Transmission pathways in reptile ticks

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Abstract

Understanding how parasites are transmitted through a population is fundamental to the understanding of the spread of wildlife diseases. Emerging threats to wildlife species include both exotic and new endemic diseases. Coupled with the spread of diseases are new paradigms due to habitat restrictions where a local outbreak of a pathogen may have greater relative impact than in other less restricted areas. Another route which is increased by restricted habitats is the spill over of previously benign parasites from other species. There are serious human health concerns from exposure to pathogens from wildlife as a result of the increased frequency of interactions between humans and wildlife.

The main aim of this study is to investigate the pathways of reptile tick transmission, by utilising GPS data collected in the lizard activity season (Sept-Dec) over four years (2008-2011) to create social networks of the Sleepy lizard (*Tiliqua rugosa*) host and genotypes of the reptile tick, *Bothriocroton hydrosauri*.

Initially this study explored the ability that transmission network models had on predicting parasite loads. Creating, transmission networks derived from the infection windows of the tick species, *Bothriocroton hydrosauri* and *Amblyomma limbatum,* among their sleepy lizard (*Tiliqua rugosa*) hosts in a natural population in South Australia. The consistent correlations over time between *B. hydrosauri* infection intensity and network derived infection risk suggest that network models can be robust to environmental variation among years. However, the contrasting lack of consistent correlation in *A. limbatum* suggests that the utility of the same network models may depend on the specific biology of a parasite species.

The second part of this study was to develop new diagnostic microsatellite DNA loci for the reptile tick, *Bothriocroton hydrosauri* using next generation sequencing. I used the alleles I identified to assign adult ticks collected in 2010 and 2011 to either the background wild population at the study site, or to larvae from other locations experimentally attached in pulses to lizards at the study site.

For the adult ticks identified as background, I asked whether ticks were more closely related to each other on hosts that were more closely linked in a parasite transmission

network. Then also asked which of three alternative network structures best explained the patterns of genetic relatedness. The three network models were social, asynchronous refuge sharing and spatial proximity. I found that adult ticks were more related to each other when they were collected from the same host, than when collected from different hosts. I also established that when adult ticks were on different lizards they had higher relatedness if those lizards had shorter path lengths connecting them on each of the three networks we explored. In each of the two study years a different network best explained the dynamics of transmission. The social contact network was the poorest predictor of tick relatedness in both years, while the spatial overlap based network (in one year) and the asynchronous shared refuges network (in the other year) were the strongest predictors.

Lastly, this study investigates which transmission pathway model could best explain the likelihood that a lizard receives a tick from a donor lizard. Using the tick samples identified as originating from experimental infection pulses and Exponential random graph models (ERGMs). My major discovery with this section was that in each year a different model was the best predicator. In 2010 the transmission network (adjacency and distance) was the best predictor and in 2011 it was a model of the social network distance between lizard pairs.

My study highlights, that changing environmental conditions might vary the relative importance of alternative processes driving the parasite transmission dynamics. This could lead to further studies specifically investigating the effects and influence that the environment has on host behaviors, in turn extending our understanding on parasite transmission. There were limitations in my study due to limited genetic markers and further work would benefit from utilizing newer genomic techniques to trace pulses of tick progeny in social networks within this amenable study system.

Declaration

'I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that 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.';

Caroline Wohlfeil

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Organisation of thesis, publications arising from this thesis and statement of candidate contributions

This thesis is composed of unpublished chapters (**chapters 1, 3 and 6**), published papers (**chapter 2**) and submitted (or in preparation for submission) manuscripts (**chapter 4 and 5**). Due to the mix of chapter types, each chapter had its own reference section.

All co-authors have given permission for the work that arose from collaboration to be included in this thesis.

Below are the estimates of relative contribution of each co-author to each publication are provided, and the chapter of the thesis containing the paper is indicated. The order of the papers is in relation to the chapters, rather than publication date

Chapter 2

Wohlfeil, C. K., Leu, S. T., Godfrey, S. S. & Bull, C. M. 2013. Testing the robustness of transmission network models to predict ectoparasite loads. One lizard, two ticks and four years. *International Journal for Parasitology: Parasites and Wildlife,* 2**,** 271-277.

CKW(70%) SSG(10%) STL(10%) CMB(10%)

CKW collected the field data, analysed data and wrote the manuscript. SSG assisted with field data collection and data prepation. STL checked the networks for problems. CMB thoroughly reviewed the manuscript. All authors reviewed and gave feedback on the manuscript.

Chapter 4

Deducing the dynamics of parasite transmission from patterns of parasite genetic relatedness across host networks

Wohlfeil, C. K., Godfrey, S. S., Leu, S. T., Gardner M.G. & Bull, C. M.

CKW(55%) SSG(25%) STL(5%) MGG(5%) CMB(10%)

CKW collected the field data,conducted all laboratry work, analysed network and genetic data and wrote the manuscript. SSG assisted with field data collection and constructed and analysed the ERGM models. STL checked the networks for issues. MGG advised on genetic procedures and analyses and reviewed the manuscript. CMB thoroughly reviewed the manuscript. All authors reviewed and gave feedback on the manuscript.

Chapter 5

Experimentally added pulses of tick infestation to test predicted transmission networks.

Wohlfeil, C. K., Godfrey, S. S., Leu, S. T., Gardner M.G. & Bull, C. M.

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Chapter 1: General Introduction

Models of parasite-host dynamics rely on understanding transmission dynamics, the way the parasite moves from one host to another. Knowledge of how parasites are transmitted through a population is important to the understanding of wildlife diseases and how they spread across a population. Exotic diseases and new forms of endemic diseases are emerging threats to wildlife species. Many of those wildlife species are now restricted to relatively small areas of habitat, where a local outbreak of a pathogen may have greater relative impact than if the species was more widely spread. Additionally, apparently benign parasites of one species can spill over into other species. For instance as a result of the increased frequency of interactions between humans and wildlife, there are serious human health concerns from exposure to pathogens from wildlife. Examples include Hendra virus in Australia and Lyme disease in the US. It is equally likely that wildlife populations could become exposed to new pathogens from contact with domestic animals or invasive species. For all these reasons, a vital component of wildlife management will be an ability to understand how parasites or diseases spread through a population, so that informed management actions can be taken to reduce the rate of spread. Managers will need to identify the risk of infection, and determine how connected or how vulnerable individuals are if a disease outbreak has occurred. In this project I will be using social network theory to investigate the dynamics of parasite transmission in one wildlife population. I will combine theory and observed infestation patterns with the use of parasite genetics to explore the interactions between ectoparasitic ticks and their sleepy lizard hosts. Although these parasites have relatively low impact on the fitness of their hosts, the system acts as a model for the transmission dynamics of other potentially more harmful parasites. The more we understand about host-parasite ecology, the better we will be placed to respond to future disease threats to wildlife populations.

Social Organisation and Networks

Social organisation describes the integration of many behaviours related to spatial aggregations within a population. These include, the interactions between individuals, the relationships between individuals (such as the content, quality and patterns of interactions) and finally the structure (which is the content, quality and patterns of relationships) (Hinde, 1979). Most animal populations have some level of social structure with higher rates of association among some individuals than others (Drewe, 2010, Aiello et al. 2016). This structure is sometimes described in terms of a social network, and it has often been suggested that the connecting links of a social network form pathways for the transmission of parasites and pathogens (Godfrey et al. 2009; 2010).

Network theory is by no means a new concept, having been around for centuries in the mathematical and social science fields. However, the use of networks to describe animal social behaviour is relatively new (Krause et al. 2009). One of the costs of group living is the increased risk of exposure to disease, and host behaviour is a key factor in parasite transmission. Early theories and models involving connectivity among conspecifics in a population, such as models of competitive interactions, assumed that all individuals moved freely within a population, with equal access to resources, and that connectivity was random. This created a homogeneous network. This has been a basic assumption of traditional SIR (Susceptible – Infectious – Recovered) models of parasite-host dynamics, that transmission from host to host is random, and that all susceptible hosts in a population are equally likely to contact an infected host and become exposed to infection themselves (Franks et al. 2009). In most animal populations random encounters are not the case, with the behaviour of individuals is heterogeneous. In real populations each individual does not interact with each other individual, creating different degrees of interaction among individuals within the population (Krause and Ruxton 2002). The heterogeneity of the network had been problematic to capture, until advances in social network theory enabled the individual behaviours, connectivity and population structures to be described and quantified (Krause et al. 2009).

Social network theory can be used to identify internal population interactions, relevant to parasite transmission (Krause et al. 2007). From social networks we can determine contact patterns and hence risks of infection of the individuals within a population, casting light on

the spread of disease and parasites through the population (Krause et al. 2007). The elements which make up a social network used by parasite ecologists can be described by the terms 'nodes' and 'edges', where a node represents an individual, and an edge represents the connecting interaction between two individuals (Croft et al. 2008).

One commonly used indirect method to infer transmission around a population has been to look for associations between social network structure and infection prevalence. A high incidence of infections among those individuals with stronger social connections has been used to deduce the transmission process in diseases of humans (Klovdahl, 1985; Christley et al., 2005), of domestic stock (Ortiz-Pelaez et al., 2006), and of wildlife (McCallum et al. 2001; Lembo et al. 2008; Clay et al. 2009, Vanderwaal et al. 2014).

The tool of social network theory can be used to link social and transmission networks. A social network describes the strength of association among individuals. A transmission network describes which hosts are likely to pass parasites to which other host individuals. By understanding the social structure of the host, ecologists can deduce the transmission networks and predict the spread of parasites and diseases through a population of animals. An example of the relationship between social networks and transmission networks of parasites is the study of an Australian lizard, *Egernia stokesii* and its ticks and blood parasites. Godfrey et al. (2009), used social networks to show there was heterogeneity in the opportunities of transmission, and sharing of common refuges contributed to the likelihood of individual infection of tick and blood parasites.

Although the social network structure is important in understanding how a disease or parasite can spread and impact the animal population, it is also critical to understand the dynamics and life history characteristics of the disease or parasite, and the mode of transmission. Specifically, directly transmitted parasites are more likely to be transmitted along edges in a social network, whereas less directly transmitted parasites, like those with some obligate off host stage, are less likely to follow along social network pathways. The importance of understanding the biology of the disease or parasite is crucial in developing transmission networks and understanding the transmission dynamics through a population. Godfrey et al. (2010) studied the effect of parasite load on an individual's position in a social network. They concluded that although the host position in the social network or its connectivity could influence transmission, it ultimately was the characteristics of the disease

or parasite which determined the properties of the transmission network, since each disease or parasite may have its own mechanisms and pathways for transmission.

An example of indirect transmission networks of a parasite comes from Fenner et al. (2011), who used indirect inferences to differentiate alternative models of the transmission of the nematode, *Pharyngodon wandillahensis,* within a population of the pygmy bluetongue lizard, *Tiliqua adelaidensis*. This nematode has a faecal-oral transmission pathway (Fenner et al., 2008). The lizards deposit scat piles close to their burrow entrances, apparently as a sign of burrow ownership (Fenner and Bull, 2011a). The scats from infected lizards include nematode eggs and presumably these eggs are ingested by another lizard as it uses its tongue for olfactory inspection of the scats (Fenner and Bull, 2011b). The level of connectedness in network derived from spatial proximity between the burrows occupied by resident and dispersing lizards explained infection status better than connectedness among all lizards. This suggested that transmission was more likely between residents and unfamiliar dispersers, as they inspected each other's scats, than among longer term residents who were familiar with each other (Fenner et al., 2011).

The application of network modelling in animal systems has increased in recent years, and Krause et al. (2014) demonstrated how an understanding of behavioural ecology has been significantly expanded by applying a network approach.

This has important relevance to models of host-parasite dynamics. Classic SIR (Susceptible, Infectious, Recovered) models of parasite host dynamics, developed by Anderson and May (1979), are still used today in models of the epidemiological spread of human diseases, such as AIDS, (Klovdahl 1985), HIV (Friedman et al. 1997) and influenza, (Hsu and Shih, 2010) and of other diseases in wildlife populations (Packer et al. 1999, Drewe 2010). These models generally assume random encounters among individuals and that all uninfected individuals are equally at risk of contacting an infected individual. However, when studying host – pathogen relationships at a finer spatial scale, and within social networks, a different general pattern becomes evident, that a small percentage of the population is responsible for the transmission of disease and parasites to a larger percentage of the population. These individuals are commonly referred to as 'Super spreaders'. It is unknown to what is the cause of an individual becoming a 'Super spreader', higher pathogen shedding and larger amounts of contacts could be contributor. Once identified, the infection can be controlled within the larger population, (Stein 2012). At this stage 'super spreaders' in most cases can only be identified retrospectively, such examples of super spreading

individuals include the SARS virus (Stein 2011) and the Ebola outbreak of 2014/2015 (Wong et al 2015).

Originally defined by Woolhouse et al. (1997), the 20/80 rule can be applied to many different vector-borne parasites and diseases. For example, Clay et al. (2009) conducted a study on the transmission of Sin Nombre virus in deer mice (*Peromyscus maniculatus*) and found that, from the contacts between individual mice, 20 percent of the individuals accounted for 80 percent of the contacts, and thus 80% of the potential virus transmission, observed within the population. Woolhouse et al. (1997) implied that the 20/80 rule could be used to predict disease spread and could contribute to control programs. Applying this rule to human diseases such as Sexually Transmitted Diseases (STDs), they suggested that the cost of using transmission data to locate and treat the major transmitters (the 20%) could be beneficial in that treatment costs would be reduced compared to treating the whole population. For parasite transmission, identifying and potentially removing many of the 20% could reduce the infection risk for the rest of the population.

Perkins et al. (2003) applied this principle in investigating the host-parasite relationship between the yellow-necked- mouse and tick-borne encephalitis, which is transmitted via co-feeding ticks. They found that the 20/80 rule applied and a distinct group of about 20% of individuals was responsible for the infection of 80% of the population. They removed the top 20% most tick infested mice and reduced the disease transmission by 74-94%. In this study it was found that sexually mature males of high body mass predominated in the 20% group, and this demonstrates the importance of focusing on the right target group for an impact on the reduction of disease within the population.

In another example, McCallum (2008) suggested that earlier detection of the Tasmanian Devil Tumour disease and culling of the infected individuals could have eliminated the threat that the disease now has on the entire Tasmanian devil population.

Much of the research about parasite transmission dynamics so far has relied on correlations between network connectedness and infection patterns to infer the pathways of parasite transmission. There are two fundamental problems with this approach. The first is that a significant correlation, for instance a higher prevalence of infection among more connected individuals, does not necessarily imply that we have modelled the most likely parasite transmission pathway. There may be better and more strongly correlated models of

transmission not yet considered. We need better understanding of the parasite ecology to develop alternative models of parasite transmission to get closer to the structure of the real transmission network, and understand that alternative networks may operate in alternative ecological conditions. The second is that a correlation does not imply causation. For example, if many individuals in a host population use the same sites for drinking, and if those moist sites are particularly good for the survival of off-host stages of a parasite, then individuals that go to those sites will a) be highly connected, and b) be at higher risk of parasite infection because more parasites have survived there. This may lead to a correlation between social network connectedness and infection rates, but not because of transmission along social networks. Social connections away from the water will not promote transmission. So we need more direct evidence to determine whether or not parasite transmission occurs along network edges. That evidence might come from adding experimental pulses of labelled parasites to some individual hosts and tracking where they are transmitted to. This is an approach attempted in this thesis.

This thesis uses a native lizard and its ectoparasitic ticks to explore more detailed understanding of parasite populations (through genetic analysis). It compares a number of alternative transmission pathway models to explain infection patterns, and uses experimentally added and genetically labelled parasite pulses.

Study Species

Figure 1: One of the host study animals a sleepy lizard, *Tiliqua rugosa*, being released after handling, with radio, GPS recorder and data logger attached to its tail.

The sleepy lizard, *Tiliqua rugosa* (Gray, 1825), belongs to the family Scincidae and is widely distributed over the southern half of Australia, except along the coast and ranges of the east and south east, (Cogger, 2014). The sleepy lizard is a large lizard, with adults growing to over 28cm snout-to vent length (SVL). It is viviparous, producing a clutch of 1-3 live young after a 4-5 month gestation (Bull 1995). Adult lizards are documented normally to weigh between 500-800 grams (Bull and Burzacott, 1993), however throughout the field seasons of this study; several lizards weighed over 900 grams (pers. Obs.). It has been found to have an omnivorous diet, which is dominated by plant material (Dubas and Bull 1991).

Sleepy lizards are long lived, forming monogamous pair bonds, with mates finding each other in successive years. In these pairs, the male and female remain together for a substantial proportion of their time (active and inactive in refuges) for up to 8 weeks before mating in the spring (Bull, 2000). After temporary separation, sleepy lizards of both sexes

use a number of cues to re-locate their partner, including following trails, detecting airborne signals or returning to familiar sites. Both the male and the female are responsible for maintaining the close associations (Leu et al. 2010a). Despite this social monogamy there is evidence of extra pair fertilization occurring in 14% of the offspring, but these cases are mostly in pairs with weaker associations (Bull et al. 1998). There are benefits to monogamous pairing, and it has been found that when paired there is an enhanced level of vigilance provided by the males for their female partners. When together the male tends to be found feeding less, and this benefited the female as the detection of approaching threats was higher than when alone (Bull and Pamula 1998). This behaviour appears to be an adaptation for predator avoidance, allowing the female to focus on foraging rather than vigilance (Bull and Pamula, 1998).

The sleepy lizard also forms long term pair bonds with the pair bond frequently exceeding 10 years and the longest recorded partnership being 27 years and still going (Leu et al. 2015). Although many long term pair bonds are formed, up to one third of pairs change partners over the course of 5 years (Bull 1988; Leu et al. 2015). There are two main trends in the change in partner. Firstly, smaller males tended to be more likely to be separated from their females in a subsequent year, and secondly, females were more likely to abandon partners from a previous year if they had a high parasite load (Bull & Burzacott, 2006). The behaviour of pairing long-term and choosing mates with lower parasite loads has the potential to reduce opportunities for parasite transmission (Bull & Burzacott, 2006).

The parasites that have been most studied in association with sleepy lizards are their ectoparasitic ticks. More details of this association are provided below. Tick load can temporarily impact host fitness, with high tick load reducing body condition, reproductive ability and sprint speed (Main & Bull, 2000). These in turn, could influence an individual's connectivity within the social network. Main and Bull (2000) found that a host with high tick loads had reduced endurance and movement within its home range compared with a host with a lower parasite load, and any reduced activity may reduce the number of times an individual contacts other conspecifics.

Direct observations of lizards are time consuming and difficult. An on board data logger was first developed, to overcome these issues, by Kerr et al. (2004b) and used to study more fine details of the behaviour of the sleepy lizard, initially studying the use of refuge sites. The technology has allowed us to study patterns of activity in relation to surrounding

microhabitat and temperature ecology when the observer was not present. This led to insights into the impact of human observer presence, on the lizards. When watched, lizards initially decreased activity significantly below the level of unobserved lizards at the same time, and then had a burst of atypically high activity to move away from the observer (Kerr et al. 2004b). As well as showing the value of these indirect methods of observing behaviour, this result also indicated the importance of conducting any handling of the lizards, for measurements, at a time when the lizards were naturally inactive. Kerr et al. (2006) used this technology also to give a more detailed understanding of the space use by the sleepy lizard, reporting they had a home range which has extensive overlap with other lizards, but a core home range with much less overlap with neighbours. There were higher incidents of male-female core overlap than female- female or male-male overlap. This reflects that this species has a monogamous mating system. Outside of the core areas, there are extensive overlapping sally zones, where space is shared with other lizards. In favourable years, when there are plenty of the annual plants the lizards feed on, lizards tend to have larger home ranges than in drought years. These larger areas facilitate more overlap with neighbouring lizards and in productive years, more cases of extra pair fertilization could take place (Kerr et al. 2006). Bull and Burzacott (1993) found that lizards in better body condition had higher tick loads. They suggested this did not mean that the ticks benefitted the lizards but that parasite loads may relate to the lizard home range quality and location. That is, good lizard habitat is also favourable to off-host tick survival, and the fittest lizards, occupying the best habitats are also exposed to more ticks. High or low parasite loadings on lizards were consistent over years, again suggesting that some home ranges had habitat consistently more favourable to the breeding cycles of the tick than others. If these habitats were also better for lizards, then that would explain the unexpected positive correlation between lizard condition and tick load.

The next technological step in revealing sleepy lizard behaviour was to add GPS receivers and data loggers to the units carried by the lizards. These recorded synchronous locations of almost all lizards in the same area at every ten minute interval and allowed us to deduce cases where two lizard locations were in close proximity and where they probably came into contact with each other. From this we could derive contact social networks as discussed above, although we could not deduce whether the contact was associative or aggressive. From these empirically derived networks, Leu et al. (2010a) showed that, even though the very close association of male and female partners was broken after mating, those partners maintained a high frequency of regular contacts even after mating. This behaviour is

not related to parental care, since the neonates are born five months later when both partners are largely inactive. The reasons that this occurs could be due to the availability of suitable refuges in the shared home range, mutual tolerance for close association, continued enhanced vigilance, and the efficient location of mates in future years (Leu et al., 2010a). Lizards leave individual chemical trails and lizards use these both to locate others they want to associate with (Lindle and Bull 2002) and probably to avoid other specific individuals, (Bull et al. 1993, Kerr and Bull 2002). Leu et al. 2010a suggested that this could reduce the risk of an encounter which could escalate into a fight, which can result in reduced lizard fitness.

Although aggressive behaviour is rarely directly observed, Godfrey et al. (2012) using social networks, explored the positions that aggressive males had within the network. Combining the networks with behavioural assays, they found that lizards that were more aggressive in the assays were more likely to have fresh scale damage in field surveys. The study also showed that the less aggressive males were more strongly connected with females within the network than the more aggressive males. The unpaired males may use aggressive behaviour to establish a position within the population, when encountering other males. It could also be that the aggressiveness of the males is dependent on the social context of the individual (Godfrey et al. 2012). They also suggested that aggressiveness is an adaptive behaviour, because when unpaired, and having limited access to partners, aggression may allow a male to establish a stable home range, while once established and when paired, abstaining from aggression may be beneficial to reduce risk of injury.

Unlike several species with kin related social groups, Godfrey et al. (2014) found no association between network connectedness and genetic relatedness in sleepy lizards, suggesting this was because both males and females were likely to disperse from their place of birth. In fact the male-female partnerships were found to be less related than if they had chosen partners at random from among neighbouring lizards (Bull et al. 1999) indicating an ability to recognise the degree of relatedness.

Sleepy lizard parasites

Sleepy lizards are hosts to a number of different parasites. These include two ectoparasitic tick species, *Bothriocroton hydrosauri* and *Amblyomma limbatum*, which are the main focus of this thesis. The sleepy lizards also is host to a number of gut bacteria, including *Salmonella enterica*, nematode worms (*Thelandros trachysauri* and *Oochoristica trachysauri*), a blood parasite, *Hemolivia mariae*, and other bacteria belonging to the *Rickettsia* genus. There have also been recent reports of a reptile specific flu-like virus in Western Australian populations of *Tiliqua rugosa* (O'Dea et al. 2016). Although those symptoms have not been confirmed at the study site of this thesis, one season lizards showed signs of cold like symptoms, (pers. obs.).

The two, three-host, ectoparasitic tick species, *Bothriocroton hydrosauri* and *Amblyomma limbatum*, attach to the host as larvae and then as nymphs, engorge and detach to moult. The females attach to a third host, and then, once mated, engorge and detach to lay eggs. Those eggs hatch into larvae which start the process again. Males stay on the lizards for many months, feeding infrequently, waiting for opportunities to mate (Andrews and Bull 1980). These ticks are transmitted from one host to another, in the process of developing to a new life stage. For example, engorged larvae detach, moult to nymphs and then are ready to attach to a second lizard host. The ticks are assumed only to survive off-host for long enough to find another lizard host if they detach within a lizard refuge. This also increases their chance of encountering another host, as multiple lizards may, asynchronously, use the same refuges (Kerr et al. 2006). Thus tick transmission occurs through the asynchronous sharing of refuge sites, (Leu et al. 2010b). Once each life stage becomes engorged and detaches from the host, it finds shelter in the leaf litter (Petney & Bull, 1983). This litter is found around the base of bushes, shrubs and around fallen logs, which the host uses as refuge sites, (Chilton and Bull 1993). Once off the host, ticks are vulnerable to many predators, such as ants, (Bull, Chilton and Sharrad, 1988). They do not actively seek for hosts, but wait in the one place until physically disturbed by a host individual.

Climate can contribute to the rate of parasite transmission, and where it takes place, as the host behaviours can change. For example lizards use different refuge types depending on

the weather, low bluebushes in cooler weather and deep wombat burrows in hot weather, and this can change the potential for tick transmission, (Kerr et al 2003).

Close to the study site the two tick species have an abrupt parapatric boundary with *B. hydrosauri* distributed in more mesic conditions to the south, and *A. limbatum* in more arid conditions to the north (Smyth 1973; Bull et al. 1989; Bull and Burzacott 2001). The northern species, *A limbatum*, has higher desiccation tolerance (Bull and Smyth 1973) although this does not explain the abruptness of the boundary between the two species. Our study site was in an overlap zone where both species could be found on lizards, although the different desiccation tolerances, different development times, and different times of survival while off-host (Chilton and Bull 1993) led to us developing different models of their off-host survival. These developmental and survival times were used by Leu et al. (2010c) to define a window of infection time for the transmission networks, from when the tick had moulted to become infectious to the time it was likely to have died from desiccation. If another lizard entered the refuge during this infection time window then it could become infected, and a link between the previously infected and the new infected host was established on the transmission network. Based on laboratory observations, the infection time window was set at 9-39 days from detachment for *A. limbatum* and 11-24 days for *B. hydrosauri*, in the analyses of Leu et al. (2010c). Of course rates of development and desiccation will vary with the climate, and the actual time window for each detached tick will vary, but for models it is convenient to choose a standard representative value. In this thesis some consideration is given as to whether alternative time windows of infection produce transmission networks that better reflect the empirically derived infection patterns.

The pattern of refuge use by the sleepy lizard is also very important for parasite transmission. Kerr et al. (2004b) showed that during the spring peak of activity, lizards used many refuges within their homes range. In summer and on hot days towards the end of spring, when the activity of the lizards slowed, the number of refuges used decreased and lizards used different refuges such as deep wombat burrows to reduce thermal stress. A consequence of multiple lizards remaining in single burrows for extended lengths of time and may use wombat burrows repeatedly to remain cool, increased chance of contact with other lizards and their ticks (Kerr and Bull 2006). In previous studies, GPS logger data were used to create networks of asynchronous refuge sharing and explore how it related to the parasite loading of the lizard. Leu et al. (2010c) reported that lizards with higher connectivity within

an asynchronous refuge sharing network had higher parasite loads, and suggested this was an appropriate transmission network for ticks.

Hemolivia mariae is a lizard blood parasite, which has a life cycle that involves both *Bothriocroton hydrosauri* and *Amblyomma limbatum* and the sleepy lizard. This protozoan parasite goes through its sexual cycle in its tick host. It is transmitted to the lizard (effectively the vector species) when a lizard ingests an engorged infected tick. It enters the lizard blood stream at very low infection intensity, and then re-infects a tick through ingestion of infected red blood cells of the vertebrate lizard host, (Smallridge and Bull 1999). Smallridge and Bull (1999) reported that *A. limbatum* was more likely than *B. hydrosauri* to develop a *H. mariae* infection and more likely to transmit it to the lizard host, and they suggested that this differential susceptibility to infection may contribute to maintaining the parapatric boundary between these two tick species. There was lower prevalence of *H. mariae* in lizards in areas which were dominated by *B. hydrosauri* than where *A. limbatum* was more common (Smallridge and Bull, 1999)

Sleepy lizards with a *Rickettsia* like infection had been documented by Bull and Burzacott (1993), with *Rickettsia* like blood cell inclusions found in some lizards in the field. *Rickettsia* is a bacterium; some species can be transferred to humans as it is present in the salivary excretions of tick. *Rickettsia honei* found in *B. hydrosauri* from Flinders Island, Tasmania, is responsible for Flinders Island Spotted Fever. Whiley et al. (2016) used qPCR to assay *B. hydrosauri* from sleepy lizard, collected from locations where cases of Spotted Fever have been reported. It was expected that the results would show the presence of *Rickettsia honei*, however a different strain of *Rickettsia* was identified. This study was the first to use PCR to positively identify *Rickettsia* in tick samples. Although, the 15 *Tiliqua rugosa* blood samples were negative for *Rickettsia*, all 41 *B. hydrosauri* samples collected were positive for the *Rickettsia* spp., identifying *B. hydrosauri* as vector for multiple *Rickettsia* spp with a high prevalence in the South Australian study area (Whiley et al. 2016).

The sleepy lizard is also host to two nematode worms, *Thelandros trachysauri* and *Oochoristica trachysauri*, both gastrointestinal parasites that are faecal-oral transmitted, with eggs found in lizard scats. In the field site, lizards have a high infestation rate, with all males and 80% of females having *Thelandros* present in their scats. The infestation of *Oochoristica* was lower, with 19% of males and 11.5% of females infected. The lower infection rate may

have been due to the *Oochoristica* needing an intermediate host to complete their life cycle, (Gyawali et al. 2013).

One final parasite of sleepy lizards that has been explored in some detail is the gut bacterial species *Salmonella enterica* that is infrequently found in most Australian wildlife species, but relatively common although apparently non pathogenic in Australian reptiles (Parsons et al 2010). Bull et al. (2012) tracked different genetic strains of this species to deduce that the parasite transmission was best predicted from the social contact network of their sleepy lizard hosts.

This thesis will first aim to replicate the transmission network study, by Leu et al. (2010c), on a larger scale, with more lizards; two tick species instead of one, and over multiple years, to investigate the generality of any transmission network models. Then it will use the genetic analysis of the reptile tick, *B. hydrosauri*, to expand the understanding of the tick transmission pathways.

Genetics and tick genetics

Combined with social networks, molecular tools such as genetics are valuable in investigating parasite transmission and the impact to the process of disease ecology (Archie et al. 2008). Thus genetic tools have become important in ecology they can be used in conjunction with a parasite transmission networks to identify the source of infection both forward, who infects who, and backwards, who infected who (Archie et al.2008).

