*Eudyptula minor*, Forster 1781), formerly known as fairy penguin in  
85   Australia and called (little) blue penguin or Kororā in New Zealand, is the smallest  
86   member of the penguin (Spheniscidae) family and the only one known to breed on  
87   mainland Australia. Due to their small size of around 33cm in standing height (Reilly  
88   1983), an average of 1.1kg in weight (Dann et al. 2000), and limited agility when on  
89   land, they are prey to introduced predators including foxes, cats and dogs in addition  
90   to their natural predators in the water.

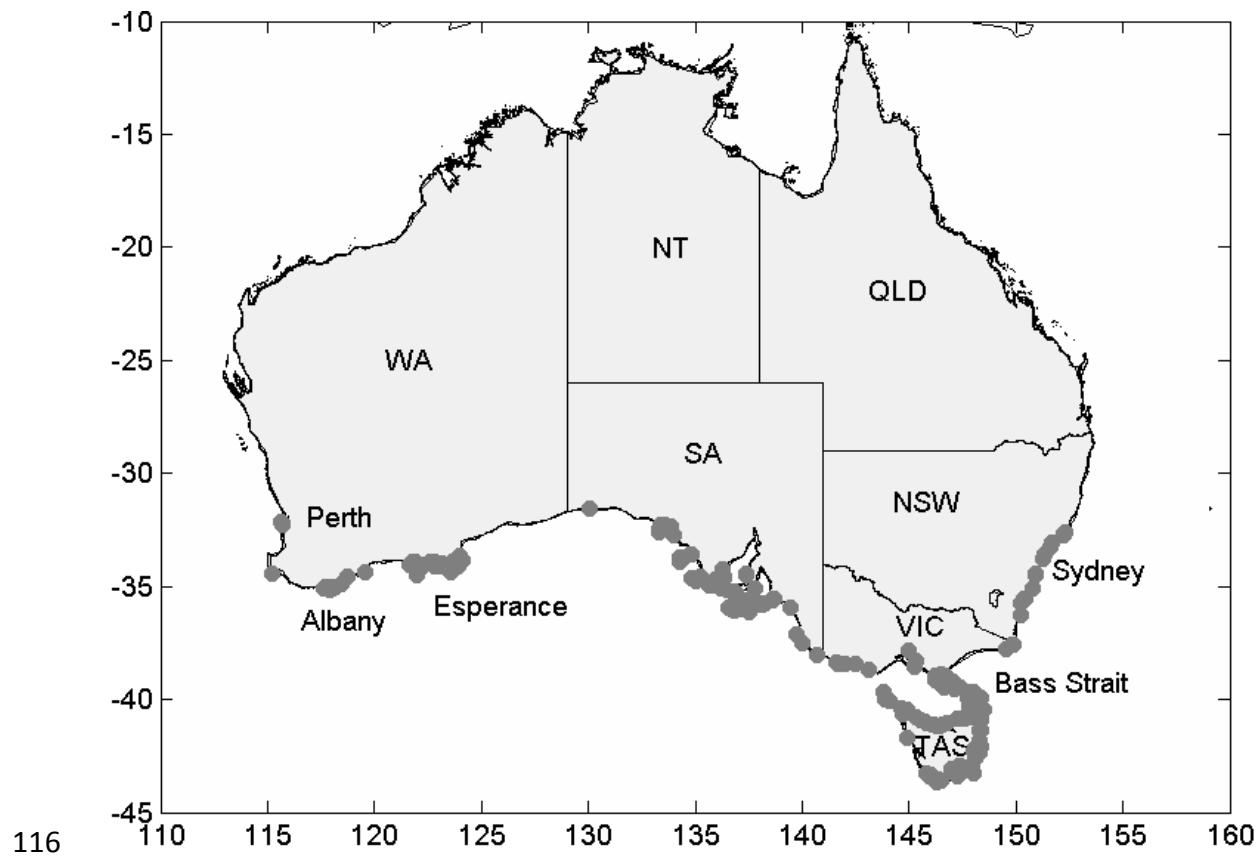
91   The little penguin life cycle starts with hatching after five weeks of incubation, during  
92   which the parents take turns to incubate the egg, which is similar in size, shape and  
93   colour to a hen's egg. The breeding season varies among locations and years, probably  
94   due to different availability of food. In New South Wales (NSW), penguins generally  
95   breed from June to February (Bourne & Klomp 2004). Double brooding, where a pair  
96   breeds twice in one season, can also be observed (e.g. Cunningham et al. 1993). At the  
97   end of the breeding season, breeders will spend some time at sea to fatten up before  
98   entering moult in February or March (Stahel & Gales 1987; Weerheim et al. 2003),  
99   whereas fledglings will travel long distances and stay can at sea for more than a year  
100   before returning to their natal colony to breed themselves (Reilly 1994). After moult,  
101   adults will again enter a feeding stage before beginning the next breeding season.

102   During the day, little penguins stay in their burrows unless they are feeding at sea,  
103   catching small shoaling fish below 12cm length and swallowing them under water or at  
104   the surface (Reilly 1994). Before sunset, they will form large groups or rafts offshore

105 until they make their way ashore in the two hours after sunset, relying on the cover of  
106 darkness.

107 **1.2. Penguin distribution and population trends in New South Wales**

108 The little penguin's breeding range extends from Fremantle near Perth, Western  
109 Australia (WA) in the West along the southern coast of Australia, including Islands of  
110 the Bass Strait and Tasmania, up to Port Stephens, NSW in the North (Fleming 1939,  
111 Fig. 1). Little penguins also occur around the coastline of New Zealand, including  
112 Chatham Islands. In June 2014, a group of penguin researchers came together to  
113 collate the most current estimates of colony sizes of little penguins in Australia (see  
114 Appendix Table 1A). The map in Fig. 1 is based on these estimates and distribution  
115 maps throughout this thesis make use of the data.



116  
117 **Fig. 1:** Map of known penguin colonies in Australia, colonies represented as grey circles  
118 NSW – New South Wales, NT – Northern Territory, QLD – Queensland, SA – South  
119 Australia, TAS – Tasmania, VIC – Victoria, WA – Western Australia  
120

121 In 1948, Keith Alfred Hindwood compiled a list of 18 coastal islands in New South  
122 Wales that were recorded as breeding places of sea birds (Hindwood 1948). The nine  
123 islands listed in Table 1 were recorded as known breeding places of little penguins. All  
124 of these were included in the 1970s Australian Bird Bander's *Sea Bird Island Series*,  
125 which was later continued by the *Corella* journal. Since then, population estimates  
126 have been updated and refined, and a remake of the *Sea Bird Island Series* is currently  
127 underway.

128   **Table 1:** Historically known breeding places of Little Penguins in New South Wales  
 129   (North to South) that were part of the Sea Bird Island Series, with historic and most  
 130   current estimates of colony size. 'pairs' refers to breeding pairs

<b>Island</b>	<b>Historic Estimate</b>	<b>Reference</b>	<b>Recent estimate</b>	<b>Reference</b>
<b>South Solitary Island</b>	"a few pairs"	Lane 1975b	No penguins reported	Carlile et al., pers. comm.
<b>Broughton Island</b>	"some pairs"	Lane 1976b	20-40 pairs	Carlile et al. 2012
<b>Cabbage Tree Island</b>	< 1000, probably few hundred pairs	Fullagar 1976	140 pairs	Priddel & Carlile 2004
<b>Bird Island</b>	"a few pairs"	Lane 1973	n/a	
<b>Lion Island</b>	300 pairs	Lane 1975	≤ 125 pairs*	Sargent et al. 2004
<b>Five Islands</b>	> 1000 burrows on Big Island	Gibson 1976	ca. 150 pairs on Five Islands group	NSW NPWS 2005
<b>Brush Island</b>	2000-3000 pairs	Morris 1974	2 200 pairs	Carlile, Priddel, Blackmore, et al. 2012
<b>Bowen Island</b>	> 1000 pairs	Lane 1976	1500-5000 pairs	Lintermans 1989
<b>Tollgate Islands</b>	< 5000 pairs	McKean & Fullagar 1976	180 ± 15 pairs	Carlile et al. submitted
<b>Montague Island</b>	1000-10 000 pairs	Fullagar 1973	5000 pairs	Weerheim et al. 2003

131   \* The estimate for 1989 mentioned in Sargent (2004) was 250 individuals, which  
 132   equals a maximum of 125 pairs if all penguins he counted were paired up and breeding

133

134   A comprehensive list of known penguin breeding colonies in Australia was compiled in  
 135   the form of a National Review of the Conservation Status and Management of  
 136   Australian Little Penguin Colonies (Dann et al. 1996). For WA, a separate management  
 137   review was also conducted (Cannell 2001), but focussed on the biggest WA penguin  
 138   colony at Penguin Island, which had previously been assigned the highest conservation  
 139   status of all major little penguin colonies within Australia (Dann et al. 1996).

140 Population declines have been reported from numerous locations including Phillip  
141 Island in Victoria (Dann 1992), some colonies in Tasmania (Stevenson & Woehler 2007)  
142 and Manly in Sydney Harbour (Fig. 1, NSW National Parks and Wildlife Service 2000).  
143 On Phillip Island, the population has since increased from ca. 12,000 to ca. 32,000  
144 breeding birds, and population increases have also been reported from islands off  
145 Wilsons Promontory, in northern-central Bass Strait (Schumann et al. 2013) and  
146 several other sites in Australia and New Zealand (Dann 2013). Due to the remote  
147 location of most little penguin breeding colonies, population sizes are inherently hard  
148 to estimate and reliable estimates are only available for few colonies. Colony size can  
149 vary from a few breeding pairs to tens of thousands (Marchant et al. 1990). Overall,  
150 the reported increases in penguin numbers likely balance the declines reported  
151 elsewhere, but the establishment of new colonies has rarely been recorded. A notable  
152 exception is the breeding colony at the St. Kilda breakwater in Melbourne, where little  
153 penguins have been recorded breeding since 1974 (Eades 1975). Consequently, while  
154 the overall number of little penguins in Australia and New Zealand may not have  
155 declined, the number of breeding sites very likely has. The little penguin's breeding  
156 range would thus be reduced or more fragmented, which might lead to reduced gene  
157 flow among colonies due to reduced connectivity.

158 Historically, Little Penguins were common on offshore islands as well as a few  
159 sheltered areas on the mainland coast, including reports from NSW (Barton 1978).  
160 Declines in mainland populations of Little Penguins are frequently attributed to  
161 expansion of human population, subsequent destruction of breeding habitat and  
162 interference by dogs. The only currently known mainland breeding colony of little

163 penguins in NSW is found at Manly in Sydney Harbour and has been the first colony of  
164 a common species to be listed as an endangered population according to the  
165 Threatened Species Conservation Act (NSW National Parks and Wildlife Service 2000).

166 **1.3. Connectivity and dispersal**

167 Adult little penguins are regarded as largely sedentary (Reilly 1994) and reported to  
168 return to breed within meters of a successful breeding burrow (Johannesen et al. 2002;  
169 Rogers & Knight 2006). Juveniles, however, can travel for more than 1 000 km from  
170 their natal colony following fledging (Reilly & Cullen 1982; Reilly 1994), and will only  
171 start to breed when they are two or three years old.

172 Studies of connectivity and dispersal that rely on marking individuals are hampered by  
173 low sample sizes of recovered individuals, particularly away from the area where they  
174 are marked, and the difficulty of distinguishing between short-term visitors and  
175 breeders. At Manly, the presence of banded penguins originating from colonies at Five  
176 Islands, Bowen Island, Montague Island and Phillip Island (Fig. 1) provided definitive  
177 evidence that interchange occurs between these colonies (Priddel et al. 2008) despite  
178 high site-fidelity usually exhibited by breeding individuals of the species. Only a few  
179 individuals, probably young that have not bred in their natal colony, disperse to other  
180 colonies (Reilly & Cullen 1982; Marchant et al. 1990; Dann 1992), but banding studies  
181 hardly allow quantification of dispersal and subsequent breeding. Most of the birds  
182 that disperse long distances after fledging are thought to eventually return to their  
183 natal colonies to breed. For example, many fledglings from Phillip Island in Victoria  
184 spend much of their first two years at the Bonney Upwelling in western Victoria and

185 South Australia, but few were recorded breeding at the local colonies there (Dann  
186 1992).

187 On the other hand, studies of genetic patterns can overcome these difficulties:  
188 selectively neutral genetic variation does not influence population health, but can be  
189 used to infer dispersal rates among populations. Recent genetic studies confirm  
190 dispersal between colonies of little penguins in south-eastern Australia. The genetic  
191 homogeneity among these colonies can be explained by low but consistent  
192 contemporary gene flow among them (Overeem et al. 2008). In this thesis, genetic  
193 methods are used to quantify this gene flow and estimate dispersal rates among  
194 Australian colonies of little penguins.

195 **1.4. Rationale of the study**

196 Little penguins (*Eudyptula minor*) are an iconic marine species in decline due to  
197 anthropogenic influences, which are particularly affecting populations close to  
198 expanding urban areas such as Sydney in NSW. The NSW marine environment is  
199 heavily impacted by natural and anthropogenic factors that threaten biodiversity  
200 including the penguins. These factors include climate change, development, pollution,  
201 predation by feral pests, and commercial and recreational activities such as tourism  
202 and fishing. In Sydney Harbour, the only known NSW mainland colony of little penguins  
203 at Manly Cove is listed as an endangered colony (NSW National Parks and Wildlife  
204 Service 2000), yet little is known about the long term viability of its population. In  
205 addition, NSW boasts penguin colonies in several NSW Marine Parks (Port Stephens,  
206 Jervis & Batemans Bay), but research is needed about their population ecology and  
207 habitat use in marine park waters. In the most recent update on the Recovery Plan for

208 the endangered Manly little penguins (Little Penguin Recovery Team 2007) one of the  
209 stated objectives was to research the ecology of the population. Specifically, research  
210 into the distribution of penguin foraging habitats and the level of migration between  
211 Manly and other NSW penguin populations was recommended, to assess the impact of  
212 threats and management strategies. The fundamental aim of this project was to  
213 facilitate choice of management strategies for the endangered population of little  
214 penguins at Manly and colonies elsewhere in NSW. To achieve this, genetic and  
215 demographic data were combined to forecast likely population changes under a range  
216 of possible scenarios incorporating natural and anthropogenic threats.

217 **1.5. Thesis overview**

218 The fundamental aim of this thesis was to combine genetic and demographic data  
219 about Australian little penguins to forecast likely population changes under a range of  
220 possible scenarios, including evaluation of anthropogenic threats. To target this aim,  
221 four separate studies were undertaken. Each of those studies is presented as a chapter  
222 in this thesis and is currently being prepared for publication in a scientific journal as  
223 indicated on the title page for each chapter. Differences in formatting style among  
224 chapters are due to the differing requirements set by the target journals.

225 Chapter 2 is titled "*Identifying a decline of flightless, burrow-nesting seabirds using*  
226 *non-invasive methods to replace mark-recapture approaches for estimating population*  
227 *size and survival rates*" and compares different methods to estimate demographic data  
228 on population size and survival rates in NSW. Estimates were obtained using data in  
229 existing attendance and sighting databases, augmented by new surveys conducted at  
230 two focal populations within NSW. Non-invasive techniques were trialled to estimate

231 population size (based on nightly beach arrivals) and survival rates (based on a novel  
232 adaptation of territory occupancy surveys, Roth & Amrhein, 2010), with the latter  
233 resulting in estimates consistent with mark-recapture based approaches.

234 In Chapter 3, which is titled "*Population genetics of Little Penguins (Eudyptula minor)*  
235 *in Australia*", size and connectivity of little penguin populations in three Australian  
236 states - NSW, SA and WA - were estimated using established measures of genetic  
237 structure and diversity. While colonies varied greatly in their census population size,  
238 smaller colonies were genetically not less diverse than larger ones. Limited genetic  
239 differentiation among colonies in NSW, however, seemed to result in a genetic  
240 diversity that is maintained at a slightly lower level than closer to the centre of the  
241 penguin distribution. Isolation by distance was not observed within NSW, and was  
242 present but weak at somewhat larger distances between NSW and SA. Differentiation  
243 was stronger at the maternally inherited mitochondrial marker than at the biparentally  
244 inherited microsatellites, which might indicate sex-biased dispersal driven by male  
245 dispersers. A complex interaction between the influence of oceanic currents and  
246 demography was furthermore suggested by contrasting dispersal patterns at different  
247 scales and using different markers. This chapter thus has an unusual focus on  
248 assessment of marginal populations, and finds ongoing dispersal among small, yet  
249 genetically diverse populations at the penguin's range edge.

250 Chapter 4 – "*Patterns of Major Histocompatibility Complex diversity, parasitism and*  
251 *mate choice in Little Penguins (Eudyptula minor)*" – uses an adaptive genetic marker of  
252 the immune system (Major Histocompatibility Complex, MHC) to complement Chapter  
253 Three's population genetics of NSW and WA penguins using established genetic

254 markers. The possible impact of pathogenic threats can be assessed by studying the  
255 highly variable MHC genes, which influence many important biological traits, including  
256 susceptibility to parasite infestation and mating preferences. Penguin mortality due to  
257 infestation with a novel *Haemoproteus* parasite has recently been described for the  
258 first time. We therefore investigated whether selective processes are helping to  
259 maintain variability of MHC genes in penguins. We compared populations of different  
260 size and connectivity, and examined the selective effects of parasite pressure and mate  
261 choice. Genetic diversity at the MHC locus largely mirrored diversity at neutral loci,  
262 indicating a strong effect of stochasticity. Nevertheless, there were signs of selective  
263 pressures acting on the MHC. In a small sample, there was non-significant suggestion  
264 that the lowest rates of parasite infestation occurred in individuals of intermediate  
265 MHC diversity. The penguins showed signs of inbreeding at the MHC locus, with  
266 significantly higher levels of inbreeding in the parental than in the offspring generation  
267 in Western Australia, but not New South Wales, despite similar overall levels of  
268 inbreeding between states. Some mechanism of inbreeding avoidance or reduced  
269 fitness of young inbred offspring was therefore suspected for Western Australian  
270 penguins. This chapter is the first population-scale study of MHC genes in little  
271 penguins and adds an example for the use of non-neutral markers in wildlife studies.

272 Finally, Chapter 5 (“*Viability of little penguin (*Eudyptula minor*) populations*  
273 *at their northern range edge*”) incorporates data from Chapters 2 and 3 into a  
274 population viability analysis (PVA, Beissinger & McCullough 2002) model to evaluate  
275 outcomes of different scenarios and recommend management directions for penguin  
276 populations in NSW and WA. Three steps were taken to achieve this aim. (1) Baseline

277 models of the current status of little penguins in NSW and WA were built using data  
278 from the literature. (2) Sensitivity analyses were conducted to investigate the effect of  
279 different survival and reproductive rates as well as changes in frequency and severity  
280 of La Niña events on model outcomes. (3) Directions for research and management  
281 were suggested based on the results of this PVA. Compared to many other studies of  
282 similar species, this chapter draws on more extensive data in two ways: it has better  
283 demographic data as reported in Chapter 2, as well as dispersal data from the genetic  
284 work in Chapter 3.

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- 402

## Appendix 1

**Table 1A:** Current information on little penguin breeding colonies, collated during and after penguin research meeting on 27<sup>th</sup> June 2014 at the Sydney Institute of Marine Sciences in Mosman, Sydney; Is - Island

Meeting participants: Gemma Carroll, MQ; Diane Colombelli-Negrel, Flinders; Peter Dann, Phillip Island; Robert Harcourt, MQ, Lisa O'Neill; Amanda Peucker, Deakin; Judy Reizes, MEC; William B. Sherwin, UNSW; Jacob Sife, Manly Council; Jennifer J. Sinclair, UNSW; Ikuko Tomo, SA Museum; Sandra Vogel (now Vardeh), UNSW

Other contributors: Nicholas Carlile, OEH; Martin Fortescue, Booderee NP; Dominic Maher, UNSW; Tracey Rogers, UNSW

Colony name	Population estimate	Year of latest estimate	Latitude	Longitude	Sources
<b>New South Wales</b>					
Inner Rock	20	2012	-32.601	152.304	Carlile 2012
Broughton Is	60	2012	-32.617	152.312	Lane 1976, Carlile et al. 2012
Little Broughton Is	26	2013	-32.621	152.333	Carlile 2013
Cabbage Tree Is	280	2004	-32.687	152.223	Fullagar 1976, Morris 1976, Priddel & Carlile 2004
Boondelbah Is	200	2004	-32.707	152.228	Morris 1976, Fullagar 1995, Priddel & Carlile 2004
Moon Is	26	1974	-33.088	151.672	Gray & Gwynne 1974
Bird Is	5	1973	-33.229	151.602	Lane 1973
Lion Is	120	2013	-33.556	151.316	Segent et al. 2006, Rogers and Knight 2006, Vogel 2013
Manly, Sydney	162	2013	-33.81	151.28	O'Neill 2014
Flinders Islet, Five Islands	30	2005	-34.456	150.93	Battam 1976, NSW NPWS 2005
Bass Islet, Five Islands	10	2005	-34.465	150.945	Battam 1976, NSW NPWS 2005
Big Island, Five Islands	100	2005	-34.49	150.927	Gibson 1976, NSW NPWS 2005
Martin Islet, Five Islands	10	2005	-34.494	150.938	Battam 1976, NSW NPWS 2005
Bowen Is	10000	1995	-35.117	150.765	Lane 1976, Lintermans 1989, Fortescue 1995

Brush Is	4400	2012	-35.53	150.42	Morris 1974, Carlile et al. 2012
Belowla Is	726	2011	-35.554	150.39	Lane 1976, Blackmore et al. 2011
Grasshopper Is	110	2012	-35.632	150.333	Lane 1976, Priddel et al. 2012
Wasp Is	220	2012	-35.667	150.311	Lane 1976, Priddel et al. 2012
Snapper Is	48	2014	-35.725	150.213	Carlile & Priddel 2014
Tollgate Is	360	2014	-35.75	150.26	McKean & Fullagar 1976, Lane 1976, Carlile et al. 2014
Montagu(e) Is	10000	2003	-36.25	150.23	Fullagar 1973, Heyligers & Fullagar 1995, Weerheim 2003
<b>Victoria</b>					
Anser Is	400	1981	-39.141	146.323	Harris & Norman 1981
Barrilliar (Barrallier) Is	8	2001	-38.276	145.313	Penguin Study Group, Unpubl. Data 1995, Dann et al. 2001 – (taken from Dann and Norman 2006)
Citadel Is	90	1981	-39.114	146.237	Harris & Norman 1981
Dannevig Is	400	1981	-39.106	146.238	Harris & Norman 1981
Gabo Is	35000	1995	-37.563	149.911	Reilly (1977), Fullagar 1995
Great Glennie Is	1000	1981	-39.087	146.235	Harris & Norman 1981
Kanowna Is	100	2006	-39.154	146.311	Harris & Norman 1981, Dann & Norman, 2006
Lady Julia Percy Is	4000	1981	-38.418	142.001	Harris & Norman 1981
Lawrence rocks	218	1981	-38.407	141.67	Harris & Norman 1981
McHugh Is	2000	1981	-39.116	146.242	Harris & Norman 1981
Merri Is	10	1995	-38.402	142.47	Penguin Study Group, Unpubl. Data 1995, Dann & Norman, 2006, Harris & Norman 1981
Middle Is	584	2003	-38.403	142.468	Penguin Study Group, Unpubl. Data 1995, Overeem and Wallis, 2003
Norman Is	900	1981	-39.022	146.242	Harris & Norman 1981
Notch Is	1000	1995	-38.94	146.676	Penguin Study Group, Unpubl. Data 1995

Phillip Is	26000	2006	-38.483	145.231	Penguin Study Group, Unpubl. Data 1995, Dann & Norman, 2006
Port Campbell	1000	1981	-38.665	143.103	Harris & Norman 1981
Portland Harbour	10	1995	-38.348	141.61	Penguin Study Group, Unpubl. Data 1995
Pyramid rock (Phillip Is)	10	1995	-38.529	145.222	Penguin Study Group, Unpubl. Data 1995, Harris & Norman 1981
Rabbit Is	4000	1995	-38.911	146.511	Thoday 1991, Penguin Study Group, Unpubl. Data 1995
Rabbit Rock	200	1981	-38.915	146.49	Harris & Norman 1981
Rag Is	400	1995	-38.954	146.682	Penguin Study Group, Unpubl. Data 1995
Seal Is	1000	2006	-38.924	146.662	Penguin Study Group, Unpubl. Data 1995, Dann & Norman, 2006.
Shellback Is	400	1981	-38.969	146.227	Harris & Norman 1981
St Kilda Breakwater	200	1995	-37.861	144.962	Penguin Study Group, Unpubl. Data 1995
The Skerries	10	1981	-37.753	149.517	Barton 1978, Harris & Norman 1981
Tullaberga Is	900	1981	-37.556	149.845	Harris & Norman 1981
Wattle Is	500	1995	-39.138	146.361	Penguin Study Group, Unpubl. Data 1995
<b>Tasmania</b>					
Albatross Is	200	1996	-40.377	144.655	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Babel Is	2000	1996	-39.948	148.332	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Battery Is	80	1996	-40.458	148.179	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Baynes Is	80	1996	-40.769	147.937	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Beagle Is	35	1996	-40.336	147.922	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Bicheno	200	1996	-41.878	148.306	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Big Black Reef	10	1996	-40.398	147.96	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Big Green Is	300	1996	-40.185	147.98	Brothers & Pemberton unpubl. data (Dann et al. 1996)

Billy Goat Reefs	300	1996	-40.223	148.267	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Bird Rock/Shag Rock	100	2002	-40.92	148.32	Tasmanian Department of Primary Industry, Parks, Water and Environment (DPIPWE), 2002
Boronia Beach	70	2007	-42.99	147.321	Stevenson and Woehler, 2007.
Boxen Is	4000	1996	-40.379	147.896	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Breaksea Is	1,000	1996	-35.064	118.054	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Briggs Islet	520	1996	-40.256	148.282	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Bruny Is	800	2007	-43.4	147.272	Brothers & Pemberton unpubl. data (Dann et al. 1996), Stevenson and Woehler, 2007.
Cat Is	400	1996	-39.95	148.356	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Cataraqui Point (King Island)	2000	1996	-40.027	143.88	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Chalky Is	7000	1996	-40.1	147.889	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Christsmas Is	100	1996	-39.689	143.832	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Coswell Beach	220	2007	-42.145	148.079	Stevenson and Woehler, 2007.
Craggy Is	2000	1996	-39.687	147.68	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Curtis Is	1000	1996	-39.471	146.646	Brothers & Pemberton unpubl. data (Dann et al. 1996)
De Witt Is	4500	1996	-43.596	146.354	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Deal Is	1100	1996	-39.477	147.329	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Derwent Estuary	354	2010	-42.935	147.386	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Dial Point	80	1996	-41.103	146.064	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Diamond Is	400	2002	-41.859	148.29	Brothers & Pemberton unpubl. data (Dann et al. 1996), Tasmania DPIPWE, 2002
Dover Is	1000	1996	-39.472	147.282	Brothers & Pemberton unpubl. data (Dann et al. 1996)
East Islet	120	1996	-39.214	147.023	Brothers & Pemberton unpubl. data (Dann et al. 1996)
East Kangaroo Is	3200	1996	-40.183	147.902	Brothers & Pemberton unpubl. data (Dann et al. 1996)

East Moncoeur Is	350	1996	-39.227	146.539	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Egg Island	40	1977	-43.102	147.014	van Tets (1977)
Erith Is	500	1996	-39.449	147.292	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Fisher Is	10	1996	-40.216	148.239	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Flat (Mutton Bird)	6000	1996	-43.421	145.968	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Flat Witch Is	2400	1996	-43.621	146.293	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Forstyh Is	20000	1996	-40.51	148.31	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Fortescue Bay	700	2007	-43.13	147.96	Stevenson and Woehler, 2007.
Fossil Bluff	1000	1996	-40.98	145.732	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Foster Islands	700	1996	-40.723	147.974	Brothers & Pemberton unpubl. data (Dann et al. 1996)
George Rocks/Georges Island	200	2002	-40.924	148.329	Brothers & Pemberton unpubl. data (Dann et al. 1996), Tasmania DPIPWE, 2002
Goose Is	2000	1996	-40.303	147.796	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Governor Is	20	2002	-41.872	148.313	Tasmania DPIPWE, 2002
Grassy Is	400	1996	-40.069	144.061	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Great Dog Is	60	1996	-40.248	148.253	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Gull Is	8000	1996	-40.435	148.497	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Hawley Beach	present	1996	-41.138	146.543	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Hogan Is	1300	1996	-39.221	146.986	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Horseshoe Reef	40	1996	-41.146	146.424	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Ille Du Golfe	250	1996	-43.569	146.525	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Inner Sister Is	80	1996	-39.694	147.914	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Isabella Is	600	1996	-40.129	147.944	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Preservation Islet (NW)	70	1996	-40.471	148.048	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Islet SE Great Dog	65	1996	-40.256	148.261	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Key Is	170	1996	-40.435	148.027	Brothers & Pemberton unpubl. data (Dann et al. 1996)

Lillico Strait (Lillico Beach, Bass Strait)	1400	1996	-41.161	146.289	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Little Anderson Is	60	1996	-40.291	148.114	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Little Badger Is	240	1996	-40.3	147.916	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Little Chalky Is	3600	1996	-40.138	147.893	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Little Chappel Is (Mount Chappel Island)	80	1996	-40.268	147.91	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Little Christmas Is	600	2007	-42.254	148.027	Tasmania DPIPWE, 2002, Stevenson and Woehler, 2007.
Little Dog Is	200	1996	-40.253	148.207	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Little Goose Is	350	1996	-40.295	147.785	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Little Green Is	100	1996	-40.226	148.255	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Little Is	900	1996	-39.822	147.825	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Little Swan Is	600	2002	-40.724	148.081	Brothers & Pemberton unpubl. data (Dann et al. 1996), Tasmania DPIPWE, 2002
Little Waterhouse Is	40	2002	-40.823	147.628	Brothers & Pemberton unpubl. data (Dann et al. 1996), Tasmania DPIPWE, 2002
Lonah/Goat Is	400	1996	-41.12	146.127	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Long Island	110	1996	-40.358	148.007	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Long Islet	350	1996	-39.207	147	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Louisa Is	1300	1996	-43.535	146.359	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Low Head	1000	1996	-41.061	146.791	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Maatsuyker Is	600	1996	-43.65	146.279	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Maclean Is	80	1996	-40.76	147.935	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Maria Is	2000	1996	-42.627	148.086	Brothers & Pemberton unpubl. data (Dann et al. 1996)

Marriot Reef	125	2007	-39.917	147.85	Brothers & Pemberton unpubl. data (Dann et al. 1996), Stevenson and Woehler, 2007.
Mid Woody	60	1996	-40.294	148.115	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Mile Is	1400	1996	-40.124	147.916	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Neds Reef	80	1996	-40.333	148.074	Brothers & Pemberton unpubl. data (Dann et al. 1996)
New Year Is	100	1996	-39.671	143.825	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Night Islet	170	1996	-43.338	146.012	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Ninth Is	14000	1996	-40.834	147.269	Brothers & Pemberton unpubl. data (Dann et al. 1996)
North East Isle	1000	1996	-39.445	147.378	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Outer Sister Is	4500	1996	-39.654	147.992	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Oyster Rocks	80	1996	-40.291	148.062	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Paddy's Island	80	2002	-41.397	148.303	Tasmania DPIPWE, 2002
Passage Is	9000	1996	-40.506	148.342	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Penguin Point (Three Sisters?)	450	1996	-41.12	146.119	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Pirates Bay	60	2007	-43.02	147.93	Stevenson and Woehler, 2007.
Preservation Is	550	1996	-40.475	148.063	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Ram Is	60	1996	-40.312	148.205	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Red Chapel Beach	6	2007	-42.908	147.345	Stevenson and Woehler, 2007.
Refuge Is	1000	2002	-42.178	148.263	Tasmania DPIPWE, 2002
Rocky Cape	100	1996	-40.894	145.534	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Rodondo Is	150	1996	-39.23	146.387	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Round Islet	300	1996	-39.228	146.997	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Roydon Is	1200	1996	-39.908	147.774	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Rum Is	250	1996	-40.494	148.074	Brothers & Pemberton unpubl. data (Dann et al. 1996)

Schouten Is	100	2002	-42.316	148.297	Brothers & Pemberton unpubl. data (Dann et al. 1996), Tasmania DPIPWE, 2002
Sentinel Is	1600	1996	-39.839	147.768	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Somerset	100	1996	-41.037	145.83	Brothers & Pemberton unpubl. data (Dann et al. 1996)
South West Isle	250	1996	-39.522	147.128	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Spences Reefs	210	1996	-40.231	148.243	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Spike Is	600	1996	-40.549	148.11	Brothers & Pemberton unpubl. data (Dann et al. 1996)
St Helens Is	10000	2002	-41.347	148.342	Brothers & Pemberton unpubl. data (Dann et al. 1996), Tasmania DPIPWE, 2002
Stinking Bay	100	2007	-42.996	147.657	Stevenson and Woehler, 2007.
Storehouse Is	5000	1996	-39.959	148.362	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Swan Is	600	1996	-40.737	148.109	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Taillerfer Rocks	240	2002	-42.36	148.315	Tasmania DPIPWE, 2002
Tasman Is	400	1996	-43.239	148.003	Brothers & Pemberton unpubl. data (Dann et al. 1996)
The Nuggets	200	2002	-42.117	148.348	Tasmania DPIPWE, 2002
The Nut	100	1996	-40.762	145.303	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Three Hummock Is	5000	1996	-40.437	144.905	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Three Sisters Is	1000	1996	-41.121	146.121	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Tin Kettle Is	200	1996	-40.295	148.147	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Trefoil Is	400	1996	-40.632	144.689	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Trumpeter Islets	1000	1996	-43.28	145.815	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Tucks Reef	35	1996	-40.26	148.245	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Twin Islets	180	1996	-39.203	146.985	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Vansittart Is	310	1996	-40.277	148.301	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Walker Is	2000	1996	-43.633	146.277	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Watchhouse Is (?)	1000	1996	?	?	Brothers & Pemberton unpubl. data (Dann et al. 1996)

Waterhouse Is	500	1996	-40.78	147.633	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Wedge Is	500	1996	-43.136	147.672	Brothers & Pemberton unpubl. data (Dann et al. 1996)
West Moncoeur Is	4000	1996	-39.232	146.506	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Wilsons Point	1000	1996	-41.668	144.923	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Wynyard Is	1000	1996	-40.995	145.769	Brothers & Pemberton unpubl. data (Dann et al. 1996)
Wright Is	40	1977	-41.154	146.417	van Tets (1977)
<b>South Australia</b>					
Albatross Is	10	1996	-35.068	136.181	Copley 1996
Althorpe Is	132	2004	-35.372	136.861	Copley 1996, Robinson et al., 1996., R. Velzeboer & S. Shepherd. Unpubl. Data (2004)
Avoid Is (aka Sudden Jerk Island)	22	1996	-34.563	135.212	Copley 1996
Baudin Rocks (Godfrey Islands)	60	2011	-37.091	139.723	Parker et al., 1979., DENR, 1994, Copley, 1996., S. Goldsworthy. Unpubl. Data - 2006
Black Rocks	23	1996	-34.614	135.288	Copley 1996
Blythe Is	50	1996	-34.568	136.292	Copley 1996
Boston Is	100	2006	-34.698	135.930	A. Peucker. Unpubl. Data - 2006
Bunda Cliffs	100	2006	-31.579	130.096	Reily 1974, B. Page. Unpubl. Data - 2006
Cape Martin	4	1996	-37.500	140.016	Copley 1996
Cape Northumberland	60	1996	-38.057	140.667	Copley 1996
Chinamans Hat Is	10	1996	-35.289	136.918	Copley 1996
Coffin Bay Islands	220	1996	?	?	Copley 1996
Curta Rocks	145	1996	-34.946	135.871	Copley 1996
Dorothee Is	200	2004	-33.742	134.284	Parker and Cox, 1974, Copley 1996, A. Weibkin. Unpubl. Data - 2004
Duffield Is	100	1996	-34.655	136.319	Copley 1996

English Is	10	1996	-34.638	136.196	Copley 1996
Evans Is	500	2005	-32.377	133.481	A. Weibkin. Unpubl. Data - 2005
Flinders Is	20	2006	-33.723	134.487	D. Armstrong. Unpubl. Data - 2006
Franklin Islands	2000	2004	-32.456	133.638	Copley 1996, A. Weibkin. Unpubl. Data - 2004
Freeling Is	53	1996	-32.481	133.345	Copley 1996
Goose Is	30	1996	-34.456	137.365	Copley 1996, Robinson et al. 1996, SANPWS
Granite Is	146	2010	-35.564	138.631	Copley 1996, N. Gilbert. Unpubl. Data 2001, R. Morcom. Unpubl. Data 2005, N. Gilbert, 2010
Green Is	10	1996	-34.462	137.404	Copley 1996
Greenly Is	1500	2004	-34.647	134.798	Copley, 1996. A Weibkin. Unpubl. data - 2004
Hareby Is	500	2008	-34.581	136.293	Copley 1996, Robinson, 1996. A Weibkin. Unpubl. Data - 2008
Kangaroo Is	8000	1996	-35.803	137.245	Copley 1996
Kingscote, KI	706	2008	-35.648	137.623	Brock and Kinloch 2008
Emu Bay, KI	298	2008	-35.593	137.538	C. Gibbons. Unpubl. Data 2008
Penneshaw, KI	216	2008	-35.721	137.943	Parker et al. 1979, S. Somerfield. C. Gibbons, Unpubl. Data 2008
Antechamber Bay, KI	178	2008	-35.800	138.081	C. Gibbons. Unpubl. Data 2008
Vivonne Bay, KI	150	2008	-36.004	137.159	Copley, 1996. C. Gibbons. Unpubl. Data 2008
Christmas Cove, KI	140	2008	-35.719	137.934	C. Gibbons. Unpubl. Data 2008
Cape Cassini, KI	116	2008	-35.581	137.329	Parker 1979, C. Gibbons. Unpubl. Data 2008
Cape Willoughby, KI	116	2008	-35.841	138.134	Parker 1979, C. Gibbons. Unpubl. Data 2008
Stokes Bay, KI	60	2008	-35.636	137.169	Parker 1979, C. Gibbons. Unpubl. Data 2008
Browns Beach, KI	32	2008	-35.798	137.842	C. Gibbons. Unpubl. Data 2008
Seal Bay, KI	23	2010	-35.991	137.303	T. Soutar. Unpubl. Data 2010
Snellings Beach, KI	16	2008	-35.667	137.073	C. Gibbons. Unpubl. Data 2008

Western River Cove, KI	16	2008	-35.674	136.971	DEP 1987, C. Gibbons. Unpubl. Data 2008
North Page Is	50	1996	-35.759	138.301	Copley 1996
Rocky River, KI	1	1979	-35.965	136.654	Parker, 1979
Maupertuis Bay, KI	1	1979	-35.999	136.684	Parker, 1979
Breakneck River, KI	1	1979	-35.932	136.581	Parker, 1979
American River, KI	1	1987	-35.781	137.772	Parker, 1979. DEP, 1987
Busby Islet	40	1996	-35.624	137.641	Copley 1996
Cape Younghusband, KI	100	1996	-36.040	136.816	Copley 1996
Beatrice Islets	1	1979	-35.649	137.682	White 1918, Parker et al. 1979
Knobby Island	1	1996	-35.994	137.288	Robinson et al 1996
Pelorus Islet	1	1996	-36.112	137.529	Robinson et al 1996
Kirkby Is	350	1996	-34.550	136.213	Copley 1996
Lewis Is	100	2006	-34.956	136.032	A. Wiebkin. Unpubl. Data 2006
Liguanea Is	665	1996	-34.986	135.623	Copley 1996
Little Waldegrave (West) Is	105	1996	-33.594	134.759	Copley 1996
Lipson Island	100	2006	-34.264	136.267	Copley, 1996. S. Harrison. Unpubl. Data 2006
Lusby Is	200	1996	-34.543	136.259	Copley 1996
Middle Island	600	1996	-35.215	136.832	Copley 1996
North Veteran Is	10	1996	-33.941	134.276	Copley 1996
Nullarbor (Bunda?) Cliffs	2000	1996	-31.579	130.096	Copley 1996
Olive Is	2290	2006	-32.721	133.969	A. Weibkin. Unpubl. Data - 2006
Owen Is	100	1996	-34.859	136.009	Copley 1996
Partney Is	540	1996	-34.523	136.255	Copley 1996
Pearson Is	12000	2006	-33.943	134.266	Parker and Cox 1978, Copley 1996, A. Weibkin. Unpubl. Data 2006

Penguin Is	400	1996	-37.498	140.015	Copley 1996
Penguin Islet (=Penguin Island?)	4	2011	-37.498	140.015	Skira & Brothers 1988, Carey et al. 2011
Pullen Is	10	2011	-35.538	138.691	DEP 1983, Copley 1996, Robinson et al. 2006, N. Gilert unpubl. Data 2011
Rabbit (Owen?) Is	10	1996	?	?	Copley 1996
Reevesby Is	1857	2009	-34.515	136.282	Copley 1996, J. van Weenan. Unpubl. Data 2009, A. Weibkin. Unpubl. Data 2009
Round Is (Coorong?)	100	1996	-35.941	139.462	Copley 1996
Roxby Is	1250	1996	-34.592	136.317	Copley 1996
Royston Is	70	1996	-35.196	136.839	Copley 1996
Seal Is	100	1996	-35.339	136.921	Copley 1996
Sibsey Is	4	2004	-34.647	136.183	A. Weibkin. Unpubl. Data 2004
Smith Is	50	1996	-34.985	136.028	Copley 1996
Spilsby Is	100	100	-34.662	136.342	W. Goedseke. Unpubl. Data 2010
St Francis Is	2663	1996	-32.509	133.291	Copley 1996
St Peter Is	1000	2005	-32.288	133.574	Copley 1996, Robinson et al. 1996, A. Weibkin. Unpubl. Data 2005
Troubridge Is	3010	2010	-35.118	137.826	Copley 1996, M. Waterman. Unpubl. Data 2009, A. Weibkin 2010
Waldegrave (East) Is	500	2006	-33.598	134.796	Copley 1996, Robinson et al. 1996, S. Goldsworthy. Unpubl. Data 2006
Wardang Is	8000	2004	-34.501	137.358	J. Lawley. Unpubl. Data 2004
Wedge Is	100	2004	-35.155	136.461	Copley 1996, Robinson et al., 1996, J. van Weenan Unpubl. Data 2004
West Is	20	2010	-35.608	138.592	Copley 1996, M. Waterman. Unpubl. Data 1992, R. Brandle. Unpubl. Data., N. Gilbert. Unpubl. Data 2010

Winceby Is	400	1996	-34.489	136.284	Copley 1996
Wright Is	300	1996	-35.583	138.609	Paton 1996
South Four Hummocks	1	1996	-34.783	135.034	Robinson et al. 1996
Egg Is	1	1996	-32.473	133.317	Robinson et al. 1996
Dog Is	1	1996	-32.488	133.333	Robinson et al. 1996
Fenelon Is	1	1996	-32.583	133.282	Robinson et al. 1996
Goat Is	1	1996	-32.309	133.512	Robinson et al. 1996
Eyre Is	1	1996	-32.367	133.826	Robinson et al. 1996
Lounds Is	1	1996	-32.273	133.367	Robinson et al. 1996
<b>Western Australia</b>					
Bald Is	100	1996	-34.918	118.462	Dann et al. 1996, Cannell et al. 2001
Bellinger Is	40	1996	-33.886	123.644	Dann et al. 1996, Cannell et al. 2001
Ben Is	100	1996	-33.899	122.754	Dann et al. 1996, Cannell et al. 2001
Bird Is	1		-32.278	115.690	Cannell et al. 2001
Boxer Is	100	1996	-34.000	121.678	Dann et al. 1996, Cannell et al. 2001
Breaksea Is	500	2001	-35.064	118.056	Dann et al. 1996, Cannell et al. 2001
Carnac Is	200	1996	-32.121	115.662	Dann et al. 1996, Cannell et al. 2001
Charley Is	10	1996	-33.922	121.876	Dann et al. 1996, Cannell et al. 2001
Cheyne Is	150	2001	-34.593	118.767	Dann et al. 1996, Cannell et al. 2001
Coffin Is	10	1996	-35.000	118.214	Dann et al. 1996, Cannell et al. 2001
Cull Is	100	1996	-33.922	121.903	Dann et al. 1996, Cannell et al. 2001
Daw Is	100	1996	-33.847	124.139	Dann et al. 1996, Cannell et al. 2001
Doubtful Islands	100	1996	-34.375	119.578	Dann et al. 1996, Cannell et al. 2001
Eclipse Is	100	1996	-35.183	117.886	Dann et al. 1996, Cannell et al. 2001
Figure of Eight Is	10	1996	-34.032	121.605	Dann et al. 1996, Cannell et al. 2001

Flat Is	10	1996	?	?	Dann et al. 1996, Cannell et al. 2001
Forrest Is	10	1996	-33.917	122.710	Dann et al. 1996, Cannell et al. 2001
Garden Is	25	1997	-32.210	115.679	Cannell et al. 2001
Goose Is	100	1996	-34.081	123.183	Dann et al. 1996, Cannell et al. 2001
Hood Is	70	1996	-34.141	122.049	Dann et al. 1996, Cannell et al. 2001
Inshore Is	10	1986	-33.916	122.829	Cannell et al. 2001
Kermadec Is	100	1996	-34.088	122.833	Dann et al. 1996, Cannell et al. 2001
Lorraine Is	10	1996	-33.949	122.564	Dann et al. 1996, Cannell et al. 2001
Mackenzie Is	10	1996	-34.198	122.106	Dann et al. 1996, Cannell et al. 2001
Marts Island	100	1996	-34.000	122.633	Dann et al. 1996, Cannell et al. 2001
Michaelmas Is	10	1996	-35.044	118.040	Dann et al. 1996, Cannell et al. 2001
Migo Is	30	2001	-35.072	117.650	Cannell et al. 2001
Mistaken Is	100	1996	-35.063	117.944	Dann et al. 1996, Cannell et al. 2001
Mondrain Is	200	1996	-34.138	122.245	Dann et al. 1996, Cannell et al. 2001
Nares Is	100	1996	-33.932	122.595	Dann et al. 1996, Cannell et al. 2001
North Twin Peak Island	100	1996	-33.989	122.843	Dann et al. 1996, Cannell et al. 2001
Observatory Is	100	1996	-33.922	121.794	Dann et al. 1996, Cannell et al. 2001
Penguin Is	1000	1996	-32.305	115.691	Dann et al. 1996, Cannell et al. 2001
Ram Is	10	1996	-34.031	122.143	Dann et al. 1996, Cannell et al. 2001
Remark Is	10	1996	-34.064	121.986	Dann et al. 1996, Cannell et al. 2001
Richards Is	10	2001	-35.076	117.651	Dann et al. 1996, Cannell et al. 2001
Rob Is	100	1996	-34.033	122.234	Dann et al. 1996, Cannell et al. 2001
Round (Recherche Is)	100	1996	-34.103	123.888	Dann et al. 1996, Cannell et al. 2001
Salisbury Is	100	1996	-34.358	123.554	Dann et al. 1996, Cannell et al. 2001
Sandy Hook Is	100	1996	-34.034	121.995	Dann et al. 1996, Cannell et al. 2001

Seal Is (King George Sound)	10	1996	-35.076	117.975	Dann et al. 1996
Seal Is (Shoalwater bay)	100	1996	-32.293	115.690	Dann et al. 1996
Shag Is	100	1996	-32.296	115.691	Dann et al. 1996
Shelter (Muttonbird) Is	300	2001	-35.051	117.694	Cannell et al. 2001
Six Mile Is	40	1996	-33.639	123.967	Dann et al. 1996, Cannell et al. 2001
Skink Is	35	1987	-33.987	123.148	Cannell et al. 2001
St Alouarn Is	100	1996	-34.403	115.196	Dann et al. 1996, Cannell et al. 2001
Stanley (Wickham) Is	200	1996	-34.021	123.291	Dann et al. 1996, Cannell et al. 2001
Station Is	100	1996	-33.960	122.523	Dann et al. 1996, Cannell et al. 2001
Termination Is	100	1996	-34.471	121.992	Dann et al. 1996
Westall (Combe) Is	100	1996	-34.079	122.966	Dann et al. 1996
Wickham (Stanley) Is	100	1996	-34.020	123.291	Dann et al. 1996, Cannell et al. 2001
Woody Is	10	2000	-33.963	122.012	Dann et al. 1996, Cannell et al. 2001

1      **Formatted for publication in Journal of Applied Ecology – Title Page**

2      **Chapter 2: Identifying a decline of flightless, burrow-nesting seabirds using  
3                          non-invasive methods to replace mark-recapture approaches  
4                          for estimating population size and survival rates**

5

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20      **Author Contributions:**

21      MF and TR provided field data; KSB helped collect and analyse demographic data in  
22      2012. JJS and WBS secured funding for and helped set up this study, and I (SV)  
23      collected most data from 2012-2014, performed all statistical analyses, and wrote the  
24      chapter.

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32           Tables: 16

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34 **Number of references:** 73

35 ***Summary/Abstract:***

36 The life history traits of seabirds including penguins make them vulnerable to  
37 anthropogenic pressures in marine and terrestrial environments. Early detection and  
38 management of adverse effects of these pressures relies on efficient monitoring of  
39 seabird populations, usually done through regular estimation of population sizes and  
40 mortality or survival rates. Here, we estimate population sizes and apparent survival  
41 rates of little penguins (*Eudyptula minor*) at two island colonies in NSW, while  
42 assessing the feasibility of replacing invasive methodology that requires the capture  
43 and microchipping of numerous individuals with non-invasive methods based on  
44 burrow occupancy records and beach counts. Beach counts alone can give an  
45 indication of how a percentage of the population is faring, but might be biased by  
46 changes in habitat utilisation both at sea (via foraging trip lengths) and on land (via  
47 shifts in breeding habitat). Results of mark-resight modelling revealed an opposing  
48 trend to beach counts on Bowen Island, but agreed with the declining trend on Lion  
49 Island. Estimates of apparent survival based on the non-invasive method agreed well  
50 with the results of mark-recapture modelling. Non-invasive studies of burrowing sea  
51 birds like penguins could therefore replace mark-recapture based studies if burrow  
52 occupancy can be monitored for a large number of burrows over several visits per  
53 season.

54 ***Synthesis and applications:*** Population declines due to exceptionally low survival at  
55 a colony of little penguins near Sydney, Australia, were detected using both invasive  
56 and non-invasive techniques. The latter could therefore replace more invasive and  
57 time-consuming surveys based on mark-recapture approaches.

58

59 ***Keywords:*** Apparent survival, *Eudyptula minor*, fairy penguin, little penguin, mark-  
60 resight

61	<b>Contents</b>	
62	Chapter 2: Identifying a decline of flightless, burrow-nesting seabirds using non-	
63	invasive methods to replace mark-recapture approaches for estimating population	
64	size and survival rates .....	35
65	Introduction .....	39
66	Methods.....	48
67	Results.....	65
68	Discussion.....	77
69	Acknowledgements.....	82
70	References.....	83
71	Appendix 2 .....	88
72		

### 73    **Introduction**

74    For many decades, estimating demographic parameters has been of significant interest  
75    to population ecologists trying to understand population dynamics (e.g. Seber 1982;  
76    Williams et al. 2002; Sandvik et al. 2012), which are affected by fluctuating numbers of  
77    births, deaths, immigration and emigration rates as a result of environmental and  
78    anthropogenic processes. Generally, smaller populations are more likely to be affected  
79    by such fluctuations as well as random individual differences in survival and  
80    reproduction compared with larger populations (Lande 1993; Mugabo et al. 2013) and  
81    thus, are more vulnerable to extinction. Measurable parameters like survival rates and  
82    abundance are therefore fundamental to studies of population demography (e.g.  
83    Lebreton & Burnham 1992; Williams et al. 2002; Mills 2012), because they can be used  
84    to describe population dynamics, which in turn are important to understand the  
85    impact of population changes over time and to track and forecast changes in animal  
86    numbers and ranges occupied.

87    Despite the ongoing interest in measuring demographic parameters in wild animal  
88    populations, there are still difficulties. On the one hand, measurement of survival still  
89    presents a particular difficulty for animal ecologists, because “marked individuals  
90    cannot be followed closely through time” (Lebreton & Burnham 1992). True survival is  
91    impossible to estimate for most studies, and commonly replaced with estimates of  
92    apparent survival. The latter equals the product of true survival and fidelity, where  
93    fidelity is  $1 - \text{permanent emigration}$ . Recent attempts have also been made to  
94    estimate apparent survival based on territory occupancy using an approach that  
95    foregoes marking of individuals altogether (Roth & Amrhein 2010). On the other hand,

96 even the seemingly straightforward estimation of animal abundance can be  
97 complicated in species that are difficult to access, such as seabirds breeding on  
98 uninhabited islands.

99 Some countries, including the UK and Ireland, have well-defined census methods and  
100 times for counting sea birds (Walsh et al. 1995). This publication describes survey  
101 methods for two burrow-nesting seabirds, Manx shearwater (*Puffinus puffinus*) and  
102 the puffin (*Fratercula arctica*), both of which are capable of flight. In Australia, such a  
103 comprehensive handbook of census methods does not exist. Burrow-nesting seabird  
104 species like the little penguin are generally quantified by counting active burrows,  
105 along transect lines or randomly placed quadrats, and species identity of birds  
106 occupying burrows needs to be established (Bibby et al. 2012), either visually or  
107 acoustically. Acoustic monitoring techniques might be suitable for low-density seabird  
108 colonies, but their resolution for larger colonies is expected to be poor (Marques et al.  
109 2013). Numbers of the flightless penguins can also be estimated by counting  
110 individuals coming ashore to breed (Cannell et al. 2011; Carlile et al. 2012) or  
111 estimating the size of rafts forming around dusk (Priddel & Carlile 2004). Seabird  
112 numbers have also been estimated by using mark-recapture methods (e.g. Nisbet  
113 2001; Crespin et al. 2006; Sidhu et al. 2007), or combinations of any of the above  
114 methods (e.g., Cannell et al. 2011).

115 The life history traits of seabirds – including long life spans, delayed reproduction and  
116 small clutches – make them vulnerable to anthropogenic pressures in marine and  
117 terrestrial environments. Seabirds are among the most threatened groups of birds and  
118 their decline has accelerated over the past few decades (Croxall & Butchart 2012). The

119 principal anthropogenic threats identified for birds at sea are commercial fisheries  
120 (through competition and by-catch), effects of climate change (in the form of rising  
121 sea-surface temperatures, changes to oceanography and acidification) and pollution  
122 (marine debris, oil and light), whereas on land, introduced predators, habitat  
123 degradation – partly due to climate change causing rising sea levels – and human  
124 disturbance are the main threats (Commonwealth of Australia 2012; Croxall & Butchart  
125 2012). Land-based threats are affecting the flightless penguins in particular, and these  
126 birds have in fact been identified as one of the two most threatened groups of seabirds  
127 (Croxall & Butchart 2012).

128 The distribution of the little penguin (*Eudyptula minor*), the smallest of all penguin  
129 species, extends from the Shoalwater Island group (most notably Penguin Island) near  
130 Perth in Western Australia, along the southern coast of Australia up to Port Stephens  
131 on the NSW East Coast (map in Fig. 1), and along the coasts of the North, South,  
132 Steward and Chatham Islands of New Zealand, with a total abundance of little  
133 penguins estimated between 350,000 – 600,000 individuals (Dann et al. 1996). Due to  
134 its high abundance and extensive range, the species is listed as “least concern” on the  
135 IUCN Red List of endangered species despite a decline in numbers both in Australia  
136 (mainly Tasmania and South Australia, Fig.1, Stevenson & Woehler 2007; Wiebkin  
137 2011) and New Zealand (South Island, Dann 1994). Quantitative data are lacking for  
138 most offshore islands, probably due to difficulties in accessing the colonies as well as  
139 time and funding it takes to reliably assess abundance. As a burrowing sea bird, little  
140 penguins breed in burrows that are about one meter long and end in a nest chamber,  
141 which makes estimating their colony sizes harder than for non-burrowing penguin

142 species. Nests can also be found under any suitable shelter, e.g. rocks, caves, houses or  
143 discarded timber (Reilly 1972). While the timing of the breeding season varies among  
144 locations and years, little penguins in NSW generally breed from mid-winter to  
145 summer (June to February, Bourne & Klomp 2004), with peak breeding activity from  
146 September to December (Morris 1974). After chicks have fledged, the adults spend  
147 some weeks at sea to fatten up prior to the moulting season, which occurs between  
148 February and April. Moulting is followed by another feeding period before  
149 commencing the next breeding cycle.

150 As a species endemic to Australia and New Zealand, the little penguin is protected by  
151 both federal and state wildlife laws in Australia (federal: Australian Government 1999)  
152 and by federal laws in New Zealand (Wildlife Act 1953, reprint as at 25 October 2013,  
153 and Fisheries Act 1996, reprint as at 1 January 2014). It has been identified as having  
154 conservation value in the Temperate East Marine Bioregion (Commonwealth of  
155 Australia 2012) off the Australian East coast, which borders the Coral Sea in the North  
156 and extends almost to the border between New South Wales (NSW) and Victoria (VIC)  
157 in the South (Fig.1). Little penguins are also listed as at-risk – declining by the New  
158 Zealand Department of Conservation (Flemming 2013).

159 In Australia, little penguin colonies are reported to have declined in numerous  
160 locations, and the colony at Manly in Sydney Harbour (Fig.1) has been listed as an  
161 endangered population (NSW National Parks and Wildlife Service 2000). Since its  
162 listing, the colony has successfully recovered from a few tens of pairs to achieve the  
163 most successful breeding season in decades, with 85 breeding pairs in 2013 (Lisa  
164 O'Neill, pers. comm.). Yearly abundance estimates are also available for this colony

165 (Little Penguin Recovery Team 2007; Priddel et al. 2008). However, population  
166 dynamics of island colonies in NSW are largely unknown. While abundance estimates  
167 are available for many island colonies, they are mostly restricted to single years and  
168 are derived from changing methodologies (e.g. Seabird Island Series, Lion Island: Lane  
169 1976; Bowen Island: Lane 1975, Fig. 1). There are a few colonies that have been  
170 surveyed over a longer time period. These include Lion Island in Pittwater and Bowen  
171 Island in Jervis Bay (Fig.1), which will be the subject of this study. Apparent survival  
172 estimates are only available for a single one of the colonies, the one at Bowen Island,  
173 which has been studied intensively in the 1980s and 1990s (Fortescue 1991). During  
174 the course of these studies, Fortescue found that the timing of breeding was much less  
175 variable on Bowen Island than, for example, Phillip Island in Victoria (Fig.1).

176 Lion Island is the closest island colony to the endangered penguin population at Manly  
177 and had previously been surveyed in the late 1950s (Lane 1975), when the penguin  
178 population was estimated to be around 300 breeding pairs based on burrow counts,  
179 and extensive monitoring was undertaken from 1991 to 2008 by Taronga Zoo and the  
180 NSW National Parks and Wildlife Service. In 1989, the population size was estimated to  
181 be approximately 250 individuals (Sargent et al. 2004) based on anecdotal evidence,  
182 and that there were at least 60 breeding pairs through the 1990s, based on the  
183 number of occupied burrows found on the island (Rogers et al. 1995; Rogers & Knight  
184 2006). Similar to Bowen Island, the timing of the little penguin breeding season on  
185 Lion Island was less variable than Phillip Island and the breeding success of the birds  
186 was much higher on Lion Island compared Phillip Island over the four years (Rogers et  
187 al. 1995) and eight years (Rogers and Knight 2006) monitored in the 1990s.

188 Bowen Island's penguin population was first estimated to consist of at least 1000  
189 individuals (Hindwood 1948; Lane 1976) based on anecdotal evidence, and later as a  
190 minimum of 1500 breeding pairs (Lintermans 1989) based on the number of occupied  
191 burrows found in a limited area and 7 000 breeding pairs (Fortescue 1991) based on  
192 the total number of breeding burrows estimated for different vegetation types.

193 The aim of this chapter is to investigate population trends and apparent survival rates  
194 of little penguins at two island colonies in NSW, while assessing the feasibility of  
195 replacing invasive methodology that requires the capture and microchipping of  
196 numerous individuals with non-invasive methods based on burrow occupancy records  
197 and beach counts. Such estimates of population trends and survival form the basis for  
198 long-term monitoring and modelling of population dynamics to improve effective  
199 management. Here, we estimate population trends and survival using both invasive  
200 and non-invasive methods. If estimates are shown to be consistent between methods,  
201 invasive surveys could be replaced by the non-invasive alternative. In other regions  
202 including the well-studied Phillip Island in Victoria (Dann 1992) and Penguin Island in  
203 Western Australia (Cannell et al. 2011), beach counts are already regularly conducted  
204 to detect population trends.

205 To test our hypotheses, we simultaneously applied two methods for abundance  
206 estimation. First, we used established methods based on mark-recapture to estimate  
207 abundances (mark-resight) from which to infer population sizes, and to estimate  
208 apparent survival rates (Cormack-Jolly-Seber, "CJS" and robust design, "RD"  
209 modelling).

210 The CJS model (CJS, Cormack 1964; Jolly 1965; Seber 1965) is a live recapture model,  
211 and has been the most general approach to survival estimation using mark-recapture  
212 since 1964 (Lebreton & Burnham 1992). Assumptions for the CJS model include that (i)  
213 every marked animal present in the population has the same probability of being  
214 captured in each sampling period – an assumption that is best met by considering only  
215 the breeding population; (ii) every marked animal present in the population during a  
216 sampling period has the same probability of survival until the following sampling  
217 period; (iii) marks are neither lost nor overlooked and are recorded correctly; (iv)  
218 sampling periods are instantaneous (or very short periods) and recaptured animals are  
219 released immediately; (v) all emigration from the sampled area is permanent; and (vi)  
220 the fate of each animal with respect to capture and survival probability is independent  
221 of the fate of any other animal.

222 The RD model (Pollock et al. 1990; Kendall & Nichols 1995; Kendall et al. 1995; Kendall  
223 et al. 1997) is a combination of an open model and closed capture models suitable  
224 when one has intense bursts of sampling followed by gaps. The key difference  
225 between the CJS and the RD model is that instead of just one capture occasion  
226 between survival intervals, multiple capture occasions are analysed in the robust  
227 design. These occasions are close together in time, meeting assumption (vii) that no  
228 mortality or emigration occurs during these short time intervals (closure assumption).  
229 Assumptions (i-vi) also apply to robust design models.

230 Secondly, to non-invasively estimate abundance with minimal disturbance to breeding  
231 penguins, we used beach counts at easily accessible parts of the colonies, past records  
232 for which date back to the late 1980s / early 1990s. Survival rates can also be

233 estimated using a novel approach previously trialled on nightingales (Roth & Amrhein  
234 2010), which relies on territory occupancy records without the necessity to individually  
235 identify occupants. For penguins, territories are defined as burrows, and burrow  
236 occupancy, “BO”, was recorded to estimate survival. In this article, the three different  
237 estimates of apparent survival will be referred to as “survival-CJS”, “survival-RD” and  
238 “survival-BO”. For the latter, we adapted the hierarchical site-occupancy model to  
239 burrowing seabirds, where burrows represent territories. The model assumes that  
240 burrow occupancy states are independent and identically distributed for each territory  
241 and breeding season. Survival-BO is defined as the probability that an individual  
242 occupying a burrow survives until the next breeding season and settles in the same  
243 burrow again and therefore similar to apparent survival estimated using mark-  
244 recapture methods, here called survival-CJS. Nest fidelity is high in penguins, as has  
245 been shown for the populations at Lion Island (Rogers & Knight 2006), Phillip Island  
246 (Reilly & Cullen 1979) and Wellington in New Zealand (Bull 2000; Pledger & Bullen  
247 1998). When applied to data on nightingale territory occupancy, estimates of survival-  
248 BO were very similar to survival-CJS estimates that were obtained through CJS  
249 modelling applied to ringing data from the same nightingale population.

250 Because both survival-BO and survival-CJS are estimates of apparent survival and  
251 cannot distinguish between death and emigration, a third method can also be used to  
252 estimate apparent survival, or survival-RD, along with estimates of migration.

253 Additionally, we hypothesise that the larger, more closely managed colony at Bowen  
254 Island currently has a more stable abundance of penguins when compared to  
255 estimates from the late 1980s, while the smaller colony at Lion Island, where invasive

256 weeds have not been controlled and surveying has stopped in 2007, could be  
257 declining. Yearly adult survival rates on both islands are expected to be around 75 %,  
258 which is within the range reported from other stable Australian colonies (e.g. Phillip  
259 Island: 75 % to 91 %, Dann et al. 2014; Bowen Island: 73 %, Fortescue 1991; Penguin  
260 Island: 72 +/- 9 % in 1986-2009, Cannell, B. unpublished data).

