Tabanid flies and potential transmission of *Trypanosoma evansi* in Queensland

A thesis submitted by

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in the
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and the
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STATEMENT ON THE CONTRIBUTION OF OTHERS

My postgraduate supervisors Dr Lee Skerratt (Principal supervisor), Prof Rhondda Jones and Dr Graham Burgess (Co-supervisors) made major contributions to the scope and design of this research. Some contribution in this regard was also made by Dr Kirsty van Hennekeler who had previously conducted some research on aspects of the ecology of tabanids in North Queensland. My supervisors also made valuable suggestions in the preparation of this thesis and articles for publication.

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DECLARATION OF ETHICS

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the Joint NHMRC/AVCC Statement and Guidelines on Research Practice (1997), the James Cook University Policy on Experimentation Ethics, Standard Practices and Guidelines (2001), and the James Cook University Statement and Guidelines on Research Practice (2001). The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review Committee (Approval number A1176)

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ABSTRACT

Australia has quarantine policies and surveillance activities considered to be among the best in the world with regards to many exotic pests and diseases, but it has very low levels of preparedness against surra, a deadly infectious disease of animals caused by the blood-borne protozoan parasite *Trypanosoma evansi*. The country is currently free of surra, but the disease is endemic to many countries in Southeast Asia, some of whose geographical proximity to northern Australia is a cause for concern. The parasite has a very wide host range and is transmitted mechanically through the bite of blood-feeding tabanid flies, particularly those species which have a tendency to switch hosts during a blood meal. Because Australia has many tabanid species and many potential host animals, surra is recognized as a major threat to local livestock industries and wildlife. Previous experimental studies have indicated that infection with *T. evansi* can cause acute disease and high mortality in marsupials. Consequently, surra is one of the priority exotic diseases listed on the North Australia Quarantine Strategy (NAQS) program. However, the major limiting factor for Australia’s biosecurity plans and disease preparedness with regards to surra is the lack of information on the transmission dynamics and relative impact among the various animal species in northern Australia because little is known about the ecology of available tabanid species and nothing is known about their vectorial capacity, host preferences and host range.

The major aims of this research project were therefore to evaluate and compare the vectorial potential of the various tabanid species among some of the major animal species in north Queensland, as well as evaluate the flies’ ecological preferences and responses to current surveillance techniques. Successful mechanical transmission of surra depends to a large extent on the blood feeding behaviour of the tabanid species attracted to particular host animals and the responses of the animals to attack by tabanid flies. Because the pathogen survives for a relatively short period on tabanid mouthparts, interrupted feeding is considered to be the most important single factor responsible for the role of tabanids in mechanical transmission. Therefore the relative vectorial potential of the different tabanid species was estimated by assessing the feeding success, feeding duration and feeding frequency of the most abundant tabanid species in the study area, using direct field observations on horses, pigs and kangaroos as well as using electrocuting nets around horses. Tabanid host preferences were evaluated by
serological analysis of trap-caught tabanids using ELISA to identify blood meals originating from cattle, horses, pigs and macropods, which are among the most abundant potential hosts in north Queensland.

Twelve tabanid species were identified during the studies, and the most abundant were *Pseudotabanus silvester* Bergroth, *Tabanus pallipennis* Macquart, *T. townsvilli* Ricardo, *T. dorsobimaculatus* Ricardo and *T. strangmannii* Ricardo. Their feeding behaviour varied with fly species and host species, and the findings predict that some species such as *T. pallipennis* will be better vectors, while others such as *P. silvester* are unlikely vectors. Surra could infect all host types studied and pigs are likely to be potential reservoirs of the infection, while macropods are a key host type for most tabanid species and probably face the highest risk in the event of surra incursion. The highest frequency of host blood identified in tabanids was from macropods (61-80% in five tabanid species) even in locations where estimated densities of macropods were relatively lower than other potential hosts. Therefore the prevalence of large populations of wallabies and feral pigs in northern Australia is an important risk factor for the rapid spread of surra and would present a major challenge for effective control of the disease.

Assessment of current surveillance techniques showed that octenol-baited Nzi traps performed well at indicating the presence of tabanid species in the area, could reliably predict the activity of some tabanid species on animals, but were not reliable for other tabanid species. Trapping tabanids in different types of habitat for two consecutive wet seasons showed that most tabanid species preferred savannah woodland to open grassland, although *T. pallipennis* maintained relatively high populations in both types of habitat while *P. silvester* was only found in woodland areas. Woodland areas proved to be the better habitat type for routine surveillance.

The findings from these studies facilitate much better prediction of the risk of surra incursion and form a basis for design of rational surveillance, quarantine and intervention strategies. Predictive risk models for surra can now be based not only on general abundance of tabanids, but also on the relative risk to specific hosts and relative vectorial capacity of different tabanid species, hence making the models more reliable. Available options to minimise attack by tabanids and transmission of surra among domestic animals are discussed, and the urgent need to develop strategies to protect the marsupials of Australia is highlighted.
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CHAPTER ONE

GENERAL INTRODUCTION

1.1. RATIONALE

Animal trypanosomosis (also called surra) caused by the protozoan parasite Trypanosoma evansi is widely distributed, and occurs in parts of Africa, America, Europe and Asia (Barnard, 2003; Dagantes et al., 2009). The disease causes significant morbidity and mortality among livestock (Lun et al., 1993; Jones et al., 2005) and can also affect wildlife (Veer et al., 2002; Herrera et al., 2005). Virtually all mammalian species investigated, except humans, have proved to be susceptible (Reid, 2002). Surra is mainly transmitted mechanically during blood-feeding by female tabanid flies which are carrying the parasite on their mouthparts (Dielman, 1986; Herrera et al., 2005). Tabanids, which are found worldwide (Mackerras, 1954) may also transmit other animal pathogens such as viruses, bacteria and helminthos (Krinsky, 1976; Foil, 1989).

The Australasian region has at least 400 species of tabanids (Daniels, 1989), and surra is endemic to most of the countries in southeast Asia (Luckins, 1988; Holland et al., 2004) (Fig 1.1) where it is an important constraint to the productivity of smallholder livestock (Lun et al., 1993; Dagantes et al., 2009). The disease is believed to have been introduced into southeast Asia in livestock imported from India (Hoare, 1972). Surra also poses a threat to livestock and native fauna in Australia where it is currently absent, but the risk of an incursion from Indonesia is high (Thompson, 2001; Reid, 2002). Tabanids are ubiquitous in Australia and there are large populations of potential reservoir hosts (Thompson et al., 2003), hence surra has the potential to become endemic in most parts of the country. This could have a devastating effect on livestock enterprises, equine industries and biodiversity of Australia (Reid 2002; Sandeman and Warner, 2002), which is a major exporter of livestock commodities and major tourist attraction because of its unique fauna.

To safeguard against surra outbreaks in Australia, Sandeman and Warner (2002) emphasise the need to maintain effective border protection and to develop early warning
systems as well as containment strategies to prevent its spread. Therefore surra has been placed as high priority on the list of the Northern Australia Quarantine Strategy (NAQS) (AFFA, 2004; Thompson et al., 2003), and recently there have been increased efforts to develop highly sensitive diagnostic tools (Reid and Copeman, 2003) as well as predictive models for surra based on environmental factors and tabanid distribution in the northern areas of Australia (van Hennekeler, 2007). The major limiting factor in the biosecurity and disease preparedness efforts is that there are so many tabanid species in northern Australia (van Hennekeler, 2007), whose vectorial capacities among the many species of potential hosts have not been investigated (Thompson et al., 2003).

As with any vector-borne disease, the capacity for prevention, management and prediction of surra relies on a clear understanding of the interaction among host, vector, parasite and the environment. However, there is little knowledge of the feeding patterns and host-oriented behaviour of tabanid flies in general compared to other haematophagous insects (Gibson and Torr, 1999), although a few studies have been undertaken in parts of North America (Mullens and Genhardt, 1979; Foil et al., 1985), South America (Barros, 2001; Ferreira et al., 2002), Africa (Hafez et al., 1970; Phelps and Holloway, 1990; Desquesnes and Dia, 2003), Europe (Kneipert, 1980; Konstantinov, 1995; Krcmar et al., 2005) and Asia (Manresa and Mondonedo, 1935). No such studies have been conducted in Australia, and therefore there is no information on the relative importance of the different tabanid species in terms of their potential for surra transmission.

A comprehensive consolidation of most of the early tabanid work in Australia was published by Mackerras in a series of papers between 1954 and 1971 with a view to ‘enable workers in the field to recognize their species and thus serve as a foundation for future research’ (Mackerras, 1956). The work focused on the taxonomy and distribution of Australian tabanid species with very few notes on their behaviour. Mackerras (1956) expressed surprise that ‘while the Australian workers were energetic collectors……they wrote so little about what the insects did.’ It was still recognised however, that ‘the habitats and behaviour of the adults vary greatly in the different groups’ (Mackerras, 1956), but no specific studies were conducted. In 1959 Mackerras admitted ‘we do not know enough about host preferences to relate the distribution of the flies to the occurrence of animals on which they feed.’ Further published work on the ecology of Australian tabanids in the 1970s was based on anectodal evidence obtained in pursuit of
other objectives (Spratt, 1972; 1974). This lack of information continued for a long time, until recent attempts to address this problem started with ecological studies looking at the effects of climate and weather (Van Hennekeler, 2007; Van Hennekeler et al., 2010) in northern Queensland.

This project aimed to close this glaring information gap by investigating Australian tabanid species to understand their feeding behaviour, host preferences, abundance and distribution among different habitat types, and responsiveness to traps. These are essential components for the formulation of rational strategies for prevention, surveillance, prediction and management of surra in northern Australia where the risk of incursion is considered to be highest (Thompson et al., 2003; van Hennekeler et al., 2008).

1.2. BACKGROUND INFORMATION

The parasite *Trypanosoma evansi* and the disease surra

*Trypanosoma evansi* (Trypanosomatidae, Kinetoplastida) has the largest geographical distribution and the widest range of hosts compared to other pathogenic trypanosomes (Dagantes *et al.*, 2009). In susceptible animals the disease is manifested by pyrexia directly associated with parasitaemia (Fig 1.2), together with progressive anaemia, loss of condition and lassitude (OIE, 2004). The clinical signs of surra in different animal species are well-reviewed by Dielman (1986), and some of the most susceptible animals are horses, cats, dogs and camels with observed mortality rates as high as 100% (Hoare, 1972; Dielman, 1986). Sheep, goats and pigs are not very susceptible and seldom show clinical signs when infected, while the disease is usually chronic in cattle and buffaloes where mortality rates are generally low (Dielman, 1986), but production losses are high (Lun *et al.*, 1993; Reid, 2002). Throughout its geographical range, surra was introduced as a parasite of domesticated livestock and any wildlife infections were acquired secondarily: the disease is often fatal in wildlife (Clark and Dunn, 1933).

Among the pathogenic trypanosomes, *Trypanosoma evansi* is the only one that does not undergo cyclic development in an insect vector, and is transferred mechanically from host to host on the mouthparts of biting insects without any multiplication of the parasite or any biological association between parasite and vector (Desquesnes *et al.*, 2009). *T. evansi* belongs to the *T. brucei* group, and is thought to have been derived from *T. brucei brucei* by the loss of the maxicircles of the kinetoplastic mitochondrial DNA, which would be responsible for its inability to undergo a cyclic development in tsetse flies (Lun and Desser, 1995; Desquesnes *et al.*, 2009) or in any insect. All attempts to demonstrate the cyclic development of *T. evansi* have failed (Hoare, 1972; Krinsky, 1976). This loss of capacity for biological transmission and reliance on mechanical transmission possibly accounts for the broad geographical distribution of *T. evansi* (Lun and Desser, 1995), since transmission is no longer restricted to a particular vector species. Consequently, many biting insects have been suspected of transmitting surra, but there is now overwhelming evidence and general acceptance that *T. evansi* is mainly transmitted mechanically by various species of tabanid flies (Foil, 1989; Reid,
To a lesser extent, ingestion of meat from infected carcasses by carnivores can also result in infection (Franke et al., 1994; Luckins, 1998).

Tabanid flies are also capable of mechanical transmission of other trypanosome species such as *T. vivax* (Desquesnes and Dia, 2004). *T. vivax* still has the capacity to be cyclically transmitted by the tsetse fly, but transmission has been sustained mechanically by tabanids in areas where tsetse are absent, such as Latin America (Gardiner & Wilson, 1987) and parts of Africa (Roeder et al., 1984; Rahman, 2005).

Many studies have been directed at understanding the pathology, diagnosis, and treatment of *T. evansi* infection (Dielmen, 1986; Franke et al., 1994; Silva et al., 1995; Reid and Copeman, 2003; Dagantes et al. 2009) and consequently, control of surra is mainly based on trypanocidal treatment of individual animals that exhibit signs of disease or parasitological evidence of infection (Luckins, 1998), and to a lesser extent the use of prophylactic drugs to prevent infection (Dielmen, 1986). However, no program to prevent and control any arthropod-borne disease anywhere in the world has ever been successful or sustainable without good understanding and some control of the vector (Gubler, 1998). Heavy dependence on drugs is likely to remain for some time, but there have been various problems with drug efficacy and availability in some Asian countries (Reid, 2002; Jones et al., 2005). Suggestions for integrating vector control into the system have been considered in Indonesia, but ‘at present there is very little information on vector (behavioural) ecology that could be used as an alternative or part of integrated control regimes’ (Jones et al., 2005). So the paucity of information on potential vectors of surra is not unique to Australia, and the potential consequences of such ignorance are not confined to Indonesia.

![Image of Trypanosoma evansi](source: www.vet.uga.edu)

**Fig 1.2.** *Trypanosoma evansi* among host blood cells (Source: www.vet.uga.edu)
Tabanid distribution and general ecology

Tabanid flies belong to the family Tabanidae within the Order Diptera of the Class Insecta (Hill and MacDonald, 2007). At least 4000 species of tabanids have been described (Foil, 1989) and the Australasian region supports just over 400 of them (Daniels, 1989), of which about 200 species belonging to 20 genera occur in Australia (Mackerras, 1956; Elliot, 2005). There are four recognised subfamilies in the Tabanidae and three of them are present in Australia, that is Pangoniinae, Chrysopinae and Tabaninae (Mackerras, 1956; Daniels, 1989) although the highest percentage of species belongs to the Tabaninae (Mackerras, 1956). Subfamily Tabaninae is comprised of tribes Haematopotini, Diachlorini and Tabanini, of which the last two occur in Australia (Mackerras, 1954) (Fig 1.3). Worldwide, some of the best-known and widely-distributed tabanid genera include Haematopota, Hybomitra, Chrysops and Tabanus, but in Australia some of the common genera also include Pseudotabanus, Dasybasis, Lilaea, Sceptia and Cydistomyia (Mackerras 1954, 1955, 1959, 1971). In many parts of the world Chrysops are commonly known as deerflies (Squitier, 2003) and the rest are collectively referred to as horse flies (Mackerras et al., 2008), but in Australia they are also called March flies (Strother, 1999). Tabanids are also known as gadflies or clegs in parts of Europe (Konstantinov and Uli’anov, 1988; Yuval, 2006).

The distribution of tabanid species is intriguing in that most infested areas studied around the world had at least 10 species at the same location in the same season (Hayakawa et al., 1988; Foil et al., 1991; Ralley et al., 1993) and at times more than 20 (Phelps & Vale, 1976; Barros, 2001; Krcmar et al., 2005), and yet individual species are generally confined to a particular region or country. For instance, none of the species in the listed studies have been recorded in more than one continent, and among the multitude of species which occur in Australia (Mackerras, 1954, 1956, 1971; Mackerras et al., 2008) just a few have been recorded in neighbouring Papua New Guinea and Solomon Islands (T. pallipennis, T. dorsobimaculatus, T. innotabilis and T ceylonicus) and only one in Indonesia and Sri Lanka (T. ceylonicus) (Mackerras, 1971; Daniels, 1999; van Hennekeler, 2007). While so many species have been described in various places, most are rarely encountered and relatively few are common. For example, the north-eastern part of Australia supports the largest number of species in the country (Mackerras, 1971) and yet recent surveys in the Cape York Peninsula over a two-year
period by van Hennekeler (2007) identified 38 species of which only 12 were considered abundant. Similarly, Smith et al. (1970) collected 36 tabanid species in a study in Ontario but ‘relatively few were sufficiently abundant to constitute serious pest problems’.

The ecological preferences of tabanid flies are very diverse, as demonstrated by their broad geographical distribution which only excludes extreme northern and southern latitudes such as Greenland, Iceland and Antarctica (Strother, 1999; Squitier, 2003). However, the localised nature of most species indicates that most are climatic specialists. For instance in Australia species dominant in the tropical northern regions are different from species found in the more temperate southern regions (Mackerras, 1959, 1971). Most locations also have strong seasonal patterns of tabanid abundance: wherever there are seasonal fluctuations in rainfall and temperature, the appearance of adult tabanids is seasonal (Mackerras, 1959, 1971; Dale and Axtell, 1976; Barros, 2001; Krcmar, 2005), and Hafez et al., (1970b) have demonstrated the importance of temperature and humidity on the development of T. taeniola in Egypt. In most areas various tabanid species usually appear in succession during the flight season (Barros, 2001; Krcmar, 2005; van Hennekeler, 2007), and sometimes the sequence is consistent and predictable. On a smaller spatial scale, abundance of different tabanid species varies between vegetation types (Phelps and Vale, 1976; Dale and Axtell, 1976; Hayakawa et al., 1986; Barros, 2001). Habitat preferences of this kind may be associated with variation in larval habitat requirements among species (Lane, 1976) Hayakawa et al., 1986, 1988). Habitat type has been observed to affect the distribution of some Australian tabanids in southeast Queensland (Spratt, 1974), and therefore the present project included an examination of the species associated with different habitat types in northern Queensland.
Fig 1.3. World distribution of the three tabanid tribes in the subfamily Tabaninae, showing the presence of tribes Diachlorini and Tabanini in Australia. (redrawn from Mackerras, 1954)
Tabanid reproduction and life cycle

Like all dipterans, the tabanid life cycle involves complete metamorphosis, including egg, larva, pupa and adult stages (Barnard, 2003) (Fig 1.4). Mating normally occurs soon after emergence of adults and usually precedes the female’s first blood-meal (Taylor et al., 1992). Eggs are laid within 3-5 days of ingesting a full blood-meal (Hafez et al., 1970a), and are deposited in clusters which vary in size among species and within species and may range between 100 and 1000 eggs per batch (Foil and Hogsette, 1994). The egg masses are deposited on various substrates but most often on vegetation. Suitable vegetation includes grass blades (as with Pseudotabanus silvester, Elliot, 2005) and foliage overhanging different forms of larval habitat, which include water, mud, moist soil and sand (Strother, 1999). The diversity of oviposition sites is amazing, and Taylor et al. (1992) observed egg masses of T. gibensis attached to the underside of a bridge in Ethiopia. After 2-7 days, depending on ambient weather conditions, the eggs hatch and the larvae drop off onto the substrate below (Squitier, 2003).

While tabanid larvae are predominantly aquatic or semi-aquatic, some species are terrestrial and can develop on the forest floor (Lane, 1976; Hayakawa et al., 1988). Many species are associated with freshwater habitats but some breed in coastal salt marshes (Dale and Axtell, 1976; Meany et al., 1976). In Australia, the larvae of most Tabanus and Mesomyia (Lileaea) are aquatic, but Dasybasis oraria breeds in the sand of sea beaches and the larvae of Scaptia muscula were found in dry sand, while some species of Cydistomyia breed in rot-holes of Casuarina trees (Mackerras, 1956). The larvae of Chrysops feed upon organic matter in the soil, but most other tabanid larvae are predators of earthworms, crustaceans and larvae of other insects (Elliot, 2005), and they can also be cannibalistic (Hafez et al., 1970b; Mackerras, 1971; Meaney et al., 1976). There are 6-13 larval instars (separated by moults) depending on the time of year in which the eggs were deposited and the conditions under which they live (Hafez et al., 1970b), and usually the mature larva enters into hibernation until environmental conditions are suitable for further development, adult survival and reproduction (Hafez et al., 1970a); although in some species any of the instars is capable of switching to the prolonged state of dormancy (Mackerras, 1971). All tabanids overwinter as larvae, and the larval stage can last anything between a few weeks (for multivoltine species) and three years (Mackerras, 1971; Ellis and Hays, 1973; Lyon, 2000).
The pupal stage is very short, lasting only 1-3 weeks before adults emerge (Strother, 1999). Adult life span is 30-60 days (Squitier, 2003). One female *Tabanus taeniola* can take up to six full blood-meals and deposit six batches of eggs in a period of 31 days at a constant temperature of 27°C (Hafez et al., 1970a). Most tabanid species have one generation per year, although some may have two or three (Apperson and Waldvogel, 2001). There is very little published information about the early stages of Australian tabanids and ‘this is a reflection, not so much of lack of interest, as of the difficulty workers have experienced in finding the larvae and pupae, and no-one has yet succeeded in inducing any of our species to lay viable eggs in captivity’ (Mackerras, 1956). The situation has not changed since that comment by Mackerras.

**Fig 1.4.** Generalised life cycle of tabanids. Modified from Scott Charlesworth, Purdue University.  www.extension.entm.purdue.edu
Tabanid feeding habits and transmission of disease

Adult male and female tabanids feed on nectar and pollen from flowers but the females of most species are also blood-feeders (Lane and Daly, 1980; Daniels, 1989), because they require protein for egg development (Taylor and Smith, 1989). In Australia many tabanid species are known to particularly like the blossoms of *Leptospermun, Melaleuca* and *Grevillea* (Mackerras, 1959; Elliot, 2005). Among the blood-feeding species of the genus *Tabanus* in Australia, general observations indicated that man was not a preferred host, but many species preferred domestic stock (Mackerras, 1971). Typically, tabanids feed outdoors and are normally active during daytime (Hill and MacDonald, 2007), although collections of a few species from light traps in Uganda (Corbet, 1970) and a few specimens of *T. innotabilis* and *Cydistomyia clavicallosa* with similar traps in Australia (Mackerras, 1956) have suggested a level of nocturnal flight activity among some species.

The mouthparts of haematophagous female tabanids have well-developed saw-like mandibles which the flies use, unlike other biting insects, to slash and cut open host skin as well as lacerate blood vessels, causing blood to pool before they lap up the blood with sponge-like labellae (Squitier, 2003). Blood ingested by some species is nearly equal to the weight of the fly itself (Hafez *et al.*, 1970a), but bite wounds can ooze quantities of blood which may exceed the amount ingested (Lane and Daly, 1980). In general, tabanid flies are aggressive feeders, their painful bites to the host cause defensive reactions that disturb the flies so that they must attack again and again, often choosing other hosts, in order to complete their blood-meal (Luckins, 1998). This form of feeding enables transmission of pathogens among hosts. In some cases, loss of blood may be significant (Mohamed-Ahmed and Mihok, 2009) and the irritation caused by intense attack by tabanids may cause animals to stop grazing and bunch-up or seek shelter, which may reduce weight gain and milk production (Ralley *et al.*, 1993).

Tabanids are capable of spreading at least 25 different animal infections (Krinsky, 1976), of which a few are transmitted biologically (also called cyclic transmission) while most are transmitted mechanically. In Australia some species of *Tabanus*, *Dasybasis* and *Lilaea* are involved in the biological transmission of the filarioid nematode, *Pelecitus roemerii*, found in the intermuscular connective tissue of kangaroos.
and wallabies (Spratt, 1974; Spratt & Varughese, 1975). Among the mechanically transmitted infections, *T. evansi* has been extensively studied in different countries in the twentieth century and the evidence incriminating tabanids as vectors is conclusive (Foil, 1989). Comprehensive reviews of studies on the experimental transmission of *T. evansi* by various tabanid species and other biting flies are given by Krinsky (1976) and Luckins (1998), and more than 20 different species of *Tabanus* and a few species of *Chrysops* and *Haematopota* have been shown to transmit *T. evansi* under experimental conditions. Other biting insects tested apart from Tabanidae are the stable flies *Stomoxys* spp., with success rates lower than tabanids (Mitzmain, 1912; Mihok *et al.*, 1995; Luckins, 1998) and their role is still somewhat controversial (Reid 2002). Very few studies have been undertaken on tabanid flies other than *Tabanus* spp. and hence their role is not well documented. Some are thought to have low efficiency as vectors under natural conditions because they generally complete their blood-meals on a single animal (Luckins, 1998), although there is not much data to support this. However, even with *Tabanus* spp., most laboratory transmission trials have been done with tabanid species that readily feed when they are captured and brought into the laboratory. These, mostly tend to be naturally persistent feeders that are reluctant to switch hosts (Foil, 1989). Therefore there is need for field-based behavioural studies to verify the conclusions such as those conducted in this study. The less persistent tabanids on an individual host are probably the most important in mechanical transmission (Foil, 1989). Some of the most abundant tabanids in Australia include both *Tabanus* and non-*Tabanus* species, and their potential role is yet to be evaluated. The only previous surra outbreak in Australia occurred in 1907 when the infection was diagnosed among 500 imported camels at Port Hedland after arrival from Pakistan (Cleland, 1907). All the infected animals were culled and surra has not been recorded in the country since then.

**Important factors in mechanical transmission of surra**

Tabanids exhibit various adaptations related to blood-feeding which, in addition to enhancing the chance of fly survival and reproduction, increase the probability of transmission of disease agents from one animal to another (Krinsky, 1976). The adaptations are described as:

1. **Autogeny**, that is, the requirement of a blood-meal for maturation of eggs. This requirement stimulates host-seeking behaviour.
2. Telmophagy, that is, feeding from a pool of blood after cutting through the skin of an animal. This allows pathogens in superficial tissues and peripheral blood to drain into the pool of tissue fluids and be imbibed by the feeding fly.

3. Large blood-meal, which increases the probability that the fly will imbibe the etiologic agent from an infected host

4. Long engorgement time, which increases the duration of exposure of the fly to infected tissue fluids

5. Interrupted feeding, which results in host-switching before completion of a blood-meal. This is described as the most important single factor responsible for the role of tabanids as efficient mechanical vectors (Mackerras et al, 2008). The number of interruptions experienced by a species before repletion also affects the probability of a tabanid to act as a mechanical vector among many hosts (Krinsky, 1976).

Foil (1989) explains the last factor (interrupted feeding) as a series of events that lead to mechanical transmission as follows:
First, the vector initiates feeding upon an infected host; second, the vector’s feeding is interrupted; third, the vector moves to a susceptible host, transporting the agent on or within the body parts that routinely contact the host, and; fourth, the vector feeds upon the susceptible host and introduces the agent via a wound created by the fly. Therefore the successful mechanical transmission of *T. evansi* by tabanids depends on the behavioural interaction between the vector and the host. This was therefore a major focus of my study.

The other factors discussed by Foil (1989) include vector mobility, the proximity of infected and susceptible hosts, host defensive movements when attacked by tabanids, the quantity of blood-meal residue that remains on the mouthparts following an interrupted feed, and the overall abundance of tabanids prevalent in an area. Vector mobility and the proximity of hosts are significant because *T. evansi* is not environmentally resistant and survival periods on the flies’ mouthparts are short. In general, there is a 4-5% chance of transmission if feeding on the recipient host takes place within an hour of the infective feed, but only about 0.1% after three hours (Luckins, 1998).
The factors above are sometimes used to describe the family Tabanidae as a whole, but this is misleading since ‘the Tabanidae is as diverse as any other Dipteran family, including the Culicidae’ (Foil, 1989). Such generalisations lead to serious misconceptions and are not helpful if practical surveillance and control strategies are to be developed. Considerable research is required to determine the relative efficiency of different vector species in ensuring successful mechanical transmission as well as the effect of the host species (Luckins, 1998).

Further reviews of literature relevant to various aspects of this study are included in the Introduction and Discussion sections of each chapter.

