Implications of movement and population structure in the endangered pygmy bluetongue lizard (*Tiliqua adelaidensis*): Lessons for conservation

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Abstract

As habitats becomes more fragmented, changes in normal population processes can become disrupted leading to populations in fragmented habitats having reduced effective population size, with loss of genetic diversity resulting from increased genetic drift and inbreeding. Dispersal is an important function in any population to counter those processes, contribute to gene flow among populations, and allow the spread of a species into available population sites. Dispersal is one of the key factors in preventing inbreeding within populations and can contribute to maintaining stable social structures and viable populations.

The endangered pygmy bluetongue lizard, *Tiliqua adelaidensis* is known only from small populations in 31 isolated fragments of once more continuous native grassland habitat in the mid north of South Australia. It is a largely solitary scincid lizard and spends the majority of its time in narrow, vertical, single entrance burrows constructed by lycosid and mygalomorph spiders, waiting to ambush passing invertebrate prey. Individual pygmy bluetongue lizards normally remain closely associated with a single burrow.

I used pitfall trapping to monitor out-of-burrow movements by pygmy bluetongue lizards, with a total of 49,440 trap-nights from three sites over 2 years. I found that male pygmy bluetongue lizards were more likely to move than were females, with neonates the second-most captured group. Most movement by males was in the breeding season, representing partner searching moves, rather than dispersal movements away from the local area. This suggests that even when they leave their burrows, most lizards do not move far, and probably remain in or return to the local area of the burrow they originally moved from.

I used genotypes at polymorphic microsatellite DNA loci to investigate the mating system and social structure within populations, and found that 75% of litters had multiple paternity. There was no evidence of active kin avoidance, with mating partners apparently chosen randomly with respect to the level of relatedness among neighbouring lizards. However, mating partners were located closer to each other than expected by chance, and most commonly within 30 m of each other. Drivers for

the polygamous mating system may be the single occupancy burrow and the central place territorial defence of those burrows.

Adult lizards within a demographic population belonged to several divergent genetic clusters at both the whole population scale (11-13ha) and within 1.2 ha sampling sites within demographic populations. Significant spatial autocorrelation suggested low natal dispersal distances for both sexes, and that resident adults had settled close to related individuals. The different genetic clusters were not spatially sorted at the local scale, implying different genetic lineages were maintained in the demographic populations possibly due to strong isolation by distance. At this stage, despite the habitat fragmentation and lack of gene flow among adjacent populations, genetic diversity within isolated populations is probably maintained by this genetic structure coupled with localised promiscuous mating.

I investigated phylogenetic relationships among 10 demographic populations across the range of the species using both mitochondrial and microsatellite DNA. These data confirmed that individual populations, separated by inhospitable farmlands, are genetically isolated. There were at least two geographically separated clusters of populations, emphasising the importance of conserving populations in multiple areas to maintain as many genotypes as possible.

This thesis provides important information to guide management strategies for maintaining genetic diversity within species and particularly the pygmy bluetongue lizard. Given the mounting pressures of climate change and of interrupted landscapes limiting dispersal, the findings of this thesis should be used to promote *in situ* adaptive management processes including informed translocations for maintaining viable populations of this endangered species.

If I have seen further it is by standing on the shoulders of giants

Isaac Newton

This thesis is dedicated to all the mealworms who died in the name of science

Contents

Declaration

I certify that this thesis does not incorporate without acknowledgment and material previously submitted for a degree or diploma in any university; and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Julie Schofield

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Organisation of thesis

This thesis is structured with six chapters, some of which were written for publication as peer-reviewed journal articles. The chapters written as articles are included as submitted, which necessitates some repetition of material presented in the introduction. Additionally, these articles necessarily use the plural "we", due to the contribution of co-authors. To ensure consistency, and prepare for future submissions, this convention has been followed in the remainder of the thesis. Because of this structure, reference lists for citations are provided at the end of each chapter rather than as a compiled list at the end of the thesis.

This thesis is an investigation into movement using different investigative scales, from the level of genes to individuals, and between populations. It explores implications of movement patterns for the conservation of the endangered pygmy bluetongue lizard.

The thesis begins with a general introduction and brief overview of the background knowledge and theory underpinning this research and places the thesis aims in context. The introduction also includes an outline of the structure of the thesis, and an introduction to the pygmy bluetongue lizard and the study area.

The second chapter starts at the organism scale investigating dispersal of adults and juveniles within populations by pitfall trapping, and identifying seasonal patterns of movement.

Chapter 3 builds on the insights gained from movements at the individual scale to investigate the movement of genes. Specifically, this chapter explores the mating system and breeding strategies of individuals within the population, and how those might contribute to gene flow and gene mixing within populations.

Continuing the theme of genetic structure within populations, Chapter 4 investigates whether the demographic populations are panmictic as would expected from the breeding strategies described in Chapter 3 and uses analysis of microsatellite DNA genotypes to investigate genetic structuring and to speculate on how far lizards have dispersed from their natal burrows to where they settle as adults.

Chapter 5 takes a broader view of the flow of genes and focuses on the current and historical genetic structure across populations to determine units of conservation significance for future management actions.

The final chapter is an overview of the knowledge gained from the research in this thesis. It includes potential ramifications for conservation of this species. The thesis ends with a summary of important areas for future research.

Publications arising from this thesis and statement of candidate contribution

This thesis is composed predominantly of manuscripts that have already been published, or that have been submitted, or that are near to being submitted for publication in peer reviewed journals. I conducted all of the field work and data collation, and most of the analyses and interpretation in all of the papers. I benefited from the advice of my supervisor Prof C. M. Bull in all papers and my co-supervisor Dr Mike Gardner in the genetically based papers. All co-authors have given permission for the work that arose from collaboration to be included in this thesis. Estimates of the relative contribution of each co-author to each publication are provided.

Chapter 2
Julie A. Schofield, Aaron Fenner, Kelly Pelgrim, and C. Michael Bull (2012) Male-biased movement in pygmy bluetongue lizards: implications for conservation. *Wildlife Research.* 39(8): 677-684
JS 70% AF 7.5% KP 2.5% MB 20%

Chapter 3
Julie A. Schofield, Michael G. Gardner, Aaron Fenner, and C. Michael Bull (2014) Promiscuous mating in the endangered Australian lizard *Tiliqua adelaidenesis*: a potential windfall for its conservation. *Conservation Genetics* 15: 177-185.
JS 70% MG 15% AF 5% MB 10%

Chapter 4

Julie A. Schofield, Michael G. Gardner and C. Michael Bull Genetic structure of the endangered pygmy bluetongue lizard (*Tiliqua adelaidensis*) within a fragmented landscape. Submitted to *Conservation Genetics*

JS 75% MG 15% MB 10%

Chapter 5

Julie A. Schofield, Michael G. Gardner and C. Michael Bull Conservation of genetic variation amongst populations of the endangered Pygmy bluetongue lizard (*Tiliqua adelaidensis*). For submission to *Wildlife Research*

JS 75% MG 15% MB 10%

In addition my observations have contributed 10 – 40% to five other publications from the pygmy bluetongue research team, which are included as an appendix to this thesis:

Pelgrim, K., Fenner, A. L., Schofield, J. A. and Bull C. M. (2014) Dynamics of a temperate grassland reptile community in the Mid-North of South Australia. *Transactions of the Royal Society of South Australia*. 138: 257-266

Ebrahimi, M., Schofield, J. A. and Bull, C. M. (2012). *Tiliqua adelaidensis* (Pygmy Bluetongue Lizard). Alternative Refuge. *Herpetological Review*, 43 (4): 652-653.

Ebrahimi, M., Schofield, J. A. and Bull, C. M. (2012) Getting your feet wet: Responses of the endangered pygmy bluetongue lizard (*Tiliqua adelaidensis*) to rain induced burrow flooding. *Herpetology Notes*. 5: 297-301

Andy Sharp, Julie Schofield and Aaron Fenner (2010) The effects of cell grazing on the longevity of spider burrows, and the potential consequences for the endangered Pygmy Bluetongue Lizard. *Ecological Management & Restoration* 11 (1): 69 – 72.

Aaron L. Fenner, Julie A. Schofield, Annabel L. Smith and C. Michael Bull (2008) Observations of snake predation on the Pygmy Bluetongue Lizard, *Tiliqua adelaidensis*. *Herpetofauna* 38 (2): 105-109

Chapter 1. Dispersal

Literature review

General Introduction

Understanding dispersal characteristics of a species can be vital knowledge for the conservation of endangered species at several levels, the genetic, the individual and the population level. This thesis explores dispersal, its consequences and conservation implications for the endangered pygmy bluetongue lizard, an Australian skink. A particular focus of the thesis is to understand the genetic consequences of reduced dispersal opportunities for this species since it is now restricted to a small number of isolated populations.

What is dispersal?

Dispersal has been defined by Clobert *et al.* (2001) as "the movement between the natal area or social group and the area or social group where breeding first takes place". It is different from breeding dispersal or migration, which are movements that take place between breeding events. As such, dispersal is a component of the demography of a population. Populations increase with the recruitment of individuals, either from births or from incoming dispersers (immigration), and they decrease with the loss of individuals, either through mortality or through dispersal away from the population site (emigration). Like other demographic processes, dispersal can vary among locations and years in the same species.
Dispersal may be a passive event such as in the dispersal of plants where pollen or seeds are transported by wind or water) or it may be active with deliberate movement by individuals as in the case for many vertebrate species. However, there is substantial variation in dispersal tendencies among species and even among individuals within a species.

Dispersal is only evolutionarily meaningful if it results in the disperser breeding in the new location. Selection may also favour dispersal as a strategy, and the environmental conditions faced by the individual can determine the extent of dispersal. The production of more offspring after dispersing (if not dispersing means not breeding) is a strategy that will favour dispersal or individuals who have a

disposition for dispersal. Dispersal can be considered as a unique behaviour with multiple causes, or as a family of behaviours with strong resemblances but with each behaviour having its own evolutionary origin (Clobert *et al.* 2008).

Andrewartha and Birch (1952) first suggested dispersal from local populations was density dependent. We now know that other processes can influence the local rate of dispersal, and that dispersal is more dynamic and complex than had been assumed (Clobert *et al.* 2001; Ims and Andreassen 2005). Most commonly explored is the role of natal dispersal, as in almost all animal species a proportion of juveniles leave their place of birth and eventually settle and breed at some other location (Sutherland *et al.* 2000). One ecological explanation for this is that dispersing individuals are avoiding competition with already established individuals, and often reducing competition among related kin (Cote and Clobert 2010; Edelman 2014,). Dispersal distances can vary among individuals, and this could be result from genetic (Pasinelli *et al*. 2014) or environmental causes (Gauffre *et al.* 2014; Scandolara *et al.* 2014; Bitume *et al.* 2013). A consequence of longer distance dispersals is that individuals can colonise (or recolonise) unoccupied patches of habitat across the landscape, and that there is the potential for some exchange of genetic material across adjacent populations (Sutherland *et al.* 2000).

Why disperse? Ecological Advantages.

There are many advantages for individuals that disperse. Firstly, individuals increase their chance of finding new less exploited resources or genetically distinct mating partners. Secondly, individuals can escape from areas with high local densities of conspecifics where there is elevated competition for resources, or from areas of high predation risk, or from areas where resources are declining naturally. Many habitats do not remain stable and local populations that occupy those habitats tend to be ephemeral, establishing, growing and then declining in abundance as initially favourable habitat becomes less favourable (Caughley 1980). This transient nature of ecological conditions has led to evolutionary advantages for dispersing individuals that might locate areas of better quality; individuals may benefit by dispersing when local resources are depleted (Comins *et al*. 1980; Perrin and Mazalov 2000).

Factors that may affect local fitness and consequently encourage dispersal may include local population density or patch capacity (Poethke and Vovesstadt 2002), habitat quality (e.g. Baguette *et al.* 2011), predation or parasitism (Poethke *et al.* 2010), and aspects of social status or of the individual's own physiological condition (Bolwer and Benton 2005; Handley and Perrin 2007). Short distance dispersal is probably sufficient for avoiding inbreeding or kin competition, whereas long distance dispersal might function to colonize a new territory or escape crowding (Perrin and Goudet 2001).

Dispersal may directly advantage the dispersing individual, or there may be indirect advantages, for instance through kin competition which results when related individuals compete for limited resources. An individual which disperses and reduces the level of kin competition, can increase its inclusive fitness by increasing the level of resources available to the close relative that does not disperse. Kin competition can occur between siblings or between parents and offspring. For example in the common lizard, *Lacerta vivipara*, mother and offspring competition leads to the dispersal of female offspring (Verken *et al.* 2007). Juvenile dispersal in this species was greatest at high densities of females and body condition of mothers was positively correlated with the extent of juvenile dispersal (Lena *et al*. 1998). Individual variation or phenotype may also play a role in the levels of kin avoidance or cooperation, such as in the side- blotched lizard, species name, where the males display one of three different breeding colours. Each reproductive colour has varying levels of aggression to other conspecifics (Sinervo and Lively 1996). Sinervo and Clobert (2003) found that orange throated males avoided each other whereas blue throated males found near each other were more genetically related than expected by chance alone. The different life strategies are advanced by the differences in dispersal, the blue throated lizards disperse to where other cooperative blue throated male are thus increasing their collective fitness whereas the high stamina aggressive competitive orange males are able disperse further and establish territories near less related individuals.

Costs of dispersal

As well as having benefits, dispersal can also have costs, which can be considered in four main groups: energy exerted; risk; time; and opportunity (Bonte *et al.* 2012). Movement is costly because it requires time and energy that cannot be used for any other activity. Active dispersal costs energy, and there is a risk that dispersal will not result in finding a better habitat than the one that was left. Moving through unfamiliar landscapes increases the exposure to detrimental conditions such as temperature extremes and increased predation. Predation risk affects animal movement decisions (Lima and Dill 1990). For example Sato *et al.* (2014) found that lizards moving through cleared areas between suitable habitat were exposed to greater risk of predation and to higher temperatures than their recorded critical maximum temperatures.

Then, once a suitable habitat has been reached, other costs include unfamiliarity with the new habitat, and the location of its resources and refuges. Thus dispersers risk competition from resident conspecifics or other species in the new habitat, predation because refuges cannot be located quickly enough, and starvation if new locations of food resources are not learned quickly enough (Greenwood 1980; Stamps 2001). In small mammals for example, survival rate can be almost 50% lower for dispersers than for philopatric individuals (Johnson and Gaines 1990).

Why disperse? Genetic Advantages.

Dispersal from the natal area usually results in individuals occupying sites with less related neighbours than if they had not dispersed. Avoiding relatives can be genetically advantageous in two ways. First it can reduce levels of kin competition for resources. Moving away from kin can indirectly help them through reducing the pressure on resources. Given that related individuals share genes, this dispersal from kin potentially increases the fitness of the disperser as it increases the chance that their genes will get passed on to the next generation by the individuals left behind (Zhan *et al.* 2007;Stevens *et al.* 2006; Stuart and West 2002). Second, avoiding relatives can reduce inbreeding depression that could result from mating between related individuals that carry similar genotypes. Inbreeding in populations can affect both individual fitness and population viability. Inbred individuals can have

increased occurrence of detrimental traits (e.g. deformities), increased susceptibility to pathogens, and lower fertility (Lambin, Aars *et al*. 2001). Three main mechanisms have been identified for avoiding inbreeding, these are kin avoidance through partner discrimination, dispersal, particularly sex biased dispersal, and multiple mating (Olsson *et al.* 1994; Mateo 2010). Partner discrimination, a mechanism used when there is reduced dispersal, is considered in this thesis, and so, although more related to lack of dispersal, it is briefly included in this dispersal focussed review. I do not discuss multiple mating in this introduction to dispersal as this is covered in chapter 3.

The mechanisms used to recognise relatives (and hence to avoid kin as mating partners) will depend on the social organisation (Pusey and Wolf 1996). Species organised in stable family groups can use familiarity as a cue for relatedness. An individual learns the distinctive signals of familiar animals around it and treats these as kin. Populations with low levels of social structure may rely on genetic markers for kin recognition (Blouin and Blouin 1988 (Bull and Cooper 1999). Recognition by familiarity requires individuals to have a period of association with one another before being recognized as kin. Use of genetic markers, such as "phenotype matching", on the other hand, requires no such period of prior association, with the relatedness of newly encountered individuals being assessed based on how similar they are to the reference phenotype. Phenotype matching, uses a reference phenotype (either self or kin) against which other individuals are judged (Heth 1998) such as disease resistance via the major histocompatibility complex (Zelano and Edwards 2002). Examples of phenotype matching include the use of olfactory (Parrott *et al.* 2007; Bull and Cooper 1999), auditory (Kulahci 2014;Akcay *et al*. 2013) or visual cues to detect relatedness to avoid breeding with related individuals (Sherman *et al.* 1997).

A potential, and commonly used strategy for species with limited kin recognition or with social structures that do not facilitate familiarity cues is random dispersal. If individuals disperse in random directions or for different distances from their natal sites, neighbouring adults are less likely to be related than average thus reducing the opportunities for inbreeding (Lambin *et al.* 2001). This effect can be enhanced by sex biased dispersal.

Sex biased dispersal

Sex-biased dispersal, is where individuals of one sex have a greater tendency to disperse away from natal sites or disperse further from those sites than members of the other more philopatric sex. The net result is that neighbouring adults, that are likely to become mating partners, are probably less related to each other than if each sex had dispersed equally. Greenwood (1980) hypothesised that differences among species in the form of sex biased dispersal, for instance which sex disperses furthest, are defined by factors promoting increased access to mates or resources. Greenwood's theory suggests that male biased dispersal is more likely in polygynous species and female based dispersal in monogamous species (Favre; *et al.* 1997). In polygynous species, because the process of transforming resource into offspring (a female task) is much more time consuming than that of fertilizing females (a male task), local mate competition among males normally exceeds local resource competition among females. This asymmetry is expected to induce a male - biased dispersal (Perrin and Mazalov 2000). In monogamous species familiarity with local resources should be more important for males, who defend nests and territories thus leading to the dispersal of females when resource competition is high (Greenwood 1980). Female biased dispersal is most commonly seen in birds (Greenwood and Harvey 1982) but is also found in mammals (Favare *et al*. 1997).
Sex biased dispersal is more complicated than the initial dichotomy proposed by Greenwood, with mating systems playing a role in favouring sex biased dispersal as limiting resources are often sex-specific (Perrin and Mazalov 2000). Indeed Handley and Perin (2007) suggested that the role of social systems emerges as a key factor in determining intensity and direction of dispersal bias among mammals. This may indeed be true for other taxa as sociality has been found to influence the magnitude of the sex bias in dispersal, and particularly long-lived, highly social, polygynous animals (Greenwood and Harvey 1982; Smale *et al.*1997).

Social systems appear to influence the dispersal of *Egernia whitii* where both monogamy and polgany have been recorded, and where there is lack of consistent pattern with regard to sex-biased dispersal, with trends varying amongst breeding seasons (Chapple and Keogh 2005). The variation in dispersal may be due to social organization related traits such group composition, group size, group stability, as social groups and levels of extra-pair paternity varied potentially representing

flexible dispersal adaptations to local conditions (Chapple and Keogh 2005; While *et al*. 2009)

Avoidance of inbreeding and kin competition will also affect sex biased dispersal as they act as strategies that separate opposite sex siblings prior to mating (Pusey & Wolf 1996). This has been shown in some species of *Antechinus* where almost all juvenile males disperse shortly after they are weaned, while females are strongly philopatric (Cockburn *et al.* 1985). Dispersing males were most likely to settle in patches where resident females were unrelated to them (Banks and Lindenmayer 2014).

Fragmentation of habitat influences the bias in the dispersal of sexes (Stow *et al.* 2001) through alteration of the processes discussed above by reducing the opportunities for successful dispersal.

Habitat fragmentation.

Fragmentation occurs when habitat is divided in to two or more pieces by some form of disturbance. It can impact on both the geographic range of a species and the demographic size of individual populations. Historically fragmentation was caused by large scale natural events such as the rise and fall of oceans or mountains, or shorter more localised events such as fires, landslides and floods. More recently fragmentation has resulted from anthropogenic activities, and now usually results from agriculture or development where extensive areas of natural habitat become converted to other states such as monoculture agriculture or cleared forestry areas. This new form of fragmentation can happen rapidly and be widespread.

The remaining fragments of natural habitat are often isolated from each other by degraded or unfavourable habitat. Any continuous population of a species that inhabited the area previously is now divided into multiple smaller populations. In many instances, fragmentation causes interruption to dispersal processes (Saunders *et al.*1991), so that populations in the fragments become isolated from each other.

Habitat fragmentation may inhibit movement of one or both of the sexes, or result in greater exposure to deleterious risks for those individuals that do attempt to disperse (Stow *et al.* 2001). The resultant isolated patches can then develop lower genetic diversity and a higher genetic similarity of individuals within sites (Gaines *et al.* 1997; Harrisson *et al.* 2013). A decrease in genetic diversity was recorded in eastern collard lizards in fragmented habitat, although this decline was subsequently turned around with management actions to reconnect the habitat (Neuwald and Templeton 2013). Fragmentation of habitat increases the proportion of inhospitable and risky area in the landscape, increases the ratio of edges to interior habitats, and decreases habitat connectivity (Saunders *et al.* 1991).

A consequence of the reduced dispersal is that small populations will have related individuals living close to each other, with higher risk of inbreeding. However, populations in fragments have been shown to respond to this risk by altering behaviours to avoid inbreeding. Peacock and Smith (1997) found mating changed from non random choice of partners in continous habitat to more random mating in fragmented habitat areas. In mountain possums the mating system was shown to change from monogamy in a natural continuous forest habitat, to polygamy (and the subsequent increase of genetic diversity within each litter) in fragmented habitat, resulting from the change of distribution of resources available to the female possums and the increased overlap with male territories in the fragmented habitat (Martin and Martin 2007). This change from monogamy to polygamy leads to increased genetic variation among offspring even if some of the parents are closely related. Fragmentation that affects the female density and distribution, also leads to change in territory and risk behaviours of the males (Haapakoski and Ylönen 2010).

The probability of inbreeding can also increase with declining population size due to there being fewer choices of mating partners from which to select (Banks *et al.* 2007). Thus small isolated populations face a possible double challenge from lower genetic variation coupled with smaller effective population sizes, reducing their capacity to evolve appropriate adaptations to new conditions, and increasing the potential for inbreeding depression (Saccheri *et al.* 1998; Keller and Waller 2002).

One of the key premises of conservation is to prevent loss of genetic variation, particularly in small or isolated populations. Species that have evolved in continuous habitat may be poorly adapted to moving between or living within small fragments, especially those species with small dispersal distances or low population densities.

When considering habitat in a fragmented landscape both the size of the patch and its distance from other patches are important in determining whether a population within the patch will be viable (Saunders 1991). The size of the patch has a direct influence on the number of individuals that can be supported within the patch and shorter distances between fragments may increase the opportunity for dispersal to augment or recolonise the fragment.

Species with low numbers of individuals in individual populations in disjunct fragments of habitat are often considered at high risk of extinction. This is partly due to demographic stochasticity having a larger impact on small populations than in larger ones. For any given period of time births and deaths will not be equal nor will the immigration or emigration from a population (Heatwole and Taylor 1987). In small populations these fluctuations increase the probability of extinction by chance alone (Shaffer1981; Newman and Pilson 1997).

Having many inter-connected populations reduces the risk of the permanent extinction of any one population, as connections allow the potential for recolonisation by individuals from other nearby populations. However should the fragmentation become so great that movement between populations is prevented then isolated populations risk permanent extinction. When dispersal between fragmented habitats is no longer possible species and populations may benefit from translocations among fragments, a management strategy that mimics dispersal.

The ability of a species to cope with fragmentation and move between suitable patches will vary among species and individuals (Hawkes 2009; Hamback *et al.* 2010; Betts *et al.* 2014). For example in common lizards (*Lacerta vivipera*) larger individuals were more likely to disperse between habitat fragments when connectivity was low whereas a greater proportion of smaller individuals dispersed when connectivity was high (Cote and Clobert 2010).

Measuring dispersal and movement

Researchers studying dispersal need quantitative measures of dispersal rates. Dispersal is inherently difficult to measure directly, particularly because a very small proportion of dispersers, which are difficult to detect, may have a disproportionally large influence on the dispersal outcomes of a population. For instance we may accurately measure how far individuals disperse over a short distance range, but the few individuals that make long-distance dispersal, and that will be hardest to detect, may have the greatest impact on gene flow among isolated populations. Many current dispersal studies do not adequately represent the actual patterns of dispersal (Koenig, *et al.* 1996; Van Houtan 2010; Marmet 2011; Byrne *et al.* 2014). These studies do not account for the fact that while short distance dispersal movements are recorded many of the long distance movements are missed. Under representation of long distance movements is a consequence of the decreasing probability of detection with increasing distance within a finite study area. Koenig *et al.* (1996) suggests that traditional plot-based methods may underestimate actual mean dispersal values in many vertebrate species by a factor of between three to nine and recent studies support this (Saurola and Francis 2004; Fedy *et al*. 2008; Estes-Zumpf *et al.* 2010). These biases can be countered or accounted for through methods such as, surveying very large areas relative to dispersal distances, monitoring nearby sites to determine the number of individuals passing between the study areas, radio tracking and genetic studies (Koenig, *et al.* 1996).

Much of the basic background information on dispersal rates, important for conserving species, is even more difficult to obtain directly, for secretive, nocturnal and fossorial species that are hard to observe. Management actions are often based on perceived geographic barriers to dispersal or fragment isolation, without specific information on how these might actually relate to population structure (Clostio *et al.* 2012). However genetic analysis of population structure, can provide indirect inferences about dispersal rates, and has much to offer for management of endangered species.

Genetic measurements

Molecular analyses of DNA from non-invasive sampling can provide information of importance to conservation and can expand on information collected from field observations. Genetic analysis can be used to determine, among other things, the sex of individuals in species where sexes are morphologically indistinguishable, population size, demographic history, mating and breeding systems, population structure, gene flow, parentage, sites for reintroductions and translocations, identity of disease organisms and diet (Allendorf and Luikart, 2006; Frankham *et al.* 2010) Molecular techniques have allowed the estimation of genetic and phylogenetic relatedness among individuals, populations, species and higher taxa (Hughes 1998). These techniques have been used in the comparison of historical and current movements across a landscape (Lada *et. al.* 2008; Chiucchi and Gibbs 2010). Specifically, for the study of dispersal, molecular genetic techniques It can be used to detect rare, but important long distance dispersal events that are often missed by trapping studies and direct observations (Koenig *et al.* 1996). Molecular techniques can also be used to detect differences in the dispersal of the sexes (Ujivari *et al.* 2008). More generally, they provide useful tools that do not rely on direct observation of the movement or mating, but can be used to trace where an individual originated from and who they have mated with and how successfully (Broquet and Petit 2009).

Within populations, molecular techniques can also reveal unexpected patterns of genetic transmission resulting from diverse behavioural tactics that individuals employ (Hughes 1998). They are particularly valuable for species that are difficult to track or have cryptic mating systems (Haig 1998) and allow insights into relatedness and kin interactions, for example detailing how reproductive success is partitioned among individuals in a group (Hughes 1998). For instance, microsatellite DNA analyses have commonly uncovered extra pair paternity in species that are socially monogamous (Griffith 2010) and have allowed identification of parents of offspring when there is little or no parental care. Accurate parentage assignment allows for the determination of the genetic payoff for observed behavioural strategies. (Hughes 1998).

Conservation Units

An additional role of molecular genetic analyses in conservation biology has been in the identification of subsets of populations that can be considered as separate conservation units. Over the past two decades, the use of population analyses of genetic markers has increased substantially as an indirect means of inferring units for conservations (Moritz 1994; Fraser and Bernatches 2001; Palsbøl *et al*. 2006). Units can be thought of as groups of one or more populations of conspecific individuals among which the degree of differentiation or connectivity, through immigration, is sufficiently low so that each unit or group of populations should be monitored and managed separately (Taylor and Dizon, 1999). There has been some debate as to how these units should be defined (Crandall 2000, Fraser and Bernatches 2001). An early suggestion by Moritz (1994) defined Ecologically Significant Units within a species as reciprocally monophyletic groups (i.e. individuals within a group share a more recent common ancestor with individuals in the same group than any individuals share with individuals in the other group) whereby evolutionary heritage within species will be maintained by managing these units separately. More recently Conservation Units have been described in a context based frame work for conservation proposed by Fraser and Bernatches (2001) whereby differentially weighted criteria used alone or in combination (Stockwell *et al*. 2003; Frankham 2010; Moritz and Potter 2013).

While there is debate about the definition, there is broader agreement that conservation units represent the range of genetic diversity in a species, and that conservation managers should now be undertaking conservation and restoration practises that maintain and increase genetic diversity within species, as well as extending the species range, thereby promoting in situ adaptive processes (Crandall 2000; Fraser and Bernatches 2001; Thomas 2011; Weeks *et al.* 2011).