This technology can be used to confirm the original source point for the disease or parasite, inferring the pathways of transmission across the globe. This was demonstrated by examples such as the H5N1 virus known as bird flu, where collecting samples over the past decade, creating a phylogeny and mapping the locations allowed reconstruction of the possible source of infection, (Archie et al. 2008).

In particular, microsatellite markers have become a popular genetic tool. With the development of polymerase chain reaction (PCR) technology, microsatellites have become even more versatile markers, (Schlotterer 2000). Microsatellites are repeats of nucleotides, usually 1- 6, these are found in most taxa and in high frequencies (Selkoe and Toonen 2006). The widespread occurrence of polymorphisms at microsatellite loci makes them useful

indicators in a range of studies of ecology and behaviour. One challenge of using microsatellites is their limitation of being species specific Chambers and MacAvoy (2000).

Parasites, such as ticks, can be vectors for wildlife diseases. Understanding the mechanisms of the population structure and genetics is helpful in combating wildlife diseases. There have been many studies which have developed tick species specific microsatellite markers. These include polymorphic loci for the raccoon tick, *Ixodes texanus* (Dharmaraajan et al. 2009a), the American dog tick*, Dermacentor variabilis* (Dharmarajan et al. 2009b), the cattle tick, *Boophilus macroplus* (Chigagure et al. 2000 and Koffi et al. 2006) and *Ixodes ricinus* (Roed et al. 2006). The cattle tick, *Rhipicephalus (Boophilus) microplus,* is an example of how understanding the population genetics can be useful in tackling parasite transmission. Cutulle et al. (2009) used microsatellite markers to investigate gene flow and found that, although there was low heterozygosity, the data could be used to trace the source of an outbreak or contamination across quarantine lines. Further investigation and a record of source populations would need to occur for this to be successful.

The black-legged tick, *Ixodes scapularis*, a vector for human disease, has also been the study of genomic research (Pagel Van Zee et al. 2007). One challenge specifically identified with tick genetics is that tick genomes consist of significant amounts of repetitive DNA. The *Ixodes* project was one of the first to study in depth the nature and genome of ticks, and 454 sequencing of additional tick species is suggested as a viable solution to understanding the large tick genomes. The unlocking of these genomes would then improve our ability to use these genetic tools to understand tick transmission and movement patterns and potential disease transmission.

Guzinski et al. (2008) first investigated the genetics of *B. hydrosauri* and *A limbatum* in the study area, with limited success in creating microsatellites using the isolation via enrichment protocol of Gardner et al (2008). They reported development of 10 microsatellite markers for *B. hydrosauri*. For *A. limbatum* they could develop no reliable microsatellite markers, due to limitation of the MRT labelled primers at the time (Guzinski unpublished thesis, 2008). From the genetic data for B. *hydrosauri* they reported a ripple effect, where larvae, nymphs and then adults spread from a viable clutch of eggs like a ripple across the host landscape. Specifically, the ticks on an individual host were more highly related to each other, than ticks in the overall sample area (Guzinski et al. 2009).

Although some marker development was completed in that earlier study, more markers need to be developed to ensure more reliable identification of individual ticks to establish a rigorous tick population dynamic network. With the relatively new genetic technique (Margulies et al. 2005), of genome sequencing, (sometimes referred to as shotgun or 454 sequencing) primers can be created. The technique allows the elimination of recombinant steps enabling the cost and time of finding microsatellite markers to be cut. This technology increases the number of microsatellites available to create the genetic accuracy, high enough to distinguish between different individual ticks on the one host.

Successful application of this new technique has been documented by Abdelkrim et al. (2009). They used GS-FLX (454 Life Sciences) sequencing techniques to develop 13 polymorphic microsatellites markers for the genome of the blue duck *(Hymenolaimus malacorhynchos)*. Gardner et al. (2011) used the GS-FLX method for development of microsatellites for 54 non-model species. This was over a range of taxonomic groups and included cases where enrichment techniques had previously failed. Using the GS-FLX method, a much larger number of microsatellites were found than if traditional methods were used, but in order to find the same number of useable loci, the amount of sequences for invertebrates was on average double that needed in plants or vertebrates. (Gardner et al. 2011)

An aim of this thesis was to combine the use of genomic sequencing techniques, with the Guzinski's previously developed loci, to allow sufficient diagnostic genetic loci to explore natural tick infection patterns and to experimentally add genetically unique clutches of tick in a population of sleepy lizards with known social network structure. As will be explained in the thesis it was still difficult to develop a wide array of polymorphic satellite DNA loci in *B. hydrosauri*, but sufficient genetic information was derived to provide detailed insights into the transmission dynamics.

Figure 2: Sleepy lizard with an engorged female, *Amblyomma limbatum*, attached on the neck.

Figure 3: Sleepy lizard with an engorging female *Bothriocroton hydrosauri*, attached behind the front limb. (Photo: T Wey)

Project Aims

The overall aim of this study was to further understand the mechanisms of host-parasite interactions by investigating transmission networks

Thesis structure:

This chapter, **Chapter 1** outlines the background and aims of the thesis study

Chapter 2 tests the robustness of transmission network models and determines if models remain consistent in different tick species in different years. This study, compared a single year smaller study of one tick species, with a larger lizard population, four years of networks and two tick species,

Chapter 3 is a technical methodology chapter. The main aim was to develop new loci for the reptile tick, *Bothriocroton hydrosauri*, using 454 sequencing. Although this was not as successful as hoped, the techniques developed sufficient new loci to identify related individuals from within the natural tick population and from uniquely different artificially infected clutches of ticks.

In **Chapter 4** we used the genotypes of the ticks that were identified in Chapter 3 to describe infection patterns in the natural population. The aim was to address two questions; firstly, are ticks on the same lizard more related than tick on different lizards? And secondly, amongst the tick on different lizards, is the relatedness of tick influenced by host social relationships, transmission network pathways or spatial relationships?

Chapter 5 this chapter focussed on experimental tick infections, to identify actual transmission pathways from larval tick additions to subsequent adult tick hosts.

Chapter 6 reviews the results and discusses the conclusions of the study and how this work might contribute to a wider understanding of parasite transmission dynamics.

Study Site

Figure 4: Shows the location of the field site in relation to Adelaide on a map of South Australia, (Map via Google Earth accessed on 2/6/2017)

The study site, Figure 5A, was an area of 1.0 x 1.5 km, located approximately 6km along the Bundey Church Road (Transect 6), West of Bundey Bore Station (33°54'S, 139°20' E) in South Australia and was chosen because both tick species were found to overlap in the area. Shown on figure 5B in yellow, the locations were the original area of the study site. This area expanded over time, the Red box, due to increased lizard number studied and their home range movement. Within the field site, there two dams, the northern dam was a permanent water source as it held water during all field seasons. The southern dam was less permanent as it only held some water during the 2010 and 2011 field seasons. There is an infrequently used track which runs through the middle of the site, as shown on figure 5B. The habitat was predominantly chenopod shrub-land (figure 6), with some areas of casuarinas scrub (figure 7).

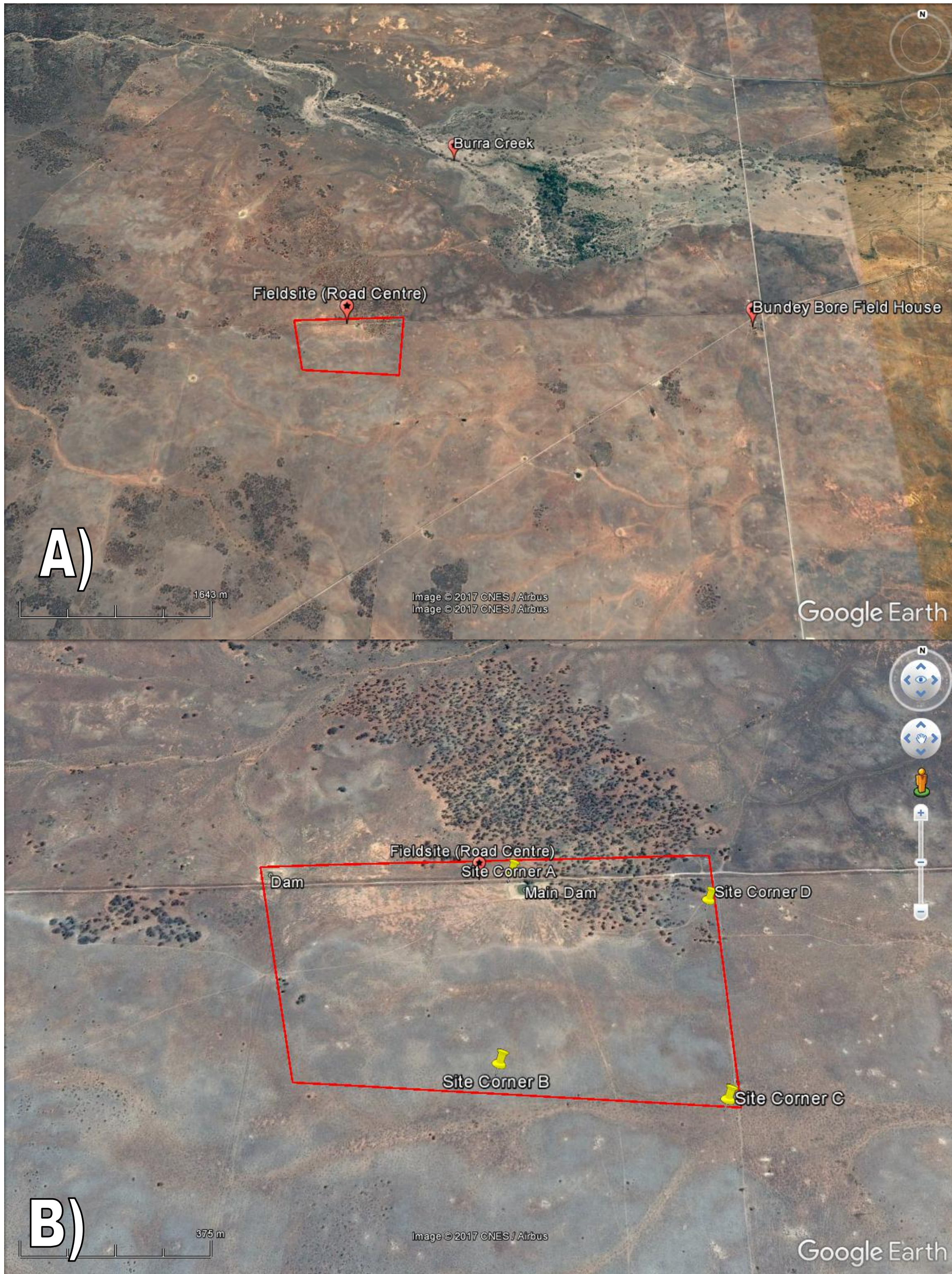

Figure 5: A) This image shows a terrain and vegetation found in the field site and the location from the field house. B) The yellow pins represent the original study location. The Red box represents the field area used the seasons 2-4. (Image, Google Earth accessed on 2/6/2017)

Figure 6: Vegetation in the central part of the study site. Taken in 2011, showing the site as predominantly being chenopod vegetation with *Casuarina* trees in the northern section of the site

Figure 7: This image shows the *Casuarina* scrub vegetation at the northern section of the field site.

Rainfall and temperature

The area has hot dry summers and cool wet winters with an average annual rainfall of about 250 mm. The study was conducted over four years, 2008-2011, during the spring and

early summer (September- December), the only period of the year when sleepy lizards are normally active (Kerr and Bull, 2006). The average monthly temperature is similar across all four field seasons, (figure 8). Figure 9 shows the total monthly rainfall over the whole year the rainfall during the field season differed between years, with 2008 being the driest season and 2010 the wettest, (figure 10). During the field season, Aug-Dec, 161.6 millimetres of rain fell in 2008, 215.7mm in 2009, 310.4mm in 2010 and 195.8mm in 2011.

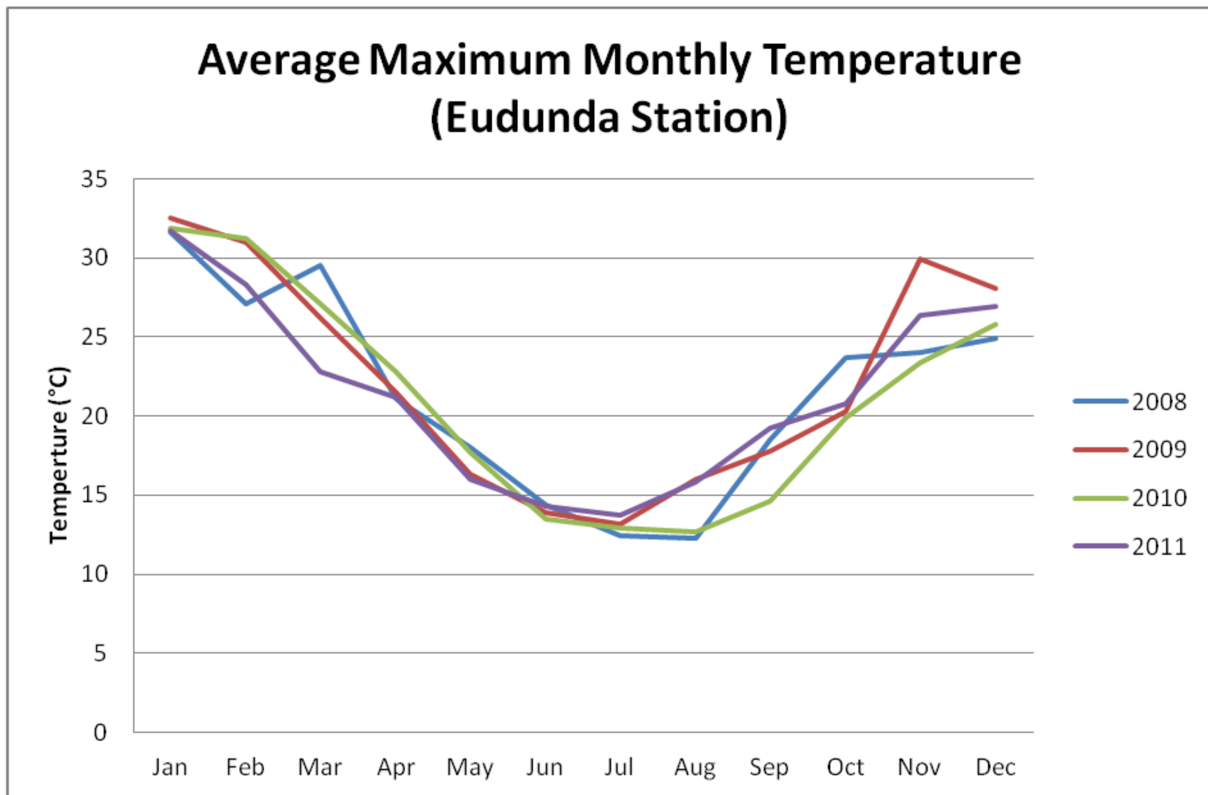

Figure 8: This shows the average maximum monthly temperature recorded at Euduna, the closest Bureau of Meteorology temperature recording station to the field site. (http://www.bom.gov.au/jsp/ncc/cdio/weatherData/av?p_nccObsCode=36&p_display_type=dataFile&p_startYear=&p_c=&p_stn_num=024511 Site accessed 20/11/2016)

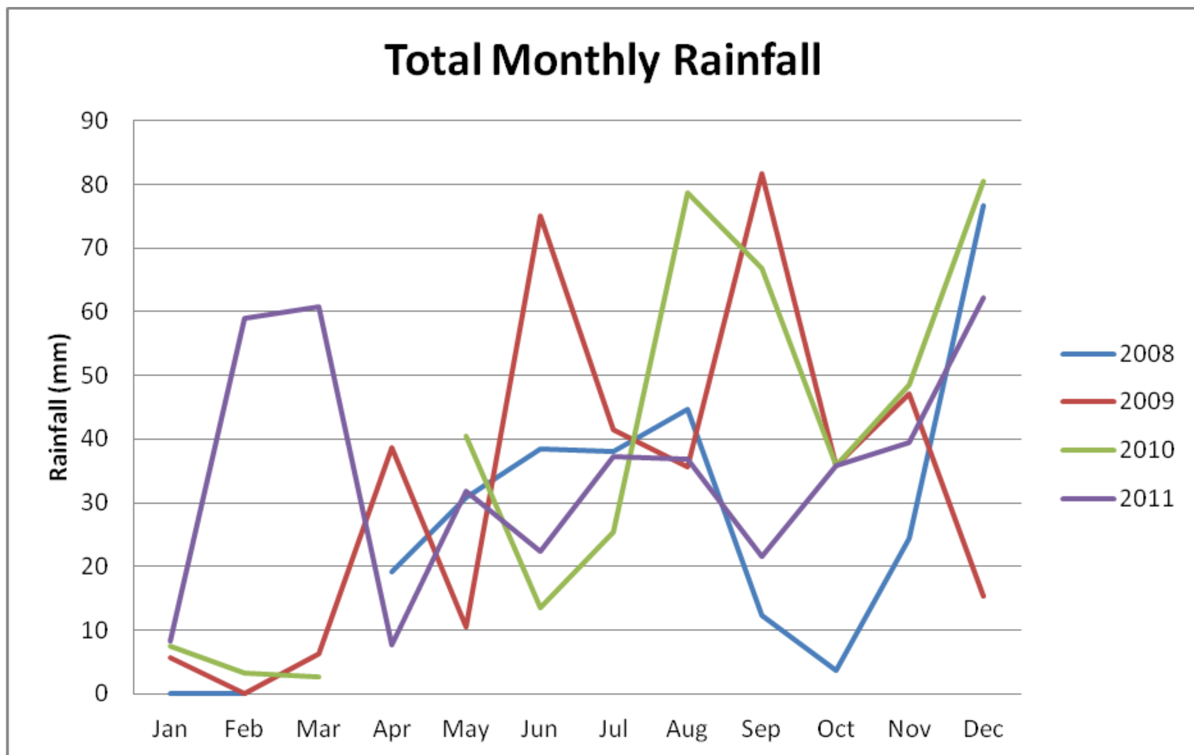

Figure 9: This shows the total monthly rainfall recorded at Robertstown, the closest Bureau of Meteorology rainfall recording station to the field site. (http://www.bom.gov.au/jsp/ncc/cdio/weatherData/av?p_nccObsCode=139&p_display_type=dataFile&p_startYear=&p_c=&p_stn_num=024528 Website accessed 20/11/2016)

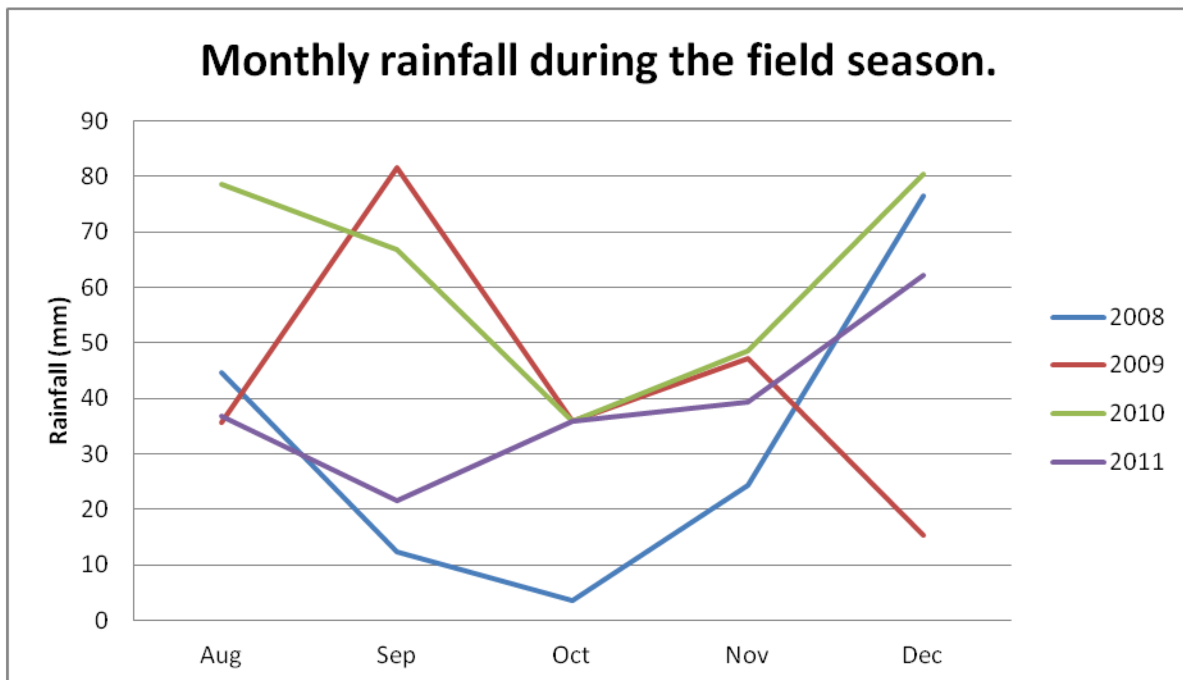

Figure 10: The monthly rainfall during the field season. (Website accessed 20/11/2016)

Field methods

Lizard capture and data recording

In late August every season (Lizards' peak activity period between late august to late December), lizards were located and a radio tag attached. At this time an initial record was made of GPS location and a range of parameters, such as activity at the time of encounter, if paired with another lizard and tick load. These parameters were repeatedly recorded at every 12 day data download event. Usually in this first week of the season enough lizards were located or relocated that GPS units were attached. After the first week lizards had GPS units attached at the initial capture. When these units were attached or changed over, data relating to the units were also collected, such as lizard encounter, unit off/on time and when the lizard was released.

In 2008, GPS units were attached to 48 lizards, in October of this season male *B. hydrosauri* were removed from lizards that had 2 or more adult males at the time of capture. These ticks were used for genetic analysis. This season was 159 days long and concluded in early February.

In 2009, 61 Lizards had GPS units attached, but no ticks were collected for genetic analysis. In October the first experimental infections of uniquely identified clutches took place, subsequent pulse additions occurred in April 2010. The methods of clutch collection and identification are in Chapter 3. This season was 127 days long and concluded in late January.

In 2010, 61 lizards had GPS units attached and in addition to the general information collected, all adult tick were collected for genetic analysis at each data download event. This season was 115 days longs and concluded in late December.

In 2011, GPS unit were attached to 61 lizards and the standard information was recorded every 12 days. Ticks were collected in three collection trips, at the first data download of each month. Due to equipment failure and having to find lizard when active to recover GPS units, the field season was shortened and finished after the last tick collection trip in November, the total season was 81 days long.

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Chapter 2: Testing the robustness of transmission network models to predict ectoparasite loads. One lizard, two ticks and four years.

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This chapter had been published in *International Journal for Parasitology: Parasites and Wildlife,* Vol 2, pages 271-277 and describes and tests the robustness of the transmission networks over four years, investigating two tick species.

Abstract

We investigated transmission pathways for two tick species, *Bothriocroton hydrosauri* and *Amblyomma limbatum,* among their sleepy lizard (*Tiliqua rugosa*) hosts in a natural population in South Australia. Our aim was to determine whether a transmission network model continued to predict parasite load patterns effectively under varying ecological conditions. Using GPS loggers we identified the refuge sites used by each lizard on each day. We estimated infectious time windows for ticks that detached from a lizard in a refuge. Time windows were from the time when a detached tick moulted and become infective, until the time it died from desiccation while waiting for a new host. Previous research has shown that *A. limbatum* moults earlier and survives longer than *B. hydrosauri*. We developed two transmission network models based on these differences in infective time windows for the two tick species. Directed edges were generated in the network if one lizard used a refuge that had previously been used by another lizard within the infectious time window. We used those models to generate values of network node in-strength for each lizard, a measure of how strongly connected an individual is to other lizards in the transmission network, and a prediction of infection risk for each host. The consistent correlations over time between *B. hydrosauri* infection intensity and network derived infection risk suggest that network models can be robust to environmental variation among years. However, the contrasting lack of consistent correlation in *A. limbatum* suggests that the utility of the same network models may depend on the specific biology of a parasite species.

Key Words: lizard; tick; social network; parasite transmission

1. Introduction

Hosts within a population typically vary in the intensity of parasite infestation. In a previous study, Leu et al., (2010b) reported that the patterns of infestation of one tick species, *Amblyomma limbatum*, on its host lizard, *Tiliqua rugosa*, in one year, could be explained by a transmission network model. Our aim in the current study was to determine whether that model was sufficiently robust to predict patterns of infection in two tick species across multiple years.

Variation among hosts in the intensity of infection can result from post-infection differences among hosts in levels of resistance or immunity to the parasite, from interactions with the already established parasite community of the host, or from differences among hosts in their exposure to infestation (Poulin, 2007). Exposure can be determined by host behaviour. For instance behavioural differences between males and females (Zuk and McKean, 1996; Grear et al., 2012) or between adults and juveniles (Griffing et al., 2007) can influence their exposure to infection and their subsequent parasite loads. Transmission of parasites from one host to another host depends on behavioural processes and is now often described in terms of social networks. These recognise the role of social structures that influence contacts among hosts. Social networks describe the links between individuals that contact each other, and that are potential pathways for directly transmitted parasites (McCallum et al., 2001; Altizer et al., 2003; Clay et al., 2009). Network properties help in modelling the spread of parasites within a population (Hamede et al., 2012).

For parasites that have indirect transmission, transmission networks can still be constructed that reflect the ecological process of a parasite moving from one host to another.

If a parasite leaves one host or sheds propagules that then wait to infest another host, then transmission can occur between hosts that share the same space, and space sharing transmission networks can be derived to predict infection patterns (Godfrey et al., 2010). Sometimes the dynamics of parasite transmission are unclear where it is difficult to make empirical observations of critical stages in the transmission process. In those cases, exploring alternative network models that reflect different plausible transmission processes, and comparing the predictions of each model with the empirically observed patterns of parasite infection can help to understand transmission ecology (Fenner et al., 2011). Our central hypothesis was that transmission networks adequately model pathways for the passage of parasites around a population. A prediction of that hypothesis is that individuals that are more connected in the network are at higher risk of infection, and are likely to have higher parasite loads.

Additionally, the dynamics of parasite transmission might vary over time, for instance in different seasons of the year (Hamede et al., 2009) or under different ecological conditions, and an important question is how robust are transmission models to ecological change. Conservation managers, who will often not know the mechanisms or dynamics of transmission of an exotic pathogen, will need to know if models of transmission derived from one time and place will translate to other times and places.

Our question in this study concerned the robustness of transmission network models. We asked whether a transmission network model continued to predict parasite load patterns effectively under alternative ecological conditions. Our study system was a natural

population of Australian sleepy lizards (*Tiliqua rugosa*), hosting two ectoparasitic ticks, *Bothriocroton hydrosauri* and *Amblyomma limbatum*. Both of these are three host ticks. The three life stages, larvae, nymphs and adults, each feed on a single lizard host, and then detach to moult to the next stage, or, for adult females, to lay a single clutch of eggs. Both ticks are reptile specific (Belan and Bull, 1995), and both have the sleepy lizard as their main host for all life stages in our study site (Smyth, 1973; Bull et al., 1981; Bull and Burzacott, 2001). Leu et al., (2010b) developed a transmission network model for *A. limbatum* on sleepy lizards based on asynchronous refuge sharing by the host lizards. In this model a lizard could become infected if it used a refuge previously used by another lizard that might have shed ticks there. The model included a time window of infection risk that was set by a pre-moult period and survival duration for the unattached tick. Leu et al., (2010b) reported a significant correlation between transmission network node in-strength (a measure of the level of network connection) and *A. limbatum* tick load in a network of 18 sleepy lizards, in one year. Lizards that more often used refuges previously used by other lizards, were at higher risk of infection and that was reflected in higher tick loads.

This study tests the robustness of that relationship by considering patterns of infestation of two tick species over four ecologically contrasting years. Our aim was to determine whether the transmission network model remained an effective descriptor of infection patterns for different species in different years. Our hypothesis was that transmission networks represent pathways for the passage of parasites, in this case ticks, around a population. Our transmission network models were constructed from our understanding of the host-parasite system, and the test of our hypothesis was whether specific predictions from the network models fitted empirical data of parasite loads. In this case, we predicted that a higher in-strength of individual lizards, that is a higher cross infection risk,

should lead to a higher infestation load. Lizards exposed to more infection risk should become more infected, and if the transmission network models are correct we should find a positive correlation between node in-strength of individual lizards and their tick loads. Leu et al. (2010b) reported a positive correlation for a smaller sample of lizards in one year, infested only with *A. limbatum*. In this study, we aimed to expand the generality of that previous result by conducting equivalent analyses on a larger sample of lizards, at a different location, over four (new) years, and with two tick species.

2. Materials and Methods.

2.1 Study site

The study was conducted in a 1.0 x 1.5 km area of semi-arid chenopod shrub land near Bundey Bore Station (33° 54' S, 139° 20' E) in South Australia. At this site, sleepy lizards are active over the spring and early summer (September – December), and almost completely inactive at other times of each year (Kerr & Bull, 2006; Kerr et al., 2008). The study took place over four consecutive activity seasons, from 2008 - 2011. The long-term average annual rainfall in the study area is about 250 mm, and the actual rainfall at Bundey Bore Station was lower than average in 2008, but average or above average in the other three years (Table1).

2.2 Data recording

The procedures for measuring lizard activity, storing GPS locations, and deriving social networks have been previously described (Kerr et al., 2004a; Leu et al., 2010a; Bull et al., 2012; Godfrey et al., 2012). Briefly, at the start of each season (September), we located

all resident adult lizards within the study site (2008, n = 48 lizards; and n = 61 lizards in each of 2009, 2010 and 2011), and attached data logger units to the dorsal surface of the tails of each lizard. These recorded synchronous GPS locations for all lizards that were active, every 10 minutes, over the following four to five months (2008, n = 159 days; 2009, n = 127 days; 2010, n = 115 days; 2011, n = 81 days). The data logger units included a radio transmitter that allowed each lizard to be located every 12 days to download data and change batteries. Data downloads were conducted at times before or after the diurnal period of lizard activity, to avoid interfering with normal behaviours and to reduce the impact of handling on lizard behaviour that was reported by Kerr et al., (2004b). Some units malfunctioned in some of the 12 day periods and some lizards were not fitted with data logger units early in the season. For analyses, we only included lizards with data from more than 30 days (2008, n = 45 lizards; 2009, n = 56 lizards; 2010, n = 60 lizards; 2011, n = 59 lizards). For most of those lizards we had over 100 days of data in each of the first three years (Table 1). The season was shorter in 2011 when equipment failure prematurely ended monitoring in late November.