261 Some of the reported survival rates were based on birds marked using flipper bands,  
262 which have been shown to reduce survival of little penguins (Dann et al. 2014).  
263 Estimates obtained using transponders, as in this study, are therefore expected to be  
264 higher than those based on flipper banded penguins.

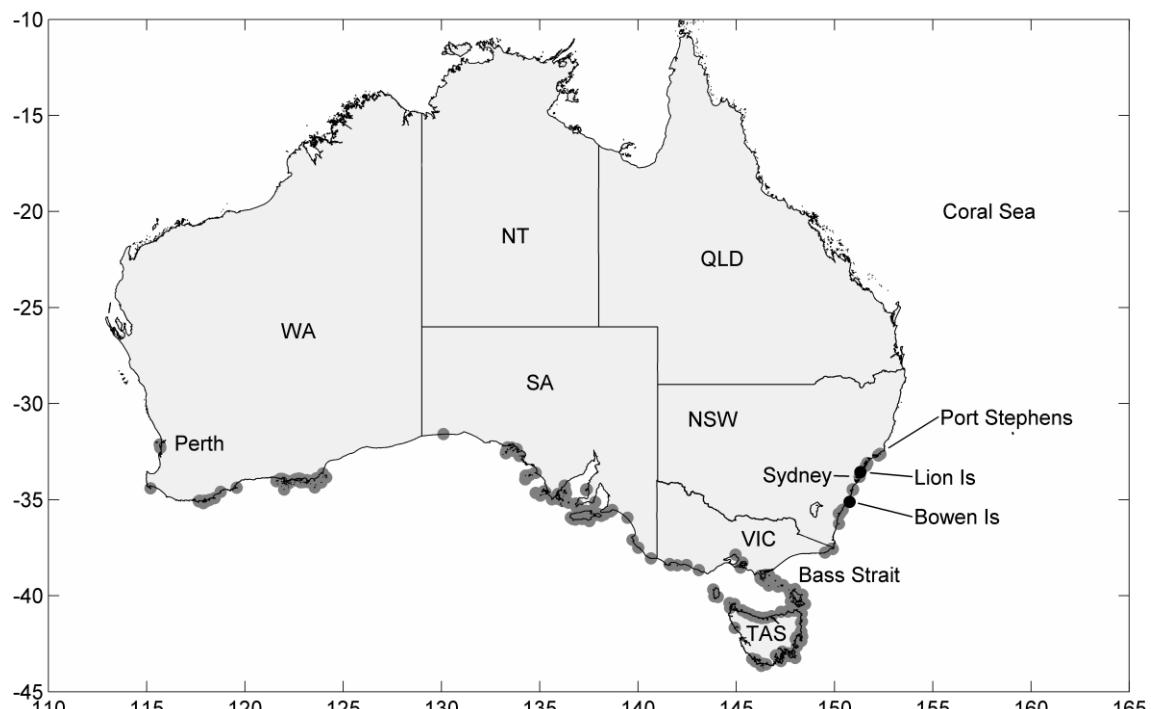
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266 **Methods**

267 ***Study sites***

268 Two little penguin colonies with earlier published estimates of demographic  
269 parameters, Lion and Bowen Islands in New South Wales (NSW, Fig.1), were chosen for  
270 surveys of population size and survival rates between 2012 and 2014 (Tables 1 & 2).

271



272  
273 **Fig. 1:** Map of all known penguin colonies in Australia (grey) with sampling locations  
274 highlighted (black). Is - Island; Longitudes and latitudes are shown.

275 NSW – New South Wales, NT – Northern Territory, QLD – Queensland, SA – South  
276 Tasmania, VIC – Victoria, WA – Western Australia

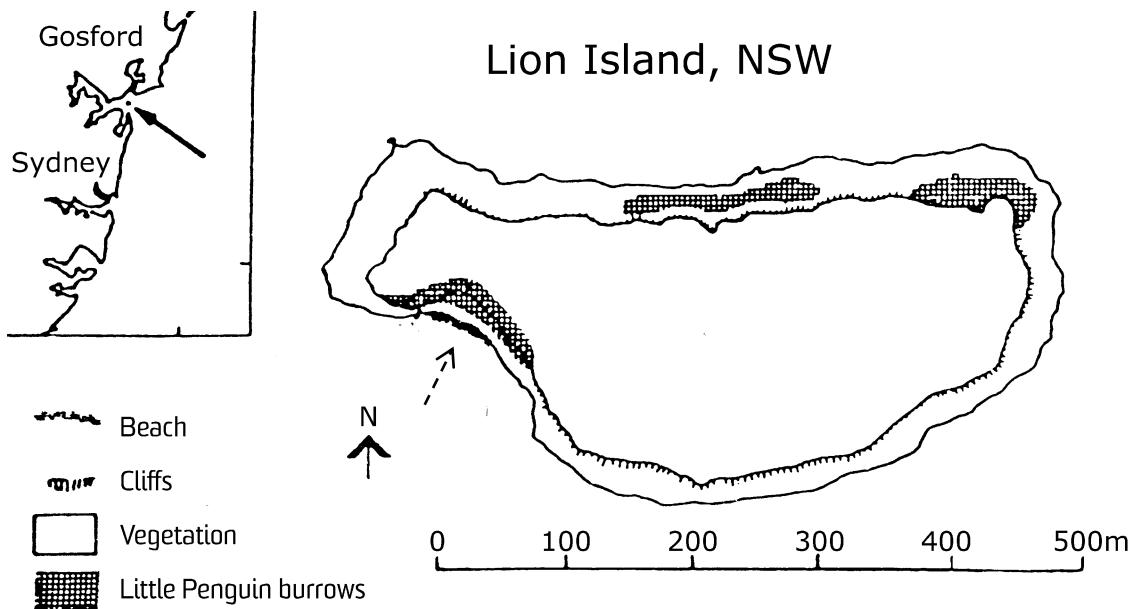
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279 Lion Island (8 ha) is a Nature Reserve situated at the mouth of the Hawkesbury River in  
280 the Pittwater estuary, at 30° 33' S, 151° 20' E, and about 300 m from the nearest  
281 mainland shoreline (Fig. 2). It forms part of Ku-ring-gai National Park approximately  
282 30km north of the centre of Sydney and is managed by the NSW National Parks &  
283 Wildlife Service (NPWS). Bowen Island (51 ha) is part of the jointly managed Booderee  
284 National Park, Jervis Bay, at 35° 07' S, 150° 46' E (Fig. 3). For both islands, past records  
285 of beach counts are available to investigate long-term trends (Table 1). These counts  
286 are from the main penguin landing beaches and were conducted after sunset, when  
287 penguins return to their burrows during the breeding season. Care was taken to not be  
288 visible to arriving penguins, by blending in with vegetation or rocks on the beaches and  
289 choosing locations where observers did not produce shadows, especially during  
290 moonlit nights. While we cannot exclude the possibility that penguin numbers were  
291 affected by observer presence, any possible effect is expected to be the same  
292 throughout the season and between years. Moreover, no differences were detected  
293 between the first and successive nights during the counts, which indicates that  
294 observer error did not affect penguin numbers counted (see also Sutherland & Dann  
295 2014). Demographic methods used to estimate survival rates and population sizes at  
296 Bowen and Lion Islands are summarised in Table 2.

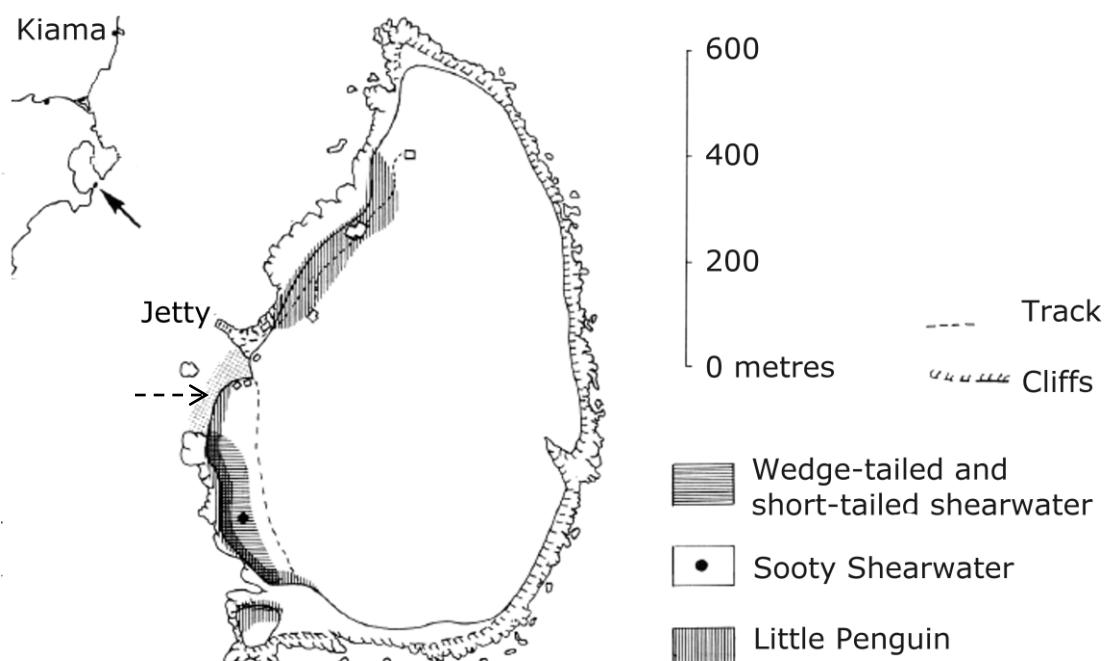
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299  
300 **Fig. 2:** Map of Lion Island (adapted from Cunningham et al. 1993). The main landing  
301 site for little penguins is associated with the hatched area at the south western side of  
302 the island. Count/sampling site indicated using dashed arrow.

303



304  
305 **Fig. 3:** Map of Bowen Island (adapted from Lane 1976). Penguin Beach is on the  
306 western side of the island, just south of the jetty. Count/sampling site indicated using  
307 dashed arrow.

308

309   **Table 1:** Timeline of beach counts at Penguin Beach, Bowen Island and the main beach,  
 310   Lion Island, between October and December each year

Colony	Month	Average Count	SD	Number of counts*	Source
Bowen Island	Nov-88	310		1	Booderee NP records
	Oct-95	236		1	Booderee NP records
	Nov-95	234		1	Booderee NP records
	Dec-95	289	12	2	Booderee NP records
	Oct-02	300		1	Booderee NP records
	Nov-02	247	34.4	3 (206-250)	Booderee NP records
	Oct-03	200		1	Booderee NP records
	Oct-06	232		1	Booderee NP records
	Dec-09	371		1	Booderee NP records
	Oct-12	171	22.9	3 (154-203)	This study
	Nov-12	151	22.5	6 (128-190)	This study
	Oct-13	97	1.8	4 (95-99)	This study
	Nov-13	99	3.5	2 (95-102)	This study
	Oct-14	106	15	3 (91-126)	This study
Lion Island	Month	Average Count	SD	Number of counts*	Source
	Nov-91	40		1	Taronga Zoo
	Dec-91	61		1	Taronga Zoo
	Oct-92	74	9.5	2 (64-83)	Taronga Zoo
	Oct-93	69		1	Taronga Zoo
	Nov-93	95		1	Taronga Zoo
	Dec-93	68		1	Taronga Zoo
	Dec-00	82	8.3	4 (75-96)	Taronga Zoo
	Dec-01	65	4	4 (59-68)	National Parks
	Nov-02	71	5.3	4 (65-79)	National Parks
	Nov-03	54	4.9	4 (46-59)	National Parks
	Dec-04	53	0	2 (53-53)	National Parks
	Dec-05	80	6	2 (74-86)	National Parks
	Dec-06	55	1	2 (54-56)	National Parks
	Dec-07	24	1	2 (23-24)	National Parks
	Dec-08	40	2	2 (38-42)	National Parks
	Nov-12	16	1	2 (15-16)	This study
	Dec-12	15	4	2 (11-18)	This study
	Oct-13	22	4	2 (18-26)	This study
	Nov-13	12	3	2 (9-14)	This study
	Dec-13	14	2	3 (12-16)	National Parks
	Oct-14	14	3	4 (9-16)	This study

311   \* minimum and maximum numbers counted are shown in brackets, where known

312   **Table 2:** Summary of demographic methods used to estimate population size and  
 313   survival rates of little penguins on Bowen and Lion Islands

		Bowen Island		Lion Island	
Previous method(s)	Population size	Beach counts	Burrow density	Burrow counts	Unknown
	Survival	Mark-recapture		n/a	
Reference method(s)	Population size	Mark-resight models		Mark-resight models	
	Survival	CJS models	Robust design models	CJS models	Robust design models
Non-invasive method	Population size	Beach counts		Beach counts	
	Survival	Burrow occupancy		Burrow occupancy	

314

315

316

317 ***Mark-recapture and mark-resight***

318 For the mark-recapture part of this study, penguins were intercepted when arriving on  
 319 the main beach (Penguin beach on Bowen Island and the main, south-western beach  
 320 on Lion Island, Fig. 2 & Fig. 3) after sunset to return to their burrows following a day of  
 321 feeding at sea. A plastic mesh fence was erected in a V-shape along the back of the  
 322 main beach. As the penguins reached a specific area along the fence, they were  
 323 corralled and counted for use in population estimates by the non-invasive method.  
 324 Penguins were captured and moved to an area with sufficient light, where a passive  
 325 integrated transponder (PIT-tag or RFID, Trovan Unique ID100) was implanted under  
 326 the skin at the back of the penguin's neck. More than fifty individual penguins were  
 327 marked per colony during the initial capture occasion(s) on both Bowen and Lion  
 328 Islands. Penguins were then recaptured and checked for transponders on consecutive  
 329 nights during several visits to each island across the survey period in 2012 and 2013, or  
 330 at the peak of the breeding cycle as identified in the two previous years in 2014  
 331 (Table 3). Differences in the field work schedule between islands and years were the  
 332 results of logistical constraints. Numbers of marked and unmarked individuals  
 333 encountered were recorded.

334

335 **Table 3:** Sampling dates in 2012 to 2014 at Bowen and Lion Islands

	2012				2013				2014	
	Bowen Island	5- 7/10	19- 21/10	9- 11/11	20- 22/11	4- 7/10	27- 28/11	20- 22/12		17- 19/10
Lion Island	13- 14/10	26- 28/10	16- 17/11	5- 6/12	21- 22/09	11- 12/10	15- 16/11	14- 15/12	10- 11/10	24- 25/10

336

337 ***Population Size***

338 **Non-invasive method**

339 Beach counts were used as a non-invasive option to estimate the number of penguins  
340 using the main landing site on each island. Population size is estimated using penguin  
341 beach counts as an index, where each penguin is counted when it crosses the fence  
342 erected on the back of the main landing beach on each island. On the same days as the  
343 mark-recapture and burrow occupancy surveys (see below), beach counts were  
344 conducted each night during the visits (Table 3), from sunset until no penguins came  
345 ashore for a period of at least 30min. All counts were completed 3 hours after sunset.  
346 Care was taken to conduct counts in the same way as the earlier surveys, i.e. using the  
347 same survey area and times. Data from 2012 to 2014 were compared to records from  
348 earlier surveys that also estimated population size. These earlier surveys indicated that  
349 the main beach was used by approximately one twelfth of the population on Bowen  
350 Island and more than half the population on Lion Island. Beach counts are not only a  
351 reflection of the population size but also of the stage of breeding of the penguins (and  
352 activity ashore for moult, courtship etc.). For example, during the guard stage (when  
353 chicks are less than two weeks old), only one parent goes out to sea at a time; when  
354 the chicks are older than two weeks, both parents would leave the chicks. The  
355 numbers crossing the beach can therefore double without the population size  
356 changing. Despite this limitation, beach counts conducted at similar times over several  
357 years can give valuable insights into population trends.

358 There was no indication of a major shift in habitat utilisation and nesting area on either  
359 of the surveyed islands (Fortescue, pers. comm.; NSW NPWS, unpublished data), so  
360 that we assume that beach counts represent an unchanging proportion of the whole  
361 population. We are therefore using multi-year beach counts as an index of population  
362 size to investigate trends, although other factors affecting beach counts, particularly  
363 annual differences in timing of breeding and participation levels may complicate their  
364 use in the absence of additional surveys. Counts from individual years should not be  
365 relied upon because of annual differences, but assessment over multiple years is  
366 expected to smooth any variation between years.

367 On Bowen Island, records were kept of penguin counts conducted at different times of  
368 year, but always at Penguin Beach (e.g. Fortescue 1991; Fortescue 1999). To  
369 investigate available data for evidence of long-term trends, only records from the  
370 second half of the breeding season (October to December, Table 1) were used to  
371 achieve comparability with our sampling period (Table 3).

372 On Lion Island, a little penguin research program was started in 1990 and the colony  
373 was monitored intermittently until 2007. In 2013, the National Parks monitoring  
374 program was revived and penguin counts are again conducted once a year, in early  
375 December (Vera O'Donovan, pers. comm.).

376 Nightly count records from the peak breeding period (October to December) were  
377 averaged and plotted against year of data collection for both islands. Trends were  
378 determined by fitting a straight line of best fit to the data, using MICROSOFT EXCEL 2010.  
379 Post-hoc power analysis was used to determine the power of the data to show a trend,

380 using sample sizes and Pearson product-moment correlation coefficient  $r$  at a  
381 significance level of  $\alpha = 0.05$ . Beach counts only yield an index of relative abundance  
382 rather than an absolute estimate, so the percentage of the colony using the main  
383 beach was calculated based on earlier, comprehensive surveys conducted on both  
384 islands, and used to infer the population size of penguins during 2012 and 2013.

385 **Mark-resight method**

386 Mark-resight methods were used to estimate the number of penguins using the main  
387 landing site at each island, using the same data that were collected for estimation of  
388 survival rates based on mark-recapture methods. The size of the population using the  
389 main beach in 2012 to 2014 was computed in program MARK, version 7.1 (White &  
390 Burnham 1999) using the mixed logit-normal mark-resight model (McClintock et al.  
391 2009) for sampling during sighting occasions without replacement (i.e., individuals may  
392 only be sighted once per distinct occasion), and a known number of marked individuals  
393 in the population available for resighting. A number of different models were  
394 investigated, with different constraints put on the following parameters: resighting  
395 probability  $p$ , individual heterogeneity level  $\sigma(\text{sigma})$  and population size  $N$ . These  
396 parameters were: constant over time (.) $; allowed to vary between years but be$   
397 constant within season ( $S$ ) $; to vary freely over time ( $t$ ) between primary occasions;$   
398 and a final treatment in which the resighting probability was allowed to vary between  
399 primary occasions and between secondary occasions within primary occasions ( $tt$ ).  
400 Models were excluded from further analyses if they did not reach non-suspect  
401 numerical convergence as per the program's internal assessment after three model  
402 runs.

403 Model selection from the full set of models was based on Akaike Information Criterion  
 404 (AIC) values (Akaike 1974). The AIC identifies the model that has the best fit with the  
 405 smallest number of parameters, thereby representing a compromise between lack of  
 406 bias (many parameters) and lack of precision (few parameters) (Burnham & Anderson  
 407 1992). The model with the lowest AIC value is assumed to be the best model because it  
 408 is most parsimonious given the data. Out of the models in the candidate model set, it  
 409 has the best fit with fewest parameters. Older estimates were used as cited in the  
 410 literature (Table 4). For comparison of older estimates to the ones obtained using  
 411 mark-resight methods, estimates from 2012 to 2014 were analysed for inclusion of the  
 412 older estimates in their 95 % confidence interval if variances were not known for older  
 413 estimates.

414

415 **Table 4:** Summary of mark-recapture or mark-resight population size estimates and  
 416 methods

<b>Island</b>	<b>Year</b>	<b>Estimate</b>	<b>Estimation method</b>	<b>Number of survey dates</b>	<b>Source</b>
<b>Bowen Island, Penguin Beach</b>	1988	320	Beach counts/burrow density*		Fortescue 1991
	2012	see results	Mark-Resight (McClintock et al. 2009)	9	This study
	2013			9	
	2014			3	
<b>Lion Island, Main Beach</b>	1992	172	Mark-Recapture (Schumacher & Eschmeyer 1943)	5	Cunningham et al. 1993
	1994	209	Mark-Recapture (Schumacher & Eschmeyer 1943)	5	Rogers, unpubl. data
	2012	see results	Mark-Resight (McClintock et al. 2009)	4	This study
	2013			7	
	2014			4	

417 \*not based on mark-recapture

418 **Survival**

419 **Non-invasive method**

420 A novel, non-invasive method was trialled to assess its suitability for estimation of  
421 survival rates of burrow-nesting sea birds. On Bowen Island, a random sample of 44  
422 natural, active penguin burrows were identified based on presence of at least one  
423 penguin and marked during the first visit to the island in September 2012. Only easily  
424 accessible parts of the island were checked to minimise disturbance to breeding  
425 penguins and prevent caving-in of burrows. Marked burrows were subsequently  
426 checked for occupancy, indicated by visible penguin presence, calls, or smells and  
427 recent scats during the second half of the breeding season in 2012 to 2014. On Lion  
428 Island, only 14 active burrows could reliably be identified and monitored throughout  
429 the duration of the project. Occupancy checks consisted of visual checks, followed by  
430 tactile checks with the researcher's outstretched arm. To confirm species identity of  
431 burrow occupants that could only be felt and not seen, they were pulled from the  
432 burrow and returned after successful identification. On both islands, most burrows  
433 were too deep to repeatedly pull out penguins, so individual identification of burrow  
434 occupants was not possible. For the hierarchical site-occupancy model, a burrow was  
435 considered active if it was found to be occupied on at least one day per visit.

436 The burrow occupancy model was run using the R statistical software package version  
437 3.0.2. (R Development Core Team 2010) with the package R2WINBUGS and WINBUGS  
438 version 1.4.3 (Lunn et al. 2000). Estimates of survival-BO based on Roth & Amrhein

439 (2010) were compared to the mark-recapture estimates of survival-CJS to assess the  
440 reliability of the non-invasive method.

441 **Mark-recapture method**

442 Two different mark-recapture methods were used to estimate apparent survival as  
443 survival-CJS and survival-RD. Additionally, CJS modelling was used as a reference  
444 method to assess the performance of the non-invasive method at estimating survival-  
445 BO.

446 Assumptions for the CJS and robust design models were discussed in the introduction.  
447 Assumption (ii), that every marked animal present in the population during a sampling  
448 period has the same probability of survival until the following sampling period, might  
449 be violated due to age-dependent survival in little penguins, but differences in survival  
450 after first breeding are low (Sidhu et al. 2007). Assumption (iii), which requires that  
451 marks are neither lost nor overlooked, and are recorded correctly, is realistic for two  
452 reasons. Firstly, the PIT-tags used in this study have a unique code and the hand-held  
453 reader used to detect them can be used at a distance, regardless of the angle of scan,  
454 light or environmental conditions. Secondly, tag loss rates have been estimated at 4%  
455 in the first year after tagging and 1% in subsequent years (Sidhu et al. 2011).  
456 Assumption (iv), that resampling is instantaneous, seems reasonable because of the  
457 use of successive nights as sampling periods. Furthermore, recaptured animals were  
458 released immediately after their tag number was recorded. To address assumption (v),  
459 that all emigration from the sampled area is permanent, CJS estimates were calculated

460 with and without pooled capture histories (see section about pooling of capture  
 461 histories below) to make them comparable to robust design estimates.

462 Only apparent survival (the probability that an individual survives and returns to the  
 463 same study site, survival-CJS) can be estimated with CJS modelling, because it cannot  
 464 distinguish between death and emigration from the population.

465 Based on CJS modelling, various models constraining the constancy of parameters for  
 466 apparent survival  $\Phi$  and recapture probability  $p$  (analogous to resighting probability  
 467 in mark-resight models) have been introduced independently by several authors (see  
 468 Lebreton & Burnham 1992). Here, I consider three levels of variation for survival and  
 469 recapture probability: none (.), where parameters are constant during the study  
 470 period; seasonal ( $S$ ), where parameters are constant within each breeding season, but  
 471 different between breeding seasons, when penguins were not observed; or fully time-  
 472 dependent ( $t$ ) with a different parameter for each primary interval (set of models  
 473 summarised in Table 5).

474 **Table 5:** Model parameters for Cormack-Jolly-Seber Models

475  $\Phi$  – apparent survival,  $p$  – recapture probability, ( $t$ ) – parameter fully time-  
 476 dependent, ( $S$ ) – parameter varies seasonally, (.) – parameter constant over study  
 477 period

Model description	Apparent survival	Recapture Probability
Apparent survival fully time dependent	$\Phi(t)$	$p(t)$ $p(S)$ $p(.)$
Apparent survival varies seasonally	$\Phi(S)$	$p(t)$ $p(S)$ $p(.)$
Constant apparent survival	$\Phi(.)$	$p(t)$ $p(S)$ $p(.)$

478

479 The robust design model allows estimation of a large number of important  
480 demographic parameters, including estimates of the immigration ( $\gamma'$ ) and emigration  
481 ( $\gamma''$ ) parameters and true survival  $S$ . Here,  $\gamma'_i$  is the probability that given that an  
482 individual was not in the sample at time ( $i-1$ ), that is also not present (i.e., not in the  
483 sample) at time ( $i$ ), and  $\gamma''_i$  is the probability of temporarily emigrating from the sample  
484 between sampling occasions ( $i - 1$ ) and ( $i$ ), and its complement ( $1 - \gamma''_i$ ) is the  
485 probability of remaining in the sample between sampling occasions ( $i - 1$ ) and ( $i$ ).

486 For the robust design models, full likelihood models for capture probability  $c$  and  
487 recapture probability  $p$  were used for the closed captures, where the population size  $N$   
488 is included in the likelihood. Models include random or Markovian probability of  
489 movement between ‘availability states’ (PMA, Markovian: constant probabilities for  $c$   
490 and  $p$ ,  $c_i = p_i = \text{const.}$  for each primary occasion  $i$ ). Markovian models lead to  
491 identifiability problems for estimates of survival parameters  $S$  and movement  
492 parameters  $\gamma$ , unless constraints are introduced (Cooch & White 2014). Constraints  
493 include assuming constant survival rate ( $S(.)$ ) – Models MarkovS(.)) and setting the last  
494 two movement parameters to be equal ( $\gamma'_k = \gamma'_{k-1}$ , and  $\gamma''_k = \gamma''_{k-1}$  – models  
495 MarkovGamma).

496 Models also included random movement (where immigration ( $\gamma'$ ) = emigration ( $\gamma''$ ))  
497 and no movement ( $\gamma' = 1$ ,  $\gamma'' = 0$ ) models, each with the same three levels of variability  
498 for survival rates  $S$  as described for CJS models (constant (.), seasonal ( $S$ ), or fully time-  
499 dependent ( $t$ )). The full set of models that were compared is summarised in Table 6.

500

501 **Table 6:** Model parameters for Robust Design Models

502  $\gamma'$  – immigration,  $\gamma''$  – emigration,  $S$  – survival,  $(t)$  – parameter fully time-dependent,  
 503  $(S)$  – parameter varies seasonally,  $(.)$  – parameter constant over study period

PMA	Constraints	Survival	Abbreviation
<b>Markovian</b>	$\gamma'_k = \gamma'_{k-1}$ , and $\gamma''_k = \gamma''_{k-1}$	$S(t)$	MarkovGammaS(t)
	$\gamma'_k = \gamma'_{k-1}$ , and $\gamma''_k = \gamma''_{k-1}$	$S(S)$	MarkovGammaS(S)
	$\gamma'_k = \gamma'_{k-1}$ , and $\gamma''_k = \gamma''_{k-1}$	$S(.)$	MarkovGammaS(.)
	Random Movement $\gamma' = \gamma''$	$S(t)$	MarkovRandomMovementS(t)
	Random Movement $\gamma' = \gamma''$	$S(S)$	MarkovRandomMovementS(S)
	Random Movement $\gamma' = \gamma''$	$S(.)$	MarkovRandomMovementS(.)
	No Movement $\gamma' = 1$ , $\gamma'' = 0$	$S(t)$	MarkovNoMovementS(t)
	No Movement $\gamma' = 1$ , $\gamma'' = 0$	$S(S)$	MarkovNoMovementS(S)
	No Movement $\gamma' = 1$ , $\gamma'' = 0$	$S(.)$	MarkovNoMovementS(.)
<b>Random</b>	Random Movement $\gamma' = \gamma''$	$S(t)$	RandomMovementS(t)
	Random Movement $\gamma' = \gamma''$	$S(S)$	RandomMovementS(S)
	Random Movement $\gamma' = \gamma''$	$S(.)$	RandomMovementS(.)
	No Movement $\gamma' = 1$ , $\gamma'' = 0$	$S(t)$	NoMovementS(t)
	No Movement $\gamma' = 1$ , $\gamma'' = 0$	$S(S)$	NoMovementS(S)
	No Movement $\gamma' = 1$ , $\gamma'' = 0$	$S(.)$	NoMovementS(.)

504

505 **Pooling of capture histories for CJS and robust design modelling**

506 Due to likely temporary emigration from the breeding colony occurring later in the  
 507 breeding season, the robust design estimates are likely to be biased because the  
 508 closure assumption is violated. Capture histories were therefore pooled when the  
 509 number of penguins dropped towards the end of the season, meaning a penguin was  
 510 considered present during the last recapture occasion before the start of the drop in  
 511 numbers if it was encountered during or after this occasion. This is to ensure that  
 512 unbiased estimators are obtained following the “emigration only” scenario in Kendall  
 513 1999. Immigration was assumed to be negligible during the second half of the breeding  
 514 season.

515 Survival rates were estimated using CJS and robust design models implemented in  
516 program MARK, version 7.1 (White & Burnham 1999). Model selection was again based  
517 on AIC values. The AIC value was also taken into account to determine which  
518 parameters to use for further analyses. Following the rules of thumb suggested by  
519 Burnham & Anderson (1992), when the difference in AIC between two models  
520 ( $\Delta AIC$ ) is  $< 2$ , both models have similar ability to explain in the data. If  
521  $2 < \Delta AIC < 7$ , then there is considerable support for a real difference between the  
522 models, and if  $\Delta AIC > 7$ , then there is strong evidence to support the conclusion of  
523 differences between the models. For the first case ( $\Delta AIC < 2$ ), parameter estimates  
524 were reported for all models equally supported by the data. The AICc Weight (called an  
525 Akaike Weight by Burnham & Anderson 1998), the likelihood of the models and their  
526 respective deviance values are also reported. The sum of all AICc weights is 1. These  
527 weights can be used for model averaging. The likelihood of a model (Model Likelihood)  
528 is the AICc Weight for the model of interest divided by the AICc Weight of the best  
529 model. This value is the strength of evidence for this model relative to other models in  
530 the set of models considered, and is the reciprocal of the evidence ratio. Deviance is  
531 defined as the difference in  $-2\log(Likelihood)$  of the current model and  $-$   
532  $2\log(Likelihood)$  of the saturated model, where the saturated model is the model with  
533 the number of parameters equal to the sample size.

534 Estimates of apparent survival based on CJS modelling (survival-CJS) are directly  
535 comparable to the estimate derived from burrow occupancy (survival-BO, see above).  
536 Robust design models, on the other hand, estimate survival and emigration  
537 independently. Survival-RD is therefore not directly comparable to either survival-BO

538 or survival-CJS. To assess the suitability of the non-invasive method to estimate  
539 survival-BO, it was compared to survival-CJS using an unpaired t-test in the GRAPHPAD  
540 software online QuickCalcs (<http://www.graphpad.com/quickcalcs/>).

541

542 ***Comparison of mark-recapture and non-invasive methods***

543 Survival estimates using mark-recapture and burrow occupancy records were obtained  
544 in the same years (2012-2014) during the same visits to the two islands, Lion Island  
545 and Bowen Island. This allows direct comparison of these estimates as confounding  
546 factors are minimised.

547 Population trends were also obtained from the same two islands, and data collected  
548 during 2012-2014 were taken during the same visits, while beach counts spanned  
549 more years than population size estimates. These additional data were included  
550 although they may compromise comparability of estimates, because higher  
551 fluctuations were expected for beach counts compared to population estimates.

552 **Results**

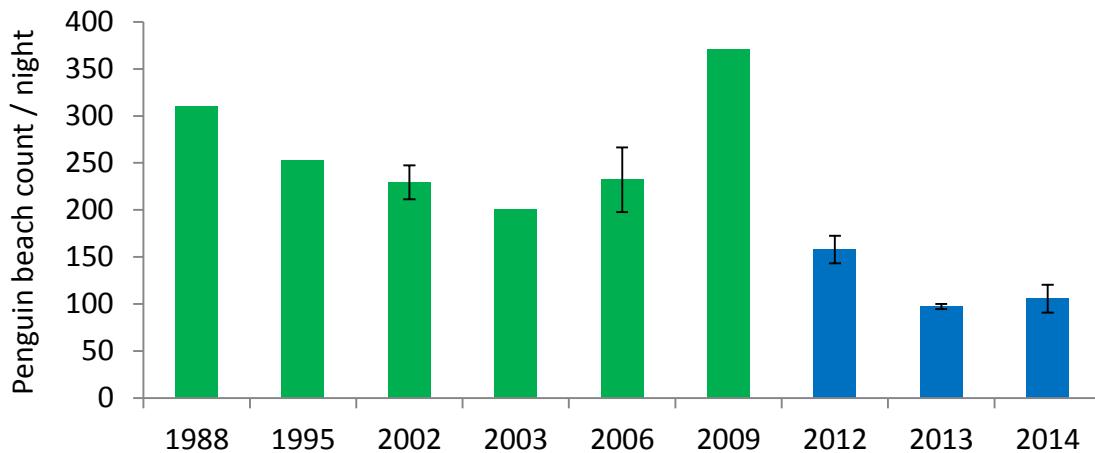
553 ***Population sizes***

554 **Non-invasive method**

555 Trends in population sizes were investigated based on beach counts of a sample of the  
556 population that used the main landing beach on each island (Fig. 4 and 5). The records  
557 for Bowen Island show significant interannual variability in penguin counts, which  
558 could be related to variable timing of breeding and a mismatch between peak breeding  
559 and timing of beach counts, or a shift in habitat use towards other parts of the island.

560 The latter does not seem likely, as the Bowen Island colony already supported the  
561 highest burrow density reported for the species (Dann et al. 1996; Fortescue 1999). It  
562 is therefore unlikely that any of the habitats on Bowen Island could now support an  
563 even higher density of penguins. There appears to be a declining trend of penguins  
564 landing at Penguin Beach during the three years this study was conducted, with the  
565 lowest numbers of penguin arrivals recorded during 2013. A linear trendline fitted to  
566 the data had a negative slope of -6.0, indicating that the relative abundance decreased  
567 by 6 penguins per year (or 6 % of the abundance in 2013). Statistical analysis revealed  
568 a non-significant correlation with a Pearson's r value of -0.586. A power analysis  
569 showed that power was only 0.536, so a possible correlation might have gone  
570 undetected.

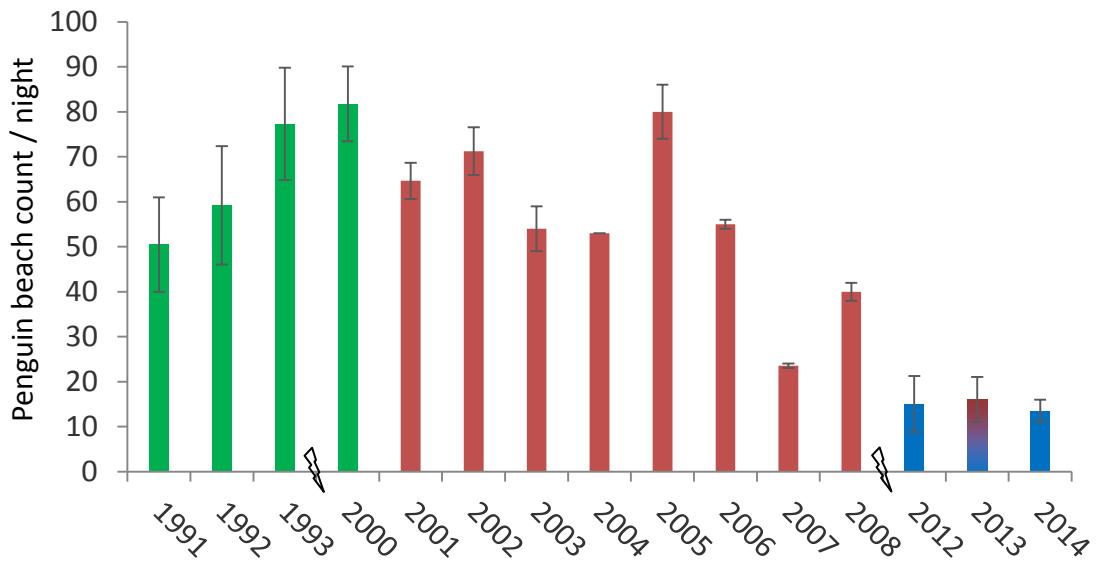
571 The penguin population on Bowen Island was estimated to be around 7 000 breeding  
572 pairs in the 1990s (Fortescue 1995) based on occupied burrow densities. Beach counts  
573 were roughly twice as high then as in 2012-14. If beach counts were representative of  
574 the whole colony, a current abundance estimate would be around 3500 breeding pairs.  
65



575

576 **Fig. 4:** Beach counts of penguins arriving on Penguin Beach, Bowen Island, at night  
 577 during the breeding season (counts conducted between October and  
 578 December); Green – Booderee National Park surveys;  
 579 Blue – surveys conducted as part of this study; error bars represent  $\pm$  standard  
 580 deviation (SD) and are only available for surveys with more than one count  
 581 conducted during that year; x-axis not scaled. A linear trendline fitted to the  
 582 time-scaled data had a negative slope of 6.0, with a Pearson's r value of 0.586  
 583 and a power of 0.536.

584 On Lion Island, a decrease in nightly penguin arrivals has been detected (Fig. 5). The  
 585 slope of a linear trendline fitted to all available records is -2.26, but given that counts  
 586 were increasing in the 1990s, a trend was also investigated for records after 2000 only.  
 587 The slope increased to -4.81, indicating a similar loss of numbers per year to that of  
 588 Bowen Island, which is more dramatic in the smaller population, where 5 individuals  
 589 correspond to almost one third of the number of penguins encountered each night in  
 590 2013. According to the analyses conducted, the Pearson's r was significant at -0.688  
 591 with a power of 0.914. A decrease in penguin numbers on Lion Island was also  
 592 evidenced by the low number of active penguin burrows found compared to earlier  
 593 surveys where there were 14 active burrows between in this study compared with an  
 594 average of 46 active burrows (range from 33 to 60 active burrows, Appendix Table 1A)  
 595 over the eight years monitored between 1990 and 1998 (Knight & Rogers 2004;  
 596 Rogers & Knight 2006).



597

598 **Fig. 5:** Beach counts of penguins arriving on the main beach, Lion Island, at night  
 599 during the breeding season (counts conducted between October - December)  
 600 Green – Taronga Zoo surveys, Red – NPWS surveys, Blue – surveys conducted  
 601 as part of this study; note breaks in scaling of x-axis. The slope of a linear  
 602 trendline fitted to all available records is -2.26, and -4.81 for records after 2000  
 603 only. For all records, Pearson's r is -0.688 with a power 0.914.

604

## 605 **Mark-resight**

606 For Bowen Island, the model with time-dependent resighting probability, constant  
 607 heterogeneity level and time-dependent variation of the population size ( $p(t)$   $\sigma(t)$   $N(t)$ )  
 608 best represented the mark-resight data based on the AIC value (Table 7). Parameter  
 609 estimates for this model are summarised in Table 8.

610 **Table 7:** Model results for the best six mark-resight models – Bowen Island

611  $p$  – resighting probability,  $\sigma$  – individual heterogeneity level,  $N$  – population size

612 ( $tt$ ) – parameter fully time-dependent, ( $t.$ ) parameter varies among primary occasions,

613 ( $S$ ) – parameter varies seasonally, (.) – parameter constant over study period

Model	AICc	Delta AICc	AICc Weights	Model Likelihood	Number of Parameters	Deviance
$p(t.) \sigma(.) N(t)$	1075.9	0	0.814	1	15	1045.45
$p(t.) \sigma(S) N(t)$	1079.2	3.291	0.157	0.193	17	1044.62
$p(t.) \sigma(S) N(S)$	1083.8	7.904	0.016	0.019	13	1057.46
$p(t.) \sigma(S) N(.)$	1084.2	8.299	0.013	0.016	11	1061.95
$p(tt) \sigma(.) N(t)$	1090.0	14.086	0.001	0.001	29	1030.33
$p(tt) \sigma(S) N(t)$	1093.2	17.322	0.000	0.000	31	1029.33

614

615

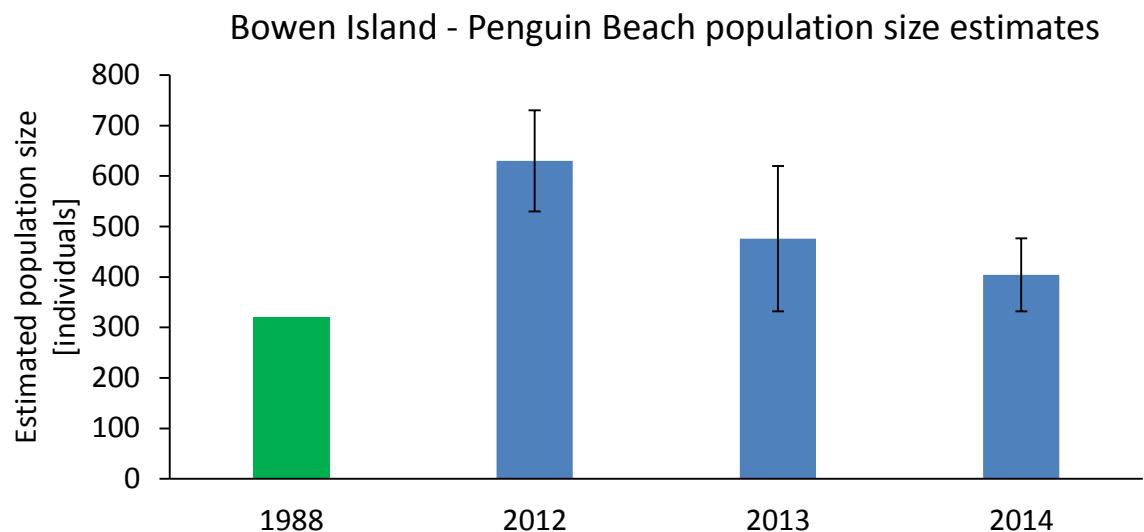
616 **Table 8:** Estimates of number of penguins  $N$  using Penguin Beach on Bowen Island,  
617 based on mark-resight model  $p(t.) \sigma(.) N(t)$ , with standard errors, 95 % confidence  
618 interval (CI) and yearly averages; Confidence intervals that did not include Fortescue's  
619 1988 estimate of 320 penguins using Penguin Beach are highlighted in bold.

Date	N	SE(N)	95% CI		Year	N (Yearly Average)	SE(N)
			Lower	Upper			
October 2012	438.54	46.66	<b>357.33</b>	<b>541.28</b>	2012	629.99	100.35
Early November 2012	725.50	120.02	<b>528.20</b>	<b>1004.37</b>			
Late November 2012	725.94	134.36	<b>509.62</b>	<b>1044.30</b>			
October 2013	372.83	58.37	277.20	508.89	2013	475.90	144.08
November 2013	614.33	140.03	<b>399.61</b>	<b>961.32</b>			
December 2013	440.55	233.85	182.32	1206.55			
October 2014	404.47	72.39	288.58	576.89	2014	404.47	72.39

620

621 Mark-resight estimates of the size of the population using Penguin Beach were  
622 compared to Fortescue (1988)'s estimate based on burrow densities and beach counts  
623 (Fig. 6). Despite differences in estimation methods, the estimates for the last two years  
624 differ by less than 30 % from Fortescue's estimate, and the estimate for 2012 is less  
625 than twice as high. No significant differences in penguin abundance could be detected  
626 between the three survey years of this study (unpaired t-test,  $t = 1.334$ ,  $p = 0.253$ ). All  
627 three estimates for 2012 resulted in a confidence interval that was above Fortescue's  
68

628 1988 estimate of 320 individuals, whereas only one of the 2013 estimates (November)  
 629 did not overlap with 320 (Table 8). The most recent estimate from October 2014 is also  
 630 not different from the 1988 estimate.



631  
 632 **Fig. 6:** Estimates of the size of the population using Penguin Beach, based on burrow  
 633 densities and beach counts (green, Fortescue 1988) and Mark-Resight (blue,  
 634 this study); error bars represent  $\pm$  standard error

635  
 636 On Lion Island, the mark-resight model best representing the data had time-dependent  
 637 resighting probability, annual variation in individual heterogeneity level and time-  
 638 dependent variation of the population size ( $p(t)$ )  $\sigma(S)$   $N(t)$ , Table 9). The parameter  
 639 estimates for this model are summarised in Table 10.

640

641 **Table 9:** Model results for the best six mark-resight models – Lion Island

642  $p$  – resighting probability,  $\sigma$  – individual heterogeneity level,  $N$  – population size  
 643  $(tt)$  – parameter fully time-dependent,  $(t.)$  or  $(t)$  parameter varies among primary occa-  
 644 sions,  $(S)$  – parameter varies seasonally,  $(.)$  – parameter constant over study period

Model	AICc	Delta AICc	AICc Weights	Model Likelihood	Num. Par	Deviance
$p(t.) \sigma(S) N(t)$	988.837	0	0.833	1	19	950.063
$p(t.) \sigma(.) N(t)$	993.610	4.773	0.077	0.092	18	956.914
$p(t.) \sigma(t) N(t)$	994.686	5.850	0.045	0.054	23	947.557
$p(tt) \sigma(S) N(t)$	995.758	6.921	0.026	0.031	29	935.968
$p(S) \sigma(t) N(t)$	997.151	8.314	0.013	0.016	16	964.599
$p(tt) \sigma(t) N(t)$	1000.113	11.276	0.003	0.004	33	931.795

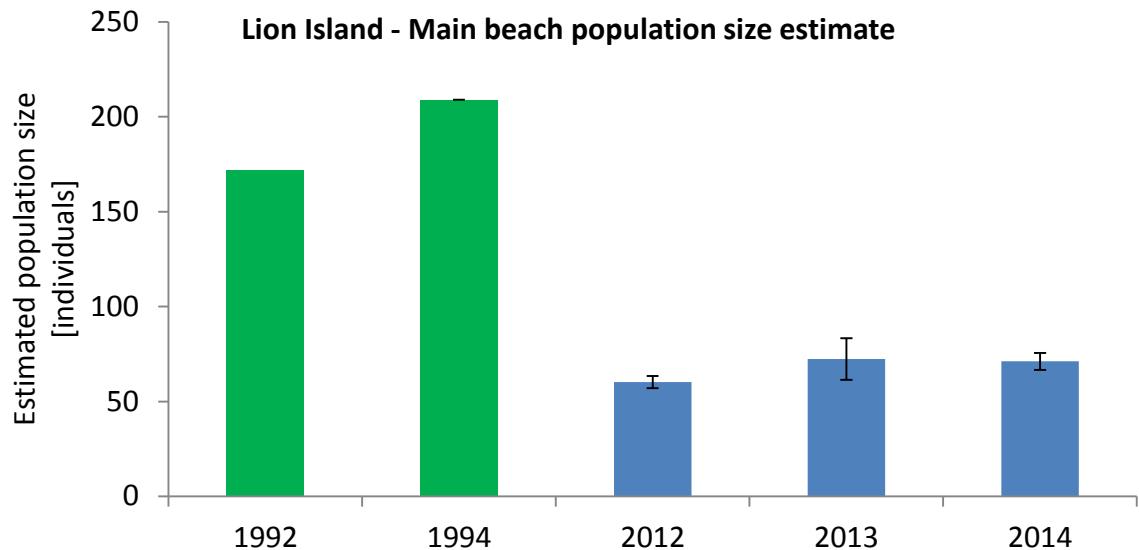
645

646 **Table 10:** Estimates of number of penguins  $N$  using the main beach on Lion Island,  
 647 based on mark-resight model  $p(t.) \sigma(S) N(t)$ , with standard error; yearly averages  
 648 include values from October to December only; confidence intervals that did not  
 649 include mark-recapture estimates from 1992 (172) and 1994 (209) highlighted in bold.

Date	N	SE(N)	95 % CI		Year	N(Yearly Average)	SE(N)
			Lower	Upper			
October 2012	52	0	<b>52</b>	<b>52</b>	2012	60.21	3.23
November 2012	69.14	6.18	<b>60.64</b>	<b>86.02</b>			
December 2012	59.48	3.50	<b>55.12</b>	<b>69.91</b>			
September 2013	58.28	4.76	<b>53.67</b>	<b>75.54</b>	2013	72.41	10.97
October 2013	68.65	5.27	<b>61.09</b>	<b>82.51</b>			
November 2013	77.90	11.30	<b>63.42</b>	<b>110.72</b>			
December 2013	70.69	16.33	<b>56.27</b>	<b>133.81</b>			
Early October 2014	73.09	5.84	<b>65.93</b>	<b>90.66</b>	2014	71.17	4.44
Late October 2014	69.25	3.04	<b>66.21</b>	<b>79.96</b>			

650

651 Estimates of the size of the population using the main (south-western) beach on Lion  
 652 Island were derived from a mark-recapture study started in 1992 (Cunningham et al.  
 653 1993) and completed by Rogers et al., 1994, where all penguins encountered were  
 654 marked, and compared to the results of the present study (Fig. 7). There was a  
 655 significantly higher abundance of penguins in 1992 and 1994 than during this study,  
 656 with none of the confidence intervals overlapping with the older estimates, but no  
 657 further drop in numbers was observed between 2012 and 2014.



658

659 **Fig. 7:** Estimates of the size of the population using Lion Island's main beach, based on  
 660 the formula by Schumacher & Eschmeyer (1943) – (green, Cunningham et al.  
 661 1993; Rogers & Eldershaw 1995) and using the mark-resight method (blue, this  
 662 study)

663

#### 664 ***Survival***

##### 665 **Non-invasive method**

666 Apparent survival rates (survival-BO) were estimated from burrow occupancy surveys  
 667 for Bowen Island and Lion Island. Due to the limited number of burrows that could  
 668 reliably be monitored, both estimates of survival-BO have large, but similar standard  
 669 deviations (Table 11). Mean apparent survival is significantly higher on Bowen Island  
 670 than Lion Island (two-sample t-test with equal variances: T-value = 3.448, p = 0.001).

671 **Table 11:** Estimate of apparent survival based on burrow occupancy, survival-BO; and  
 672 model input parameters

	Apparent Survival	SD	Number of territories (= burrows)	Number of years	Number of visits per year
<b>Bowen Is</b>	0.7643	0.2603	44	2	3
<b>Lion Is</b>	0.4925	0.2452	14	2	4

673    **Mark-recapture method**

674    The most likely CJS model for Bowen Island is seasonal variation in apparent survival  
 675    with time-dependent recapture probability ( $\Phi(S)p(t)$ , Table 12), whereas the most  
 676    likely model for Lion Island was identified to be constant apparent survival  $\Phi$  and  
 677    time-dependent recapture probability  $p$ , i.e.  $\Phi(.)p(t)$  (Table 13). Because  $\Phi(S)p(t)$  is  
 678    within  $\Delta AIC < 2$  of the best CJS model for Lion Island, results for this model were  
 679    also reported. The CJS model best fitting pooled capture histories is constant apparent  
 680    survival with seasonal variation in recapture probability -  $\Phi(.)p(S)$  for Lion Island; and  
 681     $\Phi(S)p(t)$  for Bowen Island, the same model that best fit unpooled capture histories for  
 682    that island.

683

684    **Table 12:** Model results for CJS models – Bowen Island including best model for pooled  
 685    capture histories (see robust design method). Models with fully time-dependent  
 686    apparent survival did not reach numerical convergence.

687     $\Phi$  – apparent survival,  $p$  – recapture probability,  $(t)$  – parameter fully time-  
 688    dependent,  $(S)$  – parameter varies seasonally,  $(.)$  – parameter constant over study  
 689    period

<b>Model</b>	<b>AICc</b>	<b>Delta AICc</b>	<b>AICc Weights</b>	<b>Model Likelihood</b>	<b>Number of Parameters</b>	<b>Deviance</b>
$\Phi(S)p(t)$	832.394	0	1	1	22	499.502
$\Phi(S)p(.)$	863.460	31.066	0	0	4	570.844
$\Phi(S)p(S)$	865.456	33.062	0	0	6	568.658
$\Phi(.)p(.)$	891.818	59.425	0	0	2	603.317
$\Phi(.)p(S)$	895.648	63.254	0	0	4	603.032
<hr/>						
Pooled - $\Phi(S)p(t)$	809.391		0.999	1	20	481.136

690   **Table 13:** Model results for CJS models – Lion Island including best models for pooled  
 691 capture histories (see robust design method). Models with fully time-dependent  
 692 apparent survival did not reach numerical convergence.

693   *Phi* – apparent survival, *p* – recapture probability, (*t*) – parameter fully time-  
 694 dependent, (*S*) – parameter varies seasonally, (.) – parameter constant over study  
 695 period

	<b>Model</b>	<b>AICc</b>	<b>Delta AICc</b>	<b>AICc Weights</b>	<b>Model Likelihood</b>	<b>Number of Parameters</b>	<b>Deviance</b>
Not pooled	<i>Phi(.)p(t)</i>	600.125	0	0.683	1	17	349.479
	<i>Phi(S)p(t)</i>	602.052	1.927	0.261	0.382	19	346.395
	<i>Phi(S)p(S)</i>	605.142	5.017	0.056	0.081	6	379.917
	<i>Phi(.)p(S)</i>	614.238	14.113	0.001	0.001	4	393.279
	<i>Phi(S)p(.)</i>	623.702	23.578	0.000	0	4	402.743
	<i>Phi(.)p(.)</i>	625.629	25.504	0	0	2	408.836
Pooled	<i>Phi(.)p(S)</i>	424.494	0	0.377	1	4	212.824
	<i>Phi(t)p(S)</i>	424.953	0.458	0.300	0.795	6	208.928
	<i>Phi(S)p(S)</i>	425.693	1.198	0.207	0.549	5	211.862

696

697

698   The parameter estimates from the best CJS models are summarised in Table 14. For  
 699   Bowen Island, parameters are listed for the first model in Table 12; for Lion Island  
 700   parameters are listed for the results of the first two models in Table 13, as well as the  
 701   results of the best models using pooled capture histories. The only estimates that were  
 702   significantly different from each other were those derived from the best model for Lion  
 703   Island using unpooled capture histories when compared to either of the two estimates  
 704   for Bowen Island, as well as the results from the best models for pooled capture  
 705   histories between Bowen and Lion Islands.

706

707   **Table 14:** Survival-CJS estimates of apparent survival based on best CJS models,  
 708   including T-test results for all pairs of estimates. T-values are presented below the  
 709   diagonal, corresponding p-values above the diagonal. Significant results ( $p < 0.05$ ) are  
 710   highlighted in bold.

	<b>Model</b>	<i>phi</i>	SE	BI	BI – p	<b>LI</b>		<b>LI – p</b>		
						1	2	1	2	3
<b>Bowen Island (BI)</b>	<i>Phi(S)p(t)</i>	0.701	0.098		0.535	<b>0.048</b>	0.383	0.152	0.425	0.395
<b>BI – pooled (p)</b>	<i>Phi(S)p(t)</i>	0.791	0.105	0.626		<b>0.025</b>	0.182	<b>0.046</b>	0.180	0.168
<b>Lion Island (LI)</b>	<i>Phi(. )p(t)</i>	0.453	0.069	<b>2.050</b>	<b>2.373</b>		0.841	0.740	0.311	0.371
	<i>Phi(S)p(t)</i>	0.498	0.212	0.884	1.370	0.202		0.975	0.788	0.818
<b>LI – pooled (p)</b>	<i>Phi(. )p(S)</i>	0.489	0.075	1.472	<b>2.092</b>	0.335	0.031		0.500	0.574
	<i>Phi(t)p(S)</i>	0.577	0.104	0.809	1.378	1.032	0.271	0.686		0.943
	<i>Phi(S)p(S)</i>	0.566	0.112	0.864	1.417	0.910	0.232	0.571	0.072	

711

712   For robust design modelling, Bowen Island was visited on seven primary occasions  
 713   (visits), four of which occurred in 2012 and three in 2013. In 2012, each of the visits  
 714   consisted of three secondary occasions (sampling days), whereas in 2013, their length  
 715   varied between two and four successive sampling days. There were a total of eight  
 716   visits to Lion Island, all but one three-night survey consisting of two sampling days  
 717   each. Pooling capture histories for sampling days after penguins started leaving the  
 718   colony resulted in a reduced number of 18 encounters in six primary occasions (Bowen  
 719   Island) and 11 encounters in five primary occasions (Lion Island), respectively.

720   For Bowen Island, four models had almost equal AIC ( $\Delta AIC < 2$ ), whereas unpooled  
 721   Lion Island data were equally well explained by two models, and pooled data by three.  
 722   The model indicating no movement ( $\gamma' = \gamma'' = 0$ ) best fit the data for both islands.  
 723   Unpooled capture histories were best explained by time-dependent survival rates for  
 724   Lion Island and seasonal variation in survival for Bowen Island (Table 15).

725   Using pooled capture histories, survival estimates increased significantly for Bowen  
 726   Island (two-sample t-test with equal variances, T-value = -3.059,  $p = 0.022$ ) relative to  
 74

727 the unpooled data, whereas Lion Island estimates are similar for pooled and unpooled  
 728 data (T-value = 0.043, p = 0.968).

729

730 **Table 15:** Model results for robust design models

731 Models that best explained both pooled and unpooled capture histories are  
 732 highlighted in bold; Models that allow comparison between the two islands based on  
 733 pooled capture histories are underlined

	Capture History	Model	AICc	Model Likelihood	S	SE
<b>Bowen Island</b>	Not pooled	<b>NoMovementS(S)</b>	<b>83.017</b>	<b>1</b>	<b>0.536</b>	<b>0.077</b>
		<b>MarkovNoMovements(S)</b>	<b>83.167</b>	<b>0.927</b>	<b>0.573</b>	<b>0.080</b>
		MarkovGammaS(t)	83.475	0.795	0.737	0.165
		MarkovRandomMovementS(S)	84.811	0.408	0.559	0.083
	Pooled	<b>MarkovNoMovements(S)</b>	<b>57.757</b>	<b>1</b>	<b>0.603</b>	<b>0.084</b>
		<b>NoMovementS(S)</b>	<b>58.394</b>	<b>0.727</b>	<b>0.565</b>	<b>0.080</b>
		MarkovNoMovementS(t)	58.634	0.645	0.614	0.113
		MarkovRandomMovementS(S)	59.346	0.452	0.635	0.085
<b>Lion Island</b>	Not pooled	<b>MarkovNoMovements(t)</b>	<b>48.309</b>	<b>1</b>	<b>0.586</b>	<b>0.202</b>
		NoMovementS(t)	50.514	0.37	0.509	0.198
	Pooled	<b>MarkovNoMovementS(S)</b>	<b>-64.389</b>	<b>1</b>	<b>0.538</b>	<b>0.104</b>
		MarkovNoMovements(.)	-63.371	0.601	0.482	0.074
		<b>MarkovNoMovements(t)</b>	<b>-63.283</b>	<b>0.575</b>	<b>0.615</b>	<b>0.139</b>

734

735 Comparison of non-invasive method and mark-recapture methods

736 Beach counts allowed us to investigate trends of a representative proportion of the  
 737 two penguin colonies over more than 20 years. The counts showed that Bowen Island  
 738 numbers were fluctuating and showing a declining trend, although this trend was not  
 739 significant and associated with a low statistical power (Fig. 4). Previous abundance  
 740 estimates for Penguin Beach on Bowen Island were based on different methodology to  
 741 mark-recapture, but match the results of this study and confirm that the penguin

742 population on Bowen Island is stable or increasing. This contradicts the apparent  
 743 declining trend of the beach counts.

744 Significant differences could be detected with high power between previous beach  
 745 counts on Lion Island and the results of the present study, with a steep decline in  
 746 numbers after 2007 (Fig. 5). Abundance estimates based on mark-recapture allowed  
 747 assessment of numbers of Lion Island main beach users in 1992 and 1994, and an  
 748 estimate of the same part of the population from 2012 to 2014. A similar trend can be  
 749 observed to the one based on beach counts, with a roughly four-fold drop in numbers.

750 For both islands, survival estimates were similar when comparing apparent survival  
 751 based on burrow occupancy (survival-BO) to apparent survival based on CJS modelling  
 752 (survival-CJS), but different from true survival accounting for emigration as calculated  
 753 using the robust design model (survival-RD, Table 16). Differences in apparent survival  
 754 between the two islands could be detected with statistical significance between both  
 755 survival-BO and survival-CJS, whereas true survival-RD was similar between the two  
 756 islands.

757 **Table 16:** Survival estimates for Bowen and Lion Islands based on three different  
 758 methods

	Survival-BO	Survival-CJS <i>Phi(S)p(t)</i>	Survival-RD Pooled
Bowen Island	0.764	0.701	0.603
Lion Island	0.493	0.498	0.538
T-value	3.448	2.050	0.485
p-value	0.001	0.048	0.629

759

760 **Discussion**

761 ***Abundance***

762 Mark-resight analyses showed that about 500 penguins were using Penguin Beach on  
763 Bowen Island (Fig. 6), whereas the abundance of penguins using the main beach on  
764 Lion Island is in the order of 60-70 individuals (Fig. 7). Based on the trends of beach  
765 counts (declining, Fig. 4) combined with estimates of the number of penguins using  
766 Penguin Beach (increasing, Fig. 6), the little penguin colony at Bowen Island appears to  
767 be stable. A possible explanation for the conflicting trends is that penguins might have  
768 to take longer foraging trips in some years than others, which would explain lower  
769 numbers in beach counts, while the total number of beach users stays stable or  
770 increases. Alternatively, the counts might have been conducted at different stages of  
771 the penguin breeding cycle. Even different proportions of birds in guard or post-guard  
772 stage could significantly alter the rate at which they cross the beach. Due to the lack of  
773 breeding data accompanying the earlier beach counts, this possibility cannot be  
774 excluded. However, beach counts were available for nine years, and the declining  
775 trend was mostly consistent except for one outlier in 2009, which might have been an  
776 unusual year for penguin breeding. A third possible explanation for these conflicting  
777 trends is that recent counts might not be comparable to previous beach counts for  
778 methodological reasons. A major difference was that in 2012-2014, penguins were  
779 intercepted using a mesh fence, checked for transponders and manually transferred  
780 from one side of the fence to the other, whereas earlier counts were completely non-  
781 invasive. This might have resulted in some penguins avoiding the main beach and  
782 coming ashore outside of the range of the survey.

783 The colony at Lion Island seems to be declining dramatically, with a roughly four-fold  
784 drop in numbers of both beach counts (Fig. 5) and abundance based on mark-resight  
785 (Fig. 7). Here, trends of abundance over time determined using the two different  
786 methodologies agree well. Ongoing monitoring of the colony will allow assessment of  
787 abundance of penguins on Lion Island into the future.

788 Beach counts alone can give an indication of how a percentage of the population is  
789 doing, but might be biased by changes in habitat utilisation both at sea (via foraging  
790 trip lengths) and on land (via shifts in breeding habitat) as well as interannual  
791 differences in the timing of breeding. Results of mark-resight modelling are similarly  
792 affected by the latter two factors, but revealed an opposing trend to beach counts on  
793 Bowen Island, while they agreed with the declining trend on Lion Island. In the short  
794 term, both the disturbance caused by fencing or capture and the insertion of  
795 transponders may have caused some drop in numbers (aversion, Dann et al. 2014).

796 ***Survival***

797 The annual apparent survival rate for penguins on Bowen Island is about 75 %  
798 (Table 16), which is very similar to an earlier estimate (72.8 %, Fortescue 1991) and  
799 within the range documented elsewhere (e.g. Dann 1992; Johannessen et al. 2003),  
800 particularly for the first year after tagging. The well-studied, currently stable colony at  
801 Phillip Island showed a similar penguin survival rate of 75 % during times high fox and  
802 dog depredation (Dann 1992), but higher survival of about 80% for birds fitted with  
803 transponders the previous year, and 91% for subsequent years at a time when the  
804 colony was recovering from a previous population decline (Dann et al. 2014;  
805 Sutherland & Dann 2014). Lion Island, on the other hand, has a very low apparent  
806 survival rate of only about 50 %, which is the lowest annual survival published for little  
807 penguins.