1.3. STRUCTURE OF THESIS

The lack of previous research on the potential role of Australian tabanid species for transmission of surra necessitated that the research investigate all the species that were available in reasonable quantities during the study period. The potential roles of different tabanid species were compared on the basis of the species abundance and blood-feeding behaviour on different hosts under natural conditions. The tabanids were evaluated against some of the key parameters for mechanical transmission described by Krinsky (1976) and Foil (1989) as discussed above. The project also evaluated whether catches from odour-baited stationary traps correctly represented the activity levels of tabanids around live animals; and further investigated the abundance and distribution of the various tabanid species among different habitat types.

In this thesis, materials and methods that are applicable to multiple chapters are described in Chapter Two and referred to in the relevant chapters where the specific study designs and analytical approach are covered. In general, aspects of host-vector interaction are addressed in Chapters 3-7 and aspects of trap performance and tabanid ecology in Chapters 8-9.

Chapter Three describes how the feeding success of various tabanid species was evaluated using electrocuting nets. Species that failed to complete their blood meal on a single host were considered to be more efficient mechanical vectors.
Chapter Four describes direct observations of tabanids naturally alighting and feeding on horses, kangaroos and pigs. Through these observations more information on tabanid feeding success was obtained, and the frequency of feeding attempts by different species to achieve a complete blood meal was evaluated. This helped to estimate the number of hosts potentially exposed to infection by individual flies during a feeding cycle. The responses of each host to attack by the horse flies could potentially contribute to interrupted feeding by the tabanids, and this was also investigated in this chapter.

Further investigations of the feeding pattern of the different tabanid species focused on determining the host-pREFERENCES OF THE DIFFERENT TABANID SPECIES AND THEREFORE PREDICTING WHICH ANIMAL SPECIES WERE MOST AT RISK OF Catching surra. This was done by identification of the origin of blood-meals in trap-caught tabanids using ELISA.

Chapter Five describes the process of optimisation of the assays to identify blood residues originating from four selected types of potential tabanid hosts.

Chapter Six covers the analysis of field samples that had been captured from a range of locations within the study area where various potential hosts were available. The chapter also describes an experiment to test detectability of blood traces at various levels of digestion within the tabanid abdomen.

Chapter Seven describes experiments to investigate the host-switching behaviour of tabanids using the mark-release method. Distances between two horses were to be varied and movement of marked flies between the animals recorded, and this chapter explains the challenges faced in this endeavour, the useful bits of information gleaned and the lessons learnt in the process.

In Chapter Eight, the relationship between tabanid collections from stationary traps and tabanid activity around horses was investigated among various tabanid species to evaluate whether catches from odour-baited stationary traps correctly represented the activity level of tabanids around live animals, which was estimated using electrocuting nets. This was an important validation step for interpreting trap catches in Chapter Nine.
Chapter Nine examines the abundance and distribution of the various tabanid species between two types of habitat common in tropical Queensland, i.e. savanna woodland versus open grassland, using odour-baited traps over two wet seasons. The study, which is critical in guiding surveillance strategies, also evaluated whether tabanid densities were affected by distance from rivers and creeks, presumably the preferred breeding sites of most of the species.

Lastly, in Chapter Ten a general discussion summarises the findings of the whole project in the context of the predicted roles of the various tabanid species in surra transmission among the available host species in the event of an incursion into northern parts of Australia. The Chapter offers some key recommendations on tabanid surveillance and risk assessment for surra in order to assist in quarantine efforts and disease preparedness, as well as suggest options for integrated disease management.

Most of the information presented in Chapters Three, Four, Six and Eight had been published in international peer-reviewed journals prior to submission of this thesis. Copies of the published papers are attached in the Appendix.
CHAPTER TWO
GENERAL MATERIALS AND METHODS

2.1. Introduction

Materials and methods that are common to multiple chapters are described in this chapter. Methods specific to single chapters are described there.

2.2. The Nzi trap

A significant part of the field studies involved collection of adult tabanids from their natural habitat using Nzi traps (Mihok, 2002) baited with 1-octen-3-ol (Mihok et al., 2007; Krcmar et al., 2009).

The Nzi trap (Fig. 2.1) is an effective method for sampling Australian tabanid species especially when baited with 1-octen-3-ol (hereafter called octenol) and/or CO₂ (Van Hennekeler et al., 2008). However, CO₂ is not appropriate in lengthy field activities due to lack of feasible methods for dispensing the chemical over long periods and hence octenol is the more widely used bait.

The traps were constructed as described by Mihok (2002), with same fabric and netting materials that were used by Van Hennekeler et al., (2008), that is, Sunbrella® Pacific Blue, Sunbrella® Black (Glen Raven, NC.) and white polyester mosquito mesh. Octenol was dispensed from a 100 ml plastic vial with a cotton wick protruding through a 3mm hole drilled on the bottle cap. The plastic vial was secured to one of the corner posts of the trap at about 1m above the ground, and dispensed octenol (commercial reagent grade, 98%, from Sigma-Aldrich, USA) at ~0.5 ml / day.

The type of collector bag affixed to the trap depended on the objective of a particular study: specially-designed cages (Fig 2.1B) were used as collectors when captured tabanids were required to be alive and healthy, while ‘normal’ collapsible collector bags
A wide range of traps have been used in various countries for field sampling of tabanids (Hall et al., 1998; Sasaki, 2001; Kremar et al., 2005). In Australia Van Hennekeler et al. (2008) showed that the Nzi trap consistently caught more tabanids and more species of tabanids than canopy traps. The Nzi trap also proved to be more robust and required less maintenance in the field than the canopy trap (van Hennekeller et al., 2008), and had previously captured more *Tabanus* spp. and *Chrysops* spp. than the Manitoba trap (Mihok and Carlson, 2007). The trap is also known to catch large numbers of other types of biting flies including tsetse flies *Glossina* spp. and stable flies *Stomoxys* spp. (Mihok et al., 2007). When baited with appropriate odour attractants, the Nzi trap has also captured a wide variety of tabanid species in Kenya, Canada, USA (Mihok et al., 2006; Mihok et al., 2007) and Sudan (Mohamed-Ahmed et al., 2007).

Reference to the Nzi trap is made in Chapters 4, 6, 8, 9 and 10 of this thesis.
Fig 2.1. The Nzi trap (A). Type of trap collector used when live tabanids were needed (B); and ‘normal’ collector when there was no need to keep tabanids alive (C).
2.3. The ‘incomplete ring’ of electrocuting nets

Electrocuting nets (Vale, 1974; 1983; Packer and Brady, 1990), also called ‘E-nets’ (Torr et al., 2008) were originally developed to study the behaviour and ecology of tsetse flies, and have also been used for studies on stable flies (Stomoxys sp) (Schofield and Torr, 2002; Foil and Younger, 2006; Torr et al., 2006), mosquitoes (Knols et al., 1998; Torr et al., 2008) and to a lesser extent on tabanids (Phelps and Vale, 1976; Mohamed-Ahmed and Mihok, 2009). The study described here is the first time they have been used in Australia. The nets can be made in different shapes and sizes and arranged in different formats depending on study objectives (Vale, 1980; Torr, 1988; Lindh et al., 2009), and the more common design for investigating host-oriented behaviour is the ‘incomplete ring of E-nets’ (Vale, 1977; Odulaja and Mohamed-Ahmed, 1997; Vale et al., 1999; Dransfield and Brightwell, 2001; Torr et al., 2007) with an animal at the centre of the ring (Fig 2.2). This design has been used with remarkable success in understanding the feeding and host-oriented behaviour of tsetse in Africa (Vale, 1977; Hargrove, 1980; Baylis et al., 1994; Torr & Mangwiro, 2000; Torr et al., 2007), leading to the development of effective bait technologies for tsetse control (Vale et al., 1988; Mangwiro et al., 1999).

To study the abundance and feeding status of different tabanid species flying to and from a host, an adult horse was placed in an octagonal pen surrounded by an ‘incomplete ring’ (4 m radius) of six large electrocuting nets (Vale, 1974) following the method of Vale (1977). Each E-net was comprised of a rectangular aluminium frame supporting a panel of fine black netting sandwiched between two grids of at least 100 parallel electrocuting wires 0.2 mm thick and 8 mm apart. Each E-net (Bonar Industries, Harare) was 1.5m long by 2m high, and all six covered about 35 % of the total perimeter of the ring. The grids were powered by a high-voltage transformer (supplied by Early Warning Systems, Pietermaritzburg) (Fig 2.2) that converted 12V DC to ~40kV interrupted DC. The horse in the pen (3m radius x 2.5m high) was clearly visible through the E-nets (to the human eye at least), was supplied with hay during the study sessions and was free to move about. Some of the flies approaching or departing from the horse collided with the E-nets, were instantly electrocuted and fell onto grey corrugated Laserlite® sheets on the ground. Flies caught on the outside or the inside of the ring were presumed to be approaching or leaving the horse, respectively (Torr and
Hargrove, 1998; Vale, 1977). When the system was initially used on tsetse, most flies were not able to see the nets in time to avoid them (Vale, 1974; 1977; Packer and Brady, 1990).

This method is referred to in Chapters 3 and 7.

2.4. The study area

The study was conducted in and around the small rural city of Townsville (Australian Bureau of Statistics population estimate June 2008, 175,542 people) which is located in the dry tropical region of north Queensland, Australia (19 17’S, 146 48’E) (Fig 2.3). Rainfall is seasonal (Jones and Rienks, 1987), usually stretching between November and April, with an annual average of 1143 mm (van Hennekeler et al., 2008). A wide range of tabanid species are prevalent in the area (Mackerras, 1954, 1956, 1971; van Hennekeler et al., 2008). Adult tabanids generally start appearing within a few weeks after the onset of the rainy season and disappear by around May/June. Trapping locations are shown in Fig 2.3. Reference to the trapping locations is made in Chapters 6 and 9.
Fig 2.2. The ‘incomplete ring of electrocuting nets’ with a horse at the centre (A); and a closer view of an E-net showing the top corner (B), and connections to a high-voltage transformer (brown) and 12V battery (yellow)(C).
Fig 2.3. A – The study area (within the red square) in North Queensland. Map shows the distribution of *Tabanus* in Australia (redrawn from Mackerras, 1971). Each spot is a record of one species within the area enclosed by 1° of latitude and longitude. The line marked 25 is in the 25 inch rainfall isohyte.

B- Satellite view of the study area showing the distribution of four trapping locations (yellow) [images not to scale].
2.5. Tabanid identification

All tabanids captured with either traps or E-nets in the different studies were individually identified to species whenever possible. Most species in the study area are easily distinguishable from each other. They were identified using keys by Mackerras (1956, 1959, 1961, 1971), and confirmed by comparison with preserved specimens from the same region previously identified by Van Hennekeler et al., (2008). The presence and conspicuousness of ocelli on the frons and setulae on the basicosta as well as the shape of the callus and antennal segments are some of the major distinguishing features seen with a stereomicroscope. However some species have outstanding characteristic features such as body colours and abdominal banding patterns that can be distinguished accurately by an experienced observer even from a distance of a few metres. Some examples of species common in the study area are shown in Fig 2.4. Abdominal banding is distinct in species such as *Tabanus strangmannii*, *T. pallipennis*, *T. concolor* and *Pseudotabanus silvester*. 
Fig 2.4. Pictures of some of the tabanid species encountered in the study area. (Magnified to about double the actual size).
CHAPTER THREE

Tabanid feeding success and densities on horses evaluated with electrocuting nets*


3.1. Introduction

Tabanid flies are known to cause significant annoyance and blood loss to their hosts, and can also be mechanical vectors of many livestock pathogens, including equine infectious anaemia virus and Trypanosoma evansi (surra) (Foil, 1989). Pathogen transmission occurs through interruption of insect blood-meals, resulting in dispersal of partially-fed flies to other animals in order to complete the blood-meal (Foil et al., 1985). Once feeding is initiated, some tabanid species are more persistent at trying to complete a blood meal on the same animal and they show greater tenacity in the face of defensive movements by the host. Consequently the least persistent species are probably the most efficient mechanical vectors, and species that complete their blood meals on a single animal are likely to be less important (Foil, 1989; Luckins, 1998; Barros and Foil, 2007). Therefore an understanding of the interaction between different tabanid species and their hosts is essential in predicting the role played by each species in mechanical transmission. In general, there is little knowledge on host-oriented behaviour of tabanid flies compared with other haematophagous insects (Gibson and Torr, 1999), and this may be why current control measures against tabanid populations have little effect (Roberts, 1980; Barros and Foil, 2007).

Previous studies of tabanid activity on animals involved direct visual inspection and sometimes capturing alighting flies using hand-held nets or cups (Foil et al., 1985; Leprince et al., 1994; Krcmar, 2005; Muzari et al., 2010a), but in some cases repeated visits by the same fly could not be distinguished from new visits and the effect of the
presence of observers on behaviour of tabanids was unknown. Close proximity of humans to animals influenced the behaviour of tsetse flies (Vale, 1974; Hargrove, 1976) and some North American tabanid species (Hribar et al., 1992), although effects have not been seen with some African tabanid species (Vale and Phelps, 1974).

In this study I adopted a method that overcomes the limitations of the above studies by using electrocuting nets to intercept tabanids in flight around a horse (Vale, 1974; Torr and Mangwiro, 2000). The electrocuting nets have successfully overcome similar problems in tsetse (Torr and Mangwiro, 2000), stable fly (Schofield and Torr, 2002) and mosquito (Torr et al., 2008) research, and larger proportions of flies were unable to see the nets in time to avoid them (Packer and Brady, 1990; Griffiths and Brady, 1994). My study assessed the potential role of different Australian tabanid species for mechanical transmission of surra by evaluating whether flies managed to feed and to complete their blood-meals on a free-moving horse before flying away. This lets us identify those species most likely to contribute to surra transmission should the disease establish itself in Australia.

Additionally, the study evaluated the relative densities of each tabanid species around the horse, as this also affects potential for disease transmission and further indicates the level of annoyance and blood-loss suffered by the animal. During a previous study in Africa, a combination of interrupted feeding and high insect densities among animals was implicated in the successful mechanical transmission of T. vivax among cattle by the tabanid Atylotus agrestis (Desquesnes and Dia, 2004).

3.2. Materials and methods

Feeding status of tabanid species approaching and departing from a horse

To study the abundance and feeding status of different tabanid species flying to and from an adult horse, it was placed in an octagonal pen surrounded by an ‘incomplete ring’ (4 m radius) of six large electrocuting nets (Vale, 1974) following the method of Vale (1977), as described in Chapter Two. Flies caught on the outside or the inside of the ring were presumed to be approaching or leaving the horse, respectively (Vale, 1977; Torr and Hargrove, 1998). The E-nets covered about 35% of the circumference
of the ring. The horse pen used was located in the far corner of a generally unused paddock, at least 100m away from pig housing and from cattle areas.

Each study session lasted 2-4 hours between 09:00 h and 18:00 h, depending upon prevailing weather conditions and diurnal activity times of local tabanid species. During each study session a person made five-minute visits to the site at 30-minute intervals to collect into vials any twitching tabanids that had been stunned but not killed by the electric current. Dead tabanids were collected from the corrugated trays at the end of the session (Fig 3.1). A sticky deposit could have been applied to the trays to retain all flies (Torr et al., 2006), but this was not done because of the need to maintain the quality of specimens for dissection afterwards. Tabanids were later dissected under a stereomicroscope to examine the gut for presence of blood. On this basis, each fly was classed as unfed, partially-fed or fully fed. In partially-fed flies blood was visible after dissection, but the abdomen was not distended as is characteristic of fully-engorged tabanids (Muzari et al, 2010a).

The field study was conducted for 20 days between December 2008 and April 2009, which is the hot wet season when tabanid populations are highest in the area. Seventeen of the 20 days were in February and March 2009. Days were selected according to weather conditions, with particular preference for sunny, rain-free days which are the most appropriate for operation of E-nets and for tabanid activity (van Hennekeler, 2007). Study times covered the primary activity periods of the local tabanid species as much as possible.

**Estimation of tabanid densities on a horse**

Vale (1977) assumed a random approach and departure of tsetse flies at rings of E-nets, and used the catch to estimate the numbers of tsetse attracted to the host (Torr, 1994). In the current study, it would also be possible to closely estimate the absolute numbers of tabanids of each species attracted to the horse if the data supported this assumption. The assumption could be tested by considering the ratios of flies captured outside and inside the incomplete ring of nets. Given that the E-nets covered 35 % of the ring circumference, 35% of approaching flies would be captured on the outward-facing side of the nets, allowing 65% to reach the horse through the gaps between the nets. When they leave the horse, 35% of these would be captured on the inward-facing side of the
net (which is 22.75% of the flies that originally approached the horse). Consequently, ratio of flies caught on the outside and inside of the ring is expected to be 35:22.75 (or 1:0.65). Tabanids generally do not fly over objects that are over two meters high (Mullens, 2009), and this is similar to other blood feeding diptera such as tsetse flies, of which more than 80% fly at less than 50 cm above ground level when approaching a host (Torr, 1988). Therefore the nets would have been high enough to intercept almost all flies coming in their direction. A conformity of the data to the expected outside:inside ratio would support in principle the assumption of random approach and departure, and this would enable the estimation of tabanid burden on the horse by each species, since the flies captured outside would represent 35% of the absolute number that approached the horse.

**Data analysis**

For each tabanid species on either side of the ring of nets, the numbers of unfed, partially fed and fully fed flies were each expressed as proportions of the total catch inside or outside the ring in order to evaluate the proportions likely to seek another host for completion of a blood-meal.

To test the assumption of random approach and departure in order to estimate tabanid densities on the horse, the observed ratios of tabanids caught outside (approaching) and inside (departing) were compared to the expected ratio (1:0.65) for each species using Chi-square tests. For species with relatively large sample sizes, the distribution of catches among the individual E-nets in the ring around the animal were assessed by Chi-square to further test the randomness of approaching flies. Statistical analysis was conducted with SPSS 16.0. and PASW Statistics 18.0.
Fig 3.1. Electrocuted tabanid flies lay on corrugated tray before they were collected for dissection
3.3. Results

**Completion of blood-meals**

Overall, more than 96% of tabanids approaching the horse did not have any host blood in them (Fig. 3.2), and yet more than 60% still left the horse without feeding (Fig. 3.3). The tabanid species differed significantly in the proportions obtaining some blood from the horse ($\chi^2 = 112.99$, df=4, p<0.001). Feeding success was highest for *P. silvester* (37%), *Dasybasis oculata* Ricardo (35%) and *T. pallipennis* (9%), and extremely low in *T. townsvilli* (1%) and *T. dorsobimaculatus* (0%). With regards to persistence once feeding was initiated, *P. silvester* was highest, with 89% of feeders obtaining a full meal, while 49% and 48% of *T. pallipennis* and *D. oculata* were fully engorged, respectively.

**Tabanid densities around the horse (on E-nets)**

General observations during the study indicated that some tabanids became lodged in the E-nets at the positions of impact and did not immediately drop onto the collecting trays (Fig. 3.4), and it was noted that they would generally have been flying at a height of less than 1.5m above ground level. This further confirmed the expectation that the E-nets would be tall enough to potentially intercept most tabanids flying in their direction.

Tabanid species and quantities captured inside and outside the ring of electric screens throughout the study are shown in Table 3.1. Results of $\chi^2$ tests comparing the observed outside:inside ratios to the expected ratio of 1:0.65 are also shown for each tabanid species in the table.
Table 3.1. Tabanid species, total numbers and ratios of females captured outside and inside the ring of E-nets in 20 days of sampling. Number of males is shown in parentheses next to number of females. Number next to species name indicates the number of days when the species was captured. Nc = not considered because of small sample size. (*) indicates $\chi^2$ significant difference from expected ratio of 1:0.65 at the $p=0.05$ level.

<table>
<thead>
<tr>
<th>Tabanid species</th>
<th>E-nets (Outward-facing side)</th>
<th>E-nets (Inward-facing side)</th>
<th>Total on E-nets</th>
<th>Out : In ratio (females)</th>
<th>$\chi^2$ significance (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. pallipennis</td>
<td>458 (6)</td>
<td>392 (3)</td>
<td>850 (9)</td>
<td>1: 0.86</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>P. silvester</td>
<td>143 (4)</td>
<td>123</td>
<td>266</td>
<td>1: 0.86</td>
<td>0.003*</td>
</tr>
<tr>
<td>T. townsvilli</td>
<td>145 (2)</td>
<td>92 (2)</td>
<td>237 (2)</td>
<td>1:0.63</td>
<td>0.675</td>
</tr>
<tr>
<td>T. dorsobimaculatus</td>
<td>99 (1)</td>
<td>71 (1)</td>
<td>170 (1)</td>
<td>1: 0.72</td>
<td>0.251</td>
</tr>
<tr>
<td>D. oculata</td>
<td>62 (18)</td>
<td>71 (18)</td>
<td>133</td>
<td>1:1.15</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>T. concolor</td>
<td>16 (12)</td>
<td>8 (12)</td>
<td>24</td>
<td>nc</td>
<td></td>
</tr>
<tr>
<td>T. strangmannii</td>
<td>9 (2)</td>
<td>9 (2)</td>
<td>18 (3)</td>
<td>nc</td>
<td></td>
</tr>
<tr>
<td>T. notatus</td>
<td>6 (5)</td>
<td>11 (5)</td>
<td>17</td>
<td>nc</td>
<td></td>
</tr>
<tr>
<td>Lilaea fuliginosa</td>
<td>9 (8)</td>
<td>7 (8)</td>
<td>16</td>
<td>nc</td>
<td></td>
</tr>
<tr>
<td>P. distincta</td>
<td>2 (1)</td>
<td>0 (1)</td>
<td>2</td>
<td>nc</td>
<td></td>
</tr>
</tbody>
</table>

T. pallipennis was the most abundant species captured. In all species, male tabanids comprised only 0.9% of the total numbers. The ratios of tabanids caught on the outside and inside of the ring of nets were significantly higher than expected for T. pallipennis, P. silvester and D. oculata (Table 3.1) because of relatively high catches on the inward-facing side of the nets. However, for T. townsvilli and T. dorsobimaculatus the ratios were not significantly different from what was expected (Table 3.1). Therefore the assumption of random approach was not valid for T. pallipennis, P. silvester and D. oculata. This was further confirmed by Chi-square test comparing frequencies of T. pallipennis caught on the outward-facing sides of the six E-nets, which showed significant differences among some of the nets ($\chi^2=22.722$; df=5; $p<0.001$). Consequently, the absolute numbers of tabanids attracted to the horse could only be estimated for T. townsvilli and T. dorsobimaculatus. The estimated totals approaching were 414 and 283 respectively for these two species, and averages were 21 and 14 tabanids per three-hour session. Since it was not possible to derive absolute estimates for T. pallipennis, P. silvester and D. oculata, the total catch from both sides of the E-nets was used as an index of the numbers of tabanids attracted to the animal (Torr, 1994; Vale et al., 1999; Torr et al., 2007). This gave a minimum of 44, 67 and 7 flies respectively, per three-hour session on days that the species were active.
Fig 3.2. The proportions of fully-fed, partially-fed and unfed tabanids captured on E-nets as they flew towards a horse. (*T. dorsobim* = *T. dorsobimaculatus*)

Fig 3.3. The proportions of fully-fed, partially-fed and unfed tabanids captured on E-nets as they flew away from a horse in the field.
Fig 3.4. Tabanids (*T. dorsobimaculatus*) stuck to the electric net soon after contact. Sparks can be seen where there is contact between the insect and the wires.
3.4. Discussion

**Completion of blood-meals and implications for transmission**

The importance of tabanid feeding behaviour in influencing their potential for mechanical transmission is well-documented (Foil, 1989; Luckins, 1998; Desquesnes and Dia, 2004; Barros and Foil, 2007), and interrupted feeding is regarded as the most important single factor responsible for the role of tabanids as efficient mechanical vectors (Krinsky, 1976; Magnarelli and Anderson, 1980). Among the flies that managed to obtain some blood from the host in the present experiment, *P. silvester* exhibited a higher level of persistence (89% achieved full blood-meals) compared with *T. pallipennis* (49%) and *D. oculata* (48%). Tabanid species that complete their blood meals on a single animal are likely to be less efficient mechanical vectors (Foil, 1989; Luckins, 1998), and this implies that *P. silvester* has a lower potential for mechanical transmission of surra. Interestingly, similar results were obtained previously in observational studies, where *P. silvester* achieved 88% engorgement success on a horse (Muzari *et al.*, 2010). Differences in feeding persistence among tabanid species have previously been recorded in America, where *T. lineola* was regarded as the most persistent feeder (Foil, 1983; Foil *et al.*, 1985). Conversely, *T. pallipennis* in this study was very sensitive to the host’s defensive movements and previous studies have shown that it needs as many as nine partial meals before reaching engorgement (Muzari *et al.*, 2010a). In Burkina Faso, a high incidence of *Trypanosoma vivax* observed in cattle under experimental conditions was attributed to the high rate of disrupted meals (54%) experienced by the tabanid species *Atylotus fuscipes* (Desquesnes and Dia, 2004).

The sensitivity and reaction of tabanids to host actions appeared to vary among species, with all *T. dorsobimaculatus* and almost all *T. townsvilli* (99%) leaving without a meal, compared to 63% in *P. silvester* and 65% in *D. oculata*. 
**Tabanid densities on animal**

Tabanid ratios on either side of the E-nets were not significantly different from the expected ratio for the species *T. townsvilli* and *T. dorsobimaculatus*, but ratios were significantly higher than expected in *T. pallipennis*, *P. silvester* and *D. oculata* (Table 3.1) Some authors, studying the behaviour of tsetse flies with E-nets around a warthog (Torr, 1994) or around a cow (Baylis and Nambiro, 1993) also intercepted higher than expected numbers of flies inside the ring. Torr (1994) attributed this to the fact that the E-nets are not able to capture 100% of flies colliding with them (Griffiths and Brady, 1994), therefore flies that strike the inside surface of a net and escape to fly in the opposite direction have a higher probability of colliding with another electric net than flies striking the outside surface of the nets (Torr, 1994). Griffiths and Brady (1994) noted that some 15-20 % of tsetse flies behaviourally avoided E-nets by flying round them, and it is also likely that when approaching the horse, a proportion of *T. pallipennis*, *P. silvester* and *D. oculata* were able to see the E-nets in time to avoid bumping into them, but were less able to do so when leaving the horse, as many probably left abruptly due to disturbance by the host. On the other hand, approach and departure of some species of tabanids may simply not be in random directions and might be affected by wind direction (Vale and Phelps, 1974) or other features such as big trees near the site (Torr, 1994), resulting in a distortion of the ratios.

The burdens of *T. pallipennis* and *P. silvester* recorded around a horse in our study were higher than the densities of *Atylotus fuscipes* (55 insects per eight-hour session) which resulted in successful mechanical transmission of *Trypanosoma vivax* among cattle in Burkina Faso (Desquesnes and Dia, 2004). This makes *T. pallipennis* (with at least 44 insects per three-hour session) a very likely vector for surra, other factors being equal, considering its high rate of disrupted meals (49%). The relatively high densities of *T. pallipennis*, *P. silvester* and *T. townsvilli* on the horse are also likely to result in a high level of annoyance and reduced grazing, affecting the general welfare of the animal. If similar densities occur on cattle this could cause significant production losses such as reduced weight gain or milk production (Ralley et al., 1993).
CHAPTER FOUR

Alighting and feeding behaviour of tabanid flies on horses, kangaroos and pigs *


4.1. Introduction

Tabanids are some of the most diverse and widely distributed biting flies, with at least 4000 species described (Baldwin et al., 2005). Many tabanid species are mechanical vectors of viruses, bacteria, protozoans and helminths that cause diseases in animals (Krinsky, 1976; Foil, 1989). Surra is one of the most important diseases transmitted by tabanids among livestock and wild animals, and is caused by the protozoan parasite Trypanosoma evansi. Mammalian hosts affected include a wide range such as horses, camels, cattle, buffalo, pigs, sheep, goats, dogs, cats, elephants, hyenas and tigers (Veer et al., 2002; Jones et al., 2005).

