# Exploring the host-parasite relationship between Brown Kiwi (*Apteryx mantelli)*, kiwi ticks *(Ixodes anatis)* and kiwi tick-borne haemoparasites


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# Abstract

Host and parasites have co-evolved for millions of years providing selection pressures with the parasite using the host for survival and reproduction, and the host, in turn, developing defence strategies to combat the parasites to better survive infection. North Island Brown Kiwi (NIBK, *Apteryx mantelli)*, a species of ratite endemic to New Zealand, is host to a number of host-specific parasites, one of which is the Kiwi tick *Ixodes anatis.* Like the NIBK, the kiwi tick is also endemic and therefore vulnerable to extinction. The aim of this thesis was to fathom the host-parasite relationship between the NIBK and their ticks, as almost nothing is known about this relationship. To study any host-parasite relationship, we need to know basic life history traits of both parasites and hosts. As a result, this thesis combined laboratory and field methods to determine aspects of the tick life cycle, and field methods that determined various haematological and biochemical parameters of NIBK and how to use them to ascertain the effect of heavy tick loads on the birds. We also used various different laboratory methods to determine if these ticks were vectors to protozoa that might affect NIBK. We measured moulting and oviposition times of various stages of engorged kiwi ticks collected from NIBK and kept at various temperature and relative humidity (RH) regimes. We found that engorged larval and nymphal stages of *I. anatis* preferred lower temperatures as compared to most other species of ixodid ticks with successful development occurring under RH above 94%, and temperatures of 10 to 20˚C. Whereas, in the field the different stages of the ticks were able to develop at drier humidity of 65% to 69%, under similar temperatures. We also found that the ticks were abundant in kiwi burrows throughout the year and prefer more tree and soil burrows in the forest. Using this, we were able to hypothesise a seasonal life cycle for the kiwi tick.

Before we could look at the effect of these ticks on their NIBK host, we established a method of estimating tick infestations on the birds. Along with this, we also established normal reference range for haematological and biochemical values using different populations of NIBK. The results of both the tick index and the normal parameters can be used by managers and veterinarians around NZ when determining kiwi health.

We then proceeded to remove/reduce tick infestation levels from a group of NIBK treated with parasiticides and compared their haematological and biochemical

parameters with a group of untreated control birds. The treated birds showed higher total protein values and had a higher weight gain after reduction of tick infestation with recorded lower activity than control birds leading us to conclude that the ticks negatively affected the birds. However, when the birds were left untreated for a year, they gained those ticks back and their parameters went back to values prior to experimental removal of ticks. This indicated a co-evolutionary relationship between the NIBK and the kiwi tick, *I. anatis*, as in cases of chronic infection of a parasite on its host, especially when they co-evolve together, the costs of parasitism are not as pronounced as both host and parasite are in an arms race to increase their fitness. However, we found no evidence of tick borne protozoa in any of our infected birds, suggesting that either the infections were not present, present in extremely low intensities in the blood or we need more detailed investigations into what happened to the NIBK and tick specific haematozoa that have been previously reported in NIBK.

This research contributed to our knowledge of the relationship between NIBK and the kiwi tick *I. anatis.* In the process, it also helped establish various protocols for assessing health of NIBK as well as assessing tick infestation on ground birds that can be used by a large group of individuals, including future researchers. As a result of this thesis, we recommend that wildlife managers also take parasite conservation and translocation into consideration while managing endemic host-parasite networks.


*Nature doesn't sit still. Things and individuals are changing, dying and new things are coming. They're all stories.*

*Sir David Attenborough*

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***Ehara taku toa, he takitahi, he toa takitini***

*My success should not be bestowed onto me alone, as it was not individual success but success of a collective, so I would like to dedicate this thesis to all the people that touched my life during the course of this research…*

To the army of amazing volunteers

To friends and colleagues like family

To the Rawhiti, Ponui and my whanau

x

# Preface and Thesis Outline

This thesis is written as a series of independent manuscripts meant for publication. One chapter (Chapter 2) has already been published and the others will be submitted soon. As a result, the individual chapters inevitably contain some level of repetition, particularly in the study sites, study species, and the introductions. I am the main author of each paper with my supervisors and in some cases other people as co-authors. My contribution and that of co-authors will be indicated below.

Chapter 1 is an in-depth literature review of multiple concepts associated to host parasite relationships focusing on ectoparasites, especially ticks including parasite ecology, effect of parasites on host, and parasite detection methods, all of which are further used in this thesis.

Chapters 2 and 3 help us answer questions of *Ixodes anatis* biology and to explain possible adaptations of these ticks to the New Zealand environment and their kiwi host. The main aim of these chapters was to establish aspects of the ecology of the kiwi tick *I. anatis* in the field as well as laboratory by answering these three questions:

1. What is the ideal temperature and humidity range for successful development of different stages of *I. anatis* in laboratory conditions*?*
2. What are the different temperatures and humidity provided by brown kiwi burrows, and how do they affect the development of different stages of *I. anatis* in the field?
3. What is the effect of site, habitat and shelter type on the abundance of *I. anatis* in the field, and do the ticks' life history stages follow the host (brown kiwi) reproductive cycle?

To assess the ideal microclimatic requirements of the kiwi ticks, *I. anatis*, in Chapter 2, we collected more than 750 *I. anatis* ticks from hosts in the field on Ponui Island and subjected them to various temperature and humidity regimes in the laboratory using the drying power of salt solutions to discern survival and development times of each stage. These conditions and times were then compared to those of ticks placed in artificially constructed burrows in the field. The lab experiments took six months to complete whereas the field life cycle experiments took around three months. Chapter 3 looked at the distribution and abundance of kiwi ticks in the field and how the location, habitat type, and burrow type affect their abundance. For this study we studied free-

living ticks in a brown kiwi population in the field. The study followed the tick abundance and stages in a sample of 64 burrows over a 14-month period.

Determining tick loads on a bird currently requires the use of acaricide on the bird followed by ruffling of feathers to dislodge and collect the tick for counting. This is a time consuming (15-20 minutes/ bird) and invasive procedure. Therefore, Chapter 4 aimed to find a way to quantify tick infestations on the birds using photography. We expected that taking photos would minimise handling times, by as much as ten minutes shorter, and reduce the need to use acaricides on the birds. The results from this chapter can be used by future researchers as well as by conservation managers to evaluate tick loads on birds for management of both kiwi and kiwi ticks.

The main aims of Chapter 5 were to establish the normal range of reference values for common haematological and biochemical parameters of the North Island brown kiwi (NIBK), *Apteryx mantelli,* using wild birds from different populations around the North Island of New Zealand. The second aim was to explore variations between these parameters between populations the various populations. We collected blood samples from 102 NIBK from seven different populations to establish these values over a period of two year to achieve these aims.

Chapter 6 aims to examine the effect of *I. anatis* on NIBK by comparing the haematological and biochemical values, as well as other physiological parameters, of a group of kiwi with experimentally reduced kiwi tick loads using a before-after design and against values from a control group. Our main questions for this chapter were;

1. What is the effect of parasite removal on the haematological, biochemical and physiological parameters in a highly tick infested population of kiwi?
2. How long does it take for kiwi blood parameters to recover from long term parasite infestation once parasites are removed?

Kiwi ticks have been shown to vector haemoprotozoa, in particular *Babesia* and *Hepatozoon*. These micro-parasites were reported from birds caught in a population close to where birds translocated to Ponui Island come from and also neighbouring Puketi Forest. We therefore considered it likely that these microparasites would be present on Ponui and Puketi birds. In Chapter 7 we searched for the presence of these parasites in the bloods of the birds sampled for Chapters 5 and 6. We started with relatively simple techniques such as staining dissected ticks and kiwi blood slides, and

tick histology, and then searching for parasites under the microscope. We did not find the ticks but discovered that current knowledge of tick histology and micro-parasite morphology are not developed enough to identify these parasites with confidence using these methods. This led to searching for the parasites using fluorescent *in-situ* hybridisation, various polymerise chain reactions, and next generation sequencing.

Chapter 8 presents a syn*thesis*, drawing together different ideas arising from each chapter to give a more complete picture of host-parasite relations in NIBK and *I. anatis*. While this thesis present some unique work and conclusion, I also discuss future applications of the results in the evolving work of parasite conservation and considerations for yet to come research work in this field.

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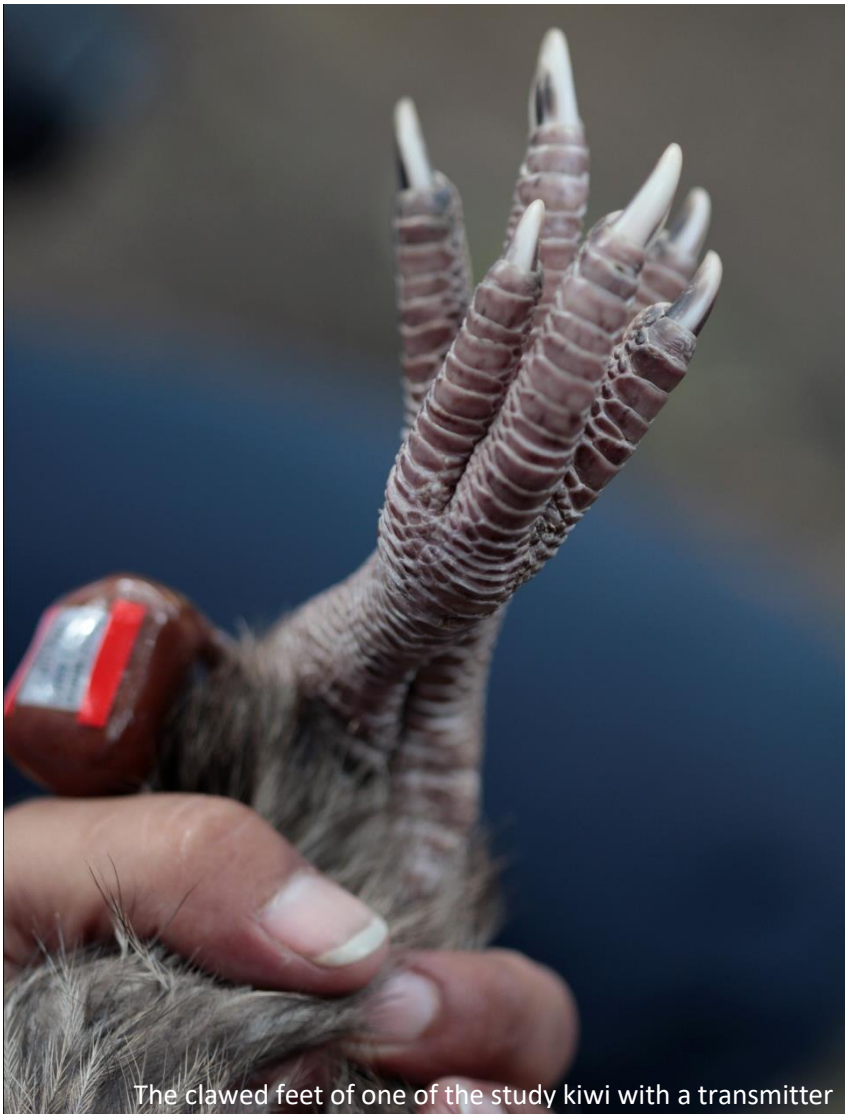
## List of abbreviations

| | |
|---|---|
| ANOVA | Analysis of Variance |
| CI | Confidence intervals |
| DLC | Differential leucocyte count |
| DOC | Department of Conservation |
| Hb | Haemoglobin |
| MANOVA | Multivariate analysis of variance |
| MCHC | Mean corpuscular haemoglobin concentration |
| NIBK | North Island Brown Kiwi |
| NZ | New Zealand |
| PCV | Packed cell volume |
| RBC | Red blood cells |
| RH | Relative Humidity |
| RSHG | Red stony hill gully |
| SD | Saturation Deficit |
| SD | Standard deviation |
| SE | Standard errors |
| TEC | Total erythrocyte counts |
| TLC | Total leucocyte count |
| TPP | Total plasma proteins |
| WBC | White blood cells |

# Chapter 1



The clawed feet of one of the study kiwi with a transmitter

Feet, what do I need you for when I have wings to fly?

Frida Kahlo

# Chapter 1: Literature Review

## 1.1 Parasite-host relationships

The nature of a host-parasite relation can be as simple as a host[1] and parasite living together, to more complex, like a function of interactions between the host and parasites at, not only the individual level, but also a species and population level (Horwitz and Wilcox, 2005). These interactions can be defined by coevolution, distribution of host and parasites, host susceptibility, the environment and genetics. The Red Queen hypothesis was developed by Van Valen (1977) and it recognises that species do not exist in isolation and must constantly adapt, evolve, and proliferate in order to survive while competing against ever-evolving opposing species. This is best illustrated in the evolution of host and parasite or predator and prey relationships. Both parasites/predators and hosts/prey are continuously undergoing a dynamic co-evolution (or arms race) (Thompson, 1999; Yoder and Nuismer, 2010). We can see the effect of this arm's race in various host-parasite systems. For example, in the evolution of ants, where *Harpagoxenus sublaevis* is a parasitic slave-maker ant dominating the arms race on its host, the ant *Leptothorax acervorum;* the slave-maker ants are more successful in parasitizing populations of their host in subsequent attacks over time when compared to a host population they have never encountered (Foitzik, Fischer, and Heinze, 2003). Another example is the avian brood parasitic cuckoos and their hosts, where the hosts try to lay polymorphic eggs of different colour and patterns and have diversity in clutches, to avoid the cuckoos being able to match their eggs (Hall, Scanlan, Morgan, and Buckling, 2011; Spottiswoode and Stevens, 2012). The effects of this co-evolution are also seen when parasite induced selection pressure affects and maintains sexual reproduction (Hamilton, Axelrod, and Tanese, 1990; Polak and Starmer, 1998). For example, where the host has the capability to maintain resistance through recombination and outcrossing, and asexual hosts are more prone to pathogens[2] and parasites (Moritz, McCallum, Donnellan, and Roberts, 1991; Morran, Schmidt, Gelarden, Parrish, and Lively, 2011)**.** The symbiotic[3] relationship between host and parasite can be

[1] Host: the larger-bodied member in a symbiotic relationship

[2] Pathogen: A microbe that can cause diseases. Pathogens include but are not limited to viruses, bacteria and protozoa. While all pathogens are parasites, not all parasites are pathogens.

[3] Symbiosis: referring to a close relationship between members of two species, in which individual of one species live near on or in those of the other species. Symbiosis does not imply mutualism.

of various types and are not only parasitic[4] but can be mutual[5], commensal[6] or a phoresy[7].

## 1.2 Effect of parasitism on hosts

The effect of a parasite on its host is shaped by various trade-offs at multiple biological levels. At the population level, the success of a parasite depends on its ability to reach and infect susceptible animals which in turn depends on a set of factors such as the ability to spread between individual hosts and how long the parasite lives within each host (Schmid-Hempel, 2011). At an individual level, the success of a parasite depends on factors such as co-infection with other competing parasites, strains and species infecting the host (Pedersen and Fenton, 2007).

If the parasite is vector-borne, it also affects the vector[8] that transmits it. Ewald (1993) predicted that parasites would have a lower virulence [9]to their vectors, as compared to their main host, since the parasite gains little by harming its main route of transmission. Elliot, Adler, and Sabelis (2003) found that though this is true in the literature, there is still an effect of parasite on its vector. For example, arboviruses have a negative effect on development times and reduce the life span of their mosquito vectors (Faran et al., 1987) likewise malarial parasites reduce mosquito survival (Ferguson and Read, 2002). The effect of parasites on their host does not stop at virulence to the vector, but parasitic manipulations also drive the evolution of their vector in order to increase their chances of transmission (Lefevre and Thomas, 2008). Parasites affect their hosts in two ways, by exploiting the host for resources that the host could have used itself (direct), and by causing the host to spend energy in parasite defence (indirect) (De Lope, Møller, and De la Cruz, 1998).

Direct effects of parasites vary, from irritation and skin damage caused by biting (e.g. lice or mites) to blood loss anaemia[10] caused by heavy infestation with blood sucking

[4] Parasitism: a relationship in which one-member (the parasite) benefits through the use of resources gathered by the other member (the host), usually at a cost to the host.
[5] Mutualism: a symbiosis in which the relationship benefits both members.
[6] Commensalism: a symbiosis in which one member benefits from the relationship, but the other neither gains nor loses.
[7] Phoresy: a symbiosis in which the smaller-bodied member uses the host primarily for transport.
[8] Vector: an organism that transmits a disease from one host to another.
[9] Virulence: the harmfulness of a disease
[10] Anaemia: a condition in which there is a deficiency of haemoglobin in the blood caused by red blood cell loss.

ectoparasites (ticks or fly larvae) or haemoparasites (e.g. *Plasmodium*). In addition, large tick infestations result in blood and nutrient loss, which may result in the failure of the body to function correctly. Internal parasites like round worms, for instance, cause iron deficiency anaemia due to reduced iron absorption by the gut, and iron loss due to diarrhoea (Roche and Layrisse, 1966). Haematozoa like *Babesia* spp*.,* cause haemolytic anaemia and reduce haemoglobin concentration due to haemolysis of RBC (Fair, Whitaker, and Pearson, 2007; Heylen and Matthysen, 2008; O'Brien, Morrison, and Johnson, 2001; Valera, Hoi, and KrištÍn, 2006). Ectoparasites such as ticks, mosquitoes and fleas can cause direct blood loss that leads to macrocytic, hypochromic, regenerative anaemia (McKilligan, 1996; Pfäffle, Petney, Elgas, Skuballa, and Taraschewski, 2009; Tyler and Cowell, 1996). Also some ectoparasites, especially ticks, produce a neurotoxin which may cause paralysis, and sometimes death, in the host (Gothe and Neitz, 1991; Luttrell, Creekmore, and Mertins, 1996).

Indirect effects of parasites on hosts are a bit more complex and difficult to measure. For example, in ungulates the body size principle[11] states that the smaller the animal, the harder it will groom to compensate for the higher cost of tick infestations (Mooring, Benjamin, Harte, and Herzog, 2000). The time spent grooming could instead be utilised for grazing or finding mates. When we apply the body size principle to birds, they have a smaller body size compared to most other animals and thus the costs of parasitism in avian hosts may be more pronounced and expressed in the form of various physiological and behavioural changes. Parasites can take away precious time the birds may need for various day to day activities. Ectoparasite-infested seabirds spent 23% of their time per minute preening as compared to 5% per minute for non-infested birds (Duffy, 1983), this time could have instead been spent foraging. Parasite infestations cause egg and nest desertion (Duffy, 1983), delayed breeding (Møller, 1990), reduced clutch sizes and chances of second clutching (Korpimaki, Hakkarainen, and Bennett, 1993; Møller, 1990; Moss and Camin, 1970), and increase in incubation time (Widemo, 1989). Young animals are more susceptible to parasitism since they have a relatively larger surface area and inefficient defence against parasites. The resulting infestation may cause anorexia,

[11] Body size principle (Hart et al. 1992): States that smaller-sized animal, because of their greater surface-to-mass ratio, should engage in more frequent tick removal grooming than larger-bodied animals in order to compensate for higher costs of tick infestation

irritation leading to reduced feeding and even death (Lehmann, 1993). In past studies, parasitism reduced nestling survival, juvenile growth rate, reduced lifetime reproductive success, and appeared to alter energy allocation of resources to reproduction, parasite defence, and body maintenance (Fitze, Tschirren, and Richner, 2004; Loman, 1980). Infection with parasites reduced body mass and weight gain in chicks, caused disrupted fledging time leading to either earlier (Berggren, 2005; Morbey, 1996) or delayed fledging time (Morbey, 1996).

Either way, the host faces trade-offs between resources lost to parasitism (health) and defence against parasites. Sometimes, the direct effects lead to indirect outcomes. For instance, when parasites directly decrease the general health of their avian hosts (Bosch and Figuerola, 1999; O'Brien et al., 2001; Tomás, Merino, Moreno, and Morales, 2007; Valera et al., 2006), this leads to exertion of a strong selection pressure on the hosts by decreasing nestling survival, reducing reproductive events and host life span (Hamilton and Zuk, 1982). Ectoparasites such as fleas and lice decreased survivorship in colonial birds such as cliff swallows by as much as 12% (Brown, Brown, and Rannala, 1995b). Parasites also directly affect behaviour of hosts, as in the case of the internal parasite *Trichostrongylus tenu* that infect red grouse and decrease cautious behaviour thus increasing the predation risk to their host (Hudson, Dobson, and Newborn, 1992).

## 1.3 Hosts' immune response to parasites

In order to protect itself, the host's body then deploys a defence against the parasites by mounting an immune response (Wakelin, 1996). The immune system is an important physiological life history trait and can be classed as innate[12] or adaptive[13] (Elgert, 1996) . Variation in immune function between individuals indicates fitness related individual differences (Elgert, 1996). A variety of factors can affect a host's immune response including age, sex differences, costs to the host, specific and context dependent

[12] *Constitutive/ Innate immunity* is defined as an immune response formed by a mixture of humoral and cellular components that do not require a previous challenge by a pathogen or antigen (or induced immune response) and is the first line of defence against pathogens

[13] *Adaptive/acquired immunity* is defined as cell mediated antigen-specific immunity in response to exposure to specific pathogens. It is more complex than the innate and includes a "memory" that makes future responses against the specific antigen more efficient.

responses and concurrent infections by different parasites (Schmid-Hempel, 2003). During an adaptive immune response, the host body produces antibodies to fight infection. An example is the study by Staszewski, McCoy, Tveraa, and Boulinier (2007) that tested adaptive immune response and found that the antibody levels in Black legged Kittiwake (*Rissa tridactyla)* were directly proportional to the level of exposure to tick (*Ixodes uriae*) infestations. In another laboratory-based study Prevot et al. (2007) found that the host system (rabbits and SWISS mice) produced antibodies against a salivary serpin produced by the tick *Ixodes ricinus.* So, while parasites may negatively affect their host, the host can combat parasites by mounting an immune response. However, this also adds to the effect of the parasite, as mounting an immune response is energetically costly and the costs are reflected in reduced activity, feeding and reproductive rate (Klasing and Leshchinsky, 1999). The costs may be more pronounced in certain situations, for example, when the host has limited energy and nutritional resources or if it is under other sources of stress. Variations in costs depending on circumstances result in trade-offs between health, immuno-competence and fitness (Schmid-Hempel, 2003; Zuk and Stoehr, 2002).

## 1.4 Detection of ectoparasite effect on hosts: Haematological Indices

Haematology is the study of number and morphology of the cellular elements found in the blood like erythrocytes, leucocytes and thrombocytes (Merck Manual, 2012). Haematology may be necessary for disease diagnosis, but is also a good indicator of the physiological and nutritional state of an animal (Khan and Zafar, 2005; Aderemi, 2004). Blood parameters, especially those that are diagnostic for anaemia, are a great way to tell acute effects of parasitic infestation on hosts (Table 1.1). The changes in physiology caused by blood sucking parasites are almost instantaneous and easy to measure through a series of tests such as packed cell volume (PCV), haemoglobin concentrations in blood, blood glucose, mean corpuscular haemoglobin concentration and complete blood profiles such as red and white blood cell counts and differential leukocyte counts (Atkinson and Van Riper 1991; Gauthier-clerc et al., 2003; Heylen and Matthysen 2008; Merino et al., 2001; Norcross et.al, 2002; Norte et al. 2013; Ots et al. 1998; Pryor and Castro, 2015; Szep and Moller, 1999; Valera et al., 2005). Immunocompetence in birds

can also be measured by a number of techniques such as leukocyte counts (Zuk and Johnsen, 1998), heterophil to lymphocyte ratios (Dufva and Allander, 1995; Ots and Horak, 1996), buffy coat layer, plasma proteins and serum proteins (Gustafsson, Nordling, Andersson, Sheldon, and Qvarnström, 1994). Studies of proximate factors demonstrate that poor health and immune responses are responsible for delayed breeding and in some cases breeding failure of animals (Moreno et al., 1998). Therefore, by measuring the health and immune parameters of a host at a given time should help us measure effects of parasitism which may lead to long term problems. However, in a normal situation, most of these blood parameters vary with various factors such as body size, metabolism, season, altitude, behaviour and stages of development (age) (Sealander, 1965). For example, haemoglobin and haematocrits are lower in juvenile animals as compared to adults (Sealander, 1965).

### Packed cell volume (PCV, also known as haematocrit)

PCV is the percentage of red blood cells in blood and is measured by centrifugation of micro capillary tubes at a high speed for five to ten minutes. Since PCV is a measure of the proportion of whole blood that is composed of erythrocytes, if an animal is dehydrated, the volume of plasma decreases thus increasing the PCV. A decrease in PCV can be due to anaemia caused by loss or destruction of erythrocytes.

### Haemoglobin (Hb)

Hb is a metalloprotein in erythrocytes that is responsible for carrying oxygen and thus the Hb concentration indicates the oxygen carrying capacity of the blood, and similar to PCV, the concentration in whole blood is affected by the hydration status. Therefore, like PCV, Hb also increases in cases of dehydrated individuals and decreases in cases of anaemia caused by blood loss. The Hb concentration in birds varies with age, with some birds showing as much as a 40% increase in their first year of life (Samour et al., 2011). In normal situations, there is not enough evidence to support sex related differences in Hb in birds (Minias, 2015), but both PCV and Hb have been shown to decrease in females during egg production and pre-laying (Minias, 2015)

### Mean corpuscular haemoglobin concentration (MCHC, calculated value)

MCHC is the concentration of haemoglobin in a given volume of red blood cells. Thus, it is calculated by the formula Hb/(PCV/100).

### Total plasma protein (TPP)

The protein component of plasma is mainly albumin and globulin. While these proteins can be measured directly by techniques such as electrophoresis, refractometry has been widely used, since 1960, to estimate TPP in many clinical and field veterinary trials. Refractometry measures the amount of total solids present in plasma by refraction of light. Total solids in plasma are comprised mainly of protein and other dissolved solids like glucose, urea, cholesterol and triglycerides. A handheld refractometer, which is portable and easy to use, can be used to measure TPP of animals in field situations, and have been usually calibrated to subtract 2g/dl off the reading to correct for non-protein solids. In the past it was believed that refractometry did not give accurate readings for some birds and reptiles. For instance, Lumeij et al., 1996 found that refractometry produced highly corelated TPP values when compared to other traditional methods in pigeons but were on the higher side and thus suggested not using refractometry as a reliable method. However, Cray, Rodriguez and Arheart (2008) and Schmidt et al., (2008) suggest this could be due to the higher fat content in pigeon blood and showed that refractometry was a reliable method to measure TPP in various other avian species. TPP decreases in cases of malnutrition or diseases causing protein loss while it can increase with dehydration and increase in the concentration of non-protein solids (like in cases of lipemia and hyperglycaemia, Merck Manual, 2012).

### White blood cell (WBC) counts

There are five types of leukocytes (or white blood cells = WBC) commonly found in bird blood; Lymphocytes, heterophils, basophils, eosinophils and monocytes. (Merck Manual, 2012). Heterophils are phagocytic cells that are the first line of defence in immune responses to infection. Lymphocytes provide specific immunity against pathogens through direct cell-mediated effects, and by production of antibodies (Minias, 2019). Together, heterophils and lymphocytes make up more than 80% of the WBC, and their numbers in circulation give some indication of the immune status of an individual. For instance, an increase in WBC counts almost always indicates stress or infection (Minias, 2019).

### Glucose

Birds tend to have higher blood glucose levels than mammals of the same size (Braun and Sweazea, 2008). The exceptionally high levels of glucose, in birds, combined with the lower body mass does not affect internal organs that are involved in erythropoiesis, causing anaemia as happens in other animals (Sweazea et al., 2008; Langslow, 1978).This in turn hampers our ability to diagnose the effect of parasites on the bird's body using glucose as an indicator. However, as a rule, a low-quality diet with lowered protein intake decreases blood glucose levels in birds (Machın et al., 2004) thus adding to the stress in already parasitized birds.

So, when considered together these tests are a reliable method to look at effects of parasitism on the health and fitness of avian hosts (Heylen and Matthysen, 2008; Quillfeldt, Masello, and Möstl, 2004). A summary of the various factors used for this study are given in Table 1.1, which also provides a detailed reference list of the papers that have used these parameters and the changes they have seen in cases of ectoparasite infestation in birds. Usually, to define the clinical effects of ectoparasites on the host, changes in specific health parameters are measured in a naïve host with no parasites to which the parasites are introduced. However, in our study, our host were NIBK a vulnerable species that has coexisted with the tick for what we think is a long time and lives with chronic parasite infestation. Therefore, using naïve hosts was not ethically and practically possible, and therefore we looked at the changes on a set of health parameters following parasite removal in a highly tick infested population of wild occurring NIBK. We expected that the changes we would see in parameters would follow the opposite direction from those given in column three in Table 1.1.

Table 1.1: Different blood test parameters that we will use to test for clinical effect of tick infestations, along with their definitions and expected changes in infested birds

| Parameter | Definition | Expected changes in case of ectoparasite infestation | Source(s) |
|---|---|---|---|
| **Packed cell volume (PCV)** | It is the percentage of red blood cells in a whole blood sample and measures the relative proportion of erythrocytes to blood volume | Decreased | Atkinson & Van Riper 1991; Heylen and Matthysen 2008 & 2011; Valera et al., 2005; Gauthier-clerc et al., 2003; Merino et al., 2001; Norcross et.al, 2002; Norte et al. 2013; Pryor and Castro, 2015 |
| **Haemoglobin (Hb)** | It is the iron-containing metalloprotein in blood responsible for oxygen transport | Decreased | Carlton 2008, Norte et al., 2013; O'Brien 2001 |
| **Blood Glucose** | The amount of glucose present in blood | Increased | Campbell 2004; Norte et al., 2013; Pryor and Castro, 2015 |
| **Total Leucocyte count** | Total number of white blood cells present in blood | Increased | Davies et al., 2008;Krams et al. 2013; Norte et al. 2009; Ots et al. 1998; Szep & Moller, 1999 |
| **Total Erythrocyte counts** | Total/differential number of red blood cells present in blood | Increase in number of immature erythrocytes | Heylen and Matthysen 2008; O'Brien et al., 2001; Pfäffle et al., 2009 |
| **Heterophils** | Phagocytic cells , part of innate immune response | Increased | Davis et al. 2008; Campbell & Ellis 2007; Krams et al,. 2013; Ots et al., 1998 |
| **Lymphocytes** | Leukocytes that assist in recognition and destruction of specific pathogens, part of adative immune response | Increased | Krams et al., 2013; Ots et al., 1998 |
| **H:L ratio** | Ratio of Heterophils to Lymphocytes | Increased | Davis et al., 2004, Norte et al., 2013 |
| **Total Plasma Proteins TPP** | Proteins, mainly albumin and globulin, present in blood plasma | Decreased | Ots et al., 1998; Quillfeldt et al., 2004 |
| **Weight** | | Decreased | De Lope et al., 1998; DJA Heylen & Matthysen, 2008; McKilligan, 1996; Norte et al., 2013; Pryor and Castro, 2015; Wanless et al., 1997 |

## 1.5 Ecto and Endo parasites

Parasites can be classified into many different categories based on their interaction with their host, and their life cycle. There are two main types of parasites, depending on the site they inhabit on the host, ectoparasites[14] and endoparasites[15]. Sometime ectoparasites act as host for other parasites, often endoparasites. When this happens, the endoparasite often completes part of its life cycle in the parasite vector. Most of these parasite-vector relationships are species, genus or family specific (Cox, 2010).

[14] An ectoparasite is one that lives outside, on the host's body and external structures

[15] An endoparasite is one that lives inside the host's body

There are many types of ectoparasites that affect avian hosts, which is far beyond the scope of this thesis. Therefore, we will only focus on ticks.

Ticks are obligate[16] ectoparasites belonging to the Phylum Arthropoda and are grouped together with mites to form the sub class Acari within the Class Arachnida.

## 1.6 History and Classification of ticks

Ticks have existed before hominids were believed to walk earth and have been famous as disease agents in some of the oldest literature known to man. While the oldest tick sample (species: *Carios jerseyi)* dates back to the Cretaceous Period (90 – 94 mya), mostly fossilised in amber (Klompen and Grimaldi, 2001), the oldest report of ticks in the literature is believed to date back to about 800 BC, where ticks were mentioned in Homer's Odyssey as "kynoraistes" on Ulysses' dog (Hoogstraal 1970 – 1984 as cited in Fuente, 2003). However, Obenchain and Galun (1982) and Hillyard (1996) cited that writings date back to 1550 BC in Egyptian papyrus scrolls mentioning tick fever and at the same time, a tick was found infesting a hyena-like animal in an Egyptian tomb (Arthur, 1965). Subsequently, ticks have also appeared in Aristotle's *Historia Animalium* where they were believed to originate in high grass since they were often found there, and Pliny the Elder described ticks as "living on blood with its head always fixed and swelling", but believed that since they had no anus, they would "burst with over-repletion and die from actual nourishment" (Arthur, 1965). Fossilised ticks have since continued to be found, from before hominid periods, in amber deposits with the latest known *Dermacentor reticulatus* found in the auditory canal of a woolly rhinoceros as late as the Pliocene period (2-5 mya) (Feunte, 2003).

Today there are around 900 species of ticks classified into three main subfamilies of Ixodidae (hard ticks), Argasidae (soft ticks) and Nuttalliellidae (which only has only one member; *Nuttalliella namaqua)* (Barker and Murrell, 2004; Beati and Klompen, 2019; Guglielmone et al., 2010). Hard ticks are further divided into Prostriata, which contains the Australasian *Ixodes* and other *Ixodes*, and Metastriata. We will focus on ticks in the sub-family Ixodidae (Beati and Klompen, 2019).

[16] Obligate parasite: A parasite that completely depends on the host

## 1.7 Biology of ticks

The biological cycle of ticks include the following processes: egg laying, hatching, development, host interaction, feeding and mortality (Hoch, Monnet, and Agoulon, 2010). Ixodid ticks can be one, two or three-host ticks showing four life stages: egg stage and three instars (larvae, nymphs and adults). In a three-host tick, each active stage finds a host, feeds and drops off to develop on the ground. Feeding lasts from 2-10 days in each stage and is followed by a digestion period and moulting into next stage (Kocan, de la Fuente, and Coburn, 2015) while adult females produce egg batches that can be from 1000 to 10,000 eggs after which they die (Chilton, 1992), however this can vary between different species and season. In an ecological capacity, ticks can be of two types, exophilic ticks that actively quest or seek their host, and endophilic (nidicolous) species that remain in nests and burrows of host animals. While exophilic ticks' mate on the host, endophilic ticks mate usually off the host (Hillyard, 1996). The length of the life cycle of Ixodid ticks is highly variable and in part is thought to depend on the life cycle of their hosts (Yuval and Spielman, 1990). Some Ixodid ticks can complete their life cycle in just a year (Dantas-Torres and Otranto, 2013), while others can take up to three years (Frenot et al., 2001).

## 1.8 Factors affecting Ixodid tick life cycles and abundance

Ixodid ticks thrive in optimal climatic conditions and may enter diapause when conditions become unfavourable (Oorebeek and Kleindorfer, 2008). A population model using data from a large number of Ixodid tick species showed that temperature and day length affected the stage to stage development; temperature, humidity, tick stage and size affected the host-seeking (more commonly referred as questing) activity; and host type and density affected attachment to host. Mortality in turn was affected by low humidity and high density of ticks (Randolph, 2004). In the next sections I will discuss what we know regarding the effect of these variables in more detail.

### 1.8.1 Temperature & Humidity

Ixodid ticks have temperature and humidity-dependent population dynamics. A great proportion of the published work about various ticks' life cycles deals with the season-dependant life cycle models of ticks (Lees and Milne, 1951; Randolph, Green, Hoodless,

and Peacey, 2002; Walker, 2001). However, using seasons to define life cycles is rudimentary and not accurate from a global perspective as the different regions where the ticks have been studied are in different geographical locations where the temperatures and humidities vary. For instance, a study in Sweden (Jaenson et al., 2012) reported that ticks of the species *I. scapularis* were more abundant in summer and spring but in contrast, in Northwest Florida, adult questing *I. scapularis* are more common in winter and early spring months (Cilek and Olson, 2000). These may sound like contradictions until we notice that due to the difference in geographic location, temperatures in Florida during winter are similar to summer and spring temperatures in Sweden. So instead of saying a tick is more abundant in summer versus winter, I will focus on the effect of temperatures on the ticks and not the various seasons.

Many studies have looked at different conditions for the development of different stages of ticks in the laboratory (Arthur and Snow, 1968; Kahl and Knülle, 1988; Lees, 1946; Padgett and Lane, 2001; Troughton and Levin, 2007). Of these, Troughton and Levin (2007) conducted the most extensive study, looking at the effect of temperature and a constant high humidity, on laboratory raised colonies of seven different tick species (Table 1.2). Their study coincided with findings from other researchers (Arthur and Snow, 1968; Padgett and Lane, 2001) and showed that the ideal conditions required for completing different life cycles lies between 18 to 26 °C, and high relative humidity (≥ 90%), depending upon the tick species. They also showed that when temperatures were reduced to 4 - 8 °C, the duration of different developmental processes increased by months, and the mortality rates of the ticks increased.

Humidity also influences different stages of the development of Ixodid ticks. *Ixodes vespertilionis,* for example, requires 100% humidity to complete its life cycle and cannot survive below 61% (Obsomer et al., 2013). Hydration is crucial to avoid desiccation, maintain water balance and questing activity in ticks (Randolph, 2004). Ticks absorb water vapour from the atmosphere by secreting a hygroscopic fluid produced in the salivary glands onto the surface of the hypostome (maxilla). Once this secretion absorbs water, it is swallowed by the pharyngeal orifice. (Kahl and Knülle, 1988; Needham and Teel, 1986). This active inward sorption of water becomes ineffective below a relative humidity termed the critical equilibrium humidity (CEH), which varies between 45% and 95% depending on the tick species, and is lower in ticks than other arthropods. Below

the CEH, passive absorption of water takes place, but is exceeded by the simultaneous transpiration of water, thus resulting in net loss of weight (Knülle and Devine, 1972; Gaede and Knülle, 1997***).*** This weight loss is probably why it was previously believed that engorged states of immature ticks (nymphs and larvae) were unable to take up water vapour. Later studies suggested that at 91% humidity and 15°C, the immature ticks absorb water vapour from the air passively and recover from partial desiccation within a few days (Kahl and Knülle, 1988). In contrast, nymphs and adults take up water vapour passively at 81-85% at 15°C and 20°C for a few days and then uptake abruptly ceases 1-3 days after initiation of apolysis (exoskeleton shedding; Kahl & Knülle, 1988).

While on the host, a feeding tick ingests large quantities of water and ions that it returns to the host via the salivary glands to maintain osmotic balance and concentrate blood portions (Needham and Teel, 1991). However, off the host, the engorged tick needs to find a way to conserve this water, and ticks have been found to be highly susceptible to desiccation requiring relative humidity of more than 85% to survive (Oorebeek and Kleindorfer, 2008; Tack, Madder, Baeten, Vanhellemont, and Verheyen, 2013). At high humidity, ticks quest for longer periods thus improving their chances of finding a host (Randolph, 2004). Especially in dry regions, tick survival is more dependent on increased humidity rather than temperature. In Australia, where summer temperatures are high and winters are milder than other parts of the world, there is high abundance of *I. hirsti* (cat tick) under conditions of high relative humidity and rainfall and lower temperature (Oorebeek and Kleindorfer, 2008).

Saturation deficits, which integrate the temperature and relative humidity of the environment to provide an estimate of the drying power of the atmosphere, are used by many scientists as a factor affecting life cycles and can be calculated using the formula in Randolph and Storey (1999).

### 1.8.2 Host dependant factors

Host specificity is measured by the degree of dependence of a tick species on a particular host species or taxonomic group (Hoogstraal and Aeschlimann, 1982). Host specificity in ticks can range from strict (as in the case of *I. lividus* Koch, 1844, which feeds exclusively on sand martins, *Riparia riparia*) to moderate (e.g. in the bat tick, *I. vespertilionis*, Koch 1844, which parasitises several species of bats) to the very broad and wide-ranging host range of generalists, such as *I. ricinus* (Hillyard, 1996).

Host availability also affects tick abundance (the number of ticks present in certain area), which peak with higher number of available hosts (Oorebeek and Kleindorfer, 2008). This is most often seen in the breeding season where there is a constant host available, in the form of parents or offspring (Oorebeek and Kleindorfer, 2008). An example of the effect of host densities on ticks was observed in sub-Antarctic King and Macaroni Penguins where there was an increase in the presence of engorged *I. uriae* during breeding and incubation, with increased host availability on shore (Frenot et al., 2001). Host range and activity also affect the number of ticks on a host, also known as tick load (or tick infestation). For example, western fence lizards (*Sceloporus occidentalis)* with larger home ranges had higher tick loads than conspecifics with smaller home range (Tälleklint-Eisen and Eisen, 1999) as they were exposed to more woodland areas increasing the exposure rate to parasites.

### 1.8.3 Vegetation & habitat

Spatial and temporal variation in tick abundance is not just associated with temperature and relative humidity, but strongly related to vegetation type. A large part of a tick's life involves questing for the host. Exophilic species of ticks are known to walk nine meters after dark, possibly to find a favourable questing site in their surroundings and others are known to walk on average 40 cm in a day around its questing post and find shelter in litter or soil cracks when no host is found (Obsomer et al., 2013). Different tick stages have their own preferences for microhabitats (Klompen et al., 1996, Goethert and Telford 2009). While the larval and nymphal stages of *I. pacificus*, may prefer insulated microhabitats such as within cracks in the soil, inside rodent burrows, or beneath rocks, to develop (Padgett and Lane, 2001), significantly more adults are found in vegetation (Cilek and Olson, 2000) and open grasslands (Tälleklint-Eisen and Eisen, 1999). Larvae and nymphs are often found to quest within the leaf litter rather than ascending vegetation since they are more prone to desiccation and mortality in open grasslands (Padgett and Lane, 2001; Tälleklint-Eisen and Eisen, 1999). Nesting and denning cavities/burrows of vertebrate hosts provide key microhabitats for ticks, and birds are known to include green plant material and feathers that reduce parasite fitness (Heeb, Kölliker, and Richner, 2000).

Table 1.2: *Developmental times for seven different species of Ixodid ticks kept at standardised laboratory conditions (at 24 ± 1°C, ≥ 90% RH and a 16:8 hours [light: dark] photoperiod). Modified from (Troughton & Levin, 2007).*

| Species | No. Generations | Female feeding | Gestation | Egg incubation | Larval feeding | Larval molt | Nymphal feeding | Nymphal molt | Normal life cycle |
|---|---|---|---|---|---|---|---|---|---|
| | | Unit: days | | | | | | | |
| *Ixodes scapularis* | 18 | 6-10 | 17-19 | 42-46 | 3-7 | 37-43 | 3-8 | 37-47 | 204-219 |
| *I. Pacificus* | 9 | 4-14 | 21-31 | 35-49 | 3-8 | 37-44 | 3-9 | 40-45 | 214-229 |
| *Amblyomma americanum* | 15 | 10-15 | 14-21 | 50-61 | 4-9 | 21-26 | 3-8 | 28-39 | 196-211 |
| *Dermacentor occidentalis* | 5 | 5-12 | 11-22 | 35-42 | 3-7 | 21-28 | 5-9 | 40-41 | 180-195 |
| *D. variabilis* | 14 | 6-12 | 10-24 | 37-49 | 2-8 | 13-35 | 4-11 | 26-35 | 176-191 |
| *Haemaphysalis leporispalustris* | 10 | 9-23 | 10-25 | 35-51 | 11-16 | 26-39 | 6-16 | 24-33 | 209-224 |
| *Rhipicephalus sanguineus* | 13 | 7-12 | 8-23 | 28-35 | 2-7 | 13-42 | 4-10 | 27-35 | 162-177 |

## 1.9 Ticks as vectors of diseases

Over the past years, there have been many new emerging/re-emerging vector-borne diseases (Beugnet et al., 2009, Colwell et al., 2011, Shaw et al., 2001). This emergence is thought to be most likely due to climate change and global warming (Daszak and Cunningham, 1999; Harvell et al., 2002; Valera et al., 2006). Climate change is especially likely to affect vector borne diseases (Gage, Burkot, Eisen, and Hayes, 2008) as the globe warms, vectors are able to access former vector free zones and increase their distribution range and abundance. Environmental changes improve the vector competence of existing species, which may result in more new infections emerging (Tompkins and Slaney, 2014).

Even though they do not fly or jump, ticks have evolved to become one of the most important groups of arthropod vectors of human and animal pathogens (Parola and Raoult, 2001). An adult tick may consume anywhere from 0.51ml (*Ixodes scapularis)* of blood to as much as 1.34ml (*Dermacentor variabilis)* (Koch and Sauer, 1984). However, they concentrate the blood meal by injecting large quantities of water and ions back into the host using the salivary glands (Koch and Sauer, 1984; Rechav et al., 1994). This mechanism is partly why they are good as vectors of diseases. Theobald Smith and Frederick Kilbourne were the first to demonstrate tick as vectors of diseases, between 1889– 1893, with their experiments on transmission of *Babesia bigemina* in cattle by the

cattle tick, *Boophilus annulatus* (Heyman et al., 2010). Today, ticks are recognised vectors of a number of well-known diseases, some of which are also zoonotic, such as Rocky Mountain spotted fever (Demma et al., 2005), Crimean-Congo haemorrhagic fever (Charrel et al., 2004), African tick bite fever (Hotez and Kamath, 2009) and Lyme disease (Spielman et al., 1987). Ticks are second only to mosquitoes as vectors of haemoparasites[17] that infect vertebrates (Prudencio et al., 2010; Sonenshine, 2018). They are particularly important in the transmission of Apicomplexan blood parasites known as Haematozoan.

Haematozoan infections depend on a number of factors like the availability of arthropod vectors, presence of the parasite themselves, population of host species, and opportunities for the vectors to feed. Haematozoans have a varying degree of specificity to their invertebrate vector as well as the vertebrate host. Maintenance and persistence of haemoprotozoa within the tick vector is ensured by trans-ovarian (parent to offspring) and trans-stadial (stage to stage) transmission which can occur over several ticks.

### 1.9.1 Detection of Haemoparasites

Traditionally, haemoparasite infections are confirmed on the basis of history, clinical symptoms, identification of parasites in blood smears and histopathology of tissue in affected hosts (Earlé, Huchzermeyer, Bennett, and Brassy, 1993; Schein, Mehlhorn, and Voigt, 1979; Yabsley et al., 2009). However, the detectable prevalence (proportion of parasite infected samples from the total number of samples) of the parasite depends on the intensity of infection (mean number of parasites per unit of sample tested), life cycle stage of the parasite, and the immune response of the host. More often than not, parasitic infections are chronic and low levels of parasitaemia make detection difficult (Rae, 1995). In some cases, the host may have concurrent mixed infections with different protozoa that show very similar morphology and aetiology (Page 1987; Peirce et al., 2004).

Reliable diagnostic tests are therefore required for detection of haematozoa in the host as well as the vector. Molecular detection technology like polymerase chain reaction

[17] Haemoparasite: An animal parasite (bacterium, apicomplexan, hemoflagellate or filarid worm) living in the blood of a vertebrate.

(PCR) that target and amplify parts of the parasite genome are now used more frequently to detect haemoparasites up to the species level (Hellgren, 2004; Krizanaskiene et al., 2006). DNA- based diagnostic methods are very effective and have the ability to detect parasites at low densities (Freed and Cann 2003). For instance, Torres et al. (2006) have been able to detect *Plasmodium* spp. infections as low as one malarial parasite/µl of blood. However low densities of parasites, in cases of chronic infections, are very often missed by PCR. Metagenomics (MGS) helps to eliminate that problem using next-generation sequencing (NGS). In MGS, a vast number of reads are sequenced in a single stroke, where a DNA fragment of around 100 to 200 base pair is used. This then undergoes massive parallel DNA sequencing using bridge PCR and fluorescent labelled nucleotides which are read by a computer program ultimately resulting in several million clusters of amplified DNA (Shendure and Ji, 2008). These can then be run through the National Center for Biotechnology Information (NCBI) gene database to identify the various parasites, if present. However, while NGS is revolutionising the future of DNA sequencing techniques, there is a lack in personnel expertise required to interpret and analyse results (Behjati and Tarpey, 2013; Metzker, 2010).

## 1.10 Ticks in New Zealand and the Kiwi Tick

New Zealand has 12 species of ticks, five are endemic, six native, and one is introduced (Table 1.3; Heath, 2012; Heath and Palma, 2017; Heath, Palma, Cane, and Hardwick, 2011). After Dumbleton (1953) first compiled the Ixodid ticks found in New Zealand, various authors have then described them taxonomically and reclassified some of them based on more detailed investigations (Heath, 1987; Heath and Palma, 2017; Murray, Palma, Pilgrim, and Shaw, 1990; Spain and Luxton, 1971).

Out of the five endemic species of ticks only *Ixodes anatis* (kiwi tick) has been found on brown kiwi in addition to the introduced cattle tick, *Haemaphysallis longicornis* (Castro, 2006; Heath, 2010). The classification of *I anatis* has been controversial, the type specimens for the first ticks found on kiwi were lost, so the type specimen used to describe it came from a grey duck (*Anas superciliosa;* Heath and Kwak, 2019). However, apart from the rare case of having been found on the grey duck and later on a Canada goose (*Branta canadensis*), the tick has been exclusively found on kiwi in large numbers

(Heath, 2010). Thus, *I. anatis* is said to be a host specific kiwi tick found on two species of kiwi, brown kiwi and tokoeka (*Apteryx australis)* (Heath, 2010). The morphology, classification and phylogenetics of *I. anatis* have been well documented (Heath, 2010; Kwak et al., 2017; Kwak and Heath, 2018). However, a single short-term observational study has looked into the prevalence of *I. anatis* in kiwi burrows (Swift, Heath, and Jamieson, 2015). Heath (2010) used published records and museum samples to describe seasonality and biology of *I. anatis.* However, seasonality cannot be based on chance collections of parasites and describing biology of a tick by drawing parallels from other species is only theory based until proven by experimentation and observations in the habitat of the tick over time. Heath (2010) presumed that the *I. anatis,* like other ticks, was a three-host tick and that copulation between male and females took place off host. *I. anatis* is suspected of transmitting *Babesia kiwiensis* and *Hepatozoon kiwi* (Jefferies et al., 2008; Pierce et al. 2003) but may also transmit other protozoans such as malaria and theileria. Kiwi have also been diagnosed with avian malaria through smear and PCR work (Howe et al., 2011; Schoener we at., 2014). There are also unconfirmed records of possible *Theileria* spp. found in NIBK blood samples (Castro pers. comm).

### 1.10.1 Babesia, Theileria and Hepatozoon

*Babesia, Theileria* and *Hepatozoon* are single- celled *Apicomplexan* parasites (*Protozoa*, Phylum *Apicomplexa*). These protozoa are intracellular parasites that have an apical complex composed of specialized organelles that are important in host cell penetration (Atkinson, 1991). They rely on ticks as their arthropod vector to complete their life cycle. For most of the species, Ixodid ticks act as intermediate hosts but *Hepatozoon* has been reported to have tick, louse, flea, or mosquito as a hosts (Watkins et al., 2006). These parasites possess sexual and asexual life cycles: the fertilisation and formation of zygotes and the asexual sporogony occur in the invertebrate host, while the sexual gametogony and asexual schizogony happen in the vertebrate host (Schnittger, Rodriguez, Florin-Christensen, and Morrison, 2012). While *Babesia* spp*.* infect only erythrocytes, *Theileria* and *Hepatozoon* spp*.* are known to infect both lymphocytes and erythrocytes of the vertebrate host and do not have transovarial transmission in ticks (Chauvin, Moreau, Bonnet, Plantard, and Malandrin, 2009; Schnittger et al., 2012).

In severe cases babesiosis causes haemolysis of red blood cells, leading to anaemia, haemoglobinuria, hyper bilirubinuria, possible organ failure and death (Altay, Dumanli, and Aktas, 2012; Aydin, Aktas, and Dumanli, 2015; Hunfeld and Brade, 2004; Hunfeld, Hildebrandt, and Gray, 2008; Peirce, 2000). There are more than 100 *Babesia* species described worldwide (Hunfeld et al., 2008), the most important cases are reported in cattle, dogs and humans. For birds, at least 13 *Babesia* spp. are described, some of these have only been found in one bird species while others seem to be specific to the birds' order or family (Peirce, 2000).

*Theileria* is a tick–borne haemoprotozoa that has a major impact on livestock (Mehlhorn, Schein, and Ahmed, 1994). Some of the main species causing diseases in cattle are *Theileria parva*, and *Theileria orientalis* (Irvin, 1987). Symptoms for theileriosis are similar to those caused by *Babesia* spp (Mehlhorn et al., 1994; Yin, Schnittger, Luo, Seitzer, and Ahmed, 2007).

While *Hepatozoon* spp. has a similar life cycle like that of *Theileria* and *Babesia* spp. and the sexual reproduction takes place in the vector, the parasites stay in the haemocoel and do not travel to the salivary glands (Smith, 1996). The infections in the vertebrate hosts take place when the host either directly or indirectly ingests the infected vector (Baneth, Samish, and Shkap, 2007). After this, the sporozoites migrate to the liver and undergo asexual reproduction and gamonts are then released into the blood stream where they infect erythrocytes (Smith, 1996). In severe cases they may cause fever, anaemia and emaciation (Baneth and Weigler, 1997).

## 1.11 North Island Brown Kiwi (NIBK)

Kiwi (order-Apterygiformes, family- Aterygidae and genus- *Apteryx*) are the smallest member of the Ratite group (infraclass – Palaeognathae), which is a group of flightless birds containing giants such as the Elephant bird, Moa, Ostrich, Rhea, Emu and Cassowaries. Kiwi are nocturnal, ground-dwelling birds endemic to New Zealand (Holzapfel et al., 2008). There are five recognised species of kiwi (Weir, Haddrath, Robertson, Colbourne, and Baker, 2016). These are, in order of declining population sizes, north island brown kiwi (NIBK*)*, southern brown kiwi or tokoeka, great spotted or roroa (*Apteryx haastii)*, little spotted or pukupuku (*Apteryx owenii)* and rowi (*Apteryx rowi)* (Weir et al., 2016).

*Table 1.3: The 12 species of ticks found in New Zealand, their distribution and status. (Modified from Heath et al., 2011)*

| Species name | Distribution | Status |
|---|---|---|
| *Ixodes uriae* | Bipolar; 40˚N to 70˚N and 40˚S to 64˚S; circumpolar at those latitudes. | Native |
| *Carios capensis* | Widespread on islands of South Atlantic, Pacific and Indian Oceans and occasionally on the mainland between 40˚N and 45˚S. | Native |
| *Haemaphysallis longicornis* | Western Pacific from 50˚N to 42˚S | Introduced |
| *Ixodes amersoni* | Pacific: Enderby Island, Phoenix Islands group; Curtis Island, Kermadec Island | Native |
| *Ixodes eudyptidis* | New Zealand and southern Australia | Native |
| *Ixodes auritulus zealandicus* | New Zealand subregion and sub-Antarctic | Endemic |
| *Ixodes kerguelenensis* | New Zealand subregion and sub-Antarctic | Native |
| *Amblyomma sphenodonti* | New Zealand | Endemic |
| *Ixodes anatis* | New Zealand | Endemic |
| *Ixodes jacksoni* | New Zealand | Endemic |
| *Carios quadridentatus* | New Zealand | Endemic |
| *Ixodes laridis* | New Zealand and Australia | Native |

### 1.11.1 Ecology, breeding and habitat use

NIBK are long lived birds with an average estimated age of 18.7 years and a maximum of 65 years (McLennan et al., 1996; Robertson, 2004). Adult NIBK are sexually dimorphic where females are larger (up to 25%) and disproportionally have longer bills (Sales, 2005; Reid and Williams, 1975; Potter, 1989). Like other nocturnal species, kiwi have poor eyesight and rely instead on tactile, olfactory and auditory cues to navigate, forage and socialise (Martin et al., 2007; Cunningham, Castro, & Alley, 2007; Martin et al., 2007; Castro et al., 2010; Corfield et al., 2014).