808 Mark-recapture estimates of survival-CJS agreed well with the results of the non-  
809 invasive method to estimate apparent survival as survival-BO in little penguins. This  
810 means that non-invasive studies of burrowing sea birds like penguins could replace  
811 mark-recapture based studies if burrow occupancy can be monitored for a large  
812 number of burrows over several visits per season. To further increase confidence in  
813 burrow occupancy data, one could follow the methods used by Sutherland & Dann  
814 (2012) on Philip Island to work out the best timing for such surveys for NSW colonies.

815 ***Replacing mark-recapture with non-invasive methods***

816 Survival estimates obtained using non-invasive methodologies (survival-BO) agree well  
817 with the results of CJS models (survival-CJS) obtained for the same years. For  
818 comparisons between years, the factors determining attendance ashore, particularly  
819 the timing of breeding as well as location and abundance of prey, would have to be  
820 comparable. In light of the importance of reducing disturbance to already declining  
821 colonies, replacing invasive methodology could help minimise the probability of  
822 already struggling colonies like Lion Island going extinct, while not compromising data  
823 quality.

824 However, it should be noted that survival estimates based on burrow occupancy are  
825 uncertain because they rely on a very limited number of natural burrows that could be  
826 reliably monitored during the course of this study. Natural burrows can have a high  
827 turnover rate and frequently collapse or get buried under debris. The proposed  
828 method would be more suitable to studies on colonies with a high density of artificial  
829 nest-boxes, which allow reliable monitoring with a detection probability close to unity,  
830 as well as offering better breeding resources for penguins than natural burrows  
831 (Perriman & McKinlay 1993) and improving survival rates of offspring as well as  
832 breeding productivity in poor breeding seasons (Sutherland et al. 2014).

833 Beach counts do not identify individuals and do not take possible differences of  
834 foraging trip lengths into account. If the same number of penguins continued to use  
835 the beach but spent a longer time at sea, beach counts would decrease while  
836 population size would stay the same. Beach counts alone are therefore a poor

837 indicator of population size when other factors like food availability or distance to  
838 foraging grounds are variable. A possible solution might be to combine beach counts  
839 with burrow density estimates and monitor differences in both over time.

840 While this paper focussed on using non-invasive methods for surveys of little penguins,  
841 beach counts can similarly be used for all the flightless penguin species, while burrow  
842 occupancy data might be useful to estimate survival rates in other burrow-nesting  
843 seabirds as well. Further studies are recommended to ensure applicability of these  
844 methods to other species.

845

846 ***Recommendations for penguin conservation***

847 The decline of the colony at Lion Island, which is the closest penguin colony to the  
848 endangered population at Manly in Sydney Harbour, could have profound impacts on  
849 that population. The colony at Manly is not isolated, either demographically or  
850 genetically, from other colonies in south-eastern Australia (Priddel et al. 2008) and in  
851 fact, four out of ten immigrants to the Manly colony were from Lion Island. The current  
852 situation seems similar to the early 1990s, when concerns over a large number of  
853 injured penguins brought into the Taronga Zoo Wildlife Clinic spawned a research  
854 programme to ensure long-term viability of the Lion Island colony (Cunningham et al.  
855 1993).

856 In contrast to Lion Island, the Manly colony currently appears to be stable (Lisa O'Neill,  
857 pers. comm.), which indicates that local rather than regional conditions affect penguin  
858 numbers. It could therefore be beneficial to develop bush regeneration program for  
81

859 Lion Island to remove invasive weeds and improve nesting habitat. Providing nest  
860 boxes for penguins has been shown to further improve breeding habitat while  
861 facilitating the monitoring of population dynamics.

862 Continued monitoring of tagged penguins from Lion Island could answer whether a  
863 significant proportion of penguins emigrate to neighbouring colonies including Manly,  
864 thus explaining some of the differences in trends at both sites. At Manly, monthly  
865 surveys of the whole known colony are continuing throughout the breeding season  
866 and all penguins are checked and fitted with transponders. Thus, detection probability  
867 of microchipped immigrants from Lion Island is high.

868

869

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1047 **Appendix 2**

1048 **Table 1A:** Summary of burrow checks on Lion Island

1049 (Knight & Rogers 2004; Rogers & Knight 2006)

Year	Number of burrows checked	Number of active burrows
1990	39	33
1991	56	39
1992	83	55
1993	70	55
1994	72	60
1995	80	39
1997	135	54
1998	112	29

1050

1051

1      **Formatted for publication in Conservation Genetics – Title Page**

2            **Chapter 3: Population genetics of Little Penguins**

3                    **(*Eudyptula minor*) in Australia**

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19      **Keywords:** *Fairy penguin, dispersal, metapopulation, microsatellites, mitochondrial*  
20      *DNA*

21      **Author Contributions:**

22      JJS and BLC provided samples and field data for WA, JJS also performed mtDNA  
23      analyses on WA samples. I (SV) performed laboratory analyses on all NSW samples,  
24      with the help of MRT for mtDNA laboratory analyses; I also performed all statistical  
25      analysis, and wrote the chapter.

26	<b>Contents</b>
27	Chapter 3: Population genetics of Little Penguins ( <i>Eudyptula minor</i> ) in Australia.... 89
28	Abstract..... 91
29	Introduction ..... 93
30	Materials and Methods..... 98
31	Results..... 107
32	Discussion..... 120
33	Acknowledgements..... 124
34	Conflict of Interest .....
35	References..... 125
36	Appendix 3 .....
37	

38    **Abstract**

39    Conservation of genetic diversity is important for the overall health of populations  
40    because decreased genetic variability can be associated with increased levels of  
41    inbreeding, reduced fitness and diminished evolutionary potential, leading to  
42    increased probability of extinction. The exchange of genetic diversity among  
43    neighbouring populations can be influenced by a suite of pressures that are  
44    particularly asymmetric at a species' range-edge, including geographic barriers,  
45    prevailing oceanic currents or winds, population sizes and reproductive rates. Here, we  
46    study several colonies at the range-edge of the little penguin (*Eudyptula minor*),  
47    populations of which have been declining in numerous locations. To ensure the  
48    species' long-term survival, it is essential to assess connectivity and dispersal among  
49    little penguin colonies. We thus investigated population structure and patterns of  
50    dispersal among eastern Australian colonies of little penguins. While colonies varied  
51    greatly in their census population size, smaller colonies were genetically not less  
52    diverse than larger ones. Limited genetic differentiation among colonies in New South  
53    Wales (NSW), however, seems to result in a genetic diversity that is maintained at a  
54    slightly lower level than closer to the centre of the penguin distribution. Isolation by  
55    distance was not observed within NSW, and weak at intermediate distances between  
56    NSW and South Australia. Differentiation was stronger at the maternally inherited  
57    mitochondrial marker than at the biparentally inherited microsatellites, which might  
58    indicate sex-biased dispersal driven by males. A complex interaction between the  
59    influence of oceanic currents and demography was furthermore suggested by  
60    contrasting dispersal estimates using different markers.

Chapter 3: Population genetics of Little Penguins  
(*Eudyptula minor*) in Australia

61

92

62    **Introduction**

63    Together with ecosystem and species variation, genetic variation is a fundamental  
64    level of biodiversity, and therefore essential to consider for biodiversity conservation  
65    (McNeely et al. 1990; Convention on Biological Diversity 1992). Conservation of genetic  
66    diversity is important for the overall health of populations because decreased genetic  
67    variability can be associated with increased levels of inbreeding, reduced fitness and  
68    diminished evolutionary potential, leading to increased probability of extinction  
69    (Newman & Pilson 1997; Eldridge et al. 1999; Frankham et al. 2014). This adaptive  
70    genetic variation is controlled by several factors, most importantly the size of the  
71    population, the genetic mutation rate and selective pressures acting on the  
72    population, as well as dispersal among populations of the same species. Minimum  
73    viable population sizes (MVP, Shaffer 1981) have been estimated in the order of  
74    several thousand individuals for 99% persistence over 40 generations in a typical  
75    outbreeding species (Traill et al. 2007), but debate is ongoing about the applicability of  
76    such general estimates and their use in conservation planning (Flather et al. 2011). The  
77    mutation rate, the fundamental source of genetic variation, is an evolved characteristic  
78    that is strongly influenced by the genetics of each organism, in addition to  
79    environmental influences (Altenberg 2011). Selective pressures can be manifold (e.g.  
80    artificial vs. natural selection, balancing vs. directional selection, sexual, parasite-  
81    mediated selection etc.), but can only act on a population if it contains genetic  
82    diversity – a prerequisite that can be compromised in small, isolated populations. Such  
83    isolation is underpinned by the philopatry that is commonly reported for sea birds  
84    including little penguins (Dann 1992).

85 Some biogeographic models furthermore predict that populations close to a species'  
86 range-edge occur at lower densities and exhibit greater variability of abundance  
87 compared to populations at the core of the distribution (Hanski 1991; Lawton 1993),  
88 which in turn affects their retention of genetic variation.

89 Studies of connectivity and dispersal among neighbouring populations that rely on  
90 marking individuals are hampered by low sample sizes of recovered individuals and the  
91 difficulty of differentiating between short-term visitors and breeders. Studies of  
92 genetic patterns, on the other hand, overcome these difficulties: selectively neutral  
93 genetic variation does not influence individual survival or reproduction, but can be  
94 used to infer dispersal rates among populations. At neutral genetic markers such as  
95 many microsatellites, the genetic structure across a species' distribution mirrors  
96 patterns of colonisation and dispersal and can provide insights into limits to the  
97 species' geographic range (Slatkin 1987; Bohonak 1999). Dispersal and the resulting  
98 gene flow will homogenise allele frequencies of genetic markers; signs of differences in  
99 allele frequencies (or occurrence of private alleles that are only found in one of the  
100 colonies) among neighbouring colonies therefore indicate limited gene flow (Slatkin  
101 1985; Slatkin 1987; Bohonak 1999). Quantification of differences in allele frequencies  
102 can furthermore be used to infer gene flow quantitatively, either as the number of  
103 dispersers or migrants per generation, or the average dispersal distance per individual  
104 (Wright 1943; Slatkin 1995; Spong & Creel 2001, but see Whitlock & McCauley 1999).

105 Such population genetic studies are usually based on markers that are not subject to  
106 natural selection, so-called neutral markers such as many microsatellites, some single  
107 nucleotide polymorphisms (SNPs) and possibly the mitochondrial control region  
108 sequences, to elucidate dispersal patterns and population history. Microsatellites are  
94

109 biparentally inherited, short tandem repeat regions that are typically co-dominant and  
110 assumed to be selectively neutral, e.g. do not affect fitness of individuals (Jarne &  
111 Lagoda 1996; Chambers & MacAvoy 2000). Due to their structure, they are fast-  
112 evolving and show a high level of intra-specific polymorphism. Microsatellites are  
113 commonly used to investigate kinship and population structure resulting from recent  
114 population history (Sunnucks 2000). Mitochondrial DNA (mtDNA) is only maternally  
115 inherited and regarded as an efficient tool for investigating phylogenetic patterns  
116 (Avise et al. 1987; Avise 1995) and disentangling different contributions to gene flow  
117 by the sexes (Prugnolle & de Meeus 2002). The hypervariable region I (HVRI) of the  
118 mitochondrial D-loop (control) region is characterised as non-coding and the most  
119 polymorphic part of the human mtDNA genome (Stoneking et al. 1991). However,  
120 mitochondrial DNA cannot be assumed to be neutral because of complete linkage of  
121 the mitochondrial genome (Ballard & Kreitman 1995; Meiklejohn et al. 2007).  
122 Additionally, the effect of sex-biased dispersal on the genetic structure of populations  
123 can be assessed by studying complementary genetic markers with different inheritance  
124 types, such as biparental nuclear microsatellites and matrilineal mitochondrial DNA  
125 (Scribner et al. 2001).

126 Patterns of gene flow can be influenced by a suite of pressures, many of which are  
127 particularly asymmetric at a species' range-edge, including prevailing oceanic currents  
128 or winds, climate, population sizes and reproductive rates. Here, we study several  
129 colonies at the range-edge of the little penguin (*Eudyptula minor*), which is the  
130 smallest penguin species and the only one breeding in mainland Australia. Their total  
131 abundance has been estimated as 350 000 – 600 000 individuals (Dann et al. 1996).

132 While declines in numbers at some colonies (e.g. Dann et al. 2000; Wiebkin 2011)  
133 might be balanced by increases in others (Sutherland & Dann 2014; Schumann et al.  
134 2013), the number of colonies has likely declined. One colony at Manly in Sydney  
135 Harbour (Fig. 1) has been listed as an endangered population (NSW National Parks and  
136 Wildlife Service 2000). Thus far, this colony at Manly has been managed as a single  
137 population that is separate from surrounding island colonies and it has successfully  
138 recovered from a few tens of breeding pairs to achieve the most successful breeding  
139 season in decades, with 85 breeding pairs in 2013 (Lisa O'Neill, pers. comm.). Despite  
140 this success, 85 breeding pairs are unlikely to be a sufficiently large population for  
141 long-term survival of an isolated colony as discussed above.

142 It is therefore essential to assess connectivity and dispersal of Little Penguin colonies in  
143 New South Wales (NSW, Fig. 1) to place the Manly colony in a broader context.  
144 Banding studies of Little Penguins (e.g. Dann 1992; Sidhu 2007; Priddel et al. 2008)  
145 have had limited success in elucidating connectivity and dispersal among neighbouring  
146 penguin colonies. Genetic studies have thus far focussed on different geographic scales  
147 and regions: On a range-wide scale, a study of mitochondrial DNA (mtDNA)  
148 phylogeography in Little Penguins suggested strong differences between individuals  
149 from Australia and the Otago Peninsula compared to the rest of New Zealand (Banks &  
150 Mitchell 2002; Banks et al. 2008). This pattern was supported by later increased  
151 sampling of Australian individuals (Overeem et al. 2008; Peucker (nee Mitchelson) et  
152 al. 2009). Within Australia, Overeem et al. (2008) found colonies of Little Penguins  
153 from around the border between NSW and Victoria (VIC) to Penneshaw on Kangaroo  
154 Island, South Australia (SA, Fig. 1), to be largely homogenous at microsatellites and a

155 mitochondrial marker. Only Penneshaw, the westernmost colony sampled in their  
156 study, showed significant differentiation from the other colonies, which lead to further  
157 research on this zone of elevated genetic structuring. It was found that the observed  
158 pattern represents a genetic cline, within which structuring exists over much shorter  
159 spatial scales than elsewhere (Burridge et al. 2015). Peucker et al. (2009) focussed on  
160 mitochondrial DNA only, and found phylogeographic structuring to be absent among  
161 Australian colonies despite non-random distribution of haplotypes among colonies.

162 To date, the small-scale genetic population structure of Little Penguins in NSW has not  
163 been addressed, and we will attempt to elucidate population structure and patterns of  
164 dispersal among Eastern Australian colonies of Little Penguins. In particular, we  
165 hypothesise that penguins disperse from the centre of their distribution around the  
166 Bass Strait, leading to predominantly northward dispersal in NSW (Fig. 1).  
167 Alternatively, major oceanic currents could drive the dispersal of penguins, which  
168 would lead to more dispersal southward than northward due to the directionality of  
169 the East Australian Current off the coast of NSW. We also expect effective and census  
170 population sizes to be lower and more variable towards the range edge of the species.  
171 Directionality of dispersal was tested using the program MIGRATE to estimate the  
172 number of migrants per generation based on microsatellites and mtDNA, while  
173 population sizes were assessed based on demographic surveys (Appendix Table 1A)  
174 and measures of genetic diversity, as well as the mutation-scaled population size  $\Theta$   
175 calculated by MIGRATE.

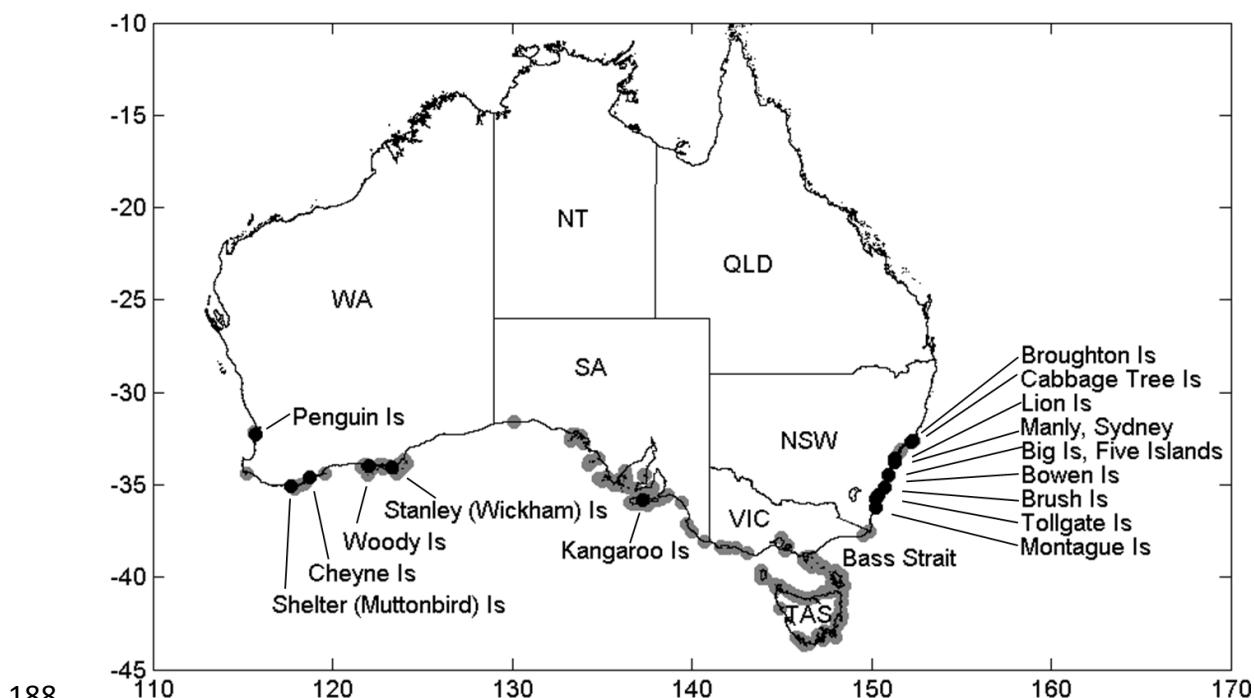
176

177 **Materials and Methods**

178 **Study sites**

179 Ten Little Penguin colonies, nine in NSW and one on Kangaroo Island in South  
180 Australia, were visited in 2012 or 2013 and genetic samples were collected from each  
181 (Fig. 1). Sampling sites were chosen so as to cover the northern range edge of Little  
182 Penguin distribution and include colonies of varying sizes and conservation status  
183 (Appendix Table 1A). Census population sizes for the sampled colonies were taken  
184 from the literature and as based on estimates obtained through demographic surveys  
185 using a suite of different methods. Additionally, samples from a previous study  
186 conducted in WA (Sinclair et al. in prep) were included in parts of this study.

187



188 **Fig. 1:** Map of all known penguin colonies in Australia (grey) with sampling locations  
189 highlighted (black); Is - Island; Latitude and Longitude are shown.  
190

191

192    ***Sampling***

193    All samples were collected in accordance with Animal Ethics protocols under the  
194    UNSW Animal Care and Ethics Committee Approval # 11/127B.  
195    To collect genetic samples, two methods were used to capture adult penguins. Where  
196    possible, penguins were intercepted when arriving on the main landing site (beach)  
197    after sunset to return to their burrows following a day of feeding at sea (Broughton,  
198    Cabbage Tree, Lion and Bowen Islands). At these colonies, a plastic mesh fence was  
199    erected in a V-shape along the back of the main beach; as the penguins reached a  
200    specific area along the fence, they were corralled. Penguins were captured and moved  
201    to an area with sufficient light, where blood samples were taken and microchips  
202    implanted for identification and to prevent double-sampling of individuals (Bowen and  
203    Lion Islands only). At all other colonies, adult penguins were taken from their burrows,  
204    where they stayed during the day if not foraging at sea. There, penguins were removed  
205    from burrows after visual and tactile checks for eggs or very young chicks. Burrows  
206    with eggs or blind chicks were not sampled to minimise disturbance to breeding  
207    penguins. The target sample size was set at 20, which is generally considered sufficient  
208    to calculate genetic estimates (Kalinowski 2005; Hale et al. 2012; Hoban et al. 2013).  
209    This target could not be met for Broughton Island and Tollgate Islands due to logistic  
210    constraints.

211    ***DNA extraction***

212    Blood samples were taken from the metatarsal vein of at least 20 individual penguins  
213    per colony (except the two islands mentioned above) in Eastern Australia (Appendix  
214    Table 1A). Samples from NSW were collected using sterile 23 gauge needles and non-

215 heparinised capillary tubes. Blood samples (50-100 µl) were stored in 1 ml of  
216 Longmire's buffer (Longmire et al. 1988) at ambient temperature until samples were  
217 processed within a few weeks. DNA was extracted from blood samples following the  
218 procedure outlined in Crandall et al. (1999). Samples from SA were collected on FTA  
219 cards and extracted following a variation of method #4 for nucleated erythrocytes  
220 (Smith & Burgoyne 2004). Specifically, a 4 mm<sup>2</sup> square of blood-soaked FTA®  
221 databasing paper for each individual was washed in: (1) 200 µL of FTA lysis buffer (100  
222 mM Tris free base, 0.1% SDS) for 30 min; (2) 200 µL DNAzol® for 10 min; (3) 200 µL  
223 molecular grade water (MGW) for 5 min twice; and (4) 200 µL 95% ethanol for 10 min.  
224 DNA was resuspended from squares in 50 µL 1x Tris Light EDTA (10 mM Tris, 0.1 mM  
225 EDTA).

226 ***Microsatellite genotyping***

227 For microsatellite genotyping, each individual was screened at 11 microsatellite loci  
228 (Appendix Table 2A) following an adaptation of the procedure outlined in (Sinclair et  
229 al. in prep). 5 µL microsatellite multiplex PCR reactions were performed containing 1 X  
230 Qiagen multiplex mix, fluorescently labelled forward and unlabelled reverse primers,  
231 0.025 µL BSA and 15-25 ng of genomic DNA. Each individual was screened in an initial  
232 multiplex reaction consisting of all markers. Some markers amplified poorly in some  
233 individuals so each individual was screened a second time with a second multiplex  
234 reaction consisting of five markers (Appendix Table 2A). Amplifications were carried  
235 out on an Eppendorf Mastercycler using an initial denaturing at 94 °C for 15 min, 50  
236 cycles of (94 °C for 30 s, annealing temperature for 90 s, 72 °C for 90 s), and a final  
237 elongation at 72 °C for 10 min. The annealing temperature followed a step-down

238 pattern with 10 cycles each of 64 °C, 50 °C, 56 °C, 53 °C, and 50 °C. PCR product  
239 fragment analysis was performed on an AB3730 DNA Analyser at the UNSW Ramaciotti  
240 Centre and alleles were scored using the microsatellite plugin for GENEIOUS v6  
241 (Biomatters Ltd.) software.

242 ***Mitochondrial DNA genotyping***

243 Mitochondrial HVRI sequences are widely used to study population structure and  
244 relationships among recently diverged taxa including Little Penguins (Overeem et al.  
245 2007; Peucker et al. 2009; Sinclair et al., in prep). It was thus chosen to investigate  
246 relationships among Eastern Australian little penguins, using a similar procedure to  
247 (Sinclair et al., in prep). The mitochondrial HVRI was amplified by PCR using the  
248 primers L-tRNAGlu (5'-CCTGCTTGGCTTTYTCCAAGACC-3') and H-Dbox (5'-  
249 CTGACCGAGGAACCAGAGGCCGC-3') (Roeder et al. 2002) in 10 µL reactions using the  
250 Qiagen Taq PCR Core Kit (1x buffer, 1x Q-solution, 0.2 mM dNTPs, 2 µM of each  
251 primer, 0.5 units Taq polymerase and 30-50 ng of genomic DNA). PCR products were  
252 purified by adding 2 µL Exosap-IT (USB Corp.) to 5 µL of each reaction product and  
253 running on an Eppendorf Mastercycler at for 15 minutes at 37 °C followed by 15  
254 minutes at 80 °C. Cycle sequencing was carried out in 20 µL reactions containing 2.5µL  
255 5x BigDye sequencing buffer (Applied Biosystems Inc.), 1.0 µL BigDye 3.1 Ready  
256 Reaction premix (Applied Biosystems Inc.), 3.2 pmol H-Dbox primer, and 35 ng purified  
257 PCR product and q.s. ddH<sub>2</sub>O. Cycle sequencing reactions were carried out on an  
258 Eppendorf Mastercycler using an initial 96 °C denaturation for 1 min, 25 cycles of  
259 (96 °C for 10 s, 50 °C for 5 s, 60 °C 4 min), and then held at 10 °C. The final product was  
260 sequenced using an ABI 3730x/ DNA analyzer (Applied Biosystems Inc.) at the

261 Australian Genome Research Facility. Mitochondrial DNA sequences were viewed and  
262 edited using GENEIOUS and alignment of a 388bp region was performed using the built-  
263 in CLUSTALW v2 plug-in (Larkin et al. 2007).

264 ***Analysis of microsatellites***

265 Genetic diversity was estimated by calculating allelic richness (number of alleles and  
266 effective number of alleles), Shannon's Information Index ( $H^s$ ), expected and observed  
267 heterozygosities - including unbiased expected heterozygosity ( $uH_e$ ), which is better  
268 suited than standard  $H_e$  for estimating heterozygosity when sample sizes are low (Nei  
269 1987; Pruett & Winker 2008) - as well as the Fixation Index for each population using  
270 GENALEX 6.5 (Peakall & Smouse 2006; Peakall & Smouse 2012). Measures of genetic  
271 diversity were analysed for, and transformed to reach, normal distribution using the R  
272 statistical software package, version 3.0.2 (R Development Core Team 2010). In cases  
273 where data were not normally distributed, even after transformation, the non-  
274 parametric Kruskal-Wallis rank sum test was used to test for differences among  
275 colonies. Deviations from a Fixation Index ( $F_{IS}$ ) of zero, as expected under random  
276 mating, were tested using the F-stat bootstrap option in ARLEQUIN ver. 3.5.1 (Excoffier  
277 & Lischer 2010). The 95% confidence interval (CI) for bootstrapped values of  $F_{IS}$  was  
278 reported. To test whether genetic diversity correlated with census population size, a  
279 linear regression was fitted between measures of genetic diversity and census  
280 population size, using R.

281 Population structure was investigated using three complementary approaches:  
282 measures of genetic differentiation, Bayesian clustering based on multi-locus  
283 genotypes using STRUCTURE, and isolation by distance (IBD, see below). The degree of

284 genetic differentiation among populations was estimated by the widely used  $F_{ST}$  (Weir  
285 & Cockerham 1984),  $\Phi_{PT}$  to allow direct comparison to patterns at a mitochondrial  
286 marker (Excoffier et al. 1992; Peakall et al. 1995; Maguire et al. 2002), and  $I = {}^S H_{ua}$ , a  
287 measure of mutual information (Sherwin et al. 2006), using GENALEX 6.5. Negative  $F_{ST}$   
288 and  $\Phi_{PT}$  values were converted to zero, because both measures are defined for a range  
289 of values between 0 and 1. Some algorithms used to calculate these values can give  
290 negatives when correcting for differences in sample sizes.

291 The software STRUCTURE version 2.3.4 (Pritchard et al. 2000; Falush et al. 2003) was  
292 used to infer the most likely number of genetic clusters K from microsatellite data.  
293 Clustering was based on the logarithmic likelihood of the populations to be divided  
294 into K groups using Structure Harvester (as described in Evanno et al. 2005). Possible  
295 values for K were allowed to range from 1 to a maximum of 10, which represents the  
296 total number of colonies sampled. The STRUCTURE approach foregoes any a priori  
297 information about population affiliation of the individuals. Individual assignment to the  
298 inferred clusters was visualised using a bar plot giving the likelihoods that each  
299 individual belongs to any of the clusters. Finally, microsatellites were analysed for  
300 evidence of isolation by distance (IBD) using the Mantel option in GENALEX 6.5. For  
301 that, codominant-genotypic genetic distance was calculated as a pairwise, individual-  
302 by-individual genetic distance matrix (Peakall et al. 1995; Smouse & Peakall 1999).

303 To counteract the problem of multiple comparisons, only results that remained  
304 significant after Bonferroni correction were regarded as highly significant. For that,  
305 tests were considered to belong to a family of hypothesis if the same test was done on

306 multiple pairwise comparisons. The significance level  $\alpha$  was then adjusted to 0.05  
307 divided by the number of pairs of populations tested.

308 ***Analysis of mitochondrial DNA haplotypes***

309 DNASP Ver. 5.10.01 (Librado & Rozas 2009) was used to generate a haplotype data file,  
310 which was then used to identify the population affiliation of the individual haplotypes  
311 that were displayed in a haplotype network generated using the software NETWORK  
312 version 4.6.0.0 (Polzin & Daneshmand 2011). Additionally, population differentiation  
313 was investigated by importing the generated haplotypes into GENALEx 6.5. Using this  
314 program,  $\Phi_{PT}$ , a measure of population genetic differentiation analogous to  $F_{ST}$ , but  
315 using sequence information, was calculated via Analysis of Molecular Variance  
316 (AMOVA). The advantage of  $\Phi_{PT}$  is that it suppresses intra-individual variation and is  
317 therefore ideal for comparisons between codominant and haploid or binary data  
318 (Maguire et al. 2002), where no intra-individual variation (heterozygosity) is available.  
319 To compare genetic differentiation of penguins from different states,  $\Phi_{PT}$  values were  
320 compared using a two-sample t-test in MICROSOFT EXCEL 2010. Finally, mitochondrial  
321 haplotypes were analysed for evidence of IBD using the Mantel option in GENALEx 6.5.

322 ***Dispersal among penguin colonies***

323 Dispersal among colonies was estimated using the program MIGRATE 3.6.4 (Beerli &  
324 Palczewski 2010). For each population, the program calculated the mutation-scaled  
325 population size  $\Theta$  (Theta,  $\Theta = x * \text{effective population size } N * \text{mutation rate } \mu$ , where  
326 the factor  $x$  depends on the ploidy and inheritance of the data; for nuclear  
327 microsatellite data it is = 4 and for the mitochondrial DNA with female-only transition  
328 it is = 1). The program also assessed directional dispersal as  $xNm$ , which represents the

329 number of immigrants per generation Nm (m for migration rate), scaled by x, where x  
330 depends on the inheritance mode, see above. In Eastern Australia, there are two  
331 competing hypotheses. First, we might expect more dispersal in from the centre of the  
332 penguin distribution in the Bass Strait towards the edge in the Sydney region (South to  
333 North), following the abundant centre model (Lawton 1993; Sagarin & Gaines 2002).  
334 Alternatively, major oceanic currents could force penguins to disperse predominantly  
335 in the opposite direction (East Australian Current, North to South). Following advice  
336 from the MIGRATE programmer (Beerli, pers. comm.), dispersal was analysed only  
337 between pairs of colonies or populations, thereby avoiding instability due to a larger  
338 number of parameters to estimate simultaneously. Migration was estimated at two  
339 different levels: local (between colonies on Bowen and Lion Islands) and regional  
340 (where colonies in NSW were pooled due to lack of genetic differentiation). In case of  
341 unequal sample sizes, a random subset equating to the smaller sample in the data set  
342 was used, because preliminary simulations showed that very unequal sample sizes led  
343 to unstable results. We thus analysed dispersal between Bowen Island and Lion Island,  
344 as well as between NSW (all colonies pooled) and SA (Kangaroo Island) based on the  
345 multilocus microsatellite dataset and mitochondrial DNA haplotypes.  
  
346 Two mathematically different strategies were used in MIGRATE to find those population  
347 sizes and dispersal rates that best fit the data. The Bayesian estimation approach was  
348 based on a uniform prior distribution for  $\Theta$  and M (with  $\Theta_{\min}=0$ ,  $\Theta_{\max}=100$ ,  $\Theta_{\delta}=10$   
349 and  $M_{\min}=0$ ,  $M_{\max}=10000$ ,  $M_{\delta}=1000$  for microsatellites; and  $\Theta_{\min}=0$ ,  $\Theta_{\max}=0.10$ ,  
350  $\Theta_{\delta}=0.01$  and  $M_{\min}=0$ ,  $M_{\max}=1000$ ,  $M_{\delta}=100$  for mtDNA). Settings included a random  
351 initial tree and the use of a static heating scheme, with the four recommended

352 temperatures (1.0, 1.5, 3.0, 1000000) that set the tendency for the program to make  
353 jumps of different lengths when searching the possible range of population sizes and  
354 dispersal rates. The search strategy used “slice” sampling with an autotune value for  
355 parameter acceptance ratios of 0.44, which is more reliable than using the Metropolis-  
356 Hastings algorithm, according to the MIGRATE documentation (Beerli 2012). One long  
357 chain was used, with a burn-in of 10 000 steps, after which results were summed over  
358 three independent replicates. Other values were left at the MIGRATE defaults. For the  
359 second, maximum likelihood approach to estimation, with three long chains combined  
360 for the estimates, we used the same heating scheme as before, and other values were  
361 left at the MIGRATE defaults.

362 For analysis of microsatellite genotypes, the Brownian motion model was used  
363 because the single-step mutation model was unstable when trying to calculate  
364 likelihoods for loci with wildly different repeat numbers. Mitochondrial haplotypes  
365 were analysed under the DNA sequence model.

366 Three independent runs were performed for each analysis and results were averaged.  
367 Asymmetrical dispersal was tested by comparing averaged median dispersal rates  
368 using the two-sample t-test for MICROSOFT EXCEL 2010.

369    **Results**

370    ***Analysis of microsatellite genotypes***

371    Despite large variation in census population sizes  $N_c$ , variation in genetic diversity at  
372    microsatellite loci was low (Table 1). None of the measures of genetic diversity were  
373    normally distributed after transformation, and a Kruskal-Wallis rank sum test did not  
374    detect any differences in the number of effective alleles ( $N_e$ ,  $p_{Ne}=0.8876$ ), expected  
375    heterozygosity ( $H_e$ ,  $p_{He}=0.8863$ ) or the Information Index ( $^S H$ ,  $p_I=0.8662$ ) among  
376    penguin colonies, although Kangaroo Island had the highest diversity values at all these  
377    measures. Unbiased expected heterozygosity also did not differ among colonies  
378    ( $p_{uHe}=0.9034$ ). There was no correlation between any of the measures of genetic  
379    diversity and census population size (minimum  $p=0.2594$  for linear regression between  
380     $H_o$  and  $N_c$ ).

381    Values of the Fixation Index did not deviate from zero (bootstrap 95% CI = [-0.05375  
382    0.06516],  $p_{FIS}= 0.5269$ ), which is consistent with random mating. AMOVA showed that  
383    only 7 % of the variation occurred among populations, whereas the remaining 93 % of  
384    variation was found within populations (Table 2).

385

386

387 **Table 1:** Genetic diversity of ten Little Penguin populations in Eastern Australia, based  
388 on eleven microsatellite loci

389 N = Sample Size,  $N_a$  = number of alleles,  $N_e$  = effective number of alleles,  $H^s$  =  
390 Information Index,  $H_o$  = Observed Heterozygosity,  $H_e$  = Expected Heterozygosity,  $H^u$  =  
391 Unbiased Expected Heterozygosity,  $F_{IS}$  = Fixation Index, and  $N_c$  = census population  
392 size, ind. - individuals

<b>Pop</b>		<b>N</b>	<b><math>N_a</math></b>	<b><math>N_e</math></b>	<b><math>H^s</math></b>	<b><math>H_o</math></b>	<b><math>H_e</math></b>	<b><math>H^u</math></b>	<b><math>F_{IS}</math></b>	<b><math>N_c</math></b>
<b>Broughton</b>	Mean	11.000	3.000	2.096	0.727	0.463	0.401	0.420	-0.125	20-40 pairs <sup>1</sup>
	SE	0.000	0.539	0.306	0.171	0.111	0.089	0.093	0.057	
<b>CabbageTree</b>	Mean	23.000	3.545	1.785	0.639	0.340	0.335	0.343	0.116	140 pairs <sup>2</sup>
	SE	0.000	0.679	0.233	0.156	0.093	0.083	0.085	0.120	
<b>LionIsland</b>	Mean	41.636	4.727	2.023	0.763	0.347	0.371	0.376	0.072	250 ind. <sup>3</sup>
	SE	0.730	1.192	0.308	0.189	0.092	0.091	0.092	0.062	
<b>Manly</b>	Mean	13.000	4.000	2.352	0.850	0.399	0.423	0.440	0.046	60 pairs <sup>4</sup>
	SE	0.000	0.853	0.374	0.211	0.096	0.100	0.104	0.037	
<b>FiveIslands</b>	Mean	16.909	4.091	2.571	0.887	0.449	0.442	0.455	-0.017	> 1000 pairs <sup>5</sup>
	SE	0.091	0.986	0.487	0.218	0.105	0.098	0.101	0.051	
<b>Bowen</b>	Mean	59.909	5.182	2.110	0.775	0.351	0.371	0.374	0.102	5000 pairs <sup>6</sup>
	SE	0.694	1.242	0.349	0.201	0.096	0.096	0.096	0.058	
<b>Brush</b>	Mean	17.818	3.818	2.138	0.771	0.384	0.395	0.406	0.009	2-3 000 pairs <sup>7</sup>
	SE	0.182	0.672	0.316	0.186	0.099	0.095	0.097	0.063	
<b>Tollgates</b>	Mean	14.909	3.545	1.926	0.691	0.345	0.350	0.362	0.015	< 5000 pairs <sup>8</sup>
	SE	0.091	0.623	0.291	0.175	0.098	0.090	0.094	0.060	
<b>Montague</b>	Mean	27.727	4.727	2.213	0.844	0.377	0.412	0.419	0.054	5000 pairs <sup>9</sup>
	SE	0.273	1.054	0.334	0.196	0.088	0.093	0.095	0.037	
<b>Kangaroo</b>	Mean	45.909	5.818	2.909	1.054	0.510	0.499	0.504	-0.028	> 2000 ind. <sup>10</sup>
	SE	0.091	1.151	0.574	0.214	0.092	0.091	0.092	0.027	

393 <sup>1</sup> Carlile et al. 2012; <sup>2</sup> Priddel & Carlile 2004, <sup>3</sup> Sergent et al. 2004, <sup>4</sup> Little Penguin Recovery Team 2007,  
394 <sup>5</sup> Gibson 1976, <sup>6</sup> Fortescue 1995, <sup>7</sup> Carlile, et al. 2012, <sup>8</sup> McKean & Fullagar 1976, <sup>9</sup> Weerheim et al. 2003,  
395 <sup>10</sup> Wiebkin 2011

396  
397 **Table 2:** AMOVA results for microsatellite genotypes of 275 individuals

398 Based on a codominant genotypic distance matrix for calculation of  $\Phi_{PT}$ ; df - degrees of  
399 freedom, Est. Var. - estimated variation

Source	<b>df</b>	<b>Est. Var.</b>	<b>% Est. Var.</b>
Among Pops	9	0.364	7%
Within Pops	265	5.084	93%
Total	274	5.448	100%

401 Genetic differentiation among Eastern Australian penguin colonies is low (max.  $F_{ST}$  =  
 402 0.122, Appendix Table 3A). The maximum occurred between Tollgate Islands and  
 403 Broughton Island, although both have lower than target sample size and might  
 404 therefore be biased. The highest  $F_{ST}$  detected among colonies with more than 20  
 405 samples was 0.081 between Bowen and Kangaroo Islands. Patterns of genetic  
 406 differentiation measured by  $\Phi_{PT}$  were similar to  $F_{ST}$ , with a maximum of 0.223 between  
 407 the neighbouring islands of Broughton and Cabbage Tree Islands (Table 3). Again, the  
 408 highest differentiation among samples with a sample size above 20 was found  
 409 between Bowen and Kangaroo Islands, with a  $\Phi_{PT}$  value of 0.142. Kangaroo Island was  
 410 significantly differentiated from all colonies in NSW, with an average  $\Phi_{PT}$  of 0.0899 ±  
 411 0.0436 (SD).

412 **Table 3:**  $\Phi_{PT}$  genetic differentiation at 11 microsatellite loci

413  $\Phi_{PT}$  values are presented below the diagonal, whilst probabilities  $p(\text{rand} \geq \text{data})$  based  
 414 on 999 permutations are shown above diagonal. Differentiation between NSW and SA  
 415 is significant at  $\Phi_{PT} = 0.0899 \pm 0.0436$ . Significant results after Bonferroni correction  
 416 ( $p \leq 0.001$ ) are highlighted in italics.

	<b>Bro</b>	<b>Cab</b>	<b>Lio</b>	<b>Man</b>	<b>Fiv</b>	<b>Bow</b>	<b>Bru</b>	<b>Tol</b>	<b>Mon</b>	<b>Kan</b>
<b>Broughton Is</b>		<i>0.001</i>	<i>0.001</i>	0.075	0.309	<i>0.001</i>	<i>0.001</i>	<i>0.001</i>	<i>0.001</i>	0.002
<b>CabbageTree Is</b>	<i>0.223</i>		0.029	0.016	<i>0.001</i>	0.010	0.002	0.002	0.012	<i>0.001</i>
<b>Lion Is</b>	<i>0.140</i>	0.022		0.152	<i>0.001</i>	0.416	0.241	0.003	0.032	<i>0.001</i>
<b>Manly</b>	0.041	<i>0.055</i>	0.017		0.416	0.015	0.230	<i>0.001</i>	0.364	0.029
<b>Five Iss</b>	0.010	<i>0.116</i>	<i>0.071</i>	0.002		<i>0.001</i>	0.072	<i>0.001</i>	0.054	0.022
<b>Bowen Is</b>	<i>0.160</i>	0.028	0.000	0.037	<i>0.091</i>		0.089	0.003	0.013	<i>0.001</i>
<b>Brush Is</b>	<i>0.109</i>	0.065	0.008	0.015	0.030	0.016		<i>0.001</i>	0.386	<i>0.001</i>
<b>Tollgate Iss</b>	<i>0.203</i>	<i>0.072</i>	0.062	<i>0.092</i>	<i>0.101</i>	0.057	<i>0.069</i>		0.038	<i>0.001</i>
<b>Montague Is</b>	<i>0.096</i>	0.040	0.020	0.004	0.024	0.024	0.002	0.032		<i>0.001</i>
<b>Kangaroo Is</b>	0.064	<i>0.148</i>	<i>0.117</i>	0.031	0.027	<i>0.142</i>	<i>0.095</i>	<i>0.125</i>	<i>0.060</i>	

417

418 Based on three different measures of genetic differentiation ( $F_{ST}$ ,  $\Phi_{ST}$  and MI), penguin  
 419 colonies on the Tollgate Islands and Kangaroo Island are significantly diverged from  
 420 most other colonies studied (see Table 3, Appendix Table 3A and Table 4A). After

421 Bonferroni correction, most comparisons still show a significant level of genetic  
422 differentiation between those two and the remaining eight colonies studied.  
423 Despite signs of low, but statistically significant levels of genetic differentiation among  
424 some pairs of penguin colonies, there was no clear population structure at  
425 microsatellites based on the Bayesian clustering approach (Fig. 2). While the most  
426 likely number of genetic clusters identified in the dataset is two, these clusters did not  
427 align with the geographic origin of individual samples. However, there is significant  
428 support for a weak pattern of isolation by distance (IBD,  $R^2=0.027$ ,  $p=0.01$ , Fig. 4),  
429 driven by the most distant colony at Kangaroo Island. Removing Kangaroo Island from  
430 the data set, thus focussing on data from NSW only, resulted in a non-significant  
431 pattern of IBD ( $R^2=0.0004$ ,  $p=0.220$ , Fig. 3).



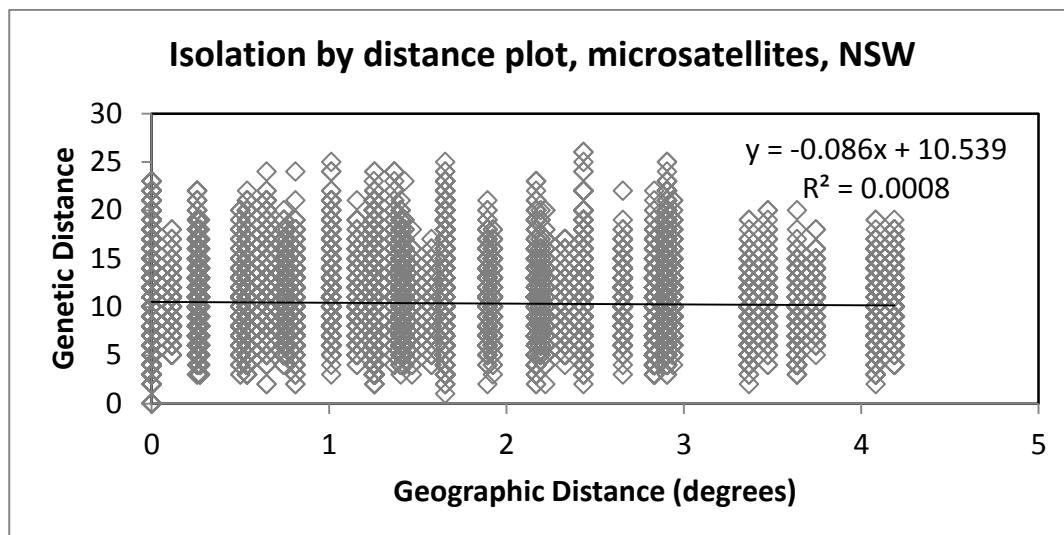
432

433 **Fig. 2:** STRUCTURE bar plot of 274 individuals from 10 colonies, sorted North to South

434 Shade of grey in bars represents cluster, while bars represent individual penguins,  
435 showing the likelihood of the individual to belong to the respective cluster; black bars  
436 indicate border between neighbouring colonies

437

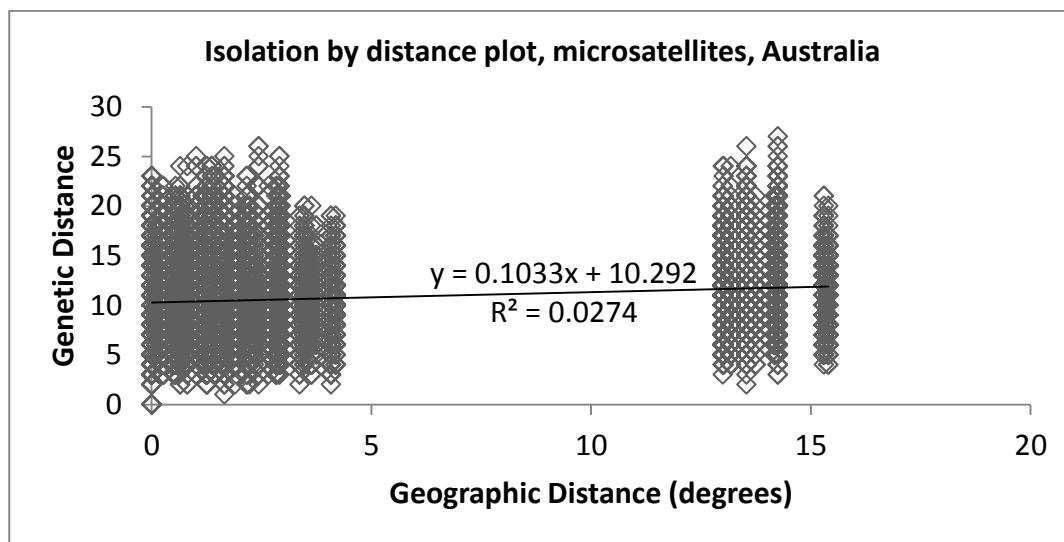
438



439

440 **Fig. 3:** Isolation by distance at microsatellite loci, NSW only

441



442

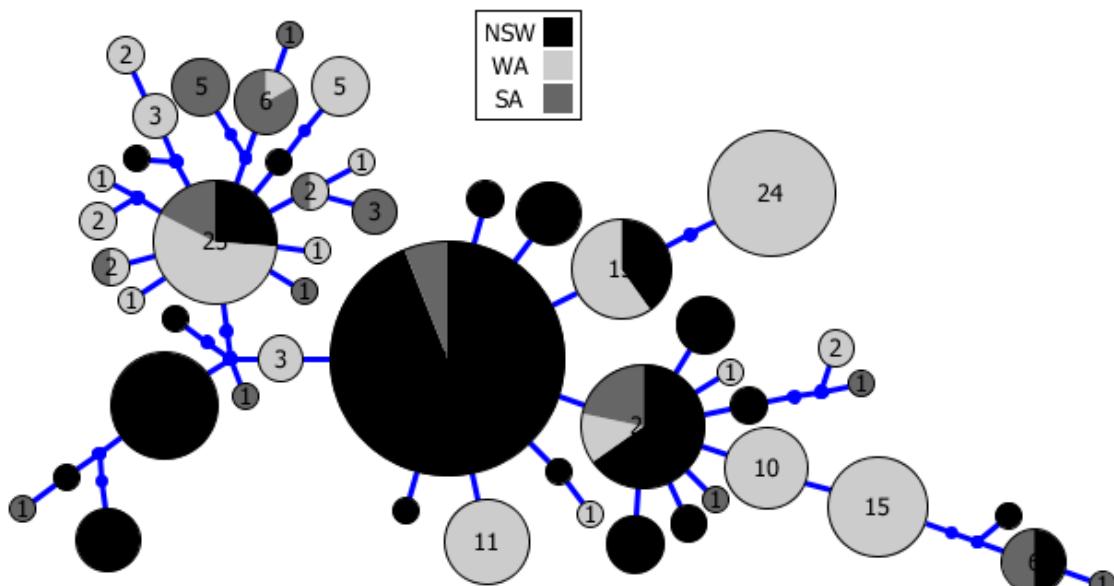
443 **Fig. 4:** Isolation by distance at microsatellite loci, all locations

444

445 **Analysis of mitochondrial DNA haplotypes**

446 Haplotypes of a 388 bp fragment of the mitochondrial control region could be  
447 obtained for a total of 311 individuals from eight populations in New South Wales, one  
448 in South Australia (collected for this study) and an additional three regions in Western  
449 Australia (Sinclair et al., in prep). Forty-eight different haplotypes were identified, and  
450 the haplotype network generated shows a mix of origins for the most common, central  
451 haplotypes as well as more diverged haplotypes unique to the western populations in  
452 South and Western Australia (Fig. 5).

453



454

455 **Fig. 5:** Haplotype network for mitochondrial control region sequences of 311  
456 individuals.

457 Colour of pie chart sectors corresponds to state of origin of individuals carrying the  
458 haplotype; size of circles corresponds to number of individuals with each haplotype;  
459 size of sectors corresponds to number of individuals from each Australian state.

460

461 AMOVA showed that 12 % of the variation of mitochondrial haplotypes occurred  
462 among populations, whereas the remaining 88 % of variation was found within  
113

463 populations (Table 4). Genetic differentiation was investigated by calculating pairwise  
464  $\Phi_{PT}$  values between each of the localities: ten Eastern Australian colonies and three  
465 populations in Western Australia (Table 5). Here, too, the most strongly divergent  
466 populations were found in South Australia and Western Australia. Very few pairs of  
467 populations from NSW were significantly differentiated at the mitochondrial marker,  
468 with a maximum  $\Phi_{PT}$  of 0.128 between Lion Island and the Tollgate Islands. All  
469 populations from NSW, however, were differentiated from Kangaroo Island in SA  
470 (average  $\Phi_{PT} = 0.2071 \pm 0.0345$ ) and all but one (Broughton Island with the lowest  
471 sample size of 11 individuals) were also significantly different from populations in WA.  
472 Genetic differentiation between NSW and SA was significantly stronger at the  
473 mitochondrial marker than at microsatellites based on average  $\Phi_{PT}$  values (two-sample  
474 t-test with equal variances, T-value = -5.9658, p < 0.0001, Tables 3 and 5). IBD was also  
475 significant at the mitochondrial marker ( $R^2 = 0.1266$ , p = 0.01, Fig. 7), and driven by the  
476 most distant colony at Kangaroo Island. Removing Kangaroo Island from the data set  
477 resulted in a non-significant pattern of IBD within NSW ( $R^2 = 0.0009$ , p = 0.230, Fig. 6).

478

479 **Table 4:** AMOVA results for mitochondrial control region sequences of 311 individuals  
480 Based on a haploid distance matrix for calculation of  $\Phi_{PT}$ ; df - degrees of freedom, Est.  
481 Var. - estimated variation

Source	df	Est. Var.	% Est. Var.
Among Pops	10	0.055	12%
Within Pops	300	0.406	88%
Total	310	0.460	100%

482

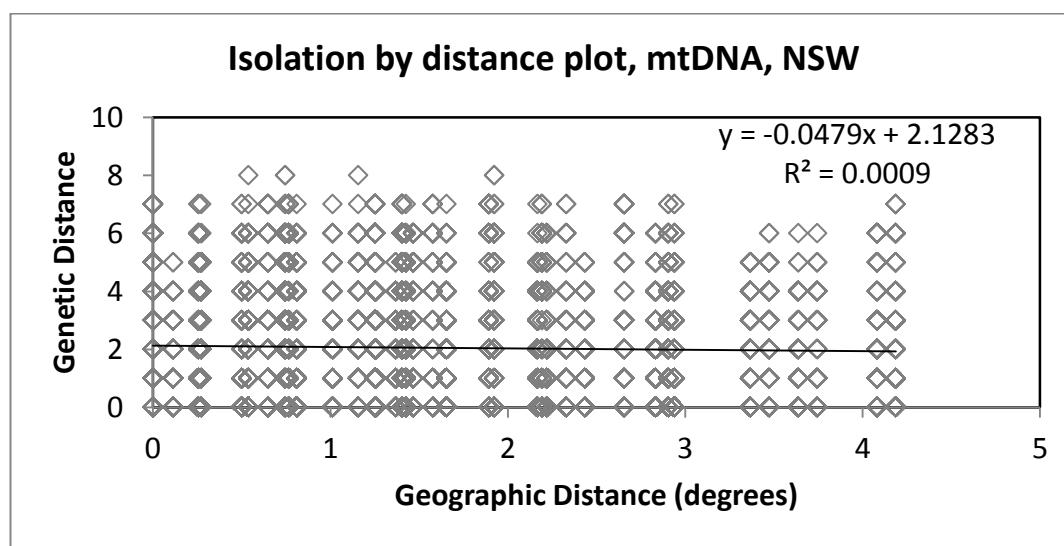
483 **Table 5:**  $\Phi_{PT}$  genetic differentiation at the mitochondrial control region

484  $\Phi_{PT}$  values are presented below the diagonal, whilst probabilities  $p(\text{rand} \geq \text{data})$  based  
485 on 999 permutations are shown above the diagonal. Negative  $\Phi_{PT}$  values were  
486 converted to zero. Differentiation between NSW and SA is significant at  $\Phi_{PT} = 0.2071 \pm$   
487  $0.0345$ . Significant results after Bonferroni correction ( $p \leq 0.001$ ) are highlighted in  
488 italics.

	Bro	Cab	Lio	Man	Fiv	Bow	Bru	Tol	Mon	Kan	WA
<b>Broughton</b>		0.403	0.126	0.424	0.362	0.381	0.309	0.424	0.360	0.004	0.052
<b>CabbageTree</b>	0.005		0.370	0.255	0.396	0.189	0.306	0.042	0.096	<i>0.001</i>	0.007
<b>LionIsland</b>	0.056	0.000		0.111	0.415	0.026	0.362	0.013	0.113	<i>0.001</i>	0.003
<b>Manly</b>	0.000	0.019	0.051		0.368	0.372	0.188	0.316	0.418	<i>0.001</i>	0.005
<b>Fivelands</b>	0.000	0.000	0.000	0.000		0.394	0.364	0.161	0.364	<i>0.001</i>	0.002
<b>Bowen</b>	0.000	0.021	0.078	0.000	0.000		0.050	0.216	0.317	<i>0.001</i>	<i>0.001</i>
<b>BrushIsland</b>	0.009	0.007	0.000	0.020	0.003	0.053		0.045	0.143	0.002	0.016
<b>Tollgates</b>	0.000	0.077	0.128	0.015	0.034	0.020	0.073		0.116	0.002	0.008
<b>Montague</b>	0.000	0.050	0.046	0.000	0.000	0.002	0.032	0.050		<i>0.001</i>	0.003
<b>Kangaroo Is, SA</b>	0.184	0.208	0.196	<i>0.197</i>	0.223	0.241	0.130	0.250	0.235		<i>0.001</i>
<b>WA</b>	0.056	0.090	0.098	0.104	0.110	0.110	0.056	0.092	0.123	0.137	

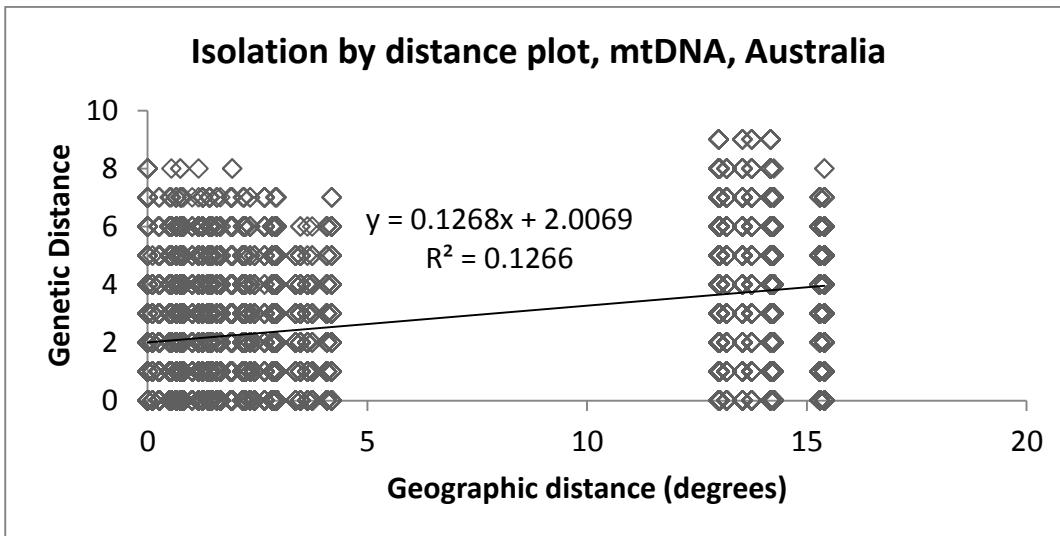
489

490



491

492 **Fig. 6:** Isolation by distance of mitochondrial haplotypes, NSW only



493

494 Fig. 7: Isolation by distance of mitochondrial haplotypes, all colonies sampled

495

496 ***Dispersal among penguin colonies***

497 Dispersal results are based on the Bayesian analyses in MIGRATE. The maximum  
498 likelihood runs resulted in very flat likelihood surfaces (Appendix Table 7A to 22A) and  
499 estimates differed strongly among runs as well as giving different results from the  
500 Bayesian analyses (Appendix Table 5A and 6A). Maximum likelihood estimates were  
501 therefore not considered for further analyses.

502 Based on the microsatellite loci, the Bayesian analysis of symmetry of dispersal  
503 between two colonies in NSW (Bowen and Lion Island) showed no evidence for  
504 directional dispersal, with just very slightly elevated immigration rates into Bowen  
505 Island (Table 6, two-sample t-test with equal variances: T-value = 1.387, p = 0.238), and  
506 identical values for the mutation-scaled population size  $\Theta$  (Table 7, T-value = -0.339,  
507 p = 0.752). On a larger scale, dispersal between NSW and SA was asymmetrical, with  
508 more immigration into South Australia in all three MIGRATE runs (T-value = -3.606,  
509 p = 0.023). Estimates of population size were slightly, but not significantly, higher for

510 South Australia than New South Wales, despite the smaller geographic range sampled  
511 in SA (T-value = -1.270, p = 0.273).

512 Dispersal rates derived from the mitochondrial haplotypes, however, were significantly  
513 higher from Lion to Bowen Island than the reverse (two-sample t-test with equal  
514 variances: T-value = 2.911, p = 0.044). The mutation-scaled population size  $\Theta$  is similar  
515 for Bowen and Lion Islands (T-value = -1.198, p = 0.297). Dispersal between NSW and  
516 SA was also asymmetrical, but in the opposite direction to dispersal identified using  
517 microsatellite markers (T-value = 4.404, p = 0.012). A higher  $\Theta$  was also estimated for  
518 SA than NSW (T-value = -6.574, p = 0.003). The effective population size  $N_e$  based on  $\Theta$   
519 values is presented in Table 8.

520 When comparing MIGRATE estimates derived from the two types of genetic markers,  
521 the estimated number of immigrants based on mitochondrial genotypes is roughly four  
522 times as high (Table 6), while effective population sizes are approximately one fourth  
523 of those estimated based on microsatellites (Table 8).

524      **Table 6:** MIGRATE estimates of the number of immigrants per generation, xNm, based  
525      on Bayesian estimation methods; lower sample size used for subsampling highlighted  
526      in bold.

Dispersal estimates	Microsatellites				Mitochondrial haplotypes			
	Recipient population				Recipient population			
Local	Bowen Island		Lion Island		Bowen Island		Lion Island	
Sample Size	61		43		36		17	
	Median	95% CI	Median	95% CI	Median	95% CI	Median	95% CI
Run 1	123.3	0.0	96.7	0.0	418.3	109.3	357.0	123.3
		253.3		206.7		986.7		755.3
Run 2	110.0	0.0	103.3	0.0	493.7	123.3	381.7	93.3
		240.0		220.0		730.7		698.7
Run 3	96.7	0.0	96.7	0.0	524.3	174.0	401.0	120.0
		213.3		206.7		995.3		876.0
Mean	<b>110.0</b>		<b>98.9</b>		<b>478.8</b>		<b>379.9</b>	
Regional	<b>NSW</b>		<b>SA</b>		<b>NSW</b>		<b>SA</b>	
Sample Size	229		46		161		39	
	Median	95% CI	Median	95% CI	Median	95% CI	Median	95% CI
Run 1	90.0	0.0	116.7	0.0	384.3	25.3	294.3	124.7
		200.0		246.7		98.0		693.3
Run 2	90.0	0.0	116.7	0.0	470.3	97.3	317.0	174.0
		193.3		246.7		467.3		488.7
Run 3	103.3	0.0	136.7	0.0	412.3	132.7	309.7	133.3
		226.7		280.0		898.0		800.7
Mean	<b>94.4</b>		<b>123.3</b>		<b>422.3</b>		<b>307.0</b>	

527

528

529 **Table 7:** MIGRATE estimates of mutation-scaled population size  $\Theta$ , based on Bayesian  
530 estimation methods; lower sample size used for subsampling highlighted in bold.

Theta estimates	Microsatellites				Mitochondrial haplotypes			
	Population				Population			
Local	Bowen Island		Lion Island		Bowen Island		Lion Island	
Sample Size	61		43		36		17	
	Median	95% CI	Median	95% CI	Median	95% CI	Median	95% CI
Run 1	1.500	0.000	1.900	0.067	0.012	0.000	0.018	0.005
		2.933		3.533		0.035		0.049
Run 2	1.767	0.000	1.833	0.000	0.014	0.001	0.013	0.001
		3.267		3.333		0.040		0.032
Run 3	2.467	0.000	2.333	0.000	0.009	0.000	0.012	0.002
		5.200		5.067		0.023		0.042
Mean	1.911		2.022		0.012		0.014	
Regional	NSW		SA		NSW		SA	
Sample Size	229		46		161		39	
	Median	95% CI	Median	95% CI	Median	95% CI	Median	95% CI
Run 1	1.367	0.000	1.567	0.000	0.012	0.000	0.017	0.005
		2.800		3.000		0.003		0.033
Run 2	1.933	0.000	3.133	0.000	0.008	0.000	0.019	0.008
		4.533		5.867		0.021		0.064
Run 3	2.067	0.000	2.600	0.000	0.009	0.001	0.019	0.003
		4.533		5.333		0.019		0.039
Mean	1.789		2.433		0.009		0.018	

531

532

533 **Table 8:** Estimates of the effective population size  $N_e$  based on MIGRATE estimates of  
534 mutation-scaled population size  $\Theta$ ; calculations assumed a mutation rate of  $10^{-4}$  for  
535 microsatellites, and  $10^{-5}$  for mitochondrial DNA.

	Microsatellites		Mitochondrial haplotypes	
	Population		Population	
Local	Bowen Island	Lion Island	Bowen Island	Lion Island
	4778	5055	1200	1400
Regional	NSW	SA	NSW	SA
	4472.5	6082.5	900	1800

536

537 **Discussion**

538 **Diversity**

539 Within a species, there are advantages and disadvantages to gene flow. Large amounts  
540 of gene flow from a central population to the edge may hamper local adaptation at the  
541 margins, by swamping marginal populations with alleles that are adaptive within the  
542 distribution centre but mal-adaptive at the edge (Kawecki 2008). On the other hand,  
543 very low amounts of gene flow to margins could result in small, isolated populations  
544 that are prone to genetic drift and susceptible to local extinction (Bridle & Vines 2007).