Surra does not occur in Australia (Reid and Copeman, 2003) but its introduction is considered a risk because it is endemic to many countries in south-east Asia (Luckins, 1988; Reid, 2002; Holland et al., 2004). Australia supports at least 190 species of tabanids (Mackerras, 1956), and most livestock and wild animals are potentially susceptible to surra (Sandeman and Warner, 2002). Experimental studies have indicated that the pathogen could cause acute disease with high mortality in wallabies and kangaroos (Reid et al., 2001). The most probable entry points of surra into Australia are along the northern coastline, particularly areas around Darwin and Cape York Peninsula (Thompson et al., 2003; van Hennekeler, 2007). To protect Australia from potential outbreaks, Sandeman and Warner (2002) emphasise the need to maintain effective border protection and to develop early warning systems as well as containment.
strategies to prevent spread. This cannot be achieved without good understanding of the potential role of different tabanid species and their interaction with vertebrate hosts.

Landing and feeding behaviour of tabanids on different vertebrate hosts have long been of interest to biologists because an understanding of this interaction could lead to better intervention strategies and improved disease management. Observations have been conducted on several tabanid species alighting on horses (Foil et al., 1985; Barros, 2001; Barros and Foil, 2007), cattle (Hollander and Wright, 1980; Phelps and Holloway, 1990; Desquesnes and Dia, 2003), water buffaloes (Mitzmain, 1912; Manresa and Mondonedo, 1935) camels and guinea pigs (Hafez et al., 1970), ducks (Limeira-de-Oliveira et al., 2002; Ferreira and Rafael, 2004), reptiles (Ferreira et al., 2002) and goats (Mohamed-Ahmed and Mihok, 2009) in various parts of the world. No such studies have been conducted on tabanid species that occur in Australia.

Recent trapping studies in the Cape York Peninsula of northern Queensland, identified 38 tabanid species, but only one-third of these were considered abundant (van Hennekeler, 2007). There is no published information on the blood-feeding behaviour of Australian tabanids, and therefore the relative importance of the different species in terms of potential for surra transmission is not known. While many different tabanid species can be found feeding on animal hosts, it is important to evaluate the relative efficiency of different species in their ability to transmit disease (Luckins, 1998).

This paper describes recent studies on the alighting and feeding behaviour of some of the more abundant tabanid species in Queensland and discusses the implications for surra transmission. Tabanid behavioural traits such as multiple-feeding events and duration of feeding affect the chances of disease transmission between animals, while landing frequency and alighting sites will affect the choice and success of intervention strategies against particular tabanid species on different host animals. All animal studies had prior approval from the James Cook University Animal Ethics Review Committee.
4.2. Materials and methods

Study area and duration

Field observational studies were conducted on horses, eastern grey kangaroos (Macropus giganteus) and pigs in Townsville, northern Queensland between March 2007 and May 2008. The observation sites for horses and pigs were on the outskirts of James Cook University (JCU), an area with tropical savannah vegetation in which several species of tabanids are abundant during the warm wet season (Van Hennekeler et al., 2008). Observations on kangaroos were conducted at a wildlife sanctuary about 20km away from JCU, in denser vegetation. Overall, there were 22 days of observation on horses, 15 days on pigs and 16 days on kangaroos. Each daily observation period lasted 2-3 hours between 0900hrs and 1800hrs, the timing depending on prevailing weather conditions and the activity times of tabanid species in the area.

Field observations on tabanid landing and feeding behaviour

Two horses were separated from the rest of the herd and taken to an isolated paddock at least 300m away from the other animals. Tabanids approached the animals naturally from their habitat, while two observers watched one horse at a time from within 3m, as the animals grazed freely. Two pigs, at least 100kg each, were used in the study, and they were permanently housed in a pen with sides 5m x 4m constructed with wire-fencing about 2.5m high. Adequate food and water was supplied daily. Observers stood outside or inside the fence depending on the position of the pigs, which were free to walk about in the pen. Kangaroos were observed individually or in groups of two or three as they browsed or lay in the shade (Fig 4.1). In total, five different horses, two pigs and eight kangaroos were used in the study.

The observers recorded the species of tabanid seen landing on the animal, the landing site on the body of the animal, landing duration, the duration of feeding and whether the fly appeared fully engorged before departure. Between them, the observers were capable of keeping track of six tabanids on an animal at a time if needed, but the tabanid burden on the animals rarely reached that level, so that all tabanids alighting were recorded. The alighting sites on the host body were classified as head and neck, belly,
back, front legs and hind legs. The tail was regarded as part of the back except on the kangaroo where it was considered as a separate site because of its relative size. Animal defensive behaviours such as skin twitch, tail flick, foot stomp, head shake and scratching (this latter behaviour only occurred in the kangaroo) were recorded if they were observed. New and repeat visits of tabanids were not distinguished. A tabanid was considered to have started feeding once it lowered its head and lodged its mouthparts into the skin of the animal. Engorgement of the tabanids was obvious as the abdomen became distended when full of blood, and in some species blood could be seen through the stretched abdominal integument.

**Tabanid identification**

The two observers were able to identify local tabanid species on alighting from a short distance without capturing them, having worked on related studies in the same area in preceding seasons. To further confirm the identities of tabanid species active in the area at any particular time of the season, tabanids were also collected twice a week from three odour-baited Nzi traps (Mihok, 2002) deployed permanently about 250m from the observation sites at JCU. All captured flies were identified using keys by Mackerras (1959, 1961, 1971) as described in Chapter Two. However, tabanid species in the genus *Dasybasis* were not readily distinguishable from a distance.
Fig 4.1 Field observation of tabanid feeding behaviour on horses (A), pigs (B) and kangaroos (C)
**Tabanids feeding on a restrained horse**

In order to evaluate the number of partial blood-meals needed by interrupted tabanids to reach full engorgement, which is a critical factor in mechanical transmission, I needed to establish the time required for flies to complete a full meal. This is most accurately determined if the fly is able to feed to engorgement during a single alighting. Some species of tabanids were rarely able to complete their blood meals on an unrestrained animal due to host defensive movements, so it was necessary to restrain the animal and protect the tabanids during blood-feeding. This part of the study was only done using a horse, which was held in 2m x 1m stocks constructed with metal bars (normally used to facilitate examination and treatment of horses), so that the horse could not move freely during the experiment. Tabanids were placed individually in transparent tubes closed with netting material and allowed to take a blood meal through the netting while the tube was firmly held against the skin of the animal (Fig 4.2). Twenty *Tabanus townsvilli* and 114 *T. pallipennis* were used. Using a stop-watch, feeding duration to engorgement was determined for each tabanid. The tabanids were also weighed individually before feeding and within an hour of engorgement to estimate the amount of blood they consumed.

**Preliminary observations on cattle**

Another important potential host for tabanids and surra in Queensland is cattle. Some preliminary observations were conducted on Brahman heifers with plans to include them among the range of hosts studied, but the cattle were eventually excluded from the detailed study due to technical reasons at the time. The cattle had a tendency to quickly walk away from observers when approached in the field, and mustering some of them to appropriate study sites required more personnel than were available. However, some general insight was obtained from the preliminary observations.
Fig 4.2 Evaluating the feeding duration of individual tabanids (in glass tube) on a restrained horse
Data analysis

In analysis of average feeding durations, means (and SE) or medians were quoted if the data were normally distributed, but if the data were significantly skewed only medians were used. Comparison of average feeding durations among species and hosts was done using either one-way ANOVAs or the non-parametric Kruskal-Wallis test depending on the shape of the distribution and homogeneity of variances on non-transformed and log-transformed data. Pair-wise comparisons within each test were conducted using Tukey’s HSD test or Mann-Whitney U test with Bonferroni-adjustment of significance levels where necessary. SPSS version 16.0 was used for the analyses. Tabanid species with fewer than 20 observations on a host were excluded from statistical analysis.

4.3. Results

Tabanid species alighting on and biting different hosts

Six species of tabanids were recorded alighting on animals during field observations. They were *T. pallipennis*, *Pseudotabanus silvester*, *T. townsvilli*, *T. dorsobimaculatus* Macquart, *P. distincta* Ricardo and *T. notatus* Ricardo. The numbers of each species seen on each host are listed in Table 4.1. The number of days of tabanid activity recorded on each host is also stated.

On horses and kangaroos most landings did not result in a bite, but on pigs at least 75% of alighting flies managed to bite.
Table 4.1 The number of tabanids by species landing, biting and engorging on different host animals, and the feeding durations recorded during field observations. Numbers in brackets indicate the number of days that tabanids were observed on a host.

<table>
<thead>
<tr>
<th>Host</th>
<th>Tabanid species</th>
<th>Landing</th>
<th>Biting</th>
<th>Engorged</th>
<th>Engorgement success (%)</th>
<th>Median feeding duration (s)</th>
<th>Feeding duration range (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse</td>
<td>T.pallipennis</td>
<td>258</td>
<td>44</td>
<td>3</td>
<td>7</td>
<td>11</td>
<td>1-120</td>
</tr>
<tr>
<td></td>
<td>P.silvester</td>
<td>114</td>
<td>43</td>
<td>38</td>
<td>88</td>
<td>180</td>
<td>15-360</td>
</tr>
<tr>
<td></td>
<td>T.townsvilli</td>
<td>103</td>
<td>21</td>
<td>6</td>
<td>29</td>
<td>180</td>
<td>60-660</td>
</tr>
<tr>
<td></td>
<td>P.distincta</td>
<td>13</td>
<td>8</td>
<td>6</td>
<td>75</td>
<td>120</td>
<td>30-180</td>
</tr>
<tr>
<td></td>
<td>T.notatus</td>
<td>10</td>
<td>3</td>
<td>3</td>
<td>100</td>
<td>240</td>
<td>150-240</td>
</tr>
<tr>
<td></td>
<td>T.dorsobimaculatus</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
<td>10</td>
<td>3-210</td>
</tr>
<tr>
<td></td>
<td>Dasybasis sp.</td>
<td>14</td>
<td>5</td>
<td>2</td>
<td>40</td>
<td>10</td>
<td>3-210</td>
</tr>
<tr>
<td>Pig</td>
<td>T.pallipennis</td>
<td>108</td>
<td>105</td>
<td>2</td>
<td>2</td>
<td>420</td>
<td>60-2460</td>
</tr>
<tr>
<td></td>
<td>P.silvester</td>
<td>65</td>
<td>63</td>
<td>6</td>
<td>10</td>
<td>300</td>
<td>15-1440</td>
</tr>
<tr>
<td></td>
<td>T.townsvilli</td>
<td>24</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>210</td>
<td>30-1920</td>
</tr>
<tr>
<td></td>
<td>P.distincta</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>149</td>
<td>147-208</td>
</tr>
<tr>
<td></td>
<td>T.dorsobimaculatus</td>
<td>10</td>
<td>10</td>
<td>1</td>
<td>10</td>
<td>330</td>
<td>180-1680</td>
</tr>
<tr>
<td></td>
<td>Dasybasis sp.</td>
<td>7</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>50</td>
<td>30-120</td>
</tr>
<tr>
<td>Kangaroo</td>
<td>T.pallipennis</td>
<td>35</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>2-5.</td>
</tr>
<tr>
<td></td>
<td>P.silvester</td>
<td>25</td>
<td>9</td>
<td>2</td>
<td>22</td>
<td>60</td>
<td>1-150</td>
</tr>
<tr>
<td></td>
<td>T.notatus</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The feeding durations of different tabanid species on free hosts

The distributions of feeding durations (log-transformed) of the three most abundant species *P. silvester*, *T. pallipennis* and *T. townsvilli* on the different host animals are shown in Fig 4.3. Feeding durations of *P. silvester* and *T. pallipennis* were longest on the pigs and shortest on the kangaroos, although *T. pallipennis* showed more substantial differences in response to the hosts (Fig 4.3). For *T. townsvilli*, feeding durations were not significantly different between the horses and pigs (Table 4.2). Higher feeding durations did not, however, necessarily result in greater engorgement among tabanid species nor for the same tabanid species among different hosts (Table 4.1).
*P. silvester* was the most persistent of the tabanid species on the defensive horses, managing to hang on for more than ten times longer and achieving at least ten times more success at engorgement than *T. pallipennis*. However, on pigs *T. pallipennis* generally persisted longer, although average feeding durations for the two tabanid species were not significantly different (Table 4.3).

![Feeding durations (log-transformed) of three most abundant tabanid species on three host animals as recorded from field observations (Box plot with whiskers).](image)

**Fig 4.3.** Feeding durations (log-transformed) of three most abundant tabanid species on three host animals as recorded from field observations (Box plot with whiskers).
Table 4.2 Comparison of average feeding durations among three tabanid species on horses, pigs and kangaroos. Significance levels of 0.0167 were computed from Bonferroni-adjustment of p=0.05 for pairwise comparisons using the Mann-Whitney test. Statistically significant differences are marked *. (Average feeding durations shown in Table 4.1)

<table>
<thead>
<tr>
<th>Host</th>
<th>Tabanid species compared</th>
<th>Test</th>
<th>Test statistic</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horses</td>
<td>All three tabanid species</td>
<td>Kruskal-Wallis</td>
<td>$X^2_{(2)}=67.3$ (n=103)</td>
<td>p&lt;0.05 *</td>
</tr>
<tr>
<td></td>
<td>$P.silvester$ vs $T.pallipennis$</td>
<td>Mann-Whitney</td>
<td>U=61, Z=-7.2 (n=82)</td>
<td>p&lt;0.0167 *</td>
</tr>
<tr>
<td></td>
<td>$P.silvester$ vs $T.townsvilli$</td>
<td>Mann-Whitney</td>
<td>U=338, Z=-0.83 (n=58)</td>
<td>p&gt;0.0167</td>
</tr>
<tr>
<td></td>
<td>$T.townsvilli$ vs $T.pallipennis$</td>
<td>Mann-Whitney</td>
<td>U=17, Z=-6.3 (n=66)</td>
<td>p&lt;0.0167 *</td>
</tr>
</tbody>
</table>

Pigs

<table>
<thead>
<tr>
<th>Host</th>
<th>Tabanid species compared</th>
<th>Test</th>
<th>Test statistic</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All three tabanid species</td>
<td>ANOVA (on Log_{10})</td>
<td>$F_{(2, 183)}=3.48$ (n=186)</td>
<td>p&lt;0.05 *</td>
</tr>
<tr>
<td></td>
<td>$P.silvester$ vs $T.pallipennis$</td>
<td>Tukey HSD</td>
<td>p&gt;0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$P.silvester$ vs $T.townsvilli$</td>
<td>Tukey HSD</td>
<td>p&gt;0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$T.townsvilli$ vs $T.pallipennis$</td>
<td>Tukey HSD</td>
<td>p&gt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

Kangaroos

<table>
<thead>
<tr>
<th>Host</th>
<th>Tabanid species compared</th>
<th>Test</th>
<th>Test statistic</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$P.silvester$ vs $T.pallipennis$</td>
<td>Mann-Whitney</td>
<td>U=5.5, Z=-1.96 (n=13)</td>
<td>p=0.05</td>
</tr>
</tbody>
</table>

Table 4.3 Comparison of average feeding durations of each of three tabanid species among three hosts. Significance levels of 0.0167 were computed from Bonferroni-adjustment of p=0.05 for pairwise comparisons using the Mann-Whitney test. Statistically significant differences are marked *. (Average feeding durations shown in Table 4.1)

<table>
<thead>
<tr>
<th>Tabanid species</th>
<th>Tabanid hosts compared</th>
<th>Test</th>
<th>Test statistic</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P. silvester$</td>
<td>All three hosts</td>
<td>Kruskal-Wallis</td>
<td>$X^2_{(2)}=34.95$ (n=109)</td>
<td>p&lt;0.05 *</td>
</tr>
<tr>
<td></td>
<td>horse vs pig</td>
<td>Mann-Whitney</td>
<td>U=519.5, Z=-4.64 (n=100)</td>
<td>p&lt;0.0167 *</td>
</tr>
<tr>
<td></td>
<td>horse vs kangaroo</td>
<td>Mann-Whitney</td>
<td>U=61, Z=-2.95 (n=46)</td>
<td>p&lt;0.0167 *</td>
</tr>
<tr>
<td></td>
<td>pig vs kangaroo</td>
<td>Mann-Whitney</td>
<td>U=34.5, Z=-4.26 (n=72)</td>
<td>p&lt;0.0167 *</td>
</tr>
<tr>
<td>$T.pallipennis$</td>
<td>All three hosts</td>
<td>Kruskal-Wallis</td>
<td>$X^2_{(2)}=96.77$ (n=154)</td>
<td>p&lt;0.05 *</td>
</tr>
<tr>
<td></td>
<td>horse vs pig</td>
<td>Mann-Whitney</td>
<td>U=44, Z=-9.52 (n=150)</td>
<td>p&lt;0.0167 *</td>
</tr>
<tr>
<td></td>
<td>horse vs kangaroo</td>
<td>Mann-Whitney</td>
<td>U=43, Z=-1.73 (n=49)</td>
<td>p&gt;0.0167</td>
</tr>
<tr>
<td></td>
<td>pig vs kangaroo</td>
<td>Mann-Whitney</td>
<td>U=0.001, Z=-3.39 (n=109)</td>
<td>p&lt;0.0167 *</td>
</tr>
<tr>
<td>$T. townsvilli$</td>
<td>horse vs pig</td>
<td>Mann-Whitney</td>
<td>U=181.5, Z=-0.214 (n=39)</td>
<td>p&gt;0.05</td>
</tr>
</tbody>
</table>
**Host defensive behaviours**

The horse’s defensive behaviour involved tail flicking, skin twitching, foot stomping, and head-shaking. The kangaroo mainly responded by scratching off the tabanids using its swift front legs which could reach almost any part of its body. The pig however, generally ignored the tabanids and did not appear to notice them alighting or biting. When tabanids left the pigs it was generally not in response to the pigs’ defensive behaviour, but most flies left of their own accord.

Of the 258 *T. pallipennis* recorded alighting on horses in grazing land, 70% were dislodged by the host within six seconds of landing while they were still probing, before they could start feeding. The majority (65%) of *T. townsvilli* recorded alighting on the horses were disturbed within two seconds of landing as the horses responded quickly to dislodge them.

**Normal feeding frequency required by tabanids to complete a blood-meal**

On an unrestricted horse, a single bite was clearly not sufficient for *T. pallipennis* and *T. townsvilli* to complete a blood-meal (Table 4.1), but was enough for *P. silvester* which took on average 180 seconds to reach engorgement. However, *P. silvester* found the pig a less suitable host and only 10% managed engorgement despite more than 50% of them sucking for more than 180 seconds and about 20% persisting for more than 10 minutes without interruption from the pig.

The feeding durations of interrupted and uninterrupted *T. pallipennis* biting unrestrained and restrained horses respectively are shown in Fig 4.4. Almost all *T. pallipennis* were interrupted within 60 seconds of starting a meal on an unrestrained horse (Fig 4.4) and were not able to complete the meal. Of these, 75% were shaken off within only 20 seconds, with a median feeding duration of 11 seconds (Table 4.1). On a restrained horse, most *T. pallipennis* that took a bite managed to complete their blood-meal, and needed a mean feeding duration of 179 (±12) seconds to do so. If *T. pallipennis* is consistently interrupted during its feeding, it would therefore need to take as many as nine partial meals before it became engorged. Of the 114 *T. pallipennis* flies that were offered the opportunity to take a blood-meal on a restrained horse, 54% did not attempt
Individuals of *T. pallipennis* weighed an average of 44 (±2) mg before feeding and 84 (±4) mg after engorgement.

*T. townsvilli* was recorded on horses and pigs, but was not seen on kangaroos. None of the *T. townsvilli* managed to feed to repletion on the pig, despite a relatively long feeding duration. On the defensive horses the median feeding duration of *T. townsvilli* was 180 seconds, but only six individuals (29%) went away with a full meal after hanging onto the animal for between 6 and 11 minutes. On a restrained horse, 17 out of 20 *T. townsvilli* declined to feed in captivity, and out of the three that took the meal, two reached engorgement taking 11 and 12 minutes and consuming 80 mg and 200 mg of blood in the process. Individual *T. townsvilli* biting a horse and getting interrupted might therefore need four to five partial meals to reach engorgement.

**Fig 4.4.** Frequency distribution of the feeding durations of ‘protected’ *T. pallipennis* that obtained full blood-meals on a restrained horse (green) and ‘unprotected’ *T. pallipennis* obtaining partial, interrupted blood-meals on defensive unrestricted horses in the field (purple)
Landing positions of tabanids on different animals

The proportions of each of the tabanid species recorded alighting on various parts of the host’s body are shown in Fig 4.5 (horses), Fig 4.6 (pigs) and Fig 4.7 (kangaroos). Figure 4.8, Fig 4.9 and Fig 4.10 are pictures of some of the tabanids biting these animals.

Fig 4.5-4.7 show that preference for landing position on each host varied among tabanid species. *T. pallipennis* mostly preferred landing on the legs of horses (71%) and pigs (76%) and on the tail of kangaroos (57%). *P. silvester* mostly preferred landing on the belly of horses (73%) and legs of kangaroos (76%). In general, tabanids landed most on the lower sections of the body (belly and legs) of horses and pigs, and rear sections (hind legs and tail) of kangaroos.

**Fig. 4.5.** The proportion of each of five tabanid species alighting on various parts of the body of horses during field observations.
Fig. 4.6. The proportion of each of four tabanid species alighting on various parts of the body of pigs during field observations.

Fig. 4.7. The proportion of each of two tabanid species alighting on various parts of the body of kangaroos during field observations. None were recorded on the belly or head and neck.
Fig 4.8. Tabanids biting a horse: A- *Pseudotabanus distincta*; B- *Tabanus townsvilli*
Fig 4.9. Tabanids biting a kangaroo: A- *Pseudotabanus silvester*; B- *Tabanus pallipennis*
Fig 4.10. Tabanids biting a pig:  A- *T. pallipennis*;  B- *P. silvester*
Observations on cattle

In preliminary observations on cattle, which were eventually excluded from the detailed study, it was noted on Brahman heifers that *P. silvester* was landing more on the belly and *T. pallipennis* landing more on the legs, a similar pattern to that recorded on the horses. It is likely that the rest of the tabanid blood-feeding activity on the horse would be similar to activity on the cow since the animals’ body size, coat texture and defensive techniques at their disposal are generally similar.

4.4. Discussion

Potential transmission of surra

Interrupted feeding is the most important single factor responsible for the role of tabanids as efficient mechanical vectors, and the probability of a tabanid acting as a mechanical vector increases with the number of interruptions in feeding (Krinsky, 1976; Magnarelli and Anderson, 1980). Nine partial meals may be required by *T. pallipennis* to reach engorgement on a horse and possibly more on a kangaroo, due to interruption to feeding by host defensive behaviours. This suggests that this tabanid species could be an important vector for surra and other mechanically transmitted animal diseases. While the required biting frequency of *T. pallipennis* could not be determined on the pig, the fact that 97% of alighting flies managed to bite and none of them obtained a full meal suggests that this fly species may be an important vector for pigs as well.

The finding that *P. silvester* could generally complete a blood-meal through one bite on the horse makes this species an unlikely vector of surra among horses. Tabanid species that complete their blood meals on a single animal are likely to be less efficient mechanical vectors (Foil, 1989; Luckins, 1998). However, *P. silvester* was not as successful in feeding to repletion on pigs and kangaroos and therefore has potential to be a significant vector among these animals, especially among pigs where 75% of
alighting flies were able to bite. As a result even horses are still at risk if they coexist with infected pigs where *P. silvester* is prevalent.

The estimated four to five bites required by most *T. townsvilli* to reach engorgement on a horse make it a good potential vector for surra, but probably to a lesser extent than *T. pallipennis*. However, *T. townsvilli* had longer feeding durations in its interrupted meals on the horse than *T. pallipennis*, and this is important because the probability of picking up an infectious agent increases with the duration of exposure of the fly to infected tissue fluids (Krinsky, 1976).

Of the three animal species studied, pigs seem to be the most vulnerable to possible infection from tabanids because they ignore the tabanids and allow them to bite freely. In addition, if a pig is infected, it can be a significant reservoir of infection because most of the flies biting it are unable to engorge, and will need to complete the meal on another host. The long feeding durations of all tabanid species on the pig give them high chances of picking up the infection. Most feral pigs in northern Australia are darker in colour than the domestic pig used in the study, and further studies are required to investigate the effect of this factor on tabanid responses.

The relatively low tabanid activity around the kangaroos may be an effect of the study site. The site where tame kangaroos were available for observation was about 20 km from the site where horse and pig observations were conducted, and tabanids are generally not uniformly distributed across wide areas (Sheppard and Wilson, 1977; Barros, 2001), so differences in microhabitat may result in differences in population densities and activity patterns.

If surra invades northern Australia, its rate of spread among livestock could be minimised by adapting animal husbandry practices in line with knowledge of transmission risk among different animal species. Separation of pigs from the rest of the livestock could be a good starting point, since pigs have high potential for infection through almost any tabanid species available. Worse still, pigs have a high tolerance for *T. evansi* and may seldom show clinical signs even though infected (Dieleman, 1986; Reid *et al.*, 1999), hence they could play a key role as reservoirs of the disease. The presence of feral pigs in parts of northern Australia is an important risk factor for the spread of surra.
Choice of alighting site by tabanids on hosts

The distribution of various tabanid species on the body regions of host animals has proved to be species-specific in other studies (Hollander and Wright, 1980; Phelps and Holloway, 1990; Hribar et al., 1992; Konstantinov, 1995; Mohamed-Ahmed and Mihok, 2009;), and species-specificity was also apparent in this study.

Several factors have been suggested as contributing to various spatial patterns on hosts:

- The reduction of inter-specific competition on the host (Hollander and Wright, 1980).
- Differences in hair length on different parts of the host’s body relative to the length of tabanid mouthparts (Mullens and Gerhardt, 1979),
- Differences in intensity of host defensive movements among different body regions (Hribar et al., 1992)
- Differences in carbon dioxide concentration between anterior and posterior ends of an animal (Mullens and Gerhardt, 1979).

The favourite landing site of *P. silvester* on horses was the horse’s belly and this probably contributed to the flies’ high engorgement success on this host, since the horse had to rely on skin twitches as a defence and most tabanids were able to continue feeding despite this. The tabanids that landed on the horse’s legs were more easily dislodged by foot-stamping and tail-flicking.

Where insecticide-treated animals are considered a significant option for reducing tabanid populations and tabanid burdens on animals, insecticide application strategies should ensure optimal insecticide concentration on the limbs and belly if *T. pallipennis, T. townsvilli* and *P. silvester* are prevalent. The differences in landing and feeding duration among the different tabanid species may also affect the effectiveness of topical insecticides and need to be considered during insecticide screening assays.

**Efficiency of the direct observation method**

The estimation of feeding success and completion of blood meals was presumably more accurate using E-nets (Chapter Three) than direct field observations, since flies
collected from E-nets could be examined later under a microscope and the potential influence of the observer on tabanid behaviour would be eliminated. However, tabanid feeding durations could only be estimated through direct field observations on different hosts, although the method also allowed crude estimation of tabanid burden and feeding success. Obviously, the E-nets approach is technically more demanding, and data from both methods provided an opportunity to assess the relative efficacy of the direct observation method. On comparison, the two methods gave nearly equal estimates for *P. silvester*, with biting (feeding) success of 38% vs 37% and engorgement success of 88% vs 89%. However, direct observations overestimated the biting success of *T. pallipennis* (17% vs 9%) and underestimated its engorgement success (7% vs 49%); and the method overestimated both the biting success (20.4% vs 1%) and engorgement success (29% vs 0%) of *T. townsvilli*. Incidentally, the general implications of the findings were the same with either method for the three species. The direct observation method was most reliable for *P. silvester* in this case, and will probably be for any other species that generally feeds to repletion through a single bite. Therefore in circumstances where the use of E-nets is not feasible, direct observations would still be useful and do give a fair indication of vectorial capacity of some tabanid species.