Historically, NIBK were considered to be monogamous. However, there are increasing records of polyandry and polygynandry in addition to monogamy (deVieco, 2019;

Taborsky and Taborsky, 1999; Ziesemann, Brunton, and Castro, 2011). Breeding occurs in winter, from June to January (Taborsky and Taborsky, 1999; Ziesemann et al., 2011), although the species has also been found breeding outside this time. The female NIBK lay up to two eggs per clutch and can have two clutches per season while the males incubate the egg(s) (Colbourne, 2002). Clutches, on average are laid 25-30 days apart and incubation lasts for 75-85 days (Ziesemann et al., 2011) during which the males are active for only 1-4 hours every night as opposed to 9-12 hours that the females do. Nest reuse is common in NIBK with more successful hatching in previously used nests (Ziesemann et al., 2011)

As long as the kiwi have a rich supply of soil invertebrates and a cover for shelter to roost in, they can be found inhabiting a wide variety of vegetation types, from a hole in the middle of a pasture, to modified pine forest or dense native bush (Dixon, 2015; Potter, 1990).

### 1.11.2 Threats and Conservation Efforts

The current conservation status of NIBK is considered as declining according to the New Zealand Threat Classification System (NZTCS) (Robertson et al., 2016) and current conservation efforts focus on extensive predator control and Operation Nest Egg (ONE). ONE is a captive scheme in which wild kiwi eggs are lifted to captive institute to hatch and these chicks are hand reared and put into predator proof crèches until they are >1000g (considered introduced mammalian predator safe) and then released back into the wild (Colbourne et al., 2005). This is important as while adult birds have low mortality due to predators, chick mortality can be high with less than ten percent of chicks reaching adulthood with stoats (*Mustela erminea*), ferrets (*Mustela putorius*) and cats (*Felis catus*) being significant predators (McLennan et al., 1996; Wilson, 2014). Intensive management of NIBK through programs such as ONE raises an increased number of immunologically naïve birds which are more susceptible to diseases such as coccidiosis ( Morgan et al., 2012).

### 1.11.13 Kiwi Haematology

Morgan (2008) provided a detailed guide to kiwi veterinary care along with a compiled range of haematological parameters to use as normal reference for NIBK, rowi and the great spotted kiwi. While this guide is very helpful, the normal reference range for NIBK (sourced from Doneley, 2006) does not provide a sample size or population source for the given values. Nevertheless, a summary of the previously reported values used in this study are given in Table 1.4 for NIBK and great spotted kiwi.

Table 1.4: Table modified from Morgan (2008) indicating the normal reference values for two species of kiwi where n indicates sample size and uk = unknown. *these are the percentages of different white blood cells reported for great spotted kiwi. **are calculated values obtained as a fraction of heterophils over lymphocytes for both species.

| **Haematological parameters** | | North Island Brown Kiwi (*Apteryx mantelli)* Doneley (2006) | | Great Spotted Kiwi (*Apteryx haasti)* Robertson (2006) | | |
|---|---|---|---|---|---|---|
| | **unit** | **n** | **mean (Range)** | **n** | **mean (Range)** | **%*** |
| PCV | % | uk | 46 (38-54) | 25 | 39.4 (35.3 - 43.5) | |
| Hb | g/L | uk | not given | | not given | |
| Glucose | mmol/L | uk | 3.0 - 3.9 | | not given | |
| Serum Proteins | g/L | uk | 54 - 62 | 25 | 46 (39 -53) | |
| Heterophils | ($x10^9$/L) | uk | 6.0 (4.0-8.2) | 29 | 8.7 (4.3-13.1) | 55 (45-65) |
| Lymphocytes | ($x10^9$/L) | uk | 4.2 (2.5- 5.9) | 29 | 4.8 (1.9-7.7) | 32 (22 -42) |
| Monocytes | ($x10^9$/L) | uk | 0.3 (0.1-0.5) | 28 | 0.5 (0 - 1.1) | 2 (0-5) |
| Eosinophils | ($x10^9$/L) | uk | 0.18 (0.7-1.29) | 28 | 0.8 (0 -1.9) | 4 (0-8) |
| Basophils | ($x10^9$/L) | uk | 0.56 (0.09 - 1.3) | 28 | 0.9 (0.4-1.4) | 6 (3.0-9.0) |
| MCHC | g/L | uk | 250 (110-333) | | not given | |
| H:L ratio | - | uk | 1.4 - 1.6** | 29 | 1.7 - 2.3 ** | |
| WBC | ($x10^9$/L) | uk | 11.6 (8.7 - 14.5) | 29 | 15.7 (7.9-23.5) | |

### 1.11.14Ponui Island, NIBK and parasites in a wild population context

Ponui Island ($36^0$55'S, $175^0$11'E), is located approximately 30 km south-east of Auckland in the Inner Hauraki Gulf, New Zealand. Ponui Island is divided into three farms two of which are owned by the Chamberlin family and one by the Spencer family (Miles and Castro, 2000). The island has been farmed since 1850's (Bellingham, 1979) mainly for sheep and beef.

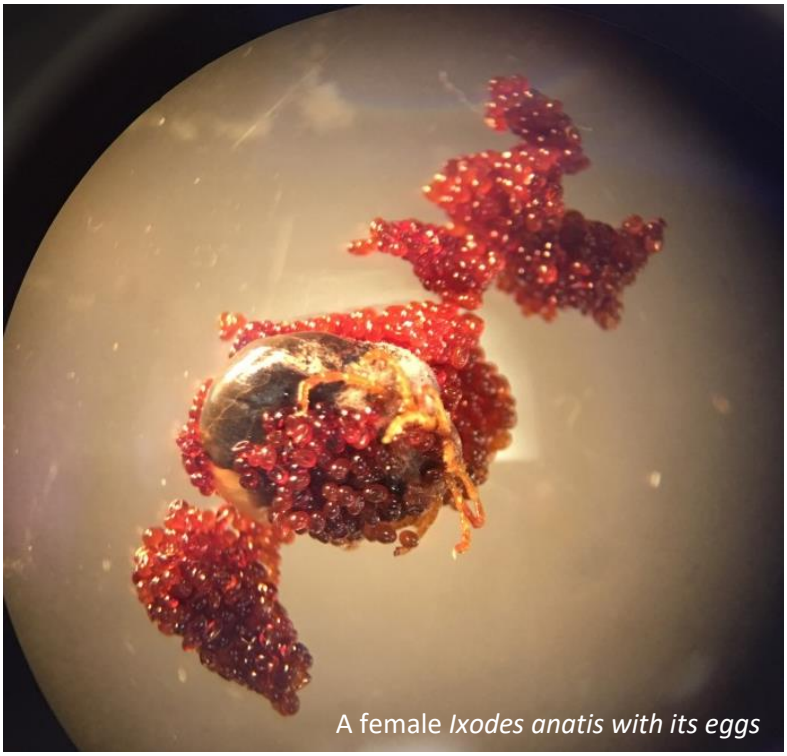
Ponui Island has a range of different habitats of which two thirds is pasture and the remaining third is a combination of scrub (consisting mainly of *Coprosma* spp.

*Leptospermum ericoides* and *Pseudopanax* spp.), swamp (largely *Typha orientalis*) and broadleaf-podocarp forests (Shapiro 2005).

The study site covered approximately 2 $km^2$ located on the southern 1/3$^{rd}$ part of the island. The NIBK population on the 18 $km^2$ island originated from 14 birds (six from Little Barrier Island and eight from Waipoua, Northland) translocated onto the island in 1964 (Miles & Castro, 2000) and the numbers have grown and are estimated to be at 100 birds/$km^2$ (Cunningham et al 2007). The NIBK on the island have been intensively monitored by the Behavioural Ecology and Conservation Group led by Dr Isabel Castro since 2004 with the help of radio transmitters attached to the tibiotarsus of individual birds. Due to this long-term monitoring and research program, many aspects of the behaviour and history of the individual birds are known.

The high density of NIBK occurring on Ponui Island has been suggested to be comparable to numbers before the arrival of humans (Ziesemann et al., 2011). These high numbers along with the sharing of roosts and nests (Ziesemann et al., 2011), provide a good habitat for the build-up of parasites. Indeed, the NIBK on Ponui have been found to carry large ectoparasite loads of kiwi ticks, cattle ticks, lice, mites and rat fleas (Castro,2006; Heath, 2010). The presence of a host population living with its parasites offers a unique opportunity to measure their relationship, in particular the effect of the kiwi tick on its host.

# Chapter 2



A female *Ixodes anatis with its eggs*

The world is full of wonders, but they become more wonderful, not less wonderful when science looks at them - David Attenborough

# Chapter 2: Aspects of the development of Ixodes anatis under different environmental conditions in the laboratory and in the field

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## 2.1 Introduction

The amount of time that each stage of ticks takes to completion is determined by the interactions between temperature and moisture (relative humidity, RH) in the off-host habitat (King, Gettinby, and Newson, 1988; Randolph, 2004; Tukahirwa, 1976). During protracted off- host (questing) and engorged periods of their life, ticks are more prone to desiccation than when feeding (e.g. Apanaskevich and Oliver 2014) and their ability to perform bodily functions largely depends on water vapour absorption (Gaede and Knülle, 1997). Thus, optimum developmental conditions ensure faster progress to the next stage of the life cycle and better chances of survival. Numerous laboratory-based studies have explored the response of different species of Ixodid ticks to microclimates and their influence on developmental times (Arthur and Snow, 1968; Heath, 1979, 1981; Kahl and Knülle, 1988; Lees, 1946; Needham and Teel, 1991; Padgett and Lane, 2001; Troughton and Levin, 2007; Yoder, Hedges, and Benoit, 2012). Most of these studies agree that, for optimum development, ixodid ticks prefer temperatures between 18 and 26°C and relative humidity between 75% and 94%. Some of these studies showed that an increase in temperature within the preferred range reduced moulting times, and that while some species were able to tolerate temperatures up to 38°C, mortality rates

increased. At lower temperatures such as 4-8°C some species continue development, but at a greatly reduced rate and with higher mortality. Similar results have been demonstrated in the small number of field studies that have been conducted with various species (Campbell and Glines, 1979; Koch and Tuck, 1986; Norval, 1977; Ogden et al., 2004; Troughton and Levin, 2007).

*Ixodes anatis* Chilton, 1904, is a host-specific ixodid tick found on apterygid birds; the North Island brown kiwi (*Apteryx mantelli,* NIBK*)* and the Tokoeka (*Apteryx australis*), and therefore is endemic to New Zealand (Dumbleton, 1953; Heath, 2010). It is an endophilic, nidicolous species which has only been recovered either off the hosts or within the burrows of these birds. *Ixodes anatis* of all stages are prevalent in kiwi burrows throughout the year (Swift et al. 2015; Bansal et al., 2019; pers. obs.).

Our aims in this study were two-fold: to determine in the laboratory the conditions of temperature and RH that ensured the best survival, and the shortest interstadial periods for the kiwi tick, and to contrast these with those of ticks of different stages placed in artificial kiwi burrows outdoors. To date, little is known about the environmental conditions that are ideal for the development of *I. anatis* and therefore our null hypothesis was that this species would behave comparably to other species with similar ecological requirements such as *I. uriae, I. aboricola*, and *I. trianguliceps* (Gray et al., 2014) or species in other genera such as *Amblyomma* and *Archaeocroton* (Barker and Burger, 2018; Gray et al., 2014) which are all examples of nidicoles.

## 2.2 Materials and Methods

### 2.2.1 Experimental Design

Two series of experiments were conducted to determine the optimum developmental conditions for *I. anatis*. In the first, engorged larvae, nymph and adult were incubated under laboratory conditions (Laboratory Experiments) and in the second, engorged ticks were maintained in artificial kiwi burrows (Field Experiments) in a forested area close to the Laboratory (40.3709° S, 175.6303° E; Figure 2.1). In all experiments, the pre-moult period was defined as the time from when an engorged tick was placed in the incubator or burrow to the time it started moulting. Moulting duration was the time from when the tick started moulting until the time the new stage first appeared. Moulting success

was the proportion of ticks that were able to successfully ecdyse. For females, preoviposition was the time from the moment the female was placed in the incubator to the time it started laying eggs, and oviposition was the time taken for the female from starting to stop laying eggs.

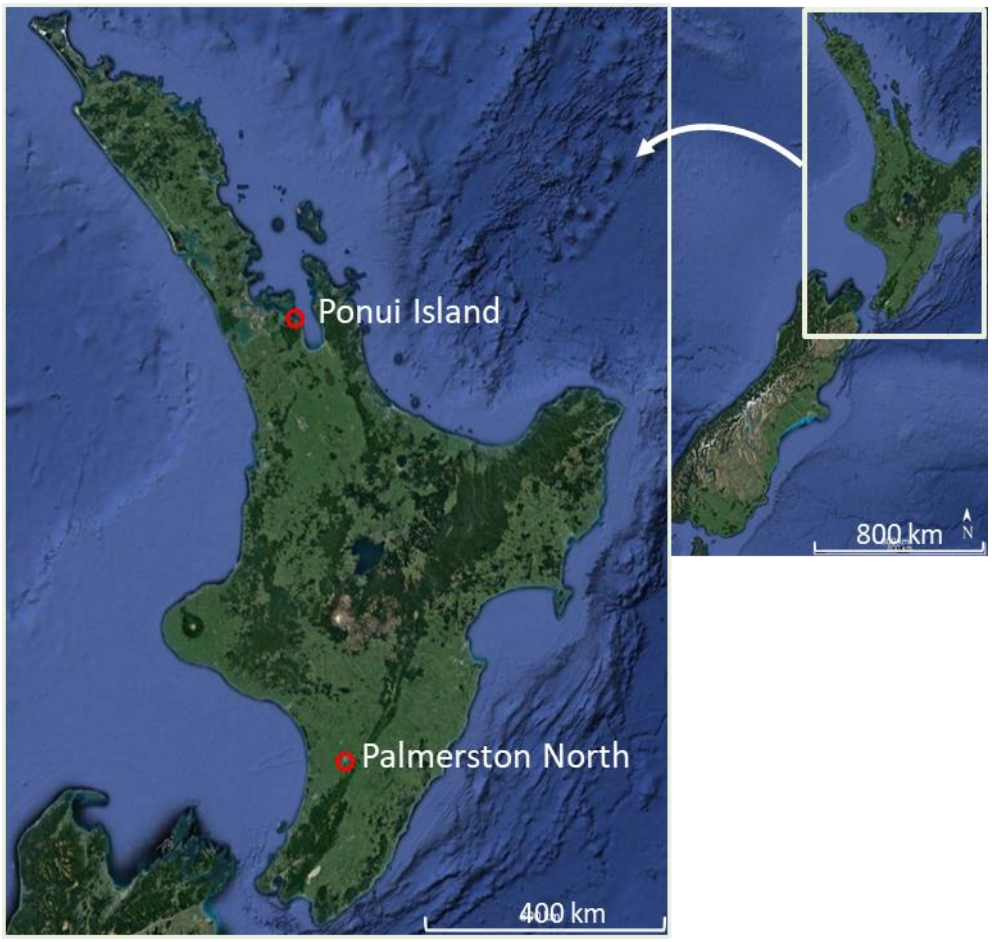



Figure 2.1: Map showing the two sites used in experiments designed to find the best temperature and humidity conditions for the development of *Ixodes anatis*, the kiwi tick. Ponui Island is where the ticks were collected, and Massey University in Palmerston north is where the Laboratory Experiments as well as the Field experiment was conducted.

### 2.2.2 Tick collection

Ticks were collected from NIBK inhabiting a high-density population of 1 bird per hectare on Ponui Island (Inner Hauraki Gulf, New Zealand; 36.8622° S, 175.1842° E; Figure 2.1) (Cunningham, Castro, and Alley, 2007). These birds had been observed to have high densities of ticks, with up to 250 individuals recorded on one host (Castro, 2006; Heath, 2010). Between April and June 2016 (for the laboratory experiments) and March 2018 (for the field experiment), detached, engorged ticks were collected off the birds, bird

handlers, and the bags used to cover the birds during handling for transmitter change or as part of other experiments. Ticks were separated into the three stadial groups (larva, nymph, adult female), placed in plastic containers with fresh vegetation to provide moisture and stored at 4$^{o}$C, for a mean duration of five days (± 5 days), until they arrived in the laboratory at Massey University, Palmerston North (546 km distant from the study site; Figure 2,1).

Tick identification

Two species of ticks are known from kiwi at the study site and were differentiated on the basis of the position of the ventral anal groove as described in Dumbleton (1953). In *I. anatis* the groove is anterior to the anus (Prostriata) and in *Haemaphysalis longicornis* is posterior to the anus (Metastriata) (Figure 2.2). Only *Ixodes anatis* were used in this study.

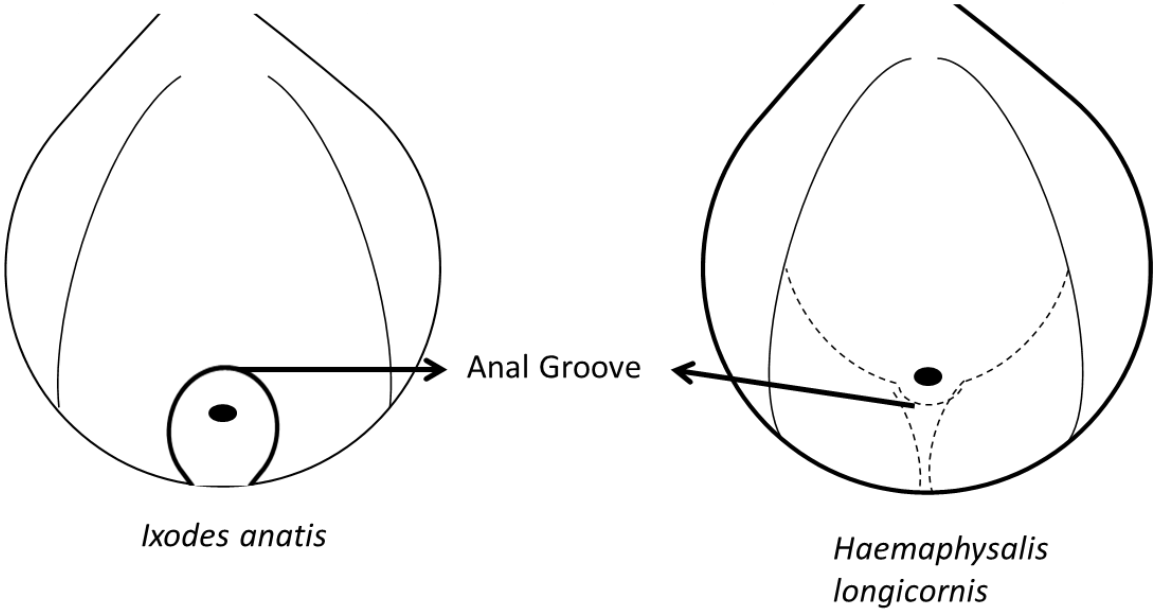



Figure 2.2: A figure showing the ventral view of the abdomen of ticks (basis capituli and legs are omitted) difference between the kiwi (left) and cattle tick (right) with respect to the placement of the anal groove used to identify the species.

### 2.2.3 Pilot Experiments

Pilot experiment 1: Before developing the final protocol for the laboratory experiment, we conducted a pilot experiment to test the effects of a range of

temperatures at a single high humidity. For this, individual engorged larvae and nymphs were placed into transparent, screw-lid, 15ml plastic bottles. A ten mm hole was drilled in the bottle lids and then covered with mesh cloth to allow passage of air. A total of 40 bottles (20 larvae and 20 nymphs) were then placed in 1000ml closed plastic storage containers with water at the bottom to produce a high relative humidity (RH). Each plastic container was placed into the respective incubators pre-set to 9.2°C, 16.5°C, 21°C and 23.5°C. Ticks were examined every second day under a stereo-microscope at 4-10X to check for movement and moulting progress. We did not find any significant difference in the moulting times for larvae and nymphs. Since only 5 engorged adult females were available for study, they were kept, one per bottle, at 16.5°C which was closer to the average environmental temperature for Ponui Island (Dixon, 2015). These were also examined every second day noting the start and completion of egg laying, however none of the females showed any oviposition at this temperature. This pilot led us to try a range of different temperatures and RHs in a second pilot.

Pilot experiment 2: To test the combined effect of temperature and humidity on the stages of the tick we needed to provide ticks with different RHs and place these at different temperature regimes. Winston and Bates (1960) developed protocols to create various RHs for exactly this purpose by dissolving enough solid salt to super saturate distilled water at boiling point. We conducted a second pilot test using salt solutions from their protocols to confirm their results with the idea to have a range of RHs for further experiments with our ticks. The salt solutions we used to achieve the required RH are given in table 2.1. These solutions were placed at the bottom of sealed plastic containers with mesh lids (Figure 2.1) and an iButton Hygrochron™ Temperature/Humidity Loggers (Model DS1923; Maxim Integrated, San Jose, California) was hung from the lid, so it was at the same level as the ticks. The entire setup was placed in fixed temperature incubators at 5˚C, 10˚C, 15˚C, 20˚C, 25˚C and 30˚C. The hygrometers were set to record temperatures every 10 minutes for a week. Despite numerous attempts, not all the salt solutions produced the RHs reported in Winston and Bates (1960) and therefore we used the actual RHs achieved (Table 2.1) as our final RHs for the main laboratory experiment.

Table 2.1: The list of saturated solutions used at different temperatures to achieve required RH. Numbers in the table represent mean RH with standard deviation.

| | 5ºC | | 10ºC | | 15ºC | | 20ºC | | 25ºC | | 30ºC | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Expected | Actual | Expected | Actual | Expected | Actual | Expected | Actual | Expected | Actual | Expected | Actual |
| **Sodium Chloride + KCl** | | | | | | | 70 | 64.90 (± 0.61) | | | 70 | 66.84 (± 0.87) |
| **Sodium Chloride** | 75 | 77.12 (± 0.62) | 75 | 62.11 (± 0.47) | 75 | 66.76 (± 1.18) | 75 | 63.29 (± 0.58) | 75 | 73.85 (± 0.56) | 75 | 72.90 (± 0.6) |
| **Potassium Bromide** | | | 85 | 83.39 (± 0.48) | | | 85 | 66.30 (± 0.71) | 85 | 63.88 (± 0.71) | | |
| **Potassium Chloride** | | | | | | | | | 85 | 83.60 (± 0.98) | 85 | 84.60 (± 0.17) |
| **Potassium Nitrate** | 95 | 92.41 (± 1.86) | 95 | 94.54 (± 0.86) | 95 | 93.86 (± 0.1.53) | | | 90 | 87.84 (± 1.2) | 90 | 81.55 (± 2.01) |
| **Potassium Sulphate** | 99 | 63.74 (± 0.50) | 99 | 94.04 (±0.45) | 99 | 96.37 (± 2.56) | 99 | 85.55 (± 1.58) | 95 | 67.48 (± 1.82) | 95 | 72.86 (± 1.60) |
| **Potassium Dichromate** | | | | | | | | | 99 | 97.57 (± 0.23) | 99 | 98.75 (± 1.80) |
| **Sodium nitrite** | | | | | | | 65 | | 65 | | 65 | |

### 2.2.4 Laboratory Experiment – Effects of a range of temperatures and humidity

Engorged larvae and nymphs were individually placed into small fabric mesh pockets which were suspended above the saturated salt solutions obtained during pilot experiment 2 (Table 2.1, Figure 2.3). These were then incubated at a range of temperatures (5°C, 10°C, 15°C, 20°C, 25°C and 30°C). There were 20 replicates of engorged larvae and 10 replicates of engorged nymphs for each humidity and temperature combination. In addition, 12 engorged adult females were available and divided into four groups of three. To measure preoviposition and oviposition time, two groups were incubated at 15°C and 93% and 96% RH respectively; one at 10°C and 94% RH and one at 20°C and 85.5% RH. Eggs obtained from these female ticks were subsequently divided into batches and placed in mesh bags (50 eggs/bag) at all temperature and RH combinations (Table 2.1). Temperatures and RH were measured every hour using iButton Hygrochron™ Temperature/Humidity Loggers (Model DS1923; Maxim Integrated, San Jose, California). The ticks were observed every two days for evidence of development, for a total of six months, by removing the box (Figure 2.3 B,C) to the counter tops at room temperature. Since the boxes were clear, they were not

opened to observe ticks thus preserving RH within the boxes. Care was taken not to keep any box out for more than five minutes. In this experiment the hypothesis was that both larvae, nymphs and adult females of *I. anatis,* would have more successful and faster developmental times at temperatures between 15°C - 20°C and RH above 90% than in conditions outside this range.

### 2.2.5 Field Experiment

Engorged larvae and nymphs were placed in artificial burrows (n=12) from June to August (Southern Hemisphere winter) 2018. At Massey University, horizontal burrows were dug in a forest environment consisting of clay/silt loam-type soil, and imitated natural burrows. These simulated burrows were approximately 120-150mm diameter and 600mm deep (Figure 2.4). A larger chamber was constructed at the end to mimic a typical kiwi-constructed burrow (de Vieco, 2019). Ten nymphs and 20 larvae were placed in each burrow in mesh pockets (one for each stage: Figure 2.3D). These ticks were checked every two to three days to record moulting. Temperature and RH were recorded every hour using iButton Hygrochron™ Temperature/Humidity Loggers. For this experiment, we expected both the stages to follow the same pattern as found in the laboratory experiment.

### 2.2.6 Statistical analysis

One-way ANOVA were carried out in R (2013) to test for significance between the number of days taken to start and complete moult for the different, where applicable. We also carried out regressions analysis to test the relationship of temperature and RH on preoviposition and oviposition times in females. The saturation deficit (SD), which is the amount of water vapour required to saturate air, (in mm of Hg) was calculated using the formula: $SD = (1- RH/100) * 4.9463e^{0.0621T}$ (where RH is relative humidity in %, e is the mathematical constant 'Euler's number' and T is temperature in °C) (Randolph and Storey,1999). For laboratory experiment and the field experiment results were reported using both RH and the corresponding SD at the given temperatures.

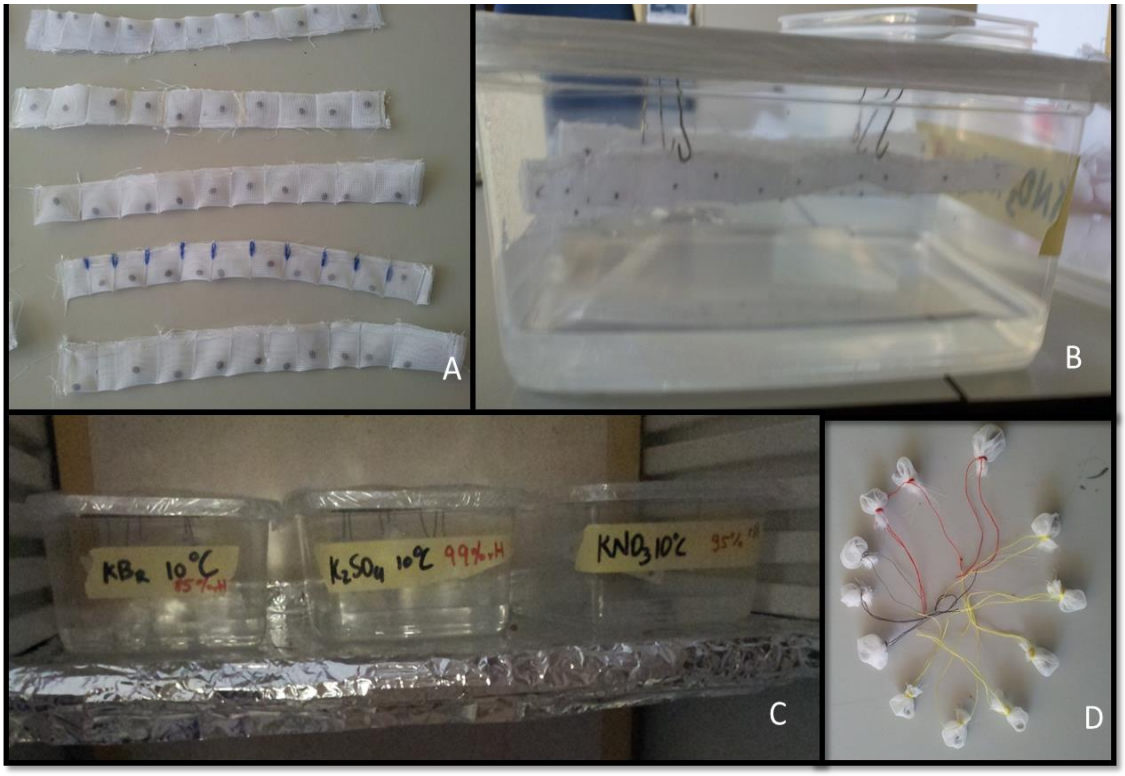



*Figure 2.3: The laboratory setup for the experiment. A- the ten chamber mesh pockets for larvae and nymphs, B- the mesh pockets suspended over the salt solution, C- the entire setup from B placed in an incubator and D- the mesh bags used for housing individual females. *D- same newly made mesh bags were also used for the field method.*

*Figure 2.4: An example of a burrow dug for the field studies at Massey University (photo by David de Vieco).*

## 2.3 Results

### 2.3.1 Laboratory Experiment

Percentage survival of larvae and nymphs and the duration range of pre-moult are summarised in Table 2.2. None of the larvae or nymphs showed signs of development at 5°C, even after 120 days of observation, and all ticks placed at 30°C died within 20

days regardless of environmental humidity. Both larvae and nymphs survived between 10°C to 20°C, with nymphs tolerating a wider range of temperatures than larvae. The greatest overall survival and shortest moulting times for both larvae and nymphs happened at 10°C and 94-95% RH, representing a SD <1 mmHg. Larvae at >5 mmHg SD did not survive, nor did nymphs at >10 mmHgSD.

The mean premoult period for larvae at 10°C and >94% RH was 60 days (range: 64 - 80) and the cumulative time for all larvae to complete a moult was a mean of 14 days (range: 5-21) (Table 2.2). At an RH >93% at 15°C, the mean premoult period for larvae was 56 days (range: 54 – 57) and mean moulting duration was 15 days (range: 14 – 17). At 20°C and between 2-6mmHg SD larvae had a mean premoult period of 73 days (range: 73-75), compared to a mean of 52 days at 5-6 mmHg, which was a statistically significant difference ($p<0.01$). At 25°C, and 64% RH (ca. 8-10mm HgSD) only 60% of larvae showed signs of development with a 35-day premoult period and seven days moulting duration. Larvae at all other experimental temperatures and RHs did not develop.

The nymphs were more tolerant to a greater range of temperature and RH than were larvae. Overall, a variable proportion of nymphs started premoult at each RH but only a small number completed the process (Table 2.2). At SD of > 3 mmHg, the nymphs showed evidence of fungal growth. All nymphs at 10°C started premoult with a mean of 75 days (range: 69-80) and completed moulting with a mean of seven days (range: 4 – 9) irrespective of RH (Table 2.2). At 15°C, the premoult period for nymphs at 67% RH (SD ~4-5 mmHg) was 71 days but only 30% of these completed moulting over a seven-day period. However, at 15°C and a SD of <1 mmHg, nymphs took significantly (ANOVA, $p <0.01$) less time to moult (Table2.2). At 20°C and an SD between 6-8mmHg all nymphs started premoult but only 20% successfully completed the process, which they did over 9 days (Table 2.2). At 66% RH (SD between 5-6 mmHg), all the nymphs moulted but with a large variation in time (mean 14 days; range: 7-42). At 86% RH (SD between 2-3mm of Hg), all nymphs had a premoult period of 38 days, taking 13 days (range: 7-14) to complete the moult. At 25°C and 88% RH (3 mm HgSD) 90% completed a moult.

All six female ticks at 15°C and 93.86% RH and the three at 20°C and 85.55% RH laid around 600 to 750 eggs each. Only one of the three placed at 10°C and only two of three placed at 15°C and 96.37% RH laid eggs. As the temperature increased, the pre-oviposition period significantly decreased ($R^2$=0.9, Fstat= 196, df = 2, p = 0.005) while

the RH had no significant effect (Table 2.3). Those that did not lay eggs, died within 60 days of being placed in the incubators. No eggs hatched under any of the experimental conditions.

Table 2.2: Survival and development time of *I. anatis* engorged larvae and nymphs under tested laboratory conditions. Pre-moult in this table refers to the number of ticks that survived and started moulting and moulting refers to the actual time of moulting from attachment to complete emergence. Saturation deficit of air in each chamber was calculated using the formula from Randolph and Storey (1999).

| Temp (°C) | RH (%) | Saturation Deficit (mm of Hg) | Larvae | | | | Nymphs | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Premoult | | Moulting | | Premoult | | Moulting | |
| | | | Time (days) | Survival % | Time (days) | Survival % | Time (days) | Survival % | Time (days) | Survival % |
| 5 | 64 (± 0.50) | 1-2 | | 0 | | 0 | | 0 | | 0 |
| | 77 (± 0.62) | 1-2 | | 0 | | 0 | | 0 | | 0 |
| | 92 (± 1.86) | <1 | | 0 | | 0 | | 0 | | 0 |
| 10 | 62 (± 0.47) | 3-4 | | 0 | | 0 | 71 ± 1.70 | 100 | 6.3 ± 1.7 | 100 |
| | 83 (± 0.48) | 1-2 | | 0 | | 0 | 71 ± 0.84 | 100 | 7.4 ± 0.8 | 100 |
| | 94 (±0.45) | <1 | 64 ± 4.39 | 100 | 9.8 ± 3.7 | 100 | 78 ± 0.84 | 100 | 6.6 ± 0.8 | 100 |
| | 95(± 0.86) | <1 | 78 ± 1.23 | 100 | 18.3 ± 5.02 | 100 | 78 ± 1.33 | 100 | 7 ± 1.3 | 100 |
| 15 | 67 (± 1.18) | 4-5 | | 0 | | 0 | 71 | 100 | 7 | 30 |
| | 94 (± 0.1.53) | <1 | 57 ±1.41 | 100 | 14.9 ± 1.4 | 100 | 50 ± 0.95 | 100 | 6.7 ± 0.9 | 100 |
| | 96 (± 2.56) | <1 | 57 ± 1.33 | 100 | 14.75 ± 1.3 | 100 | 36 ± 0.84 | 100 | 7.4 ± 0.8 | 100 |
| 20 | 63 (± 0.58) | 6-8 | | 0 | | 0 | 62 ± 1.51 | 100 | | 0 |
| | 65 (± 0.61) | 6-8 | | 0 | | 0 | 66 ± 1.03 | 100 | 9 | 20 |
| | 66(± 0.71) | 5-6 | 52 ± 0.67 | 100 | 14.15 ± 0.7 | 100 | 38 ± 0 | 100 | 12.6 ± 2.95 | 100 |
| | 86 (± 1.58) | 2-3 | 73 ± 0.82 | 100 | 6.6 ± 0.8 | 100 | 66 ± 1.93 | 100 | 14 ± 4.8 | 100 |
| 25 | 64 (± 0.71) | 8-10 | 35 | 60 | 7 | 60 | 35 ±15.65 | 50 | 8.8 ± 2.5 | 10 |
| | 67(± 1.82) | 8 | | 0 | | 0 | 56 | 10 | | 0 |
| | 74 (± 0.56) | 6 | | 0 | | 0 | 70 | 100 | | 0 |
| | 84 (± 0.98) | 3-4 | | 0 | | 0 | 35 ± 1.64 | 50 | 7 | 30 |
| | 88 (± 1.2) | 3 | | 0 | | 0 | 70 ± 2.54 | 90 | 9 ± 1.7 | 90 |
| | 98 (± 0.23) | <1 | | 0 | | 0 | 28 | 70 | 7 | 40 |
| 30 | 67 (± 0.87) | 10-12 | | 0 | | 0 | | 0 | | 0 |
| | 73(± 1.60) | 8-10 | | 0 | | 0 | | 0 | | 0 |
| | 73 (± 0.6) | 8-10 | | 0 | | 0 | | 0 | | 0 |
| | 82 (± 2.01) | 5-6 | | 0 | | 0 | | 0 | | 0 |
| | 85 (± 0.17) | 4-5 | | 0 | | 0 | | 0 | | 0 |
| | 99 (± 1.80) | <1 | | 0 | | 0 | | 0 | | 0 |

Table 2.3: The development times for the 12 female engorged ticks (three at each chamber) at given temperature and RH regimes.

| °C | % RH | SD Mm of Hg | preoviposition period (in days) | oviposition period (in days) | success rate % |
|---|---|---|---|---|---|
| 10 | 94 | <1 | 33 | 7 | 33.3 |
| 15 | 94 | <1 | 27 | 9 | 100.0 |
| 15 | 96 | <1 | 27 | 9 | 66.7 |
| 20 | 86 | 2-3 | 19 | 9 | 100.0 |

### 2.3.3 Field Experiment

Of the 12 burrows, only 11 were included in the analysis because Burrow 4 collapsed 16 days into the experiment. The mean (± Standard Deviation) temperature over all burrows was 13°C (± 0.27) for June, 11°C (± 0.15) for July and 10°C (± 0.09) for August. The mean RH over all the burrows was 66% ± 0.26 for June 68% ± 0.08 for July and 68% ± 0.17 for August (Figure 2.5). The corresponding SD of all the burrows ranged between 3-4 mmHg. Of the 220 engorged larvae placed in the burrows 218 (99.1%) moulted to nymphs. Of 110 engorged nymphs, 101 (91.8%) survived to moult, the remaining nine died after 40 days without completing the development. Larvae in 10 out of 11 burrows had a premoult period of 66 days and took seven days to complete the moult. In Burrow 10, the larval premoult period was 70 days with a duration overall of five days. Nymphs in six burrows had a 70-day pre-moult period with 75 days for the remainder. All nymphs with exception of those in Burrows 3 and 5 finished moulting in eight days. Nymphs took 8 days to moult, with exception of those in Burrows 3 and 5 which took six days (Figure 2.6).

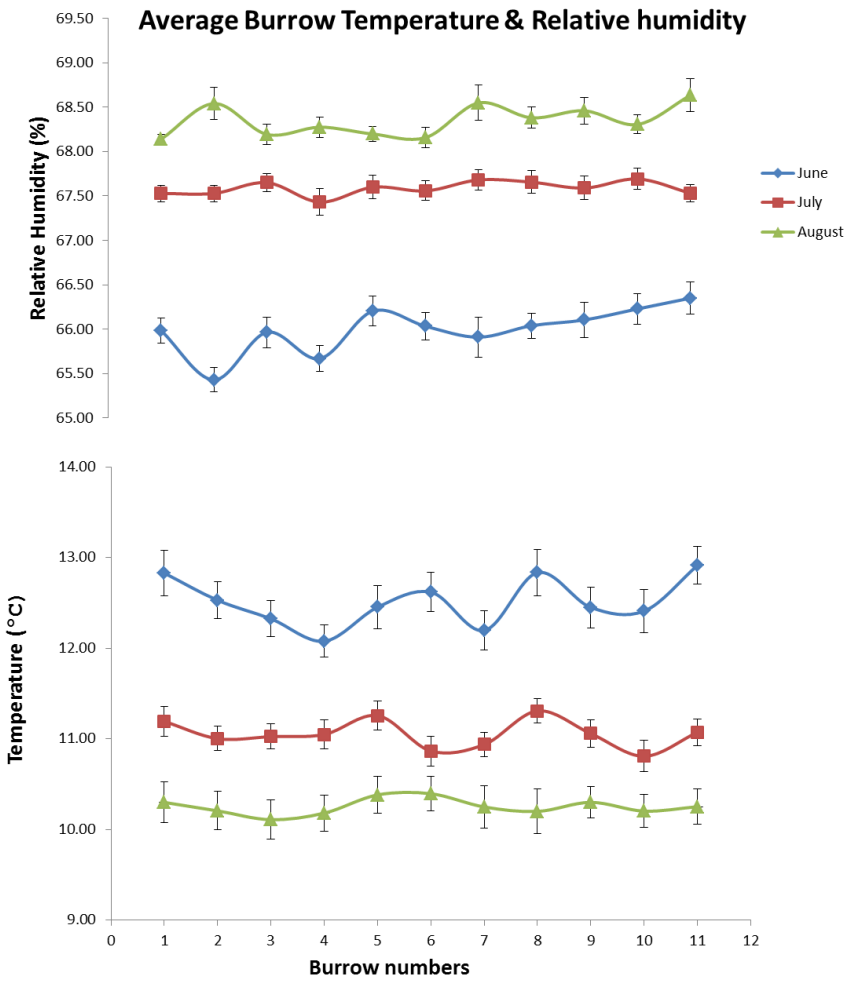



Figure 2.5: Average temperature and RH (± SE) in artificial kiwi burrows during June (blue), July (red) and August (green), 2018.

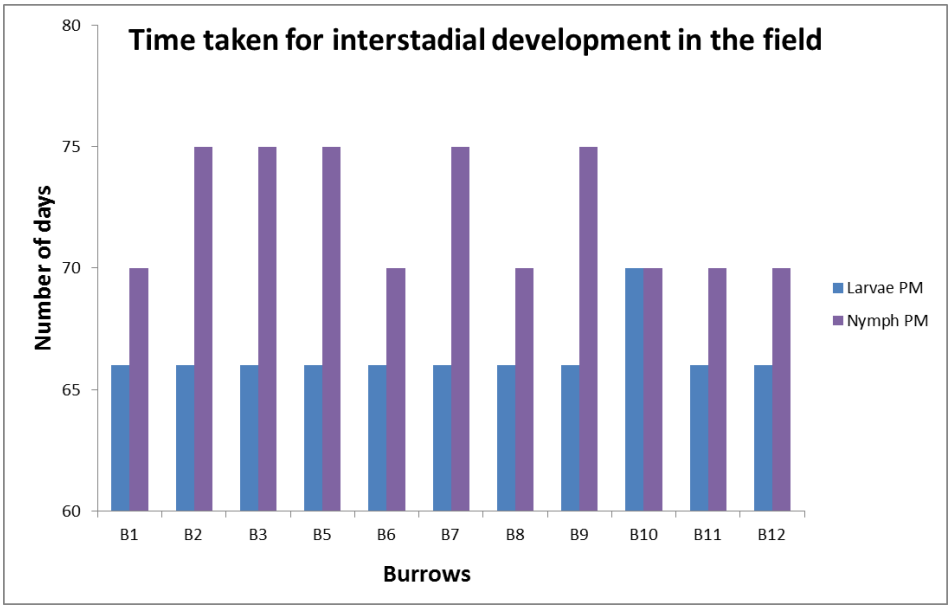



Figure 2.6: Time taken (in days) for development of immature stages of *I. anatis* in the field experiments

## 2.4 Discussion

Our experiment did not support our initial hypothesis that *Ixodes anatis* would act comparably to most other nidicolous tick species in terms of conditions of preferred temperature and humidity. Furthermore, the laboratory conditions that favoured survival and shorter moulting were very different to those in the field experiments.

Under laboratory conditions, the requirements for larvae were narrower than for nymphs. Engorged larvae showed optimum development (moulting times and survival) at 10-20°C when SDs were <1-2 mmHg (RH>94%). Engorged nymphs survived and moulted up to 25°C but, like larvae, appeared to favour a range of 10-20°C, although with the ability to survive a somewhat drier atmosphere, tolerating a SD range of <1-10 mmHg. Females laid eggs at all temperatures and the range of humidity tested, although the pre-oviposition period was from six to 14 days longer at SD of <1 mmHg as compared to 2-3 mm of Hg. However, due to the small number of females tested, this result serves only as a loose guideline and needs to be further explored. The failure of eggs to hatch at the various temperatures and RHs combinations could be due to several reasons. The longer duration of oviposition at the lower temperature may have exposed the eggs to a greater decline in their water balance than would have occurred at higher temperatures. Also, breaking the eggs into smaller batches would have possibly increased the surface area of the egg mass and exposed them to increased dehydration. However, the failure of eggs to hatch requires further investigation with a larger sample size of females.

Under field conditions, the temperatures in the burrows varied slightly across the 3 months with a mean temperature of 11°C (range: 10-13), mean RH of 67% (range: 65-69) and a calculated SD between 3-4mm of Hg, which were at the lower end of the favourable range for both larvae and nymphs, but ambient humidity was a little drier than the larvae would seem capable of tolerating. Having said this, the RH was measured in the burrow air, not at the soil surface which may have been slightly more humid. From previous studies (deVieco, 2019) as well as data collected during this study (Appendix 2.1) while external temperature fluctuates, the diurnal temperature within the burrow remains relatively constant. In addition, over the year, the microclimate in a burrow is

not as extreme as in the external environment and remains within a range of ± 6 units for both temperature and humidity.

In the burrows, the developmental success rate for both larvae and nymphs was very high (99% and 91.8% respectively). In the laboratory however, larvae exposed to similar conditions (10°C and 62.1%-83% RH; SD 1-4 mmHg), did not survive. It is possible that engorged larvae in burrows were in closer contact to available soil moisture, and able to absorb it in through the cuticle or experience reduced water loss. Larvae in laboratory chambers were surrounded by humid atmospheric air, but at a level perhaps lower than that experienced by larvae in burrows. Ogden et al., (2004) reported that even small fluctuations or changes in temperature and humidity can affect the developmental times in ticks. Therefore, it is also possible that these differences between lab and field results may have been caused by our routine checks as larvae are less tolerant to minor changes in temperature and RH (Chilton and Bull, 1993). As the boxes with the ticks were removed from the incubators to a setting at room temperature, even for five minutes, it could have caused a sudden fluctuation in the temperature and RH in the boxes, exposing the ticks to more severe changes of temperature and humidity when extracted than those in the field as some were kept at considerable higher or lower temperatures than the laboratory environment. Another study by Padgett and Lane (2001) found that when larvae were left undisturbed, they had a higher success of moulting than the ones that were disturbed more often, supporting this possibility.

In studies with kiwi-occupied burrows (Bansal et al., 2019; Swift et al., 2015; Chapter 3) larvae were most prevalent from January to June (summer and autumn), and lowest in October (spring; usually a damper season). Nymphs, on the other hand, were less prevalent in January, with highest numbers from June to December. In the present study the artificial ' burrows' did not have any kiwi, which is very likely to have influenced temperature and humidity levels, both from physiological exhalations, body warmth (Calder, Parr and Karl, 1978) and deposited vegetable and waste matter.

In general, in many species of Ixodidae, immature stages survive better at moderate to high RH (>90%) and between 18°C to 25 °C but die off rapidly at 75% RH at similar temperature conditions (Ginsberg et al., 2017; Needham and Teel, 1991; Padgett and Lane, 2001; Troughton and Levin, 2007). We found that the optimum temperature preferred by *Ixodes anatis* to complete development is between 10°C to 15°C which is

lower than many other species of ixodid ticks. We suggest the extended developmental times as a function of low temperature preference may be an adaptation for survival in burrows which are unoccupied for long periods as well as to the cold temperatures in New Zealand throughout the year. However as in other species, the bioclimatic requirements of larvae are at the lower end of the range tolerated by the species overall. To a large extent this determines both seasonal patterns and habitat suitability for the species because, if larvae are disadvantaged, the life cycle can be disrupted. Nymphs, however, are generally more desiccation resistant and have a better tolerance of higher temperatures than do larvae, with engorged females capable of withstanding even greater bioclimatic extremes (Chilton and Bull, 1993; Heath, 1975, 1981; Needham and Teel, 1991).

The best survival strategy for the kiwi tick is to have a mix of stages in each burrow, ready to take advantage of the return of a host. A quicker development cycle for engorged larvae over the warmer time of year provides unfed nymphs that are able not only to withstand cooler times of the year but also the attendant added risks of dehydration. Unfed stages were not tested in these experiments and such a study would throw additional light on the biology of *I. anatis* in relation to its host.

The kiwi is a nocturnal animal and can range widely in search of food as well as use a multitude of burrows within its range (Dixon, 2015, Jamieson et al., 2016). The tick too is exclusively host-specific (aberrant hosts are very rare; see Heath 2010) and this suggests it would be an advantage for the tick to be sedentary and to be capable of sustained quiescence in the event of the spasmodic presence of hosts. There has been no success in finding questing *I. anatis* outside of kiwi burrows, reinforcing the inference of the tick's sedentary nature and thus its adaptation to stable, but relatively cool and damp conditions in the burrows and reflecting the findings in this study as well as the evolutionary consequences of its association with the kiwi.

This insight into the conditions required for the kiwi tick to develop off-host, only answers one of a three-part question about the life cycle of the ticks and cannot be used by itself to infer ecology of the tick. To get a complete picture of its ecology, it is also necessary to look at the type of habitat available to these ticks off host and the actual abundance of these parasites present in the wild which we address in the next chapter (Chapter 3).

# Chapter 3

Gordon, the kiwi, in a nest box in the daytime

M ā te huruhuru, Ka rere te manu

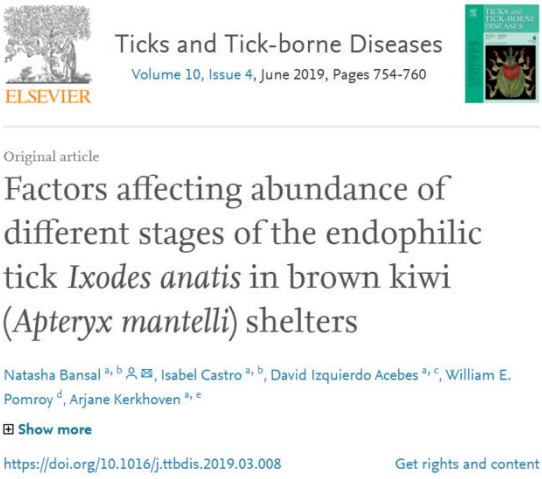
Adorn this bird with feathers to enable it to fly

# Chapter 3: Factors affecting abundance of different stages of the endophilic tick *Ixodes anatis* in brown kiwi (*Apteryx mantelli*) shelters

This chapter has been published in Tick- and Tick-Borne Diseases.

Reference for paper:

"Bansal, N., Castro, I., Acebes, D. I., Pomroy, W. E., & Kerkhoven, A. (2019). Factors affecting abundance of different stages of the endophilic tick Ixodes anatis in brown kiwi (Apteryx mantelli) shelters. *Ticks and Tick-borne Diseases*."




**Author contributions:** Pilot and preliminary experiments: IC and DI. Experimental design: NB and IC. Fieldwork: NB, AK, IC, and DI. Writing: NB, with comments from IC, WP, DI and AK. Analysis: NB, IC.


## Abstract:

Hosts' burrows are a key habitat for their ectoparasites and can influence host-parasite interactions and communities. Knowledge of the structure of host-parasite relationships enables a better understanding and prediction of the likely spread of vector-borne diseases. *Ixodes anatis* is a species of endophilic nidicolous tick found on brown kiwi (*Apteryx mantelli*) and in their shelters and nests. These ticks are also suspected to carry haemoprotozoa such as *Babesia kiwiensis* and *Hepatozoon kiwi* however very little is known about these ticks or their population dynamics and relationships with their hosts. Therefore, a 14-month study was conducted from May 2013 to June 2014 on Ponui Island, in the Inner Hauraki Gulf of New Zealand, to evaluate the seasonal abundance of the different life stages of *I. anatis* in brown kiwi

shelters and how that changed in various locations, habitats (forest, scrub and pasture) and shelter types (tree, soil and surface). In total, 12,172 ticks were collected from 63 shelters which were sampled monthly. Un-engorged larvae predominated over other stages and accounted for 87.2% of the samples collected. Season, shelter location, habitat and shelter type all had effects on tick abundance. We found that location, habitat in which the shelters were located, and the type of shelter were significant predictors of *I. anatis* abundance. Forest habitats and tree shelters had significantly higher tick abundance and shelters had significantly more ticks than surface shelters. The significant interactions between habitat and shelter type as well as location and shelter type indicate the relationships are complex and we suggest that a combination of these factors associated with the activity of the hosts influences tick development and reproduction.

Keywords: ectoparasites; off-host aggregation; parasite-host interaction; tick life cycle; exophilic.

## 3.1 Introduction

Ticks are generally considered important for their role in vectoring diseases that can be transmitted to humans or that can affect human health. However, most ticks do not affect humans but parasitize other animals. In wild animal populations, ticks may play a role in controlling population size and driving host evolution by disease transmission and the direct effects of parasitism (de la Fuente et al., 2016). To understand the relationship between ticks and hosts, including patterns of occurrence in disease transmission, it is necessary to study their life cycle and how this relates to the hosts'.

Ticks have evolved two feeding strategies, they can either be (1) exophilic (non-nidicolous), actively questing or seeking their host, or (2) endophilic (nidicolous), remaining in or near nests and burrows of hosts (Sobrino et al., 2012). While exophilic ticks may be highly exposed to environmental conditions and dependent on host accessibility, endophilic ticks are generally better protected from fluctuating environments and have more accessible hosts (Ruiz-Fons and Gilbert, 2010; Wilson,

Ducey, Litwin, Gavin, and Spielman, 1990). Their dependency on climatic conditions makes ticks' life cycles dependent on their host's population dynamics and the stability of the habitats they inhabit. There are numerous studies focussing on the biology and ecology of exophilic ticks (Dantas-Torres and Otranto, 2013; Fourie, Snyman, Kok, Horak, and Van Zyl, 1993; Padgett and Lane, 2001; Ruiz-Fons and Gilbert, 2010; Wilson et al., 1990), due to their impact on livestock and human health. However, studies looking into endophilic ticks, especially in wildlife, are few (Frenot et al., 2001; Sobrino et al., 2012; Van Oosten, Heylen, and Matthysen, 2014).

Ixodid ticks (246 species) are common parasites of animals having a wide range of host species. These ticks have season-dependent population dynamics with temperature, humidity, and tick stage known to affect the stage-to-stage development and host-seeking activity (or questing) (Randolph, 2004). As a general rule, tick abundance increase in warmer temperatures (Lindgren, Tälleklint, and Polfeldt, 2000; Padgett and Lane, 2001) but there is much variation in the relative abundances of the various life stages (Dantas-Torres and Otranto, 2013; Estrada-Peña, 2001). In temperate regions, temperatures in winter are too low to allow moulting and questing activity (Tack et al., 2013) and ticks go into morphogenetic diapause (Fourie et al., 1993; Lindgren and Gustafson, 2001; Oorebeek and Kleindorfer, 2008). Whereas in the warmer period including spring and summer, hatching and moulting increases with rising temperatures, until a critical temperature is reached that stops development and leads to desiccation (Padgett and Lane, 2001). As with temperature, humidity influences different stages of the development of Ixodid ticks. When not feeding, ticks in general have been found to be highly susceptible to desiccation and require a relative humidity of more than 85% to thrive (Oorebeek and Kleindorfer, 2008; Tack et al., 2013). For example, bat ticks *Ixodes vespertilionis,* prosper in 100% humidity and cannot survive below 61% humidity (Obsomer et al., 2013). Hydration is crucial for questing activity too. At high humidity, ticks quest for longer periods thus improving their chances of finding a host (Obsomer et al., 2013). When conditions become unfavourable, ticks, especially larval and nymphal stages may enter diapause (Oorebeek and Kleindorfer, 2008).

*Ixodes anatis* is a host-specific endophilic tick found on two species of kiwi, *Apteryx mantelli* (Brown Kiwi) and *A. australis australis (*Southern Brown Kiwi or Tokoeka*)*, and as its host it is endemic to New Zealand (Dumbleton, 1953; Heath, 2010). Little is known

about their relationship with the host, their abundance, life cycle and the factors controlling it. In the only population where research on these ticks has taken place, Ponui Island (Swift et al., 2015), hosts are heavily parasitized with individual birds carrying up to hundreds of ticks (Castro, 2006; Heath, 2010). These findings led us to investigate the life cycle of the tick and its relationship with the host. Therefore, Swift, Heath, and Jamieson (2015) examined the prevalence and intensity of *I. anatis* in different types of brown kiwi shelters over a six-month period during the host breeding season. They found that brown kiwi shelter use was a significant predictor of mean intensity and prevalence of *I.* anatis, and that ticks were more abundant in roosts that were holes in the ground and under trees. Since *Apteryx spp.* use shelters for nesting and roosting (Jamieson et al. 2016; Dixon, 2015) as well as surface shelters, and kiwi move between roosts, each *I. anatis* instar may feed on the same or different bird (Heath, 2010). The aim of this study was to investigate the effect of brown kiwi shelter type, overall habitat type at shelter location, and site on the abundance of the various stages of *I. anatis* throughout the year to better understand the life cycle of this enigmatic tick.