545 While demographically, we found more small colonies closer to the range edge of the  
546 little penguin distribution (Appendix Table 1A), individually, these colonies were  
547 genetically not less diverse. The limited genetic differentiation among colonies in NSW,  
548 however, seems to result in a genetic diversity that is maintained at a slightly lower  
549 level than closer to the centre of the distribution (Table 1), as theory would predict for  
550 range margins (Cahill & Levinton 2015).

551 These results tie in well with previous findings for Australian little penguins, where  
552 genetic homogeneity was found among Victorian colonies east of Kangaroo Island  
553 (Overeem et al. 2008; Burridge et al. 2015). Genetic study of Adélie penguins has  
554 similarly revealed a lack of genetic structuring despite a wide distribution of the  
555 species and strong natal philopatry (Roeder et al. 2001). The possible explanations for  
556 a lack of genetic structuring in Adélie penguins is threefold: First, the species occurs in  
557 very large colonies, which reduces the possible impact of genetic drift; second,  
558 geographical, environmental and behavioural barriers to gene flow are weak; and  
559 third, its population history of re-colonisation of ice-free areas on the Antarctic

560 continent after the last glacial maximum might indicate a single refugium from which  
561 re-colonisation occurred. In contrast to Adélie penguins, little penguins inhabit the  
562 temperate coasts of Southern Australia and New Zealand, and have a more dynamic  
563 population history (Grosser et al. 2015). Colony sizes vary substantially, from a few  
564 breeding pairs close to their northern distribution limit, to tens of thousands of pairs  
565 closer to their centre of distribution. The observed genetic homogeneity within NSW is  
566 therefore most likely due to ongoing dispersal among colonies.

567 Despite strong differences in abundance of penguins among colonies (Appendix Table  
568 1A), their genetic diversity was largely uniform, as indicated by similar values of  $H_e$  and  
569  $H^s$  (Table 1). Especially, there were no signs of reduced genetic diversity of small  
570 colonies. This could further indicate ongoing dispersal among colonies despite the high  
571 philopatry of little penguins (Dann 1992). Estimates of the mutation-scaled population  
572 size  $\Theta$  confirm this pattern, with Lion and Bowen Island having similar  $\Theta$  estimates to  
573 each other at both microsatellite and mitochondrial markers.

574 **Divergence**

575 Ongoing dispersal is further evidenced by the fact that only some penguin colonies  
576 show significant genetic differentiation within NSW, without a clear geographic pattern  
577 (Table 3 and Appendix Tables 3A and 4A). Levels of differentiation at the biparentally  
578 inherited microsatellites between NSW and SA are also low, despite large geographic  
579 distances between colonies. Isolation by distance was not observed at close  
580 geographical proximity within NSW, and weak at intermediate distances between NSW  
581 and SA (Fig. 4).

582 Penguins are known for their (serial) monogamy, and are therefore expected to follow  
583 patterns of philopatry and dispersal commonly found in monogamous birds. Philopatry  
584 is usually stronger in males, whereas dispersal is expected to be driven by female  
585 dispersers (Liberg & von Schantz 1985). Here, differentiation between penguin  
586 colonies in NSW and SA was stronger at the maternally inherited mitochondrial marker  
587 than at the biparentally inherited microsatellites (Table 3 and Table 5), which might  
588 indicate sex-biased dispersal driven by male dispersers, although this pattern could  
589 alternatively be explained by the larger effective population size of the nuclear  
590 microsatellite markers compared to the mitochondrial marker (Table 8).

591 ***Directionality of dispersal***

592 Little penguins in Eastern Australian occur in the sphere of influence of the East  
593 Australian Current, which is likely to affect their dispersal direction (Cook & Crisp  
594 2005). Based on microsatellites, directionality of dispersal was not detected at the  
595 small geographic scale between Lion and Bowen Islands, but between NSW and SA,  
596 slightly more penguin dispersal was found southward than northward (Table 6).  
597 Interestingly, dispersal estimates based on the mitochondrial marker showed  
598 asymmetrical dispersal in the opposite direction, predominantly from SA to NSW, and  
599 elevated dispersal rates from Lion to Bowen Island. Low immigration rates into Lion  
600 Island might in part explain the declining population trend on this island (see Chapter 2  
601 in this thesis). Large variation of estimates is in part due to the fact that estimation of  
602 dispersal direction is particularly difficult for single loci (Beerli & Palczewski 2010), and  
603 our analyses relied on a single mitochondrial locus. While confidence intervals (CIs)  
604 were large for estimates of the number of immigrants per generation (Table 6), sample

605 sizes would generally be considered large enough for MIGRATE analyses. However, one  
606 could improve the power of the approach by analysing a larger number of loci, for  
607 example by looking at SNPs in a representative part of the genome. For microsatellites,  
608 dispersal estimates are low, and CIs include zero, so these results should be  
609 interpreted cautiously.

610 The comparison of dispersal estimates between microsatellite and mitochondrial  
611 markers contradicts the suspected male-biased dispersal indicated by stronger  
612 differentiation at mitochondrial DNA. Dispersal estimates are approximately four times  
613 as high for mitochondrial genotypes as for microsatellites, indicating strong female-  
614 biased dispersal. This interpretation, however, is also complicated by the large  
615 variation in microsatellite estimates and the limited conclusiveness of estimates based  
616 on a single mitochondrial locus. Further investigation of sex-biased dispersal in little  
617 penguins could benefit from the use of genetic markers linked to sex chromosomes.

618 The observed patterns might indicate a complex interaction between the East  
619 Australian Current and demography. The East Australian Current may be facilitating  
620 dispersal of both sexes in a southerly direction, while populations closer to the centre  
621 of the penguin distribution supply similar numbers of female penguins northward. This  
622 is consistent with the apparently higher mutation-scaled population size  $\Theta$  for SA than  
623 NSW, when based on mitochondrial DNA (Table 7). Thus, the South Australian  
624 population at Kangaroo Island, which is closer to the centre of the penguin distribution  
625 than colonies from NSW, has slightly elevated genetic diversity compared to NSW.

626

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636

637 **Conflict of Interest**

638 The authors declare no conflict of interest.

639

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862 **Appendix 3**

863 **Table 1A:** Sampling locations in Eastern Australia

864 Coordinates of penguin colonies, sample size of individual genetic material, years  
865 sampled and the most recent published estimates of population sizes.

866 <sup>1</sup> Carlile et al. 2012; <sup>2</sup> Priddel & Carlile 2004, <sup>3</sup> Sergent et al. 2004, <sup>4</sup> Little Penguin  
867 Recovery Team 2007, <sup>5</sup> Gibson 1976, <sup>6</sup> Fortescue 1995, <sup>7</sup> Carlile, et al. 2012, <sup>8</sup> McKean  
868 & Fullagar 1976, <sup>9</sup> Weerheim et al. 2003, <sup>10</sup> Wiebkin 2011

Location (N to S)	Coordinates	Sample Size	Year sampled	Estimated census population size - N <sub>c</sub>
Broughton Island	32° 37' S, 152° 18' E	11 <sup>A</sup>	2013	20-40 pairs <sup>1</sup>
Cabbage Tree Island	32° 41' S, 152° 13' E	37	2012	140 pairs <sup>2</sup>
Lion Island	33° 33' S, 151° 19' E	63	2012	250 individuals <sup>3</sup>
Manly	33° 48' S, 151° 17' E	20	2012	60 pairs <sup>4</sup>
Big Island, Five Islands	34° 29' S, 150° 55' E	20	2012	> 1000 pairs <sup>5</sup>
Bowen Island	35° 07' S, 150° 46' E	86	2012	5000 pairs <sup>6</sup>
Brush Island	35° 32' S, 150° 25' E	20	2012	2000-3000 pairs <sup>7</sup>
Tollgate Islands	35° 45' S, 150° 15' E	16 <sup>A</sup>	2012	> 5000 pairs <sup>8</sup>
Montague Island	36° 15' S, 150° 13' E	36	2012	5000 pairs <sup>9</sup>
Kangaroo Island, SA	35° 50' S, 137° 13' E	45	2013	> 2000 individuals <sup>10</sup>
<b>Total</b>		<b>358</b>		

869

870 **Table 2A:** Details of microsatellites used for genotyping (Sinclair et al., in prep)

871 The M13 prefix in the primer sequence denotes a 5'-M13 tail  
872 (CACGACGTTGTAAAACGAC), attached to the forward primer sequence.

873 *References:* 1. Akst et al. 2002; 2. Billing et al. 2006; 3. Roeder et al. 2002

Marker	Label	Primer Mix1 [mM]	Primer Mix2 [mM]	Ref	Primer Sequence
<b>Am13</b>	Vic	110	100	3	Fwd 5'-TTTTCCCATCTC TCTCCTG Rev 5'-CAGTTTCAACAATCCTTCC
<b>B3-2</b>	Pet	130	-	1	Fwd 5'-GGTGGTTATAGATGCCAGC Rev 5'-CAGTGCCCAGGAATCCAGTT
<b>Emm1</b>	Fam	80	-	2	Fwd M13-CAATGCTGTAGTCCACTG Rev 5'-TGGGTGAAGTGCCTTGAG
<b>Emm2</b>	Ned	120	100	2	Fwd M13-AGCCTACATGACTGCAAAGC Rev 5'-TGGCTCTACACATCTTCTGG
<b>Emm3</b>	Vic	90	100	2	Fwd M13-TGCACAACAGGTGTATGACG Rev 5'-CTGAAGCTCTGAACTGTGC
<b>Emm4</b>	Pet	140	100	2	Fwd M13-GGGAGGGCCTAACAAACTAC Rev 5'-TTAGATGCCTGGTCATTGG
<b>Emm5</b>	Pet	140	100	2	Fwd M13-ATTAACCTGGCCTGGGTT Rev 5'-TTTATGCTCCCTCATTCCAC

<b>Emm6</b>	Ned	80	-	2	Fwd M13-TTGTTGGTCTGTATCACAGG Rev 5'-CAGGGAACTGTCAGTAAATGG
<b>Emm7</b>	Fam	80	-	2	Fwd M13-AGATAAACTGGGTGTGAGACG Rev 5'-GAAAGGGAAGCGTTGTATG
<b>Emm8</b>	Fam	80	-	2	Fwd M13-TGCACACTAGCAGATACGG Rev 5'-GACAAATTGTGCTTGTACGC
<b>G3-11</b>	Vic	80	-	1	Fwd 5'-ATGATTCAAGGCAGGTGGA Rev 5'-CAGAAGCTTCAGGAAGGGCA

874

875 **Table 3A:**  $F_{ST}$  genetic differentiation at 11 microsatellite loci

876 Below diagonal are  $F_{ST}$  values; probabilities  $p(\text{rand} \geq \text{data})$  based on 999 permutations  
877 are shown above diagonal. Differentiation between NSW and SA is significant at  
878  $F_{ST} = 0.053$ . Significant results after bonferroni correction ( $p > 0.001$ , are highlighted in  
879 italics below; Is – Island, Iss - Islands

	<b>Bro</b>	<b>Cab</b>	<b>Lio</b>	<b>Man</b>	<b>Fiv</b>	<b>Bow</b>	<b>Bru</b>	<b>Tol</b>	<b>Mon</b>	<b>Kan</b>
<b>Broughton Is</b>		<i>0.001</i>	<i>0.001</i>	0.080	0.310	<i>0.001</i>	0.003	<i>0.001</i>	<i>0.001</i>	0.006
<b>Cabbage Tree Is</b>	<i>0.122</i>		0.038	0.022	<i>0.001</i>	0.012	0.005	0.002	0.009	<i>0.001</i>
<b>Lion Is</b>	<i>0.084</i>	0.013		0.098	<i>0.001</i>	0.413	0.206	0.003	0.023	<i>0.001</i>
<b>Manly</b>	0.021	0.029	0.010		0.434	0.014	0.202	0.004	0.337	0.029
<b>Five Iss</b>	0.005	<i>0.063</i>	<i>0.042</i>	0.001		<i>0.001</i>	0.064	<i>0.001</i>	0.047	0.029
<b>Bowen Is</b>	<i>0.094</i>	0.016	0.000	0.021	<i>0.053</i>		0.082	0.002	0.006	<i>0.001</i>
<b>Brush Is</b>	0.059	0.035	0.005	0.008	0.016	0.009		0.006	0.390	<i>0.001</i>
<b>Tollgate Iss</b>	<i>0.112</i>	0.039	<i>0.037</i>	0.052	<i>0.056</i>	0.033	0.039		0.030	<i>0.001</i>
<b>Montague Is</b>	<i>0.054</i>	<i>0.022</i>	0.012	0.003	0.014	0.014	0.001	<i>0.018</i>		<i>0.001</i>
<b>Kangaroo Is</b>	0.033	<i>0.080</i>	<i>0.066</i>	0.016	0.014	<i>0.081</i>	0.051	<i>0.067</i>	0.032	

880

881 **Table 4A:** Shannon's mutual information index ( $I$ , see Sherwin et al. 2006) genetic  
882 differentiation at eleven microsatellite loci

883 Below diagonal are  $I$  values, probabilities  $p(\text{rand} \geq \text{data})$  based on 999 permutations  
884 are shown above diagonal. Significant results after bonferroni correction ( $p > 0.001$ )  
885 are highlighted in italics; Is – island, Iss - Islands

	<b>Bro</b>	<b>Cab</b>	<b>Lio</b>	<b>Man</b>	<b>Fiv</b>	<b>Bow</b>	<b>Bru</b>	<b>Tol</b>	<b>Mon</b>	<b>Kan</b>
<b>Broughton Is</b>		<i>0.001</i>	<i>0.001</i>	0.079	0.062	<i>0.001</i>	<i>0.001</i>	<i>0.001</i>	<i>0.001</i>	0.001
<b>Cabbage Tree Is</b>	<i>0.068</i>		0.066	0.005	<i>0.001</i>	0.002	0.002	0.003	0.004	<i>0.001</i>
<b>Lion Is</b>	<i>0.055</i>	0.025		0.047	<i>0.001</i>	0.694	0.066	0.003	0.017	<i>0.001</i>
<b>Manly</b>	0.053	0.049	0.030		0.310	0.018	0.034	<i>0.001</i>	0.233	0.003
<b>Five Iss</b>	0.047	<i>0.060</i>	<i>0.045</i>	0.041		<i>0.001</i>	0.007	<i>0.001</i>	0.020	0.002
<b>Bowen Is</b>	<i>0.040</i>	0.026	0.012	0.025	<i>0.039</i>		0.010	<i>0.001</i>	<i>0.001</i>	<i>0.001</i>
<b>Brush Is</b>	<i>0.081</i>	0.046	0.028	0.052	0.052	0.025		0.002	0.052	<i>0.001</i>
<b>Tollgate Iss</b>	0.092	0.049	0.036	<i>0.069</i>	<i>0.070</i>	0.030	0.060		0.081	<i>0.001</i>
<b>Montague Is</b>	0.055	0.040	0.028	0.035	0.039	<i>0.025</i>	0.036	0.036		<i>0.001</i>
<b>Kangaroo</b>	0.045	<i>0.064</i>	<i>0.060</i>	0.041	0.038	0.065	0.060	<i>0.058</i>	0.043	

886 ***MIGRATE Estimates – Summary tables***

887 **Table 5A:** MIGRATE estimates of the number of immigrants per generation, xNm

Dispersal estimates		Microsatellites		Mitochondrial haplotypes	
		Recipient population		Recipient population	
Local dispersal		Bowen Island	Lion Island	Bowen Island	Lion Island
Sample Size		61	<b>43</b>	36	<b>17</b>
Bayesian	Run 1	123.3	96.7	418.3	357.0
	Run 2	110.0	103.3	493.7	381.7
	Run 3	96.7	96.7	524.3	401.0
<b>Bayesian Mean</b>		<b>110.0</b>	<b>98.9</b>	<b>478.8</b>	<b>379.9</b>
Maximum Likelihood	Run 1	1.4	0.3	272.8	466.9
	Run 2	0.0	10.6	505.0	341.4
	Run 3	0.8	0.6	320.5	435.4
ML Mean		0.7	3.9	366.1	414.6
<hr/>					
Regional dispersal		NSW	SA	NSW	SA
Sample Size		229	<b>46</b>	161	<b>39</b>
Bayesian	Run 1	90.0	116.7	384.3	294.3
	Run 2	90.0	116.7	470.3	317.0
	Run 3	103.3	136.7	412.3	309.7
<b>Bayesian Mean</b>		<b>94.4</b>	<b>123.3</b>	<b>422.3</b>	<b>307.0</b>
Maximum Likelihood	Run 1	1.5	2.2	416.0	333.7
	Run 2	2.1	0.3	171.9	122.2
	Run 3	2.7	1.6	220.1	383.6
ML Mean		2.1	1.4	269.3	279.8

888

889

890 **Table 6A:** MIGRATE estimates of mutation-scaled population size  $\Theta$

Theta estimates		Microsatellites		Mitochondrial haplotypes	
		Recipient population		Recipient population	
Local		Bowen Island	Lion Island	Bowen Island	Lion Island
Sample Size		61	<b>43</b>	36	<b>17</b>
Bayesian	Run 1	1.500	1.900	0.012	0.018
	Run 2	1.767	1.833	0.014	0.013
	Run 3	2.467	2.333	0.009	0.012
<b>Bayesian Mean</b>		<b>1.911</b>	<b>2.022</b>	<b>0.012</b>	<b>0.014</b>
Maximum Likelihood	Run 1	1.196	1.997	0.002	0.006
	Run 2	0.983	0.870	0.003	0.004
	Run 3	1.715	3.123	0.002	0.005
ML Mean		1.298	1.997	0.002	0.005
<hr/>					
Regional		NSW	SA	NSW	SA
Sample Size		229	46	161	39
Bayesian	Run 1	1.367	1.567	0.012	0.017
	Run 2	1.933	3.133	0.008	0.019
	Run 3	2.067	2.600	0.009	0.019
<b>Bayesian Mean</b>		<b>1.789</b>	<b>2.433</b>	<b>0.009</b>	<b>0.018</b>
Maximum Likelihood	Run 1	2.143	7.004	0.002	0.012
	Run 2	2.449	2.068	0.001	0.001
	Run 3	0.700	1.018	0.001	0.001
ML Mean		1.764	3.363	0.001	0.004

891

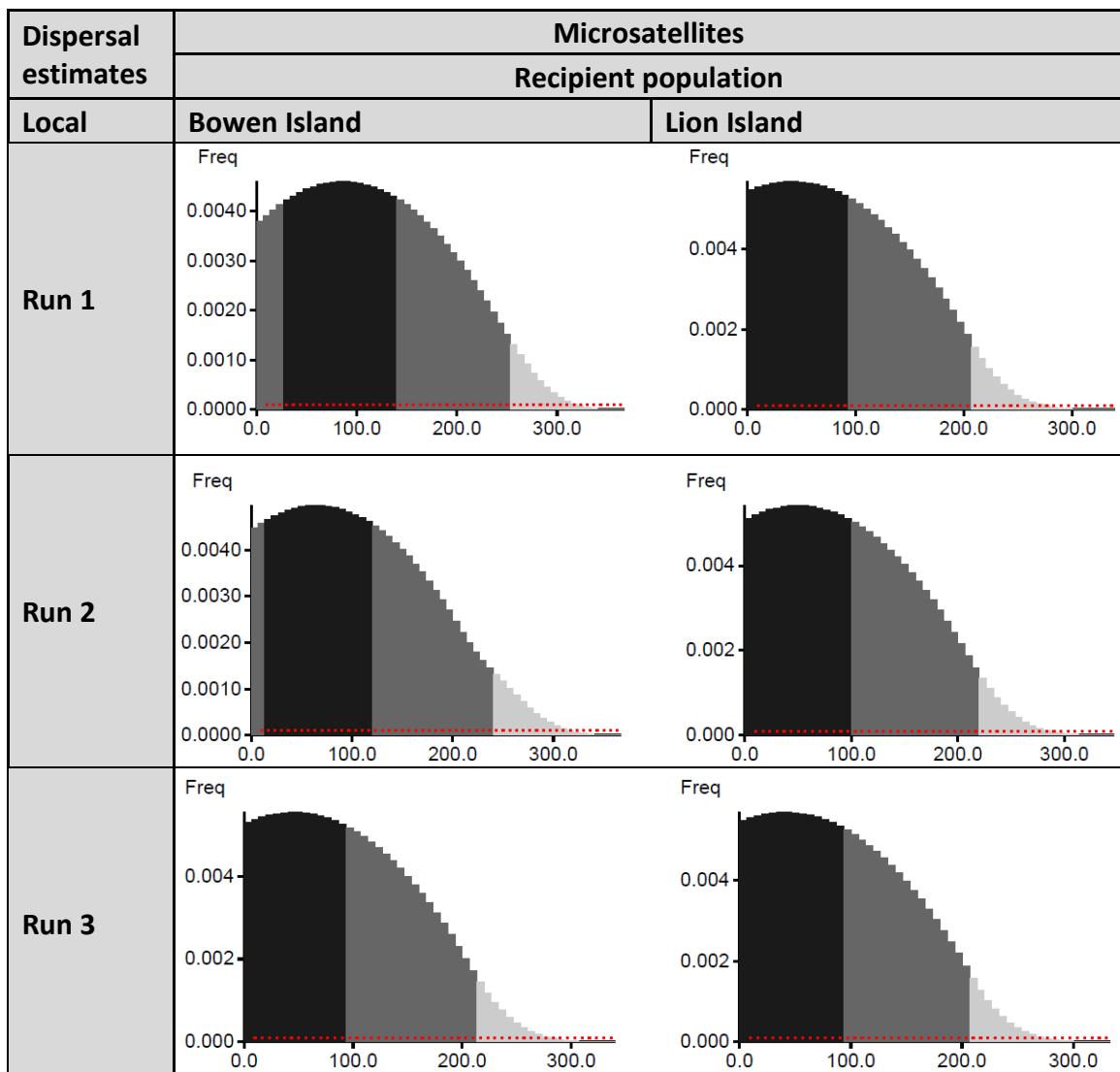
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895 **MIGRATE Estimates – Likelihood plots for number of migrants per generation,**  
896 **xNm, after Bayesian inference**  
897

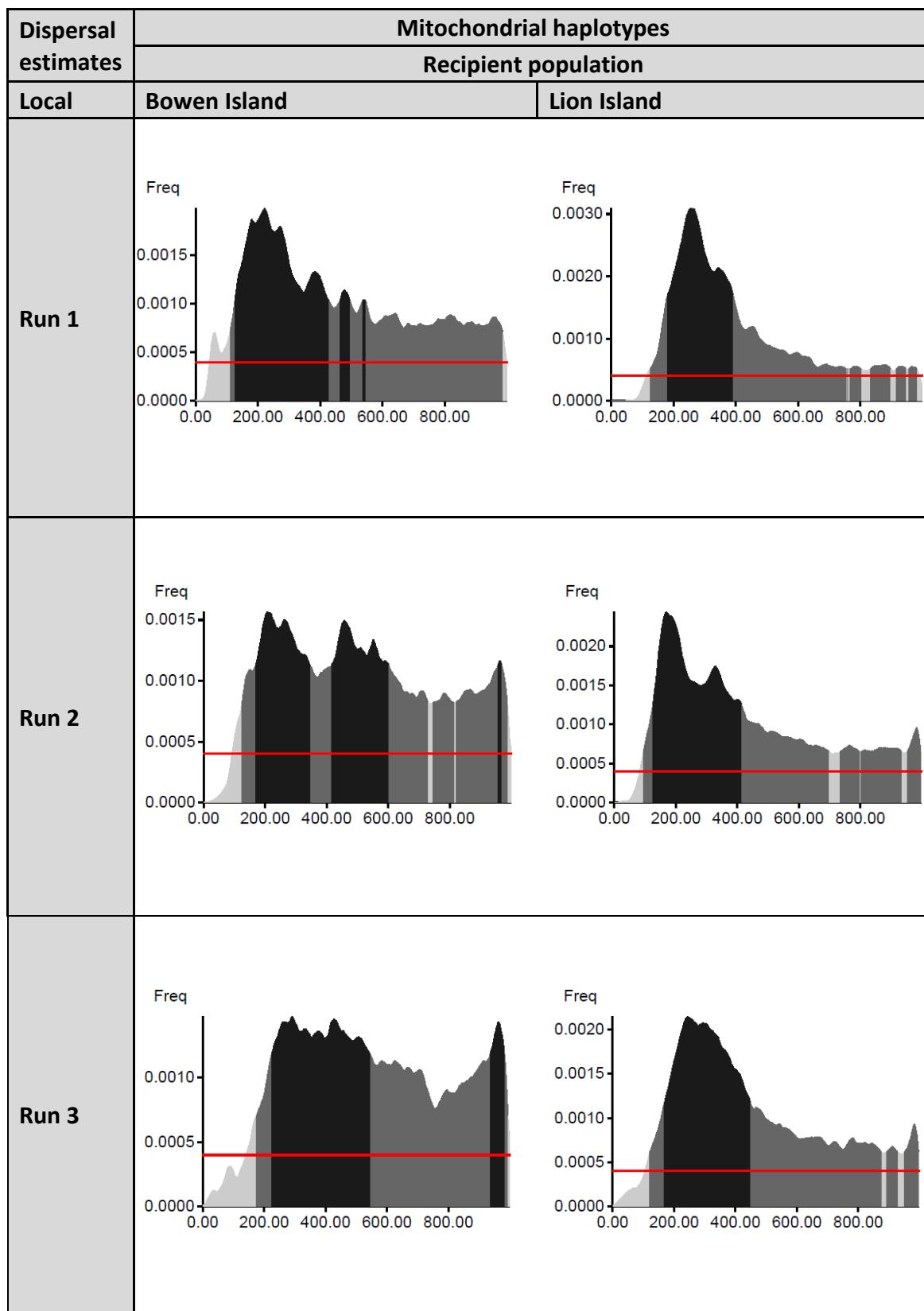
898 **Table 7A:** Likelihood plots of the number of immigrants per generation, xNm, into  
899 Bowen or Lion Island; posterior distribution over eleven microsatellite loci after  
900 Bayesian inference



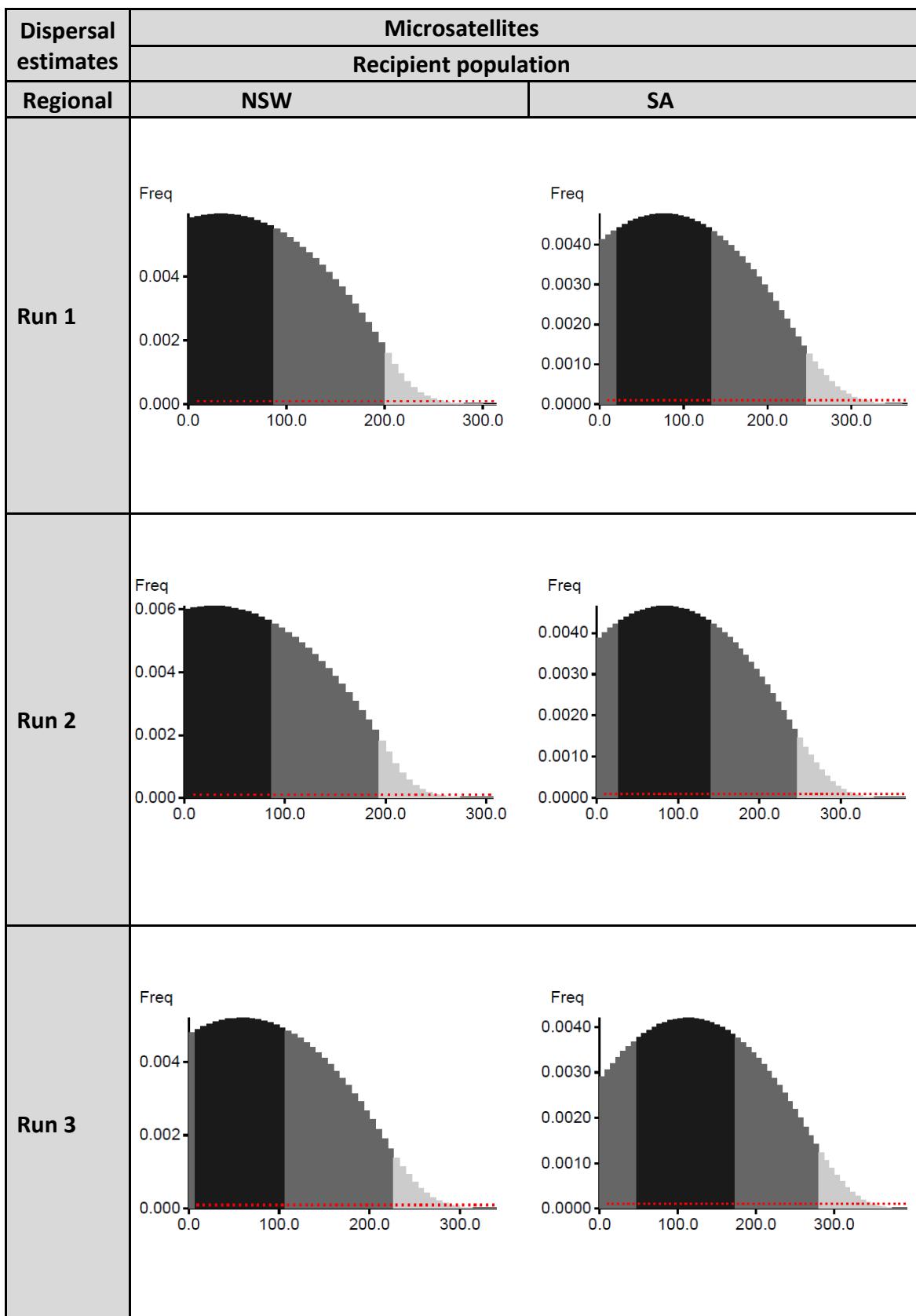
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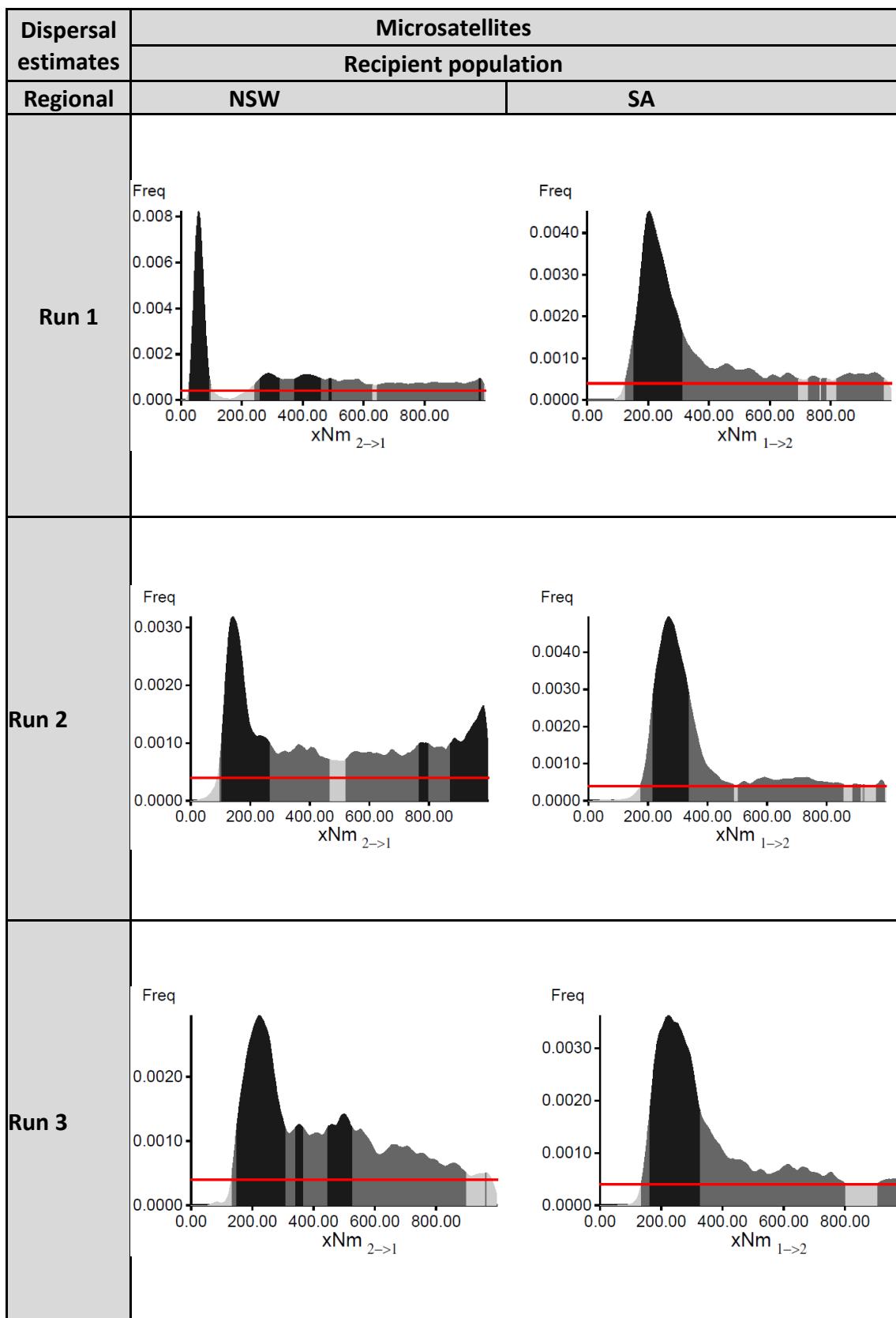
903 **Table 8A:** Likelihood plots of the number of immigrants per generation,  $xNm$ , into  
 904 Bowen or Lion Island; posterior distribution at one mitochondrial marker locus after  
 905 Bayesian inference



907 **Table 9A:** Likelihood plots of the number of immigrants per generation,  $xNm$ , into NSW  
908 or SA; posterior distribution at eleven microsatellite loci after Bayesian inference

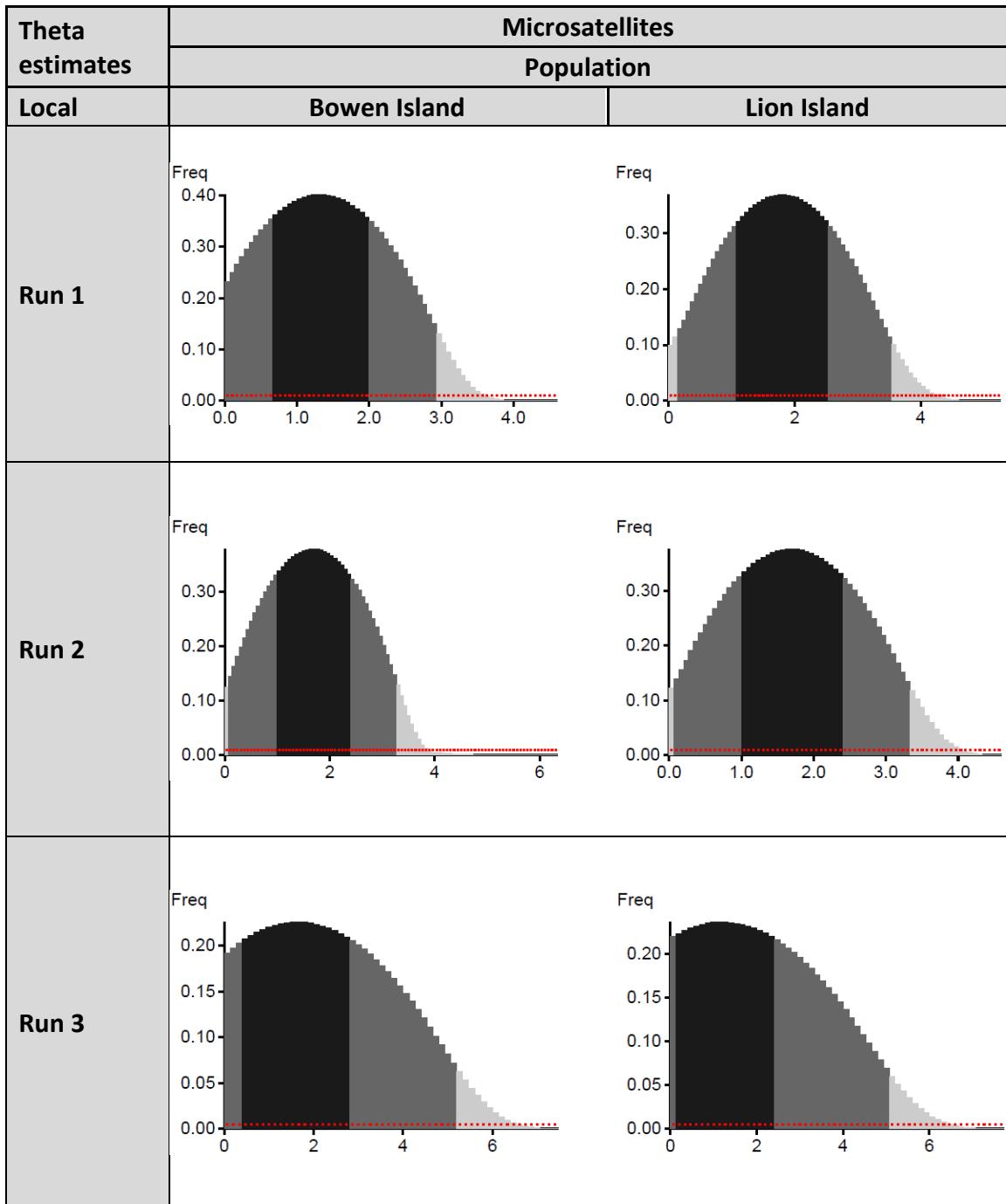


909 **Table 10A:** Likelihood plots of the number of immigrants per generation,  $xNm$ , into  
 910 NSW or SA; posterior distribution at one mitochondrial marker locus after Bayesian  
 911 inference



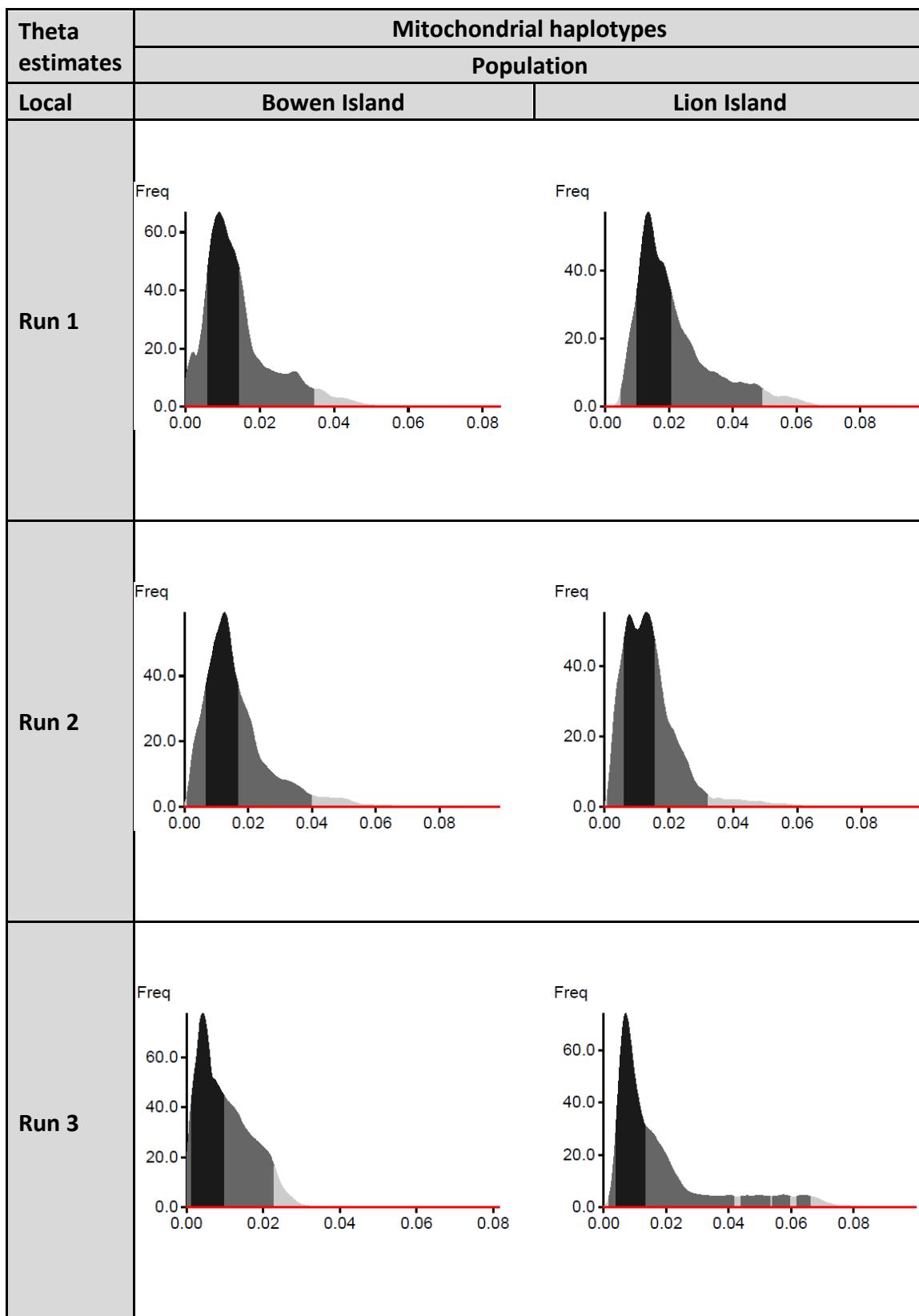
912 **MIGRATE Estimates – Likelihood plots for mutation-scaled population size  $\Theta$**   
913 **after Bayesian inference**  
914

915 **Table 11A:** Likelihood plots of the mutation-scaled population size  $\Theta$ , at Bowen and  
916 Lion Island; posterior distribution at eleven microsatellite loci after Bayesian inference



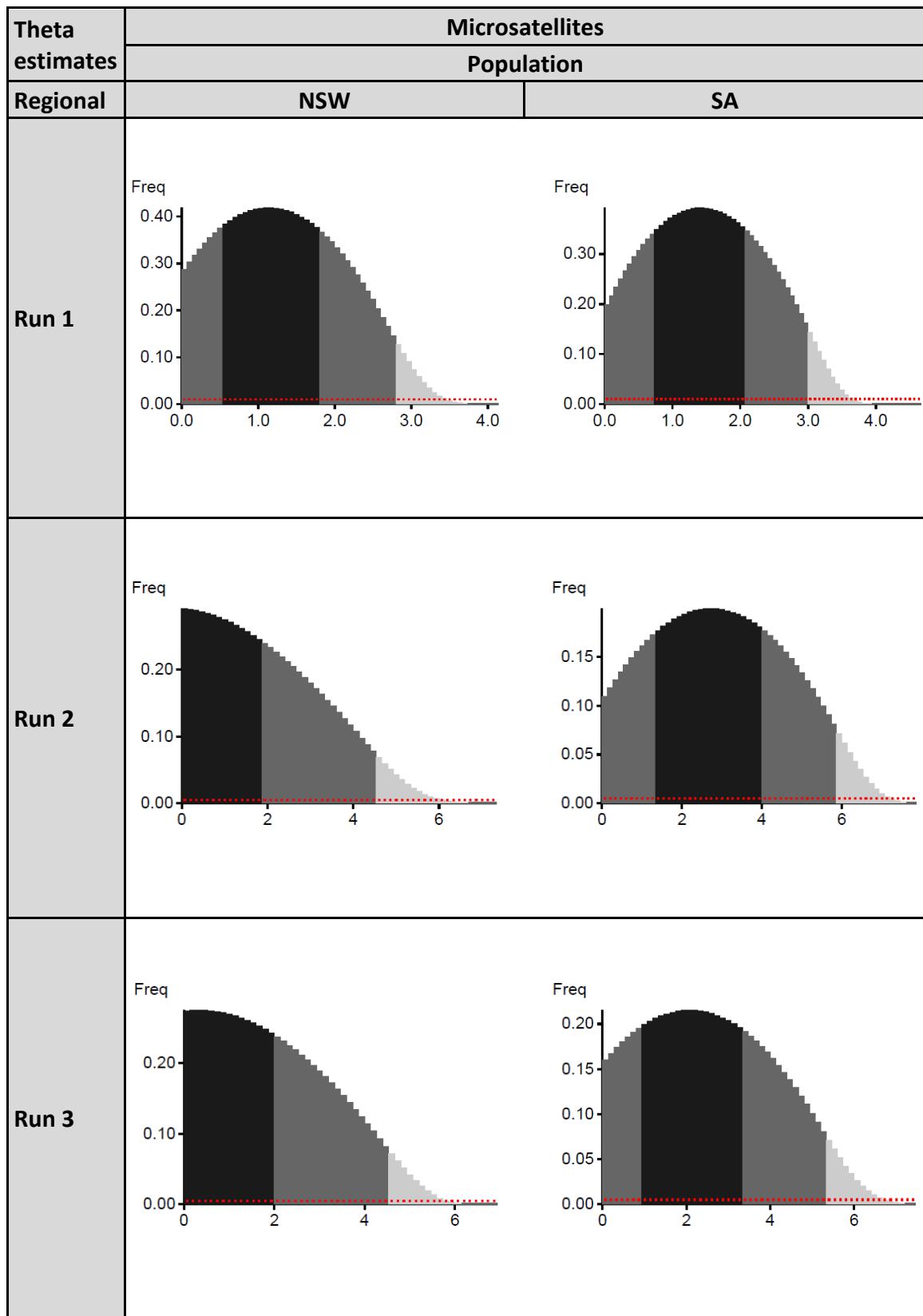
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918 **Table 12A:** Likelihood plots of the mutation-scaled population size  $\Theta$ , at Bowen and  
 919 Lion Island; posterior distribution at one mitochondrial locus after Bayesian inference



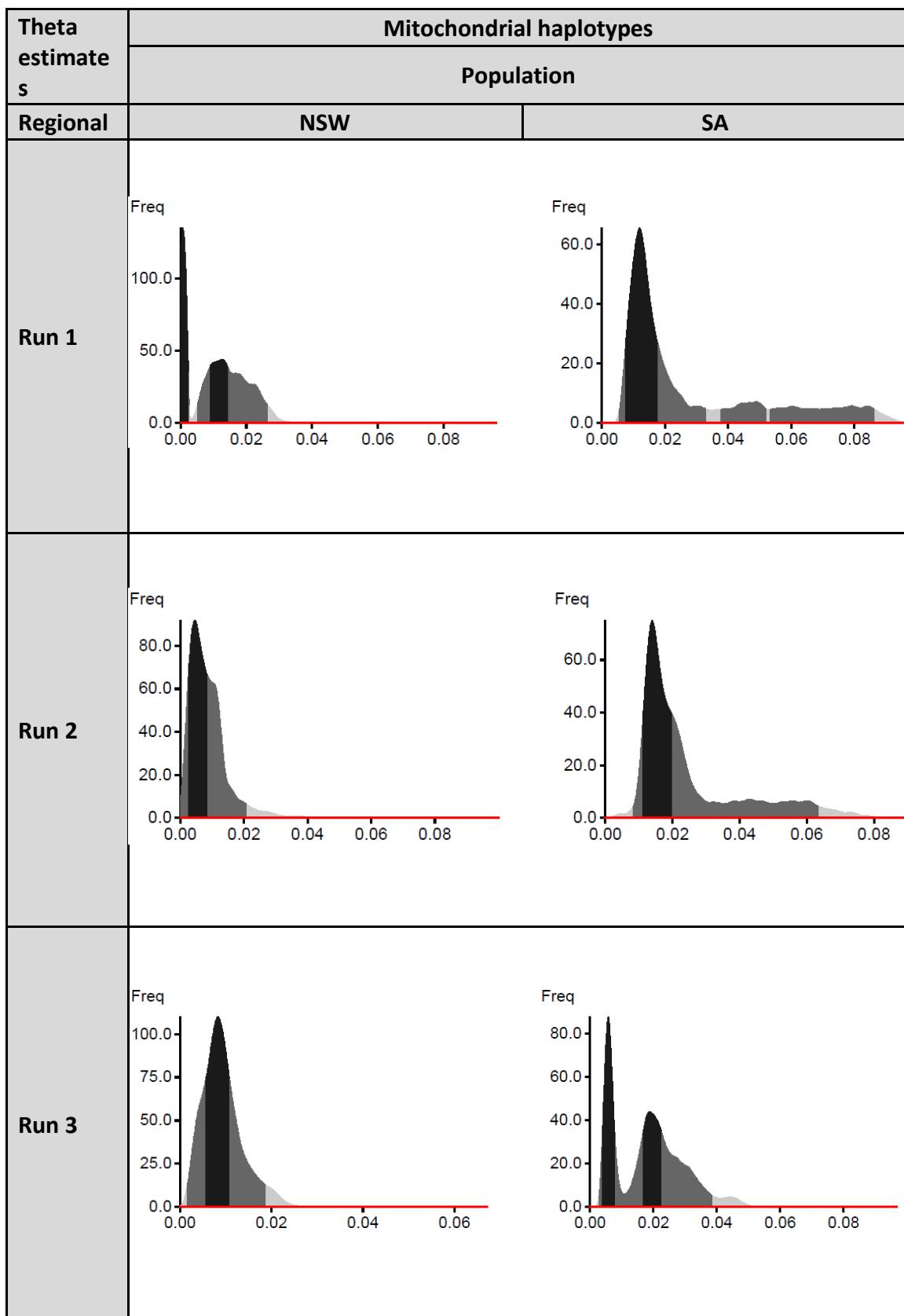
920

921 **Table 13A:** Likelihood plots of the mutation-scaled population size  $\Theta$ , in NSW and SA;  
922 posterior distribution at eleven microsatellite loci after Bayesian inference



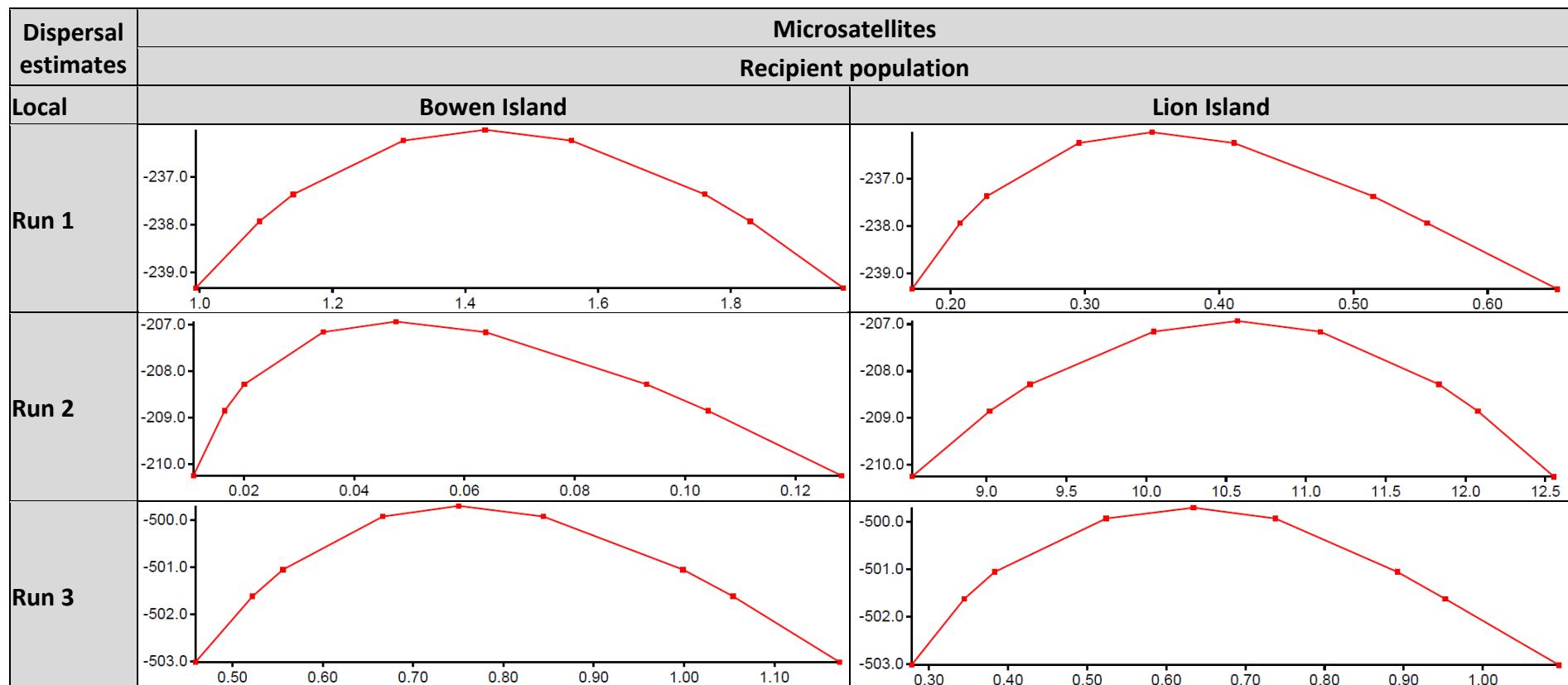
923

924 **Table 14A:** Likelihood plots of the mutation-scaled population size  $\Theta$ , in NSW and SA;  
 925 posterior distribution at one mitochondrial locus after Bayesian inference



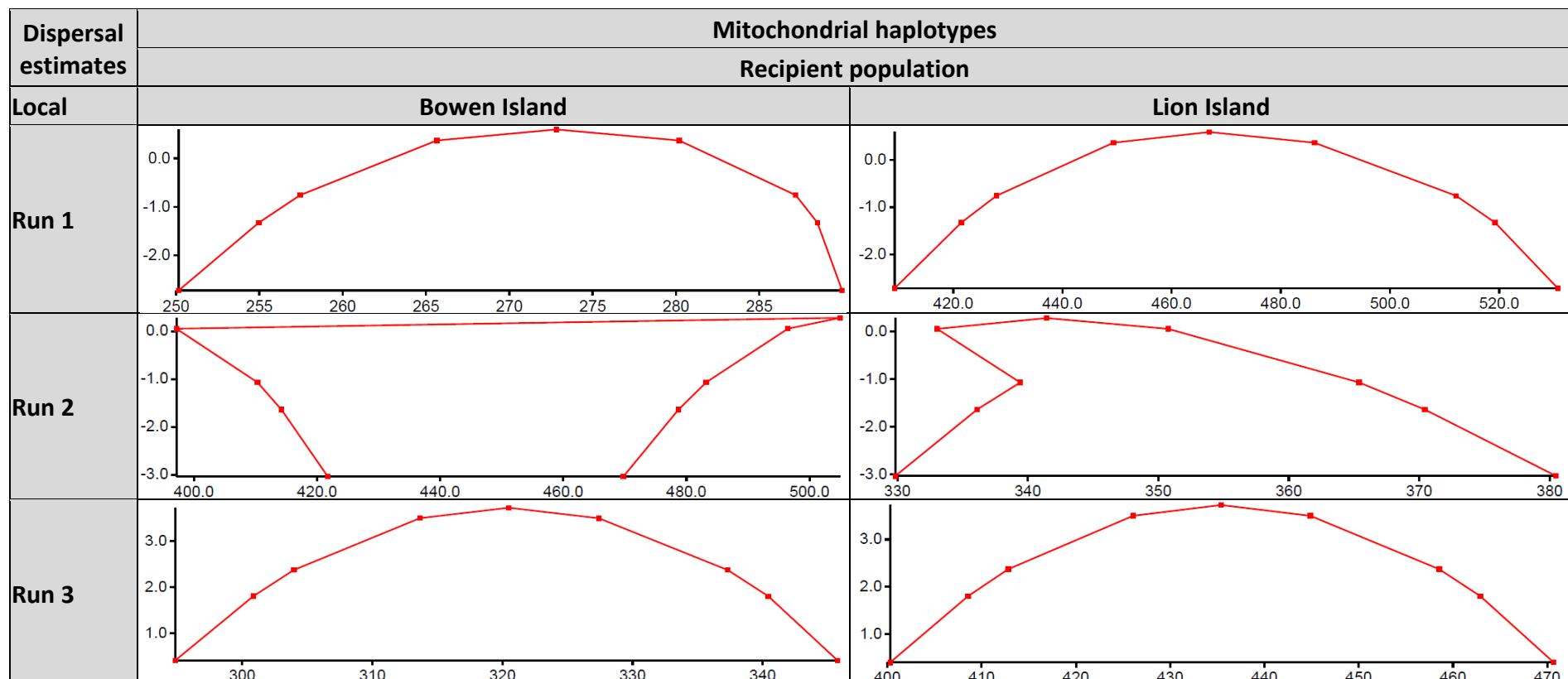
927 **MIGRATE Estimates – Likelihood plots for number of migrants per generation, xNm, after Maximum Likelihood inference**

928 **Table 15A:** Likelihood plots of the number of immigrants per generation,  $xNm$ , into Bowen or Lion Island; posterior distribution at eleven  
929 microsatellite loci after Maximum Likelihood inference



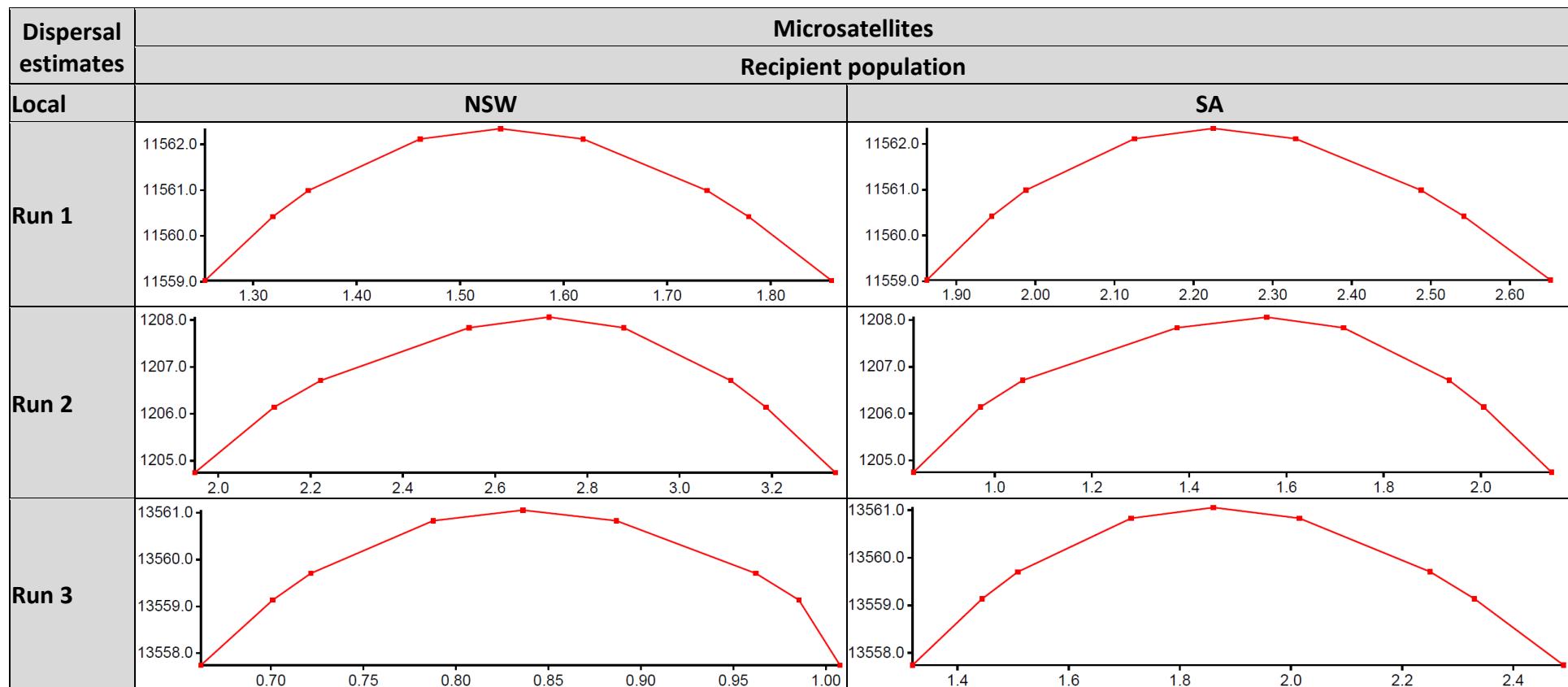
930

931 **Table 16A:** Likelihood plots of the number of immigrants per generation,  $xNm$ , into Bowen or Lion Island; posterior distribution at one  
932 mitochondrial locus after Maximum Likelihood inference



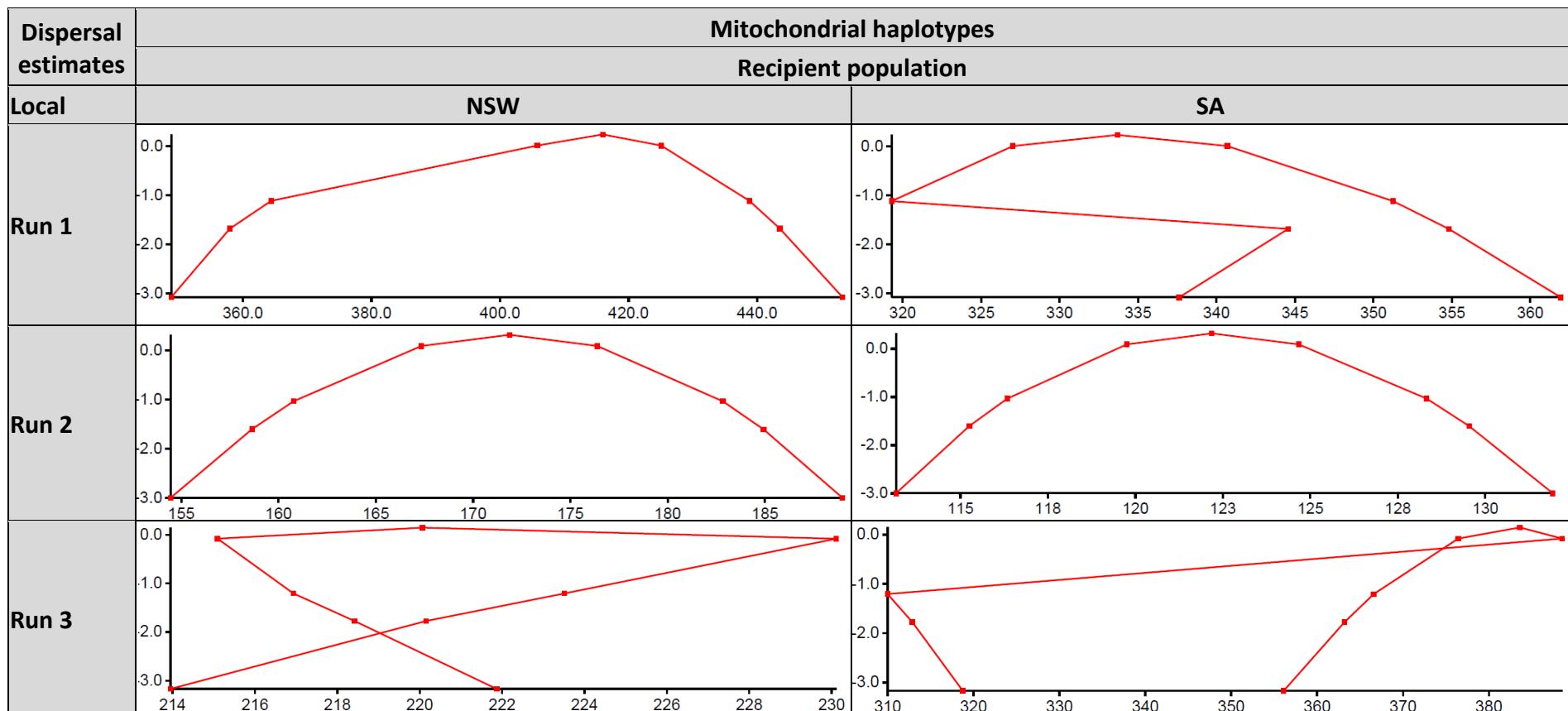
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934 **Table 17A:** Likelihood plots of the number of immigrants per generation,  $xNm$ , into NSW or SA; posterior distribution at eleven microsatellite  
935 loci after Maximum Likelihood inference



936

937 **Table 18A:** Likelihood plots of the number of immigrants per generation,  $xNm$ , into NSW or SA; posterior distribution at one mitochondrial  
938 locus after Maximum Likelihood inference