The greater disparity between the percentages was with *T. townsvilli*, and the extremely low feeding success recorded by microscopic examination of samples from E-nets may significantly underestimate risk, since some specimens might have had blood residues on their mouthparts and nothing in the gut but they would still be a potential risk in pathogen transmission. To investigate such an anomaly in future, analysis of the mouthparts using quantitative ELISA is recommended, although it would also be necessary to determine the amount of blood residue on mouthparts that is required to achieve successful transmission of *T. evansi*. Foil *et al* (1987) estimated a moderate-sized tabanid such as *T. fuscicostatus* would have an average of ~10 nl of blood residue on its mouthparts following feeding, and that amount could contain sufficient viral quantity to successfully transmit equine infectious anaemia (EIA). It has not been determined whether such an amount can lead to successful transmission of *T. evansi*. 
CHAPTER FIVE

Optimisation of ELISA for identification of the sources of tabanid blood meals

5.1. Introduction

An understanding of the transmission dynamics of arthropod-borne infectious diseases is important to public health because it forms the basis for developing rational prevention, surveillance and control strategies (Meece et al., 2005). One of the most important parameters in pathogen transmission by haematophagous insects is feeding preference (Ponlawat and Harrington, 2005). Knowledge on host-feeding habits and preference of the vectors is important in indicating which animals are most at risk and which are the most-likely reservoirs of disease in an area. In Australia, the need for tabanid host-preference studies is demonstrated by the broad diversity of tabanid species sharing the same habitats (Mackerras, 1971; van Hennekeler, 2007) and the availability of a wide range of potential hosts.

In the past, host preference by biting insects has been studied through direct field observations of landing and feeding behaviour (Manresa and Mondonedo, 1935), choice experiments between traps baited with odours from different concealed hosts in the field (Zimmerman and Turner, 1983; Costantini et al., 1998), flight responses of insects to different host odours in a laboratory wind tunnel (Pates et al., 2001, Gikonyo et al., 2003) and laboratory identification of ingested blood from engorged insect specimens captured from their habitats (Blackwell et al., 1994; Arunachalam et al., 2005). While each of these approaches may have advantages under certain circumstances, the last one has emerged as the most useful and most widely adopted, since it provides information on preference among a wide host range under natural conditions.

The identification of ingested blood in field-caught insects dates back to the early twentieth century, and the procedures have been improved over time. Serological techniques are the mainstay of all the conventional tests such as the precipitin test (Bull
agarose gel diffusion assay (Crans, 1969; Collins et al., 1986; Arunachalam et al., 2005) and Enzyme-linked Immunosorbent assay (ELISA) (Burkot et al., 1981; Blackwell et al., 1994). These tests rely on host-specific antisera that are produced against the antigens of each potential host species to identify the source of blood meal from the guts of wild-caught flies (Gill, 1984; Clausen et al., 1998). ELISA is considered the most sensitive and most specific of these techniques, being 1,000 times more sensitive than the precipitin test (Washino and Tempelis, 1983) and able to identify as little as 0.02 µl of fresh blood (Service et al., 1986). As a result, ELISA is currently the most widely used technique and has been used on a wide variety of biting insects (van den Hurk et al., 2003; Richards et al., 2006; Marassa et al., 2006).

In addition to ELISA, some laboratories have successfully utilised molecular techniques in recent years for identifying insect blood-meals, and these are based upon polymerase chain reaction (PCR) amplification and identification of specific fragments of cytochrome-B gene sequences from host samples (Boakye et al., 1999; Ngo and Kramer, 2003; Meece et al., 2005). They are the most sensitive and their advantage over immunological assays is that they can distinguish beyond family and genus level, which some antibodies used in ELISA cannot achieve (Simoa et al., 2005; van den Hurk et al., 2007). However, the molecular techniques are far more costly, and in many cases reliable primer sets for the entire range of potential hosts have not been developed (Ponlawat and Harrington, 2005). Consequently, they are only used when extremely necessary such as when the sensitivity of ELISA is not sufficient for the intended objective. Otherwise ELISA remains the most widely used assay for identifying the sources of insect blood meals.

A range of different types of ELISA has been developed, and the technique is versatile in that one can choose a type depending on types of antigens and antibodies available. ELISA types include direct, indirect, a combination of direct and indirect, and sandwich (Chow et al., 1993). All ELISA systems require that the reagents used be optimised to assess the working concentration of each component of the test (Crowther, 2001), and due to variability between batches, it is essential that a standardisation procedure be developed so that each lot of reagents can be compared (Chow et al., 1993). In the present study, reagents for ELISA were optimised to identify tabanid blood meals originating from horses, pigs, macropods and cattle. The optimisation process is
described in this chapter, and the application of the assays on field-collected tabanid samples is covered in Chapter Six.

5.2. Materials and methods

Types of ELISA and evaluation of optimal concentrations of reagents

The potential tabanid hosts selected for this study were cattle, horses, pigs and macropods, and three types of ELISA assay were developed: Direct ELISA for bovine IgG, direct sandwich ELISA for equine and porcine IgG and indirect sandwich ELISA for macropod IgG (Crowther, 2001). The original plan had been to use direct sandwich ELISA for all host types, but other ELISA types were later adopted for macropods and bovine (cattle) due to either unavailability of some reagents or unsatisfactory sensitivity of this assay type with available reagents. Optimal dilutions of antisera and conjugates were obtained by checkerboard titration and selection was based on high sensitivity, low background and high specificity. Optimisation ensures maximum discrimination between positive and negative specimens. Sensitivity of each assay was evaluated on serial dilutions of homogenised blood-fed flies, or their equivalent mix of host serum and fly extract. Appropriate negative and/or positive controls were used at each stage. Specificity was assessed by comparing simultaneous serial dilutions of sera from a selected range of seven alternative hosts. The general procedures for each of the ELISA types are described below.

Direct ELISA for detection of bovine IgG

The procedures for the direct ELISA were modified from Reid and Copeman (2003) and Beier et al. (1988), and were conducted using polystyrene U-bottom 96-well microtitre plates (Sarstedt, USA). For the bovine assay 50 µl of bovine serum (or other test solution) diluted in coating buffer (0.05 M Carbonate/ bicarbonate (pH 9.6)) (TropBio) was loaded into designated wells of the microtitre plates before incubating at 4°C to allow adsorption overnight. The following day plates were washed four times with TEN-Tween (Tris 0.05M, EDTA 0.006M, Sodium chloride 0.003M with 0.05% Tween 20) (TropBio) using an automatic plate washer (Multidrop Combi, Thermo
Electron Corporation), and 50 µl of anti-bovine IgG conjugated with horseradish peroxidase, diluted in TEN-TC (TEN-Tween with 0.2% casein) (TropBio) was added to each well. After incubating at room temperature (25-26°C) for one hour in a humid plastic box, plates were washed six times and then 100 µl of ABTS (2,2’-azino-di-(3-ethyl-benzthiazoline-6-phosphate) peroxidase substrate solution (KPL, Gaithersburg, Maryland) was added to each well to detect enzyme activity. Plates were then incubated in the dark at room temperature for one hour after which Optical Density (OD) was measured at dual wavelengths 414nm and 492nm (Hall et al., 1987; Reid and Copeman, 2003) using an automatic ELISA plate-reader (Multiskan Ascent V1.24, Thermo Electron Corporation) interfaced to a computer with Ascent Software Version 2.6.

In the checkerboard titration, the OD results were then assessed to determine the optimal reagent concentrations for this assay.

**Direct sandwich ELISA for detection of horse and pig IgG**

Procedures for the sandwich ELISAs, used for the horse and pig assays, were modified from Blackwell et al (1994) and Crowther (2001). Initially, wells were coated with 50 µl capture antibody consisting of affinity-purified host-specific anti-species IgG diluted in coating buffer (Carbonate/ bicarbonate) and incubated overnight at 4°C. The following day the plates were washed four times using TEN-Tween then 50 µl of host serum (or test suspension) was added to designated wells before incubation at room temperature for one hour. After washing again four times, 50 µl enzyme-conjugated anti-species antibody diluted in TEN-TC was added to the wells and again kept at room temperature for one hour before washing six times. Lastly, 100 µl of ABTS substrate was added to each well and incubated in the dark at room temperature for one hour before OD readings were taken.

**Indirect sandwich ELISA for detection of macropod IgG**

The indirect sandwich ELISA followed the procedure as described for the direct sandwich ELISA except for an extra step after the capture antibody, which involved
addition of a second antibody (Crowther, 2001), followed by one hour incubation and four washes before the enzyme-conjugated antibody was added.

**Tabanid sample preparation for ELISA**

During optimisation of the ELISAs, sensitivity of each assay was evaluated by testing the derived optimal reagent concentrations on real tabanid flies that were known to be either positive or negative for the host IgG to be detected by the assay. Similar sets of tabanids were also to be used as either positive control or negative control during testing of field samples after the optimisation process. The tabanid samples were prepared for analysis as follows:

Tabanid abdomens were removed from the insect and individually placed in 500 µl of TEN-Tween and 0.5g of 0.1mm diameter zirconia/silica beads (Daintree Scientific, Tasmania) then ground-up for two minutes using an automatic Mini Bead-beater (Biospec Products, Bartlesville). The resulting homogenate was centrifuged for five minutes and, for the analysis, required volumes for each test were drawn from the supernatant.

Tabanids used as negative control specimens in the ELISA were obtained by capturing hungry *Tabanus pallipennis* females from the wild using odour-baited Nzi traps and keeping them under insectary conditions for six days on a diet of water and diluted sucrose solution to allow digestion of residues from previous blood-meals, if any. The specimens were then stored at -20°C to be used as and when required. All such specimens eventually tested negative for IgG of potential hosts. This was the best option in the absence of unfed laboratory-reared flies which researchers on other insects have used as negative controls (Blackwell *et al*., 1994; Gomes *et al*., 2001; Ponlawat and Harrington, 2005). No success has been achieved with laboratory-rearing of Australian tabanids (Mackerras, 1956).

Positive controls were prepared by feeding individual *T. pallipennis* on blood of host animals (horses or cattle) until fully engorged (Beier *et al*., 1988; Blackwell *et al*., 1994), or by using serum of known animals (Bongiorno *et al*., 2003; Ponlawat and Harrington, 2005). In the latter case, 20 µl of pig or macropod serum were mixed with tabanid suspension prepared similarly to the negative controls, and this serum quantity
was based on previous findings that a fully-fed *T. pallipennis* contains on average 40 µl of blood (Muzari *et al.*, 2010), and the assumption that 50% of the blood meal is serum (Chow *et al.*, 1993).

5.3. Results

5.3.1. Direct Sandwich ELISA for identification of horse blood

Determining optimal concentrations of capture antibody and conjugated antibody for identification of horse blood

The initial stage of the checkerboard titration of reagents for identification of horse blood tested rabbit anti-horse IgG (the capture antibody) at 10 dilutions ranging between 1/200 and 1/102400, peroxidase-conjugated goat anti-horse IgG (labelled antibody) at concentrations of 1/2,000 and 1/5,000, horse serum (positive antigen) at dilutions of 1/1000 and 1/5,000, bovine serum (negative antigen) at dilutions of 1/1,000 and 1/5,000. The starting ranges of dilutions were based partly on manufacturer’s recommendations and partly on past experience with use of similar reagents.

The microtitre plate layout and resultant colours are shown in Fig 5.1. Graphs of the OD values from positive (horse) and negative sera (bovine) were plotted for the two levels of conjugate antibody tested (Fig 5.2 and 5.3), in order to identify a dilution of capture antibody which gave the highest contrast between positive and negative serum samples, indicating maximum discrimination.
Fig 5.1. Layout and resultant image of microtitre plate obtained in checkerboard titration assessing reactions of a range of dilutions of Capture Antibody (rabbit anti-horse) with positive (horse) and negative (bovine) sera.

<table>
<thead>
<tr>
<th>Capture Ab dilution</th>
<th>OD values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse serum 1/1000</td>
<td>Horse serum 1:1000</td>
</tr>
<tr>
<td>Horse serum 1/1000</td>
<td>Horse serum 1:5000</td>
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<td>Bovine serum 1/1000</td>
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<td>Bovine serum 1/5000</td>
<td>Bovine serum 1:1000</td>
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<tr>
<td>Bovine serum 1/5000</td>
<td>Bovine serum 1:5000</td>
</tr>
</tbody>
</table>

Fig 5.2. Results of checkerboard titration assessing effect of a range of dilutions of Capture Antibody (rabbit anti-horse) on positive (horse) and negative (bovine) sera. Capture antibody dilutions started from 1/200 and conjugate dilution was constant at 1/2,000.
Fig 5.3 Results of checkerboard titration assessing a range of dilutions of Capture Antibody (rabbit anti-horse) on positive (horse) and negative (bovine) sera. Capture antibody dilutions started from 1/200 and conjugate dilution was constant at 1/5,000.

The results (Fig 5.1, Fig 5.2 and Fig 5.3) indicated progressively higher levels of discrimination as dilution factor of capture antibody increased across the plate, but also indicated that the range of dilutions initially used did not stretch far enough to reach the titration end-point with reference to the background ODs seen in the blank (control) columns 11 and 12. Based on these results, a new range of two-fold dilutions for the capture antibody (rabbit anti-horse IgG) was tested and this time dilutions ranged between 1/3,200 and 1/1,636,400. The plate layout and results of the new range of dilutions are shown Fig 5.4. Dilutions for the antigen (IgG) and conjugated antibody remained the same as in the initial stage. The graphs from the new range of capture antibody concentrations (Fig 5.5 and Fig 5.6) showed that the optimal dilution with high discrimination and low background was between 1:25,600 and 1:51,200. Therefore the selected optimum was 1: 50,000.
Fig 5.4 Image of microtitre plate results obtained in checkerboard titration to assess reactions among different reagent concentrations in the development of ELISA assay for identification of horse blood (Capture Antibody dilutions start at 1/3,200).

Discrimination between horse IgG (rows A-D) and bovine IgG (rows E-H) can be seen in columns 4-7.
Fig 5.5 Results of checkerboard titration assessing a range of dilutions of Capture Antibody (rabbit anti-horse) on positive (horse) and negative (bovine) sera. Capture antibody dilutions started from 1/3,200 and conjugate dilution was constant at 1/2,000.

Fig 5.6 Results of checkerboard titration assessing a range of dilutions of Capture Antibody (rabbit anti-horse) on positive (horse) and negative (bovine) sera. Capture antibody dilutions started from 1/3,200 and conjugate dilution was constant at 1/5,000.
The graphs of the OD values obtained at two conjugate dilutions (1/2,000; Fig 5.5 and 1/5,000; Fig 5.6) show that there is no difference between a serum dilution of 1/1,000 and a serum dilution of 1/5,000 at the selected capture antibody dilution (1/50,000) if conjugate dilution is 1/2,000 (Fig 5.5). A conjugate dilution of 1/2,000 was therefore selected as the optimum, since it resulted in higher OD values even at a higher horse serum dilution of 1/5,000. A high OD signal at a high serum dilution is a sign of good sensitivity.

As a result of the whole checkerboard titration, the optimal reagent concentrations for the horse-blood identification assay were as follows:

- Capture antibody (rabbit anti-horse): 1/51,200 (rounded off to 1/50,000)
- Labelled antibody (conjugated goat anti-horse): 1/2,000
- Positive control IgG (horse serum): 1/5,000

**Testing the specificity (cross-reactions) of the horse-blood assay at the selected dilutions of reagents**

Selection of optimal dilutions of the antibody and conjugate for the developed horse-blood assay was based on minimal cross-reactions with bovine serum as the negative antigen. It was, however, necessary to check whether the selected dilutions did not cross-react with sera from other possible hosts that occur in the study area. To do this, the assay was performed using the selected concentrations of reagents, with sera of horse, bovine, kangaroo, pig, sheep, human, chicken and magpie goose all tested on the same microtitre plate. The sera from all these animals were tested at dilutions ranging from 1/1,000 to 1/1,024,000, performed by serial double-dilutions across the plate up to column 11. The detailed layout of the plate and colours arising from the reaction are shown in Fig 5.7. The results from OD readings of the plate are plotted in Fig 5.8.
Fig 5.7 Layout and image of microtitre plate after testing for cross-reactions among reagents of the horse identification assay at the optimal dilutions and sera from seven other animal species.

Fig 5.8 Comparison of OD values obtained after testing the optimal horse ELISA for cross-reactions with sera from several potential host animals. Dilution of all sera started at 1/1000.

The results indicate no discernible cross-reaction between the reagents in this assay and any of the other host sera tested at the selected optimal dilutions.
Testing the sensitivity of the horse-blood identification ELISA on blood-fed tabanids at optimal dilutions of reagents

After the assay was developed and optimal dilutions selected on the basis of reactions with horse serum samples, it was necessary to then test the sensitivity of the assay on actual blood-fed tabanids. Four blood-fed specimens of *Tabanus pallipennis* were tested against four non-fed specimens of the same species on the same microtitre plate. The blood-fed specimens had been allowed to feed to engorgement on a restrained horse, while the non-fed tabanids had been kept on a diet of sugar and water for six days after collection from an odour-baited trap, to allow for total digestion of any traces of blood in the gut (Chapter 4).

Prior to the test, tabanid abdomens had been crushed and homogenised in 500 µl of TEN TC and this constituted the sample solution at neat concentration. For this test of sensitivity, the sample solutions from the four blood-fed fed flies were allocated the first four rows, while the sample solutions from the unfed flies were allocated the lower four rows on a microtitre plate. All wells had been pre-coated with rabbit anti-horse IgG. By serial double-dilutions, the sample solutions were distributed across the plate at dilution factors ranging from Neat to 1,024 from column 1 to column 11 (Fig 5.9), with volumes of 50 µl per well.

The results are shown in Fig 5.9 and Fig 5.10. They show that the assay clearly discriminates between blood-fed and non-fed tabanids at all dilution levels tested.
Fig 5.9 Layout and image of Microtitre plate after testing the sensitivity of the ELISA assay to identify presence of horse blood in four blood-fed and four non-fed tabanids (Sample dilutions start from Neat and continue in serial double-dilutions).

Fig 5.10 Results of ELISA testing the sensitivity of the horse IgG assay to identify presence of horse blood in four blood-fed and four non-fed tabanids

Because the end-point of sensitivity was not reached with the dilutions between neat and 1:1,024, a further test was conducted with higher blood-fed homogenate dilutions, ranging between 1/100 and 1/1,02,400 (Fig 5.11).
Fig 5.11 ELISA Plate results in assessment of sensitivity of horse assay on four blood-fed (Rows A-D) and four non-fed (Rows E-F) tabanid homogenates at increasing dilutions across the plate.

Fig 5.12 Comparison of OD values for four blood-fed tabanids and four non-fed tabanids at different dilutions of homogenised tabanid sample
From Fig 5.12, it is clear that discrimination could still be achieved up to a dilution of about 1/1,000 of the neat homogenate. Because the neat homogenate is itself a dilution of ~ 40 µl blood meal in 500 µl diluent (TEN TC), the assay in fact detected 1/12,500 µl (0.0001 µl) of blood.

5.3.2 Direct Sandwich ELISA for identification of pig blood

Determining optimal concentrations of pig serum, capture antibody and conjugated antibody for identification of pig blood

The checkerboard titration of reagents for identification of pig (swine) blood started by testing goat anti-swine IgG (the capture antibody) at 10 dilutions ranging between 1/200 and 1/102,400, peroxidise-conjugated goat anti-swine IgG (labelled antibody) at concentrations of 1/2,000 and 1/5,000, pig serum (positive antigen) at dilutions of 1/1,000 and 1/5,000 and bovine serum (negative antigen) at dilutions of 1/1,000 and 1/5,000 (Fig 5.13).

Results of this initial stage are shown in Fig 5.13, Fig 5.14 and Fig 5.15 comparing positive and negative sera at the two levels of conjugate antibody dilution tested.

<table>
<thead>
<tr>
<th>Capture Ab dilution</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td></td>
</tr>
<tr>
<td>400</td>
<td></td>
</tr>
<tr>
<td>800</td>
<td></td>
</tr>
<tr>
<td>1600</td>
<td></td>
</tr>
<tr>
<td>3200</td>
<td></td>
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<tr>
<td>6400</td>
<td></td>
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<tr>
<td>12800</td>
<td></td>
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<tr>
<td>25600</td>
<td></td>
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<tr>
<td>51200</td>
<td></td>
</tr>
<tr>
<td>102400</td>
<td></td>
</tr>
</tbody>
</table>

Fig 5.13 Layout and resultant image of microtitre plate used in checkerboard titration to assess reactions among different reagent concentrations in the development of assay for identification of pig blood.
Fig 5.14. Distinction between pig (positive) and bovine (negative) sera at different dilutions of anti-pig capture antibody in the pig-detection assay using conjugate dilutions of 1/2,000.

Fig 5.15 Distinction between pig (positive) and bovine (negative) sera at different dilutions of anti-pig capture antibody in the pig-detection assay using conjugate dilutions of 1/5,000.
From the graphs, it is clear that the anti-swine reagents did not react with the negative serum (bovine) at all dilution levels. In the end, the preferred dilution of anti-swine capture antibody was 1/1,600 (later rounded off to 1/1,500) because of its ideal OD of about 1.8 units produced with a conjugate dilution of 1/5,000 and a pig serum dilution of 1/5,000. Crowther (2001) recommends conjugate dilutions yielding OD units of around 1.8 on a good titration curve, as this gives ‘the most controllable results’ in ELISA.

**Testing the specificity (cross-reactions) of the pig-blood assay at the selected optimal dilutions of reagents**

The optimal dilutions of the antibody and conjugate for the pig-blood assay were selected based on comparison with bovine serum as the negative antigen. To check for cross-reactions with other possible hosts, the selected concentrations of reagents were also tested with sera of horse, bovine, kangaroo, pig, sheep, human, chicken and magpie goose. The sera from all these animals were tested at dilutions ranging from 1/1,000 to 1/1,024,000, performed by serial double-dilutions across the plate up to column 11 (Fig 5.16).

![Fig 5.16 Layout and image of microtitre plate used for testing the selected optimum reagents for identification of pig blood against sera from seven other potential hosts to check for cross-reactions.](image)
Testing for cross-reactions between the pig assay reagents and sera from different potential hosts over a range of serum dilutions

Fig 5.17 Pattern of OD values obtained when testing the optimal pig assay for cross-reactions with sera from several potential host animals

The selected optimal dilutions of anti-swine capture antibody and conjugate did not cross-react with sera from the other potential host animals tested.

**Testing the sensitivity of the pig-blood identification ELISA on blood-fed tabanids at optimal dilutions of reagents**

Optimal dilutions of reagents had been selected on the basis of reactions with pig serum samples, and it was necessary to then test the sensitivity of the assay on actual blood-fed tabanids. To simulate blood-fed tabanids, homogenate from crushed abdomens of non-fed *Tabanus pallipennis* were thoroughly mixed with pig serum in the proportion 500 µl to 20 µl. The serum amount was based on separate observations that *T. pallipennis* imbibe on average 40 µl of blood to get a full blood meal (Chapter Four; Muzari et al, 2010), and it was assumed that half of this amount is composed of serum (Chow, 1993). In this way, four ‘fed’ tabanids were tested against four non-fed specimens of the same species on the same microtitre plate.
For this sensitivity test, the sample solutions from the four ‘blood-fed’ flies were allocated the first four rows, while the sample solutions from the unfed flies were allocated the lower four rows on a microtitre plate. All wells had been pre-coated with goat anti-swine IgG. By serial double-dilutions, the sample solutions were distributed across the plate at dilution factors ranging from neat to 1,024 from column 1 to column 11 (Fig 5.18), with volumes of 50 µl per well. The ELISA results as seen on the microtitre plate are shown in Fig 5.18 and plotted in Fig 5.19.

Fig 5.18. ELISA results for comparison of sample solutions from four ‘pig-fed’ tabanids in the first four rows, and unfed tabanids in the lower four rows. Sample dilution factors ranged from neat to 1,024.
Sensitivity of Pig Assay, comparing 4 positive (with pig serum) with 4 negative (no serum) tabanid samples over a range of dilutions

<table>
<thead>
<tr>
<th>Column Number (serial dilutions of sample starting at Neat)</th>
<th>OD reading</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Tabanid 1</td>
<td></td>
</tr>
<tr>
<td>Positive Tabanid 2</td>
<td></td>
</tr>
<tr>
<td>Positive Tabanid 3</td>
<td></td>
</tr>
<tr>
<td>Positive Tabanid 4</td>
<td></td>
</tr>
<tr>
<td>Negative Tabanid 1</td>
<td></td>
</tr>
<tr>
<td>Negative Tabanid 2</td>
<td></td>
</tr>
<tr>
<td>Negative Tabanid 3</td>
<td></td>
</tr>
<tr>
<td>Negative Tabanid 4</td>
<td></td>
</tr>
</tbody>
</table>

Fig 5.19 Checking the sensitivity of the Pig-blood ELISA: Comparison of sample solutions from four positive (blood-fed) tabanids and four negative (unfed) tabanids over a range of dilutions

The assay discriminated between blood-fed tabanids and unfed tabanids over a wide range of dilutions. The highest tested dilution was 1/1,024 of the neat homogenate, which in itself had a dilution of ~ 40 µl blood meal in 500 µl diluent, implying that this assay was able to detect 1/12,800µl (~0.0001 µl) of pig blood. The high OD recorded at the 1/1,024 dilution indicates that the assay is sensitive enough to detect even lower volumes of blood than the 0.0001 µl tested.

5.3.3 Indirect Sandwich ELISA for identification of macropod blood

Determining optimal concentrations of reagents for macropod assay

An indirect sandwich ELISA had to be adopted for the macropod assay because enzyme-conjugated anti-macropod antibody was not available from commercial suppliers. With this approach, the macropod antigen would be sandwiched between a capture antibody (mouse anti-kangaroo) and a second (indicator) antibody (rabbit anti-macropod), which would then be detected using enzyme-conjugated anti-rabbit antibody (goat anti-rabbit).
**Titration for Capture Antibody (mouse anti-kangaroo)**

The first optimal dilution to be determined was for the capture antibody (mouse anti-kangaroo). Ten different dilution levels were tested between 1/400 and 1/204,800; while the indicator antibody was initially held constant at 1/2,000 in all the rows and conjugated antibody set at two levels, 1/2,000 and 1/5,000. The positive antigen (kangaroo serum) and negative antigen (horse serum) were allocated four rows each, with two rows at a dilution of 1/1,000 and two at 1/5,000 (Fig 5.20). Choice of fixed dilutions (indicator and conjugated antibodies) was guided by manufacturers’ recommendations and knowledge from preliminary attempts. The complete layout of the microtitre plate and resultant well colours are given in Fig 5.20, and results from OD readings are plotted in Fig 5.21 and Fig 5.22.

Fig. 5.20 Layout and resultant colour image of microtitre plate after testing a range of 10 different dilutions of Capture Antibody (mouse anti-kangaroo) in the indirect sandwich ELISA for detection of macropod antigens.
Fig 5.21 Performance of the Capture Antibody (mouse anti-kangaroo) at different dilutions with Conjugated Antibody at two dilution levels and animal sera (kangaroo and horse) diluted at 1/1,000.

Fig 5.22 Performance of the Capture Antibody (mouse anti-kangaroo) at different dilutions with Conjugated Antibody at two dilution levels and animal sera (kangaroo and horse) diluted at 1/5,000.
From the graphs, the best dilutions of the Capture Antibody were between 1/6,000 and 1/12,000 just before the steepest decline in OD values (Fig 5.21 and Fig 5.22). Therefore titrations of other reagents were later done with the capture antibody at two dilutions, 1/6,000 and a convenient 1/10,000 (replacing 1/12,000). With serum dilutions, there was no obvious difference in performance between 1/1,000 (Fig 5.21) and 1/5,000 (Fig 5.22). However, the higher serum dilution (1/5,000) was selected for use in further titration of other reagents, since it implied better sensitivity of the assay. The situation was the same for Conjugate Antibody dilutions, and the higher dilution of 1/5,000 was selected.