Translocations

Translocations can be considered as a form of assisted dispersal, moving individuals along paths that would be difficult for them to disperse along independently, and are often used where there is little or no immigration between populations (Hedrick 1995). Relocating animals within their range or to a former part of their range has

become an increasingly commonly used tool in the conservation of threatened species (Fischer and Lindenmayer 2000). Some ecologists suggest that carefully planned translocations are the best way to conserve threatened species, or species with dispersal corridors too tenuous to allow a natural dispersal and colonization process (Hulme 2005). One strategy often used in the conservation of endangered species is to capture the animals from the wild, hold them for a period of time in a controlled and protected environment and then release them in suitable habitat in the wild. For example juveniles can be collected and reared and then released back into a population at a size and age when they are less vulnerable to predators (Priddel and Wheeler1996; Alberts 2007). Or individuals could be taken from a site that is impacted by sudden adverse effects (such as drought, pollutants or fire) and released back when those threats have passed. Alternatively release at another site could augment an existing low density population, or establish a new population where the species is not currently found (Liu *et al*. 2012). In all cases it is desirable to minimise the impact on the source population and to maximise the chance of establishment at the release site.

Translocations, when necessary, should be targeted at increasing gene flow between isolated populations of a species or populations to maintain their adaptive potential as well as reducing future environmental challenges to species (Weeks *et al.* 2011; Breed 2013). The use of translocations to accomplish gene flow between populations that reverses inbreeding, recovers genetic diversity, to improve reproductive fitness, or future proof a species against climate change is sometimes limited due to concerns about outbreeding depression (Edmands 2007). However the probability of outbreeding is much less than the probability of population extirpation due to inbreeding depression and loss of genetic diversity in separate, small, isolated populations (Frankham 2011).

For translocations to 'future proof' species against climate change conservation managers should now be undertaking conservation and restoration practises that maintain and increase genetic diversity within species, as well as extending the species range, thereby promoting in situ adaptive processes (Thomas 2011; Weeks *et al.* 2011). In the case of range extension source material from more distant

(geographically and ecologically) populations may harbour adaptations that more closely match the environment of the focal restoration site today and into the future.

Before translocations of an endangered species are planned, we need detailed information about the natural dispersal behaviour, about the genetic structure and breeding systems within natural populations, and about the broader genetic relationships among existing populations of the species. This thesis explores those questions in an endangered Australian lizard, the pygmy bluetongue lizard, *Tiliqua adelaidensis*.

Pygmy bluetongue lizards

Background

The pygmy bluetongue lizard is an endangered lizard found to live exclusively in the fragmented native grassland remnants of the mid north of South Australia (Armstrong *et al*. 1993; Hutchinson *et al*. 1994) Historically the pygmy bluetongue lizard's geographic range extended over at least 150km, from the Adelaide plains to Burra in the mid north (Ehmann, 1982). Despite rigorous searching there were no new records collected for 30 years after 1960, until a specimen was discovered dead in the stomach of a brown snake, *Pseudonaja textilis* (Armstrong and Reid 1992).

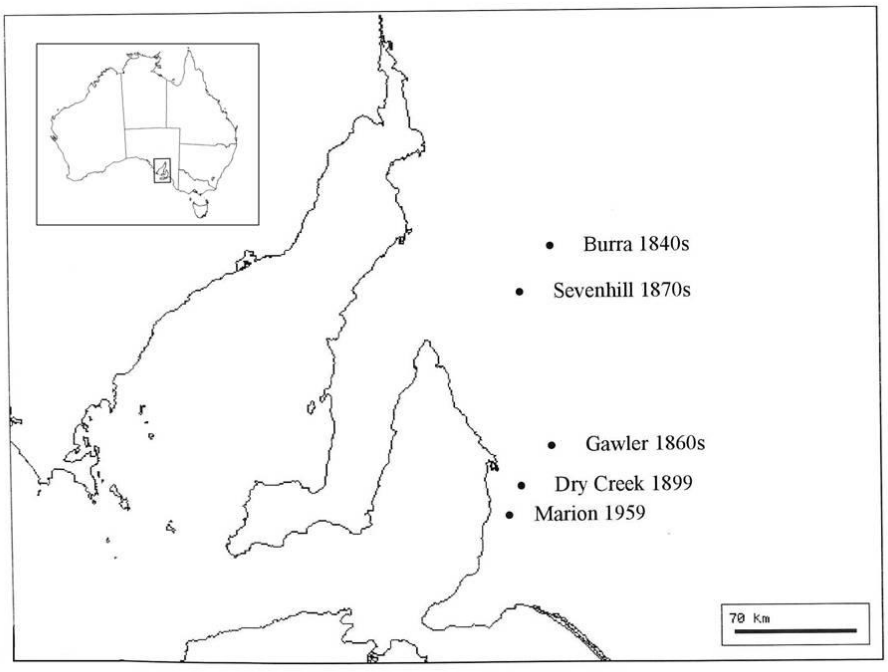

Figure 1. Historical sightings of Pygmy Bluetongue lizards. Sourced from Milne 1999

Since their rediscovery, 31 small populations have been located within the mid north of South Australia. They represent a much reduced geographical range, and all known populations are on private land, potentially threatened by agricultural disturbance of the native grassland (Milne 1999)(Fig. 3). Habitat loss and fragmentation are one of the major threats to the conservation of biodiversity as discussed above. This is particularly pertinent for species that are less mobile, such as small reptiles, and as such less able to move between existing habitat fragments. The pygmy bluetongue lizard is classified as endangered and in the future translocations may be an important tool for the conservation management of this species (Fordham *et al.* 2012).

Figure 2. Example of native grasslands used by pygmy blutongue lizards fragmented by uninhabitable cropped areas .

Burrow usage

The pygmy bluetongue lizard has been found to shelter almost exclusively in near vertical, single entrance burrows constructed by lycosid and mygalomorph spiders (Fig. 3). The lizards rely extensively on these burrows for survival and thus preservation of the burrows is essential for the long-term sustainability of this species (Milne *et al.* 2003). For the pygmy bluetongue lizards there is a suggestion of social structuring where individuals maintain areas that contain resources and defend the area from conspecifics (Fenner and Bull 2010, Fenner and Bull 2011) however this social structuring has not yet been confirmed.

Figure 3. Pygmy bluetongue lizard at the entrance of its burrow.

The pygmy bluetongue lizard exhibits a territorial defence of their burrow when conspecific intruders are close, but will not move away from their burrow entrance to defend against conspecifics (Fenner and Bull 2011). Mating occurs in spring (Oct – early Nov) and litters of 2-4 live young are born late in the following summer (Feb – March) inside the mothers burrow (Milne 1999). Neonates disperse from the natal burrow within a few weeks (Milne and Bull 2002).

Lizards are more frequently found in areas with greater number of deep spider burrows (Fig. 4) suggesting burrows are one limiting factor in populations (Souter 2007). The dimensions of the burrows are important to the lizards (Milne 2003). Observations by Fellows *et al.* (2009) have shown that deeper holes are occupied preferentially to other shallow holes. However lizards will use artificial burrows, straight holes dug into the ground, and the placement of artificial burrows has also been shown to increase the numbers of lizards within an area. Studies have shown juvenile pygmy bluetongue lizards will occupy artificial burrows once they leave the maternal burrow, and that local recruitment of juvenile lizards can be increased with extra burrows (Souter *et al.* 2004), suggesting that suitable burrows may be a limiting factor leading to juvenile dispersal.

Figure 4. Spider burrow now occupied by a pygmy bluetongue lizard.

Previous studies that have investigated the length of time an individual will stay in a burrow have found that some adult lizards can retain the same burrow for over two years (Bull *et al*. 2014), but that males are more likely to move especially during the mating period in November (Milne 1999; Fellows 2008). Milne (1999) reported that on average females remained in the same burrow for 8.6 weeks whereas males averaged 4.3 weeks. Juveniles remained in the same burrow on average for 8.1 weeks. These findings are consistent with the findings of Fellows (2008) who found males were more likely to spend shorter periods of time in a given burrow.

Distances moved

Pygmy bluetongue lizards have been observed to spend most of their time associated with their burrow, either sheltering inside the burrow or active at their burrow entrance either basking or ambushing passing invertebrate prey. This suggests a largely sedentary life style, although some studies have observed lizards leaving burrows (Milne 2003; Fenner and Bull 2010) and individuals are sometimes caught in pitfall traps away from their burrows (Milne 1999). Lizards are dormant over the winter months (June, July, August) with activity beginning in September and peaking in November. Activity drops in December and decreases further in January. It then increases from February to April, after neonates are born, and decreases again in May with the onset of winter (Milne 1999)

The lizards seem to remain in the same area for long periods of time with only small movements, usually to locate a more suitable burrow (Fellows 2008). Milne (1999)

rarely recorded female lizards moving more than 20 meters from the burrow they were originally located in. Other genetic studies undertaken by Smith (2006) suggested possible male dispersal bias and reported that related females were more clustered than expected by random chance. Both genetic and field studies showed that females can move up to 200m however these longer distance movements are uncommon (Milne 1999; Smith 2006).

The longer distance movements for male lizards are less clear. Among males that had left their burrows, few males were recaptured within a 1ha monitoring area but it is unknown if the low recapture rate was because individuals were dispersing from the area or if they have been subject to predation (Milne 1999; Fellows 2008).

The movement and dispersal of juveniles is also less clear. A proportion of the annual recruits may have to disperse from the site, to find appropriate burrows. In all studies to date the recapture of juveniles has been rare, but the furthest observed distance is 60 m over a two year period (Milne 1999). Factors which may increase the dispersal of juveniles from a site may be territoriality and density of resident adult lizards within the area. Factors limiting the success of dispersal may be the time taken to find a new burrow, and the subsequent lack of refuge for protection against temperature extremes and predation.

Research Aims

The 31 known populations of the pygmy bluetongue lizard are on private land and as such potentially threatened by agricultural practices or land use change on the native grassland habitat (Milne 1999). All known sites are isolated by ploughed fields, which are areas of high mortality risk for dispersing lizards (Souter 2003). Climate change has been shown to reduce available habitat and metapopulation connectivity in other lizard species (Massot *et al.* 2008). Fordham *et al.* (2012) predicted that climate change will negatively impact current populations of pygmy bluetongue lizards as well as shifting the habitable range of the lizard. They suggested that long-term persistence of this species would require translocations.

The major aim of this project was to investigate the levels of dispersal within populations and among localities within the fragmented habitat of the endangered

pygmy bluetongue lizard *Tiliqua adelaidensis* to provide information to conservation managers on dispersal, mating systems, genetic structure both within and among populations, and population dynamics of this species. This information will be critical for management decisions to maintain current populations and to prepare for future translocation efforts if they are warranted.

Research questions:

Chapter 2: What is the different movement patterns of different age classes or sexes? Does the amount of movement differ between the start and the end of the active season?

Chapter 3: What is the mating system of the pygmy bluetongue lizard? Do the lizards choose less related individuals to mate with?

Chapter 4: Is there genetic clustering within populations?

Chapter 5: Is there phylogenetic structuring between the populations of pygmy bluetongue lizards ? and is this structuring recent or historic?

Chapter 6: How do the results of this thesis help inform the conservation management of the endangered pygmy bluetongue lizard?

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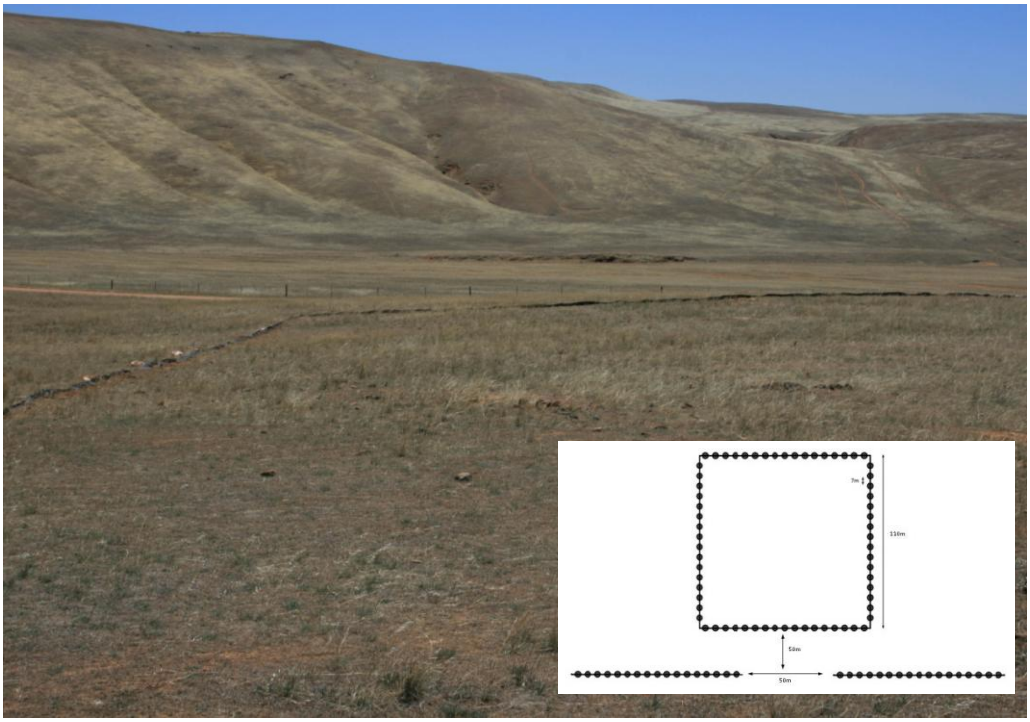
Chapter 2. Movement of individuals

Dynamic and complex factors drive movement and dispersal, and can influence individuals within populations differently. Movements can either reflect local activity, or longer-distance dispersal to a different area. Within a species, there can be variation among age classes and between sexes in the amount of movement activity.

Previous studies on *Tiliqua adelaidensis* had suggested a largely sedentary life style with occasional movements by adult lizards. This chapter investigates the movement patterns of different sex and age classes within a population, and their timing. Understanding movement of cohorts can inform conservation actions through understanding dispersal and the potential loss of individuals from the population from predation. It may also help inform the selection of individuals if translocation becomes necessary.



Sampling grid arrangement described in this chapter, where black circles represent pitfall traps and the lines denote plastic drift fence.

Conceived and designed the pitfall trapping surveys: JS AF MB. Performed the surveys: JS AF KP. Analyzed the data: JS. Contributed to the writing of the manuscript: JS MB.

Male-biased movement in pygmy bluetongue lizards: implications for conservation. Wildlife Research 39, 677-684

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Abstract

Context. Translocation has become an increasingly common tool in the conservation of species. Understanding the movement patterns of some species can be important to minimise loss of individuals from the translocation release site.

Aims. To describe seasonal and sex-biased movements within populations of an endangered Australian lizard.

Methods. We monitored seasonal movement in the endangered pygmy bluetongue lizard (*Tiliqua adelaidensis*) by using pitfall trapping, with a total of 49 440 trap-nights from three sites over 2 years. Other studies have shown that individual pygmy bluetongue lizards normally remained closely associated with their spider burrow refuges, with very little movement.

Thus, we interpreted any captures detected through pitfall trapping as out of burrow movements. We investigated whether there was any seasonal, age or sex bias in moving individuals.

Key results. We found that male pygmy bluetongue lizards were more likely to move than were females. After adults, neonates were the second-most captured age class. Spring was the peak movement time for adults, whereas movement of neonates occurred in autumn.

Key conclusions. The majority of movement can be attributed to males in the breeding season, whereas females move very little.

Implications. The present study provides some baseline data that would allow more informed decisions about the most appropriate individuals in a population to choose for a translocation program and the times to conduct translocations to allow the maximum chance for establishment.

Introduction

Translocations are now commonly considered as an option to address abundance declines in endangered species. There have been previous successes in introducing animals onto islands and in predator-exclusion areas (Towns and Ferreira 2001; Moseby *et al*. 2011). However, in species that are limited by habitat availability, it is important to understand the movement dynamics of a species before undertaking such conservation actions to allow the management of translocation releases, to ensure that the maximum number of individuals remain near the release site. In a review published by Germano and Bishop (2009), after poor habitat selection, the most common reason for translocation failure was due to homing and migration of introduced individuals out of release sites.

Dynamic and complex factors drive movement and dispersal, and can influence the individuals within populations differently (Clobert *et al*. 2001, 2009; Ims and Andreassen 2005). Evolutionary explanations for movement are often about dispersal from natal sites and include the reduction of inbreeding and kin-competition (Clobert *et al*. 2001). Ecological explanations suggest that movement is a more immediate response to variation in levels of resources, parasitism or predation (Andrewartha and Birch 1984; Dobson and Jones 1985).

Movements can either reflect local activity, or longer-distance dispersal to a different area. Within a species, there can be variation among age classes and between sexes in the amount of movement activity. Natal dispersal occurs when young leave their place of birth, whereas breeding dispersal is the movement of adult individuals between successive breeding events (Greenwood 1980; Clobert *et al*. 2001). The timing and distance of each of these events can vary with sex and age.

In both types of dispersal, the sex that is more likely to disperse also varies among species. Among bird species, it is more common for females to disperse, and to disperse for longer distances than males do, whereas male-biased dispersal is the more common pattern in mammal species (Greenwood 1980). The more dispersive sex or age group is also likely to show more short-term movement activity within a population site (King and Duvall 1990; Croft *et al*. 2003), and a lower likelihood of remaining at translocation release sites.

Male-biased dispersal has been the most commonly reported pattern in reptiles, including crocodiles, turtles and snakes (Tucker *et al*. 1998; Paquette *et al*. 2010). Within lizards, male-biased dispersal has been reported, for example, in adults of *Anolis sagrei* (Polychrotidae) (Calsbeek 2009) and *Lacerta agilis* (Lacertidae) (Olsson *et al*. 1996), and in juveniles of *Uta stansburiana* (Phrynosomatidae) (Doughty *et al*. 1994), *Eulamprus leuraensis* (Scincidae) (Dubey and Shine 2010) and *Chlamydosaurus kingii* (Agamidae) (Ujvari *et al*. 2008). In contrast, female-biased dispersal has been reported in two species of lizard, namely, *Lacerta agilis* (Olsson *et al*. 1996) and *Niveoscincus microlepidotus* (Olsson and Shine 2003).

Patterns of age- or sex-related movement and dispersal, across taxa in general, are not necessarily consistent among congeners or even among different populations of the same species (Herzig 1995; Matthysen 2005; Clobert *et al*. 2008; Lane and Shine 2011). In the Australian scincid genus *Egernia*, juveniles of *E. stokesii* usually remain within natal groups for several years, and reach adulthood before dispersing (Gardner *et al*. 2001), whereas juvenile dispersal in the first year after birth is most common in *E. whitii* (Chapple and Keogh 2006). In the rock crevice-dwelling *E. cunninghamii*, the bias towards male dispersal varies among populations, depending on the extent of ground cover around their rock outcrop population sites (Stow *et al*. 2001). This implies that it will be difficult to derive generalised predictions about movement patterns from members of the *Egernia* lineage of Australian scincid lizards.

The endangered pygmy bluetongue lizard, *Tiliqua adelaidensis*, belongs to the *Egernia* lineage (Gardner *et al*. 2008), and we aimed to describe differences in movement patterns across time, among age classes and between sexes in that species. It is a medium-size skink, endemic to a small area in the mid-northern region of South Australia. It was thought to be extinct and was then rediscovered in 1992 (Armstrong and Reid 1992). Currently, the main threatening processes to this species are land clearance and habitat fragmentation (Milne 1999). Pygmy bluetongue lizards spend most of their time associated with their home burrows, constructed by lycosid or mygalomorph spiders (Hutchinson *et al*. 1994; Milne 1999; Fenner and Bull 2011). They refuge in the burrows and use burrow entrances as basking sites and ambush sites to make short brief excursions to capture passing prey (Milne *et al*.

2003; Fenner *et al*. 2007; Fenner and Bull 2011). Pygmy bluetongue lizards have high site fidelity, with lizards using the same burrow for periods from 4 weeks to over a year (Fellows 2008). Video camera observations (Milne 1999; Milne *et al*. 2003) have shown that resident lizards rarely move more than 20 cm from their burrow for prey capture, and then generally return to the same burrow. Any movement further than 20 cm of the burrow usually results in the lizard not returning to that burrow (Milne 1999). When pygmy bluetongue lizards have been recorded to move between burrows, the distance moved has normally been less than 20m (Milne 1999; Fellows 2008). These observations may underestimate the actual movement distances because they are based on relatively few cases and because the surveys were spatially confined. Nevertheless, genetic analyses of population structure (Smith *et al*. 2009) showed a level of clustering of related individuals within local populations, which suggested both that life-time dispersal distances within populations were likely to be small, and that movements within these populations were infrequent.

Predictions of future demographics of this endangered lizard species suggest that translocations (or re-introductions to presumed previous parts of its range) will be an essential component for the managed persistence of this species under realistic climate-change scenarios (Fordham *et al*. 2012). Successful translocation relies on individuals remaining at the release sites long enough to become familiar with those sites. Thus, to maximise translocation success, we should choose less dispersive lizards and we should choose times when lizards are least dispersive. Our aim in the present study was to document movement patterns within a natural population of pygmy bluetongue lizards, to provide insights relevant to translocations.

In natural populations, pygmy bluetongue lizards are likely to undertake two main types of movements. First, movements could be local; for instance, when an adult male is moving around to find a mate, or when any lizard is seeking an alternative local burrow. Second, movements could be more dispersive when a lizard is moving away from its natal population so as to occupy and breed in a new area. Current populations of the pygmy bluetongue lizards are restricted to small, isolated fragments of native grassland, a habitat that was once more widespread (Hyde 1995). Those fragments are surrounded by a matrix of unsuitable, ploughed farmland

(Souter 2004). Dispersal attempts between adjacent populations by any age class or by either sex, are likely to be unsuccessful, as confirmed by the lack of evidence for recent gene flow between populations as close as 1 km apart (Smith *et al*. 2009). In the present study, we aimed to describe the extent and nature of movements within a population of pygmy bluetongue lizards. We assessed whether different sex or age classes differed in their temporal patterns of movement.

Materials and methods

The study was conducted over two spring–summer seasons (2008/09 and 2009/10) in two populations of *T. adelaidensis*, 11 km apart. In the present paper, we use the term 'season' to refer to the entire period from early spring to late summer (September–March) when these lizards are normally active (Hutchinson *et al*. 1994). Each population was located in remnant native-grassland habitat, in the mid-north of South Australia, within 13 km of Burra (33_42◦S, 138_56◦E). The region has a Mediterranean climate with cool, wet winters and hot, dry summers, with an average annual rainfall at Burra of 430mm (Bureau of Meteorology 2012). We used pitfall trapping to detect temporal patterns of movement within the lizard population. Because lizards are normally active only in the immediate vicinity of their burrow entrance, we interpreted any pitfall capture as a movement away from the burrow.

We constructed trapping grids at three sites, one (Site 1) within one population, and the other two (Sites 2 and 3) 1 km apart within the second population. Each grid had four 110-m-long trap lines set in a square (and thus enclosing an area of 1.2 ha), and two additional 110-m-long trap lines, parallel to one edge of the square and 50m from it. These additional trap lines formed a 270-m-long line with a 50-m central break. Each trap line had a 15-cm-high black-plastic drift fence and 16 bucket traps (20 L, 38 cm deep, 28.5 cm diameter), placed immediately under the drift fence, and spaced at 7-m intervals along its length. Bucket traps were divided in half with a plastic divider to differentiate between captures of lizards approaching the drift fence from either side. There were 96 bucket traps at each site. Traps remained open over individual trapping sessions that lasted from 5 to 10 days, and were inspected each morning and evening in those sessions. We used plastic lids to close the traps between trapping sessions. Fences remained in place between trapping sessions but were removed at the end of each season.

In the 2008/09 season, only Sites 1 and 2 were sampled for the whole season, and traps were open in those grids for 109 days (11 trapping sessions, 20 544 trap days) from 2 October 2008 until 15 March 2009. In the 2009/10 season, trapping took place over 79 days at all three sites (12 trapping sessions; 22 752 trap days) from 3 September 2009 to 15 March 2010. There was at least one trapping session in each of the 6 months October–March in each of the two seasons of field work.

Each new captured lizard was individually marked by toe clip and its sex, mass and snout-to-vent length (SVL) were recorded. Lizards were classified into neonates (SVL <50 mm), sub adults (SVL 50–80 mm) or adults (SVL >80 mm) (Milne 1999). The neonate class included individuals from the time when they were born (usually during February) until the end of that season (March). Thus, no neonates were recorded from September to January in either season. Sex was determined by the larger head size and shorter body length of males, and both adults and sub adults were sexed using this method. After processing, lizards were released on the opposite side of the fence line to reflect the direction they had been going when trapped. In analyses, data for the numbers of lizards captured each month were standardised to captures per 1000 trap days. We called this parameter the trapping rate.

The three sites were each 1 km or more apart. We considered that this was beyond the normal short-term movement range of a pygmy bluetongue lizard and we treated the sites as independent replicates. No lizard marked in one site was found in any other site over the 2 years of the study. Because trapping on site three was incomplete in the first season, we conducted two separate sets of analyses, with one including data only from Sites 1 and 2 but for both seasons, and the other including data from all three sites but only for the second season. We assessed the effect of sex, age class, month and season on trapping rate using repeated-measures ANOVA analyses (in SPSS16.0.1 IBM Corporation). Repeated measures were month (September–March) and season (2008/09 or 2009/10), and sex and age class were between-subject factors. The sites (Sites 1, 2 or 3) were used as replicates. We conducted separate analyses for sex (including only adults and sub adults; neonates are too small to accurately sex), and for age class (including adults, sub adults and neonates). When interpreting the results, we used significant interactions between sex (or age class) and time (month or season) to recognise different temporal patterns of

trapping rates (and thus of movement) among different groups of individuals in the population. Where the data were nonspherical the Greenhouse–Geisser correction was used in the analyses.

Results

During the study there were 104 captures in the pit-fall traps of 91 individual pygmy bluetongue lizards. There were 27 captures in the 2008/09 season and 77 in the 2009/10 season. Among the captures, 66 (63.5%) were adults, 23 (22.1%) sub adults and 15 (14.4%) neonates. Of the adult and sub adult captures, 69 (86.25%) were males and 11 (13.75%) were females. Captures were concentrated in October/November and February/March and were less frequent in the middle of each season (December/January) (Fig. 1).

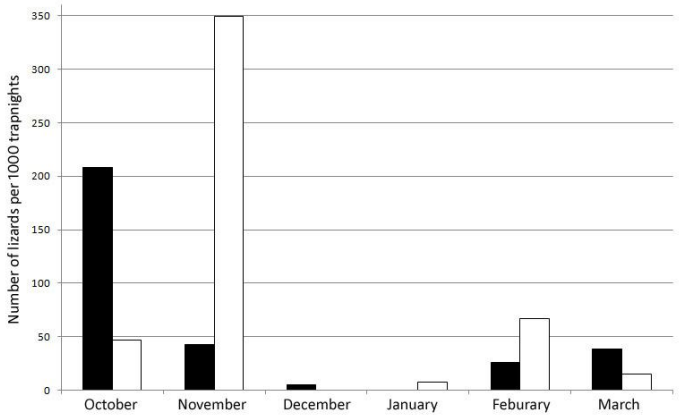


Figure 1. Trapping rates from all sites in each month of the study. Black bars indicate captures in the 2008/09 season; white bars indicate captures in the 2009/10 season

Recaptures

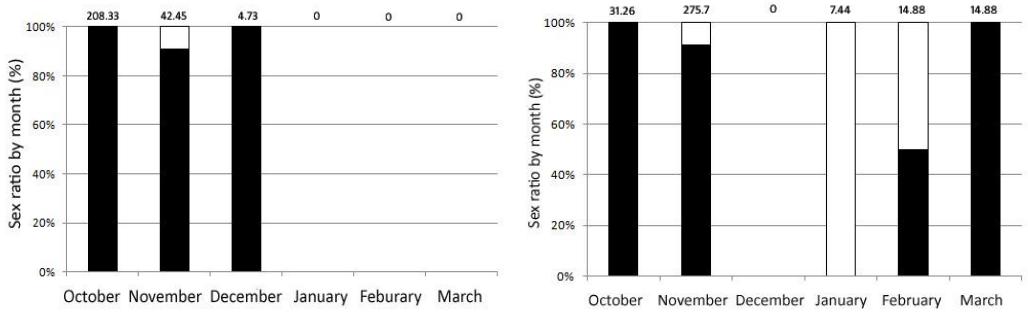
There were 12 recaptures of 10 individual lizards (eight adults, one sub adult and one neonate) over the two trapping seasons. Two adults and one sub adult were captured three times. Seven of the eight recaptured adults were males, as was the one recaptured sub adult. Thus, 88.9% of recaptures for which the sex could be identified were males. The mean time between recaptures was 97.4 days (s.e. = 162, range 1–369 days), and in all but three cases, the recapture was from the opposite side of the fence from the initial capture. The mean distance between the pitfall traps of the initial capture and the recapture was 11m (s.e. = 2.5, median = 7, range = 0–28 m).