## 3.2 Material and Methods

### 3.2.1 *Apteryx* spp.

There are five recognized species of kiwi and all of them are endemic to New Zealand (Weir et al., 2016). All species are nocturnal and ground dwelling; the brown kiwi is the most common of all kiwi species. Old records suggest brown kiwi densities of 40-100birds/km2 (Buller and Keulemans, 1888) however current densities on most mainland populations rarely exceed four adult birds/km2 (McLennan and Potter, 1992). In our study site the density is very high, with an estimated 100 birds/km2 (Cunningham et al., 2007). Brown kiwi use burrows dug in the soil, tree cavities and thickets of vegetation, for shelter during the day (Dixon, 2015; Jamieson, Castro, Jensen, Morrison, and Durrant, 2016). Generally brown kiwi use soil and tree shelters in the forest more often than other shelter types (Dixon, 2015; Jamieson et al., 2016). However, shelter use is seasonal, with birds using forest habitats relatively more often in winter and spring, and scrub, swamp and pasture in summer and autumn (Dixon, 2015). Besides season, age and pairing status also affects shelter use with younger and single birds more likely

to use a variety of habitats when compared to mated birds (Dixon, 2015). They do not necessarily use the same shelter on consecutive nights, but often share them with their partners (Ziesemann et al., 2011). Brown kiwi breed from June to February between mid-winter to mid-summer (Potter and Cockrem, 1992; Ziesemann et al., 2011). Eggs are incubated for 74 -84 days (Calder, Parr, and Karl, 1978) with first clutches hatching in October and second clutches in February. Some shelters and tree cavities are used for both nesting and roosting (Dixon, 2015; Jamieson et al., 2016).

### 3.2.2 Study Site

Ponui Island (36055'S, 175011'E) is located approximately 16km SE of Auckland in the Inner Hauraki Gulf, New Zealand (Figure 3.1). The brown kiwi population originated from 13 birds from two different source populations translocated onto the island in 1964 (Miles and Castro, 2000). In 2004, a long-term study of 35-50 tagged birds started on the island, allowing us to know where shelters were located and whether they were actively visited by birds. Ponui Island displays a collage of different habitats of which two thirds is pasture and the remaining third is a combination of scrub (consisting mainly of *Coprosma* spp., *Leptospermum ericoides* and *Pseudopanax* spp.), swamp (largely *Typha orientalis*) and broadleaf-podocarp forests (Shapiro, 2005).

The study site, on the southern half of the island, covered approximately 200ha and was spread over three main gullies (Figure 3.1). These were designated Kauri Bush, Red Stoney Hill gully (RSHG) and Pipe Gully. Ponui Island lies on a North/South axis and therefore RSHG and Pipe Gully are located on the eastern side while Kauri Bush lies on the western side of the central ridgeline. This northern North Island region receives South-westerly winds. The maximum summer temperatures range from 22 to 26°C whilst in winter the maximum temperature drops to an average of 10-12°C. Over winter, there is usually more rainfall, especially over the months of June, July and August (NIWA, 2014). Brown kiwi on Ponui Island use all types of habitat in all the three gullies on a regular basis (Cunningham and Castro, 2011). The study birds have non-breeding home ranges between 5.32 ± 1.7 ha for males and 6.19 ± 2.48 ha for females with great overlap between all the study birds (Ziesemann, 2011) and this leads to many birds using the same shelters (pers. obs.). All the gullies had areas of forest and scrub vegetation; however, pasture was only present in Kauri Bush and RSHG (Figure 3.1).

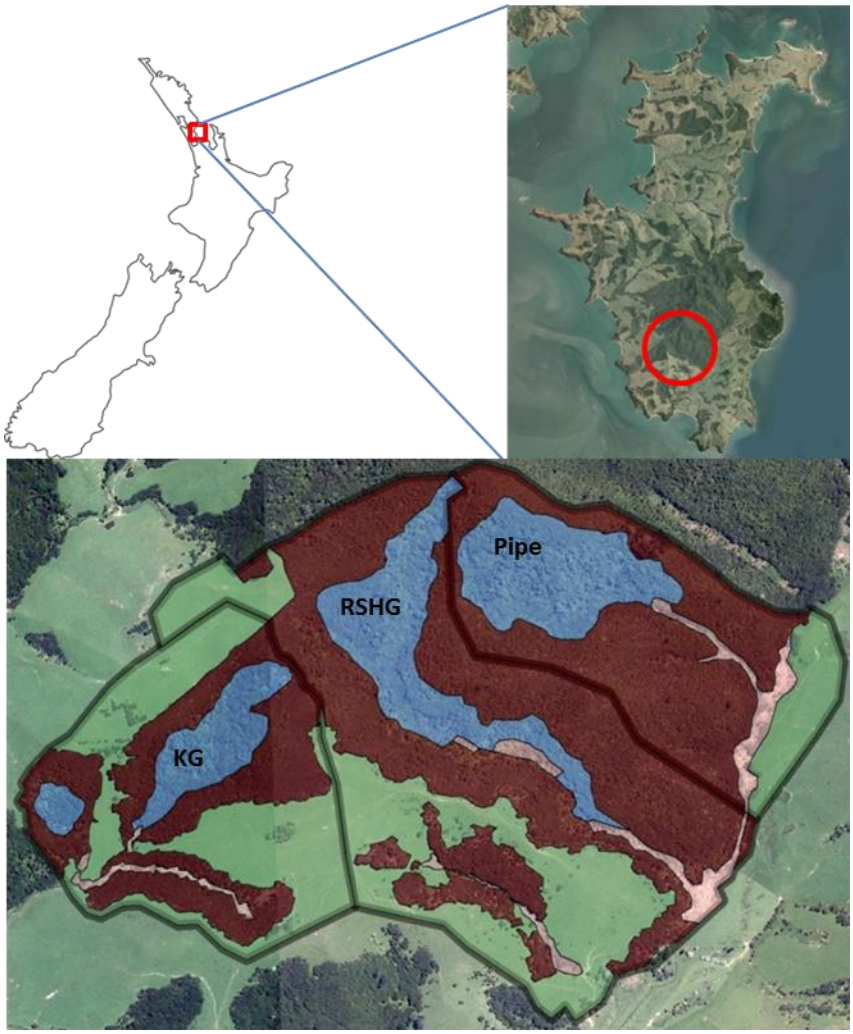



Figure 3.1: Map of the study area on Ponui Island, marked by the red circle on the top right image and expanded underneath, where KG is Kauri gully, RSHG is red stony hill gully and Pipe is pipe gully. Modified from T. Dixon, 2015.

### 3.2.3 Experimental Protocol

We checked 63 shelters for ticks at monthly intervals from May to November 2013, in January 2014 and from April to June 2014. However, not all shelters were sampled each month (Table 3.1). The main reason for this is that during the main part of the breeding season (June to December), known nests and shelters with birds in them were avoided to prevent disturbing the birds. In addition, by the end of the project, two of the surface shelters had collapsed and were not sampled.

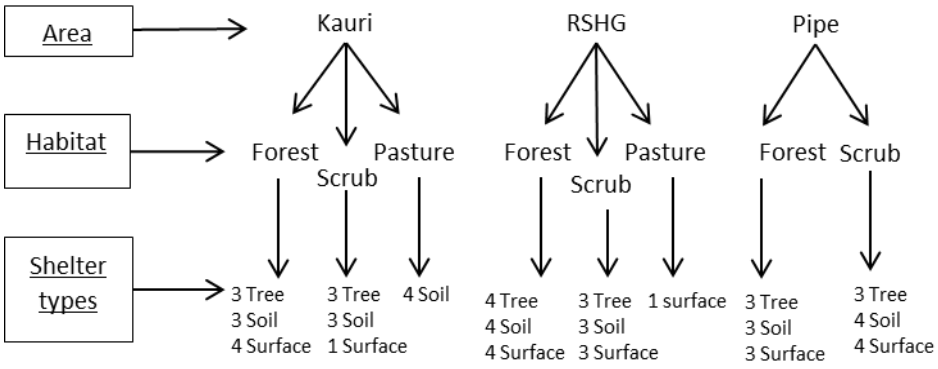



Figure 3.2: Experimental design: The number of shelters separated by Location, Habitat and Shelter type.

Shelters were clustered into three main types: *soil shelters*, comprising dug or naturally occurring burrows (for example as a result of tree roots decomposing and leaving a hole); *tree shelters* (including any hole in a tree, branch or living root); and *surface shelters* (including shelters under sparse vegetation or in the open) (Figure 3.2 and 3.3). Only shelters used by birds in the last five years were used in this study.

### 3.2.4 Field work

For sampling we used a non-destructive sampling technique, of attracting ticks to a warm-bodied host, first described in Swift et al., (2015) with the following improvements: Instead of large bottles, we used mini rubber hot water bottles (21 x 12 cm, 500ml) because the larger bottles were too big to go through most of the shelter entrances even after folding them. These larger bottles rubbed against the sides of the shelters damaging them, as well as losing ticks when they were pulled out. The water bottles were placed inside a black polar fleece sleeve instead of a white one that made it easier to spot the different tick stages since most of the stages are light coloured. Thermos flasks were used to carry boiling water to the chosen shelter sites and the hot water bottles were filled immediately before being placed into the shelter as far as the hand could reach. The reason for this was to avoid loss of temperature before the hot water bottles were in place. Prior to the main study, we investigated the temperature loss of the water bottles, under the protocol described above, to determine how long the bottles held temperatures useful for collecting ticks (body temperature).

Table 3.1: Number of shelters sampled separated by location, habitat and shelter type per month.

| | Location | | | Habitat | | | Shelter type | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Months** | **Kauri** | **Pipe** | **RSHG** | **Forest** | **Scrub** | **Pasture** | **Tree** | **Soil** | **Surface** | **Totals** |
| **May-13** | 17 | 18 | 21 | 25 | 26 | 5 | 19 | 22 | 15 | 56 |
| **Jun-13** | 21 | 20 | 22 | 31 | 27 | 5 | 19 | 24 | 20 | 63 |
| **Jul-13** | 18 | 17 | 20 | 28 | 23 | 4 | 14 | 22 | 19 | 55 |
| **Aug-13** | 14 | 15 | 16 | 26 | 18 | 1 | 12 | 14 | 19 | 45 |
| **Sep-13** | 16 | 13 | 18 | 27 | 16 | 4 | 14 | 17 | 16 | 47 |
| **Oct-13** | 18 | 15 | 19 | 26 | 22 | 4 | 16 | 19 | 17 | 52 |
| **Nov-13** | 21 | 17 | 22 | 30 | 25 | 5 | 19 | 24 | 17 | 60 |
| **Jan-14** | 21 | 17 | 22 | 30 | 25 | 5 | 19 | 24 | 17 | 60 |
| **Apr-14** | 20 | 17 | 19 | 28 | 24 | 4 | 17 | 24 | 15 | 56 |
| **May-14** | 20 | 17 | 19 | 28 | 24 | 4 | 17 | 24 | 15 | 56 |
| **Jun-14** | 20 | 17 | 19 | 28 | 24 | 4 | 17 | 24 | 15 | 56 |

We placed water bottles in 15 shelters, measured the surface temperature of the bottles and counted the number of ticks visible on the polar-fleece cover hourly for five hours. The water bottles were then left in the shelters to obtain a final measurement 24 hours after the start of the experiment (as in Swift et al., 2015). We found that water temperature at the time of placement ranged from 45-50°C but had fallen to 30-35°C after the first two hours (close to kiwi body temperature of 38 °C). We also found that the majority of the ticks on the fleece were collected in the first two hours after placement. Consequently, our final protocol was to leave the hot water bottles in the shelters for one and a half hours. On arrival to each site, we recorded the state of the shelter, presence/absence of spider webs or birds in shelters, fresh digging marks and

any other relevant observations. After the allocated time, the sleeve-covered bottle was removed from the shelter and put into a re-sealable plastic bag. However, if the fleece appeared clean, it was removed, and it alone went into the Ziploc bag. During placement and removal, presence of ticks on clothes and hands of the person carrying out the experiment was noted. On return to the camp, the plastic bags containing the sleeves were stored at 4°C for at least one hour to slow down the activity of the ticks before counting them.

Each sleeve was inspected for ticks using a stereo microscope (Olympus VMZ) and hand-held magnifying lenses. Numbers of individual ticks in the different stages of their life cycle (larvae, nymphs or adults) as well as sex, where possible, were recorded. Each tick was carefully removed with entomology tweezers after counting and placed into separate smaller plastic bags labelled for each shelter and containing some leaf litter. After counting and classifying ticks, they were returned to their respective shelters within 24 hours.

### 3.2.5 Statistical Analysis

Due to the non-Gaussian distribution of our aggregated count data, Generalised Estimating Equations (GEE) were used to model the effect of location (RSHG, Kauri and Pipe), habitat (forest, scrub and pasture) and shelter type (soil, tree and surface) on the abundance of ticks collected. GEE also accounted for repeated measure sampling of the same shelters every month. We used a negative binomial distribution model with the log link function as our data was over-dispersed (variance greater than mean) count data. Pairwise comparisons were carried out at 95% confidence intervals with Sidak corrections to account for multiple comparisons. Results were considered significant

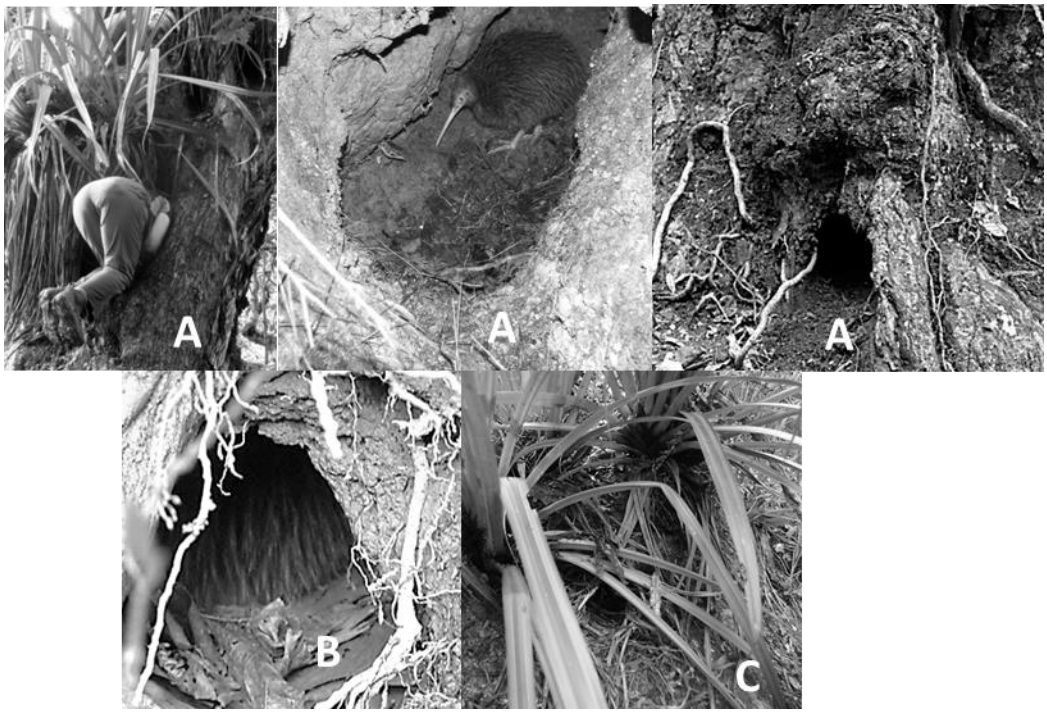



*Figure 3.3: Different shelter types that the Brown kiwi use which were used for this experiment. A represents the tree shelters, (from left to right), a person looking for a bird in a tree shelter, a kiwi inside a huge tree, another type of tree shelter in the roots of a tree. B represents dug out soil shelters. C represents surface shelters, in this case just under a fallen clump of* Astelia spp.

when $p < 0.05$. Analyses were undertaken for counts of all stages combined because there were too few adults to make meaningful comparisons. All tests were carried out using IBM SPSS Statistics 22.

## 3.3 Results

We counted a total of 12,172 ticks from the 63 shelters over the 14-month period; 87.2% (n=10,612) were larvae, 10.2% (n=1244) were nymphs and 2.6% (n=316) were adults.

All stages of ticks were present throughout the year but their abundance in shelters varied over time. The larvae numbers were high between January to June with three observed peaks in May 2014, August 2014 & April 2015 (Figure 3.4). The nymph numbers showed corresponding peaks from June onwards and were relatively high. Adult ticks increased as time progressed, however most of the adults were collected when a bird was present in the shelter or had been in there recently. In general, the numbers of each successive tick stage declined over time.

Birds were present in 14 of the 63 shelters through the study period and the prevalence of ticks in these shelters varied dramatically between 100%, in a shelter where a bird was found twice, to 27.27%, in a shelter where a bird was found once, indicating that the presence of birds was not a predictor of tick prevalence. The distribution of ticks in the shelters was not uniform, with 39.68% of shelters having ticks more than 50% of the times and only three shelters recorded with no ticks throughout the study.

Location, habitat and shelter type all had a significant effect on tick numbers (GEE model; Table 3.2) Kauri and RSHG had similar tick counts (n=5124 and 4791 respectively) when compared to Pipe (n=2257); however, this difference was not significant (Wald chi-square = 1.892, df = 2, p-value =0.388). There were also significant interactions between location and shelter type and habitat and shelter type. Tree shelters had higher tick abundance than the other types (Table 3.3). Shelters within the forest had significantly higher number of ticks as compared to scrub and pasture (Table 3.3). Pasture had the lowest tick abundance with only 165 ticks retrieved from the five pasture shelters sampled.

Table 3.2: The GEE model showing the various factors and interactions used with Chi-square values and significance levels.

| Predictor Variables | Wald Chi-Square | df | Sig. |
|---|---|---|---|
| (Intercept) | 194.157 | 1 | <0.0001 |
| Location | 19.260 | 2 | <0.0001 |
| Habitat | 23.951 | 2 | <0.0001 |
| S.type | 83.896 | 2 | <0.0001 |
| Location * Habitat | 3.674 | 2 | 0.159 |
| Location * S.type | 35.085 | 4 | <0.0001 |
| Habitat * S.type | 19.446 | 2 | <0.0001 |
| Location * Habitat * S.type | 6.844 | 4 | 0.144 |

Table 3.3: *Pairwise tests between the different fixed effect variables with means, significance levels and 95% confidence intervals. Bolded values denote significant differences.*

| Variable | | Mean | Pairwise test | Sidak Sig. (p value) | 95% Wald Confidence Interval for Difference | |
|---|---|---|---|---|---|---|
| | | | | | Lower | Upper |
| Location | Kauri | 5.95 | Pipe | 0.426 | -2.01 | 7.47 |
| | RSHG | 5.05 | Kauri | 0.932 | -3.08 | 4.87 |
| | Pipe | 3.22 | RSHG | 0.759 | -6.8 | 3.13 |
| Habitat | Forest | 11.11 | Pasture | **0.007** | 1.98 | 16.11 |
| | Scrub | 3.63 | Forest | **0.002** | 2.32 | 12.65 |
| | Pasture | 2.07 | Srub | 0.779 | -5.93 | 2.82 |
| Shelter type | Soil | 6.29 | Surface | 0.068 | -0.25 | 9.78 |
| | Tree | 11.50 | Soil | 0.396 | -13.94 | 3.53 |
| | Surface | 1.53 | Tree | **0.008** | -17.87 | -2.08 |

Table 3.4: Prevalence of ticks in shelters sampled each month divided according to location, habitat, and shelter type represented in percentage.

| | Location | | | Habitat | | | Shelter Type | | |
|---|---|---|---|---|---|---|---|---|---|
| | Kauri | RSHG | Pipe | Forest | Scrub | Pasture | Tree | Soil | Surface |
| **May-13** | 52.94 | 47.62 | 66.67 | 68.00 | 46.15 | 40.00 | 73.68 | 59.09 | 26.67 |
| **Jun-13** | 57.14 | 68.18 | 45.00 | 58.06 | 59.26 | 40.00 | 63.16 | 50.00 | 60.00 |
| **Jul-13** | 27.78 | 55.00 | 35.29 | 50.00 | 30.43 | 33.33 | 57.14 | 47.62 | 21.05 |
| **Aug-13** | 42.86 | 68.75 | 40.00 | 50.00 | 55.56 | 0.00 | 91.67 | 35.71 | 36.84 |
| **Sep-13** | 62.50 | 55.56 | 53.85 | 62.96 | 62.50 | 0.00 | 92.86 | 46.15 | 50.00 |
| **Oct-13** | 61.11 | 47.37 | 46.67 | 53.85 | 54.55 | 33.33 | 93.75 | 33.33 | 35.29 |
| **Nov-13** | 42.86 | 54.55 | 52.94 | 0.60 | 45.83 | 20.00 | 63.16 | 52.17 | 35.29 |
| **Jan-14** | 28.57 | 36.36 | 35.29 | 30.00 | 44.00 | 0.00 | 57.89 | 37.50 | 0.00 |
| **Apr-14** | 0.45 | 68.75 | 46.67 | 42.86 | 60.87 | 25.00 | 58.82 | 56.52 | 26.67 |
| **May-14** | 0.55 | 47.37 | 29.41 | 50.00 | 39.13 | 50.00 | 58.82 | 39.13 | 40.00 |
| **Jun-14** | 16.67 | 26.32 | 35.29 | 28.57 | 26.09 | 0.00 | 29.41 | 30.43 | 13.33 |

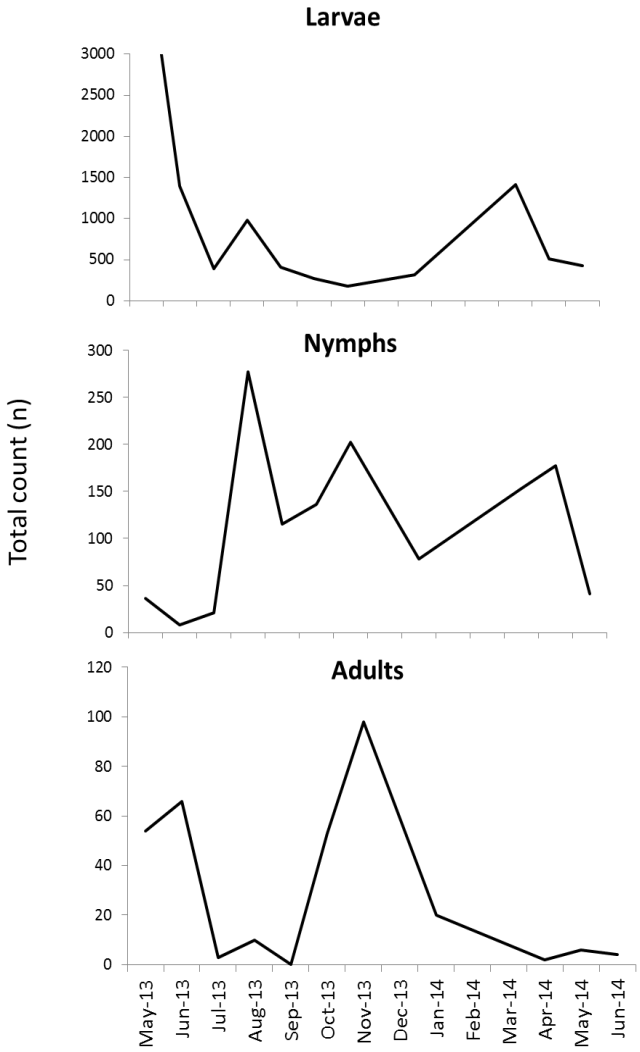



Figure 3.4: Total Numbers of different stages of I. anatis found over 14-month sampling of 63 brown kiwi (A. mantelli) shelters on Ponui Island, New Zealand. Three types of shelters (soil, tree and surface) located in three different habitat types (forest, scrub and pasture) from three different adjacent locations (Kauri, RSHG and Pipe) were sampled.

## 3.4 Discussion

We found that location, habitat in which the shelters were located, and the type of shelter were significant predictors of *I. anatis* abundance. However, there seems to be complex significant interactions related to the location and the habitat where the shelters are found.

### 3.4.1 Seasonal stage variations

Larval abundance peaked at the start of the study in May 2013, a small peak in August 2013 and again in April 2014 with numbers declining towards winter of 2013 and at the end of the study towards the winter of 2014. They remained low through the winter of

2013 and then slowly rose from January. This pattern implies there is likely to be just one generation per year with only a few ticks cycling outside this pattern. It is not uncommon for larvae to show peaks when conditions are favourable (Oorebeek & Kleindorfer, 2008) as this provides the opportunity for eggs to develop and hatch. The occurrence of larvae at these times indicates a seasonal hatching of previously laid eggs, likely in the presence of ideal hatching conditions. It may be that these ideal egg hatching conditions control *I. anatis* life cycles, but this remains to be confirmed.

The larval peaks and consequent declines are followed closely by peak in nymph numbers. The first two peaks of nymphs were observed in the winter and spring months of August and November of 2013, 2-3 months after the larval peak implying that larvae had moulted during winter and spring but probably quite slowly due to low temperatures. The next peak occurred in May 2014 immediately after the April 2014 larval peak. We believe these ticks might be the few cycling outside the yearly seasonal pattern. In 2014, May and April had an average temperature of 16.8 °C and average humidity of 95.3% (NIWA, 2014). This combination of mild temperature and high humidity, with little fluctuation, may have been ideal for quick moulting of larval stages of *I.* anatis (Chapter 1). Adult ticks were present in small numbers throughout the study but peaked in November 2013. These adults were mostly present in shelters when there were birds present (or had been recently used) suggesting that they may have come off the birds themselves. Their presence could also be the result of recently engorged nymphs that freshly moulted into adults and were questing soon after. We think that it is unlikely that adult ticks would come off the birds in these shelters once those adults had started to feed because fed adults would not be interested in attaching to a warm sleeve, rather they would seek seclusion whilst they were developing eggs.

Larvae and nymphs were present in the shelters throughout the sampling period indicating a high prevalence of ticks in shelters irrespective of brown kiwi usage. However, the abundance of adults seemed to spike if the shelter had recently been used. Generally, Ixodid ticks follow a seasonality that depends on host densities and their breeding cycles because this ensures that the ticks can complete their own life cycle (Frenot et al., 2001; Oorebeek and Kleindorfer, 2008). Where density of hosts populations are naturally high throughout the year, as records indicate for brown kiwi shortly after human arrival in New Zealand (Buller and Keulemans, 1888), and as it was

the case for our study site, a parasite may not need to have a life cycle that synchronises with its host's. Therefore, we believe that the seasonality we observe in this study has more to do with variations in the microclimate of the shelters rather than host breeding cycles.

### 3.4.2 Effect of location, habitat and shelter type

There was a significant effect of location, habitat and shelter type on the abundance of *I. anatis*. However, at different times of the month, the macro climate of the different locations may affect the microclimate within shelters which in turn is influenced by the nature of the vegetation and habitat (Pringle, Webb, and Shine, 2003). Furthermore, vegetation may also affect the abundance and distribution of host species, which in turn determine tick abundance. Therefore, the interactions between these factors are complex and it is unlikely that any one factor alone explains tick abundance.

That said, closed shelters have been known to provide more of a stable microclimate less prone to fluctuations (Kerr and Bull, 2006) and Ixodid ticks are dependent on high relative humidity and optimal temperature in their microhabitat for survival (Oorebeek and Kleindorfer, 2008). Tree shelters had a higher tick prevalence indicating that these shelters may be providing stable optimal conditions for different stages of ticks. Soil shelters had the second highest tick prevalence even though brown kiwi in our study area use almost the same number of soil and tree shelters (Dixon, 2015; Jamieson et al., 2016). Surface shelters had significantly low prevalence of all stages of ticks. Surface shelters range from an open spot on the ground to areas under a few fallen branches of trees or some decaying vegetation, that provides temporary cover to the birds and make these shelters more prone to microhabitat fluctuations.

We found a significantly higher number of ticks in shelters located within the forest, corresponding with brown kiwi roosting behaviour (Dixon, 2015). This build-up of parasite numbers is probably a result of frequent use of these shelters by the birds thus providing continuous host availability and robust tick transmission. Pasture shelters only had larval stages whose numbers significantly decreased with monthly sampling. This decline in larvae numbers could be related to the movement of brown kiwi from pasture to scrub and forest shelters with the approach of the breeding season

(Dixon, 2015). However, the number of pasture shelters limited us since we could only find five.

Location was a significant factor in determining tick abundance; we believe that this significance is likely to be due to environmental differences between the sites due to differences in exposure to the weather. Future research needs to focus on finding what microclimates are available within shelters and how these fluctuate with season, habitat and locations.

The results of this study along with the results obtained from chapter 2 provide a more complete picture of the off-host ecology of the kiwi tick including abundance in different habitat, seasonal variations and temperature and RH requirements. When put together with host movement and ecology, it shows that the parasites seem to be ever present in the burrows regularly used by the birds, but numbers of the different stages vary seasonally. High parasite numbers will result in the birds being more susceptible to the negative effects of parasitism, especially during periods of stress such as the breeding season (July to January, with peak levels of adults and nymphs), or during summer droughts (December to March, decreasing numbers of adults and nymphs). Annual variations in tick numbers related to the tick responses to environmental conditions remain to be studied but are needed to fully understand the life-long effects of this tick on its host. Results from this study will also help managers working with wild populations of the birds to know when NIBK could be more susceptible to higher tick infestation and thus more prone to secondary infections with other diseases.

**Declaration of interest**

The authors report no conflicts of interest.

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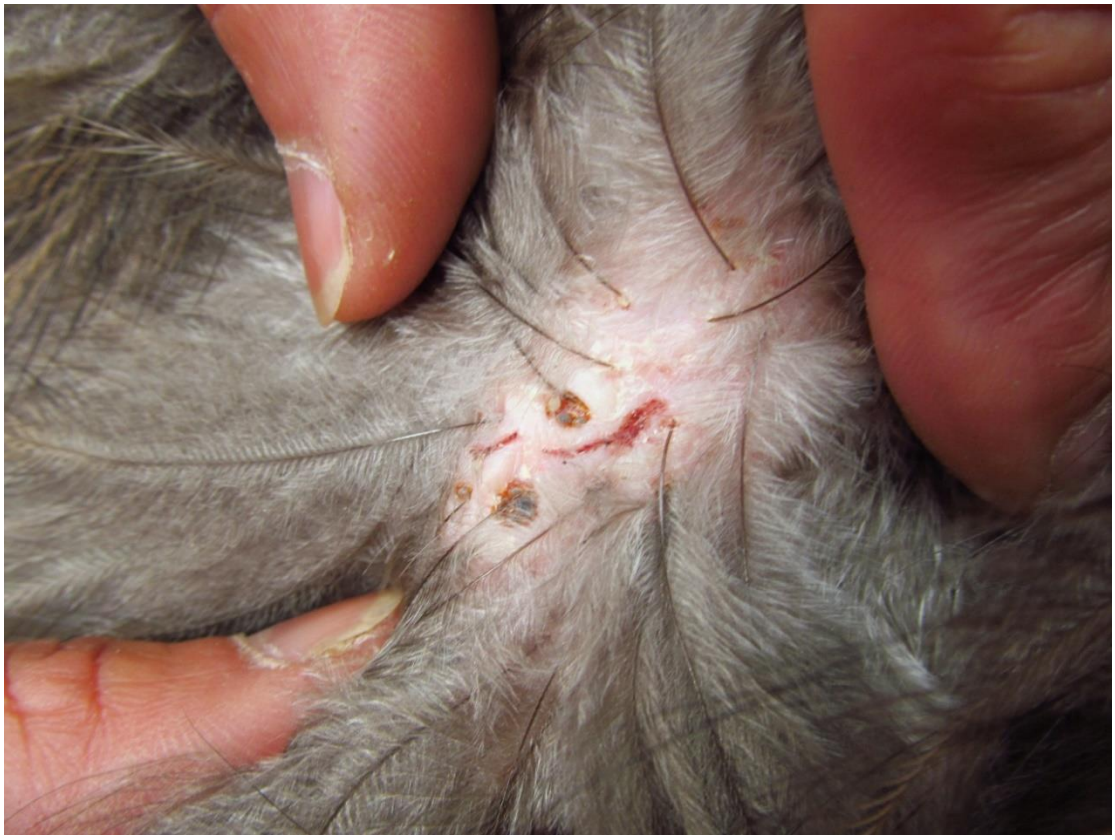
# Chapter 4



The damn vermin are so numerous that I am afraid to sneeze, for fear the damned parasite would regard it as gong for dinner, and eat me up - Robert Cobb Kennedy

# Chapter 4: A new method to quantify levels of tick infestation in flightless ground birds using photography.

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Experimental design: NB and IC. Fieldwork: NB and IC. Writing: NB with comments from IC and ADR. Analysis: NB and ADR.

## 4.1 Introduction

Estimating ecto-parasite numbers on and off-host is generally difficult since most arthropods show aggregated distributions, where the majority of hosts have few parasites while a few have many (Clayton and Moore 1997). There are various methods of off-host (Haas, 1966; Krasnov, Khokhlova, and Shenbrot, 2004; Needham and Teel, 1991; Reinhardt, Naylor, and Siva-Jothy, 2007) and on-host parasite estimation (Krasnov et al., 2004; Lehmann, 1994) but most, if not all, require direct handling of animals for timed spot counts, treatment to remove parasites (Walther and Clayton, 1997), putting animals in enclosures to allow the parasites to drop off, or even euthanasia (Clayton and Drown, 2001; Koop and Clayton, 2013). Traditionally in all cases, the animals need to be handled, however prolonged handling causes stress (Buttler et al., 2011; Heath and Dufty, 1998), especially for wildlife, and researchers have to consider the welfare implications of such sampling.

The advent of new technologies provides the possibility of using non-invasive, or at least less invasive methods with minimal to no animal handling, to detect parasites. For instance, Cortivo et al. (2016) and Barbedo et al. (2017) have been successful is using infrared thermal cameras combined with image processing to estimate tick and fly numbers in livestock, all without any need for animal handling. As long as the method provides an estimate of parasites that is correlated to the total intensity of the parasite burden and is repeatable it could be used instead of more invasive methods (Clayton and Tompkins, 1994; Moller, 1991).

*Ixodes anatis* is a species-specific tick found on two species of kiwi; the North Island Brown kiwi (*Apteryx mantelli)* and the Tokoeka (*Apteryx australis*) (Heath, 2010). Previous research described the off-host abundance and intensity of these ticks (Chapter 3; Bansal et al., 2019; Swift et al., 2015), and the on-host abundance (Castro, 2006) using the dust-ruffling method (Walther and Clayton, 1997). We wanted to minimise stress to kiwi by reducing handling time and avoiding the use of chemicals when quantifying tick loads. Therefore, the aim of this study was to determine if there is a correlation between tick numbers counted using the dust-ruffling method and counts of ticks from photographs taken of areas in the kiwi's body where ticks are commonly found.

## 4.2 Methods

### 4.2.1 Experimental design

Ponui Island holds a high-density population of North Island brown kiwi (Cunningham et al., 2007) and also their ticks (Castro, 2006; Heath, 2010). These birds have been utilised for many different studies with 30-50 brown kiwi marked with radio transmitters annually since 2004. Fifty wild radio-transmitterised kiwi were caught on Ponui Island (36.8622° S, 175.1842° E) from their roosting burrows in 2017 between January and May as part of other research (Chapters 4 and 5) some tagged birds required tick removal. We designed this study to take advantage of the opportunity and took photos of each bird before we removed its ticks to allow direct comparison of counts by both methods.

### 4.2.2 Photos for quantifying ectoparasites

Kiwi ticks tend to be found buried mostly around the face and the flanks of the bird (Castro et al. In prep.; Figure 4.2). On the flank region, they are most usually embedded in the skin at the base of the feather follicles (Castro et al. In prep.). Overall, we took a set of five photos of each bird to estimate tick numbers. On the face most ticks tended to be around the bill, sometimes on the corner of the bill as well as inside the ears and around the eyes. Three photos were taken from the face: two from either side (Figure 4.3a), and one from the front showing the forehead (Figure 4.3a). On the body, the area under the vestigial wings is greatly reduced compared to other birds (Calder, 1978) and tends to be featherless. Ticks are usually found in this area and we took two photos from the space beneath each wing (Figure 4.2A, 4.2B and

4.3b). For all pictures we used an oval scale with an area of 0.21$m^2$ (11cm by 6cm; Figure 4.3b) to define the area where to search for ticks, and only ticks visible within this area were counted. The scale was used as a guide because birds were of different sex and age, but we did not intend to calculate ticks per unit of area. These photos were used to count ticks regardless of their life stage by zooming into the images (Figure 4.2) using the open source software ImageJ (Schneider, Rasband, and Eliceiri, 2012). Only birds with good quality photos (for e.g. Figure 4.2) were used to estimate tick numbers making our final sample size of 40 birds. Photo taking took a mean of minutes (± 1.5 minute; n = 40).

### 4.2.3 Manual ectoparasite collection

The birds were de-parasitized using a modified version of the dust-ruffling method described by (Walther and Clayton, 1997) and used in the past in kiwi on Ponui Island (Castro, 2006): the birds were powdered over the body surface and under the feathers (avoiding the face) with Vitapet™ flea powder (containing 1.95% Permethrin, a synthetic pyrethroid). We used a neck cone to prevent powder falling on the head, eyes, nose and ears of the birds (Figure 4.1). The birds were then left resting in a bag (pillowcase) for 10 minutes, with their head out. During this period, all ticks on the face of the birds were manually plucked using forceps to grab the ticks close to the skin and slowly pull while twisting (Duscher, Peschke, and Tichy, 2012). After this period the feathers of the bird were carefully ruffled over a pillowcase for one and a half minutes to extract and collect all the parasites. The bags were then sealed and taken to the lab to count and identify the ectoparasites. After this procedure we examined the bird to make sure we had removed all visible ticks. The total handling time from start of this method to the end for the bird was 15 minutes (± 1 minute; n = 40).


*Figure 4.1: A) shows a kiwi bird with a neck cone to keep the insecticide away from the head area, sitting in a bag for 10 minutes for the insecticide to act. B) The permethrin-based insecticide used in this study from VitaPet™.*

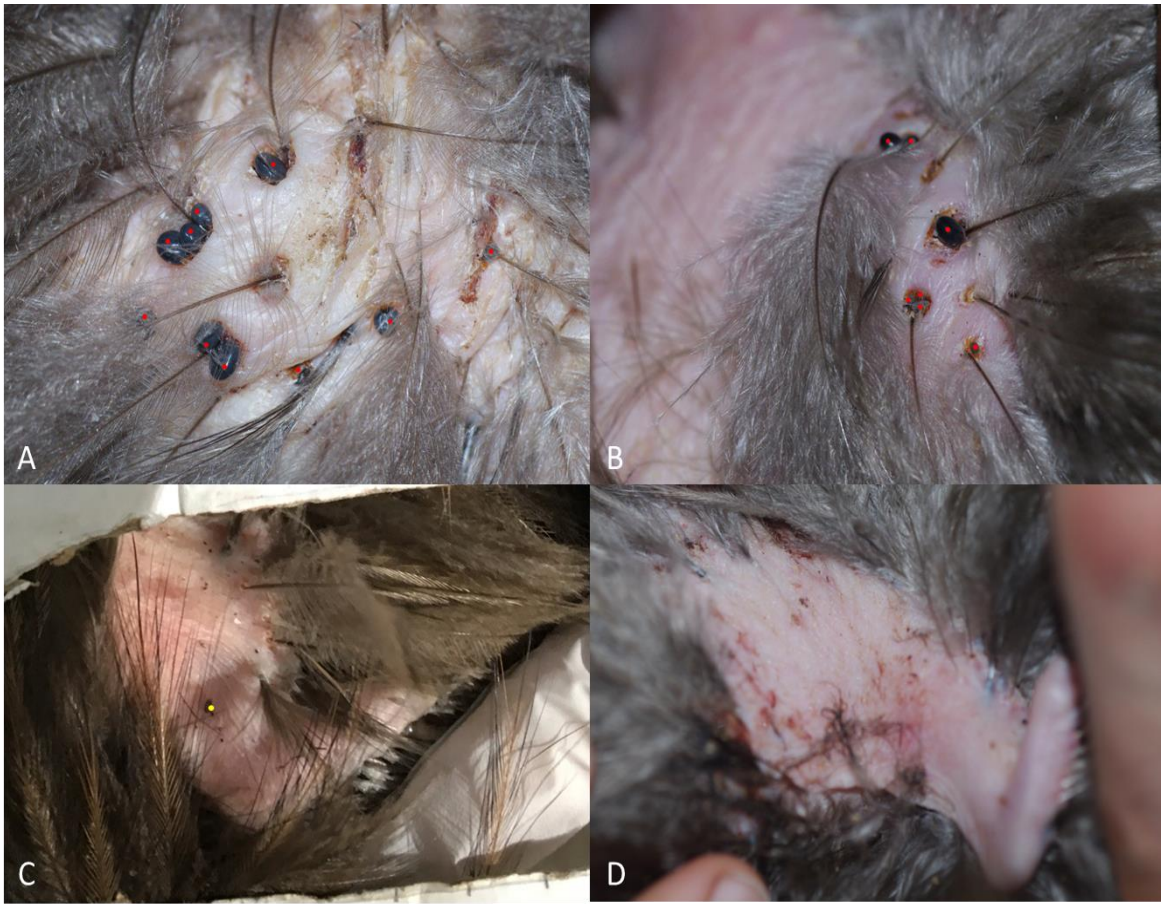


*Figure 4.2: A and B are examples of good in focus photos from which tick were counted. The red dots represent the ticks labelled using the software ImageJ. C and D are examples of bad photos which were not included into the count data. In case of C, the yellow dot is a tick however since the photo does not cover the entire featherless underwing area, it could not be used. D is an example of an unclear and unfocused photo*

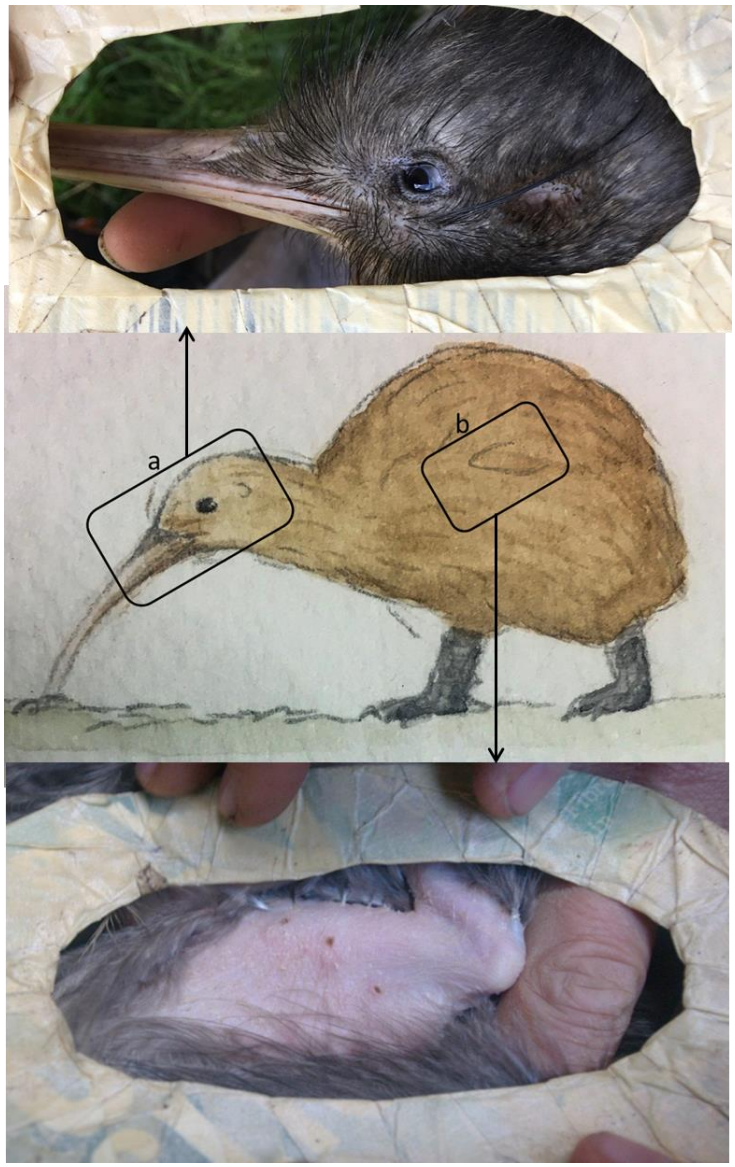


*Figure 4.3: This figure illustrates the sites on the bird where the photos were taken. Three photos were taken at 'a' to cover the head and neck region (2 sides and one from top) and two photos were taken at 'b' to cover the featherless region below the wings. (Artwork by Camille Rostan)*

### 4.2.4 Statistical analysis

We plotted the number of ticks on photos against the number of ticks collected from the dust-ruffling technique to visualise and explore out data (Figure 4.4). Clearly there was a non-linear relationship between the two data sets. Therefore, we carried out a generalised linear model on the real number of ticks ($R_q$) found on each ($q_{th}$) individual using negative binomial likelihood (1) in a Bayesian framework. The negative binomial probability mass function (2) technically allows for inferring the number of 'successes' (k) before a number of 'failures' (r). As these were tick counts, a negative binomial

variable can be viewed as representing the number of successful Bernoullian events (k in our case equals "finding a tick") before a failed one (i.e. no more ticks found). For this study, we chose r = 1 to represent the not–finding–more–ticks' event. Having set r = 1 simplifies (2) to $p^k q$ (Gelman et al., 2013). We designed a fairly straightforward generalised (link function (3)) linear model (4) to investigate the relationship between the number of ticks found in the different pictures taken from the birds ($pic_q$), and the real number found collected from dust-ruffling the birds ($R_q$).

In the chosen minimal linear expression (4), a global offset (or intercept, int) is summed to the product of an estimated multiplier (β) by the real un–scaled number of ticks counted from pictures ($pic_q$). Because we have the same units of measures and built a simple model, the choice of not scaling the number of ticks found in the pictures allows for a more direct interpretation of the results. In an ideal world, the β point estimate (median) value would represent the most likely value that, multiplied by the number of ticks found in the pictures, and subsequently added to the global offset, would result in the rate parameter of a corresponding negative binomial distribution, giving some estimate of the real number of ticks on the related individual bird.

$$R_q \sim \text{dnegbin}\ (l_q, 1) \tag{1}$$

$$Pr(X = k) = \binom{k+r-1}{k} p^k q^r \tag{2}$$

where q = 1 − p

$$l_q = 1/(1 + e^{-m_q}) \tag{3}$$

$$mq = \text{int} + \beta * pic_q \tag{4}$$

We used the external Gibbs sampling engine offered by *Just Another Gibbs Sampler* (J.A.G.S.; Plummer, 2003), in R environment (Team, 2013) to run three distinct Markov Chain Monte Carlo (MCMC) simulations. After 10000 burn–in iterations (that is, discarded iterations to allow for the chains to start exploring higher posterior probability areas), each simulation ran five independent chains, for $4*10^5$ iterations, retaining one in every five simulated values (thin=5) (Plummer, Best, Cowles, and Vines, 2006). Each simulation considered the number of ticks found on (a) facial pictures (Figure 4.3a), (b) underwing body pictures (Figure 4.3b), and (a+b) the sum of the two, respectively, and how each correlated with the total number of ticks counted after dust-ruffling.

Following each simulation, we visually inspected the trace plots to check for good mixing, estimated each variable's effective size (the number of really independent simulated values per variable), and ran Heidelberger's diagnostic with t = 0.01 and p = 0.05 (Heidelberger and Welch, 1983). Additionally, we divided the number of ticks in the photographs into three groups based on number of ticks: 0-10, 11-20 and above 21 and ran the models again to identify the minimum number of ticks we needed in a photo to provide a reliable estimate. Then, we performed a fivefold cross validation for each of the simulations. In each cross validation run, 20% (or 1/5) of the data was set to not available (NA). We later checked to what extent the model was able to correctly estimate the 95% high density intervals for the missing data points.

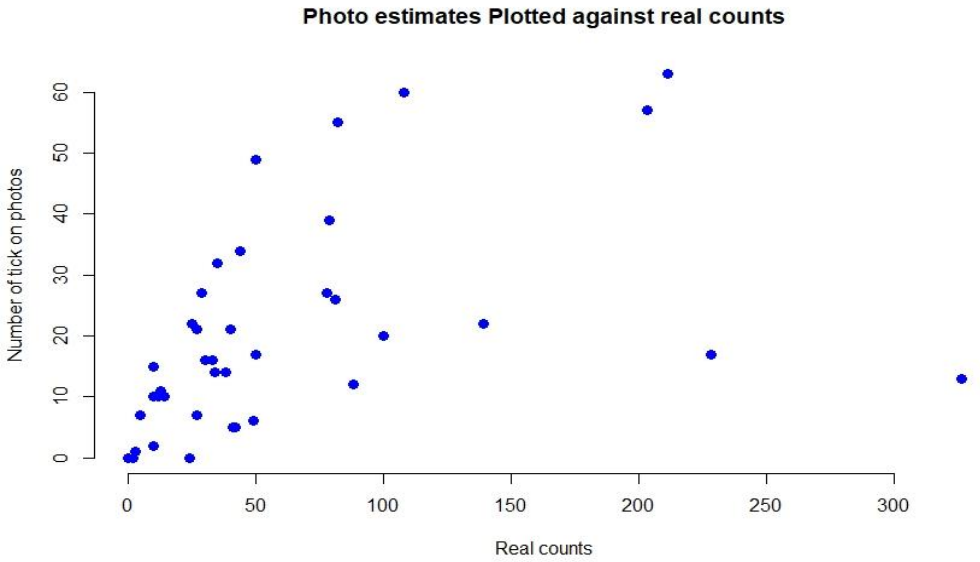


*Figure 4.4: Scatter plot of the photo count of tick numbers plotted against the number of ticks on birds collected after DR.*

## 4.3 Results and Discussion

The trace plots and the Heidelberger's diagnostic (Heidelberger and Welch, 1983) produced by the generalised linear model were satisfactory, including when the photo count data was divided into the three groups: 0-10 ticks, 11-20 ticks and above 21 ticks. All the variables in all the five chains passed both the Stationarity, as well as the Half-width test for the *face* and *body* simulations. When cross-validated, all simulations performed reasonably well as the estimated 95% high density intervals for the

removed values included the real numbers of ticks on the birds regardless of it being face, body, or total simulation.

To estimate the real tick numbers on the birds with some accuracy we looked for the models providing the narrower distributions of estimated parameters (Figure 4.5). We thus employed the model using only the underwing model for the cases in which 0 to 20 ticks were spotted on such pictures. Alternatively, the collective number found on face and body gave more accurate results for higher (21-100) real number of ticks (Figure 4.3).

The projected estimated range of values for real tick infestations on the birds using the coefficients generated during our analysis is given in Table 4.1 only using underwing for group 0-2, and Table 4.2 using a total of face and underwing.