At each data download we also counted the numbers of naturally attached adult, nymphal and larval ticks of each of the two tick species on each lizard. Then we determined the maximum tick count both for all stages of each tick species, and for just larvae plus nymphs, for each lizard over all of the fortnightly counts in the season. We used those maximum numbers as a measure of the tick load of each lizard in subsequent analyses to test whether lizards that were more strongly connected in a transmission network had higher tick loads. Adult ticks (and particularly male ticks) of these two species can remain attached to a host lizard for over 12 months and in this paper we report on patterns of larval and nymphal attachments, which we considered were more likely to be affected by short-term behaviours

of the hosts. The analyses of total tick loads (adults, nymphs and larvae) produced identical trends but are not reported here.

All lizards were treated using procedures formally approved by the Flinders University Animal Welfare Committee in compliance with the Australian Code of Practice for the Use of Animals for Scientific Purposes and conducted under permits from the South Australian Department of Environment and Heritage to Undertake Scientific Research.

2.3 Transmission networks

Our hypothesis was that transmission networks represent pathways for the passage of parasites, in this case ticks, around a population. We constructed models of transmission networks for the ticks following Leu et al., (2010b). Engorged larval and nymphal ticks detach from their lizard host and then take a period of time to moult to the next infectious stage. Unattached ticks will subsequently die from desiccation or predation if the next host does not come past within another period of time. Our network models assumed that tick transmission from one host to the next takes place in the refuges used by lizards, since ticks exposed outside those shelters will quickly desiccate in the hot summer conditions (Chilton and Bull, 1993a). Thus there is a time window after ticks have detached from one lizard, when there is a risk of infection for a second lizard that uses the same refuge. The time window begins after ticks have moulted, and ends when they have died. Transmission can occur through the asynchronous use of the same refuge site by the second lizard within that time window of infection.

Leu et al. (2010b) estimated that the duration of this infectious time window for *A. limbatum* was 9-39 days. This was derived from reports that, after detachment, engorged

larval *A. limbatum* take an average of 8 days to molt to unfed (and infectious) nymphs (Chilton et al., 2000), and that unfed nymphs survive at 30°C for an average of 31 days after molting (Chilton and Bull, 1993a). Similarly, for this study, we estimated a time window of infection for *B. hydrosauri* of 11-24 days. This was based on previous data reporting that *B. hydrosauri* at 30°C takes two days longer than *A. limbatum* to molt, and survives desiccation for a shorter period than *A. limbatum* (Bull and Smyth, 1973; Chilton and Bull, 1993; Chilton et al., 2000). We also assumed that the time window for infection by larvae was similar, based on previous measures of the period from detachment of engorged females to egg laying (Chilton and Bull, 1994), the development time of eggs to hatching (Chilton and Bull, 1994), and the survival time of larvae (Chilton and Bull, 1993), Again these values are highly variable as temperature and humidity change, but larvae of *A. limbatum* become infectious consistently faster than *B. hydrosauri*, and can become infectious within 14 days and survive desiccation for up to 14 days at warm temperatures (Chilton and Bull, 1993; 1994).

Although we imposed two specific time windows into our transmission network models, we recognize that molting and survival times for each tick species will be different under different climatic conditions over an activity season, and for different life stages, and with different levels of predation pressure, largely from ants (Dawes-Gromadski and Bull, 1997a; 1997b). Thus, although we developed two transmission network models, one with short (11-24 days) and the other with long (9-39 days) windows of infection, these are only two of a range of possible models that we could have used to determine relationships between network connections and tick loads on lizards.

2.4 Refuge locations

To integrate infectious time windows into a transmission network, we needed to know when lizards were using individual refuge sites. We deduced that the last active GPS location for each day was close to the site of the refuge that the lizard used for that night (Kerr et al., 2004a). In most cases in this study, and in that of Leu et al. (2010b), lizards occupied a refuge for one night, and then moved into another refuge after activity on the next day. If a lizard remained inactive in the same refuge over consecutive days, for instance when climatic conditions inhibited normal diurnal activity, the refuge location was only recorded once. In those cases, our models assumed that all transmission events, such as detachment of engorged ticks and attachment of waiting, unfed ticks, happened during the first night of refuge use. We have previously reported that these ticks do not move to seek new hosts, but stay close to where they detached (Petney et al., 1983), and our models assumed that unfed ticks remained in the lizard refuge where they had detached. In our models, most transmission occurred when a lizard moved into a refuge that contained waiting, unfed ticks.

Then, in each model, we compared the refuge location of each lizard on each night with the refuge locations of each other lizard on all of the subsequent nights within the time window of infection. Following Leu et al. (2010b), and taking into account the dimensions of refuge bushes and the 6 m precision of the GPS locations (Leu et al., 2010a), two lizards in refuges recorded as up to 14 m apart, were considered to have occupied the same refuge. This conservative approach probably overestimated both the level of refuge sharing and the opportunities for parasite transmission. We also estimated the number of different refuges used by each lizard by inspecting the distances between all possible pairs of recorded refuge locations over the activity season, and by assuming that records within 14 m of each other

were the same refuge. Again this probably underestimated the actual number of different refuges used.

2.5 Analysis of network models

A prediction of our hypothesis was that lizards that were more connected in the network would have higher parasite loads. The following analyses were designed to test that prediction. For each model (short and long time windows of infection) we constructed a weighted directed transmission network, based on the asynchronous overnight refuge sharing events (described above), and on the infection risk of each of these events. The network consisted of directed edges from lizard A to lizard B, if lizard A used a refuge and then lizard B used the same refuge on at least one night within the time window of infection. Edge weight was defined as the probability of transmission of ticks from lizard A (node of origin) to lizard B (node of destination). At each refuge use (or re-use) ticks could detach from lizard A and, after they had molted, be waiting to attach to lizard B. For lizard B, the probability of infection with ticks from lizard A, from a single night in a refuge, increased with the number of nights that lizard A had previously used this refuge, up to the duration of the time window of infection. The total probability that lizard B would become infected with ticks from lizard A (the edge weight) was the sum of all the infection risks of all of the refuge sharing events over the activity season, when lizard B used a refuge after lizard A. The asynchronous timing of refuge use required for transmission meant that edge weights in opposite directions between two individual lizards were different.

We then calculated node strength, a parameter that defines the connectedness of each individual in the network, and incorporates both the number of edges from the node (i.e. how many other lizards that lizard has interacted with) and the weight of those edges (as described above). Node strength is defined as the total weight of all edges connected to a node (Croft *et*

al. 2008). In our analyses we used node in-strength, the sum of the weights of all edges directed towards the node, as a measure of the total risk of an individual lizard to become infected from sharing refuges with any other lizard in the population.

Measurements of both node strength and node in-strength can increase with the number of days of observation. Therefore, because individual lizards were observed for different numbers of days, we standardized the in-strength value by dividing by the number of overnight refuge records, and called this standardized in-strength the "cross-infection risk", as in Leu et al. (2010b). We used PopTools (Hood, 2008) to analyze our transmission networks.

In all correlation analyses, we used Spearman rank correlations because the data were not usually normally distributed. Because network derived measurements, such as node in-strength, are relational, non-independent data (Croft et al., 2008), we used randomization tests to estimate the probability that the observed correlation coefficient was obtained by chance. Since our network was based on dyadic interactions (asynchronous refuge sharing by pairs of lizards), we randomized node labels (tick load or number of different refuges) among nodes (lizards) and re-calculated the correlation coefficient (James et al., 2009). We repeated this procedure 10,000 times to achieve a consistent frequency distribution for values of the randomly generated correlations (Bejder et al., 1998). Following Croft et al. (2008), we calculated Monte Carlo *P*-values as the quotient of the number of times the randomly generated values exceeded or were below the observed value, depending on our hypothesis. P-values were corrected for multiple testing as described by Holm (1979). We considered that each year produced independent datasets, and corrected for multiple testing separately for the set of four analyses within each year. Corrected P-values are presented in the results.

3. Results.

The surveyed lizards were recorded using an average of 12 -16 different refuges in the activity season of each year of the study (Table 1). Values were not directly comparable because there were different numbers of days of observation among lizards and years. Individual refuges were used by a lizard on up to 56 nights within the observation period (Table 1). Each year, between 79 – 95% of surveyed hosts were infected with ticks of each species (Table 2). The mean intensity of infection (measured as the mean of the maximum recorded tick load per lizard) for *B. hydrosauri* (larvae and nymphs) peaked in 2010, and was higher than for *A. limbatum* in the last two years (Table 2). In each year some lizards were recorded with loads of each tick species very much higher than the mean, and in the last three years, there were significant positive correlations between the infection intensity of *B. hydrosauri* and of *A. limbatum* on each host (Table 2).

To test our prediction, that stronger connections in the transmission network led to higher infection levels, we derived eight transmission networks, one for each model (short or long time window of infection), in each of the four years. Two of those, for the long and short time window of infection in one year, are shown in Fig 1. In each season, the models differed in the distribution of nodal in-strengths with higher values for the long time window of infection models (Table 3). The nodal in-strengths generated by the two models were highly correlated within years ($r = 0.99$; $P = 0.009$). There were significant positive correlations between the standardised in-strength of each lizard node in the network (the cross infection risk) and the *B. hydrosauri* tick load, for each transmission network model, in the activity season of each year (Table 4), as predicted by our hypothesis. There was no consistent trend

across the four study years for the correlations to be stronger when we applied one or the other model.

For *A. limbatum* there were significant positive correlations between the standardised in-strength of each lizard node in the network and tick load for the lizards in 2011, but not in the other three years (Table 4).

4. Discussion

Our hypothesis was that transmission networks represent pathways for the passage of parasites, in this case ticks, around a host population, in a range of ecological conditions. Leu et al. (2010b) reported a transmission network based on asynchronous sharing of refuge sites that predicted the patterns of tick infestation in a population of sleepy lizards. That previous study reported that the tick load on lizards was positively correlated with the node in-strength of the network, called the cross infection risk. The conclusions of Leu et al. (2010b) were derived from one tick species, *A. limbatum*, in a social network of 18 lizards, in one year.

Here, we considered a larger network of lizards, over four (different) years and with two tick species. Although the study site was about 5 km north of the previous study, average climatic conditions, vegetation and lizard behaviour were similar across the two sites. The only major difference was that there were two tick species at the new site, while there was only one at the site of the previous study. In the current study, we also developed two model transmission networks based on a short and a long duration of the time window of infection. These alternative time windows were derived from empirical data on the survival times of one life stage of each of the two tick species, under desiccation at one temperature. They generated models with different frequency distributions of in-strength values. Specifically,

there was a consistent trend for the short time window of infection model, where ticks had a lower time of survival while waiting for hosts, to have fewer edge connections and lower in-strength values. In theory, a less connected network should be less efficient in transmission of the tick parasites from host to host. It is unlikely that either model accurately represented the time that infective stages of either tick species can persist in every refuge under every climatic condition experienced during the four lizard activity seasons of the study. But the results of Leu et al. (2010b) suggested that in one year and for one tick species, the long time window for transmission (that we also used in the current study) was sufficiently close to reality to allow the derived model to predict a pattern of tick infestation on lizards close to that observed. An aim of the current study was to test the robustness of that model.

For one tick species, the current results provided strong support for the conclusions of the previous study. For the tick *B. hydrosauri,* there were strong and significant positive correlations between the cross-infection risk derived from the model and the empirically observed tick load. The relationship was consistent across years and with both versions of the transmission network model. Those four years differed substantially in rainfall. Previous studies have suggested that low annual rainfall has resulted in a poorer germination and persistence of the annual plants that make up a major part of the diet of these herbivorous lizards, and that in turn has led to lower levels of lizard activity in drier years (Kerr and Bull, 2006). Tick transmission might be adversely affected in low rainfall years both from this reduced lizard activity and from a faster desiccation rate of unfed ticks waiting for hosts in drier conditions. Combining these two effects means lizards are less likely to move into a new refuge, and ticks waiting there will have a shorter time window of infectivity before they die, in a drier year.

However, across those climatically different years there was a consistent pattern. Lizards that more often used refuges that other lizards had used had higher loads of the tick species *B. hydrosauri*. The pattern of infection among host lizards closely matched the predictions of the transmission network models and provided support for the assumed mechanisms of parasite transmission among hosts. The pattern of infection suggested that the transmission network models reliably describe the dynamics of the transmission of this tick among its lizard hosts.

But for the other tick species, *A. limbatum*, the pattern was different, and we only found significant correlations between tick load and cross-infection risk in one of the four study years. In that year there was a significant correlation between tick infection and both network models. Two questions arise. One is why the pattern differed between the two tick species. An explanation may be that, while waiting off the host for the next lizard to come by, nymphs and adults of *A. limbatum* tolerate desiccation for longer (Bull and Smyth, 1973; Chilton and Bull, 1993), and have lower mortality from predation (Dawes-Gromadski and Bull, 1997a; 1997b) than *B. hydrosauri*. This is the reason we allocated a longer time window of infection for that species originally. But another consequence may be that detached *A. limbatum* ticks can persist for longer in a wider range of microhabitats while waiting for their next host. Host refuges provide shelter for detached ticks from predation and desiccating high temperatures (Kerr et al., 2003), but *B. hydrosauri* may be more reliant than *A. limbatum* on host refuges as survival and transmission locations, over a wider range of conditions. As a result, the transmission network models that incorporate asynchronous refuge sharing may be more robust for *B. hydrosauri* than for *A. limbatum*. One conclusion from the current study is that network based models of the transmission dynamics of parasites among host individuals

will not necessarily be consistent for different parasites, even for those with very similar life cycles and transmission mechanisms.

A second question is why the results from three of the four years in this study differed from those of the previous study that reported a significant positive correlation between *A. limbatum* tick load and node in-strength (cross-infection risk). Possible explanations include that the environmental conditions differed between the first three years of the current study and 2007, when the previous study was conducted. Another factor is that microhabitats available as sites for ticks to wait for hosts may differ between the two sites, such that host refuges are not as important in the site used for the current study. Another possible explanation is that only one tick species infected lizards at the site of the first study, while both tick species were present at the current study site. These two species have a broadly parapatric distribution (Bull and Burzacott, 2001). The current study site, 6km west from the field house on Bundey church road, was located in their narrow overlap zone. Although the ecological processes that generate this sharp distributional boundary are not known, some form of negative interspecific interaction between the two tick species must occur (Bull, 1991; Bull and Possingham, 1995). Possible mechanisms include reproductive interference (Andrews et al., 1982; Chilton et al., 1992), and density dependent competition during feeding (Tyre et al., 2003). Whatever the mechanism, it is possible that, for *A. limbatum,* the effects of interspecific interactions on host infection patterns might override those generated by the transmission networks. Our data showing significant positive associations between infection intensities of each species might contradict this interpretation, but a second conclusion we can derive from the current study is that models of the transmission dynamics of parasites among host individuals will not necessarily be consistent for an individual parasite species in different circumstances.

The study tested two network models. Like other investigations of the transmission of parasites and pathogens around wildlife populations, our interpretation relies on correlational patterns, higher parasite loads for hosts with stronger network connections. In most fields of ecology, correlations and associations are the first evidence required to support a model of the ecological process. Significant correlations provide an indication that a particular model process is one possible explanation of the pattern observed. Further studies are then required to explore alternative explanations. A lack of correlation does not necessarily eliminate the model, if other processes mask the effects of the ecological mechanism that is proposed. For instance, in this case, tick loads might be higher in one part of the study area than others, perhaps because, in a heterogeneous habitat, tick survival while waiting for a host is higher in some microhabitats than others. In that case, a positive relationship between tick load and node in-strength might be masked by high in-strength values for some nodes in local areas where tick densities are low.

Our understanding of the ecological processes that generate the dynamics of host-parasite interactions lags behind our understanding of many other ecological processes. Perhaps this is because of the difficulty of directly observing the process of parasite transmission, and our reliance on patterns of host infection to infer transmission. Nevertheless, the theoretical models of parasite-host dynamics, including those involving social networks, now demand empirical data on actual transmissions to test their rigour.

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LEGEND TO FIGURE:

Figure 1: Transmission networks generated with a) a short time window of infection; and b) a long time window of infection, from the GPS location data of the lizards in the study population in 2010. Nodes represent individual lizards and edges between nodes are directed towards the lizard that is at risk of infection. The edges are weighted as described in the main text and the thicker the line the more weight is associated with that edge.

Table 1 The rainfall in each year, and the mean, standard error and range of the number of days each lizard was surveyed, the number of different refuges used by each lizard, and the number of times each lizard used its most commonly occupied refuge.

	2008	*2009*	*2010*	*2011*
Rainfall (mm)	159	266	332	459
No of Lizards	45	56	60	59
Days surveyed				
Mean (SE)	79.2 (3.2)	81.9 (2.2)	86.7 (2.0)	56.4 (1.8)
Range	33 - 122	44 – 112	53 - 112	30 – 79
Number of different refuges used				
Mean (SE)	15.6 (0.8)	14.6 (0.7)	14.5 (0.6)	12.2 (0.6)
Range	6 - 27	6 - 26	7 - 27	4 - 27
Number of times in most commonly used refuge				
Mean (SE)	20.0 (1.4)	19.8 (1.2)	23.9 (1.4)	15.3 (0.9)
Range	7 - 56	8 - 50	8 – 51	5 - 34

Table 2. The prevalence (percentage of lizards that are infected) and intensity of infection (mean number of larvae and nymphs per lizard (SE)) for each tick species in each season of the study. Intensity was measured as the maximum tick load recorded on each lizard over a season. Also shown is the range of maximum tick loads recorded on all lizards for each tick species in each season, and the Spearman rank correlation values for total tick infestation intensity of the two tick species.

	2008	*2009*	*2010*	*2011*
No of Lizards	45	56	60	59
B. hydrosauri				
Prevalence	82.2%	78.6%	95.1%	82.3%
Intensity (L+N)	3.09 (0.89)	4.50 (1.21)	23.08 (6.56)	7.10 (1.49)
Range of loads	0 - 32	0 - 37	0 - 237	0 - 58
A. limbatum				
Prevalence	91.1%	94.6%	88.5%	79.0%
Intensity (L+N)	2.04 (0.58)	4.38 (2.22)	5.33 (1.19)	3.63 (1.38)
Range of loads	0 - 21	0 - 130	0 - 55	0 - 77
Correlation of intensities	r = 0.05 P = 0.73	r = 0.45 **P < 0.001**	r = 0.53 **P < 0.001**	r = 0.67 **P < 0.001**

Table 3. Mean (SE) and range of nodal in-strength values for all lizards in the transmission networks derived in each season with a short (11 – 24 days after tick detachment) or a long (9 – 39 days after tick detachment) infective time window.

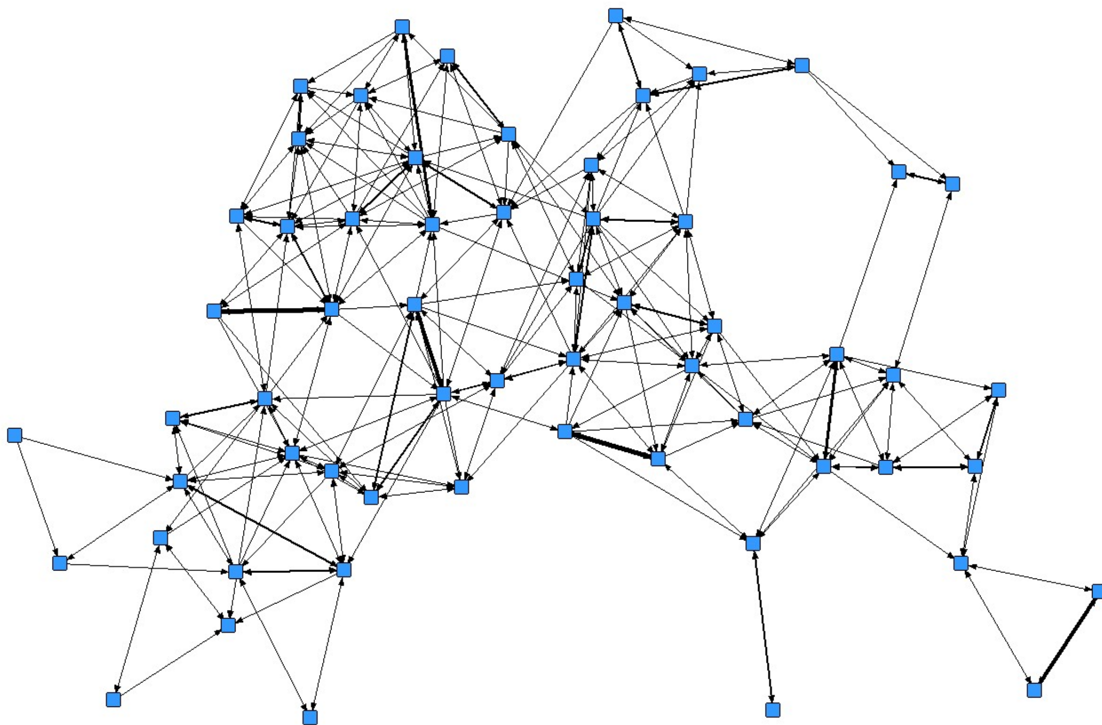
	2008	*2009*	*2010*	*2011*
Short Infection Window				
Mean (SE)	1.19 (0.18)	0.86 (0.07)	0.94 (0.08)	0.80 (0.07)
Range	0 – 5.6	0 – 2.2	0 – 2.8	0 – 1.9
Long Infection Window				
Mean (SE)	2.33 (0.34)	1.63 (0.13)	1.90 (0.17)	1.51 (0.12)
Range	0 – 10.2	0 – 4.6	0.1 – 5.9	0 – 3.5

Table 4 Analyses of correlation between tick load and cross-infection risk, for each model (Short = short time window of infection; Long = long time window of infection), and for each tick species, in each season. r_{sp} = Spearman's rank correlation value; 95% CI = the 95% confidence intervals around correlation derived from 10,000 network randomizations; *P* = probability that the observed r was outside those confidence limits. For analyses, *P*-values were corrected for multiple testing within each year using the Holm method (Holm 1979). *P*-values in bold were significant.

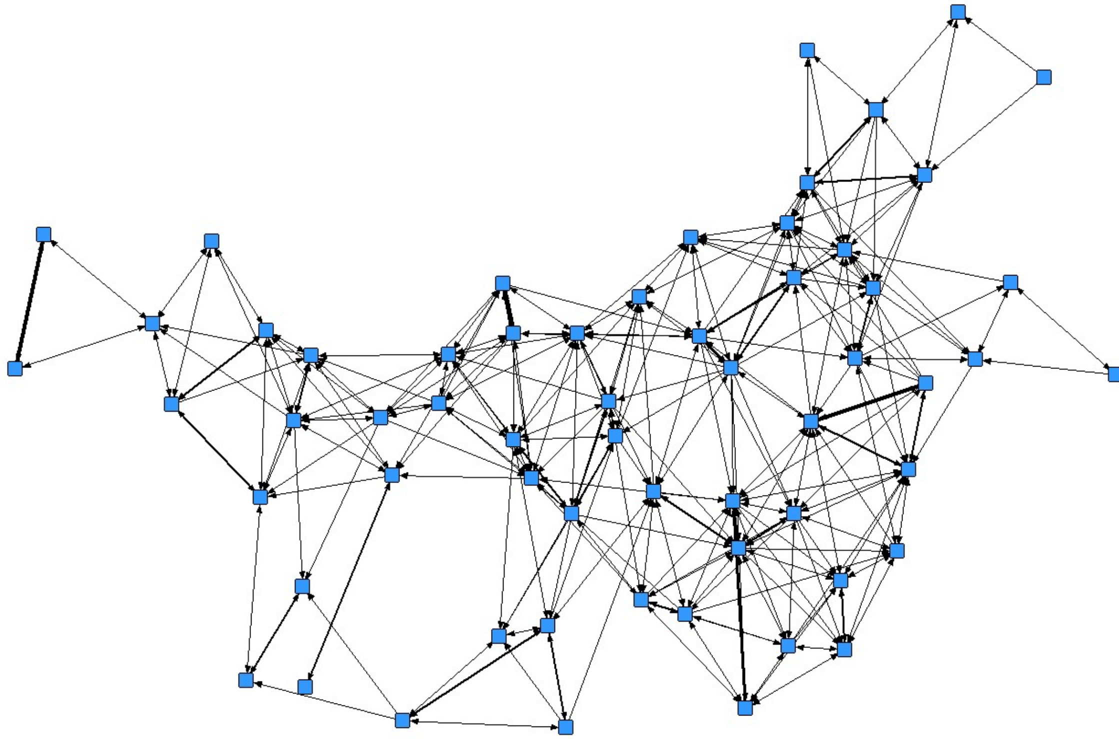
	B. hydrosauri			*A. limbatum*		
	r_{sp}	95% CI	*P*	r_{sp}	95% CI	*P*
2008						
Short	0.467	-0.295: 0.293	**0.0048**	-0.014	-0.297: 0.297	0.99
Long	0.401	-0.289: 0.294	**0.0093**	-0.019	-0.289: 0.290	0.99
2009						
Short	0.289	-0.265: 0.259	**0.0369**	0.144	-0.264: 0.262	0.15
Long	0.370	-0.262: 0.258	**0.0100**	0.218	-0.267: 0.260	0.10
2010						
Short	0.257	-0.258: 0.253	0.0705	0.204	-0.251: 0.255	0.12
Long	0.293	-0.249: 0.259	**0.0444**	0.192	-0.263: 0.252	0.12
2011						
Short	0.359	-0.260: 0.256	**0.0076**	0.268	-0.255: 0.248	**0.0366**
Long	0.348	-0.259: 0.258	**0.0135**	0.246	-0.256: 0.256	**0.0366**

Figure 1: Transmission networks generated with a) a short time window of infection; and b) a long time window of infection, from the GPS location data of the lizards in the study population in 2010. Nodes represent individual lizards and edges between nodes are directed towards the lizard that is at risk of infection. The edges are weighted as described in the main text and the thicker the line the more weight is associated with that edge.

a)



b)



Chapter 3: Further development of microsatellite markers for the reptile tick, *Bothriocroton hydrosauri*.

This chapter describes the methodology used to develop new diagnostic microsatellite DNA loci for the reptile tick, *Bothriocroton hydrosauri* and their application to determine the identification and relatedness of individual ticks from their genotypes. I used the alleles I identified to assign ticks collected in 2010 and 2011 to either the background wild population at the study site, or to larvae from other locations experimentally attached in pulses to lizards at the study site. Further analyses involving these identifications are described in later chapters.

Introduction

The advent of molecular DNA markers has allowed substantial new insights into many aspects of behaviour and ecology of populations, including the identification and tracking of individuals through the landscapes they occupy. Microsatellites, which are repeats of simple short sequence motifs, have been one of the most used molecular markers to date due to an abundance of highly polymorphic microsatellite loci in the genomes of many taxa (Goldstein and Schlötterer 1999; Li et al. 2002; Selkoe and Toonen, 2006).

Recent advances in capacity of next generation DNA sequencers have made the identification and development of relevant microsatellite loci easier and faster. This has been a recent boon for studies of species in which it has been more difficult to obtain a useful suite of microsatellite markers (e.g. *Lepidoptera*, Zhang 2004). For instance, many bird species are known to be depauperate in useful microsatellite loci (Primmer et al. 1997), but, tackling one problematic species, Abdelkrim et al. (2009), used next generation sequencing, specifically the GS-FLX technology (454 Life Sciences), to overcome previous challenges and successfully develop polymorphic microsatellite markers for the blue duck, *Hymenolaimus malacorhynchos*. This species had previously proved difficult to obtain more than limited genetic data, and the availability of microsatellite markers provided important information for conservation management, due to its fragmented populations and endangered status. Gardner et al. (2011) further investigated the GS-FLX method and found that roughly twice the number of sequences was required when searching for and developing the same number of useful microsatellite loci from invertebrates compared to vertebrates.

Ticks (Acari) are another group for which the development of microsatellite markers has presented challenges. Pagel Van Zee et al. (2007) provided one explanation for the

difficulties when they identified that tick genomes consist of unusually high amounts of repetitive DNA.

My study focused on the reptile tick, *Bothriocroton hydrosauri*. Previously, Guzinski et al. (2008) developed several microsatellite markers for this species using an enrichment protocol (Gardner et al. 2008), but those loci had relatively low individual discriminatory power (Guzinski et al. 2009). To overcome this, I used the next generation sequencing protocol of Gardner et al. (2011) to develop new loci for this tick, with the aim of identifying highly polymorphic loci with greater discriminatory power to be available for studies of genetic structure and transmission dynamics in populations of this ectoparasite. *Bothriocroton hydrosauri* is a three host tick species. It has three life stages, larvae, nymphs and adults that each attach, engorge and then detach from separate (or sometimes the same) host individuals. Female ticks mate on their third host and then engorge, detach and lay eggs that hatch into larvae. By collecting lizards with mated female ticks attached, it is easy to harvest the females as they detach, and then collect the eggs and subsequent larvae from single clutches. Our design, in experiments described later, was to add a pulse of genetically unique tick larvae, all from one clutch, to a single host, and then, by genetically identifying the subsequent adults that those larvae develop into after attaching to three hosts, to deduce the transmission pathways of tick infestation among host individuals. For this I needed genetic markers to uniquely identify the pulses of added larvae.

This chapter reports the first stage of this experiment. I aimed to develop new loci, so that, in combination with the previously developed loci, I could identify individual adults derived from different pulse clutches of experimentally infected tick larvae, and differentiate those ticks from the naturally occurring individuals of that species in our study population. Our loci needed to be sufficiently variable to allow us to find ticks with alleles that were not

currently represented among ticks at the study site, yet not too variable so that individuals within a clutch of larvae had a common detectable genetic signature. I report here the development of the new marker loci and the patterns of allelic diversity that I found both within the study population and among the sources of infection pulses. Specifically the aims of this chapter were first to obtain a set of microsatellite loci to assay in the reptile tick, *Bothriocroton hydrosauri*, and then to confirm that the loci I used were sufficiently diagnostic first to differentiate between the experimentally added pulses of larvae and the background natural tick population, and second to differentiate among the different clutches in the pulses that were added.