**Titration for indicator antibody (rabbit anti-macropod)**

As determined from the preceding stage, capture antibody was set at two levels (1:6,000 and 1:10,000), and to obtain the optimum dilution for indicator antibody (rabbit anti-macropod), it was tested at 10 levels (by serial dilutions across the plate starting at 1:500) (Fig 5.23). Conjugated antibody was set at two levels (1:2,000 and 1:5,000), and both positive antigen (kangaroo serum) and negative antigen (horse serum) were held at a constant 1:5,000 dilution.
Fig 5.24 Results of titration for Indicator Antibody (rabbit anti-macropod) at 10 different dilution levels discriminating between kangaroo serum and horse serum at a conjugate dilution of 1/2,000.

Fig 5.25 Results of titration for Indicator Antibody (rabbit anti-macropod) at 10 different dilution levels discriminating between kangaroo serum and horse serum at a conjugate dilution of 1/5,000.
Indicator antibody (rabbit anti-macropod) showed best discrimination between kangaroo serum and horse serum at dilution levels between 1:16,000 and 1:32,000 (Fig 5.24 and Fig 5.25). After testing them separately, the final choice was for 1:32,000 as it gave lower background OD values. Conjugate dilutions of 1:2,000 and 1:5,000 did not show any difference and final choice was for 1:5,000. The capture antibody (mouse anti-kangaroo) performed just as good at a dilution level of 1:10,000 as it did at 1:6,000 (Fig 5.24 and Fig 5.25).

**Testing the specificity (cross-reactions) of the macropod-blood assay at the selected optimal dilutions of reagents**

The macropod assay, based on the selected reagent dilution levels from the checkerboard titration, was tested for possible cross-reactions with sera from seven other host species. Serial dilutions of serum for each species were conducted along the rows of the plate, with each species occupying one row and starting at a dilution of 1:1,000 (Fig 5.26). Specificity level of the macropod assay was good, with no cross-reactions of the reagents with sera from non-macropod species (Fig. 5.26 and Fig 5.27).

![Fig 5.26 ELISA plate result showing specificity of macropod assay when performed on sera of different host animals. Reactions were only observed in the row with kangaroo serum.](image)
Fig 5.27 The capacity of the macropod ELISA (indirect sandwich) to differentiate between kangaroo serum and sera from other animal species over a range of serum dilutions

**Testing the sensitivity of the optimal macropod-blood assay in ‘blood-fed’ tabanids**

To test the sensitivity of the selected reagent dilutions on tabanids, four ‘kangaroo-fed’ tabanids were tested against four non-fed specimens of the same species on the same microtitre plate (Fig 5.28). ‘Kangaroo-fed’ tabanid homogenate was obtained by mixing kangaroo serum with crushed non-fed tabanid specimens.

The ELISA results as seen on the microtitre plate are shown in Fig 5.28 and the OD results plotted on Fig 5.29.
Fig 5.28 ELISA results for comparison of homogenate solutions for four ‘kangaroo-fed’ tabanids in the first four rows, and unfed tabanids in the lower four rows. Homogenate dilution factors ranged from Neat to 1,024; with constant Capture Ab dilution of 1:10,000; Indicator Antibody of 1:16,000 and Conjugate dilution of 1: 5,000.

Fig 5.29 Sensitivity of the macropod-blood ELISA on sample solutions from four positive (blood-fed) tabanids and four negative (unfed) tabanids over a range of double-dilutions starting at Neat.

Discrimination between fed and non-fed tabanids was perfect throughout the whole range of homogenate dilutions tested. Again, the highest dilution of neat homogenate tested was 1/1,024, giving a final blood volume of ~0.0001 µl detected convincingly,
but the pattern on Fig 5.29 indicates that sensitivity of the assay could go beyond the level tested.

5.3.4 Direct ELISA for Identification of Bovine Blood

*Development of direct ELISA for detecting presence of bovine antigen (IgG)*

The (bovine) assay initially demonstrated insufficient sensitivity when sandwich ELISAs were used (Details in section 5.3.5). Therefore a direct non-sandwich ELISA was employed and this excluded both the capture and indicator antibody stages. In this method, bovine antigen attached directly onto the microtitre wells is detected with conjugated anti-bovine antibody followed by ABTS substrate solution to produce the colour reaction.

Bovine serum was used as the source of positive IgG and horse serum as negative IgG to test the capacity of the direct ELISA to discriminate between the two host species. The appropriate serum dilution to be used as a positive control in subsequent tests as well as the suitable conjugate dilution was first determined by checkerboard titration using the layout shown in Fig 5.30. Whole bovine serum at a range of dilutions was used for coating the wells. The conjugate was goat anti-bovine HRP (procured with minimum cross-reactivity to Armenian hamster, human, mouse and rat serum proteins) and was tested at 1:2,000 and 1:5,000 dilutions (Fig 5.30) based on manufacturer’s recommendations.

The direct ELISA checkerboard titration clearly discriminated between bovine and horse sera at all serum dilutions tested and at both conjugate concentrations tried (Fig. 5.30 and Fig 5.31). Conjugate dilution of 1:5,000 was chosen as the preferred concentration.
Fig 5.30 Microtitre plate showing results of checkerboard titration in the development of direct ELISA for bovine assay, with positive antigen (bovine serum) in rows A-D and negative antigen (horse serum) in rows E-H.

Fig 5.31 Discrimination between positive (bovine) and negative (horse) sera over a wide range of serum dilutions
Checking for cross-reactions in the direct ELISA for bovine antigen identification

After obtaining satisfactory discrimination with equine serum as negative antigen, other potential host sera were also tested as negative antigens to check for any possible cross-reactions with the anti-bovine conjugate. Sera from different animals were tested one in each row as shown in Fig 5.32. All sera were tested at a range of double-dilutions between 1:1,000 and 1:1,024,000.

The anti-bovine conjugate in the direct ELISA did not react with serum from any other hosts tested (Fig 5.32 and Fig 5.33), and the assay discriminated clearly between bovine serum and that of other hosts.

Fig 5.32 Layout and results from microtitre plate testing for cross-reactions between anti-bovine conjugate with sera from different animals. Bovine serum was in row B, which gave the only positive colour reaction.
Testing sensitivity of direct ELISA (bovine detection assay) on blood-fed and non-fed tabanids

After demonstrating good specificity of the direct ELISA for bovine IgG detection using sera from bovine and other different hosts, the assay’s level of sensitivity was assessed on samples of ‘bovine-fed’ tabanids. In this instance, the ‘bovine-fed’ tabanids were made-up by adding 20µl of bovine serum to crushed abdomens of non-fed *T. pallipennis* homogenised in 500 µl TEN TC. Serial dilutions of these positive homogenates were performed across the plate along the first four rows. Negative homogenates, which consisted of crushed and homogenised non-fed tabanids, were also diluted across the plate along the bottom four rows. The homogenised sample dilutions initially ranged from neat to 1:1,024 (Fig 5.34 and 5.35) by serial double-dilutions. Surprisingly, OD values increased with increasing sample dilutions for the ‘cow-fed’ tabanids (Fig 5.35). To investigate the full extent of this trend, dilutions were later extended to range from neat to 1: 4,194,304 (Fig 5.36 and 5.37). In the extended dilution range, serial dilutions for positive homogenates started in first two rows (A and B) up to column 11 and continued in the next two rows (C and D). Column 12 was used for positive controls (bovine serum at 1:5,000, 12A-12D) and blanks (reagent diluents only, 12E-12H).
Fig 5.34 Layout and image of microtitre plate showing results of direct ELISA on tabanid homogenate with added cow serum (top four rows) and without cow serum (bottom four rows) with serial dilutions of the homogenised sample going up to 1/1,024.

Fig 5.35 Sensitivity of the bovine antigen detection assay over a narrow range of dilutions of four ‘cow-fed’ and four non-fed tabanids.

Apparently, the presence of tabanid homogenate at relatively high concentrations in the first few columns had a negative effect on the colour development, as were the excessively low concentrations on the tail end of the dilution range (Fig 5.36 and Fig 5.37). The highest colour intensities were mid-way through the dilution range.
Fig 5.36 Layout and image of microtitre plate showing results of direct ELISA on tabanid homogenate mixed with bovine serum (top two rows A and B extending to rows C and D) and without bovine serum (rows E and F extending to rows G and H). Serial dilutions of the homogenate ranged from Neat to 4,194,304.

Fig 5.37 Sensitivity of the bovine assay at different sample (tabanid homogenate) dilutions (extended dilution range) in the presence and absence of bovine serum
The presence of crushed tabanid material in the sample had a clear effect of reducing the detectability of bovine serum if dilution levels of neat homogenate were less than 1:100. The observed positive gradient with increasing sample dilution in that range was not expected, but implies that good detectability of bovine IgG with this assay can only be achieved if the homogenised tabanid sample is further diluted by a factor of at least 100, but less than 4,000.

A similar trend was obtained when the same assay with same dilution levels was applied to homogenised samples of tabanids that had blood-fed to engorgement on a bovine (Fig 5.38), although the colour intensity in positive wells was about two times lower when blood was involved instead of just bovine-serum. The ODs remained lower when the test was repeated with normal blood in place of serum.

![Graph showing detectability of bovine antigen in homogenised samples of blood-fed and non-fed tabanid by direct ELISA at different concentrations of homogenate.](Fig 5.38)

However, it was apparent from Fig 5.38 that the optimal dilution levels of the homogenised sample would have to be between 1:100 and 1:4,000. Further tests with blood-fed tabanid samples at homogenate dilutions of 1:2,000, 1:1,000 and 1:200 were conducted, and it emerged that the 1:200 dilution gave the best positive signal. This was adopted as the optimal dilution for positive control and for the homogenised test samples captured from field sites.
Therefore, while field-collected samples would be tested as neat homogenate for horse, pig and kangaroo assays, they would need an extra 1:200 dilution for the bovine assay. However, this did not compromise the sensitivity of the bovine assay, because even a higher homogenate dilution of 1:1,000 (implying a total blood meal dilution of 1:12,500 considering the initial 40:500 at grinding) was very convincingly detectable (Fig 5.38) with this assay. This was equivalent to ~0.0001 µl of blood which was equally detectable with the two sandwich assays for horse, pig and kangaroo. Interestingly, the 1:200 dilution of tabanid sample increased the detectability of the bovine IgG in the blood meal.

5.3.5 Previous (unsuccessful) attempts to optimise the bovine IgG detection assay by direct sandwich and indirect sandwich ELISA

The Direct ELISA technique that was used to identify bovine antigens was initially assumed to be less sensitive than the Sandwich ELISAs because of the requirement for the antigen to bind non-specifically to the wells of the microtitre plate. So the initial attempts to develop an assay for bovine antigen were based on Direct Sandwich ELISA and later on Indirect Sandwich ELISA, but both did not produce a good enough assay with the reagents used. The Direct ELISA was the last option. However, the assay was still very sensitive as described earlier. The initial attempts, which were eventually abandoned, are described briefly below.

Attempt at using Direct Sandwich ELISA for detection of bovine IgG

Direct Sandwich ELISA for bovine antigens was tried using the normal procedure of checkerboard titration for determining optimal concentrations of capture and conjugated antibodies. The capture antibody (rabbit anti-bovine) was first tried over a range of 10 different dilutions starting from 1/200 and conjugated antibody initially at two dilutions 1/2,000 and 1/5,000, while positive serum (bovine) and negative serum (horse) were each set at 1/1,000 and 1/5,000. These ranges of concentrations had worked well as starting points for other sandwich ELISAs in this study, and were within the concentrations recommended by the manufacturers.
From the results of the titration, no discrimination was achieved between positive antigen (bovine serum) and negative antigen (horse serum), suggesting possible cross-reaction at all levels. This necessitated an investigation into the extent of cross-reactions with sera of the rest of the other potential host animals.

The assay showed very little discrimination between the bovine serum and sera from horse, pig, kangaroo, sheep, human, chicken and goose, but also produced a strong positive reaction where capture antibody was present with no serum added. This suggested that the conjugate might be binding directly to the capture antibody, but still with the possibility of cross-reaction between the conjugate and sera from all the other seven non-bovine hosts. It was therefore necessary to investigate further and check the specificity of the goat-anti-bovine conjugate, i.e whether the conjugate was reacting with sera from the non-target hosts. This was tested by omitting the capture antibody and coating the sera directly to the plate, and the results showed that the specificity of the conjugate was very good and it was not reacting with the negative sera.

The results of the conjugate specificity test indicated that the goat anti-bovine conjugate in fact had good specificity level and it did not react significantly with all the other non-bovine hosts. This implied that any colour reactions previously observed were probably a result of reaction between the conjugate and the capture antibody. On the basis of these results, the capture antibody for this assay had to be changed, and after this the rabbit anti-bovine IgG was replaced by other alternatives as described below.

Based on previous indication that rabbit anti-bovine IgG was not a suitable capture antibody for the assay, it was replaced with goat anti-bovine IgG in the next attempt. This also meant that the capture IgG and conjugated IgG both originated from goat, hence this could further minimise chances of undesirable attachment between the capture antibody and conjugated antibody. Similar checkerboard titration procedures were performed as described above, using the new combination of reagents. Surprisingly, there was still no discrimination between positive and negative antigen over a wide range of dilution levels, and the results still suggested some reaction between capture antibody and conjugated antibody.

Because of the unexpected results with anti-bovine IgG originating from goat, an attempt was made to develop the assay using sheep anti-bovine IgG and peroxidise-
conjugated sheep anti-bovine IgG. Again, the same procedure for checkerboard titration was followed, but the resultant colour from the positive antigen showed insufficient discrimination with negative antigen. The strategy of using anti-bovine antibodies from the same host species in the same assay was therefore not effective in both cases, and this was unexpected. A different strategy had to be adopted.

**Attempts at using the Indirect Sandwich ELISA for identification of bovine antigen**

Unsatisfactory results in development of direct sandwich ELISA for identification of bovine blood were possibly due to unexpected attachment between anti-bovine capture antibody and anti-bovine conjugated antibody. To try and address this problem, an indirect sandwich ELISA was tried using sheep anti-bovine IgG as capture antibody, rabbit anti-bovine IgG as indicator antibody and HRP-conjugated goat anti rabbit IgG as the enzyme-conjugated antibody. It was hoped that the additional step of the indicator antibody within the process plus the fact that the conjugated antibody was not an anti-bovine would prevent the undesirable attachment of the conjugated antibody to anything other than the indicator antibody, which in turn would have attached to the bovine antigen if the antigen was present in the test solution. Again, this approach did not improve the level of discrimination between positive (bovine) and negative (horse) antigen in this assay at all dilution levels.

Therefore it was logical to avoid using a capture antibody for the bovine assay, and the direct ELISA was tried next and it gave very good specificity and sensitivity as described earlier.

**5.3.6 Summary of optimal dilutions of the different antibodies used in all the ELISAs**

For the four types of host animals that the assays were developed to detect, optimal dilutions of reagents as obtained from the series of investigations described in this chapter are shown in Table 5.1.
5.4. Discussion

The three types of ELISA proved to be suitable assays for the host types they were optimised to identify. They were all sensitive enough to detect as little as 0.0001 µl of host blood and all reagents at the optimal concentrations showed no cross-reactions with sera of non-intended hosts.

Direct ELISA has been considered to be less sensitive than sandwich ELISAs because of non-specific binding of test antigen to microtitre plate (Chow et al., 1993). This appeared true when homogenised samples were tested at equal dilutions of up to 1/32 (Fig 5.18, pig; Fig 5.28, kangaroo; and Fig 5.36, bovine), and the poor sensitivity of direct ELISA in this range suggested that the binding of host antigens was limited due to competition from other proteins from the insect (Fig 5.36) and also from host blood (Fig 5.38). This problem is somewhat similar to what was encountered by Beier et al (1988) in trying to detect Plasmodium falciparum sporozoites by direct ELISA on blood-fed mosquitoes. However in the present study, sensitivity of this assay significantly increased after further dilution of the test sample to between 1/100 and 1/4,000 (Fig 5.36), thereby reducing the concentration of competing proteins to levels where antigen binding was sufficiently enhanced. Overall, the results agree with the suggestion of Beier et al (1988) that direct ELISA can be just as accurate as the sandwich ELISAs depending on study objectives and level of sensitivity desired. However, the increased dilution of test sample adopted in the present study has the potential to reduce availability of antigen from the test sample and the effect of this might be noticed if the blood meal was extremely small and the assay was desired to detect blood quantities of less than 0.0001 µl.

In the initial attempts to use sandwich ELISA for bovine antigens (section 5.3.5), the reasons for failure of commercially available anti-bovine reagents to discriminate between different hosts were not immediately clear. Changing the supplier did not provide a satisfactory alternative.

The study further confirmed that the indirect sandwich ELISA is a suitable alternative to the direct sandwich ELISA if conjugated antibodies of a required host (macropod) are not commercially available, and this is quicker and most probably cheaper than trying to develop one’s own conjugated antibodies (Hall et al., 1987).
Table 5.1 Reagents and their optimal concentrations used in the ELISAs to identify sources of tabanid blood-meal

<table>
<thead>
<tr>
<th>Host tested and type of ELISA</th>
<th>Capture Antibody (Affinity-purified IgG)</th>
<th>Second Antibody (Affinity-purified IgG)</th>
<th>Enzyme-conjugated Antibody (IgG)</th>
<th>Antibody Dilution</th>
<th>Antibody Dilution</th>
<th>Antibody Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine (Direct ELISA)</td>
<td>Goat anti-bovine*</td>
<td>Goat anti-bovine*</td>
<td>1:5000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Horse (Direct sandwich ELISA)</td>
<td>Rabbit anti-horse*</td>
<td>Goat anti-horse*</td>
<td>1:2000</td>
<td>1:50,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pig (Direct sandwich ELISA)</td>
<td>Goat anti-swine*</td>
<td>Goat anti-swine*</td>
<td>1:5000</td>
<td>1:1,500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macropod (Indirect sandwich ELISA)</td>
<td>Mouse anti-kangaroo**</td>
<td>Rabbit anti-macropod**</td>
<td>1:5000</td>
<td>1:10,000</td>
<td>1:32,000</td>
<td>1:5000</td>
</tr>
</tbody>
</table>

Source of reagent: * Jackson ImmunoResearch Laboratories, USA. ** TropBio Pty Ltd, Australia.
CHAPTER SIX

Host preferences of tabanid flies based on identification of blood meals by ELISA*

*modified version of: Muzari, M.O., Burgess, G.W., Skerratt L.F., Jones R.E. and Duran T. (2010), Host preferences of tabanid flies based on identification of blood meals by ELISA. *Veterinary Parasitology* **174**: 191-198

6.1. Introduction

*Trypanosoma evansi* is endemic throughout southeast Asia and much of the Indonesian archipelago (Reid *et al*., 1999). It does not occur in Australia, but there is a risk of it entering the country from the north (Reid and Copeman, 2003; Van Hennekeler *et al*., 2008). *Trypanosoma evansi* is mainly spread mechanically on the mouthparts of blood-feeding tabanids (Herrera *et al*., 2005; Desquesnes *et al*., 2008). There are many species of tabanids in Australia (Mackerras, 1971), but very little is known about their feeding habits and host range. With vector-borne diseases, knowledge of the main hosts is a key element in understanding the potential importance of the vector and epidemiology of the disease (Van den Bossche and Staak, 1997).

Blood meal analysis has been used to determine main hosts, host preferences and feeding patterns in many biting insects; for example *Anopheles* mosquitoes (Mukabana *et al*., 2002; Mwangangi *et al*., 2003; Parida *et al*., 2006), *Culex* mosquitoes (Mboera and Takken, 1999; Lee *et al*., 2002; Zinser *et al*., 2004), *Aedes* mosquitoes (Ponlawat and Harrington, 2005; Richards *et al*., 2006), savannah tsetse flies (Snow *et al*., 1988; Clausen *et al*., 1998), rain-forest tsetse flies (Spath, 2000; Njioku *et al*., 2004), *Lutzomyia* sand flies (Marassa *et al*., 2006), *Phlebotomus* sand flies (Bongiorno *et al*., 2003; Svobodova *et al*., 2003), *Simulium* blackflies (Hunter and Bayly, 1991; Boakye *et al*., 1999) and biting midges, *Culicoides* sp. (Tempelis and Nelson, 1971; Blackwell *et al*., 1994). Tabanid flies remain among the least studied vectors, and analysis of feeding patterns by blood-meal identification have only been conducted in Louisiana (USA)
(Wilson and Richardson, 1969), Connecticut (USA) (Magnarelli and Anderson, 1980) and Congo (Gouteux et al., 1989). The study in Congo only focused on two anthropophilic species of *Chrysops* which are known vectors of *Loa loa* among humans. No work has been done on host preferences of tabanid flies in Australia, although many species have been observed feeding on various animals (Mackerras, 1971; Muzari et al., 2010c). General observations indicated that humans were not preferred hosts but several species fed on domestic stock (Mackerras, 1971). This lack of knowledge remains a critical gap in our ability to evaluate the likely impact of tabanid-transmitted diseases like surra.

This study investigated the feeding patterns and host preferences of different tabanid species by identifying and comparing prevalence of blood-meals from different host types among field-caught tabanids in Queensland, north-eastern Australia.

### 6.2. Materials and methods

**Study area and tabanid trapping**

Tabanid flies were captured from their natural habitat in the Townsville area of Queensland using Nzi traps (Mihok, 2002) baited with octenol. Most of the area consisted of savanna vegetation dominated by *Eucalyptus* trees, with sections of open grassland generally used for grazing. Nine traps were deployed at permanent positions in February - March 2007 and insects were collected from them three times per week. Tabanids were identified to species based on the keys of (Mackerras, 1959, 1961, 1971) and stored individually in microtubes at -20°C until further analysis.

The trapping area was conveniently divided into four locations based on estimated relative densities of four potential hosts: horses, cattle, pigs and macropods. The most common macropod species in the area was the agile wallaby (*Macropus agilis*) (Fig 6.1) although other macropods also occur, such as allied rock wallabies (*Petrogale assimilis*), whiptail wallabies (*Macropus parryi*), swamp wallabies (*Wallabia bicolor*), eastern grey kangaroos (*Macropus giganteus*) and common wallaroos (*Macropus robustus*). Blood samples from species within this family are not distinguishable by serological analysis (van den Hurk et al., 2003). The locations, namely Alligator Creek,
Oonoonba, Stuart and JCU (Chapter 2, Fig 2.4) were 8-20 km apart and traps within a location were at least 250 m apart. Vegetation was denser at Alligator Creek and JCU than at Oonoonba and Stuart, and so was the apparent population of macropods. Estimated relative densities of animals in this study (Table 6.1) were based on prior familiarisation with the areas (Fig 6.2) and information from locals (Spath, 2000). For instance, macropods could be seen daily at Alligator Creek browsing in groups throughout the area just before dusk, while at Stuart or Oonoonba an occasional macropod might be encountered once in 2-3 days. Pigs were rare (Fig 6.1) on the study locations, but were considered because they are more densely populated in other parts of northern Queensland (van den Hurk et al., 2003) where they could be important hosts for the same tabanid species. Other possible hosts in the area included sheep, rabbits, dogs, birds, flying foxes (Pteropus spp.) and dingos (Canis lupus dingo). Sheep were only present on two research properties and are not common in northern Queensland.

<table>
<thead>
<tr>
<th></th>
<th>Alligator Creek</th>
<th>Oonoonba</th>
<th>Stuart</th>
<th>JCU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horses</td>
<td>High</td>
<td>Low</td>
<td>High</td>
<td>Very low</td>
</tr>
<tr>
<td>Cattle</td>
<td>Low</td>
<td>Very high</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Macropods</td>
<td>Very high</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Pigs</td>
<td>Low</td>
<td>Very low</td>
<td>Very low</td>
<td>Low</td>
</tr>
</tbody>
</table>
Fig 6.1. Some of the potential tabanid hosts in north Queensland:
A- agile wallaby;    B- feral pig
[Photos courtesy of James Cook University: www.jcu.edu.au]
Fig 6.2. Tabanid traps at different locations in the study area, with different kinds of dominant host: A- Oonoonba (cattle); B- Stuart (horses); C- JCU (cattle)
**Tabanid sample preparation and ELISA procedures for blood meal analysis**

Tabanid abdomens were removed from the insects and individually placed in 500 µl of TEN-Tween (Tris 0.05M, EDTA 0.006M, Sodium chloride 0.003M with 0.05% Tween 20) and 0.5g of 0.1mm diameter zirconia/silica beads (Daintree Scientific, Tasmania) then ground-up for two minutes using an automatic Mini Bead-beater (Biospec Products, Bartlesville). The resulting homogenate was centrifuged at 10,000 rpm for five minutes and, for the analysis, required volumes for each test were drawn from the supernatant. Samples with visible traces of blood after grinding were noted. Each homogenised tabanid sample was later tested for the presence of antigens originating from horses, pigs, cows and macropods.

Direct ELISA for bovine antigens, direct sandwich ELISA for equine and porcine antigens and indirect sandwich ELISA for macropod antigens (Crowther, 2001) were used to identify tabanid blood meals. The procedures for the three types of ELISA are described in Chapter 5. Optimal dilutions of antisera and conjugates for the assays (Table 5.1) were obtained by checkerboard titration and selection was based on high sensitivity, low background and high specificity, and the optimisation procedures are also described in Chapter 5.

Blood meal extracts were tested in duplicate wells and each plate also had four wells for negative controls and four for positive controls. The cut-off value for a negative result in all types of ELISA was calculated as the mean OD of negative controls plus two standard deviations (Beier et al., 1988; Gomes et al., 2001; Reid and Copeman, 2003). A sample was considered positive if ODs for duplicate wells were both above the negative cut-off value.

**Effect of blood-meal digestion on antigen detection by ELISA**

To determine ELISA sensitivity in relation to blood-meal digestion, 40 *T. pallipennis* were fed to repletion on a restrained horse (Muzari et al., 2010), and groups of ten flies were killed at 0, 48, 72 and 144 hours after feeding. Before they were killed, the fed tabanids were kept in the insectary at 29-30°C and ~70% relative humidity with
alternating 12-hour dark and light periods, and were supplied with water and sucrose solution for nourishment (Fig 6.3). An additional batch of 10 control flies was prepared as earlier described under the same insectary conditions. Each of the five groups was killed by freezing and stored at -20°C before analysis of abdominal contents (Fig 6.4) by ELISA to test for the presence of antigens from horse blood.

Fig 6.3. Tabanids in a netting cage in the insectary. Purple sponges contain glucose solution and water for nourishment
Fig 6.4. Appearance of homogenised contents of tabanid abdomens when the flies were killed at different times post-feeding

**Data Analysis**

Tabanid species with fewer than 40 specimens collected from traps were excluded from blood-meal assays and statistical analysis. Proportions of blood meals of each host type among the major tabanid species were compared by Chi-square analysis or Fisher’s Exact test as appropriate. The effect of sampling location on the proportion of host blood-meals prevalent in different tabanid species was investigated by logistic regression. In testing effect of blood-meal digestion on host antigen detectability by ELISA, average optical density records were compared among all the groups using Kruskal-Wallis test, and pair-wise between groups using Mann-Whitney tests. The statistical packages used were SPSS 16.0 and PASW Statistics18.
6.3. Results

Prevalence of host blood-meals among different tabanid species

After optimisation, all the assays were sensitive enough to detect a minimum of 0.001 µl of blood residue in tabanid abdomen, and there were no cross-reactions among any tested hosts at the optimal reagent concentrations. When fully engorged, a medium-sized tabanid such as *T. pallipennis* contains ~40 µl of blood (Muzari *et al*., 2010).

A total of 1597 tabanids belonging to ten species were captured over a five-week trapping period, and 1498 specimens from six most abundant species were analysed for their source of blood meal. A total of 697 (47 %) of the specimens gave positive host identification in the assays for horses, cattle, pigs and macropods, although traces of blood had been visible in only 3.2 % of the field-collected specimens after homogenisation of abdominal contents. In the six tabanid species, the proportions of positive specimens were between 19-55% (Fig. 6.5). *Tabanus strangmannii* had the highest proportion and *Tabanus concolor* the lowest. Mixed blood meals from two or three host types were encountered in all tabanid species, and comprised more than 20% of all detected meals in *Pseudotabanus silvester*, *T. dorsobimaculatus* and *T. pallipennis* (Fig. 6.5).