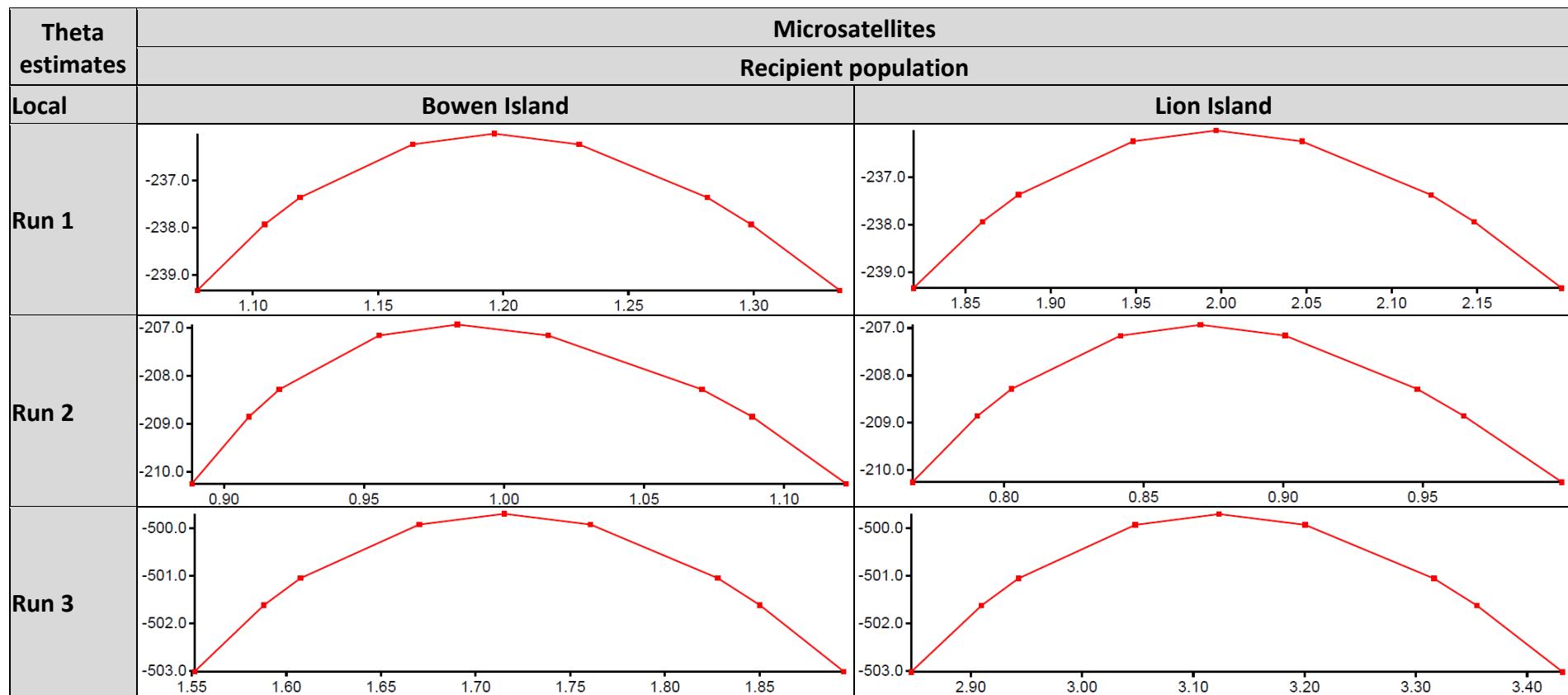
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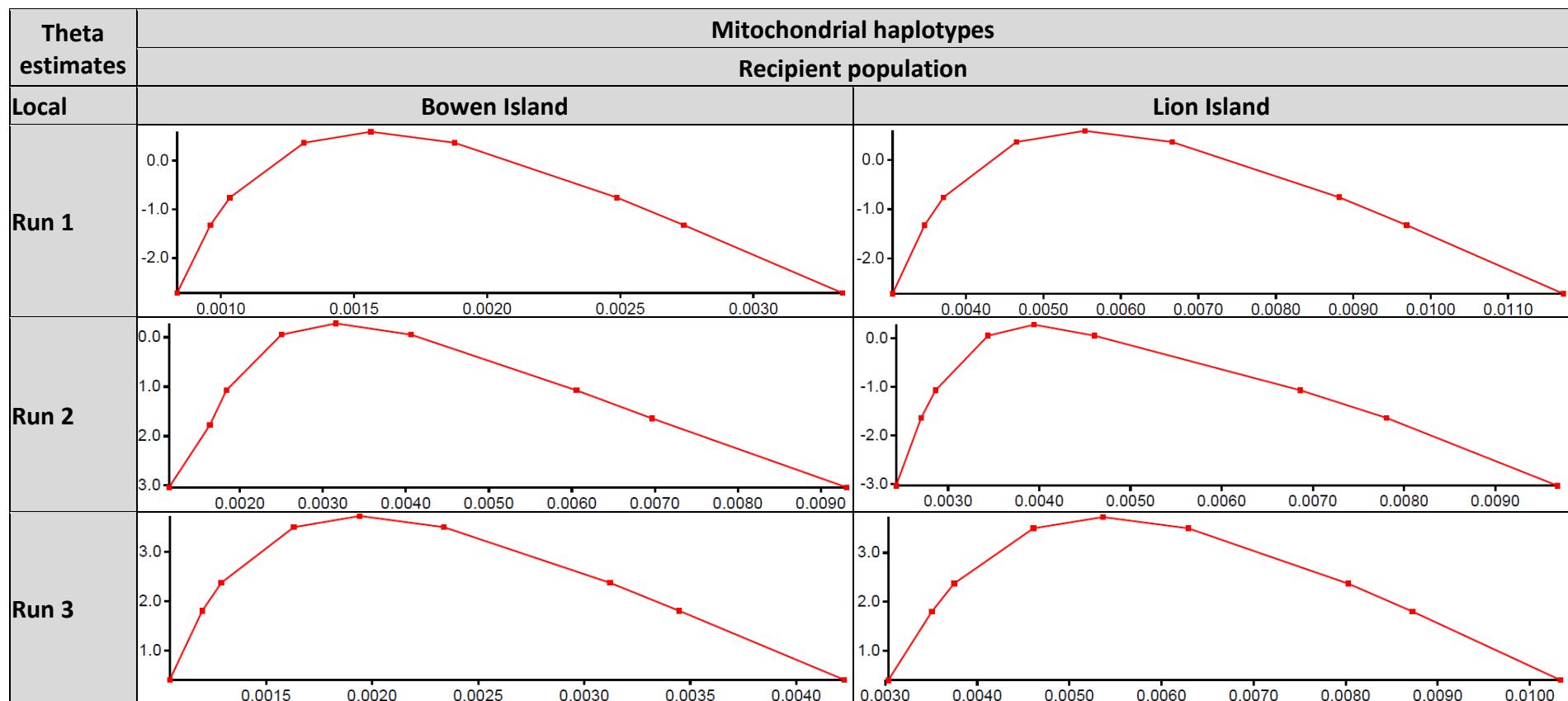
941 **MIGRATE Estimates – Likelihood plots for number of migrants per generation,  $xNm$ , after Maximum Likelihood inference**

942

943 **Table 19A:** Likelihood plots of the mutation-scaled population size  $\Theta$ , at Bowen and Lion Island; posterior distribution at eleven microsatellite  
 944 loci after Maximum Likelihood inference



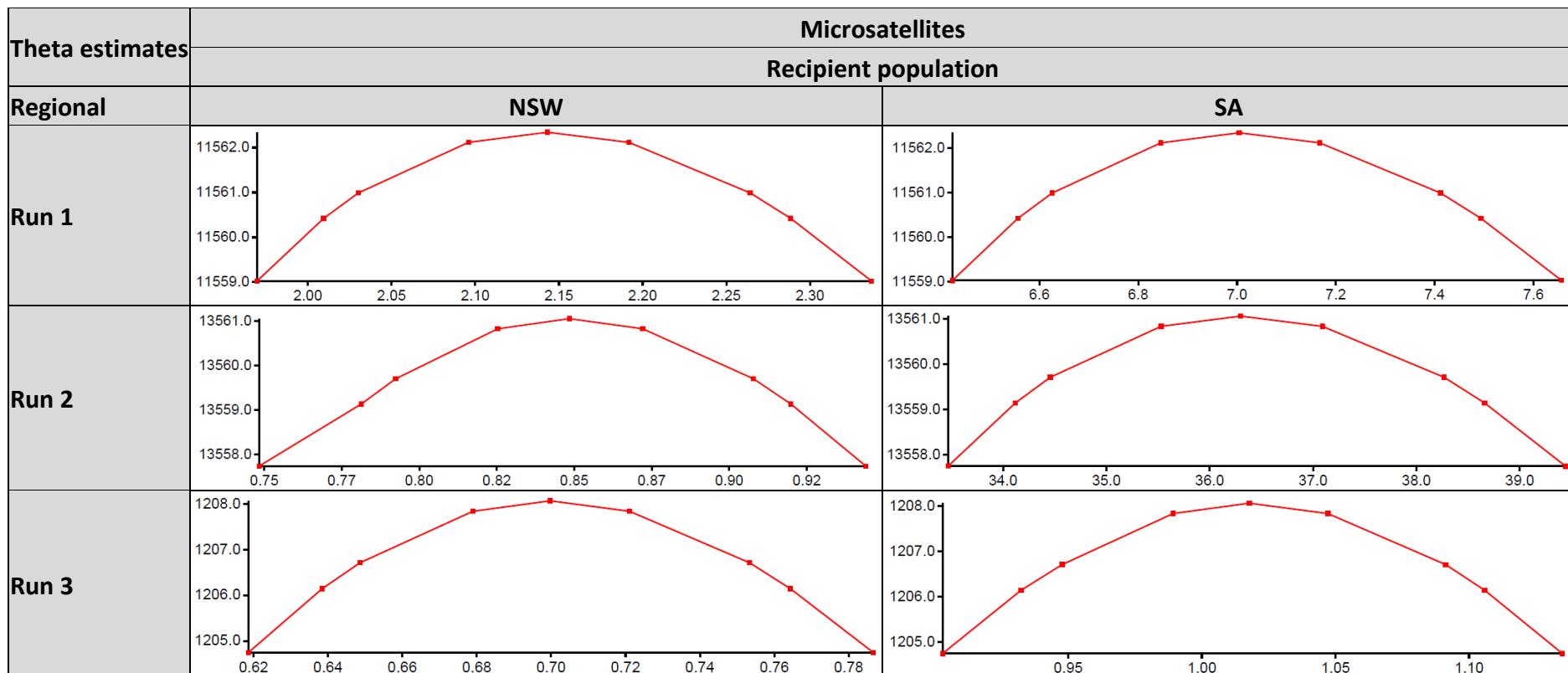
945 **Table 20A:** Likelihood plots of the mutation-scaled population size  $\Theta$ , at Bowen and Lion Island; posterior distribution at one mitochondrial  
 946 locus after Maximum Likelihood inference



947

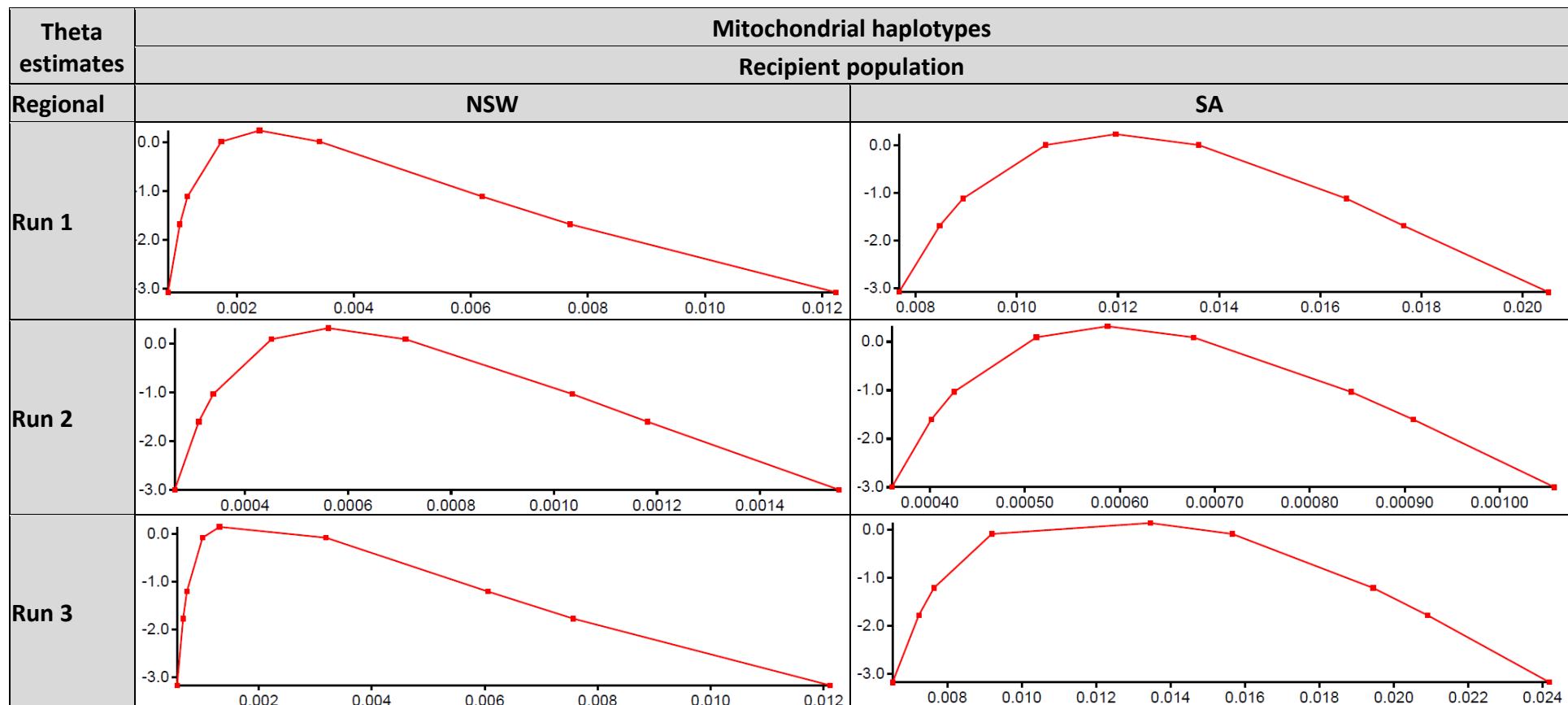
948

949 **Table 21A:** Likelihood plots of the mutation-scaled population size  $\Theta$ , in NSW and SA; posterior distribution at eleven microsatellite loci after  
950 Maximum Likelihood inference



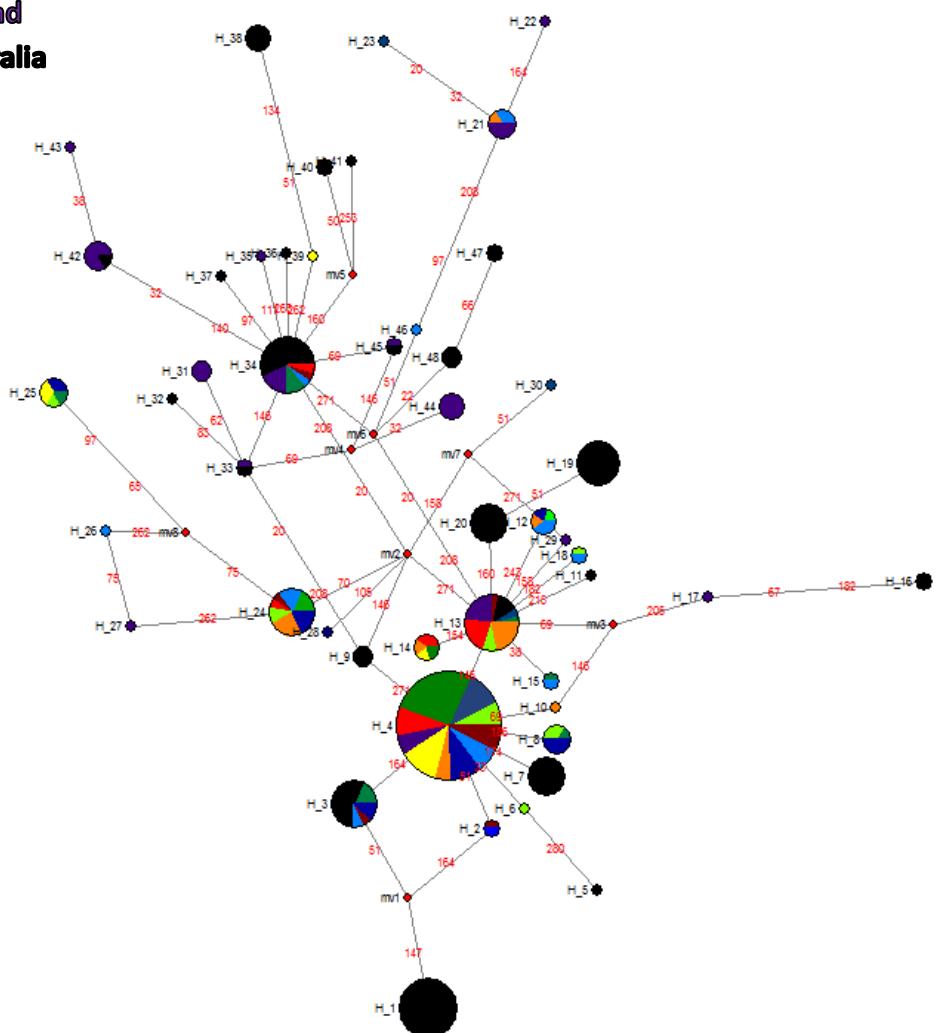
951

952 **Table 22A:** Likelihood plots of the mutation-scaled population size  $\Theta$ , in NSW and SA; posterior distribution at one mitochondrial locus after  
953 Maximum Likelihood inference



954

**Broughton Island**  
**Cabbage Tree Island**  
**Lion Island**  
**Manly**  
**Five Islands**  
**Bowen Island**  
**Brush Island**  
**Tollgate Islands**  
**Montague Island**  
**Kangaroo Island**  
**Western Australia**



955

**Figure 1A:** Haplotype network for mitochondrial control region sequences of 311 individuals

957 Colour of pie chart sectors corresponds to colony of origin of individuals carrying the haplotype, size of sectors corresponds to number of individuals from each colony.

151

Chapter 3: Population genetics of Little Penguins  
(*Eudyptula minor*) in Australia

958

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## 2 Chapter 4: Patterns of Major Histocompatibility Complex diversity, 3 parasitism and mate choice in Little Penguins (*Eudyptula minor*)

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**20    Keywords:** immune gene variability, host-pathogen coevolution, MHC

## **21    *Author contributions:***

22 JJS and BLC provided samples and field data for WA; DCN contributed samples and  
23 parasite data for SA. I (SV) performed laboratory analyses on all samples, with the help  
24 of MC (single-read sequencing) and GC (MiSeq); I also performed all statistical analysis,  
25 and wrote the chapter.



27 **Summary:** 235 words

28 Processes relevant for safeguarding wild populations from extinction rely on adaptions

29 within populations and are therefore best studied by looking at genes under selection

30 for which environmental pressures are known or suspected. In particular, the possible

31 impact of pathogenic threats can be assessed by studying highly variable Major

32 Histocompatibility Complex (MHC) genes, which influence many important biological

33 traits, including susceptibility to parasite infestation and mating preferences.

34 Populations of one of Australia's most iconic native bird species, the little penguin

35 (*Eudyptula minor*), are declining in numerous locations, and penguin mortality due to

36 infestation with a novel *Haemoproteus* parasite has recently been described for the

37 first time. We therefore investigated whether selective processes are helping to

38 maintain variability of MHC genes in penguins. We compared populations of different

39 size and connectivity, and examined the selective effects of parasite pressure and mate

40 choice. Genetic diversity at the MHC locus largely mirrors diversity at neutral loci,

41 indicating a strong effect of stochasticity. Nevertheless, there were signs of selective

42 pressures acting on the MHC. The lowest rates of parasite infestation were suspected

43 for individuals of intermediate MHC diversity. The penguins showed signs of

44 inbreeding at the MHC locus, with significantly higher levels of inbreeding in the

45 parental than in the offspring generation in Western Australia, but not New South

46 Wales, despite similar overall levels of inbreeding between states. Some mechanism of

47 inbreeding avoidance was therefore suspected for Western Australian penguins.

## 49 **Contents**

50	Chapter 4: Patterns of Major Histocompatibility Complex diversity, parasitism and	
51	mate choice in Little Penguins ( <i>Eudyptula minor</i> ) .....	153
52	Introduction .....	157
53	Materials and methods.....	162
54	Results.....	173
55	Discussion.....	184
56	Acknowledgements.....	189
57	References.....	190
58	Appendix 4 .....	196
59		

60 **Introduction**

61 An increasing number of studies indicate that genetic diversity plays an important role  
62 in buffering populations against environmental change including disease outbreaks  
63 (Coltman et al. 1999; Spielman et al. 2004; Frankham & Kingslover 2004). Therefore,  
64 studying the genetic effects of population changes is of central importance for  
65 conservation biology (Frankham 2005; Eizaguirre & Baltazar-Soares 2014). Population  
66 genetic studies often focus on genetic markers that are not subject to natural  
67 selection, so-called neutral markers such as many microsatellites, some single  
68 nucleotide polymorphisms (SNPs) and possibly the mitochondrial control region  
69 sequences. These markers are useful to elucidate dispersal patterns and population  
70 history (see Chapter 3), or to study molecular clocks, analyse paternity and classify  
71 individuals by relatedness (Sommer 2005). However, processes relevant for  
72 safeguarding wild populations from extinction rely on adaptations within populations  
73 and are therefore best studied by looking at genes that are under selection and for  
74 which environmental pressures are known or suspected. In particular, the possible  
75 impact of pathogenic threats can be assessed by studying a highly variable group of  
76 genes shown to influence many important biological traits, including immune  
77 recognition influencing susceptibility to parasite infestation (e.g. Wegner et al. 2003),  
78 infectious and autoimmune diseases (Fernando et al. 2008), mating preferences  
79 through individual odours (Penn & Potts 1998), kin recognition, cooperation (Manning  
80 et al. 1992; Grafen 1992) and pregnancy outcomes (Ober 1998). This group of genes is  
81 called the Major Histocompatibility Complex (MHC).

82 The MHC consists of genes that encode for cell-surface proteins that bind to antigens  
83 and present these to T-lymphocytes, thus initiating an immune response (Benacerraf  
84 1981). There are two classes of MHC genes: class I genes confer resistance against  
85 intracellular parasites and pathogens and are expressed on nearly every cell type,  
86 while the peptides coded by MHC class II genes can bind antigens of extracellular  
87 parasites and are only expressed by specialised antigen-presenting cells (e.g.,  
88 macrophages or dendritic cells - Janeway et al. 2001). MHC genes have been shown to  
89 directly relate to parasitism, and resistance alleles increase rapidly in frequency, with  
90 effects observable among successive generations (Eizaguirre et al. 2012).

91 Here, we investigate exon 2 of the DRB1-like MHC Class II  $\beta$ 1 domain in little penguins  
92 (Tsuda et al. 2001). This gene is well conserved, with orthologous DRB loci found in  
93 birds (chickens: Pharr et al. 1998; blackbirds: Edwards et al. 2000) and primates  
94 including humans (Gaur & Nepom 1996). The gene has previously been shown to be  
95 highly polymorphic in several penguin species (Tsuda et al. 2001; Kikkawa et al. 2005;  
96 Bollmer et al. 2007; Kikkawa et al. 2009), and under positive selection in Magellanic  
97 penguins (Knafler et al. 2012).

98 The exceptional variability of MHC genes is believed to be maintained by one or more  
99 of several selective processes that either give an advantage to heterozygous  
100 individuals (Doherty & Zinkernagel 1975), or else favour rare genotypes (frequency-  
101 dependent selection, Slade & McCallum 1992). These processes are expected to have a  
102 stronger effect in populations more heavily affected by parasites (Cohen 2002). The  
103 heterozygote advantage or frequency-dependent selection might also be due to sexual

104 selection acting through MHC-based mate choice, possibly as a secondary evolutionary  
105 response to the selection by parasites (Milinski 2006).

106 One of Australia's most iconic native bird species is the little penguin (*Eudyptula*  
107 *minor*), populations of which are declining in numerous locations (e.g. Dann et al.  
108 2000; Wiebkin 2011). Common threats to which declines are attributed include  
109 predation by introduced and native predators, habitat destruction and modification, as  
110 well as direct and indirect effects of fishing activity (e.g. Croxall & Butchart 2012;  
111 Cannell et al. 2011; Wiebkin 2011). The effects of climate change put further stress on  
112 little penguins, with higher sea surface temperatures being linked to poorer breeding  
113 success in south-western Australia (Cannell et al. 2012) and rising sea levels affecting  
114 availability of nesting habitat (Schumann et al. 2013). There might also be positive  
115 effects of a warming climate, with higher sea-surface temperatures linked to increased  
116 breeding success and first year survival in south-eastern Australia (Dann & Chambers  
117 2013; Sidhu et al. 2012; Cullen et al. 2009).

118 Recently, a novel *Haemoproteus* parasite was observed in little penguins, the first time  
119 it was recorded in any member of the Spheniscidae family (Cannell et al. 2013).

120 Mortality could be ascribed to the parasite's presence, and involvement of anomalous  
121 environmental conditions was postulated to have led to a potential increase in local  
122 vectors. Additionally, the likelihood of disease outbreaks and parasite infestations  
123 might be altered as an effect of climate change (Cannell et al. 2013; Burge et al. 2014).

124 Little penguins are the smallest penguin species and the only one breeding in Australia.  
125 These flightless seabirds attract large numbers of tourists to several Australian states,  
126 and are important to local economies (Bool et al. 2007; Whiteoak 2009). Their total  
159

127 abundance has been estimated as 350,000 – 600,000 individuals (Dann et al. 1996),  
128 but might now be lower due to recent declines in some colonies (e.g., Dann et al. 2000;  
129 Wiebkin 2011a). One colony at Manly in Sydney Harbour has even been listed as an  
130 endangered population (NSW National Parks and Wildlife Service 2000). Following its  
131 listing and subsequent intensive management, this colony has successfully recovered  
132 from a few tens of pairs to the most successful breeding season in decades, with 85  
133 breeding pairs in 2013 (Lisa O'Neill, pers. comm.). However, other penguin colonies  
134 around Australia are not doing as well, with declines and even local extinctions  
135 reported in South Australia (Colombelli-Négrel & Kleindorfer 2014) and Tasmania  
136 (Stevenson & Woehler 2007).

137 Here, we investigate whether the aforementioned selective processes lead to penguin  
138 populations of different size, connectivity and parasite pressure maintaining a similar  
139 variability of MHC genes. In penguins, MHC have thus far only been studied in a limited  
140 number of individuals, and only at one MHC class II gene. In 2001, Tsuda et al.  
141 reported their phylogenetic analysis of an MHC locus in 16 wild and captive individuals  
142 from four penguin species, followed by an analysis of the sequence variations in the  
143 endangered Humboldt penguins at the same locus, as well as using three other primer  
144 pairs amplifying parts of the same DRB1-like gene (Kikkawa et al. 2005). A third  
145 publication from the same group revealed trans-species polymorphism of this gene in  
146 banded penguins (genus *Spheniscus*, Kikkawa et al. 2009). Bollmer et al. (2007) also  
147 reported low MHC variation in the endangered Galápagos penguin using a  
148 combination of cloning and sequencing, and MHC diversity and mate choice were  
149 similarly investigated in 50 pairs of Magellanic Penguin (Knafler et al. 2012). Little

150 penguins have thus far been represented in these studies with only four captive-bred  
151 individuals, so we present the first study of wild little penguins MHC diversity and its  
152 relation to parasitism and mate choice. The study of immunogenetic patterns at a large  
153 geographic scale allows us to compare different populations and identify signatures of  
154 selection to elucidate the roles of mate choice and parasite pressure. In particular, we  
155 asked four questions. (1) We tested whether genetic diversity at the MHC locus  
156 mirrors diversity at neutral loci (see Chapter 3 for methods). (2) To disentangle the  
157 impacts of parasite pressure and mate choice, we tested whether individuals with  
158 higher MHC diversity had lower parasite loads. (3) Using information on penguin  
159 pedigrees, we also investigated whether parent pairs were more or less matched at  
160 the MHC than expected by chance. (4) We investigated whether the parental  
161 generation had a different level of inbreeding to their offspring.

162

163 **Materials and methods**

164 **Study sites**

165 Genetic samples were collected from a total of 13 little penguin populations (Fig. 1).

166 Nine islands with Little Penguin colonies, eight in New South Wales (NSW) and

167 Kangaroo Island in South Australia (SA), were visited in 2012 or 2013. The colony at

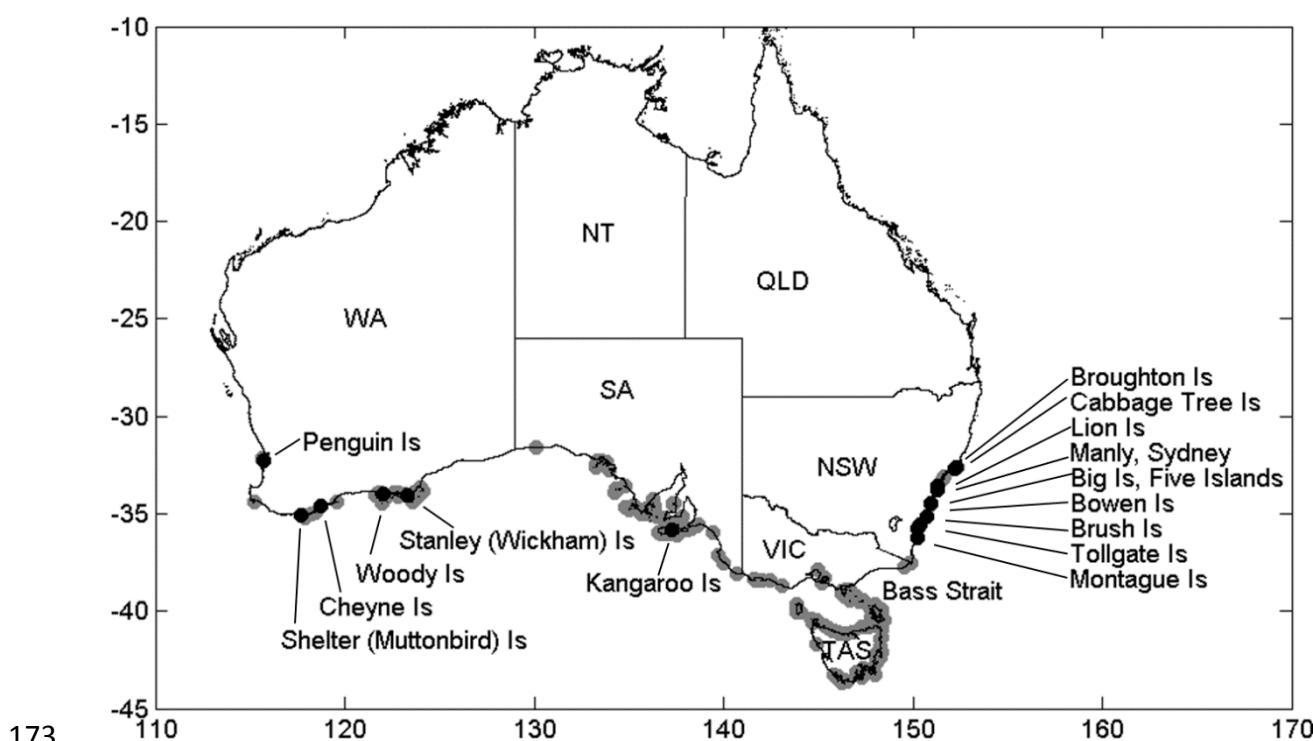
168 Manly was the only mainland colony of little penguins sampled. Additional samples

169 from three populations in Western Australia (WA) were collected between 2000 and

170 2009. Sampling sites cover the northern range edge of the Little Penguin distribution in

171 both NSW and WA, and include colonies of varying sizes and conservation status

172 (Appendix Table A1).



173 174 **Fig. 1:** Map of all known penguin colonies in Australia (grey) with sampling locations  
175 highlighted (black); Is - Island; Latitude and Longitude are shown.

176

177

178 Two different capture methods were followed to obtain genetic samples. The first  
179 method was used at the following colonies in NSW: Broughton, Cabbage Tree, Lion and  
180 Bowen Islands. At each of those colonies, penguins were intercepted when arriving on  
181 the main landing site (beach) after sunset to return to their burrows following a day of  
182 feeding at sea. A plastic mesh fence was erected in a V-shape along the back of the  
183 beach. Penguins were corralled as they reached a specific area along the fence, then  
184 captured and moved to an area with sufficient light, where blood samples were taken.  
185 On two islands (Bowen and Lion Islands) that were revisited several times, microchips  
186 were additionally implanted for identification and to prevent double-sampling of  
187 individuals. For all other colonies in NSW, penguins were taken from their burrows or  
188 nest boxes, where they stayed during the day when not feeding at sea. They were  
189 taken from burrows after visual and tactile checks for eggs or very young chicks, and  
190 blood samples were taken from adults or chicks with emerging adult plumage. Burrows  
191 with eggs or blind chicks were not sampled to minimise risks to offspring. For sampling  
192 in WA, a mix of the two capture methods was used. A summary of data collected for  
193 each colony can be found in Table 1.

194

195   **Table 1:** Sampling locations with genetic sample size, methods used and availability of  
 196   parasite and pedigree data (number in brackets indicates number of families sampled);  
 197   more detailed data on sampling locations including geographic coordinates can be  
 198   found in Appendix Table A1

<b>Location (N to S)</b>	<b>Sample Size</b>	<b>Year sampled</b>	<b>MHC sequencing Method</b>	<b>Parasite data</b>	<b>Pedigree info</b>
Broughton Island	11	2013	MiSeq	None	None
Cabbage Tree Island	37	2012	MiSeq	None	None
Lion Island	63	2012	MiSeq	None	Yes (5)
Manly	20	2012	MiSeq	none	None
Big Island, Five Islands	20	2012	MiSeq	none	None
Bowen Island	86	2012	MiSeq	none	Yes (12)
Brush Island	20	2012	MiSeq	none	None
Tollgate Islands	16	2012	MiSeq	none	None
Montague Island	36	2012	MiSeq	none	None
<hr/>					
Kangaroo Island, SA	45	2013	MiSeq	Blood parasites	None
<hr/>					
Albany, WA	24	2007	Single-read and MiSeq	none	None
Esperance, WA	25	2007-2009	Single-read and MiSeq	none	None
Perth, WA	31	1999-2009	Single-read and MiSeq	none	Yes (11)
<b>Sum</b>	<b>407</b>				

199

200   **Blood sampling and DNA extraction**

201   Blood samples were taken from the metatarsal vein of at least 20 Little Penguin  
 202   individuals per colony, except Broughton Island and Tollgate Islands, where target  
 203   sample size could not be met due to logistical constraints (Appendix Table A1).

204   Samples from NSW and WA were collected using sterile 23-gauge needles and non-  
 205   heparinised capillary tubes. Blood samples (50-100 µl) were stored in 1 ml of  
 206   Longmire's buffer (Longmire et al. 1988) at ambient temperature until samples were  
 207   processed within a few weeks. DNA was extracted from blood samples following the

208 procedure outlined in Crandall et al. (1999). Samples from SA were collected with a 25-  
209 gauge needle from the foot vein, stored on Whatman FTA cards (GE Healthcare) and  
210 extracted following a variation of method #4 for nucleated erythrocytes (Smith &  
211 Burgoyne 2004). Specifically, for each individual, a 4 mm<sup>2</sup> square of blood-soaked FTA  
212 databasing paper was washed in: (1) 200 µL of FTA lysis buffer (100 mM Tris (free  
213 base), 0.1% SDS) for 30 min; (2) 200 µL DNAzol for 10 min; (3) 200 µL molecular grade  
214 water (MGW) for 5 min twice; and (4) 200 µL 95% ethanol for 10 min. DNA was  
215 resuspended from discs in 50 µL 1x Tris Light EDTA (10 mM Tris, 0.1 mM EDTA).

216 ***MHC genotyping***

217 An MHC fragment of the class II β1 domain (exon 2) was amplified with the PCR  
218 primers pen-1 (5'-AACGGCACCGAGCAGGGTGAGGT-3') and pen-4 (5'-  
219 CCCGTAGTTGTGTTGGCAG-3') (Tsuda et al. 2001) in 10 µL reactions using the Qiagen  
220 Taq PCR Core Kit (1x buffer, 1x Q-solution, 0.2 mM dNTPs, 2 µM of each primer, 0.5  
221 units Taq polymerase and 30-50 ng of genomic DNA) under a thermal profile adapted  
222 from Tsuda et al. 2001 and consisting of an initial denaturation of 94 °C for 10 min,  
223 followed by 35 cycles of 94 °C for 30 secs, 54 °C for 30 secs and 72 °C for 2 min each,  
224 after which the reaction was held at 4 °C.

225 A sample of 78 little penguins from Western Australia was initially screened for  
226 successful amplification using agarose gel electrophoresis to confirm the length of the  
227 product (198 base pairs, bp). Products were purified by adding 2 µL Exosap-IT (USB  
228 Corp.) to 5 µL of each reaction product, then running on an Eppendorf Mastercycler for  
229 15 min at 37 °C followed by 15 min at 80 °C. Cycle sequencing was carried out in 20 µL  
230 reactions containing 2.5 µL 5x BigDye sequencing buffer (Applied Biosystems Inc.),

231 1.0 µL BigDye 3.1 Ready Reaction premix (Applied Biosystems Inc.), 3.2 pmol H-Dbox  
232 primer, and 35 ng purified PCR product and q.s. ddH<sub>2</sub>O. Cycle sequencing reactions  
233 were carried out on an Eppendorf Mastercycler using an initial 96 °C denaturation for  
234 1 min, 25 cycles of (96 °C for 10 s, 50 °C for 5 s, 60 °C 4 min), and then held at 10 °C.  
235 The genetic software GENEIOUS version 5.0.3 (Drummond et al. 2011) was used to  
236 compile and edit the DNA sequences and the EM and ELB algorithms in ARLEQUIN  
237 version 3.5 (Excoffier & Lischer 2010) were used to infer MHC haplotypes. In a second  
238 step, haplotypes were inferred for the overlapping region between forward and  
239 reverse sequence reads (between 60 and 140 bp) to reduce ambiguities. Both of these  
240 algorithms relied on the assumption that all single nucleotide polymorphisms (SNPs)  
241 were detected. This assumption is likely met when a single locus gets amplified, but  
242 might be violated when multiple loci are involved. The reason for the possible violation  
243 is that nucleotides that only occur in the minority of variants at a particular site might  
244 be overlooked.

245 Number of inferred haplotypes as well as expected and observed heterozygosities  
246 were calculated using GenAIEx ver. 6.5 (Peakall & Smouse 2006). Unbiased expected  
247 heterozygosity ( $uH_e$ ) is better suited than standard  $H_e$  for estimating heterozygosity  
248 with low sample sizes (Nei 1987; Pruett & Winker 2008), and is also calculated. All  
249 results are reported for the three Western Australian penguin populations.

250 The extensive polymorphism and possible multiple loci detected in the initial screening  
251 process made reliable inference of haplotypes of the full 198bp region impossible  
252 using single-read sequencing alone. Therefore, a second sequencing strategy was  
253 pursued. The use of next-generation sequencing technology in the form of the Illumina

254 MiSeq platform can be seen to be the equivalent to the sequencing of clonally  
255 amplified products derived from a single DNA molecule in a cell-free system (Babik  
256 2010). In the past, next-generation sequencing studies of MHC genes have mostly  
257 relied on 454 technology (Babik et al. 2009) because it provides a read length of 250–  
258 500 bp, sufficient to cover entire MHC exons. Recent improvements to the Illumina  
259 sequencing range are making similar read lengths accessible on the MiSeq and HiSeq  
260 platforms, using their paired-end sequencing approach. This new technology is more  
261 cost-effective than 454 sequencing while reducing artefact formation due to its  
262 lowered error rate (Loman et al. 2012). For sequencing the 198 bp MHC fragment, we  
263 chose a 150 bp paired-end run on the MiSeq, using a two-step multiplexing approach  
264 to sequence a total of 304 individual samples, all of them initially set up as duplicates.  
265 The first multiplexing step consisted of the integration of a 4 bp multiplex  
266 identification tag (MID tag) into the sequencing primers, which also contained the  
267 MiSeq adapter sequences. The second step made use of Illumina's Nextera Index Kit,  
268 which allows analysis of 96 samples in one MiSeq run. In this case, each of the 96  
269 reactions indexed with the Nextera kit contained DNA from 4 individuals, which could  
270 be identified based on their MID tag. To prepare DNA samples for the next-generation  
271 sequencing process, the initial amplification step was identical to the one for single-  
272 read sequencing (see above), but with a reduced number of 25 PCR cycles followed by  
273 a reconditioning PCR step, during which the modified primers containing MID tags and  
274 MiSeq adapter sequences were introduced, while keeping PCR conditions the same.  
275 Reconditioning PCR has been shown to decrease the formation of random artefacts  
276 (Lenz & Becker 2008). All products of this step were verified using agarose gel  
277 electrophoresis and successful amplicons were normalised to a common concentration

278 of 25 ng / 20 µl using the SequalPrep 96-well Normalization Plate Kit (Applied  
279 Biosystems), and the products were submitted to the UNSW Ramaciotti Centre for  
280 Genomics for Illumina MiSeq analysis.

281 Sequences obtained were analysed using the open-source software MOTHUR v.1.34.0  
282 (Schloss et al. 2009), designed for microbial community analyses, but well suited for  
283 analyses of amplicon sequencing studies of animal populations. Forward and reverse  
284 reads were first filtered according to their qual (quality score) values, cutting off reads  
285 when the qual score dropped to less than 25 at any position, using the qthreshold  
286 option in the trim.seqs command. They were then sorted by individual of origin and  
287 the matching forward and reverse reads were combined into contigs using the  
288 make.contigs command and an oligos file containing the MID tag for each individual.

289 Only sequences with perfect MID tag and primer sequences were kept. The  
290 screen.seqs command was used to remove contig sequences that contained any  
291 ambiguities or were shorter than 150 bp or longer than 160 bp. Unique sequences  
292 were identified using the unique.seqs command. These were then screened for  
293 chimeric sequences, which are common artefacts in amplicon studies, with the help of  
294 the command chimera.uchime. In the last step, singletons (sequences that were only  
295 found once in the whole MiSeq run) were removed using the split.abund command  
296 with a cutoff of 1. Finally, a count table with the number of reads of each unique, non-  
297 chimeric sequence per individual was generated using the count.table command. From  
298 there, further adjustments were made on the count table using MICROSOFT EXCEL 2010.  
299 First, individual singletons (sequences that were only found once per individual) were

300 removed. Secondly, all individuals with less than 100 reads per individual were  
301 removed from the analysis.

302 Sequences were considered true variants of an individual if they were found in more  
303 than 5 % of its reads, as a compromise between minimising the probability of including  
304 artefacts while ensuring that low frequency variants are still maintained in the dataset  
305 (Sommer et al. 2013). Here, I will refer to variants when addressing the different  
306 sequences of MHC amplified, considering that those may stem from different loci. Due  
307 to the frequently observed copy number variation at MHC genes (Malaga-Trillo et al.  
308 1998; Reusch & Lange fors 2005), it is often impossible to assign MHC variants to loci.  
309 This is also why more than two variants might be observed per individual and MHC  
310 heterozygosity values cannot be calculated in the usual way. MHC diversity was  
311 therefore calculated using three approaches: as nucleotide diversity  $\pi$  using the  
312 software ARLEQUIN ver. 3.5.1.2 (Excoffier & Lischer 2010); as the mean number of MHC  
313 variants per individual (MHC/ind); and as the average total number of different MHC  
314 variants in a population divided by sample size (MHC/pop). The latter two quantities  
315 were calculated in MICROSOFT EXCEL 2010. To test for differences in MHC diversity  
316 among populations, F-tests were performed to test equality of variances and two-  
317 sample t-tests used to test for differences among sample means using the ANALYSIS  
318 TOOLPAK Add-in for MICROSOFT EXCEL 2010.

319 The variant pools from the different states were investigated for signatures of  
320 selection or demographic history, first by analysing their pN/pS ratios using the dN/dS  
321 option in the programme DNAsP 5.10.01 (Librado & Rozas 2009). The pN/pS (p for  
322 polymorphism) ratio characterises non-synonymous and synonymous variation within

323 a population, in contrast to the more commonly used dN/dS ratio for fixed differences  
324 between species (McDonald & Kreitman 1991). Additionally, we calculated Tajima's D  
325 in DNASP and Fu's  $F_S$  in ARLEQUIN.

326 Differences in variant pools among penguins from different localities were investigated  
327 using the ANalysis Of SIMilarity (ANOSIM) option in the software PRIMER 6, an analogue  
328 of univariate ANOVA which tests for differences between groups of multivariate  
329 samples from different locations (Clarke 1993; Jäger et al. 2007). ANOSIM was run on  
330 Bray-Curtis similarity matrixes using 999 permutations.

331 While  $F_{ST}$  is considered an appropriate measure for biallelic markers, it is not generally  
332 used in studies of highly polymorphic markers derived from an uncertain number of  
333 loci, such as MHC variants. However, for comparability with other studies of multilocus  
334 MHC genotypes, pairwise  $F_{ST}$  values were calculated in ARLEQUIN by entering the  
335 nucleotide sequence of each MHC variant and number of individuals with that variant  
336 in each population as haplotype data (e.g. Strand et al. 2012).  $F_{ST}$  values were only  
337 reported in the appendix, as their value for studies of multilocus genotypes is  
338 uncertain.

339 To counteract the problem of multiple comparisons, only results that remained  
340 significant after Bonferroni correction were retained. Tests were considered to belong  
341 to a family of hypotheses if the same test was done on multiple pairwise comparisons.  
342 The significance level  $\alpha$  was then adjusted to 0.05 divided by the number of pairwise  
343 comparisons tested.

344 **Parasite load**

345 Blood parasite information was only recorded in the SA colonies. To estimate blood  
346 parasite load, blood samples were collected as described above during sampling for  
347 genetic analyses. For each individual, one drop of blood was placed on a slide to  
348 prepare one blood smear. Blood smears were then air-dried, fixed in 99% ethanol for  
349 ca. 5 min, and later stained with Wright–Giemsa at the Children and Women Hospital  
350 (Adelaide, SA). The whole area of each smear was microscopically examined under a  
351 100 x oil immersion lens for presence of blood parasites. Parasite load is presented as  
352 per 10,000 cells. Parasite load and MHC diversity were analysed for correlations by  
353 comparing average parasite loads among penguins with different MHC allelic richness.  
354 Statistical significance of differences in parasite load was investigated using F-tests to  
355 test equality of variances and two-sample t-tests to test for differences among sample  
356 means. Individual parasite load was then plotted against the individual number of MHC  
357 variants to investigate correlations between the two measures.

358 **Mate Choice**

359 The genetic signals of mating preferences were analysed in three ways. Firstly, MHC  
360 variant similarity was calculated as the MHC variant sharing value D, with  $D = 2F_{AB}/(F_A$   
361  $+ F_B)$ , where  $F_A$  and  $F_B$  are the numbers of variants in individuals A and B, and  $F_{AB}$  is the  
362 number shared in both (Wetton et al. 1987; Eizaguirre et al. 2009). This value of  
363 variant similarity was calculated and averaged for breeding pairs as well as all possible  
364 combinations of breeders (individuals that had been encountered with a mate) from  
365 WA and from NSW. If disassortative mating was to occur at the MHC, we would expect  
366 variant similarity to be lower between breeding pairs than between random pairs. We

367 therefore compared D-values for observed breeding pairs to those of simulated  
368 potential pairs within each locality.

369 Secondly, we investigated mating preferences based on approximate equivalent  
370 measures to the inbreeding coefficient  $F_{IS}$  for variants derived from an unknown  
371 number of loci. Two measures were calculated: Firstly,  $O$  is the negative of the average  
372 number of variants per individual ( $I$ ) divided by the total number of variants in the  
373 population ( $P$ ) (W. Sherwin, pers. comm.).  $O$  behaves similarly to  $F_{IS}$  in that it is higher  
374 for elevated rates of inbreeding, and we expect it to be lower where disassortative  
375 mating produces offspring that are more diverse at the MHC than if mating was  
376 random. Secondly,  $I'$  uses the maximum number of variants per individual ( $2L$ ) to  
377 estimate the number of loci ( $L$ ) from which these variants stem. It is calculated as  
378  $I' = (3L - 2I) / L$  and ranges from approximately -1 (strong outbreeding) to +1 (complete  
379 inbreeding), thus mimicking  $F_{IS}$  (W. Sherwin, pers. comm.). Estimates of standard error  
380 were obtained using repeated subsampling and equation A.9.14 in Crow & Kimura  
381 1970 (see appendix). Values of inbreeding coefficients were then compared between  
382 states (NSW and WA) and between the two generations (parents and their offspring)  
383 sampled within states.

384 For comparability with the  $F_{IS}$  equivalents  $O$  and  $I'$ , we also analysed the genotypes  
385 obtained through single-read sequencing of the parent and offspring generation of the  
386 Perth colony in WA by calculating the inbreeding coefficient  $F_{IS}$  (Wright 1969) in  
387 ARLEQUIN. Because this measure is generally not appropriate for variants derived from  
388 an unknown number of loci,  $F_{IS}$  results were only reported in the appendix.

389 **Results**

390 ***Single-read sequencing***

391 Single-read sequencing produced 78 alignments of forward and reverse reads. Most of  
392 these alignments contained ambiguous bases and therefore represented pseudo-  
393 haplotypes. We initially assumed that pseudo-haplotypes were the result of  
394 sequencing a maximum of two alleles from one MHC locus and analysed them using  
395 ARLEQUIN's ELB algorithm, which produced 96 inferred haplotypes, whereas the EM  
396 algorithm inferred 94 haplotypes. When comparing the inferred haplotypes with  
397 known parent-offspring trios from the Perth colony, only 5 of the 8 trios had one  
398 parent sharing one allele with an offspring, and in only one trio did each parent  
399 contribute one haplotype to the offspring. This, in addition to the conflicting results of  
400 the two algorithms, could have been due to three problems with the haplotype  
401 inference process: haplotype inference through the aforementioned algorithms could  
402 have been inaccurate; pseudo-haplotypes might have been flawed due to incomplete  
403 or wrong identification of ambiguous bases; or the observed pseudo-haplotypes may  
404 stem from more than one locus. To investigate whether the algorithms give consistent  
405 results with less ambiguity and when mis-identification of ambiguities is unlikely,  
406 genetic analyses were repeated on the overlapping region between forward and  
407 reverse reads only, which fell between the 60<sup>th</sup> bp and the 140<sup>th</sup> bp of the sequence.  
408 The inferred haplotypes generated from this region proved more reliable, with both  
409 the EM and ELB algorithms producing the same number of haplotypes and sequences,  
410 with all 8 trios sharing the same alleles, which were consistent with Mendelian  
411 inheritance. Using the aforementioned methods on this short region, no more than

412 two haplotypes were inferred per individual. Haplotypes were thus treated as alleles at  
413 the same locus despite uncertainty about the true number of loci amplified. Observed  
414 heterozygosities were very similar in the three sub-populations (see Table 2). However,  
415 an excess of homozygotes was observed when comparing  $H_o$  and  $H_E$  values, leading to  
416 neutral or positive inbreeding coefficients, which suggest that disassortative MHC-  
417 based mate choice is not observable at this small region of the MHC locus when  
418 analysed by this method. Only 27 out of 78 individuals (34.6%) were heterozygous,  
419 which contrasts with an examination of the same 80 base pair region of the sequences  
420 reported in Tsuda et al. (2001), a study of several penguin species with a very small  
421 sample size of *E. minor*, where all four individuals sequenced were heterozygous.

422 **Table 2:** Haplotype diversity at an 80 bp sequence of the MHC gene

423 No. Homoz – Number of Homozygotes, No. Haplo – Number of Haplotypes,  $H_o$  –  
424 Observed heterozygosity,  $H_E$  – expected heterozygosity,  $uH_E$  – unbiased expected  
425 heterozygosity,  $F_{IS}$  – inbreeding coefficient

Population	Sample Size	No. Homoz	No. Haplo	$H_o$	$H_E$	$uH_E$	$F_{IS}$ value	$F_{IS}$ p-value
Perth	40	26	7	0.350	0.345	0.350	0.342	0.017
Albany	17	11	6	0.353	0.516	0.531	0.001	0.572
Esperance	21	14	6	0.333	0.298	0.305	0.093	1.000
Total	78	51	13	0.345	0.386	0.395	0.083	0.148

426

427 **MiSeq – next-generation sequencing**

428 The total number of paired reads generated for all populations using the MiSeq  
429 approach was 870,682. After quality filtering and combination of forward and reverse  
430 reads, 553,009 contigs remained. Of those, 490,996 sequences met the screening  
431 criteria and were subsequently sorted into 30,538 unique sequences. 1,835 unique  
432 chimeric sequences were identified and removed, and a total of 8,766 unique

433 sequences remained after additionally removing all global singletons, i.e. sequences  
434 that were only found once in the data set. Removal of individual singletons, i.e.  
435 sequences that were only found once per individual, further reduced the number of  
436 unique sequences to 5,052. After applying the 5 % threshold for accepting reads as  
437 true variants and removing individuals that did not meet the minimum read number  
438 requirement, 96 MHC variants were identified in the whole dataset. Unfortunately, our  
439 stringent filtering lead to a greatly reduced number of individuals with results  
440 sufficient for further analyses, and only samples from WA still had replicate MiSeq  
441 results. These were used to estimate the error in calling variants that remained after  
442 the filtering steps. While only 38 % of the 49 individuals with replicates had exactly  
443 identical genotypes in the replicate, 75 % of variants called were also found in the  
444 replicate. This indicates that despite the large coverage and stringent filtering,  
445 artefacts still remain in the data set and/or not all true variants were picked up in both  
446 replicates. Individual genotypes were therefore not analysed. Sample sizes and  
447 numbers of MHC variants are summarised in Table 3.

448

449 **Table 3:** Allelic diversity of MHC genotypes according to MiSeq analyses

450 MHC/ind – average number of MHC variants per individual, MHC/loc - total number of  
451 different MHC variants in a location divided by sample size, Pi – nucleotide diversity

	Sample Size	MHC/ind	SD (MHC/ind)	Number of variants	MHC/loc	Pi
<b>NSW</b>	<b>83</b>	<b>2.40</b>	<b>1.18</b>	<b>66</b>	<b>0.80</b>	<b>12.09</b>
Bowen Island	18	2.00	1.26	22	1.22	
Brush Island	7	2.43	1.40	12	1.71	
Broughton Island	5	1.60	0.80	6	1.20	
Cabbage Tree Island	9	2.67	1.25	17	1.89	
Five Islands	10	2.40	0.92	10	1.00	
Lion Island	7	2.71	1.16	14	2.00	
Montague Island	11	2.82	0.94	16	1.45	
Manly	9	2.67	1.15	12	1.33	
Tollgate Islands	7	2.57	0.90	12	1.71	
<b>SA</b>	<b>28</b>	<b>2.71</b>	<b>1.16</b>	<b>18</b>	<b>0.64</b>	<b>12.79</b>
Kangaroo Island	28	2.71	1.16	18	0.64	
<b>WA</b>	<b>54</b>	<b>2.17</b>	<b>1.23</b>	<b>41</b>	<b>0.76</b>	<b>11.36</b>
Albany	15	1.60	0.61	15	1.00	
Esperance	20	1.82	0.72	18	0.90	
Perth	18	2.78	1.44	22	1.22	
<b>Australia</b>	<b>165</b>			<b>96</b>		<b>11.94</b>

452

453 Numbers of variants per individual varied between 1 and 5, with differences in the  
454 number of variants per individual that might be explained by one or more of: variation  
455 in loci number within the species, variant sharing between loci and homozygosity at  
456 some loci (Babik et al. 2009; Sepil et al. 2012). Next-generation sequencing frequently  
457 identifies more variants per individual than single-read sequencing (e.g. Sommer et al.  
458 2013), even when cloned variants are re-sequenced in a more elaborate setup than the  
459 pilot study described above (Table 2). The average number of MHC variants per  
460 individual is similar between NSW and the two other states, but significantly higher in  
461 SA than WA (two-sample t-test with equal variances, t-stat = 2.036, p = 0.045). Within

462 WA, both variances of variant numbers and average numbers of variants are  
 463 significantly higher in Perth than Albany or Esperance ( $F_{PER-ALB} = 5.458$ ,  $p = 0.001$ ;  
 464  $F_{PER-ESP} = 4.067$ ,  $p = 0.001$ ;  $t\text{-stat}_{PER-ALB} = 3.062$ ,  $p = 0.005$ ;  $t\text{-stat}_{PER-ESP} = 2.514$ ,  $p = 0.020$ ),  
 465 but similar between Albany and Esperance.

466 A comparison of MHC variant pools using an analysis of similarity among the three  
 467 Australian states showed differences between NSW and WA (ANOSIM  $R = 0.117$ ,  
 468  $p = 0.001$ ), but not between NSW and SA or SA and WA. Within the states, no  
 469 statistically significant differences in variant pools were identified in NSW or SA, but in  
 470 WA, differences were detected both at the level of populations and colonies  
 471 (Table 4).

472 **Table 4:** Differences in variant pools among Western Australian populations (a) and  
 473 colonies (b),  $R$  = ANOSIM statistic, below diagonal;  $p$  = significance value, above  
 474 diagonal.

475 Significant results ( $p < 0.017$  for 4.a,  $p < 0.005$  for 4.b, significant after Bonferroni  
 476 correction) are highlighted in italics.

4.a	$R \setminus p$	Albany	Esperance	Perth
Albany		0.13	<b>0.005</b>	
Esperance	0.029		0.064	
Perth	<b>0.098</b>	0.044		

477

4.b	$R \setminus p$	Albany		Esperance		Perth
		MBI	CHI	WDI	WKI	PGI
Albany	MBI		0.18	<b>0.01</b>	0.762	0.073
	CHI	0.086		0.167	0.228	<b>0.002</b>
Esperance	WDI	<b>0.15</b>	0.033		0.171	0.055
	WKI	-0.003	0.032	0.052		0.116
Perth	PGI	0.109	<b>0.12</b>	0.053	0.073	

478

479

480 To assess statistical power of ANOSIM analyses, numbers of permutations were varied  
481 from 999 to 100 000, and results are summarised in Appendix Table A2. The default of  
482 999 permutations was sufficient to reliably calculate the test statistic R and assess  
483 statistical significance of differences among allele pools.

484 The pN/pS ratio was above unity for all Australian states (Table 7). None of the  
485 confidence intervals included 1, which would indicate selective neutrality.

486

487 **Table 5:** Average pN/pS ratio and 95 % confidence intervals by state

	NSW	SA	WA	Australia
Average pN/pS	3.24	1.76	2.96	3.10
SD(pN/pS)	2.37	1.35	2.10	2.45
Upper CI	3.34	1.98	3.10	3.17
Lower CI	3.14	1.55	2.82	3.03

488

489 Other statistical tests for deviation from selective neutrality or demographic stability -  
490 Tajima's D and Fu's F<sub>S</sub>, did not reject the null hypothesis of selective neutrality or  
491 demographic stability for samples from SA or WA. However both Tajima's D and Fu's F<sub>S</sub>  
492 are significant for samples from NSW and for all samples pooled, which are also the  
493 groups with the highest pN/pS ratio observed.

494

495 **Table 6:** Neutrality test results by state; D<sub>S</sub> – Tajima's D for synonymous sites; D<sub>N</sub> –  
496 Tajima's D for non-synonymous sites  
497 Significant results (p < 0.05) are highlighted in bold, highly significant ones for Fu's F<sub>S</sub>  
498 (p < 0.01, significant after Bonferroni correction) bold and in italics

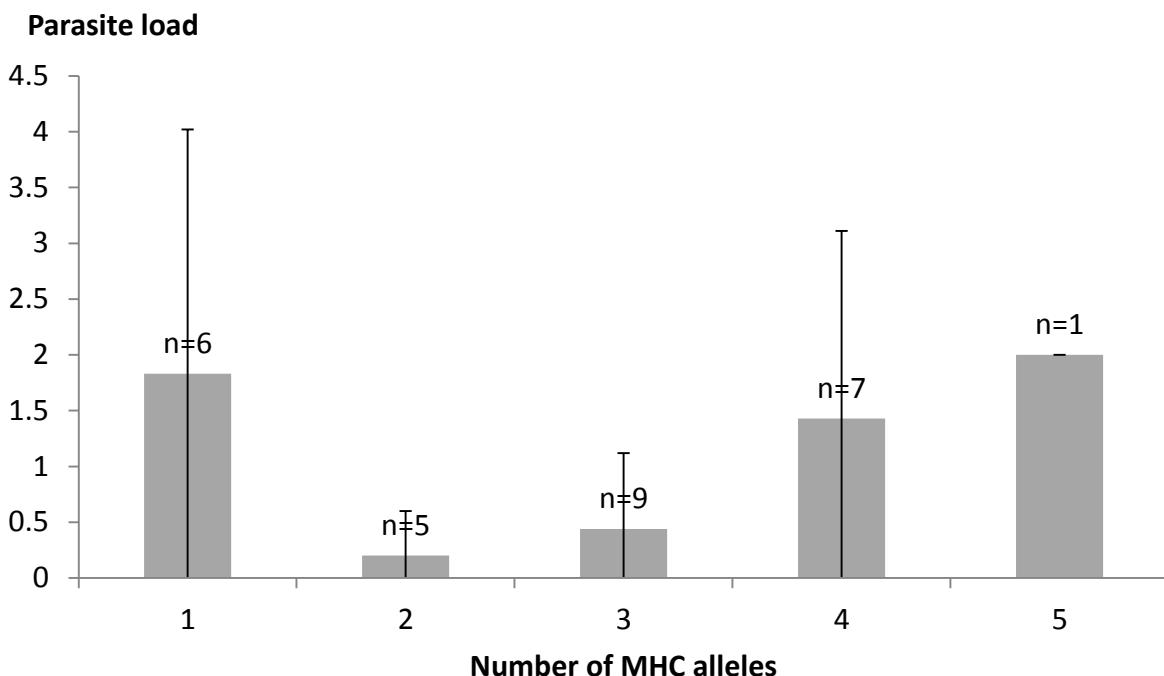
	NSW	SA	WA	Australia
Tajima's D	<b>2.090</b>	1.346	1.487	<b>2.280</b>
D p-value	<b>&lt; 0.05</b>	> 0.1	> 0.1	<b>&lt;0.05</b>

	NSW	SA	WA	Australia
Tajima's D <sub>S</sub>	-0.050	1.687	-0.324	0.216
D <sub>S</sub> p-value	> 0.1	> 0.1	> 0.1	> 0.1
Tajima's D <sub>N</sub>	<b>2.246</b>	1.360	1.434	<b>2.150</b>
D <sub>N</sub> p-value	<b>&lt; 0.05</b>	> 0.1	> 0.1	<b>&lt; 0.05</b>
Fu's F <sub>S</sub>	<b>-20.170</b>	2.513	-7.171	<b>-23.780</b>
F <sub>S</sub> p-value	<b>0.004</b>	0.83	0.075	<b>0.006</b>

499

500 ***MHC diversity and parasitism***

501 Eleven of the 28 penguins from Kangaroo Island were infested with apicomplexan  
 502 blood parasites. Of those, the maximum number of parasites counted in the blood  
 503 smears was 6 blood parasites per 10,000 cells, with an average of 2.55 and a standard  
 504 deviation of 1.44. While there appears to be a general tendency for penguins with low  
 505 or high MHC diversity to have high parasite loads, and variance in parasite load to be  
 506 reduced in individuals of intermediate MHC diversity, this relationship is not  
 507 statistically significant (Fig. 2). Due to the low sample size in each group, none of the  
 508 pairwise comparisons of parasite load were statistically significant (two-sample  
 509 t-tests).



510

511 **Fig. 2:** Number of MHC variants and average individual parasite loads  $\pm$  SD

512 **MHC-based mate choice**

513 Of the 72 penguin individuals assigned to 28 families, only 30 have successfully been  
514 genotyped at the MHC. Those individuals belonged to 18 different families, of which  
515 only nine had more than one member with MHC genotype information and only three  
516 had MHC genotypes for both parents and at least one offspring (Table 9).