*Table 4.1: Table with estimated number of ticks on birds calculated using photo counts between 0-20 (only underwing)*

| Number of ticks on photos | Estimated Median | Estimated Mean | Estimated range of ticks on Kiwi |
|---|---|---|---|
| 0 | 21 | 30.6 | 0-43 |
| 1 | 22 | 32.1 | 1-45 |
| 2 | 23 | 33.6 | 2-47 |
| 3 | 24 | 35.2 | 3-49 |
| 4 | 25 | 36.9 | 4-51 |
| 5 | 27 | 38.7 | 5-54 |
| 6 | 28 | 40.6 | 6-56 |
| 7 | 29 | 42.5 | 7-59 |
| 8 | 31 | 44.6 | 8-62 |
| 9 | 32 | 46.8 | 9-65 |
| 10 | 34 | 49.0 | 10-68 |
| 11 | 35 | 50.2 | 11-70 |
| 12 | 36 | 52.5 | 12-73 |
| 13 | 38 | 54.9 | 13-76 |
| 14 | 40 | 57.5 | 14-80 |
| 15 | 42 | 60.1 | 15-83 |
| 16 | 43 | 62.8 | 16-87 |
| 17 | 45 | 65.7 | 17-91 |
| 18 | 48 | 68.9 | 18-96 |
| 19 | 50 | 71.9 | 19-100 |
| 20 | 52 | 75.3 | 20-104 |

*Table 4.2: Table with estimated number of ticks on birds calculated using photo counts between 21 - 100 (using a total of face and underwing).*

| Number of ticks on photos | Estimated Median | Estimated Mean | Estimated range of ticks on Kiwi |
|---|---|---|---|
| 21 | 39 | 57.2 | 21-79 |
| 22 | 41 | 59.1 | 22-82 |
| 23 | 42 | 60.9 | 23-85 |
| 24 | 43 | 62.8 | 24-87 |
| 25 | 45 | 64.8 | 25-90 |
| 26 | 46 | 66.8 | 26-93 |
| 27 | 48 | 68.9 | 27-96 |
| 28 | 49 | 71.1 | 28-99 |
| 29 | 51 | 73.4 | 29-102 |
| 30 | 52 | 75.5 | 30-105 |
| 31 | 54 | 77.9 | 31-108 |
| 32 | 56 | 80.5 | 32-112 |
| 33 | 57 | 83.0 | 33-115 |
| 34 | 59 | 85.7 | 34-119 |
| 35 | 61 | 88.4 | 35-123 |
| 36 | 63 | 91.1 | 36-127 |
| 37 | 65 | 94.1 | 37-131 |
| 38 | 67 | 97.0 | 38-135 |
| 39 | 69 | 100.2 | 39-139 |
| 40 | 71 | 102.9 | 40-143 |
| 41 | 74 | 106.4 | 41-148 |
| 42 | 76 | 110.0 | 42-153 |
| 43 | 78 | 113.4 | 43-157 |
| 44 | 81 | 116.6 | 44-162 |
| 45 | 83 | 120.5 | 45-167 |
| 46 | 86 | 124.2 | 46-172 |
| 47 | 89 | 128.0 | 47-178 |
| 48 | 91 | 132.0 | 48-183 |
| 49 | 94 | 136.4 | 49-189 |
| 50 | 97 | 140.4 | 50-195 |
| 51 | 100 | 144.9 | 51-201 |
| 52 | 104 | 149.9 | 52-208 |
| 53 | 107 | 154.3 | 53-214 |
| 54 | 110 | 159.2 | 54-221 |
| 55 | 114 | 164.2 | 55-228 |
| 56 | 117 | 169.4 | 56-235 |
| 57 | 121 | 174.7 | 57-243 |
| 58 | 125 | 180.4 | 58-250 |
| 59 | 128 | 185.6 | 59-257 |
| 60 | 133 | 191.8 | 60-266 |
| 61 | 137 | 197.6 | 61-274 |
| 62 | 141 | 203.8 | 62-283 |
| 63 | 146 | 210.9 | 63-293 |
| 64 | 150 | 216.8 | 64-301 |
| 65 | 155 | 223.8 | 65-310 |
| 66 | 160 | 230.5 | 66-320 |
| 67 | 164 | 237.9 | 67-330 |
| 68 | 171 | 246.0 | 68-341 |
| 69 | 176 | 253.7 | 69-352 |
| 70 | 181 | 261.5 | 70-363 |
| 71 | 187 | 269.4 | 71-374 |
| 72 | 193 | 278.3 | 72-386 |
| 73 | 199 | 287.0 | 73-398 |
| 74 | 205 | 295.8 | 74-410 |
| 75 | 211 | 305.4 | 75-424 |
| 76 | 219 | 315.2 | 76-437 |
| 77 | 225 | 324.8 | 77-451 |
| 78 | 232 | 334.5 | 78-464 |
| 79 | 239 | 345.7 | 79-479 |
| 80 | 247 | 356.4 | 80-495 |
| 81 | 254 | 367.7 | 81-510 |
| 82 | 264 | 379.6 | 82-526 |
| 83 | 271 | 391.2 | 83-543 |
| 84 | 279 | 403.5 | 84-560 |
| 85 | 288 | 416.5 | 85-578 |
| 86 | 297 | 429.2 | 86-595 |
| 87 | 306 | 442.4 | 87-613 |
| 88 | 317 | 456.6 | 88-633 |
| 89 | 327 | 471.0 | 89-653 |
| 90 | 336 | 484.9 | 90-673 |
| 91 | 347 | 501.3 | 91-695 |
| 92 | 358 | 516.5 | 92-716 |
| 93 | 369 | 533.4 | 93-740 |
| 94 | 381 | 550.1 | 94-763 |
| 95 | 393 | 567.9 | 95-787 |
| 96 | 405 | 584.5 | 96-811 |
| 97 | 419 | 604.1 | 97-838 |
| 98 | 431 | 621.6 | 98-862 |
| 99 | 446 | 641.8 | 99-890 |
| 100 | 459 | 662.1 | 100-919 |

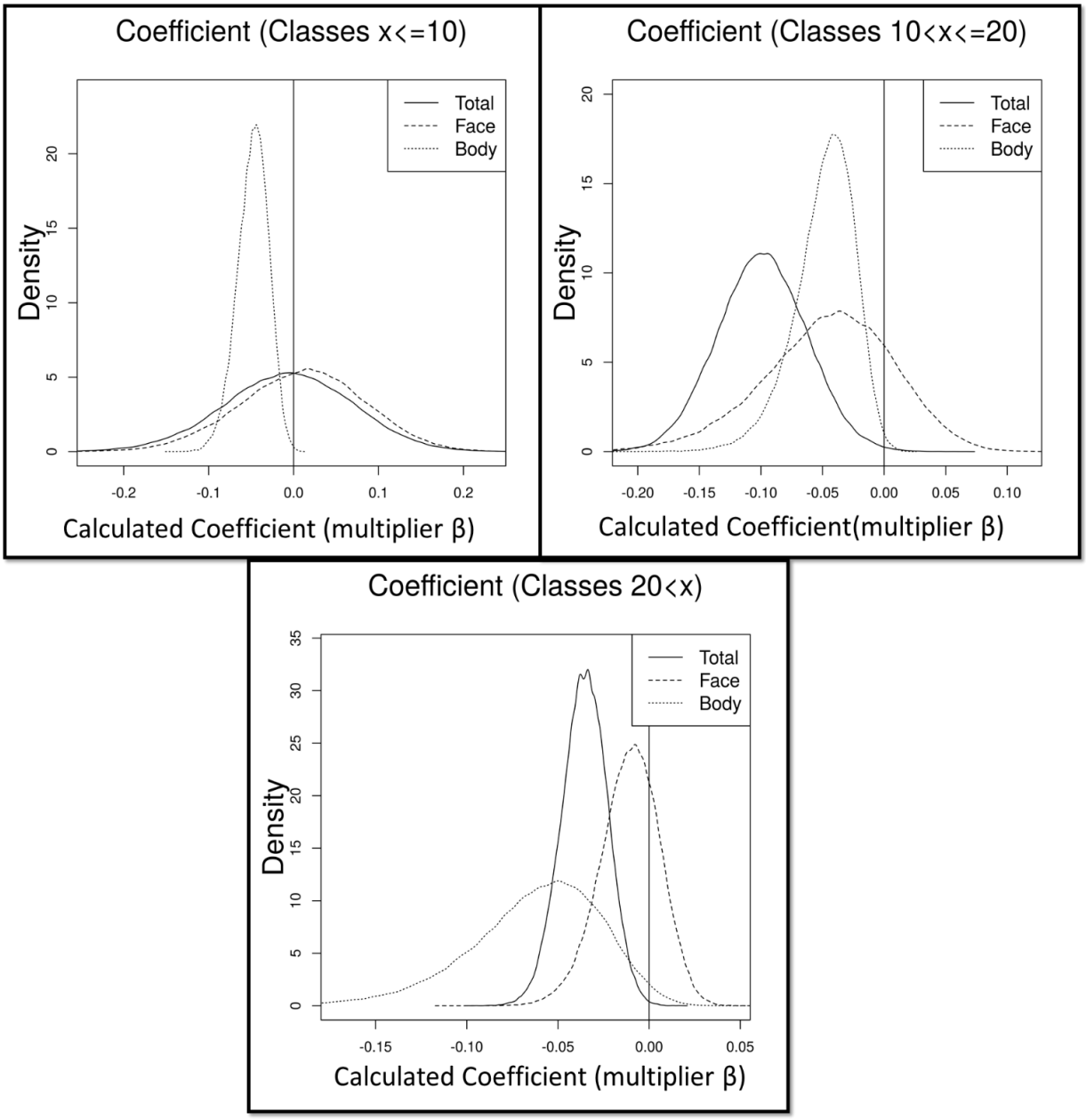


*Figure 4.5: These three graphs represent the density of the coefficients (estimated multiplier β ) that we ned to multiply with the ticks in photos to get an estimate of ticks on the bird for the various groups; A= 0-10 ticks in the photos, B=11-20 ticks on the photos and C=more than 21 ticks in the photos. The line in the three graphs represents zero. To read the graph, look at the curves with a smaller width and narrower peak that fall to only one side of the zero line, either positive or negative. If the coefficients are all negative or all positive (narrower peaks), it is a better fit to estimate the total ticks on the birds, but if the coefficient falls in the negative and positive, the resulting estimates are unrealistic. , in A & B, the curve for body fall to the negative side of the zero line and has a sharper peak indicating that the body counts would give the best estimates for classes represented by A and B. In C the curve for Total falls to one side of the zero line and has a sharper peak thus indicating that for classes represented by graph C, the coefficient of total counts will give a better estimate of actual tick numbers on brown kiwi.*

This study aimed to quantify actual tick levels on NIBK using photos. We found that we could estimate the 95% high density intervals of tick numbers on the birds using photos with confidence. Photos of the underwing were most reliable at low tick counts (<20) (Figure 4.4) whereas a combination of underwing and face was more reliable to estimate tick counts when the numbers were >20 in the photo (Figure 4.4). Thus, we were successful in reducing the handling time from 15 minutes to 2 minutes to estimate the tick infestation in kiwi by using photos, which we hope will reduce stress in the birds for future monitoring. They will also provide a tool for managers to monitor level of infestation on kiwi and ticks in their populations. The only limitations of this method is that we were not able to tell the different stages or species of the ticks unless better quality cameras are used for the photos. However, this method could be applicable to other birds with similar morphology as the kiwi.

## 4.4 Recommendations and Conclusion

In conclusion, our proposed method as well as the estimate table can be used instead of more time consuming, invasive methods to determine tick infestations in NIBK and other related species after appropriate calibration of the method. This method using photographs provides an easy way for wildlife managers and researchers to measure and monitor levels of ticks on kiwi.

I suggest that any managers that try out the DR method in the future, also take photos of the birds, thus enabling them to pool the data with the data we have collected to increase the accuracy of the model and predictions. We recommend that managers that would like to estimate the number of ticks on their managed kiwi take photos as described in this paper from the underwing of the kiwi and calculate the tick infestation using Table 4.1. if they find 20 or fewer ticks on the photos, or Table 4.2 if they find more than 20 ticks in the photos. The tables don't only provide a mean number of ticks that might be present on the bird but also a range.

The third part of the three-part question into the ecology of the parasite involves the on-host life cycles. This is always difficult when working with wild endangered birds. However, the results of this chapter will make it easier to collect data, with minimal stress to the birds which in turn will help us discern the on-host life cycle of the kiwi ticks making it easier to connect the dots and have a more robust picture of the life

cycle of the kiwi tick. Unfortunately, this was out of the scope of this study, however since we wanted to know if and how these ticks affect NIBK using routine health parameters, we had to first establish what the normal values for health parameters were in kiwi which we do in chapter 5.

# Chapter 5

Author with a chick caught during the sampling

When I get angry, I go all kiwisaurus rex on people. Like this… rawr, rawr, rawr. It really messes people up – Sebastien Millon

# Chapter 5: Establishing normal reference-range-values for blood parameters of North Island Brown Kiwi (*Apteryx mantelli*, NIBK), and investigating variation between different populations.

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**Author contribution:**

Experimental design: NB with input from IC. Fieldwork: NB, IC, MU and AH. Writing: NB with comments from IC, MU and AH. Analysis: NB.

## 5.1 Introduction

Haematology and biochemical values reflect the health of an organism, and therefore play a crucial role in understanding proximate factors that affect the immune response in avian hosts (Ots, Murumagi and Horak, 1998). Poor health and associated low immune response are responsible for delayed breeding, breeding failure, and lowered survival of animals (Moreno et al., 1998). A variety of factors can affect an animal's immune response including age, sex, costs to the individual, specific and context dependent responses, and concurrent infections by different parasites (Schmid-Hempel, 2003). This makes it difficult to find a simple measure of immunocompetence in hosts. Mounting an immune response is energetically costly and the costs are reflected in reduced activity, feeding, and reproductive rate (Klasing and Leshchinsky, 1999). The costs may be more pronounced in certain situations, for example, when the host has limited energy and nutritional resources or if it is under other sources of stress (Svensson et al., 1998). Variations in costs depending on circumstances result in trade-offs between health, immuno-competence, and fitness (Zuk and Stoehr, 2002; Schmid-Hempel, 2003). Traditionally, haematological and biochemical values of a host are measured (Table 1.1) and then compared to normal values to explain sources of variation. In a normal situation, blood parameters such as haemoglobin, haematocrit, and mean corpuscular

haemoglobin concentration are inversely proportional to body size, which means the smaller the species, the higher the values (Sealander, 1965). These higher values are correlated to higher metabolism in small sized animals. Other factors that cause variation in the various haematological parameters can be environmental, such as season, year, altitude, habitat, weather and food availability, or individual related, like age, sex, parasitic infestation, reproductive stage and genetic makeup (Fair, Whitaker and Pearson, 2007; Jerzak et al., 2010; Lill, 2011; Norte et al., 2009; Ots, Murumagi and Horak, 1998). Therefore, it is vital to know the normal reference ranges for the various parameters for the study species.

The north island brown kiwi (NIBK), *Apteryx mantelli*, is one of the five species of kiwi (Weir et al., 2016) all of which are endemic to New Zealand. There is only one published record of NIBK haematological values by Doneley (2006), which has been republished by Morgan (2008), and is the main guide used for reference values for NIBK in New Zealand. However, the original source population and sample size of NIBK for these values is unknown, and the data could have originated from a sample of captive birds, or even birds that were treated at a veterinary facility. Therefore, the main aim of this study was to investigate the variation in various haematological parameters (Table1.1) of different populations of NIBK and establish a reference range of parameters for wild birds. We also compared these values to Doneley (2006) and values from other birds in the ratite group.

## 5.2 Material and Methods

### 5.2.1 Study Sites

We sampled seven different populations of NIBK around the North Island of New Zealand (Table 5.1, Figure 5.1, 5.2 and 5.3). Ponui Island, Whinray Scenic Reserve and Kuaotunu Kiwi Sanctuary were sampled in 2017 and the rest of the populations were sampled in 2019.

*Table 5.1: The timeline for sampling of different populations with the total sample sizes of each, including the female (f) and male (m) kiwi numbers where available. *we do not have data on number of kiwi to calculate the density therefore it was based on author*

| Site | NIBK habitat size (ha) | Cover type | NIBK density/ha | NIBK sampled | References |
|---|---|---|---|---|---|
| Ponui Island | 1800 | Mixture of pasture, scrub, swamp and broadleaf-podocarp forest | 1 | 41 (20f, 21m) | Cunningham et al., 2007; Miles and Castro, 2000; Shapiro, 2005 |
| Whinray Scenic Reserve (Motu) | 430 | Pristine native podocarp forest | 0.07 | 5 (3f, 2m) | S Sawyer pers. Comm. |
| Kuaotunu Kiwi Sanctuary | 2850 | Mixture of regenerating coastal forests, pine plantations, pasture | 0.2 | 5 (3f, 2m) | DOC (n.d); Project Kiwi trust (n.d); pers. Obs. |
| Purerua Peninsula | 3600 | Mixed pasture, vineyards, scrub, swamp, … | High* | 21 | Craig, 2019; pers. Obs. |
| Moturua Island | 164 | Regenerating scrub dominated by Manuka & Kanuka | 0.3 | 18 | Craig, 2019; DOC (n.d) |
| Motuarohia Island | 63.4 | Pine forest, kikuyu grasslands and scrub | 0.6 | 7 | Craig, 2019; DOC (n.d) |
| Puketi Forest | 5000 | Ancient subtropical Kauri forest | 0.06 | 5 | Craig, 2019; DOC (n.d) |

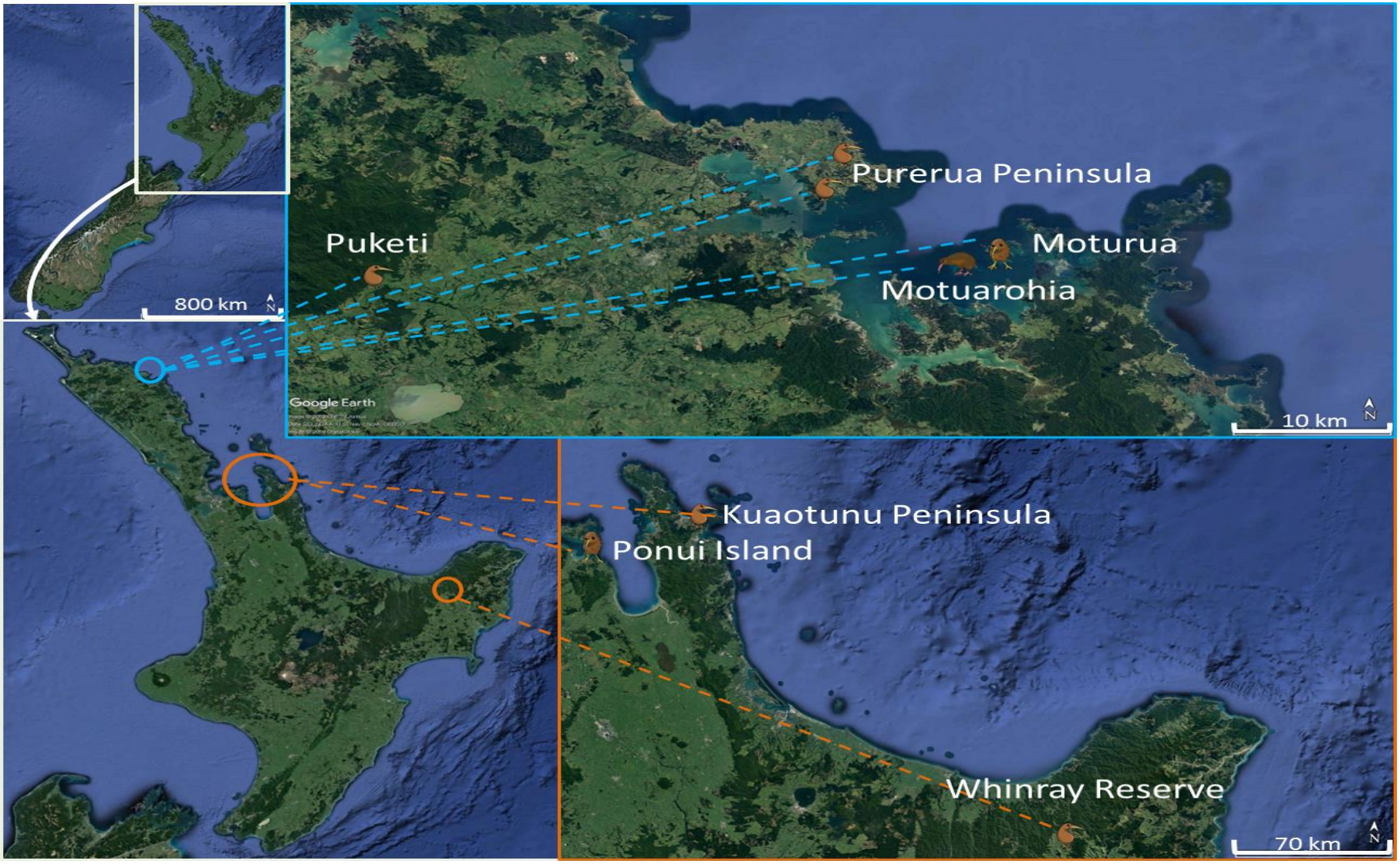


*Figure 5.1: Map of new Zealand 's North Island enlarged to show the Bay of Islands with the four sampling sites there (Purerua, Moturua, Motuarohia and Puketi) and the Hauraki gulf showing the three populations sampled there (Ponui, Kuaotunu and Whinray reserve (also known as Motu))*


*Figure 5.2: A representative photos of the habitat at each site; Ponui island with grazed regenerating bush, Motu (Whinray reserve) with old undisturbed forest (photos from Wikimedia Commons) and Kuaotunu (Tunu) showing variety of disturbed habitats from pastureland, coastal regenarting bush to pine plantations (photos from google earth).*

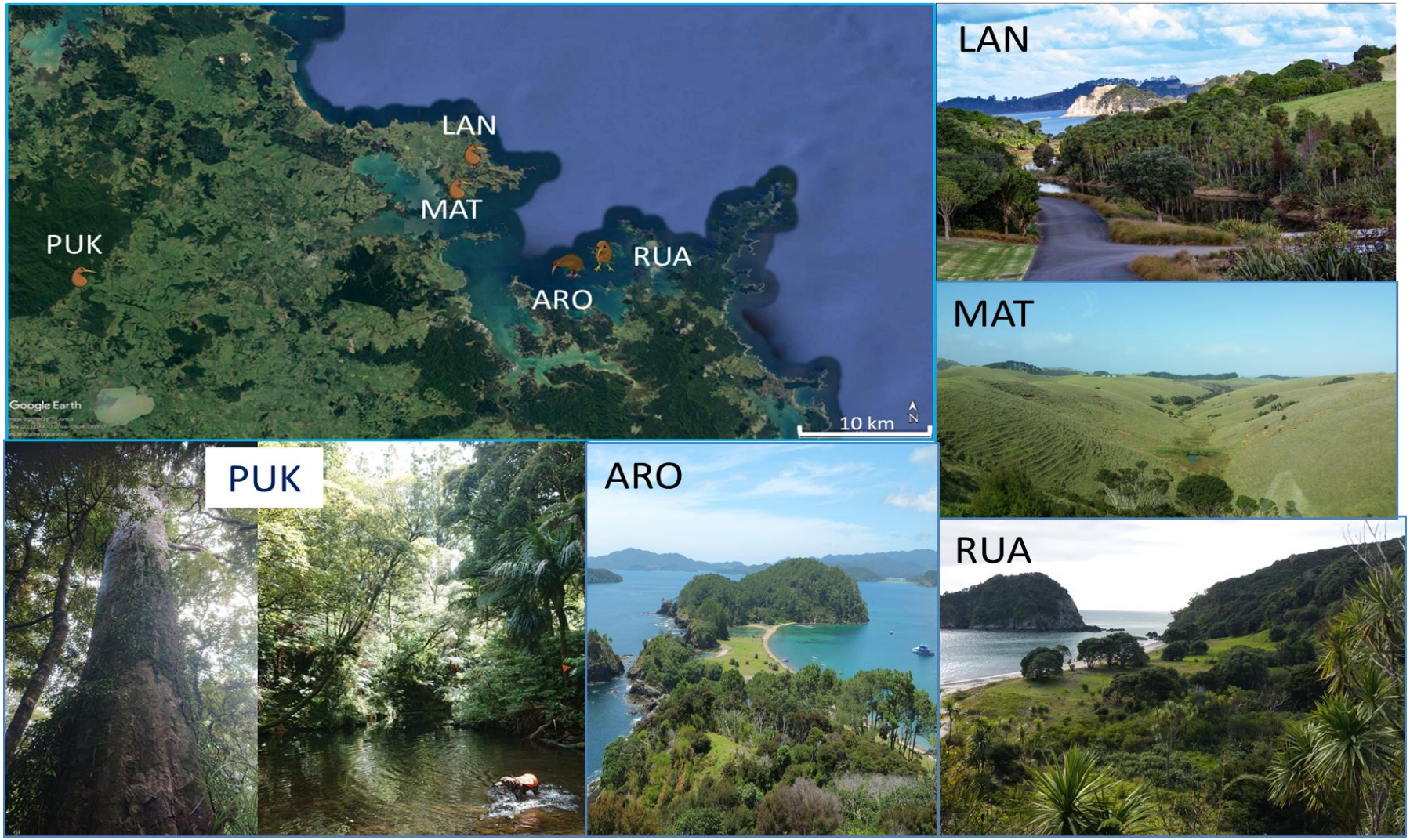


*Figure 5.3: A representative photos of the habitat at each site in the Bay of Islands; Lan = The Landing (Purerua Peninsula) has well-kept manicured lawns and gardens, MAT = Mataka Station (Purerua Peninsula) is mainly pasture with pockets of bush and cliffsides covered in Muhlenbekia spp. (photo by J. Klut), Rua = Moturua Island (photo by J. Klut) and ARO = Motuarohia island (photo from wiki commons) both of which have a mixture of kikuyu grassland and regenerating scrub and PUK = Puketi forest which, like Motu, has old undisturbed Kauri forest (photo by M. Undin).*

### 5.2.2 Field sampling

For three of our populations, Ponui Island, Kuaotunu Peninsula, and Motu, birds could be tracked down and sampled during the day, since they were fitted with radio transmitters as part of long-term monitoring by other studies. At all other locations, birds without transmitters were caught either during the day using a kiwi detection dog (Puketi Forest, Motuarohia Island, and Moturua Island), or at night by hand while they were out foraging (Purerua Peninsula and Moturua Island). After birds were caught, up to 0.5 ml of blood was collected from the metatarsal vein, using a one ml disposable syringe and a 25G needle following the guidelines in the Kiwi first aid and veterinary care manual as well as the Kiwi best practice manual (Morgan, 2008; Robertson and Colbourne, 2017). Collected blood was used to fill two heparinised haematocrit tubes (75ul) and to make two blood smears from each bird. Haemoglobin and blood glucose were measured on site using EasyTouch® GHB. The smears were airdried and then fixed in 70% ethanol in the field. In the laboratory they were stained with Giemsa stain (Appendix 5.1) and used to count WBCs and RBCs using a compound microscope.

Within eight hours of sample collection, haematocrits were centrifuged in a micro haematocrit centrifuge at 10,000 rotations per minute (rpm) for five minutes and packed cell volume (PCV) was measured. The plasma from these centrifuged haematocrits was used to measure total plasma proteins (TPP) using a refractometer (Atago® Hand-held Refractometer). Clotted blood was stored for future studies initially at -4, and once in the lab, at -80.

### 5.2.5 Statistical Analysis

We used box plots to visualise the various haematological parameters and how they vary between the different populations. We used Shapiro-Wilk test to determine normality for each variable individually and transformed the values using square root transformations where necessary and any remaining outliers were removed before analysis. In total we sampled 102 birds (Table 5.1) and knew the sex of birds from three populations (total of 51). Out of these 102, 56 birds were caught in the day and 32 at night. Therefore, sex and time of catch were also used as factors along with year, month and the source population. To establish reference values for blood parameters, a

minimum of 40 samples is required to be able to use parametric statistical tests (Friedrichs et al., 2012; Pavlov, Wilson and Delgado, 2012; Siest et al., 2013) and thus we believe our sample of 102 bird would provide for an adequate estimation of reference range. To test if the variation between populations for any of our variables was significant, we conducted a multivariate analysis of variance (MANOVA) using population, year, sex, time of capture and month as independent variables, and the haematological parameters (PCV, TPP, Hb, Glucose, MCHC and H:L ratios) as dependent variables. I ran a separate linear model with all the independent factors and weight as the dependent variable because kiwi are sexually dimorphic and therefore sex and weight are confounding variables. As a post-hoc test and to see where our differences lay, we used multiple ANOVAs on each of the different variables (Wilkinson, 1975) and this approach provides correction for an experiment-wise compound error (Hummel and Sligo, 1971).Sample means were used to report the new normal set of values with 95% confidence intervals (CI). All statistics were carried out in R (version 3.6.0).

### 5.2.6 Permits

This study was conducted under Animal Ethics Protocol MUAEC 16/92 and DOC Wildlife Permit 50249-FAU (Ponui, Motu and Kauotunu birds), and Animal Ethics Protocol MUAEC 18/82 and DOC Wildlife Permit 70826-CAP (other sites).

## 5.3 Results

General findings

While certain blood parameters like packed cell volume (PCV), haemoglobin (Hb) and consequently mean corpuscular haemoglobin concentration (MCHC) did not significantly vary between the populations, others such as total plasma proteins (TPP), Glucose, heterophil to lymphocyte ratio (H: L) and weight showed significant differences between populations (Table 5.2). The average Hb of all birds was relatively similar although the values had a larger variance, especially for Kuaotunu (15 ± 5.8 CI) and Puketi (14 ± 3.9 CI) birds but this is most likely due to the small sample of birds from these populations (five each). The TPP of birds from Puketi (4.6 ± 0.4 CI) was significantly lower, while that of Kuaotunu (6.6 ± 1 CI) was significantly higher than that of all the

other populations (Table 5.2). On the other hand, glucose of the Puketi birds along with birds from Motu, was significantly higher and that of Kuaotunu birds was significantly lower than all other populations (Table 5.2, Figure 5.4). Conversely, birds from Kuaotunu Peninsula had significantly lower glucose levels than all other populations (Table 5.2, Figure 5.4).

Individual kiwi from all sites with exception of the Bay of Islands had their sex known as they were part of other studies. Although NIBK females are generally larger and have disproportionally longer bills than males when adults, it is impossible to discriminate between juvenile males and females using morphometric data alone and genetic sexing is needed. Therefore, we could not control for differences in weight due to sex or age for the birds sampled from the Bay of Islands' populations (Figure 5.3). If we assume that birds of all ages are equally likely to be caught at each site, we would expect that when examined as a whole there should be no significant differences in the morphometrics of the various populations. However, the birds from Moturua Island were significantly lighter than the birds from all other sites with an average weight of 1781.9 grams (± 118.5 CI). This was after removal of two chicks, which weighed less than 1000 grams, from the analysis.

There was no significant effect of year (MANOVA, Wilks $\lambda$=0.97, p=0.62),month (MANOVA, Wilks $\lambda$=0.81, p=0.11, Table 5.2), sex (MANOVA, Wilks $\lambda$=0.9, p=0.21) or time of capture (MANOVA, Wilks $\lambda$=0.97, p=0.65) on any of the parameters (Table 1.1). Overall, only the difference between populations was statistically significant (MANOVA, Wilks $\lambda$=0.43, p=0.001, Table 5.2).

Comparison with Doneley 2006 values

When compared to the values reported by Doneley (2006), there were significant differences between the various parameters. The most striking difference were on glucose values, as our populations fell between 75 to 124 mg/dl, except for Kuaotunu birds that had a mean of 55 (± 19) mg/dl and would have been the only ones within in the normal values reported by Doneley (2006) (Table 5.4). Another major difference was in the MCHC value, ours being higher than the value reported by Doneley (2006). While the other values reported did not differ drastically, we did report a set of different ranges than them.

Comparison with other ratites

When compared to other ratites (Table 5.4), the haematological values from our birds, especially PCV, Hb, differential leucocyte counts, MCHC and H: L ratio, are similar to Masai Ostrich values published by Mushi et al. (1999). However, the biochemical parameters such as glucose and TPP are within the range reported for Southern Cassowary by Biggs (2013) and Emu by Menon et al., (2013). While values for Rhea are either higher (PCV and glucose) or lower (Hb, TPP and MCHC) than our reported NIBK values (Table 5.4).

Proposed new values

There is usually normal variation between blood parameters in different populations of the same species of birds which can be due to age, sex, environmental factors and reproductive stage (Fair, Whitaker and Pearson, 2007; Jerzak et al., 2010; Norte et al., 2009). However, when establishing normal reference ranges for such values, researchers need to consider this variation. Therefore, we propose a new set of reference range values for NIBK (Table 5.3) and recommend that any future research on blood parameters use our values and add to them.

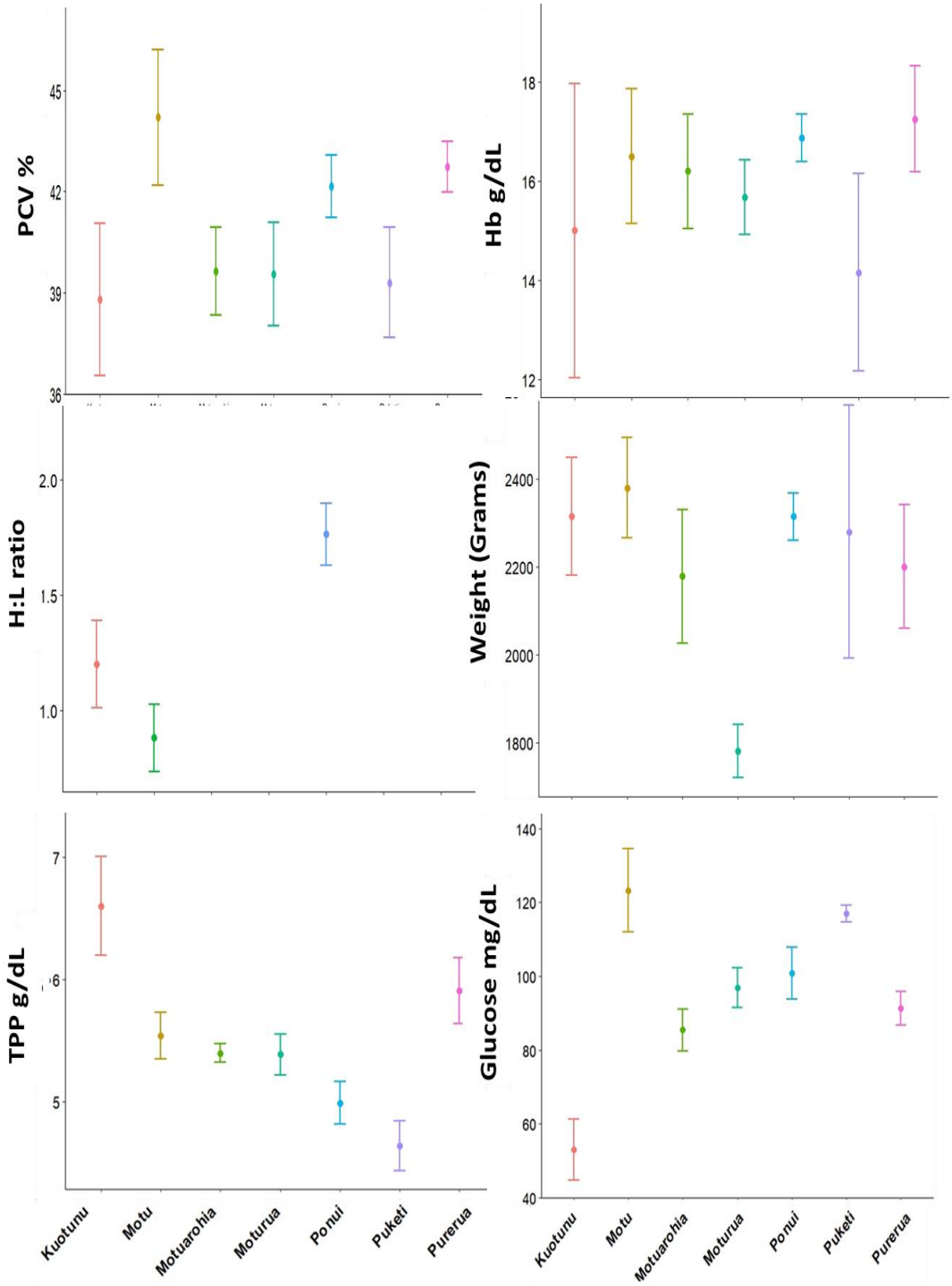


Figure 5.4: *This graph shows the line plots of six blood parameters packed cell volume (PCV), haemoglobin (Hb), heterophil to lymphocyte ratio (H: L), total plasma proteins (TPP), glucose and weight of north island brown kiwi, measured in the different populations. The bold dots represent the mean value for each population with the bars representing the standard error.*

*Table 5.2: The results from the MANOVA model and the post hoc ANOVA tests, showing the various factors and interactions used with Wilk's lambda values and significance levels.*

| MANOVA | | Predictor | Wilk's | F | df | Sig |
|---|---|---|---|---|---|---|
| Overall | | Populations | 0.35022 | 2.976 | 6 | 1.49E-05 |
| | | Month | 0.80611 | 1.65 | 2 | 0.1182 |
| **ANOVA** | **Predictor** | **Sum of squares** | **Mean squares** | **F** | **df** | **Sig** |
| PCV | Populations | 272.57 | 45.428 | 2.307 | 6 | 0.045 |
| | Month | 116.5 | 58.251 | 2.958 | 2 | 0.06 |
| TPP | Populations | 18.275 | 3.046 | 4.316 | 6 | 0.001 |
| | Month | 0.763 | 0.381 | 0.54 | 2 | 0.59 |
| Hb | Populations | 46.78 | 7.796 | 0.448 | 6 | 0.84 |
| | Month | 27.32 | 13.658 | 0.785 | 2 | 0.46 |
| Glucose | Populations | 16482.8 | 2747.14 | 6.109 | 6 | 4.68E-05 |
| | Month | 909.1 | 454.54 | 1.011 | 2 | 0.37 |

Table 5.3: The proposed values for North Island brown kiwi from this study and how they compare to previously reported values. **converted values from mmol/L to mg/dL. **calculated values of H: L ratios from absolute heterophil and lymphocyte counts.*

| Haematological parameters | unit | North Island Brown Kiwi (Apteryx mantelli) This study | | North Island Brown Kiwi (Apteryx mantelli) Doneley (2006) |
|---|---|---|---|---|
| | | n | mean (Range) | |
| Packed Cell Volume | % | 97 | 41.58 (36.2 - 47) | 46 (38-54) |
| Haemoglobin | g/dL | 87 | 16.51 (12.9-20.1) | not given |
| Glucose | mg/dL | 68 | 99.85 (75-124) | 54 - 70* |
| Total plasma protein | g/dL | 93 | 5.4 (4.5 -6.3) | 5.4 - 6.2 |
| Heterophils | % | 45 | 62.7 (51-74) | 60 (40-82) |
| Lymphocytes | % | 45 | 32.8 (24-42) | 42 (25- 59) |
| Monocytes | % | 45 | 2 (0-4) | 3 (1-5) |
| Eosinophils | % | 45 | 2.1 (1 - 3) | 1.8 (7-12.9) |
| Basophils | % | 45 | 0.5 (0-1.8) | 5.6 (0.9 - 13) |
| MCHC | g/dL | 88 | 39.4 (29-49) | 25 (11-33.3) |
| H:L ratio | - | 45 | 1.66 (0.8-2.5) | 1.4 - 1.6** |
| RBC | $10^{12}$/µL | 60 | 3.76 (3.3-4.2) | Not given |

*Table 5.4: This table shows how our reported values compare to values reported in other ratites. n is the sample size. * represents the study by Uhart et al. 2006 and is the only wild population of bird apart from our birds. The values with the same numbers in the Masai ostrich column correspond to the reference article that reported those values.*

| Haematological parameters | | North Island Brown Kiwi (*Apteryx mantelli*) This study | | *Rhea americana* (Gallo et al., 2015; Uhart et al., 2006*) | | Masai Ostrich (*Struthio camelus*) (Mushi 1999[1]; Palomeque et al., 1991[2]) | | Southern Cassowaries (*Casuarius casuarius*) (Biggs 2013) | | Emu *(Dromaius novaehollandiae*) (Menon et al., 2013) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | unit | n | mean (range) | n | mean (range) | n | mean (SD) | n | mean (range) | n | mean |
| Packed cell Volume | % | 97 | 41.58 (36.2 - 47) | 22 | 49.5 (44-54)* | 50 | 43.25 ± 1.9[1] | 8 | 48.1 (33.5-58) | 46 | 51 |
| Haemoglobin | g/dL | 87 | 16.51 (12.9-20.1) | 56 | 10.44 ±1.39 (7.80-13.33) | 50 | 16.68 ± 0.93[1] | 3 | 17.4 (13.5-20) | 46 | |
| Glucose | mg/dL | 68 | 99.85 (75-124) | 20 | 212.4 (177.84 - 260.6)* | 4 | 207.4 ± 33.68[2] | 7 | 173.34 (98.1 - 230.4) | 46 | 171 |
| Total plasma protein | g/dL | 93 | 5.4 (4.5 -6.3) | 22 | 4.8 (2.9- 5.4)* | 4 | 3.87 ± 0.58[2] | 8 | 5.6 (4.5 -7.5) | 46 | 5 |
| Heterophils | % | 45 | 62.7 (51-74) | 58 | 64.10 ±9.90 (37-88) | 50 | 60 ± 2.1[1] | | | 46 | 62 |
| Lymphocytes | % | 45 | 32.8 (24-42) | 58 | 26.93 ±9.62 (6-50) | 50 | 32 ± 2.0[1] | | | 46 | 32 |
| Monocytes | % | 45 | 2 (0-4) | 58 | 6.40 ±2.99 (0-15) | 50 | 1 ± 0.5[1] | | | 46 | 4.4 |
| Eosinophils | % | 45 | 2.1 (1 - 3) | 58 | 2.05 ±2.06 (0-10) | 50 | 1 ± 0.2[1] | | | 46 | 1.1 |
| Basophils | % | 45 | 0.5 (0-1.8) | 58 | 0.52 ±1.27 (0-6) | 50 | 6 ± 1.4[1] | | | 46 | 0.5 |
| MCHC | g/dL | 88 | 39.4 (29-49) | 56 | 25.64 ±2.49 (18.18-33.06) | 50 | 38.56 ± 2.0[1] | | 45.1 (44.4-45.7) | 46 | |
| H:L ratio | - | 45 | 1.66 (0.8-2.5) | | | 50 | 1:02[1] | | | 46 | 0.8-3.3 |

## 5.4 Discussion

We found that there was a variation in blood parameters between the various populations. We also found that some of the values measured in our study differed considerably from what was previously reported normal for NIBK (Doneley, 2006; Morgan, 2008).

### 5.4.1 Health Parameters and what they say about the populations

We found statistically significant differences in four of the seven parameters measured, and these warrant further discussion and investigation.

#### *5.4.1.1 Total Plasma Protein*

TPP is composed of proteins (albumin, globulin, fibrinogen) as well as non-protein substances. The role of these proteins is in coagulation, immune response, maintenance of osmotic pressure, and transport of other proteins. Elevated gamma globulins are indicative of an active immune response (Ots et al., 1998, Townsend et al., 2018). TPP is also known to increase in cases of acute inflammation, dehydration and haematozoa (blood parasites) infections (Ots et al., 1998; Townsend et al., 2018; Williams, 2005), but decreases with increase in nutritional or heat stress, age (Berrong and Washburn, 1998; Costa, McDonald and Swan, 1993), and infections with coccidian parasites (Conway et al., 1993). Although Puketi birds had a significantly lower TPP level and Kuaotunu birds had a higher TPP level than other populations, they still fall in the average normal range for other ratites (Gallo et al., 2015; Menon et al., 2013). Thus, this variation could just be a normal variation between populations, a result of any of the above-mentioned reasons or an error due to the small sampling size for both those populations. Future research should use methods such as electrophoresis to measure the ratio of albumin to globulin in the plasma proteins.

#### *5.4.2 H: L ratio*

The H:L ratio of Ponui birds was significantly higher than Kuaotunu and Motu birds. H:L ratio in birds increase with ectoparasite infestation (Davis et al., 2008; Norte et al., 2013; Ots et al., 1998). Ponui birds have a high level of ectoparasite infestation (Castro, 2006; Heath, 2010) Motu birds have no ticks (S Sawyer pers. comm.; pers. obs.) and Kuaotunu birds had a low tick infestation (pers. obs.). This could be the reason we found elevated

H:L ratio in the Ponui birds, however, this could also be due to the small sample sizes of Motu and Kuaotunu birds and if data was available for the other populations with higher ectoparasite infestations, it would give a better idea as to why we see this difference.

### *5.4.3 Blood Glucose*

Blood glucose levels of Kuaotunu birds were significantly lower, than other populations, while Motu and Puketi birds had significantly higher glucose levels compared to all the other populations. It is difficult to gauge the meaning of glucose levels in birds as it is regulated differently to mammals; for example, it is not affected by starvation, and is inversely proportional to body mass. High levels of glucose with lower body mass do not affect internal organs that are involved in erythropoiesis, causing anaemia as happens in other animals (Braun and Sweazea, 2008; Langslow, 1978). However, it is known that a low-quality diet with lowered protein intake decreases blood glucose levels in birds (Machın, Simoyi, Blemings, and Klandorf, 2004).

NIBK consume a relatively higher protein-based diet (53%) as compared to other insectivorous animals, with very little plant material (Minson, 2013). The percentage of crude protein for the various invertebrates that brown kiwi eat range from 44-50% for larvae of various beetles, 55-60% for earthworms, crickets and grasshoppers, 60-65% for adult beetles and >70% for spiders (Minson, 2013). The abundance and diversity of these native invertebrates in significantly higher in native forest (116 species) as compared to pine plantations (45 species) and native forest seem to have a higher abundance of earthworms and various beetles (Anderson and Death, 2000) that are especially more abundant during cold winter months where other invertebrates are lacking. Given that both Motu and Puketi are remnants of ancient podocarp forest, where there has been little to no disturbance, it is possible that the invertebrate fauna available to NIBK living in these forests is more representative of their historical diet with a higher abundance and richness of invertebrates throughout the year, resulting in better quality of protein intake and thus in significantly higher blood glucose. Kuaotunu on the other hand is a mixture of livestock farms, pine plantations, native bush, and urban developed areas, which would affect the composition and variety of invertebrates available possibly resulting in lower blood glucose levels. Future studies should investigate with the effect of various diets in the amount of crude protein in the brown

kiwi diet and how that affects plasma glucose. Another possible explanation for the discrepancy in glucose could be that the kiwi at Motu and Puketi forests do not have to compete for food with each other as both have a lower density of kiwi per hectare. Densities are higher at Kuaotunu. If density was a likely explanation for the differences observed, then we would also expect that Ponui Island birds, in particular, would also have low glucose, but this was not the case. A third possibility is that Kuaotunu birds are genetically different to the other populations and have distinct metabolism that explains these differences. In support of this possibility, Coromandel birds have genetic differences that separate them from other NI brown kiwi populations (Weir et al., 2016). However, understanding these differences will require a more detailed study of food composition and availability for these populations which was out of the scope of the current study.

#### *5.4.4 Weight*

The weight of Moturua birds was significantly lower than other populations. As mentioned earlier we did not know the sex of the birds from the Bay of Islands populations including Moturua Island, therefore we cannot attribute this difference to sex. Apart from sex as an influencing factor for weight in NIBK, age of the birds could also affect the weight with a possibility that the birds from Moturua Island were mostly juveniles.

### 5.4.1 Comparing blood parameters to other ratites

Blood parameters of animas are usually related with animal size with smaller sized species showing larger normal values (Dickerson, 1978; Sealander, 1965). These large values are due to the higher metabolism in smaller animals, but NIBK have a very slow metabolic rate (Calder and Dawson, 1978; McNab, 1996). Therefore, even though they are smaller than other larger ratites, their values are not exceptionally high and are comparable with these closely related species. Although we must keep in mind that while our values are similar to other ratites, the values for all the ratites are based on captive farm or zoo animals with the only free ranging birds being the Rhea values given by Uhart et al. (2006).

Future studies should focus on determining whether the population level differences in health parameters we observed can be linked to population age, sex ratio, genetic diversity or population size history, with larger sample size per population, while using our values as a baseline.

# Chapter 6

Replete female kiwi tick on one of the study birds

Don't forget that the flavour of wine and cheese depend upon the types of infecting microorganisms – Martin Fischer

# Chapter 6: Effect of experimental removal of kiwi ticks, *Ixodes anatis*, from a chronically infected host species, *Apteryx mantelli*.

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## 6.1 Introduction

Parasites utilise hosts' resources for their survival and reproduction, so it is therefore unsurprising that most studies of host-parasite relationships report a negative association of the parasite on the host (Fair et al., 2007; O'Brien et al., 2001; Saino et al., 1998; Simon et al., 2004; Słomczyński et al., 2006; Szép and Møller, 2000; Valera et al., 2006). For example, parasitized hosts may present reduced fecundity (Asghar et al., 2011; Brown et al., 1995), decreased incubation and hatching success (Brown et al., 1995; Clayton and Tompkins, 1994; Duffy, 1983; Mangin et al., 2003), decreased chick growth and increased chick mortality (Berggren, 2005; Morbey, 1996), increased nest desertion (Lehmann, 1993), and changes in anti-predatory and mating behaviour (Bosholn et al., 2016; Garcia-Longoria et al., 2014). Haematophagus ectoparasites, like ticks, can cause blood loss anaemia in hosts, causing a decrease in body mass and body condition (Kleindorfer et al., 2006; O'Brien et al., 2001; Saino et al., 1998; Simon et al., 2004) as well as suppress immune function by decreasing inflammatory responses (Norte et al., 2013; Saino et al., 1998).

Although parasites are often viewed as negative because of their effect on their hosts, they are themselves important parts of biodiversity (Cunningham, 1996). In addition,

the seemingly 'negative effects' of parasites are selection pressures that play a role in the maintenance of the host's fitness and thus are important to the host's evolution (Poulin, 2007; Hamilton, 1980). Some of the effects of chronic parasitism are thought to occur over the life time of the hosts and require long term studies and large sample sizes (e.g. Asghar et al., 2011; Puente et al., 2010) and thus may be difficult to detect in the short term in the absence of direct experimentation. However, there have been a few studies that demonstrate the effect of parasites on adult avian hosts (Heylen and Matthysen, 2008; Kleindorfer et al., 2006; Knowles, Palinauskas and Sheldon, 2010; Morris et al., 2007; Norte et al., 2013; Wanless et al., 1997). Though, all these studies have found some form of negative effect of parasites on host health parameters (Table 1.1), the effects are not immediately life threatening but may ultimately affect the hosts' fitness. However, removal of parasites results in nearly instantaneous changes in blood parameters and this may be a way to demonstrate the effect of the parasite on the host (e.g. Carleton, 2008; Graczyk et al., 1994).

*Ixodes anatis* is a host-specific endophilic tick so far found on two species of kiwi, (Aves, Ratites) *Apteryx mantelli* (North Island Brown Kiwi or NIBK) and *A. australis (*Southern Brown Kiwi or Tokoeka*)*, and as with its host, it is endemic to New Zealand (Dumbleton, 1953; Heath, 2010). Populations of both host species have become reduced and fragmented and very few areas have populations believed to be as numerous as when humans first arrived in New Zealand (Germano et al., 2018). Recent studies have addressed ecological and environmental effects on tick stages and numbers in the wild and in the lab, aimed at discerning the tick's life cycle (Bansal et al., 2019; Chapter 2), but the relationship between tick and host remains unknown. This project started as a result of observing a high infestation of kiwi ticks in a high-density population of brown kiwi (Castro, 2006; Heath, 2010). All stages of this tick feed on blood from the host, and therefore it could be assumed that heavy infestations would result in higher blood loss and be reflected in blood parameters as well as other aspects of the behaviour and fitness of the hosts. Given the serious conservation status of kiwi species, including the reduction in genetic diversity, reduction in numbers, and habitat shrinkage (Germano et al., 2018), we were interested in studying the relationship between kiwi ticks and NIBK. The ultimate objectives of such research are to understand the role of these ticks in the selection processes on the host, understand and mitigate the impact of the host's

endangerment by the ticks, and be able to inform managers on what to do with the ticks, when hosts are translocated as part of conservation efforts. This study was a step towards addressing these issues.

Since the main objective of this study was to measure the effect of kiwi ticks on their adult host in the wild, we developed an experimental design that would allow us to measure this before and after tick control, and between treated birds and a control group. This design would allow us to detect changes over a short time as well as consider local environmental effects. To do this we measured the effect of ticks on a set of haematological parameters that are regularly used to evaluate animal health, and in addition we measured host activity levels and as well as some of the measures of reproductive effort by the host. We carried out this project on a NIBK population that has been studied since 2004 and therefore we were able to use weight and behaviour data from several years to confirm some of our findings.

Table 1.1: Different blood test parameters that were used in this study to test for clinical effect of tick infestations, along with their definitions and expected values in infested and non-infested birds.

| Parameter | Definition | Expected changes in case of ectoparasite infestation | Source(s) |
|---|---|---|---|
| **Packed cell volume (PCV)** | It is the percentage of red blood cells in a whole blood sample and measures the relative proportion of erythrocytes to blood volume | Decreased | Atkinson & Van Riper 1991; Heylen and Matthysen 2008 & 2011; Valera et al., 2005; Gauthier-clerc et al., 2003; Merino et al., 2001; Norcross et.al, 2002; Norte et al. 2013; Pryor and Castro, 2015 |
| **Haemoglobin (Hb)** | It is the iron-containing metalloprotein in blood responsible for oxygen transport | Decreased | Carlton 2008, Norte et al., 2013; O'Brien 2001 |
| **Blood Glucose** | The amount of glucose present in blood | Increased | Campbell 2004; Norte et al., 2013; Pryor and Castro, 2015 |
| **Total Leucocyte count** | Total number of white blood cells present in blood | Increased | Davies et al., 2008;Krams et al. 2013; Norte et al. 2009; Ots et al. 1998; Szep & Moller, 1999 |
| **Total Erythrocyte counts** | Total/differential number of red blood cells present in blood | Increase in number of immature erythrocytes | Heylen and Matthysen 2008; O'Brien et al., 2001; Pfäffle et al., 2009 |
| **Heterophils** | Phagocytic cells , part of innate immune response | Increased | Davis et al. 2008; Campbell & Ellis 2007; Krams et al,. 2013; Ots et al., 1998 |
| **Lymphocytes** | Leukocytes that assist in recognition and destruction of specific pathogens, part of adative immune response | Increased | Krams et al., 2013; Ots et al., 1998 |
| **H:L ratio** | Ratio of Heterophils to Lymphocytes | Increased | Davis et al., 2004, Norte et al., 2013 |
| **Total Plasma Proteins TPP** | Proteins, mainly albumin and globulin, present in blood plasma | Decreased | Ots et al., 1998; Quillfeldt et al., 2004 |
| **Weight** | | Decreased | De Lope et al., 1998; DJA Heylen & Matthysen, 2008; McKilligan, 1996; Norte et al., 2013; Pryor and Castro, 2015; Wanless et al., 1997 |

## 6.2 Materials and Methods

### 6.2.1 *Apteryx mantelli*

The North Island brown kiwi (NIBK), *Apteryx mantelli*, is the most abundant of the five species of kiwi (Weir et al., 2016), a group of endemic and flightless birds from New Zealand (NZ). Past records of densities of NIBK were 40-100birds/km$^2$, however current densities on most mainland populations rarely exceed four adult birds/km$^2$ (McLennan and Potter, 1992). Kiwi are nocturnal and ground dwelling and they use burrows dug in the soil, tree cavities, and thickets of vegetation for roosting during the day (Dixon, 2015; Jamieson et al., 2016; McLennan et al., 1987). NIBK breed from June to January between winter to mid-summer (Potter and Cockrem, 1992; Ziesemann et al., 2011) and eggs are incubated for 74 -84 days with first clutches hatching in October and second clutches in January. NIBK are under threat mainly due to predation by invasive mammalian predators, and most conservation efforts focus on predator control (McLennan et al., 1996; Pierce et al., 2006).

### 6.2.2 Study Site

Ponui Island (36°55'S, 175°11'E) is located approximately 16km SE of Auckland in the Inner Hauraki Gulf, New Zealand (Figure 3.1). The NIBK population on the island originated from 13 birds translocated onto the island in 1964 (Miles and Castro, 2000) and as to 2007 the numbers are estimated at one per ha (Cunningham et al., 2007). The study site comprised a forested area covered in regenerating native vegetation, on the southern half of the island, covering approximately 100 ha. It is moderately steep hill and gully country, and the study area was spread over two gullies (Kauri Bush and Red Stoney Hill Gully (RSHG); Figure 3.1). A preliminary study investigating acaricide efficacy was conducted in a third gully (Pipe Gully, Appendix 6.2). Ponui Island kiwi are part of a long-term study by our team, Behaviour Conservation Group, Massey University, with 30-50 kiwi being followed since 2004. Previous studies at this site (e.g. Dixon, 2015) have shown that there is movement of kiwi between the gullies during foraging, but the individual birds' roosting sites are within one of the gullies only. Previous studies also show that birds use a number of burrows with great degree of overlap in burrow use by different individuals (Jamieson et al., 2016; Dixon, 2015). At the time of this study, there were 50 birds with radio transmitters within the study area. Ponui Island brown kiwi are

hosts of *I. anatis* (and on some rare occasions cattle ticks, *Haemophysalis longicornis*) (Castro, 2006; Swift et al., 2015; Bansal et al., 2019).

### 6.2.3 Experimental design

To achieve our objective, we removed kiwi ticks from a group (N=14) of radio tagged hosts roosting in Kauri Bush gully (Figure 3.1, Chapter 3), subsequently maintaining ticks at the lowest densities possible on the hosts for four months, and then allowing tick numbers to build up again over a one-year span. We sampled these birds for parasite loads and measured haematological and physiological parameters known to be associated with parasitaemia twice, once before treatment and then again two months after tick removal (Figure 6.1). We also measured activity of the birds during the entire treatment period. This before and after design allowed us to control for individual kiwi response to parasites. To control for wider location, temporal effects, and the effect of tick manipulations we also collected information on kiwi tick loads and haematological and physiological parameters from a sample of birds in the adjacent gully, RSHG in which no birds had been treated (Figure 3.1, Chapter 3). All brown kiwi in this study wore Very High Frequency transmitters (VHF) especially made for NIBK called Chick Timer transmitters™ (Kiwitrack Ltd., Havelock North, NZ) that allowed us not only to locate the birds, but also record their daily hours of activity (section 6.2.8). The treatment group at Kauri Bush was formed by 14 radio tagged birds while the independent control group in RSHG was 17 radio tagged birds. There were 18 birds wearing radio transmitters at Pipe Gully of which we used nine in our pilot study (Appendix 6.2). Birds are captured generally twice per year to replace transmitters, and to check on transmitter attachment and we use these opportunities to gather morphometric data from the birds.

### 6.2.4 Burrow treatment to reduce tick load (2017)

All known burrows in Kauri Bush were sprayed with alpha-Cypermethrin 1.5%, diluted to a 0.5% solution (Ripcord Plus, BASF®), a product used to rid animals from ectoparasites. While this chemical has not been previously used on kiwi, the active ingredient has been used on other birds including ostriches (*Struthio camelus*), a relative of the kiwi, with negligible harmful effects at low doses (Anwar, 2003; Aslam et al., 2010; Martínez-Haro et al., 2007; Suzan, 2012; Verocai et al., 2008). As a precaution to ensure the kiwi's safety, we blocked burrows for ten days after spraying, the period that we

found the Ripcord™ kill ticks after a single spray (Appendix 6.1). We sprayed and blocked the burrows in a rotational fashion to ensure that birds always had some roosting burrows available that did not have active acaricide in them. To achieve this, burrows were divided into three groups with group two sprayed ten days after group one, and group three sprayed ten days after group two (Figure 6.1). Each group was sprayed two times, during March, and during May-June. A backpack spray unit with a long nozzle was used for this purpose. The nozzle was inserted into the burrow as far as the hand could go, the handle was pushed and while keeping uniform pressure on the pump, the nozzle was slowly pulled out. We repeated this three times for deep burrows and sprayed only one time in shallow small burrows. We tested the effect of our spraying by quantifying the level of tick infestation in 20 burrows in each gully between the two spray sessions in April 2017, using the method described in Bansal et al. 2019 (Chapter 3). To control for possible seasonal effects, we also tested these burrows in April 2018, (Figure 6.1). We treated burrows just before treating the birds (Figure 6.1). We assumed that this consecutive treatment of both burrow and birds would maximise the number of ticks killed and keep infestation level low enough for us to see a change in the haematological and physiological parameters.