Study site

The main study site was an approximately 1.0 x 1.5 km area of semi-arid chenopod shrub land, interspersed with some areas of casuarina woodland near Bundey Bore Station (33°54'S, 139°20' E) in the mid-north region of South Australia. The ecology and behaviour of about 60 adult sleepy lizards, *Tiliqua rugosa*, and their parasites that occupy this study site have been extensively reported from previous studies (Bull *et al.* 2012; Godfrey *et al*. 2013; 2014; Parsons *et al*. 2015; Spiegel *et al*. 2016). These lizards are the most common hosts and the most commonly infected by *B. hydrosauri* in this geographical region (Belan and Bull 1995).

Tick Collection and DNA extraction

To develop new genetic markers and to characterise the allelic composition of naturally occurring ticks at this site I collected 13 adult male *B. hydrosauri* from 11 different host lizards over the 2008-09 spring summer seasons of lizard activity. These ticks are referred to as "background" ticks, representing the background natural population into which

the experimental pulses were to be added. For the experimental releases of genetically unique *B. hydrosauri* larvae, I collected 12 sleepy lizards, from regions outside of the field site during the two periods Feb 2009-March 2009 and Oct- Dec 2009. They were located 22 – 137 km from the study site and were each at least 5 km apart from each other (Figure 1). One of these lizards had two mated or engorging female ticks attached (labeled A/B1 and A/B2), while the other 11 lizards each had one female tick (labeled C – M). Female ticks were identified as mated by the presence of a spermatophore in the genital pore or by an engorging condition which only starts after mating has taken place (Bull and Burzacott 1994). The ticks came from near Mt Mary (34° 06’ S; 139° 26’ E; two females; 22 – 26 km from study area), Karoonda (35° 05’ S; 139° 53’ E; nine females; 125 – 158 km from study area), and Monarto (35° 05’ S; 139° 06’ E; two females; 137 km from study area). Table 1 shows the locations and dates that each clutch was collected, hatched and attached to the respective donor lizards.

Table 1: This table shows each clutch, the region it is from, the GPS location, the dates the lizards with female ticks were collected, the dates that the larvae hatched, the dates that larvae were attached to the donor lizards shown in the last column.

Pulse	Clutch	Region	Easting	Northing	Date Collected	Date Hatched	Date attached	Donor Lizard
Oct 2009	A/B1	Karoonda	414072	6107519	March 3, 2009	July 14, 2009	October 12, 2009	11130
Oct 2009	A/B2	Karoonda	414072	6107519	March 3, 2009	July 14, 2009	October 12, 2009	12434
Oct 2009	C	Mount Mary	356795	6229637	February 19, 2009	July 14, 2009	October 10, 2009	9532
Oct 2009	F	Mount Mary	356502	6228418	February 19, 2009	August 6, 2009	October 12, 2009	40012
Oct 2009	G	Karoonda	398937	6119163	March 2, 2009	August 12, 2009	October 10, 2009	12847
April2010	D	Monarto	332964	6114523	November 20, 2009	January 18, 2010	March 15, 2010	40009
April2010	E	Monarto	329503	6111724	November 20, 2009	January 25, 2010	March 15, 2010	9390
April2010	H	Karoonda	390210	6122573	December 9, 2009	February 11, 2010	March 15, 2010	10039
April2010	I	Karoonda	398926	6119130	November 29, 2009	February 11, 2010	March 15, 2010	13535
April2010	J	Karoonda	392792	6134003	November 17, 2009	January 25, 2010	March 15, 2010	9310
April2010	K	Karoonda	398861	6119088	December 6, 2009	February 11, 2010	March 15, 2010	12030
April2010	L	Karoonda	392566	6121138	December 9, 2009	February 11, 2010	March 15, 2010	9291
April2010	M	Karoonda	386920	6126007	December 9, 2009	February 18, 2010	March 15, 2010	40015

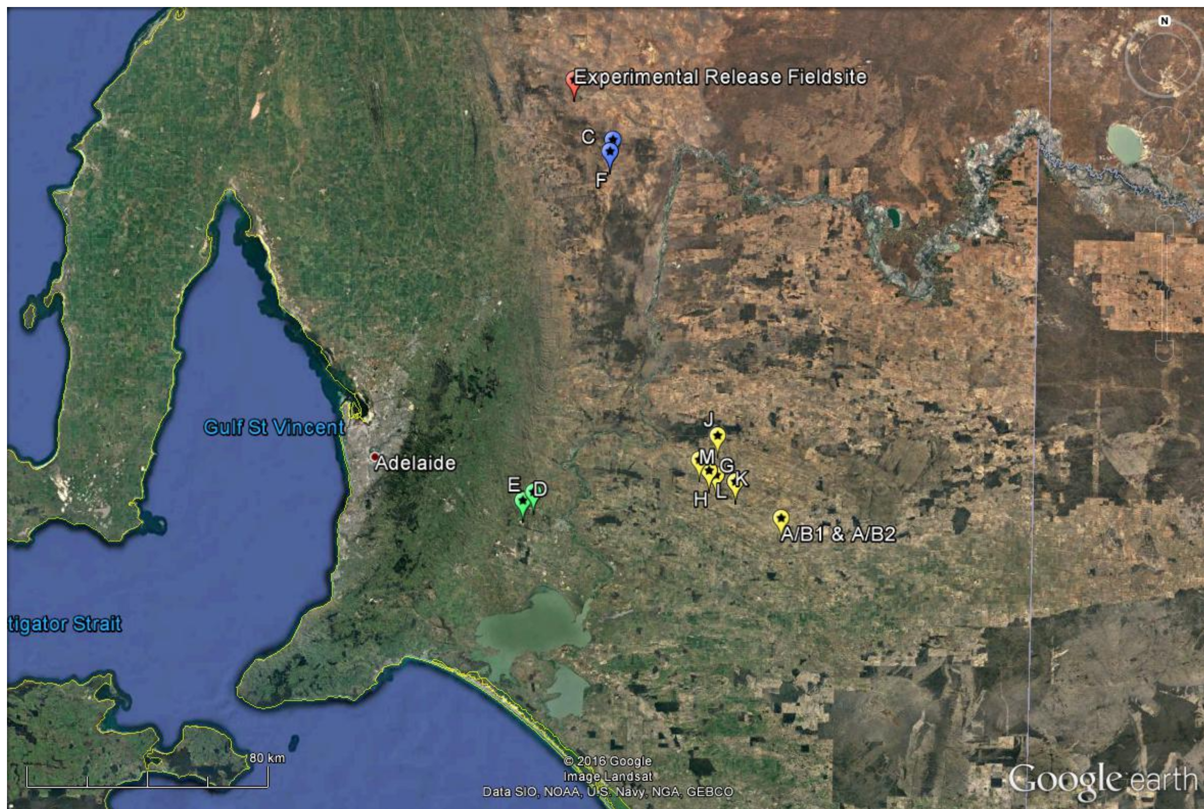


Figure 1: Locations of the lizards that female *Bothriocroton hydrosauri* were collected from. The experimental release field site is marked in red, blue markers are lizard collected from the Mount Mary East region; Green markers are the Monarto region and yellow markers represent the Karoonda region.

I transported lizards back to the Flinders University campus and kept them in individual cages in the animal house at 25°C with a 12:12 photo-period. Lizards were provided with shelter, heat lamps during the day, and ample food and water, and held on a raised 400 x 600 mm wire mesh grid floor. Absorbent paper under the grid allowed engorged female ticks, when they detached from the lizard, to fall through and shelter until they could be collected. Female ticks were then placed in individual 200 ml plastic vials, with a fine gauze top to allow air circulation but prevent tick escape. The vials were held over saturated KCl, within a sealed chamber to provide 85% relative humidity, within a 28°C constant temperature room.

Eggs were laid early in 2009 from the first set of female collections (females A/B1, A/B2, C, F and G) and in late 2009 or early 2010 from the second set of female collections

(females D, E, H-M) on average 43 days after engorged females were placed in the desiccators. Larvae first hatched from the eggs an average of 41 days later. Most clutches produced between 1,000 – 2,000 larvae. After the eggs hatched I collected legs from the female tick to later attempt DNA extraction, although this was subsequently unsuccessful. I also collected normally 20 whole larvae from each clutch for DNA extraction to use in clutch identification. The larval ticks were then held at 25°C, and at 85% relative humidity, until the addition to field based lizards.

Pulses of larvae were added to lizards in the study site in October 2009 (clutches A, B, C, F and G) and April 2010 (clutches D, E, H-M). These times were at the beginning (spring) and end (late summer) of the 2009-2010 lizard activity season. To develop into adults, in laboratory conditions, one possible time frame is up to 176 days, (Sharrad, unpublished thesis, 1979); however in field conditions it can take up to 18 months for *B. hydrosauri* to complete one complete lifecycle, (Bull and Sharrad, 1980). This meant that the October pulses would be adult in the 2010 season. The larvae attached in April 2009 'wintered' (no tick development) on the donor lizards, I expected that this pulse to have reached adulthood by the 2011 season.

I subsequently collected 156 adult *B. hydrosauri* from 57 different host lizards over the 2010-11 spring summer season of lizard activity. These ticks are referred to as "2010" ticks, representing the ticks collected in that lizard activity season. They will have been a mixture of background ticks from natural infections, and adults that were derived from the first set of pulsed additions from females A/B, A/B2, C, F and G. All adult ticks were collected (and removed) from each lizard at the times of fortnightly GPS data downloads. I collected an additional 207 adult *B. hydrosauri* from 50 different host lizards over the 2011

spring season of lizard activity. These ticks are referred to as "2011" ticks, and may have included background ticks and adult ticks derived from both experimental pulses, although it was expected they would predominantly be from the second group of attachments from females D, E and H-M.

DNA Extraction and Problems with DNA Amplification

DNA was extracted from the various tick sources using a variety of methods. First I tried the Gentra Puregene extraction kit for tissue samples (Qiagen, Doncaster, Australia) method. The 2010 samples, DNA from adult *B. hydrosauri ticks* were were extracted using the Gentra extraction protocol. I describe below subsequent other protocols which I tried due to poor amplification using the Gentra kit.

Refinement of DNA extraction

I tried a CCDB Glass Fibre Plate DNA extraction method, which had been modified for use with the vacuum manifold and final elution centrifugation (Ivanova et. al., 2006). Following the success of that extraction method it was used for all subsequent samples and also to re-extract previous background samples where available. In tests to establish the optimal DNA dilution for amplification, only neat DNA amplified. Hereon in, neat DNA was used for all PCR amplifications.

Final PCR conditions

All development PCR amplifications were conducted in Corbett Thermo Palm cyclers and for the 2010 and 2011 samples in the Mastercycler Pro S thermal cyclers at a volume of

10µl. Each reaction was made using 1X MRT buffer, 400 nM of each primer, 0.2 U of Immolase (*Bioline)* and 40 nmoles of neat DNA.

The cycling conditions were as follows: initial denaturation step 94° for 10 mins, followed by 34 cycles at 94°C for 45 secs, annealing temperature (See Table 5) for 45 secs and extension of 72°C for 1 min, followed by a final extension of 72°C for 30 mins and 25°C for 1 min.

To check if samples worked, 6 samples from each plate were run on a 1.5% Agrose gel at 104 Volts for 20 mins, and then stained in Gel Red stain for 20 mins.

Samples were then pooled together based on their Fluorescent tag, 5 µl of PET, 4 µl of FAM, 3.5 µl of VIC and 2.5 µl of NED. From this pool a 1:20 dilution was sent to the Australian Genomic Research Facility (AGRF) for genotyping by Fragment Analysis, Capillary Separation. Samples were then sized and scored using the program GeneMapper version 3.7 (*Applied Biosystems*).

Microsatellite loci: Development for individual discrimination

In March 2009 genomic DNA (a total of 5µg) was isolated from 28 individuals of *Bothriocroton hydrosauri,* consisting of leg tissue of 12 ticks, (five from the Bundey Bore field site, and seven from the Karoonda region); and from whole body extracts of 16 adults (eight from the Karoonda region and eight from Mount Mary East). Leg tissue did not yield enough DNA for sequencing. So although not optimal, because host tissue would also be

included, I used whole bodies, with the Gentra Puregene extraction kit for tissue samples (Qiagen, Doncaster, Australia) using the manufacturer's protocol, with added Proteinase K (20mg/ml) and Glycogen (20mg/ml). DNA was sent to the Australian Genomic Research Facility (AGRF) in Brisbane, Australia for shotgun sequencing on a Titanium GS-FLX (454 Life Sciences/Roche FLX) following the protocol in Gardner et al. (2011). Raw sequences were screened for di-hexanucleotide motif classes with eight or more tandem repeats using MSATCOMMANDER v. 1.03 (Faircloth, 2008) and primers were designed using default parameters (as automated within MSATCOMMANDER) using Primer3 (Rozen & Skaletsky 2000). To identify and eliminate motifs with similar flanking regions, which may represent the same loci, all sequences were analysed in the program MicroFamily (Meglécz 2007).

Following further the procedure outlined in Gardner et al. (2011), 40 new loci were then chosen for further development. Initially those loci were trialed for amplification in a single individual 15µl reaction containing 1X buffer, using 0.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 200 nM each forward and reverse locus-specific primers and 10-50 ng gDNA. The following PCR conditions were used: 94°C for 9 min followed by 36 cycles at 94ºC for 45 sec, 52ºC for 45 sec, and 72ºC for 1 min; and a final elongation step at 72ºC for 30 min. PCR products were visualised on a 1.5% agarose gel stained with ethidium bromide. Fourteen of the loci failed to amplify at the first stage of amplification (Table 2), leaving twenty six of the 40 loci that amplified an unambiguous product of the expected size.

TABLE 2: Loci which failed at initial stages of primer development

Locus Name	Forward Primer sequence (5'-3')	Reverse Primer sequence (5'-3')	Repeat Motif	Length	Temp	Type	Florescent Tag
Bhy03	**TTATGTTCCCGGCACCACG**	**GAGAACAAGCTGCAAGCCG**	**(ATGGT)^17**	**153**	**F**	**Pentanucleotide**	**N/A**
Bhy05	**TCATCATCGTTGTCGTCGC**	**AGTCCGTTGTCGTAGGAGG**	**(ATT)^29**	**156**	**F**	**Trinucleotide**	**N/A**
Bhy15	**GACCAAGTGGAAGCGAACG**	**GATTGCCAACACCCCTGTC**	**(GATT)^23**	**193**	**F**	**Tetranucleotide**	**N/A**
Bhy17	**GGTGGCGAGTTGATGGTTG**	**CCCCTTGGTTCTAAAACGGC**	**(AT)^8**	**199**	**F**	**Dinucleotide**	**N/A**
Bhy18	**TTGCTGTAACCTGTTGGCG**	**CCGACTCCGAAAACTGTGC**	**(ACTC)^12**	**200**	**F**	**Tetranucleotide**	**N/A**
Bhy20	**GGCAACAGGTATGGCAAGC**	**TTTCAGGTTGTCCGGTGTG**	**(AC)^8**	**204**	**F**	**Dinucleotide**	**N/A**
Bhy24	**AGGACTCTTGCTGGAAGGC**	**CTAAGGCTAGTACCGACCTG**	**(ATGGT)^24**	**219**	**F**	**Pentanucleotide**	**N/A**
Bhy25	**GCACCCTCAGCCTCACTC**	**TGCAGACTGGTTCAGGAGAC**	**(GATT)^30**	**221**	**F**	**Tetranucleotide**	**N/A**
Bhy30	**GTCGTACTGAGAAATAAGTCTGTC**	**AGAGGCTTTGACAGGTCCC**	**(AAT)^24**	**228**	**F**	**Trinucleotide**	**N/A**
Bhy33	**TGAACAACAAAACCCACGACC**	**GTTAATCTTCGCTGACGGGC**	**(AAT)^24**	**237**	**F**	**Trinucleotide**	**N/A**
Bhy35	**CATCGCACAGGCGGTTTC**	**GTGTGTGTGTGCGCGTG**	**(AC)^12**	**244**	**F**	**Dinucleotide**	**N/A**
Bhy36	**CACCCTCTTCACCCTTCCC**	**ATGCCATGCCCGTCAAATG**	**(AC)^9**	**244**	**F**	**Dinucleotide**	**N/A**
Bhy39	**GGTGCAGGGATTGCTGTTG**	**GCTCAGCATGACGATGTGG**	**(GATTT)^14**	**248**	**F**	**Pentanucleotide**	**N/A**
Bhy40	**AAGACGACCCACCACAGTC**	**TCAGGGTAAATGGGCAGGC**	**(AC)^10**	**232**	**F**	**Dinucleotide**	**N/A**
Locus failed after tag was attached							
Bhy19	**TCACACGGGCACTGATCTC**	**CTCGAAGGGCTCCCAGAAG**	**(CG)^8**	**202**	**50**	**Dinucleotide**	**NED**

The genomic DNA from the 28 individuals sent for shotgun sequencing occupied 14.29% of a plate and produced 102,121 individual sequences, with an average fragment size of 332, of which 1.88 % (1920 sequences) contained microsatellites (Gardner *et al*. 2011). Using the computer software MICROFAMILY, primers could be designed for 83 unique loci of which I tested 40, with 14 failing to amplify. Also listed on this table is one locus that failed to amplify in the later stages of primer development.

I then tested the amplifications on a temperature gradient to establish the optimal annealing temperature for each. The 26 remaining loci were then tested for polymorphism using the optimised PCR conditions, with six tick individuals, one from the study site, one from Monarto, and four from Karoonda. The optimised PCR amplifications were conducted in Palm-Cycler™ (Corbett Life Science, Sydney Australia) in a total volume of 10µl. Each reaction consisted of 1X AmpliTaq Gold Buffer, 3mM MgCl2, 0.2mM of each dNTPs, 400 nM of each primer, 0.5 U of AmpliTaq Gold enzyme, 0.2 mg/ml of BSA (Roche) and 10-50 ng gDNA. The cycling conditions were: initial denaturation step 94° for 9 mins, followed by 34 cycles at 94°C for 45 secs, optimal annealing temperature (see tables 3-6) for 45 seconds and extension of 72°C for 1 minute, followed by an final extension of 72°C for 30 mins and 25°C for 30 secs. The resulting PCR products were separated on a 3% agarose gel for two hours and stained with Gel Red. If a locus showed inconsistent amplification (nine loci, Table: 3) or ambiguous alleles (six loci, Table: 4), it was discarded. Eleven of the original 40 loci then remained.

TABLE 3: Loci which were inconsistent in amplification

Locus Name	Forward Primer sequence (5'-3')	Reverse Primer sequence (5'-3')	Repeat Motif	Length	Temp	Type
Bhy06	AAGGGAGATGGTGTGCGAG	CAAGTAATCGAGTCACAGGCG	(AT)^10	167	52	Dinucleotide
Bhy07	AGGCTCATTTGCGCGATG	AAGTAGGGCGAGGATGCAG	(CTGT)^13	170	52	Tetranucleotide
Bhy08	CCTTCCTCACCTGCAGAGC	TCACTCCGATAGGCAGCAC	(AC)^9	173	50	Dinucleotide
Bhy09	CGTTACAAAGGGGATGCCG	AAGTGTAGTTGAGTGTATTGTACTG	(AATC)^25	176	54	Tetranucleotide
Bhy10	TCCCGGTTTCCTCAACCAC	ACTGCTACCTCTGTATGACGC	(AT)^13	177	52	Dinucleotide
Bhy11	AGGCCACCTCGTTTAGTGG	GCACCCCATCTTGCCTTTC	(AT)^9	177	56	Dinucleotide
Bhy13	AACGCGCCAAATATCCAGC	ACAGTAGCCACTACCCTGC	(AGAT)^18	184	54	Tetranucleotide
Bhy14	AAGACGGGAAAACCAAGCG	CCTTTCTTTCGCCGGTGTC	(AG)^8	188	52	Dinucleotide
Bhy16	CGGACGCTTCGCACTTAAC	ACGGTCGTAAGTCAGTCAG	(GATT)^20	197	54	Tetranucleotide

TABLE 4: Loci which had ambiguous alleles

Locus Name	Forward Primer sequence (5'-3')	Reverse Primer sequence (5'-3')	Repeat Motif	Length	Temp	Type
Bhy29	CTGCTTTCCCGCTGGTTTC	AACGCATGCACCAGTACAC	(AG)^9	226	54	Dinucleotide
Bhy02	AAGTTGAGGCCGTTCCCTC	TAGATCCTTGCGGCACTCC	(CT)^8	149	52	Dinucleotide
Bhy04	TGGACTAAAGACGCCTCCC	TTGGACTTCATCCCAGCCG	(CT)^11	156	54	Dinucleotide
Bhy22	CGCAGCGAGGTTCTCAATC	GGCCAACTCGTCGCTTTTC	(GATT)^33	214	56	Tetranucleotide
Bhy31	AAGTCCAAGGGCTCCAAGG	CAGCGCGTAGGGCAAAAG	(AATC)^24	230	54	Tetranucleotide
Bhy34	AGTACGGCTTTCAGCCCC	TGGCCTAGTGCAGTTCCTAC	(GATT)^30	242	52	Tetranucleotide

Each forward primer of the identified 11 suitable loci, was 5′ labelled with a fluorescent tag: FAM (GeneWorks), NED, PET or VIC (Applied Biosystems) as shown on Table 5 and 6 Initially, trials using the six test DNA samples were conducted to test if the loci could be used in multiplex amplifications, however all loci failed and had to be amplified individually. The locus BHY19 failed to amplify after the fluorescent tag was attached and was also discarded (Table 2), leaving ten loci.

Clutch identification and Allele analysis

The remaining ten loci were each then screened for their specific value in providing unique genetic signatures for each clutch. Those loci had to show allelic combinations among the 13 clutches that was distinctly different from the background natural population and had to have genotypes that were reliably passed on to their offspring larvae. I assayed 85 individual larvae from the 13 clutches (mean 6.5 per clutch) for their genotypes at these ten loci. DNA failed to amplify from 14 individual larvae, leaving 71 larvae for the clutch identification (mean 5.5 per clutch; range = 4 – 7 (Table 7)). Female ticks die after completing oviposition, but to avoid disturbance of clutches, the mothers were left with their eggs until they hatched, before their legs were sampled for maternal DNA. As mentioned previously, DNA extraction was then unsuccessful on these samples potentially due to the degradation of the tissue after death. Hence no DNA was available for the mothers of the progeny that were attached to the lizards in the experimental section of this project. This meant that reliable and useable DNA from the clutch mothers was not sampled. However, Oliver and Bremner (1968) reported a diploid karyotype for *B. hydrosauri*, and I inferred diploid Mendelian inheritance of alleles at each locus by comparing genotypes of the offspring larvae within clutches. I also assumed single paternity based on observations of mating behaviour where the first male to mate with a female inserts a copulatory plug after

mating to prevent further copulations (Andrews and Bull 1980). In that case there should be no more than four different alleles present at any one locus within a sample of larvae from a single clutch. With this larger scale testing, seven of the ten loci were discarded as there was little to no variation among samples from different clutches and locations; these were categorized as non-diagnostic (Table 5). This left three of the newly developed loci that could potentially be used to discriminate among different tick sources.

I then trialled the nine previously described *Bothriocroton hydrosauri* loci (Guzinski et al. 2008), following their published conditions. However the published multiplex mixtures produced inconsistent amplification, so each primer pair was amplified individually using Immolase and an MRT buffer consisting of 5X Immolase buffer, 1 mM each dNTP, 7.5 mM $MgCl_2$, 2.5X BSA (Hayden et. al. 2008). Because there was a high amplification failure with all loci, I changed the PCR protocol to utilise Immolase (*Bioline)* enzyme and a MRT buffer (Hayden et al. 2008) which had provided more success with the newly developed loci. I continued to trial the nine previously developed loci, however only one, BOHY03 (Guzinski et al. 2008), had enough allelic variation to be potentially diagnostic with unique alleles for some clutches. However, amplification of this locus continued to fail in most samples. I attempted to rectify this by conducting a temperature gradient and found that the optimal annealing temperature needed to be reduced to 52°C from the 59°C, stated in Guzinski et al.(2008). As amplification was still inconsistent with all samples regardless of loci used, I tested DNA concentrations using a fluorimeter, (Qubit, Life technologies) in 44 DNA samples from a combination of larvae and adult tick samples, testing both extraction methods. DNA was found to be present, at an average concentration of 13.64 ng/µl, indicating that there should have been a sufficient amount of DNA for amplification. However with many samples still inconsistently amplifying, it was concluded that there were possible inhibitors in the DNA. Identification of the specific molecular issues responsible for

blocking the successful assay of many potential loci was beyond the scope of the project. Nevertheless, changing the extraction method had a profound effect on amplification success, with the majority of PCR amplifications working after switching to the CCDB Glass Fibre Plate DNA extraction method. Unfortunately DNA extractions had been attempted from many ticks using the previous method, and for some there was no remaining tissue. This meant that I was unable to get DNA from some samples using the new method.

I then continued genotyping with the four loci (three new and one previous) that were variable and potentially diagnostic amongst the clutches used in the pulses of tick addition. Amplicons for the useable loci were separated on an AB3730 capillary Genetic Analyser (Applied Biosystems) at the Australian Genomics Research Facility (AGRF Adelaide).

TABLE 5: Loci which were non-diagnostic for clutch identification

Locus Name	Forward Primer sequence (5'-3')	Reverse Primer sequence (5'-3')	Repeat Motif	Length	Temp	Type	Florescent Tag
Bhy01	CATGACCTCCGCACCAAAC	CCTTTCCGCAGGAGGGTAG	(AG)^8	149	56	Dinucleotide	FAM
Bhy21	TCAGTGCGGCAGGAGATAG	GTTGCACCAGCACTCAGAC	(AATC)^18	206	56	Tetranucleotide	PET
Bhy23	CAATACACGCCGCTCTCTC	TATGGTGGGCATCCCTTCG	(CT)^12	217	56	Dinucleotide	VIC
Bhy26	TACCGGAGTGGCGATGAAC	GTATTCAAAGGCGACGCGG	(AATC)^26	221	59	Tetranucleotide	NED
Bhy32	CGACTTCTCCGCCTTGTTG	TGAAGACCTCGGCTCTATGC	(GATT)^21	231	52	Tetranucleotide	NED
Bhy37	AATCACTGCAGGACCACGG	GACACGTGACAAGCGACAC	(GATT)^23	244	52	Tetranucleotide	FAM
Bhy38	ATCCGCGATGTATGCGAAC	TCGTCAACACTACGTCCTCG	(AT)^13	248	52	Dinucleotide	VIC
From Guzinski et al. 2008							
BOHY01	AGTCGGGCTTCAAAGGTTCA	CCTACCCAGTCCCATTAAAGA	(AAAG)18	224	59	Tetranucleotide	PET
BOHY02	CACTACCTCCTGTTGCACACA	GGGACTTGTCGTTTTTGCTGT	(AAAG)9 ... (AAAG)2 ... (AAAG)1 ... (AAAG)2	206	59	Tetranucleotide	VIC
BOHY04	CGTCACACTTGATACGTTGTC	AGGCGTAATTAATGACCGCT	(AAAG)11	231	59	Tetranucleotide	FAM
BOHY06	TGTGGCCAATCACTCTTTGT	TTAGACTGCACTCGATGGCGT	(AAAG)14 ... (CAAG)10	200	59	Tetranucleotide	VIC
BOHY07	ATGTGGAGGTAGTGGGTTCGA	GTTTTTGAGCTGTTTTATGCG	(AAAG)7	127	59	Tetranucleotide	FAM
BOHY08	TACGCAGCGGATAGGCAAC	TGGGTGATATTGTCAAAGGCT	(AAAG)16 ... (AAAG)3	246	59	Tetranucleotide	FAM
BOHY09	TCTGTATTGGAACGTGTGACG	CCAAGGAAGAGAGGTCATCAT	(AC)19	161	59	Dinucleotide	NED
BOHY10	GCGAGCCGATGTAGTGAAA	CTGCACATAAATGTAGATAGC	(AC)28	192	59	Dinucleotide	PET

From the 11 (42.3%) loci that were polymorphic, and were assigned a fluorescently tagged forward primer, and when compared to the background, eight were considered non-diagnostic, (have some variation, but not unique to a specific location or clutch.) and subsequently not used.

TABLE 6: Loci used in analysis

Locus Name	Forward Primer sequence (5'-3')	Reverse Primer sequence (5'-3')	Repeat Motif	Length	Temp	Type	Florescent Tag
Bhy12	**CACCAATGCAGTTCCGTCC**	**TTGACGTACCACCCCAACC**	**(GATT)^18**	**181**	**54**	**Tetranucleotide**	**VIC**
Bhy27	**CGCCACTCCTTTCTGTAATATCC**	**TGACAGAAGTCGCTCCACG**	**(AGAT)^22**	**223**	**52**	**Tetranucleotide**	**PET**
Bhy28	**TACAGCATGGTTCTGTGCG**	**GCCACGAGGCATGATAACG**	**(AAAT)^16**	**225**	**50**	**Tetranucleotide**	**FAM**
Modified from Guzinski et al. 2008							
BOHY03	**CCGACACCTTCGTTACCGA**	**ATGTGGAACAGCGCCTCATTA**	**(AAAG)^11**	**261**	**52**	**Tetranucleotide**	**Ned**

The final loci (Table 5) which I used to determine the identification of a tick sample as coming from and individual clutch or from a background natural tick infestation.