Sex

Analysis of the trapping rates from two sites over 2 years (Table 1) showed a significant interaction between year, month and sex and a significant interaction between month and sex. Males were trapped significantly more than females in the early months of the season in both years, and although there were no captures of adult or subadult lizards in the later months of Season 1, males and females moved equally often later in Season 2 (Fig. 2).

Table 1. Results of repeated measures ANOVA on the effects of lizard sex, month and year on trapping rates of adult and sub adult *Tiliqua adelaidensis* over two seasons at sites 1 and 2.

	df	**F**	**Sig.**
Sex	1, 6	19.38	<0.001
Month	5, 30	24.61	<0.001
Year	1, 6	1.11	0.33
Month * Sex	5, 30	19.87	<0.001
Year * Sex	1, 6	0.23	0.65
Year *Month	5, 30	13.07	<0.001
Month * Year * Sex	5, 30	8.08	<0.001



2 (a) 2(b)

Figure 2. Sex ratio of pygmy bluetongue lizards captured in pitfall traps at site 1 and 2 during (a) 08/09 (b) and 09/10 active seasons. (Black bars percent male lizards; white bars percent female lizards). Trapping rate of lizards per 1000 trap days per month above graph.

The trapping of more males than females earlier in the season was also reflected in the significant interaction effect of month and sex (Tables 1, 2). There were also significant main effects of month and of sex, with most trapping records of adult and subadult lizards in the early months of the season and by males moving in that period (Fig. 2). Analysis of all three sites in the second year showed similar significant trends (Table 2, Fig. 3).

Table 2. Results of repeated measures ANOVA on the effect of lizard sex and month on trapping rates of *Tiliqua adelaidensis* at all 3 sites during the 2009/10 season.

	df	**F**	**Sig.**
Sex	1, 10	6.85	0.03
Month	5, 50	14.13	<0.001
Month * Sex	5, 50	8.30	<0.001

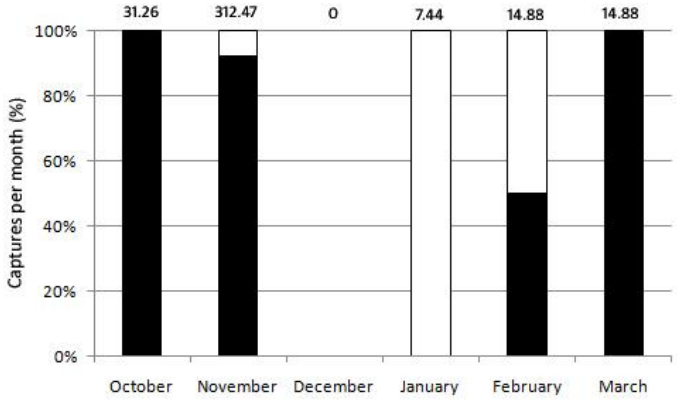


Figure 3. Sex ratio of adult pygmy bluetongue lizards captured in pitfall traps at all sites during 09/10 active seasons. (Black bars percent male lizards; white bars percent female lizards). Trapping rates of lizards per 1000 trap days per month above graph.

Age class

Neonates are born in late January and early February and were trapped only in February and March of each season. Nevertheless, the analyses (Tables 3, 4) detected no significant effects of age class, or interactions of age class with month or year, to indicate that adults, sub adults or neonates differed in the times when they were trapped. The analysis for the two sites over 2 years confirmed a significant interaction

between year and month and a significant effect of month for overall trap rates (Table 3). Again this reflected an overall difference of the timing of movement, with most lizards trapped early in the season, although the pattern differed between seasons (Fig. 1).

Table 3. Results of repeated measures ANOVA on the effects of month and year of the trapping rates of the different age classes of *Tiliqua adelaidensis* over two seasons and two monitoring sites

	df	**F**	**Sig.**
Age class	2, 9	1.44	0.29
Month	1.30, 11.71	5.37	0.03
Year	1, 9	0.23	0.64
Month * Age class	2.6, 11.71	1.65	0.23
Year * Age class	2, 9	1.95	0.20
Year * Month	2.13, 19.24	5.63	0.01
Month * Year * Age class	4.28, 19.24	1.72	0.18

Table 4. Results of repeated measures ANOVA on the effect of month on the trapping rates of the different age classes of *Tiliqua adelaidensis* at 3 sites during the 09/10 activity season

	df	**F**	**Sig.**
Age class	2, 15	1.67	0.22
Month	1.43, 21.42	7.80	0.01
Month * Age class	2.86, 21.42	2.57	0.08

In the analysis of all three sites over 1 year, there was a marginally significant interaction effect of month and age class, with more adults trapped earlier in the season (an expected result because there were no neonates present then), and more neonates trapped later in the season (Fig. 4). The significant effect of month (Table 4) reflected the higher trapping rates earlier in the season.

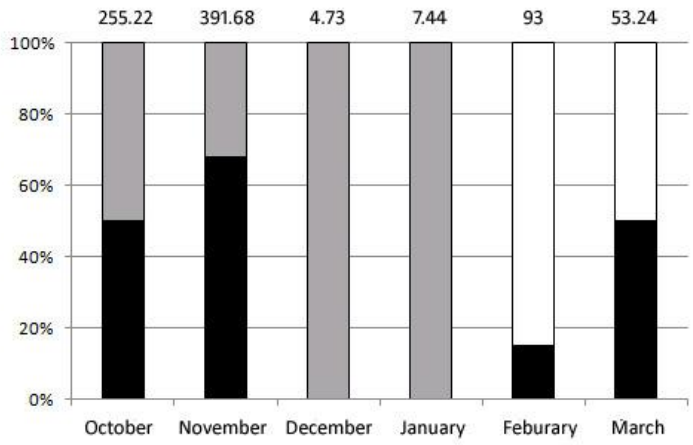


Figure 4. Percent of pygmy bluetongue lizards captured by age class in pitfall traps at all sites during 08/09 and 09/10 active seasons (Black bars percent adult lizards, Grey bars percent sub adults and white bars percent neonate lizards). Trapping rates of lizards per 1000 trap days per month above graph.

Discussion

We interpreted a capture in the pit-fall traplines to indicate that a lizard was moving from its burrow. This was based on previous observations that resident lizards rarely move from their burrows (Milne *et al*. 2003; Fenner and Bull 2011), and vigorously defend their burrows from potential conspecific intruders, but only if they do not have to move out of the burrow area (Fenner and Bull 2011). The movements we detected in the present study may have been either local movements that were restricted to the immediate area, or dispersal movements away from the local area. Of the individuals that were trapped,11% were subsequently recaptured, close to their original capture site but on the opposite side of the trapline. These probably represent local movements. This suggests that even when they leave their burrow, some lizards do not move far, and probably remain in or return to the local area of the burrow they originally moved from. Our data are likely to have underestimated the actual number of trapped lizards returning, because there were periods between trapping sessions when the traps were closed. Nevertheless, we still considered a trap capture to indicate that a lizard had abandoned its original burrow and was moving around in search of a new burrow site, or of mating opportunities. During any movement, a lizard will be at greater risk from predation because it is more exposed. Fenner *et al*. (2008) used the frequency of tail damage to infer a high rate of

predation attempts on pygmy bluetongue lizards. Snakes and raptors are confirmed predators of pygmy bluetongue lizards (Armstrong and Reid 1993; Hutchinson *et al*. 1994). An additional risk for moving lizards is that they may move into suboptimal habitat where few suitable refuge burrows are available. Now that populations persist only in smaller fragments surrounded by poor habitat, the risk of not finding a suitable burrow may have increased.

We inferred from trapping-rate differences that male pygmy bluetongue lizards are more likely to move than females. This is consistent with previous indirect observations of movement in this species. Male lizards were the first individuals found (out of their burrows) when the species was rediscovered in 1992 (Armstrong and Reid 1992; Milne 1999). Fellows (2008) reported that male pygmy bluetongue lizards occupied burrows for a shorter time and moved more frequently than did females. Similarly, previous genetic analysis (Smith *et al*. 2009) suggested that males moved farther than did females during dispersal.

We also inferred a temporal influence on movement, with the strong male bias occurring in the trapping records in early spring. The original rediscovery of the species in October 1992, in the early part of that season, involved a male pygmy bluetongue lizard in the stomach of a brown snake, and the lizard was probably away from its burrow when it was preyed on (Armstrong and Reid 1992). In many other reptile species, males move around seeking female partners early in their activity season (Spoecker 1967; Kerr and Bull 2006; Weaver 2010). Similarly, male pygmy bluetongue lizards have been observed away from their own home burrows and approaching occupied female burrows early in the season (Milne 1999; Fenner and Bull 2009). We have not observed females moving to gain mating opportunities. We do not know whether males that are searching for mates return to their original burrow or move to a new burrow after they have finished mating. Some other spring movement, by both sexes, could be associated with the deterioration of their current burrow over winter.

In the hotter and drier months of December to January, we detected little movement through pitfall captures. If individuals are not moving during that period, they

probably retain a stable spatial arrangement, and this reflects the previously reported strong stability of burrow association in this species (Fellows *et al*. 2009). Movement during late summer (February/March) was inconsistent between seasons. The Burra region had one of the driest years on record in 2008, and in the 2008/09 season, there was no detected movement of adults in the late summer period. The lack of vegetation cover or of prey items during dry seasons may reduce the movements of lizards (Fenner and Bull 2007). In the 2009/10 season, with more normal rainfall, adult males and females were equally mobile in late summer, although they were trapped at much lower rates than were males in spring. These individuals may have been searching for more suitable burrows to over-winter in, or to establish new burrows for the following spring.

We found that movement of neonates occurred in late summer (February/March), following parturition. Dispersal from the maternal burrow, soon after birth, has previously been reported in pygmy bluetongue lizards, and neonates will move into artificial burrows during these months (Milne *et al*. 2002, 2003; Souter *et al*. 2004). Neonates have also been observed making exploratory movements near their maternal burrow before eventually dispersing (J. Schofield, pers. obs.). Thus, we would expect to trap moving neonates during those months.

An unexpected result was the relatively low trapping rate of neonates, even though they disperse from their natal burrows soon after birth (Milne *et al*. 2002). There are several possible explanations. Neonates may have avoided traps if their movement distances were short, if they detected and avoided the traps more effectively than did adults, or if they moved predominantly during non-trapping periods. Alternatively, moving neonates may have suffered a higher predation mortality than did adults, leaving fewer to be captured in traps. Milne (1999) found that annual first year mortality was 64–93.3% over his 4-year study.

Implications for conservation

The movements that we have reported in the present study are characteristic of many other lizard species (Spoecker 1967; Doughty *et al*. 1994; Olsson *et al*. 1996; Calsbeek 2009; Dubey and Shine 2010), and are part of the normal population processes in response to temporal changes in resource availability, and that would

lead to reduced levels of inbreeding and kin competition. In a continuous habitat, or one with habitat patches connected by dispersal corridors, it would be expected that some of the annual recruitment would disperse to adjacent sites, and that this dispersal might buffer populations from local demographic loss. In addition, the genetic mixing from population exchanges should provide the variation to allow each population to adapt to changing conditions. Those processes are less frequent in fragmented habitat.

One potential conservation strategy to prevent the decline of population abundance and genetic diversity may be to replicate 'natural' movement between adjacent populations through the managed exchange of individuals. This would stabilise population size and maintain the genetic diversity. As yet, there is no evidence of genetic bottlenecks or inbreeding in the six populations of pygmy bluetongue lizards that have been genetically sampled (Smith *et al*. 2009). However, should genetic variation decline, replication of dispersal patterns could be achieved through reciprocal translocations (Moritz 1999; Bouzat *et al*. 2009).

Knowledge of movement patterns allows more insights into the choice of individuals and the potential timing for these translocations. If one group within the population is more mobile, conservationists could capture for translocation individuals from that group to mimic natural dispersal of the species. In isolated fragments of habitat, like those occupied by pygmy bluetongue lizards, individuals that move around may be more prone to predation or may disperse into the hostile surrounding matrix. Thus, they might be considered as a harvestable surplus because their selection as translocation stock would come at little cost to the resident population. Because adult females are the least dispersive group, their use in a translocation program is likely to have the greatest adverse impact on the source population in terms of social groups (Temeles 1994; Shier and Swaisgood 2012) and lost reproductive potential. However, females are essential for reproductive growth, so translocation programs may need to consider taking juvenile females from the source population and raising them in captivity to adults for release.

Using more mobile members of a population might reduce the impact on the source population; however, it could compromise the success of the translocation. Translocations in reptiles are not always successful (Germano and Bishop 2009) and part of the problem may be persuading translocated individuals to stay where they are released. If we understand why individuals disperse from their home burrows, we may be able to determine when to conduct releases to minimise the initial movement of individuals away from their release site, and allow them time to adjust to local conditions there. Our study suggests that early spring is the time when translocated adults might be most likely to move around, and probably disperse from a release site, and that translocation programs will be more effective if delayed until later in the season (February/March).

Many other factors need to be considered in translocation programs, including avoiding the introduction of new parasites and pathogens (Cunningham 1996) and the inappropriate mixing of separate genetic lineages (Moritz 1999); however, the present study provides some baseline data that would allow more informed decisions about the most appropriate individuals in a population to choose for a translocation program and the times to conduct translocations to allow the maximum chance for establishment.

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Chapter 3. Movement of genes

Dispersal is only evolutionarily meaningful if the genetic material is being passed on. In this chapter we investigate how the high levels of male movement in the breeding season (Chapter 2) influences the mating system of the pygmy bluetongue lizard. Understanding the mating system provides vital insight for conservation managers in terms of social interactions, managing genetic variation and inbreeding avoidance.

Female pygmy bluetongue lizard with her litter of three live born offspring

Conceived and designed the sampling: JS MG . Performed the sampling: JS AF. Analyzed the DNA and the data: JS. Contributed to the writing of the manuscript: JS MG MB.

Promiscuous mating in the endangered Australian lizard Tiliqua adelaidensis: a potential windfall for its conservation. Conservation Genetics 15, 177-185

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Abstract

Studies have revealed an unsuspected complexity in social systems within a few lizard species, including group living, long-term monogamy and individual recognition of partners or offspring. Comparisons among these species and their relatives could provide valuable insights, allowing us to investigate traits that are shared across social systems and identify general principles relating to the evolution of sociality. The endangered pygmy bluetongue lizard, *Tiliqua adelaidensis,* is a member species in the *Egernia* group, but is thought to show a more solitary social structure than other members in this group. Within this study we used microsatellite markers to determine the mating system of *T. adelaidensis*. Unlike many other species in the *Egernia* group, we found a predominately promiscuous mating system in *T. adelaidensis.* We detected multiple paternity in 75% of litters. Of the 70 males identified as having fathered juveniles, only five were identified as mating with the same female in more than one year and only three were identified as the father of juveniles with the same female in consecutive years. The genetic evidence suggested that partners were chosen randomly with respect to the level of relatedness among neighbouring lizards. However, mated lizards were geographically closer to each other than expected by random chance. Multiple paternities rely on the opportunity for males to encounter multiple females during the period when they are receptive to mating, and this may depend on population densities. Drivers for the polygamous mating system may be the single occupancy burrow and the central place territorial defence of those burrows in *T. adelaidensis*. We propose a fourth mating system for the *Egernia* group: polygyny within stable non-social colonies.

Introduction

Species within many taxonomic groups display a range of social behaviours from those that live in highly social groups, often providing some level of parental care to their young, to species where individuals are normally isolated, contacting only for reproduction, and providing little or no parental care. The mating system of a species is defined by the number and frequency of mating partners, and is often linked to the form of social organization. The mating system can also influence mating success, gene flow within and between populations, and the ability to recolonize newly available habitat and avoid inbreeding (Greenwood 1980). Many species have evolved social mechanisms and mating systems in large, continuous habitats, but now occupy isolated fragments of habitat, with consequential impacts on their dispersal and mating systems (Stow *et al.* 2001; Levy *et al.* 2010). Knowledge of the mating system and how it has changed with restricted dispersal is an important component in the sustained management of these species.

How have different mating systems and levels of sociality evolved among related species? Studies of variation among the members of a single clade can provide insights into the relevant selective factors (Oliver and Sachser 2011). Over two decades of field studies have revealed that one Australian lizard clade, the *Egernia* group of skinks, includes species with a variety of social systems (Bull 2000; Gardner *et al.* 2002; O'Connor and Shine 2003; Stow and Sunnucks 2004; Chapple 2003). The *Egernia* group is a monophyletic lineage that includes six primarily Australian genera *Egernia*, *Liopholis*, *Bellatorias*, *Lissolepis*, *Tiliqua*, and *Cyclodomorphus*, and one Melanesian genus *Corucia* (Gardner *et al.* 2008).

A large proportion of species in the *Egernia* group live in mixed sex social aggregations, often comprising related individuals, with shared refuges or home sites (Chapple 2003). Monogamy appears to be the most common mating strategy in the social, sedentary species, such as those that live in social groups on isolated rocky outcrops (e.g. *Egernia stokesii* (Gardner *et al.* 2002), *E. saxatilis* (O'Connor and Shine 2003), *E. cunninghami* (Stow *et al*. 2004), and *Liopholis whitii* (Chapple and Keogh 2005), those that have large multi generational groups co-occurring in extended burrow systems (e.g. *Liopholis kintorei* (McAlpin *et al.* 2011)), or those that have limited movement (e.g. *Tiliqua rugosa* (Bull 2000)). Uller and Olsson

(2008) predicted that females of species with low population densities during the reproductive season and of species with strong pair-bonding, should have fewer encounters with alternative mating partners during the female receptive phase, and thus have lower levels of multiple paternity. In several well studied social species in the *Egernia* group, monogamous mate fidelity is high among years, and multiple paternity is low within years (Chapple 2003; Uller and Olsson 2008).

The genus *Tiliqua*, embedded within the *Egernia* clade, does not appear to share the high levels of social grouping of its sister taxa. Field work on *T. rugosa* has shown monogamous parings during breeding seasons that can persist for over 20 yrs (Bull 2000), but no evidence of more extended kin group associations (Bull and Baghurst 1998). The more mobile *T. scincoides* appears to be primarily asocial, with males occupying individual territories and mating polygamously with overlapping females (Cogger 2000; Koenig *et al*. 2001).

The pygmy bluetongue lizard (*Tiliqua adelaidensis*) is a cryptic species found in native grasslands in the geographical region referred to as the mid north of the state of South Australia. Individual lizards live up to 9 yrs (Milne 1999) and spend the majority of their time alone, refuging in abandoned spider burrows, or basking, and at the burrow entrance from where they ambush passing invertebrate prey (Hutchinson *et al.* 1994; Milne *et al.* 2003). Each burrow is occupied by one individual and in both males and females their range of normal activity extends no more than 5cm from their burrow entrance (Fenner and Bull 2011). Mating occurs in the spring months in October and November (Milne 1999; Milne *et* al. 2003; Fenner and Bull 2009). Adult males move away from their burrows, seeking females to mate with during this period (Schofield *et al.* 2012). Video recorded matings have been brief encounters between a burrow resident and another lizard moving up to the burrow, apparently in search of a mate (Milne *et al.* 2003; Fenner and Bull 2009; Ebrahimi pers.comm.). Females produce one litter of up to four live young per year and can breed in consecutive years (Milne *et al.* 2002). Some neonates begin dispersing from the natal burrow within a week after birth and by 5 weeks most of the juveniles have left the natal burrow, leading to early separation of the mother and her offspring (Milne *et al.* 2002). Alternatively, some females move to a new burrow soon after the birth, leaving the juvenile to inhabit the natal burrow (Milne *et al.*

2002). Apart from the brief contacts during mating, and the short shared occupancy of natal burrows, there are no records of social aggregations in this species.

Smith *et al.* (2009) reported restricted gene flow even between closely adjacent populations, and moderate levels of genetic differentiation among sites with FST varying from 0.021 – 0.091. They found no evidence of population genetic bottlenecks and little evidence of inbreeding due to consanguineous mating. Individual populations had observed heterozygosities ranging from 0.75 to 0.82 (Smith *et al*. 2009).

However, the secretive lifestyle of this species makes it difficult to observe whether there are any social associations among neighbouring lizards, or whether the mating system is polygamous as may be predicted if this is a more asocial species. We used genetic analysis to identify the parents of juvenile pygmy bluetongue lizards in populations from two locations, and to determine the mating system used by this species. We had two aims. The first was to provide an additional comparative case within the *Egernia* clade to allow new insights into the evolution of sociality within that group. The second was to provide vital information for modeling population genetics and demography, and determing conservation strategies, within isolated populations of this endangered species.

Methods

Field sampling

Lizards were sampled from two localities in native grassland, 11kms apart, and both within 20 km east of Burra, South Australia (33^{o} 42'S; 138^{o} 56'E). In the spring and summer of 2005/2006 we searched 11 – 12 ha at each locality and captured 160 lizards from locality 1 and 63 lizards from locality 2. In a second sampling period which included the two spring and summer seasons of 2008/2009 and 2009/2010, we captured 353 individual lizards within three 1.2 ha enclosures. One enclosure site was at locality 1 and two enclosure sites, 1 km apart, were at locality 2. Each enclosure site had four 110 m long trap lines set in a square (and thus enclosing an area of 1.2 ha) (Fig1). Each trap line had a 15 cm high black-plastic drift fence and 16 bucket traps (20 litre, 38 cm deep, 28.5 cm diameter), placed immediately under

the drift fence, and spaced at 7 m intervals along its length (Figure 1). We attempted to capture most of the resident lizards in each site first by setting the pitfall traplines and trapping for 43,000 trap days over the entire sampling period (Schofield *et al*. 2012). We also searched the inside of each enclosure each month for any occupied burrows that we could detect, and attempted to lure individuals to the surface with mealworms following the method of Milne *et al.* (1999). We sampled blood from those resident lizards that we were able to capture.

Each captured lizard, was individually marked by toe clip and its sex, mass, snout-to-vent length (SVL), and GPS location were recorded. Lizards were classified into neonates up to 6 months old (SVL <50mm), sub adults up to 18 months old (SVL51-80mm) or adults (SVL> 80mm), following Milne (1999). Among adults, sex was determined by the larger head size and shorter body of males (Hutchinson *et al* 1994). Between late Jan and early March, females produce a live litter of up to four offspring which remain in the maternal burrow with their mother for periods varying from a few days to several weeks (Milne *et al.* 2002). We recorded each case where neonates were found in the same burrow as an adult female, and, where possible, we also sampled blood or toe clips from these individuals. Sub-adults within enclosures could have moved there before the enclosure walls were erected, and their parents may not necessarily have been within the sampling area.

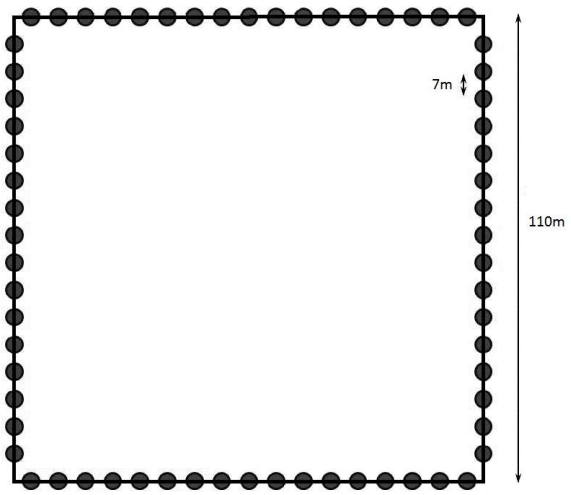

Figure 1. Sampling grid used at the 3 study sites, where black circles represent pitfall traps and the lines denote plastic drift fence.

DNA extraction and PCR amplification

A blood sample from a clipped toe was stored on FTA paper (Whatman, Maidstone), and DNA was extracted following the procedure for nucleated erythrocytes (Smith and Burgoyne 2004). Individual genotypes for 561 lizards were determined at 15 previously described polymorphic microsatellite loci: Est12 (Gardner *et al.* 1999), TrL9, TrL12, TrL14, TrL15, TrL16, TrL19, TrL21, TrL27, TrL28, TrL29, TrL32, TrL34,TrL35 and TrL37 (Gardner *et al.* 2008). Multiplex PCR conditions followed Gardner *et al*. (2008) with amplicons genotyped on an ABI 3730 capillary electrophoresis DNA analyser (Applied Biosystems, Foster City, CA). A fluorescently labelled size standard (GS500 (-250) LIZ) was run with the samples and alleles were scored using GeneMapper software version 3.7 (Applied Biosystems) with manual checking.

Hardy-Weinberg disequilibrium and linkage

We tested whether any individual locus had null alleles or deviated from Hardy-Weinberg equilibrium (HWE), and whether there was any linkage disequilibrium (LD) between pairs of loci, using GENEPOP 4.0.10 (Raymond and Rousset 1995; Rousset 2008). We obtained a larger sample for these tests by combining our data from this study with genotypes for 34 additional lizards reported by Smith *et al.* (2009) from a separate but nearby locality (locality 6 of Smith *et al.* (2009) 1 km from locality 1, and 6km from locality 2). We ran the HWE and LD tests separately on adults from each locality to determine if there were consistent patterns. P-values were adjusted for multiple testing by the sequential Bonferroni method (Holm 1979) when appropriate.

Parentage analysis

For each juvenile, whether it was captured in the first or second sampling period, we searched for potential parents from among all of the adults sampled from the same location over both sampling periods. We used adult genotypes at the 15 microsatellite loci in the program CERVUS 3.03 to assign parents to genotyped juveniles. The following simulation parameters were used: 100,000 cycles, 70% of the candidate parents sampled, 88% of loci typed and a genotyping error rate of 1%. We accepted that we had sampled the true parents when the confidence level exceeded 95%. Adults that were assigned as parents but that mismatched their

presumed offspring at > 2 loci were disregarded as inferred parents. Juveniles were assumed to be siblings from the same litter if they were allocated the same mother and were born in the same year. The simulations were also performed using an input parameter of 50% of candidate parents sampled but as there was no difference we only present the results for 70%.

To assess the levels of monogamy and polygamy among adult lizards, we used CERVUS 3.03 and COLONY 2.0 to determine sib-groups and to predict the number of unsampled parents. We assumed a polygamous mating system with no inbreeding as the populations were in HWE. The marker type, allelic dropout rate and other error rates that were used can be found in Online Resource 1. The probability that a parent was in the sample was tested at 50% and 70%. The results were the same for both, therefore probabilities were set at 70%. A probability of 70% was chosen because the cryptic nature of the lizards made it unlikely we had sampled of all the adults. Paternal and maternal relationships derived from the CERVUS results were entered as Known Paternal and Known Maternal data sets. We then used COLONY to simultaneously infer sibship and parentage using a full-pedigree likelihood method (Jones and Wang 2009). Not all potential parents were sampled during our studies and our estimates may not be an accurate reflection of all of the matings that had taken place. However as we had good discrimination with our loci (Smith *et al.* 2009) any potential bias would be minimal. The mean heterozygosities of litters fathered by single or multiple fathers were compared using a paired sample t-test. Allelic richness could not be estimated with confidence due to the small sample size.

Where we identified both parents and knew their actual locations when sampled, we investigated whether individual parents showed any evidence for a preference for less related individuals as mating partners. To do this we compared the relatedness of the partners to their relatedness to other geographically close alternative partners. The only sample set that was large enough for this analysis was in enclosure site 2 (locality 2) in the second sampling period. Relatedness (*r*) was estimated, using the program Coancestry 1.0 (Wang 2011), with a moment estimator which assumes no inbreeding (Wang 2002). We compared relatedness of the two parents with the relatedness of opposite sex individuals located closest to them. For each parent we considered either its relatedness to the nearest, or its mean relatedness to the four

nearest, non-partner individuals of the opposite sex. We then compared the relatedness of partners and non-partners by paired t-test, separately for each sex.

Results

Hardy-Weinberg and linkage disequilibrium

Genotype frequencies deviated significantly from HWE at five of the 15 loci, but in each case the deviations were only detected at single localities, one (TrL32) at locality 1, three (TrL12, TrL15 and TrL37) at locality 2, and one (TrL32) at locality 6. No locus showed significant deviation from HWE at more than one of the three localities (locality 1, sample size N=142; locality 2, N=220; locality 6, N =34). Similarly, only two pairs of loci were significantly linked in locality 1 (TrL 15/ TrL 16 and TrL 15/ TrL 21), three were linked in locality 2 (TrL 16/ TrL 19, TrL 19/ TrL 37, and Est12/ TrL 21) and none were linked in locality 6. Null alleles were detected at five loci, but again no locus showed null alleles consistently over all localities: locality1 (TrL16) locality 2 (TrL15, 21, 28 and Est12) and locality 6 (TrL 16). All 15 loci were used in subsequent analyses as patterns of disequilibrium and null alleles were not consistent across localities and deviations may have been due to population level processes (e.g. birth and death rates; different founding individuals).