517

518 **Table 7:** Families with MHC genotype information

519 P – Parent, C – Chick / Offspring

Family	Colony	Number of family members sampled	Number of members with MHC genotypes	Individuals with MHC genotype
WA_01	Penguin Island	3	1	1 P
WA_02	Penguin Island	2	0	
WA_03	Penguin Island	2	0	
WA_04	Penguin Island	3	2	1 P, 1 C
WA_05	Penguin Island	3	0	
WA_06	Penguin Island	3	3	2 P, 1 C
WA_07	Penguin Island	3	1	1 C
WA_08	Penguin Island	3	3	2 P, 1 C
WA_09	Penguin Island	3	1	1 P
WA_10	Penguin Island	3	2	1 P, 1 C
WA_11	Penguin Island	3	2	1 P, 1 C
B06	Bowen Island	3	3	2 P, 1 C
B17	Bowen Island	3	0	
B19	Bowen Island	4	2	2 C
B20	Bowen Island	2	2	1 P, 1 C
B22	Bowen Island	2	0	
B29	Bowen Island	2	1	1 P
B31	Bowen Island	2	0	
B33	Bowen Island	2	1	1 C
B36	Bowen Island	3	1	1 C
B39	Bowen Island	2	0	
B41	Bowen Island	3	2	2 P
B45	Bowen Island	2	1	1 C
L01	Lion Island	2	0	
L06	Lion Island	2	0	
L07	Lion Island	3	1	1 P
L08	Lion Island	2	0	
L13	Lion Island	2	1	1 P
<b>Total</b>		<b>72</b>	<b>30</b>	<b>18 Families</b>

520

521 The genetic signals of mating preferences were analysed in three ways. Firstly, due to

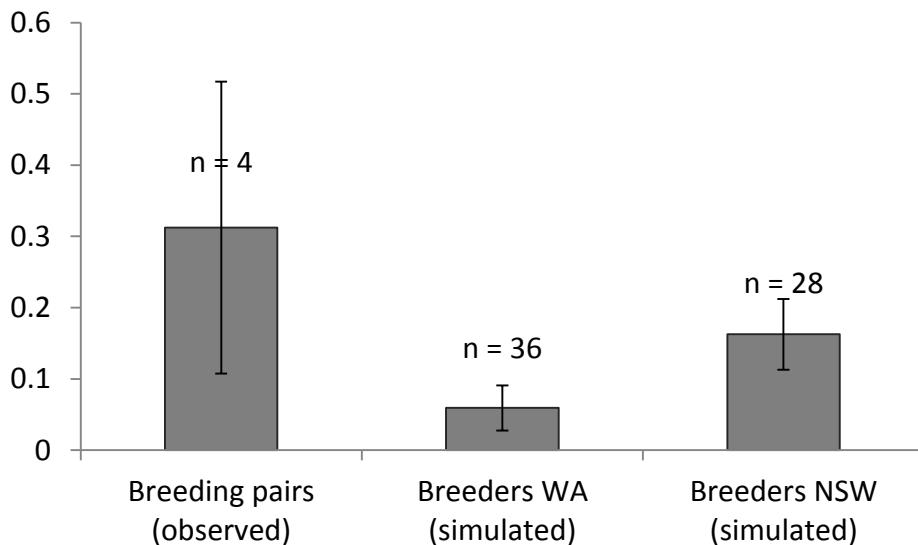
522 the low number of families with multiple members for which the MHC genotype was

523 known, sample sizes for the calculation of the MHC variant sharing value D were very

524 low, leading to insufficient statistical power to detect deviations from random mating

525 if it exists (Fig. 3). The expected variant sharing value for breeders from WA or NSW

526 under random mating was included in the range of observed variant sharing values for  
527 breeding pairs.



528  
529 **Fig. 3:** Average MHC variant sharing value D for breeding pairs and simulated random  
530 mating of breeders by state of origin; error bars represent standard error of the mean;  
531 n – sample size

532  
533 For the second investigation of mating preferences based on the next-generation  
534 sequencing data, we used comparison of average O and I' (inbreeding) values between  
535 the parent and offspring generations, for which sample sizes were slightly larger (Table  
536 11). Values of I', which behave similarly to the inbreeding coefficient  $F_{IS}$ , are  
537 significantly higher than zero for all groups of penguins analysed, suggesting that  
538 inbreeding, selection against heterozygotes or recent admixture might be observed at  
539 the MHC. Both measures of inbreeding, I' and O, were similar between NSW and WA.  
540 In NSW, parents and offspring had I' values above zero, and NSW neither I' nor O were  
541 significantly different between parents and offspring. In WA, I' and O showed a similar  
542 trend between generations: both I' and O were elevated in the parental generation,

543 indicating more inbreeding than what is observed in the offspring generation. For  
544 comparison of I' and O with traditional  $F_{IS}$ , these were reported in Appendix Table A5.

545

546 **Table 8:** Inbreeding coefficients O and I' (Sherwin, pers. comm.) by state of origin (pop)  
547 and generation (gen); inbreeding coefficients  $\pm$  SE highlighted in bold

548 L – estimated number of loci derived from maximum number of variants (2L); I –  
549 average individual number of variants; I' – Inbreeding coefficient:  $(3L - 2I) / L$ ; P –  
550 number of variants in population; O – Inbreeding coefficient: I / P

	NSW		WA	
	Parents	Offspring	Parents	Offspring
2L		5		7
L		3		4
I <sub>Pop</sub>		2.07 $\pm$ 1.26		2.73 $\pm$ 2.46
I <sub>Gen</sub>	2.25 $\pm$ 1.69	1.86 $\pm$ 0.69	1.89 $\pm$ 0.54	4.00 $\pm$ 2.67
I' <sub>Pop</sub>	<b>1.62 <math>\pm</math> 0.20</b>		<b>1.63 <math>\pm</math> 0.20</b>	
I' <sub>Gen</sub>	<b>1.50 <math>\pm</math> 0.33</b>	<b>1.76 <math>\pm</math> 0.28</b>	<b>2.06 <math>\pm</math> 0.20</b>	<b>1.00 <math>\pm</math> 0.39</b>
P <sub>Pop</sub>	20		22	
P <sub>Gen</sub>	13	10	13	15
O <sub>Pop</sub>	<b>-0.10 <math>\pm</math> 0.01</b>		<b>-0.12 <math>\pm</math> 0.02</b>	
O <sub>Gen</sub>	<b>-0.11 <math>\pm</math> 0.02</b>	<b>-0.09 <math>\pm</math> 0.02</b>	<b>-0.09 <math>\pm</math> 0.01</b>	<b>-0.18 <math>\pm</math> 0.03</b>

551

552 The main contrast between the two methods of mating-pattern analysis was that no  
553 indication for deviation from random mating could be detected using the first, but  
554 some indications of non-random mating were suggested by the second. The variant  
555 sharing values for observed breeding pairs from WA or NSW overlapped with the  
556 variant sharing values for random pairs of breeders. The second method, which used a  
557 new method to calculate inbreeding coefficients for multiallelic genotypes based on  
558 next-generation sequencing, resulted in I' values above zero and significant differences  
559 between generations for WA penguins. At the same time, none of the traditional  
560 inbreeding coefficients  $F_{IS}$  deviated from zero for single-read MHC sequences  
561 (Appendix Table A5).

562 **Discussion**

563 ***MHC diversity and divergence among localities***

564 Little penguins from different colonies in NSW were largely similar concerning their  
565 MHC variant pools, which agrees well with the limited genetic differentiation at neutral  
566 microsatellites and at a mitochondrial marker (see Chapter 3). When comparing  
567 samples from NSW to those from SA, only some pairwise comparisons of colonies  
568 showed significant differentiation, while samples from all colonies in NSW pooled were  
569 highly differentiated from those in SA and WA. Despite the large coverage and  
570 stringent filtering adhered to for the analysis of next-generation sequencing data,  
571 artefacts still remained in the data set and/or not all true variants were picked up in  
572 both replicates and individual genotypes were therefore not analysed. However, the  
573 strong differences among variant pools from different states' populations were still  
574 detectable despite the high amount of noise caused by the retention of artefacts  
575 causing problems with reproducibility. Due to the similarity between neutral markers  
576 and the MHC, for which the same individuals were genotyped, we cannot exclude the  
577 possibility that stochastic effects shape this pattern of MHC diversity.

578 ***Selection on MHC***

579 The MHC appeared to be under different influences to microsatellites. The  
580 microsatellite markers (Chapter 3) showed no signs of being under selection or  
581 involved in non-random mating – microsatellites were in Hardy-Weinberg  
582 equilibrium for most colonies studied. Of course,  $pN/pS$  cannot be calculated for  
583 microsatellites because the alleles do not differ from one another by base-substitution,  
584 and they are usually in non-coding regions. In contrast, there were signs of selection

585 and non-random mating for MHC. The pN/pS ratio of MHC sequences was above unity  
586 for all Australian states, indicating that the latter might be under positive selection or  
587 affected by demographic changes (Table 7). However, values of pN/pS ratios within  
588 populations are less straightforward than between-species dN/dS ratios (Kryazhimskiy  
589 & Plotkin 2008), so small deviations from unity should not be relied upon. Despite this,  
590 there is a second indication that populations in NSW may be under selection or have  
591 undergone a recent demographic expansion, indicated by a large, negative Fu's  $F_S$   
592 value (Fu 1997) and significant values of Tajima's D. In contrast, for SA and WA, these  
593 statistical tests gave no indication that the MHC locus studied might be deviating from  
594 neutrality or demographic stability (Table 8).

### 595 Possible selective effects of parasites

596 To investigate the impacts of parasite pressure, we tested whether individuals with  
597 higher MHC diversity had lower parasite loads. This was not the case, but despite low  
598 sample sizes, a reduced variance and average of parasite load was suggested with  
599 intermediate individual MHC diversity (Fig. 2). This pattern agrees with the theory that  
600 an intermediate, not maximum number of individual MHC variants optimises parasite  
601 resistance, as found for sticklebacks in a situation where multiple duplicate loci were  
602 present (Wegner et al. 2003; Reusch et al. 2001; Woelfing et al. 2009).

### 603 Mating patterns

604 Using information on penguin pedigrees, we also investigated whether parent pairs  
605 were more or less diverse at the MHC than expected by chance, and whether the  
606 offspring generation showed a different degree of inbreeding to the generation of  
607 their parents. Two of our three approaches to this question produced usable results,

608 but unfortunately, low sample sizes did not allow conclusions to be drawn regarding  
609 MHC allele sharing between known breeding partners (Fig. 3).

610 In the second approach to analysis of breeding patterns, multi-locus inbreeding  
611 coefficients indicate inbreeding at the MHC (Table 11). In NSW, parents and offspring  
612 were inbred according to  $I'$ , but no differences between parents and offspring were  
613 detected. In WA, the offspring generation in had significantly lower average inbreeding  
614 coefficient values for  $I'$  and  $O$  than the corresponding parent generation, indicating  
615 some mechanism of inbreeding avoidance might be at work. It might also indicate that  
616 inbred chicks have higher survival than their outbred conspecifics, leading to a higher  
617 inbreeding coefficient in older penguins.

618 One might ask how these new statistics compare with  $F_{IS}$  values for MHC and  
619 microsatellites. The results of the new statistics disagree with the  $F_{IS}$  values for the  
620 80 bp single-read biallelic data and with the  $F_{IS}$  values for the microsatellite data. This  
621 disagreement might be due to limitations of the single-read data that might not  
622 accurately represent the polymorphism at the full 198 bp sequence and the existence  
623 of multiple loci, and due to the nature of microsatellite data being chosen for their  
624 selectively neutral and non-coding properties. The most likely reason for this  
625 disagreement is that  $F_{IS}$  is not a suitable measure unless allelism is known. However, it  
626 could also indicate problems with the new methods, which allow the use of more  
627 comprehensive next-generation sequencing data, but which will need further testing  
628 before they can be applied to other studies.

629 ***Suitability of MHC marker for population genetic studies***

630 MHC genotyping based on single-read sequencing and haplotype inference algorithms  
631 proved unreliable for the highly polymorphic and apparently multilocus exon 2 of the  
632 class II  $\beta$ 1 domain, while next-generation sequencing allowed genotyping of a large  
633 number of individuals in one MiSeq run. The large number of sequences generated  
634 made stringent quality filtering necessary to ensure only true variants are included in  
635 the final data set, and individual replicates should always be analysed. Despite  
636 problems with final sample sizes and incongruencies between the two intra-individual  
637 amplicon replicates, which could have been due to DNA cross-contamination or  
638 unnoticed exchange of barcodes before the second amplification, our workflow can  
639 serve as a powerful tool to analyse highly variable, functional genes in population  
640 genetic studies.

641 Using this approach, we were able to show that MHC variant pools of little penguins  
642 from different colonies in NSW were largely similar (Table A2), which agrees well with  
643 the limited genetic differentiation at neutral microsatellites and a mitochondrial  
644 marker (Chapter 3). This comparison is relevant because a subset of individuals  
645 genotyped at the neutral markers was chosen for MHC analyses. However, when all  
646 MHC samples from NSW were pooled together, they were significantly differentiated  
647 from those in SA and WA (Table A2). Due to the similarity between neutral markers  
648 and the MHC, the possibility that stochastic effects shape this pattern of MHC diversity  
649 cannot be excluded, although selective neutrality tests indicated an influence of  
650 selection or demographic changes at the MHC locus (Table 8). Based on the results of  
651 the present study, there is no reason to believe that low population size has

652 compromised genetic diversity at this immunogenetic marker in any of the little  
653 penguin populations studied.

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667

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- 867

868 **Appendix 4**

869 **Table A1:** Sampling locations in eastern Australia and Western Australia

870 Coordinates of penguin colonies, sample size of individual genetic material, years  
871 sampled and the most recent published estimates of population sizes

872 <sup>1</sup> Carlile et al. 2012; <sup>2</sup> Priddel & Carlile 2004, <sup>3</sup> Sergent et al. 2004, <sup>4</sup> Little Penguin  
873 Recovery Team 2007, <sup>5</sup> Gibson 1976, <sup>6</sup> Fortescue 1995, <sup>7</sup> Carlile, et al. 2012, <sup>8</sup> McKean  
874 & Fullagar 1976, <sup>9</sup> Weerheim et al. 2003, <sup>10</sup> Wiebkin 2011, <sup>11</sup> Cannell 2001

875 <sup>A</sup> also known as Shelter Island

876 <sup>B</sup> also known as Stanley Island

877

Location (N to S)	Coordinates	Sample Size	Year sampled	Estimated population size
Broughton Island	32° 37' S, 152° 18' E	11 <sup>A</sup>	2013	20-40 pairs <sup>1</sup>
Cabbage Tree Island	32° 41' S, 152° 13' E	37	2012	140 pairs <sup>2</sup>
Lion Island	33° 33' S, 151° 19' E	63	2012	250 individuals <sup>3</sup>
Manly	33° 48' S, 151° 17' E	20	2012	60 pairs <sup>4</sup>
Big Island, Five Islands	34° 29' S, 150° 55' E	20	2012	> 1000 pairs <sup>5</sup>
Bowen Island	35° 07' S, 150° 46' E	86	2012	5000 pairs <sup>6</sup>
Brush Island	35° 32' S, 150° 25' E	20	2012	2000-3000 pairs <sup>7</sup>
Tollgate Islands	35° 45' S, 150° 15' E	16 <sup>A</sup>	2012	< 5000 pairs <sup>8</sup>
Montague Island	36° 15' S, 150° 13' E	36	2012	5000 pairs <sup>9</sup>
Kangaroo Island, SA	35° 50' S, 137° 13' E	45	2013	> 2000 penguins <sup>10</sup>
Albany, WA	Muttonbird Island <sup>A</sup> 35° 3' S, 117° 41' E Cheyne Island 34° 36' S, 118° 46' E	24	2007	Muttonbird Island 200-400 penguins <sup>11</sup> Cheyne Island: 100-200 penguins <sup>11</sup>
Esperance, WA	Woody Island 33° 58' S, 122° 1' E Wickham Island <sup>B</sup> 34° 01' S, 123° 17' E	25	2007-2009	Woody Island 10-12 penguins <sup>11</sup> Wickham Island 10-20 penguins <sup>11</sup>
Perth, WA	Penguin Island 32° 18' S, 115° 41' E	31	1999-2009	Penguin Island 1000 penguins <sup>11</sup>
<b>Sum</b>		<b>407</b>		

878

879 
$$V_{I'} = \frac{4V_I}{L^2} + \frac{4I^2V_L}{L^4}$$
      Equation A.9.14 (Crow & Kimura 1970) for variance

880  $V_I$  calculation of inbreeding coefficient I

881 **Table A2:** ANOSIM results with different numbers of permutations, where Global R is  
882 the ANOSIM test statistic, sign is the significance value, and N° ≥ R is the number of  
883 permuted statistics greater than or equal to Global R; significant results are highlighted  
884 in italics.

Number of permutations	999	999	999	999	999	10000	100000
Within NSW	Global R	0.049	0.049	0.049	0.049	0.049	0.049
	sign	0.06	0.072	0.063	0.072	0.064	0.069
	N° ≥ R	59	72	62	71	63	686
Between States	R	<i>0.076</i>	<i>0.076</i>	<i>0.076</i>	<i>0.076</i>	<i>0.076</i>	<i>0.076</i>
	sign	<i>0.001</i>	<i>0.001</i>	<i>0.001</i>	<i>0.001</i>	<i>0.001</i>	<i>0.00005</i>
	N° ≥ R	0	0	0	0	0	4
Within WA Colonies	R	<i>0.07</i>	<i>0.07</i>	<i>0.07</i>	<i>0.07</i>	<i>0.07</i>	<i>0.07</i>
	sign	<i>0.006</i>	<i>0.005</i>	<i>0.002</i>	<i>0.007</i>	<i>0.003</i>	<i>0.003</i>
	N° ≥ R	5	4	1	6	2	26
Within WA Populations	R	<i>0.054</i>	<i>0.054</i>	<i>0.054</i>	<i>0.054</i>	<i>0.054</i>	<i>0.054</i>
	sign	<i>0.01</i>	<i>0.012</i>	<i>0.008</i>	<i>0.009</i>	<i>0.008</i>	<i>0.007</i>
	N° ≥ R	9	11	7	8	7	68

885

886 Genetic differentiation was also investigated by calculating  $F_{ST}$  values between each  
887 pair of localities: nine colonies from NSW, one from SA and three populations in  
888 Western Australia (Appendix Table A3), as well as differentiation between states  
889 (Appendix Table A4). Only one pair of colonies within NSW is significantly differentiated  
890 at the MHC locus after Bonferroni correction – Bowen Island and Brush Island,  
891 although the latter was represented by a very low sample of only 7 individuals.  
892 Kangaroo Island is differentiated from the same two colonies, but appears similar to  
893 the rest of NSW. In WA, Perth is significantly differentiated from all other locations  
894 studied, while Esperance is different from most colonies in NSW and SA, but not  
895 Albany. Interestingly, Albany, being situated between Esperance and Perth, is only  
896 different from Kangaroo Island, but not the more distant colonies in NSW. When  
897 pooling samples from the same state, all pairwise comparisons of MHC haplotypes  
898 show highly significant genetic differentiation measured by  $F_{ST}$  (Appendix Table A2).

899 **Table A3:** Genetic differentiation of Australian penguin populations at the MHC;  
900  $F_{ST}$  values below diagonal, corresponding p-values above. Significant results  
901 ( $p < 0.0006$ , significant after Bonferroni correction) are highlighted in italics

$F_{ST} \setminus p$	NSW										SA	WA		
	BI	Brl	Bro	CI	FI	LI	MI	M	TI	KI	ALB	ESP	PER	
BI		<i>0.000</i>	0.144	0.063	0.018	0.648	0.063	0.018	0.108	<i>0.000</i>	0.216	0.009	<i>0.000</i>	
Brl	<i>0.118</i>		0.027	0.009	0.126	0.027	0.117	0.063	0.036	<i>0.000</i>	0.018	<i>0.000</i>	<i>0.000</i>	
Bro	0.039	<i>0.127</i>		0.405	0.297	0.459	0.171	0.018	0.504	0.306	0.045	0.009	<i>0.000</i>	
CI	0.025	0.062	0.003		0.117	0.720	0.162	0.009	0.153	0.009	0.144	0.009	<i>0.000</i>	
FI	0.046	0.040	0.017	0.033		0.315	0.531	0.648	0.522	0.630	0.018	<i>0.000</i>	<i>0.000</i>	
LI	-0.012	<i>0.084</i>	-0.003	-0.013	0.001		0.738	0.261	0.288	0.027	0.324	0.090	<i>0.000</i>	
MI	0.017	0.029	0.028	0.013	-0.008	-0.012		0.495	0.684	0.081	0.126	0.009	<i>0.000</i>	
M	0.049	0.032	0.096	0.055	-0.012	0.013	-0.004		0.108	0.063	0.054	0.009	<i>0.000</i>	
TI	0.032	<i>0.087</i>	-0.002	0.027	-0.005	0.001	-0.013	0.037		0.351	0.063	<i>0.000</i>	<i>0.000</i>	
KI	<i>0.072</i>	<i>0.093</i>	0.018	0.059	-0.006	0.028	0.020	0.032	0.001		<i>0.000</i>	<i>0.000</i>	<i>0.000</i>	
ALB	0.005	0.090	0.051	0.020	0.046	0.003	0.019	0.037	0.037	<i>0.059</i>		0.252	<i>0.000</i>	
ESP	0.047	<i>0.137</i>	0.094	0.044	<i>0.075</i>	0.028	0.063	0.062	<i>0.084</i>	0.072	0.009		<i>0.000</i>	
PER	<i>0.165</i>	<i>0.306</i>	<i>0.270</i>	<i>0.154</i>	<i>0.240</i>	<i>0.141</i>	<i>0.213</i>	<i>0.205</i>	<i>0.275</i>	<i>0.233</i>	<i>0.124</i>	<i>0.067</i>		

902

903

904 **Table A4:** Genetic differentiation of Australian penguins at MHC, by state of origin;

905  $F_{ST}$  values below diagonal, corresponding p-values above. Significant results ( $p < 0.01$ ,  
906 significant after Bonferroni correction) are highlighted in italics.

$F_{ST} \setminus p$	NSW	SA	WA
NSW		<i>0.0000</i>	<i>0.0000</i>
SA	<i>0.0246</i>		<i>0.0000</i>
WA	<i>0.0878</i>	<i>0.1227</i>	

907

908 For investigation of mating preferences, we also analysed the reduced 80 bp single-  
909 read sequences of the parent and offspring generation of the Perth colony in WA using  
910 the inbreeding coefficient  $F_{IS}$  (Appendix Table A5). None of the groupings showed an  
911 inbreeding coefficient that deviated from zero.

912   **Table A5:** Inbreeding coefficient  $F_{IS}$  and the significance value for deviation from zero  
913   for known penguin families from Perth (pop) and the two generations (gen); sample  
914   sizes in brackets

Perth (30)			
$F_{IS}$ - Pop		p-value	
-0.0919		1	
Parents (19)		Offspring (11)	
$F_{IS}$ - Gen	p-value	$F_{IS}$ - Gen	p-value
-0.0976	1	-0.0811	1

915

916

917

Chapter 4: Patterns of Major Histocompatibility Complex diversity,  
parasitism and mate choice in Little Penguins (*Eudyptula minor*)

918

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1      **Formatted for publication in Pacific Conservation Biology – Title page**

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3      **Chapter 5: Viability of little penguin (*Eudyptula minor*) populations**

4                   **at their northern range edge**

5

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16

17     **Keywords:** PVA, sensitivity analysis, reproduction, survival, La Niña, ENSO

18

19     **Author contributions:**

20     JJS and BLC provided field data for WA, I (SV) performed modelling of NSW and WA  
21     populations; I also performed statistical analyses, and wrote the chapter.

22

## 23 Table of Contents

24	Chapter 5: Viability of little penguin ( <i>Eudyptula minor</i> ) populations at their northern	
25	range edge .....	201
26	Summary: .....	203
27	Introduction .....	205
28	Method.....	210
29	Results.....	223
30	Discussion.....	242
31	Acknowledgements.....	246
32	References.....	246
33	Appendix 5 .....	252
34		

**35 Summary:** 202 words

Demographic and environmental stochasticity as well as catastrophes can result in extinction of small, isolated populations, which are predominantly found away from the centre of a species' distribution. Sea birds like the little penguin are recognised as potential indicators of marine environments due to their high trophic level, and are among the most threatened groups of birds. A population viability analysis in VORTEX was conducted to assess the current status of little penguins in NSW and WA and estimate the sensitivity of model outcomes to changes in survival and reproductive rates as well as frequency and severity of La Niña events. Declining population trends of Australian little penguins were forecast for their northern range edge, both in Western Australia and New South Wales. Forecasts were more sensitive to mortality than reproductive rates, and frequency and severity of La Niña events significantly affected model outcomes for the northern population in Western Australia. It is recommended that attempts to halt the projected population declines should focus on reduction or prevention of increases in mortality and further research is needed to confirm population-specific data on reproduction and mortality rates. Continued population monitoring will ensure timely detection of population declines at the penguin's northern distribution limit.

53

Chapter 5: Viability of little penguin (*Eudyptula minor*)  
populations at their northern range edge

54

55    **Introduction**

56    Genetic, demographic and environmental stochasticity as well as catastrophes can  
57    result in extinction of small, isolated populations (Beissinger, 2002; Lande, 1993).  
58    Exceptional environmental conditions such as those encountered during years of  
59    extremes of the El Niño Southern Oscillation (ENSO), so-called El Niño or La Niña  
60    events, are impacting survival and reproductive rates of sea birds (Chambers et al.  
61    2014), including the little penguin (*Eudyptula minor*) in Australia (Dann & Chambers  
62    2013). Large-scale climate patterns such as ENSO affect terrestrial and marine  
63    environments differently: on land, El Niño events are often associated with drier than  
64    normal conditions across eastern and northern Australia, while La Niña events are  
65    associated with wetter than normal conditions across the continent (Australian Bureau  
66    of Meteorology 2015). Because little penguins breed in burrows, the wetter conditions  
67    during La Niña are particularly likely to affect their breeding success. At sea, ENSO  
68    affects Australia's marine waters to differing degrees around the coast (Holbrook et al.  
69    2009). The effect of ENSO is particularly strong and very significant with regards to the  
70    southward flowing Leeuwin Current, which is intensified during La Niña years in  
71    Australia's west, while being further transmitted along the south coast of Australia. In  
72    the southward flowing East Australian Current (EAC), ENSO is observed as a weaker  
73    signal than in the West (Holbrook et al. 2009).

74    Little penguins in south-eastern Australia are expected to increase productivity with  
75    warmer oceans as projected under various climate change scenarios (IPCC 2013),  
76    resulting in earlier breeding, heavier chicks and an increased chance of double

77 brooding, at least in the short-term (Chambers et al. 2014). Long-term trends will also  
78 be affected by changes in climatic oscillations.

79 In a recent study, the frequency of extreme La Niña events was forecast to almost  
80 double in the near future (Cai et al. 2015), from once every 23 to once every 13 years,  
81 with ca. 75 % of the increase expected to occur in years following extreme El Niño  
82 events, thus projecting more frequent swings between opposite extremes from one  
83 year to the next.

84 Because many sea birds are top-level predators, their population dynamics integrate  
85 variabilities in abundance of their prey species as well as environmental conditions  
86 over a wide range of spatial and temporal scales. Seabirds are therefore recognised as  
87 potential indicators of marine environments (Woehler 2012). Additionally, seabirds are  
88 among the most threatened groups of birds and their decline has accelerated over the  
89 past few decades (Croxall & Butchart 2012). The flightless penguins are particularly  
90 affected by land-based threats, and these birds have in fact been identified as one of  
91 the two most threatened groups of seabirds (Croxall & Butchart 2012). Little penguins  
92 are an iconic seabird species native to Australia and New Zealand, and are declining in  
93 some locations, especially in mainland Tasmania and South Australia (e.g., Wiebkin  
94 2011), but increasing in others, including Phillip Island (Sutherland & Dann 2014). In  
95 New South Wales (NSW, Fig. 1), the only known remaining mainland colony at Manly  
96 in Sydney Harbour (Fig. 1) has been listed as an endangered population (NSW National  
97 Parks and Wildlife Service 2000). Since its listing, the colony has successfully recovered  
98 from a few tens of pairs to achieve the most successful breeding season in decades,  
99 with 85 breeding pairs in 2013 (Lisa O'Neill, pers. comm.). While the colony is managed

100 as a separate population to surrounding island colonies, it is not genetically isolated  
101 (Chapter 3). Rather, the NSW metapopulation is genetically largely homogeneous, with  
102 the metapopulation size estimated to be around 27 000 individuals (Appendix Table  
103 1A). In Western Australia (WA), the penguin population is estimated at slightly more  
104 than 5 000 pairs (Appendix Table 2A) and the colony at Penguin Island near Perth (Fig.  
105 1) has been extensively studied over the last few decades (e.g. Klomp & Wooller 1988;  
106 Klomp & Wooller 1991; Wienecke et al. 1995; Cannell 2001). It has been characterised  
107 as the largest colony of little penguins in Western Australia (Cannell et al. 2011) and  
108 has the highest conservation status of all major little penguin colonies within Australia  
109 (Dann et al. 1996). Both NSW and WA therefore harbour little penguin colonies of  
110 special significance for conservation, and each of them is close to the species' northern  
111 distribution limit (Fig. 1), so that their loss would decrease the species' range. It is  
112 therefore important to assess these populations' current status, population viability  
113 and sensitivity to environmental change to facilitate the development of management  
114 recommendations. To do this, population viability analysis (PVA) models can be useful,  
115 and PVAs have been employed to model the effect of El Niño on the persistence of  
116 small populations of the Galápagos penguin (*Spheniscus mendiculus*, Vargas et al.  
117 2007). In a broader seabird context, sooty shearwaters (*Puffinus griseus*) have been  
118 used as a case study to test the usefulness of PVA models for conservation advice  
119 (Hamilton & Moller 1995), concluding that "models can assist by formalising how  
120 uncertain our current understanding is". Since then, PVAs have been applied to  
121 several Australian bird species (Burgman 2000), but with the notable exception of a  
122 study on the impact of reduced juvenile mortality in Laysan albatross (*Phoebastria*  
123 *immutabilis*, Finkelstein et al. 2010), their use in seabird research has been sparse. This

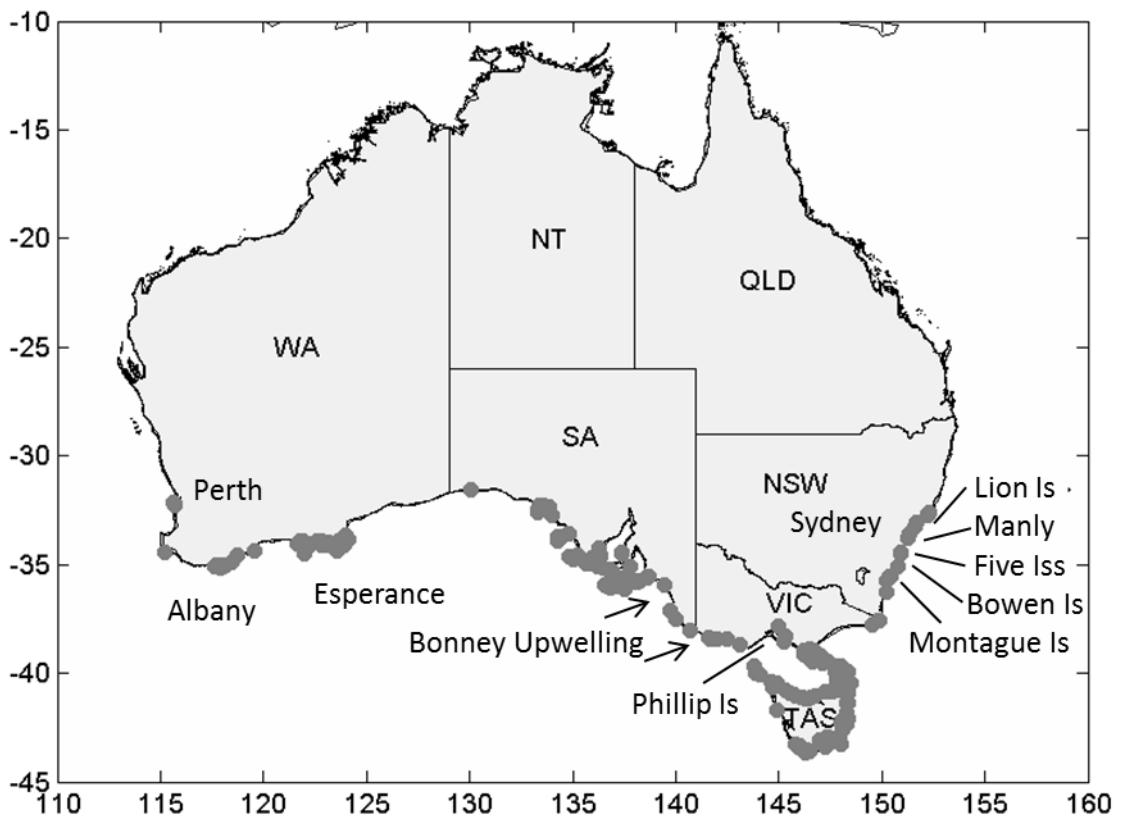
124 is probably due to a lack of input data for most seabirds. Compared to the study  
125 reported in this thesis, some researchers have therefore used alternative, less data  
126 intensive approaches to estimate population viability or assess sensitivity to  
127 environmental threats. Examples include a population model for razorbills (*Alka torda*,  
128 Lavers et al. 2009) and a sensitivity analysis of the endangered Hutton's shearwater  
129 (*Puffinus huttoni*, Cuthbert et al. 2001), both of which resulted in valuable results for  
130 prioritising conservation research and management.

131 To get meaningful results from PVAs, it is recommended to address a specific question  
132 involving a focal (e.g., threatened, indicator, sensitive, or umbrella) species, to ensure  
133 the level of detail is consistent with available data, and to focus on relative (i.e.,  
134 comparative) rather than absolute results, and risks of decline rather than risks of  
135 extinction alone (Akçakaya & Sjögren-Gulve 2000). This study follows those  
136 recommendations.

137 The PVA programme VORTEX was originally designed to model bird and mammal  
138 populations, and its most recent version 10.0.8 (Lacy & Pollak 2014) was used to  
139 forecast penguin population size over 50 years, in increments of one year. For each set  
140 of input parameters, models were run as 100 iterations to estimate population size (N)  
141 over time and to assess the probability of extinction (PE). Three steps were taken to  
142 characterise the little penguin population of NSW: (1) Baseline models of the current  
143 status of little penguins in NSW and WA were built using collected data and  
144 information from the literature; (2) Sensitivity analyses were conducted to investigate  
145 the effect of different survival and reproductive rates as well as changes in frequency

146 and severity of La Niña events on model outcomes; and (3) Directions for research and  
147 management were suggested, based on the results of this PVA.

148



149  
150 **Fig. 1:** Map of known penguin colonies in Australia, colonies represented as grey circles  
151 NSW – New South Wales, NT – Northern Territory, QLD – Queensland, SA – South  
152 Australia, TAS – Tasmania, VIC – Victoria, WA – Western Australia

153

154

155 **Method**

156 VORTEX 10 (Lacy & Pollak 2014) is a stochastic population modelling programme for  
157 PVA that can model many of the extinction vortices that threaten the persistence of  
158 small populations. Population dynamics are modelled as discrete, sequential events,  
159 whereby VORTEX simulates a population by following a series of events describing the  
160 annual cycle of a typical sexually reproducing, diploid organism. Population simulations  
161 are iterated many times to generate the distribution of fates the population might  
162 experience (Lacy 1993; Lacy 2000). The programme also allows incorporation of the  
163 effects of catastrophes, such as environmental factors associated with ENSO, on  
164 reproductive and survival rates.

165 The best available data of little penguin biology were incorporated in the VORTEX  
166 models. Estimates of vital rates were obtained from the published literature,  
167 unpublished technical reports of annual censuses and the results of Chapter 2 in this  
168 thesis (Table 1-4). Where data were not available for the little penguin, information on  
169 related species of penguins was used.

170 ***ENSO classification and climate scenarios***

171 Values of the Southern Oscillation Index (SOI), obtained through the Australian Bureau  
172 of Meteorology, were used to classify years from 1994 to 2009 as normal, El Niño or La  
173 Niña. Breeding data for Penguin Island in WA were then analysed for differences  
174 among years following the three different types of ENSO events (B. Cannell,  
175 unpublished data).

176

177 **Vital parameters for population modelling**

178 Below, we briefly describe the parameters for the different little penguin populations  
179 that were used in the VORTEX model. Species-specific model settings are summarised  
180 in Table 1, population-specific parameters in Table 2 for NSW and Table 3 for WA.

181 **Table 1:** Simulation model parameters used to conduct the population viability analysis  
182 EV – environmental variation

Model parameter	Value	Source
Number of years modelled	50	n/a
Number of iterations	100	
Sequence of events in each time cycle	EV, Breed, Mortality, Age, Disperse, Harvest, Supplement, rCalc, Ktruncation, UpdateVars, Census	default (see Vortex 10 User's manual, Lacy et al. 2015)
Extinction definition	One sex remains	default
Inbreeding depression	none	Chapter 3
Correlation of EV among populations	0.5	default
Dispersing sex(es)	Both	Reilly 1994
Dispersing age range (years)	1 - 3	Reilly 1994
Percent survival of dispersers	100	default
Reproductive system	Monogamy	Reilly 1994
Age of first offspring, both sexes	2	Dann & Cullen 1990
Maximum age	26	Dann et al. 2005
Sex ratio at birth (% males)	50	default (monogamy)
Concordance of variation in reproduction and survival	Yes	default (Lacy et al. 2015)

183

184 **Table 2:** Population-specific parameters of VORTEX population viability analysis for NSW  
185 R – Reproduction, S - Survival

Population specific parameters	NSW - Baseline	Source
Number of populations	2	Geographic distribution and genetic structure (Chapter 3)
Dispersal rates	Table 4.a	Bayesian analysis in MIGRATE

<b>Population 1: Lion Island (North)</b>		
Percent of adult females breeding each year, mean $\pm$ SD	100 %	Default
Distribution of number of broods per year	43.30 % 0 broods	Vargas et al. 2007
	33.03 % 1 brood	Cunningham et al. 1993
	23.67 % 2 broods	
Normal distribution of brood size, mean $\pm$ SD	1.41 $\pm$ 0.21	Cunningham et al. 1993
Initial population size	1 049	Penguin meeting - Northern Colonies (Appendix Table 1A)
Carrying Capacity (K)	2 098	Twice initial population size
Annual mortality rates (as percents, male = female):		
Age 0 to 1, mean $\pm$ SD	83.5 $\pm$ 0.7	Sidhu et al. 2007
Age 1 to 2, mean $\pm$ SD	28.9 $\pm$ 2.5	
After age 2, mean $\pm$ SD	20.9 $\pm$ 10.5	Chapter 2
Catastrophe: La Niña		
Impact	Global	Australian Bureau of Meteorology 2015
Frequency	15%	
Multiplicative impacts on reproduction*survival	0.90	
<b>Population 2: Bowen Island (South)</b>		
Percent of adult females breeding each year, mean $\pm$ SD	100 %	Default
Distribution of number of broods per year	43.3 % - 0 broods	Vargas et al. 2007
	46.4 % - 1 brood	Fortescue 1991
	10.3 – 2 broods	
Normal distribution of brood size, mean $\pm$ SD	1.62 $\pm$ 0.4	Fortescue 1991
Initial population size	25 864	Penguin meeting 2014
Carrying Capacity	51 728	Twice initial population size
Annual mortality rates (as percents, male = female):		
Age 0 to 1, mean $\pm$ SD	83.5 $\pm$ 0.7	Sidhu et al. 2007
Age 1 to 2, mean $\pm$ SD	28.9 $\pm$ 2.5	
After age 2, mean $\pm$ SD	42.3 $\pm$ 10.4	Chapter 2
Catastrophe: La Niña		
Impact	Global	Australian Bureau of Meteorology 2015
Frequency	15%	
Severity (proportion of normal values)	R * S = .9	R: 0.95, S: 0.95

186   **Table 3:** Population-specific parameters of VORTEX population viability analyses for two  
187   possible stratifications of the WA data set: Stratification 1 is based on two populations  
188   (North, around Perth, and South, including Albany and Esperance), whereas  
189   Stratification 2 splits the WA metapopulation in three populations (Perth, Albany and  
190   Esperance)

191   R – Reproduction, S - Survival

<b>Population specific rates</b>	<b>WA - Stratification 1</b>	<b>WA - Stratification 2</b>	<b>Source</b>	
Number of populations	2	3	Genetic structure (Sinclair et al., unpublished data)	
Dispersal rates	Table 4.b	Table 4.c	Bayesian analysis in MIGRATE	
<b>Population 1: Perth (North)</b>				
% adult females breeding, mean $\pm$ SD	100%		default	
Distribution of number of broods per year	51.6 % - 0 broods		B. Cannell, unpubl. data	
	25.67 % - 1 brood			
	22.73 % - 2 broods			
Normal distribution of brood size, mean $\pm$ SD	$1.22 \pm 0.22$		B. Cannell, unpubl. data	
Annual mortality rates (as percents, male = female):				
Age 0 to 1, mean $\pm$ SD	$83.5 \pm 0.7$		Sidhu et al 2007	
Age 1 to 2, mean $\pm$ SD	$28.9 \pm 2.5$			
After age 2, mean $\pm$ SD	$15.1 \pm 9.9$		B. Cannell, unpubl. data	
Catastrophe: La Niña				
Impact	Global		Australian Bureau of Meteorology 2015	
Frequency	15%			
Severity (proportion of normal values)	$R * S = 0.8$ (R: 0.9, S: 0.9)		B. Cannell, unpubl. data	
Initial population size	2069		Cannell et al. 2011	
Carrying Capacity (K) $\pm$ SD	$4138 \pm 1531$		2x pop size, B. Cannell	
<b>Population 2 (and 3):</b>	<b>Esperance / Albany (South)</b>	<b>Albany (A) and Esperance (E)</b>		
% adult females breeding each year, mean $\pm$ SD	100%		default	
Distribution of number of broods per year	51.60 % - 0 broods		B. Cannell, unpubl. data	
	25.67% - 1 brood			
	22.73 % - broods			
Normal distribution of brood size, mean $\pm$ SD	$1 \pm 0.4$		Dann & Cullen 1990	
Initial population size	2306	4423 (A), 974 (E)	genetic data: 10x N <sub>e</sub>	
Carrying Capacity	4612	8846 (A), 1948 (E)	2x pop size	

Annual mortality rates (as percents, male = female):		
Age 0 to 1, mean ± SD	83.5 ± 0.7	Sidhu et al. 2007
Age 1 to 2, mean ± SD	28.9 ± 2.5	
After age 2, mean ± SD	15.1 ± 9.9	B. Cannell, unpubl. data
Catastrophe: La Niña		
Impact	Global	Australian Bureau of Meteorology 2015
Frequency	15%	
Severity (proportion of normal values)	0.95	Reproduction: 0.975 Survival: 0.975

192

193

#### 194 Inbreeding depression

195 In Chapter 3 of this thesis, the genetic diversity of little penguin colonies of different  
196 size and conservation status was compared and no significant differences detected.  
197 We therefore conclude that none of the populations have previously undergone  
198 population bottlenecks leading to reduced genetic diversity. Furthermore, population  
199 sizes (Appendix Table 1A) are large enough to make inbreeding unlikely, so we did not  
200 include inbreeding depression in the models.

201 With population sizes forecast to decline, inbreeding is likely to start having an effect,  
202 but running models with and without inbreeding depression did not give significantly  
203 different results (see Appendix Table 3A).

#### 204 Dispersal and survival of dispersers

205 Dispersing sex(es): Both sexes of little penguins have been observed to disperse (Reilly  
206 & Cullen 1982), although sex-biased dispersal is indicated by results of genetic studies  
207 (Sinclair et al. in prep; Chapter 3). Little penguins mainly disperse after fledging until  
208 they are reproductively mature and start breeding, after which they generally return to  
209 the same colony each year (Reilly 1994). The dispersing age range was therefore set as  
214

210 one to three years. Percent survival of dispersers was set as 100 % because mortality is  
211 high during the first years of a penguin's life, as implemented in the model, and  
212 additional mortality is unlikely to affect dispersers specifically.

213 Dispersal rates and effective population size  $N_e$  were estimated based on  
214 mitochondrial DNA data used as input for Bayesian modelling in the program  
215 MIGRATE-N (NSW: Chapter 3 in this thesis, WA: J. Sinclair, unpublished data). From this,  
216 the percentage of annual dispersers was calculated as the number of migrants per  
217 generation estimated in MIGRATE-N, divided by the product of  $N_e$  and the generation  
218 time of 7 years, as calculated by VORTEX. Percentages of individuals that disperse  
219 between each pair of populations are shown in Table 4.

220

221 **Table 4:** Dispersal rates from source (row) to recipient population (column), based on  
222 mitochondrial DNA (Chapter 3); LI – Lion Island, BI – Bowen Island

Table 4.a

New South Wales		
	LI (North)	BI (South)
LI (North)		3.312
BI (South)	3.278	

Table 4.b

Western Australia - Stratification 1		
	Perth (North)	South
Perth (North)		1.378
South	0.002	

Table 4.c

	Perth	Albany	Esperance
Perth		0.235	0.180
Albany	0.021		0.048
Esperance	0.025	0.105	

223

224 **Reproductive system and ages**

225 Little penguins are generally monogamous (Reilly 1994). On Lion Island in NSW, mate  
226 fidelity was 89%, with a significant positive relationship between breeding success in  
227 one year and mate fidelity in the next (Rogers & Knight 2006). The divorce rate of little  
228 penguins in New Zealand was estimated to be as low as 3% (Bull 2000). We therefore  
229 chose a monogamous reproductive system for the little penguins. The age of first  
230 offspring for little penguins is between two and three years for both sexes, after the  
231 end of the juvenile stage (Dann & Cullen 1990). Little penguins reach a maximum age  
232 of 26 years (Dann et al. 2005) and are assumed to continue breeding until death. The  
233 model outcome was not sensitive to maximum age of breeding because, based on  
234 annual mortality rates, most individuals were projected to die before they reach  
235 age 26.

236 **Sex ratio of offspring at birth**

237 It was assumed that the sex ratio at birth was 50% males, which is the default setting  
238 for monogamous reproductive systems.

239 **Concordance of variation in reproduction and survival**

240 Environmental variation (EV) is the annual variation of probabilities of reproduction  
241 and survival that arise from random variation in environmental conditions. All  
242 individuals in the population are simultaneously impacted by EV. The sources of this  
243 environmental variation are not part of the population's characteristics and include  
244 examples like weather (including ENSO status), densities of predator and prey  
245 populations, and parasite loads (Lacy et al. 2015). These factors are likely to affect

246 reproduction and survival at the same time, resulting in good years for reproduction  
247 that are also good years for survival.

248 Due to the close geographic proximity of the penguin populations studied, we assume  
249 an intermediate value of 0.5 for the correlation of EV among populations, which is also  
250 the default setting in Vortex.

251 **Maximum number of broods per year and progeny per brood**

252 Little penguins generally produce two eggs per clutch (Stahel & Gales 1987), and rates  
253 of double brooding can be as high as 31 % in good years (Cunningham et al. 1993).  
254 Breeding parameters for each population are summarised in Table 2 and 3.

255 **Percent of adult females breeding**

256 Reliable data for the percentage of females attempting to breed each year were not  
257 available for any Australian colony, and NSW population size estimates were based on  
258 numbers of breeding pairs found during one or more breeding seasons (see below,  
259 Appendix Table 1A) so the default value of 100 % was used. Due to scarcity of  
260 information, it was not possible to estimate density dependence of reproductive rates.

261 **Breeding success**

262 In years following non-LaNiña events between 1994 and 2009, an average of 48.4 % of  
263 adult females attempting to breed successfully raised chicks in a breeding season on  
264 Penguin Island in WA (Cannell, pers. comm.). These penguins produced an average of  
265 1.22 offspring per pair, with standard deviation (SD) of 0.22 (B. Cannell, pers. comm.).  
266 For the other population(s) in WA, the same brood size as reported for Phillip Island  
267 was assumed (Dann & Cullen 1990) as  $1 \pm 0.4$ .

268 For NSW, we chose the percentage of adult females successfully breeding, i.e.  
269 producing one or more clutches, of 56.7 %, taken from Galapagos and African penguins  
270 (Vargas et al. 2007).

271 Average brood size was  $1.41 \pm 0.21$  per pair for Lion Island (Cunningham et al. 1993)  
272 and 1.62 per pair for Bowen Island (Fortescue 1991). To calculate SD for the number of  
273 offspring at Bowen Island, a coefficient of variation of 0.15 was calculated from the  
274 Lion Island estimate. This coefficient was applied to the mean for Bowen Island,  
275 resulting in an SD of 0.24.

## 276 **Mate monopolisation**

277 Degree of monopolisation of breeding opportunities is not known and was left at the  
278 default setting of 100 %.

## 279 **Mortality**

280 In a study of little penguins banded at Phillip Island, it was found that sex did not  
281 significantly affect survival probability (Sidhu 2007). Male and female little penguins  
282 generally do not behave differently, as both sexes are known to disperse and care for  
283 offspring (Reilly & Cullen 1979). Furthermore, there is little sexual dimorphism in little  
284 penguins, so mortality is unlikely to be different among sexes. Age-specific survival  
285 estimates for the first two years of life (Sidhu et al. 2007) were converted into  
286 mortality rates (average  $\pm$  SD) of  $83.5 \pm 0.7$  after fledging for the first year, and  $28.9 \pm$   
287 2.5 for the second year of age. In WA, annual adult (after age two) mortality was  
288  $15.1 \pm 9.9$  based on data for the Penguin Island population (B. Cannell, unpubl. data),  
289 and  $42.3 \pm 10.4$  for Lion and  $20.9 \pm 10.5$  for Bowen Island in NSW, respectively

290 (Chapter 2 of this thesis). Unfortunately, these mortality estimates may include ENSO  
291 affected years.

292 **Number of populations and population sizes**

293 Little penguins were divided into different numbers of populations based on a  
294 combination of geographic penguin distribution and genetic structure. In WA, three  
295 genetically distinct genetic clusters had previously been identified (Sinclair et al., in  
296 prep). These agreed with the geographic origin of individuals and included Penguin and  
297 Garden Islands as well as Bunbury ("Perth"), Muttonbird (Shelter) and Cheyne  
298 (Mistaken) Islands ("Albany"), and Wickham (Stanley) and Woody Islands  
299 ("Esperance") – see map in Fig. 1. Due to the low level of differentiation among the  
300 latter two localities, simulations were also run using two populations, Perth and  
301 surrounds ("North") versus the southern populations around Albany and Esperance  
302 combined ("South"). The two different groupings of colonies within WA were referred  
303 to as "stratifications", with stratification 1 treating the WA metapopulation as two  
304 populations, the northern population around Perth and the south grouped together,  
305 and stratification 2 referring to the three different populations described above. In  
306 NSW, genetic structure is well defined (Chapter 3). We therefore relied on geographic  
307 clustering of colonies and assumed the smaller colonies to the North, from  
308 Wollongong to Port Stephens, to be demographically similar to Lion Island (LI, colonies  
309 collectively referred to as "North"), whereas the larger, southern colonies south of  
310 Wollongong were assumed to resemble Bowen Island (BI, "South"). For NSW, initial  
311 population sizes ( $N_b$ , number of breeders, with stable age distribution) were summed  
312 over the colonies found within the geographic ranges described (Appendix Table 1A

313 and Table 2A): 1049 for northern NSW, 25 864 for southern NSW. These estimates of  
314  $N_b$  are mainly based on the number of breeding pairs and do not represent the whole  
315 population of potential breeders  $N_p$  in NSW. WA population sizes were derived from a  
316 recent census for northern WA ( $N_p = 2\ 069$  pairs, Cannell et al. 2011) and from genetic  
317 data for southern WA. For the latter, estimates of the effective population size  $N_e$   
318 derived from MIGRATE-N modelling of microsatellite data were multiplied by ten to  
319 obtain an estimate of census population size  $N_c$  (Frankham 1995), which was assumed  
320 to represent the number of potential breeders  $N_p$  in the population.

### 321 Carrying capacity

322 The population carrying capacity  $K$  was set at the double of the initial population size  
323 for each subpopulation due to known historic and recent declines in penguin numbers.  
324 We wanted to be able to identify if the populations are capable of growing and  
325 therefore did not constrain them with low values for  $K$ , which was also assumed to be  
326 constant (standard deviations of  $K$  left at zero).

### 327 Harvesting and Supplementation

328 Neither harvesting nor supplementation was implemented in the model.

### 329 Catastrophes

330 Although there is a possibility that subpopulations experience partly independent  
331 causes of mortality and reproductive success, annual variation in environmental  
332 conditions is probably largely concordant across the northern distribution limit of little  
333 penguins. La Niña events were therefore modelled as global catastrophes in VORTEX.

334 During the last century, 15 La Niña events were recorded (National Centers for  
335 Environmental Prediction 2015), with average intervals of almost 7 years between  
336 them. The frequency of La Niña catastrophes was therefore set at 15 %. During normal  
337 years (neither El Niño nor La Niña), penguins survive and reproduce according to the  
338 input data listed above. While reproductive output has been reduced by 20 % in years  
339 after La Niña years on Penguin Island, WA (B. Cannell, unpubl. data), the influence of  
340 La Niña on colonies in southern WA and in NSW is less clear. ENSO does not affect the  
341 East Australian Current as strongly as it does the Leeuwin Current (Holbrook et al.  
342 2009), so the effect on the product of survival and reproductive rates was assumed to  
343 be only -10 % for NSW and -5 % for southern WA.

344

345 ***Sensitivity testing***

346 We used sensitivity testing to test the effect of alternative vital parameters believed to  
347 be important to the persistence of the species and for which some degree of variability  
348 and uncertainty were suspected. Estimates of standard deviation (SD) due to  
349 environmental variation were adapted using the coefficient of variation (CV) for the  
350 respective parameters by calculating CV as the ratio of SD to the baseline mean, then  
351 multiplying CV by the adjusted mean. Significance of differences between scenarios  
352 was assessed by comparing population trends and estimating whether averages  $\pm$  their  
353 standard errors overlapped. Because our principal conservation goal is to minimise the  
354 probability of extinction and stabilise long-term population size, we conducted  
355 sensitivity testing of the following key parameters that would be responsive to changes  
356 in management strategies.

357 **Mortality rates**

358 Different estimation methods for survival rates resulted in slightly different estimates  
359 of mortality for two colonies in NSW (Chapter 2 in this thesis). To estimate the  
360 influence of survival (and conversely, mortality) on model outcomes, juvenile and adult  
361 mortality rates were simultaneously varied by  $\pm 5\%$  as well as  $\pm$  one SD of the baseline  
362 values for each population.

363 **Reproductive rates**

364 The sensitivity of the probability of extinction to variation in breeding success was  
365 assessed by varying the number of broods simultaneously by  $\pm 5\%$  of the baseline  
366 values.

367 **La Niña – Catastrophes**

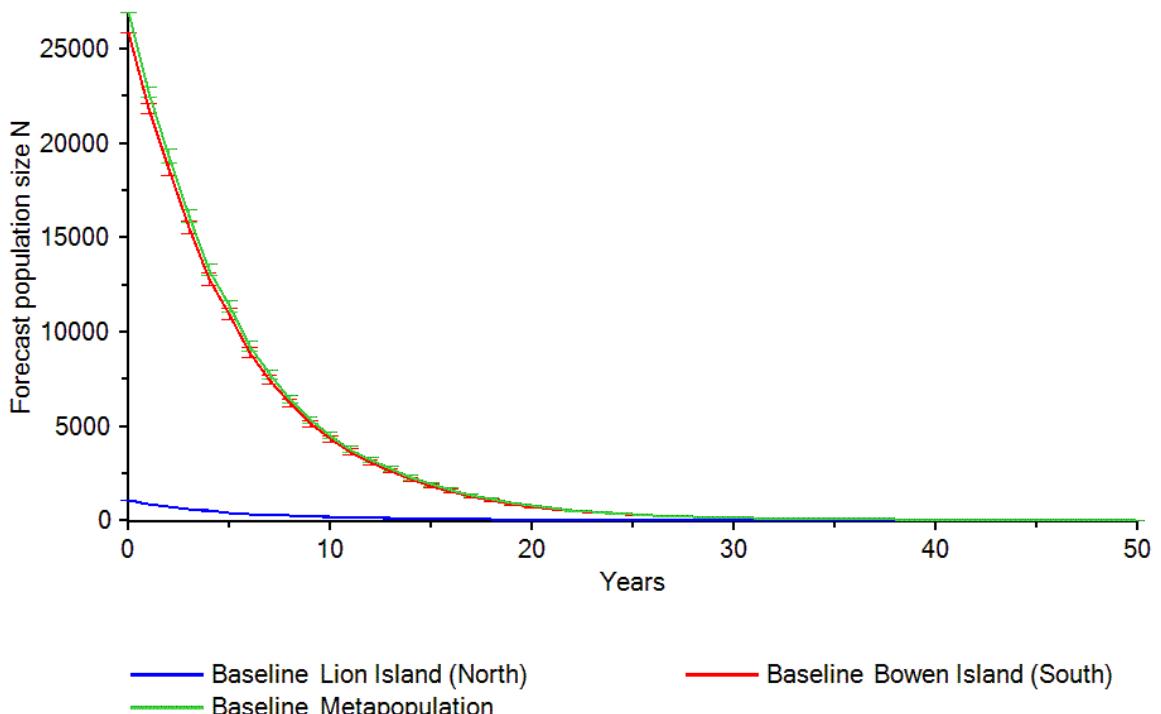
368 Because the effect of La Niña on the penguins of NSW is less clear than its effect on the  
369 colonies around Perth, sensitivity of Vortex models to the reduction of survival and  
370 reproductive rates was also investigated. We varied effect of La Niña in NSW and  
371 southern WA using a 5 % (light), 10 % (baseline), 15 % (severe) and 20 % (extreme)  
372 combined reduction in survival and reproduction.

373 Because climate scenarios predict more frequent severe weather events with global  
374 warming, we also investigated the sensitivity of penguin populations to the frequency  
375 of La Niña events by setting their occurrence to either 10 per century (10 %, low  
376 frequency) or 20 % (high frequency).

377 **Results**

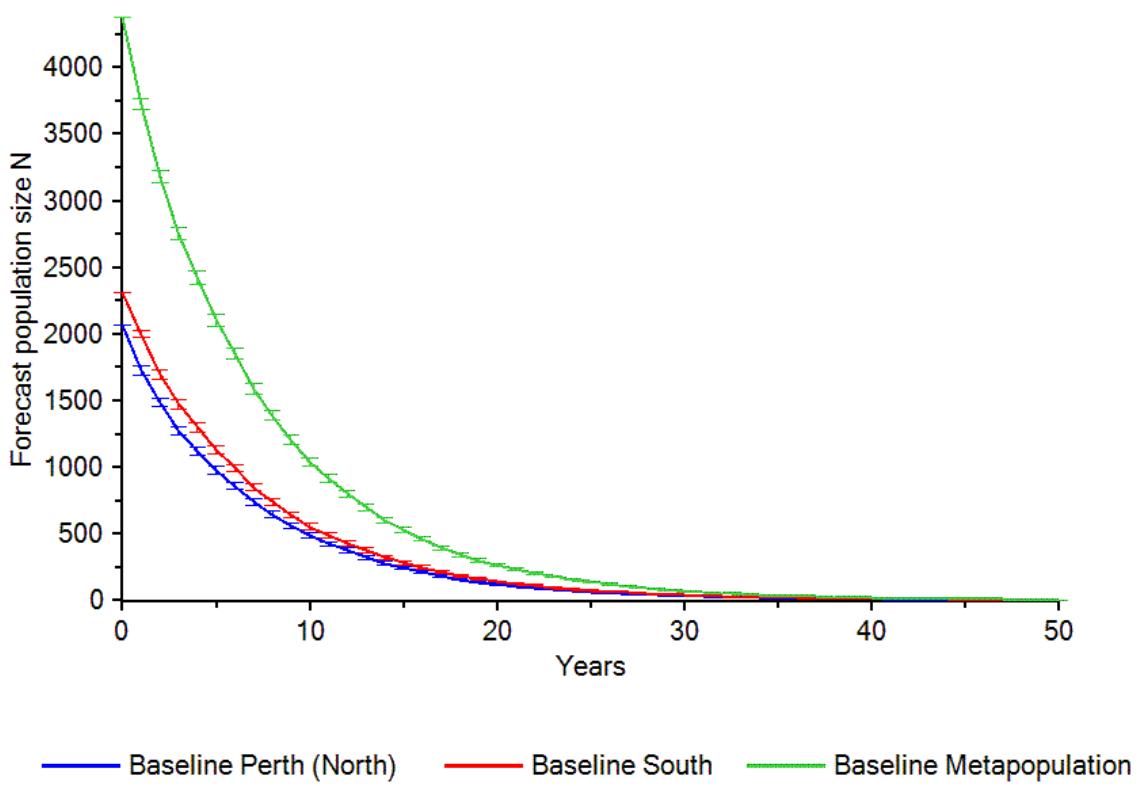
378 **Baseline Scenarios for NSW and WA**

379 The baseline scenario for NSW showed a marked decrease in population size N over  
380 time for both the northern and southern population (Fig. 2). Due to its much larger  
381 size, the population trend of the southern population dominated the overall trend of  
382 the NSW metapopulation (North and South combined). The probability of extinction  
383 after 50 years was 56 % for the South and the NSW metapopulation, and 100 % for the  
384 northern population (Table 6). The generation time calculated for NSW is 4.79 years.



389 In the baseline scenario for WA divided into two populations (stratification 1), a  
390 marked decrease in population size N over time was observed for the northern  
391 populations around Perth as well as the South (Fig. 3). The initial population sizes of

392 both populations differ by less than 300 individuals and follow a similar trend despite  
393 differences in brood sizes and dispersal rates, leading to the metapopulation following  
394 a similar trend, too. The probability of extinction after 50 years was 78 % for the Perth  
395 populations and 70 % for the South, with an extinction probability of 50 % for the WA  
396 metapopulation (Table 5). The generation time of 9.42 years for WA is almost twice as  
397 long as in NSW.



399 **Fig. 3:** Forecast of population size N over 50 years according to baseline stratification 1  
400 for two populations in WA; error bars represent standard error of 100 replicate  
401 scenarios

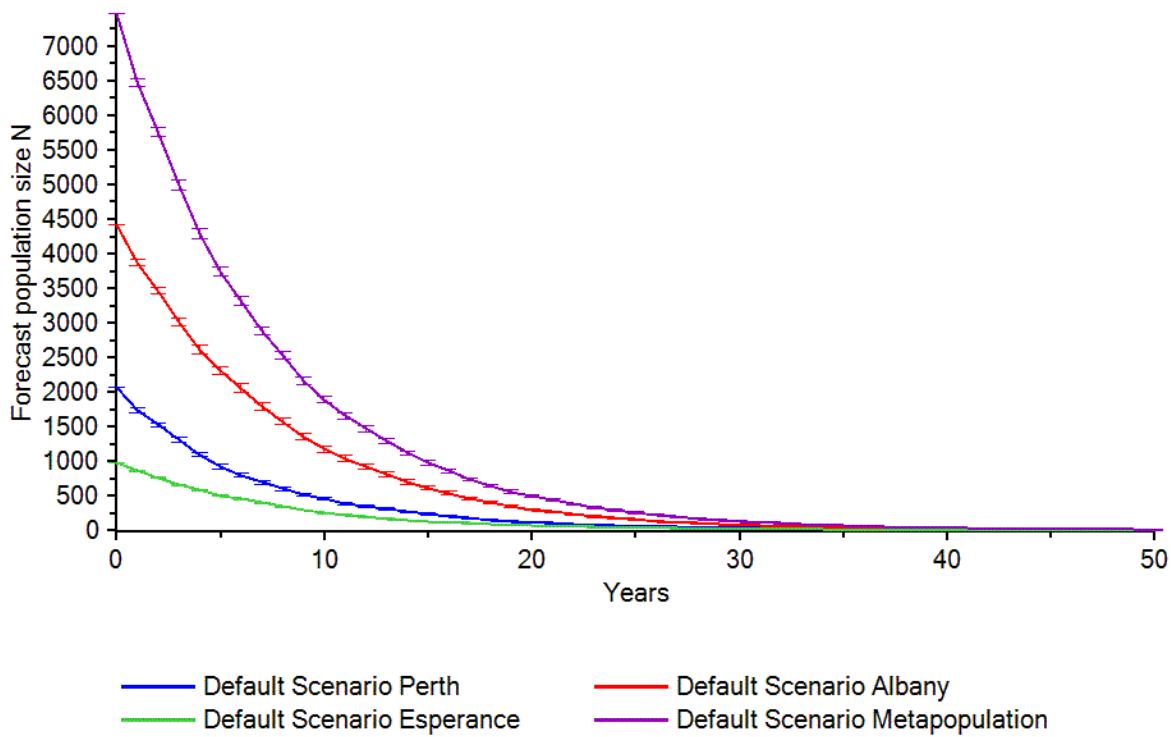
402

403   **Table 5:** Probability of extinction ± standard error for stratifications 1 and 2 with two  
404   and three WA populations, respectively, under different reproductive scenarios;  
405   Metapop – WA Metapopulation

WA Stratification 1	North (Perth)	South		Metapop
Baseline	0.78 ± 0.04	0.70 ± 0.05		0.50 ± 0.05
High Repro (+5%)	0.67 ± 0.05	0.69 ± 0.05		0.38 ± 0.05
Low Repro (-5%)	0.86 ± 0.03	0.80 ± 0.04		0.71 ± 0.05
WA Stratification 2	Perth	Albany	Esperance	Metapop
Baseline	0.74 ± 0.04	0.33 ± 0.05	0.90 ± 0.03	0.19 ± 0.04
High Repro (+5%)	0.65 ± 0.05	0.38 ± 0.05	0.87 ± 0.03	0.15 ± 0.04
Low Repro (-5%)	0.79 ± 0.04	0.46 ± 0.05	0.89 ± 0.03	0.23 ± 0.04

406

407   When the WA metapopulation was split in three populations (stratification 2), a  
408   marked decrease in population size N over time was observed for all three populations  
409   and consequently for the WA metapopulation. Over time, there was a decline in  
410   differences among populations, and all population sizes fell below 10 individuals after  
411   50 years. The probability of extinction was highest for Esperance (90 %) and 74 % for  
412   Perth, 33 % for the colonies around Albany and 19 % for the WA metapopulation  
413   (Table 5).