Analysis of Allele Inheritance

I compared offspring genotypes within clutches, to attempt to identify diagnostic alleles uniquely associated with each clutch (Table 7). It was expected that in a Mendelian inheritance, diploid system, there would be no more than four alleles per locus. However I regularly found clutches with five-seven alleles, (Table 8). Possible explanations for the increased number of alleles present could be mutations at the locus or presence of duplicate loci. As the assays of variation at these loci were simply to discriminate amongst clutches and background ticks it was not a major concern that greater than four alleles per clutch were found. The important finding was that each of the genotypes consistently showed the expected no more than two alleles per individual (Table: 7)

Table 7: Clutch Alleles of four microsatellite loci in individual larvae from 13 uniquely identifiable clutches. Only samples which amplified were included in the table and used to identify the unique alleles for each clutch. A 0/0 value is given when no genotype was derived for that locus in that individual larva

Clutch ID	Sample	BHY12	BHY27	BHY28	BOHY03
A/B1	Offspring	188/188	0/0	0/0	0/0
A/B1	Offspring	176/188	231/231	202/210	274/276
A/B1	Offspring	172/172	231/252	202/218	274/276
A/B1	Offspring	172/188	231/252	202/210	0/0
A/B1	Offspring	172/188	231/231	202/202	274/274
A/B2	Offspring	172/172	252/332	202/202	276/276
A/B2	Offspring	172/172	0/0	210/218	0/0
A/B2	Offspring	172/172	0/0	202/210	0/0
A/B2	Offspring	172/172	252/252	202/210	274/274
A/B2	Offspring	172/204	0/0	0/0	0/0
C	Offspring	196/196	248/248	182/182	288/288
C	Offspring	196/196	248/259	194/214	274/312
C	Offspring	196/200	0/0	214/214	274/312
C	Offspring	196/196	259/259	182/214	274/312
D	Offspring	188/192	0/0	0/0	0/0
D	Offspring	172/208	232/232	194/194	274/274
D	Offspring	172/208	232/308	194/194	274/292
D	Offspring	208/216	308/312	186/186	274/274
D	Offspring	188/192	308/320	195/195	274/292
D	Offspring	0/0	312/312	0/0	0/0
E	Offspring	172/200	0/0	186/186	0/0
E	Offspring	172/200	0/0	186/194	274/274
E	Offspring	172/200	304/304	186/194	274/274
E	Offspring	172/200	0/0	0/0	0/0
E	Offspring	172/196	232/304	186/194	274/274
F	Offspring	196/200	224/224	182/182	274/274
F	Offspring	196/200	232/340	182/182	274/274
F	Offspring	0/0	286/320	0/0	274/276
F	Offspring	196/200	0/0	182/182	274/274
F	Offspring	196/200	232/340	182/182	274/274
F	Offspring	196/200	224/232	182/182	274/274
G	Offspring	192/204	254/312	202/202	274/276
G	Offspring	180/188	0/0	0/0	0/0
G	Offspring	176/180	254/312	202/214	274/276
G	Offspring	176/180	0/0	202/206	274/276
G	Offspring	180/192	254/286	206/214	274/276
H	Offspring	188/196	231/253	202/206	274/274

H	Offspring	168/176	0/0	0/0	274/276
H	Offspring	188/204	224/235	186/186	315/315
H	Offspring	188/204	0/0	186/206	0/0
H	Offspring	172/192	238/292	202/206	276/274
I	Offspring	0/0	254/254	0/0	0/0
I	Offspring	0/0	0/0	182/186	0/0
I	Offspring	168/176	238/254	186/214	274/276
I	Offspring	176/176	238/254	186/214	274/276
I	Offspring	168/176	223/238	186/214	274/276
J	Offspring	176/176	216/304	202/206	274/278
J	Offspring	176/184	216/237	186/214	274/278
J	Offspring	0/0	0/0	186/214	0/0
J	Offspring	176/180	232/304	186/214	274/278
J	Offspring	172/184	304/332	186/206	274/274
J	Offspring	176/184	235/304	186/202	274/274
K	Offspring	176/200	254/324	206/214	274/276
K	Offspring	172/200	254/308	202/222	274/276
K	Offspring	180/192	324/340	202/224	276/276
K	Offspring	176/200	0/0	0/0	276/276
K	Offspring	176/192	254/308	202/214	274/276
K	Offspring	176/192	254/324	202/222	276/276
K	Offspring	176/200	254/308	206/214	276/276
L	Offspring	176/200	0/0	0/0	0/0
L	Offspring	188/196	294/294	187/214	274/274
L	Offspring	188/196	242/294	186/202	274/274
L	Offspring	184/188	248/270	202/206	274/276
L	Offspring	184/188	0/0	0/0	0/0
L	Offspring	196/196	251/270	206/214	274/276
M	Offspring	172/188	255/308	206/206	274/308
M	Offspring	172/176	228/255	202/206	276/276
M	Offspring	172/188	228/258	202/206	274/276
M	Offspring	172/184	0/0	202/206	276/276
M	Offspring	172/176	258/308	202/206	276/276
M	Offspring	172/180	258/258	0/0	0/0

Table 8: Summary of number of alleles present in each clutch at each locus

	BHY12	BHY27	BHY28	BOHY03
AB1	3	2	3	2
AB2	2	**2**	3	**2**
C	2	2	3	3
D	5	4	3	2
E	3	**2**	2	**1**
F	2	5	1	2
G	5	**3**	3	2
H	7	6	3	3
I	**2**	3	3	2
J	4	7	4	3
K	5	5	5	2
L	5	5	5	5
M	4	4	2	3

Differentiation between natural background population and pulse additions

Thirteen adult male ticks were collected in the 2008 - 09 season, a year before any other ticks were released, for pre-pulse identification of the existing tick genetic variation. Hereafter this sample is called the “Pre-pulse background”. During this season, at the fortnightly time of each data download event (see Chapter 2) only one male tick was removed if the lizard had two or more *Bothriocroton hydrosauri* adult males present. Thirteen ticks were sampled from eight lizards in October – November 2008; two lizards had 3 tick each removed at 3 different data download events, one had two ticks taken from it at two data download events, and the other five lizards each had one male tick sampled from them. Table 9 shows the alleles present at each of the four loci among these 13 ticks.

This low number of ticks collected does not allow me to assay the entire genetic diversity of the study site, but gave a representation of the alleles present in the back ground population.

Table 9: Alleles of 4 microsatellite loci in the pre-pulse collection

		BHY12	BHY27	BHY28	Bohy03
Pre-Pulse	T6B-1	200/216	228/228	182/182	274/288
Pre-Pulse	T6B-2	200/216	228/228	182/186	274/274
Pre-Pulse	T6B-3	200/200	228/228	182/186	274/274
Pre-Pulse	T6B-4	198/202	227/227	182/192	288/288
Pre-Pulse	T6B-5	216/216	228/228	182/194	274/288
Pre-Pulse	T6B-6	200/216	228/228	182/182	274/288
Pre-Pulse	T6B-7	200/200	229/229	182/186	274/288
Pre-Pulse	T6B-8	192/200	228/232	194/194	274/288
Pre-Pulse	T6B-9	200/200	228/232	182/182	274/274
Pre-Pulse	T6B-10	196/200	240/240	182/182	274/282
Pre-Pulse	T6B-11	200/216	228/228	182/186	274/277
Pre-Pulse	T6B-12	200/200	228/232	186/186	288/288
Pre-Pulse	T6B-13	200/216	228/228	182/182	274/288

I then compared the alleles in the offspring of the 13 pulse clutches (Table 7) with those of the 13 pre-pulse background ticks.I then investigated each sample (pre-pulse adult, or pulse clutch) individually, locus by locus, to identify alleles which were unique to a specific clutch or to the background, and developed a set of diagnostic rules for each locus (Tables 10-13).

Table 10: The 15 alleles detected from all samples at locus BHY12. (x) indicates if the allele was present in the pre pulse background sample or an individual clutch. Bold RED (**X**) indicates that an allele was only found in one group of ticks. These alleles were considered to be diagnostic and were used as unique identifiers.

BHY12 Allele	**Background**	**A/B1**	**A/B2**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**
168									x	x				
172		x	x		x	x			x			x		x
175											**X**			
176		x						x	x	x	x	x	x	x
180								x			x			x
184											x		x	x
188		x			x			x	x				x	x
192	x				x			x	x		x	x		
196	x			x		x	x		x				x	
198	**X**													
200	x			x		x	x					x	x	
202	**X**													
204			x					x	x					
208					**X**									
216	x				x									

To identify genotypes at BHY12 as background or pulse I established the following rules:

1) if the allele was 198 or 202 it must be background tick
2) if the allele was 175 or 208 it must be a specific clutch (clutch J or D) tick,
   a. 175 was Clutch J
   b. 208 was Clutch D.
3) if the allele was 168, 172, 176, 180, 184, 188 or 204 it must be a clutch tick
4) if the allele was 192, 196, 200, or 216 it could be from either the clutch or background.

This development of diagnostic rules was then repeated for each of the other three loci.

Table 11 shows the alleles present for BHY27

Table 11: The 33 alleles detected from all samples at locus BHY27. (x) indicates if the allele was present in the pre-pulse background sample or an individual clutch. Bold RED (**X**) indicates that an allele was only found in one group of ticks. These alleles were considered to be diagnostic were used as unique identifiers.

BHY27 Allele	Background	A/B1	A/B2	C	D	E	F	G	H	I	J	K	L	M
216											**X**			
223										**X**				
224							x		x					
227	**X**													
228	x													x
229	**X**													
231		x							x					
232	x				x	x	x				x			
235									x		x			
237											**X**			
238									x	x				
240	**X**													
242													**X**	
248				x									x	
251													**X**	
252		x	x											
253									**X**					
254								x		x		x		
255														**X**
258														**X**
259				**X**										
266			**X**											
270													**X**	
286							x	x						
292									**X**					
294													**X**	
304						x					x			x
308					x							x		x
312					x			x						
320					x		x							
324												**X**		
332			x								x			
340							x					x		

To identify genotypes of BHY27 as background or pulse I established the following rules:

1) if the allele was 227, 229 or 240 it must be background tick
2) if the allele was 216, 223, 237, 242, 251, 255, 258, 259, 266, 270, 292, 294 or 324 it must be a specific clutch tick
    a. 216 was Clutch J
    b. 223 was Clutch I
    c. 237 was Clutch J
    d. 242 was Clutch L
    e. 251 was Clutch L
    f. 255 was Clutch M
    g. 258 was Clutch M
    h. 259 was Clutch C
    i. 266 was Clutch AB2
    j. 270 was Clutch L
    k. 292 was Clutch H
    l. 294 was Clutch L
    m. 324 was Clutch K
3) If the allele was 224, 231, 235, 238, 248, 252, 253, 254, 286, 304, 308, 312, 320, 332 or 340, it must clutch tick. If the allele was 228, 232 or 240 it could be from either the clutch or background.

Table 12 shows the alleles present for BHY28

Table 12: The 13 alleles detected from all samples at locus BHY28. (x) indicates if the allele was present in the pre-pulse background sample or an individual clutch. Bold RED (X) indicates that an allele was only found in one group of ticks. These alleles were considered to be diagnostic were used as unique identifiers.

BHY28 Allele	**Background**	**A/B1**	**A/B2**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**
182	x			x			x			x				
186	x				x	x			x	x	x		x	
187													**X**	
192	**X**													
194	x			x	x	x								
195					**X**									
202		x	x					x	x	x	x	x	x	x
206								x	x		x	x	x	x
210		x	x											
214				x				x		x	x	x	x	
218		x	x											
222												**X**		
224												**X**		

To identify genotypes at BHY28 as background or pulse I established the following rules:

1) if the allele was 192 it must be background tick
2) if the allele was 187, 195, 222 or 224 it must be a specific clutch tick
    a. 187 was Clutch L
    b. 195 was Clutch D
    c. 222 was Clutch K
    d. 224 was Clutch K
3) if the allele was 202, 206, 210, 214 or 218 it must clutch tick
4) if the allele was 182, 186 or 194 it could be from either the clutch or background.

Table 13 shows the alleles present for BOHY03

Table 13: The 10 alleles detected from all samples at locus BOHY03. (x) indicates if the allele was present in the pre-pulse background sample or an individual clutch. Bold RED (**X**) indicates that an allele was only found in one group of ticks. These alleles were considered to be diagnostic were used as unique identifiers.

BOHY03 Allele	Background	A/B1	A/B2	C	D	E	F	G	H	I	J	K	L	M
274	x	x	x	x	x	x	x	x	x	x	x	x	x	x
276		x	x				x	x	x	x		x	x	x
277	**X**													
278											**X**			
282	**X**													
288	x			x										
292					**X**									
308														**X**
312				**X**										
315									**X**					

To identify genotypes at BOHY03 as background or pulse I established the following rules:

1) if the allele was 277 or 282 it must be background tick
    a. if the allele was 288 it must be a background tick or from Clutch C, If no clutch alleles were present at other loci ticks with allele 288 were identified as background ticks.
2) if the allele was 278, 292, 308, 312 or 315 it must be a specific clutch tick
    a. 278 was Clutch J
    b. 292 was Clutch D
    c. 308 was Clutch M
    d. 312 was Clutch C
    e. 315 was Clutch H

If the allele was 274 or 276 it could be from either the clutch or background.

Identification of adult ticks sampled after pulses added

Pulses of larvae were added in October 2009 (clutches A/B1, A/B2, C, F and G) and April 2010 (clutches D, E, H-M). I expected that most surviving larvae would have reached the adult stage by the following spring so that samples of adult ticks from 2010 (n = 156) would largely be from the background or from the first pulses of added larvae (clutches A/B1, A/B2, C, F or G) and from 2011 (n = 207) would contain background ticks and mostly adult ticks derived from the second pulse additions, although some from the first additions may still have been present. All adults sampled were sorted into either background or pulse added groups in a spreadsheet, using conditional formatting and applying the rules above.

In the tables below alleles at each locus which were identified as unique to the background population were coded yellow, and allele 288 at BOHY03, which might be shared between Clutch C and background, was coded pale green. Any ticks with at least one yellow or with one pale green and with non Clutch C alleles at other loci was considered as belonging to the background tick population at the site.

Any allele unique to a specific clutch was coded blue, alleles present in clutches only were coded dark green and lastly any allele identified as common to both background and clutches was coded red. Alleles from these samples which had not previously been identified in the diagnostic testing were left white. Thus ticks with some blue or dark green codes and no yellow were considered to belong to one of the added pulses of infestations.

Among these pulse origin ticks, if there were no unique clutch alleles, but shared clutch alleles present, the combination of all loci was investigated and if possible the individual was assigned to a clutch based on a majority of loci. The resulting tick sample identification was used in later analysis in the thesis. As explained above, I also eliminated clutches from the second pulse addition as possible sources of ticks sampled in year 2010.

Table 14: The 2010 samples that were identified as background.

Sample Name	BHY12	BHY12	BHY27	BHY27	BHY28	BHY28	BOHY03	BOHY03	Identified
T6-102	236	236	232	240	182	194	274	288	BACKGROUND
T6-105	192	200	228	228	182	182	0	0	BACKGROUND
T6-113	200	216	228	228	182	186	274	288	BACKGROUND
T6-114	200	216	228	228	182	186	274	288	BACKGROUND
T6-13	196	216	228	228	182	182	274	288	BACKGROUND
T6-133	200	224	228	240	186	198	288	288	BACKGROUND
T6-164	196	200	228	228	186	186	288	288	BACKGROUND
T6-169	200	200	228	228	182	186	274	288	BACKGROUND
T6-175	200	200	228	228	186	194	274	288	BACKGROUND
T6-179	200	204	228	228	182	186	288	288	BACKGROUND
T6-180	200	200	228	228	182	182	288	288	BACKGROUND
T6-182	200	216	228	228	182	182	274	288	BACKGROUND
T6-200	196	200	228	228	182	186	274	288	BACKGROUND
T6-201	196	200	228	228	182	186	288	288	BACKGROUND
T6-202	200	200	228	228	186	206	274	288	BACKGROUND
T6-21	216	216	228	228	182	182	288	288	BACKGROUND
T6-221	196	200	228	228	186	186	288	288	BACKGROUND
T6-225	216	216	228	228	182	182	274	288	BACKGROUND
T6-229	196	200	228	228	182	182	274	288	BACKGROUND
T6-23	200	204	228	228	182	182	288	288	BACKGROUND
T6-241	200	204	228	228	182	186	288	288	BACKGROUND
T6-242	200	216	0	0	0	0	288	288	BACKGROUND
T6-252	200	216	228	228	182	186	274	288	BACKGROUND
T6-254	200	216	228	228	182	186	274	288	BACKGROUND
T6-260	200	204	228	228	182	186	288	288	BACKGROUND
T6-270	200	216	228	228	182	182	274	288	BACKGROUND
T6-28	200	200	228	228	182	182	274	288	BACKGROUND
T6-290	196	200	228	228	182	182	274	288	BACKGROUND
T6-301	200	200	0	0	182	186	274	288	BACKGROUND
T6-35	200	220	228	228	0	0	288	288	BACKGROUND
T6-38	200	204	228	228	0	0	274	288	BACKGROUND
T6-40	216	236	228	239	0	0	274	288	BACKGROUND
T6-46	200	204	228	228	0	0	288	288	BACKGROUND
T6-55	216	216	228	239	182	194	288	288	BACKGROUND
T6-62	200	252	228	228	182	186	274	277	BACKGROUND
T6-66	200	216	228	228	186	194	274	288	BACKGROUND
T6-86	200	200	228	228	182	186	288	288	BACKGROUND
T6-87	200	216	228	228	182	182	274	288	BACKGROUND

Table15: these 2010 samples had alleles common to background and clutches, but their allele combination did not match that of any clutches and the samples were subsequently identified as background

Sample Name	BHY12	BHY12	BHY27	BHY27	BHY28	BHY28	BOHY03	BOHY03	Identified
T6-108	200	200	228	228	186	194	274	274	Background
T6-110	200	200	228	232	182	182	274	274	Background
T6-130	200	200	228	232	186	194	0	0	Background
T6-165	200	216	228	228	182	182	274	274	Background
T6-17	200	216	228	228	182	186	274	274	Background
T6-18	216	216	228	228	182	182	0	0	Background
T6-2	196	204	228	228	194	194	0	0	Background
T6-203	200	216	228	228	186	186	274	274	Background
T6-215	200	216	228	228	182	182	274	274	Background
T6-228	200	216	228	228	182	182	274	274	Background
T6-243	200	216	228	228	0	0	274	274	Background
T6-298	192	200	232	232	182	182	274	274	Background
T6-52	200	200	228	228	182	182	0	0	Background
T6-76	200	216	228	228	182	186	274	274	Background

Table 16: These 2010 samples were identified as a particular clutch due to the presences of a unique allele or a combination of clutch only alleles.

Sample Name	BHY12	BHY12	BHY27	BHY27	BHY28	BHY28	BOHY03	BOHY03	Identified
T6-163	188	188	231	252	202	210	276	276	A/B1
T6-239	188	188	231	252	210	218	274	276	A/B1
T6-271	172	188	231	252	202	210	276	274	A/B1
T6-293	172	188	252	252	210	218	276	276	A/B1
T6-177	172	204	224	266	202	210	274	276	A/B2
T6-178	172	172	252	332	202	202	274	274	A/B2
T6-302	172	172	224	266	210	218	274	276	A/B2
T6-41	172	204	266	332	0	0	274	276	A/B2
T6-217	192	200	228	248	0	0	274	288	C
T6-240	200	200	228	248	0	0	274	288	C
T6-266	196	196	248	259	182	214	288	312	C
T6-267	196	200	259	259	194	214	288	312	C
T6-78	196	196	259	259	182	182	288	312	C
T6-79	196	196	248	259	182	182	274	312	C
T6-80	196	200	248	248	214	214	288	312	C
T6-81	196	196	248	248	182	214	288	288	C

T6-84	196	200	248	259	214	214	0	0	C
T6-275	172	196	232	304	194	194	274	276	E
T6-303	172	200	232	232	186	186	276	274	E
T6-100	196	200	232	340	182	182	0	0	F
T6-101	192	200	232	340	182	182	274	274	F
T6-106	192	200	224	224	182	182	0	0	F
T6-150	196	200	224	224	182	182	274	274	F
T6-152	196	200	224	232	182	182	274	274	F
T6-166	192	200	232	340	182	214	274	274	F
T6-216	196	200	224	340	182	182	274	274	F
T6-246	196	200	232	340	182	182	274	274	F
T6-249	176	180	286	320	202	214	276	276	F
T6-300	196	200	224	340	0	0	274	274	F
T6-43	192	200	224	232	182	182	274	274	F
T6-44	196	200	224	232	182	182	274	274	F
T6-45	196	200	224	232	182	182	274	274	F
T6-117	192	204	286	320	206	214	0	0	G
T6-226	176	180	254	286	0	0	276	276	G
T6-227	176	204	0	0	206	214	274	276	G
T6-27	192	204	254	312	202	214	274	276	G

Table17: The 2011 samples that were identified as background.

Sample Name	BHY12	BHY12	BHY27	BHY27	BHY28	BHY28	BOHY03	BOHY03	Identified
T6-382	196	252	228	228	186	186	274	288	BACKGROUND
T6-361	200	216	228	228	182	186	274	288	BACKGROUND
T6-362	200	216	228	228	182	182	274	288	BACKGROUND
T6-368	200	216	228	228	182	186	274	288	BACKGROUND
T6-481	200	200	228	228	186	186	274	288	BACKGROUND
T6-347	200	216	228	228	182	186	274	288	BACKGROUND
T6-389	200	216	228	228	182	182	274	288	BACKGROUND
T6-495	196	200	228	228	182	182	288	288	BACKGROUND
T6-403	200	200	228	228	182	182	274	288	BACKGROUND
T6-498	200	216	228	228	182	182	274	288	BACKGROUND
T6-410	200	200	228	228	186	194	288	288	BACKGROUND
T6-462	200	216	228	228	182	186	288	288	BACKGROUND
T6-373	200	200	228	228	182	186	288	288	BACKGROUND
T6-377	200	200	228	228	182	186	288	288	BACKGROUND
T6-455	214	214	0	0	182	186	0	0	BACKGROUND
T6-392	196	252	228	228	186	206	274	288	BACKGROUND
T6-475	196	200	228	228	182	186	288	288	BACKGROUND
T6-367	196	200	228	228	182	186	288	288	BACKGROUND
T6-504	196	200	228	228	182	186	274	288	BACKGROUND

Sample	BHY12	BHY12	BHY27	BHY27	BHY28	BHY28	BOHY03	BOHY03	Identified
T6-350	200	216	228	228	182	182	274	288	BACKGROUND
T6-352	200	216	228	228	182	216	274	288	BACKGROUND
T6-479	200	216	228	228	182	194	274	288	BACKGROUND
T6-480	200	200	228	228	182	182	274	288	BACKGROUND
T6-322	196	216	228	228	182	194	274	288	BACKGROUND
T6-461	200	252	228	228	186	206	288	288	BACKGROUND
T6-402	196	200	228	228	182	186	288	288	BACKGROUND
T6-405	200	200	228	228	0	0	288	288	BACKGROUND
T6-406	200	200	228	228	0	0	274	288	BACKGROUND
T6-493	200	216	228	228	186	186	274	288	BACKGROUND
T6-441	200	216	228	228	186	194	274	288	BACKGROUND
T6-442	200	252	228	228	182	186	274	288	BACKGROUND
T6-411	196	200	228	228	182	186	288	288	BACKGROUND
T6-469	196	216	228	239	182	206	288	288	BACKGROUND
T6-466	196	200	228	228	182	186	288	288	BACKGROUND
T6-313	196	216	228	239	186	206	274	288	BACKGROUND
T6-467	200	216	228	228	182	186	288	288	BACKGROUND
T6-372	200	200	228	228	182	182	274	288	BACKGROUND
T6-379	200	252	228	228	182	182	288	288	BACKGROUND
T6-380	200	216	228	228	182	186	274	288	BACKGROUND
T6-456	216	216	228	228	182	186	274	277	BACKGROUND
T6-489	200	200	228	228	182	182	288	288	BACKGROUND
T6-429	200	252	228	228	182	186	274	277	BACKGROUND
T6-438	200	216	228	228	182	186	274	288	BACKGROUND

Table 18: these 2011 samples had common alleles, but the allele combination did not match an any clutches and the samples were subsequently identified as background

Sample Name	BHY12	BHY12	BHY27	BHY27	BHY28	BHY28	BOHY03	BOHY03	Identified
T6-360	200	216	228	228	182	182	274	274	Background
T6-415	192	204	228	228	186	194	274	274	Background
T6-421	200	204	228	228	182	186	274	274	Background
T6-505	196	200	228	228	182	182	274	274	Background
T6-454	200	216	228	228	182	182	274	274	Background
T6-414	192	204	228	228	182	194	274	274	Background
T6-400	192	200	228	232	186	194	274	274	Background
T6-332	200	200	228	228	182	186	274	274	Background
T6-445	192	204	228	232	182	194	274	274	Background
T6-446	200	200	228	232	182	186	274	274	Background
T6-503	216	216	228	228	182	182	274	274	Background
T6-409	200	200	228	228	0	0	274	274	Background
T6-312	200	204	228	228	182	186	0	0	Background
T6-376	200	216	228	228	182	182	274	274	Background
T6-451	192	204	228	228	182	194	0	0	Background

Table 19: These 2011 samples were identified as being from a particular clutch due to the presences of a unique allele or a combination of clutch only alleles.

Sample Name	BHY12	BHY12	BHY27	BHY27	BHY28	BHY28	BOHY03	BOHY03	Identified
T6-487	188	188	231	252	202	202	274	276	A/B1
T6-331	172	204	266	332	202	218	276	276	A/B2
T6-341	196	200	248	259	0	0	274	288	C
T6-342	0	0	248	248	0	0	274	312	C
T6-349	196	200	248	248	182	194	288	312	C
T6-346	188	200	228	248	194	194	274	288	C
T6-381	196	196	248	259	182	194	288	312	C
T6-364	208	216	312	320	194	194	274	292	D
T6-325	188	208	308	320	194	194	274	292	D
T6-497	172	208	312	312	194	194	0	0	D
T6-387	172	208	308	312	194	194	276	292	D
T6-452	172	208	308	312	194	194	274	274	D
T6-453	172	208	232	312	194	194	274	274	D
T6-335	172	208	312	312	194	194	274	276	D
T6-397	172	208	232	308	194	194	274	292	D
T6-401	172	208	232	312	194	194	274	274	D
T6-407	172	208	232	312	0	0	274	276	D
T6-463	180	192	312	320	202	214	274	276	D
T6-371	188	208	312	320	194	194	274	274	D
T6-370	172	196	312	312	194	194	274	292	D
T6-396	172	192	232	312	194	194	276	292	D
T6-385	192	200	224	224	182	182	274	274	F
T6-343	192	200	224	232	182	182	274	274	F
T6-465	196	200	232	340	182	182	274	274	F
T6-476	192	200	224	340	182	182	274	274	F
T6-448	196	200	224	340	182	182	274	274	F
T6-426	192	200	224	224	182	182	274	274	F
T6-425	196	200	232	340	182	182	274	274	F
T6-351	176	204	254	286	202	214	274	276	G
T6-354	175	192	254	286	202	214	276	276	G
T6-378	176	180	254	286	202	202	276	276	G
T6-477	192	204	224	235	206	206	315	315	H
T6-384	176	176	223	238	186	214	274	276	I
T6-460	168	176	254	254	186	214	274	276	I
T6-472	168	176	223	254	186	214	274	276	I
T6-338	168	176	238	254	186	214	274	276	I
T6-340	176	176	223	254	186	214	274	276	I
T6-458	176	176	238	254	186	214	274	276	I
T6-398	168	176	223	254	186	214	274	276	I
T6-394	176	176	238	254	186	214	274	276	I
T6-471	168	176	254	254	186	214	274	276	I
T6-500	168	176	238	254	186	214	274	276	I

T6-488	168	176	254	254	186	214	274	276	I
T6-490	168	176	223	254	186	214	274	276	I
T6-457	168	176	254	254	186	214	274	276	I
T6-336	176	176	223	238	186	214	274	276	I
T6-484	180	184	232	237	186	214	278	278	J
T6-363	176	192	216	304	206	214	274	274	J
T6-328	176	184	216	304	206	214	274	278	J
T6-388	176	192	216	304	202	206	274	278	J
T6-390	184	192	235	237	186	214	274	278	J
T6-323	184	192	235	237	206	214	274	278	J
T6-324	184	192	235	304	206	214	274	274	J
T6-326	176	192	235	237	186	186	0	0	J
T6-436	176	184	235	304	206	214	274	274	J
T6-437	180	184	232	237	186	214	278	278	J
T6-486	176	192	216	304	206	214	274	274	J
T6-496	176	192	0	0	202	206	274	278	J
T6-499	168	184	232	304	202	206	278	278	J
T6-494	176	184	235	304	202	206	274	274	J
T6-428	176	176	216	304	206	214	274	278	J
T6-492	176	192	254	308	206	206	276	276	K
T6-319	176	192	254	324	206	222	276	276	K
T6-434	176	200	254	324	206	222	274	276	K
T6-320	176	192	308	324	206	206	276	276	K
T6-321	176	192	254	308	202	214	276	276	K
T6-450	176	192	254	308	206	222	274	276	K
T6-432	172	192	254	324	206	214	276	276	K
T6-424	184	188	251	270	194	214	274	276	L
T6-329	188	196	242	242	206	214	274	274	L
T6-314	172	180	255	304	202	206	276	276	M
T6-423	172	184	258	304	202	206	274	274	M
T6-383	172	180	258	304	202	206	274	274	M
T6-310	172	180	258	304	202	206	274	274	M
T6-337	172	180	255	304	202	202	276	276	M
T6-427	172	188	228	258	202	206	274	274	M
T6-482	172	184	258	304	202	206	274	276	M

For the samples that could not be assigned to a clutch or the background by the simple rule set, each was examined by its individual alleles. Each allele was assigned to all possible clutches or background it was found in. Candidate sources were then tallied to identify any most likely identification that the sample could be given. If there was any particular clutch that six or more of the eight alleles could be assigned to, the sample was re-identified to that clutch.

In the 2010 year samples, there were a total of 22 cases of ambiguous samples, of these only four could be reassigned to a potential clutch. In the 2011 year there were 53 cases of ambiguous tick samples, of which 13 could be reassigned to a potential clutch. Two samples, T6- 275 and T6-303, were from Clutch E, which was released in April 2010, it is plausible that they could have been fast developers from this realise as they were collected in late December 2010.

Table 20, shows examples of how the ambiguous samples were broken down and identified.

Table 20: Example of Identification of ambiguous samples.
The allele association was which clutch or background (Bg) that the allele was found in, Allele Id summary was the amount of the found in a particular clutch or background, allowing me to give a potential identification to the ambiguous sample.