Parentage analysis

Table 1. Capture summary of the individuals from which successful genotypes were derived over the two sampling periods

Sampling period	Juveniles	Males	Females	Total
1 (2005 - 2006)	27	83	91	201
2 (2008 – 2010)	174	106	80	360

We derived genotypes from 561 of 576 captured lizards (360 adults and 201 juveniles) (Table 1). Among the adults there were 189 males (52.5%) and 171 females (47.5%). Among the 201 juveniles (130 neonates, 71 sub adults), 140 (69 neonates, 71 sub adults) were captured alone either in burrows or in pitfall traps, and 13, all neonates, were found as the only juvenile in a burrow with an adult female. Juveniles found in groups of two to four individuals were all from burrows and were all neonates. There were 11 groups of two, six groups of three, and two groups of four individual juveniles together, with or without an accompanying adult female

(Table 2). A total of 39 neonate juveniles were found in burrows with an adult female (Table 2). No juveniles were found in a burrow with an adult male lizard.

Using CERVUS, 113 (56.2%) of the juveniles could be assigned to one (80 juveniles) or both (33 juveniles) parents. Parentage was assigned to 51% of the neonates, and to 64% of the subadults. Despite an intensive survey regime at each site, only 35% (location 1) and 69% (location 2) of the parents of captured juveniles were sampled. Where only one parent was identified, it was the mother in 47 cases and the father in 33 cases.

Table 2. The number of groups containing juvenile lizards, and the total numbers of juvenile lizards in each group size category that were detected with or without a female present in the same burrow.

Number of juveniles per group	**1**	**2**	**3**	**4**
Number of groups with female present	13	5	4	1
Number of groups with no female present	140	6	2	1
Total number of groups	153	11	6	2
Total juvenile lizards	153	22	18	8

Relatedness of lizards in the same burrow

We were able to obtain a sample for DNA analysis from 17 of the 23 females located with neonates in the same burrows. In 15 cases (88.2%) CERVUS inferred the co-located female as the mother of all of the accompanying neonates. We deduced these were mothers with their litters, and found a mean relatedness of 0.468 (range 0.221-0.677) between these females and their neonates.

In the two other cases, females were not assigned as the parent of a neonate located in the same burrow, and relatedness values were low ($r = -0.17$ and $r = -0.06$). Both neonates were sampled during the period of birth and neonate dispersal (26 Jan and 24 Feb). The first was a single neonate and female. The second was a female with two neonates, one related and one unrelated.

No sub adults were found sharing a burrow, either with juvenile or with adult lizards. In 18 of the 19 groups of two or more neonates located together (Table 2), CERVUS

assigned group members to the same mother, and relatedness values among the group members suggested they were siblings or half siblings (mean r = 0.412; range = 0.121 - 0.785). The other group of two neonates found in the same burrow with an adult female, has been discussed above. In that group the two neonates appeared to be unrelated to each other (r = -0.0004).

Paternal contribution

CERVUS identified from among the 201 sampled juveniles 56 sets of 2 – 4 siblings born in the same year with the same mother (described as a family in this paper). For some of those groups the mother was not identified from among the adults that were sampled. In 37 of those sets the siblings were sampled occupying separate burrows. In 24 of these 56 families, both the mother of all of the sibs, and the father of at least one sib could be identified from among the adults sampled. In 18 (75%) of those 24 litters, COLONY suggested that an additional male fathered one or more of the other juveniles (Table 3). For 17 litters there were at least two fathers, while one litter of four sibs had at least three fathers. For the remaining 32 maternal families we used COLONY to deduce the possible male parent contributions to the litters and inferred that at least 22 (69%) of those families had multiple fathers. There was no significant difference between the mean heterozygosities of litters with single paternity (7.4) and litters with multiple paternity (6.6) (Table 4)

Table 3. Number of litters with multiple fathers as identified by CERVUS and inferred multiple father litters by COLONY in brackets

	Full sibs groups	**Range of litter size**	**half sib groups**	**Range of litter size**	**Total number of families**
Sampled families	6	2-3	18	2-4	24
Inferred families	10	2	22	2-4	32

Table.4 Levels of heterozygosity found in litters with multiple and single paternities

	Number of individuals	**Number of litters**	**Observed Heterozygosity**
Single paternity	11	5	7.38
Multiple paternity	9	4	6.57

We identified 43 female individuals that produced litters, and deduced that 18 of those (41.9%) had produced more than one litter over the duration of this study, 11 of them in consecutive years. During the second sampling period of two years, sub adult juveniles sampled in the first year (2008) were assumed to have come from matings in 2007, thus we had data for matings in four different consecutive years, even though sampling took place in three. Most females that produced multiple litters had two litters over consecutive years, while two females were detected to have produced litters in 3 and 4 consecutive years respectively.

CERVUS identified 70 (37%) of the 189 adult males sampled in the study as fathers of sampled juveniles. Seven (10%) of those males fathered juveniles with two different females in the same season. This may under-represent the rate of polygyny since both males and litters in the sampled populations would have been incompletely sampled. Five males were identified as fathering juveniles with the same female in multiple years in the second sampling period, with three of those cases (60%) being in consecutive years. Those five males were also among the seven polygynous males, mating with more than one female in at least one season.

Relatedness among mating partners

Within individuals sampled at enclosure site 2, CERVUS identified 20 juveniles for which both the mother and the father could be identified. The 20 juveniles came from 17 maternal litters, and were fathered by 17 males. Three of the males fathered juveniles from two of the females, one female had offspring fathered by two of the males, and another female had offspring fathered by three of the males. Each male-female parent combination was only responsible for one juvenile, so that none of the juveniles from the 20 sampled had an identical mother and father.

The mean relatedness between the male and female parents for each of the 20 juveniles ($r = 0.063$; Table 5) did not differ significantly from the mean relatedness of all male-female combinations among the sampled adults in site 2 (Mann Whitney $U = 34560.5$, $z = -1.64$, sig 0.101). There was no evidence that lizards were choosing mating partners that were less related than random. Furthermore for both males and females, relatedness to their mating partner was not significantly different from relatedness to the nearest other individual of the opposite sex, or from the average relatedness of the four nearest other individuals of the opposite sex (Table 6). That is,

there was no evidence that partners were chosen non-randomly from among neighbouring lizards with respect to relatedness.

Table 5. Comparison of distance to partner and relatedness between the mated pairs and the 4 nearest individuals of the opposite sex.

Female partner	Male partner	Relatedness	Relatedness of female to nearest male non partner	Average relatedness of female 4 nearest males	Relatedness of male to nearest female	Average relatedness of male to 4 nearest females	Distance between mated pairs
2325	2707	-0.0451	-0.160	-0.006	-0.115	-0.133	13.345
2351	2403	-0.0748	-0.036	-0.051	0.002	0.140	5.099
2352	2713	0.3614	0.059	0.116	-0.106	-0.086	30.806
2400	2706	0.1707	0.288	0.069	-0.087	0.053	64.899
2401	2431	-0.1018	-0.092	0.016	0.026	0.052	11.705
2410	2616	-0.1817	-0.026	-0.042	0.059	-0.078	10.630
2413	2355	0.2933	-0.085	-0.006	0.288	0.086	11.705
2413	2335	-0.0081	-0.085	0.178	-0.028	-0.044	107.331
2413	2632	0.4858	-0.074	-0.005	0.451	0.069	21.213
2453	2709	-0.1315	0.573	0.175	-0.077	-0.086	19.2094
2453	2707	-0.1128	0.573	0.175	-0.007	-0.118	22.3607
2500	2761	0.2117	-0.106	-0.038	-0.132	0.027	82.0549
2524	2626	0.171	-0.178	-0.005	0.064	0.040	8.5440
2533	2340	-0.127	0.025	-0.042	0.140	0.106	22.361
2540	2761	0.1311	0.093	0.005	0.037	0.104	17.117
2559	2431	-0.111	-0.205	-0.118	0.026	0.007	10.198
2619	2639	0.0259	-0.047	-0.072	0.138	0.018	31.6228
2651	2330	0.1096	0.003	0.057	-0.034	0.053	31.064
2683	2627	0.1291	-0.103	-0.075	-0.062	0.060	3
2744	2694	0.0597	-0.141	-0.065	0.197	0.259	20.615
Mean		0.06258	0.014	0.013	0.039	0.027	27.244

Further evidence that relatives were not discriminated against as mating partners, came from three offspring resulting from matings between partners with relatedness values of 0.500, 0.365 and 0.297 (Table 5). In each case other less related individuals were available as partners from among neighbouring lizards (Table 5). Genotypes and lizard locations were derived from samples collected when the lizard was first captured, and this may have been before or after the October/ November period when mating occurs. The mean distance between burrows occupied by males and females that had produced young was 27.24m (N = 20; SE = 6.04; range = 3 – 107m) (Table 5). The mean distance between all possible male and female pairs within enclosure site 2 was 64.7m (N = 4028; SE = 0.51; range = 1 – 160). Mated males and females were found closer to each other than if males and females within the enclosure had mated at random with respect to geographic distance (Mann Whitney U = 13226.50 ,z = -5.189, sig. < 0.001).

Table 6. Paired t-test comparing the relatedness among breeding individuals and the 4 nearest of the opposite sex.

Relatedness of mate pairs to:	**df**	**t**	**sig (2-tailed)**
Mated male and average of 4 nearest females	19	0.95	0.353
Mated female and average of 4 nearest males	19	1.17	0.257
Mated male and nearest females	19	0.522	0.608
Nearest males	19	0.746	0.465

Discussion

Compared with other lizards in the *Egernia* group our genetic results suggest a high level of multiple mating within the sampled localities of pygmy bluetongue lizards. Within a season it was common for females to be mated by two or more males, and males could mate with more than one female in this period. Mating appeared to be indiscriminate with regard to degree of genetic relatedness, and male and female mating partners could be located in burrows over 100 m apart. The distribution of the number of matings per male could not be estimated because some litters, and thus some matings, were unsampled. Even in the sampled litters, a male that had mated with the female may not have contributed to the progeny.

Chapple and Keogh (2005) proposed three distinct mating systems for the *Egernia* group (i) a combination of polygyny and within season monogamy (ii) long-term genetically monogamous pairings during the breeding season and (iii) long term genetic monogamy within temporally stable social aggregations. Unlike other members of the group, pygmy bluetongue lizards appear to be promiscuous and to display a polygamous mating system.

Several previous observations support our interpretation of the genetic analysis. Video recordings of female occupied burrows (Milne *et al*. 2003; Fenner and Bull 2009), suggest that males move across the population to seek out females in their burrows, and that individual females are visited by multiple males (Ebrahimi unpubl. data 2013).

In our study, males were recorded as far as 100 m away from the females they mated with. Records of mating have all been in the spring (October) (Milne *et al.* 2003; Fenner and Bull 2009). This is the time when other observations have suggested that males of this normally sedentary lizard are actively moving around (and exposed to predation). It is the time of year when a male lizard was found inside a brown snake stomach in 1992 when the species was re-discovered (Armstrong and Reid 1992), and also the time of year when Schofield *et al.* (2012) reported maximum capture rates of adult lizards (86% males) in pit-fall traps.

Combining those observations with the genetic data from the current study suggests that, during a short mating period in spring, males move around the population seeking females in burrows, and can mate with multiple partners. And at the same time, females in their burrows accept matings from several different males. During this period the males will be exposed to enhanced predation risk (Fenner and Bull 2009), and this increased predation may explain the absence of some of the fathers of the juveniles from the genetic sample in this study. It would also explain the disappearance of more males than females from lizard populations over a spring-summer period (Fellows 2008).

One explanation for why females accept multiple matings may be the high risk of inbreeding. Previous genetic analyses (Smith *et al.* 2009) have shown clustering of related individuals within populations, indicative of low dispersal rates. Furthermore, Fenner and Bull (2010) failed to find evidence that individual pygmy bluetongue lizards discriminated among scent cues from related and non-related individuals. Thus there is a high chance of a related male and female coming together and of the partners not being inhibited by that relatedness in their mating behaviour. Results from the current study confirm that some juveniles were produced from matings between highly related males and females. In these circumstances, females that mate with more than one male increase the chance that some of their offspring will be more outbred.

One aim of our study was to contribute to understanding how mating systems evolved within the *Egernia* group of Australian lizards. The promiscuous and indiscriminate mating system of pygmy bluetongue lizards differs substantially from related species which have stable, long-term monogamous partnerships (Bull 2000; Gardner *et al.* 2002), which show a highly developed olfactory discrimination among related and non-related individuals (Bull *et al.* 2001) and which tend to choose single, unrelated mating partners (Bull and Cooper 1999; Gardner *et al.* 2002).

One ecological factor that might drive this difference is that pygmy bluetongue lizards do not dig their own refuge burrows, but instead rely on burrows dug by spiders. These are usually too small for persistent sharing of burrows by more than one lizard, and the short supply of burrows of optimal depth (Fellows *et al.* 2009) has led to a system of single occupancy burrows and central place territorial defence (Fenner and Bull 2011). Specifically there is no opportunity for the social aggregations commonly reported in other *Egernia* group species, and for the development of within group interactions that might favour less polygamous mating systems.

There are at least two conservation implications of our results for this endangered lizard. First, the indiscriminate partner choice and close spatial proximity of relatives in existing populations, suggest that individual lizards will not actively avoid mating with highly related partners. This means there may be a greater risk of inbreeding as

populations decline, and as the genotypic range of potential partners is reduced. Continued monitoring of genetic diversity in populations, particularly those with low population density, will be important. Our result contrasts with the earlier studies on another *Egernia* group member, *E. cunninghamii*. Stow and Sunnucks (2004) reported a reduction in mating between relatives in highly fragmented areas where potential partners were limited. Second, and conversely, that a promiscuous mating system may prove advantageous during any translocations or reintroductions. This is because it could ensure the rapid mixing of genotypes among founder individuals at unoccupied sites, or the rapid integration of new genetic material into existing populations.

The success of translocations could be measured by a high reproductive output with the maintenance of genetic diversity over time (Griffith *et al.* 1989; Gregory *et al.* 2012). In polygnous mating systems females are the limiting factor. The introduction of more females than males could reduce the male search time for a mate and thus reduce predation risk to males especially in species that mate indiscriminately. However when considering reproductive potential in monogamous or pair bonding species equal numbers of each sex would result in maximal reproduction (Sigg *et al.* 2005). To ensure breeding compatibility in these species the translocation of previously mated individuals would be ideal. In species with kin recognition and mating avoidance or long term genetic monogamy within temporally stable social aggregation a selection of less related individuals would benefit reproduction and genetic diversity in translocations (Gregory *et al*. 2012). The success of captive breeding and translocation efforts for any species may hinge upon understanding both the baseline genetic diversity of source and translocated populations and the mating systems they display (Haig 1998; Sigg *et al.* 2005; Grueber and Jamieson 2008; Gregory *et al*. 2012).

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Chapter 4. Movement within populations

Significant spatial structuring within populations (Smith *et al.* 2009) and promiscuous mating among neighbours (Chapter 3) suggest there may be local population processes to retain genetic diversity, which perhaps evolved prior to habitat fragmentation, to protect diversity in a species with infrequent natural gene flow.

This study explores in more detail how genetic diversity is structured across two demographic populations of pygmy bluetongue lizards, and uses that pattern to infer dispersal behaviour. This chapter will increase understanding of the population processes allowing genetic diversity to be maintained despite habitat fragmentation.

Adult female pygmy bluetongue lizard about to enter its burrow

Conceived and designed the sampling survey: JS MG. Performed the survey and characterised DNA: JS. Analyzed the data: JS.. Contributed to the writing of the manuscript: JS MG MB.

Genetic structure of the endangered pygmy bluetongue lizard (*Tiliqua adelaidensis*) within a fragmented landscape. (submitted to Conservation Genetics)

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Abstract

Many species are now distributed across fragmented landscapes. Fragmentation affects gene flow among populations, and genetic structure within populations. This study explores how genetic diversity is structured across two demographic populations of the endangered pygmy bluetongue lizard that occupy remnant fragments of its native grassland habitat in South Australia. We found divergent genetic clusters at both the larger scale (11-13ha) and within 1.2 ha sampling sites. Significant spatial autocorrelations suggested low dispersal distances for both sexes, and that resident adults had settled close to related individuals. But the different genetic clusters were not spatially sorted at the local site scale, implying different genetic lineages were mixing in the population. We suggest genetic diversity within populations is maintained by a localised promiscuous mating strategy, that despite lack of gene flow, individual populations are currently at low risk of loss of genetic diversity, and that conservation management should focus on sustaining high population densities.

Introduction

Populations can be defined as a group of conspecific individuals that is, genetically, or spatially disjunct from other groups of individuals (Wells and Richmond 1995).Gene flow between populations is disrupted by habitat fragmentation, and this interruption can have impacts both within and between habitat fragments. Species that have evolved in large patches of continuous habitat may be poorly adapted to living within, or moving between small, widely separated habitat fragments (Habel and Schmitt 2012). Particularly for some species with small dispersal distances or low population densities, living in isolated patches in fragmented habitats may inhibit dispersal of one or both of the sexes (Stow *et al.* 2001), and enhance

processes that result in lower genetic diversity within sites and higher genetic divergences among sites (Levy *et al.* 2013; Harrisson *et al.* 2013).

Of particular concern are endangered species that persist as small populations in isolated fragments. Maintaining genetic diversity within populations of those species is a conservation challenge. Habitat fragmentation can lead to the loss of genetic diversity within remnant populations because those populations are often smaller making them more vulnerable to genetic drift. Additionally, because these populations are now more isolated from their neighbors gene flow will be reduced, preventing replenishment of genetic diversity. The loss of genetic diversity in turn affects the ability of local populations to respond to environmental challenges from climatic and habitat changes within the remnant fragments. Thus these populations face a possible double challenge from lower genetic variation coupled with smaller effective population sizes, reducing their ability to evolve appropriate adaptations to new conditions, and increasing the potential for inbreeding depression (Saccheri *et al.* 1998; Keller and Waller 2002).

However, species with naturally low dispersal rates prior to habitat fragmentation, may have previously evolved local processes to maintain genetic diversity without relying on high levels of gene flow from neighboring locations. Whigham *et al.* (2008) showed that populations with tightly clustered social structures can preserve different alleles locally, and take longer to lose neutral genetic variation than less structured populations. With reduced gene flow, these species can avoid outbreeding depression, or the loss of locally adapted genetic combinations (Blouin and Blouin 1988). However avoiding outbreeding depression is most likely to be relevant in species with naturally low levels of gene flow. Local genetic diversity can be preserved by various mechanisms including sex biased dispersal, kin avoidance in mating, or mating with multiple partners (Blouin and Blouin 1988). If these species can retain higher population densities, geographical isolation through habitat fragmentation may not be such a substantial genetic challenge. Thus the predicted loss of genetic diversity resulting from habitat fragmentation may be moderated in some species that have evolved with low levels of gene flow among local populations. Their populations may be characterized by high levels of local genetic structuring. A key question for conservation managers is how great an influence

habitat fragmentation has on the ability of a population to retain its genetic variation. To address this question we need to understand the processes within individual populations that sustain or diminish that variation.

The pygmy bluetongue lizard *Tiliqua adelaidensis* is an endangered scincid species found in a few small, isolated remnants of native grassland habitat in the mid-north region of South Australia. Habitat fragmentation has occurred over the last 150 years with an increasing matrix of cereal cropping and ploughed lands replacing native grasslands with habitat completely inhospitable for the lizards (Milne 1999; Souter 2004). Managing genetic diversity within the remnant populations of the pygmy bluetongue is an important component of their conservation management.

Adult lizards occupy abandoned, single entrance spider burrows and spend most of their time either sheltering in the burrow or half-emerged at the burrow entrance, from where they ambush passing invertebrate prey. Once they are established in a burrow the lizards tend to be sedentary except that males make mating forays in the spring breeding season (Fellows 2008; Schofield *et al*. 2012). Apart from those moves, established adults have a tiny surface home range, normally travelling no more than 10cm from the burrow entrance (Fenner and Bull 2011). Although they have no obvious social associations, they retain a stable spatial organization, in which individuals occupy the same adjacent burrows for many months (Milne 1999; Fellows 2008).

Previous broad scale population genetic analysis showed limited recent gene flow between populations as close as 1km apart (Smith *et al.* 2009), suggesting that the habitat fragmentation has led to almost complete disconnection between the populations. Despite that, Smith *et al.* (2009) found no evidence of population bottlenecks and little evidence of inbreeding within populations. Genetic diversity appears to have been maintained after up to 150 years of habitat fragmentation (15-25 generations, based on Milne 1999).

Significant spatial structuring within populations (Smith *et al.* 2009) and promiscuous mating among neighbours (Schofield *et al.* 2014) suggest there may be local population processes to retain genetic diversity, perhaps evolved, prior to

habitat fragmentation, to protect diversity in a species with infrequent natural gene flow.

This study explores in more detail how genetic diversity is structured across two demographic populations (where demographic populations are defined by all individuals of a species living in an area of continuous available habitat) of pygmy bluetongue lizards occupying remnant native grassland habitat in the mid-north region of South Australia, and uses that pattern to infer dispersal behaviour. The central aim was to increase understanding of the population processes allowing genetic diversity to be maintained despite habitat fragmentation.

Methods

Individual lizards were captured at two isolated locations (Location 1 and Location 2), 11kms apart, and both within 20 km east of Burra, South Australia (33^{o} 42'S; 138^{o} 56'E). At each location a single population of pygmy bluetongue lizards occupied a continuous patch of native grassland habitat. In a previous study, no recent gene flow was detected between the populations at these two locations (called site 1 and site 2 in that study) (Smith *et al*. 2009).

At these locations, adult lizards were sampled, either by luring them from their burrows using a fishing technique described by (Milne and Bull 2000), or by pitfall trapping as described by Schofield *et al.* (2012). There were two sampling periods. In the austral spring/summer of 2005/2006 we captured 118 adult lizards over 12 ha at Location 1 and 60 lizards over 11 ha at Location 2. In the second sampling period, including two spring/summer seasons, 2008/ 2009 and 2009/ 2010, we focussed on sampling intensively from smaller areas within the two populations. We captured 24 adult lizards from a 1.2 ha site (site 1) within Location 1, and 133 and 34 lizards from two 1.2 ha sites (site 2 and site 3), 1.0 km apart, within Location 2. We attempted to sample all individual adult lizards within the three smaller sites by searching intensively for burrows in each site every month from Sept – March in each of the two spring/summer seasons of this second sampling period. Because we captured nearly four times more adults in site 2 than site 3 we deduced that site 2 supported a higher lizard density. We marked each new burrow that was detected and then regularly inspected those burrows for lizard occupants, and attempted to capture all of those lizards.

Every new captured lizard was individually marked by toe clip and its sex, mass and snout-to vent length (SVL) were recorded. Only adults (SVL> 80mm) were used in this study. Adult sex was determined by the larger head size and shorter body length of males. Lizards were sampled for DNA analysis on their first capture. These samples were either the clipped toe which we stored in 70% ethanol, or a blood sample from the clipped toe, that we stored on FTA paper (Whatman, Maidstone). The GPS location of each captured lizard at the time of its first capture was recorded and spatial distances between capture points of pairs of individual lizards were derived. All lizards were released back to their home burrow within ten minutes of capture.

We extracted DNA from the samples, and genotyped each lizard for 14 previously validated microsatellite loci following Schofield *et al.* (2014).

Population Structuring

Smith *et al.* (2009) had previously shown the demographic populations from Location 1 and Location 2 formed genetic clusters distinct from each other. In this paper, we used STRUCTURE (Pritchard *et al.*2000) to determine if there were finer scale genetic clusters of adult lizards within each location and within the smaller sampling sites. We considered each location represented a whole population, while the sites were sub-population units. Firstly, we used, as input to STRUCTURE, the genotypes of all adult lizards sampled within each of the two sampling locations, and over both sampling periods. Since these lizards take 2 years to reach maturity, pooling of samples from within the sampling periods is reasonable as different generations will not be represented. Secondly, from the second sampling period we used genotypes of all adults from within sampling site 2 at Location 2 and, in a separate analysis, all adults from within sampling site 3 at Location 2. There were not enough captures during the second period at site 1 within Location 1 to run this analysis. STRUCTURE was run with the admixture model, no prior population information, and a MCMC length of 1 million iterations with the first 50,000 iterations discarded as burn in. In each case, we simulated the number of clusters from K = 1 to K = 6 and performed 10 independent simulations of each K-value to check for consistency across runs. We used STRUCTURE HARVESTER (Earl and von Holdt 2012) to infer the number of clusters, within each location and site by

calculating ΔK from the rate of change between successive simulations (Evanno *et al*. 2005). We assigned individuals to a cluster on the basis of the highest probability of membership and checked the consistency of assignments across the 10 runs at the most likely number of clusters.

Spatial autocorrelation

Because the presence of isolation by distance can produce erroneous clustering in STRUCTURE (Frantz *et al.* 2009), we applied independent tests for spatial autocorrelation between individuals within Location 1 and within each site at Location 2. We combined data from both sampling periods for Location 1 to obtain a large enough sample size for analysis. Sample sizes in the second sampling period were sufficient for spatial autocorrelation in site 2 but not in site 3 within Location 2.

To investigate the spatial structuring of adults we used autocorrelation analysis implemented in SPAGeDi (Hardy and Vekemans 1999) which incorporates spatial information to infer philopatry of individuals. This analysis examines the distribution of Relatedness (Li *et al.* 1993) among individuals, through space (Hardy and Vekemans 1999; Smouse and Peakall 1999). The analysis was performed separately on adult genotypes from each location (both sampling periods). In SPAGeDi, euclidean spatial distances were calculated between all pairs of individuals within each sample. Spatial distances were divided into classes to maximise the number of lizard pairs in each class while still providing fine scale resolution. Individuals were permutated among spatial locations 10,000 times to obtain 95% confidence intervals. Standard errors of mean observed relatedness estimates were generated by jackknifing over loci (Hardy and Vekemans 2002). Significant genetic structure was inferred if the standard errors of observed relatedness fell outside the confidence intervals of the permutated data.

Sex biased dispersal

To determine if sex biased dispersal was contributing to the population genetic structure, we used Favre *et al.*'s (1997) Assignment Index correction (AIc) following the method of Mossman and Waser (1999) in GENALEX 6 (Peakall and Smouse 2006).The AIc gives the likelihood that the individual is philopatric where individuals with more negative AIc scores are considered migrants. This method has the advantage of allowing each site or population to be tested separately, as it does

not rely on the permutation of several populations (as is the case with F_{ST} based tests; Lane and Shine 2010). We used a t-test to test the significance as exploration showed these data were normally distributed.

We also explored spatial autocorrelation patterns in each sex separately. We predicted that if one sex dispersed more than another the two sexes would show different spatial patterns of relatedness.

Results

Population structuring

Within Location 1 the combined STRUCTURE output over the two sampling periods indicated the presence of two genetic clusters (Fig 1a). The samples from the second sampling period, collected over a smaller 1.2 ha area, still showed the same two genetic clusters (Fig 1a). Within Location 2, with analysis that combined samples from both sampling periods, there were three genetic clusters identified in each of the two sampling periods (Fig 1b), although the proportional representation of each cluster differed between site 2 and site 3 (Fig 1b) about 1 km apart, indicating some spatial patterning within Location 2. Further separate analysis of just the 1.2 ha site 2 within Location 2, and just considering collections in the second sampling period, showed four genetic clusters (Fig 1c), although there was no apparent spatial differentiation of the four genetic clusters either when lizards were assigned cluster membership based on a >50% probability (Fig 2a) or a >90% probability (Fig 2b). No clear genetic clustering was detected from a separate analysis of the smaller sample of adult lizards from site 3 in the second sampling period.