414

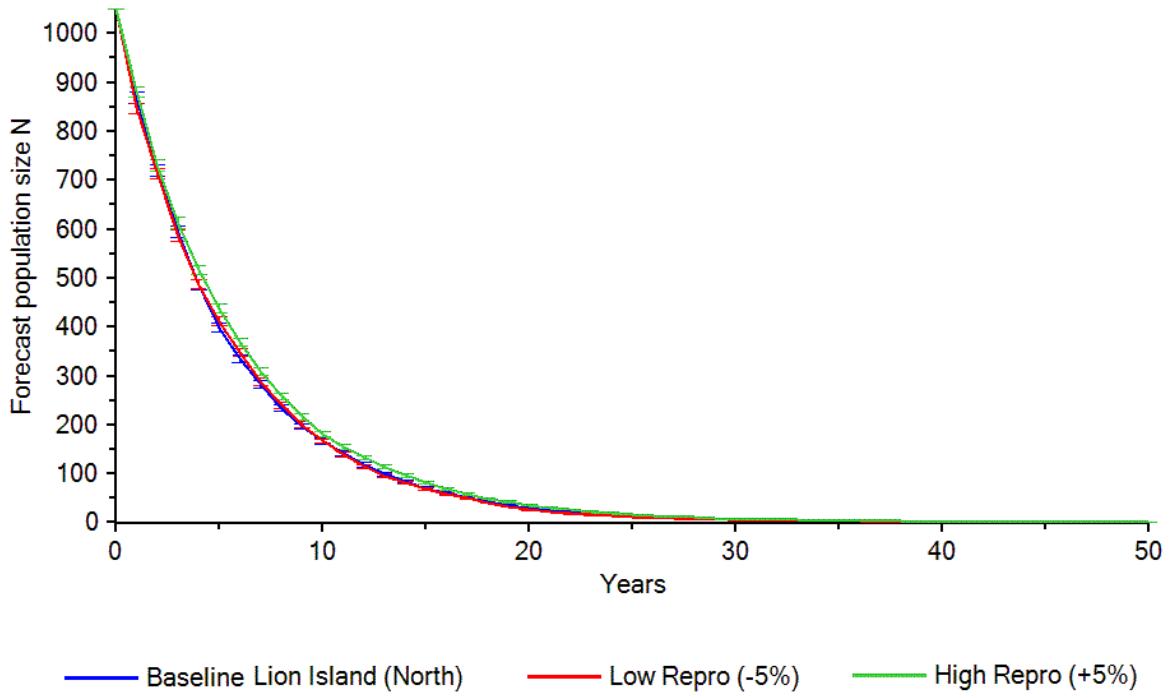
415 **Fig. 4:** Forecast of population size N over 50 years according to baseline stratification 2  
416 for three populations in WA; error bars represent standard error of 100 replicate  
417 scenarios

418

#### 419 **Sensitivity analyses**

#### 420 **Reproductive rates**

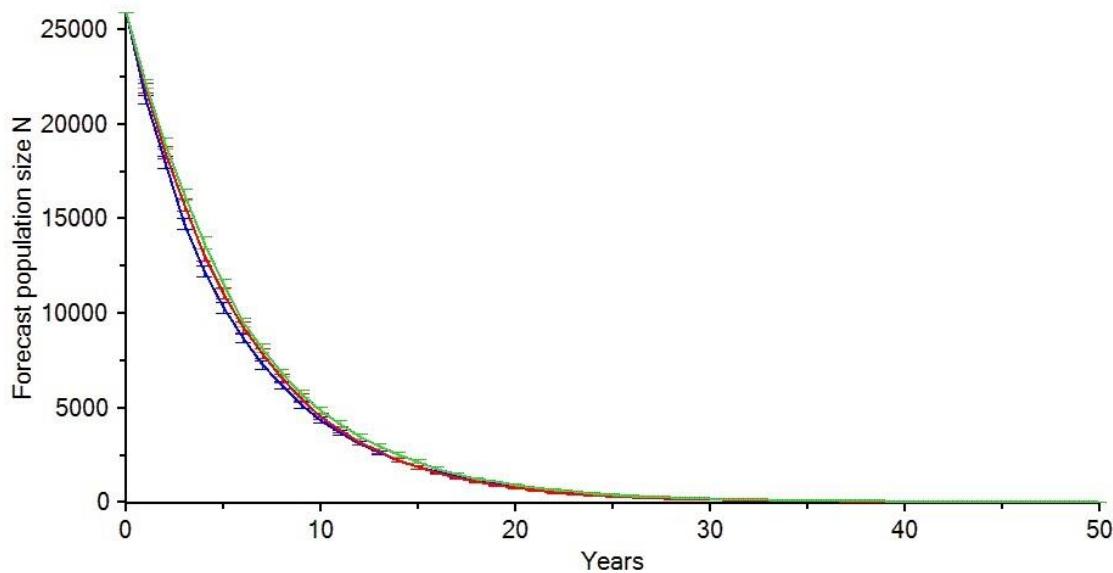
421 Varying number of broods per year by  $\pm 5\%$  did not affect population trends for the  
422 northern population in NSW significantly, with a strong decline of population size  
423 forecast for all scenarios (Fig. 5). The probability of extinction remained at 100 % for  
424 reduced reproduction, while an increase in reproductive rates decreased PE to 95 %.



**Fig. 5:** Sensitivity analysis - forecast of population size N for northern NSW over 50 years with high and low reproductive rates, varied by  $\pm 5\%$  from the baseline; error bars represent standard error of 100 replicate scenarios

429

430 Population trends for the southern population in NSW were also not significantly  
431 affected by variation of broods per year by  $\pm 5\%$  (Fig. 6). The probability of extinction  
432 increased from 56 % to 62 % with a decrease in reproduction, and reduced to 36 %  
433 when reproduction was increased (see Table 6). Strong declines in population size  
434 were forecast for all scenarios. Forecasts for the NSW metapopulation did not differ  
435 significantly from forecasts for the South alone, so they were not investigated  
436 separately (see Appendix Fig. 1A).



437            — Baseline Scenario Bowen Island (South) — Low Repro (-5%) — High Repro (+5%)  
438 **Fig. 6:** Sensitivity analysis - forecast of population size N for southern NSW over 50  
439 years with high and low reproductive rates, varied by  $\pm 5\%$  from the baseline; error  
440 bars represent standard error of 100 replicate scenarios

441

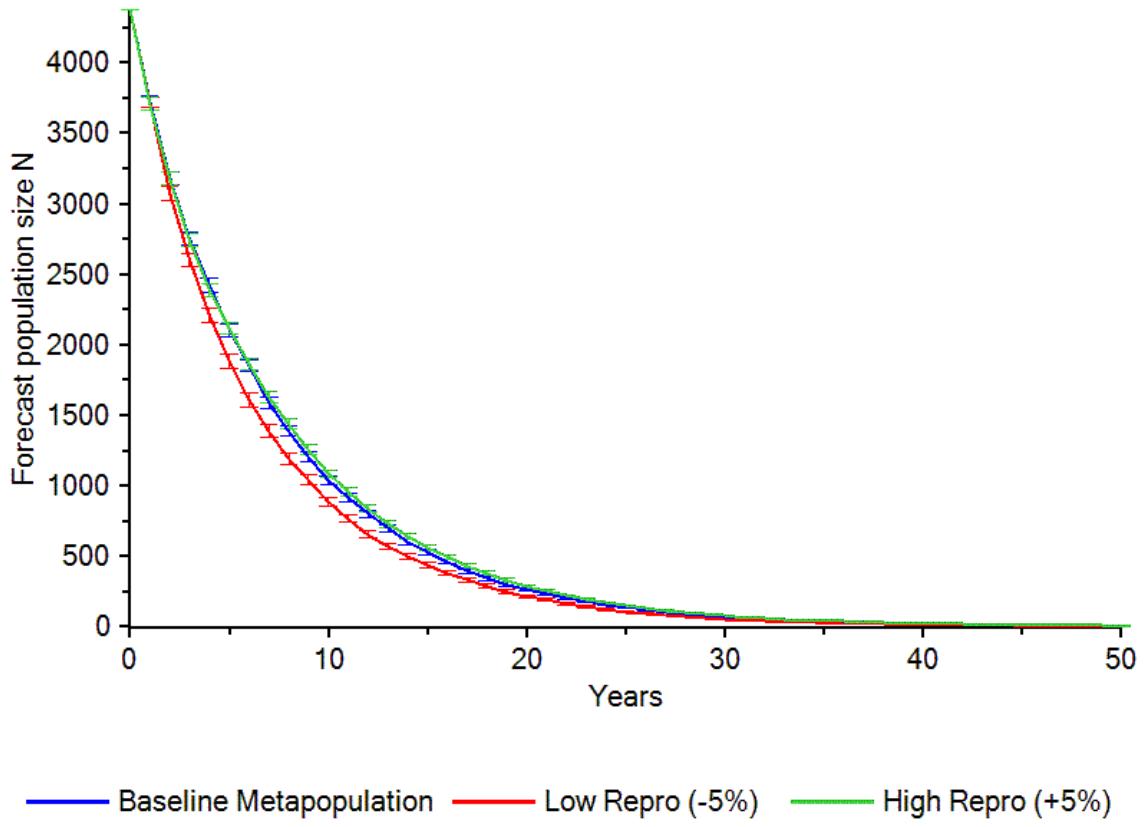
442 **Table 6:** Probability of extinction  $\pm$  standard error for NSW under different  
443 reproductive scenarios; Metapop – NSW Metapopulation

NSW	Lion Is (North)	Bowen Is (South)	Metapop
Baseline	1	$0.56 \pm 0.05$	$0.56 \pm 0.05$
High Repro (+5%)	$0.95 \pm 0.02$	$0.36 \pm 0.05$	$0.36 \pm 0.05$
Low Repro (-5%)	1	$0.62 \pm 0.05$	$0.62 \pm 0.05$

444

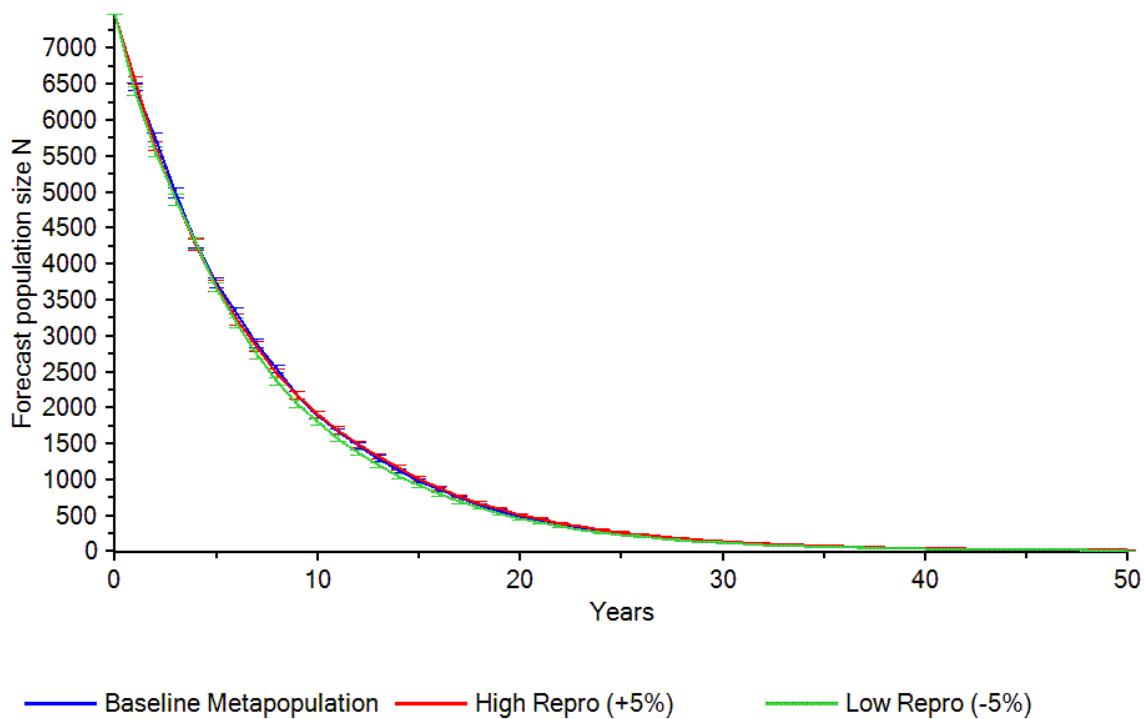
445 For WA stratification 1, when two populations were modelled, non-significant  
446 differences in population size of the WA metapopulation were modelled for 5 %  
447 increased number of broods per year (Fig. 7). Decreasing broods per year, however,  
448 resulted in significantly lower population sizes compared to the baseline, and  
449 probabilities of extinction were also significantly affected by changes in reproduction

450 (Table 5). These trends were similar for both populations within WA (see Appendix Fig.  
451 2A).



453 **Fig. 7:** Sensitivity analysis - forecast of population size N for WA metapopulation in  
 454 stratification 1 (2 populations) over 50 years with high and low reproductive rates;  
 455 error bars represent standard error of replicate scenarios

456  
457 When WA was split in three populations, forecasts showed that a 5 % change in  
458 reproduction did not affect population sizes significantly (Fig. 8). The probabilities of  
459 extinction were not significantly affected by changes in reproduction for Esperance  
460 and the WA metapopulation, but had a significant effect around Perth and Albany  
461 (Table 5). Forecasts for the three separate populations in WA showed very similar  
462 trends to the metapopulation (see Appendix Fig. 3A).



463

— Baseline Metapopulation — High Repro (+5%) — Low Repro (-5%)

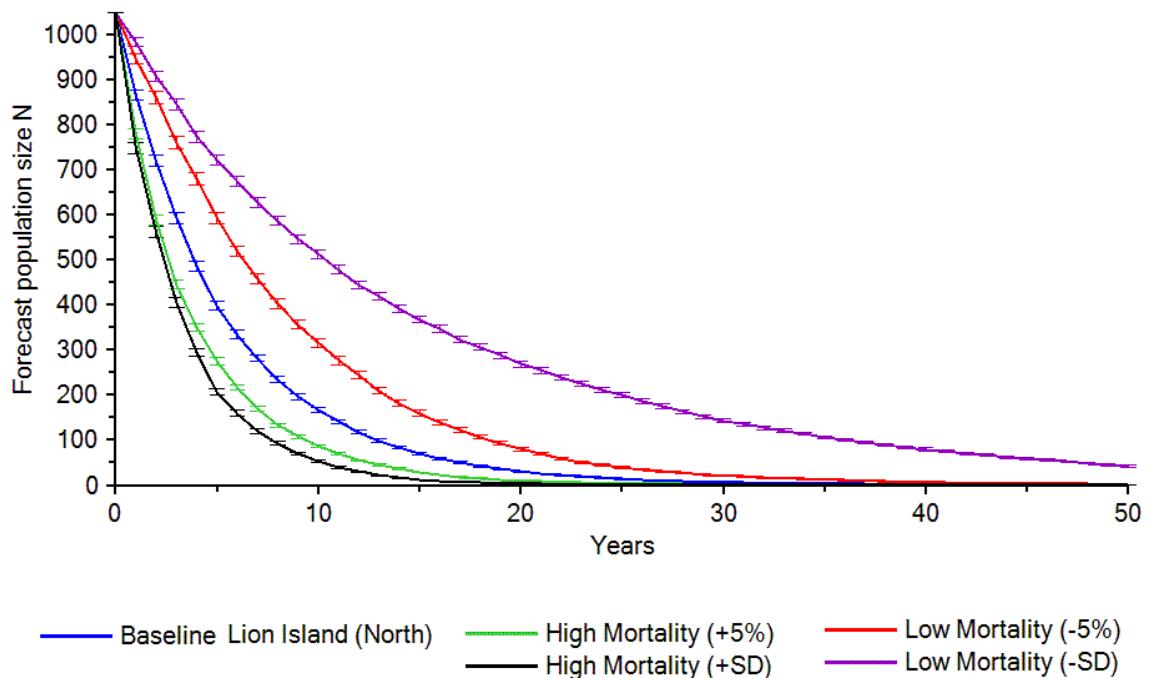
464

Fig. 8: Sensitivity analysis - forecast of population size N for WA metapopulation in stratification 2 (3 populations) over 50 years with high and low reproductive rates; error bars represent standard error of 100 replicate scenarios

467

#### 468 Mortality rates

469 Variation of mortality rates by  $\pm 5\%$  and  $\pm SD$  affected population trends for the  
470 northern population in NSW significantly, with a marked decline of population size  
471 forecast for all scenarios (Fig. 9).

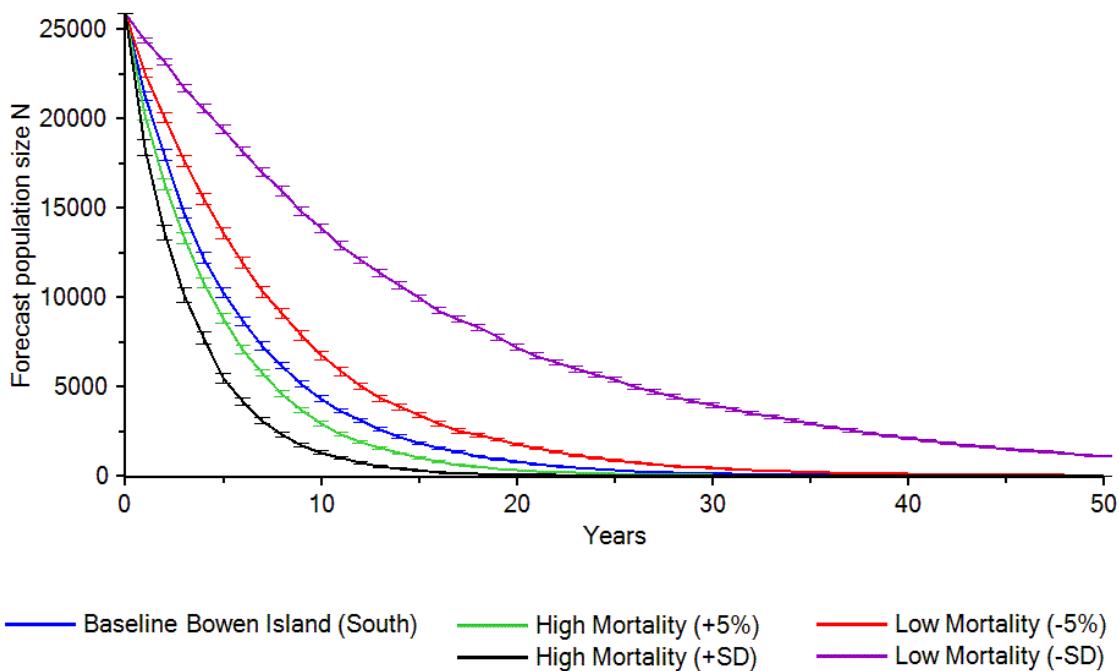


472

473 **Fig. 9:** Sensitivity analysis - forecast of population size N for the northern NSW  
474 population over 50 years with high and low mortality rates, varied by  $\pm 5\%$  and  $\pm SD$ ;  
475 error bars represent standard error of 100 replicate scenarios

476

477 The southern population in NSW behaved similarly to the north, with variation of  
478 mortality rates by  $\pm 5\%$  or  $\pm SD$  affecting population trends significantly and a marked  
479 drop in population size, approaching zero, forecast for all except the lowest mortality  
480 scenario (Fig. 10). In the latter scenario, the decline of population size was significantly  
481 slower. Again, forecasts for the NSW metapopulation did not differ significantly from  
482 forecasts for the South alone, so they were not investigated separately (see Appendix  
483 Fig. 4A).



484

485 **Fig. 10:** Sensitivity analysis - forecast of population size N for the southern NSW  
486 population over 50 years with high and low mortality rates, varied by  $\pm 5\%$  and  $\pm SD$ ;  
487 error bars represent standard error of 100 replicate scenarios

488

489 Probabilities of extinction of NSW populations were also affected significantly by  
490 variation of mortality rates (Table 7). An increase in mortality by 5 % resulted in  
491 significantly higher probabilities of extinction in all populations, while reduced  
492 mortality affected PE in the opposite way, leading to almost certain survival for the  
493 South and the NSW metapopulation, but a still high PE for the north. Increasing  
494 mortality rates by their standard deviation resulted in certain extinction for both  
495 populations and the NSW metapopulation. However, with mortality 1 SD lower than  
496 the baseline, none of the iterations went extinct (Table 7).

497

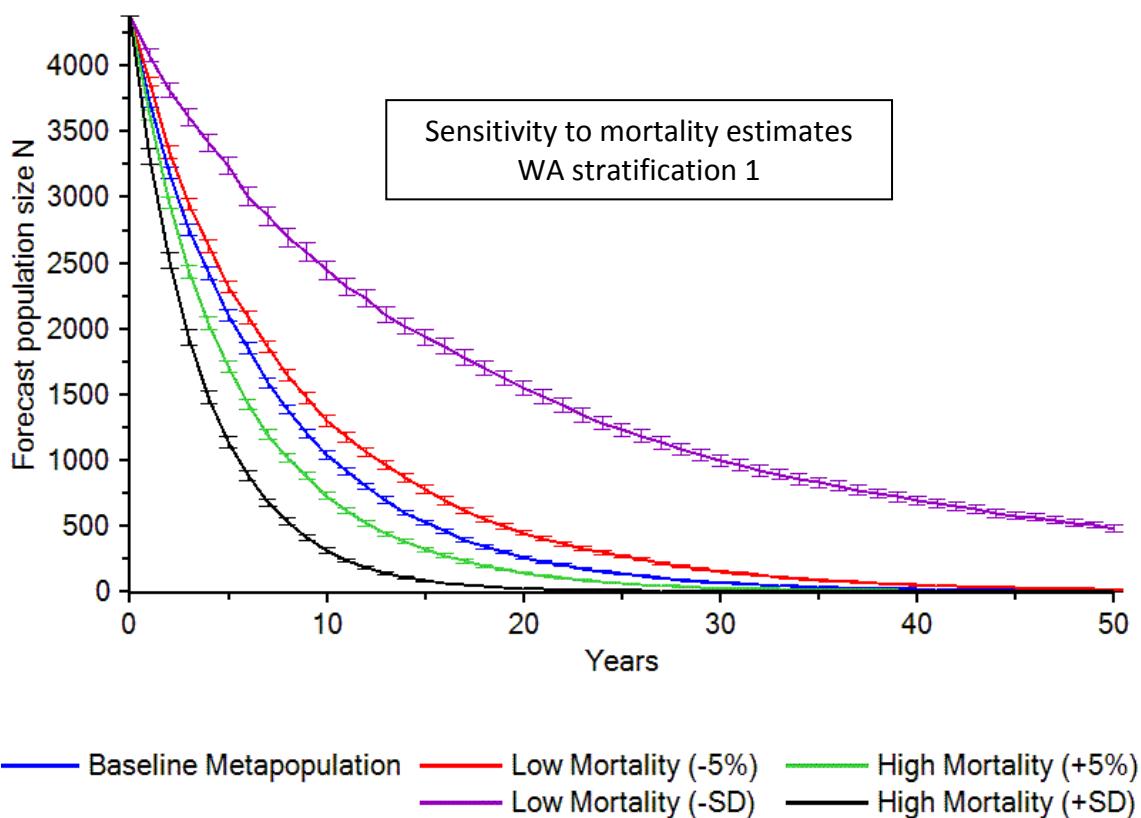
498 **Table 7:** Probabilities of extinction for NSW populations under different mortality  
499 scenarios

NSW Scenarios	Lion Island (North)	Bowen Island (South)	NSW Metapopulation
Baseline Scenario	1	0.56 ± 0.05	0.56 ± 0.05
Low Mortality (-5%)	0.82 ± 0.04	0.06 ± 0.02	0.06 ± 0.02
High Mortality (+5%)	1	0.91 ± 0.03	0.91 ± 0.03
Low Mortality (-SD)	0	0	0
High Mortality (+SD)	1	1	1

500

501 Changes of mortality rates by ± 5 % and ± SD affected population trends for the WA  
502 metapopulation under stratification 1 (two populations) significantly, with a decline of  
503 population size forecast for all scenarios (Fig. 11). All mortality scenarios showed an  
504 initial steep decline in population and slowly approached extinction, but the scenario  
505 with mortality reduced by its standard deviation declined significantly more slowly.  
506 Population trends were similar for the two populations within WA and are therefore  
507 not shown separately (see Appendix Fig. 5A). Probabilities of extinction were  
508 significantly reduced by reduced mortality, and increases in mortality lead to increased  
509 probabilities of extinction (Table 8). Especially the highest mortality scenario (+ SD)  
510 significantly increased extinction probabilities to certain extinction within 50 years for  
511 the separate populations within WA and the WA metapopulation.

512



513

514 **Fig. 11:** Sensitivity analysis - forecast of population size N for the WA metapopulation  
 515 under stratification 1 (two populations) over 50 years with high and low mortality  
 516 rates, varied by  $\pm 5\%$  and  $\pm SD$ ; error bars represent standard error of 100 replicate  
 517 scenarios

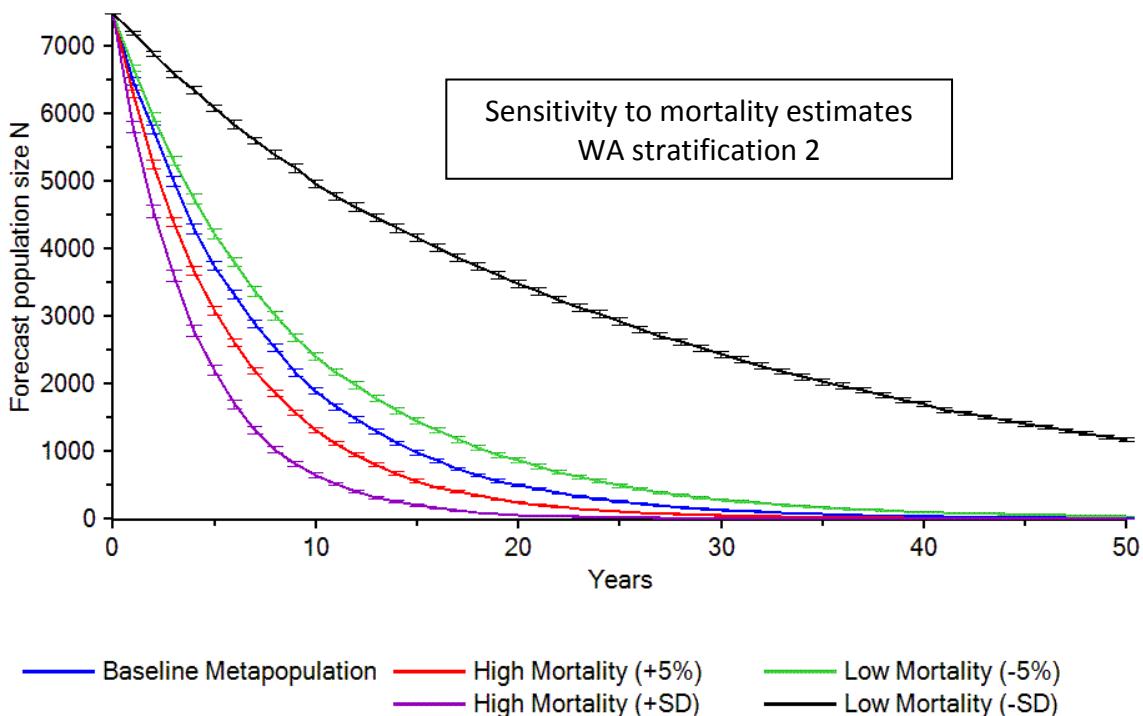
518

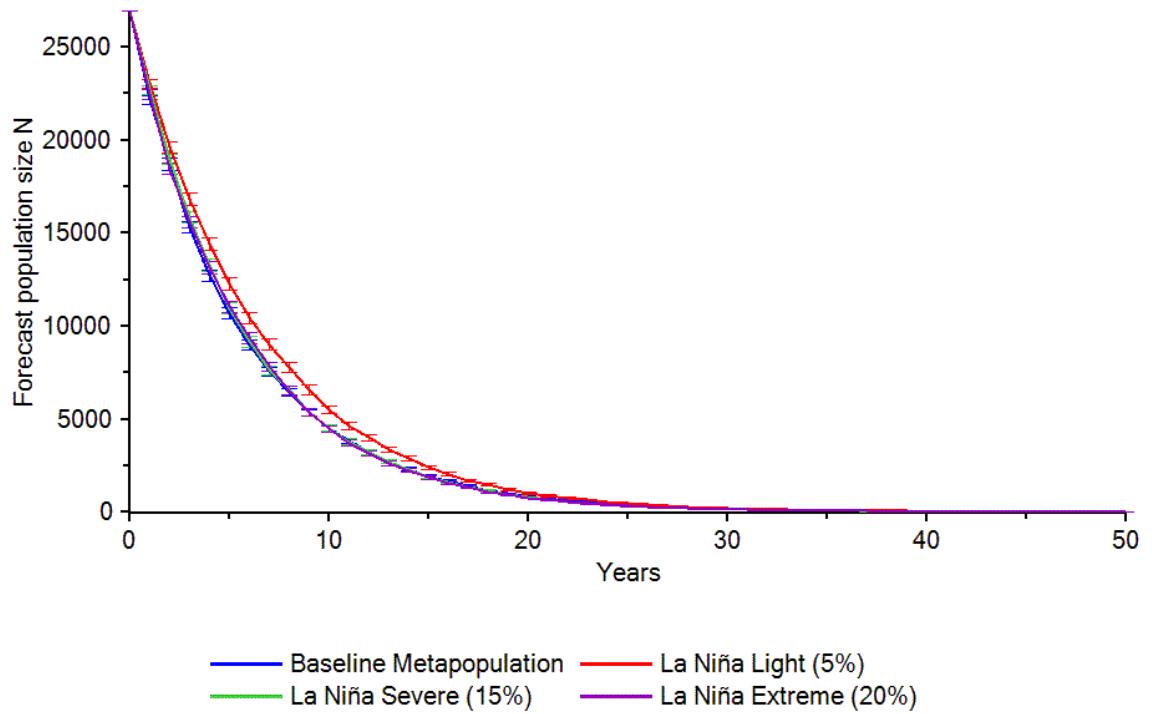
519 **Table 8:** Probabilities of extinction  $\pm$  standard error for WA stratifications 1 and 2, with  
 520 two and three populations, respectively, under different mortality scenarios; Metapop  
 521 – WA metapopulation

WA Stratification 1	North (Perth)	South	Metapop	
Baseline	$0.78 \pm 0.04$	$0.70 \pm 0.05$	$0.50 \pm 0.05$	
Low Mortality (-5%)	$0.33 \pm 0.05$	$0.30 \pm 0.05$	$0.09 \pm 0.03$	
High Mortality (+5%)	$0.96 \pm 0.02$	$0.94 \pm 0.02$	$0.91 \pm 0.03$	
Low Mortality (-SD)	$0.14 \pm 0.03$	$0.07 \pm 0.03$	$0.05 \pm 0.02$	
High Mortality (+SD)	1	1	1	
WA Stratification 2	Perth	Albany	Esperance	
Baseline	$0.74 \pm 0.04$	$0.33 \pm 0.05$	$0.90 \pm 0.03$	$0.19 \pm 0.04$
Low Mortality (-5%)	$0.38 \pm 0.05$	$0.04 \pm 0.02$	$0.52 \pm 0.05$	$0.01 \pm 0.01$
High Mortality (+5%)	1	$0.89 \pm 0.03$	$0.97 \pm 0.02$	$0.86 \pm 0.03$
Low Mortality (-SD)	$0.13 \pm 0.03$	0	0	0
High Mortality (+SD)	1	1	1	1

234

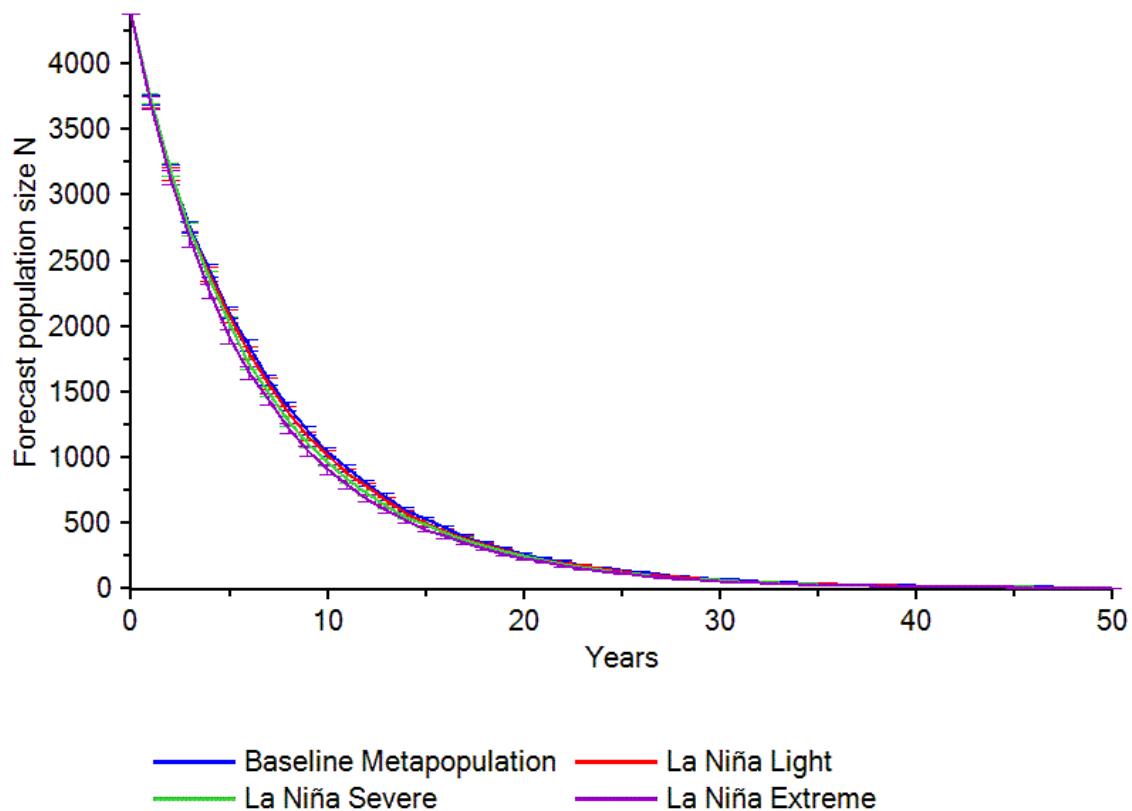
522 Under stratification 2, which assumed three populations in WA, changes of mortality  
523 rates by  $\pm 5\%$  and  $\pm SD$  affected population trends for the metapopulation  
524 significantly, with a decline of population size forecast for all scenarios (Fig. 12). The  
525 lowest mortality scenario (baseline less SD) showed a much more slowly declining  
526 trend of the population size, but continued to decline until the end of the forecasting  
527 period. The three separate populations in WA showed similar population trends to the  
528 WA metapopulation. Individual populations were therefore not shown separately (see  
529 Appendix Fig. 6A). Probabilities of extinction were reduced to zero by reducing the  
530 mortality for the two southern populations, and to significantly smaller values for the  
531 northern population around Perth (Table 8). Increases in mortality by 5 % lead to  
532 slightly increased probabilities of extinction (not significant for Perth, but significantly  
533 higher for Esperance), although Albany and the WA metapopulation were not forecast  
534 to go extinct. The highest mortality scenario (+SD) resulted in a high PE of 78 % for the  
535 WA metapopulation and up to 96 % for the penguin population around Esperance.





547

548 **Fig. 13:** Sensitivity analysis - forecast of population size N for the NSW metapopulation  
549 over 50 years with different strength of La Niña effects on survival and reproductive  
550 rates; Light – 97.5 %, Baseline – 95 %, Severe – 92.5 %, Extreme – 90 % of baseline  
551 values of reproduction and survival in La Niña years, error bars represent standard  
552 error of 100 replicate scenarios



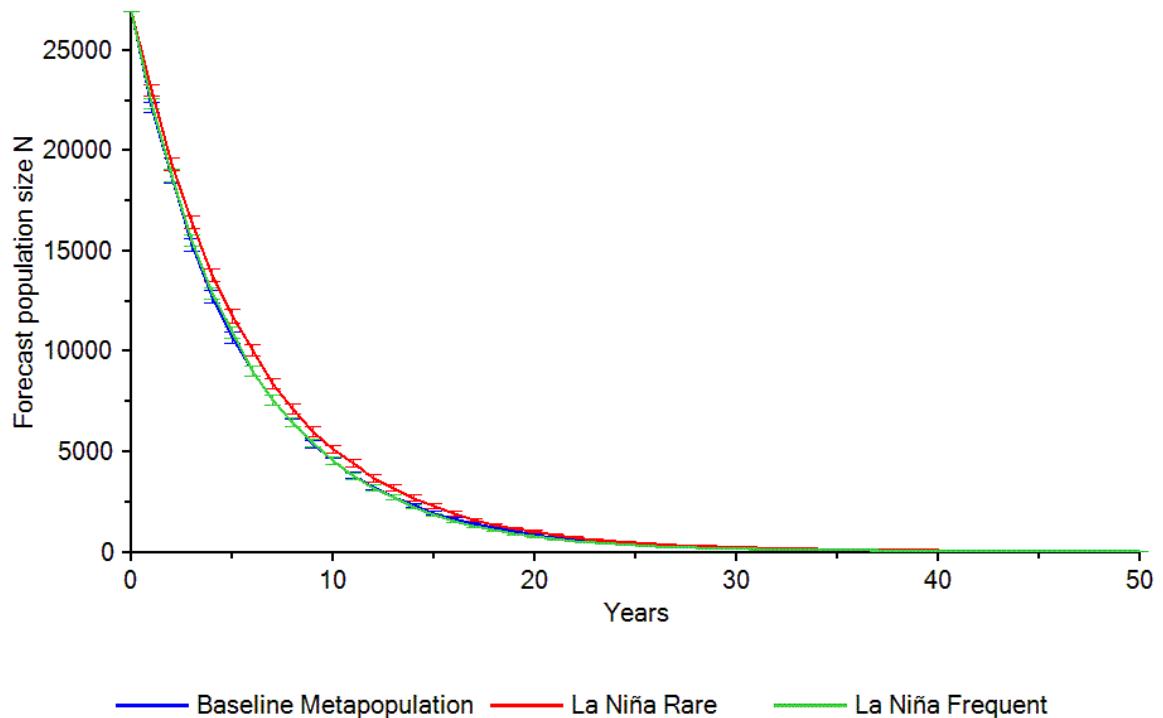
553  
554 **Fig. 14:** Sensitivity analysis - forecast of population size N for the WA metapopulation  
555 under stratification 1 over 50 years with different strengths of La Niña effects on  
556 survival and reproductive rates; error bars represent standard error of 100 replicate  
557 scenarios

558

559 **Effect of La Niña frequency on little penguin population viability**

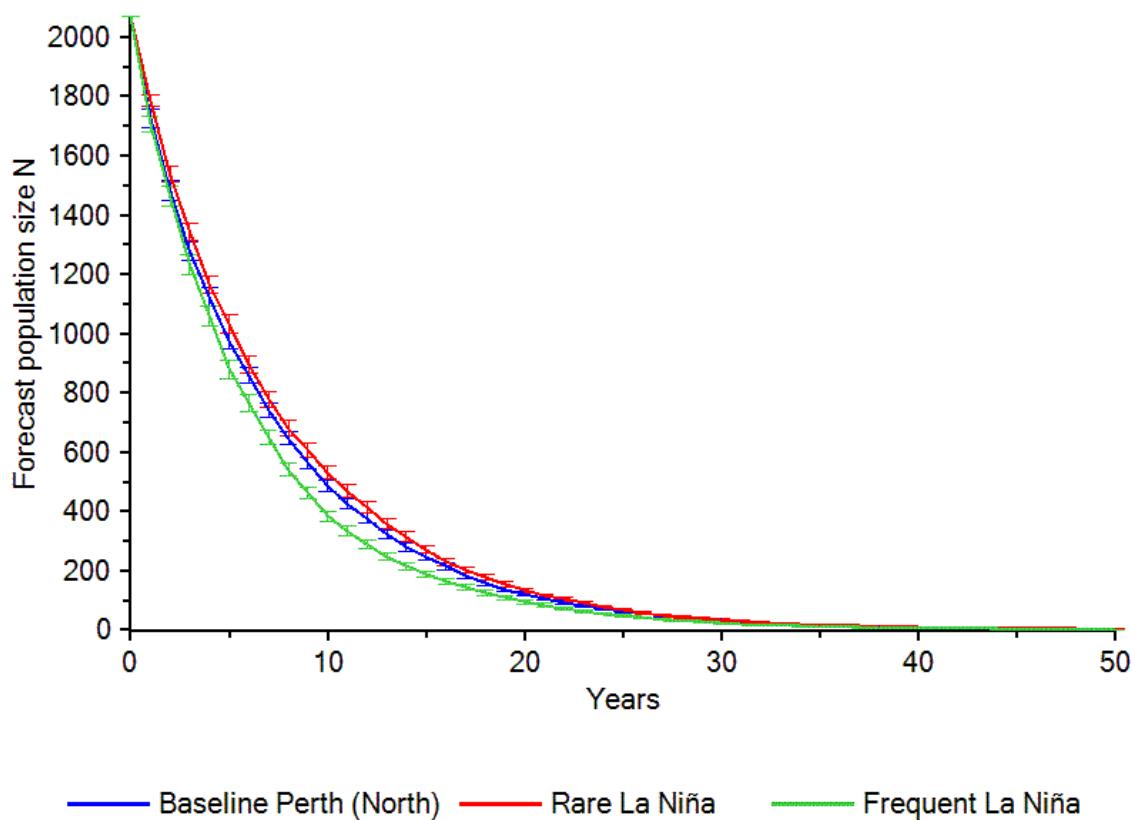
560 The frequency of La Niña catastrophes did not affect metapopulation trends in NSW or  
561 WA significantly (Fig. 15 and Fig. 16). None of the individual population forecasts  
562 within the states were significantly different under different La Niña frequency  
563 scenarios (see Appendix Fig. 7A - Fig. 9A), but probabilities of extinction were affected  
564 by variation in La Niña frequency and severity (Table 9). The effect of La Niña  
565 frequency was strongest on the northern population in WA under stratification 1,  
566 where the probability of extinction decreased by 7 % for the rare La Niña scenario and

567 increased by 4 % for frequent La Niña events, so that the rare and frequent La Niña  
568 scenarios are significantly different regarding their probability of extinction.



569  
570 **Fig. 15:** Forecast of population size N for the NSW metapopulation over 50 years with  
571 different frequencies of La Niña catastrophes; Baseline – 15 La Niña events, Rare –  
572 10 La Niña events, Frequent – 20 La Niña events per century; error bars represent  
573 standard error of 100 replicate scenarios

574



575  
576 **Fig. 16:** Forecast of population size N for the WA metapopulation under stratification 1  
577 over 50 years with different frequencies of La Niña catastrophes; Baseline – 15 La Niña  
578 events, Rare – 10 La Niña events, Frequent – 20 La Niña events per century; error bars  
579 represent standard error of replicate scenarios

580

581   **Table 9:** Probabilities of extinction (PE)  $\pm$  standard error for all La Niña scenarios;  
582   Metapop – metapopulation

		Baseline Scenario	La Niña Rare	La Niña Frequent	La Niña Light	La Niña Severe	La Niña Extreme
New South Wales	NSW North	1	0.99 $\pm$ 0.01	1	1	0.99 $\pm$ 0.01	1
	NSW South	0.56 $\pm$ 0.05	0.43 $\pm$ 0.05	0.60 $\pm$ 0.05	0.48 $\pm$ 0.05	0.58 $\pm$ 0.05	0.60 $\pm$ 0.05
	NSW Metapop	0.56 $\pm$ 0.05	0.43 $\pm$ 0.05	0.60 $\pm$ 0.05	0.48 $\pm$ 0.05	0.58 $\pm$ 0.05	0.60 $\pm$ 0.05
Western Australia - Stratification 1	North (Perth)	0.78 $\pm$ 0.04	0.73 $\pm$ 0.04	0.83 $\pm$ 0.04	0.84 $\pm$ 0.04	0.76 $\pm$ 0.04	0.79 $\pm$ 0.04
	South	0.70 $\pm$ 0.05	0.72 $\pm$ 0.04	0.77 $\pm$ 0.04	0.71 $\pm$ 0.05	0.82 $\pm$ 0.04	0.85 $\pm$ 0.04
	WA Meta	0.50 $\pm$ 0.05	0.54 $\pm$ 0.05	0.61 $\pm$ 0.05	0.55 $\pm$ 0.05	0.59 $\pm$ 0.05	0.65 $\pm$ 0.05
Western Australia - Stratification 2	Perth	0.74 $\pm$ 0.04	0.72 $\pm$ 0.04	0.79 $\pm$ 0.04	0.81 $\pm$ 0.04	0.69 $\pm$ 0.05	0.78 $\pm$ 0.04
	Albany	0.33 $\pm$ 0.05	0.39 $\pm$ 0.05	0.46 $\pm$ 0.05	0.52 $\pm$ 0.05	0.57 $\pm$ 0.05	0.54 $\pm$ 0.05
	Esperance	0.90 $\pm$ 0.03	0.88 $\pm$ 0.03	0.86 $\pm$ 0.03	0.88 $\pm$ 0.03	0.92 $\pm$ 0.03	0.97 $\pm$ 0.02
	WA Metapop	0.19 $\pm$ 0.04	0.23 $\pm$ 0.04	0.23 $\pm$ 0.04	0.33 $\pm$ 0.05	0.28 $\pm$ 0.04	0.37 $\pm$ 0.05

583

584 **Discussion**

585 **Current status – baseline models and sensitivity to changes in mortality and  
586 reproductive rates**

587 Little penguin populations in NSW were forecast to decline, but we currently do not  
588 observe the forecast declines in the southern populations, which indicates that some  
589 of the input parameters are not known with sufficient certainty to trust predicted  
590 population sizes or estimates of extinction probability. This discussion therefore  
591 focuses on differences between scenarios rather than absolute results. The  
592 populations in NSW are significantly more sensitive to changes in mortality rates than  
593 reproductive rates when both were varied by  $\pm 5\%$ , so attempts to halt the projected  
594 population declines should focus on eliminating known causes for mortality rather  
595 than managing reproductive output. When increasing mortality rates by their standard  
596 deviations, the impact was even more severe, with a significantly increased probability  
597 of extinction forecast for all populations.

598 WA little penguin population sizes were also forecast to decline in both stratifications  
599 modelled, with a particularly high probability of extinction for the colonies around  
600 Perth, which include the Penguin Island colony of highest conservation status. WA  
601 estimates of extinction probability were similarly affected by 5 % percent changes to  
602 mortality and reproductive rates to NSW estimates, as was the declining population  
603 trend under both scenarios for the state. In WA, too, sensitivity of forecasts to changes  
604 in mortality rates was higher than to reproductive rates. The major difference between  
605 the two stratifications of the WA data set was that under stratification 2, where the  
606 WA penguin metapopulation was split in three populations, reduced reproduction had

607 a significant effect on the population trajectory of the WA metapopulation, while the  
608 same scenario did not significantly affect population sizes in stratification 1.

609 **Sensitivity to variation in La Niña severity and frequency**

610 La Niña events are climatic phenomena that vary in strength and frequency and are  
611 predicted to increase in both. Their variability makes an assessment of their overall  
612 effect on penguin populations complex, but the results of this investigation suggest  
613 their effect on the already declining populations in WA and NSW might be limited.

614 None of the population trends of the NSW models were sensitive to variation in La  
615 Niña frequency, with population size trajectories and final population sizes within 2  
616 standard errors of the baseline scenario mean (Fig. 15). An increase in severity of La  
617 Niña did not significantly affect NSW population forecasts either, but reduced La Niña  
618 severity improved outcomes significantly. It is therefore likely that current La Niña  
619 occurrences already affect little penguin populations in NSW. For WA, an increased  
620 frequency of La Niña years significantly affected the population size trajectories, while  
621 reduced frequencies or changes in severity did not have a significant effect.

622 The probability of extinction (PE) decreased with decreased frequency and severity of  
623 La Niña for the southern NSW population and NSW metapopulation (Table 9). Only  
624 strong increases in severity affected the WA population sizes, but PEs remained  
625 relatively stable for the WA populations. Overall, sensitivity to changes in La Niña  
626 severity was low. We therefore conclude that an increase of La Niña severity or  
627 frequency within the range modelled would not have a devastating impact on the  
628 penguin populations studied, but caution that there are other factors that might  
629 interact with changes to ENSO patterns and ultimately threaten the little penguin's

630 long-term survival. We also confirm that the population in northern WA, including  
631 Penguin Island, might be impacted adversely by stronger and more frequent La Niña  
632 events (Cannell et al. 2012).

633 El Niño events might have a similar impact to La Niña, but unfortunately the effect of El  
634 Niño on reproductive and survival rates of little penguins is not known. Another non-  
635 antarctic penguin species, the Galapagos penguin, however, has been shown to be  
636 very susceptible to even marginal increases in frequency of El Niño events (Vargas et  
637 al. 2007).

### 638 Recommended directions for research and management

639 Climate change in the form of altered patterns of ENSO has been indicated to  
640 adversely affect the penguins on Penguin Island (Cannell et al. 2012) and the need for  
641 proactive management of anthropogenic effects on fish prey availability to maintain  
642 the wellbeing of the colony has been highlighted. The present work confirms that this  
643 colony is more sensitive to changes in ENSO patterns than colonies in southern WA  
644 and NSW. Declines in the southern populations, however, might be attributed to  
645 different environmental factors, such as increased mortality due to depredation of  
646 chicks and adults, e.g. nest predation by goannas and adult penguin predation by long-  
647 nosed fur seals (*Arctocephalus forsteri*), as observed in South Australian penguin  
648 populations (Colombelli-Négrel 2015).

649 All populations that were investigated were forecast to decline within the next 50  
650 years, although most model input data were collected over a timescale of more than  
651 20 years and a strong decline has not yet been evident, except on Lion Island  
652 (Chapter 2). We therefore recommend further research into population-specific data  
244

653 on reproduction and mortality rates and population monitoring to ensure timely  
654 detection of population declines at the penguin's northern distribution limit. In  
655 particular, the models were sensitive to variation in reproductive rates, estimates of  
656 which were based on studies conducted on few of the colonies. These were assumed  
657 to be representative of the larger population, but this needs to be confirmed.  
658 Furthermore, the percentage of adult females breeding each year was unknown for all  
659 populations and will have to be investigated if more robust results on population  
660 viability were required for little penguins. The factor that impacted model outcomes  
661 even more than reproduction was mortality, estimates of which were similarly based  
662 on studies conducted at few colonies. Survival estimates are usually apparent survival  
663 and might be underestimates if emigration is high. For this study, dispersal estimates  
664 were based on results of Chapter 3 in this thesis, and lower than the variation  
665 modelled in the sensitivity analysis. Within NSW, significant differences in annual adult  
666 survival rates have been found between Lion Island near Sydney and Bowen Island in  
667 Jervis Bay (Chapter 2). To clarify whether these are representative of the larger  
668 population, additional studies of survival in different colonies would be helpful. Studies  
669 of captive little penguin populations might furthermore help understand parameters  
670 that are difficult to measure in wild populations while minimising impact on the  
671 penguins, such as sex ratio at birth.

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862 **Appendix 5**

863 **Table 1A:** Population size estimates and location of known NSW penguin colonies; Is - Island

<b>Colony name</b>	<b>Population estimate</b>	<b>Year of latest estimate</b>	<b>Latitude</b>	<b>Longitude</b>	<b>Sources</b>
Inner Rock	20	2012	-32.601	152.304	Carlile et al. 2013
Broughton Is	60	2012	-32.617	152.312	Lane 1976c; Carlile, Priddel & Callaghan 2012
Little Broughton Is	26	2013	-32.621	152.333	Carlile & Callaghan 2013
Cabbage Tree Is	280	2004	-32.687	152.223	Fullagar 1976; Priddel & Carlile 2004b
Boondelbah Is	200	2004	-32.707	152.228	Morris 1976; Priddel & Carlile 2004a
Moon Is	26	1974	-33.088	151.672	Gray & Gwynne 1974
Bird Is	5	1973	-33.229	151.602	Lane 1973
Lion Is	120	2013	-33.556	151.316	Lane 1975; Rogers & Eldershaw 1995; Sergent et al. 2004; Vardeh, Chapter 2
Manly, Sydney	162	2013	-33.81	151.28	Bourne & Klomp 2004, O'Neill, pers. comm.
Flinders Islet, Five Islands	30	2005	-34.456	150.93	Battam 1976b; NSW National Parks and Wildlife Service 2005
Bass Islet, Five Islands	10	2005	-34.465	150.945	Battam 1976a; NSW National Parks and Wildlife Service 2005
Big Island, Five Islands	100	2005	-34.49	150.927	Gibson 1976; NSW National Parks and Wildlife Service 2005
Martin Islet, Five Islands	10	2005	-34.494	150.938	Battam 1976c; NSW National Parks and Wildlife Service 2005
<b>NSW North - Total</b>	<b>1 049</b>				
Bowen Is	10 000	1995	-35.117	150.765	Lane 1976b; Lintermans 1989; Fortescue 1995
Brush Is	4 400	2012	-35.53	150.42	Morris 1974; Carlile, Priddel, Blackmore, Craven, et al. 2012
Belowla Is	726	2011	-35.554	150.39	Lane 1976a; Blackmore et al. 2011

Chapter 5: Viability of little penguin (*Eudyptula minor*)  
populations at their northern range edge

Grasshopper Is	110	2012	-35.632	150.333	Lane 1974; Priddel et al. 2012
Wasp Is	220	2012	-35.667	150.311	Lane 1976d; Carlile, Priddel, Blackmore, Jarman, et al. 2012
Snapper Is	48	2014	-35.725	150.213	Carlile & Priddel submitted
Tollgate Is	360	2014	-35.75	150.26	McKean & Fullagar 1976, Carlile et al. submitted
Montagu(e) Is	10 000	2003	-36.25	150.23	Fullagar 1973; Heyligers & Fullagar 1995; Weerheim et al. 2003
<b>NSW South - Total</b>	<b>25 864</b>				
<b>NSW Total (rounded)</b>	<b>27 000</b>				

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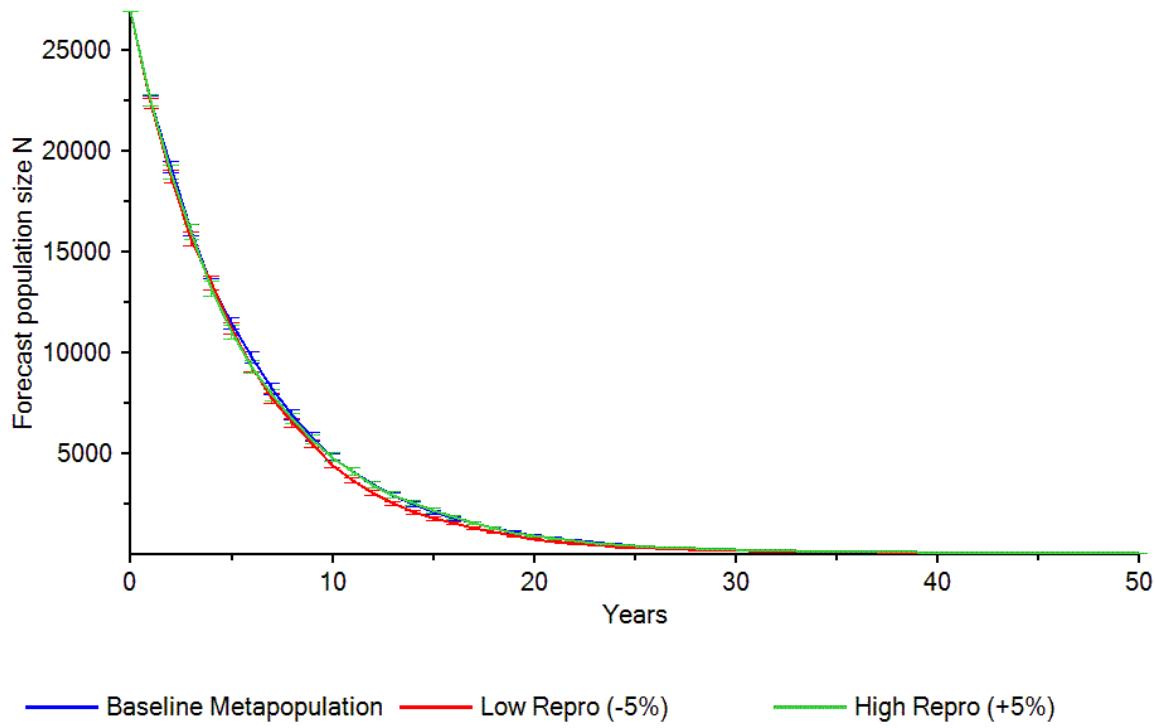
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866 **Table 2A:** Population size estimates and location of known WA penguin colonies; Is - Island

Colony name	Population estimate	Year of latest estimate	Latitude	Longitude	Sources
Bald Is	100	1996	-34.918	118.462	Dann et al. 1996, Cannell et al. 2001
Bellinger Is	40	1996	-33.886	123.644	Dann et al. 1996, Cannell et al. 2001
Ben Is	100	1996	-33.899	122.754	Dann et al. 1996, Cannell et al. 2001
Boxer Is	100	1996	-34.000	121.678	Dann et al. 1996, Cannell et al. 2001
Breaksea Is	500	2001	-35.064	118.056	Dann et al. 1996, Cannell et al. 2001
Carnac Is	200	1996	-32.121	115.662	Dann et al. 1996, Cannell et al. 2001
Charley Is	10	1996	-33.922	121.876	Dann et al. 1996, Cannell et al. 2001
Cheyne Is	150	2001	-34.593	118.767	Dann et al. 1996, Cannell et al. 2001
Coffin Is	10	1996	-35.000	118.214	Dann et al. 1996, Cannell et al. 2001
Cull Is	100	1996	-33.922	121.903	Dann et al. 1996, Cannell et al. 2001
Daw Is	100	1996	-33.847	124.139	Dann et al. 1996, Cannell et al. 2001
Doubtful Islands	100	1996	-34.375	119.578	Dann et al. 1996, Cannell et al. 2001
Eclipse Is	100	1996	-35.183	117.886	Dann et al. 1996, Cannell et al. 2001
Figure of Eight Is	10	1996	-34.032	121.605	Dann et al. 1996, Cannell et al. 2001
Forrest Is	10	1996	-33.917	122.710	Dann et al. 1996, Cannell et al. 2001
Garden Is	25	1997	-32.210	115.679	Cannell et al. 2001
Goose Is	100	1996	-34.081	123.183	Dann et al. 1996, Cannell et al. 2001
Hood Is	70	1996	-34.141	122.049	Dann et al. 1996, Cannell et al. 2001
Inshore Is	10	1986	-33.916	122.829	Cannell et al. 2001
Kermadec Is	100	1996	-34.088	122.833	Dann et al. 1996, Cannell et al. 2001
Lorraine Is	10	1996	-33.949	122.564	Dann et al. 1996, Cannell et al. 2001
Mackenzie Is	10	1996	-34.198	122.106	Dann et al. 1996, Cannell et al. 2001
Marts Island	100	1996	-34.000	122.633	Dann et al. 1996, Cannell et al. 2001
Michaelmas Is	10	1996	-35.044	118.040	Dann et al. 1996, Cannell et al. 2001

Chapter 5: Viability of little penguin (*Eudyptula minor*)  
populations at their northern range edge

Migo Is	30	2001	-35.072	117.650	Cannell et al. 2001
Mistaken Is	100	1996	-35.063	117.944	Dann et al. 1996, Cannell et al. 2001
Mondrain Is	200	1996	-34.138	122.245	Dann et al. 1996, Cannell et al. 2001
Nares Is	100	1996	-33.932	122.595	Dann et al. 1996, Cannell et al. 2001
North Twin Peak Is	100	1996	-33.989	122.843	Dann et al. 1996, Cannell et al. 2001
Observatory Is	100	1996	-33.922	121.794	Dann et al. 1996, Cannell et al. 2001
Penguin Is	1000	1996	-32.305	115.691	Dann et al. 1996, Cannell et al. 2001
Ram Is	10	1996	-34.031	122.143	Dann et al. 1996, Cannell et al. 2001
Remark Is	10	1996	-34.064	121.986	Dann et al. 1996, Cannell et al. 2001
Richards Is	10	2001	-35.076	117.651	Dann et al. 1996, Cannell et al. 2001
Rob Is	100	1996	-34.033	122.234	Dann et al. 1996, Cannell et al. 2001
Round (Recherche Is)	100	1996	-34.103	123.888	Dann et al. 1996, Cannell et al. 2001
Salisbury Is	100	1996	-34.358	123.554	Dann et al. 1996, Cannell et al. 2001
Sandy Hook Is	100	1996	-34.034	121.995	Dann et al. 1996, Cannell et al. 2001
Seal Is (King George Sound)	10	1996	-35.076	117.975	Dann et al. 1996
Seal Is (Shoalwater bay)	100	1996	-32.293	115.690	Dann et al. 1996
Shag Is	100	1996	-32.296	115.691	Dann et al. 1996
Shelter (Muttonbird) Is	300	2001	-35.051	117.694	Cannell et al. 2001
Six Mile Is	40	1996	-33.639	123.967	Dann et al. 1996, Cannell et al. 2001
Skink Is	35	1987	-33.987	123.148	Cannell et al. 2001
St Alouarn Is	100	1996	-34.403	115.196	Dann et al. 1996, Cannell et al. 2001
Station Is	100	1996	-33.960	122.523	Dann et al. 1996, Cannell et al. 2001
Termination Is	100	1996	-34.471	121.992	Dann et al. 1996
Westall (Combe) Is	100	1996	-34.079	122.966	Dann et al. 1996
Wickham (Stanley) Is	100	1996	-34.020	123.291	Dann et al. 1996, Cannell et al. 2001
Woody Is	10	2000	-33.963	122.012	Dann et al. 1996, Cannell et al. 2001
<b>WA Total (rounded)</b>	<b>5 200</b>				



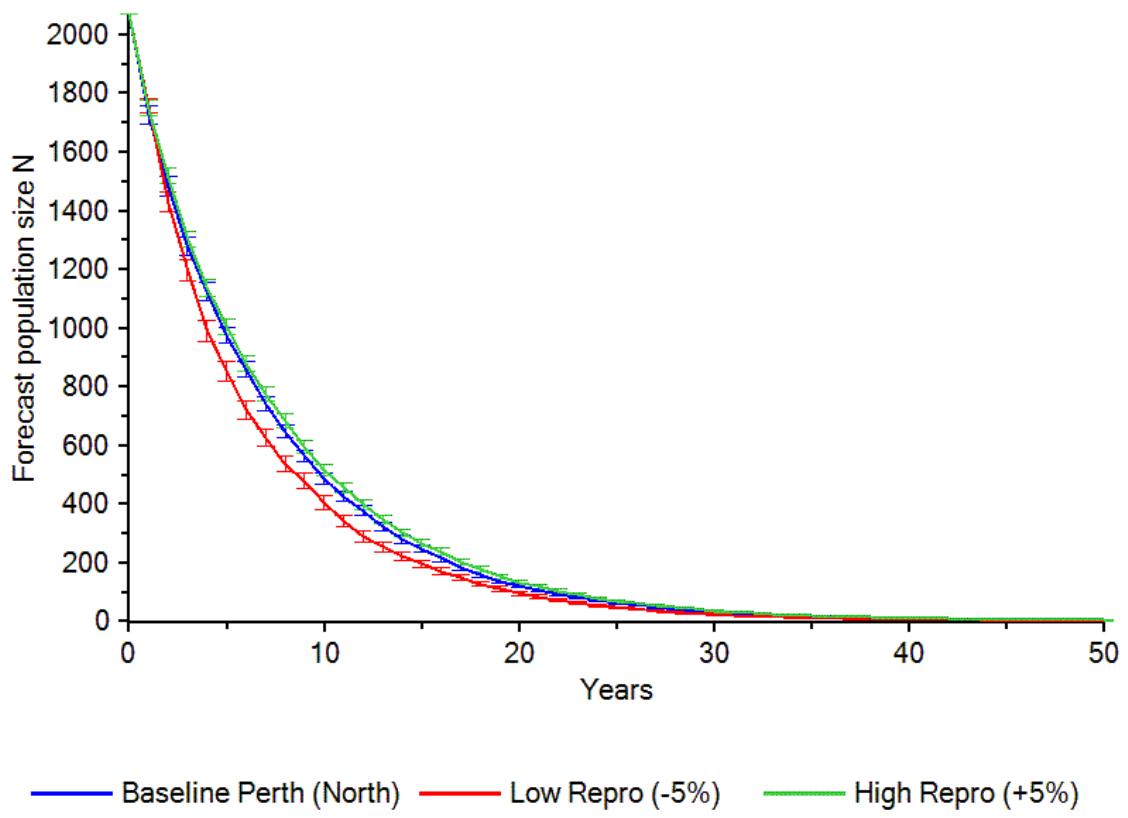
867      ————— Baseline Metapopulation    ——— Low Repro (-5%)                ——— High Repro (+5%)