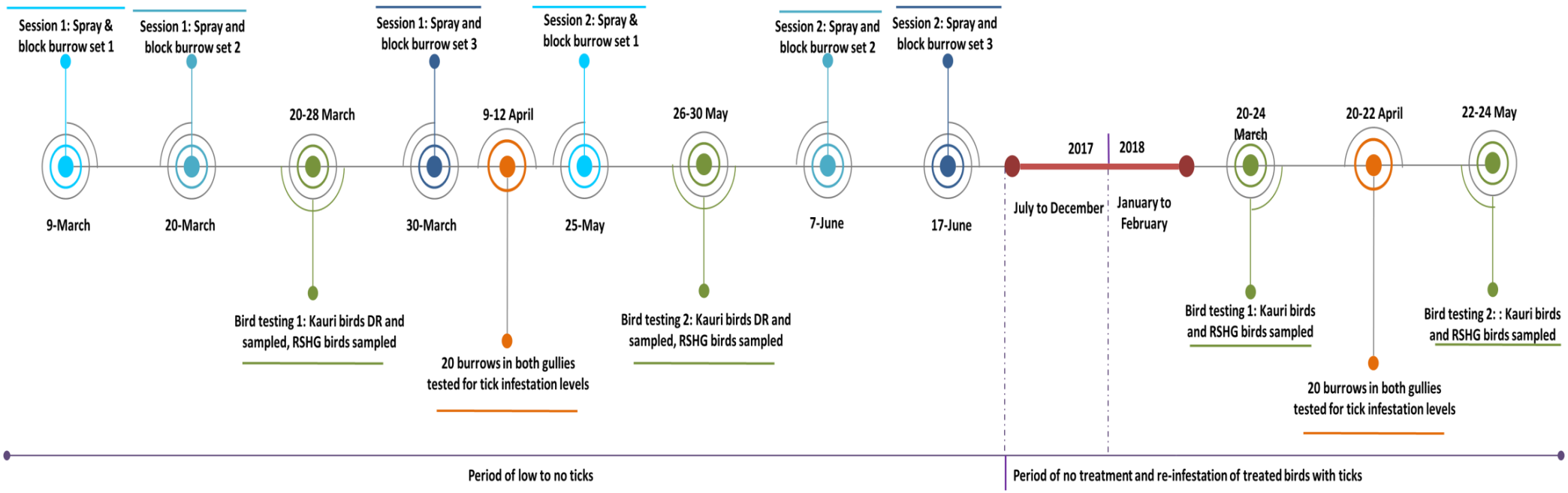


*Figure 6.1: Experimental design used for this study with detailed timeline of burrow and bird treatments as well as expectations of tick levels. The blue circles represent when the burrows were treated with Cypermethrin to reduce ticks, green represent the two sampling periods for the birds where the Kauri birds (treatment group) were dust-ruffled (DR) with pyrethrin to reduce and collect ticks (only 2017). At the same time, both* Red stony hill gully (control group, RSHG) and Kauri birds were sampled to collect blood for testing haematological parameters and weighed, which was repeated in 2018 but without treatment. The *orange circles represent when the burrows* were tested for the level of tick infestation.


*Figure 6.2: A) Author radio tracking kiwi to locate them, B) the equipment used to collect and process blood in the field and C) Blood collection from the medial meta-tarsal vein in kiwi*


*Figure 6.3: Dust-ruffling for ectoparasites where A) holding bird upside down over a cloth bag, B) dusting the bird with flea powder, C) Bird sits with body in cloth bag for 10 mins (note: the collar is used to stop the powder from going onto the face, D) removing ticks from face and neck and E) ruffling bird over bag to remove ectoparasites.*

### 6.2.5 Capture and sampling of birds

The birds were sampled four times, in March and May in 2017 and again in March and May in 2018 (Figure 6.1). Birds were located using a Telonics TR4 receiver fitted with a Yagi antenna in their day roosts (section 6.2.8; Figure 6.2A). After birds were caught, up to one ml of blood was collected from the metatarsal vein (Figure 6.2C) following the guidelines in the Kiwi First Aid and Veterinary Care Manual and the Kiwi Best Practice Manual (Morgan, 2008; Robertson and Colbourne, 2017). Blood was used to fill two heparinised haematocrit tubes, make two blood smears, and measure haemoglobin and blood glucose (Figure 6.2B for gear, EasyTouch® GHB). The remaining whole blood was stored in Eppendorf tubes, transferred to a thermos with ice until we reached the field lab and then stored at -4°C until they could be transferred to a -80 °C freezer at Massey University, to be used for DNA analysis (Chapter 7). Smears were aridried before being stored in a slide box for transfer to the lab.

### 6.2.6 Laboratory methods

Every evening, after returning from the field to the hut, the haematocrits collected were centrifuged at 10,000 rpm for five minutes and packed cell volume (PCV) was measured using a PCV chart. The plasma from these centrifuged haematocrits was then carefully separated from the packed red cells and used in a refractometer (Atago® Hand-held Refractometer) to measure total plasma proteins (TPP). Packed red cells were frozen for future work. The blood smears were fixed in 99% ethanol for one minute, air dried and stored for later staining with a Giemsa stain (Appendix 5.1). Smears were observed under a compound microscope to record differential leucocyte counts (DLC) following the method used by England et al., (1994) (see Appendix 5.2 for SOP). The percentage of red blood cells (RBC) suspected of containing Haematozoa infections were also noted for use in Chapter 7.

### 6.2.7 Treating birds to reduce and collect ectoparasites

The birds from Kauri Bush were treated two times, in March and May in 2017 (Figure 6.1). After blood sampling the birds were examined for injuries and body condition. The Kauri group birds were de-parasitized using a modified version of the dust-ruffling (DR) method described by Walther and Clayton, 1997 and used previously on Ponui Island

(Castro, 2006, Chapter 4): the birds were powdered over the body surface and under the feathers (avoiding the face) with Vitapet™ flea powder (containing 1.95% Permethrin, a synthetic pyrethrin; Figure 6.3A & B). We used a neck cone to prevent powder falling on the head, eyes, nose and ears of the birds (Figure 6.3C). The birds were then left resting in a bag (pillowcase) for 10 minutes, with their head out (Figure 6.3C). During this period, all ticks on the face of the birds were manually plucked using forceps (Figure 6.3D) by grabbing the ticks close to the skin and slowly pulling while twisting (Duscher et al., 2012). After this period, the feathers of the bird were carefully ruffled over a pillowcase for one and a half minutes to extract and collect all the parasites (Figure 6.3E). The bags were then sealed and taken to the lab to count and identify the ectoparasites (Chapter 4). After these manipulations the birds were examined to ascertain all the visible ticks had been removed and then the birds were returned to their roost.

### 6.2.8 Measuring activity of NIBK

In this study, we used the activity data from the Chick Timer transmitters to compare the activity of control and treated kiwi monthly, for the duration of the study. Chick Timer transmitters are 'smart transmitters' that give out beeps that can be picked up using a TR4 radio receiver providing information about the overall activity of the birds wearing them. The transmitter sends information on bird activity through an output that includes changes in rate of beeps with changes in activity of the birds. Bird activity is provided through an increase in beep rate from 30 (not incubating) to 48 beeps per minute if the birds reduce their activity to less than five hours a day (possible incubation), double beeps (indicates nest desertion), and an increase to 80 beeps per minute if the transmitter has not moved for 48 hours (possible mortality or transmitter drop). Activity data per day is also logged in the transmitter as total seconds of activity per day and can be obtained daily through an output of beeps provided by the transmitter every 10 minutes. Data for up to nine months can be stored in the data logger within the transmitter and downloaded after removal of the device from the bird. The recording of decreased activity is used to detect when the male kiwi start incubating eggs, as they reduce their foraging times from, a normal average of, 9-12 hours to 2-4 hours.

### 6.2.9 Statistical Analysis

*Hypothesis*

Based on previous literature, we hypothesized that the treated birds from Kauri, with reduced parasite loads, will be in a better health condition than untreated birds and this will be reflected in changes in their blood parameters and these will be close to normal values (Chapter 5). When compared to the RSHG birds, we expected the various haematological values of the Kauri birds to follow the opposite of the trends in Table 1.1 (Chapter 1), after they were treated. As parasitized birds spend a large amount of time on preening as a cost of parasitism (Dufy, 1983), we also expected the activity of the Kauri group to be lower than the RSHG group birds.

*Statistical tests*

We used box plots to visualise how the various variables changed before and after treatments between the control and treatment group birds, as well as for both the years. We used Shapiro-Wilk test to determine normality with each individual variable group and all except haemoglobin and H:L values were nomally distributed. Therefore, we transformed these variables using log transformations. As we had several different variables and wanted to test the effect of treatment on different haematological parameters, we conducted multivariate analysis of variance (MANOVA) for each year (2017 vs 2018) using treatment (March vs May, before or after tick removal on the Kauri birds), sex (male or female) and group (RSHG or Kauri) as independent variables, and the haematological parameters as matrix of dependent variables. To further explore our MANOVA we used multiple ANOVAs for the MANOVA fit models on each of the different variables (Spector, 1977; Wilkinson, 1975), and while this approach provides some protection from an experimentwise compound error (Hummel and Sligo, 1971), we applied Bonferroni procedure, one of the more conservative approaches, to get adjusted p-values (Timm, 1975). We also carried out paired t tests for comparing tick loads on the individual birds before and after dust-ruffling and to test weight gain in birds before and after treatment as well as independent t tests to compare activity of males and females in the treatment and control group, before and after treament. We analysed weight separately as average weight gain after treatment

which helped us compare the weight gain from previous years to see if this was just a normal trend or weather out treatment actually caused a difference. All statistics were carried out in R (version 3.6.0).

### 6.2.10 Permits

This study was conducted under Animal ethics protocol number 16/92 and DOC wildlife permit number 50249-FAU.

## 6.3 Results

Of the 17 birds in the RSHG, eight were female and nine were male and of the 14 birds in the Kauri seven were females and seven males. During handling for this study birds appeared normal, without signs of disease or injury and we did not detect any negative effects from our manipulations.

### 6.3.1 Treatment year: 2017

#### *6.3.1.1 Blood parameters and weight*

There were no ticks found in the burrows sprayed in Kauri Gully in April 2017, but nine of the 20 control burrows tested in RSHG, had ticks present, ranging from two to 57. The Kauri birds had significantly lower tick numbers on their body after treatment compared to before treatment (paired t-test, t= 2.13, n=12, p=0.03; sample size reduced due to two birds from Kauri losing their transmitters and therefore it was not possible to recapture them) (Figure 6.6).

There was no significant difference between the haematological parameters of the Kauri and RSHG birds before treatment (MANOVA, Wilks λ=0.94, p=0.4, n = 29). However, after treatment, the parameters of the Kauri birds were significantly different than those of the control birds (MANOVA, Wilks λ=0.68, p=0.03). The difference was because Kauri birds had significantly higher TPP values (ANOVA, F =21.03, df=1, adjusted p=0.02) than RSHG birds (Table 6.1) after they were de-parasitized. The Kauri birds were also heavier after their treatment (T-Test, t-stat = -1.8, df = 26, p=0.04, Figure 6.5). There was no significant difference in the H: L ratio (heterophil to lymphocyte ratio) between or within the two groups (Table 6.1). There was no effect of sex on any of the parameters tested in the treatment year (Table 6.4).

#### *6.3.1.2 NIBK activity*

There were significant differences in the activity of females and males between the RSHG and Kauri groups after the Kauri birds' treatment (T-test, females, t-stat = -2.7, df = 8, p= 0.03; males, t-stat = -2.24, df = 6, p= 0.06). Following treatment to remove ectoparasites in March, during April, May and June 2017, the activity of Kauri females was on average an hour longer than before the treatment, and also an hour shorter than that of RSHG birds (Table 6.2). The activity of Kauri males was also shorter than RSHG males during May, June and July. Kauri males started incubating eggs almost a month earlier than the RSHG birds in our treatment year (2017 – 2018) as compared to the previous year (2016-2017). In 2018-2019, the kauri birds still incubated earlier than the RSHG birds, although this difference was not as pronounced. Therefore, their average activity for July and August 2017, reduced drastically when compared to males from the control group (Table 6.2, Figure 6.7).

With exception of TPP, which on average was higher than normal values for the kauri birds after tick removal, all average blood parameters whether before or after tick removal and for both treated and control birds were within normal values (Figure 6.4).

### 6.3.2 Follow-up year: 2018

#### *6.3.2.1 Blood parameters*

In 2018 we used a less invasive method to detect tick infestation on the birds using a photographic method (Chapter 4). Infestation levels had returned to similar or higher values than in May 2017 (Table 6.1).

Kauri birds had significantly higher H: L ratios in March of 2018 (ANOVA, df=1, F = 15.63, adjusted p=0.005) when compared to RSHG birds.

The sex of birds and month sampled had no significant effect on any parameters in the follow up year. Although, males in RSHG had slightly higher PCV values in May, but the mean was still within the normal values for kiwi (Figure 6.8). No other values showed significant changes (Table 6.1). All other parameters were within normal range (Figure 6.4).

*Table 6.1: The table shows average values ± standard errors for various blood parameters of NIBK in 2017, 2018 and normal values for NIBK reported in Chapter 5. RSHG represents the control group and Kauri represents the treated group, that had ticks removed in 2017. In 2017, March represents before treatment and May represents after tick removal. The bold values are the ones that deviate from the normal values for NIBK. * represents values from a sample size of one; dash means that no samples were available.*

| | | 2017 | | 2018 | | Normal values for NIBK (Chapter 5) |
|---|---|---|---|---|---|---|
| | | *RSHG* | *Kauri* | *RSHG* | *Kauri* | |
| **PCV (%)** | *March* | 45.17 ± 1.18 | 42.63 ± 1.42 | 41.46 ± 1.37 | 44.05 ± 0.91 | 41.58 (36.2-47) |
| | *May* | 43.44 ± 1.14 | 44 ± 0.74 | 46.85 ± 0.83 | 44.4 ± 1.32 | |
| **TPP (g/dL)** | *March* | 5.39 ± 0.23 | 5.06 ± 0.32 | 5.8 ± 0.23 | 5.62 ± 0.16 | 5.4 (4.5-6.3) |
| | *May* | 5.92 ± 0.18 | **6.76 ± 0.23** | 6.36 ± 0.26 | 6.56 ± 0.30 | |
| **Hb (g/dL)** | *March* | 16.75 ± 0.71 | 17.25 ± 0.41 | 17.7 ± 0.91 | 16.47 ± 2.18 | 16.5 (12.9 – 20.1) |
| | *May* | 18.11 ± 1.18 | 16.77 ± 1.27 | 14.13 ± 2.24 | 16.97 ± 0.81 | |
| **Glucose (mg/dL)** | *March* | 158.00* | - | 111.2 ± 14.70 | 116.00* | 99.85 (75 – 124) |
| | *May* | 95.38 ± 7.85 | 100.46 ± 11.30 | 87.13 ± 6.70 | 84.91 ± 5.58 | |
| **H: L ratio** | *March* | 1.86 ± 0.39 | 2.37 ± 0.42 | 1.68 ± 0.21 | 2.48 ± 0.45 | 1.66 (0.8 – 2.5) |
| | *May* | 2.27 ± 0.7 | 2.04 ± 0.33 | 1.82 ± 0.32 | 2.26 ± 0.44 | |

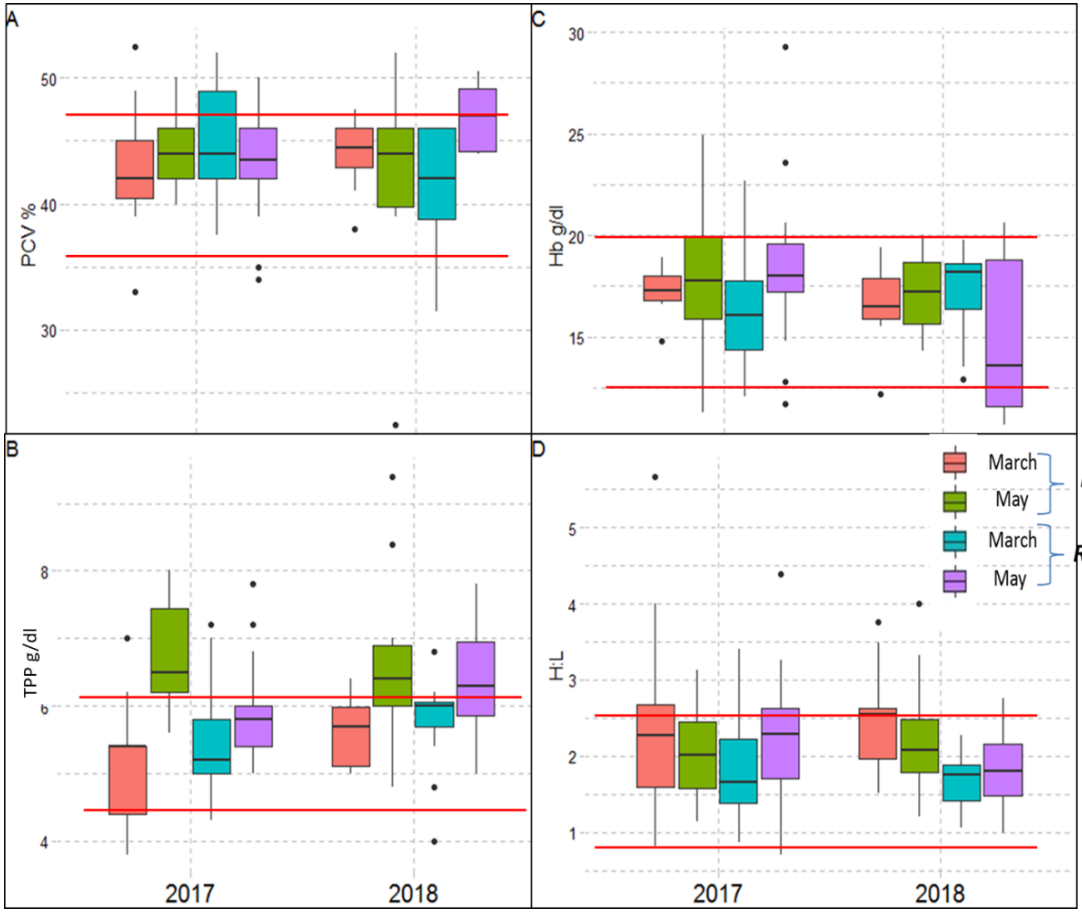


*Figure 6.4: Boxplots indicating distribution of four blood parameters in 2017-Treatment year and 2018- no treatment year. Whiskers above and below the box indicate the 10 th and 90 th percentiles. Points above and below the whiskers indicate outliers.* The red lines indicate normal range of NIBK values (From Chapter5).

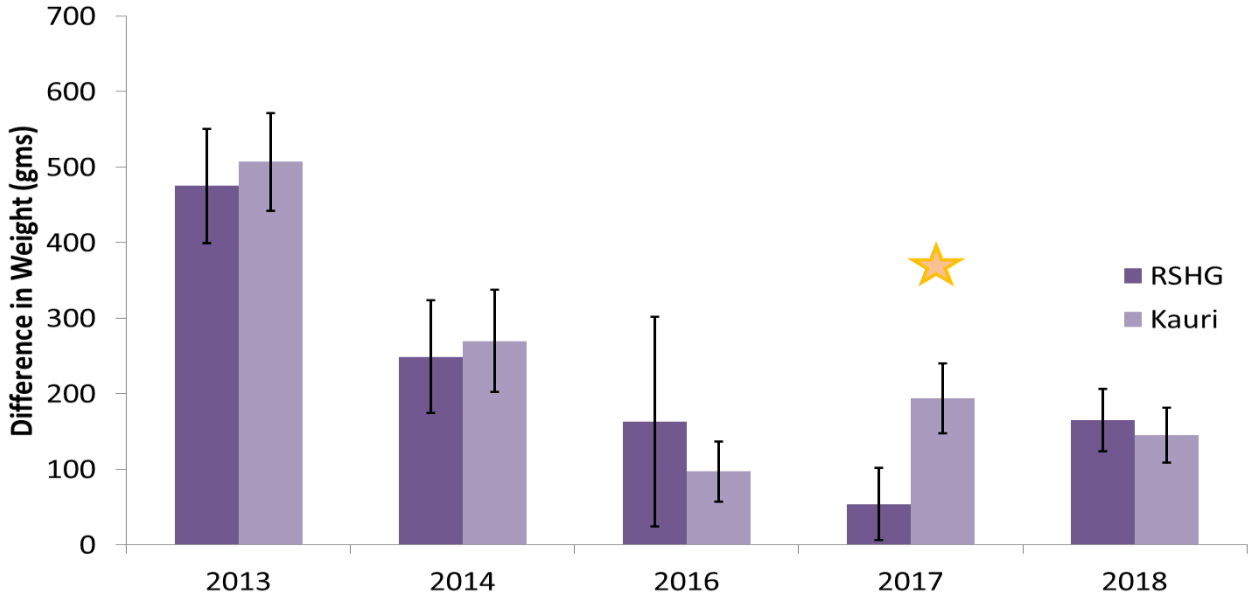


*Figure 6.5: Bar graph depicting the difference in weights between March and May of individual birds from Kauri (treated group) and RSHG (control group) gully since 2013, (2015 was not considered because the birds were not weighed in May that year). The yellow star shows where the weight gain was significant. Note: 2017 is the year the Kauri birds were treated for tick removal.*

*Table 6.2: Average active hours ± standard error for the male and female kiwi during the treatment year 2017. The data on the right of the dotted line represents activity of the birds after treatment of the Kauri birds.*

| Sex | Gully | January | February | March | April | May | June | July | August |
|---|---|---|---|---|---|---|---|---|---|
| Female | Kauri | 9.7 ± 0.66 | 9.4 ± 0.12 | 9.4 ± 0.20 | 10.2± 0.47 | 10.4± 0.31 | 10.2± 0.16 | 11.5± 0.36 | 11.5± 0.18 |
| | RSHG | 9.9 ± 0.77 | 9.5 ± 0.07 | 10.3± 0.11 | 11.2± 0.20 | 11.7± 0.31 | 11.5± 0.37 | 11.71± 0.47 | 11.73± 0.20 |
| Male | Kauri | 7.3± 0.99 | 7.9± 1.10 | 9.3± 0.17 | 10.6 ± 0.19 | 11.0 ± 0.13 | 10.3± 0.11 | 9.5± 0.27 | 7.2± 1.30 |
| | RSHG | 9.6 ± 0.32 | 9.6± 0.20 | 10.5± 0.16 | 10.4± 0.37 | 12.0± 0.26 | 11.8± 0.38 | 11.4± 0.41 | 10.5± 0.74 |

*Table 6.3: The total number ticks on the birds. In the treatment year (2017) the different stages of ticks were collected off the Kauri birds before and after treatment where L= larvae, N= nymphs and A = adult stages. In 2018, which was the follow-up year, the tick numbers were estimated from ticks in photos (Chapter 4). Dashes indicate that birds were not sampled while 0 indicate that no ticks were collected.*

| Kauri birds | 2017 (Treatment year) | | | | | | | | 2018 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | L | N | A | March | L | N | A | May | March | May |
| Bow | 66 | 13 | 9 | **88** | 0 | 3 | 2 | **5** | - | 65 |
| Daphnae | 28 | 10 | 0 | **38** | 7 | 3 | 0 | **10** | - | - |
| Dario | 123 | 79 | 9 | **211** | 5 | 3 | 2 | **10** | 67 | 69 |
| Kobi | 12 | 11 | 12 | **35** | 26 | 12 | 12 | **50** | 43 | 132 |
| Louise | 5 | 2 | 3 | **10** | 4 | 3 | 7 | **14** | 61 | - |
| Paul | 0 | 0 | 2 | **2** | 0 | 0 | 3 | **3** | 88 | - |
| Clea | 93 | 1 | 6 | **100** | 0 | 17 | 8 | **25** | 50 | 53 |
| Ponui | 46 | 21 | 11 | **78** | 9 | 17 | 3 | **29** | - | 69 |
| Max | 300 | 18 | 8 | **326** | 0 | 5 | 9 | **14** | - | 69 |
| Tashu | 219 | 3 | 6 | **228** | 20 | 17 | 7 | **44** | - | - |
| Minnie | 92 | 47 | 0 | **139** | 19 | 3 | 5 | **27** | 46 | 58 |
| Tako | - | - | - | **-** | 18 | 10 | 6 | **34** | 34 | 41 |
| Salome | - | - | - | **-** | 123 | 77 | 3 | **203** | 69 | 47 |
| Tweety | 0 | 2 | 10 | **12** | 3 | 2 | 4 | **9** | 53 | 72 |

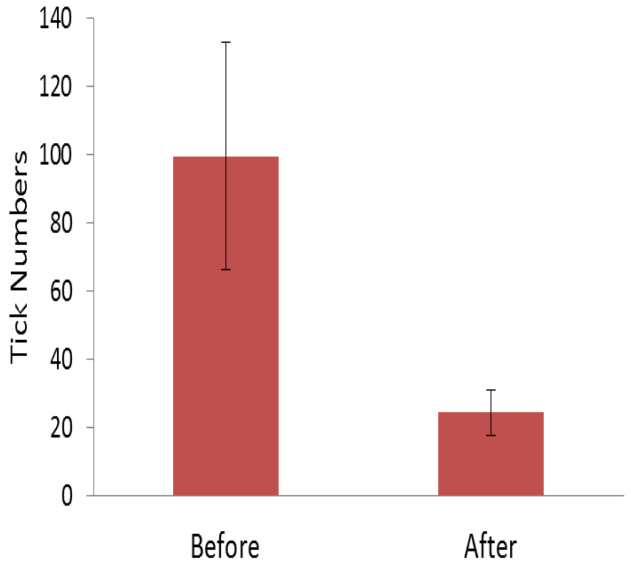


*Figure 6.6: This graph represents the average number of ticks on the Kauri birds (treated) in 2017, collected using dust-ruffling. The bars represent standard error.*

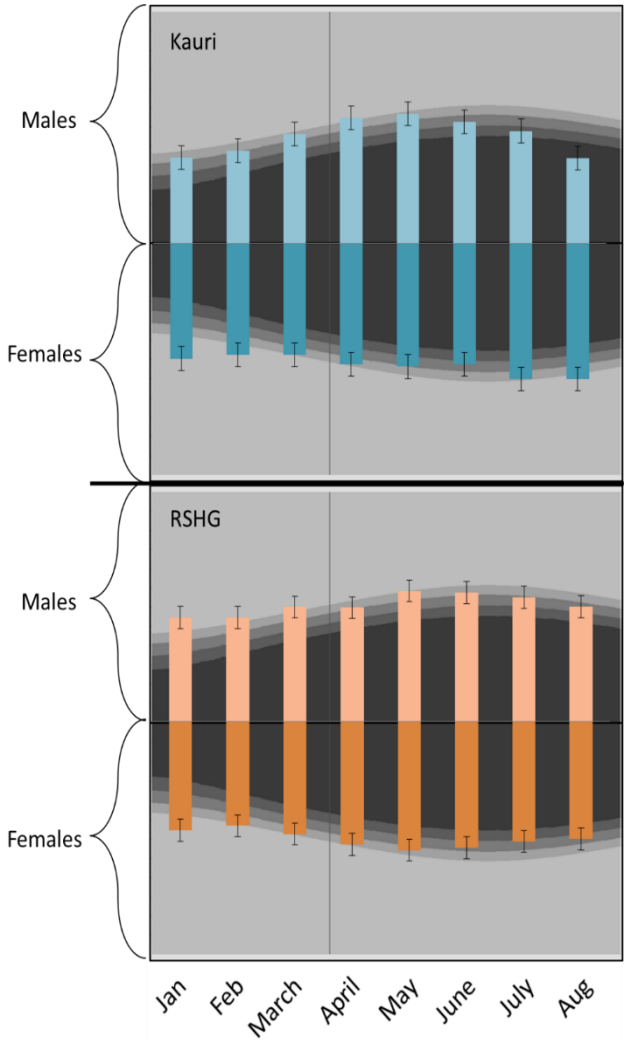


*Figure 6.7: Average activity of KG (blue) and RSHG (orange) birds in treatment year 2017.The black background indicates the length of night hours; light grey is the number of daylight hours and the various shades of grey in between indicate twilight hours (dawn and dusk). The bars indicate standard error. The single grey ling running vertical is the daylight savings, which incidentally also coincides with the month after treatment of Kauri Gully birds.*

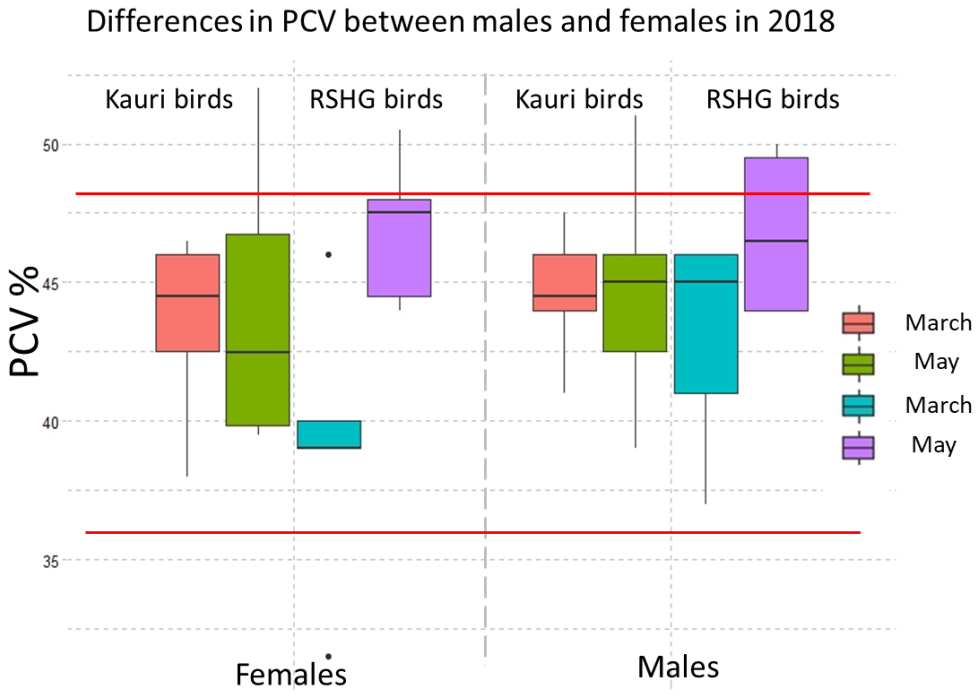


*Figure 6.8: Box plot showing the sexual difference in PCV vales in 2018 in the different gullies and different months. The red bars represent normal NIBK value range for PCV.*

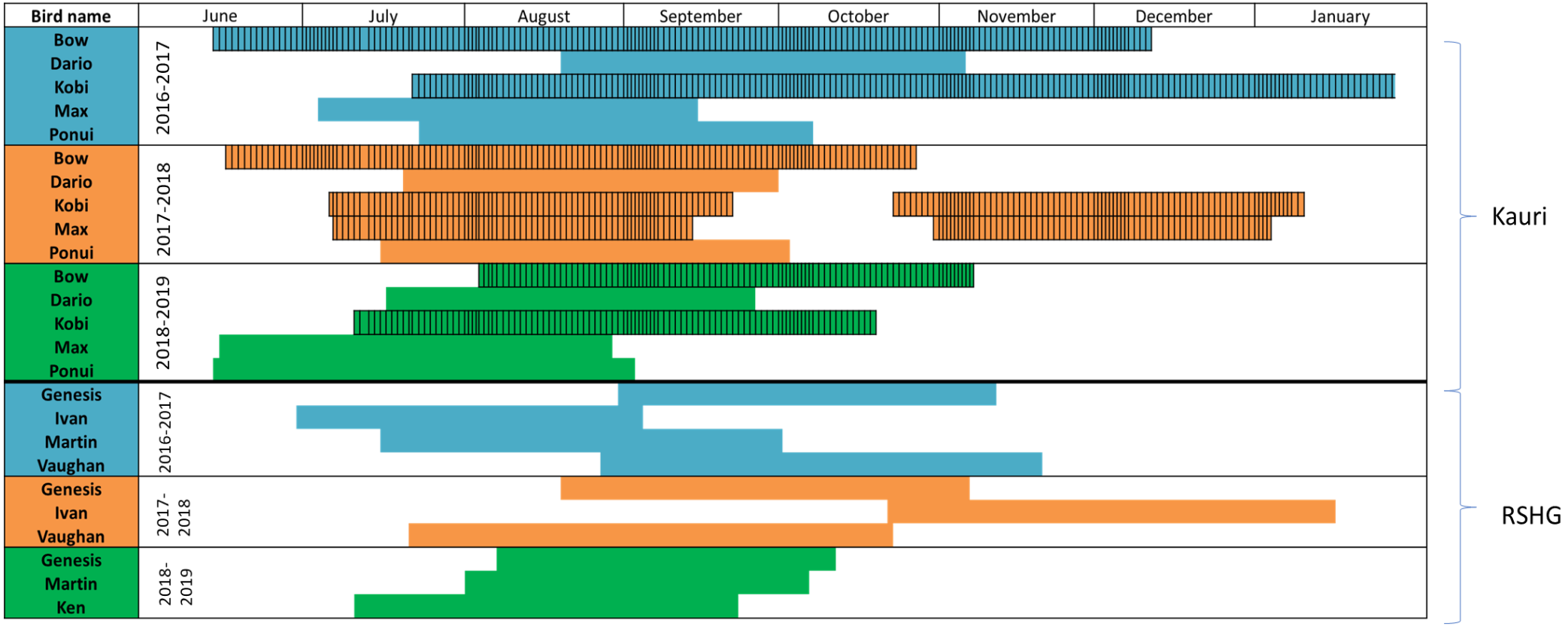


Figure 6.9: This figure shows start of incubation of Kauri gully and RSHG birds where blue is for the year 2016-2017 preceding treatment, orange is for 2017 – 2018 the year of treatment and green is for 2018-2019, the year after treatment. The colours with the black vertical stripes indicate birds that had two clutches whereas the colour bars without lines indicate a single clutch.

Table 6.4: The table gives the results from the MANOVA model showing the various factors and interactions used with Wilk's lambda values and significance levels. The numbers in bold represent significant values.

| MANOVA | Predictor variable | Wilk's | F | df | Sig |
|---|---|---|---|---|---|
| **2017** | Treatment | 0.49881 | 3.189 | 3 | **5.00E-04** |
| | Group | 0.93547 | 0.897 | 1 | 0.47266 |
| | Sex | 0.70689 | 1.486 | 3 | 0.1215 |
| **2018** | Treatment | 0.28755 | 3.189 | 3 | **5.30E-04** |
| | Group | 0.92465 | 0.815 | 1 | 0.5 |
| | Sex | 0.54118 | 3.603 | 4 | **0.02652** |

## 6.4 Discussion

The most important findings of this study are that following parasite removal, birds from Kauri Bush had higher TPP, significantly different activity levels, significant weight increase, and early onset of nesting when compared to the same birds before tick removal, and to the control birds. These results supported our hypotheses and demonstrate a likely effect of the ticks on their hosts.

Our experimental design was successful in reducing tick loads by using pyrethroid derivatives on the birds from March to June 2017 as well as keeping the level of infestation in the burrows low during this period. We believe that any significant differences observed after treatment of Kauri birds in May of 2017 was an effect of our successful treatment which lowered tick infestation levels in Kauri gully. This decrease in the tick infestation was reflected by a change in the parameters measured, and reduced activity levels and earlier onset of nesting in our treated birds. However, like other studies, some but not all haematological parameters were affected before and after treatment in Kauri birds (Bosch and Figuerola, 1999; Gauthier-Clerc et al., 2003; Norte et al., 2013).

### 6.4.1 Treatment year: 2017

TPP in birds can increase for a number of reasons, from an increase in dietary protein (Dawson and Bortolotti, 1997; Leveille and Sauberlich, 1961) to dehydration, acute inflammation or increase in non-protein substances in plasma in conditions such as

lipemia and hyperglycemia (Merck Manual, 2012). In both years and both groups of birds, the TPP increased from March to May of the same year. This increase indicates a seasonal rise in TPP values for NIBK, which could be related to changes (including increase) in food availability from March to May, for instance, studies on invertebrates on Ponui have shown that during January-March, the composition of invertebrates is predominantly Orthoptera and Araneae whereas from April to August, a large proportion of the invertebrates present are beetles and their larvae from various families (Dixon, 2015; Wilson, 2014). In addition, reproductive activities such as egg forming for females in preparation for egg laying in June/July. However, this increase is significantly higher only in Kauri birds for the year 2017 after treatment. This could be due to a number of reasons. Studies show that over time, long term parasitic infections supress inflammatory and immune responses of the host (Quillfeldt et al., 2004; Saino et al., 1998; Szép and Møller, 2000) and since a large part of TPP is made up of albumin and globulin,which are indicative of an immune response (Ots et al., 1998; Townsend et al., 2018), it is possible that a significant reduction in parasitic loads, as achieved in this study, would elicit a better immune response reflecting in the significant rise in TPP in the treated birds. Another reason for the drastic rise in TPP could be acute inflammation. As we know, our treatment kept the birds parasite free (Appendix 6.2) for at least three to four weeks, it is possible that the re-attachment of the ticks on parasite free birds just before our May sampling elicited an acute inflammatory response which was expressed in the elevated TPP of Kauri birds in May.

Studies on other bird species have found that increased ectoparasite infestation decreases weight and body mass of birds (De Lope et al., 1998; Heylen and Matthysen, 2008; McKilligan, 1996; Norte et al., 2013; Wanless et al., 1997). Thus, when compared to weight gain in previous years, the significant increase in weight gain we found in our Kauri birds after treatment was likely due to the reduced parasite load.

In long billed bids with unwieldy bills, preening is difficult, and these birds may spend 16.2% of their time scratching compared to other short billed birds (that only spend 2.3% of time scratching) (Clayton and Cotgreave, 1994). NIBK have long unwieldy bills that they use to preen, and pluck engorged adult ticks from their bodies (Pers. obs.) and long claws which they use to scratch themselves when infested with ectoparasites. We found that the control group of birds in RSHG had an average of one extra hour of activity when

compared to the treated Kauri birds We suggest that the extra activity is the result of scratching. As the infested kiwi use their claws to scratch, the transmitters placed on the legs of the birds will sense that activity, which can be vigorous. The Kauri birds on the other hand, with no to low parasite loads, would have had less need to scratch themselves thus reducing the activity registered by the transmitters.

Also, the relatively higher weight gain of the Kauri birds could have arisen from investing the time and energy saved on scratching for parasites on better foraging success after tick removal. However, our activity timers did not reveal what activity the birds spent this extra time on, and this requires further investigation.

The energy saved from less time scratching as well as the weight gain would then probably be put into breeding which is why the Kauri males, in the treatment year, also started incubating almost a month earlier than the RSHG males as compared to a year before and after treatment. This coincides with studies that have found delayed breeding and egg laying in parasite infested birds (Moller, 1990; Moreno et al., 1998). However, our sample sizes for incubating males were too small to make any definite claims that this early incubation start was a result of our treatment and consequent reduction in tick loads.

### 6.4.2 Follow-up year: 2018

Since we did not treat the birds and burrows after May 2017, the Kauri birds started re-acquiring and building tick levels up from July 2017, therefore, resulting in similar levels of tick infestations by March 2018 to before treatment. Thus, we expected that the blood parameters and activity values would not differ between the birds from the two gullies.

However, the Kauri birds had significantly higher H: L ratio values when compared to RSHG birds. H: L ratios are known to increase in cases of acute infection with ectoparasites (Davis et al., 2008; Norte et al., 2013; Ots et al., 1998), and stay elevated for some time after infection before returning to normal (Davis et al., 2008). Having re-acquired high infestation levels after a period of tick reduction, this could be one of the reasons why the Kauri birds had elevated H:L ratio values as compared to RSHG birds. Along with ectoparasites, haemoparasites can also cause an increase in H: L values

(Norte et al., 2013) and NIBK infested with kiwi tick have been shown to have haemoparasites (Howe et al., 2011; Pierce et al., 2003). Some of our study birds presented intracellular bodies that could be haemoparasites of the Genus Plasmodium (Chapter 7), but we did not pursue this line of investigation further. For future studies, a comparison between levels of haemoparasites in blood cells between the two groups may help elucidate these differences in H: L levels.

Tick removal in this study affected some haematological parameters of NIBK like TPP levels, weight gain, activity and H: L ratios. Luttrell et al. (1996) demonstrated that one to 14 ticks on one individual in passerines causes partial or complete paralysis and sometimes death. Koch and Sauer (1984) estimated that on average an adult female can take between 0.55 to 1.45 ml, a nymph can take 24.2 to 27.4 µL and a larva can take from 0.78 to 1.34 µL, of blood per feeding. We cannot say exactly how much the kiwi tick might consume, but assuming the lower limits from Koch and Sauer (1984), heavily infested birds such as Max (326 ticks) or Minnie (139 ticks, see Table 6.4) could loose from 5-7ml of blood to tick infestations which is ~2.8% of the bird's blood volume. In the case for Ponui birds where the birds can have heavy tick infestations (Castro, 2006; Heath, 2010; this study) throughout their lives and still manage to breed every year and have what we consider normal blood parameters, is an amazing feat.

We found in this study that birds suffer heavy costs when parasitized with ticks. The costs were reflected in the response of the birds to experimentally lowered tick loads which resulted in increased protein levels, decreased activity levels, increased weight gain, and earlier onset of breeding. These differences if sustained over time would likely have a significant impact on survival and reproduction and affect the life history parameters of these birds.

# Chapter 7


Histology section of a whole kiwi tick stained with H&E stain

Whāia te iti kahurangi ki te tūohu koe me he maunga teitei.
Aim for the highest cloud so that if you miss it, you will hit a lofty mountain. - Ron Mader

# Chapter 7: Detection and prevalence of tick borne haemoparasites in the blood NIBK blood and in *Ixodes anatis.*

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**Author contribution:**

Pilot experiments: NB. Lab work (except DNA analysis): NB. DNA based lab work: TM. Writing: NB and TM (PCR work) with comments from IC.

## 7.1 Introduction

New Zealand has a unique fauna of birds and parasites that have co-evolved together since the Zealandia landmass separated from Gondwana around 40 million years ago (Lafferty and Hopkins, 2018). Human arrival led to local extinctions of a number of avian hosts leaving most parasites with a smaller host pool, while some host specific parasites, like ticks, became extinct with their host (Lafferty and Hopkins, 2018).

Currently, New Zealand has 12 confirmed species of ticks of which 11 are endemic or native and one is introduced (Heath, 2012; Heath and Palma, 2017; Heath et al., 2011). Some of these are known vectors of haemoprotozoa, for example, the introduced tick *Haemophysalis longicornis* transmits *Theileria orientalis* Ikeda (James et al., 1984), or various species of *Babesia* spp. on seabirds are transmitted by the native ticks *Carios capensis* and *Ixodes eudyptidis* (Paparini et al., 2014). Once infected, hosts remain infected with these vector-transmitted haemoprotozoa for life (Valkiunas, 2004), with low levels of parasitaemia which relapse to high parasite levels in the case of stressful, immune- compromising events such as breeding or translocations (Morales et al., 2004; Valkiunas et al., 2004). Signs of clinical and sub-clinical diseases by haemoprotozoa in avian hosts are usually non-specific, thus other diagnostic methods are needed to detect these parasites.

The kiwi tick *Ixodes anatis* was first suspected of transmitting *Babesia kiwiensis* and *Hepatozoon kiwi* by Pierce et al. (2003) when they found and described the suspected organisms in the blood smear of a kiwi from Whangarei kiwi sanctuary. The *B. kiwiensis* was then confirmed by Jefferies et al. (2008) but *Hepatozoon* spp. could not be confirmed by molecular methods using polymerise chain reaction (PCR). Kiwi have also been diagnosed with avian malaria through smear and PCR work (Howe et al., 2011; Schoener we at., 2014).

The aims of this study were: 1) Determine the prevalence and severity of infection of *B. kiwiensis* in North Island Brown kiwi (NIBK); 2) Determine the prevalence and severity of infection of *B. kiwiensis* in *I. anatis*, 3) To find how this severity changed with the level of *I. anatis* infestation on NIBK and 4) To detect any other tick borne haemoprotozoa present in NIBK. Historically, evaluation of a blood smear was, and is, widely used to detect haemoprotozoa and in some cases, like *Plasmodium* spp., identification can be made up to the species level (Valkiunas, 2004). Therefore, to achieve our aims, we started with evaluating blood smears, however we were not able to detect the organism that Pierce et al. (2003) described. Therefore, this investigation evolved to use multiple staining and histology techniques to detect haemoprotozoa in kiwi ticks and NIBK blood samples, which were then followed by DNA based molecular techniques. All the procedures used to detect the haemoprotozoa, during the course of this thesis have been described and discussed in this chapter.

## 7.2 Materials and methods

### 7.2.1 Sample collection

Most of the samples for haemoprotozoa detection were collected during the experiments in the previous Chapters 5 and 6. As the total number of samples and times, of both kiwi blood and kiwi ticks, used for each method varied, they will be specified at the beginning of each method. For NIBK blood, up to one ml of blood was collected from each kiwi from the metatarsal vein following the guidelines in the Kiwi First Aid and Veterinary Care Manual as well as the Kiwi Best Practice Manual (Morgan, 2008; Robertson and Colbourne, 2017). The whole blood was stored in Eppendorf tubes, containing heparin to prevent coagulation, and transferred to a thermos with ice until

we reached the field hut, where the samples were then stored at 4 °C. Samples were transported to Massey University within 15 days and stored at -80°C. At the same time, two thin blood smears were made for each bird. The smears were air-dried and then fixed in 99% ethanol for one minute and air dried.

Free walking ticks at all stages of development were collected from the birds during handling. These were stored in 10ml screw cap bottles with 99% ethanol. Engorged and unengorged ticks were collected into separate vials. The ticks were transported to Massey University within 15 days and stored at 4°C.

### 7.2.2 Smear based detection from host's blood samples

We used one smear per bird made during the experiment in Chapter 6 from RSHG, Kauri and Pipe from March and May 2017 (N=80). The fixed smears were stained with 10% Giemsa stain for 50 mins then washed with distilled water and air dried before being examined under 200X magnification to detect any parasites (Appendix 5.1 and 7.1).

### 7.2.3 Detection using tick salivary gland staining

For this section 19 adult ticks (10 females, 9 males) and 5 nymphs were collected off the birds in March 2015 and used for dissection and salivary glands staining. After the ticks ingest blood from the host, the haemoprotozoa undergo sexual reproduction in the digestive tract of the tick and are transported to the salivary glands for subsequent infection and transmission to other susceptible hosts (Moltmann, Mehlhorn and Friedhoff, 1982; Riek, 1964). These parasites persist in concentrated numbers in the salivary glands of the vector for weeks and can be detected through staining techniques (Moltmann et al., 1982; Riek, 1964).

*Dissection of ticks*

We embedded the tick appendages into a paraffin filled petri dish. We then covered the tick with a drop of Phosphate Buffer Solution. This solution makes the organs inside float and thus easier to identify and separate. An incision was made using Ophthalmic micro-scalpel (Feather Safety Razor Co. Ltd®, Japan) just posterior to the mouth parts and then extended along the lateral carapace to remove the dorsal scutum. Next, the gut was removed which made it easier to get to the salivary glands. The anterior and posterior

salivary glands were then identified and removed (Figure 7.1). These salivary glands were mounted on slides with a little PBS solution and teased out to separate the glands from the rest of the tissue. The slides were then air dried. Two protocols were used to stain the slides.

*Protocol 1*

The first protocol was taken from Walker et al., (1979). After air-drying, 12 of the slides were fixed with absolute ethanol for 10 mins, washed with distilled water for two mins and then stained with Methyl green – Pyronin Y (MGP, Table 7.1) stain for 45 mins. This was followed by washing with 70% ethanol for two mins. The slides were then air dried and mounted in Dibutylphthalate Polystyrene Xylene (DPX) (which prevents stains from fading).

*Protocol 2*

This protocol was taken from Irvin et al., (1981), in which the remaining 12 slides with air-dried salivary glands were first fixed in Carnoy's fixative (Table 7.1) for five minutes. This was followed by washing in 70% ethanol for two minutes and then in distilled water for two minutes. The slides were air dried again and then stained by Methyl green – Pyronin Y (MGP, Table 7.1) for seven minutes and then rinsed with distilled water. After air drying again, slides were mounted in DPX. According to Irvin et al. (1981), methyl green should only stain DNA and pyronin Y should almost only stain RNA and the infected acini appear purple while uninfected acini appear blue.

### 7.2.4 Detection using tick Histology

We used 500 ticks collected during March 2016 for histology, although only 199 of these processed samples on the slides were analysed due to time limitations. As ticks have a thick chitin layer, we tried multiple protocols to obtain a satisfactory histology slide, which maintained the structural integrity of the tick and its organs so we could observe endo-parasites within tissue cells.

*Protocol 1*

For this protocol, we fixed 12 ticks in formalin directly overnight and then ran them through a short dehydration cycle (Leica automated vacuum tissue processor). Ticks

were then removed and embedded in wax. We cut 5 µm thick sections and stained them with Haematoxylin–eosin stain (Figure 7.2a).

*Protocol 2*

To soften the tick tissues further, we fixed another set of 12 ticks in Formalin-acetic alcohol (FAA, Table 7.1) solution (Carranza et al., 1987) for three days and then ran them

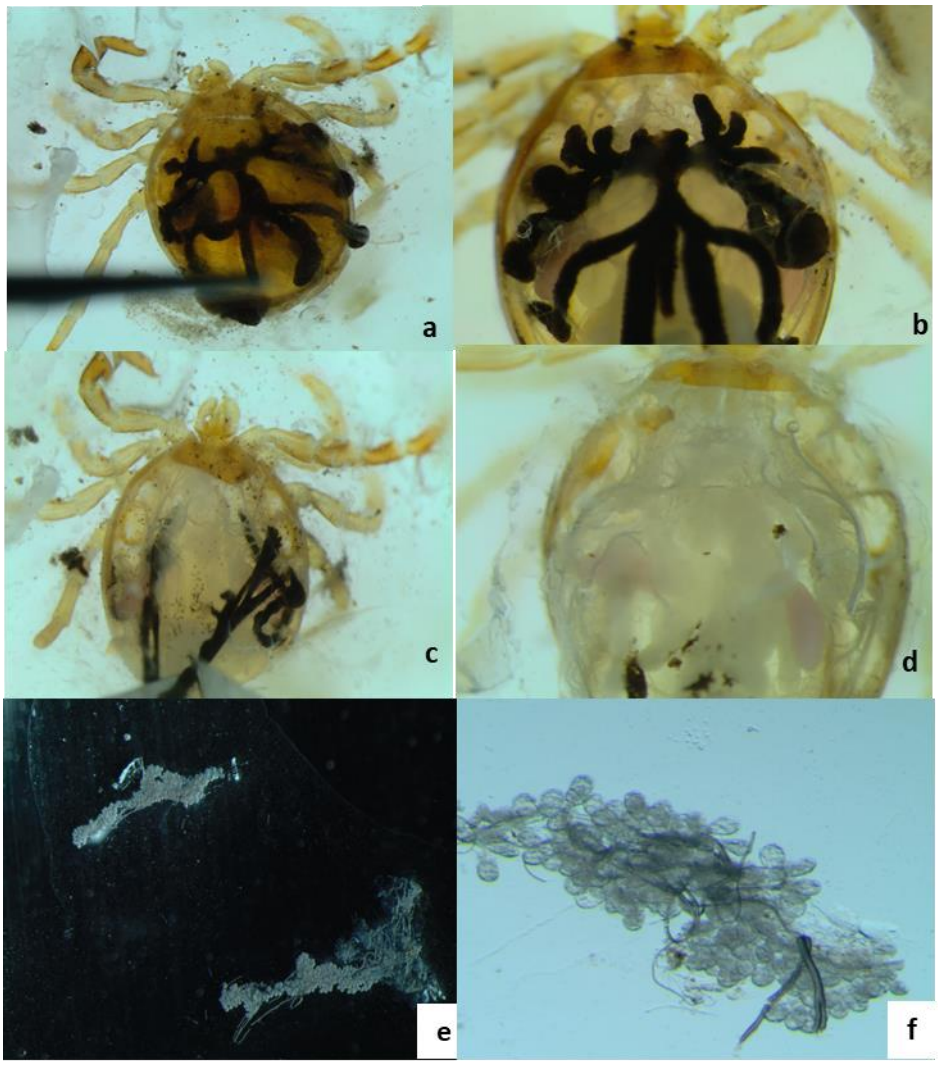



Figure 7.1: Stepwise dissection of a tick. a & b) Cutting and removing of dorsal capitulum. The gut is the dark part in the picture; c) Removal of gut to get to the salivary glands; d) A view of the salivary glands (in white) and the testis (light pink); e & f) The unstained salivary glands

through a long dehydration cycle. The embedding, sections and staining was the same as in Protocol 1 (Figure 7.2b).

*Protocol 3*

The remaining 476 ticks were processed using Protocol 3. This protocol involved three separate preparations of ticks. We did one set (N=53) where we made an incision on the

dorsal scutum of the tick. A second set of ticks (N=349) was prepared by injecting FAA (Table 7.1) into the tick (Veronez, de Castro, Bechara, and Szabó, 2010) and for a third set (N= 74) we removed the dorsal scutum. We also removed legs from all three sets, and they were then fixed in FAA for nine days. After this treatment the preparations went through the long dehydration cycle and sections were prepared as previous protocols (Figure7.2c). We also stained some of these slides with Giemsa stain (Appendix 5.1 and 7.1).

Table 7.1: A tabulated list of solutions and their respective compositions, used for histology.

| Solution | Ingredients |
|---|---|
| **FAA** | Formaldehyde [37-40%] (10%)<br>Distilled water (35%)<br>Absolute ethanol (50%)<br>Glacial acetic acid (5 %) |
| **Carnoy's fixative** | Absolute ethanol (60 %)<br>Chloroform (30%)<br>Glacial acetic acid (10 %) |
| **MGP stain** | 1 ml of 5% Pyronin Y Stock<br>10 ml of 2% Methyl Green Stock<br>250 ml of distilled water |

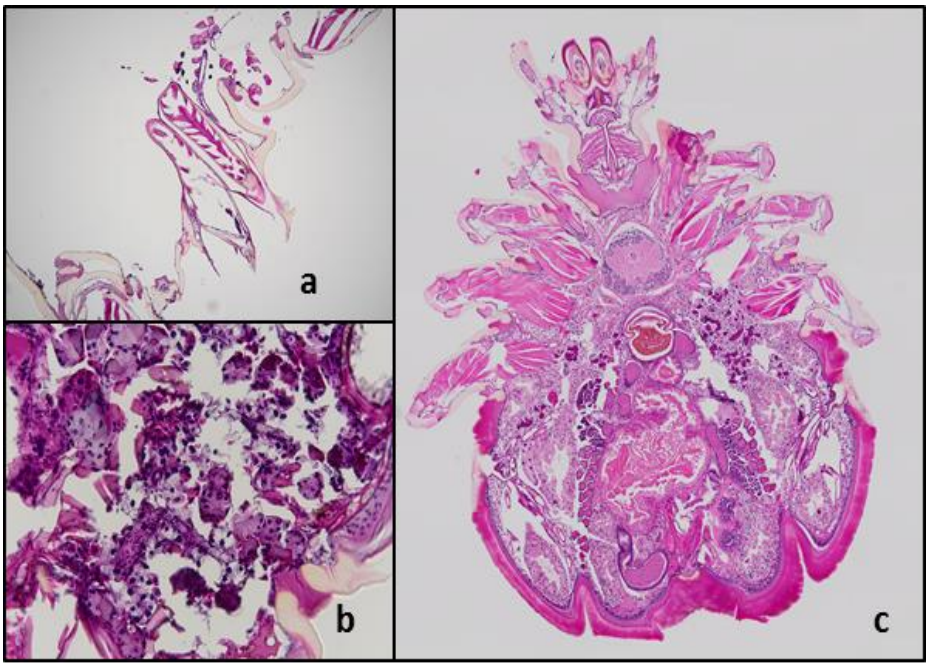



**Figure 7.2: Tick Histology a) The tick histology section after protocol 1 showed many empty spaces as the microtome did not cut through the chitin. b) Tick sections after protocol 2 showed more tissue but did not maintain structure as in some places the blade bounced off the chitin. c) A whole tick section after protocol 3, which was more complete with the tissue structure intact**

### 7.2.5 Fluorescence in situ hybridization (FISH)

We used thin smears of kiwi blood fixed and air dried on slides from six birds thought to contain a parasite from observations under the microscope. We used the protocol from Yamasaki et al. (2011) for FISH for thin smears. Since we used a 5' labelled CY3 oligonucleotide probe (Figure 7.3), we denatured the probe using the method from Ge et al., (1995).