2010

Sample	T6-212	Recipient lizard	12434	
Allele	Allele association	Allele Id summary		Potential identification
172	AB1,AB2,D,E,H,K,M	8		Lizard is AB2 donor, potential self infection
172	AB1,AB2,D,E,H,K,M	7	AB1,AB2	
224	F,H	6		
252	AB1,AB2	5	H	
210	AB1,AB2	4	D,E,K,M	
218	AB1,AB2	3	F	
274	ALL	2		
274	ALL	1		
Sample	T6-272	Recipient	40044	
Allele	Allele association	Allele Id summary		Potential identification
200	Bg,C,E,F,K,L	8		From either background or clutch C, unable to identify further
204	AB2,G,H	7		
228	Bg,M	6	Bg,C	
248	C,L	5		
194	Bg,C,D,E	4		
194	Bg,C,D,E	3	E	
288	Bg,C	2	D,L	
288	Bg,C	1	AB2,F,K,M	

2011				
Sample	T6-418	Recipient	31849	
Allele	Allele association	Allele Id summary		Potential identification
200	Bg, C,E,F	8		Potentially from clutch C, however has a large amount of Background alleles as well.
204	AB2,G,H	7		
224	F,H	6	C	
248	C,L	5	Bg	
194	Bg,C,D,E	4	E	
194	Bg,C,D,E	3	D,F,H	
274	ALL	2	AB2,G,L	
288	Bg,C	1	AB1,I,J,K,M	
Sample	T6-353	Recipient	10827	
Allele	Allele association	Allele Id summary		Potential identification
0				This tick could either be Clutch G or Clutch K, however the partner of this lizard was the Clutch G donor. Clutch G is likely.
0				
254	G,I,K	6	G,K	
254	G,I,K	5	I	
202	AB1,AB2,G,H,I,J,K,L,M	4	L,M	
206	G,H,J,K,L,M	3	AB1,AB2,J	
274	ALL	2	F	
276	AB1,AB2,F,G,H,I,K,L,M	1	Bg,C,D,E	
Sample	T6-348	Recipient	12138	
Allele	Allele association	Allele Id summary		Potential identification
196	Bg,C,E,F,H,L	8		Although Clutch E has most alleles, it only has one more identifier allele than the background and Clutch L. No further identification possible
200	Bg,C,G,H,K,L	7		
304	E,J,M	6	E	
304	E,J,M	5	Bg,L	
186	Bg,D,E,H,I,J,L	4	C,H,J,M	
194	Bg,C,D,E	3	D,F,G,I,K	
274	ALL	2	AB1,AB2,	
276	AB1,AB2,F,G,H,I,K,L,M	1		
Sample	T6-355	Recipient	11885	
Allele	Allele association	Allele Id summary		Potential identification
172	AB1,AB2,D,E,H,K,M	8	E	All alleles are found in Clutch E, however shares many with the
200	Bg,C,E,F	7		
304	E,J,M	6		
304	E,J,M	5	M	
194	Bg,C,D,E	4	Bg,C,D	
194	Bg,C,D,E	3	AB1,AB2,F,H,J,K	

274	ALL	2	G,I,L	Clutch M. The recipient lizard overlaps the home range of the Clutch E donor. Clutch E is likely.
276	AB1,AB2,F,G,H,I,K,L,M	1		

In summary, after the ambiguous samples were reassigned, in 2010, of the 156 samples collected 54 were classified as background, 38 were assigned to a clutch, 18 remained ambiguous and 42 did not amplify nor had insufficient genetic data. In 2011, of the 207overall samples collected, 57 were background ticks, 88 were assigned to a clutch, 9 tick samples did not amplify and had insufficient data for genetic analysis, and 40 remained ambiguous. The overall result was a sample of 111 ticks in the two years that could be assumed to have come from natural background infestations of *B. hydrosauri*, and 126 ticks over the same collection period that were considered to have originated from one of the 13 pulsed tick additions. These ticks were the basis for much of the subsequent analyses of the transmission dynamics in this tick population.

Discussion

This chapter established the origin of tick samples used in subsequent chapters. I was able to build on the previously developed loci of Guzinski *et al*. (2008) by utilizing the technology of 454 sequencing. I was successful in developing three more loci for the reptile tick, *Bothriocroton hydrosauri.* Although problems existed with the loci, with the four loci that were finally used, the aim of discriminating among different clutches, and between clutch derived ticks and background infestations was achieved for 65% of the collected adult ticks based on diagnostic alleles. These loci were not highly polymorphic, but I can say with confidence that I could successfully assign alleles to loci. Using the 454 development technique, I expected to be able to develop many more loci. Using MICROFAMILY, 83 microsatellites loci that were identified and for which primers could be designed, suggested the potential for a large numbers of duplicate diagnostic loci (Gardner *et al*. 2011). However, when I tested 40 new loci, 14 did not amplify, and I encountered further problems with the 26 that amplified (Table 1), with inconstant amplification (Table 2), ambiguous products amplifying (Table 3) or non-diagnostic alleles (Table 4) being the main limitations. There are potential challenges that could have inhibited the development of further *Bothriocroton*

hydrosauri loci, however discovering if apparently aberrant inheritance patterns were due to mutations, the incidence of null alleles, duplicated loci or some other issue was beyond the scope of this project.

For instance, while testing primers for clutch identification I found that some of the new loci had cases of more the four alleles represented within a clutch from supposedly diploid, single mating parents, although no individual tested had more than two alleles at a locus. Multiple paternity is one potential explanation, although the presence of the copulatory plug after mating (Andrews and Bull 1980) makes this unlikely. However, using genetic methods multiple paternity has been found in other tick species, *Rhipicephalus* (*Boophilus*) *microplus*, the cattle tick, (Cutulle et. al. 2010), *Ixdoes uriae, the* seabird tick (McCoy and Tirand, 2002) and *Ixdoes ricinus*, the sheep tick (Hasle et.al. 2008). There could also be potential shared mutations, (Jones et. al. 1999) or non-Mendelian heritance. In previous development of *Bothriocroton hydrosauri* loci for microsatellite characterisation, one locus showed evidence of non-Mendelian inheritance that could not be explained by the presence of null alleles, (Guzinski et al. 2008). Non-Mendelian inheritance had been previously reported in other tick species. Roed et al. (2006) isolated and developed 17 microsatellite loci for the tick, *Ixodes ricinus*, several of which showed patterns of non-Mendelian inheritance, but which could be explained by a high frequency of null alleles. Five loci were studied further and none of them were fully Mendelian in any families tested, although again the deviations from inheritance could be explained by null allele presences in the mother. Noel et al. (2012) isolated nine new polymorphic loci, from the same tick species and encountered null alleles, some of which could be explained by the Wahlund effect, while others were explained by technical issues of mutations at the flanking regions of the microsatellites. For *I. ricinus* another previous study investigated the non-Mendelian transmission of alleles at microsatellite loci (de Meeus et al. 2004). Of five loci, three displayed patterns that could not be explained by null alleles. These authors found that maternal alleles were poorly amplified at some loci, and confirmed short allele dominance in this tick species, a phenomenon that had not been recorded in tick species previously although it is known for microsatellites generally (Pemberton et al. 1995). It is also possible that many of the non-Mendelian inheritance that I uncovered and those of other researchers could be due to the presence of duplicated loci, complicating the allocation of alleles to single loci. In summary, ticks appear to be a difficult group for conventional population genetics analyses. I am not unique in experiencing patterns difficult to explain. However, although the

ticks proved difficult for extraction of a large number of diagnostic loci, there was sufficient variation among the four loci to identify many of the adult ticks that were later collected with a degree of confidence.

Although the inheritance patterns of the ticks I sampled are an interesting topic in their own right, the principal aim of this study was to identify diagnostic genotype combinations that would allow us to use the genotype as an effective marker of individual groups of ticks that persisted across the different life stages from larvae to adult. And, despite these limitations, the loci I genotyped allowed a substantial proportion (58.97% in 2010 and 70.04% in 2011) of all sampled individuals to be allocated with some confidence to a particular clutch or to the background natural tick infestations.

With the current 454 sequencing data, further development of loci for *Bothriocroton hydrosauri* could be achieved. However the complications of using microsatellites could be circumvented by using newer techniques, such as RADseq, (Davey 2010) but problems with this technique might arise due to poor DNA yields or inhibitors.

The genotypes of individuals collected in 2010 and 2011 and their subsequent assignment to the background or experimentally infected pulse population will be used with social network modelling to further study background relatedness of ticks within the lizard population and compare the patterns of relatedness with transmission networks and to allow us to follow their movement within the host population to see if transmission is via pathways predicted by the transmission models.

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Chapter 4: Deducing the dynamics of parasite transmission from patterns of parasite genetic relatedness across host networks

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This paper has been submitted and the genetic loci developed in chapter 3 were used to distinguish patterns of genetic relatedness from the background tick, combined with the networks patterns of host behaviours. Showing how these can be integrated to develop strong inferences about the dynamics of host to host tick transmission.

Abstract

A major question for understanding the ecology of parasite infections and diseases in wildlife populations concerns the transmission pathways from one host individual to the next. Our study addressed two questions in a lizard – tick system. First, we asked whether adult ticks were more closely related to each other on hosts that were more closely linked in a parasite transmission network. Second, we asked which of three alternative network structures best explained the patterns of genetic relatedness. The host species was the Australian sleepy lizard *Tiliqua rugosa*, and we used synchronous GPS locations of over 50 lizards, each 10 min across the whole three month activity period, over two years, to construct three alternative parasite transmission networks. One alternative was based on the extent of home range spatial overlap, another on the frequency of social contacts, and the last on the frequency of asynchronous shared refuge use between pairs of lizards. The parasite was the three host ixodid tick, *Bothriocroton hydrosauri*. In each year adult ticks from natural infestations were removed from lizards and their genotypes were determined at four polymorphic microsatellite loci. We found that adult ticks were more related to each other when they were collected from the same host, than when collected from different hosts. We also found that when adult ticks were on different lizards they had higher relatedness if those lizards had shorter path lengths connecting them on each of the three networks we explored. In each of the two study years a different network best explained the dynamics of transmission. The social contact network was the poorest predictor of tick relatedness in both years, while the spatial overlap based network (in one year) and the asynchronous shared refuges network (in the other year) were the strongest predictors. We speculate on how changing environmental conditions might change the relative importance of alternative processes driving the parasite transmission dynamics.

Key words: Tick, Lizard, Social network, Parasite transmission, *Bothriocroton hydrosauri*

Introduction

In this paper, we explore how patterns of genetic relatedness within a population of ectoparasitic ticks and patterns of host behaviours can be integrated to develop strong inferences about the dynamics of host to host tick transmission. A major question for understanding the ecology of parasite infections and diseases in wildlife populations concerns the transmission pathways from one host individual to the next. Classic SIR models of parasite host dynamics, developed by Anderson and May (1979), are still used today in models of the epidemiological spread of human diseases, such as AIDS, (Klovdahl 1985), HIV (Friedman et al. 1997) and influenza, (Hsu and Shih, 2010) and of other diseases in wildlife populations (Packer et al. 1999, Drewe 2010).

A basic assumption of those SIR models is that transmission from host to host is random, and that all susceptible hosts in a population are equally likely to contact an infected host and become exposed to infection themselves (Franks et al. 2009). However, most animal populations have some level of social structure with higher rates of association among some individuals than others (Drewe, 2010, Aiello et al. 2016). This structure is sometimes described in terms of a social network, and it has often been suggested that the connecting links of a social network form pathways for the transmission of parasites and pathogens (Godfrey *et al*. 2009; 2010). Diseases, such as bat white nose syndrome, (Blehert et al. 2009) and Tasmanian devil facial tumour disease (McCallum *et al*. 2009), are a common threat to wildlife populations. Understanding the role of networks in transmission dynamics is an important foundation for subsequent modelling of parasite-host systems (Krause et al. 2014), with relevance to conservation management and the control of parasites and pathogens emerging in wildlife populations. Yet in many parasite host systems the transmission process

from one host to another is hard to observe and quantify. Instead we rely on inferences about transmission derived from empirically observed patterns of infection.

One commonly used indirect method to infer transmission around a population has been to look for associations between social network structure and infection prevalence. A high incidence of infections among socially connected individuals has been used to deduce the transmission process in diseases of humans (Klovdahl, 1985; Christley *et al*., 2005), of domestic stock (Ortiz-Pelaez et al., 2006), and of wildlife (Lembo et al. 2008; McCallum *et al*. 2001; Clay et al. 2009).

For example Drewe (2010) showed that the level of social interaction among meerkats (*Suricata scuricatta*) was the driving force behind the spread of the infectious bacteria *Mycobacterium bovis* through a population, and that the type and direction of interaction were key to understanding infection patterns. In this example, the spread of infection from one discrete social group to another was most likely caused by floater individuals, peripheral to more than one group (Drewe 2010).

In many of the studies referred to above, researchers derived their inferences of transmission pathways by observing the spread of a new infection, or a new outbreak of an established pathogen, across a population. When a parasite has already established with a stable prevalence within a population it is more difficult to determine the transmission dynamics. We can look at indirect evidence from correlations between levels of social connectedness and infection status. For example, in two reptiles, gidgee skinks, *Egernia stokesii* (Godfrey et al. 2009) and tuatara, *Sphenodon punctatus* (Godfrey et al. 2010), individuals that were more highly connected in their social network had higher parasite loads, allowing the deduction that they had a higher infection risk and that transmission occurred along social network connections.

Evidence for this transmission pathway would be stronger if we could track individual transmissions. While this process can be difficult to observe directly, we can track transmission indirectly by identifying genetically unique strains within parasite populations and deducing that hosts carrying identical, or closely related strains are likely to represent cases of host to host transmissions (Vanderwaal et al. 2014). Bull et al. (2012), used common genotypes of *Salmonella enterica* detected in cloacal swabs from the sleepy lizard, *Tiliqua rugosa*, and reported that pairs of lizards that shared genetic strains were more strongly connected to each other in the social network, but were no closer to each other spatially, than pairs of lizards that did not share *Salmonella* strains. By comparing the fit to two alternative network transmission models, based on social contact and on home range overlap, they deduced that *Salmonella* was more likely to be transmitted by direct lizard to lizard contact than via some common non-lizard infection source (Bull et al. 2012).

We now expand this approach to explore the transmission pathways of a three host, ectoparasitic ixodid tick, *Bothriocroton hydrosauri*, around a population of the sleepy lizard (*T. rugosa*). After each feeding stage of its life cycle, this tick species detaches and there is a time period while moulting, from larvae to nymphs or from nymphs to adults, before the next stage becomes infective again. We have previously suggested a structure for the transmission network for ticks in populations of this lizard, where a network connection forms if one lizard uses a refuge that another lizard has previously used and where that lizard's ticks have previously detached (Leu et al. 2010b; Wohlfiel *et al*. 2013). Our current study uses tick genetic relatedness estimates to compare this transmission model with two alternative models, one that assumes tick transmission through direct host contact, and one that assumes that the risk of infection depends on the extent of spatial overlap of neighbouring lizard home ranges.

Guzinski et al. (2009) used the genetic population structure of sleepy lizard ticks to propose a “ripple” model for tick transmission. In their model, a detached female laying a clutch of eggs is the centre of a ripple that spreads across adjacent hosts at each new life stage from larvae to adults. An inference of that model was that tick transmission was related to spatial proximity of their hosts. In the current paper we use empirical observations of patterns of genetic relatedness among ticks attached to neighbouring sleepy lizards to differentiate among three alternative network models, including one where ticks are most frequently transmitted to spatially adjacent hosts, which they describe transmission pathways for this spreading ripple. Each of these networks might represent a plausible biological model for the transmission dynamics of parasites among hosts, although their relative importance would be difficult to measure by direct observation. Instead we can infer the transmission process by comparing how well each of the models predicts the actual patterns of genetic structure that we observe.

2. Methods

2.1 Study Site

The study site, on Bundey Church Road, 6km west of Bundey Bore Station (33°54’S, 139°20’ E) in South Australia, was a 1.0 x 1.5 km area, previously described by Godfrey et al. (2012; 2013). The habitat was predominantly chenopod shrub-land, and the lizards within the study site were part of a larger continuous population. The area has hot dry summers and cool wet winters with an average annual rainfall of about 250 mm. The study was conducted during the spring and early summer (September- December) over two years, 2010 and 2011.

This is the only period of the year when sleepy lizards are normally active (Kerr and Bull, 2006, Kerr et al., 2008). The annual rainfall at Bundey Bore Station was 332 mm in 2010, and 459 mm in 2011 (Wohlfiel *et al*. 2013).

2.2 GPS locations and tick collection

At the start of each season, in August or early September, we located all adult resident lizards within the study site (n = 61 in each year), and attached data logger units to the dorsal surface of their tails, as previously described (Kerr et al., 2004a; Leu *et al*., 2010b; Bull *et al*., 2012; Godfrey et al., 2012; 2013; Wohlfeil et al., 2013). The units recorded synchronous GPS locations for all lizards that were active, every 10 min, over the following 4–5 months (115 days in 2010; 81 days in 2011). Units on one lizard in 2010 and on two lizards in 2011 malfunctioned after less than 30 days, and those lizards, and their ticks, were excluded from the analysis. The units included a radio transmitter so that we could locate each lizard every 12 days to download data and change batteries. We did this either before or after the main daily activity period of the lizards to reduce any behavioural impacts from handling (Kerr et *al.* 2004b). At each data download in 2010, and at three data downloads in 2011 (the first of each month, we also removed all natural infections of adult male and female *Bothriocroton hydrosauri* ticks and preserved them in ethanol for subsequent DNA analysis. More than half of the tracked lizards each year (57% in 2010; 56% in 2011) had no natural infections of adult *B. hydrosauri* ticks on any of the data download observation times over an entire season. For our analyses, 54 adult ticks were collected from 26 lizards in 2010 (mean 2.08 ticks/infected lizard ± 0.23 SE, range = 1 - 5), and 58 adult ticks were collected from 27 lizards in 2011 (mean 2.15 ticks/infected lizard ± 0.33 SE, range = 1 – 8).

2.3 DNA methods and analysis

For each adult tick we determined genotypes at each of four polymorphic microsatellite loci, one (Bohy03) modified from Guzinski *et al*. (2008) and three (Bhy12, Bhy27, Bhy28) from Wohlfeil (2016). In 2010 we extracted DNA from body tissue and legs of adult ticks using the Gentra Puregene extraction kit for tissue samples (Qiagen, Doncaster, Australia) following the manufacturer's protocol, with added Protonase K (20mg/ml) and Glycogen (20mg/ml). In 2011 we used the Modified CCDB Glass Fibre Plate DNA extraction method (Ivanova et. al., 2006), modified for use with the vacuum manifold and final elution centrifugation.

All PCR amplifications were carried out in Mastercycler Pro S thermal cyclers at a volume of 10µl. Each reaction was made using 1X MRT buffer, 400 nM of each primer, 0.2 U of Immolase (*Bioline)* and 40 nmoles of neat DNA. The cycling conditions were as follows: initial denaturation 94° for 10mins, followed by 34 cycles at 94°C for 45 secs, annealing temperature of 54° (Bhy12), 52° (Bhy27 and Bohy03), 50°(Bhy28) for 45 secs and extension of 72°C for 1 min, followed by a final extension of 72°C for 30mins and 25°C for 1min.

Samples were sent to the Australian Genome Research Facility (AGRF) for genotyping by Fragment Analysis, Capillary Separation, then sized and scored using the program GeneMapper version 3.7 (*Applied Biosystems*). Relatedness was then calculated from the genotypes at the four loci, for each pair of adult ticks collected in one season, using Wang's relationship coefficient (Wang, 2002) derived in COANCESTRY Version 1.0.1.2 (Wang, 2011).

2.4 Developing the lizard networks

We used the synchronous GPS locations, each 10 min, for the host lizards, to develop, in each of the two years, three different transmission networks to represent alternative mechanisms of tick transmission. One network, derived from spatial proximity of adjacent home ranges, was based on the assumption that infection risk increased if pairs of hosts had high proximity of space use. For this we derived a matrix of geographic distances between the home range centres of each pair of lizards. We assumed that pairs of lizards with shorter distances had higher spatial proximity and stronger network connections, and thus were more likely to carry genetically related ticks. The second network, a social contact network, assumed direct transmission, and that the highest frequency of tick transmission was between lizards that were closest to each other on that network. We measured the proportion of observations each 10 min, when two lizards were in close proximity to each other while active. Following Leu et al. (2010a), we considered that lizards within 2 m of each other at the same time (allowing for GPS precision errors of 6m for each device) were in contact, or had probably made, or would soon make a social contact. Lizards that made at least one contact had a connecting edge in the network, and the more frequently they contacted the greater the strength of that edge.

However, ticks are not normally directly transmitted from host to host, but require a period of time, after detaching from one host, to moult before they become infective to the next host. To account for this lag phase between two hosts, a third, asynchronous refuge use network has been developed to describe a more biologically plausible transmission pathway (Leu et. al. 2010b; Wohlfiel et. al. 2013). Lizards use multiple refuges within their home ranges, and often use a different refuge each day (Leu et al. 2010b). This network model assumes that ticks detach when their host lizard is in a refuge, that they moult to the next life

cycle stage there, and then can infect another host that uses the same refuge at a later time. It assumes a time window available for transmission that starts after a detached tick moulted into the next infective stage and ends when the tick dies due to desiccation while still waiting for a host. Using empirically observed life history parameters for *B. hydrosauri* we have previously suggested a realistic time window in this network to be from 11 – 24 days after detachment (Leu et. al. 2010b; Wohlfiel et. al. 2013). This network model assumes higher infection risk for lizards that more often use refuges that have previously been used by other lizards. In the current study we derived an asynchronous refuge use network from the GPS locations of overnight refuge sites, the last GPS location for the day (Kerr et. al 2004a; Leu et al. 2010b). A pair of lizards with asynchronous refuge sharing within the infective time window was given a directional network edge connection (from the first user to the second user), and the edge weight was derived from the number of refuge use sharing nights.

2.5 Calculating network distances

Our hypothesis was that ticks that were more closely related would be found on lizards closer to each other on a transmission network, and our question was which of the three network models best explained the genetic pattern of tick infestations. To address this question we calculated the network distance between each pair of lizards on each of the three networks, separately in each of the two years. For the spatial proximity network we used the actual geographic distance between lizard host home range centres. Each pair of lizards was directly connected, but with different distances apart on this network. Distance between pairs of individuals on a more conventional network is normally measured by calculating path-length between two nodes, as the number of edges along the shortest path between those two nodes. We calculated the path-length between each pair of lizards for both the social contact

network and the asynchronous refuge sharing network in this way. Those path lengths can be weighted by edge strength, but in our analyses we present results from the simpler matrices derived from unweighted path lengths between each pair of lizards.

2.6 Statistical analysis

We examined how patterns of tick relatedness were influenced by the distances between their lizard hosts in two ways. First we asked if relatedness of adult ticks was higher if they were located on the same than on a different lizard host. This analysis was to confirm a previously reported finding of this pattern (Guzinski et al. 2009). For each year separately, we calculated the difference in mean relatedness between pairs of ticks collected from the same lizard (although not necessarily at the same time) and pairs of ticks on different lizards. In cases where more than on adult tick was collected from a lizard, their genotypes contributed to pairwise relatedness estimates both with ticks on the same lizard and with ticks on different lizards. . We performed a randomization test to determine if the observed difference in relatedness was significantly greater than expected by chance. To do this we randomized which ticks were on each lizard, while retaining the structure of the dataset by keeping the number of ticks on each lizard constant. We performed the randomization test with 1000 permutations, using customized script in R version 3.1.1 (R Core Team, 2014).

Additionally, we used COLONY, following the default options of the program (Jones and Wang, 2009) to infer full sibship among adult ticks collected in each year. In particular, we documented the frequency of occurrence of two or more full sib adult ticks on the same host lizard. We used all adult ticks sampled in each year (2010, n = 54; 2011, n = 58) to establish population allele frequencies, taking family structure into account. Then we analysed separately each lizard with more than two ticks (2010, n = 16 lizards, 2011, n = 13

lizards) using the allele frequencies to derive the probability (0 – 1) of inclusion in a full sib group among co-attached ticks on each lizard.

Second, we asked how lizard network connections shaped the genetic relatedness patterns of ticks that were on different lizards. Our three alternative transmission models, and the derived network distances between each pair of lizards used data from all of the tracked lizards, independent of whether or not they had attached ticks. Then our analyses, focused on those distances between pairs of lizards that had adult *B. hydrosauri* ticks attached. Again in each year separately, we compared the derived matrices of network distances (on each of the three networks) between all pairs of tick carrying lizards, and the matrix of mean relatedness values of the ticks on those lizards. Our hypothesis was that lizards that were closer to each other on a network would carry ticks that were more highly related to each other. Because some lizards had more than one tick, we calculated for each pair of lizards (and used in the analyses) the average genetic relatedness from all of the pair-wise relatedness values between ticks on the two different lizards.

More specifically we asked which of the three network models best explained the patterns of tick relatedness among lizards. We did this, separately for data from each year, by comparing the path length matrices derived from each network model against the matrix of mean tick relatedness. For these comparisons of matrices we used a multivariate Multiple Regression Quadratic Assignment Procedure (MRQAP) with Double Semi-Partialing (DSP) following Dekker et al. (2007). MRQAP analysis was performed using 10,000 permutations with the function mrqap.dsp in the package asnipe (Farine, 2013) in R (R Core Team, 2014). This analysis assesses how strongly each of several independent matrices (in this case the three path length matrices) is associated with a dependent matrix (the mean tick relatedness matrix). It determines the significance of the relationship of each independent matrix while

controlling for the influence of the others by using a semi-partialing approach. In this approach the residuals from a regression between the focal matrix and the other independent matrices are used to assess the relationship between each independent matrix and the dependent matrix. This means that related path length matrices may cancel each other out through the partialing process. Although the MRQAP indicates how strongly associated the matrices are, it does not identify which independent matrix best explains the patterns of tick relatedness. To derive that, we also performed a separate univariate Quadratic Assignment Procedure (QAP) for each independent path length matrix, to test the influence of each separately on the mean tick relatedness matrix. This analysis also produces R^2 values to assess the fit of each matrix to the patterns of tick relatedness. Higher R^2 values imply a better fit.

3. Results

Ticks from the same lizard were significantly more related to each other than were ticks from different lizards in both years (2010: mean difference in relatedness (observed) = 0.323, mean difference in relatedness (randomised) = 0.004 (95% CI, -0.149 – 0.152), $P < 0.001$; 2011: mean difference in relatedness (observed) = 0.148, mean difference in relatedness (randomised) = -0.002 (95% CI = -0.122 – 0.122), $P = 0.029$) (Fig 1).

In 2010, 16 lizards had two or more ticks collected from them, and sibship analysis of these ticks showed that 13 lizards had (2-5) ticks with a high probability of inclusion in the same full sib group. Seven lizards had some adult ticks from each of two different full sib groups.

In 2011, 13 lizards had two or more ticks collected from them, and five of those lizards had (2-4) ticks from the same full sib groups. Seven of the 13 lizards had adult ticks from two different full sib groups and one lizard had three full sib groups of adult ticks (see appendix 1).

The multivariate MRQAP analyses (Table 1) showed that, when the effects of other network matrices were accounted for, the matrix of genetic distances between the ticks on pairs of lizards was significantly related to the distances between those lizards along only one of the three alternative networks in each year. However in each year it was a different network; in 2010 the shared refuge network, and in 2011 the spatial proximity network (Table 1). The univariate QAP showed each distance matrix was significantly related to the tick genetic distance matrix when the effects of the other matrices were not accounted for, but confirmed the network matrices with the highest R^2 values were the same in each year as those identified in the MRQAP analyses (Table 1).

Mean tick relatedness decreased with increasing path length along the refuge sharing network in 2010 (Fig 2), and with increasing distance between lizard home range centres in both years (Fig 3). In each year the social contact network had relatively poor power to predict tick relatedness.

4. Discussion

We found two clear and significant results. The first was that adult ticks were more related to each other when they were collected from the same host, than when collected from different hosts. This was also reflected in the high number of full sibships inferred among co-attached adult ticks. This result confirmed a previously reported result from the same tick species on the same host in a larger geographic range (Guzinski et al. 2009). The previous study included host lizards sampled over a 29 km transect, while the current study considered the spatial patterns of tick relatedness over a much smaller area, and at a finer spatial scale. Because we sampled all of the hosts living in our study area, and still found higher relatedness among ticks on the same host than on different hosts, even on adjacent lizards, our results suggest that many sibling ticks from the same egg clutch disperse very little during their three-host life cycle.

The second result was that when adult ticks were on different lizards they had higher relatedness if those lizards had shorter network path lengths connecting them on all of the three network structures we explored. The strongest trends were for mean tick relatedness to decline with increased path length along the shared refuge network in 2010, and with increased distance between home range centres in 2011. A broad spatial pattern of declining tick relatedness with greater spatial separation of hosts has previously been reported by Guzinski *et al*. (2009) although again at a larger spatial scale. What we report newly in this paper is an exploration of alternative transmission pathways of the parasites among individual hosts at a fine spatial scale. Specifically, in each year, the social network derived from social contacts between pairs of lizards was a relatively poor predictor of the genetic relatedness of their attached ticks, compared to other networks.

We previously explained the spatial pattern of genetic relatedness of parasitic ticks on sleepy lizards by a "ripple" model (Guzinski *et al*. 2009). This descriptive model assumed individual lizards used multiple refuges (Kerr *et al*. 2003), and that ticks are most likely to survive while waiting for another host if they had detached in a lizard refuge. This is because of the high mortality of exposed ticks from predation (Bull *et al*. 1988, Dawes-Gromadzki & Bull 1997a, 1997b) and desiccation (Chilton & Bull 1993). When an engorged female tick has detached from a host and lays eggs in a lizard refuge, a clutch of hatched sibling larvae will wait as an aggregated group for another lizard host (Petney & Bull 1981), and many of those related tick larvae will then attach as a group to the first host that next enters the refuge. However, in our semi-arid study area, the hot, dry spring and summer conditions, when the lizard are most active, can cause rapid dehydration and mortality particularly for the larval stages of this tick (Chilton et al. 2000). Thus clutches of larvae will either have a high success with many siblings attaching to a single host, if that host enters the refuge soon after the ticks become infective, or they will all die if they have to wait too long before the next lizard uses the refuge (Guzinski et al. 2009).