868      **Fig. 1A:** Sensitivity analysis - forecast of population size N for NSW metapopulation  
869      over 50 years with high and low reproductive rates; error bars represent standard  
870      error of 100 replicate scenarios

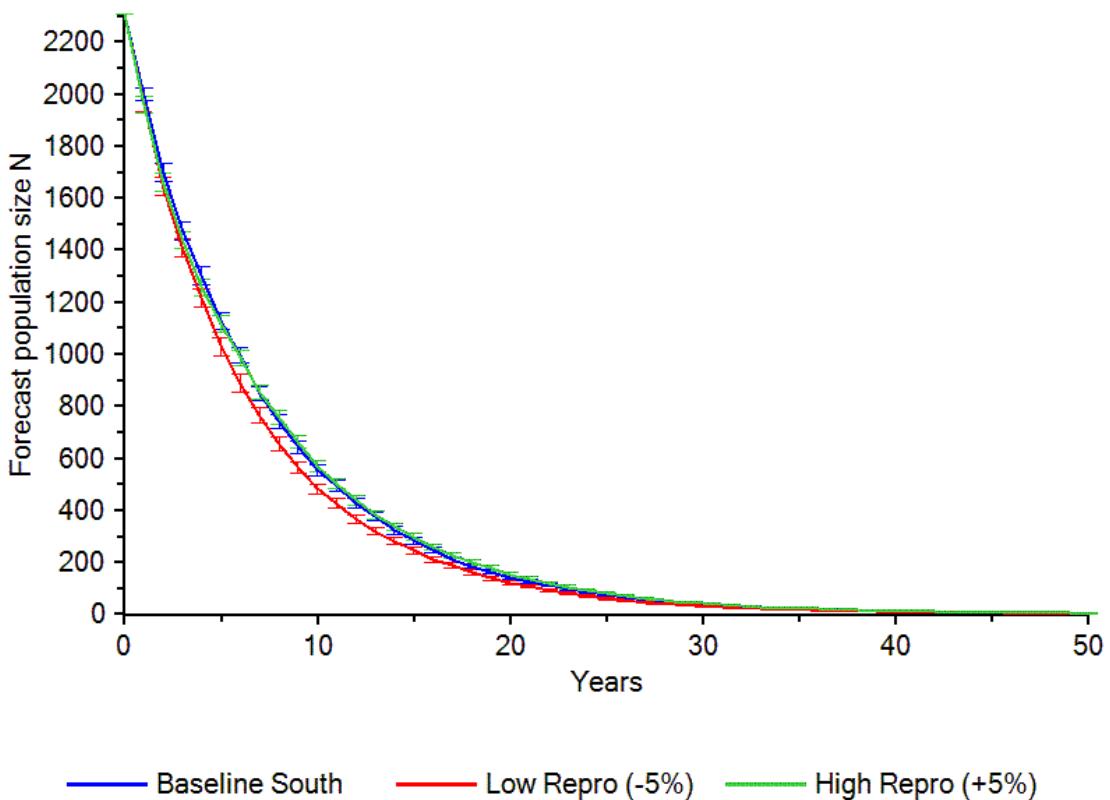
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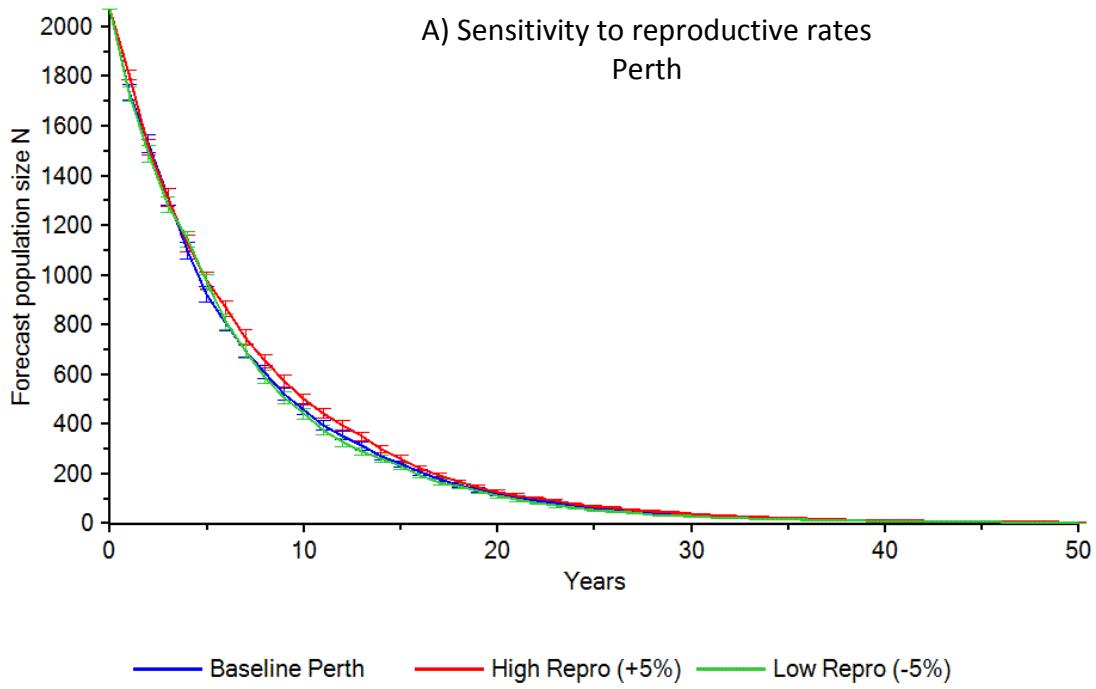
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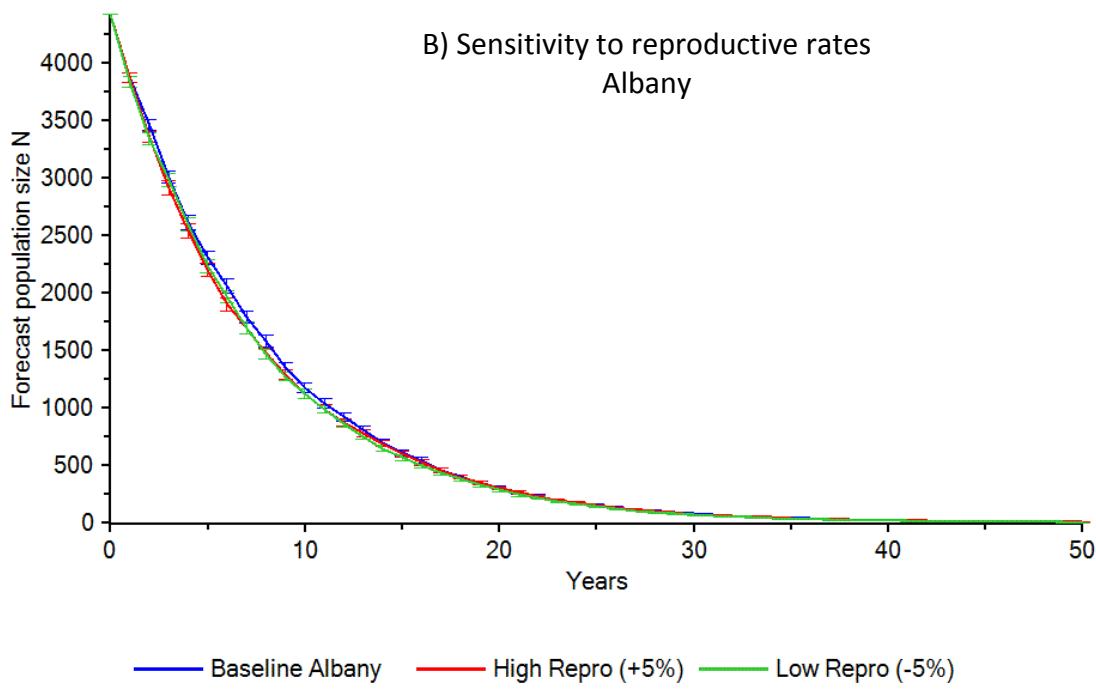
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875 **Fig. 2A:** Sensitivity analysis - forecast of population size N for WA stratification 1 with  
876 two populations (North and South) over 50 years with high and low reproductive rates;  
877 error bars represent standard error of replicate scenarios

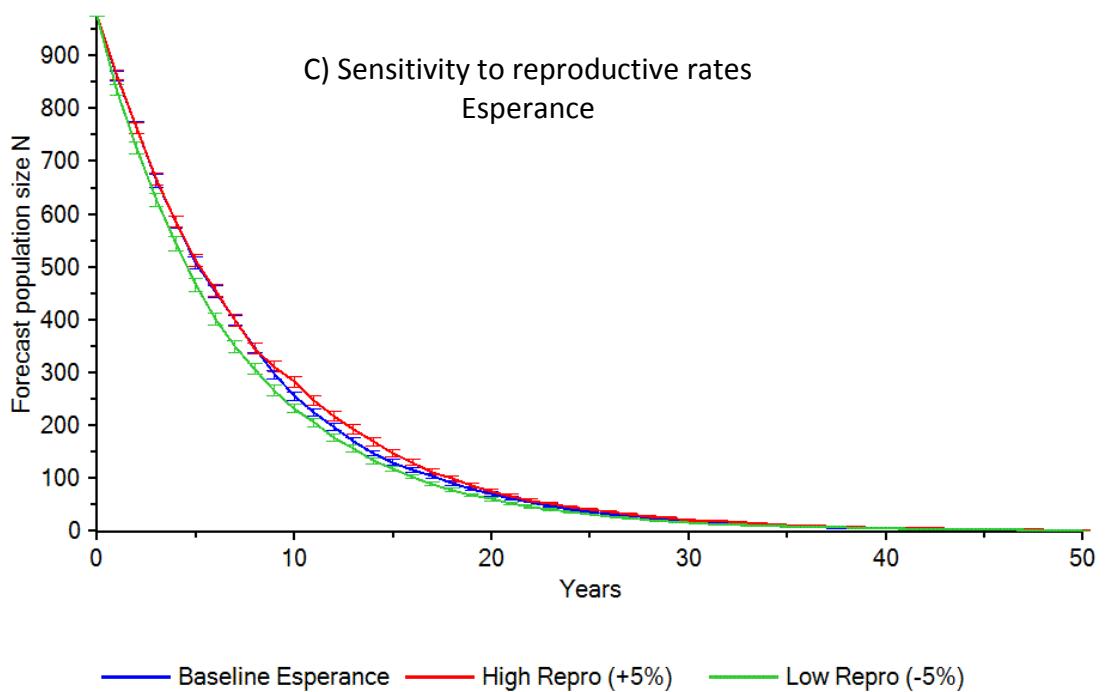


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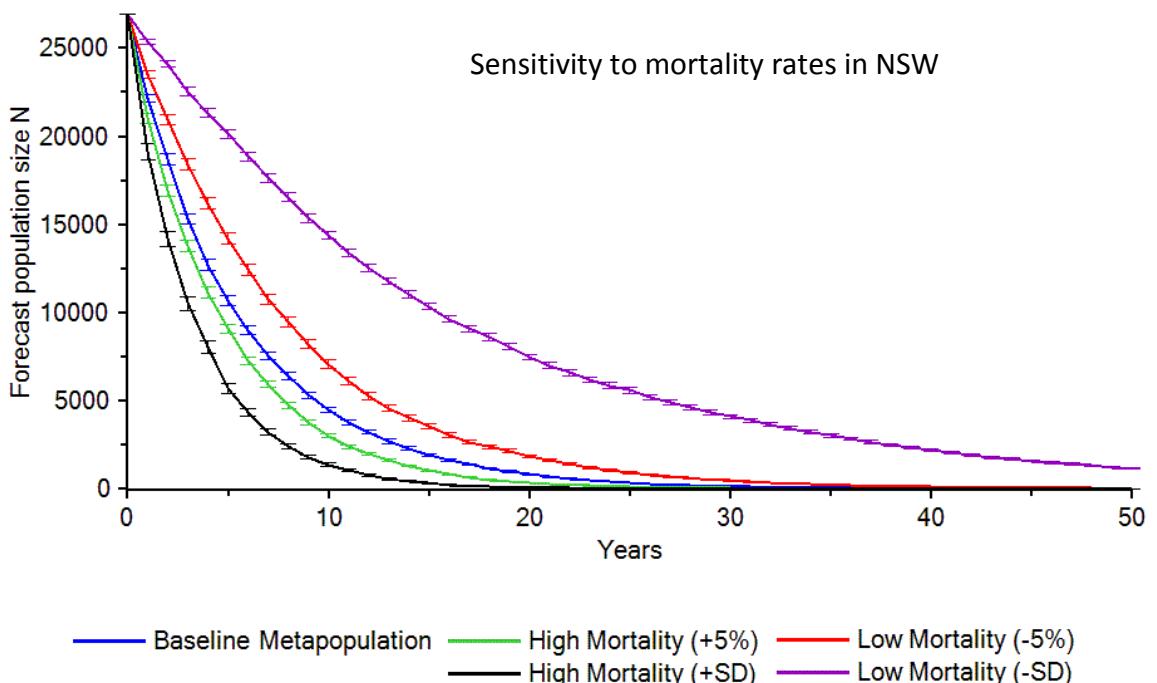
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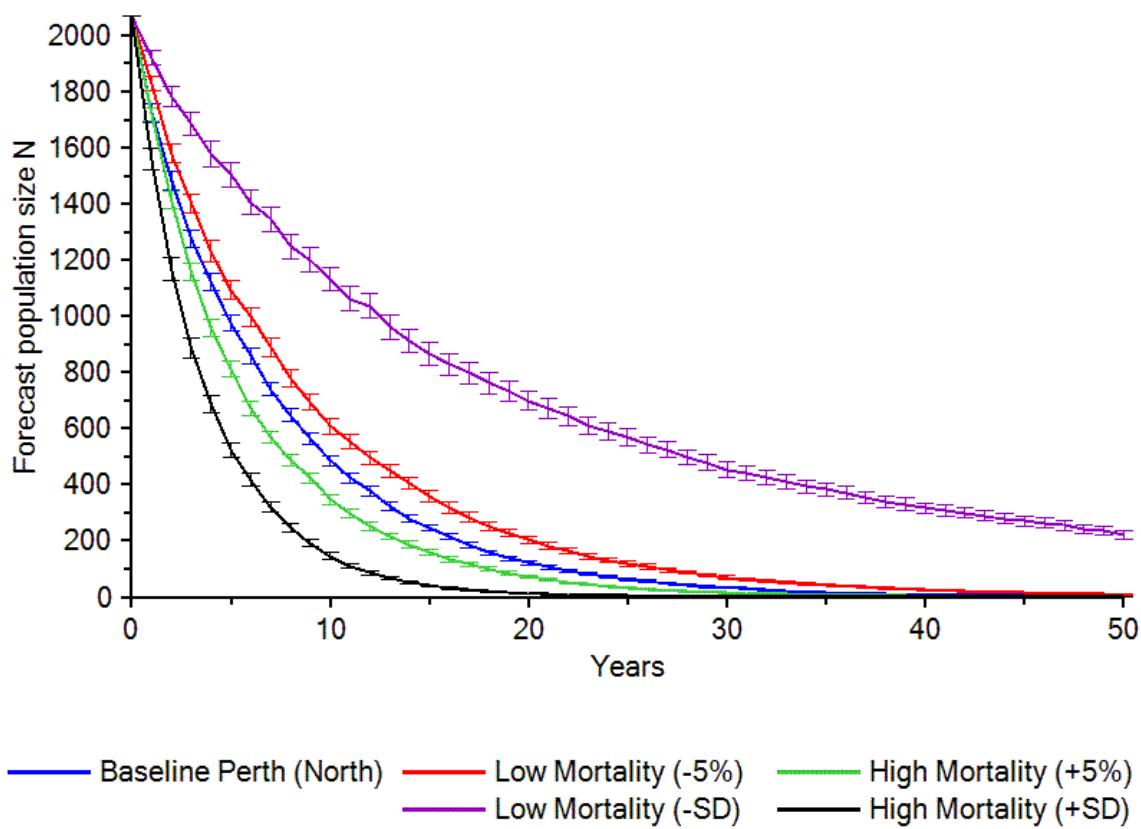
881 **Fig. 3A:** Sensitivity analysis - forecast of population size N for WA stratification 2 with  
882 three populations (A) Perth, B) Albany and C) Esperance) over 50 years with high and  
883 low reproductive rates; error bars represent standard error of 100 replicate scenarios

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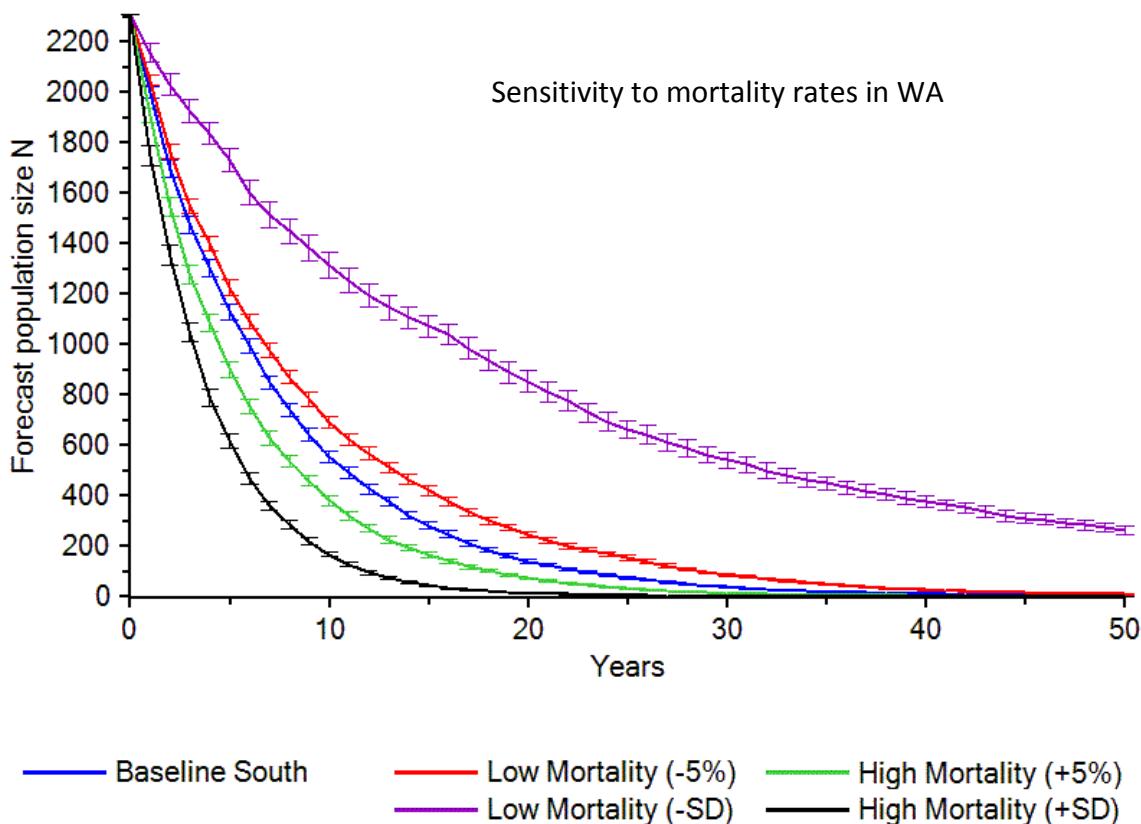


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886 **Fig. 4A:** Sensitivity analysis - forecast of population size N for NSW metapopulation  
887 over 50 years with high and low mortality rates, varied by  $\pm 5\%$  and  $\pm SD$ ; error bars  
888 represent standard error of 100 replicate scenarios



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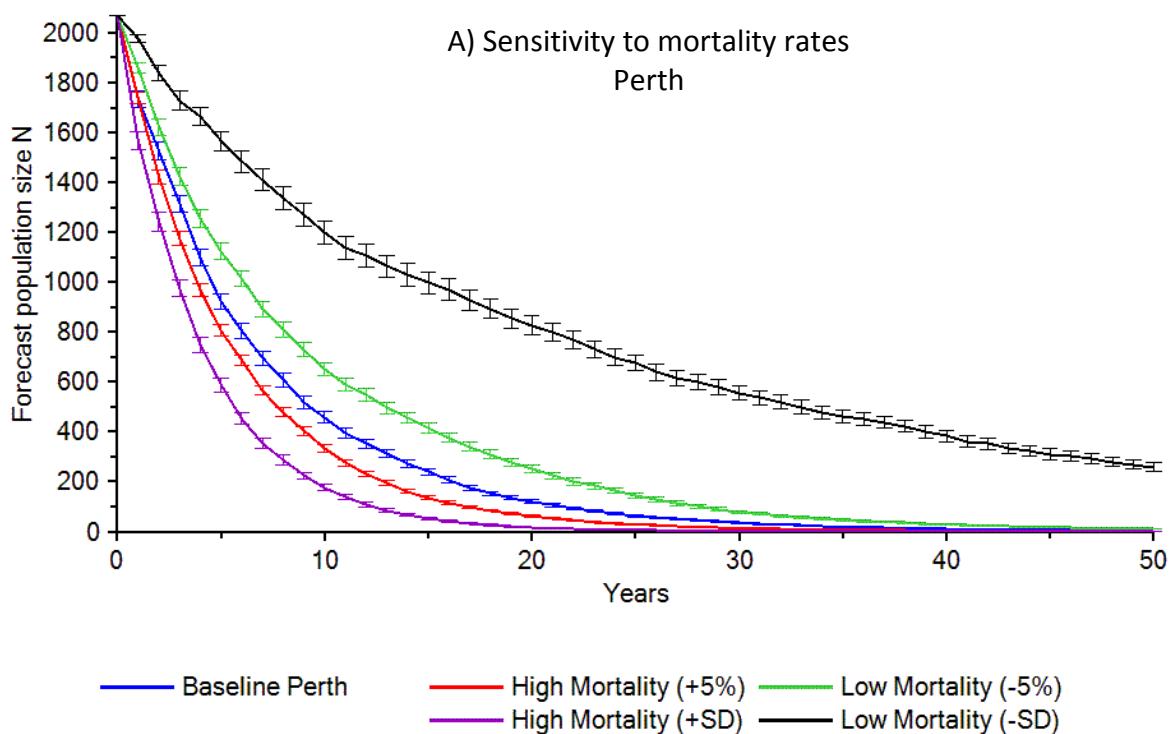


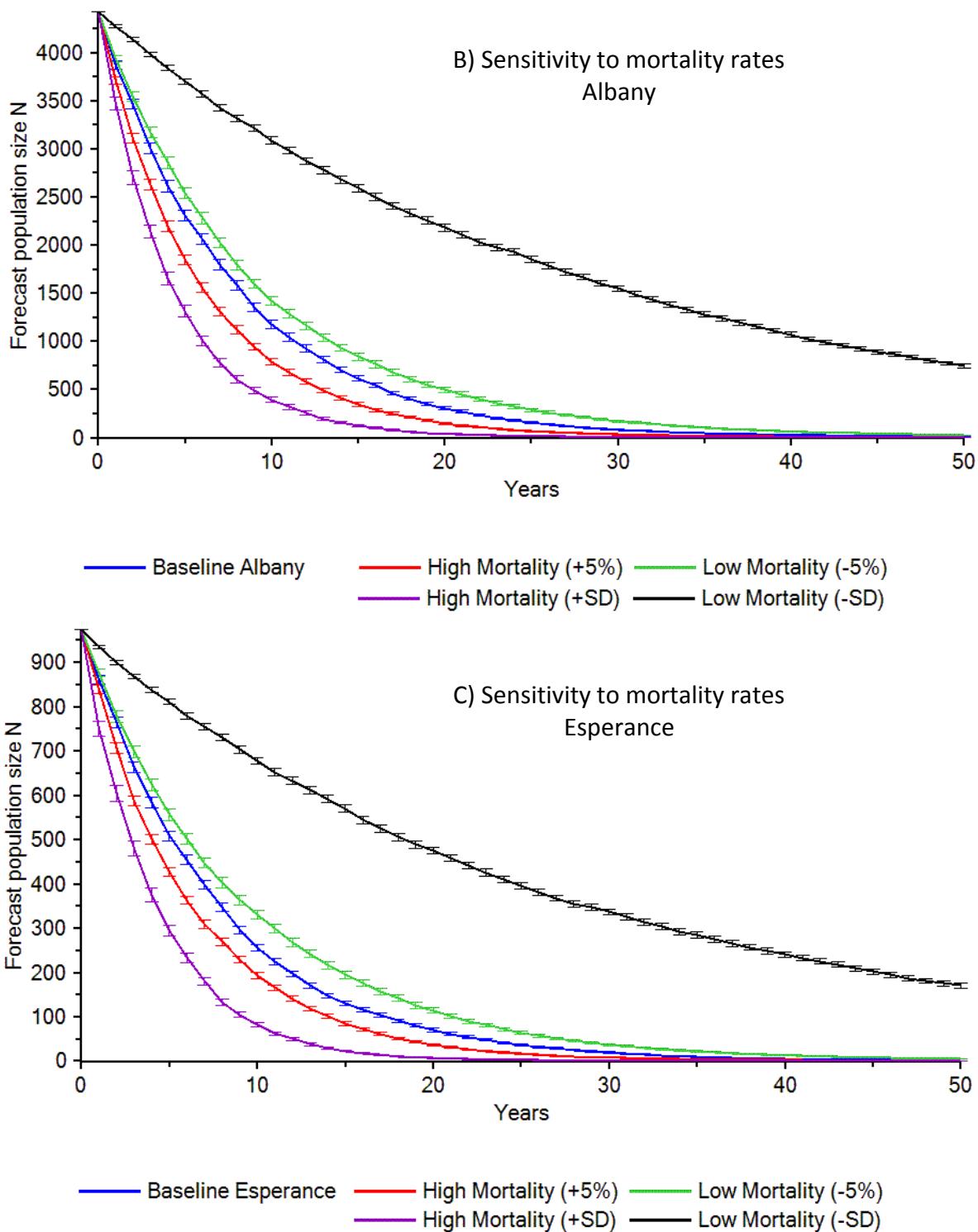
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891 **Fig. 5A:** Sensitivity analysis - forecast of population size N for two WA populations  
892 under stratification 1 over 50 years with high and low mortality rates, varied by  $\pm 5\%$   
893 and  $\pm SD$ ; error bars represent standard error of replicate scenarios

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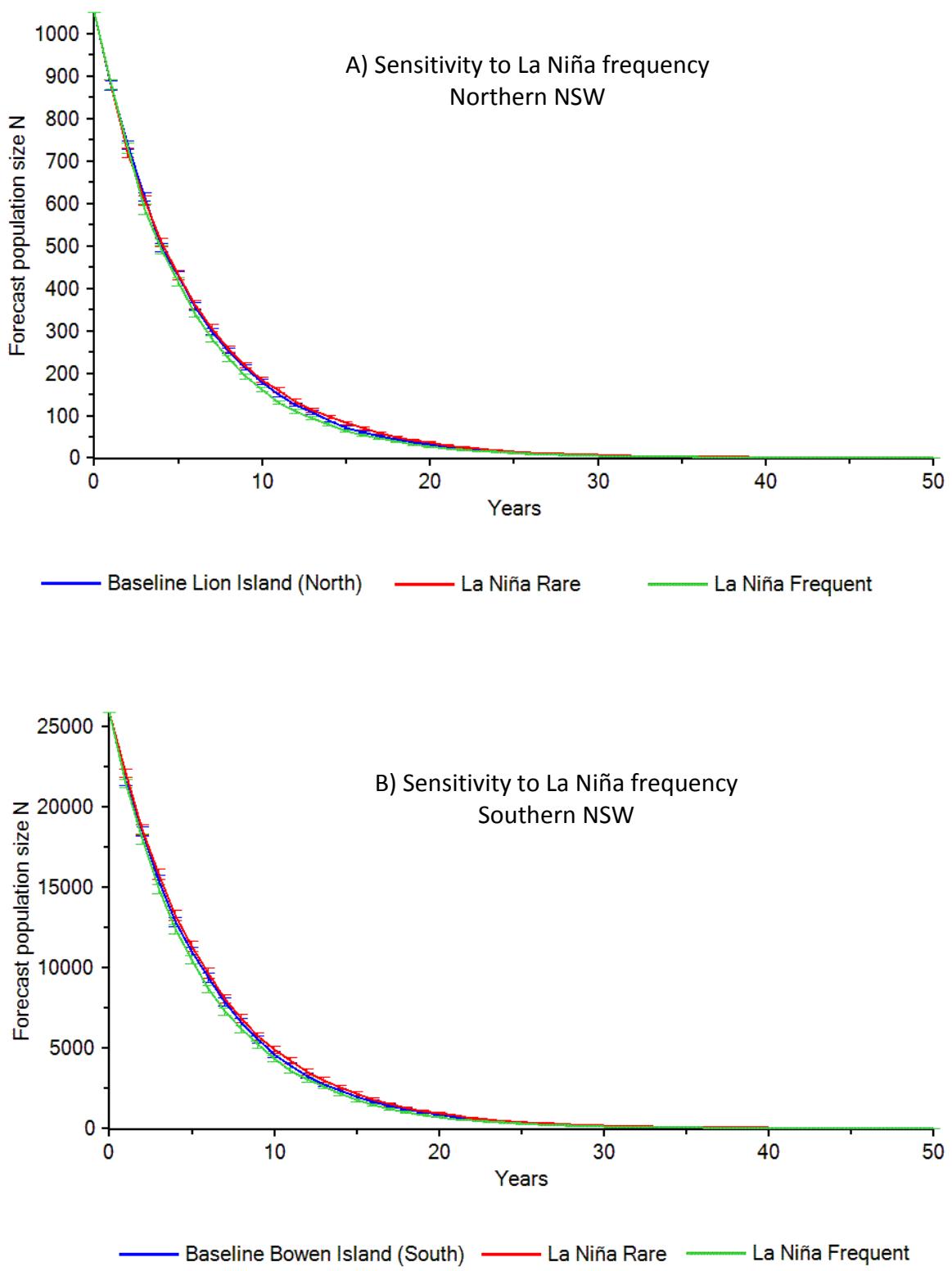




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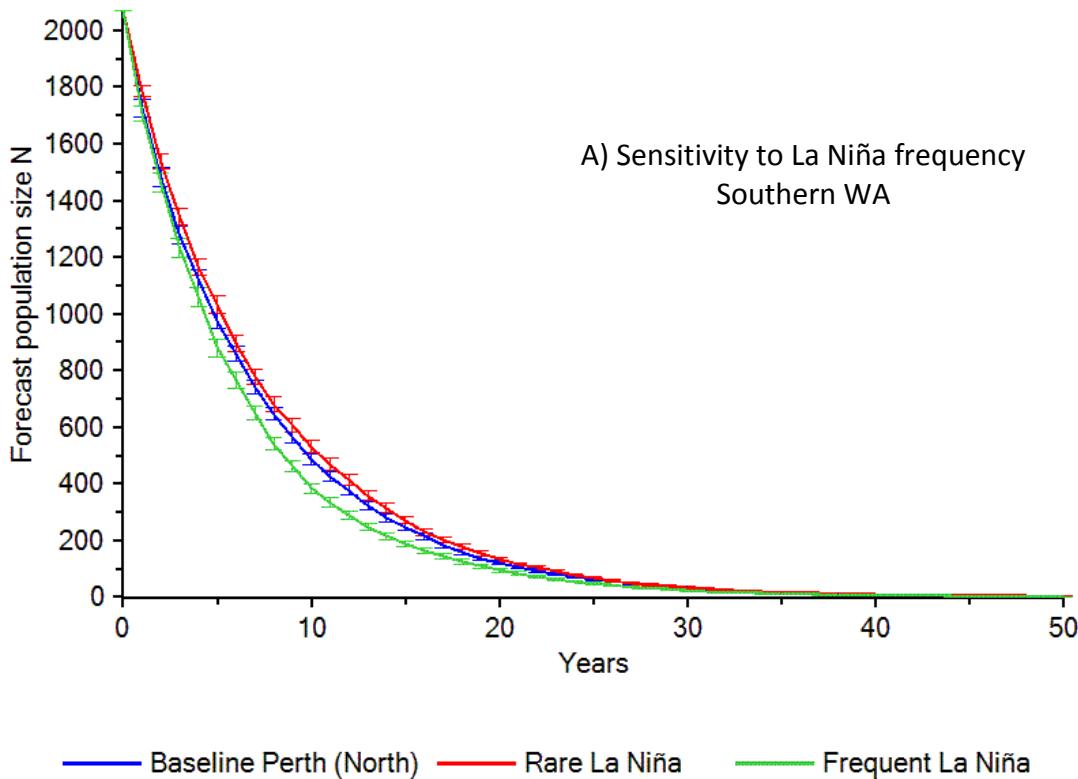
922 **Fig. 6A:** Sensitivity analysis - forecast of population size N for three WA populations  
923 under stratification 2 (A) Perth, B) Albany and C) Esperance) over 50 years with high  
924 and low mortality rates, varied by  $\pm 5\%$  and  $\pm SD$ ; error bars represent standard error  
925 of 100 replicate scenarios



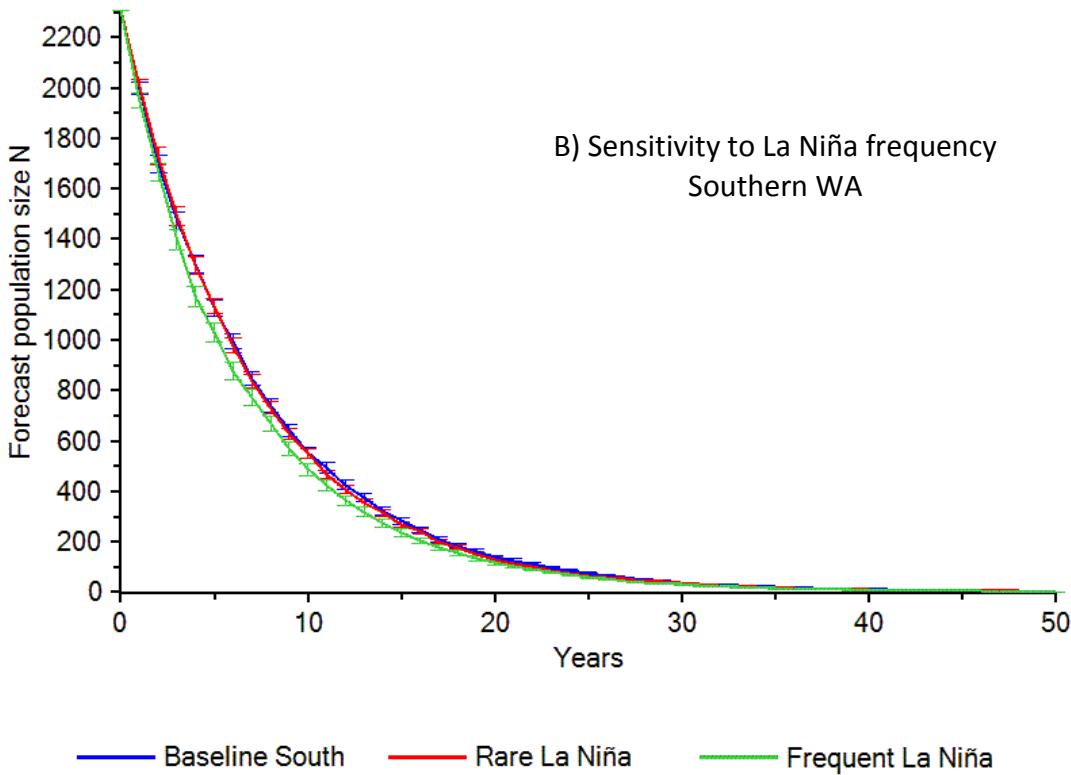
929 **Fig. 7A:** Forecast of population size N for two NSW populations [A) North (Lion Island)  
930 B) South (Bowen Island)] over 50 years with different frequencies of La Niña  
931 catastrophes; Baseline – 15 La Niña events, Rare – 10 La Niña events, Frequent – 20 La  
932 Niña events per century; error bars represent standard error of 100 replicate scenarios

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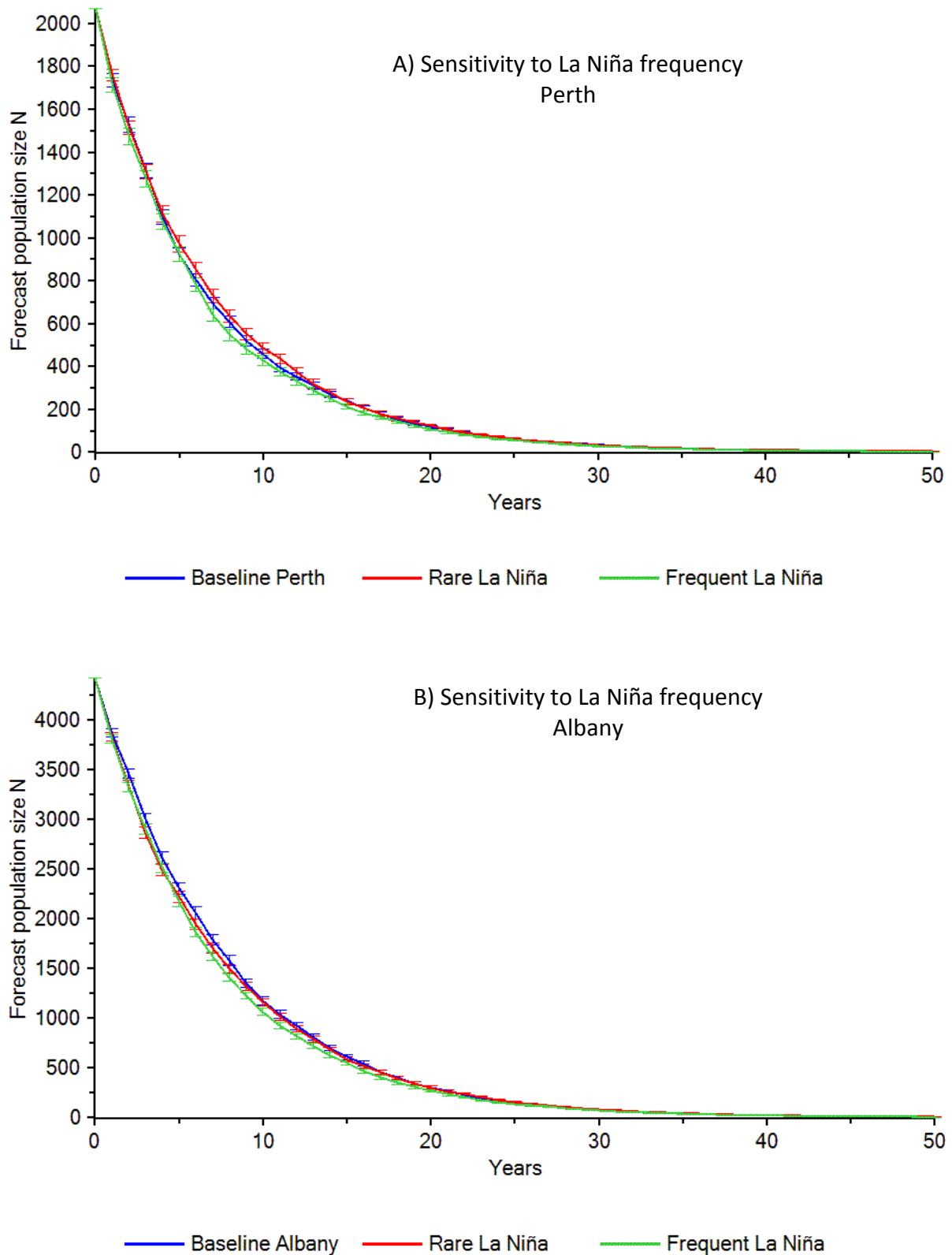


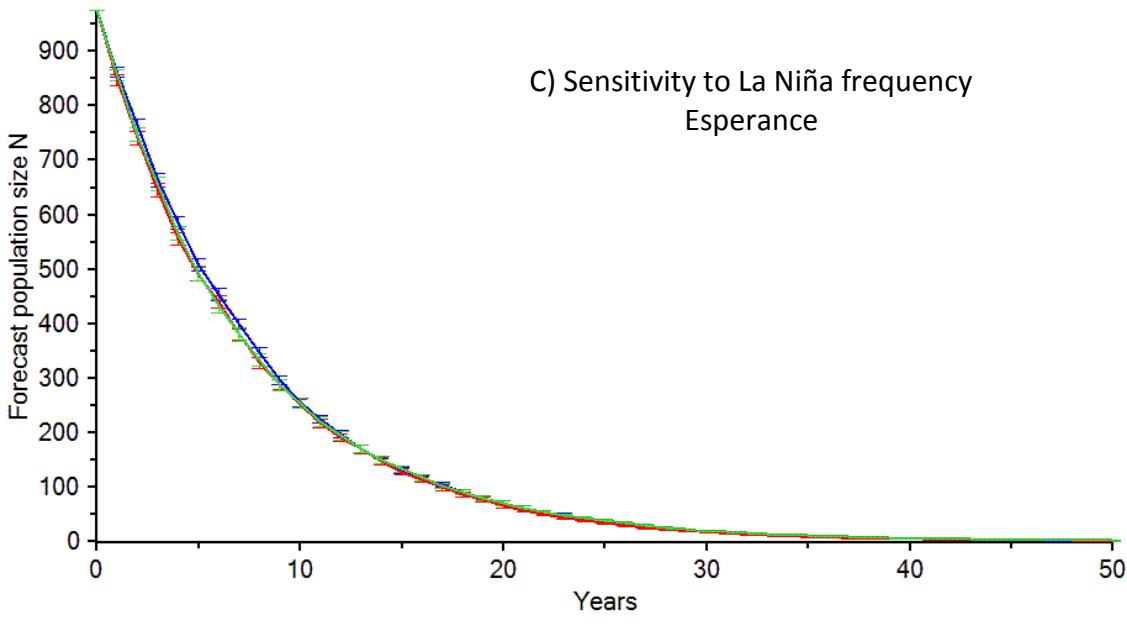
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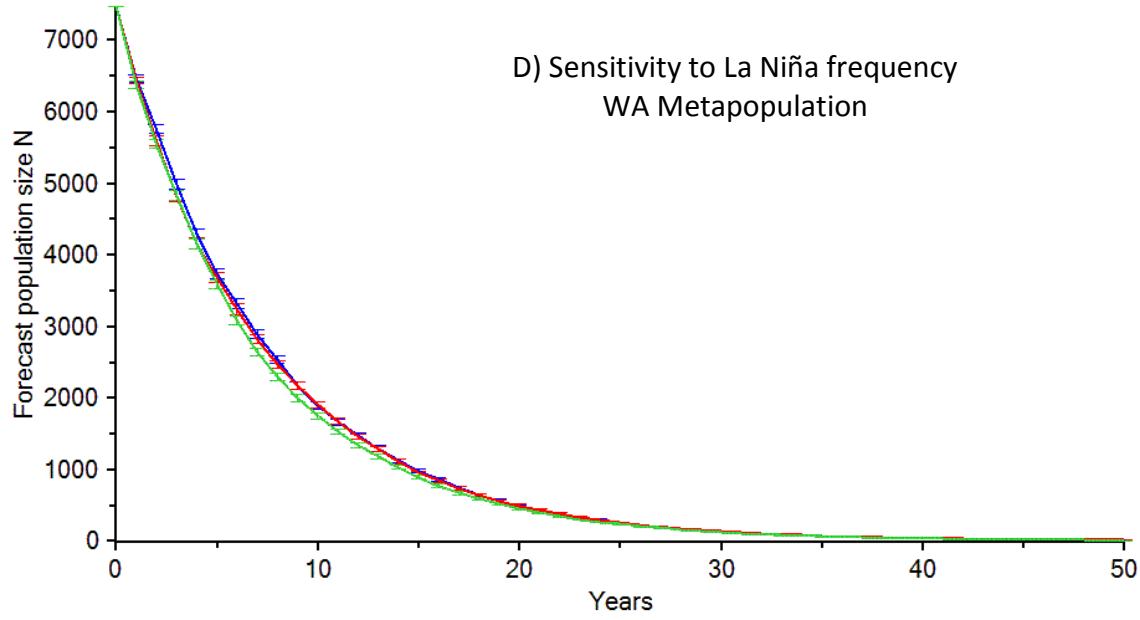
**Fig. 8A:** Forecast of population size N for two WA populations (A) Northern WA, B) Southern WA) under stratification 1 over 50 years with different frequencies of La Niña catastrophes; Baseline – 15 La Niña events, Rare – 10 La Niña events, Frequent – 20 La Niña events per century; error bars represent standard error of 100 replicate scenarios





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945 **Fig. 9A:** Forecast of population size N for three WA populations and WA  
946 metapopulation (A) Perth, B) Albany, C) Esperance, D) WA Metapopulation) under  
947 stratification 2 over 50 years with different frequencies of La Niña catastrophes;  
948 Baseline – 15 La Niña events, Rare – 10 La Niña events, Frequent – 20 La Niña events  
949 per century; error bars represent standard error of 100 replicate scenarios

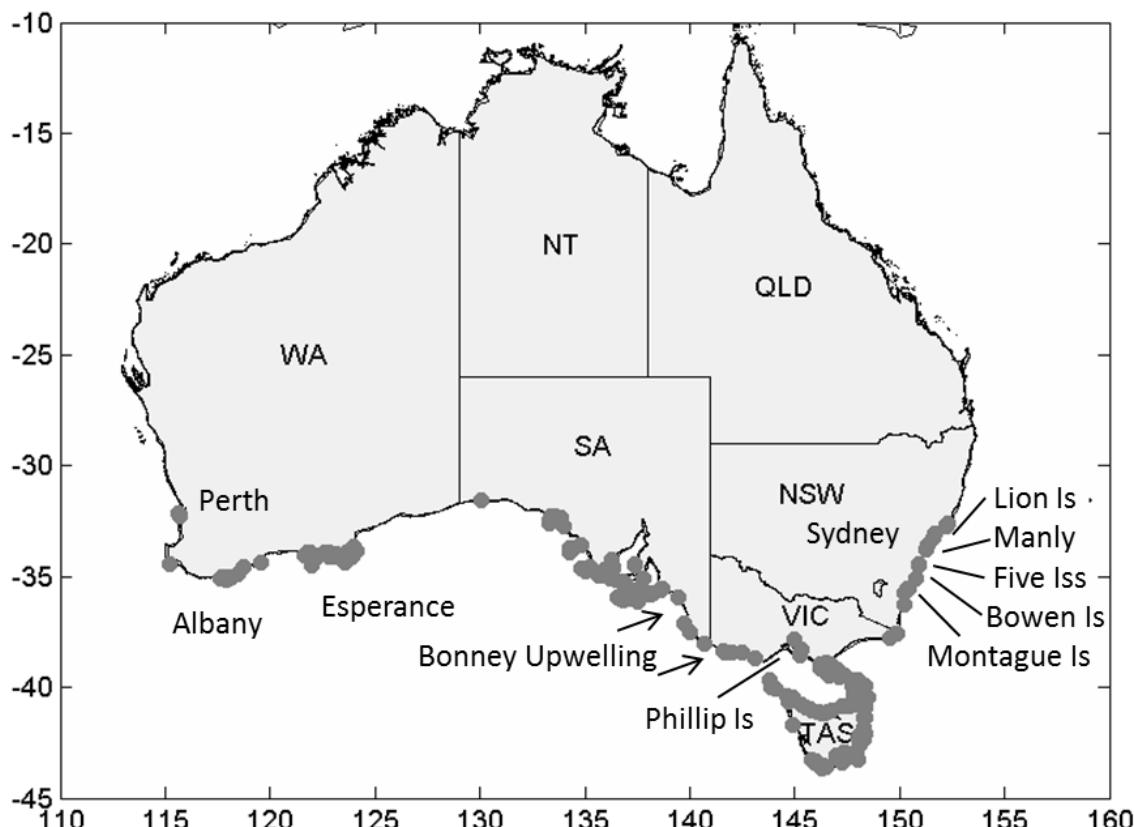
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**1    Chapter 6: Discussion / Final conclusions and recommendations for  
2    research and management of little penguins (*Eudyptula minor*)**

3    Little penguins are one of Australia's most iconic native bird species, and their  
4    populations are declining in numerous locations including colonies in South Australia  
5    (Wiebkin 2012; Colombelli-Négrel 2015), some colonies in Tasmania (Stevenson &  
6    Woehler 2007) and Manly in Sydney Harbour (NSW National Parks and Wildlife Service  
7    2000). Declines have been attributed to anthropogenic influences including climate  
8    change, development, pollution, predation by feral pests, and commercial and  
9    recreational activities such as tourism and fishing, most of which are particularly  
10   affecting populations close to expanding urban areas such as Sydney in New South  
11   Wales (NSW) and Perth in Western Australia (WA). Incidentally, both of those major  
12   Australian cities are home to special colonies of little penguins. The small, but stable  
13   penguin colony at Manly has been the first colony of a common species to be listed as  
14   an endangered population (NSW National Parks and Wildlife Service 2000), while the  
15   colony at Penguin Island near Perth has been assigned the highest conservation value  
16   of any penguin colony in Australia (Dann et al. 1996). Additionally, the two colonies are  
17   situated close to the little penguin's range edge, so that their loss would decrease the  
18   little penguin distribution range (Fig. 1), particularly because there is no opportunity  
19   for a southerly range extension south of Tasmania.

20   The fundamental aim of this thesis was to combine genetic and demographic data  
21   about Australian little penguins to forecast likely population changes under a range of  
22   possible scenarios, including evaluation of anthropogenic threats. To target this aim,  
23   four separate studies were undertaken, the results of which are summarised below.

24



25      Fig. 1: Map of known penguin colonies in Australia, colonies represented as grey circles

26      NSW – New South Wales, NT – Northern Territory, QLD – Queensland, SA – South  
27      Australia, TAS – Tasmania, VIC – Victoria, WA – Western Australia, Is – Island

28

30 **Main Findings**

31 In Chapter 2, which is titled "*Identifying a decline of flightless, burrow-nesting seabirds*  
32 *using non-invasive methods to replace mark-recapture approaches for estimating*  
33 *population size and survival rates*", we compared different methods to estimate  
34 demographic data on population size and survival rates in NSW. Mark-resight analyses  
35 showed that about 500 penguins were using Penguin Beach on Bowen Island (Fig. 1),  
36 whereas the number of penguins using the main beach on Lion Island was in the order  
37 of 60-70 individuals. For Bowen Island, a declining trend of beach counts over the last  
38 few decades conflicted with increasing estimates of the number of penguins using  
39 Penguin Beach during the same time period, so that we conclude that the little  
40 penguin colony at Bowen Island appears to be stable. The colony at Lion Island, on the  
41 other hand, seems to be declining dramatically, with a roughly four-fold drop in  
42 numbers of both beach counts and abundance based on mark-resight. At this smaller  
43 colony, trends of population size over time agree well between the two different  
44 methodologies. Ongoing monitoring of the colony will be necessary to track the  
45 population size of penguins on Lion Island into the future.

46 The low-impact method of using beach counts alone can thus give an indication of how  
47 a percentage of the population is doing, but might be biased by changes in habitat  
48 utilisation both at sea (via foraging trip lengths) and on land (via shifts in breeding  
49 habitat). It would therefore not be recommended to rely on beach counts alone, but  
50 rather to combine them with less frequent, more comprehensive studies based on  
51 methods that require individual marking of penguins.

52 Survival rates were also different between Bowen and Lion Islands. The annual  
53 'apparent survival' rate for penguins on Bowen Island was about 75 % and very similar  
54 to an earlier estimate (Fortescue 1991). This is within the lower range of apparent  
55 adult survival rates documented elsewhere (e.g. Dann 1992; Johannesen et al. 2003).  
56 Lion Island, on the other hand, has a very low apparent survival rate of only about  
57 50 %, which is the lowest annual survival rate published for little penguins.

58 Mark-recapture estimates of survival agreed well with the results of the novel, non-  
59 invasive method to estimate apparent survival based on burrow occupancy (Roth &  
60 Amrhein 2010) in little penguins. This indicates that non-invasive studies of burrowing  
61 sea birds like penguins could replace mark-recapture based studies if burrow  
62 occupancy can be monitored for a large number of burrows over several visits per  
63 season. However, given that population sizes are still best estimated by conducting  
64 mark-recapture or mark-resight studies, one could make use of the same data to  
65 confirm survival rates using these methods.

66 Chapter 3, titled "*Population genetics of Little Penguins (Eudyptula minor) in*  
67 *Australia*", used genetic methods to estimate size and connectivity of penguin  
68 populations in three Australian states - NSW, SA and WA. While colonies varied greatly  
69 in their census population size (from a few breeding pairs, e.g. Broughton Island, NSW;  
70 to thousands, e.g. Bowen Island, NSW; see also Marchant et al. 1990), it was found  
71 that the smaller colonies studied were genetically as diverse as the larger ones.  
72 However, genetic diversity within NSW is maintained at a slightly lower level than  
73 closer to the centre of the penguin distribution as a result of lower between-locality  
74 variation. This agrees with the theory that abundance and diversity are decreasing with

75 increasing distance from the centre of a species' distribution (Hanski 1991; Lawton  
76 1993). Isolation by distance was not observed at small distances within NSW, and was  
77 present but weak at somewhat larger distances between NSW and SA. Unexpectedly,  
78 differentiation was found to be stronger at the maternally inherited mitochondrial  
79 marker than at the biparentally inherited microsatellites. One possible explanation of  
80 this would be sex-biased dispersal driven by male dispersers, but others were also  
81 discussed. On land, male little penguins have been observed to have lower metabolic  
82 rates than females (Baudinette et al. 1986), and sexual differences in diving behaviour  
83 (mean dive depth, mean and maximum dive duration) have also been documented  
84 (Hoskins et al. 2008). These differences could explain why male little penguins are able  
85 to disperse larger distances than females. This chapter has an unusual focus on  
86 assessment of marginal populations, and finds ongoing dispersal among small, yet  
87 genetically diverse populations at the penguin's range edge.

88 Chapter 4 investigated "*Patterns of Major Histocompatibility Complex diversity,*  
89 *parasitism and mate choice in Little Penguins (*Eudyptula minor*)*" by using an adaptive  
90 genetic marker of the immune system (Major Histocompatibility Complex, MHC) to  
91 complement the population genetic study in Chapter 3 using genetic markers that are  
92 likely to be selectively neutral. Genetic diversity at neutral loci was largely mirrored by  
93 the MHC locus, which indicated a strong effect of stochasticity. Despite this, signs of  
94 selective pressures acting on the MHC were detected. Indications of inbreeding were  
95 found at the MHC locus, with similar overall levels of inbreeding between states.  
96 However, significantly higher levels of inbreeding in the parental than in the offspring  
97 generation were observed in Western Australia, but not New South Wales. This

98 suggests that for Western Australian penguins, either inbreeding avoidance has  
99 increased within the time-period of a single generation, or else there is reduced fitness  
100 of young inbred offspring. The possible association between MHC variation and  
101 pathogenic threats was assessed. The lowest rates of parasite infestation occurred in  
102 individuals of intermediate MHC diversity, but due small sample size we were unable  
103 to determine if this pattern is statistically robust. To further investigate these  
104 interesting patterns and test the applicability of the MHC gene as an additional genetic  
105 marker for population genetic studies, a higher sample size of individual penguins with  
106 known parasite status, pedigree information and MHC genotype would be necessary.  
107 This would allow investigation of parasite-driven selection and non-random mating in  
108 the same population. This chapter reports the first population-scale study of MHC  
109 genes in little penguins and adds an example for the use of non-neutral markers in  
110 wildlife studies.

111 Chapter 5 ("Viability of little penguin (*Eudyptula minor*) populations  
112 at their northern range edge") incorporated data from the literature as well as  
113 Chapters 2 and 3 into population viability analysis (PVA, Beissinger & McCullough  
114 2002) models to evaluate outcomes of different scenarios and recommend research  
115 and management directions for penguin populations in NSW and WA. All baseline  
116 models are based on estimates thought to represent the current situation, and are  
117 based on more extensive demographic data and dispersal estimates compared to  
118 many other studies of similar species. These models predicted severe population  
119 declines in NSW and Western Australia and high probabilities of extinction. Forecasts

120 were particularly sensitive to changes in mortality estimates, and the impact of  
121 severity and frequency of La Niña events was limited.

122

### 123 **Management recommendations**

124 The results of Chapter 2 indicate that to estimate apparent survival of burrowing sea  
125 birds like penguins, non-invasive surveys of burrow occupancy could replace mark-  
126 recapture based studies. This would necessitate burrow occupancy be monitored for a  
127 large number of burrows over several visits per season and is expected to work best in  
128 colonies with permanently marked natural burrows or artificial nest boxes. Population  
129 sizes, however, are still best estimated by conducting mark-recapture or mark-resight  
130 studies, which would enable managers to make use of these same data to also confirm  
131 survival rates using these more invasive methods. Additionally, a decline of population  
132 size at Lion Island was detected. In contrast to Lion Island, the Manly colony currently  
133 appears to be stable or increasing (Lisa O'Neill, pers. comm.). This indicates that local  
134 rather than regional conditions affect penguin numbers. It might therefore be  
135 beneficial to concentrate on ensuring the penguin's breeding habitat and ensure they  
136 have adequate nesting opportunities while being safe from predation. Providing nest  
137 boxes for penguins has been shown to improve breeding success while facilitating the  
138 monitoring of population dynamics (Perriman & McKinlay 1993).

139 In Chapter 3, population genetic patterns indicated that the amount of dispersal  
140 among colonies in NSW is strong enough to result in sufficient gene flow to prevent  
141 genetic differentiation among the colonies. Thus far, the endangered little penguin  
142 colony at Manly has been managed as a single population that is separate from

143 surrounding island colonies, but this might have to be re-evaluated in light of the  
144 strong genetic connectivity. In Chapter 2, a decline in penguin numbers on the closest  
145 island colony to Manly, at Lion Island in Sydney Harbour, was also detected. This  
146 decline could well affect the stability of the Manly colony through reduced  
147 immigration. If the Lion Island colony was to go extinct, it could furthermore increase  
148 genetic isolation of the Manly colony, leading to reduced genetic variability, which is in  
149 turn expected to increase its probability of extinction (Frankham 2005). It would  
150 therefore be advisable to closely monitor the colony at Lion Island to detect and  
151 ameliorate further drops in penguin numbers through improved management. Due to  
152 the exceptionally low apparent survival rates measured for Lion Island, and the high  
153 sensitivity of population viability to changes in mortality estimates modelled in  
154 Chapter 5, the most promising approach would be to identify and reduce possible  
155 causes of mortality in order to stabilise penguin numbers on Lion Island.

156 Chapter 4 concluded that little penguins from different colonies in NSW were largely  
157 similar concerning their MHC allele pools, which agrees well with the limited genetic  
158 differentiation at neutral microsatellites and at a mitochondrial marker (Chapter 3).  
159 However, samples from all colonies in NSW pooled were highly differentiated from  
160 those in WA at the MHC. Due to the similarity of patterns of neutral markers and the  
161 MHC, the possibility that stochastic effects shape this pattern of MHC diversity cannot  
162 be excluded, although selective neutrality tests indicated an influence of selection or  
163 demographic changes at the MHC locus. Based on the results of this study, there is no  
164 reason to believe that genetic diversity of little penguins at this immunogenetic marker  
165 is compromised by low population size in any of the populations studied. However,

166 given the population declines forecast for NSW and WA (Chapter 5), it would be  
167 advisable to re-investigate this pattern after a few decades in small, isolated  
168 populations. The current research forms a baseline for this future study.

169 The final Chapter 5 in this thesis combined data from Chapters 2 and 3 with  
170 information from the literature to assess little penguin population viability and  
171 forecast likely changes in population size. Because declines were forecast for all  
172 scenarios modelled, key little penguin populations should be monitored yearly to  
173 detect changes and make early intervention possible if population size drops below a  
174 pre-defined threshold. Due to the higher sensitivity of forecasts to changes in mortality  
175 estimates compared to reproductive rates, it would be advisable to concentrate efforts  
176 on reducing sources of mortality rather than trying to increase reproduction.

177 Altered patterns of ENSO as a symptom of climate change have previously been  
178 indicated to adversely affect the penguins on Penguin Island (Cannell et al. 2012). In  
179 the previous study, which focussed on the area around Perth, the need for proactive  
180 management of anthropogenic effects on fish prey availability to maintain the  
181 wellbeing of the colony has been highlighted. The present work confirms that this  
182 colony is more sensitive to changes in ENSO patterns than colonies in southern WA  
183 and NSW and is therefore adding evidence to the former study.

184 **Further research**

185 In Chapter 2, a decline of population size at Lion Island was detected, along with  
186 exceptionally low apparent survival rates. Continued monitoring of tagged penguins  
187 from Lion Island could answer whether a significant proportion of penguins emigrate  
188 to neighbouring colonies including Manly, where monthly surveys are continuing  
189 throughout the breeding season.

190 Chapter 3 focussed on population genetics of little penguins to elucidate population  
191 structure and dispersal among colonies, and some indication for different dispersal  
192 rates between the sexes was found. Differentiation was stronger at the maternally  
193 inherited mitochondrial marker than at the biparentally inherited microsatellites,  
194 which indicates sex-biased dispersal driven by male dispersers. However, this needs to  
195 be confirmed using other approaches such as sex-chromosomes, to help overcome the  
196 problem of interpretation posed by different effective population sizes.

197 A small study of little penguins on land has found male little penguins to have lower  
198 metabolic rates than females (Baudinette et al. 1986), and sexual differences in diving  
199 behaviour (mean dive depth, mean and maximum dive duration) have also been  
200 documented previously (Hoskins et al. 2008). Further research into the effect of these  
201 differences on penguin energetics and dispersal distance would help understand  
202 differences in patterns between the sexes.

203 An interesting pattern of reduced parasite load at intermediate MHC diversity was  
204 suggested in penguins from WA, but could not be statistically confirmed due to low  
205 sample sizes. If further research confirmed this pattern, it would agree with the theory

206 that an intermediate, not maximum number of individual MHC alleles optimises  
207 parasite resistance (Reusch et al. 2001; Wegner et al. 2003; Woelfing et al. 2009).

208 All populations that were investigated in Chapter 5 were forecast to decline within the  
209 next 50 years, although most model input data were collected over a timescale of  
210 more than 20 years and strong declines have only been evident in a few cases,  
211 including Lion Island (Chapter 2). We therefore recommend further research into  
212 population-specific data on reproduction and mortality rates and population  
213 monitoring to ensure timely detection of population declines at the penguin's northern  
214 distribution limit. In particular, some of the models were sensitive to variation in  
215 reproductive rates, estimates of which were based on studies conducted in few of the  
216 colonies. Furthermore, the percentage of adult females breeding each year was  
217 unknown for NSW populations and will have to be investigated to obtain more robust  
218 results on population viability of little penguins. Mortality rates impacted model  
219 outcomes even more than reproduction, and estimates of mortality were similarly  
220 based on studies conducted at few colonies. Within NSW, significant differences in  
221 annual adult survival rates have been found between Lion Island near Sydney and  
222 Bowen Island in Jervis Bay (Chapter 2). To clarify whether these are representative of  
223 the larger population, additional studies of survival in different colonies would be  
224 helpful. Studies of captive little penguin populations might furthermore help  
225 understand parameters like their sex ratio at birth, which is difficult to measure in wild  
226 populations while minimising impact on the penguins.

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