Targeted genetic sequence: 3' GTT TGT GTC GGG ATC GCT TTT G 5'
Probe sequence: 5' CAA AAG CGA TCC GCA CAC AAA C 3'
22bp length
GC content= 50%
Melting temp= 54.8°C
Location on genome= ~530

Figure 7.3: Probe design of *B. kiwiensis* probe for FISH.

### 7.2.6 DNA based techniques

*DNA extraction*

DNA was extracted from nine samples of RBC, collected after centrifugation, from NIBK sampled from Ponui Island and from five samples of whole blood from Cape Brett and Puketi, Northland (Figure 7.4), using the High Pure PCR Template Purification kit from Roche Diagnostics. Briefly, 5ul of RBC/whole blood was mixed to a final volume of 200ul

The suite of DNA samples prepared for PCR:

1. Engorged tick ($Tick_{eng}$) (95ng/ul)
2. Unengorged tick ($Tick_{uneng}$) (53ng/ul)
3. Mix 1: DNA from bloods 1,2,10 from Ponui (70ng/ul)
4. Mix 2: DNA from bloods 3,4,5,6,7,8 from Ponui (10ng/ul)
5. Mix 3: DNA from bloods 1,2,3,4,5 from Cape Brett (40ng/ul)
6. Mix 4: DNA from bloods 1-4 from an earlier sampling of Ponui kiwi (100ng/ul)
7. *Babesia gibsonii* positive control (800pg)
8. Cow blood with *Theileria orientalis,* Theileria positive control, 3ng/ul
9. Kiwi blood spiked with *Babesia gibsonii* (29ul Amanda DNA @10ng/ul with 1ul Bg+ @8ng/ul, final concentration of Bg is 260pg).

Figure 7.4: The set of nine DNA prepared for the PCR tests.

with Phosphate Buffered Saline (PBS -1X, 137mM NaCl, 27mM KCl, 10mM $Na_2HPO_4$, 18mM $KH_2PO_4$, pH7.4). A further 200ul of Binding Buffer and proteinase K (40ul of 20mg/ml) were added and the mixture was incubated at 70$^oC$ for 10mins. Following the incubation, isopropanol (100ul) was added, the mixture was applied to a spin column and centrifuged in a microcentrifuge (Hereaus Biofuge fresco) at 8000g for 1 min. The eluate was discarded, and the column washed with 500ul of Inhibitor Removal Buffer and two subsequent washes of Wash Buffer. The column was then dried with a 1min spin at 13000g and transferred to a clean 1.7ml tube. The DNA was eluted in 60ul of Elution Buffer (10mM Tris, pH8). The yields of DNA were between 6 ng/ul and 141ng/ul as determined by reading on a QUBIT 2.0 fluorometer (Invitrogen Life Technologies).

Tick DNA was extracted from both engorged and unengorged ticks using the DNeasy Blood and Tissue kit from Qiagen. Briefly, 3 ticks were cut up with a sterile scalpel, put into 180ul of ATL Buffer and proteinase K (20ul of 20mg/ml). The solution was incubated at 56$^oC$ overnight after which 200ul of AL buffer was added and mixed well. A further 200ul of absolute EtOH was added and mixed and the solution was applied to a spin column. The column was centrifuged for 1min at 8000g and the eluate discarded. The column was washed with 500ul of AW1 and a further 2 washes with 500ul of AW2. The column was dried with a 2min spin at 13000g and the DNA eluted in 60ul of elution buffer AE. The yield for the tick DNA was 95ng/ul for engorged ticks and 53ng/ul for the unengorged ticks (Figure 7.4).

A sample of DNA from *Babesia gibsonii* was kindly provided by Dr. Amanda Duarte Barbosa, a Post-Doctoral Research Fellow in the Vector and Water-Borne Pathogens Research Group at Murdoch University in Perth. The concentration of this DNA was determined by Qubit and was 8ng/ul.

A sample of DNA from blood from a cow infected with *Theleria orientalis* was kindly provided by Dr. Kristene Geyde, a Research Officer in the School of Veterinary Science at Massey University, Palmerston North. The concentration of this sample was 3ng/µl.

All primers were made by Integrated DNA Technologies. They came as dried pellets, were reconstituted to 1nmol/µl in sterile water and stored at -20$^oC$. A working stock of 10pmol/ul (10uM) was made and kept at -20$^oC$. All PCR was carried out on a Biometra T1Thermocycler.

PCR products (typically 5µl) were run on 1.5% (w/v) agarose gels with 1x SyBr® Safe (Invitrogen) in 1xTAE (40mM Tris, 20mM Acetate and 1mM EDTA, pH 8.6) running buffer. The gels were run at 100V for 40 or 45mins and viewed and photographed on a BIORAD GelDoc EZ System.

Considerable efforts were made to avoid contamination of the PCR reactions during the amplification process. Primers (working stocks, 10pmol.µl) were stored in 10µl aliquots and discarded after use. DNA from blood and ticks was aliquoted into smaller amounts and stored at -20$^{o}$C in a box separate from the positive control DNA. PCR reactions were prepared in a designated PCR area, into which DNA samples were never taken. A master mix containing the appropriate primers was made and aliquoted for single reactions. DNA templates were added to the master mix on the lab bench (not the designated PCR area) and amplified in a separate PCR machine. The same master mix was used to amplify the positive controls, but these reactions were prepared after the test samples had been done, so the control DNAs (or the first-round positive control PCR products) were never on the lab bench at the same time as the test DNA. These precautions were essential given the high number of PCR cycles.

*Primers*

1. *The Jefferies primers – 18S*

The primers and PCR cycling protocols from Jefferies et al. (2008) were used to try and amplify the parasite from kiwi blood (Table 7.2). The expected size of the fragment from the primer pair BT1F1/R1 is 938bp and for BTF2/R2 is 803bp. PCR was performed using EmeraldAmp GT PCR Master Mix from Takara. For the first round, 10uM (1ul) of BT1F1 and BTR1 were mixed with 10ul of Master Mix and 2ul of each template (as above) plus a negative control with no DNA, and the mixture made up to a final volume of 10ul. The PCR program for the first round was as follows: 94$^{o}$C for 3min, 58$^{o}$C for 1 min, 72$^{o}$C for 2mins followed by 45 cycles of 94$^{o}$C for 30 sec, 58$^{o}$C for 20 sec and 72$^{o}$C for 40 sec, a final extension at 72$^{o}$C for 1min and a HOLD at 10$^{o}$C. Following the first round of PCR, a second round of PCR was done using the primers BTF2 and BTR2 and the same conditions as above EXCEPT: 1ul of product from the first round of PCR was used as template (with the exception of the negative control which was made fresh) and the annealing temperature for the second round of PCR was increased from 58$^{o}$C to 62$^{o}$C. The PCR products were run on a gel as described above and photographed.

*2. Mitochondrial primers*

Nested primers were designed for the inter-genic spacer region between CO1 and CO3 (Table 7.2); there was no publicly available sequence data for this region from *B. kiwiensis* at the time of our study. Sequences were downloaded from GenBank that were assumed to be more similar to *B. kiwiensis* on the basis of where they group in the 18S phylogenetic tree [REF: AB685182, AB685183, KP666169 (*B. gibsonii*), KC207822 (*B. canis canis*), KC 207823 (*B. canis rossi*), AB499086 (*B. caballi*) and KC207824 (*Babesia* spp*. coco)]*. These were aligned in Geneious 9.1.2 (Biomatters Ltd.) and primers designed from consensus regions, using Primer3 2.3.4 (Primer3.org) in Geneious. The primers were also aligned to *Theileria orientalis* (AB499090) and *Theileria parva* (AB499089) mitochondrial sequences and it is expected that they could amplify a mitochondrial product from *Theileria* spp. The expected size of the fragment from the primer pair Babmt_154F/919R is 761bp and for Babmt_404F/890R is 464bp. The Jefferies protocol for PCR as outlined above was followed except that the annealing temperature for the second round was 60$^{o}$C.

**Table 7.2: List of primers used for PCR tests.**

| | Name | Sequence 5'-3' | Tm ($^{o}$C) |
|---|---|---|---|
| **Jefferies et al., 2008 primers** | BT1F1 | GGC TCA TTA CAA CAG TTA TAG | 48 |
| | BTR1 | CCC AAA GAC TTT GAT TTC TCT C | 51 |
| | BTF2 | CCG TGC TAA TTG TAG GGC TAA TAC | 55 |
| | BTR2 | GGA CTA CGA CGG TAT CTG ATC G | 56 |
| **Mitochondrial primers** | Babmt_154F | ACACAAGGCATGCAATACCG | 56 |
| | Babmt_404F | CATAAGTAACCTCTGTGAAAGTG | 51 |
| | Babmt_890R | ATATACTGTTTTATAATCCCATGCTGA | 52 |
| | Babmt_919R | GGTAACAGAGTAGAGACATTGATACG | 54 |
| **Apicoplast Primers** | 23S_730F | GGTAGCGAAATTCCTTGTCGGG | 58 |
| | 23S_854F | GGACAGAAAGACCCTATGAAGCTTTAC | 57 |
| | 23S_1406R | GAACCCAGCTCACGTTTCGC | 59 |

*3. Apicoplast primers 23S*

Most apicomplexans carry an apicoplast, a ~5kb plastid which is a functional remnant of a chloroplast genome (Yurina, Sharapova, and Odintsova, 2017). Primers were designed to the 23S gene carried on the apicoplast with the assumption that the 23S gene would be quite different from the kiwi genome and would provide a broader target for the detection of other Apicomplexans (Table 7.2). The following sequences from GenBank and aligned in Geneious : AB471862 (*Plasmodium fieldi*), AB471870 (*Plasmodium simiovale*), LK022892 (*Plasmodium chabaudi chabaudi*), LT608270 (*Plasmodium berghei*), LT594636 (*Plasmodium maleriae*) LN999985 (*Plasmodium falciparum*), NC031963 (*Plasmodium gallinaceum*), AP013071 (*Leucocytozoon caulleryi*), AP011950 (*Theileria orientalis*), MH107388 (*Babesia duncani*), KT428643 (*Babesia orientalis*), LK934757 (*Babesia divergens*) KX881914 (*Babesia* spp *Xinjiang*), KX881915 (*Babesia sp Lintan*), LK028575 (*Babesia microti*), AB564273 (*Eimeria tenella*), AY283806 (*Atoxoplasma sp*), KX273389 (*Cyclospora cayetanensis*), AF304319 (*Eimeria falciformis*), U87145 (*Toxoplasma gondii*). Semi-nested primers were designed from consensus regions, using Primer3 2.3.4 (Primer3.org) in Geneious.

The expected size of the fragment from the primer pair 23S_730F/1406R is 657bp and for 23S_854F/1406RR is 534bp. The Jefferies protocol for PCR as outlined above was followed and the annealing temperature for the second round was 60$^{o}$C.

*DNA Sequencing*

Where there was a single PCR product in the reactions, sequencing clean-up was able to be done enzymatically: Shrimp Alkaline Phosphatase (rSAP from New England Biolab, 0.5U) and Exonuclease 1 (Exo1, New England Biolab, 4U) were added directly to the PCR reaction. The reaction was incubated at 37$^{o}$C for 15 mins and heat killed at 80$^{o}$C for 10 mins. Where there was more than one PCR product, the band of interest was cut out of the gel and purified using using a Zymoclean $^{TM}$ Gel DNA Recovery kit from Zymo Research and the protocols within.

The products were sequenced by the Massey Genome Service. The sequence data was edited in Sequencher 5.4.6 (Gene Codes Corporation) and compared to the GenBank database using the program BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the option blastn.

Table 7.3: Concentrations of dilutions of the different positive samples for sensitivity determination

| | |
|---|---|
| **1** | 800pg/µl |
| **2** | 80pg/µl |
| **3** | 8pg/µl |
| **4** | 800fg/µl |
| **5** | 80fg/µl |
| **6** | 8fg/µl |
| **7** | NEG |

*Sensitivity determination*

To determine the sensitivity of our primers, the control DNA from *Babesia gibsonii* was diluted serially to a set of seven final concentrations (Table 7.3). The DNA samples were then used for PCR as outlined above, two rounds of 45 cycles using at 58$^{o}$C and 60$^{o}$C annealing temperatures respectively, nested (18S, mt markers) and semi-nested (23S) primers, to determine detection limit for the primers. One ul of template DNA was used in the PCR reaction

Table 7.4: The list of pooled samples for NGS

| Sample | Conc. (ng/uL) | Volume (uL) | 260/280 ratio | 260/230 ratio |
|---|---|---|---|---|
| **1 (Tick)** | 27.9 | 20 | 2.05 | 2.16 |
| **2 (Ponui)** | 27.8 | 15 | 1.98 | 1.93 |
| **3 (PCB)** | 39 | 18 | 1.92 | 1.76 |

Next Gen Sequencing using metagenomic sequencing (MGS)

Three samples were prepared for “metagenomic” sequencing (Table 7.4); Pooled tick DNA, pooled DNA from Kiwi blood (Ponui) and pooled DNA from kiwi blood (Puketi and Cape Brett, PCB). DNA from individuals was pooled at roughly equimolar proportions and the total concentration determined using a QUBIT 2.0 (Invitrogen, Life Technologies). DNA quality (260/280 and 260/230 ratios) were read on a Nanodrop 1000 (Thermo Scientific).

Three libraries were prepared using Illumina NextEra XT library preparation and these were run on an Illumina MiSeq™ 2X 150 base paired-end Micro run using version 2 chemistry at the Massey Genome Centre, Palmerston North.

## 7.3 Results

### 7.3 1 Smear based detection

We found a few slides infected with intra-erythrocytic haemoparasites (Figure 7.5) that looked like *Plasmodium* spp. (Schoener pers. coms). However, we could not confirm the species of protozoa we were looking at based on slide data alone.

### 7.3.2 Tick salivary gland staining

The MGP staining in protocol 1 was very weak and did not stain the gland dark enough for us to see endoparasites even after increasing staining time (Figure 7.6). We obtained more stained cells with protocol 2 and more contrast between cells with nuclei that were dark enough to see. However, the salivary glands showed no visible infection with *Babesia* spp. or *Theileria* spp. as described by Irvin et al., (1981).

### 7.2.3 Tick histology

Protocol 3, where we injected FAA into the tick body produced the best results for histology sectioning. The shattering was minimum, and we were able to get whole sections of the tick (Figures 7.2). To find the abnormal structures in tick histology, we had to first define what was normal under our staining method (Figure 7.7), which was a difficult task due to the sparse literature present on invertebrate histology and the lack

of uniformity in previous studies. However, we extrapolated from other arthropod studies and were also able to identify the different parts of the tick histology (Figure 7.7). Figure 7.8 shows possible microorganisms within the tissues of the gut and salivary glands of the kiwi tick.

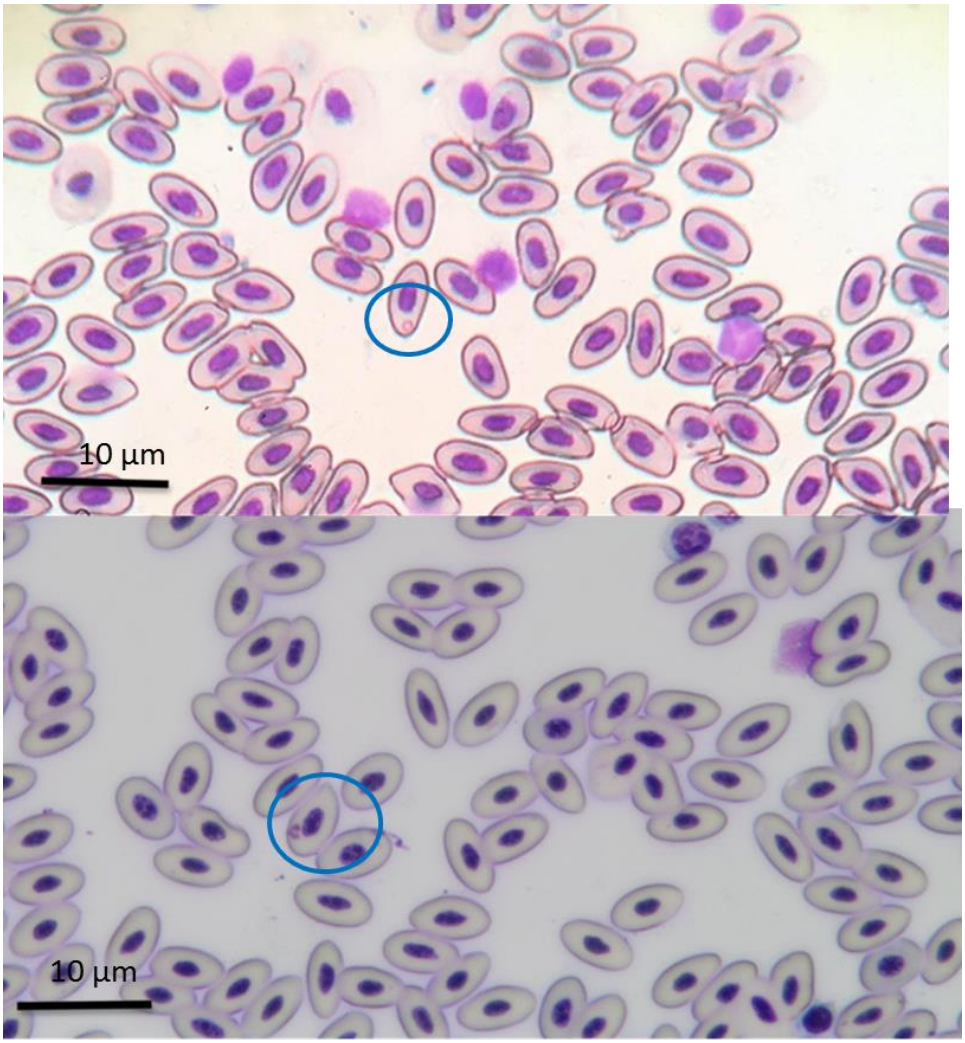



Figure 7.5: Examples of inclusion bodies that could represent haemoparasites, seen in the RBC of NIBK (within blue circles). Stain used was the Giemsa stain.

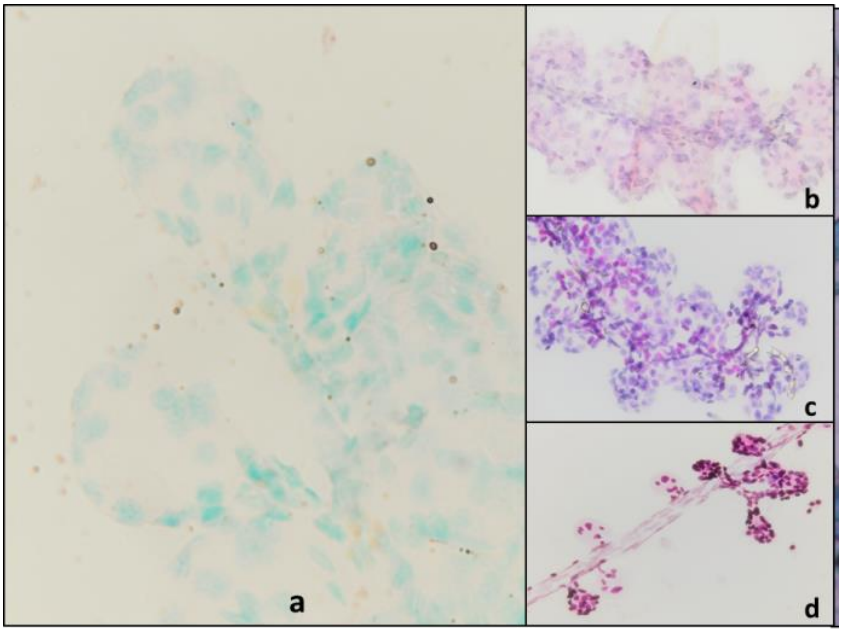



Figure 7.6: Salivary gland staining where (a) used MGP stain and protocol 1 and, (b), (c) and (d) used MGP stain with protocol 2 (each slide has stained at a different intensity due to unknown reasons). Photos were taken at 60X oil imersion. No evidence of haemoprotozoa was seen in our slides.

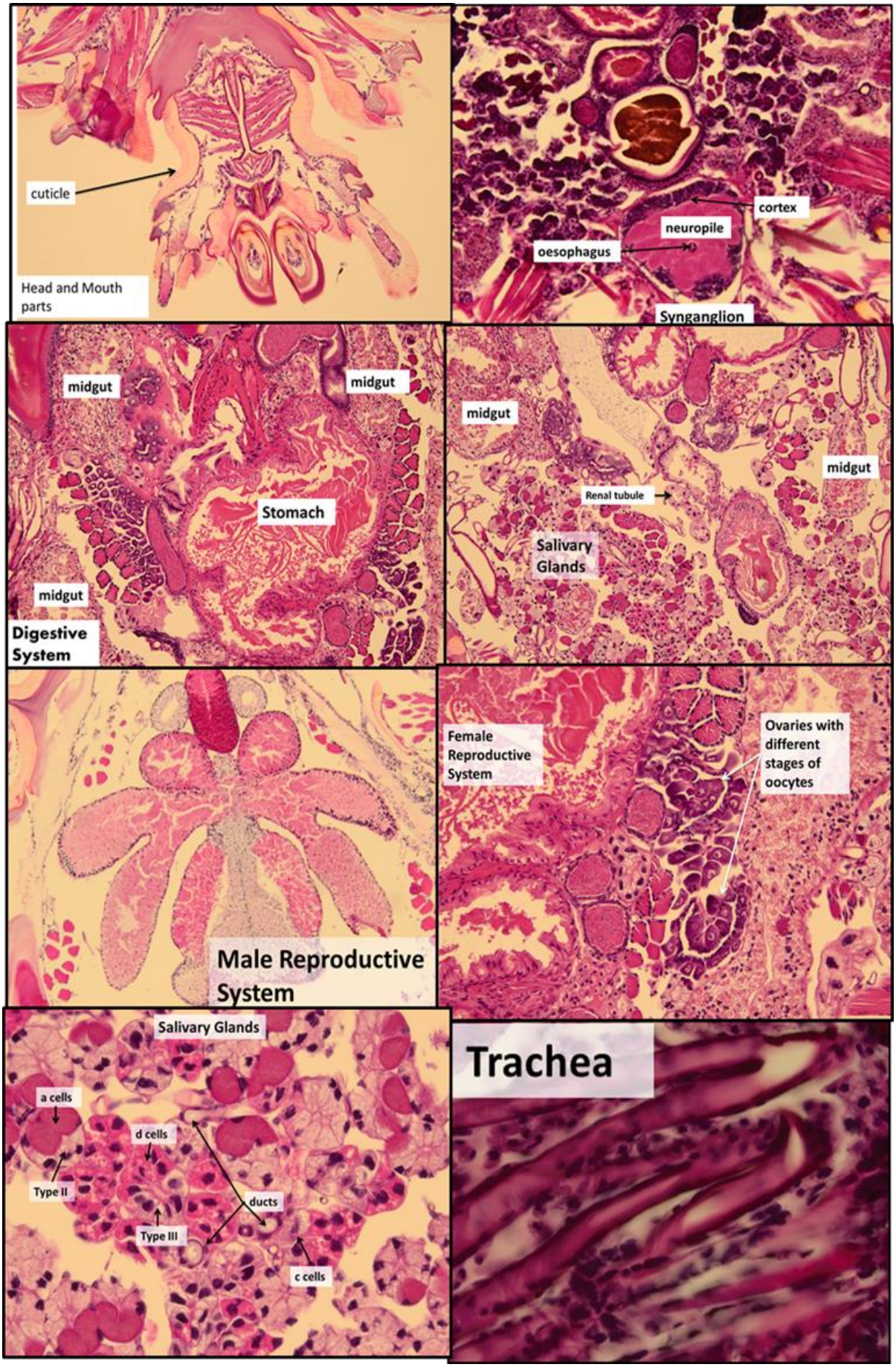



Figure 7.7: Normal histology sections of different parts of tick *Ixodes anatis*.

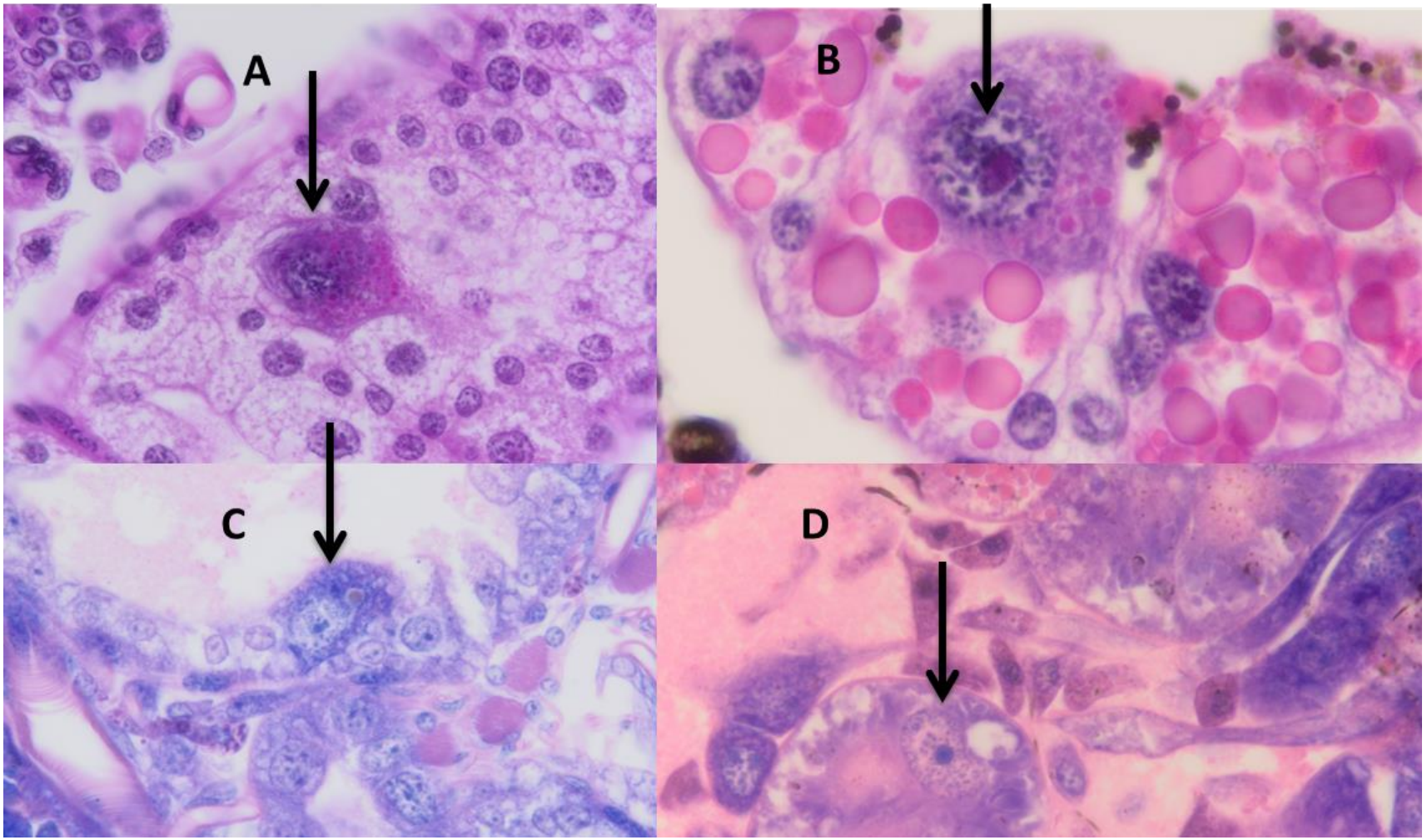



Figure 7.8: This figure shows possible infections in histology sections of the kiwi tick (based on extrapolated data from the literature available). A & B are stained with H&E stain and C & D are stained with Giemsa. A& C are in the gut lining of the tick and B & D are in the salivary glands.

### 7.3.4 Fluorescence in situ hybridization (FISH)

We tested only a few samples with FISH. The first sample gave us general immune fluorescence and we had to then adjust for the natural inflorescence produced by haemoglobin present in blood cells (Figure 7.9). After attempting to correct for that, we still got inconclusive results with no positive infection detected.

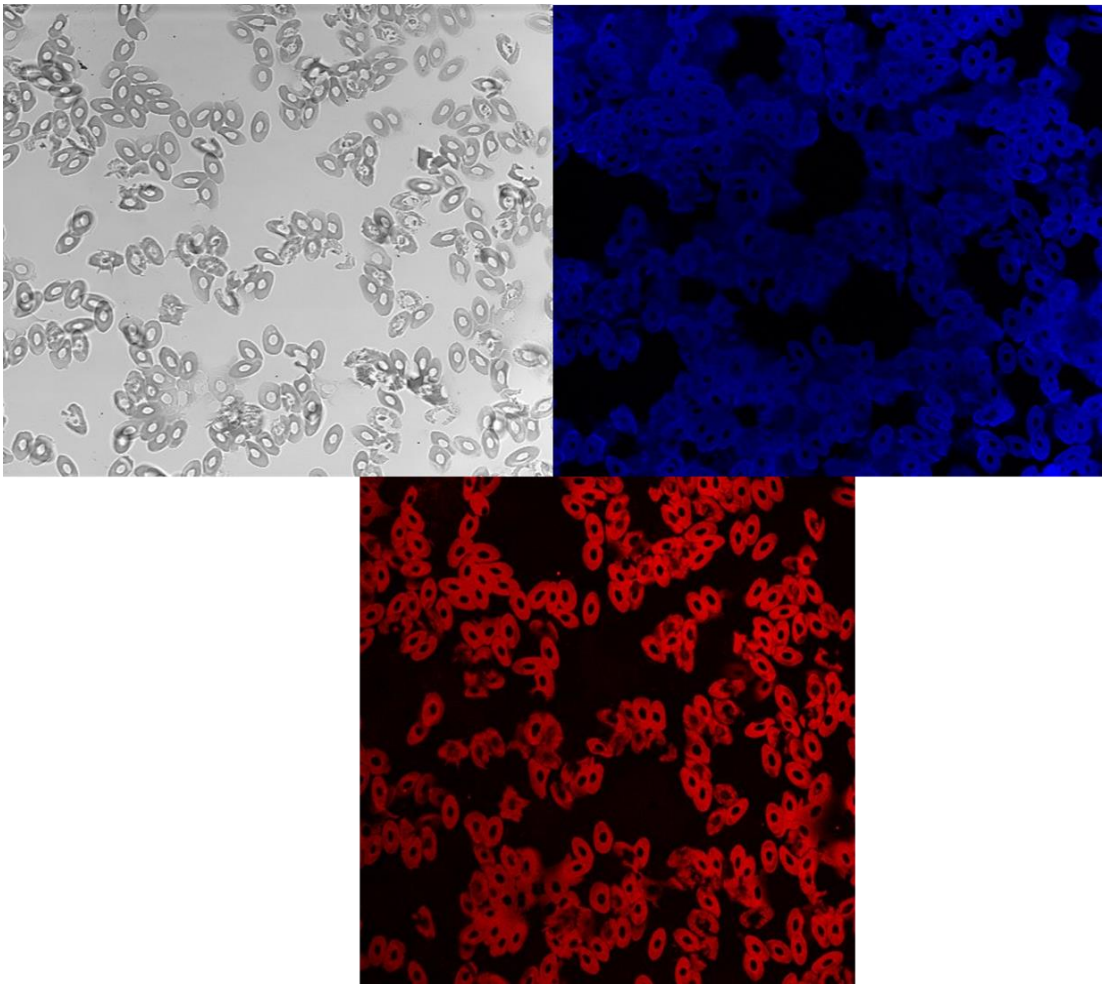


Figure 7.9: FISH of the slides produced using a scanning confocal microscope which showed generalised fluorescence. Cyanine 3 (CY3) labelled fluorescence probe should have hybridised with the *Babesia* to produces red colour, however, in the bottom photo, all blood cells are producing red coloured fluorescence. The blue image was taken when the slide was counter stained with DAPI to stain nuclear material

### 7.3.5 DNA based techniques

1. The Jefferies primers- 18S

After a first round of PCR, only the positive controls produced a band (Figure 7.10). The Jefferies primers and PCR conditions amplified both the *Babesia* and the *Theileria* positive controls (as expected). The spiked DNA sample (*Babesia* spiked into kiwi blood) produced an amplification product, indicating that the kiwi blood was not inhibiting the PCR. After a second round of PCR there were faint bands in both the tick samples and all of the blood mixes. The fragments were larger than the products in the positive controls. The sequences from the tick DNA samples 1 and 2 (Figure 7.4) matched tick *Ixodes uriae* 18S (99%). The sequences from the largest fragments from DNA samples 3 to 6 (Figure 7.4) matched kiwi 18S (100%). The 3 positive controls (Figure 7.4) matched *B. gibsonii* (99%) and *T. orientalis* (99%) sequences from GenBank as expected. The apicomplexan *B. kiwiensis* was not detected.

2. Mitochondrial primers

No products were detected in the first round for the positive controls (Figure 7.10). There were two bands present in the lane containing Mix 4. The bands at about 1kb in the samples Mix 3 and 4 were cut out of the gel and purified. However, the sequencing was unsuccessful. The positive controls Bg+ and Th+ matched *B. gibsonii* (99.78%) and *Theileria orientalis* (99.55) mitochondrial sequences on GenBank (www.ncbi.nlm.nih.gov), as expected.

3. Apicoplast primers 23S

Only the positive *Theileria* control produced a fragment in the first round of amplification (Figure 7.10). All lanes except for the negative control showed a band of approximately the same size (Figure 7.10). These products were prepared for sequencing enzymatically as outline above.

The sequence of the 23S region of the apicoplast of *B. gibsonii* was not available on GenBank and the closest match was to *B. orientalis.* Most other fragments were derived from plants as indicated in the Table 7.5. These fragments may have amplified from the template DNA, from contaminating DNA from the environment or the reagents. These 23S primers were too non-specific to be useful for further studies.

*Table 7.5: The results of the sequencing of positives seen using apicoplast primers.*

| | |
|---|---|
| $Tick_{eng}$ | Hespercyparis sp. 99% (white cedar) |
| $Tick_{uneng}$ | Zoysia sp 99% and other species of grass |
| Mix 1 | Lejeunea cavifolia 99% (pouncewort) |
| Mix 2 | Pyracantha fortuneana 99% (firethorn) |
| Mix 3 | Pinus sp 99% (pine) |
| Mix 4 | mixed poor sequence |
| Bg+ | Babesia orientalis 93% |
| Th+ | Theileria orientalis 100% |
| $Bg+_{Blood}$ | Babesia orientalis 93% |

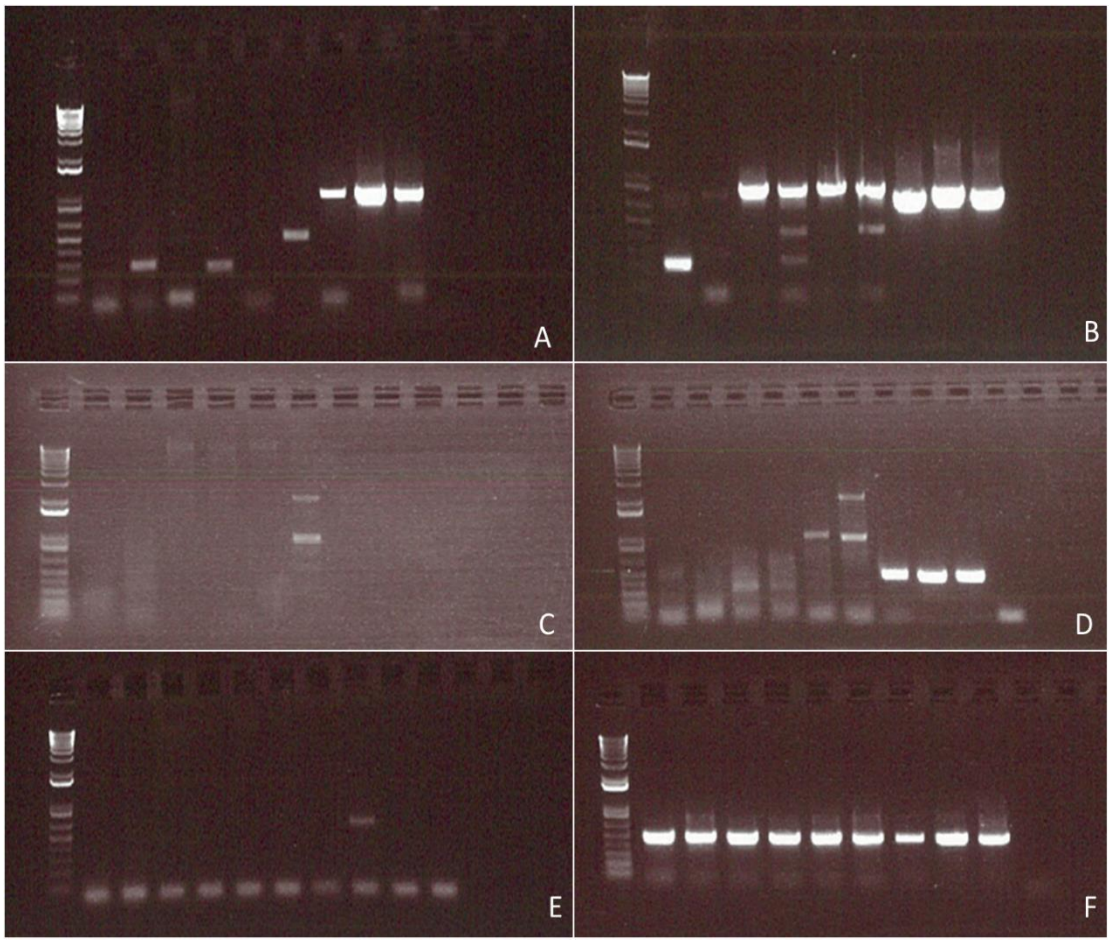



Figure 7.10: The gel results for the three primers. A & B show amplification products obtained with the first and second round of the Jefferies primers respectively. C & D show results from the first and second round of the mitochondrial primers respectively. E & F show results from the first and second round of the apicoplast primers respectively. The order for all figures in the lanes is as follows: Lane 1)- 1kb+Ladder (Invitrogen), Lanes 2) $Tick_{eng}$ 3) $Tick_{uneng}$ 4) Mix 1 5) Mix 2 6) Mix 3 7) Mix 4 8) Bg+ 9) Th+ 10) $Bg+_{in\ blood}$ 11) neg.

*Sensitivity determination*

There was some nonspecific amplification at 80fg and 8fg (Figure 7.11). The limit of detection for the 18S marker using a 58°C annealing temperature was 8pg (between 800fg-8pg) and this limit was not extended by another round of PCR at 60°C (or at 58°C, results not shown). The limit of detection for the mt marker was 800fg (80fg-800fg) for 45 cycles of PCR using a 58°C annealing temperature. This limit increased to 80fg (8fg-80fg) with another 45 cycles at 60°C. The limit for detection for the 23S marker was 800fg (80-800fg) for 90 cycles of PCR using annealing temperatures of 58°C in the first round and 60°C in the second round.

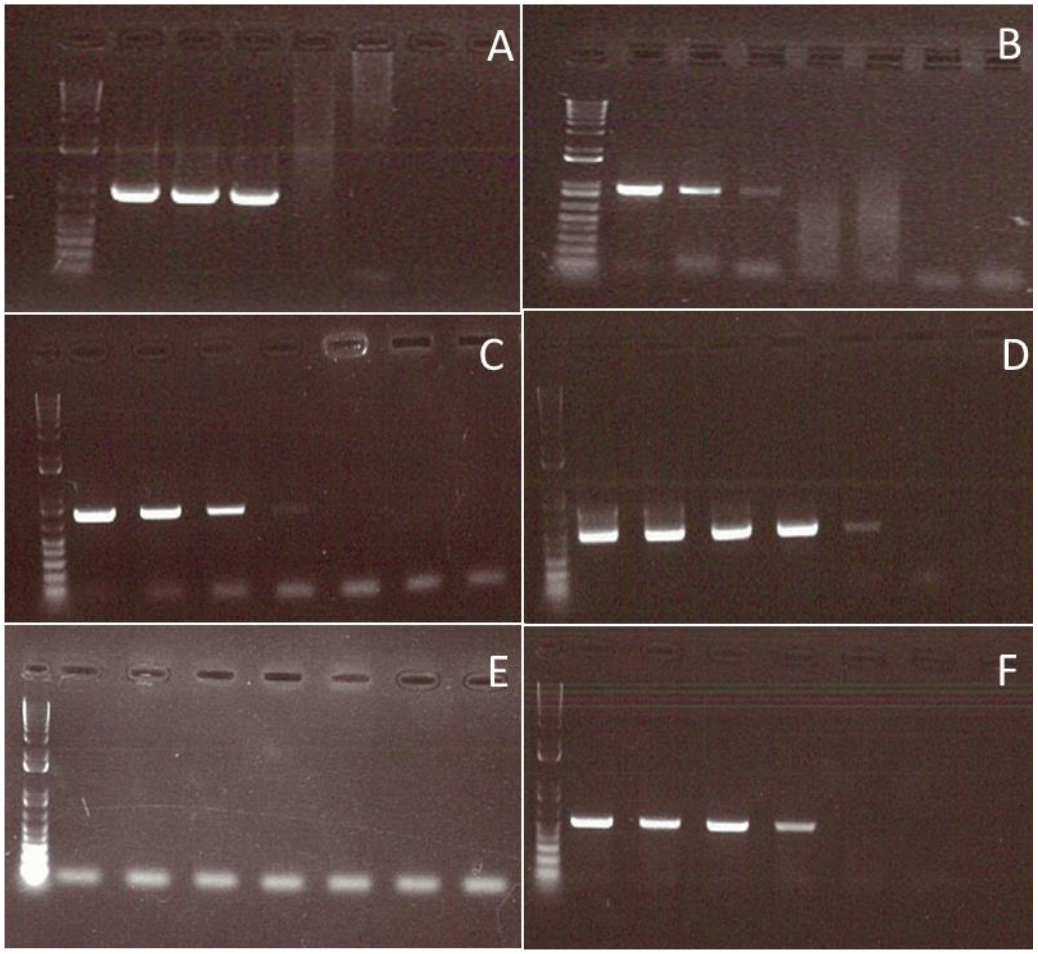



Figure7.11: A & B show PCR amplification products obtained with the first and second rounds of the Jefferies primers respectively. C & D show results obtained with the first and second rounds of the mitochondrial primers respectively. E & F show results obtained with the first and second rounds of the apicoplast primers respectively.

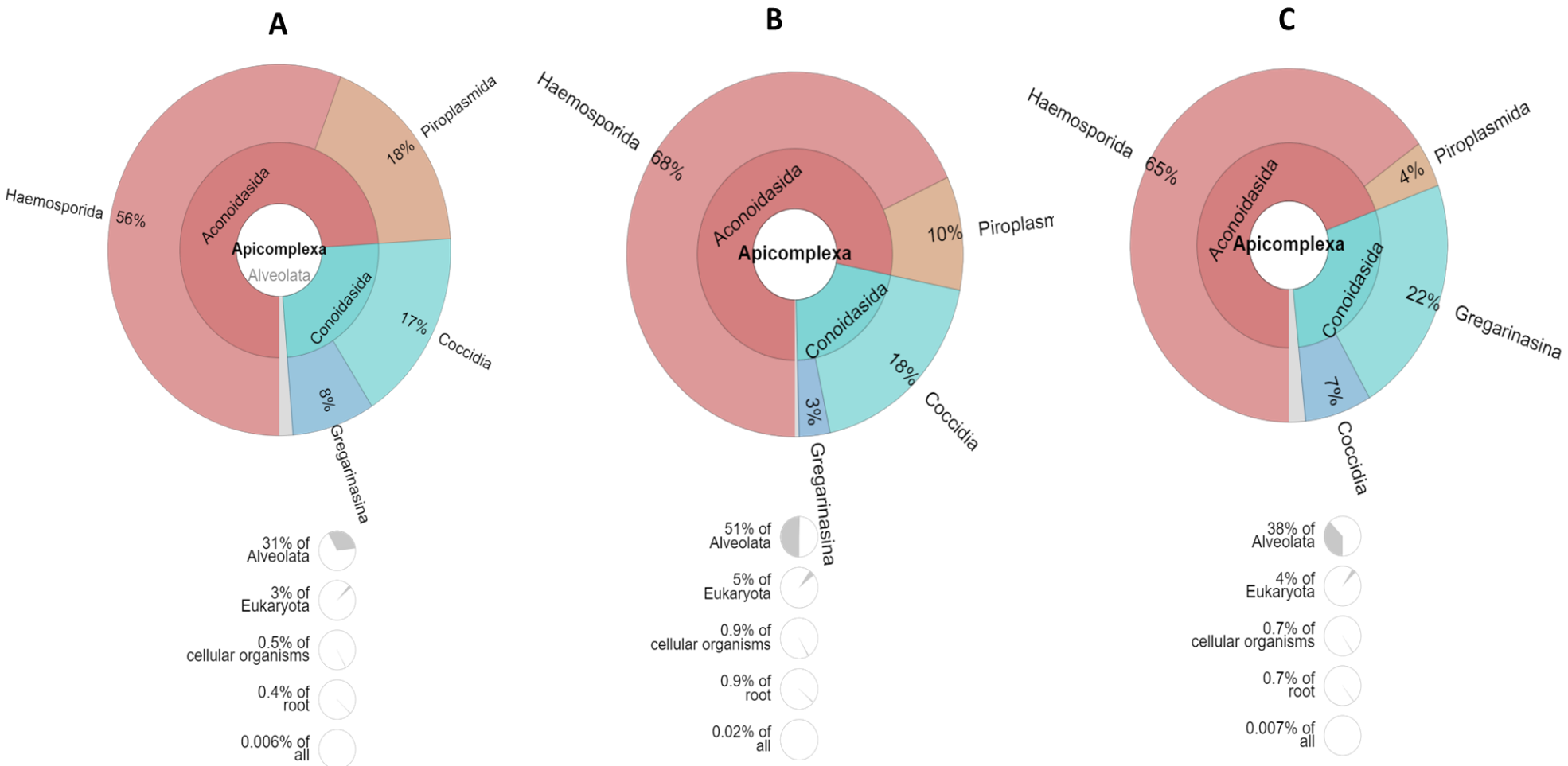



Figure 7.12: The Metagenomic classification of Apicomplexa where A) was from PCB, B) from Ponui kiwi and C) was from kiwi ticks. Ponui kiwi have a higher percentage of Apicomplexa than PCB or the ticks. However, C from the ticks shows a higher concentration of Gregarinasina, which is a parasite that inhabits invertebrates (like ticks). Even so, the total percentages of all these are still very small (between 0.5-0.9% of Eukaryotes).

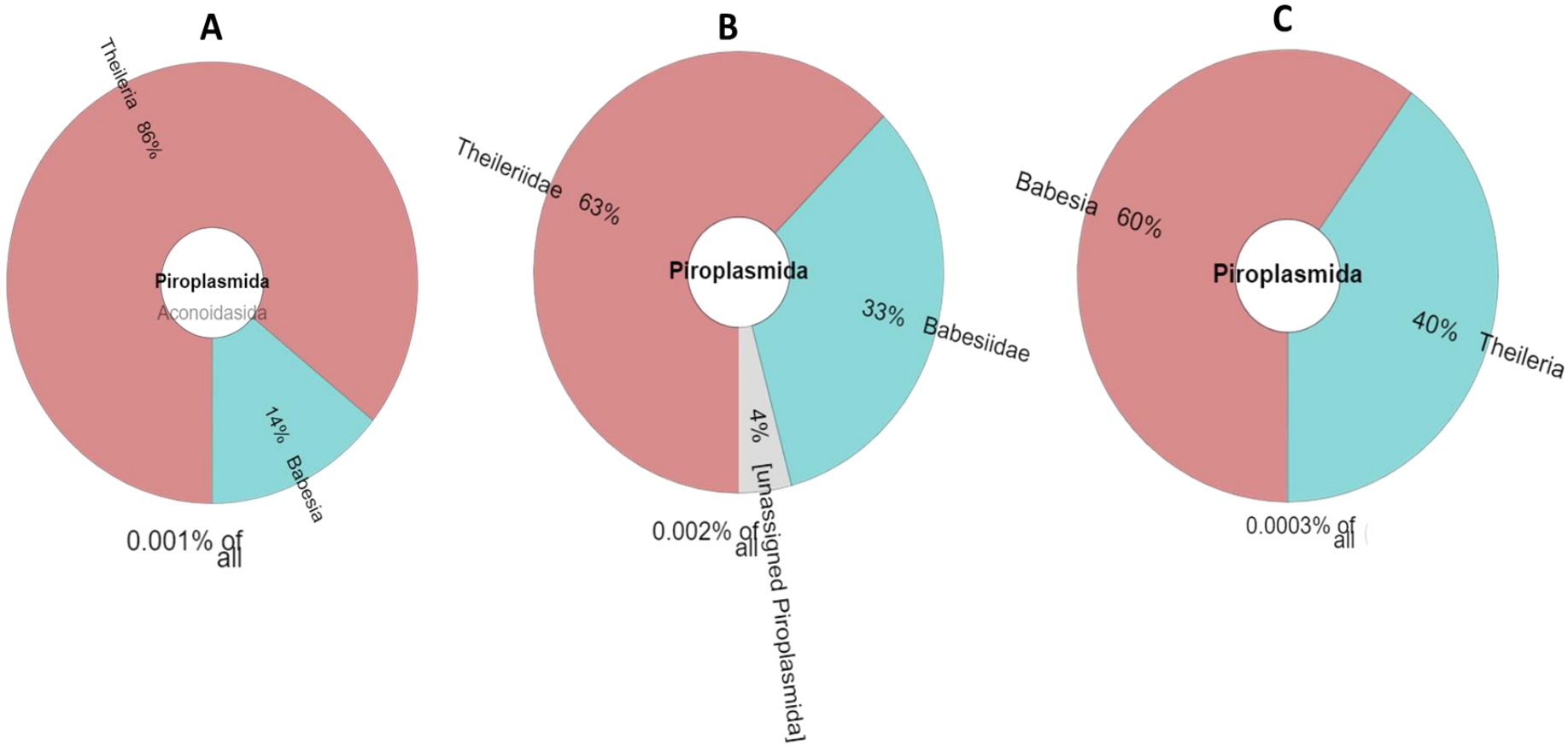



Figure 7.13: The Metagenomic classification of Piroplasmids which include *Babesia* spp and *Theileria* spp. from A) the PCB, B) Ponui kiwi and C) kiwi ticks (engorged and unengorged mixed) visualised using Krona charts. The piroplasmids make up a very small percentage of all the cellular organisms.

*Next Gen Sequencing (NGS)*

When we analysed the metagenomic results we found that the birds and ticks had similar abundance of the different metagenomic taxa (Appendix 7.2, 7.3 and 7.4). The taxa Apicomplexa made up only ~5% of Eukaryotes. In all three groups, Haemosporida (containing *Plasmodium* spp.) was highly abundant and relatively confident classification compared to other taxa within Apicomplexans (Figure 7.12). PCB had somewhat similar levels of Piroplasmida and Eimeriorina which was higher than both Ponui birds and the ticks. The ticks on the other hand showed a higher amount of *Gregarina* spp., which is a parasite that inhabits the intestine of invertebrates (Figure 7.12). There also remained some non-specific DNA which could not be matched to existing database. The Piroplasmids such as *Babesia* and *Theileria* in all groups has similar concentrations and was present in miniscule amounts (only 7% of Apicomplexans, Figure 7.13).

## 7.4 Discussion

While we did see some inclusion bodies in the smears of the birds as well as some indication of infection, these could not be conclusively identified as *B. kiwiensis, Hepatozoon* spp. or *Theileria* spp. None of the other tests produced any conclusive proof to the presence of *B. kiwiensis, Hepatozoon* spp. or *Theileria* spp. This led us to move onto DNA based molecular techniques and yet again we were unsuccessful at finding any haematozoa in our samples. As a result, we tried to measure the sensitivity of our primers and found that our mitochondrial primers were more sensitive than the Jefferies primers picking up infections as low as 80fg/µl. Despite this increased sensitivity, we were not able to detect any positive apicomplexans. When we used metagenomic sequencing, we found that there was a presence of Apicomplexan DNA within our samples, but the reads were very low. Furthermore, there was a higher abundance of matches to *Plasmodium* spp., than the Piroplasmids, from the samples leading us to speculate that the haemoparasites we saw in our smears may belong to species of *Plasmodium* spp. There was also some non-specific DNA that did not match to anything in the database which could be due to degradation of the DNA. Unfortunately, metagenomics sequencing is only as good as the data base available for it to match

sequences against and the small number of reads of Apicomplexans cannot positively indicate their presence.

While a very low prevalence of protozoa or a previous infection with said protozoa could explain our results, we believe that the chance that our ticks or our kiwi contained any of the tick-borne protozoa that we were looking for is very low. The few blood smears that contained haemoparasite-like structures most strongly resembled meronts of *Plasmodium*, which we did not confirm using PCR. However, while the Illumina sequencing results at such low levels cannot confirm the presence of *Plasmodium* spp., requires more detailed examination.

*B. kiwiensis* was first reported by Pierce et al. (2003) from NIBK chicks that had been brought into captivity soon after their birth from the Whangarei region. This report was then confirmed by Jefferies et al. (2008) from juvenile NIBK caught in the same region. Apart from these two instances, no clinical cases have ever been reported for *Babesia or Theileria* leading us to believe that these could also be emerging infections seen in NIBK when under stress and in captivity as is seen in the case of *Plasmodium* (Banda et al., 2013).

Thus, in conclusion, the negative PCR results and overwhelming match to kiwi DNA, do not support the findings of Jefferies et al. (2008) who identified *B. kiwiensis* in 12 of 13 wild caught juvenile birds and one tick obtained from the Whangarei region. Future studies therefore need to sample from the same population that Jefferies et al. (2008) sampled to look at whether there has been a loss in parasite abundance and diversity, and this is a cost we are paying for captive breeding and conserving endangered NIBK.