Among the six tabanid species, proportions of blood meals from each type of host was variable (Figs. 6.6-6.9), with statistically significant differences in prevalence of meals from macropods ($\chi^2 = 35.22; df = 5; p<0.001$), horses ($\chi^2 = 47.11; df = 5; p<0.001$), cattle ($\chi^2 = 17.41; df = 5; p = 0.004$) and pigs (Fisher’s exact = 14.21; $p=0.007$). Meals of macropod origin were highest in all tabanid species, reaching 27-40% of tested specimens (and 61-80% of positive specimens) in five of the species. *Tabanus dorsobimaculatus*, *T. pallipennis* and *Tabanus strangmannii* showed the highest relative preference for macropod blood (Fig. 6.6), *P. silvester* and *T. strangmannii* the highest relative preference for horse blood (Fig. 6.7) and *P. silvester*, *T. pallipennis* and *T. strangmannii* highest for cow blood (Fig. 6.8). Meals from pigs were least frequent (Fig 6.9), with highest prevalence being only 4.8% in *Tabanus dorsobimaculatus*. All
tabanid species fed on at least three of the host types tested, and the least specialised was *P. silvester*, showing 13.1%, 20% and 27% meal prevalence from cattle, horses and macropods, respectively and 3.3 % from pigs.

**Fig. 6.5.** Proportions of tabanid specimens with single or mixed host blood detected in their abdomens when tested by ELISA at all locations in Townsville. For each species, number of specimens analysed is shown in parenthesis.
Fig. 6.6. Prevalence of blood meals originating from macropods as identified in different tabanid species captured with traps in their natural habitat at all four locations in Townsville.
Fig. 6.7. Proportions of blood meals originating from horses in different tabanid species captured in their natural habitat.
Fig. 6.8. Prevalence of tabanid blood meals originating from cows. The tabanids were captured with traps at all four locations in Townsville.
Prevalence of host blood-meals among different locations

Logistic regression to investigate effect of sampling location on the proportions positive for various host types was only conducted for *T. pallipennis* and *T. dorsobimaculatus* because the two species were distributed among all the four locations. The analysis indicated that sampling location did not affect the tabanid species differences in proportion positive for macropod blood ($\chi^2 = 4.627; \text{df} = 3; p = 0.201$), horse blood ($\chi^2 = 2.278; \text{df} = 3; p = 0.517$) and cow blood ($\chi^2 = 0.966; \text{df} = 3; p = 0.809$). Tabanid numbers with blood-meals from pigs were too low for the analysis.

The only locations where all six tabanid species were captured were JCU and Alligator Creek, and logistic regression also showed that there was no significant interaction
between sampling location and proportion of each tabanid species positive for macropod blood ($\chi^2 = 8.420; \text{df} = 5; p = 0.135$), horse blood ($\chi^2 = 6.528; \text{df} = 5; p = 0.258$) and cow blood ($\chi^2 = 7.090; \text{df} = 5; p = 0.214$).

*Pseudotabanus silvester* was the most abundant species captured, but it was only present at two locations, Alligator Creek and JCU. Comparison of blood meal prevalence among locations was therefore only conducted for *T. pallipennis*, which was present on all four trapping locations and had at least 50 blood-positive specimens per site. From all the flies tested, only two *T. pallipennis* specimens were positive for pig blood. The proportion of macropod blood meals in *T. pallipennis* was highest at Alligator Creek (50%) and lowest at Stuart (32%) (Fig 6.10), but the difference among all four sampling locations was not statistically significant ($\chi^2 = 7.28; \text{df} = 3; p = 0.064$). Therefore the apparent difference in densities of macropods at the trapping locations had a slight but insignificant effect on prevalence of macropod blood meals. The proportions of horse blood meals in *T. pallipennis* correlated well with the estimated density of horses at the various locations, higher at Alligator Creek and Stuart (12-16 %) than at Oonoonba and JCU (3-4 %) (Fig 6.11), and the difference was statistically significant ($\chi^2 = 19.56; \text{df} = 3; p<0.001$). The prevalence of blood meals from cattle was lowest at Alligator Creek and highest at Oonoonba (Fig 6.12) as expected, but the differences were small and not statistically significant ($\chi^2 = 0.83; \text{df} = 3; p = 0.84$).
Fig. 6.10. Prevalence of macropod blood meals in *T. pallipennis* captured at different locations in Townsville.
Fig. 6.11. Prevalence of horse blood meals in *T. pallipennis* captured at different locations in Townsville.
Fig. 6.12. Prevalence of blood meals from cattle in *T. pallipennis* captured at different locations in Townsville.
Blood-meal digestion by tabanids and detection by ELISA

Horse blood antigens were identified in tabanid specimens up to 144 hours (6 days) post-ingestion (Fig. 6.13). Within 48 hours after engorgement all the samples tested positive, 90% were positive at 72 hours and 80% at 144 hours. There was a significant difference in average OD readings among the five groups (Kruskal-Wallis test, $\chi^2 = 39.354; \text{df} = 4; p < 0.001$), which included the control group. The median OD levels decreased with time after blood-feeding, but most markedly between 48 and 72 hours (Fig. 6.13) when the only significant difference between adjacent post-feeding periods was noted (Mann-Whitney Test, $U=0.0, Z = -3.78, n=20, p<0.001$). The median OD for the control group was significantly different from the 144 hours group (Mann-Whitney Test, $U=1.0, Z = -3.368, n=20, p<0.01$) and hence was obviously different from all the other groups with shorter post-feeding periods.

Fig. 6.13. The detectability by ELISA of horse blood antigens in the abdomen of fully fed tabanids at different periods after engorgement. OD was measured at wavelengths 414 nm and 492 nm.
6.4. Discussion

Prevalence of host blood-meals among different tabanid species and locations

The tabanid specimens analysed in this study were all captured using odour-baited traps, which generally catch flies that are in search of a blood-meal (Odulaja and Madubunyi, 1997; Muzari and Hargrove, 2005), and this represents only a subset of the blood-fed tabanid population. This consequently underestimates the prevalence of tabanid attacks on each host type, but more so on the less defensive hosts and in relation to tabanid species that are more persistent at trying to complete the blood-meal without switching hosts. In particular, the relative prevalence of host blood meals in *T. dorsobimaculatus* would have been higher in the natural population than was identified in this study, because in other studies fly collections from octenol-baited Nzi traps did not correlate with activity levels of this species on animals (Muzari *et al*., 2010b). Therefore the recorded prevalences are only minimum estimates of what exists in the whole tabanid population, which may be considerably higher.

The prevalence of positive blood-meals (38 – 55 % in five of the tabanid species) (Fig. 6.5) is quite high considering that all captured flies were tested without prior selection of flies with visible blood meals. While visual selection has been commonly used on other insects before blood meal identification (Van den Bossche and Staak, 1997; van den Hurk *et al*., 2003; Zinser *et al*., 2004), it was inappropriate with our tabanids because none of them showed obvious signs of engorgement and most of the species naturally had dark abdominal integument which made it impossible to visually distinguish partially-fed flies. Dissection of individual flies to examine the midgut for blood traces before analysis (Magnarelli and Anderson, 1980; Mohamed-Ahmed and Odulaja, 1997) was not a preferred option because it is time-consuming and inaccurate when small amounts of blood are expected. The relatively low proportion of specimens with visible traces of blood (3.2%) after homogenisation confirmed this, and suggests that exclusion of unfed tabanids by visual inspection may result in loss of important information. Similarly, Magnarelli and Anderson (1980) recorded only 10% positive flies by dissection of several tabanid species collected from odour-baited traps in an area with a wide variety of hosts.
The high prevalence of positive specimens in the present study confirmed that the selected host types constituted significant food sources for most tabanid species in the study area, but possibly less so for *T. concolor* which had the lowest proportion of positively-identified blood meals (19%). Tabanids that tested negative for the four host types examined were either still seeking for their first ever blood-meal, had completely digested all previously-ingested blood or had fed on some other host type besides the four tested. Though, none of them had visible blood after grinding.

The detection of blood meals from all host types within the same tabanid species indicates that host choice was probably related to host availability. This, and the presence of mixed meals in all species imply various levels of opportunistic feeding, and that surra transmission could occur among all the animals considered. Opportunistic feeding has also been recorded among tabanids in America including *T. atratus, T. lineola*, and *Hybomitra lasiophthalma* (Magnarelli & Anderson, 1980) and among some species of *Chrysops* in Africa (Gouteux *et al.*, 1989). However, the preponderance of macropod blood meals in nearly all tabanid species (61-80% of positive samples) and in all locations in the current study, even where macropod density appeared very low, suggests that macropods are preferred hosts, particularly for *T. pallipennis*. The tabanids seem to be efficient at locating the few macropods in their habitat. If preference exists, then a preference for native hosts would not be surprising, since all the tabanids are endemic to Australia and exotic hosts in northern Queensland have only been widespread for approximately 150 years. In northern Australia macropods would face the highest risk of catching surra if it was introduced, and the disease could spread readily because the animals are widely distributed. Chances of mechanical transmission among macropods are further enhanced because the most abundant tabanid species *T. pallipennis* and *P. silvester* experience a high proportion of interrupted feeds when getting a blood meal from kangaroos (Muzari *et al.*, 2010). In a previous study in Louisiana the primary hosts of most tabanid species included both wildlife (deer) and domestic animals (cattle and horses) (Wilson and Richardson, 1969).

The fact that all tabanid species fed least on pigs could be attributed to the very low availability of this host at the trapping locations, but the occurrence of pig blood meals in five tabanid species suggests that pigs may be a significant host in other parts of
Queensland where their populations are higher. Pigs have a high tolerance for infection with *T. evansi* and can be reservoir hosts for the pathogen (Reid *et al*., 1999).

In other studies in northern Queensland where large populations of feral pigs and macropods coexist, macropods were the preferred host of the mosquito *Culex annulirostris*, and were suspected to be diverting host-seeking mosquitoes from pigs and thus probably impeding the establishment of Japanese Encephalitis (JE) on mainland Australia since marsupials are poor hosts of JE virus (van den Hurk *et al*., 2003). However, the preference of tabanids for macropod hosts would most likely promote the establishment and spread of surra because wallabies have shown high, persistent parasitaemia resulting in acute disease and mortality when experimentally infected with *T. evansi* (Reid *et al*., 2001).

**Blood-meal digestion by tabanids and detection by ELISA**

The capacity of the ELISA technique to detect host blood-meals in *T. pallipennis* up to six days after blood-feeding was remarkable, and the fact that 80% of the specimens were still positive after this period suggests that blood meals might still have been detectable a few days longer if this had been tested. However, the limit of detection is expected to be shorter if only partial blood-meals had been acquired as expected for the negative controls which were collected from traps and starved for 6 days. The blood-fed tabanids killed after six days would most probably have oviposited if appropriate conditions had been provided. Hafez *et al* (1970) reported *T. taeniola* laying eggs 3-5 days after blood-feeding, and one female taking six full blood-meals and depositing six batches of eggs in a period of 31 days at a constant temperature of 27°C. Therefore host blood-meals recognised in field-caught tabanids probably included residues from previous gonotrophic cycles, and mixed blood-meals identified are not all necessarily from host-switching activity during the most recent feeding attempts. However, some of the mixed blood meals are still likely to be a consequence of the difficulties flies face in freely engorging on a single host due to host defensive movements (Bongiorno *et al*., 2003).

In other studies, host blood-meals were identifiable by ELISA up to 32 hours post-engorgement in *Anopheles stephensi* (Beier *et al*., 1988), and by PCR heteroduplex
analysis up to three days post-ingestion in *Simulium damnosum* (Boakye *et al*., 1999) and up to seven days after feeding in *Culex tarsalis* (Lee *et al*., 2002). Consequently, Lee *et al*. (2002) suggested the possibility in their study that an individual wild-caught mosquito might contain residues from two different blood-meals. The detection of blood residues from previous gonotrophic cycles might also have contributed to the relatively high prevalence of identified blood-meals in the current study.
CHAPTER SEVEN

Evaluation of host switching behaviour of tabanids using the mark-release method

7.1. Introduction

Successful mechanical transmission of surra by any species of tabanid flies depends on the likelihood of that species to immediately switch between hosts when interrupted during blood-feeding. Tabanid species that resist the defensive movements of the host and persist with their feeding on the same host until repletion are not important as vectors of pathogens (Foil, 1989) such as T. evansi which have short survival periods on the mouthparts of the fly and do not undergo biological development in the insect (Luckins, 1998). However, many species continually make choices about whether to persist or fly to an alternative host to complete feeding. The choice to switch hosts probably depends on a number of things but two factors likely to be important are the mobility of the vector and the proximity of the hosts (Foil, 1989).

The most widely used method for tracking the movements of individual insects is the mark-release recapture method. The technique has been used in studies of mosquitoes (Nutsathapana et al., 1986; Muir & Kay, 1998; Watson et al., 2000), termites (Su and Scherer, 2003; Evans, 2004), ants (Buczkowski and Bennett, 2006), tsetse flies (Hargrove, 2001) and tabanids (Foil, 1983; Barros & Foil, 2007). In this study, the method was used for the first time on Australian tabanid species to investigate their host-switching behaviour.

7.2. Materials and methods

The experimental set-up was modified from the method of Barros and Foil (2007) whereby movement of marked flies between four stationary horses was recorded using
the mark-release-recapture method. Our study involved only two horses. The two horses were taken to an isolated paddock (Fig 7.1) where tabanid activity was known to be relatively high, at least 300m away from the main herd. Five positions were allocated in a straight line so that the horses could be separated by distances of 5m, 10m, 15m and 20m in a direction parallel to the wind as and when required. It was assumed that tabanids interrupted while feeding on the downwind horse would seek their next host using visual and olfactory cues and hence navigate upwind towards the second horse. Tabanid flies feeding upon the downwind horse were captured with a hand net, marked with individually identifiable colour coding on the dorsal surface of the thorax (Fig 7.3) and released to either resume feeding on the same horse or seek an alternative host of which the nearest was the upwind horse. A non-toxic water-based paint was used for marking the flies with dots of different colours at different positions on the dorsal side of the thorax so that each fly could be individually identified. Only *Tabanus pallipennis*, the most abundant species at the time was marked for the initial testing of the procedure. The marked flies were released about 0.5 m away from the same horse.

In another part of the study, host-switching of tabanids was investigated between two pigs, male and female, that were housed in adjoining fenced compartments (Fig 7.2). The pigs were free to move about in the compartments, and distance between them during the time of the observations was 1-4 m. The marking procedure was modified to include chilling of the captured flies (Salvato *et al.*, 2003; Leskey and Wright, 2004) in a kaylite box with ice-pack for about 10 minutes before release in order to diminish escape responses (these are when flies leave the immediate area in response to an adverse event such as prior capture). *Tabanus pallipennis* were captured using a glass tube while feeding, and when chilled and marked they were released 5m downwind of both pigs. To boost the numbers of tabanids coming to the site, octenol was released from an open bottle at the edge of the pig housing during the experiment.

Tabanids were easier to capture on the pigs than on the horses. Therefore in a further attempt to estimate distance from which tabanids were able to locate a horse soon after interrupted feeding, tabanids captured on pigs were chilled in the kaylite box, marked and transferred to a release point that was 20m downwind of two stationary horses. After release of the marked tabanids, the two horses were observed for 30 minutes to check whether any of the marked flies came to them.
Fig 7.1 Field site for mark-release studies on horses

Fig 7.2 Pig housing used for testing tabanid host-switching responses
Fig 7.3  *T. pallipennis* marked on thorax with dots of different colours
7.3. Results

In the first experiment with two horses separated by ten metres, 14 *T. pallipennis* were captured on the downwind horse in two two-hour sessions on consecutive days. At least another 30 catching attempts were not successful because the flies were very sensitive to disturbance by movement of the hand-net. The tabanids captured on the downwind horse, marked and released near the same horse, did not return to the horse and also were not seen on the horse 10 m upwind. Once released, the tabanids immediately flew away in random directions, typical of a frantic escape response. They were not seen again.

A further logistical problem was encountered when the two horses repeatedly became restless and difficult to control during the experiment. Due to the behaviour of the horses and the marked tabanids, it was not feasible to continue to test other distances in the same way.

The second experiment, involving the two pigs in adjoining fenced compartments gave slightly better results. Over a period of three days, 35 tabanids were marked, chilled and released in two-hour sessions. Each tabanid remained stationary for about 5 minutes after release and then flew off in random directions but at lower speeds than had been observed without the chilling treatment. Six of the marked flies reappeared after 24, 28, 21, 64, 69 and 29 minutes of release and attempted to feed on the pigs (Fig 7.4). They were all first seen on the female pig, but three of them switched to the male pig, with one later switching back to the female pig. They each fed for a few minutes but none of them looked engorged before they eventually left the pigs.

In the third experiment, 22 tabanids captured on the pigs and released 20 m downwind of a pair of horses were not seen around the horses during the 30 minutes of observation immediately after release. Surprisingly, two of these tabanids were later seen again at the pigs, which were about 100m downwind from where the flies had been released near the horses. One of them was seen 50 minutes post-release and the other about 24 hours later.
7.4. Discussion

Some interesting insights on host-switching by tabanids emerged from the behaviour of marked flies around the pigs, although problems with the horses and the escapist behaviour of the marked tabanids hindered execution of the experimental protocol as initially designed.
The immediate escape response of marked *T. pallipennis* captured from the horses was not expected, given findings from similar studies where 11 tabanid species in USA (Foil, 1983) and five species in Brazil (Barros & Foil, 2007) either returned immediately to the same horse or moved on to the next animal a few meters away after the mark-release procedure. In both of these experiments the capturing and marking did not affect the feeding behaviour of the tabanids, and recapture rates were 52% and 36% respectively. However, the response of *T. pallipennis* in the current study was somewhat similar to the response of *Hybomitra* in Russia, where a ‘chaotic flying away of horse flies was observed’ in experiments on the capture-recapture of the flies at different distances from a single pasturing cow (Konstantinov, 1993). The response of tabanids to disturbance by capturing and marking seems to depend on the particular species of horse fly. *Tabanus pallipennis* is a particularly sensitive species when attempting to feed on a defensive horse, with median landing durations of only 11 seconds (Muzari *et al.*, 2010a), and it would not be surprising if capturing and handling exacerbated the escape responses. It was also particularly difficult to catch this species off a horse because of this behaviour. However, the flies were not as sensitive on the pig, which made no attempt to dislodge them (Muzari *et al.*, 2010a).

Foil (1983) estimated that less than 0.1% of tabanids would transfer between hosts separated by a distance of 50 m following an interrupted feed. Consequently, a spatial barrier of 200 m between infected and susceptible animals was suggested as an effective means of preventing mechanical transmission between the two groups (Foil, 1983). Such barriers may be effective in Australia, but the effective distance for species like *T. pallipennis* needs to be evaluated.

The return of six out of 35 marked flies to the pigs after 21-69 minutes was amazing. The fact that the six could locate the two pigs again suggested that they did not fly too far off after release, and the relatively long time taken before return suggests that they might have taken more time to fully recover from the chilling treatment.

The observed switching of some of the marked flies between the male and female pig demonstrated how *T. pallipennis* can be a good vector of surra, particularly within a herd. The high likelihood of mechanical transmission within a herd has been expressed
by Konstantinov and Veselkin (1989). However, the disease can readily spread among herds if they mingle at sites of common interest such as water-holes.

Confirmation of the host-switching distance of tabanids between horses is still necessary for Australian species, and this can be done through improvement of the original protocol based on lessons learnt from this study. Most importantly, better-trained horses are needed so that they remain calm at the specific stations for required periods of time. However, because of the escape response of the marked *T. pallipennis* in random directions even after chilling them in a kaylite box, it might be necessary to set up more horse stations with observers surrounding the release point to improve the chances of recapture. This will obviously require more resources. The best chilling duration needs to be evaluated in order to facilitate quick recovery and minimise effect on tabanid behaviour.

Given the differences in sensitivity between tabanid species, it may also be worthwhile to try totally different methods of marking *T. pallipennis* to what has been used on tabanids elsewhere. For instance, handling of the insect could be avoided by quickly spraying or dusting its wings with a dye while it is still on the animal.
CHAPTER EIGHT

Responses of different tabanid species to odour-baited stationary traps and live animals in the field*


8.1. Introduction

In order to understand the ecology and distribution of the various tabanid species and to formulate risk models for surra transmission among the animal species prevalent in northern Australia, tabanid surveillance has been conducted using stationary traps (van Hennekeler, 2007). The Nzi trap (Mihok, 2002) is the most effective sampling method tested so far, especially when baited with octenol and/or CO₂ (Van Hennekeler *et al.*, 2008). Currently, estimation of the relative risk of disease transmission by different tabanid species is based on fly abundance as indicated by collections from traps, among other factors, but it is not known whether trap catches correctly reflect tabanid activity on animals. Some studies elsewhere have shown that different tabanid species may not respond to traps in similar ways (Mohamed-Ahmed *et al.*, 2007) and that their response to artificial sampling devices may be different from their response to live animals (Phelps and Holloway, 1992; Krcmar, 2005). Therefore the present study investigated the relationship between tabanid activity around horses in the field and tabanid catches from octenol-baited Nzi traps.

This study further investigated whether tabanids approached a host from any particular direction in relation to the prevailing wind. Vale (1977) discovered that hungry tsetse flies can detect ox odour from as far downwind as 90m, then orient and fly upwind in the odour plume towards its source. A tendency for upwind flight in the presence of odour attractants was also identified in the black fly *Simulium arcticum* (Sutcliffe *et al.*, 1995) and in several species of mosquito (Gillies *et al.*, 1978). However, Phelps and Vale (1976) noted variable responses among tabanid genera and realised that the
upwind flight of some *Tabanus* species (mainly *T. pertinens* and *T. leucostomus*) towards an ox was not as pronounced as in *Philoliche zonata* or in tsetse flies. The effective range of the odour plume for *Tabanus* was only about 15m, which apparently was almost the same as the visual range of the tabanids. Therefore the odour-mediated response of some tabanids may not be the same as for other biting flies, and the odours probably do not play a major role in long-range orientation of host-seeking tabanids, although they might cause higher proportions to enter a trap. Not much has been done to investigate this concept, but researchers commonly make the assumption that tabanids approach baits from the downwind side. For instance, it has been suggested that competing traps should be lined in a crosswind direction (Hall *et al.*, 1998; Sasaki, 2001; van Hennekeler *et al.*, 2008) to avoid bias in comparative experiments. This may not always be convenient depending on the physical features of the study area. The same assumption was used in setting up the current study, but however, the positioning of electrocuting nets also allowed for this assumption to be examined.

8.2. Materials and methods

*Tabanid activity around a horse (on E-nets) and in traps*

Tabanid activity on an animal was estimated using an ‘incomplete ring of E-nets’ surrounding a horse in the field (Fig 8.1a) as described in Chapter Two. In order to investigate the relationship between tabanid numbers captured in traps and tabanid activity on an animal, two Nzi traps were deployed on opposite sides of the site that had the horse and incomplete ring of E-nets, so that each trap was ~60m away from the E-nets site, in a crosswind direction (Fig 8.1b). This assumed tabanids would approach traps and the E-nets from downwind and hence trapping sites would not impact on each other. Insect collection cages were affixed to the traps at the start of each study session and removed at the same time that the E-nets were switched off, and this was done for 20 days.

The study was conducted between December 2008 and April 2009, which is the hot wet season when tabanid populations are highest in the area. Most of the days were in February and March 2009. Each study session lasted 2-4 hours between 09:00 h and 18:00 h, depending upon prevailing weather conditions and diurnal activity times of
local tabanid species. Days were selected according to weather conditions, with particular preference for sunny, rain-free days which are the most appropriate for operation of E-nets and for tabanid activity (van Hennekeler, 2007).

In order to allow for differences in tabanid abundance between sites, odour-baited traps were operated concurrently on all three sites, without animal or E-nets for 19 days conveniently selected throughout the season. On those days insects were collected from traps at 24-hour intervals.

**Host-oriented flight responses**

The general flight orientation of tabanids approaching a host was examined by comparing the tabanid collections from E-nets (outward-facing sides) on different sides of the host in relation to the prevailing wind direction. The net positions were consecutively numbered 1-6, with adjacent screens 1 and 6 positioned on the general upwind side of the horse, adjacent screens 3 and 4 on the downwind side and opposite screens 2 and 5 facing each other in a cross-wind orientation (Fig 8.1b). Records from 18 days of study between February and April 2009 were analysed for *T. pallipennis* which was the most abundant species. The wind in the study area generally blows from a north-easterly direction during the day (van Hennekeler *et al*., 2008), and is indicated with arrows in Fig 8.1b.

**Data analysis**

Relationships between tabanid abundance in traps and on horses were investigated by regression analysis on individual species after logarithmic transformation of daily catches on E-nets and in traps. Total tabanid catch from the ring of E-nets was used as an index of the number of flies that approached the horse during each session (Torr, 1994; Vale *et al*, 1999; Torr *et al*, 2007)

To account for site differences before the regression analysis, the total trap catch collected at each site when E-nets and horses were absent was expressed as a proportion of the largest of the totals from the three sites, to derive a correction factor for each site and species. Daily tabanid counts from the main experiment were then divided by the
appropriate correction factor, and the corrected values were used in the regression analysis.

Frequency of tabanids approaching the horse from different directions was compared using Chi-square test on catches from the outward-facing sides of the E-nets.

Fig 8.1a. A horse inside the ring of E-nets. Nzi traps were located 60m away from this site.
Fig 8.1b. Set-up of experiment to compare tabanid activity around a horse with catches from Nzi traps in the field. E-net positions numbered 1-6. (Not drawn to scale).
8.3. Results

Tabanids captured around a horse (on E-nets) and in traps

The same species of tabanids were captured in the trap as on E-nets around a horse (Table 8.1), although for almost all species the E-nets caught more flies. The trap collected between 4.5-56.4% of what the E-nets captured for all tabanid species except for *T. strangmannii* (100%). Male tabanids were less than 1% of the total captured.

**Table 8.1.** Tabanids captured on E-nets around a horse and in odour-baited Nzi traps during 20 days of sampling. Number of males is shown in parentheses. - when the species was captured on at least one of the three sites during the sampling period

<table>
<thead>
<tr>
<th>Tabanid species</th>
<th>E-nets (Outward-facing side)</th>
<th>E-nets (Inward-facing side)</th>
<th>Total on E-nets</th>
<th>Nzi trap (mean of two sites)</th>
<th>Days with fly activity a</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. pallipennis</em> Macquart</td>
<td>458 (6)</td>
<td>392 (3)</td>
<td>850 (9)</td>
<td>128</td>
<td>20</td>
</tr>
<tr>
<td><em>P. silvester</em> Bergroth</td>
<td>143</td>
<td>123</td>
<td>266</td>
<td>150</td>
<td>4</td>
</tr>
<tr>
<td><em>T. townsvilli</em> Ricardo</td>
<td>145</td>
<td>92 (2)</td>
<td>237 (2)</td>
<td>14</td>
<td>20</td>
</tr>
<tr>
<td><em>T. dorsobimaculatus</em></td>
<td>99</td>
<td>71 (1)</td>
<td>170 (1)</td>
<td>73</td>
<td>20</td>
</tr>
<tr>
<td><em>D. oculata</em> Ricardo</td>
<td>62</td>
<td>71</td>
<td>133</td>
<td>6</td>
<td>18</td>
</tr>
<tr>
<td><em>T. concolor</em> Walker</td>
<td>16</td>
<td>8</td>
<td>24</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td><em>T. strangmannii</em> Ricardo</td>
<td>9 (2)</td>
<td>9 (1)</td>
<td>18 (3)</td>
<td>21</td>
<td>16</td>
</tr>
<tr>
<td><em>T. notatus</em> Ricardo</td>
<td>6</td>
<td>11</td>
<td>17</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td><em>Lilaea fuliginosa</em> Taylor</td>
<td>9</td>
<td>7</td>
<td>16</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td><em>P. distincta</em> Ricardo</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Relationship between trap catches and tabanid activity around a horse

Background information on the differences among the three experimental sites had been obtained by using Nzi traps on all sites prior to using E-nets on the middle site.