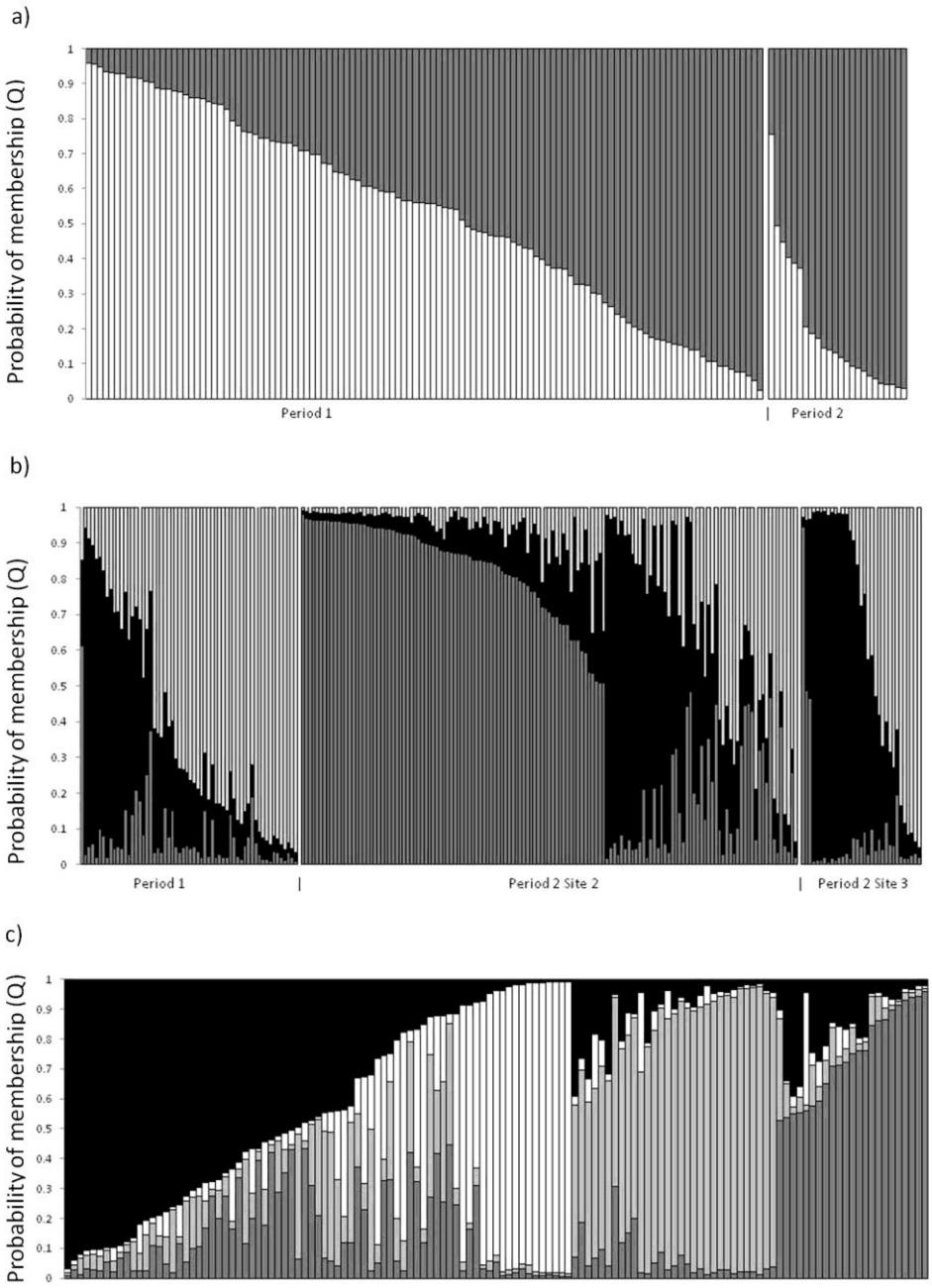

Figure 1. Probability of assignment to a cluster (*Q*) arranged by *Q value* for all sampled *Tiliqua adelaidensis* individuals arranged according to *Q* value in a) Location 1 over two sampling periods; *K*= 2 clusters b) Location 2 over two sampling periods; *K*= 3 clusters; and c) site 2 within Location 2 over sampling period 2 (note that results differ from those in panel (b) because a separate analysis was conducted for this smaller sample); *K*=4 clusters. Each bar represents one individual. The different shades represent the clusters identified by STRUCTURE.

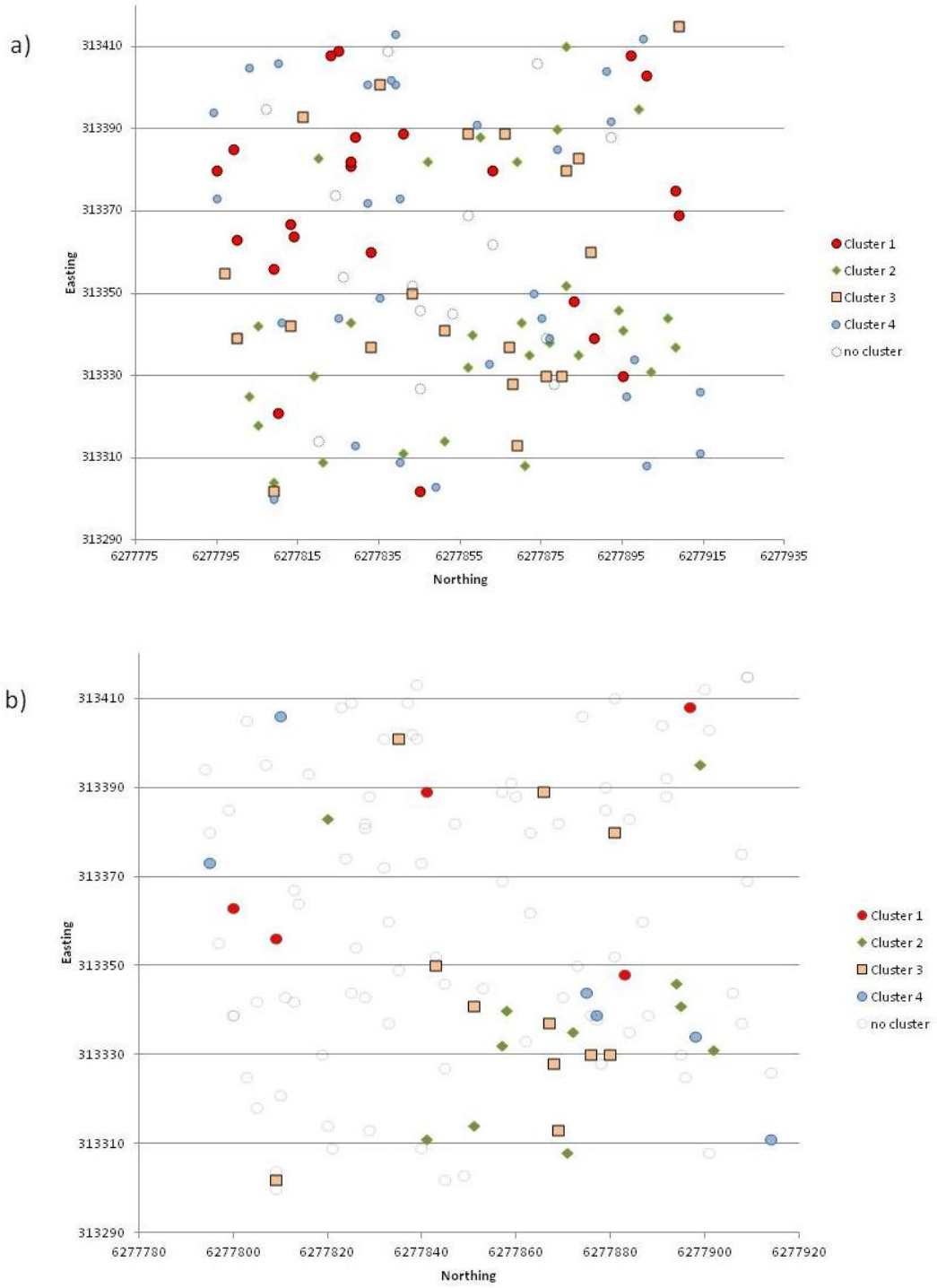

Figure 2. Spatial distribution of individuals from each of four genetic clusters at site 2 in sampling period 2 (a) all individuals assigned to a cluster with a >50% cluster probability (b) all individuals assigned to a cluster with a >90% cluster probability by STRUCTURE.

Spatial autocorrelation

Adult lizards exhibited significant spatial structuring (individuals more related than expected by chance) at distances up to 30 m apart at Location 1(Fig 3a), and up to 45 m apart at Location 2 (Fig 3b).

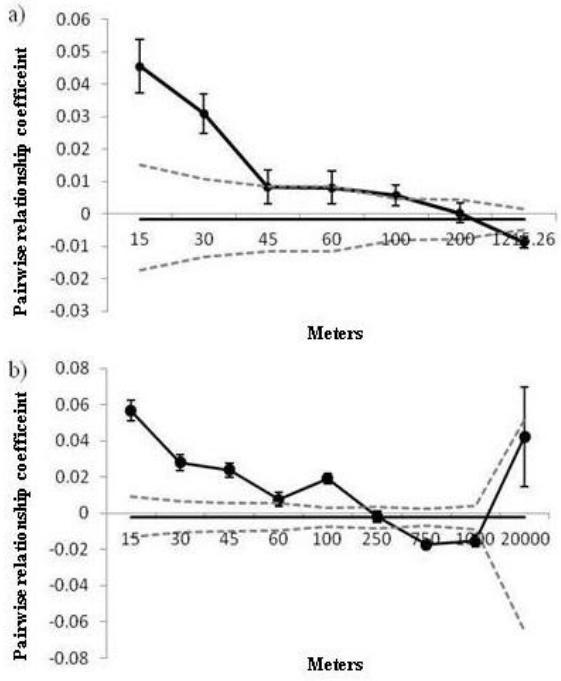

Figure 3 Results of spatial autocorrelation analyses by SpaGeDI of all adult individuals at a) Location1 and b) Location 2.

Sex biased dispersal

The AIc tests indicated no significant evidence for sex biased dispersal at either location (Fig 4; Table 1). Females showed significant spatial structuring at distances of 40 m at Location 1 (Fig 5a) and 60 m at Location 2 (Fig 5b). Equivalent figures for males were 20 m at Location 1 (Fig 6a) and 40 m at Location 2 (Fig 6b).

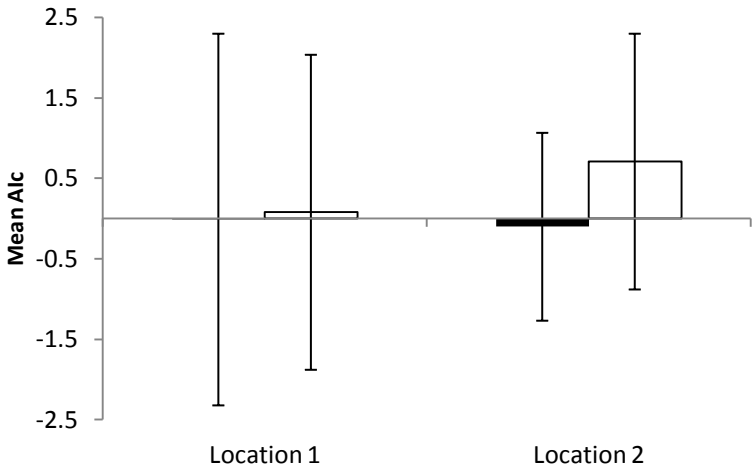

Figure 4. Mean Assignment Index correction values for females (in black) and males (white) at 2 locations, standard deviation shown by error bars.

Table 1. Results of t- test of Assignment Index correction(AIc) values for male and female pygmy bluetongue lizards.

	t	**df**	**Sig (2-tailed).**
Location 1	-.332	45	742
Location 2	0.505	59	0.616

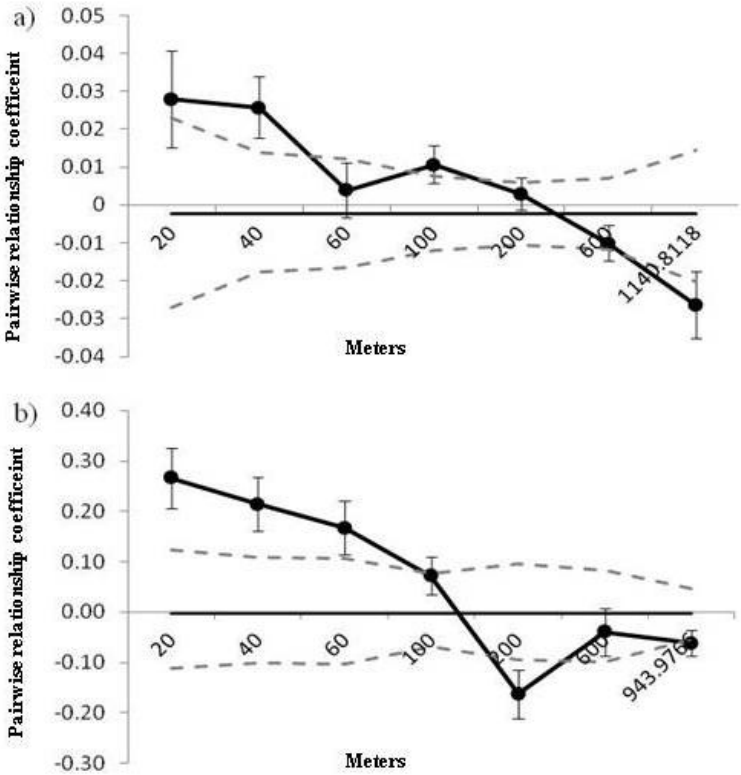

Figure 5. Results of spatial autocorrelation analyses by SpaGeDI of all females individuals at a) Location1 and b) Location 2.

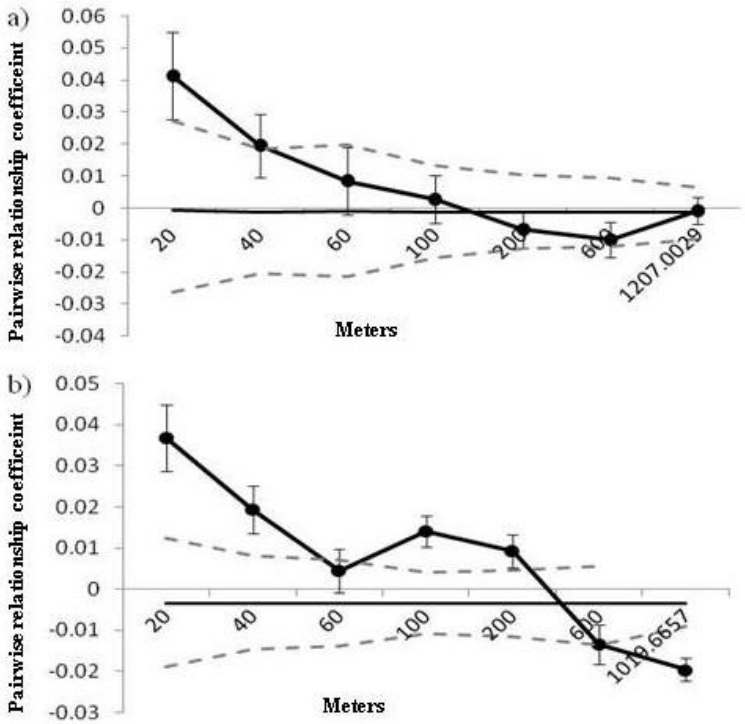

Figure 6. Results of spatial autocorrelation analyses by SpaGeDI of all males individuals at a) Location1 and b) Location 2.

Discussion

Genetic structure of pygmy bluetongue lizard populations.

The distinctive genetic clusters that we detected in this study, at both the whole population (Location) and sub population (Site) scale, imply that broad genetic divergences are being maintained within pygmy bluetongue lizard populations, despite their contraction to isolated fragments of their native grassland habitat. This confirms a similar conclusion, with smaller sample sizes, by Smith *et al.* (2009). The very different cluster compositions between site 2 and site 3 within Location 2 imply broad spatial patterning of the genetic structure within localities. Two contrasting results about spatial patterns help us to understand the population processes involved.

On the one hand the pattern of spatial autocorrelation implies that individual adult lizards are likely to occupy burrows close to genetically related adults, and that this spatial structuring can be detected among pairs of adult lizards at distances up to 30 – 45 m from each other. This pattern was consistent across separate populations at two

locations and was consistent with previous results derived from lower sampling intensity (Smith *et al.* 2009). At location 1 there was a limited significant pattern of spatial autocorrelation at short distances, with some relatedness at 40 m for females and at 20 m for males. this could have been to the smaller sample size or the lower density of individuals at Location 1. Lower densities may lead to an increased potential availability of resources (such as unoccupied burrows further from relatives or neighbors) may have allowed individuals to disperse further.

One likely explanation for this high level of genetic structuring is that many established adults in the population have not dispersed far from their natal burrows. If lizards moved further and settled randomly across the landscape within their populations we would expect a more panmictic population structure. The pygmy bluetongue lizard may be a naturally short distance disperser. Selection may have favoured short dispersal distances that reduce the time spent out of burrows and thus the exposure to predators. Milne (1999) recorded high levels of juvenile mortality and Fenner *et al*. (2008a,b) suggested there is high predation by endemic predators (snakes and birds) when lizards are away from burrows. The further they disperse, the more their exposure to this predation.

Alternatively lizards, particularly juveniles, may attempt long dispersal, but few of those longer distance dispersers survive to be included in the current adult samples. The second, apparently contradictory result, was that within the one 1.2 ha site with an adequate sample size (site 2) we detected four separate genetic clusters among the lizards, but with no apparent spatial patterning of those clusters. Thus not all neighboring lizards were related. A possible explanation is that different genetic lineages are maintained within the population, and overlap within local areas. This lack of panmixia suggests that the promiscuous matings found by Schofield *et al.* (2014) are confined to individuals within local neighborhoods and not continuous across the landscape.

We found no evidence of a significant sex bias in dispersal from assignment index values, and nor were there clear differences in the patterns of spatial autocorrelation between the sexes. This suggests that males and females tend to disperse a similar small distance from their natal burrows to the burrows they occupy as adults.

Although field observations from pitfall trapping (Schofield *et al.* 2012) and genetic evidence from paternity studies (Schofield *et al.* 2014) have suggested that males move about, over short distances and more often than females, this surface movement of males is seasonal and reproduction based (Schofield *et al.* 2012), and does not imply longer distances of dispersal by males.

Similarly a greater loss of resident males than females (Fellows *et al.* 2009) is likely to be due to higher rates of predation on more mobile males than from higher rates of male dispersal. The potential risk of inbreeding within populations resulting from low distances dispersed coupled with the absence of any dispersal sex bias, is probably averted by the promiscuous mating system in this species (Schofield *et al.* 2014). Females accept multiple matings (Schofield *et al.* 2014), and among the potential male partners in close proximity are members of alternative genetic clusters.

Implications for pygmy bluetongue lizard conservation.

This study and others (Smith *et al.* 2009; Rogers 1998) show high genetic diversity and significant genetic structuring have been maintained across small and large scales within populations of pygmy bluetongue lizards, despite a substantial decline in area of its native grassland habitat into tiny isolated fragments. Frankham (1996) suggested that, although habitat fragmentation has the potential to cause loss of genetic diversity, losses can be minimized if high numbers of individuals can be retained in small population sites during the fragmentation. It appears that there is resistance to the genetic effects of fragmentation within populations of pygmy bluetongue lizards. And although there are now relatively few extant populations of this species, their divergent genetic composition as shown by Smith *et al.* (2009) suggests potential sources of genetic rescue through translocation or population supplementation if alleles are lost from an individual population.

Future conservation actions for the pygmy bluetongue lizard should include continued monitoring of the genetic diversity of the populations with the aim to maintain or re-establish diversity in failing populations. The spider holes that the lizards use as burrows are a limiting resource and artificial burrows can be added to

the populations to increase local densities (Souter *et al.* 2004). Strategies for genetic rescue will depend on the circumstances that have led to a decline in genetic diversity.

If population densities become too low and the opportunities for multiple mating with allelic diverse range of individuals become restricted (i.e. inbreeding is too high) then the addition of genetically different individuals across the population might be appropriate. Conversely if the availability of neighbouring relatives becomes low, and outbreeding becomes too high, then the targeted release of clustered groups of related individuals in adjacent burrows may be a solution. Frankam (2012) has suggested, however, that there may be low risk of outbreeding depression in contemporary fragmented landscapes (<200 years), as neither adaptive differentiation nor fixed chromosomal differences are likely to have evolved within the fragments.

From a broader perspective, this study suggests that the risk of loss of genetic diversity from habitat fragmentation may vary among species depending on their evolutionary history. Species with naturally low dispersal may have evolved mechanisms, for instance in their mating systems, that maintain genetic diversity even when dispersal corridors for potential gene flow are closed. For these species the genetic risks from habitat fragmentation may be lower than for more mobile species. Management actions for each species should be considered carefully and should take into account the mating systems and population structures that best reduce diversity loss.. For these lizards, and other species that have established population processes to retain high levels of genetic diversity even within small remnant populations, management actions should focus on sustaining population density rather than direct actions to increase genetic diversity. However careful ongoing monitoring to detect any sudden changes in genetic structure will be essential.

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Chapter 5. Movement among demographic populations

Understanding genetic structure and evolutionary relationships within a species is an important evaluation step to identify populations with low genetic diversity, to delineate genetically distinct units for conservation and to pinpoint suitable source populations for future translocation efforts.

This chapter further extends the investigation of Chapter 4 by investigating the between population genetic structure across the range of *Tiliqua adelaidensis.* Here we investigate current and historic genetic structure in a now fragmented habitat. Understanding the flow of genes across the landscape will aid in the delineation of conservation units and management actions

Neonate pygmy bluetongue lizards out of its burrow.

Conceived and designed the surveys: JS MG. Performed the sample collection and DNA analyses : JS. Analyzed the data: JS MA.. Contributed to the writing of the manuscript: JS MG MB.

Conservation of genetic variation amongst populations of the endangered Pygmy bluetongue lizard (*Tiliqua adelaidensis*)

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Abstract

By maintaining as many of the important genetic building blocks within the species as possible, conservationists give species the best chance to adapt to changing conditions. Often evolutionary significant units (ESUs) or conservation units are described to ensure that disparate genetic populations continue separate evolutionary trajectories. This study explores the genetic variation in historic (mitochondrial) and recent (microsatellite) markers in the context of management units in a species with limited dispersal. We found most of the nuclear marker variation was among populations. A high portion of the locations sampled had a unique genetic marker combination. The greatest marker diversity was found in the southern part of the pygmy bluetongue lizard's range. As no sampling locations were reciprocally monophyletic, ESUs could not be used to delineate groups for management, rather a more flexible conservation unit is required to preserve the genetic variability of the pygmy bluetongue lizard.

Introduction

A key component of the management of threatened species is the maintenance of evolutionary processes that may allow the species to adapt to future environmental challenges (Weeks *et al*. 2011). To maintain evolutionary processes, a thorough understanding of the genetic population structure of the endangered species is required. Early work in endangered species management that incorporated a population genetic component focused on the concept of evolutionarily significant units (ESUs) (Ryder 1986). This delineation aimed to provide an objective approach to prioritizing population units for conservation. The concept of ESUs were further refined by recognizing ESUs as reciprocally monophyletic groups (i.e. individuals

share a most recent common ancestor with other individuals in the same geographic area) to ensure that evolutionary heritage within species will be maintained by managing these long isolated populations separately (Moritz 1994). However these definitions tend to focus on preserving historic reproductive isolation and there needs to be a focus on preservation of functional, and hence adaptive, diversity rather than of historical legacy (Crandall 2000). In order for efforts to be directed at maintaining networks that capture the adaptive diversity within species, Crandall (2000) argued that the terminology of ESUs should be replaced with a more holistic concept of species, based on the concepts of 'ecological exchangeability'. The idea of ecological exchangeability proposes that individuals can be moved between populations and can occupy the same ecological niche or selective regime. This proposal by Crandall (2000) allows that species be conserved with populations with varying levels of gene flow evolving through drift and natural selection and that conservation efforts should be directed at maintaining networks that capture the adaptive diversity within species.

There is agreement however, that the main conservation goal should be to preserve both evolutionary processes and the ecological viability of populations (Moritz 1999). By maintaining as many of the important genetic building blocks within the species as possible the goal is to ensure that the process of evolution is not excessively constrained (Waples 1995). However a rigid, universal definition of an ESU across all species may not be possible, and no single approach will work best in all situations. Each approach has its strengths and weaknesses under different circumstances (Fraser and Bernatches 2001) with the core aim of preserving the adaptive genetic variance within species.

Fragmentation is a process that can disrupt the adaptive diversity of a species through altering genetic drift and selection. With increased fragmentation of habitats, it is imperative to determine population structure due to historical species' and recent genetic erosion (inbreeding and loss of genetic diversity: Nei 1973; Keyghobadi *et al*. 2005). This genetic erosion may ultimately reduce individual fitness, such as lowering disease resistance and the ability of populations to adapt to local environmental changes (Keller & Waller 2002). In situations where recent local processes have influenced the population structure, augmentation of gene flow

between populations could alleviate the loss of genetic diversity in many cases (Frankham 2012), providing genetic or evolutionary rescue (Carlson *et al*. 2014). Thus, understanding genetic structure and evolutionary relationships within a species is an important evaluation step to identify populations with low genetic diversity, to delineate genetically distinct units for conservation and to pinpoint suitable source populations for future translocation efforts (Clostio *et al.* 2012) if they are required.

Consideration of both past and recent effects that lead to population structure will help enable conservation managers to undertake conservation and restoration practices that maintain and increase genetic diversity within species by maintaining gene flow between populations, as well as extending the species range. Doing so will thereby promote *in situ* adaptive processes (Thomas 2011, Weeks *et al.* 2011).

Here we seek to understand the historical and recent population structure of an endangered scincid lizard the Australian pygmy bluetongue lizard, *Tiliqua adelaidensis*. The pygmy bluetongue lizard is endangered with a restricted distribution. The species was considered extinct after the last specimen was discovered in 1959, until its rediscovery in 1992 (Armstrong and Reid 1992). These lizards are restricted to a small number of natural, native grassland fragments in South Australia (Fig 1). Land-use changes over the last century have converted perennial native grasslands into croplands, pastures and urban areas, causing substantial contraction and fragmentation of the species range due to loss of essential habitat. Previous microsatellite studies showed low levels of dispersal between adjacent populations, and isolation by distance occurring at <1km (Chapter 4), and four distinct genetic clusters within the Burra Region (Smith *et al*. 2009; Chapter 4). In this study our definition of demographic population is as in Chapter 4 all individuals of a species living in an area of continuous available habitat surrounded by an unsuitable habitat. Preliminary mitochondrial DNA (mtDNA) analysis (Rogers 1998) suggested genetic differences between northern and southern populations. These areas are broadly separated by a gully of lower elevation which may have been forested pre European settlement, possibly hindering dispersal of the lizards over long time frames. However little is known about the local extent of long term refugial locations in that region.

Based on the limited dispersal and extensive genetic differences found among demographic populations that are relatively close geographically, we hypothesise that there will be both historical and more recent genetic differentiation among current population locations. We assessed historical separations using mitochondrial DNA, and more recent gene flow with microsatellite DNA. Specifically we

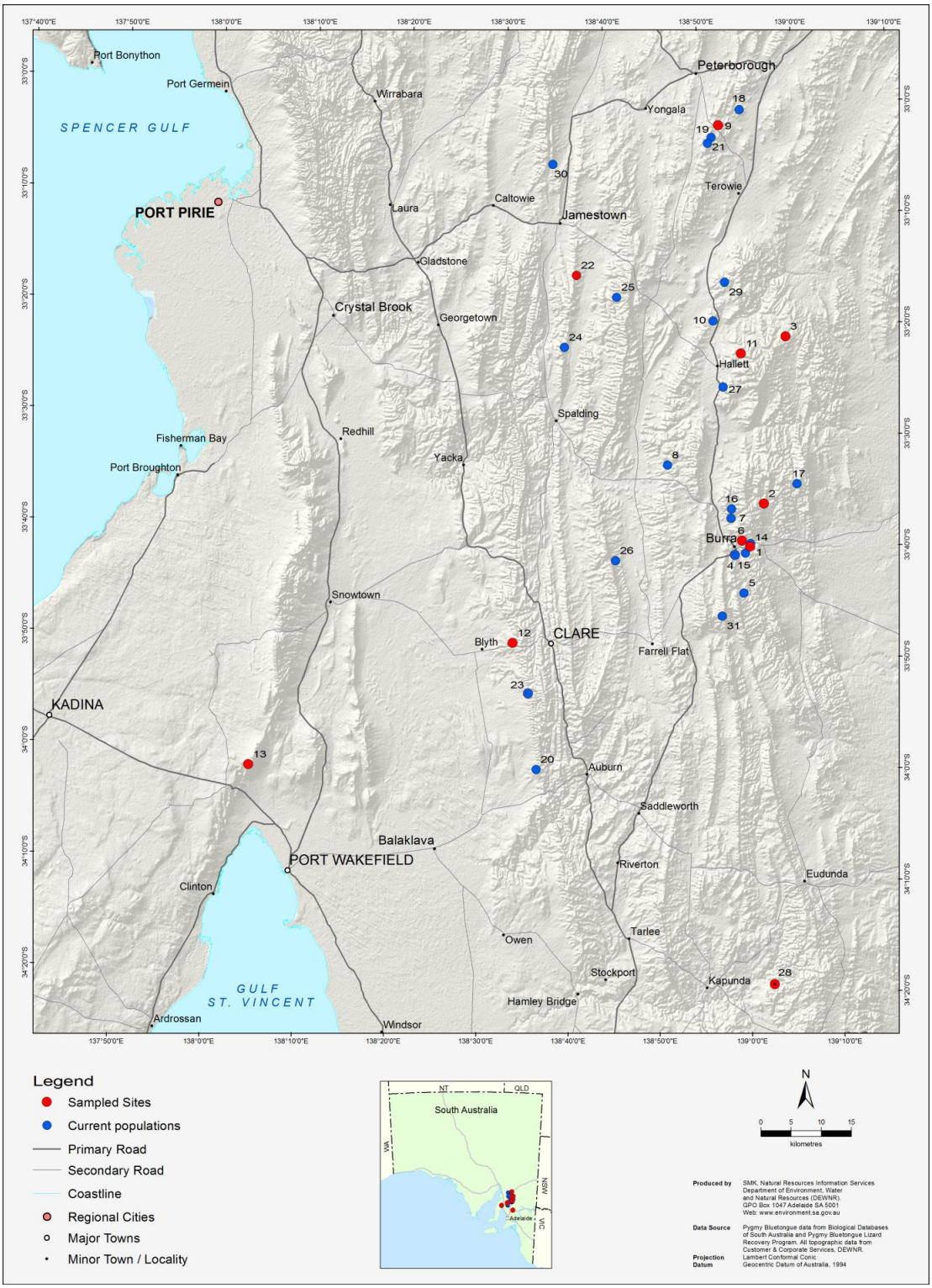

Figure 1. Distribution of pygmy Bluetongue lizards including known locations (blue) and sampled locations (red) in South Australia.

considered these geographically separate populations located to the north and to the west of the main concentration of populations around Burra will be genetically differentiated.