In the ripple model, similar processes occur after engorged larvae and then nymphs detach, moult and wait for the next host, so that, in a landscape of high tick mortality, the genetic pattern is set by the rare success of a small subset of reproductive females whose progeny have spread (in a ripple) across the home ranges of three successive hosts (Guzinski et al. 2009). However, because sleepy lizards have, within their overall home ranges, core areas that are rarely encroached by conspecific lizards of the same sex (Kerr and Bull 2006), it is likely that, for many ticks, the next host will be the same host individual as previously, and that self-infection will be common. Indeed, Leu et al. (2010b) reported that self infection risk was more strongly correlated with empirically observed tick loads than with infection risk from refuge sharing with other lizards. An outcome of the model is that ticks on the

same host or on spatially adjacent hosts are likely to be more highly related to each other than are ticks on different hosts or from more spatially separated hosts.

In this respect the results of our current study have confirmed the insights of Guzinski et al. (2009) although at a finer spatial scale. However our results have also addressed a new question about the mode of transmission of these ectoparasitic ticks. We compared three alternative network models of tick transmission, and reported which of them best explained the observed patterns of mean tick relatedness among lizard hosts. We expected that ticks would be more closely related to each other if they were more closely connected along actual transmission pathways. Our analysis identified a different pathway that best explained the pattern in each of two years.

Additionally, the analysis of genotypes and tick relatedness patterns has provided an important new insight, that networks derived from social contacts between lizards are poorer predictors of the tick relatedness patterns than are the other two networks. Although there is the opportunity for host switching by ticks when two lizards make body contact, often for prolonged periods when pairs of lizards share refuges together, our results , suggest that ticks are rarely transmitted from one lizard host to the next by direct contact. Although this may seem a rather complex way to reach this simple conclusion, it is a check on the reliability of the analyses, and shows the potential power to differentiate among more subtle transmission pathways in cases where we have less information about the transmission dynamics.

Because we analysed relatedness patterns among adult ticks that are three hosts (and two transmission events) from where they initially aggregated with their larval siblings, it is possible that some of the genetic signal of the transmission path has been diluted with overlapping “ripples” from different maternal sources. We may have derived stronger signals

if we had focussed on analysis of nymphal ticks which have had just one transmission event from the larval to the nymphal host. Indeed Guzinski *et al*. (2009) reported that the mean relatedness among ticks on the same host decreased from larvae to nymphs to adults. However, adult ticks were easy to sample, are generally more abundant on hosts because they can remain attached and unfed for long periods of time (Andrews and Bull 1980), and they still showed patterns that revealed aspects of transmission ecology.

Using infection patterns to differentiate among alternative models of transmission pathways, through different network structures, may prove to be a powerful future tool in parasite ecology where direct observations of transmission dynamics are rarely possible and where inferences must often be derived from snap-shot observations. It is worth noting, from our study, that each of the 'best fit' models in the univariate QAP analyses was also significant in the MR-QAP DSP analyses, when the influence of the other factors was accounted for. This means that in 2010 the importance of the shared refuge network remained strong. The effect of path length along this network was not confounded by spatial overlap of adjacent lizard home ranges, and its effect remained significant even when distance between home range centres was accounted for. Similarly, for 2011, the distance between host home range centres remained a significant predictor of genetic distance between ticks, even when the two other network effects were accounted for.

The difference between years in the best transmission pathways may be explained by changing ecological conditions. There was almost 40% more rain in 2011 than in 2010. In the drier year, desiccation may have been a greater threat to waiting ticks. In that dry year we suggest that more successful transmissions would have relied on ticks that had detached and survived inside the relatively sheltered lizard refuges, and the asynchronous refuge sharing

network best explained the patterns of infection. In 2011 more ticks may have survived after detachment in locations outside of lizard refuges, and persisted for long enough to infest other host lizards that simply used the same space as the original host. Here the spatial proximity network might have provided the best model for transmission. Alternatively lizard movement patterns, and the resulting network structures are known to vary among years with differing rainfall (Godfrey *et al*., 2013) and this may have influenced transmission dynamics. Although these are speculations that would require confirmation in other wet and dry years, our results suggest that a single transmission network for parasites and pathogens is not a realistic biological model across varying ecological conditions, but that transmission may occur along different structural networks in different circumstances. This has important implications for our understanding of pathogen and parasite transmission in natural systems, and how we model and make assumptions about pathogen transmission in wildlife populations. Those models need to incorporate temporally variable transmission dynamics as ecological conditions vary.

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Figures

Figure 1. Mean relatedness of ticks from the same (black symbols) and different (hollow symbols) lizards.

Figure 2. The relationship between the mean relatedness of ticks on different lizards in 2010 (black symbols) and 2011 (hollow symbols) and the path length along the Asynchronous refuge sharing

Figure 3. The relationship between the mean relatedness of ticks on different lizards in 2010 (black symbols) and 2011 (hollow symbols) and the geographic distances between the home range centres of those lizards.

Table 1 – Summary of QAP and MRQAP-DSP analyses of mean relatedness among ticks on lizards, and matrices describing the unweighted network distances between lizards in networks derived from spatial proximity, social contact and asynchronous refuge sharing.

	Univariate QAP			**Multivariate MR-QAP**	
	Coef	***P***	**R^2**	**Coef.**	***P***
2010 (n=26)					
Spatial Proximity	-0.0006	**0.004**	0.027	2.08e-5	0.949
Social Contact	-0.124	**<0.001**	0.036	-0.035	0.548
Asynchronous refuge sharing	-0.115	**<0.001**	0.049	-0.096	**0.005**
2011 (n=23)					
Spatial Proximity	-0.0009	**<0.001**	0.122	-0.001	**<0.001**
Social Contact	-0.116	**0.031**	0.009	0.114	0.065
Asynchronous refuge sharing	-0.061	**<0.001**	0.060	0.013	0.646

Figure 1

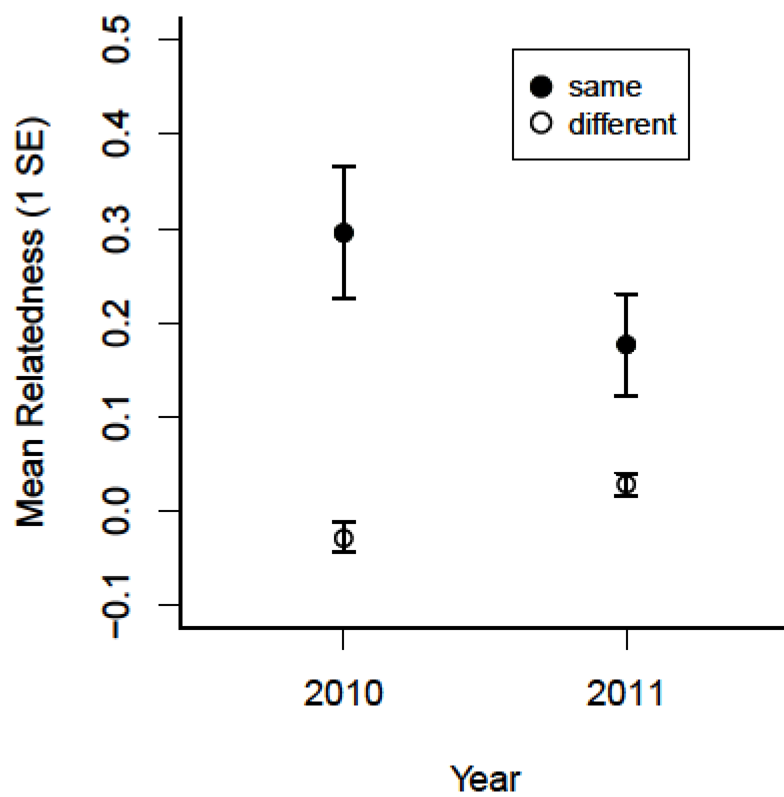


Figure 2

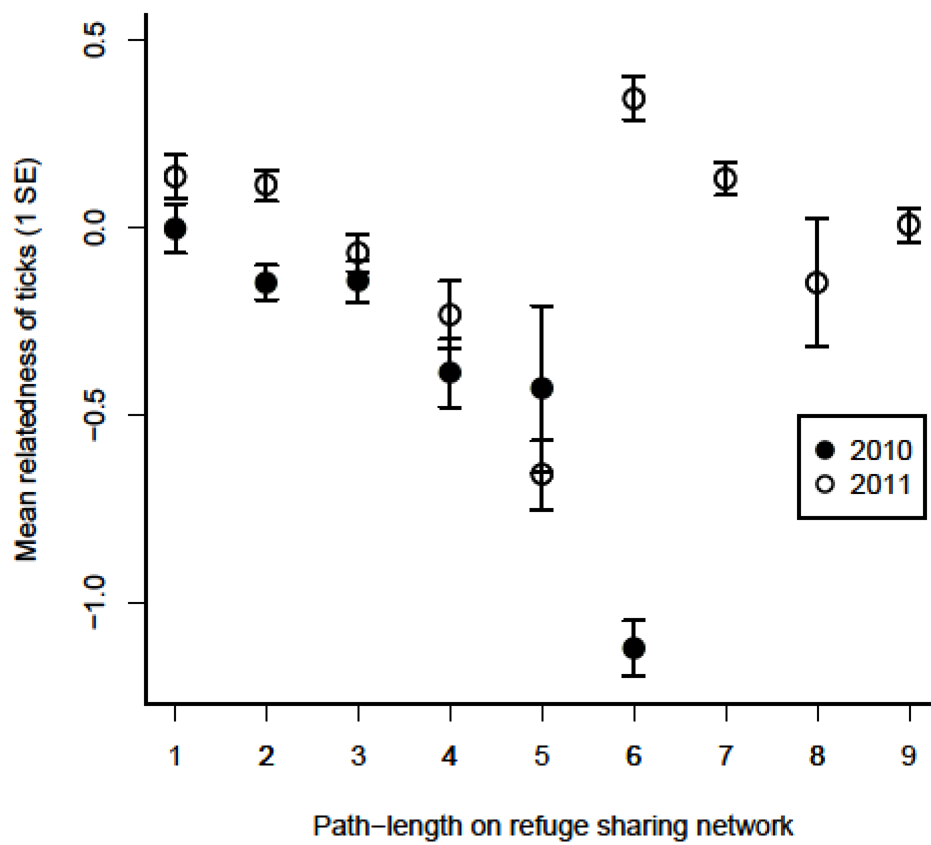
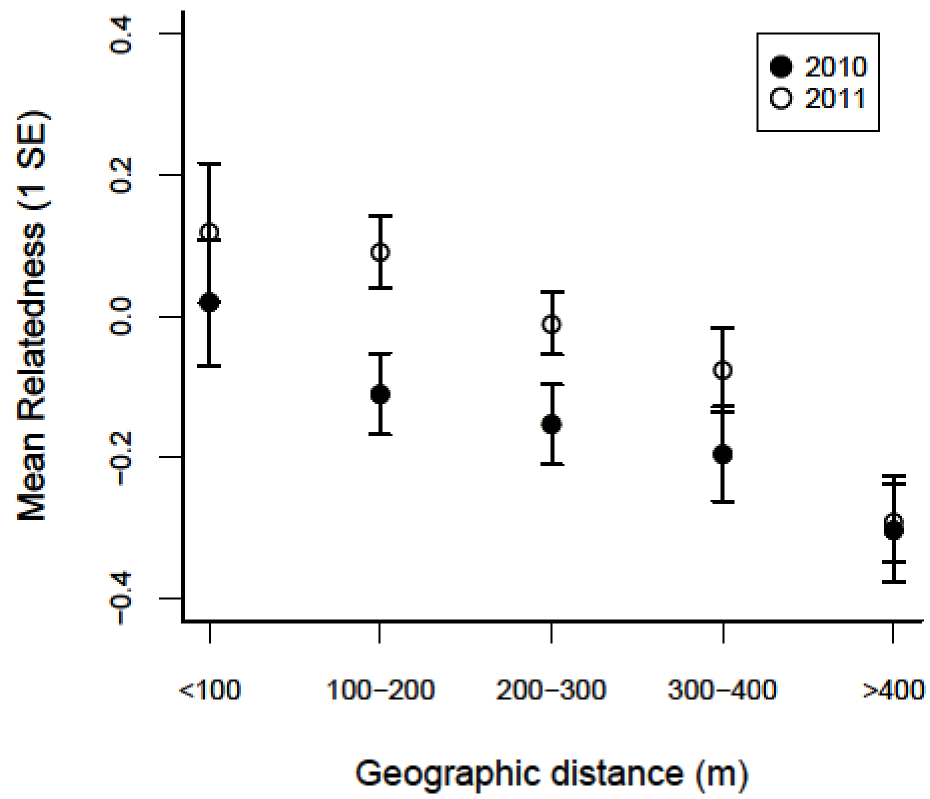


Figure 3



Appendix 1: Full sibship groups on lizards with more than two ticks. Number of individuals in each full sib group (probability of inclusion in the full sib group). Using the updated allele frequency of all background ticks that year (2010 n = 54, 2011 n= 58).

2010 Lizards (n= 16)	**Sib group 1**	**Sib group 2**	**2011 Lizards (n= 13)**	**Sib group 1**	**Sib group 2**	**Sib group 3**
2368	3 (0.429)		9372	4 (0.413)		
3549	2 (0.569)		9390	2 (0.523)	2 (0.730)	
9372	2 (0.460)		10827	2 (0.703)	1 (1.000)	
9390	3 (0.407)	2 (0.799)	12847	2 (0.754)		
10827	2 (0.695)	2 (0.462)	13535	2 (0.509)		
11157	2 (0.624)		40013	4 (0.138)	1 (1.000)	
11885	2 (0.841)	1 (1.000)	40014	2 (0.345)	1 (1.000)	
12434	2 (0.625)		40019	2 (0.366)	1 (1.000)	
12847	5 (0.270)		40022	2 (0.569)	1 (1.000)	
12893	2 (0.558)		40070	2 (0.628)		
40009	2 (0.572)		40100	2 (0.406)	1 (1.000)	
40012	2 (0.408)	1 (1.000)	40101	3 (0.278)	4 (0.135)	1 (1.000)
40040	1 (1.000)	1 (1.000)	40102	2 (0.558)		
40070	1 (1.000)	1 (1.000)				
40074	2 (0.601)					
40101	2 (0.796)	1 (1.000)				

Chapter 5: Experimentally added pulses of tick infestation to test predicted transmission networks.

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The genetic loci developed in chapter 3 were used to distinguish between unique clutches attached to donor lizards and to identify the recipient lizards of these clutches. This chapter the major experimental component of the thesis and combines the use of genetics with network modelling to gain an understanding of the processes that influence parasite transmission.

Abstract

Networks models are being used in an increasingly predictive way to inform disease management, yet many of the assumptions underlying these models remain untested. Here we provide the first large-scale field-based experimental test of the role of networks in the transmission of a tick through a sleepy lizard (*Tiliqua rugosa*) population (n = 60). Genetically distinct clutches of larval ticks (*Bothriocroton hydrosauri*) were reared and then released onto 13 different individual lizards in two separate 'pulses' (October 2009, n = 5; April 2010, n = 8), and recaptured in the wider lizard population as adult ticks (in the spring-early summer of 2010 and 2011). Using genetic markers, we identified which 'donor' lizard the adult ticks originated from, to trace the transmission of the ticks through the lizard population. We asked whether transmission networks were able to predict which host individuals those ticks ended up on. We developed several alternative models that predicted that alternative types of contact were important to transmission, including a social network (based on inferred direct contacts), a refuge sharing network (based on lizards using the same refuge within an 'infectious' period), and the spatial proximity of lizards to each other. We tested each of these hypotheses using Exponential Random Graph Models (a statistical model that enables the analysis of network data) within an AIC model selection framework. In each of the two years, different networks (and different contact types) were ranked as better explanations of transmission patterns. For the October pulse, asynchronous refuge sharing networks were the strongest explanation of transmission patterns, where lizards that shared refuges more often with a donor lizard were more likely to receive ticks from them. In contrast, for the April pulse, the social network was the strongest explanation of transmission patterns, where lizards that were closer to a donor lizard in the social network were more likely to receive ticks from them. We suggest that either the timing of the pulses, or variation in environmental conditions between years could contribute to these differences. Overall, this

study provides a novel test of the influence of networks on parasite transmission, and highlights the complexity of host-parasite interactions.

Introduction

Networks are increasingly being used to understand the transmission of parasites and diseases (Craft et al., 2011; Hamede et al., 2012). These studies have provided deeper insights into the way host behaviour influences the transmission of parasites. Structural elements of networks, especially their 'connectedness' (or centrality), have frequently been found to be associated with infection patterns in wildlife populations (Godfrey et al., 2010; Leu et al., 2010a). Because of the recognized importance of accounting for the heterogeneities in contact patterns in disease transmission, networks are now playing a role in disease management, by enabling targeting of influential individuals for vaccination or culling (e.g. Rushmore et al. 2013). However, while these tools have provided useful insights into the transmission pathways of parasites and diseases, many of the assumptions that lie with their use remain untested.

An experimental approach provides a stronger insight into whether networks do indeed influence the transmission of parasites; but thus far experimental tests have been limited, particularly in field-based systems. Otterstatter et al. (2007) carried out experimental tests in small captive networks of bumblebees, and demonstrated that transmission occurred most rapidly in strongly connected networks. Similarly, Corner et al. (2007) showed using experimental infections in captive possums that the transmission of tuberculosis among possums was highest among possums that shared dens. However, carrying out large-scale field-based experiments to test the role of networks in parasite transmission is often logistically infeasible. In this current study, we utilize a lizard-tick host-parasite system to experimentally test the influence of networks on tick transmission.

The sleepy lizard (*Tiliqua rugosa*) is host to a three-host reptile-specific tick (*Bothriocroton hydrosauri*), with the sleepy lizard being the dominant host in this study system (see Bull et al. in press for further details on the host-parasite system). Previously the transmission of these ticks has been modelled using networks that represent opportunities for transmission occurring between lizards that share common refuges within a period when the ticks are seeking a new host (Leu et al. 2010a). Connectedness of individual lizards in these networks is predictive of tick load (Leu et al. 2010a), and this pattern is consistent in most years (Wohlfeil et al. 2013). However, other factors may also influence the transmission of ticks; here we utilize a hypothesis testing framework to determine if (a) lizard behaviour is associated tick transmission, and (b) what type of network best reflected the transmission of experimentally added ticks.

By rearing genetically distinct clutches of larval (1st stage) ticks and releasing them on different individual 'donor' lizards, we can trace the factors most influential in the transmission of these ticks by recapturing them as adults (3rd stage), and determining how far they have travelled, both in space, and also in context of the network positions between their lizard hosts. In this study, we asked which aspects of host behaviour were most influential to tick transmission, and tested the hypothesis that networks are influential in the transmission of ticks through lizard populations.

Methods

Study site

The study site was a 1.0 x 1.5 km area of mostly chenopod scrubland located near Bundey Bore Station (33°54'S, 139°20' E) in South Australia. It was previously described by Wohlfiel et al. (2013). The study was conducted over four years, 2008 - 2011, during the spring and early summer (September- December) of each year, when sleepy lizards are normally active. Lizards become mostly inactive outside of this time period (Kerr and Bull, 2006). The area has hot dry summers and cool wet winters with an average annual rainfall of about 250 mm.

Pulse clutch collection

In 2008 and 2009, we captured 13 sleepy lizards with attached, mated females of *B. hydrosauri* from various regions in South Australia that were 10 – 150 km away from the study area, and each more than 5 km from each other (Table 1 in Chapter 3). Previously Guzinski et al. (2008) had reported significant genetic differentiation at microsatellite DNA loci between these ticks on hosts 5 km apart. We transported lizards back to the animal house on the Flinders University campus and kept them at 25°C on a 12:12 photo-period. Lizards were housed in individual enclosures with shelter and ample food and water. The floor of each enclosure was a raised wire mesh, which allowed detached ticks to drop through the mesh and hide in towelling paper in the bottom of the pen. Engorged female ticks detached from their hosts after an average of 25 days, and were collected during twice daily inspections of the bottom of the pen. The females were then held in 50ml sample jars with gauze covering to prevent escape, in containers maintained at 85% humidity above a saturated potassium chloride solution, at 25°C and with a 12:12 photo-period. Egg laying

started an average 43 days after female detachment, and the first larvae hatched an average 41 days later. Female *B. hydrosauri* usually lay clutches of over 1,000 eggs (Chilton et al. 2000). From each batch of hatched larvae, 20 were retained to determine the genotypic characteristics of that clutch, allowing us to genotypically differentiate clutches from each other, and from naturally occurring, “background” ticks from the study area (refer to Chapter 4).

Pulse attachment

Table 1: This table shows each Clutch origin, donor lizard, the dates that the larvae were attached to the donor. The number of tick initially infected onto the lizard, the number of larvae seen on the lizard at time of release, the number of larvae seen dead or unattached in the lizard and the number of larvae which were unaccounted for (these may have been attached and not seen on the lizard or potentially eaten by the lizard)

Clutch	Pulse	Origin	Donor Lizard	Initial infection	Number seen attached	Dead or unattached in bag	Unaccounted for larvae
A/B1	Oct	Karoonda	11130	502	104	17	381
A/B2	Oct	Karoonda	12434	508	132	3	373
C	Oct	Mount Mary East	9532	436	63	174	199
D	April	Monarto	40009	250	106	51	93
E	April	Monarto	9390	252	81	47	124
F	Oct	Mount Mary East	40012	508	132	9	367
G	Oct	Karoonda	12847	481	103	41	337
H	April	Karoonda	10039	250	29	90	131
I	April	Karoonda	13535	258	74	81	103
J	April	Karoonda	9310	260	102	14	144
K	April	Karoonda	12030	257	95	2	160
L	April	Karoonda	9291	291	41	7	243
M	April	Karoonda	40015	257	58	3	196

On two occasions during the 2009 – 2010 spring and summer, in October 2009 and then once in April 2010, we took batches of hatched larvae from the genetically unique clutches, to attach to 13 lizards, one unique clutch per lizard, at the field site. Five clutches were added to five lizards in Oct 2009, and eight different clutches were added to eight lizards in April 2010. We called these attachments experimental pulses, and we called the lizards 'donors' because they donated later life stages of ticks from the experimental pulse to other lizards.

For the tick attachments, we captured individual donor lizards from refuges at the field site and held them overnight in individual sealed cotton bags with 250 larvae from their assigned clutch. The following morning we removed the lizard from its bag and released it to its previous refuge, and then counted all unattached larvae remaining in the bag to deduce the number attached. We also counted as many of the small larvae as we could observe under scales of each donor lizard, although we were not confident that this was the actual number attached because they are very small and hard to see. To ensure sufficient larvae had attached, in October, we repeated the tick attachment procedure to the same five donor lizards, two weeks later with another 250 larvae from the same larval clutch.

Following the second October 2009 attachment we were able to observe an average of 53 (±6.62SE) larvae attached to each of the five donor lizards; while the eight lizards in April 2010 each had an average of 73(±10.05SE) observed attached larvae. We assumed that the very fastest life cycle development might allow some of the October 2009 attached larval ticks to reach the adult stage by the end of the same season (by March or April 2010), and that those adults would then remain attached without feeding over the subsequent winter to be still attached in the spring of 2010 (Chilton & Bull 1993a). Other ticks from the October pulse, with longer delays from one life stage to the next (because of longer times waiting for

the next host), should appear as adult ticks on hosts in the following spring (Oct – Dec 2010). Thus we assumed that all ticks from the Oct 2009 pulse, that became attached adults, would be observed and collected by the end of the 2010 season, Similarly ticks from the April 2010 attachment would mostly delay development over winter and reach adult stages after the end of the 2010 collections, and be first detected and collected in the 2011 spring. Thus we assumed collections of adults of any experimentally added ticks were the products of the October pulse in 2010, and of the April pulse in 2011. Other ticks not identified belonging to a pulse were considered pre-existing 'background' ticks and were not included in this analysis.

GPS data and Post Pulse infection tick collection

The donor lizards were part of a larger population of 61 adult lizards resident in the study site. In each of the two years after the pulse addition, 2010 and 2011, we located all adult resident lizards in September, at the start of each season and attached data logger units to the dorsal surface of their tails, as previously described (Wohlfeil et. al., 2013). The units recorded synchronous GPS locations for all lizards that were active, every 10 min, over the following 4–5 months (115 days in 2010; 81 days in 2011), and were used to derive tick transmission networks as described below. Units on one lizard in 2010, and on two lizards in 2011 malfunctioned after less than 30 days recording, and those lizards were excluded from the network analysis. The units included a radio transmitter so that we could locate each lizard every 12 days to download data and change batteries in the data logger units. At each data download in 2010, and at three data downloads in 2011 (the first of each month), we removed all adult male and female *Bothriocroton hydrosauri* ticks and preserved them in ethanol for subsequent DNA analysis. As described above we anticipated that some of the ticks added as larval pulses would be detected as adults in all of these collections. Our aim

was to identify, from the genotype, those adult ticks that were derived from the larval pulse additions, and the clutch they came from, and thus the donor lizard they were attached to as larvae.

DNA Methods

We extracted the DNA from ticks using the Gentra Puregene extraction kit for tissue samples (Qiagen, Doncaster, Australia) following the manufacturer's protocol, with added Protonase K (20mg/ml) and Glycogen (20mg/ml). For 2010 collections we extracted DNA from tick samples using the Gentra Puregene extraction kit for tissue samples (Qiagen, Doncaster, Australia) following the manufacturer's protocol, with added Protonase K (20mg/ml) and Glycogen (20mg/ml). For 2011 samples we used the CCDB Glass Fibre Plate DNA extraction method (Ivanova et. al., 2006), modified for use with the vacuum manifold and final elution centrifugation.

All PCR amplifications for the clutch mothers were conducted in Palm-Cycler™ (Corbett Life Science, Sydney Australia) in a total volume of 10µl. Each reaction consisted of 1X AmpliTaq Gold Buffer, 3mM MgCl2, 0.2mM of each dNTPs, 400 nM of each primer, 0.5 U of AmpliTaq Gold enzyme, 0.2 mg/ml of BSA (Roche) and 40NMoles. PCR amplifications for the larvae, and for the 2010 and 2011 adult tick collections were carried out in the Mastercycler Pro S thermal cycler at a volume of 10µl. Each reaction was made using 1X MRT buffer, 400 nM of each primer, 0.2 U of Immolase (*Bioline)* and 40 nmoles of neat DNA. The cycling conditions were: initial denaturation 94° for 10mins, followed by 34 cycles at 94°C for 45 secs, annealing temperature of (54° (Bhy12), 52° (Bhy27 and Bohy03), 50°(Bhy28)) for 45 secs and extension of 72°C for 1 min, followed by an final extension of 72°C for 30mins and 25°C for 1min.

Pulse identification and assignment of pulse ticks

DNA was extracted from 20 single larvae from each of the 13 pulse clutches, and from all of the adult ticks collected from the field sampling at the study site. Samples which were successful in amplifying were sent to AGRF for genotyping at four microsatellite DNA loci (refer to methods chapter) by Fragment Analysis, Capillary Separation. Samples were then sized and scored using the program GeneMapper version 3.7 (*Applied Biosystems*). We established unique combinations of alleles at the four loci that allowed us to distinguish between background natural infestations and experimentally added ticks, and in the latter group to distinguish among the 13 clutches (see Chapter 3, Tables 10-13).

Modeling observed tick transmission: connections between donor and final hosts

Among those adult ticks that we could identify as derived from a specific experimentally added larval clutch, we then had empirically derived transmission pathways from the original donor to the final host the adult tick was located on. We used an unweighted, bipartite network approach to represent the actual (observed) transmission pathways, as an M x N matrix. The donor lizards were in the rows, and all of the lizards that subsequently received one or more adult ticks from those donors were in the columns. If there was one (or more) tick transmissions from the experimental pulse from a donor to a recipient lizard, a value of 1 was assigned to that donor-recipient pair. If there was no transmission that pair had a value of 0. A separate network was developed for each 'pulse' of ticks that were released (corresponding to adults collected in the two different years of the study (2010 and 2011)). These empirically derived networks were compared with equivalent networks based on lizard behaviour or spatial proximity to determine which network model most closely predicted the observed patterns of tick transmission.

Our sample did not capture all tick transmission events. Some ticks were likely transmitted to host lizards outside of our sample area, particularly if the donor lizards were on the edges of the sampling area, and some ticks that were collected could not be definitively assigned to a particular clutch. However, our aim was not to estimate rates of tick transmission, but to derive a representative sample of tick transmission pathways in our lizard population, and to determine how closely those actual pathways were represented by various descriptive models of tick transmission. Since all tracked hosts were sampled equally often, we assumed we had an unbiased sample of transmission pathways.

We asked, what processes best explained the likelihood of a lizard receiving a tick from a donor lizard? We proposed four alternative transmission pathway hypotheses. First, ticks may be passed from host to host during host social contact, and the host contact social network is the transmission pathway. Second, ticks may detach in a lizard refuge and then attach to another lizard using the same refuge some time later, and asynchronous refuge sharing networks predict tick transmission. Third, if ticks are less reliant on refuges for their development and transmission, then spatial proximity of hosts may be a better predictor of transmission. Finally, differences among individual hosts in their behaviour may influence the likelihood of receiving donor-ticks. In this fourth hypothesis, behavioural attributes such as home range size or the number of refuges used by a lizard might better explain tick transmission. We considered each of these four hypotheses and combinations of them, using a model selection approach.

Contact social network construction

We developed a social network to represent the social relationships among lizards, and their potential influence on tick transmission. The contact social network was developed using the GPS location data to derive the proportion of time each dyadic combination of

lizards spent in proximity, while they were both active. Following Leu et al. (2010b), we considered that two lizards within 2 m of each other at the same time, allowing for errors in GPS precision, had probably made, or would soon make a social contact. We calculated the Simple Ratio Index (SRI) for each dyad, as the number of recorded contacts divided by the total number of observations when both lizards were active. The SRI formed weighted edges in the social network. Edges were bi-directional, because we could not infer the direction of the social contact. We developed a separate social network for each year of the study.

Asynchronous refuge use networks

We similarly developed networks for each of 2010 and 2011, based on probable transmission pathways for ticks detaching from one lizard host, moulting, and then attaching to the next host that uses the same refuge (Leu et al. 2010a; Wohlfiel et al. 2013). This network model, first described by Leu et al. (2010a), assumes that ticks detach when their host lizard is in a refuge, that they moult to the next life cycle stage there, and then can infect another host that uses the same refuge at a later time. We subsequently call this the transmission network. The model assumes there is an infection time window within the refuge that starts after the tick has moulted and ends if the tick dies while still waiting for a host. We have previously used a time window of 11-24 days, based on previous studies of *B. hydrosauri* average time to moult (11 days) and average off host survival time (13 days) for larvae and nymphs at 30°C (Chilton and Bull 1993). The network was derived from the GPS locations of overnight refuge sites, close to the last active location for the day. Network links were formed when one lizard used a refuge, and another lizard used the same refuge on a subsequent day within the infectious time window (Leu et al. 2010a; Wohlfiel et al. 2013).