Correction factors to account for site differences were derived from these trap catches, and interestingly they were different among the tabanid species. For example, correction factors for *T. pallipennis* on the three sites were 1.0, 0.3 and 0.3, while for *T. townsvilli* they were 0.9, 0.3 and 1.0. On the other hand the correction factors for *P. silvester* were 0.6, 0.9 and 1.0.
There was a significant linear relationship between trap catch and abundance of *P. silvester* around a horse (F=642.25; df=1; p<0.001), and trap catch explained 94.4% of the variance in *P. silvester* activity around the horse (Fig. 8.2). With the regression models for *T. pallipennis* (F=26.56; df=1; p<0.01) and *T. townsvilli* (F=21.08; df=1; p<0.01) only 43.1% and 55.4% of the variation could be explained by catches from the traps, respectively (Fig 8.3 and 8.4b). However, the relationship for *T. townsvilli* only existed when trap catches were more than zero (Fig 8.4b), implying that traps sometimes gave false negatives by failing to detect presence of the species while there was activity around the animal (Fig 8.4a). For *T. dorsobimaculatus* (Fig 8.5) there was no significant relationship between the number of flies caught in traps and the numbers caught from E-nets around a horse (F=0.434; df=1; R²=0.14; p=0.517).

**Flight direction towards the host**

Tabanids (*T. pallipennis*) approached the horse from all directions, but there was a significant difference among the sides ($\chi^2 = 14.595$, df=2, p=0.001), with the lowest number coming from crosswind (Fig 8.6) and accounting for 25% of all flies captured. The numbers of flies coming from the downwind (172) and upwind (169) sides were almost equal, and the difference between them was not statistically significant ($\chi^2 = 0.026$, df=1, p=0.871).
Fig 8.2. Relationship between catches of *P. silvester* in traps and on E-nets (surrounding a horse) on a logarithmic scale

Fig 8.3. Relationship between catches of *T. pallipennis* in traps and on E-nets (surrounding a horse) on a logarithmic scale
Fig 8.4a. Scatterplot of abundance of *T. townsvilli* in traps and on E-nets (surrounding a horse)

Fig 8.4b. Relationship between catches of *T. townsvilli* in traps and on E-nets (surrounding a horse) when trap catches are above zero
Fig 8.5. Scatterplot of the abundance of *T. dorsobimaculatus* in traps and on E-nets (surrounding a horse) on a logarithmic scale

Fig 8.6. Distribution of *T. pallipennis* approaching a horse in relation to prevailing wind direction
8.4. Discussion

Relationship between trap catches and tabanid activity around a horse

The octenol-baited Nzi traps captured fewer tabanids per site than the E-nets surrounding a horse for nearly all tabanid species (Table 8.1), perhaps reflecting a difference in attractiveness and catching efficiency of the two systems. The trap, however, captured generally the same tabanid species as did the E-nets, and may therefore still be a reliable means of determining the presence of any of the species.

The results from the regression analysis showed that the trap was a very good indicator of *P. silvester* activity around a live animal, gave a reasonable indication for *T. pallipennis* and *T. townsvilli* but was unable to reflect the activity levels of *T. dorsobimaculatus*. Therefore surra-transmission risk models based on trap data may be reliable where *P. silvester*, *T. pallipennis* and *T. townsvilli* are concerned, but should be used with caution with regards to other species. With *T. townsvilli*, the occurrence of false negatives from traps on some days means that the traps need to remain in position for at least a week or two before a conclusion on the presence of this species can be reached.

The big disparity between abundance of *T. townsvilli* in traps and on E-nets (Table 8.1) demonstrates that the performance of the Nzi trap on this species is not as good as on other species, and the same problem occurs with *D. oculata*. Differences in trappability with stationary baits have also been noted among tabanids in Sudan (Mohamed-Ahmed et al., 2007) and differences in tabanid response to traps and live animals have been recorded in Zimbabwe (Phelps and Holloway, 1992), in Croatia (Krcmar, 2005) and in America (Leprince et al., 1992). For instance, Leprince et al. (1994) observed that *T. americanus*, *T. fuscicostatus* and *T. lineola* were captured more frequently in CO₂-baited canopy traps than on Jersey bullocks, but the reverse was observed for *Leucotabanus annulatus*, and yet no significant differences were evident for the species *T. pallidescens* and *T. wilsoni*. The results agree with the general statement by Foil and Hogsette (1994) that ‘the accuracy of extrapolation from trap catches to the tabanid burden on livestock can be limited’. Apparently, different kinds of traps demonstrate this variability in performance in trapping various tabanid species, and the trap
efficiency can also be affected by the type of chemical used as odour attractant (Krcmar et al., 2005; Mihok et al., 2007). It is therefore recommended that for tabanid species that are deemed to be significant vectors in an area, the correlation of trap catches to tabanid activity on livestock be examined in order to guide the interpretation of trap results in an epidemiological context.

The very large overall proportion of females captured on the E-nets suggests that most of the intercepted flies were likely to be seeking a host rather than just being caught during random flight, since only females need a blood-meal. However, the presence of some males on the E-nets confirms that the nets are a good way of intercepting unsuspecting tabanids during their natural flight, unlike the traps which require extra effort for the insects to enter, which is not undertaken by males. A similar trend was observed in Sudan, where only female horse flies were caught in traps, while flies intercepted by electrocuting nets also included males (Mohamed-Ahmed et al., 2007).

*Host-oriented flight responses*

The results showed a multi-directional flight of *T. pallipennis* towards a host, in which the main directions are upwind and downwind. The almost equal proportions from upwind and downwind sides were not expected, since wind-borne odour attractants from the host are blown downwind. While general wind direction in the area is known from previous studies (van Hennekeler, 2007), government meteorological records and personal observations, it was not measured at the site and was probably not constant at all times during the experiments. Nevertheless it is unlikely that the natural variations would be so extreme as to have the wind blowing with equal frequency in opposite directions during the daytime.

The attraction of flies from upwind may depend on visual stimulation only (Phelps and Vale, 1976), and the equal proportion with flies from downwind indicates that the tabanid species probably relies almost solely on visual cues to locate its host. This suggests that the role of odours such as octenol when used to significantly increase trap catches may be a short range effect that induces the tabanids to stay longer around the bait and investigate more closely, thus resulting in a higher proportion of flies entering the trap (Mihok et al., 2007; van Hennekeller et al., 2008; Krcmar et al., 2009). Even in
the laboratory, the presence of octenol has been shown to increase the proportion of *T. nigrovittatus* blood-feeding through a parafilm membrane (Downer and Stofolano Jr, 2006), suggesting a short-range stimulation effect. In the field, there is no obvious reason for the general preference of *T. pallipennis* to be flying downwind and upwind in search of hosts rather than crosswind, but the effect of the wind currents in the aerodynamic flight movements of flies might play a role in this.

*Tabanus pallipennis* seems to fly about in a random search for hosts in the absence of any host stimuli, a behaviour described as ‘ranging’ flight (Vale, 1980), until they can see a host. This probably explains why most tabanid species are mostly active on bright, sunny days when visibility is very high. Since odour attractants appear to play only a short range role, any attempts to use artificial baits such as traps and insecticide-treated targets for diverting tabanids from livestock (Foil and Hogsette, 1994) would require a very high density of the baits to have a significant effect (Lindh et al, 2009). This behaviour of tabanids is different to that of obligate blood feeding diptera such as tsetse flies (and probably stableflies). This is probably because failure to locate a host within a few days is not life-threatening for tabanids as it is with tsetse, since tabanids can survive for long periods on plant juices.

The findings here also indicate that it is appropriate to line up experimental sites in a crosswind orientation where possible, but suggest that this is not essential as long as distances between the trap sites are beyond the visual range of the tabanids, which is not expected to exceed 15-20 m (Phelps and Vale, 1976; Mihok et al, 2007). In study areas with mixed habitat types, the distance of each site from a patch of woodland is likely to affect tabanid catches more than the positioning of traps in relation to wind direction. Krcmar et al (2005) rightly placed experimental traps at sites in a line ‘along the lightly shaded edges of the forest’, although their 400 m distance between adjacent sites was probably longer than necessary. Shorter distances between trap sites may give the advantage of sampling flies from the same population group during experiments involving several different treatments, especially when Latin Square design is not a practical option (Perry et al., 1980; Hall et al., 1998; Sasaki, 2001; Krcmar et al, 2005).

The 30 m intervals between traps in a study by Hall et al (2005) in Hungary and the 20 m trap intervals by Sasaki (2001) in Japan were probably sufficient, but any closer would most likely result in biased results.
CHAPTER NINE

The relative abundance and distribution of tabanid species among different types of habitat

9.1. Introduction

The abundance and distribution of tabanids in space and time is generally associated with the presence of water and relatively high temperatures, which are key requirements in their life cycle (Hafez et al., 1970; Krcmar, 2005). Hence the adults appear seasonally in many parts of the world, including Australia (Reid 2002, van Hennekeler, 2007). It has been noted, however, that even where moisture and temperature conditions are favourable, tabanids are not uniformly distributed across habitats but can be significantly affected by vegetation distribution patterns in an area (Smith et al, 1970; Hayakawa et al., 1986; Barros, 2001).

In investigating the seasonal abundance and natural distribution of various tabanid species in different parts of the world, researchers have used different approaches, such as comparing number of adult tabanids collected in traps (Phelps and Vale, 1976; Hayakawa et al., 1986, Krcmar, 2005), number of adult tabanids captured by man as they try to feed off a domestic animal (Manresa and Mondonedo, 1935; Barros 2001; Krcmar 2005)(Barros, 2001; Manresa and Mondonedo, 1935) and number of larvae and pupae encountered in soil samples (Lane, 1976; Meany et al., 1976; Hayakawa et al., 1988). While the early stages of tabanid larvae are generally considered to be aquatic, some tabanid larvae have been found on terrestrial sites in Japan (Hayakawa et al., 1988), and of these some were collected from forest areas and others from grassland areas. In a study in California, river banks yielded higher densities and diversity of immature tabanids than other habitats (Lane, 1976). Researchers in Massachusetts found some tabanid species to be specifically associated with coastal salt marshes (Meany et al., 1976). However, from a disease transmission point of view, the distribution of adult tabanids is more important, and it is logical to expect higher adult
population densities closer to their breeding sites. Hayakawa et al. (1986, 1988) demonstrated that the relative abundance of adult tabanids of different species among different habitats in Japan was dependent on availability of preferred larval sites in each habitat, and not directly related to the richness of blood sources.

Mackerras (1954) described the distribution of some of Australia’s tabanid species on a broad scale based on information provided by individual insect collectors who sent specimens for identification. More recently, van Hennekeler (2007) used Nzi traps to study the relative abundance and seasonal fluctuations of tabanids in the Cape York Peninsula of North Queensland in Australia. However, the traps were so sparsely distributed (sometimes hundreds of kilometers apart) that it was not possible to obtain any information on habitat preferences of the various tabanid species in the area (tabanid species vary by area, Chapter 1). An understanding of habitat preferences by different species is essential for optimal vector surveillance, and may be useful in attempts to minimise exposure of livestock to attack by tabanids (Foil and Hogsette, 1994).

Queensland harbours a great diversity and high populations of tabanids, particularly along the coastal and adjacent areas (Mackerras, 1956). Large parts of these coastal regions consist of flat grassland valleys with a few scattered trees and major rivers meandering towards the Pacific Ocean. A bit further inland, the terrain is generally dissected by many smaller creeks and the vegetation becomes thicker, characterised by savannah-type woodlands of variable densities dominated by native eucalyptus trees. The purpose of the investigations in the present paper was to describe and compare the abundance and distribution of various tabanid species among different habitats during the wet season over two consecutive years. Firstly, the study compared the savannah woodland habitat to the open grassland habitat; and then secondly within these habitats the study investigated how distance from potential breeding sites affected the abundance and distribution of various tabanid species. Permanent and semi-permanent rivers were regarded as potential breeding sites in this study. The study was conducted around Townsville near the north Queensland coast.
9.2. Materials and methods

Tabanid distribution between savannah woodland and open grassland

Tabanid relative abundance and distribution in savannah woodland and open grassland habitats (Fig 9.1) was investigated by regular collections from Nzi traps baited with octenol during two wet seasons. The traps were deployed and maintained at the same positions and tabanids were collected weekly. The first collection season was February – May 2007, and the second was January- April 2008.

Each habitat had two sampling locations and each location had up to three traps. Specifically, three traps at Alligator Creek and one trap at JCU comprised the collections from woodland habitat, while three traps at Oonoonba and one trap at Stuart comprised collections from open grassland habitat in the first season. The locations (Chapter 2, Fig 2.3) were 8-20km apart and traps within a location were at least 200m apart. The sets of four traps per habitat were used to evaluate the activity patterns of individual species between years and habitats. In 2008 two additional traps were deployed at JCU when it became apparent that this site could also be used to study the effect of distance from breeding sites on adult abundance. So the woodland habitat had six traps in the second season, and all 10 traps were used in the overall comparison of mean weekly catches per trap between the two habitat types.

Tabanid densities at different distances from rivers

The distribution of tabanids in relation to permanent and semi-permanent riverways, which were regarded as potential breeding sites, was investigated in three locations. The sampling locations were Alligator Creek, JCU and Oonoonba and within each location three traps were positioned at different distances from a river, the closest trap being within 30m from the river bank (Fig 9.1.C), the second 300-500 m and the furthest 700 - 1000 m away from the river bank. The selected rivers were Ross River (at Oonoonba), Alligator Creek (at Alligator Creek) and Goondaloo Creek (at JCU campus). The Ross River has water throughout the year, and water level varies seasonally. Alligator Creek generally flows for at least six months each year and has small to large pools of stagnant water for the rest of the year. Goondaloo Creek flows only during the wet season for about four months, then has small pools for another four months before drying out.
completely for the remaining quarter of the year. Tabanids were collected weekly from each of the traps for 13 weeks in early 2008.

**Fig 9.1.** Nzi traps in different types of potential tabanid habitat: A- open grassland; B-savannah woodland; C- close to river bed
Data analysis

Data analysis was conducted per species on the seven most abundant tabanid species collected over the two wet seasons. Tabanid collections obtained weekly from eight traps in 2007 and 2008 were log-transformed for assessment of distribution patterns between habitats and between years for each species.

Differences in overall tabanid abundance between habitats were assessed by comparing mean weekly catches among the four sampling locations over the two seasons by two-way ANOVA on log-transformed data, which also assessed interaction between location and season. Multiple comparisons among the four locations were conducted using Tukey HSD to group sampling locations into homogeneous subsets. The derived subsets indicated whether distribution of the locations conformed to the composition of savannah woodland and open grassland habitats. Location means and standard errors were detransformed for reporting.

To evaluate relative densities of tabanids at different distances from rivers, average catches from the traps were compared using ANOVA or Kruskal Wallis test to identify any significant differences among them and whether their relative catches suggested an influence of distance. Regression analysis was used to test for any probable relationship between catch and distance from closest major river bank.

9.3. Results

Tabanid species and abundance in savannah woodland and open grassland areas

From the eight Nzi traps deployed in 2007 and 10 traps in 2008, 12 tabanid species belonging to five genera were collected (Table 9.1), of which the most abundant were *P. silvester*, *T. pallipennis*, *T. townsvilli*, *L. fuliginosa*, *T. strangmannii* and *T. dorsobimaculatus*. In general the same tabanid species were captured in both wet seasons (Table 9.1), although the proportions of each differed somewhat.
Table 9.1. Tabanid species and quantities collected from Nzi traps baited with octenol in two wet seasons: 2007 (8 traps) and 2008 (10 traps)

<table>
<thead>
<tr>
<th>Tabanid species</th>
<th>Feb - May 2007 (13 weeks)</th>
<th>Jan - Apr 2008 (16 weeks)</th>
<th>Total both seasons</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Tabanus pallipennis</em> Macquart</td>
<td>1,695</td>
<td>2,593</td>
<td>4,288</td>
</tr>
<tr>
<td><em>Pseudotabanus silvester</em> Bergroth</td>
<td>887</td>
<td>9,171</td>
<td>10,058</td>
</tr>
<tr>
<td><em>T. townsvilli</em> Ricardo</td>
<td>258</td>
<td>593</td>
<td>851</td>
</tr>
<tr>
<td><em>Lilaea fuliginosa</em> Taylor</td>
<td>255</td>
<td>299</td>
<td>554</td>
</tr>
<tr>
<td><em>T. dorsobimaculatus</em> Macquart</td>
<td>181</td>
<td>386</td>
<td>567</td>
</tr>
<tr>
<td><em>T. concolor</em> Walker</td>
<td>178</td>
<td>111</td>
<td>289</td>
</tr>
<tr>
<td><em>T. strangmannii</em> Ricardo</td>
<td>112</td>
<td>521</td>
<td>633</td>
</tr>
<tr>
<td><em>Dasybasis aculata</em> Ricardo</td>
<td>95</td>
<td>171</td>
<td>266</td>
</tr>
<tr>
<td><em>T. notatus</em> Ricardo</td>
<td>16</td>
<td>50</td>
<td>66</td>
</tr>
<tr>
<td><em>P. distincta</em> Ricardo</td>
<td>16</td>
<td>47</td>
<td>63</td>
</tr>
<tr>
<td><em>Cydistomyia dodii</em> Taylor</td>
<td>5</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td><em>D. clavicallosa</em> Ricardo</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Unidentified species (3 genera)</td>
<td>33</td>
<td>79</td>
<td>112</td>
</tr>
</tbody>
</table>

Weekly collections from four traps in savannah woodland and four traps in open grassland during much of the wet season in 2007 (Fig 9.2 and Fig 9.3) and in 2008 (Fig 9.4 and Fig 9.5) showed some differences in activity pattern among some of the most abundant tabanid species between the two habitat types. The most conspicuous was *P. silvester*, which occurred in large numbers in the savannah woodland but was absent in open grassland habitat during both years. This species dominated catches at the start of the wet season for up to four weeks, but numbers fell drastically by the sixth week and the species completely disappeared after that, less than halfway through the wet season. After the short-lived *P. silvester* dominance, *T. pallipennis* had the highest peak activity and maintained highest prevalence for the rest of the season in both woodland and grassland habitats for both years. *Tabanus townsvilli* exhibited a moderate but consistent level of abundance in the woodland areas. In 2008, most species had a bimodal distribution which was not seen in 2007 (Fig 9.4 and Fig 9.5).
Fig 9.2. Weekly abundance of different tabanid species collected from four traps in savannah woodland habitat in the wet season of 2007.

Fig 9.3. Weekly abundance of different tabanid species collected from four traps in open grassland habitat in the wet season of 2007.
Fig 9.4. Weekly abundance of different tabanid species collected from four traps in savannah woodland habitat in the wet season of 2008

Fig 9.5. Weekly abundance of different tabanid species collected from four traps in open grassland habitat in the wet season of 2008
Habitat preference was obvious in *P. silvester*, with all (10,058 specimens, Table 9.1) collected in woodland areas and none from the open grassland locations (Fig 9.6). The rest of the major species were present in both types of habitat but higher proportions were collected from woodland habitats except for *T. pallipennis* which had nearly equal proportions overall (Fig 9.6). Within the season, the relative abundance of *T. pallipennis* between habitats fluctuated from month to month by a factor of 10-30% (Fig 9.7 and Fig 9.8).

**Fig 9.6** Relative frequencies of the most abundant tabanid species captured with four traps in Savannah woodland and four traps in Open grassland over two wet seasons. Total number of flies is shown in parentheses for each species.
Fig 9.7 Monthly distribution of *T. pallipennis* between savannah woodland and open grassland habitats in the wet season of 2007. Total catches in each month are shown in parentheses.

Fig 9.8 Monthly distribution of *T. pallipennis* between savannah woodland and open grassland habitats in the wet season of 2008. Total catches in each month are shown in parentheses.
For most of the species, there was no significant interaction between year and locations (Alligator Creek, JCU, Oonoonba and Stuart), as indicated by two-way ANOVA on log-transformed weekly catches for *T. pallipennis* \( (F=1.293; df=3; p=0.330) \), *T. townsvilli* \( (F=0.007; df=3; p=0.999) \), *T. dorsobimaculatus* \( (F=2.481; df=3; p=0.121) \), *L. fuliginosa* \( (F=0.197; df=3; p=0.896) \) and *T. concolor* \( (F=0.439; df=3; p=0.73) \).

The only significant interaction between location and year was noted in *T. strangmannii* \( (F=4.637; df=3, p=0.028) \), with the highest mean catch being at JCU in 2007 and shifting to Stuart in 2008 (Table 9.2). Consequently for this species multiple-comparisons with Tukey HSD did not group the mean catches from woodland locations (JCU and Alligator Creek) and grassland locations (Stuart and Oonoonba), indicating no significant difference between the two types of habitat for *T. strangmannii*.

The detransformed marginal means for each sampling location are shown for the different tabanid species in Table 9.2. No significant difference among the four trapping locations was detected for *T. pallipennis* \( (F=2.909; df=3; p=0.087) \), hence showing no significant difference between habitats. The species showing significant preference for woodland habitats were *T. townsvilli* \( (F=8.764; df=3; p=0.004) \), *L. fuliginosa* \( (F=4.575; df=3; p=0.029) \) and *T. concolor* \( (F=4.579; df=3; p=0.029) \).

**Table 9.2** Detransformed mean catches per trap week (± SE) obtained over two wet seasons from four sampling locations in two types of tabanid habitat. For species that were only available for part of the season, means were derived from only the weeks that the species were available.

<table>
<thead>
<tr>
<th>Species</th>
<th>Savannah Woodland</th>
<th>Open Grassland</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alligator Crk</td>
<td>JCU</td>
</tr>
<tr>
<td><em>P. silvester</em></td>
<td>59.97 ±1.18</td>
<td>146.55 ±1.26</td>
</tr>
<tr>
<td><em>T. pallipennis</em></td>
<td>5.51 ±1.43</td>
<td>33.34 ±1.65</td>
</tr>
<tr>
<td><em>T. townsvilli</em></td>
<td>3.55 ±1.27</td>
<td>8.43 ±1.41</td>
</tr>
<tr>
<td><em>L. fuliginosa</em></td>
<td>4.24 ±1.32</td>
<td>3.35 ±1.48</td>
</tr>
<tr>
<td><em>T. dorsobimac.</em></td>
<td>2.13 ±1.19</td>
<td>5.31 ±1.28</td>
</tr>
<tr>
<td><em>T. concolor</em></td>
<td>2.83 ±1.22</td>
<td>1.83 ±1.33</td>
</tr>
<tr>
<td><em>T. strangmannii</em></td>
<td>2.12 ±1.12</td>
<td>6.04 ±1.17</td>
</tr>
</tbody>
</table>
Tabanid densities at different distances from rivers

Tabanid species diversity was the same at all distances from major water courses within the study locations. Densities of the most abundant species in the 2008 season, when three locations each had a linear transect of three traps, were not affected by distance from rivers at any of the three study locations. Collections of *T. pallipennis* at JCU (Fig 9.9) showed no significant difference among mean catches at the three distances tested (ANOVA, $F=0.260$, df=2, $p=0.772$), and the same was noted for the same species at Alligator Creek (Fig 9.10) (Kruskal Wallis Test, $\chi^2=4.33$, df=2, $p=0.115$). Results at Oonoonba were surprising because the highest average catch was recorded at the furthest distance away from the river (Fig. 9.11), although this was not significantly different from the trap closest to the river (Mann-Whitney, $U=106$, $Z=-0.839$, $p=0.402$). The middle trap had the lowest average catch and the difference was statistically significant (Kruskal Wallis, $\chi^2=7.046$, df=2, $p=0.03$), but clearly demonstrated no effect of distance on the catches (Fig 9.11).

![T. pallipennis LogCatch vs distance from river at JCU](image)

**Fig 9.9** Distribution of *T. pallipennis* catches among three permanent traps sited at different distances from the Goondaloo Creek at JCU. (Points marked 165 and 166 are outliers and should be ignored).
**Fig 9.10** Distribution of *T. pallipennis* catches among three permanent traps sited at different distances from the river bank at Alligator Creek.

**Fig 9.11** Distribution of *T. pallipennis* catches among three permanent traps sited at different distances from the Ross River at Oonoonba. (Point marked 282.0 is an outlier and should be ignored.)
Fig 9.12 Distribution of *P. silvester* catches among three permanent traps sited at different distances from the Goondaloo Creek at JCU

Fig 9.13 Distribution of *P. silvester* catches among three permanent traps sited at different distances from the river bank at Alligator Creek
Catches of \textit{P. silvester} also showed no statistically significant difference among the three traps at JCU (Kruskal Wallis, $\chi^2=0.277$, df=2, p=0.870) as well as Alligator Creek (Kruskal Wallis, $\chi^2=0.074$, df=2, p=0.963), and the distributions at each location (Fig 9.12 and 9.13) indicated that the catches were not affected by distance from the rivers.

\textbf{9.4 Discussion}

\textit{Tabanid relative abundance in savannah woodland and open grassland areas}

Total tabanid numbers from the two seasons showed clearly that \textit{P. silvester} was the most abundant species in the study area, followed by \textit{T. pallipennis} and \textit{T. townsvilli} (Table 9.1). The wide range of species captured during the two wet seasons demonstrated the sympatric tendency that characterises tabanid populations as described in studies elsewhere (Smith \textit{et al.}, 1970; Gonzalez, 1993; Strickler and Walker, 1993; Barros, 2001; Al-Talafha \textit{et al.}, 2005; Krcmar, 2005) where at least 10 species occurred at the same location within a season. The dominance of different species at different times of the wet season was also noted in some of the earlier studies mentioned above, albeit with totally different species of tabanids to the ones captured in this study. This was most clearly demonstrated by \textit{P. silvester} and \textit{T. pallipennis} in woodland habitats in both seasons (Fig 9.2 and 9.4), and to some extent by \textit{T. dorsobimaculatus} in 2007 in both types of habitat. However apart from \textit{P. silvester}, the flight period of most species extended to almost the whole of the wet season although there were fluctuations in abundance (Figs 9.2-9.5). The bimodal distribution noted for most species in 2008 (Fig 9.4 and Fig 9.5) did not appear in 2007 (Fig 9.2 and 9.3), and was most likely caused by a three-week long dry spell in February which coincided with a temporary drop in tabanid abundance. This also demonstrated the sensitivity of adult tabanids to current environmental conditions.

The reasons for succession patterns among tabanid species are not well understood. Barros (2001) suggested that succession may be associated with a strategy to reduce interspecific competition, but this is not convincing since their resources such as food and space are generally not limiting factors for blood sucking insects (Waage and
Davies, 1986). In those species where the succession pattern is clearly exhibited, the consistency between years is remarkable, and demonstrates the complexity and accuracy of their biological clocks in relation to prevailing climatic conditions. It is highly likely that short-season species like *P. silvester* produce only one generation per year, while species like *T. pallipennis* and *T. townsvilli* may produce more generations in the same season (Jones, 2001), given the apparent stability of their adult populations through most of the flight season (Fig 9.2 and 9.4). Alternatively, they might be among the species where any of the larval instars is capable of switching to the dormant state when winter sets in (Mackerras, 1971), such that the following wet season finds them all at different stages of development and hence maturing at different times throughout the season.