# Chapter 8: Syn*thesis*

Author with Clea, the kiwi

"That's it on the maps; nature doesn't acknowledge frontiers. Neither can ecology… Where to begin to understand what we've only got a computer-speak label for, ecosystem? Where to decide it begins…" - Nadine Gordimer

# Chapter 8: Syn*thesis*

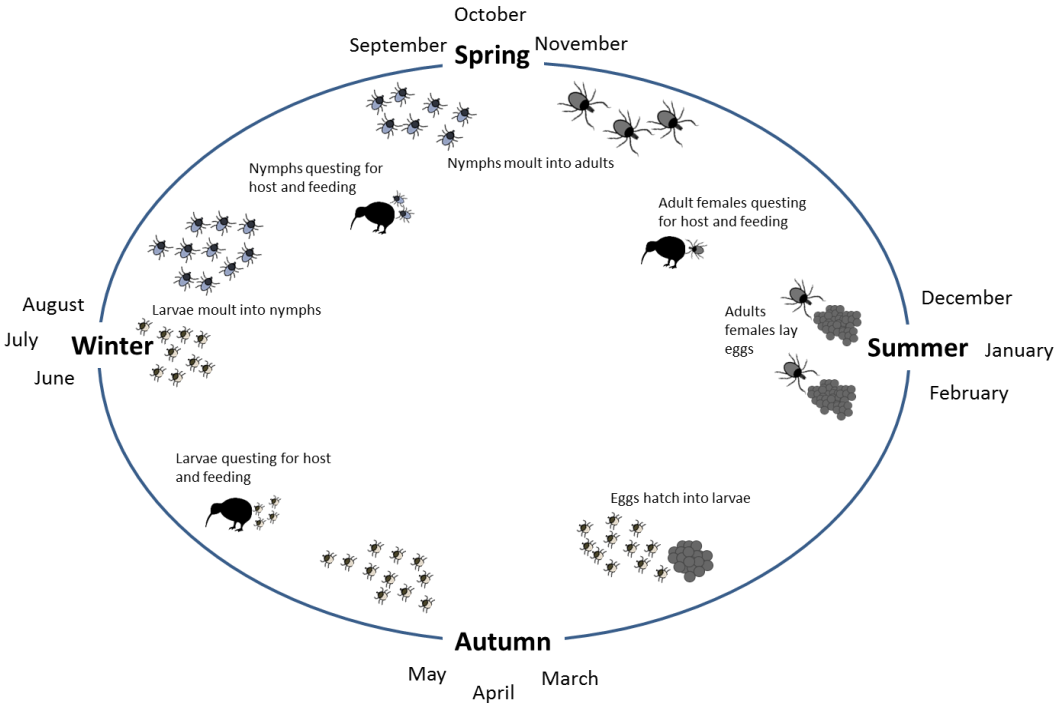
This thesis actually began when we saw ~2000gms birds on Ponui Island with massive loads of more than 200 ticks along with other parasites like lice, mites and fleas, and these birds seemed to be foraging, mating and reproducing year after year with no obvious detrimental health effects. We aimed to understand more about this host-parasite relationship and the effect these species-specific parasites had on the birds and it quickly became clear that this was not a straightforward question. To study any host-parasite system, it is imperative to know about the ecology of both host and parasite and very little was known about the kiwi tick*, Ixodes anatis,* before this study. Thus, we embarked on a journey which resulted in this thesis. We employed a combination of laboratory techniques as well as field experiments to achieve our aims.

## 8.1 *Ixodes anatis* life cycle, ecology and patterns of abundance

Chapter 2 and chapter 3 helped us unravel the life cycle and abundance patterns of *I. anatis.* To learn more about the ecology of the parasite, we tested the abundance and seasonal pattern of *I. anatis* in kiwi burrows over a year (Chapter 3) and found that the ticks were present throughout the year with larvae most abundant from January to June and nymphs most abundant from June to December. We also found that that tree and soil burrows in the forest had higher tick levels than scrub and pasture burrows. To ascertain whether this difference in the tick abundance was associated with microclimates in the burrows as well as to find the ideal microclimate requirements of *I. anatis* ticks, we conducted life cycle studies (Chapter 2). We found that *I. anatis* seemed to do better in lower temperatures, which makes sense as New Zealand is located in a temperate climate zone and would explain the high numbers of ticks that we found during the colder winter and autumn months. Ideally to determine a life cycle for the kiwi tick, we would require studying abundance patterns over a period of two-three years. However, the combination of our two chapters (2 and 3) allowed us to propose a life cycle pattern for *I. anatis* (Figure 8.1) in NIBK. Though this life cycle pattern will differ in other places where the kiwi ticks are found such as Stewart Island or Fiordland National Park, which are located on the lower South Island of NZ and are

colder with some months of snowfall. Being nidicolous would provide the ticks with stable environments as kiwi burrows have relatively constant microclimates (Chapter 2; de Vieco, 2019). However, microclimate may be different in the burrows of the Southern kiwi species for reasons beyond external environmental conditions. Kiwi species in the South Island have a more cooperative breeding system with several birds of different ages sharing burrows. Anecdotally (I. Castro pers. comm.) burrows in Stewart island are much larger than those used by brown kiwi and the nesting materials used by Rakiura Tokoeka (*Apteryx australis*) are different to those used by brown kiwi. Therefore, more work should be done to establish any potential differences in microhabitat for the ticks related to kiwi spp. burrow characteristics and the species natural histories. We were not able to get eggs of the ticks to hatch and future studies could look more closely at the climatic requirement for hatching of *I. anatis* eggs. Another interesting topic for future research could be the genetic variation of *I. anatis* in different climatic regions and on kiwi hosts throughout the country and how this may affect their abundance and ecology.

Figure 8.1: Proposed season-based life cycle of the one host tick, *I. anatis*, in brown kiwi. This figure shows the dominant stages during the various seasons found in shelters on Ponui Island, New Zealand.



## 8.2 Establishing health parameters of *Apteryx mantelli*

After discerning more about the ecology of the ticks, the next step before we could look at the effect of *I. anatis* on their North Island Brown Kiwi (NIBK) host, was to establish a method to be able to quantify these ticks in different populations as well as establish what normal health parameters for NIBK are. We addressed these questions in Chapter 4 and 5. Chapter 4 helped us streamline a method to estimate parasite loads from birds, in an ethical way that would reduce stress and handling time. Using Bayesian modelling, we found that the there was a strong correlation between the number of ticks in the photos and the real number of ticks on the birds, however only when a bird had more than 20 ticks. For less than 20 ticks on the bird, photos were not a reliable method to estimate infestation, and it would probably be easier to count these ticks individually directly on the birds. We also calculated a multiplying factor that can be used for estimating tick loads in kiwi.

In Chapter 5, we examined common haematological and biochemical (henceforth together blood) parameters (Table 5.1) between different populations of NIBK to try and define what is normal. We examined and attempted to describe the sources of variation in the blood parameters, which was apparent between some populations (Chapter 5). However, more research needs to go into ascertain why there is variation between the populations. This might include looking at kiwi physiology, age, sex, food availability and type as well as genetics of the different birds.

While there was a previously published manual for normal reference values of NIBK, we found that it lacked information and had no evidence on the source population and sample sizes used to determine these values. Therefore, as a result of this work (Chapter 5), we are able to provide an improved table (Table 5.3) of reference blood parameters for future researchers as well as veterinary practitioners for wild NIBK based on various different populations and a good sample size.

## 8.3 Host-parasite relationship: effect of *I. anatis* as parasites and vectors on *A. mantelli*

After Chapters 2 to 5 we were finally ready to investigate the effect of chronic infestations of kiwi tick on their hosts. We have done this in Chapter 6, by conducting an

experiment to remove/reduce the tick loads in a set of treatment birds and comparing their blood parameters as well as activity patterns with an untreated control group of NIBK. We found that the total plasma protein of the treatment birds was higher than the control birds and the treated birds gained more weight while reducing their activity by one hour per day. However, how this one extra hour of activity undertaken by the control birds is spent requires further study. When the same group of birds were re-examined the following year, the infestations levels of the birds in the treatment and control groups was similar (calculated using results of Chapter 4) and the blood parameters of the treatment group was within the normal range of blood parameters proposed in Chapter 5. This shows that the ticks do have a negative influence on NIBK and the increased TPP, weight gain and one extra hour of activity that the birds save per day is one less trade-off and can go into increasing the reproductive output or even the life expectancy of the birds. However, for the level of parasitism seen on the birds, these effects were not as pronounced as we had assumed and we wonder if this is due to adaptation, their long standing co-evolution history with the ticks or another factor that we did not measure for. This requires further investigation, maybe also using the information we have gained to add to a model for host parasite interaction between NIBK and the ticks. Furthermore, kiwi have other endemic ectoparasites and it would be very interesting to look at interactions between these various ectoparasites and how they relate to their kiwi host.

In previous studies, Pierce et al., (2003) found that *I. anatis* was a vector for two haemoprotozoa, *Babesia kiwiensis* and *Hepatozoon kiwi,* first described from NIBK blood smears. In another investigation conducted but not published, these ticks were also suspected to carry *Theileria* spp. (I Castro pers. Comm.). In Chapter 7, we wanted to look at the prevalence and intensity of these haemoprotozoa and how it changed in the birds treated (Chapter 6) and in the different populations described in Chapter 5. We utilised a range of different methods, from dissecting the ticks to histology and *in-situ* hybridisation to describe the life cycle, prevalence and intensity of these haemoprotozoa in the ticks. However, after trying and perfecting a number of different methods including PCR techniques, we did not get any conclusive results. This result was surprising, particularly as a previous study (Jeffries et al., 2008) had confirmed *B. kiwiensis* in blood samples from a population of NIBK close to where our study birds in

Chapters 5 and 6 originated. As *I. anatis* is kiwi specific and is suspected of transmitting *Babesia kiwiensis*, ideally, we expected to be able to detect at least some *Babesia* spp. in our tested birds or ticks as both the parasite and vector would have evolved with the host, kiwi. However, as kiwi have undergone multiple bottle necks over many generations due to habitat loss and fragmentation, it is safe to say that their ticks may have suffered the same fate including loss of genetic diversity, especially with current conservation practices like translocations.

For future research, it would be helpful to include birds from the population that Jefferies et al., (2008) sampled and investigate how the parasites and their vector interact. Unfortunately, the lack of corroborating information and expertise on tick histology, smear staining and assessing protocols as well as data interpretation was one of our biggest challenges in Chapter 7. We hope that the standard operating procedures that were collected and added to the thesis can be of value in future studies that look into haemoparasites detection.

## 8.4 An incorporation of parasite conservation into ecosystem management

Parasites come in incredibly diverse shapes and forms, (Poulin, 2010) and recent studies show that they make a large part of the earth biomass (Kuris et al., 2008). Several studies show that they provide fundamental services in the ecosystem and play key roles in food web stability (for eg. Dunne et al., 2013; Lafferty et al., 2006; 2008, Hudson et al., 2006; Hatcher, Dick and Dunn, 2012). Parasites may play the same role in ecosystems as that of predators (Raffel et al., 2008; Strona, 2015) and although their roles are not as obvious as those of predators relationships, they can influence trophic interactions in the community (Combes, 1999). Parasites evolve more rapidly than their hosts, and have developed strategies to maximise their fitness using arms race processes. This higher speed in accumulating genetic changes can also be used as a tool to study host population history and structure (Dougherty et al., 2016; Gompper & Williams, 1998; Pizzi, 2009; Whiteman & Parker, 2005).

Increasingly, parasitologists are rightfully calling for the conservation of parasites (Dougherty et al., 2016; Gompper & Williams, 1998). The plight for saving parasites

was first put forward by Windsor in 1990 but was largely ignored for 20 years (Strona, 2015).

Now conservationists are going as far as to suggest establishing parasite habitats to conserve them (for eg: Stringer and Linklater, 2014). Since parasites, like insects and invertebrates, are difficult to monitor, loss of entire species often goes undetected (Strona, 2015). As we are believed to be in the sixth mass extinction process (Wake and Vrendenburg, 2008), now more than ever, parasites are facing higher extinction rates due to factors such as climate change and loss of habitat in the form of environment and host, on a scale much larger and different than any pressures that helped shape various host-parasite systems throughout millions of ears of co-evolution (Strona, 2015). There are numerous examples in the literature that show that parasites are a major factor that shapes the evolution of species by providing the much-needed selection pressures for 'survival of the fittest' (Pizzi, 2009; Wood & Johnson, 2015). The ability of the parasite to cull weak individuals, by either making them more susceptible to predation (eg. Johnson et al., 1999; Lafferty and Morris, 1996) or other diseases (Dunn et al., 2009) may help to keep host densities in check thus promoting more resource availability and species co-existence (Hatcher et al., 2006). For example, two lizard species in the Caribbean, *Anolis gingivinus* and *Anolis wattsi*, that are found together only when *A. gingivinus* is heavily parasitized by malarial parasite *Plasmodium azurophilum,* even though both are hosts for the parasite and can be parasitized simultaneously (Schall, 1992). These abilities of the parasite to affect community and populations represent a delicate balance in the functionality of the ecosystem, to which disturbances caused by, say, human interference may lead to unprecedented effects (Strona, 2015).

One of the main reasons conserving parasites is not very popular is the fear that they may threaten the existence of endangered hosts. Additionally, parasites have the potential to become zoonotic and pose a risk to human health. Lastly, parasites are not as charismatic to people as mega-fauna and public perception of parasites tend to be negative (Durden & Keirans, 1996; Gómez, Nichols, & Perkins, 2012; Wood & Johnson, 2015). This concept of prioritising conservation based on human perceptions needs a rethink. When we talk of conserving ecosystems, we need to think about the parasites

as well as the host, because the loss of parasites may affect food web stability. For instance, a study by Lafferty et al., (2006) showed that parasites were involved in 78% of connectivity in a salt marsh-based food web and a disruption in this connectance would then affect the stability of this web.

In New Zealand, kiwi are endemic and endangered hence, by association, so is the kiwi tick. Most strategies for conservation of kiwi focus on predator control, captive rearing and release, and unfortunately today many populations of NIBK around NZ, especially those established through in-situ chick raising and release, seem to be free of ectoparasites that are dependent on kiwi for survival (for example our test Population from Motu, Gisborne that was established in a new area previously devoid of kiwi using captive reared and hatched kiwi). There is still hope for kiwi parasites though, as current strategies for kiwi translocation do not explicitly treat kiwi for ectoparasites and therefore at least some parasites get translocated with the birds (McInnes pers. comm.). However, this is not enough for parasites with high level of endemism to survive and can cause future problems like bottle necking parasite populations and reduced genetic diversity. While working with parasites with inadequate knowledge of their life histories and ecology can have unforeseen consequences, I hope that this thesis sheds enough light on the ecology of the kiwi tick and their effect on NIBK to be able to model and reconsider the intrinsic value of parasites and advocate for their conservation. I propose that host-parasite interaction of the kiwi tick and brown kiwi be treated as a unit of conservation, with translocation of kiwi specific ectoparasites as part of the conservation strategy, preservation of which will hopefully contribute to effectively stronger host conservation. Thus, advocating for a framework that includes host and its parasites as a species assemblage and sets the ground rules for future conservation projects.

## 8.5 Concluding Remarks

So, while parasites may negatively affect their host, they are important for the smooth functioning of an ecosystem and for co-evolutionary processes of their host species. Parasite conservation around the world is on the rise; as a result, conservation

management plans in New Zealand need to integrate parasite management to protect our biodiversity. Only through multi-disciplinary approaches like those undertaken in this study can we begin to understand more about the complex host- parasite dynamic processes that are necessary to successfully preserve native species decline and restore balance to our ecosystems.

# References


Aderemi, F. A. (2004). Effects of replacement of wheat bran with cassava root sieviate supplemented or unsupplemented with enzyme on the haematology and serum biochemistry of pullet chicks. *Tropical Journal of Animal Science*, *7*(1), 147-153.

Altay, K., Dumanli, N., & Aktas, M. (2012). A study on ovine tick-borne hemoprotozoan parasites (Theileria and Babesia) in the East Black Sea Region of Turkey. *Parasitology research, 111*(1), 149-153.

Anderson, S. J., & Death, R. G. (2000). The effect of forest type on forest floor invertebrate community structure.

Anwar, K. (2003). Cypermethrin, a pyrethroid insecticide induces teratological and biochemical changes in young chick embryos. *Pak J Biol Sci, 6*, 1698-1705.

Apanaskevich, D. A., & Oliver Jr, J. H. (2014). Life cycles and natural history of ticks. *Biology of ticks*, *1*, 59-73.

Arthur, D. R. (1965). Ticks of the genus Ixodes in Africa. *Ticks of the Genus Ixodes in Africa.*

Arthur, D. R., & Snow, K. R. (1968). Ixodes pacificus Cooley and Kohls, 1943: its life-history and occurrence. *Parasitology, 58*(4), 893-906.

Asghar, M., Hasselquist, D., & Bensch, S. (2011). Are chronic avian haemosporidian infections costly in wild birds? *Journal of Avian biology, 42*(6), 530-537.

Aslam, F., Khan, A., Khan, M. Z., Sharaf, S., Gul, S. T., & Saleemi, M. K. (2010). Toxico-pathological changes induced by cypermethrin in broiler chicks: Their attenuation with Vitamin E and selenium. *Experimental and Toxicologic pathology, 62*(4), 441-450.

Atkinson, C. T., & Van Riper III, C. (1991). Vectors, epizootiology, and pathogenicity of avian species of Haemoproteus (Haemosporina: Haemoproteidae). *Bulletin of the Society for Vector Ecology*, *16*, 109-126.

Aydin, M. F., Aktas, M., & Dumanli, N. (2015). Molecular identification of Theileria and Babesia in ticks collected from sheep and goats in the Black Sea region of Turkey. *Parasitology research, 114*(1), 65-69.

Banda, M. E., Howe, L., Gartrell, B. D., McInnes, K., Hunter, S., & French, N. P. (2013). A cluster of avian malaria cases in a kiwi management programme. *New Zealand veterinary journal*, *61*(3), 121-126.

Baneth, G., Samish, M., & Shkap, V. (2007). Life cycle of Hepatozoon canis (Apicomplexa: Adeleorina: Hepatozoidae) in the tick Rhipicephalus sanguineus and domestic dog (Canis familiaris). *Journal of Parasitology, 93*(2), 283-299.

Baneth, G., & Weigler, B. (1997). Retrospective case-control study of hepatozoonosis in dogs in Israel. *Journal of Veterinary Internal Medicine, 11*(6), 365-370.

Bansal, N., Castro, I., Acebes, D. I., Pomroy, W. E., & Kerkhoven, A. (2019). Factors affecting abundance of different stages of the endophilic tick Ixodes anatis in brown kiwi (Apteryx mantelli) shelters. *Ticks and Tick-borne Diseases*.

Barbedo, J. G. A., Gomes, C. C. G., Cardoso, F. F., Domingues, R., Ramos, J. V., & McManus, C. M. (2017). The use of infrared images to detect ticks in cattle and proposal of an algorithm for quantifying the infestation. *Veterinary parasitology*, *235*, 106-112.

Barker, S. C., & Burger, T. D. (2018). Two new genera of hard ticks, Robertsicus n. gen. and Archaeocroton n. gen., and the solution to the mystery of Hoogstraal's and

Kaufman's" primitive" tick from the Carpathian Mountains. *Zootaxa*, *4500*(4), 543-552.

Barker, S., & Murrell, A. (2004). Systematics and evolution of ticks with a list of valid genus and species names. *Parasitology, 129*(S1), S15-S36.

Basaria, S., & Dobs, A. S. (2003). Androgens and the hematopoietic system. In *Androgens in Health and Disease* (pp. 233-242). Humana Press, Totowa, NJ.

Beati, L., & Klompen, H. (2019). Phylogeography of ticks (Acari: Ixodida). *Annual review of entomology, 64*, 379-397.

Behjati, S., & Tarpey, P. S. (2013). What is next generation sequencing? *Archives of Disease in Childhood-Education and Practice, 98*(6), 236-238.

Berggren, Å. (2005). Effect of the blood-sucking mite Ornithonyssus bursa on chick growth and fledging age in the North Island robin. *New Zealand Journal of Ecology*, 243-250.

Berrong, S. L., & Washburn, K. W. (1998). Effects of genetic variation on total plasma protein, body weight gains, and body temperature responses to heat stress. *Poultry Science*, *77*(3), 379-385.

Bosholn, M., Anciães, M., Gil, D., Weckstein, J. D., Dispoto, J. H., & Fecchio, A. Individual variation in feather corticosterone levels and its influence on haemosporidian infection in a Neotropical bird. *Ibis*.

Bosch, M., & Figuerola, J. (1999). Detrimental effects of ticks Ornithodoros maritimus on the growth of yellow-legged gull Larus michahellis chicks.

Beugnet, F., & Marié, J. L. (2009). Emerging arthropod-borne diseases of companion animals in Europe. *Veterinary parasitology*, *163*(4), 298-305.

Biggs, J. R., & Zoo, C. T. (2013). Captive Management Guidelines for the Southern Cassowary. *On-line pdf). Accessed October 9*, 2013.

Braun, E. J., & Sweazea, K. L. (2008). Glucose regulation in birds. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 151*(1), 1-9.

Brown, C. R., Brown, M. B., & Rannala, B. (1995). Ectoparasites reduce long-term survival of their avian host. *Proceedings of the Royal Society of London B: Biological Sciences, 262*(1365), 313-319.

Buller, S. W. L., & Keulemans, J. G. (1888). *A history of the birds of New Zealand* (Vol. 2): Selbstverl.

Buttemer, W. A., & Astheimer, L. B. (2000). Testosterone does not affect basal metabolic rate or blood parasite load in captive male white-plumed honeyeaters Lichenostomus penicillatus. *Journal of Avian Biology*, *31*(4), 479-488.

Buttler, E. I., Gilchrist, H. G., Descamps, S., Forbes, M. R., & Soos, C. (2011). Handling stress of female common eiders during avian cholera outbreaks. *The Journal of Wildlife Management*, *75*(2), 283-288.

Calder III, W. A., & Dawson, T. J. (1978). Resting metabolic rates of ratite birds: the kiwis and the emu. *Comparative Biochemistry and Physiology Part A: Physiology*, *60*(4), 479-481.

Calder, W. A., Parr, C., & Karl, D. (1978). Energy content of eggs of the brown kiwi Apteryx australis; an extreme in avian evolution. *Comparative Biochemistry and Physiology Part A: Physiology, 60*(2), 177-179.

Campbell, T. W., & Ellis, C. K. (2007). Hematology of birds. *Avian and Exotic animal hematology and cytology*, 3-50.

Campbell, A., & Glines, M. (1979). Development, survival, and oviposition of the rabbit tick, Haemaphysalis leporispalustris (Packard)(Acari: Ixodidae), at constant temperatures. *The Journal of parasitology*, 777-781.

Campbell, T. W. (2004). Mammalian hematology: laboratory animals and miscellaneous species. *Veterinary hematology and clinical chemistry (1st ed). Lippincott Williams & Wilkins, Philadelphia, USA*, 211-224.

Carleton, R. E. (2008). Ectoparasites affect hemoglobin and percentages of immature erythrocytes but not hematocrit in nestling Eastern Bluebirds. *The Wilson Journal of Ornithology*, *120*(3), 565-569.

Castro, I. (2006). North Island brown kiwi, Apteryx mantelli, on Ponui Island, Hauraki Gulf: Preliminary reports from two studies. *Kokako, 13*(2), 27-30.

Charrel, R. N., Attoui, H., Butenko, A., Clegg, J., Deubel, V., Frolova, T., . . . Labuda, M. (2004). Tick-borne virus diseases of human interest in Europe. *Clinical microbiology and infection, 10*(12), 1040-1055.

Chauvin, A., Moreau, E., Bonnet, S., Plantard, O., & Malandrin, L. (2009). Babesia and its hosts: adaptation to long-lasting interactions as a way to achieve efficient transmission. *Veterinary research, 40*(2), 1-18.

Chilton, N. B. (1992). An index to assess the reproductive fitness of female ticks. *International journal for parasitology, 22*(1), 109-111.

Chilton, N. B., & Bull, C. M. (1993). A comparison of the off-host survival times of larvae and nymphs of two species of reptile ticks. *International journal for parasitology, 23*(5), 693-696.

Cilek, J., & Olson, M. (2000). Seasonal distribution and abundance of ticks (Acari: Ixodidae) in northwestern Florida. *Journal of medical entomology, 37*(3), 439-444.

Clayton, D. H., & Cotgreave, P. (1994). Comparative analysis of time spent grooming by birds in relation to parasite load. *Behaviour*, *131*(3-4), 171-187.

Clayton, D. H., & Drown, D. M. (2001). Critical evaluation of five methods for quantifying chewing lice (Insecta: Phthiraptera). *Journal of Parasitology, 87*(6), 1291-1301.

Clayton, D. H., & Tompkins, D. M. (1994). Ectoparasite virulence is linked to mode of transmission. *Proceedings of the Royal Society of London. Series B: Biological Sciences, 256*(1347), 211-217.

Colbourne, R. (2002). Incubation behaviour and egg physiology of kiwi (Apteryx spp.) in natural habitats. *New Zealand Journal of Ecology*, 129-138.

Colbourne, R., Bassett, S., Billing, T., McCormick, H., McLennan, J., Nelson, A., & Robertson, H. (2005). The development of Operation Nest Egg as a tool in the conservation management of kiwi. *Science for conservation, 259*, 24.

Colwell, D. D., Dantas-Torres, F., & Otranto, D. (2011). Vector-borne parasitic zoonoses: emerging scenarios and new perspectives. *Veterinary parasitology*, *182*(1), 14-21.

Combes, C. (1996). Parasites, biodiversity and ecosystem stability. *Biodiversity & Conservation*, *5*(8), 953-962.

Conway, D. P., Sasai, K., Gaafar, S. M., & Smothers, C. D. (1993). Effects of different levels of oocyst inocula of Eimeria acervulina, E. tenella, and E. maxima on plasma constituents, packed cell volume, lesion scores, and performance in chickens. *Avian Diseases*, 118-123.

Cortivo, P. D., Dias, E., Barcellos, J. O. J., Peripolli, V., Costa Jr, J. B. G., Dallago, B. S. L., & McManus, C. M. (2016). Use of thermographic images to detect external parasite load in cattle. *Computers and Electronics in Agriculture*, *127*, 413-417.

Costa, N. D., McDonald, D. E., & Swan, R. A. (1993). Age-related changes in plasma biochemical values of farmed emus (Dromaius novaehollandiae). *Australian veterinary journal*, *70*(9), 341-344.

Craig, E., Gardiner, C., Renwick, N., & Sporle, W. (2011). Taxon plan for Northland brown kiwi (Apteryx mantelli).

Cray, C., Rodriguez, M., & Arheart, K. L. (2008). Use of refractometry for determination of psittacine plasma protein concentration. *Veterinary clinical pathology*, *37*(4), 438-442.

Cox, F. E. (2010). History of the discovery of the malaria parasites and their vectors. *Parasites & vectors, 3*(1), 5.

Cunningham, A. A. (1996). Disease risks of wildlife translocations. *Conservation biology*, *10*(2), 349-353.

Cunningham, S., & Castro, I. (2011). The secret life of wild brown kiwi: studying behaviour of a cryptic species by direct observation. *New Zealand Journal of Ecology*, 209-219.

Cunningham, S., Castro, I., & Alley, M. (2007). A new prey-detection mechanism for kiwi (Apteryx spp.) suggests convergent evolution between paleognathous and neognathous birds. *Journal of Anatomy, 211*(4), 493-502.

Dantas-Torres, F., & Otranto, D. (2013). Seasonal dynamics of Ixodes ricinus on ground level and higher vegetation in a preserved wooded area in southern Europe. *Veterinary parasitology, 192*(1), 253-258.

Daszak, P., & Cunningham, A. (1999). Extinction by infection. *Trends in Ecology & Evolution, 14*(7), 279.

Davis, A. K., Maney, D. L., & Maerz, J. C. (2008). The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. *Functional Ecology*, *22*(5), 760-772.

Dawson, R. D., & Bortolotti, G. R. (1997). Total plasma protein level as an indicator of condition in wild American kestrels (Falco sparverius). *Canadian Journal of Zoology, 75*(5), 680-686.

De la Fuente, J., Villar, M., Cabezas-Cruz, A., Estrada-Peña, A., Ayllón, N., & Alberdi, P. (2016). Tick–Host–Pathogen Interactions: Conflict and Cooperation. *PLoS pathogens, 12*(4), e1005488.

De Lope, F., Møller, A., & De la Cruz, C. (1998). Parasitism, immune response and reproductive success in the house martin Delichonurbica. *Oecologia, 114*(2), 188-193.

Demma, L. J., Traeger, M. S., Nicholson, W. L., Paddock, C. D., Blau, D. M., Eremeeva, M. E., . . . Zaki, S. R. (2005). Rocky Mountain spotted fever from an unexpected tick vector in Arizona. *New England Journal of Medicine, 353*(6), 587-594.

deVieco, D, (2019). *The egg and the nest: obtaining information about the reproductive biology of* Apteryx *spp. (Family: Apterygidae), a criptyc avian taxon through eggshells.* (Unpublished doctoral dissertation). Massey University, Palmerston North, New Zealand.

Dickerson, G. E. (1978). Animal size and efficiency: basic concepts. *Animal Science*, *27*(3), 367-379.

Dixon, T. (2015). *What they do in the shadows: habitat utilisation and diet of brown kiwi (*Apteryx mantelli*) adults within a high-density island population: a thesis submitted in partial fulfilment of the requirements for the degree of Master of Science in Ecology, Massey University, Palmerston North, New Zealand.* Massey University.

Doneley, R. (2006). Management of captive ratites. In L. T. Harrison GJL (Ed.), *Clinical avian medicine* (pp. 957-990). Florida: Spix Publishing Inc.

Dougherty, E. R., Carlson, C. J., Bueno, V. M., Burgio, K. R., Cizauskas, C. A., Clements, C. F., . . . Harris, N. C. (2016). Paradigms for parasite conservation. *Conservation Biology, 30*(4), 724-733.

Duffy, D. C. (1983). The ecology of tick parasitism on densely nesting Peruvian seabirds. *Ecology*, 110-119.

Dufva, R., & Allander, K. (1995). Intraspecific variation in plumage coloration reflects immune response in great tit (Parus major) males. *Functional Ecology*, 785-789.

Dumbleton, L. J. (1953). *The ticks (Ixodoidea) of the New Zealand sub-region*: Department of Scientific and Industrial Research.

Dunne, J. A., Lafferty, K. D., Dobson, A. P., Hechinger, R. F., Kuris, A. M., Martinez, N. D., ... & Stouffer, D. B. (2013). Parasites affect food web structure primarily through increased diversity and complexity. *PLoS Biol*, *11*(6), e1001579.

Dunn, R. R., Harris, N. C., Colwell, R. K., Koh, L. P., & Sodhi, N. S. (2009). The sixth mass coextinction: are most endangered species parasites and mutualists? *Proceedings of the Royal Society B: Biological Sciences*, *276*(1670), 3037-3045Durden, L. A., & Keirans, J. E. (1996). Host–parasite coextinction and the plight of tick conservation. *American Entomologist, 42*(2), 87-91.

Duscher, G., Peschke, R., & Tichy, A. (2012). Mechanical tools for the removal of Ixodes ricinus female ticks—differences of instruments and pulling or twisting? *Parasitology research, 111*(4), 1505-1511.

Earlé, R., Huchzermeyer, F., Bennett, G., & Brassy, J. (1993). Babesia peircei sp. nov. from the jackass penguin. *African Zoology, 28*(2), 88-90.

Elgert, K. D. (1996). *Immunology: understanding the immune system*: Wiley-Liss.

Elliot, S. L., Adler, F. R., & Sabelis, M. W. (2003). How virulent should a parasite be to its vector? *Ecology, 84*(10), 2568-2574.

England, J. M., Rowan, R. M., Van Assendelft, O. W., Bull, B. S., Coulter, W. H., Fujimoto, K., & Shinton, N. K. (1994). Guidelines for the evaluation of blood cell analysers including those used for differential leucocyte and reticulocyte counting and cell marker applications. *Clinical & Laboratory Haematology*, *16*(2), 157-174.

Estrada-Peña, A. n. (2001). Forecasting habitat suitability for ticks and prevention of tick-borne diseases. *Veterinary parasitology, 98*(1-3), 111-132.

Ewald, P. W. (1993). *Evolution of infectious disease*: Oxford University Press.

Fair, J., Whitaker, S., & Pearson, B. (2007). Sources of variation in haematocrit in birds. *Ibis, 149*(3), 535-552.

Faran, M. E., Turell, M. J., Romoser, W. S., Routier, R. G., Gibbs, P. H., Cannon, T. L., & Bailey, C. L. (1987). Reduced survival of adult Culex pipiens infected with Rift Valley fever virus. *The American journal of tropical medicine and hygiene, 37*(2), 403-409.

Ferguson, H. M., & Read, A. F. (2002). Why is the effect of malaria parasites on mosquito survival still unresolved? *Trends in parasitology, 18*(6), 256-261.

Fitze, P. S., Tschirren, B., & Richner, H. (2004). Life history and fitness consequences of ectoparasites. *Journal of Animal Ecology, 73*(2), 216-226.

Foitzik, S., Fischer, B., & Heinze, J. (2003). Arms races between social parasites and their hosts: geographic patterns of manipulation and resistance. *Behavioral Ecology, 14*(1), 80-88.

Fourie, L., Snyman, A., Kok, D., Horak, I., & Van Zyl, J. (1993). The appetence behaviour of two South African paralysis-inducing ixodid ticks. *Experimental & applied acarology, 17*(12), 921-930.

Freed, L. A., & Cann, R. L. (2003). On polymerase chain reaction tests for estimating prevalence of malaria in birds. *Journal of Parasitology*, *89*(6), 1261-1265.

Frenot, Y., De Oliveira, E., Gauthier-Clerc, M., Deunff, J., Bellido, A., & Vernon, P. (2001). Life cycle of the tick Ixodes uriae in penguin colonies: relationships with host breeding activity. *International journal for parasitology, 31*(10), 1040-1047.

Friedrichs, K. R., Harr, K. E., Freeman, K. P., Szladovits, B., Walton, R. M., Barnhart, K. F., & Blanco-Chavez, J. (2012). ASVCP reference interval guidelines: determination of de novo reference intervals in veterinary species and other related topics. *Veterinary clinical pathology*, *41*(4), 441-453.

Gaede, K., & Knülle, W. (1997). On the mechanism of water vapour sorption from unsaturated atmospheres by ticks. *Journal of Experimental Biology*, *200*(10), 1491-1498.

Gage, K. L., Burkot, T. R., Eisen, R. J., & Hayes, E. B. (2008). Climate and vectorborne diseases. *American journal of preventive medicine, 35*(5), 436-450.

Gallo, S. S. M., Ederli, N. B., Bôa-Morte, M. O., & Oliveira, F. C. R. (2015). Hematological, morphological and morphometric characteristics of blood cells from rhea, Rhea Americana (Struthioniformes: Rheidae): a standard for Brazilian birds. *Brazilian Journal of Biology*.

Garcia-Longoria, L., Garamszegi, L. Z., & Møller, A. P. (2014). Host escape behavior and blood parasite infections in birds. *Behavioral ecology*, *25*(4), 890-900.

Gauthier-Clerc, M., Mangin, S., Le Bohec, C., Gendner, J.-P., & Le Maho, Y. (2003). Comparison of behaviour, body mass, haematocrit level, site fidelity and survival between infested and non-infested king penguin Aptenodytes patagonicus by ticks Ixodes uriae. *Polar Biology, 26*(6), 379-382.

Gelman, A., Stern, H. S., Carlin, J. B., Dunson, D. B., Vehtari, A., & Rubin, D. B. (2013). *Bayesian data analysis*: Chapman and Hall/CRC.

Germano, J., Barlow, S., Castro, I., Colbourne, R., Cox, M., Gillies, C., ... & Robertson, H. (2018). Kiwi Recovery Plan 2018–2028 Mahere Whakaora Kiwi 2018–2028.

Ginsberg, H. S., Albert, M., Acevedo, L., Dyer, M. C., Arsnoe, I. M., Tsao, J. I., . . . LeBrun, R. A. (2017). Environmental factors affecting survival of immature Ixodes scapularis and implications for geographical distribution of Lyme disease: the climate/behavior hypothesis. *PloS one, 12*(1), e0168723.

Goethert, H. K., & Telford III, S. R. (2009). Nonrandom distribution of vector ticks (Dermacentor variabilis) infected by Francisella tularensis. *PLoS pathogens*, *5*(2), e1000319.

Gómez, A., Nichols, E. S., & Perkins, S. L. (2012). *Parasite conservation, conservation medicine, and ecosystem health*: Oxford Univ. Press.

Gompper, M. E., & Williams, E. S. (1998). Parasite conservation and the black-footed ferret recovery program: JSTOR.

Gothe, R., & Neitz, A. W. (1991). Tick paralyses: pathogenesis and etiology *Advances in disease vector research* (pp. 177-204): Springer.

Graczyk, T. K., Shaw, M. L., Cranfield, M. R., & Beall, F. B. (1994). Hematologic characteristics of avian malaria cases in African black-footed penguins (Spheniscus demersus) during the first outdoor exposure season. *The Journal of parasitology*, 302-308.

Gray, J. S., Estrada-Peña, Agustin, & Vial, Laurence (2014). Ecology of nidicolous ticks. *Biology of ticks*, *2*, 40-60.

Guglielmone, A. A., Robbins, R. G., Apanaskevich, D. A., Petney, T. N., Estrada-Peña, A., Horak, I. G., . . . Barker, S. C. (2010). The Argasidae, Ixodidae and Nuttalliellidae (Acari: Ixodida) of the world: a list of valid species names. *Zootaxa, 2528*(6), 1-28.

Gustafsson, L., Nordling, D., Andersson, M., Sheldon, B., & Qvarnström, A. (1994). Infectious diseases, reproductive effort and the cost of reproduction in birds. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, 346*(1317), 323-331.

Haas, G. E. (1966). A technique for estimating the total number of rodent fleas in cane fields in Hawaii. *Journal of medical entomology, 2*(4), 392-394.

Hall, A. R., Scanlan, P. D., Morgan, A. D., & Buckling, A. (2011). Host–parasite coevolutionary arms races give way to fluctuating selection. *Ecology letters, 14*(7), 635-642.

Hamilton, W. D. (1980). Sex versus non-sex versus parasite. *Oikos*, 282-290.

Hamilton, W. D., Axelrod, R., & Tanese, R. (1990). Sexual reproduction as an adaptation to resist parasites (a review). *Proceedings of the National Academy of Sciences, 87*(9), 3566-3573.

Hamilton, W. D., & Zuk, M. (1982). Heritable true fitness and bright birds: a role for parasites? *Science, 218*(4570), 384-387.

Harvell, C. D., Mitchell, C. E., Ward, J. R., Altizer, S., Dobson, A. P., Ostfeld, R. S., & Samuel, M. D. (2002). Climate warming and disease risks for terrestrial and marine biota. *Science, 296*(5576), 2158-2162.

Hatcher, M. J., Dick, J. T., & Dunn, A. M. (2006). How parasites affect interactions between competitors and predators. *Ecology Letters*, *9*(11), 1253-1271.

Heath, A. (1979). The temperature and humidity preferences of Haemaphysalis longicornis, Ixodes holocyclus and Rhipicephalus sanguineus (Ixodidae): studies on eggs. *International journal for parasitology, 9*(1), 33-39.

Heath, A. (1981). The temperature and humidity preferences of Haemaphysalis longicornis, Ixodes holocyclus and Rhipicephalus sanguineus (Ixodidae): studies on engorged larvae. *International journal for parasitology, 11*(2), 169-175.

Heath, A. (1987). A review of the origins and zoogeography of tick-borne disease in New Zealand. *Tuatara, 29*(1&2), 19-29.

Heath, A. (2010). A review of ectoparasites of Apteryx spp.(kiwi) in New Zealand, with new host records, and the biology of Ixodes anatis (Acari: Ixodidae). *Tuhinga, 21*, 147-159.

Heath, A. C. (1975). An investigation into the temperature and humidity preferenda of ixodid ticks: and their distribution in relation to bioclimatic zones in Australia.

Heath, A. C. (2012). A new species of soft tick (Ixodoidea: Argasidae) from the New Zealand lesser short-tailed bat, Mystacina tuberculata Gray. *Tuhinga, 23*, 29-37.

Heath, J. A., & Dufty, Jr, A. M. (1998). Body condition and the adrenal stress response in captive American kestrel juveniles. *Physiological Zoology*, *71*(1), 67-73.

Heath, A. C., & Kwak, M. L. (2019). Ensuring the nomenclatural stability of Ixodes anatis Chilton, 1904 with the discovery of lost type material and the designation of a lectotype. *New Zealand Entomologist*, *42*(1), 21-22.

Heath, A. C., & Palma, R. L. (2017). A new species of tick (Acari: Ixodidae) from seabirds in New Zealand and Australia, previously misidentified as Ixodes eudyptidis. *Zootaxa, 4324*(2), 285-314.

Heath, A. C., Palma, R. L., Cane, R. P., & Hardwick, S. (2011). Checklist of New Zealand ticks (Acari: Ixodidae, Argasidae). *Zootaxa, 2995*(1), 55-63.

Heeb, P., Kölliker, M., & Richner, H. (2000). Bird-ectoparasite interactions, nest humidity, and ectoparasite community structure. *Ecology, 81*(4), 958-968.

Heidelberger, P., & Welch, P. D. (1983). Simulation run length control in the presence of an initial transient. *Operations Research, 31*(6), 1109-1144.

Hellgren, O., Waldenström, J., & Bensch, S. (2004). A new PCR assay for simultaneous studies of Leucocytozoon, Plasmodium, and Haemoproteus from avian blood. *Journal of Parasitology*, *90*(4), 797-803.

Heylen, D., & Matthysen, E. (2008). Effect of tick parasitism on the health status of a passerine bird. *Functional Ecology, 22*(6), 1099-1107.

Heylen, D. J., Madder, M., & Matthysen, E. (2010). Lack of resistance against the tick Ixodes ricinus in two related passerine bird species. *Int J Parasitol, 40*(2), 183-191. doi:10.1016/j.ijpara.2009.07.011

Heylen, D. J., & Matthysen, E. (2011). Differential virulence in two congeneric ticks infesting songbird nestlings. *Parasitology, 138*(8), 1011-1021.

Heyman, P., Cochez, C., Hofhuis, A., Van Der Giessen, J., Sprong, H., Porter, S. R., . . . Niedrig, M. (2010). A clear and present danger: tick-borne diseases in Europe.

Hillyard, P. D. (1996). *Ticks of north-west Europe*. Field Studies Council.

Hoch, T., Monnet, Y., & Agoulon, A. (2010). Influence of host migration between woodland and pasture on the population dynamics of the tick Ixodes ricinus: a modelling approach. *Ecological Modelling, 221*(15), 1798-1806.

Hoogstraal, H., & Aeschlimann, A. (1982). Tick-host specificity. *Bulletin de la société entomologique suisse*, *55*, 5-32.

Holmes, J. (1983). Evolutionary relationships between parasitic helminths and their hosts.

Holzapfel, S., Robertson, H. A., McLennan, J. A., Sporle, W., Hackwell, K., & Impey, M. (2008). Kiwi (Apteryx spp.) recovery plan. *Threatened species recovery plan, 60*.

Horwitz, P., & Wilcox, B. A. (2005). Parasites, ecosystems and sustainability: an ecological and complex systems perspective. *International journal for parasitology, 35*(7), 725-732.

Hotez, P. J., & Kamath, A. (2009). Neglected tropical diseases in sub-Saharan Africa: review of their prevalence, distribution, and disease burden. *PLoS neglected tropical diseases, 3*(8), e412.

Howe, L., Castro, I., Schoener, E., Hunter, S., Baraclough, R., Alley, M. 2011. Malaria parasites (*Plasmodium* spp.) infecting introduced, native and endemic New Zealand birds. Parasitology Research. 110(2): 913–923. 10.1007/S00436-011-2577-Z.

Hudson, P. J., Dobson, A. P., & Newborn, D. (1992). Do parasites make prey vulnerable to predation? Red grouse and parasites. *Journal of Animal Ecology*, 681-692.

Hudson, P. J., Dobson, A. P., & Lafferty, K. D. (2006). Is a healthy ecosystem one that is rich in parasites?. *Trends in ecology & evolution*, *21*(7), 381-385.

Hummel, T. J., & Sligo, J. R. (1971). Empirical comparison of univariate and multivariate analysis of variance procedures. *Psychological Bulletin*, *76*(1), 49.

Hunfeld, K.-P., & Brade, V. (2004). Zoonotic Babesia: possibly emerging pathogens to be considered for tick-infested humans in Central Europe. *International Journal of Medical Microbiology Supplements, 293*, 93-103.

Hunfeld, K.-P., Hildebrandt, A., & Gray, J. (2008). Babesiosis: recent insights into an ancient disease. *International journal for parasitology, 38*(11), 1219-1237.

Irvin, A. (1987). Characterization of species and strains of Theileria *Advances in parasitology* (Vol. 26, pp. 145-197): Elsevier.

Irvin, A., Boarer, C., Dobbelaere, D., Mahan, S., Masake, R., & Ocama, J. (1981). Monitoring Theileria parva infection in adult Rhipicephalus appendiculatus ticks. *Parasitology, 82*(1), 137-147.

Jaenson, T. G., Jaenson, D. G., Eisen, L., Petersson, E., & Lindgren, E. (2012). Changes in the geographical distribution and abundance of the tick Ixodes ricinus during the past 30 years in Sweden. *Parasites & vectors*, *5*(1), 8.

James, M., Saunders, B., Guy, L. A., Brookbanks, E., Charleston, W., & Uilenberg, G. (1984). Theileria orientalis, a blood parasite of cattle. First report in New Zealand. *New Zealand veterinary journal, 32*(9), 154-156.

Jamieson, S. E., Castro, I., Jensen, T., Morrison, K. W., & Durrant, B. (2016). Roosting preferences of north island brown kiwis (apteryx mantelli). *The Wilson Journal of Ornithology, 128*(4), 857-866.

Jefferies, R., Down, J., McInnes, L., Ryan, U., Robertson, H., Jakob-Hoff, R., & Irwin, P. (2008). Molecular characterization of Babesia kiwiensis from the brown kiwi (Apteryx mantelli). *Journal of Parasitology*, *94*(2), 557-561.

Jerzak, L., Sparks, T. H., Kasprzak, M., Bochenski, M., Kaminski, P., Wiśniewska, E., ... & Tryjanowski, P. (2010). Blood chemistry in white stork Ciconia ciconia chicks varies by sex and age. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *156*(2), 144-147.

Johnson, P. T., Lunde, K. B., Ritchie, E. G., & Launer, A. E. (1999). The effect of trematode infection on amphibian limb development and survivorship. *Science*, *284*(5415), 802-804.

Jones, P. J. (1983). Haematocrit values of breeding Red-billed queleas Quelea quelea (Aves: Ploceidae) in relation to body condition and thymus activity. *Journal of Zoology*, *201*(2), 217-222.

Kahl, O., & Knülle, W. (1988). Water vapour uptake from subsaturated atmospheres by engorged immature ixodid ticks. *Experimental & applied acarology, 4*(1), 73-83.

Kempster, B., Zanette, L., Longstaffe, F. J., MacDougall-Shackleton, S. A., Wingfield, J. C., & Clinchy, M. (2007). Do stable isotopes reflect nutritional stress? Results from a laboratory experiment on song sparrows. *Oecologia, 151*(3), 365-371.

Kerr, G. D., & Bull, C. M. (2006). Interactions between climate, host refuge use, and tick population dynamics. *Parasitology research, 99*(3), 214-222.

Khan, T. A., & Zafar, F. (2005). Haematological study in response to varying doses of estrogen in broiler chicken. *International Journal of Poultry Science*, *4*(10), 748-751.

King, D., Gettinby, G., & Newson, R. (1988). A climate-based model for the development of the ixodid tick, Rhipicephalus appendiculatus in East Coast fever zones. *Veterinary parasitology, 29*(1), 41-51.

Klasing, K., & Leshchinsky, T. (1999). Functions, costs, and benefits of the immune system during development and growth. *Ostrich, 69*, 2817-2832.

Kleindorfer, S., Lambert, S., & Paton, D. C. (2006). Ticks (Ixodes sp.) and blood parasites (Haemoproteus spp.) in New Holland Honeyeaters (Phylidonyris novaehollandiae): evidence for site specificity and fitness costs. *Emu-Austral Ornithology, 106*(2), 113-118.

Klompen, H., & Grimaldi, D. (2001). First Mesozoic record of a parasitiform mite: a larval argasid tick in Cretaceous amber (Acari: Ixodida: Argasidae). *Annals of the Entomological Society of America*, *94*(1), 10-15.

Klompen, J. S. H., Black, W., Keirans, J. E., & Oliver Jr, J. H. (1996). Evolution of ticks. *Annual review of entomology*, *41*(1), 141-161.

Knowles, S. C. L., Palinauskas, V., & Sheldon, B. C. (2010). Chronic malaria infections increase family inequalities and reduce parental fitness: experimental evidence from a wild bird population. *Journal of evolutionary biology*, *23*(3), 557-569.

Knülle, W., & Devine, T. L. (1972). Evidence for active and passive components of sorption of atmospheric water vapour by larvae of the tick Dermacentor variabilis. *Journal of insect physiology*, *18*(9), 1653-1664.

Kocan, K. M., de la Fuente, J., & Coburn, L. A. (2015). Insights into the development of Ixodes scapularis: a resource for research on a medically important tick species. *Parasites & vectors, 8*(1), 592.

Koch, H. G., & Sauer, J. R. (1984). Quantity of blood ingested by four species of hard ticks (Acari: Ixodidae) fed on domestic dogs. *Annals of the Entomological Society of America, 77*(2), 142-146.

Koch, H. G., & Tuck, M. D. (1986). Molting and survival of the brown dog tick (Acari: Ixodidae) under different temperatures and humidities. *Annals of the Entomological Society of America, 79*(1), 11-14.

Koop, J. A., & Clayton, D. H. (2013). Evaluation of two methods for quantifying passeriform lice. *Journal of Field Ornithology, 84*(2), 210-215.

Korpimaki, E., Hakkarainen, H., & Bennett, G. (1993). Blood parasites and reproductive success of Tengmalm's owls: Detrimental effects on females but not on males? *Functional Ecology*, 420-426.

Krams, I. A., Suraka, V., Rantala, M. J., Sepp, T., Mierauskas, P., Vrublevska, J., & Krama, T. (2013). Acute infection of avian malaria impairs concentration of haemoglobin and survival in juvenile altricial birds. *Journal of Zoology*, *291*(1), 34-41.

Krasnov, B., Khokhlova, I., & Shenbrot, G. (2004). Sampling fleas: the reliability of host infestation data. *Medical and veterinary entomology, 18*(3), 232-240.

Križanauskienė, A., Hellgren, O., Kosarev, V., Sokolov, L., Bensch, S., & Valkiūnas, G. (2006). Variation in host specificity between species of avian hemosporidian parasites: evidence from parasite morphology and cytochrome b gene sequences. *Journal of Parasitology*, *92*(6), 1319-1325.

Kuris, A.M., Hechinger, R.F., Shaw, J.C., Whitney, K.L., Aguirre-Macedo, L., Boch, C.A., Dobson, A.P., Dunham, E.J., Fredensborg, B.L., Huspeni, T.C. and Lorda, J. (2008). Ecosystem energetic implications of parasite and free-living biomass in three estuaries. *Nature*, *454*(7203), 515-518.

Kwak, M. L., Beveridge, I., Koehler, A. V., Malipatil, M., Gasser, R. B., & Jabbar, A. (2017). Phylogenetic analysis of the Australasian paralysis ticks and their relatives (Ixodidae: Ixodes: Sternalixodes). *Parasites & vectors, 10*(1), 122.

Kwak, M. L., & Heath, A. C. (2018). Redescription of the kiwi tick Ixodes anatis (Acari: Ixodidae) from New Zealand, with notes on its biology. *Experimental and applied acarology, 74*(2), 207-223.

Lafferty, K.D., Allesina, S., Arim, M., Briggs, C.J., De Leo, G., Dobson, A.P., Dunne, J.A., Johnson, P.T., Kuris, A.M., Marcogliese, D.J. and Martinez, N.D., 2008. Parasites in food webs: the ultimate missing links. *Ecology letters*, *11*(6), pp.533-546.

Lafferty, K. D., Dobson, A. P., & Kuris, A. M. (2006). Parasites dominate food web links. *Proceedings of the National Academy of Sciences*, *103*(30), 11211-11216.

Lafferty, K. D., & Hopkins, S. R. (2018). Unique parasite aDNA in moa coprolites from New Zealand suggests mass parasite extinctions followed human-induced megafauna extinctions. *Proceedings of the National Academy of Sciences, 115*(7), 1411-1413.

Lafferty, K. D., & Morris, A. K. (1996). Altered behavior of parasitized killifish increases susceptibility to predation by bird final hosts. *Ecology*, *77*(5), 1390-1397.

Langslow, D. R. (1978). Gluconeogenesis in birds: Biochem. Soc. Trans. 6:1148-1152..

Lees, A. (1946). The water balance in Ixodes ricinus L. and certain other species of ticks. *Parasitology, 37*(1-2), 1-20.

Lees, A., & Milne, A. (1951). The seasonal and diurnal activities of individual sheep ticks (Ixodes ricinus L.). *Parasitology, 41*(3-4), 189-208.

Lefevre, T., & Thomas, F. (2008). Behind the scene, something else is pulling the strings: emphasizing parasitic manipulation in vector-borne diseases. *Infection, Genetics and Evolution, 8*(4), 504-519.

Lehmann, T. (1993). Ectoparasites: direct impact on host fitness. *Parasitology Today, 9*(1), 8-13.

Lehmann, T. (1994). Reinfestation analysis to estimate ectoparasite population size, emergence, and mortality. *Journal of medical entomology, 31*(2), 257-264.

Leveille, G., & Sauberlich, H. (1961). Influence of dietary protein level on serum protein components and cholesterol in the growing chick. *The Journal of Nutrition, 74*(4), 500-504.

Lill, A. (2011). Sources of variation in blood glucose concentrations of free-living birds. *Avian Biology Research*, *4*(2), 78-86.

Lindgren, E., & Gustafson, R. (2001). Tick-borne encephalitis in Sweden and climate change. *The Lancet, 358*(9275), 16-18.

Lindgren, E., Tälleklint, L., & Polfeldt, T. (2000). Impact of climatic change on the northern latitude limit and population density of the disease-transmitting European tick Ixodes ricinus. *Environmental health perspectives, 108*(2), 119.

Loman, J. (1980). Reproduction in a population of the hooded crow Corvus cornix. *Ecography, 3*(1), 26-35.

Lumeij, J. T., & Maclean, B. (1996). Total protein determination in pigeon plasma and serum: comparison of refractometric methods with the biuret method. *Journal of Avian medicine and Surgery*, 150-152.

Luttrell, M., Creekmore, L., & Mertins, J. (1996). Avian tick paralysis caused by Ixodes brunneus in the southeastern United States. *Journal of wildlife diseases, 32*(1), 133-136.

Lynn, S. E., Hunt, K. E., & Wingfield, J. C. (2003). Ecological factors affecting the adrenocortical response to stress in chestnut-collared and McCown's longspurs (Calcarius ornatus, Calcarius mccownii). *Physiological and Biochemical Zoology*, *76*(4), 566-576.

Machın, M., Simoyi, M., Blemings, K., & Klandorf, H. (2004). Increased dietary protein elevates plasma uric acid and is associated with decreased oxidative stress in rapidly-growing broilers. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 137*(3), 383-390.

Mangin, S., Gauthier-Clerc, M., Frenot, Y., Gendner, J. P., & Le Maho, Y. (2003). Ticks Ixodes uriae and the breeding performance of a colonial seabird, king penguin Aptenodytes patagonicus. *Journal of Avian biology, 34*(1), 30-34.

Manual, M. (2012). Haematologic reference ranges. *The Merck Veterinary Manual*.

Martínez-Haro, M., Viñuela, J., & Mateo, R. (2007). Exposure of birds to cholinesterase-inhibiting pesticides following a forest application for tick control. *Environmental toxicology and pharmacology, 23*(3), 347-349.

McKilligan, N. (1996). Field experiments on the effect of ticks on breeding success and chick health of cattle egrets. *Australian Journal of Ecology, 21*(4), 442-449.

McLennan, J., & Potter, M. (1992). Distribution, population changes and management of brown kiwi in Hawke's Bay. *New Zealand Journal of Ecology, 16*, 91-91.

McLennan, J. A., Rudge, M. R., & Potter, M. A. (1987). Range size and denning behaviour of Brown Kiwi, Apteryx australis mantelli, in Hawke's Bay, New Zealand. *New Zealand journal of ecology*, 97-107.

McLennan, J., Potter, M., Robertson, H., Wake, G., Colbourne, R., Dew, L., Miller, P. (1996). Role of predation in the decline of kiwi, Apteryx spp., in New Zealand. *New Zealand Journal of Ecology*, 27-35.