Measuring network connections between donor and recipient lizards.

Each tick has three hosts in its life cycle from larva to nymph to adult. In our empirical data set we only have the first and the third host to describe possible network pathways. For each of the two network models, we considered two different network representations of a transmission pathway; adjacency and distance. We used an adjacency matrix to represent direct relationships between lizards, where each edge represented the raw edge weight (which may be zero) between a donor-recipient pair. We used a distance matrix to represent indirect relationships between lizards, where each edge represented the smallest number of network edges (an unweighted measure of distance) between the donor-recipient pair. Because there was an additional (but unmeasured) host involved in transmission, we expected the distance matrices to better explain transmission than the adjacency matrices.

Behavioural attributes

We also calculated two attributes of behaviour for each individual lizard; home range area and the number of refuges used. Using the GPS locations derived for each individual, home range area (m^2) was calculated using 95% Minimum Convex Polygons. Overnight refuges were identified as described above for the refuge sharing networks. We counted the number of spatially distinct overnight refuges, again allowing for GPS precision as in Leu et al. (2010a), that a lizard used during an entire activity season. While we may have underestimated refuge numbers, by counting different refuges that were close together, as the same, we applied the same potential bias to all surveyed lizards.

Spatial proximity

To examine how spatial proximity influenced tick transmission, we calculated the geographic distance between each donor-recipient pair of lizards. The average location of a lizard was identified as the centre point of its 95% Minimum Convex Polygon home range, and the distance between the centres of each pair of home ranges was used to represent spatial proximity.

Exponential Random Graph Models

Then, we used a network modeling approach to examine what factors were most influential to the overall transmission of experimentally added tick pulses. Exponential Random Graph Models are a statistical model similar to generalized linear models (GLMs) that enable the analysis of network data (Goodreau et al. 2008). The presence of edges in the network can be modeled against different predictor variables, including node-based variables, edge-based variables, and attributes of the network itself.

We utilized a model selection approach to modeling the observed transmission network to identify which set of predictor variables best explained which lizards received ticks from donor lizards in our study population. Specifically, we were interested in whether network predictors (based on asynchronous refuge sharing or social contact) were better predictors of tick transmission than spatial covariates (spatial proximity or home range size). We included each edge-based covariate (transmission network adjacency and distance, social network adjacency and distance, and geographic distance) and node-based covariate (home range area and number of refuges used) separately in univariate models to determine which of these individual predictors best explained tick transmission. We also considered the combined importance of both direct (adjacency in the social or transmission network) and indirect (distance between lizards in space or on the transmission network) relationships

between lizards in multivariate models to explore the combined importance of these variables (i.e. perhaps one single factor does not explain tick transmission entirely).

To evaluate the goodness of fit of each of our models, we used two approaches; Aikaike's Information Criteria (AIC) model selection and goodness of fit assessment from model simulations. AIC values can be estimated from Exponential Random Graph Models, however these are not normally used because of the inclusion of dyadic dependent terms in the models. This means that only pseudo-likelihood can be estimated. However, because our models were dyadic independent (that is, we were not interested in the properties of the network itself in influencing its structure), the pseudo-likelihood is equivalent to the likelihood (Hunter et al. 2007), and AIC values should be informative for model selection. We developed an AIC model selection table for each set of models for each year.

To assess the goodness of fit of each of our models we calculated the proportion of edges that were correctly assigned during a simulation of the Exponential Random Graph Model. In each simulation, the probability of edges occurring between a pair of lizards is based on the covariate(s) included in the model. We compared the networks generated from the simulations to the observed donor-recipient networks edges, and calculated the proportion of edges that were correctly assigned. If the model is a good fit to the data, then simulations of that model should yield more accurate edge assignments. Simulations were constrained for the number of edges and the 'degree' of donors; ie the number of other lizards that a donor was connected to in the network. That meant that each donor gave the same number of ticks to recipients as in the observed network. Each model was simulated 10,000 times. The mean proportion and 95% confidence intervals were calculated for each set of simulated models to determine how well each model performed. We included these values alongside our AIC values to enable comparisons of model fit using the different criteria.

Because network data are not independent, we also used a permutation test to determine the significance of the network parameters influencing tick transmission. Donor-recipient relationships were shuffled (so that the same lizards received ticks, but from different donors), and in each permutation, the Exponential Random Graph Model was recalculated, and the coefficients from the randomized models were compared to the observed model. The permutation was run 1000 times for each Exponential Random Graph Model. A two-sided p-value was calculated from the number of randomized coefficients that exceeded the observed model coefficient.

Results

A total of 363 adult ticks were collected from lizards in the study population across the two years (Table 2). Of these, 112 ticks could each be definitively assigned to one of the 13 clutches of added ticks and to one of the 13 donor lizards (Table 3). Details of tick assignment to clutches are detailed in Chapter 3. Of those ticks, 48 came from the October additions (two-thirds of which were collected in 2010), and 64 from the April additions (with more than 80% collected in 2011) (Table 3).

Table 2. A summary of adult ticks removed in 2010 and 2011. *N* is the total number of lizards surveyed, with *n* being the number of lizards the ticks were removed from. Each month the number of adult ticks, the mean and standard error of ticks removed from n total of lizards and the maximum number of ticks removed from lizards.

Month	Total ticks removed (*n*=)	Mean ticks removed (SE)	Max ticks
2010 (N=60)			
Sep	79 (36)	2.194 (0.236)	7
Oct	23 (23)	1.643 (0.156)	4
Nov	22 (15)	1.467 (0.132)	4
Dec	32 (19)	1.684 (0.160)	4
Total	156 (**48**)	3.250 (0.364)	10
2011 (N=59)			
Sep	108 (34)	3.176 (0.436)	10
Oct	59 (27)	2.185 (0.300)	6
Nov	40 (23)	1.739 (0.239)	6
Total	207 (**53**)	3.906 (0.536)	18

Table 3. A summary of ticks collected from lizards in 2010 and 2011, including which ticks were identified as belonging to one of the two pulses.

Sampling Date	Sep-10	Oct-10	Nov-10	Dec-10	Sep-11	Oct-11	Nov-11
No. of lizards Total	60	60	60	60	59	59	59
No. of lizards with tick	36	23	15	19	34	27	23
No. of adult tick	79	23	22	32	108	59	40
No. lizards with Pulse tick	11	5	8	6	20	12	11
No. lizards with Background tick	21	10	9	5	16	13	9
Tick from Background	28	11	10	5	31	17	10
Tick from Oct 2009 Pulse	15	4	8	5	11	4	1
Tick from April 2010 Pulse	5	1	0	2	30	14	12

Relationships between donor and recipient lizards

For ticks released in the October (2010) pulse, the model including an effect of transmission network adjacency and geographic distance was the best-fitting model, and was moderately supported (ω_i = 0.443, Table 4). A model including transmission network adjacency and transmission network distance was half as likely as the top model (ω_i = 0.214). The model

simulations demonstrated that both models predicted about 28% of tick assignments correctly (Table 4). Adjacency in the transmission network appeared in all other models that were ranked above the null model (Table 4). Recipient lizards that had stronger edge weights to donor lizards were more likely to receive ticks from them than recipient lizards that had weaker (or no) edges to donor lizards (Figure 1). Similarly, lizards that were closer to a donor lizard in geographic space or in the transmission network were more likely to receive a tick from them. Permutation tests supported the significance of these relationships, with adjacency in the transmission network ($coef_{Obs}$ = 0.10, $coef_{Rand}$ = 0.01 (95% CI = 0 – 0.01), P = 0.004), distance in the transmission network ($coef_{Obs}$ = -0.37, $coef_{Rand}$ = 0.07 (95% CI = -0.12 – 0.26), $P < 0.001$), and geographic distance ($coef_{Obs}$ = -0.0035, $coef_{Rand}$ = 0.0001 (95% CI = -0.0015 – 0.0015), P = 0.004) all having significant effects in the Exponential Random Graph Models.

For ticks released in 2011, the top-ranking model included both an effect of social adjacency and distance, and this model was moderately supported (ω_i =0.425, Table 4). A model including adjacency in the transmission network and geographic distance was half as likely as the top model (ω_i =0.185). Geographic distance was the only variable that was included in most (5/7) models that were ranked above the null model. The simulations supported the strength of the top models in their ability to predict the destination of just over 20% of donor ticks. Despite models combining adjacency and distance ranking highly in the model selection, only variables describing measures of distance were significant in the permutation tests (social distance: $coef_{Obs}$ =-1.73, $coef_{Rand}$ = -0.48 (95% CI = -0.65 - -0.32), $P < 0.001$; geographic distance: $coef_{Obs}$ = -0.01, $coef_{Rand}$ = -0.002 (95% CI = - 0.003 - -0.0009), $P < 0.001$). Both measures of distance had a negative influence on the likelihood of receiving ticks from a donor lizard (Figure 1). Adjacency measures included in the top 2 models were not significant in permutation tests (social adjacency: $coef_{Obs}$ = 4.6, $coef_{Rand}$ = 1.5 (95% CI =

-0.64 – 4.56), $P = 0.098$); transmission adjacency: $coef_{Obs} = 0.02$, $coef_{Rand} = 0.01$ (95% CI = -0.01 – 0.03), $P = 0.394$).

Table 4. Summary of model selection of Exponential Random Graph Models, comparing the fit using AIC values and model simulation (to derive goodness of fit) that compare alternative hypotheses about the factors influencing tick transmission. K is the number of parameters in the model, AIC is the Akaike Information Criteria, ΔAIC is the difference in AIC between the top model and all subsequent models, and ω_i is the Akaike weight of the model. Models within 2 AIC of the top model are bolded.

	Exponential Random Graph Model selection				Model simulations		
	K	AIC	ΔAIC	w_i	Prop^n matched	95% Conf. Intervals	
2010							
Refnet+geodist	**6**	**133.78**	**0.00**	**0.443**	**0.28**	**0.20**	**0.40**
Refnet+refdist	**6**	**135.23**	**1.45**	**0.214**	**0.28**	**0.20**	**0.40**
Refnet	**4**	**135.76**	**1.98**	**0.164**	**0.27**	**0.20**	**0.35**
Refnet+HRsize	6	136.89	3.11	0.093	0.27	0.20	0.35
Refnet+no.refuges	6	137.58	3.80	0.066	0.27	0.20	0.35
Null	2	162.12	28.35	0.00	0.08	0.00	0.20
2011							
Socnet+socdist	**6**	**220.75**	**00.00**	**0.425**	**0.21**	**0.11**	**0.31**
Refnet+geodist	**6**	**222.41**	**1.67**	**0.185**	**0.22**	**0.14**	**0.31**
Geodist+no.refuges	6	223.07	2.32	0.133	0.19	0.11	0.28
Socnet+geodist	6	223.99	3.24	0.084	0.21	0.14	0.31
Geodist	4	224.41	3.49	0.074	0.19	0.11	0.28
Geodist+HRsize	6	224.24	5.17	0.032	0.19	0.11	0.28
Socdist	4	226.31	5.56	0.026	0.16	0.08	0.25
Null	2	289.83	69.08	0.00	0.07	0.00	0.14

Geodist = geographic distance (m); HRsize = home range size (m^2); no.refuges = number of refuges used; Null = null model (no variables); Refnet = transmission network (adjacency); Refdist = transmission network (distance); Socnet = social network (adjacency); Socdist = social network (distance).

Discussion

This study is among the first to experimentally test the role of networks in the transmission of a parasite in a natural (field-based) host-parasite system. Our study showed that transmission networks (modeled as the asynchronous sharing of refuges by lizards) were able to predict tick transmission in one of the two years only. In the second year, other aspects of host ecology or behaviour were more highly ranked in the model selection. This study highlights the complexity of host-parasite interactions, and emphasizes the importance of considering alternative/additional routes of transmission in addition to networks in explaining parasite transmission.

Different networks were classed as the best explanation of where experimentally added ticks ended up in each of the two different years. In 2010, a model containing both transmission network adjacency and geographic distance was the most highly ranked model, while in 2011 a model containing both adjacency and distance in the social network was the most highly ranked. These results suggest that different factors were important in influencing transmission in the two different years. One of the key differences between the two years is that the pulses were released at different times. When the first pulse was released in October, lizards are active and moving around and beginning to seek out mates and feed. This is a period of high lizard activity and coincides with when lizard behaviour is being monitored (and consequently is represented using network models). In contrast, in the April pulse, lizards are starting to slow down because there is little food available, and lizards spend most of their time in their refuges. The release was carried out at this time, believing that attached ticks would overwinter on their hosts, and then when lizards emerged in the spring they would spread their ticks around (at a time when we were also measuring their behaviour). However, it is possible that tick development (and transmission) may have occurred overwinter, leading to transmission between lizard pairs that overwintered together. This may

explain the influence of distance in the social network on transmission in 2011; anecdotally, lizards captured after the mating season have been observed sharing refuges together.

Alternatively, there were differences in climate between years; 2011 had a higher annual rainfall than 2010. Ticks are highly susceptible to desiccation in the off-host environment, so environmental conditions are likely to have a significant influence on their ability to survive and spread. During drier years, tick movement and survival may be more dependent on being located within a suitable microclimate while in the off-host environment. In 2010 we observed a stronger influence of the refuge sharing network on tick transmission, which supports this hypothesis. During wetter years when more moisture is available, ticks may be less reliant on these microhabitats for their survival and able to move without relying on refuges for their survival and transmission (e.g. they may disperse more broadly through space). This would explain the high ranking of the geographic distance influencing tick transmission in most models for 2011. *B. hydrosauri* is adapted to live in a cooler climate, in drier conditions it is vulnerable to desiccation (Chilton & Bull 1993b) and high mortality of exposed ticks from predation (Bull *et al*. 1988, Dawes-Gromadzki & Bull 1997a, 1997b). Thus, climatic influences may shape the role the network has in tick transmission in different years.

This study provides a novel test of the influence of networks on parasite transmission. The results provide experimental evidence that lizard behaviour is influential in tick transmission in a lizard host; however it also highlights the complexity of host-parasite systems. In the study of giraffes, Vanderwaal et al. 2014, showed while the process could be difficult to observe directly, transmission could be tracked indirectly by identifying genetically unique strains within parasite populations and deducing that hosts carrying identical, or closely related strains are likely to represent cases of host to host transmissions Bull et al. (2012) is another example of the use of genetics and networks to invesgtie

transmission. Here, common genotypes of *Salmonella enterica* detected in cloacal swabs from the sleepy lizard, *Tiliqua rugosa*, were used. It was reported that pairs of lizards that shared genetic strains were more strongly connected to each other in the social network, but were no closer to each other spatially, than pairs of lizards that did not share *Salmonella* strains. By 'comparing the fit' to two alternative network transmission models, based on social contact and on home range overlap, they deduced that *Salmonella* was more likely to be transmitted by direct lizard to lizard contact than via some common non-lizard infection source (Bull et al. 2012).

Interactions between the host, parasite, and environment may shape what aspects of host behaviour are most important at different times of the year, and between years. There are many aspects to understanding transmission pathways, one of which requires further study is the investigation of individuals within a population, as there is evidence that this transmission pathway would be stronger if individual transmissions could be tracked.

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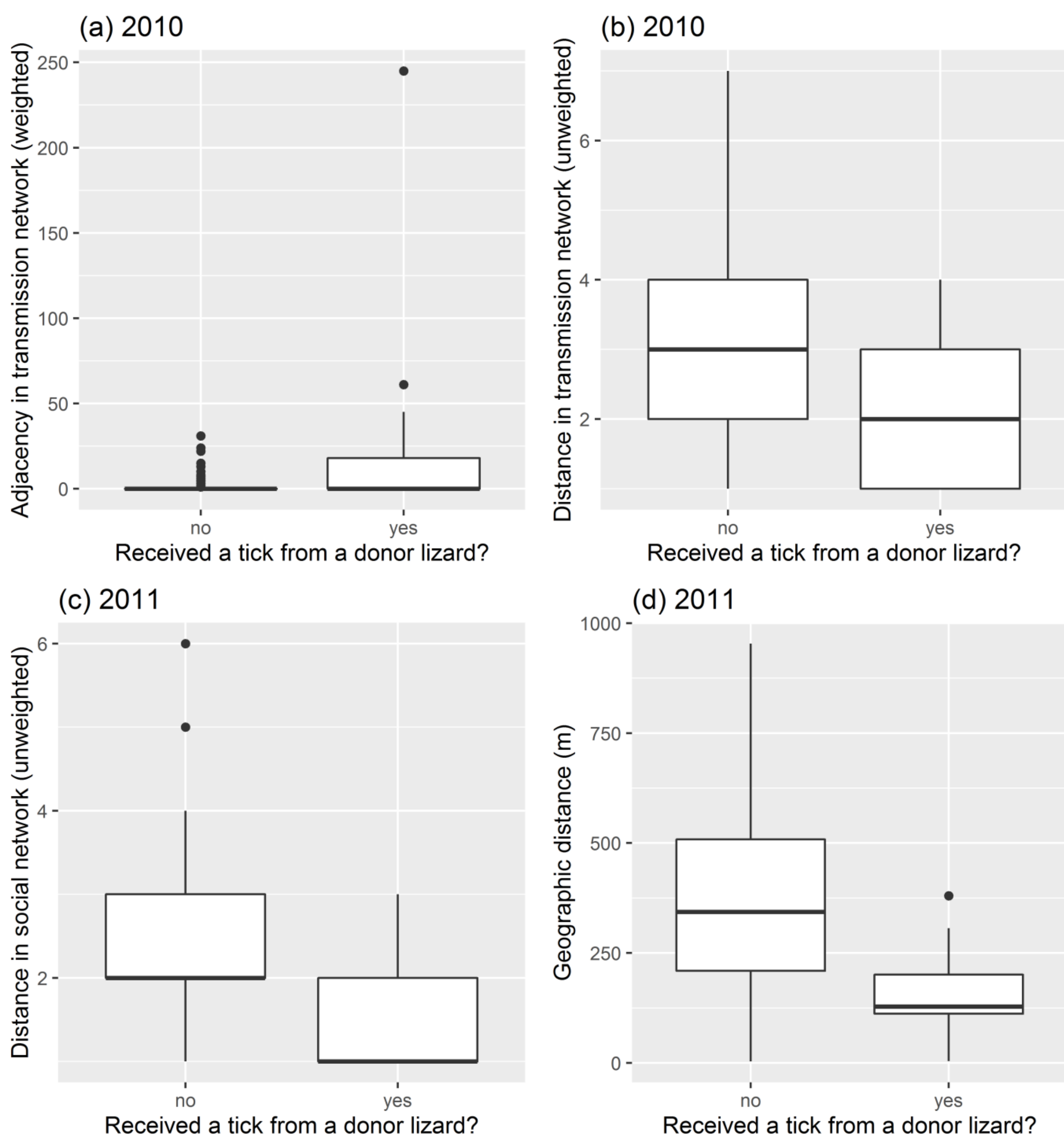
Figure 1: Boxplots portraying the results from the top ranking models for 2010 (a & b) and 2011 (c & d); (a) the median weight of adjacent edges in the transmission network, (b) the median distance between lizards in the transmission network, (c) the median distance between lizards in the social network, and (d) the median geographic distance (m) between lizard home range centres, compared between lizards that did or did not receive a tick from a donor lizard.



Appendix – Full list of models considered using Exponential Random Graph Model AIC model selection, including the type of model, type of contact that the model considered, the edge covariates and the node covariates.

Model type	Contact type	Edge covariates	Node covariates	Model number
Null model	None	-	-	1
Behaviour only	Refuge use	-	No. Refuges	2
	Space use	-	HRsize	3
Network only	Social contact	SocialAdjacency	-	4
		SocialDistance	-	5
	Refuge use	RefugeAdjacency	-	6
		RefugeDistance	-	7
	Space use	SpatialProximity	-	8
Network only (adjacency + distance)	Social contact	SocialAdjacency + SocialDistance	-	9
	Social + space use	SocialAdjacency + SpatialProximity	-	10
	Refuge use	RefugeAdjacency + RefugeDistance	-	11
	Refuge + space use	RefugeAdjacency + SpatialProximity	-	12
Network + behaviour	Social contact	SocialAdjacency	HRsize	13
		SocialDistance	HRsize	14
		SocialAdjacency	No. Refuges	15
		SocialDistance	No. Refuges	16
	Refuge use	RefugeAdjacency	HRsize	17
		RefugeDistance	HRsize	18
		RefugeAdjacency	No. Refuges	19
		RefugeDistance	No. Refuges	20
	Space use	SpatialProximity	HRsize	21
		SpatialProximity	No. Refuges	22

Chapter 6: General Discussion

The overall aim of this thesis was to investigate the dynamics and transmission relationships between a lizard host, the sleepy lizard (*Tiliqua rugosa)*, and its parasites, specifically ticks. I explored the relationship of asynchronous refuge sharing of the sleepy lizard and the ticks, *Amblyomma limbatum* and *Bothriocroton hydrosauri*. I developed microsatellite loci for *B. hydrosauri* and experimentally infected lizards with genetically distinguishable *B. hydrosauri* larvae to a) examine the dynamics of parasite transmission, b) understand the influence that different networks have on parasite transmission.

In **CHAPTER 2**, using detailed GPS data from a set of 45 lizards (season 1) and 60 lizards (season 2-4), I created long and short infection window transmission networks, based on asynchronous refuge sharing. This built on Leu at.al. (2010) by using a larger samples size over a longer period of time and examined two reptile tick species. The aim of this chapter was to test the robustness of transmission models and whether the transmission networks models continue to be effective in predicting parasite load patterns. The major finding was that for *B. hydrosauri* there was a positive correlation between the cross-infection risk derived from the model and the empirically observed tick load. This correlation was consistent across all four years in both versions of the transmission model. However, this relationship did not occur in the other tick species, *A. limbatum*. Instead, cross infection risk was positively correlated with observed *A. limbatum* load in only one year, and in this particular year, the load of both tick species was positively correlated. There are many factors that could contribute to this finding, such as environmental or climatic changes, with rainfall differing between years. Environmental conditions can impact the lifecycles of the tick, as

while waiting off the host for the next lizard to come by, nymphs and adults of *A. limbatum* tolerate desiccation for longer (Bull and Smyth, 1973; Chilton and Bull, 1993), and have lower mortality from predation (Dawes-Gromadski and Bull, 1997a; 1997b) than *B. hydrosauri*. But another consequence may be that detached *A. limbatum* ticks can persist for longer in a wider range of microhabitats while waiting for their next host. Host refuges provide shelter for detached ticks from predation and desiccating high temperatures (Kerr et al., 2003), but *B. hydrosauri* may be more reliant than *A. limbatum* on host refuges as survival and transmission locations, over a wider range of conditions. As a result, the transmission network models that incorporate asynchronous refuge sharing may be more robust for *B. hydrosauri* than for *A. limbatum.* The next step was to conduct field experiments with controlled releases of tick to test if network models continued to predict transmission in real world conditions.

I needed to utilise genetic markers in order to trace the movement of ticks through the lizards refuge networks, and allow me to distinguish between among clutches of experimental infections onto sleepy lizards and between clutch derived ticks and background tick samples. In **CHAPTER 3**, I first examined the previously developed *Bothriocroton hydrosauri* microsatellite loci developed by Guzinski et.al. (2008). Finding these inadequate and unreliable, I then aimed to develop more loci, using 454 sequencing. The results of which were used in subsequent chapters. Although using the combination of existing and the 454 sequencing technique I expected to have many loci, I encountered many issues with the existing loci and during loci development. However, I was successful in developing three more loci and I used one of Guzinski et al.'s (2008) loci.

The main limitations with the loci development were that 1) loci did not amplify, 2) there was inconsistent amplification, 3) ambiguous products amplified and finally 4) the loci

were non-diagnostic. There were many potential challenges that could have inhibited further loci development, such as mutation, null alleles, duplicate loci or an unidentified issue; these however were out of the scope of the project.

Although the inheritance patterns of the ticks I sampled are an interesting topic in their own right, the principal aim of this chapter was to identify diagnostic genotype combinations that would allow us to use the genotype as an effective marker of individual groups of ticks that persisted across the different life stages from larvae to adult. With the four loci I finally used, I was successfully able to identify the origin of 65% of the collected adult ticks based on diagnostic alleles. These loci were not highly polymorphic, but I can say with confidence that I could successfully assign alleles to loci.

Further development of loci for *Bothriocroton hydrosauri* could be achieved with the current 454 sequencing data to obtain greater assignment of recovered ticks to progeny released. However the complications of using microsatellites could be circumvented by using newer techniques, such as RADseq, (Davey 2010). This technique also may have its problems with the poor DNA yields or inhibitors I uncovered and also the need to separate host derived fragments from tick fragments.

In **CHAPTER 4**, I used the tick samples collected in 2010 and 2011 that I identified as background from chapter 3 to observe the patterns of genetic relatedness among ticks attached to neighbouring lizards and also compare alternate models (social, asynchronous refuge sharing and spatial proximity) of tick transmission to explore which model best explained tick transmission. Firstly, with patterns of relatedness there were two significant results. The first was that adult ticks were more related to each other when they were collected from the same host, than when collected from different hosts. This relationship was also reflected in the high number of full sibships inferred among co-attached adult ticks. My

results therefore confirmed a previously reported result from the same tick species on the same host in a larger geographic range (Guzinski et al. 2009).

The second significant result was that when adult ticks were on different lizards, they had higher relatedness if those lizards had shorter network path lengths connecting them on all of the three network structures we explored. The strongest trends were for mean tick relatedness to decline with increased path length along the shared refuge network in 2010, and with increased distance between home range centers in 2011. A broad spatial pattern of declining tick relatedness with greater spatial separation of hosts has previously been reported by Guzinski *et al*. (2009). Compared to other networks, the poorest predictor of genetic relatedness in both years was the social network derived from social contacts between pairs of lizards. This potentially is due the life cycle of the tick (Chilton et al. 1993a)

It was expected that ticks would be more closely related to each other if they were more closely connected along actual transmission pathways. However analysis identified a different pathway that best explained the pattern in each of two years, suggesting that there are other factors influencing tick transmission, such as changing ecological conditions. There was a higher amount of rainfall in 2011 compared with 2010; influencing factors such as desiccation could be a greater threat to ticks in the drier year. Alternatively lizard movement patterns, and the resulting network structures are known to vary among years with differing rainfall (Godfrey *et al*., 2013) and this may have influenced transmission dynamics.

In **CHAPTER 5**, my aim was to investigate the transmission pathways of the reptile tick and establish what type of network model was the best at predicting parasite transmission, using larval ticks that were experimentally attached onto donor lizards within the field site. These ticks were collected as adults in 2010 and 2011, and were genetically distinguishable from each other and from the background population. Using exponential

random graph models (ERGMs), I asked which of the network models best predicted the destination of a unique tick clutch. The tick genotypes were used to identify the recipient of originating clutch from a donor lizard. In each year a different model was the best predictor of which lizards received ticks from donor lizards. In 2010, a model including adjacency and distance in the transmission network was the best model, and in 2011 a model including adjacency and distance between lizard pairs on a social network was the best model. This highlights that there are many factors that influence transmission, rather than simply the relationship that the parasite and host share. There are ecological factors that can influence transmission, in regards to *B. hydrosauri*, climate can be important to the lifecycle, as this species is adapted to live in a cooler climate, in drier conditions it is vulnerable to desiccation (Chilton & Bull 1993b) and high mortality of exposed ticks from predation (Bull *et al*. 1988, Dawes-Gromadzki & Bull 1997a, 1997b). It could also be suggested that either the timing of the pulses, or variation in environmental conditions between years could contribute to these differences. This study provides a novel test of the influence of networks on parasite transmission, and highlights the complexity of host-parasite interactions.

There are many aspects to understanding transmission pathways, one aspect which requires further study is the investigation of individuals within a population, as there is evidence that this transmission pathway would be stronger if individual transmissions could be tracked. In the study of giraffes, Vanderwaal et al. 2014, showed while the process could be difficult to observe directly, transmission could be tracked indirectly by identifying genetically unique strains within parasite populations and deducing that hosts carrying identical, or closely related strains are likely to represent cases of host to host transmissions Another example is Bull et al. (2012), where common genotypes of *Salmonella enterica* detected in cloacal swabs from the sleepy lizard, *Tiliqua rugosa*, were used. These authors reported that pairs of lizards that shared genetic strains were more strongly connected to each

other in the social network, but were no closer to each other spatially, than pairs of lizards that did not share *Salmonella* strains. By comparing the fit to two alternative network transmission models, based on social contact and on home range overlap, they deduced that *Salmonella* was more likely to be transmitted by direct lizard to lizard contact than via some common non-lizard infection source (Bull et al. 2012).

In summary, this study highlights, that changing environmental conditions might vary the relative importance of alternative processes driving the parasite transmission dynamics. This could lead to further studies specifically investigating that effects and influence that the environment has on host behaviors, in turn extending our understanding of parasite transmission. There were limitations in my study due to limited genetic markers and further work would benefit from utilizing newer genomic techniques to trace pulses of tick progeny in social networks within this amenable study system. Identifying the characteristics of super spreaders is another key area requiring further work.

Overall this study has been successful in expanding on the knowledge and understanding of tick transmission in a sleepy lizard population. It builds from simple empirical comparisons which have been the mainstay of most parasite-wildlife network studies up until now, and using genetic tools, offers new insights into the role of networks in parasite transmission in wildlife populations. Importantly, it also provides the first large-scale field-based experimental test of the hypothesis that networks (modelled on asynchronous refuge sharing) influence parasite transmission. The results from this experimental study demonstrate that host-parasite interactions are more complex than our models could predict, and I suggest that other factors (such as environmental conditions) may influence the relative importance of networks in explaining transmission. My study provides an important contribution to our understanding of the transmission patterns of parasites in wildlife populations. It builds onto

theoretical systems, providing an example of their use in the natural world. This study also contributes to the knowledge of the factors that shape transmission processes, as it investigates the complex systems that are involved in parasite transmission.

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Thanks Mike Bull

A true inspiration, his enthusiasm and knowledge about sleepy lizards and their parasites has left its mark on many people.