Methods

Sample collection

We sampled 101 lizards from 10 population locations across the current known geographic range of the species between 2005 and 2011 (Figure 1, Table 1). The location of each lizard was recorded using a GPS. Each newly captured lizard was individually marked by toe clip (with the clipped toes stored in ethanol) to avoid resampling. DNA from each lizard was extracted from the clipped toes (28 lizards) or from a blood sample stored on FTA paper (Whatman, Maidstone) that was taken as the toe was clipped (67 lizards). DNA was extracted from toe tissue using the Gentra systems PUREGNE DNA Isolation kit. DNA was extracted from the FTA stored blood according to the procedure for nucleated erythrocytes in Smith and Burgoyne (2004).

Table 1 Location and number of lizards sampled for mitochondrial and microsatellite analysis

Location number	Number of individuals sampled
1	12
2	10
3	13
6	5
9	13
11	7
12	9
13	6
22	13
28	7

mtDNA variation

We amplified the mtDNA ND4 region for 95 individuals using the primers TGACTACCAAAAGCTCATGTAGAAGC (Forstner *et al.* 1995) and TTTTACTTGGATTTGCACCA (Arevalo *et al.* 1994). Polymerase chain reactions (PCR) were performed in a total volume of 25µL containing 1x Buffer, 2mM MgCl, 0.8mM total DNTP's, 200nm of each primer and 20ng of DNA. The PCR amplification conditions were as follows: 94C° for 9 mins + (94C° for 45sec; 48C° for 45sec; 72C° for 1min) x34 + (72C° for 6min;25C° for 30sec) x1. PCR products were purified using MultiScreen$_{384}$ PCR Filter Plates (Millipore) following the manufacturer's instructions.

Amplified PCR products were sequenced in both directions using the same primers as those used for initial PCR. The sequencing reaction was performed in a total volume of 20µL with 10mM Big Dye, 1 x Big Dye buffer, 20ng PCR product 200nm of primer. PCR amplification conditions were 96C° for 2min + (96C° for 30sec; 50C° for 15sec; 60C° for 4min) x 30 + 25C° for 1min. products were purified using MultiScreen$_{384}$-Seq Filter Plates (Millipore) and then run on an ABI Prism 3730xl capillary sequencer at the AGRF, Adelaide. Sequences were edited using SEQED V 1.0.3 (Applied Biosystems) and aligned manually using SE-AL v. 1.0.3 (Rambaut, 1995).

Detection of nuclear paralogues

Nuclear paralogues were detected in sequences from individuals at two of the sampling locations (12 and 13) from a mis-match of the forward and reverse sequence and double peaks in the electrophoreagram caused by an insertion of a nucleotide in the paralogous version. We cloned amplicons from six samples from each of these two sampling localities following the Strata Clone PCR Cloning kit (Stratgene #240205). The resulting clones were directly sequenced using both forward and reverse vector primers T3 and T7 (Strata Clone PCR Cloning kit) with the following reaction conditions: 25µL reaction 1x Buffer, 2mM MgCl, 0.8mM dNTPs, 200nm of T3 or T7 primer, 0.5U Taq Gold, 20ng DNA. amplification conditions were as follows 94C° for 9 mins + (94C° for 45sec; 60C° for 45sec; 72C° for 1min) x 34 + (72C° for 6min; 25C° for 30sec). Purification and sequencing was conducted using the same method as per the amplicons mentioned previously.

The resulting 95 *ND4* mtDNA sequences were edited, aligned using MEGA v. 6.0 (Tamura, *et al*., 2013) and performed Bayesian phylogenetic analyses using MrBayes v 3.2 (Ronquist *et al.*, 2012). For these Bayesian analyses, we performed two independent runs, using the general model GTR + I + Γ and the default value of four Markov chains per run. Each chain was run for 30 million generations with a sample frequency of 10,000 and a burn-in period of 100,000 generation. We then measured the effective sample size of each parameter, checked for chain convergence, and visualized the plots using the program tracer v. 1.5 (Rambaut and Drummond, 2009). In addition, ten sequences of species closely related to *T. adelaidensis* were included in our analyses. These were *T.rugosa, T. multifasciata, T.occipitalis, Egernia cunninghami, E.striolata, E.saxatilis, E. hosmeri* and *E.stokesii*) from Gardner *et al*. (2008a).

A unrooted haplotype median-joining (MJ) network was constructed using NETWORK v.4.6.1.2 (Bandelt *et al*., 1999) following the software instructions. The genetic variance based on the mtDNA between and within populations was tested using an AMOVA in Arlequin.

Genotyping of Microsatellite DNA

We derived genotypes from 101 individuals at 15 DNA microsatellite loci (Smith *et al*. (2009) following the conditions of Gardner *et al*. (2008b)). Amplicons were separated on an ABI 3730 capillary electrophoresis DNA analyser (Applied Biosystems, Foster City, CA) at the Australian Genomics Research Facility (AGRF Adelaide node). A fluorescently labelled size standard (GS500 (-250) LIZ) was run with the samples to facilitate allele sizing. Alleles were scored using GeneMapper software version 3.7 (Applied Biosystems) with manual checking. The resulting genotypes were checked for conformance to Hardy-Weinberg and linkage equilibrium using GENEPOP 4.0.10 (Raymond and Rousset 1995, Rousset 2008).

Microsatellite DNA genetic structure

We analysed the microsatellite genotype data to determine whether individuals sorted into separate genetic clusters using the program STRUCTURE (Pritchard *et al.* 2000). We used an admixture model with no prior population information, a burn in length of 50 000 and a MCMC length of 1 million iterations. We simulated the

number of clusters from K=1 to K = 12, (based on the number of locations sampled) and performed 10 independent simulations of each K-value to check for consistency across runs. We used STRUCTURE HARVESTER (Earl and von Holdt 2012) to infer the number of clusters by calculating ΔK which is the rate of change between successive simulations (Evanno *et al.* 2005). Individuals were assigned to a genetic cluster on the basis of the highest probability of membership and checked for consistency of assignments across the 10 runs at the most likely number of clusters. We further explored finer scale genetic clustering within each of the genetic clusters identified in the initial STRUCTURE analysis.

Results

MtDNA variation

Six individuals from location 12 and four from location 13 were successfully cloned and used in subsequent analysis. We translated the sequences into amino acids using the program Se-al and assumed the sequences that did not translate were the paralogues. The phylogenetic trees constructed from the mtDNA sequences had similar topologies for both the neighbour joining and Bayesian methods (Appendix 1). The results supported a separation of the northern-most locations (9 and 22) from the remaining sites but no sampling locations were reciprocally monophyletic as two individuals physically located in southern locations (location 3 and location 12) clustered with individuals from the northern clade.

There was reasonable support (0.88) for the separation of the southern locations into two further groups. The first consisted of locations 6, 13, 12, and 27 and the second with locations 1, 2, 3, and 11. Within the second group, one sample from location 3 appeared in the location 11 grouping and one sample from location 2 was grouped with the location 3 samples. There was also good support for differentiation of location 2 (0.97) from locations 1, 3 and 11.

We found 31 different haplotypes with five of these recorded in more than one population location (Fig 2.). Locations 3 and 11 showed the least genetic variation, while locations 22 and 9 had the greatest with 5 different haplotypes.

The among location level explained most of the variation in mtDNA sequence (about 74%, AMOVA $p<0.0001$) and with variation within locations accounting for the remainder (Table 2).

Table 2 Results of an AMOVA testing the variation in mtDNA sequence in pygmy bluetongue lizard populations.

Source of variation	Sum of squares	Variance components	Percentage variation
Among locations	270.496	3.15152	74.03671
Within locations	90.557	1.10518	25.96329
Total	361.052	4.25669	

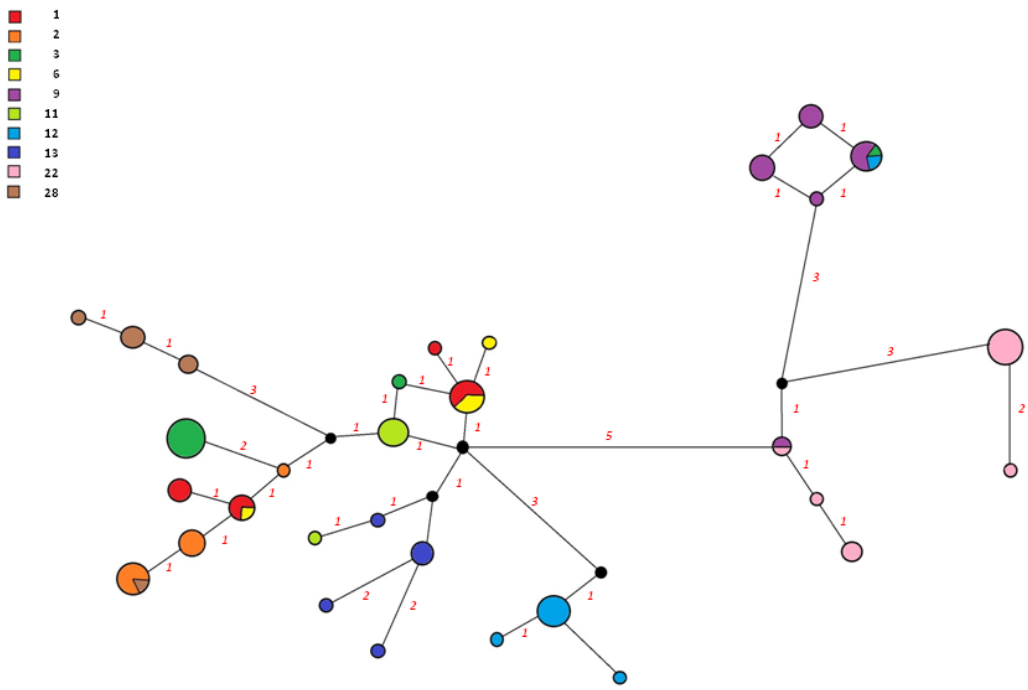

Figure 2. An unrooted haplotype median-joining (MJ) network of the mtDNA ND4 region. Circle size is proportional to the number of individuals with that haplotype. Colours relate to the location where individuals were sampled. Numbers relate to the number of nucleotide mutations

Microsatellite variation

No loci were found to consistently deviate from HWE nor were any locus to locus pairs linked in more than one population. A sample-wide analysis using all genotypes of all individuals from all 10 sampling locations in STRUCTURE identified seven genetic clusters. There was a single genetic cluster identified at each of locations 3, 9, 11 and 12 and 13 (Fig 3.). Individuals from locations 1 and 28 showed the most admixture and these locations contained individuals from several of the identified genetic clusters (Fig 3.) mostly reflecting the genetic clusters of neighbouring locations.

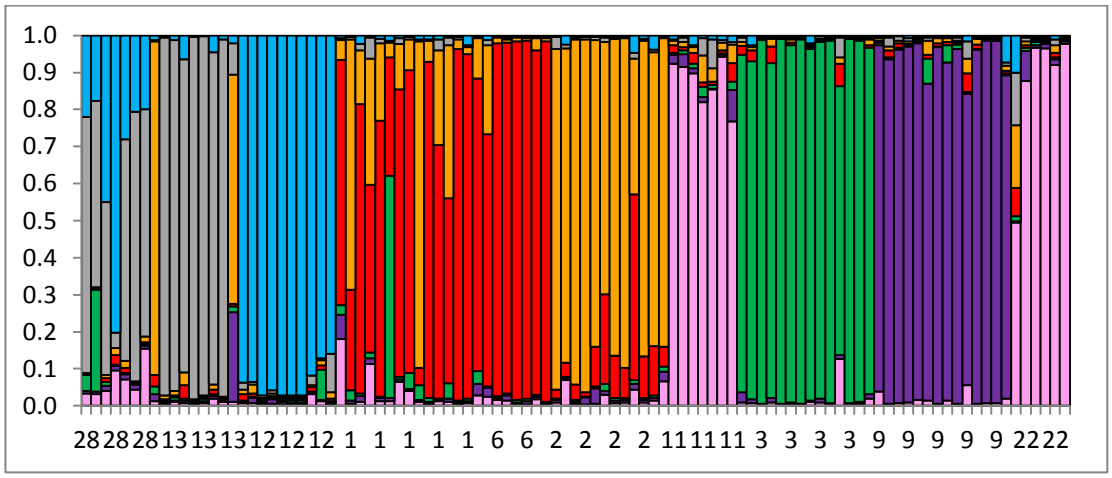

Figure 3. Probability of assignment to a cluster (Q) arranged by Q value for all sampled Tiliqua adelaidensis individuals arranged from south to north across the distribution. Each bar represents one individual. The different shades represent the clusters (k=7) identified by STRUCTURE.

Location 1 and 6 are geographically close (<1km apart), and were dominated by the same cluster, however 2 individuals from locality 1 were assigned with high probability to the same genetic cluster as location 2 and one individual from location one was assigned to the same genetic cluster as location 3 possibly indicating some longer range dispersals.

Individuals with the same haplotype were found at each of location 1 and 6. Individuals with these haplotypes were also assigned to the same microsatellite cluster. In contrast, individuals from locality 22 and its next nearest neighbour, locality 11 (33km) were grouped in same microsatellite genetic cluster despite being separated in the haplotype network (Fig 4.). Individuals from localities 13 and 28

were found in different clades on the haplotype network however in the microsatellite analysis, most of the individuals from location 13 and 28 were assigned to the same genetic cluster with the exception of one individual from each locality. Individuals from locality 13 were more similar to the microsatellite cluster comprising animals from locality 2. Individuals from locality 28 were of mixed ancestry with one individual classified to the genetic cluster of individuals from locality 2 and one to the cluster at comprising primarily locality 12 animals. Other individuals from locality 28 appeared to be admixed.

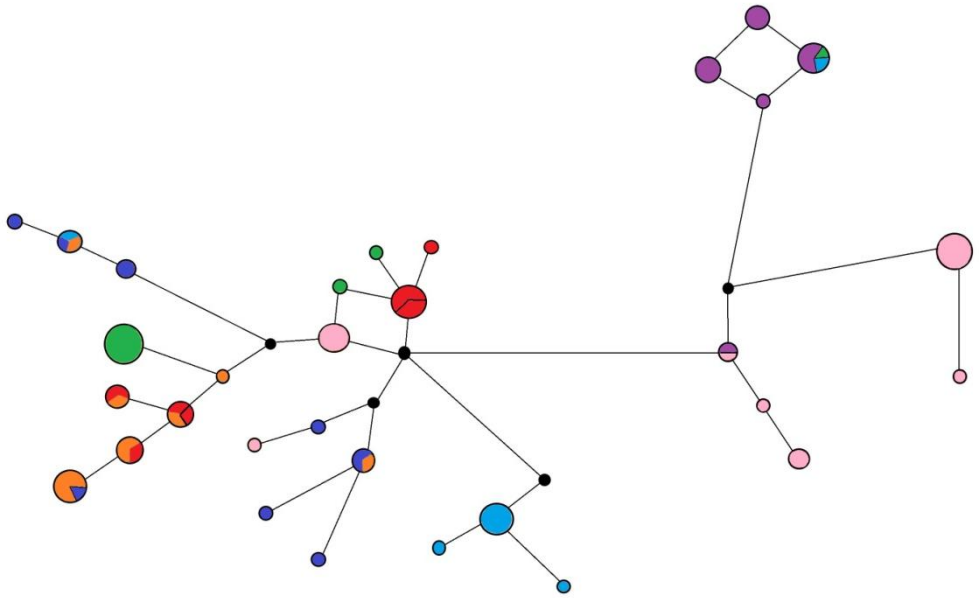

Figure 4 Network based on ND4 layout with individuals coloured according to microsatellite clusters as assigned by STRUCTURE (see Figure 3).

Discussion

Geographic patterns

Despite having a small current range the pygmy bluetongue lizards still displayed a high level of genetic structuring in the ten sampled populations. This study showed that most of the genetic variation was among the sampled populations, and not within them. There were at least 31 mitochondrial haplotypes across the sampled range with 26 of the haplotypes each recorded in only a single population. The mitochondrial

tree supports a divergence between population 9 and 22 (northern most samples) and the rest of the sampled populations (southern samples). The analysis of microsatellite DNA identified seven different genetic clusters from the ten sampled population locations across the range of the pygmy bluetongue lizards, with each location having one or more unique genetic combinations.

When individuals are assigned to a different microsatellite clade to that of the mitochondrial results this may be evidence for migrations. This study detected cases where these differences occurred.

Two of the individuals captured in southern locations were allocated to population 9 using mitochondrial data, and assign to the location of their capture by the microsatellite results. This suggests a recent migration to population 9, however give the distances between the populations we consider this unlikely. it is possible that this result could be due to mislabelled samples. A similar pattern was repeated at 1, 6 and 2 suggestion recent migration was also evident between these populations. The populations are within 10 kms of each other and they appear to be in close enough proximity for genetic information still to be shared. For other neighbouring sample locations there were few to no shared genotypes for both for mitochondrial and microsatellite DNA. Given the short dispersal distances and the isolation by distance evident in current populations (Chapter 4) it is likely that the gene flow between populations is relictual rather than current and is overlayed on historical population separations.

The level of concordance between the two DNA marker types indicates that there has been historical separation of the sampling localities possibly relating to the existence of multiple long term refugia in the region. Some localities appeared to have individuals with nuclear (microsatellite) signatures in common with other localities but have nearly unique mtDNA patterns. Potentially these are examples of incomplete lineage sorting with the mtDNA having sorted more quickly than nuclear components of the lizards' genomes.

Conservation Implications

Understanding genetic structure and evolutionary relationships within a species is an important evaluation step to identifying populations with low genetic diversity, delineating genetically distinct management units for conservation and pinpointing suitable source populations for future translocation efforts (Clostio *et al.* 2012). The distribution of mtDNA haplotypes for *Tiliqua adelaidensis* was not reciprocally monophyletic between the northern and southern populations and therefore cannot be classified as separate evolutionary significant units as defined by Moritz (1994).

However, there is a strong argument for monitoring and managing each population separately in order to preserve both local evolutionary processes and the ecological viability of populations (Moritz 1999) by maintaining as many of the important genetic building blocks within the species as possible so that the process of evolution for the species as a whole is not excessively constrained (Waples 1995). Indeed some of the locations such as location 9 and 12 have mitochondrial haplotypes and microsatellite clusters that are not represented in any other populations.

With the high among location genetic diversity and the probable lack of dispersal between locations of we feel it is appropriate to adopt the definition of management units by Palsbøll *et al*. (2006) in which are defined by the "populations of conspecific individuals among which the degree of connectivity is sufficiently low so that each population should be monitored and managed separately". Smith *et al.* (2009) found levels of heterozygosity in populations ranging from 0.75 to 0.82, and negative but non significant F_{IS}. They also reported and lower allelic richness values than were recorded in the current study for the two most northern populations (9 and 22). Since there are unique haplotypes in most of the populations we suggest further sampling of the populations to better map out the genetic movement and diversity across this species range. Potentially recent genome wide techniques could elucidate the extent of local adaptations further.

The long generation times of the lizards (up to 15 years) may mean the detected shared genotypes may be relictual and that there is no longer any genetic flow between the populations. Therefore these current results are probably indicative of the historical population structure and the current signatures of contemporary

structure may not yet be defined as recent fragmentation of the grassland habitat has only occurred in the past 50- 100 years and it may take 10's of generations for changes significant enough to cause out breeding depression or lead to speciation (Hendry *et al.* 2007, Frankham *et al*. 2011). Whilst the complete divergence of populations may not have occurred yet, the small population sizes and restricted gene flow amongst populations may result in isolated populations that are well below adequate effective population sizes for maintaining their adaptive potential (Weeks *et al*. 2011). Indeed a effective population of over 100 may be needed to avoid inbreeding depression and an effective population size of over 1000 individuals to maintain the adaptive potential (Frankham *et al. 2014*)

Future conservation actions

Fordham *et al*. (2012) argues in the face of climate change managed relocations are critical for safeguarding lizard population persistence. The northern populations are most likely to be first impacted by the heating and drying effects of climate change. If no action is taken it is likely the two haplotypes represented only in the northern populations will be lost from the gene pool. We strongly suggest that future translocation efforts should include moving animals with the northern haplotypes to more climatically stable areas.

There does not appear to be any current fitness or phenotypic differences between populations (Shaminoori 2014) suggesting at present there may not be obvious adaptive differences among distant populations. This reduced the necessity for source populations to be 'genetically matched' to recipient locations to ensure that genotypes adapted to local conditions at the recipient location are translocated (Weeks *et al.* 2011). However nothing currently is known of the genetic differences in adaptive areas of the genome which may indicate more caution is required.

This study has shown that there is a greater level of levels of marker diversity between the populations of pygmy bluetongue lizards than first expected. This study highlights the need for more intense sampling across the distribution of species with short dispersal distances not only to detect the genetic variation but also further to evaluate historic and current gene flow. This is especially important when trying to preserve the maximum genetic variability for conservation management.

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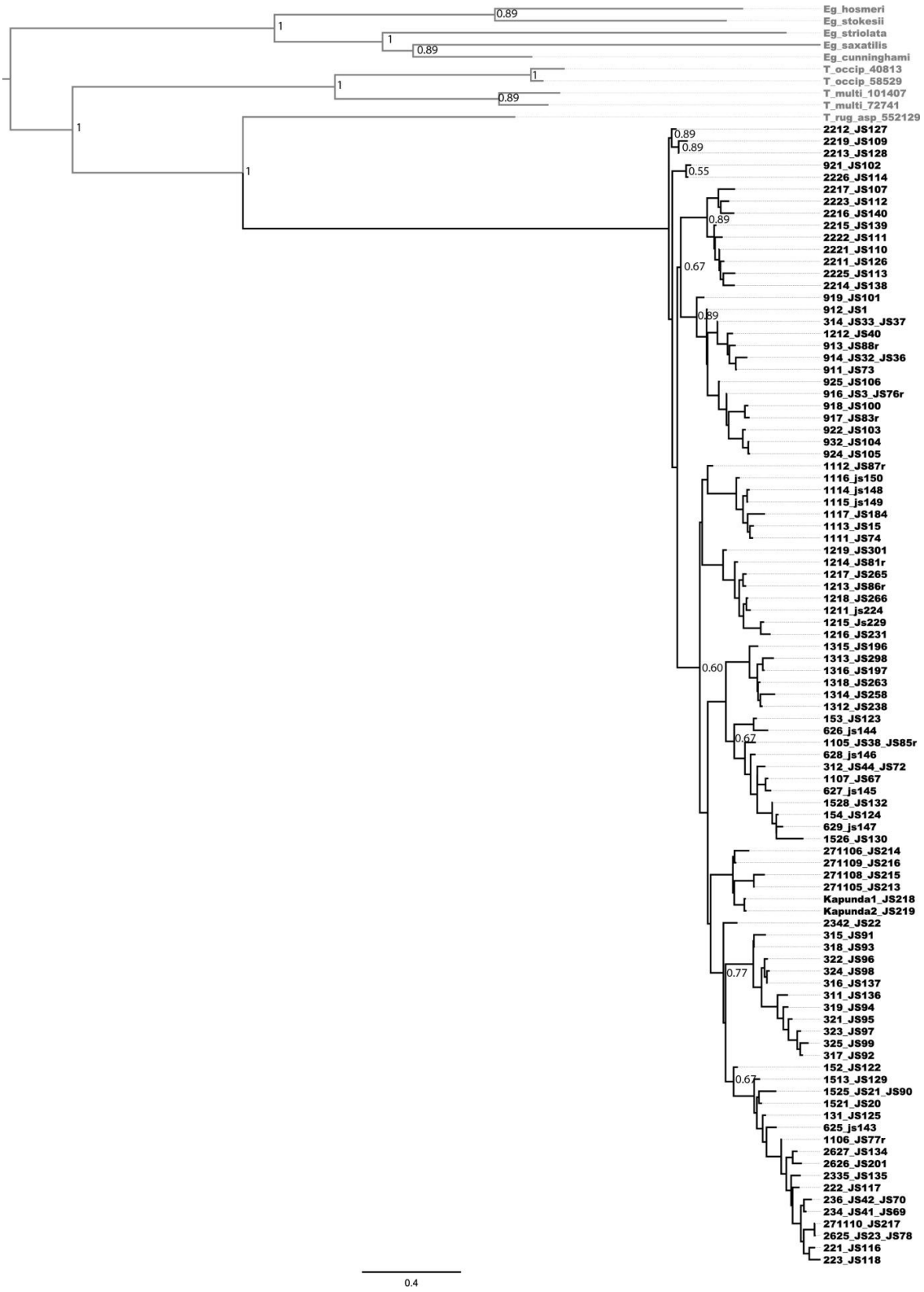
Appendix 1. Neighbour joining Phylogenetic tree constructed from the mtDNA sequences



Appendix 2. Probability of assignment to a cluster (Q) arranged south to north across the distribution for all sampled *Tiliqua adelaidensis* individuals, as identified by STRUCTURE (see figure 3).