McNab, B. K. (1996). Metabolism and temperature regulation of kiwis (Apterygidae). *The Auk*, *113*(3), 687-692.

Mehlhorn, H., Schein, E., & Ahmed, J. S. (1994). Theileria *Parasitic protozoa* (pp. 217-304): Elsevier.

Menon, D. G., Bennett, D. C., Schaefer, A. M., & Cheng, K. M. (2013). Hematological and serum biochemical profile of farm emus (Dromaius novaehollandiae) at the onset of their breeding season. *Poultry science*, *92*(4), 935-944.

Merino, S., Martínez, J., Møller, A. P., Barbosa, A., de Lope, F., & Rodríguez-Caabeiro, F. (2001). Physiological and haematological consequences of a novel parasite on the red-rumped swallow Hirundo daurica. *International journal for parasitology*, *31*(11), 1187-1193.

Merino, S., Moreno, J., José Sanz, J., & Arriero, E. (2000). Are avian blood parasites pathogenic in the wild? A medication experiment in blue tits (Parus caeruleus). *Proceedings of the Royal Society of London. Series B: Biological Sciences, 267*(1461), 2507-2510.

Metzker, M. L. (2010). Sequencing technologies—the next generation. *Nature reviews genetics, 11*(1), 31.

Miles, J., & Castro, I. (2000). Survey of Northern brown kiwi (Apteryx mantelli) on Ponui Island, Hauraki Gulf–1999. *Unpublished Report. Department of Conservation, New Zealand*.

Minias, P. (2015). The use of haemoglobin concentrations to assess physiological condition in birds: a review. *Conservation physiology*, *3*(1), cov007.

Minson, C. J. (2013). *Diet-related factors in the conservation of kiwi (*Apteryx mantelli*): a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Zoology at Massey University, Manawatu, New Zealand* (Doctoral dissertation, Massey University).

Møller, A. P. (1990). Effects of a haematophagous mite on the barn swallow (Hirundo rustica): a test of the Hamilton and Zuk hypothesis. *Evolution, 44*(4), 771-784.

Møller, A. P. (1991). Parasite load reduces song output in a passerine bird. *Animal Behaviour*, *41*(4), 723-730.

Moltmann, U. G., Mehlhorn, H., & Friedhoff, K. T. (1982). Electron microscopic study on the development of Babesia ovis (Piroplasmia) in the salivary glands of the vector tick Rhipicephalus bursa. *Acta tropica*, *39*(1), 29-40.

Morales, J., Moreno, J., Merino, S., Tomás, G., Martínez, J., & Garamszegi, L. Z. (2004). Associations between immune parameters, parasitism, and stress in breeding pied flycatcher (Ficedula hypoleuca) females. *Canadian Journal of Zoology*, *82*(9), 1484-1492.

Mooring, M. S., Benjamin, J. E., Harte, C. R., & Herzog, N. B. (2000). Testing the interspecific body size principle in ungulates: the smaller they come, the harder they groom. *Animal Behaviour, 60*(1), 35-45.

Morbey, Y. (1996). The abundance and effects of ticks (Ixodes uriae) on nestling Cassin's Auklets (Ptychoramphus aleuticus) at Triangle Island, British Columbia. *Canadian Journal of Zoology, 74*(8), 1585-1589.

Moreno, J., De Leon, A., Fargallo, J., & Moreno, E. (1998). Breeding time, health and immune response in the chinstrap penguin Pygoscelis antarctica. *Oecologia, 115*(3), 312-319.

Morgan, K., Alley, M., Pomroy, W., Castro, I., & Howe, L. (2012). Enteric coccidiosis in the brown kiwi (Apteryx mantelli). *Parasitology research, 111*(4), 1689-1699.

Morgan, K. J. (2008). *Kiwi first aid and veterinary care*: Science and Technical Pub., Department of Conservation.

Moritz, C., McCallum, H., Donnellan, S., & Roberts, J. (1991). Parasite loads in parthenogenetic and sexual lizards (Heteronotia binoei): support for the Red Queen hypothesis. *Proceedings of the Royal Society of London. Series B: Biological Sciences, 244*(1310), 145-149.

Morran, L. T., Schmidt, O. G., Gelarden, I. A., Parrish, R. C., & Lively, C. M. (2011). Running with the Red Queen: host-parasite coevolution selects for biparental sex. *Science, 333*(6039), 216-218.

Morris, S. R., Ertel, M. C., & Wright, M. P. (2007). The incidence and effects of ticks on migrating birds at a stopover site in Maine. *Northeastern Naturalist, 14*(2), 171-183.

Moss, W. W., & Camin, J. H. (1970). Nest parasitism, productivity, and clutch size in purple martins. *Science, 168*(3934), 1000-1003.

Morton, M. L. (1994). Hematocrits in montane sparrows in relation to reproductive schedule. *The Condor*, *96*(1), 119-126.

Murray, M., Palma, R., Pilgrim, R., & Shaw, M. (1990). Ectoparasites of Australian, New Zealand and Antarctic birds. *Handbook of Australian, New Zealand and Antarctic Birds. Volume 1 Ratites to Ducks*, 1365-1374.

Mushi, E. Z., Binta, M. G., Chabo, R. G., Isa, J. F. W., & Kapaata, R. W. (1999). Selected hematologic values of farmed ostriches (Struthio camelus) in Botswana. *Journal of Veterinary Diagnostic Investigation*, *11*(4), 372-374.

Needham, G. R., & Teel, P. D. (1986). Water balance by ticks between bloodmeals. *Morphology, physiology, and behavioral biology of ticks/editors, John R. Sauer and J. Alexander Hair*.

Needham, G. R., & Teel, P. D. (1991). Off-host physiological ecology of ixodid ticks. *Annual review of entomology, 36*(1), 659-681.

NIWA. (2014). CliFlo: NIWA's National Climate Database on the Web. Retrieved from http://cliflo.niwa.co.nz/

Norcross, N. L., & Bolen, E. G. (2002). Effectiveness of nest treatments on tick infestations in the Eastern Brown Pelican. *The Wilson Journal of Ornithology*, *114*(1), 73-79.

Norte, A., Lobato, D., Braga, E., Antonini, Y., Lacorte, G., Gonçalves, M., . . . Ramos, J. (2013). Do ticks and Borrelia burgdorferi sl constitute a burden to birds? *Parasitology research, 112*(5), 1903-1912.

Norval, R. (1977). Studies on the ecology of the tick Amblyomma hebraeum Koch in the Eastern Cape Province of South Africa. II. Survival and development. *The Journal of parasitology*, 740-747.

Obenchain, F. D., & Galun, R. (Eds.). (2013). *Physiology of ticks: current themes in tropical science*. Elsevier.

O'Brien, E. L., Morrison, B. L., & Johnson, L. S. (2001). Assessing the effects of haematophagous ectoparasites on the health of nestling birds: haematocrit vs haemoglobin levels in House Wrens parasitized by blow fly larvae. *Journal of Avian biology, 32*(1), 73-76.

Obsomer, V., Wirtgen, M., Linden, A., Claerebout, E., Heyman, P., Heylen, D., Tack, W. (2013). Spatial disaggregation of tick occurrence and ecology at a local scale as a preliminary step for spatial surveillance of tick-borne diseases: general framework and health implications in Belgium. *Parasites & vectors, 6*(1), 190.

Ogden, N., Lindsay, L., Beauchamp, G., Charron, D., Maarouf, A., O'callaghan, C., . . . Barker, I. (2004). Investigation of relationships between temperature and developmental rates of tick Ixodes scapularis (Acari: Ixodidae) in the laboratory and field. *Journal of medical entomology, 41*(4), 622-633.

Oorebeek, M., & Kleindorfer, S. (2008). Climate or host availability: what determines the seasonal abundance of ticks? *Parasitology research, 103*(4), 871-875.

Ots, I., & Horak, P. (1996). Great tits Parus major trade health for reproduction. *Proceedings of the Royal Society of London. Series B: Biological Sciences, 263*(1376), 1443-1447.

Ots, I., Murumägi, A., & Horak, P. (1998). Haematological health state indices of reproducing great tits: methodology and sources of natural variation. *Functional Ecology*, *12*(4), 700-707.

Padgett, K. A., & Lane, R. S. (2001). Life cycle of Ixodes pacificus (Acari: Ixodidae): timing of developmental processes under field and laboratory conditions. *Journal of medical entomology, 38*(5), 684-693.

Page, C. D., Greiner, E. C., & Schmidt, R. E. (1987). Leucocytozoonosis in crested oropendolas (Psaracolius decumanus). *AAV Today*, *1*(4), 155-157.

Palomeque, J., Pinto, D., & Viscor, G. (1991). Hematologic and blood chemistry values of the Masai ostrich (Struthio camelus). *Journal of Wildlife Diseases*, *27*(1), 34-40.

Paparini, A., McInnes, L. M., Di Placido, D., Mackereth, G., Tompkins, D. M., Clough, R., . . . Irwin, P. J. (2014). Piroplasms of New Zealand seabirds. *Parasitology research, 113*(12), 4407-4414.

Parola, P., & Raoult, D. (2001). Tick-borne bacterial diseases emerging in Europe. *Clinical microbiology and infection, 7*(2), 80-83.

Pavlov, I. Y., Wilson, A. R., & Delgado, J. C. (2012). Reference interval computation: which method (not) to choose?. *Clinica Chimica Acta*, *413*(13-14), 1107-1114.

Pedersen, A. B., & Fenton, A. (2007). Emphasizing the ecology in parasite community ecology. *Trends in ecology & evolution*, *22*(3), 133-139.

Peirce, M. (2000). A taxonomic review of avian piroplasms of the genus Babesia Starcovici, 1893 (Apicomplexa: Piroplasmorida: Babesiidae). *Journal of Natural History, 34*(3), 317-332.

Peirce, M. A., Lederer, R., Adlard, R. D., & O'Donoghue, P. J. (2004). Pathology associated with endogenous development of haematozoa in birds from southeast Queensland. *Avian Pathology*, *33*(4), 445-450.

Peirce, M. A., Jakob-Hoff, R. M., & Twentyman, C. (2003). New species of haematozoa from Apterygidae in New Zealand. *Journal of Natural History*, *37*(15), 1797-1804.

Pfäffle, M., Petney, T., Elgas, M., Skuballa, J., & Taraschewski, H. (2009). Tick-induced blood loss leads to regenerative anaemia in the European hedgehog (Erinaceus europaeus). *Parasitology, 136*(04), 443-452.

Pierce, R. J., Gardiner, C., Moodie, H., Robertson, H. A., & Sporle, W. (2006). Sustainable management of brown kiwi and other threatened birds in Northland. *Wildland Consultants Report No. 1193*.

Pizzi, R. (2009). Veterinarians and taxonomic chauvinism: the dilemma of parasite conservation. *Journal of Exotic Pet Medicine, 18*(4), 279-282.

Plummer, M. (2003). *JAGS: A program for analysis of Bayesian graphical models using Gibbs sampling.* Paper presented at the Proceedings of the 3rd international workshop on distributed statistical computing.

Plummer, M., Best, N., Cowles, K., & Vines, K. (2006). CODA: convergence diagnosis and output analysis for MCMC. *R news, 6*(1), 7-11.

Polak, M., & Starmer, W. T. (1998). Parasite–induced risk of mortality elevates reproductive effort in male Drosophila. *Proceedings of the Royal Society of London. Series B: Biological Sciences, 265*(1411), 2197-2201.

Potter, M. (1990). Movement of North Island brown kiwi (Apteryx australis mantelli) between forest remnants. *New Zealand Journal of Ecology, 14*, 17-24.

Potter, M., & Cockrem, J. (1992). Plasma levels of sex steroids in the North Island brown kiwi (Apteryx australis mantelli) in relation to time of year and stages of breeding. *General and comparative endocrinology, 87*(3), 416-424.

Poulin, R. (2007). Are there general laws in parasite ecology? *Parasitology*, *134*(6), 763-776.

Poulin, R. (2010). Parasite manipulation of host behavior: an update and frequently asked questions *Advances in the Study of Behavior* (Vol. 41, pp. 151-186): Elsevier.

Prevot, P.-P., Couvreur, B., Denis, V., Brossard, M., Vanhamme, L., & Godfroid, E. (2007). Protective immunity against Ixodes ricinus induced by a salivary serpin. *Vaccine, 25*(17), 3284-3292.

Pringle, R. M., Webb, J. K., & Shine, R. (2003). Canopy structure, microclimate, and habitat selection by a nocturnal snake, Hoplocephalus bungaroides. *Ecology, 84*(10), 2668-2679.

Prudencio, C. R., de la Lastra, J. M. P., Canales, M., Villar, M., & de la Fuente, J. (2010). Mapping protective epitopes in the tick and mosquito subolesin ortholog proteins. *Vaccine, 28*(33), 5398-5406.

Pryor, L. J. E., & Casto, J. M. (2015). Blood-feeding ectoparasites as developmental stressors: Does corticosterone mediate effects of mite infestation on nestling growth, immunity and energy availability? *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, *323*(7), 466-477.

Puente, J. M. D. L., Merino, S., Tomás, G., Moreno, J., Morales, J., Lobato, E., ... & Belda, E. J. (2010). The blood parasite Haemoproteus reduces survival in a wild bird: a medication experiment. *Biology letters*, *6*(5), 663-665.

Quillfeldt, P., Masello, J. F., & Möstl, E. (2004). Blood chemistry in relation to nutrition and ectoparasite load in Wilson's storm-petrels Oceanites oceanicus. *Polar Biology, 27*(3), 168-176.

Rae, M. (1995, July). Hemoprotozoa of caged and aviary birds. In *Seminars in avian and exotic pet Medicine* (Vol. 4, No. 3, pp. 131-137). WB Saunders.

Raffel, T. R., Martin, L. B., & Rohr, J. R. (2008). Parasites as predators: unifying natural enemy ecology. *Trends in ecology & evolution*, *23*(11), 610-618.

Randolph, S. (2004). Tick ecology: processes and patterns behind the epidemiological risk posed by ixodid ticks as vectors. *Parasitology, 129*(S1), S37-S65.

Randolph, S. E. (1994). Population dynamics and density-dependent seasonal mortality indices of the tick Rhipicephalus appendiculatus in eastern and southern Africa. *Medical and veterinary entomology, 8*(4), 351-368.

Randolph, S. E., & Storey, K. (1999). Impact of microclimate on immature tick-rodent host interactions (Acari: Ixodidae): implications for parasite transmission. *Journal of medical entomology*, *36*(6), 741-748.

Rechav, Y., Strydom, W., Clarke, F., Burger, L., Mackie, A., & Fielden, L. (1994). Isotopes as host blood markers to measure blood intake by feeding ticks (Acari: Ixodidae). *Journal of medical entomology, 31*(4), 511-515.

Rehder, N. B., & Bird, D. M. (1983). Annual profiles of blood packed cell volumes of captive American kestrels. *Canadian journal of zoology*, *61*(11), 2550-2555.

Reinhardt, K., Naylor, R. A., & Siva-Jothy, M. T. (2007). Estimating the mean abundance and feeding rate of a temporal ectoparasite in the wild: Afrocimex constrictus (Heteroptera: Cimicidae). *International journal for parasitology, 37*(8-9), 937-942.

Richner, H., Oppliger, A., & Christe, P. (1993). Effect of an ectoparasite on reproduction in great tits. *Journal of Animal Ecology*, 703-710.

Riek, R. F. (1964). The life cycle of Babesia bigemina (Smith and Kilborne, 1893) in the tick vector Boophilus microplus (Canestrini). *Australian Journal of Agricultural Research*, *15*(5), 802-821.

Robertson, H., & Colbourne, R. (2017). Kiwi Best Practice Manual.

Robertson, H., Karen Baird, John E. Dowding, Graeme P. Elliott, Rodney A. Hitchmough, Colin M. Miskelly, Nikki McArthur, Colin F.J. O'Donnell, Paul M. Sagar, R. Paul Scofield; Graeme A. Taylor. (2016) *New Zealand Threat Classification Series 19. 27* p.

Roche, M., & Layrisse, M. (1966). Nature and causes of hookworm anemia. *American Journal of Tropical Medicine and Hygiene, 15*(6 Pt 2), 1032-1102.

Ruiz-Fons, F., & Gilbert, L. (2010). The role of deer as vehicles to move ticks, Ixodes ricinus, between contrasting habitats. *International journal for parasitology, 40*(9), 1013-1020.

Saino, N., Calza, S., & Møller, A. P. (1998). Effects of a dipteran ectoparasite on immune response and growth trade-offs in barn swallow, Hirundo rustica, nestlings. *Oikos*, 217-228.

Samour, J., Naldo, J., Libanan, N., Rahman, H., & Sakkir, M. (2011). Age-related hematology and plasma chemistry changes in captive Masai ostriches (Struthio camelus massaicus). *Comparative Clinical Pathology*, *20*(6), 659-667.

Schall, J. J. (1992). Parasite-mediated competition in Anolis lizards. *Oecologia*, *92*(1), 58-64.

Schein, E., Mehlhorn, H., & Voigt, W. (1979). Electron microscopical studies on the development of Babesia canis (Sporozoa) in the salivary glands of the vector tick Dermacentor reticulatus. *Acta tropica, 36*(3), 229-241.

Schmid Hempel, P. (2011). *Evolutionary parasitologythe integrated study of infections, immunology, ecology, and genetics* (No. 574.5249 S2).

Schmid-Hempel, P. (2003). Variation in immune defence as a question of evolutionary ecology. *Proceedings of the Royal Society of London B: Biological Sciences, 270*(1513), 357-366.

dos Santos Schmidt, E. M., Paulillo, A. C., Dittrich, R. L., Hoppe, E. G. L., Bertoli, R. S., Unesp, J., & UniRP, S. J. D. R. P. (2008). Determination of ring-necked pheasant (Phasianus colchicus) serum protein concentrations by refractometry and the biuret method. *Int. J. Poult. Sci*, *7*, 672-673.

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature methods, 9*(7), 671.

Schnittger, L., Rodriguez, A. E., Florin-Christensen, M., & Morrison, D. A. (2012). Babesia: a world emerging. *Infection, Genetics and Evolution, 12*(8), 1788-1809.

Schoener, E. R., Banda, M., Howe, L., Castro, I. C., & Alley, M. R. (2014). Avian malaria in New Zealand. *New Zealand veterinary journal*, *62*(4), 189-198.

Sealander, J. A. (1965). The influence of body size, season, sex, age and other factors upon some blood parameters in small mammals. *Journal of Mammalogy, 45*(4), 598-616.

Shapiro, L. M. (2005). *Diet Overlap and Potential Competition Between North Island Brown Kiwi Chicks (Apteryx Mantelli) and Ship Rats (Rattus Rattus) for Limited Resources on Ponui Island, New Zealand: A Thesis Presented in Partial Fulfilment of the Requirements for the Degree of Master of Science in Ecology at Massey*

*University, Palmerston North, New Zealand.* Massey University, Palmerston North.

Shaw, S. E., Day, M. J., Birtles, R. J., & Breitschwerdt, E. B. (2001). Tick-borne infectious diseases of dogs. *Trends in parasitology*, *17*(2), 74-80.

Shendure, J., & Ji, H. (2008). Next-generation DNA sequencing. *Nature biotechnology, 26*(10), 1135.

Siest, G., Henny, J., Gräsbeck, R., Wilding, P., Petitclerc, C., Queraltó, J. M., & Petersen, P. H. (2013). The theory of reference values: an unfinished symphony. *Clinical Chemistry and Laboratory Medicine (CCLM)*, *51*(1), 47-64.

Simon, A., Thomas, D., Blondel, J., Perret, P., & Lambrechts, M. M. (2004). Physiological ecology of Mediterranean blue tits (Parus caeruleus L.): effects of ectoparasites (Protocalliphora spp.) and food abundance on metabolic capacity of nestlings. *Physiological and Biochemical Zoology, 77*(3), 492-501.

Słomczyński, R., Kaliński, A., Wawrzyniak, J., Bańbura, M., Skwarska, J., Zieliński, P., & Bańbura, J. (2006). Effects of experimental reduction in nest micro-parasite and macro-parasite loads on nestling hemoglobin level in blue tits Parus caeruleus. *Acta Oecologica, 30*(2), 223-227.

Smith, T. G. (1996). The genus Hepatozoon (Apicomplexa: Adeleina). *The Journal of parasitology*, 565-585.

Sobrino, R., Millán, J., Oleaga, Á., Gortázar, C., de la Fuente, J., & Ruiz-Fons, F. (2012). Ecological preferences of exophilic and endophilic ticks (Acari: Ixodidae) parasitizing wild carnivores in the Iberian Peninsula. *Veterinary parasitology, 184*(2-4), 248-257.

Sonenshine, D. (2018). Range expansion of tick disease vectors in North America: implications for spread of tick-borne disease. *International journal of environmental research and public health, 15*(3), 478.

Spain, A., & Luxton, M. (1971). Catalog and bibliography of the Acari of the New Zealand subregion. *Pacific insects monograph, 25*, 179-226.

Spector, P. E. (1977). What to do with significant multivariate effects in multivariate analyses of variance. *Journal of Applied Psychology*, *62*(2), 158.

Spielman, A., Ribeiro, J., Mather, T., & Piesman, J. (1987). Dissemination and salivary delivery of Lyme disease spirochetes in vector ticks (Acari: Ixodidae). *Journal of medical entomology, 24*(2), 201-205.

Spottiswoode, C. N., & Stevens, M. (2012). Host-parasite arms races and rapid changes in bird egg appearance. *The American Naturalist, 179*(5), 633-648.

Staszewski, V., McCoy, K. D., Tveraa, T., & Boulinier, T. (2007). Interannual dynamics of antibody levels in naturally infected long-lived colonial birds. *Ecology, 88*(12), 3183-3191.

Stringer, A. P., & Linklater, W. (2014). Everything in moderation: principles of parasite control for wildlife conservation. *BioScience*, *64*(10), 932-937.

Strona, G. (2015). Past, present and future of host–parasite co-extinctions. *International Journal for Parasitology: Parasites and Wildlife*, *4*(3), 431-441.

Suzan, A. A.-A. (2012). The pathological effect of cypermethrin on domestic pigeons (Culumba livia gaddi) at Basrah City/Southern Iraq. *International Journal of Poultry Science, 11*(4), 302-310.

Svensson, E., Råberg, L., Koch, C., & Hasselquist, D. (1998). Energetic stress, immunosuppression and the costs of an antibody response. *Functional Ecology*, *12*(6), 912-919.

Sweazea, Karen L., Blair O. Wolf, Eldon J. Braun, and Benjimen R. Walker. "High blood glucose is not associated with oxidative stress or vascular dysfunction in birds." *The FASEB Journal* 22 (2008): 1239-24.

Swift, R. J., Heath, A. C., & Jamieson, S. E. (2015). The kiwi tick, Ixodes anatis Chilton, 1904 (Acari: Ixodidae): aspects of its biology and ecology. *Systematic and Applied Acarology, 30*(1), 1-12.

Szép, T., & Møller, A. (2000). Exposure to ectoparasites increases within-brood variability in size and body mass in the sand martin. *Oecologia, 125*(2), 201-207.

Taborsky, B., & Taborsky, M. (1999). The mating system and stability of pairs in kiwi Apteryx spp. *Journal of Avian biology*, 143-151.

Tack, W., Madder, M., Baeten, L., Vanhellemont, M., & Verheyen, K. (2013). Shrub clearing adversely affects the abundance of Ixodes ricinus ticks. *Experimental and applied acarology, 60*(3), 411-420.

Tälleklint-Eisen, L., & Eisen, R. J. (1999). Abundance of ticks (Acari: Ixodidae) infesting the western fence lizard, Sceloporus occidentalis, in relation to environmental factors. *Experimental & applied acarology, 23*(9), 731-740.

Team, R. C. (2013). R: A language and environment for statistical computing.

Thompson, J. N. (1999). The raw material for coevolution. *Oikos*, 5-16.

Timm, N. H. (1975). *Multivariate analysis with applications in education and psychology* (No. 04; QA278, T5.). Monterey, California: Brooks/Cole Publishing Company.

Tomás, G., Merino, S., Moreno, J., & Morales, J. (2007). Consequences of nest reuse for parasite burden and female health and condition in blue tits, Cyanistes caeruleus. *Animal Behaviour, 73*(5), 805-814.

Tompkins, D., Dobson, A., Arneberg, P., Begon, M., Cattadori, I., Greenman, J., Pugliese, A. (2002). Parasites and host population dynamics. *The ecology of wildlife diseases*, 45-62.

Tompkins, D. M., & Slaney, D. (2014). Exploring the potential for Ross River virus emergence in New Zealand. *Vector-Borne and Zoonotic Diseases, 14*(2), 141-148.

Torres, K. L., Figueiredo, D. V., Zalis, M. G., Daniel-Ribeiro, C. T., Alecrim, W., & de Fátima Ferreira-da-Cruz, M. (2006). Standardization of a very specific and sensitive single PCR for detection of Plasmodium vivax in low parasitized individuals and its usefulness for screening blood donors. *Parasitology research*, *98*(6), 519-524.

Townsend, A. K., Wheeler, S. S., Freund, D., Sehgal, R. N., & Boyce, W. M. (2018). Links between blood parasites, blood chemistry, and the survival of nestling American crows. *Ecology and evolution*, *8*(17), 8779-8790.

Troughton, D. R., & Levin, M. L. (2007). Life cycles of seven ixodid tick species (Acari: Ixodidae) under standardized laboratory conditions. *Journal of medical entomology, 44*(5), 732-740.

Tukahirwa, E. (1976). The effects of temperature and relative humidity on the development of Rhipicephalus appendiculatus Neumann (Acarina, Ixodidae). *Bulletin of Entomological Research, 66*(2), 301-312.

Tyler, R., & Cowell, R. (1996). Classification and diagnosis of anaemia. *Comparative Haematology International, 6*(1), 1-16.

Uhart, M., Aprile, G., Beldomenico, P., Solis, G., Marull, C., Beade, M., ... & Moreno, D. (2006). Evaluation of the health of free-ranging greater rheas (Rhea americana) in Argentina. *Veterinary record*, *158*(9), 297-303.

Valera, F., Hoi, H., & Krištín, A. (2006). Parasite pressure and its effects on blood parameters in a stable and dense population of the endangered Lesser grey shrike. *Biodiversity & Conservation, 15*(7), 2187-2195.

Valkiunas, G. (2004). *Avian malaria parasites and other haemosporidia*. CRC press.

Valkiūnas, G., Bairlein, F., Iezhova, T. A., & Dolnik, O. V. (2004). Factors affecting the relapse of Haemoproteus belopolskyi infections and the parasitaemia of Trypanosoma spp. in a naturally infected European songbird, the blackcap, Sylvia atricapilla. *Parasitology research*, *93*(3), 218-222.

Van Oosten, A., Heylen, D., & Matthysen, E. (2014). Host specificity of a bird-specialised endophilic ectoparasite, the tree-hole tick Ixodes arboricola. *Parasitology research, 113*(12), 4397-4405.

Van Valen, L. (1977). The red queen. *The American Naturalist, 111*(980), 809-810.

Verocai, G., Lopes, L., Burlini, L., Cruz-Vieira, V., Melo, R., & Coumendouros, K. (2008). Efficacy of cypermethrin on the control of Struthiolipeurus spp.(Phthiraptera: Philopteridae) in ostrich. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia, 60*(5), 1274-1276.

Veronez, V. A., de Castro, M. B., Bechara, G. H., & Szabó, M. P. (2010). Histopathology of Rhipicephalus sanguineus (Acari: Ixodidae) ticks fed on resistant hosts. *Experimental and applied acarology, 50*(2), 151.

Vleck, C. M., & Vleck, D. (2002). Physiological condition and reproductive consequences in Adélie penguins. *Integrative and Comparative Biology*, *42*(1), 76-83.

Wake, D. B., & Vredenburg, V. T. (2008). Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proceedings of the National Academy of Sciences*, *105*(Supplement 1), 11466-11473.

Wakelin, D. (1996). *Immunity to parasites: how parasitic infections are controlled*: Cambridge University Press.

Walker, A. (2001). Age structure of a population of Ixodes ricinus (Acari: Ixodidae) in relation to its seasonal questing. *Bulletin of Entomological Research, 91*(1), 69-78.

Walther, B., & Clayton, D. (1997). Dust-Ruffling: A Simple Method for Quantifying Ectoparasite Loads of Live Birds (Sacudidas de Polvo: Un Metodo Sencillo para Cuantificar las Cargas de Ectoparasitos en Aves Vivas). *Journal of Field Ornithology*, 509-518.

Wanless, S., Barton, T., & Harris, M. (1997). Blood hematocrit measurements of 4 species of North Atlantic seabirds in relation to levels of infestation by the tick Ixodes uriae. *Colonial Waterbirds*, 540-544.

Watkins, R. A., Moshier, S. E., & Pinter, A. J. (2006). The flea, Megabothris abantis: an invertebrate host of Hepatozoon sp. and a likely definitive host in Hepatozoon infections of the montane vole, Microtus montanus. *Journal of wildlife diseases*, *42*(2), 386-390.

Weir, J. T., Haddrath, O., Robertson, H. A., Colbourne, R. M., & Baker, A. J. (2016). Explosive ice age diversification of kiwi. *Proceedings of the National Academy of Sciences, 113*(38), E5580-E5587.

Whiteman, N. K., & Parker, P. G. (2005). *Using parasites to infer host population history: a new rationale for parasite conservation.* Paper presented at the Animal Conservation forum.

Widemo, F. (1989). *Effect of blood parasites on the Collared Flycatcher Ficedula albicollis.* Honors thesis, Department of Zoology, Uppsala University, Uppsala, Sweden.

Wilkinson, L. (1975). Response variable hypotheses in the multivariate analysis of variance. *Psychological Bulletin*, *82*(3), 408.

Williams, T. D. (2005). Mechanisms underlying the costs of egg production. *Bioscience*, *55*(1), 39-48.

Wilson, A. L. (2014). *The triumphs, challenges and failures of young North Island brown kiwi (Apteryx mantelli): a study of behaviour, growth, dispersal and mortality: a thesis in partial fulfilment of the requirements for the degree of Master of Science in Zoology at Massey University, Palmerston North, New Zealand.* Massey University.

Wilson, M. L., Ducey, A. M., Litwin, T. S., Gavin, T. A., & Spielman, A. (1990). Microgeographic distribution of immature Ixodes dammini ticks correlated with that of deer. *Medical and veterinary entomology, 4*(2), 151-159.

Windsor, D. A. (1990). Heavenly hosts. *Nature*, *348*(6297), 104-104.

Wood, C. L., & Johnson, P. T. (2015). A world without parasites: exploring the hidden ecology of infection. *Frontiers in Ecology and the Environment, 13*(8), 425-434.

Yabsley, M. J., Greiner, E., Tseng, F. S., Garner, M. M., Nordhausen, R. W., Ziccardill, M. H., . . . Zabolotzky, S. (2009). Description of novel Babesia species and associated lesions from common murres (Uria aalge) from California. *The Journal of parasitology*, 1183-1188.

Yin, H., Schnittger, L., Luo, J., Seitzer, U., & Ahmed, J. S. (2007). Ovine theileriosis in China: a new look at an old story. *Parasitology research, 101*(2), 191-195.

Yoder, J. A., Hedges, B. Z., & Benoit, J. B. (2012). Water balance of the American dog tick, Dermacentor variabilis, throughout its development with comparative observations between field-collected and laboratory-reared ticks. *International Journal of Acarology, 38*(4), 334-343.

Yoder, J. B., & Nuismer, S. L. (2010). When does coevolution promote diversification? *The American Naturalist, 176*(6), 802-817.

Yurina, N., Sharapova, L., & Odintsova, M. (2017). Structure of plastid genomes of photosynthetic eukaryotes. *Biochemistry (Moscow), 82*(6), 678-691.

Yuval, B., & Spielman, A. (1990). Duration and regulation of the developmental cycle of Ixodes dammini (Acari: Ixodidae). *Journal of medical entomology, 27*(2), 196-201.

Ziesemann, B., Brunton, D. H., & Castro, I. C. (2011). Nesting success and breeding ecology in a high-density population of Brown Kiwi (Apteryx mantelli). *Emu, 111*(2), 148-154.

Zuk, M., & Johnsen, T. S. (1998). Seasonal changes in the relationship between ornamentation and immune response in red jungle fowl. *Proceedings of the Royal Society of London. Series B: Biological Sciences, 265*(1406), 1631-1635.

Zuk, M., & Stoehr, A. M. (2002). Immune defense and host life history. *The American Naturalist, 160*(S4), S9-S22.

# Appendix 2.1: Graphs showing temperature and relative humidity from kiwi burrows

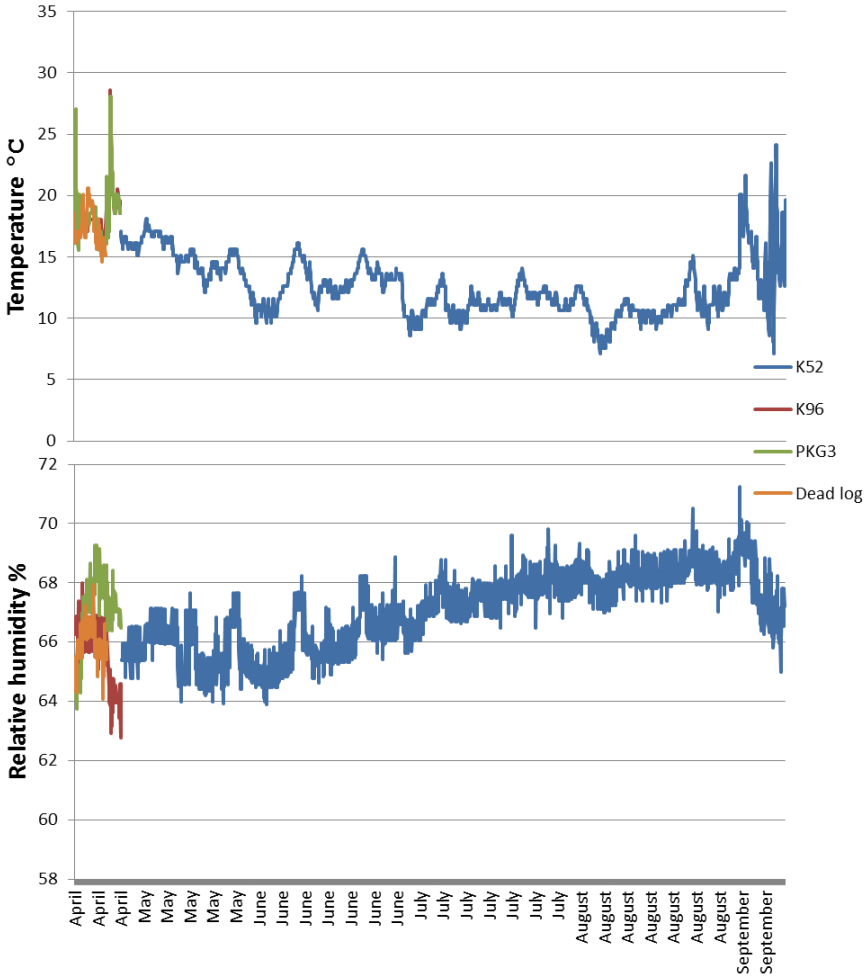



Figure2.2.1: Showing hourly temperature (above) and RH (below) in four kiwi burrows located on Ponui Island measured every hour using iButton data loggers over the period of April to September 2016. The few spikes in the temperature coincide with the presence of kiwi in the burrows but otherwise the fluctuation in the burrows is very minimal.

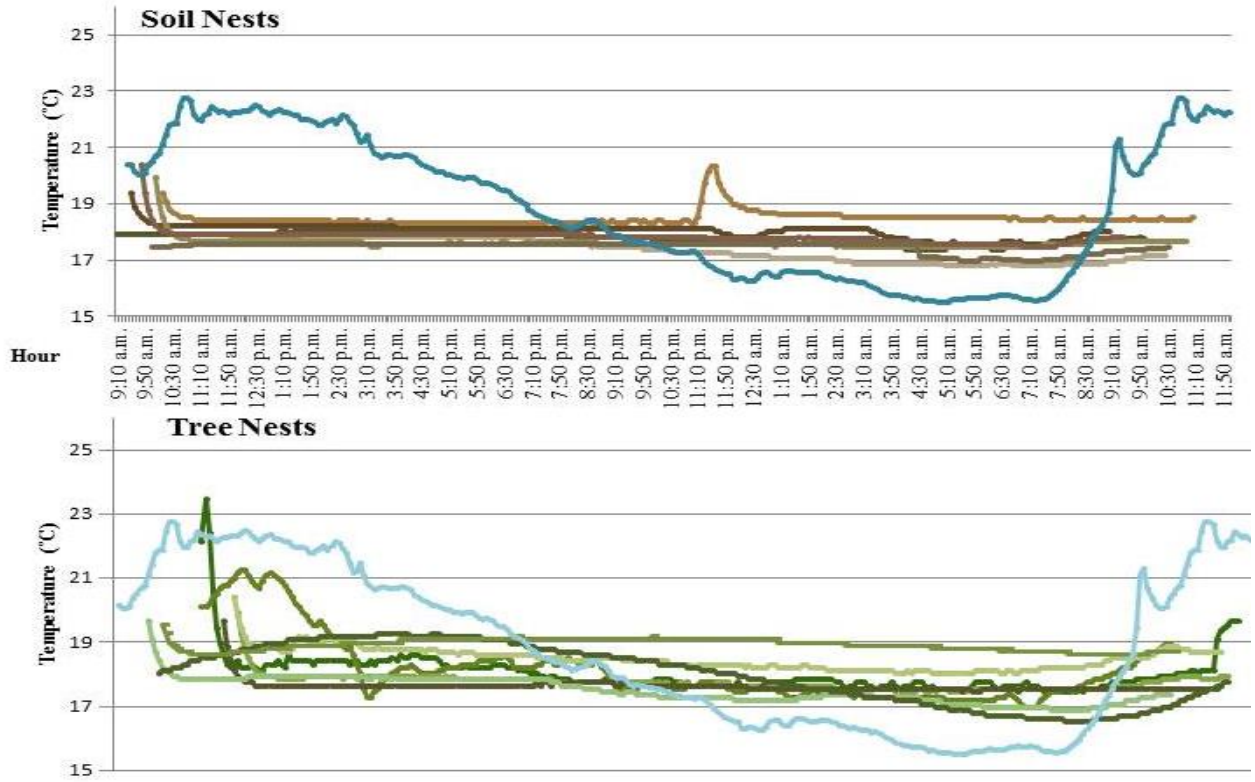



Figure2.2.2: From de Vieco, (2019) Two graphs showing the hourly temperature fluctuation inside soil nests (n= 7) and tree (n=8) nests with respect of ambient temperature (Blue lines).

# Appendix 5.1 Staining solutions

**Giemsa stain stock Preparation (WHO MM-SOP-02)**

- Dissolve 3.8g of powder in 100mL methanol. (shake vigorously for 3 minutes)
- Add 250mL of glycerol, shake for 5 minutes.
- Add remaining 150mL of methanol, shake for 3 minutes.
- Shake every day, twice for 7 days.

**Buffer Solution for Giemsa**

- Mix 9.5g sodium phosphate in 1000ml DW
- Mix 9.07g Potassium phosphate monobasic anhydrous in 1000ml DW
- Finally mix 39ml Potassium phosphate solution with 61 ml of Sodium phosphate solution in 900ml DW
- Adjust pH to 7-7.2

**Carnoy's fixative ingredients**

1. Absolute ethanol (60 %)
2. Chloroform (30%)
3. Glacial acetic acid (10 %)

**Methylene Green stock:**

- 2 g methyl green
- 100 ml distilled water
- Wash with chloroform to remove impurities and filter

**Pyronin Y stock:**

- 2 g pyronin
- 100 ml distilled water
- Wash with chloroform to remove impurities and filter

**Making Methylene Green - Pyronin stock solution [based on article: *Walker et al. 1979*]**

1. 1 ml of Pyronin Y Stock (5% aqueous solution, chloroform washed)
2. 10 ml of Methyl Green Stock (2% aqueous solution, chloroform washed)
3. 250 ml of distilled water

# Appendix 5.2 Blood cell assessing protocols

**WBC and RBC Count Protocol:**

- Take photos of the thinnest part of smear (feather) where cells are equally distributed
- Count from 10 different fields
- Total RBC/WBC = total of 10 fields/10 X $10^9$/L

**Differential WBC count:**

- Count different cells until you reach 100 cells.
- Using MsCounter2 app for iPhone.
- Abs no.= % X WBC/100

**Thrombocytes**

- TBC= average of 5 fields of oil immersion (1000 mag)

# Appendix 5.3 Mean haematology values of the different populations

*Table 5.2: The table gives the mean values, with 95% confidence intervals in brackets, for the various parameters tested with p values for the MANOVA, where significantly different groups are denoted with different letters*

| Parameters | Unit | Ponui (n=41) | Moturua (n=18) | Kuaotunu (n=5) | Motu (n=5) | Motuarohia (n=7) | Puketi (n=5) | Purerua (n=21) |
|---|---|---|---|---|---|---|---|---|
| **Weight** | Grams | 2314.9(± 101.6)$^{b}$ | 1781.9 (± 118)$^{a}$ | 2315 (± 234.5)$^{b}$ | 2380 (± 224)$^{b}$ | 2178.6 (± 298)$^{b}$ | 2280 (± 563)$^{b}$ | 2201 (± 273.7)$^{b}$ |
| **Packed Cell Volume** | % | 42.2 (± 1.8) | 39.6 (± 3) | 38.8 (± 4.4) | 44.2 (± 3.9) | 39.6 (± 2.6) | 39.3 (± 3.2) | 42.7 (± 1.5) |
| **Total plasma protein** | g/dL | 5.2 (± 0.3)$^{b}$ | 5.4 (± 0.3)$^{b}$ | 6.6 (± 1)$^{b}$ | 5.5 (± 0.4)$^{b}$ | 5.4 (± 0.1)$^{b}$ | 4.64 (± 0.4)$^{a}$ | 5.9 (± 0.5)$^{b}$ |
| **Haemoglobin** | g/dL | 16.9 (± 0.8) | 15.7 (± 1.5) | 15 (± 5.8) | 16.5 (± 2.7) | 16.2 (± 2.1) | 14.2 (± 3.9) | 17.3 (± 2.1) |
| **Glucose** | mg/dL | 104.9 (± 8.8)$^{c}$ | 96.9 (± 10.4)$^{c}$ | 55 (± 19)$^{b}$ | 123.2 (± 22.1)$^{a}$ | 85.5 (± 10.3)$^{c}$ | 117 (± 4.5)$^{a}$ | 93.4 (± 9.5)$^{c}$ |
| **MCHC** | g/dL | 40.7 (± 2.9) | 36.62 (± 5.5) | 37.85 (± 12.3) | 37.56 (± 6.9) | 40.86 (± 5.5) | 35.9 (± 9.6) | 40.6 (± 5) |
| **H:L** | - | 1.76 (± 0.3)$^{a}$ | | 1.2 (± 0.4)$^{b}$ | 0.85 (± 0.3)$^{b}$ | | | |

# Appendix 6.1: Testing duration of effectiveness of Ripcord plus (Cypermethrin) spray in burrows

**Aim:** To test the duration of effect of Ripcord spray on ticks at different concentrations to determine the smallest dose and time required to keep burrows tick free.

**Material and Method**

I dug 12 holes at Massey University to imitate kiwi burrows. I put 6 adult male and female kiwi ticks in a mesh bag in the burrows. I tied the bags down. Then I proceeded to spray the burrows with different concentrations of Ripcord Plus as shown in (Figure 5.1.1). If the ticks had died, I replaced them with a new batch to see how long the effect of the spray lasted.

**Result and Conclusion**

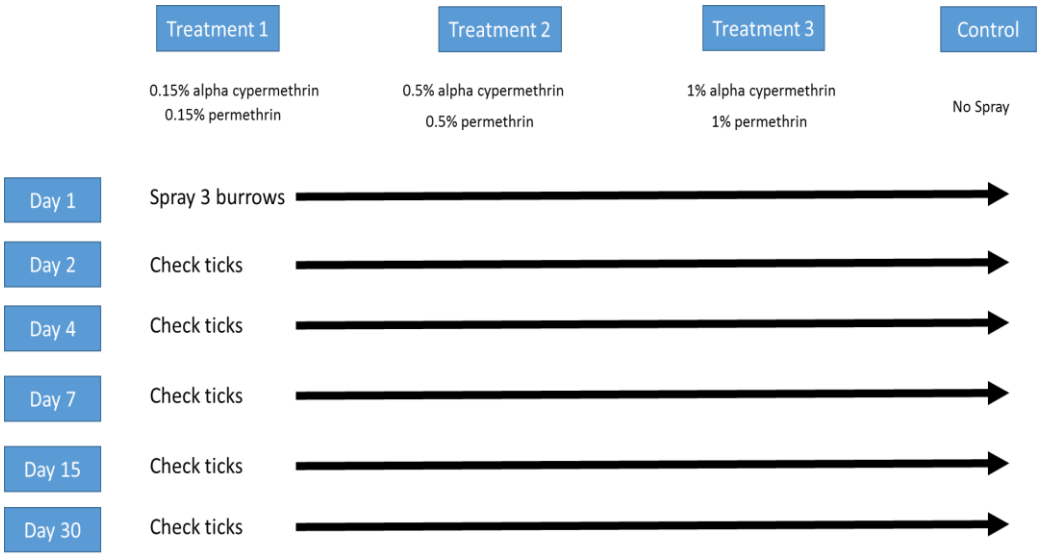



**Figure 5.1.1: The experimental design to test effectiveness of Cypermethrin based spray to kill ticks.**

I found that Ripcord plus killed all ticks up to a mean of eight days (± 2) after application. After 15 days the death rate of ticks had dropped to 45% and by day 30, no ticks the death rate was only 8%. Based on this result, we decided to keep our burrows blocked for the main experiment in chapter 5 to ten days to allow for the

parasiticide to act while at the same time avoid any contact with kiwi during the most effective period.

# Appendix 6.2: To test the efficacy of Vitapet to remove ticks off birds

***Aim:***

To test duration of effectiveness of Vitapet flea powder on North Island Brown Kiwi (NIBK) to keep the birds parasite free.

**Materials and Methods:**

We caught nine birds and treated them with Vitapet Flea powder (1.95% Permethrin) using the dust ruffle method described by Walter and Clayton (1997) and previously used by Castro (2006), with no harmful effects on the birds. We divided the birds into three groups. The first group of three birds was checked after a week, the next after two weeks and the third group after three weeks (Table 1). Ectoparasite reinfection levels were measured by visually checking the birds for ectoparasites as well as dust ruffling again.

Table 1: Experimental design for pilot studies.

| | Week 1 | Week 2 | Week 3 | Week 4 |
|---|---|---|---|---|
| **Burrows** | Treated 12 burrows with 0.5% ripcord plus | Check for ticks | Check for ticks | Check for ticks |
| **Birds** | Treated 9 birds with Vitapet Flea powder using the dust ruffling method | Check 3 birds for parasites | Check 3 different birds for parasites | Check last 3 birds for parasites |

**Results and Conclusion:**

The birds showed no re-infection in the first two weeks and only had very few ticks in the third week (Avg of 52 ticks per bird before starting and two ticks on one birds in week three). So we knew the birds would be tick free for at least 21 days. When combined with burrow spraying, we expected a dramatic reduction in tick infestation levels in treated birds.

# Appendix 7.1 Estimation of parasitaemia

**Reagents:**

- Giemsa stain
- 99% Ethanol
- Microscope with x100 oil immersion lens and 10x10 grid eyepiece

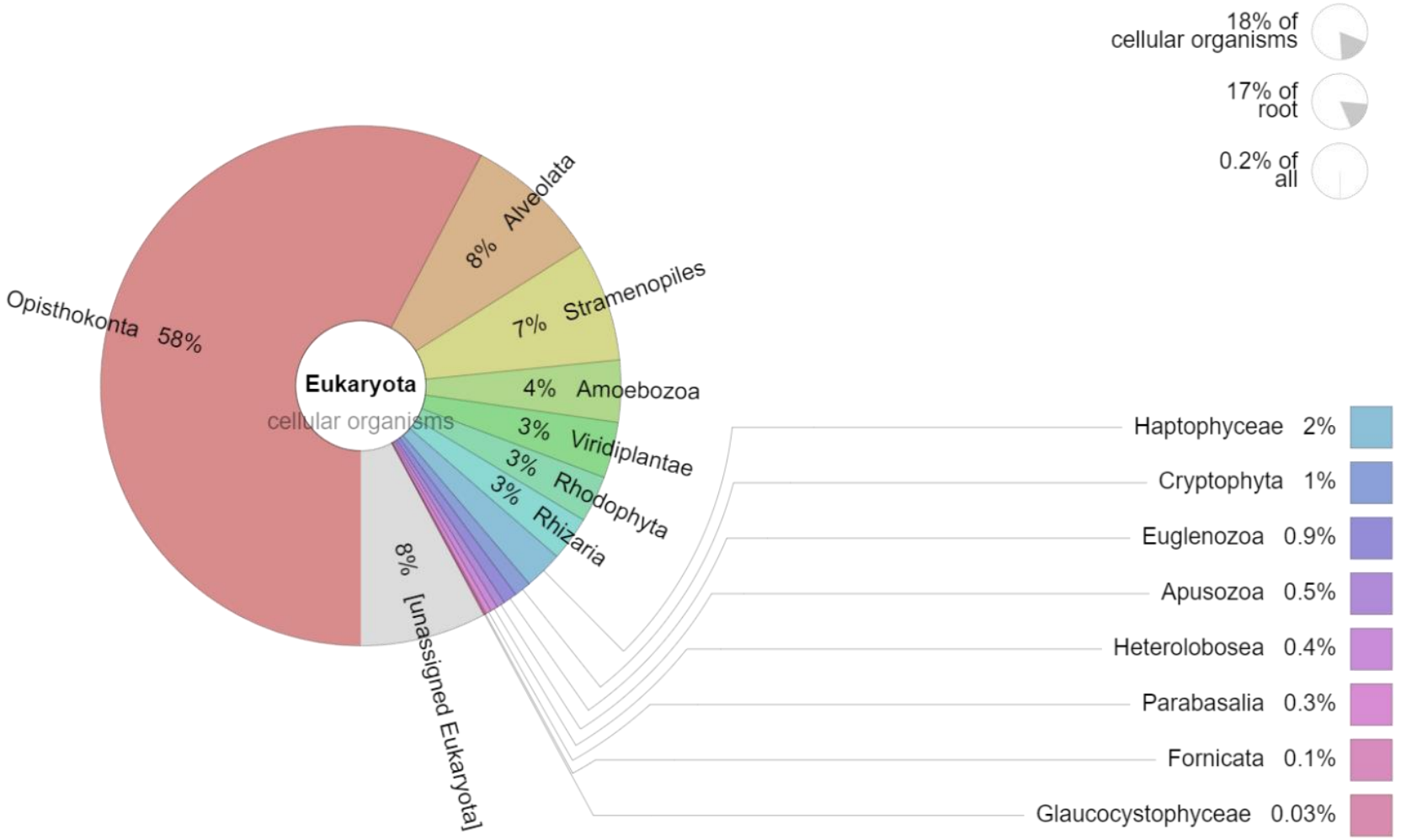
**Protocol: Giemsa Staining of Blood Smeared Slide**

1) Fix slides in 99% methanol for one minute and rinse off in tap water.
2) Make up a fresh solution of 10% Giemsa stain by mixing 9ml phosphate buffer solution with 1ml Giemsa stock.
3) Stain for 45 minutes.
4) Rinse off slide in tap water and air dry.

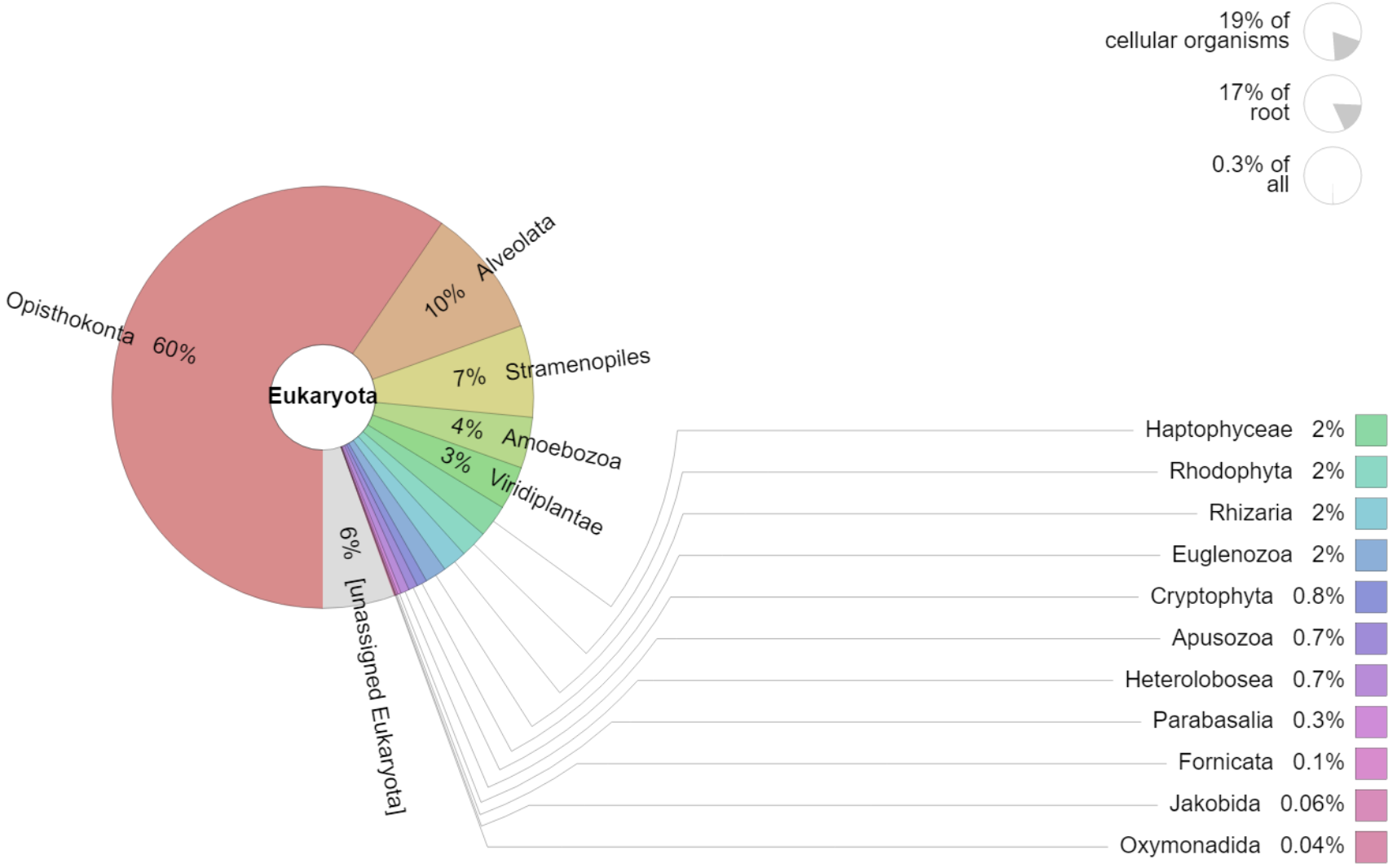
**Estimation of Parasitaemia**

View slide under 40/60x and when an infected cell is seen, look at the slide under oil immersion with a 100x objective to confirm. Estimate parasitaemia by counting the number of infected cells per 100 red blood cells. Repeat 3 times and take an average.

**Appendix 7.2 The diversity of species distribution after Metagenomics sequencing from PCB birds at the Eukaryote level visualised using a Krona chart**



**Appendix 7.3 The diversity of species distribution after Metagenomics sequencing from Ponui kiwi at the Eukaryote level visualised using a Krona chart**



**Appendix 7.4 The diversity of species distribution after Metagenomics sequencing from kiwi ticks (engorged and unengorged) at the Eukaryote level visualised using a Krona chart**