Location	Indiv.	Haplo-type	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6	Cluster 7
28	kap1	h40	0.03	0.01	0.04	0.00	0.00	**0.69**	0.22
28	kap2	h41	0.03	0.01	0.28	0.00	0.00	**0.50**	0.18
28	1105k	h38	0.04	0.01	0.01	0.01	0.01	**0.47**	0.45
28	1106k	h38	0.10	0.01	0.00	0.03	0.02	0.04	**0.80**
28	1107k	-	0.07	0.01	0.00	0.01	0.02	**0.60**	0.28
28	1108k	h39	0.04	0.01	0.00	0.00	0.00	**0.73**	0.21
28	1109k	h38	0.16	0.01	0.00	0.01	0.02	**0.61**	0.20
28	1110k	h10	0.01	0.02	0.02	0.03	**0.90**	0.00	0.01
13	1312	h32	0.01	0.01	0.00	0.00	0.01	**0.97**	0.01
13	1313	h33	0.01	0.00	0.01	0.01	0.01	**0.95**	0.01
13	1315	h35	0.01	0.01	0.01	0.04	0.04	**0.84**	0.07
13	1321	-	0.00	0.00	0.00	0.00	0.00	**0.98**	0.00
13	1311	-	0.01	0.00	0.01	0.00	0.01	**0.97**	0.00
13	1314	h34	0.02	0.01	0.00	0.01	0.01	**0.90**	0.05
13	1316	h32	0.01	0.00	0.00	0.00	0.01	**0.96**	0.01
13	1318	h32	0.01	0.24	0.02	0.01	**0.62**	0.09	0.02
12	1217	h27	0.01	0.01	0.00	0.02	0.01	0.02	**0.94**
12	1211	h26	0.01	0.01	0.00	0.01	0.02	0.01	**0.94**
12	1212	h13	0.01	0.00	0.00	0.00	0.00	0.01	**0.97**
12	1213	h24	0.01	0.01	0.00	0.01	0.01	0.01	**0.96**
12	1214	h25	0.00	0.01	0.00	0.01	0.01	0.00	**0.97**
12	1215	h27	0.01	0.00	0.01	0.01	0.00	0.01	**0.97**
12	1216	h27	0.01	0.00	0.01	0.00	0.01	0.01	**0.97**
12	1218	h27	0.03	0.00	0.00	0.01	0.01	0.03	**0.92**
12	1219	h27	0.01	0.00	0.08	0.01	0.01	0.01	**0.87**
12	1221	-	0.01	0.00	0.00	0.01	0.02	0.11	**0.86**
1	131	h2	0.18	0.07	0.03	**0.66**	0.06	0.01	0.01
1	152	h2	0.01	0.01	0.03	0.27	**0.68**	0.01	0.00
1	153	h3	0.01	0.02	0.01	**0.78**	0.15	0.02	0.02
1	154	h4	0.11	0.02	0.02	**0.45**	0.34	0.06	0.01
1	1101	-	0.01	0.01	0.00	**0.75**	0.21	0.01	0.01
1	1105	h5	0.01	0.01	**0.60**	0.32	0.04	0.01	0.01
1	1106	h1	0.07	0.01	0.01	**0.77**	0.12	0.01	0.01
1	1107	h43	0.04	0.01	0.04	**0.82**	0.08	0.01	0.00
1	1513	h1	0.01	0.01	0.04	0.05	0.88	0.01	0.01
1	1521	h1	0.01	0.01	0.01	**0.91**	0.06	0.00	0.01
1	1525	h1	0.01	0.00	0.00	**0.69**	0.26	0.03	0.01
1	1526	h6	0.01	0.01	0.04	**0.50**	0.41	0.02	0.01
1	1527	-	0.01	0.00	0.01	**0.95**	0.03	0.01	0.00
1	1528	h6	0.01	0.00	0.01	**0.93**	0.02	0.01	0.03
6	614	-	0.03	0.03	0.03	**0.79**	0.11	0.00	0.00
6	625	h2	0.03	0.02	0.00	**0.68**	0.24	0.01	0.01

6	626	h36	0.02	0.01	0.01	**0.95**	0.02	0.00	0.00
6	627	h36	0.02	0.01	0.01	**0.95**	0.01	0.01	0.01
6	628	h37	0.01	0.00	0.01	**0.97**	0.01	0.00	0.00
6	629	h36	0.00	0.01	0.01	**0.97**	0.01	0.00	0.00
6	642	-	0.02	0.01	0.01	**0.93**	0.03	0.00	0.00
6	643	-	0.00	0.00	0.00	**0.97**	0.01	0.00	0.01
2	221	h10	0.01	0.01	0.00	0.03	**0.92**	0.03	0.00
2	222	h11	0.07	0.00	0.00	0.04	**0.85**	0.01	0.03
2	223	h10	0.01	0.01	0.01	0.04	**0.93**	0.01	0.01
2	233	-	0.00	0.02	0.00	0.01	**0.95**	0.01	0.00
2	234	h9	0.01	0.04	0.01	0.11	**0.83**	0.01	0.00
2	236	h9	0.03	0.01	0.02	0.24	**0.68**	0.01	0.01
2	2335	h8	0.01	0.01	0.01	0.12	**0.85**	0.01	0.00
2	2625	h10	0.01	0.01	0.01	0.08	**0.89**	0.00	0.00
2	2626	h8	0.04	0.02	0.01	**0.50**	0.37	0.02	0.05
2	2627	h8	0.01	0.01	0.01	0.11	**0.85**	0.01	0.01
2	2694	-	0.01	0.01	0.01	0.13	**0.79**	0.01	0.04
2	2696	-	0.07	0.03	0.01	0.05	**0.83**	0.00	0.01
11	1111	h22	**0.92**	0.02	0.01	0.02	0.01	0.01	0.01
11	1112	h22	**0.92**	0.03	0.01	0.01	0.01	0.01	0.01
11	1113	h21	**0.90**	0.01	0.01	0.03	0.02	0.01	0.03
11	1114	h23	**0.82**	0.01	0.03	0.01	0.07	0.05	0.01
11	1115	h23	**0.85**	0.00	0.01	0.01	0.04	0.08	0.01
11	1116	h23	**0.94**	0.01	0.01	0.01	0.02	0.01	0.01
11	1117	h22	**0.77**	0.08	0.02	0.05	0.05	0.01	0.02
3	311	h15	0.01	0.03	**0.91**	0.02	0.01	0.01	0.01
3	312	h14	0.01	0.01	**0.91**	0.03	0.01	0.01	0.03
3	313	-	0.00	0.00	**0.98**	0.00	0.00	0.00	0.00
3	314	h13	0.01	0.01	**0.91**	0.04	0.02	0.00	0.01
3	315	h15	0.00	0.00	**0.98**	0.00	0.00	0.00	0.00
3	316	h15	0.01	0.00	**0.97**	0.01	0.01	0.01	0.01
3	317	h15	0.00	0.00	**0.99**	0.00	0.00	0.00	0.00
3	318	h15	0.01	0.00	**0.95**	0.01	0.00	0.01	0.02
3	319	h15	0.01	0.01	**0.96**	0.00	0.00	0.01	0.00
3	321	h15	0.00	0.00	**0.98**	0.01	0.00	0.00	0.00
3	322	h15	0.13	0.01	**0.73**	0.06	0.02	0.06	0.01
3	323	h15	0.00	0.00	**0.98**	0.00	0.00	0.00	0.00
3	324	h15	0.01	0.01	**0.97**	0.00	0.00	0.00	0.00
3	325	h15	0.02	0.01	**0.94**	0.01	0.02	0.01	0.01
9	911	h42	0.04	**0.94**	0.01	0.01	0.01	0.01	0.00
9	912	h13	0.01	**0.93**	0.01	0.02	0.01	0.02	0.01
9	913	h18	0.01	**0.96**	0.00	0.01	0.01	0.01	0.00
9	914	h13	0.01	**0.96**	0.00	0.00	0.01	0.01	0.01
9	915	-	0.02	**0.96**	0.00	0.01	0.00	0.00	0.00
9	916	h16	0.01	**0.86**	0.07	0.01	0.04	0.01	0.01
9	917	h19	0.01	**0.96**	0.00	0.01	0.02	0.00	0.00
9	918	h16	0.02	**0.91**	0.05	0.01	0.01	0.00	0.00
9	919	h13	0.01	**0.96**	0.01	0.01	0.01	0.01	0.01

9	921	h20	0.06	**0.79**	0.01	0.05	0.04	0.05	0.02
9	922	h17	0.01	**0.96**	0.00	0.01	0.02	0.00	0.01
9	923	-	0.01	**0.98**	0.00	0.01	0.00	0.00	0.00
9	924	h17	0.01	**0.98**	0.00	0.00	0.00	0.00	0.00
9	925	h16	0.02	**0.87**	0.00	0.01	0.01	0.01	0.07
22	2211	h31	**0.50**	0.00	0.01	0.08	0.17	0.14	0.10
22	2212	h28	**0.88**	0.08	0.01	0.01	0.01	0.01	0.01
22	2213	h28	**0.97**	0.01	0.01	0.01	0.01	0.00	0.00
22	2214	h31	**0.96**	0.01	0.00	0.01	0.00	0.01	0.01
22	2215	h31	**0.92**	0.02	0.01	0.01	0.02	0.02	0.01
22	2216	h31	**0.98**	0.01	0.00	0.00	0.00	0.00	0.00

Appendix 3. haplotypes found in the 10 sampled locations in this study.

h1
CTACGGTATTATCCGAATCACCCCTCTACTAAACCCCCTTTCAACAAAACTATACTACCCTTTTATTATTTTGGCT
ATTTGAGGCATCTTTATAACCAGCTCAATTTGTCTTCGACAGGCCGACTTAAAGTCTCTAATCGCCTACTCATCAG
TAAGCCACATAGGCCTTGTGATCACAGCCACTTTAATTCAAACCCCATGAAGCATCTCTGGGGCAATTCTACTAA
TAATCGCCCACGGCCTCACATCCTCAATACTCTTCTGCCTAGCAAACACGAATTATGAACGCACCCACAGCCGAA
CCCTTCTCCTAGCCCGGGGCTTACAACTTTTACTTCCCCTAATAACACTATGGTGACTCCTAGCTAATTTAATAAA
CATAGCCCTTCCCCCCACAATCAACCTCACTGGTGAACTCCTAATCATTTCATCCGCATACAATTGATCCCCCCTC
ACAATCGTATTAACAGGCGCCGGCACACTTCTAACAGCAATCTACTCCCTTCACATATTCTTAATAACACAACGA
GGTAAACTCCCACAACACATCATCAAAATAGCCCCAACACACACCCGAGAACA

h2
CTACGGTATTATCCGAATCACCCCCCTACTAAACCCCCTTTCAACAAAACTATACTACCCTTTTATTATTTTGGCT
ATTTGAGGCATCTTTATAACCAGCTCAATTTGTCTTCGACAGGCCGACTTAAAGTCTCTAATCGCCTACTCATCAG
TAAGCCACATAGGCCTTGTGATCACAGCCACTTTAATTCAAACCCCATGAAGCATCTCTGGGGCAATTCTACTAA
TAATCGCCCACGGCCTCACATCCTCAATACTCTTCTGCCTAGCAAACACGAATTATGAACGCACCCACAGCCGAA
CCCTTCTCCTAGCCCGGGGCTTACAACTTTTACTTCCCCTAATAACACTATGGTGACTCCTAGCTAATTTAATAAA
CATAGCCCTTCCCCCCACAATCAACCTCACTGGTGAACTCCTAATCATTTCATCCGCATACAATTGATCCCCCCTC
ACAATCGTATTAACAGGCGCCGGCACACTTCTAACAGCAATCTACTCCCTTCACATATTCTTAATAACACAACGA
GGTAAACTCCCACAACACATCATCAAAATAGCCCCAACACACACCCGAGAACA

h3
CTACGGTATTATCCGAATCACCCCCCTACTAAACCCCCTTTCAACAAAACTATACTACCCTTTTATTATCTTGGCT
ATTTGAGGCATCTTTATAACCAGCTCAATTTGTCTTCGACAGGCCGACTTAAAGTCTCTAATCGCCTACTCATCAG
TAAGCCACATAGGCCTTGTGATCACAGCCACTTTAATTCAAACCCCATGAAGCATCTCTGGGGCAATTCTACTAA
TAATCGCCCACGGCCTCACATCCTCAATACTCTTCTGCCTAGCAAACACGAATTATGAACGTACCCATAGCCGAA
CCCTTCTCCTAGCCCGGGGCTTACAACTTTTACTTCCCCTAATAACACTATGGTGACTCCTAGCTAATTTAATAAA
CATAGCCCTTCCCCCCACAATCAACCTCACTGGTGAACTCCTAATCATTTCATCCGCATACAATTGATCCCCCCTC
ACAATCGTATTAACGGGCACCGGCACACTTCTAACAGCAATCTACTCCCTTCACATATTCTTAATAACACAACGA
GGTAAACTCCCACAACACATCATCAAAATAGCCCCAACACACACCCGAGAACA

h4
CTACGGTATTATCCGAATCACCCCCCTACTAAACCCCCTTTCAACAAAACTATACTACCCTTTTATTATCTTGGCT
ATTTGAGGCATCTTTATAACCAGCTCAATTTGTCTTCGACAGGCCGACTTAAAGTCTCTAATCGCCTACTCATCAG
TAAGCCACATAGGCCTTGTGATCACAGCCACTTTAATTCAAACCCCATGAAGCATCTCTGGGGCAATTCTACTAA
TAATCGCCCACGGCCTCACATCCTCAATACTCTTCTGCCTAGCAAACACGAATTATGAACGYACCCATAGCCGAA
CCCTTCTCCTAGCCCGGGGCTTACAACTTTTACTTCCCCTAATAACACTATGGTGACTCCTAGCTAATTTAATAAA
CATAGCCCTTCCCCCCACAATCAACCTCACTGGTGAACTCCTAATCATTTCATCCGCATACAATTGATCCCCCCTC
ACAATCGTATTAACGGGCACCGGCACACTTCTAACAGCAATCTACTCCCTTCACATATTCTTAATAACACAACGA
GGTAAACTCCCACAACACATCATCAAAATAGCCCCAACACACACCCGAGAACA

h5

CGACAGGCCGACTTAAAGTCTCTAATCGCCTACTCATCAGTAAGCCACATAGGCCTTGTGATCACAGCCACTTTA
ATTCAAACCCCATGAAGCATCTCTGGGGCAATTCTACTAATAATCGCCCACGGCCTCACATCCTCAATACTCTTCT
GCCTAGCAAACACGAATTATGAACGTACCCATAGCCGAACCCTTCTCCTAGCCCGGGGCTTACAACTTTTACTTC
CCCTAATAACACTATGGTGACTCCTAGCTAATTTAATAAACATAGCCCTTCCCCCCACAATCAACCTCACTGGTG
AACTCCTAATCATTTCATCCGCATACAATTGATCCCCCCTCACAATCGTATTAACGGGCACCGGCACACTTCTAAC
AGCAATCTACTCCCTTCACATATTCTTAATAACACAACGAGGTAAACTCCCACAACACATCATCAAAATAGCCCC
AACACACACCCGAGAACA

h6

GTCTCTAATCGCCTACTCATCAGTAAGCCACATAGGCCTTGTGATCACAGCCACTTTAATTCAAACCCCATGAAG
CATCTCTGGGGCAATTCTACTAATAATCGCCCACGGCCTCACATCCTCAATACTCTTCTGCCTAGCAAACACGAAT
TATGAACGCACCCACAGCCGAACCCTTCTCCTAGCCCGGGGCTTACAACTTTTACTTCCCCTAATAACACTATGGT
GACTCCTAGCTAATTTAATAAACATAGCCCTTCCCCCCACAATCAACCTCACTGGTGAACTCCTAATCATTTCATC
CGCATACAATTGATCCCCCCTCACAATCGTATTAACAGGCGCCGGCACACTTCTAACAGCAATCTACTCCCTTCA
CATATTCTTAATAACACAACGAGGTAAACTCCCACAACACATCATCAAAATAGCCCCAACACACACCCGAGAAC
A

h7
CTACGGTATTATCCGAATCGCCCCCCCTACTAAACCCCCTTTCAACAAAACTATACTACCCTTTTATTATCTTGGCT
ATTTGAGGCATCTTTATAACCAGCTCAATTTGTCTTCGACAGGCCGACTTAAAGTCTCTAATCGCCTACTCATCAG
TAAGCCACATAGGCCTTGTGATCACAGCCACTTTAATTCAAACCCCATGAAGCATCTCTGGGGCAATTCTACTAA
TAATCGCCCACGGCCTCACATCCTCAATACTCTTCTGCCTAGCAAACACGAATTATGAACGTACCCATAGCCGAA
CCCTTCTCCTAGCCCGGGGCTTACAACTTTTACTTCCCCTAATAACACTATGGTGACTCCTAGCTAATTTAATAAA
CATAGCCCTTCCCCCCACAATCAACCTCACTGGTGAACTCCTAATCATTTCATCCGCATACAATTGATCCCCCCTC

ACAATCGTATTAACGGGCACCGGCACACTTCTAACAGCAATCTACTCCCTTCACATATTCTTAATAACACAACGA
GGTAAACTCCCACAACACATCATCAAAATAGCCCCAACACACACCCGAGAACA

h8

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h9

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h10

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h11

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h12

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h13

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h14

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h15
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h16
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h17
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h18
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h19
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h20

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h21

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h22

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h23

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h24

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h25

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h26

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h27

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h28

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h29

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h30

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h31

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h32

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h33

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h34

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h35

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h36
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h37
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h38
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TAATCGCCCACGGCCTCACATCCTCAATACTCTTCTGCCTAGCAAACACRAATTATGAACGTACCCATAGCCGAA
CCCTTCTCCTAGCCCGGGGCTTACAACTTTTACTTCCCCTAATAACACTATGGTGACTCTTAGCTAATTTAATAAA
CATAGCCCTTCCCCCCACAATCAACCTCACTGGTGAACTCCTAATCATTTCATCCGCATACAATTGATCCCCCCTT
ACAATCGTATTAACGGGCGCCGGCACACTTCTAACAGCAATCTACTCCCTTCACATGTTCTTAATAACACAACGA
GGTAAACTCCCACAACACATCATCAAAATAGCCCCAACACACACCCGAGAACA

h39
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TAAGCCACATAGGCCTTGTGATCACAGCCACTTTAATTCAAACCCCATGAAGCATCTCTGGGGCAATTCTACTAA
TAATCGCCCACGGCCTCACATCCTCAATACTCTTCTGCCTAGCAAACACRAATTATGAACGTACCCATAGCCGAA
CCCTTCTCCTAGCCCGGGGCTTACAACTTTTACTTCCCCTAATAACACTATGGTGACTCTTAGCTAATTTAATAAA
CATAGCCCTTCCCCCCACAATCAACCTCACTGGTGAACTCCTAATCATTTCATCCGCATACAATTGATCCCCCCTT
ACAATCGTATTAACGGGCGCCGGCACACTTCTAACAGCAATCTACTCCCTTCACATGTTCTTAATAACACAACGA
GGTAAACTCCCACAACACATCATCAAAATAGCCCCAACACACACCCGAGAACA

h40
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TAAGCCACATAGGCCTTGTGATCACAGCCACTTTAATTCAAACCCCATGAAGCATCTCTGGGGCAATTCTACTAA
TAATCGCCCACGGCCTCACATCCTCAATACTCTTCTGCCTAGCAAACACRAATTATGAACGCACCCACAGCCGAA
CCCTTCTCCTAGCCCGGGGCTTACAACTTTTACTTCCCCTAATAACACTATGGTGACTCCTAGCTAATTTAATAAA
CATAGCCCTTCCCCCCACAATCAACCTCACTGGTGAACTCCTAATCATTTCATCCGCATACAATTGATCCCCCCTC
ACAATCGTATTAACAGGCGCCGGCACACTTCTAACAGCAATCTACTCCCTTCACATATTCTTAATAACACAACGA
GGTAAACTCCCACAACACATCATCAAAATAGCCCCAACACACACCCGAGAACA

h41
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TAAGCCACATAGGCCTTGTGATCACAGCCACTTTAATTCAAACCCCATGAAGCATCTCTGGGGCAATTCTACTAA
TAATCGCCCACGGCCTCACATCCTCAATACTCTTCTGCCTAGCAAACACRAATTATGAACGTACCCATAGCCGAA
CCCTTCTCCTAGCCCGGGGCTTACAACTTTTACTTCCCCTAATAACACTATGGTGACTCCTAGCTAATTTAATAAA
CATAGCCCTTCCCCCCACAATCAACCTCACTGGTGAACTCCTAATCATTTCATCCGCATACAATTGATCCCCCCTC
ACAATCGTATTAACGGGCACCGGCACACTTCTAACAGCAATCTACTCCCTTCACATATTCTTAATAACACAACGA
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h42
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TAAGCCACATAGGCCTTGTGATCACAGCCACTTTAATTCAAACCCCATGAAGCATCTCTGGGGCAATTCTACTAA
TAATCGCCCACGGCCTCACATCCTCAATACTCTTCTGCCTAGCAAACACRAATTATGAACGTACCCATAGCCGAA
CCCTTCTCCTAGCCCGGGGCTTACAACTTTTACTTCCCCTAATAACACTATGGTGACTCCTAGCTAATTTAATAAA
CATAGCCCTTCCCCCCACAATCAACCTCACTGGTGAACTCCTAATCATTTCATCCGCATACAATTGATCCCCCCTC

ACAATCGTATTAACGGGCACCGGCACACTTCTAACAGCAATCTACTCCCTTCACATATTCTTAATAACACAACGA
GGTAAACTCCCACAACACATCATCAAAATAGCCCCAACACACACCCGAGAACA

h43
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TAAGCCACATAGGCCTTGTGATCACAGCCACTTTAATTCAAACCCCATGAAGCATCTCTGGGGCAATTCTACTAA
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GGTAAACTCCCACAACACATCATCAAAATAGCCCCAACACACACCCG------

h44
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ACAATCGTATTAACGGGCGCCGGCACACTTCTAACAGCAATCTACTCCCTTCACATATTCTTAATAACACAACGA
GGTAAACTCCCACAACACATCATCAAAATAGCCCCAACACACACCCGAGAACA

h45
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TAAGCCACATAGGCCTTGTGATCACAGCCACTTTAATTCAAACCCCATGAAGCATCTCTGGGGCAATTCTACTAA
TAATCGCCCACGGCCTCACATCCTCAATACTCTTCTGCCTAGCAAACACRAATTATGAACGTACCCATAGCCGAA
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CATAGCCCTTCCCCCCACAATCAACCTTACTGGTGAACTCCTAATCATTTCATCCGCATACAATTGATCCCCCCTC
ACAATCGTATTAACGGGCGCCGGCACACTTCTAACAGCAATCTACTCCCTTCACATATTCTTAATAACACAACGA
GGTAAACTCCCACAACACATCATCAAAATAGCCCCAACACACACCCGAGAACA

h46
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TAATCGCCCATGGCCTCACATCCTCAATACTCTTCTGCCTAGCAAACACRAATTATGAACGTACCCATAGCCGAA
CCCTTCTCCTAGCCCGGGGCTTACAACTTTTACTTCCCCTAATAACACTATGGTGACTCCTAGCTAATTTAATAAA
CATAGCCCTTCCCCCCACAATCAACCTTACTGGTGAACTCCTAATCATTTCATCCGCATACAATTGATCCCCCCTC
ACAATCGTATTAACGGGCGCCGGCACACTTCTAACAGCAATCTACTCCCTTCACATATTCTTAATAACACAACGA
GGTAAACTCCCACAACACATCATCAAAATAGCCCCAACACACACCCGTGAACA

h47
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CCCTTCTCCTAGCCCGGGGCTTACAACTTTTACTTCCCCTAATAACACTATGGTGACTCCTAGCTAATTTAATAAA
CATGGCCCTTCCTCCCACAATCAACCTTACTGGTGAACTCCTAATCATTTCATCCGCATACAATTGATCCCCCCTC
ACAATCGTATTAACGGGCGCCGGCACACTTCTAACAGCAATCTACTCCCTTCACATATTCTTAATAACACAACGA
GGTAAACTCCCACAACACATCATC----------

h48
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TAAGCCACATAGGCCTTGTGATCACAGCCACTTTAATTCAAACCCCATGAAGCATCTCTGGGGCAATTCTACTAA
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CATAGCCCTTCCCCCCACAATCAACCTTACTGGTGAACTCCTAATCATTTCATCCGCATACAATTGATCCCCCCTC
ACAATCGTATTAACGGGCGCCGGCACACTTCTAACAGCAATCTACTCCCTTCACATATTCTTAATAACACAACGA
GGTAAACTCCCACAACACATCATCAAAATAGCCCCAACACACACCCGTGAACA

h49
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TAAGCCACATAGGCCTTGTGATCACAGCCACTTTAATTCAAACCCCATGAAGCATCTCTGGGGCAATTCTACTAA
TAATCGCCCACGGCCTCACATCCTCAATACTCTTCTGCCTAGCAAACACRAATTATGAACGTACCCATAGCCGAA
CCCTTCTCCTAGCCCGGGGCTTACAACTTTTACTTCCCCTAATAACACTGTGGTGACTCCTAGCTAATTTAATAAA
CATAGCCCTTCCCCCCACAATCAACCTCACTGGTGAACTCCTAATCATTTCATCCGCATACAATTGATCCCCCCTC

ACAATCGTATTAACGGGCGCCGGCACACTTCTAACAGCAATCTACTCCCTTCACATATTCTTAATAACACAACGA
GGTAAACTCCCACAACACATCATCAAAATAGCCCCAACACACACCCGAGAACA

h50
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TAATCGCCCACGGCCTCACATCCTCAATACTCTTCTGCCTAGCAAACACRAATTATGAACGTACCCATAGCCGAA
CCCTTCTCCTAGCCCGGGGCTTACAACTTTTACTTCCCCTAATAACACTGTGGTGACTCCTAGCTAATTTAATAAA
CATAGCCCTTCCCCCCACAATCAACCTCACTGGTGAACTCCTAATCATTTCATCCGCATACAATTGATCCCCCCTC
ACAATCGTATTAACGGGGGCCGGCACACTTCTAACAGCAATCTACTCCCTTCACATATTCTTAATAACACAACGA
GGTAAACTCCCACAACACATCATCAAAATAGCCCCAACACACACCCGAGAACA

h51
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TAAGCCACATAGGCCTTGTGATCACAGCCACTTTAATTCAAACCCCATGAAGCATCTCTGGGGCAATTCTACTAA
TAGTCGCCCACGGCCTCACATCCTCAATACTCTTCTGCCTAGCAAACACRAATTATGAACGCACCCACAGCCGAA
CCCTTCTCCTAGCCCGGGGCTTACAACTTTTACTTCCCCTAATAACACTATGGTGACTCCTAGCTAATTTAATAAA
CATAGCCCTTCCCCCCACAATCAACCTCACTGGCGAACTCCTAATCATTTCATCCGCATACAATTGATCCCCCCTC
ACAATCGTATTAACAGGCGCCGGCACACTTCTAACAGCAATCTACTCCCTTCACATATTCTTAATAACACAACGA
GGTAAACTCCCACAACACATCATCAAAATAGCCCCAACACACACCCGAGAACA

h52
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TAAGCCACATAGGCCTTGTGATCACAGCCACTTTAATTCAAACCCCATGAAGCATCTCTGGGGCAATTCTACTAA
TAATCGCCCACGGCCTCACATCCTCAATACTCTTCTGCCTAGCAAACACRAATTATGAACGCACCCATAGCCGAA
CCCTTCTCCTAGCCCGGGGCTTACAACTTTTACTTCCCCTAATAACACTGTGGTGACTCCTAGCTAATTTAATAAA
CATAGCCCTTCCCCCCACAATCAACCTCACTGGTGAACTCCTAATCATTTCATCCGCATACAATTGATCCCCCCTC
ACAATCGTATTAACGGGSGCCGGCACACTTCTAACAGCAATCTACTCCCTTCACATATTCTTAATAACACAACGA
GGTAAACTCCCACAACACATCATCAAAATAGCCCCAACACACACCCGAGAACA

h53
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TAAGCCACATAGGCCTTGTGATCACAGCCACTTTAATTCAAACCCCATGAAGCATCTCTGGGGCAATTCTACTAA
TAATCGCCCACGGCCTCACATCCTCAATACTCTTCTGCCTAGCAAACACRAATTATGAACGCACCCATAGCCGAA
CCCTTCTCCTAGCCCGGGGCTTACAACTTTTACTTCCCCTAATAACACTGTGGTGACTCCTAGCTAATTTAATAAA
CATAGCCCTTCCCCCCACAATCAACCTCACTGGTGAACTCCTAATCATTTCATCCGCATACAATTGATCCCCCCTC
ACAATCGTATTAACGGGCGCCGGCACACTTCTAACAGCAATCTACTCCCTTCACATATTCTTAATAACACAACGA
GGTAAACTCCCACAACACATCATCAAAATAGCCCCAACACACACCCGAGAACA

Appendix 4. Genotypes of individual pygmy bluetongue lizards used in this study.

individual	EST12old		TRL14		TRL15		TRL16		TRL32		TrL21		TrL27		TrL28		TrL29		TrL30		TrL34		TRL12		TrL19		TrL35		TrL37	
131	309	328	127	129	252	270	99	99	138	146	275	279	149	168	143	143	139	154	0	0	129	131	169	185	185	188	124	124	158	177
152	282	282	136	138	264	320	105	128	138	146	260	267	0	0	147	150	130	154	95	95	136	171	156	206	177	185	130	130	177	189
153	282	305	138	153	264	320	105	110	140	140	272	271	0	0	147	152	130	144	95	97	136	171	156	167	174	175	124	124	162	175
154	279	288	129	138	295	341	105	105	140	150	256	279	0	0	143	161	146	192	95	95	138	138	164	205	177	183	124	124	160	200
221	285	285	138	143	267	282	105	122	140	152	256	267	155	161	145	169	130	159	95	98	143	151	204	236	181	195	124	124	179	179
222	305	313	127	131	291	332	87	113	148	150	271	281	149	159	145	145	133	154	95	95	135	141	209	223	172	185	124	130	150	179
223	282	285	131	138	267	332	108	128	140	148	260	267	121	121	155	169	130	130	95	95	131	149	225	225	172	172	124	124	166	174
233	282	313	129	138	313	316	113	113	140	140	256	279	155	161	155	155	130	130	95	95	119	149	166	221	181	185	124	130	166	179
234	266	285	138	143	313	341	102	105	140	152	287	288	121	161	152	158	159	161	95	95	119	163	166	210	185	191	130	130	164	177
236	274	274	131	131	279	332	105	110	150	152	288	287	153	159	150	159	135	153	95	95	141	163	158	164	172	195	130	130	166	179
311	282	340	127	129	298	301	108	110	140	140	277	277	155	159	150	167	135	142	95	95	131	143	186	197	183	185	124	130	160	177
312	266	309	131	138	332	341	108	108	138	140	281	285	149	157	147	150	135	135	95	95	136	145	186	201	167	186	124	130	160	175
313	344	344	129	138	279	332	99	108	140	160	269	271	157	159	150	167	142	144	95	95	136	141	186	190	179	195	124	130	170	170
314	282	297	129	143	276	353	99	108	140	160	267	277	149	151	147	150	130	142	95	95	129	129	184	190	179	186	124	130	158	168
315	282	309	129	129	301	341	99	105	140	160	260	273	131	142	139	147	131	142	95	95	136	145	184	186	186	200	124	130	160	164
316	282	340	129	138	279	295	99	108	138	140	271	283	127	131	133	139	128	131	95	95	145	171	150	184	186	195	130	130	170	177
317	282	282	138	143	267	276	108	110	138	160	271	277	142	143	139	150	142	144	95	95	125	165	150	184	167	195	130	130	158	164
318	309	344	129	138	279	322	99	110	138	140	260	271	131	143	139	167	131	144	95	95	141	157	186	197	175	195	124	124	168	170
319	282	282	129	131	276	303	99	110	140	140	285	285	145	159	150	150	128	144	95	95	133	165	186	190	167	179	124	130	160	168
321	282	282	131	143	288	301	99	105	138	138	260	273	149	176	139	139	130	142	95	95	145	163	167	184	165	200	124	130	160	164
322	282	340	129	143	303	344	105	108	140	140	260	283	138	159	133	150	137	163	95	95	131	131	170	188	183	186	130	130	160	168
323	282	309	127	143	288	341	99	105	138	138	260	273	159	176	139	139	130	142	95	95	157	163	184	190	165	165	124	130	164	164
324	301	309	127	138	276	341	99	99	138	140	260	269	155	159	133	167	128	142	95	95	125	165	184	186	179	179	124	124	164	181
325	282	309	127	138	267	279	105	108	138	138	267	271	149	157	150	167	142	142	95	95	125	136	190	191	177	179	124	124	168	174
614	297	355	138	157	307	320	87	105	140	140	269	281	121	149	153	155	137	139	95	95	141	171	184	201	185	195	124	130	181	183
625	297	332	129	135	322	344	96	110	140	150	279	279	121	121	139	150	144	192	95	95	127	127	166	204	172	172	124	130	175	179
626	355	355	129	135	298	298	110	110	140	152	271	271	149	153	153	173	130	163	0	0	131	136	158	194	191	191	130	130	160	183
627	305	336	135	138	249	325	105	110	140	140	258	267	138	153	158	163	159	159	95	95	127	133	167	187	174	186	124	124	175	177
628	297	355	143	157	252	276	108	110	150	152	258	271	121	168	173	173	130	159	95	95	131	136	187	209	174	195	124	124	170	175
629	297	320	138	157	276	307	99	99	140	146	267	271	121	153	155	158	142	191	95	95	136	143	185	205	174	181	124	124	175	177
642	274	332	135	138	301	356	105	110	140	140	258	271	151	153	155	173	130	154	95	95	131	141	171	202	179	185	130	130	172	175
643	285	285	127	135	307	325	108	110	150	150	264	267	121	153	152	163	130	144	95	95	135	136	185	187	179	181	124	130	170	175
911	266	270	138	138	279	325	102	105	140	146	279	279	149	174	137	137	142	159	95	95	133	133	192	224	167	177	130	130	172	174
912	309	320	138	141	258	325	105	108	140	140	273	279	161	165	137	143	130	169	95	95	127	141	192	221	165	177	124	130	168	183
913	297	301	131	138	258	356	102	108	140	146	273	279	141	161	137	143	142	171	95	95	121	131	198	211	165	185	130	130	168	198
914	266	305	131	141	313	320	105	108	140	150	279	279	155	155	137	139	126	137	95	95	133	143	177	217	165	197	130	130	156	168
915	266	301	138	138	298	298	102	105	140	148	258	279	147	153	139	143	157	159	95	95	143	143	195	199	177	185	124	130	158	164
916	270	297	131	141	255	320	99	113	140	140	277	279	145	147	139	153	123	169	95	95	125	149	181	184	165	197	124	130	156	158
917	289	289	131	138	298	320	102	105	140	144	260	267	145	153	137	152	123	159	95	95	141	143	217	221	172	181	130	130	174	179
918	309	325	131	143	258	310	99	108	140	148	264	285	153	174	139	175	137	137	95	95	131	143	199	221	165	197	130	130	164	179
919	301	309	129	138	303	310	102	108	140	140	279	279	141	147	139	143	135	159	95	95	123	143	175	192	174	188	124	130	174	179
921	270	282	138	138	298	353	102	105	140	152	265	294	147	163	137	139	126	144	95	97	133	133	158	185	174	192	124	124	174	179
922	270	288	131	138	320	320	105	105	140	140	260	262	145	153	152	158	159	159	95	95	135	143	198	217	172	185	124	130	162	174
923	289	328	138	138	307	353	105	105	140	146	272	281	145	161	137	137	142	165	95	97	123	127	198	198	185	188	130	130	152	177
924	266	328	138	138	353	353	105	105	140	144	272	281	145	161	137	143	157	165	97	97	123	127	169	198	185	188	124	130	152	177
925	293	297	131	141	255	279	105	105	0	0	279	279	153	163	139	158	157	159	0	0	119	135	213	216	165	165	124	124	164	164

1101	274	285	138	138	329	356	105	110	140	150	271	279	137	151	150	158	130	163	95	95	129	141	206	227	185	188	124	124	158	177
1105	282	282	129	143	325	332	110	110	140	140	267	277	149	149	143	147	138	142	95	95	129	145	168	184	174	185	130	130	160	185
1106	266	282	127	129	276	338	105	110	140	146	264	281	137	159	141	147	156	159	95	95	135	138	157	166	174	182	124	130	175	179
1107	282	285	129	138	325	358	87	110	140	150	264	277	149	168	141	147	142	159	95	95	117	129	168	237	174	177	124	130	158	160
1216	305	309	129	147	264	291	96	110	140	164	260	271	145	157	150	150	131	167	95	100	133	159	152	152	179	185	124	124	156	179
1217	285	293	129	138	264	322	102	113	146	148	265	283	128	147	143	150	131	153	95	97	136	159	154	179	169	185	124	124	162	179
1218	317	344	129	138	255	334	102	110	146	148	265	281	155	157	147	163	130	130	95	95	159	159	179	213	175	185	124	124	175	175
1219	282	289	127	138	285	285	102	110	148	152	252	283	143	151	159	163	135	167	95	95	136	159	186	208	169	179	124	124	162	185
1311	277	285	129	141	313	332	96	96	152	150	289	297	171	171	147	147	123	147	95	95	133	163	188	203	179	181	124	124	168	181
1312	277	309	129	129	282	350	113	116	0	0	273	273	159	161	137	145	130	156	95	95	121	133	188	203	183	193	124	124	168	183
1313	277	309	129	131	350	332	113	113	150	152	252	269	149	159	143	147	131	147	97	97	121	133	200	227	175	179	124	124	179	189
1314	274	277	129	131	290	314	96	96	148	152	273	277	157	184	137	147	123	130	95	95	133	135	204	229	175	181	124	124	162	177
1315	274	285	129	138	301	329	96	113	148	152	277	287	163	169	137	150	156	166	95	97	111	143	216	227	174	183	124	124	175	181
1316	285	309	131	138	310	332	96	113	0	0	265	295	171	171	137	152	133	156	95	95	135	136	207	229	181	183	124	124	172	189
1318	309	313	131	141	291	338	113	113	0	0	262	287	151	161	137	150	123	157	0	0	125	157	217	217	177	179	124	124	166	177
1321	277	285	129	132	315	344	96	122	150	152	252	297	165	165	143	150	156	157	95	97	133	136	188	229	175	183	124	124	174	190
1513	282	282	129	129	273	329	105	110	140	140	256	273	0	0	150	159	131	153	95	95	127	157	166	181	185	186	124	124	158	177
1521	282	320	138	153	264	298	99	105	140	150	256	264	0	0	150	163	135	142	95	95	135	135	157	166	172	185	124	130	175	192
1525	274	285	127	143	252	359	110	113	140	150	264	286	0	0	145	152	153	163	95	95	129	131	160	166	185	188	124	124	175	198
1526	282	309	143	145	252	288	87	87	140	146	264	271	0	0	150	159	157	157	95	95	129	131	166	214	179	179	124	124	168	198
1527	274	317	138	153	252	264	105	110	140	140	264	269	158	182	133	150	130	139	95	95	135	143	164	187	172	179	124	130	190	192
1528	274	317	138	153	322	325	105	105	138	148	258	267	0	0	147	158	139	163	95	95	135	163	164	167	169	177	124	130	175	175
2335	282	282	129	141	282	325	105	128	140	140	265	269	0	0	155	155	154	159	95	95	119	138	181	206	172	177	124	130	170	177
2625	282	282	129	143	291	362	105	110	140	140	256	267	151	163	145	159	139	165	95	95	141	147	209	220	179	191	124	136	0	0
2626	0	0	129	138	255	325	108	110	140	154	267	273	121	147	155	161	131	144	95	95	129	141	166	208	179	185	130	136	162	183
2627	282	297	136	143	267	335	105	105	146	152	271	281	0	0	152	155	130	131	95	95	133	171	181	206	172	179	124	130	0	0
2694	282	282	138	141	0	0	102	105	0	0	256	264	149	151	147	152	139	142	95	95	138	171	166	179	177	181	124	124	166	179
2696	282	285	143	143	273	353	87	105	0	0	270	271	145	149	139	150	131	159	0	0	131	135	181	219	172	173	130	130	166	175
1111	328	340	127	127	270	338	110	110	148	162	271	279	147	153	139	163	133	142	95	95	131	133	158	162	165	181	124	124	177	179
1112	289	325	129	138	279	335	105	110	140	162	269	289	173	174	143	147	131	159	95	95	131	141	170	222	165	200	130	130	172	172
1113	325	355	127	129	279	322	105	105	140	140	273	279	147	151	141	143	130	131	95	95	131	141	184	193	189	200	124	124	172	181
1211	266	301	138	141	291	329	102	113	150	150	254	271	147	161	150	163	130	130	95	95	161	161	152	168	189	199	124	124	164	175
1212	309	344	127	141	298	338	102	113	148	164	260	283	151	157	137	159	130	131	95	95	135	135	178	186	189	193	124	124	185	185
1213	293	344	138	147	279	335	96	96	148	148	265	267	151	157	137	163	131	142	95	97	135	161	175	213	181	185	124	124	162	175
1214	266	274	127	138	329	335	102	102	148	168	281	283	128	151	137	150	142	167	95	95	157	161	169	186	189	199	124	124	156	175
2211	293	305	129	138	288	329	105	110	146	152	258	271	159	165	143	169	131	131	95	95	136	138	200	209	184	186	124	124	154	181
2212	328	328	131	138	313	316	105	110	140	148	258	270	143	159	137	163	126	189	95	95	131	136	195	198	167	195	124	130	181	181
2213	266	289	138	138	338	344	105	110	140	150	258	260	145	149	139	145	131	144	95	95	129	136	191	191	167	172	124	124	172	187
2214	301	317	131	131	255	344	105	110	148	150	270	271	145	168	137	169	126	159	95	95	136	138	183	183	175	200	124	130	172	204
2215	266	328	138	138	279	303	102	110	140	148	270	283	149	149	145	152	133	139	95	95	117	133	182	189	165	177	124	124	149	187
2216	289	317	131	138	344	344	105	105	148	150	258	271	145	145	137	139	144	159	95	95	136	138	183	189	167	200	124	124	172	204
kap1	332	344	138	159	295	303	96	113	0	0	273	289	142	157	165	171	142	157	95	95	143	143	160	197	197	199	124	124	164	168
kap2	340	344	138	138	295	303	96	102	0	0	273	283	131	157	167	171	131	157	95	95	125	143	163	196	197	200	124	124	164	189
1105k	305	320	138	138	316	344	102	102	0	0	273	283	0	0	150	150	142	153	95	95	143	143	160	216	193	200	124	124	158	162
1106k	289	297	131	153	322	329	102	102	0	0	265	275	0	0	161	169	130	131	95	95	133	135	170	216	189	199	124	124	172	179
1107k	289	317	138	138	0	0	102	102	148	154	265	289	130	142	135	158	130	142	95	95	133	133	160	160	181	185	124	124	166	172
1108k	285	309	138	138	295	295	102	102	0	0	273	289	0	0	155	165	152	157	95	95	123	138	160	160	203	203	124	124	152	168
1109k	305	309	138	159	255	303	102	102	0	0	0	0	155	165	155	155	152	152	95	95	136	138	160	162	188	189	124	124	166	189

1110k	282	282	127	143	329	329	87	131	0	0	256	272	0	0	145	150	144	167	95	95	135	141	156	221	167	177	130	130	164	174
1114	293	309	129	159	282	335	108	110	140	148	274	293	147	149	139	145	144	152	0	0	129	129	158	165	183	198	130	130	168	177
1115	282	355	127	138	285	285	102	110	148	148	273	277	149	151	145	155	133	152	95	95	119	129	176	184	175	180	124	124	179	181
1116	282	355	138	138	270	270	105	105	140	140	271	273	138	152	139	145	131	152	95	95	129	169	181	183	184	189	124	130	172	189
1117	328	340	138	138	338	344	105	110	140	150	274	281	149	151	150	159	133	142	95	95	127	143	157	177	165	195	124	130	164	179
1215	305	305	127	138	322	335	99	105	140	148	252	281	152	157	150	163	130	131	95	95	159	161	153	186	180	187	124	124	162	175
1216	305	309	138	138	322	322	102	110	150	156	260	271	146	157	150	150	131	167	95	95	133	159	153	153	179	184	124	124	156	179
1217	285	293	131	138	255	322	102	105	140	140	0	0	0	0	0	0	0	0	0	0	0	0	155	179	169	184	124	124	162	179
1218	317	344	131	131	255	344	105	110	148	150	265	281	156	157	147	163	130	130	95	95	159	159	179	213	175	184	124	124	175	175
1219	282	289	129	141	301	322	108	110	140	148	252	283	143	152	159	163	136	167	95	95	136	159	186	208	169	179	124	124	162	185
1221	285	305	129	141	313	332	96	96	150	152	252	264	147	155	150	163	131	133	95	95	135	158	178	178	184	193	124	124	185	185
1311	277	285	138	138	313	313	108	116	140	140	289	297	171	171	147	147	123	147	95	95	133	163	189	203	179	180	124	124	168	181
1314	274	277	131	138	344	344	105	105	148	150	273	277	157	184	137	147	123	130	95	95	133	135	204	229	175	180	124	124	162	177
1315	274	285	127	127	335	347	110	110	140	148	277	287	163	169	137	150	156	166	95	97	111	143	216	227	174	182	124	124	175	181
1316	285	309	129	131	290	313	96	96	148	152	265	295	171	171	137	152	133	156	95	95	135	136	207	229	180	183	124	124	172	189
1318	309	313	131	138	313	335	108	113	140	140	262	287	151	161	137	150	123	158	0	0	125	157	215	217	177	179	124	124	166	177

Chapter 6. Conclusion

Review of findings

The major aim of this project was to investigate the levels of dispersal and movement within populations and among localities within the fragmented habitat of the endangered pygmy bluetongue lizard *Tiliqua adelaidensis,* an Australian skink. I investigated movement at different scales, at the level of individual lizards, from short term movements, to dispersal , to longer-term effects of gene flow on the whole of the currently known range of the species. I further discuss the consequences and conservation implications of the dispersal patterns, for the pygmy bluetongue lizard. There were two primary focal areas of the thesis: first to understand the genetic consequences of reduced dispersal opportunities within isolated populations; second to better inform management decisions to maintain current populations and to prepare for future translocation efforts if they are warranted.

Gene flow

Dispersal is only evolutionarily meaningful if it results in the movement of genetic material. To better understand gene flow within populations I investigated the mating system of the pygmy bluetongue lizard (Chapter 3). I showed that the mating system of the pygmy bluetongue lizards is polygynous and indiscriminate with respect to partner relatedness. Multiple paternity was detected in 75% of the litters, with some litters having up to three fathers. Males mate with multiple females within a year and do not necessarily mate with the same females in consecutive years. In this study there was a low capture rate of parents (only 35% (location 1) and 69% (location 2). Since both males and litters in the sampled populations would have been incompletely sampled, my estimate of polygyny may be too low.

The genetic evidence suggested that partners were chosen randomly with respect to the level of relatedness among neighbouring lizards. However, mated lizards were geographically closer to each other than expected by random chance. Thus while the short distance between locations of mating partners may reduce the degree of genetic mixing in the broader population, the promiscuous mating system is likely to maintain genetic diversity within localities in the population.

Given the low rate of discovery of the genetic parents of captured juveniles within exhaustively sampled sites (Chapter 3) and the low recapture rate of previously captured individuals (Chapter 1) this study highlights need to account for missing

individuals. Missing individuals could be due to cryptic individuals in the population (Levine *et al*. 2015) or through the loss of individuals via predation or other mortality or the movement of individuals through the population. Understanding how each of these effect a population could give us a more complete idea of the roles of these individuals in breeding and thus a more complete picture of population demographics.

Individual movement

The roles of breeding dispersal and juvenile dispersal are commonly explored (Sutherland *et al.* 2012,Clobert *et al.* 2001). I investigated the roles of both of these types of dispersal. I measured this individual movement in two ways, through direct observations from pitfall captures of individual moving on the surface, and by indirect inference from comparisons of individual genotypes at microsatellite DNA loci.

A key property of the pygmy bluetongue lizard is its sedentary life style. Individual lizards spend most of their time within or at the entrance of single burrows. Therefore we assumed movements, that may increase the genetic mixing within populations, are likely to take place among neonates as they leave their natal burrows, among adults and sub-adults that move to change burrows, and amongst reproductive individuals seeking mating partners.

My study adds to the mounting evidence that traditional plot based methods underestimate dispersal of individuals (Koening *et al*. 1996; Saurola and Francis 2004; Fedy *et al*. 2008; Van Houtan 2010; Estes-Zumpf *et al.* 2010; Marmet *et al.* 2011; Byrne *et al.* 2014). I did not detect high levels of juvenile dispersal with neonate and sub adult movements under represented in the pitfall captures, these moves were more common later in the season. Indeed it is likely that plot based studies are more useful for detecting seasonal movements of individuals rather than actual dispersal events.

When I examined the evidence from pitfall traps (Chapter 1) I found that male lizards were the most mobile individuals, with high capture rates early in the spring, during the breeding season. The low but equal frequency of male and female captures later in the season, are probably lizards that have abandoned their original burrows and are moving around in search of a new burrow site.

We had a low recapture rate of individuals (11%) despite long periods of pitfall trapping, and all were close to their original capture site but on the opposite side of the trapline. These probably represent local movements, suggesting that even when they leave their burrow, some lizards do not move far, and probably remain in or return to the local area of the burrow they originally moved from. For instance, males that have moved to locate a female partner, may attempt to return to their original burrows after the mating has been completed.

Despite the higher level of movement by adult males detected by trapping, the genetic evidence suggested that there was no bias in dispersal distances of the two sexes (Chapter 4). This finding is at odds with previous genetic analysis (Smith *et al*. 2009) which suggested that males moved farther than did females during dispersal. However, the finding in the current thesis is built on a larger sample size over more time and more sites. It is also consistent with comparable spatial autocorrelation patterns between males and females.

Within population dispersal

In Chapter 4 I also demonstrated distinct genetic clusters within small sites in a population and different cluster compositions between sites across a population. These trends imply broad spatial patterning of the genetic structure within populations. The results showing significant spatial autocorrelation among pairs of adult lizards at distances up to 30 – 45 m from each other (Chapter 4) was consistent with previous results derived from lower sampling intensity (Smith *et al.* 2009). These results imply that individual adult lizards are likely to occupy burrows close to genetically related adults. This pattern was consistent across separate populations at two locations.

Two contrasting results about spatial patterns help us to understand the population processes involved. If lizards moved further and settled randomly across the landscape within their populations we would expect a more panmictic population structure. One likely explanation for the high level of genetic structuring is that many established adults in the population have not dispersed far from their natal burrows. Alternatively some lizards, particularly juveniles, may attempt long dispersal, but few of those longer distance dispersers survive to be included in the current adult samples. A third explanation could be due to density dependant dispersal (Matthysen

2005). Lower densities may lead to an increased potential availability of resources (such as burrows further from relatives or neighbors) may have allowed individuals to disperse further. Populations with relatedness over greater distances had higher densities of individuals suggesting that the patterns of dispersal may be density dependant.

This study like other suggests that dispersal is influences by a multitude of factors and not just due to a sex or age of a individual (Clobert *et al.* 2001; Ims and Andreassen 2005) Further research needs to be undertaken to tease out the mechanisms that cause populations with highly structured local concentrations of related individuals clustering together.

Among population dispersal

In Chapter 5 I used microsatellite and mitochondrial markers to investigate the incidence of wider dispersal, both historically and recently, and the level of genetic structuring among populations. These results support the trends reported in Chapter 4.The short dispersal distances of individuals within populations means that habitat fragmentation historically and currently has imposed a significant barrier to any dispersal among even closely adjacent populations, posing a potential conservation issue for the spread of genotypes. The results of this study indicate that most of the genetic variation is found among populations and each sampling location has one or more unique genetic combinations.

Chapter 5 shows a division between the two northern-most populations sampled and the southern populations suggesting there may have been historical barriers to dispersal. There is also some support for a divide between the southern sites sites, 6, 13, 12, and 27 and sites 1, 2, 3, and 11.

This study suggests strong genetic structure was occurring prior to fragmentation and it is likely due to the sedentary lifestyle of the lizards. In the sub set of the sites used in this study (Smith *et al*. 2009) found no evidence of population bottle necks and a heterozygosity of 0.75 to 0.82. The microsatellite clusters in this study indicate that there are still dispersal barriers, the number of genetic divisions between populations may increase into the future as the effects of the last 100 years of landscape change become evident. However habitat fragmentation may be less of an issue than populations size (Frankham *et al.* 2014).

Broader conservation implications

In situ conservation

A broader key question for conservation managers is how great an influence habitat fragmentation has on the ability of a population to retain its genetic variation. Part of the answer comes from understanding the processes within individual populations that sustain or diminish that variation.

Chapter 5 showed there is currently low flow of genetic information between the populations with high levels of genetic differences between locations. Mitochondrial data in this thesis suggest the populations should be divided into at least three conservation units, on northern and two southern ones. Microsatellite data shows a high level of variation even within these units and as such management actions should aim to conserve this diversity where possible.

In a continuous habitat, or one with habitat patches connected by dispersal corridors, it would be expected that some of the annual recruitment would disperse to adjacent sites, and that this dispersal might buffer populations from local demographic loss. In addition, the genetic mixing from population exchanges should provide the variation to allow each population to adapt to changing conditions. These processes are less frequent in fragmented habitat.

In our study at least it is not certain that the current differentiation between populations is a result of anthropogenic habitat modification. Our results add to the number of studies that show the importance of historical gene flow in moulding patterns of genetic variation within and between populations of species that occupy now fragmented landscapes (Chiucchi and Gibbs 2010).

The mitochondrial results suggest historically there was low gene flow, even before the current habitat fragmentation. This finding is consistent with other observations and inferences of movements within populations found in this thesis. When that is added to the currently low gene flow imposed by the more recent fragmentation there may be a potential further loss of genotypes from current populations thorough extinction debt.

The low dispersal found within populations of pygmy bluetongue lizards (Chapter 4) has led to the clustering of related individuals and isolation by distance at small spatial scales (under 1km). Given the sedentary nature of pygmy bluetongue lizards, conservation strategies developed for plant populations may be appropriate models for conservation actions.

The spatial clustering further highlights the need to prevent further fragmentation of current populations in order to maintain a high number of individuals, local gene flow and population viability. Although this was not investigated in this study, it may be there is some minimum area of occupancy below which the population processes described above would not operate to sustain genetic diversity. This may be resolved by comparing populations know to occupy different areas.

For the pygmy bluetongue lizard, the indiscriminate partner choice and close spatial proximity of relatives in existing populations, suggest that individual lizards will not actively avoid mating with highly related partners. This means there may be a greater risk of inbreeding as populations decline, and as the genotypic range of potential partners is reduced. However, it appears that there is resistance to the genetic effects of fragmentation within populations of pygmy bluetongue lizards, as genetic diversity is maintained despite local isolation of populations (Smith *et al.* 2009). The promiscuous mating system, combined with the local co-existence of several apparently different genetic lineages, appears to account for this pattern. This leads to the suggestion that maintaining population sizes will be of higher priority than attempting to adjust genetic diversity in this species Indeed Frankham *et al*. (2014) argue that effective population sizes over 1000 are needed to maintain genetic fitness over time.

One potential conservation strategy that is widely discussed as a means to prevent the decline of population abundance and of genetic diversity may be to replicate ‘natural’ movement between adjacent populations through the managed exchange of individuals. This would stabilise population size and maintain the genetic diversity. Given the patterns of historic genetic diversity found in the phylogentic trees fragmentation of populations may be of lesser concern and other factors such as populations size and densities and climate change may be more important factors to address.

Given predicted climate change and the reduced ability for the lizards to migrate south, to where the optimal conditions will be found in the future, to preserve the genetics of the northern populations translocations may be necessary.

Ex situ conservation including translocations

Simulation modeling of climate change scenarios has suggested that translocation will be an essential long-term conservation management strategy for this species (Fordham *et al.* 2012). Translocations in reptiles are not always successful (Germano and Bishop 2009) and part of the problem may be getting translocated individuals to stay at the site of release. Studies by Ebrahimi (2013) suggest that dispersal, in the period immediately after release, can be reduced when lizards had one or a combination of, higher vegetation, supplementary food, greater burrows availability, or clusters of burrows , and when lizards were confined to the release area for a short time period.

This thesis emphasises that the knowledge of movement patterns allows insights into the choice of individuals and the potential timing for translocations (Chapter 2). If one group within the population is more mobile, conservationists could capture for translocation individuals from that group to mimic natural dispersal of the species. Additionally, since natural dispersal may lead individuals to move out of their habitat patch, they may be a doomed surplus such that there capture and use for translocation will not affect the donor population. however, if they are naturally dispersive, they may be less likely to remain at the release site.

Our study suggests that early spring is the time when translocated adults might be most likely to move around, and probably disperse from a release site, and that translocation programs will be more effective if delayed until later in the season (February/March).

Using more mobile members of a population might reduce the impact on the source population; however, it could compromise the success of the translocation. individuals that move around may be more prone to predation or may disperse into the hostile surrounding matrix. Thus, they might be considered as a harvestable surplus because their selection as translocation stock would come at little cost to the resident population. Because adult females are the least dispersive group, their use in

a translocation program is likely to have the greatest adverse impact on the source population in terms of lost reproductive potential. however, females are essential for reproductive growth, so translocation programs may need to consider taking juvenile females from the source population and raising them in captivity to adults for release.

The introduction of more females than males could reduce the male search time for a mate and thus reduce predation risk to males especially in species that mate indiscriminately. However when considering reproductive potential in monogamous or pair bonding species equal numbers of each sex would result in maximal reproduction.

The success of captive breeding and translocation efforts for any species may hinge upon understanding both the baseline genetic diversity of source and translocated populations and the mating systems they display (Haig 1998; Sigg *et al.* 2005; Grueber and Jamieson 2008 Gregory *et al.* 2012).

The promiscuous mating system in pygmy bluetongue lizards (Chapter 3) may prove advantageous during any translocations or reintroductions. Promiscuity could ensure the rapid mixing of genotypes among founder individuals at unoccupied sites, or the rapid integration of new genetic material into existing populations. Thus high diversity within the translocated stock may not be as high a priority as in some other species. Specifically, within any translocation program it will be important to maintain the genetic distinctness of the northern and southern lineages (Chapter 5).

Future research

There are still currently many gaps as to which life stages add most to the dispersal within a populations. While some of low capture rates of parents in Chapter 3 may result from predation, an alternative is that there is a cryptic portion of individuals with the population. While some research has focussed on the amazingly sedentary nature of many resident adults, there is some evidence that there may be a transient portion of the population (Bull *et al*. 2014). This suggests there is further to be discovered about the dynamics of the populations.

Tracking the movement of individuals is also a key area to further research on dispersal in this species. At present it is unknown whether males return to their

original burrows after the mating season, or if they have fixed territories they visit for mating opportunities each year or whether mating is opportunistic and random.

Unravelling the survivorship and movement of juveniles in the populations will have important ramifications in their conservation. Neonates are readily captured in and around their maternal burrows, early in their life, however there is limited detection of neonates and sub adults after this point. More needs to be known about how sub-adults and neonates move around and eventually become established.

The availability of resources including spider burrows and the spiders that create them is another under studied area that will influence the densities of the lizard populations and the amount of refuges available to moving individuals.

Given the small number of locations sampled and the high genetic diversity between sites further sampling across the range of the lizards is needed, this research could include investigating whether there are site specific adaptations including at the major histocompatibility complex and other genes important for the lizards' survival. Increasing the sample sizes and numbers of nuclear markers will allow for the understanding historical gene flow between populations.

Nevertheless, we now know more about the ecology, behaviour and population structure of this endangered lizard species than we do about many other species, and the short term prognosis for its persistence is good. Important management decisions can be made as a result of the results of this thesis and related studies, coupled with close and regular monitoring of both its abundance and genetic diversity.

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Appendix

Additional Publications

Pelgrim, K., Fenner, A. L., Schofield, J. A. and Bull C. M. (2015) Dynamics of a temperate grassland reptile community in the Mid-North of South Australia. accepted for publication in the *Transactions of the Royal Society of South Australia*.

Ebrahimi, M., Schofield, J. A. and Bull, C. M. (2012). *Tiliqua adelaidensis* (Pygmy Bluetongue Lizard). Alternative Refuge. *Herpetological Review*, 43 (4) 652-653.

Ebrahimi, M., Schofield, J. A. and Bull, C. M. (2012) Getting your feet wet: Responses of the endangered pygmy bluetongue lizard (*Tiliqua adelaidensis*) to rain induced burrow flooding. *Herpetology Notes*. 5:297-301

Andy Sharp, Julie Schofield and Aaron Fenner (2010) The effects of cell grazing on the longevity of spider burrows, and the potential consequences for the endangered Pygmy Bluetongue Lizard. *Ecological Management & Restoration* 11 (1) 69 – 72.

Aaron L. Fenner, Julie A. Schofield, Annabel L. Smith and C. Michael Bull (2008) Observations of snake predation on the Pygmy Bluetongue Lizard, *Tiliqua adelaidensis*. *Herpetofauna* 38 (2) 105-109