The results indicated that the breeding sites of *P. silvester* and *T. townsvilli* *L. fuliginosa* and *T. concolor* may be associated with woodland habitats, although only *P. silvester* is exclusively restricted to this particular habitat type. The findings are somewhat similar to those of Smith *et al.* (1970) who observed that, with few exceptions, the habitat preferences of Tabanidae in Ontario were not rigid but there were differences in the probability of encountering a given species in a given habitat. They however, noted that a few species were ‘exclusively sylvan’ (forest-dwellers), or ‘preferentially sylvan’ while others were ‘ubiquitous’ (abundant in all habitat types examined). Similarly in Japan, *T. chrysurus* and *Haematopota tristis* were most abundant in ‘forestlands’, while *T. nipponicus* and *T. trigonus* were predominantly abundant in ‘pasturelands’ although none were found exclusively in one habitat type, and general abundance and species diversity was higher in ‘forestlands’ (Hayakawa *et al.*, 1986). Studies by Barros (2001) in Brazil did not demonstrate any habitat specificity among the most abundant species with regards to grassland and woodland areas, but this is not surprising given that his sampling was done among a mosaic of vegetation types in which a grassland site was located only 10m from the edge of a forest (savanna) patch.

The relatively uniform distribution of *T. pallipennis* makes it likely that this species can breed and live comfortably in both types of habitat. In a previous study investigating the density and diversity of tabanid larvae in California, some species were apparently restricted to one habitat type while others were found in two or more habitats (Lane, 1976): with *T. punctifer* displaying the most diverse breeding habits and occurring in several habitats including temporary ponds, permanent ponds, seepage areas, creek
margins and river banks. None were found in woodland soil and leaf litter although the author acknowledges the inconclusiveness of this finding since the vastness of terrestrial habitats relative to semiaquatic habitats precluded sampling comparable proportions of the woodland (Lane, 1976).

The major implication of the current findings is that contact of adult flies with potential hosts is likely to occur regardless of whether the animals are in woodland or grassland areas. However, the absence of *P. silvester* from grassland areas means that domestic animals may experience at least five times less attack by tabanids if they are confined to grassland areas for the first 6-8 weeks of the wet season. General surveillance for tabanids can also be optimised by placing traps in woodland areas.

The finding that *T. pallipennis* densities at all four locations were not statistically different was unexpected, given that tabanids are generally not uniformly abundant across wide areas. This species seems to be adapted to the wide range of micro-environmental conditions prevalent in the study area. The extent of *T. pallipennis* distribution on a larger scale was shown by Van Hennekeler (2007) in Cape York Peninsula when it was captured at 11 of 12 study sites distributed across hundreds of kilometres. In some of the sites, the species also had a small peak of activity in the dry season resulting in extended duration of activity duration of 7-8 months in a year (Van Hennekeller, 2007). This is probably the most widespread species in northern Australia, and considering that it was active in relatively large numbers for nearly the whole wet season, this species could pose the biggest challenge in attempts to prevent the spread of surra.

**Tabanid densities at different distances from rivers**

The results for *P. silvester* showed that there was no significant difference among the trap catches at various distances from rivers within the 1 km transect investigated at both JCU and Alligator Creek, the only locations where the species was captured. The same was true for *T. pallipennis* at those locations. The catch variations among some of the traps at Oonoonba were not related to distance from the Ross River, since there was no significant difference between the trap closest to and furthest away from the river bank.
The findings suggest that either the tabanid species analysed did not breed preferentially along the rivers or if they did, that the flies dispersed quickly and consistently away from the breeding sites over the distance examined (1 km). In this latter case any effect of distance might be detectable well beyond the one kilometre that was investigated. However, it would be almost impossible to demonstrate this over longer distances within the same study area which has undulating terrain, since effects of other water courses present can not be avoided. It is more likely, however, that these species use the temporary pools and puddles scattered along the forest floor during the wet season as breeding sites. Comparable results were obtained in Africa by Phelps and Vale (1976), who collected tabanids weekly from Manitoba and Morris traps along three transects each nearly 1400 m long. The transect which passed in the vicinity of several small water bodies and was nearest to a river gave lowest catches, though the difference was not statistically significant. The authors found the result surprising given the supposed importance of water bodies in the breeding biology of Tabanidae (Phelps and Vale, 1976). Many tabanid species therefore do not seem to necessarily rely on the larger water bodies for breeding, and trap deployment for adult flies need not be necessarily close to big rivers.

One of the major challenges of comparing different ecological studies from the literature is that prevailing physical conditions are almost always different in each case, and authors use a wide range of terminology to describe types and distribution of vegetation in different parts of the world.

**Potential impact on disease transmission**

Based on abundance only, *P. silvester* and *T. pallipennis* (Table 9.1) would potentially play the biggest role in disease transmission in the study area. However, the impact of *P. silvester* would be limited by its short flight period (Fig 9.2 and 9.4), and confinement to woodland areas (Fig 9.6). *Tabanus pallipennis* and *T. townsvilli* would be relatively more important in those respects because they maintained relatively high population densities for most of the wet season, and were distributed more widely. Species such as *T. notatus* and *P. distincta* would be considered almost irrelevant because of their very low catches in both years (Table 9.1). However, the potential role
of different species in disease transmission must also be considered in relation to their blood feeding behaviour (Chapters 3, 4 and 7) and host preferences (Chapter 6).
CHAPTER TEN

GENERAL DISCUSSION

10.1. Introduction

This chapter summarises the findings of the whole study and links the different chapters in the context of the overall objectives of the research project. First, it describes how the different facets of the project (host-vector interaction and tabanid abundance and distribution) culminate in the prediction of the relative vectorial capacity of the various tabanid species in the transmission of surra. Secondly, the major potential hosts of tabanid flies that were investigated are considered in terms of the relative risk of infection with surra through their interaction with the different tabanid species prevalent in the area. Consequently, the potential impact of a surra incursion can now be evaluated.

The risk of a surra incursion into northern Australia has been widely recognised and well-documented (Thompson, 2001; Sandeman and Warner, 2002; Reid, 2002; AFFA, 2003; Reid and Copeman, 2003; van Hennekeler et al., 2008). However, it has not been possible to formulate rational strategies for integrated disease management based on scientific evidence prior to this project because of the serious lack of information. Therefore this chapter also discusses the options available to Australian farmers and government in terms of tackling this challenge. Most of the options discussed are based on linking the new information on tabanid blood-feeding behaviour with current methods for management of animal ectoparasites in Australia and elsewhere. The need to protect wild-life biodiversity complicates the Australian scenario and this is also discussed.

Lastly, options for effective vector surveillance are considered on the basis of the relative trappability of different tabanid species and their distribution among different types of habitats.
10.2. Relative vectorial potential of different tabanid species

The range of investigations conducted over the three wet seasons in this study showed that the potential for mechanical transmission varies among tabanid species, and sometimes among host species. Overall findings indicated that *T. pallipennis* would probably prove to be the most efficient species for surra transmission in northern Australia. The species had one of the highest proportions (49%) failing to complete a blood meal on a single host once feeding was initiated (Chapter 3; Muzari *et al.*, 2010b), required the highest number of partial feeds (nine) to reach full engorgement (Chapter 4; Muzari *et al.*, 2010) and fed on blood from all host species investigated (Chapter 6; Muzari *et al.*, 2010c). Multiple interrupted tabanid blood meals promote the spread of infectious agents among animals (Krinsky, 1976; Magnarelli and Anderson, 1980). *Tabanus pallipennis* also had one of the longest flight periods in each season, was prevalent equally in both woodland and grassland habitats and was one of the two most abundant species encountered during the three years (Chapter 9). Tabanid abundance is one of the factors most closely associated with disease prevalence (Foil, 1989). In addition, the widespread distribution of *T. pallipennis* in northern Australia (van Hennekeller, 2007) potentially exposes most animals to attack.

Among the most common species, *P. silvester* appears to pose the lowest risk in terms of mechanical transmission, particularly among horses and possibly cattle as well. It had the highest proportion (89%) of meals completed on a single animal, and generally obtained the full blood meal through only one bite. This species was not easily dislodged by horses once feeding started because the flies preferentially landed on the animal’s belly. If not for these factors, *P. silvester* would have been a major potential vector because it displayed the most opportunistic feeding behaviour (fed on any available host species) and had the highest seasonal abundance. However, its distribution was restricted to woodland habitats and its flight period was only about 6-8 weeks long, with at least 80% of the flies appearing within a period of only three weeks. The period of host-vector interaction is therefore relatively short, and the habitat range is more restricted than most other species in the area.

Overall, *Tabanus townsvilli* was the third most abundant species and may pose moderate but significant risk of mechanical transmission. The species experienced interrupted feeding and required up to five partial feeds to complete a meal, had
relatively long feeding durations (median 180 s on horses and 210 s on pigs), fed on all host types tested and maintained a moderate population at almost a constant level throughout the season, particularly in woodland areas. Longer feeding durations imply a longer duration of exposure to infected tissue fluids, leading to higher probability of picking up infectious agents (Krinsky, 1976).

Species of low to moderate risk include *Dasybasis oculata*, whose population density was among the lowest and had a short flight period, although it demonstrated a relatively high percentage (48%) of interrupted meals.

The tabanid species recorded in the Townsville area during the three-year study period are prevalent in the rest of northern Queensland, as indicated by earlier trapping studies conducted by Van Hennekeler (2007) on 11 sites in Cape York Peninsula (CYP), North Queensland. Many of them are also found in the Northern Territory (Mackerras, 1971), and hence the findings in this study are applicable to much of northern Australia, where the threat of surra incursion is considered highest (Thompson, 2003). However, further studies are required on some of the other species whose numbers were too low in the Townsville area but are more abundant elsewhere, particularly *T. notatus*, which was the most abundant and widespread species recorded in CYP in recent years (van Hennekeler, 2007). Indications are that this species may not be a very efficient vector due to high engorgement success through a single bite on a single host, but this was based on only three flies seen feeding on a horse (Table 4.1; Muzari et al., 2010). Further investigation on *T. notatus* is warranted because it constituted more than 60% of *Tabanus* catches at Coen, Lockhart River, Lakefield and Rutland Plains in CYP (van Hennekeler, 2007).

Another potentially important species present along Australia’s northern border but not seen in Townsville is *Tabanus ceylonicus*, which was previously captured in Bamaga, Weipa, Mapoon and Heathlands (van Hennekeler, 2007). It is one of the few Australian species also found in some Asian countries where surra is endemic (Chapter 1; Mackerras, 1971; Daniels, 1989; van Hennekeler, 2007) and it has been experimentally incriminated in surra transmission in Indonesia (Nieszultz, 1925). While *T. ceylonicus* distribution seems to be restricted to the northernmost parts of Queensland, it may play an important role in allowing surra to establish in the immediate post-incursion stage, after which other species may spread the disease further south. Therefore its potential
role in Australia also needs to be evaluated through studies on blood-feeding behaviour and host preference.

10.3. Relative risk of surra infection among different hosts

From the field observational studies, it was clear that different host species responded differently to attack by tabanids, but the most surprising response was that of the pigs which simply ignored the tabanids and seemed to feel no pain from the bites. They allowed five different species of tabanids to bite freely for lengthy durations although generally the flies still failed to obtain a full blood meal. Pigs would therefore face the highest risk of being decimated by surra if not for their high tolerance for *T. evansi*, seldom showing clinical signs even when infected (Dielman, 1986; Reid *et al.*, 1999). Consequently, feral pigs could play a key role as reservoirs of the disease, which could then spread to other wild animals and domestic stock.

Macropod responses to tabanid attack were easily elicited, quick and aggressive and hence very likely to promote interrupted feeding. Macropods were also a favoured host with the highest frequency of host blood identified in trap caught tabanids (61-80% in five tabanid species), even in locations where estimated densities of macropods were relatively lower than other potential hosts (Muzari *et al.*, 2010c). By far the most common macropods in the study area were agile wallabies, which were presumably the main source of the macropod blood meals detected in the flies. Even tabanid species such as *P. silvester*, which could complete a meal through a single bite on a horse, could generally not feed without interruption when feeding on macropods. Therefore macropods are at serious risk of infection with surra because they are a major host of most tabanid species and because they promote interrupted feeding which is necessary for mechanical transmission of the disease. Chances of host-vector contact would also be enhanced by the apparent preference of macropods for woodland areas where tabanid densities and species diversity are highest.

In other studies in northern Queensland where large populations of feral pigs and macropods coexist, macropods were the preferred host of the mosquito *Culex annulirostris* (van den Hurk *et al.*, 2003). They were suspected to be diverting host-seeking mosquitoes from pigs and thus probably impeding the establishment of
Japanese Encephalitis (JE) on mainland Australia since marsupials are poor hosts of JE virus (van den Hurk et al., 2003). However, the preference of tabanids for macropod hosts would most likely promote the establishment and spread of surra. Unlike pigs, macropods infected with *T. evansi* experience persistent parasitaemia resulting in acute disease and high mortality (Reid et al., 2001). Therefore the high populations of feral pigs (Ritchie et al., 2007) and agile wallabies in northern Queensland are a major risk factor for surra incursion and establishment. After a surra incursion, the disease could easily get out of control and potentially drive the macropod population to the brink of extinction. This has occurred for other mammals such as the endemic Christmas Island (Indian Ocean) rat *Rattus macleari* which was wiped out within 25 years when a highly infectious and fatal trypanosomiasis (*Trypanosoma lewisi*) was introduced onto the island through the flea-infested black ship rat *Rattus rattus* (Wyatt et al., 2008; Thompson et al., 2009). Similarly, it has occurred for other Australian vertebrates such as frogs where there were reservoir hosts for an introduced virulent disease, chytridiomycosis (Schloegel et al., 2006; Skerratt et al., 2007). Local species of haematophagous insects have probably evolved a preference for macropods because the animals are native to Australia and have sustained these insects for millennia.

Horses were studied more extensively than other animals on aspects of host-vector interaction in this project because they were generally more convenient to deal with. Consequently the variability in blood-feeding behaviour among tabanid species was mostly demonstrated on this host. The findings indicated that the horses faced the most significant threat from *T. pallipennis* due to a high proportion of interrupted feeds (49%), many partial feeds (up to nine) required by this species to complete a meal (Chapter 4; Muzari et al., 2010), high tabanid densities of at least 44 flies around a horse per three-hour session (Chapter 3), a relatively high proportion of horse blood among *T. pallipennis* specimens (8.9%) (Chapter 6; Muzari et al., 2010c) and the wide distribution of this species among different habitats including grassland pastures (Chapter 9). The highest relative preference for horse blood was, however, noted in *P. silvester* (20%) and *T. strangmannii* (16.7%). The level of interrupted feeding in *T. strangmannii* could not be evaluated in behaviour experiments due to low numbers of this species. While *P. silvester* was not considered a major potential vector among horses due to meal completion from single feeds, horses would face a risk of infection from this species if they lived in close proximity with infected pigs upon which *P. silvester* feeds freely but is unable to complete blood meals. The highest tabanid
burdens on horses were inflicted by this species (67 flies per three-hour session) (Chapter 3), although the flight period only lasted for a few weeks and the species was confined to woodland areas. Other species in which residues of horse blood were detected included *Lilaea fuliginosa*, *T. concolor* and *T. dorsobimaculatus*, but populations of these species were generally low in the study area and their feeding behaviours were therefore not adequately observed.

Cattle in northern Australia could also be significantly affected by surra. Through blood meal analysis, at least six tabanid species were seen to have fed on cattle, with the highest relative preference for cattle blood recorded in *P. silvester* (13.1%), *T. pallipennis* (9.6%) and *T. strangmannii* (7.8%). The blood feeding behaviour of tabanids was not sufficiently evaluated on cattle, but general observations indicated tendencies similar to those observed on horses, particularly with *P. silvester* and *T. pallipennis*. Therefore the broad distribution of *T. pallipennis* would also expose cattle to bites and potential infection from this species even in open grassland pastures. *Pseudotabanus silvester* is not expected to cause a major risk unless cattle live close to infected pigs.

Overall, the tabanid species studied are opportunistic feeders who can take successive meals from different kinds of host species. This can lead to cross-infection among host species. The results indicated that all four host types studied could potentially become infected with surra through transmission from most of the tabanid species prevalent in the area, although macropods probably face the highest risk.

### 10.4. Techniques for management of surra transmission in northern Australia

*The challenge of tabanid population reduction*

Any attempts at wide scale control of tabanid populations in the vast, mostly remote, areas of North Queensland would be unmanageable and ineffective. One of the major challenges is the abundance of multiple tabanid species, some with different biological characteristics. Control of the immature stages is not an option because of the diversity
of breeding habitats among and within species, and the fact that tabanid larvae and pupae are typically inaccessible in the soil. Even for species like *P. silvester* and *T. townsvilli* that have been shown to prefer woodland habitat, the vastness of such habitat renders targeted control not feasible with current methods.

While significant control of other biting flies can be achieved by exploiting their host-oriented behaviours (Gibson and Torr, 1999), adult tabanids are generally not as susceptible to such methods because only the females need vertebrate blood for egg production and host contact takes only a few minutes per fly during blood feeding, which may occur only once every three to five days. The flies are not livestock-dependent, as many of them are opportunistic feeders with a wide host range which includes wild-life. As discovered here, most of the Australian species studied seem to prefer wild-life (macropods) even when livestock are available. Moreover, tabanids can feed on plant juices and survive for several weeks without taking a blood meal. In reality it is almost impossible to significantly reduce tabanid populations for more than a few days in a wide area. Therefore the main approach to protect livestock would be to reduce biting rates and limit the rate of transmission and spread of the disease. Therefore alternative strategies are discussed below.

*Selective grazing during wet season*

Animal husbandry practices based on some of the findings from this project would be useful to avoid or minimise host-vector contact. Of the tabanids collected from traps during two wet seasons, more than 50% were *P. silvester*, whose distribution was confined to savannah woodland areas. The other tabanid species were either found equally in both habitat types or showed a distinct preference for the savannah woodland. Therefore restricting livestock to open grassland pastures, particularly for the first 6-8 weeks of the wet season, would definitely reduce the tabanid burden on the animals. A seven-year study on feral horses in Maryland showed that the horses naturally tended to avoid the forest habitat when tabanid (and other biting flies) densities were highest (Powell *et al.*, 2006). Queensland has got vast areas of open pasture which could be utilised if this strategy was to be adopted by farmers. The recommendation for ‘pastures away from wooded areas’ has also been given in the USA as a strategy against attack on horses by tabanids and biting midges (Foil and Hogsette, 1994).
**Spatial barriers between animals**

Creating spatial barriers between pigs and other livestock should reduce the risk of transmission. This is because pigs are potential reservoirs of *T. evansi* infection, and are likely to promote transmission of infection by only allowing tabanids to obtain partial blood meals. Foil (1983) recommended a 200 m quarantine distance between infected and susceptible animals, and the same recommendation could be made for spatial barriers between pigs and other livestock to limit the spread of surra. It was shown with nine Brazilian species that 100% of the flies that re-fed on a horse following interrupted feeding returned to the original host when other horses were 50m away (Barros and Foil, 2007). Although the host-switching distance depends on the tabanid species present, it is unlikely that any of the Australian species would readily switch hosts across such distances.

Foil’s (1983) suggestion of 200 m barriers could also be applied to other situations and host species in Australia, not just pigs and other livestock.

**Protective stabling of livestock**

Tabanids generally bite outdoors and rarely enter buildings, and therefore stabling of animals where possible during peak fly periods can be a useful way of shielding them from tabanid attack (Foil and Foil, 2001). This is normally recommended for horses, but pigs can also be kept in their housing. In contrast, cattle are normally only sheltered during transit to ports or abattoirs in Australia (Doherty *et al*., 2004) and there is a lack of shelter facilities. A combination of shelters and chemical treatment has been beneficial to prevent against attack by *Culicoides* spp. on cattle (Doherty *et al*., 2004, Melville *et al*., 2005). Covering gaps in animal shelters with insecticide-treated netting (Meiswinkel *et al*., 2000) would be even more beneficial to prevent tabanid bites. While it may be logistically difficult to provide housing and insecticide-treated netting barriers for very large herds, animals suspected of being infected may be selected and sheltered to minimise further transmission. These techniques are most readily applied to animals of high intrinsic market value such as thoroughbred horses (Carpenter *et al*., 2007).
Insecticide-treated animals

Residual insecticides have been used on animals in different countries to control blood-sucking arthropods such as ticks (Stendel et al., 1992; Eisler et al., 2003), tsetse flies (Baylis and Stevenson, 1998; Hargrove et al., 2003), stable flies (Baldwin et al., 2005), mosquitoes (Hewitt and Rowland, 1999; Mahande et al., 2007), horn flies (Gugielmone et al., 2000), buffalo flies (Waltisbuhl et al., 2005), and to a lesser extent tabanids (Foil and Hogsette, 1994). In Louisiana, the application of synergized pyrethrins and synthetic pyrethroids on beef cattle has been useful in providing partial protection against tabanids (Baldwin et al., 2005). This method could be used to complement spatial barriers in preventing the spread of mechanically transmitted infections by killing infective tabanids before they switch hosts. This would obviously require that effective doses of insecticide are applied to animals, particularly the infected ones.

Farmers in Queensland and other parts of northern Australia generally apply residual insecticides to cattle and horses to protect them from tick-borne diseases and to control lice and biting flies, especially buffalo flies Haematobia irritans exigua (Waltisbuhl et al., 2005) during the wet season. Application methods used include plunge dipping, spraying, pour-ons, back-rubbers and ear-tags, and the insecticides used include organophosphates, synthetic pyrethroids and macrocyclic lactones (Waltisbuhl et al., 2005). In different parts of Australia, insecticides are also applied on pigs to control mange mites, lice and nuisance flies and on sheep to control blowflies Lucilia cuprina and body lice Bovicola ovis (Pillips, 2005). While tabanids are not specifically targeted in the control of ectoparasites by Australian farmers, presumably due to lack of concern and effective control techniques, the new knowledge from this project could be used to adapt the existing practices and infrastructure to limit the spread of surra in the event of an incursion.

A key factor to consider is that ticks, lice and buffalo flies spend excessively long times attached to the host animal (Veer et al., 2002; Jensen et al., 2004) and hence can be killed by relatively low concentrations of residual insecticide, which may not be sufficient to kill tabanids given their short duration on the host. In the current studies, most Tabanus pallipennis were in direct contact with a horse for less than 20s at a time and for a total of about three minutes, and T. townsvilli had an average contact time of 180 s when meals were interrupted, although durations were higher on the pig. Feeding
durations may become even shorter when the host is treated with a topical insecticide (Habtewold et al., 2004). To be effective, lethal insecticide doses against these tabanids should be picked up within these short durations. Fortunately this is achievable. Leprince et al., (1992) recorded >96% mortality of T. fuscicostatus that had been placed for 15-60 s on the back of bullocks which had lambdacyhalothrin-impregnated ear tags. Similarly, tsetse fly species G. pallidipes and G. morsitans about the size of T. pallipennis, have been shown to pick up lethal doses of deltamethrin from cattle through landing bouts averaging 30 s duration (Torr et al., 2007) and from insecticide-treated netting within 1 s of contact (Mangwiro et al., 1999). This may still be too long to prevent treated cattle from being infected, but it should prevent further transmission through the same fly.

Researchers need to determine the optimal insecticide-treatment regimes against tabanids that are still effective but ensure no side-effects for the host animal. To prevent transmission of surra, it is necessary that tabanid knockdown (paralysis) after contact with an insecticide-treated animal occurs before the fly has time to switch hosts and continue feeding. Alternatively, the immediate effect of the chemical should prevent the affected fly from further feeding attempts until eventual knockdown and death. Such ‘behavioural aberrations, such as uncontrollable activity’ preventing further feeding are apparent in tsetse flies ‘within one minute’ after contact with pyrethroid-treated cattle (Baylis and Stevenson, 1998), and are likely to occur in tabanids as well.

The effectiveness of topical insecticides on animals is also affected by the residual persistence of the insecticide at the sites where the vectors prefer to land. In the case of T. pallipennis, T. townsvilli, P. distincta and T. notatus it would be critical to ensure that the legs of the treated animal receive and maintain an effective dose of the insecticide, while for P. silvester, particular attention must be paid to the belly of the animal. In studies where deltamethrin was applied as a pour-on to control biting flies and ticks, the legs usually ended up with the lowest concentration of insecticide compared to the back and other parts of the animal’s body (Stendel et al., 1992; Vale et al., 1999). Carpenter et al. (2007) also showed that, after application of pyrethroid pour-on, hair from the belly of cattle caused lower Culicoides mortality than hair from the backline. Similar results were found when insecticide-releasing ear-tags were used against tsetse flies, with higher mortality rates recorded on insects landing on the head and neck than on those alighting on the legs (Thomson, 1987). In North Queensland insecticidal ear tags
are commonly used on cattle in summer and are very effective against buffalo flies (Waltisbuhl et al., 2005), and it would not be surprising if they had little to no effect against tabanids that alight on the lower limbs of cattle and horses. Choice of application method is therefore essential. Dipping or spraying would guarantee a more even distribution of insecticide on the host and are probably the better options compared with ear-tags or pour-ons against tabanid species such as T. pallipennis, T. townsvilli, P. silvester and others. Dipping frequency must be based on the persistence of the insecticide on the legs rather than the back or head of the animal.

**Insect repellents**

The use of repellents or partial repellents is considered to be an effective strategy to reduce the incidence of tabanids on livestock (Foil and Hogsette, 1994). Generally, the repellents are in the form of certain synthetic pyrethroids (Foil and Hogsette, 1994), some of which also have a good killing effect if they contact the fly (Leprince et al., 1992; Eisler et al., 2003). However, there is mixed evidence for repellency of biting flies for animals treated with pyrethroids, and most authors believe that the effect is minimal (Baylis and Stevenson, 1998). Some chemicals such as methoxyphenol, pentanoic acid and acetophenone, isolated from ox odours, have shown some repellent effect on tsetse flies although they are not likely to provide a useful degree of protection where vectors are abundant (Torr et al., 1996). They have not been tested against tabanids. Other general insect repellents, particularly those containing DEET, are known to reduce insect bites on humans (Lance Sholdt et al., 1989), but they require at least daily application and are thought to be unsuitable for livestock application for a variety of other reasons (Carpenter et al., 2008). Moreover, they have demonstrated little or no effect against tabanids (Hill and McDonald, 2007).

**Artificial baits / attractants**

Following the successful use of odour-baited traps and targets to control tsetse (Vale, 1993; Torr, 1994), there has been increasing interest in the potential of this approach for control of other biting flies (Mihok et al., 1995; Hall et al., 2003; Foil and Younger, 2006), including tabanids (Mihok et al., 2006; Kremar et al., 2009), with testing of
different odour attractants and trap designs. Some traps have captured thousands of flies in a day (Mihok et al., 2006), including our octenol-baited Nzi trap in this study which at one time caught nearly 2000 *P. silvester* in 24 hours. While there is no evidence to suggest that trapping this amount of tabanids would have consistently significant impact on biting rates over a wide area, it is likely that a good density of traps close to stables or animal housing would divert significant proportions of tabanids away from the animals (Foil and Hogsette, 1994).

### 10.5. Tabanid and surra control on wild-life

The finding that macropods are a key host for most tabanid species in North Queensland is a major cause for concern, and confirms the long-standing fears that the unique Australian fauna could face devastation in the event of a surra incursion. The situation is worsened by the prevalence of large populations of feral pigs in the same general habitat as the macropods. The methods suggested for protecting livestock, such as pasture selection, spatial separation of animals and application of residual insecticides are not easily applicable to macropods and feral pigs. While one could imagine a massive, though technically very challenging, culling exercise against feral pigs (which are classified as pest animals) to reduce the abundance of reservoir hosts, the feasibility of such an exercise is doubtful. Moreover, the pig population levels may quickly build up again afterwards.

No current control method has the potential to protect macropods once surra is established in Australia, and therefore the best and only option available is to prevent its entry and establishment. While there might be a slim chance of containment if the disease was detected early, the situation could easily get out of hand and turn into an epidemic. Current efforts to improve the sensitivity, accuracy and versatility of infection detection techniques (Reid, 2003; Smuts, 2009) are steps in the right direction, and the number of sentinel herds along the northern borders may need to be increased in order to spread monitoring across a wider area. Since pigs have high chances of being infected through almost any tabanid species, the inclusion of pigs among the sentinel animals along the northern borders might be a good way to improve early detection of an incursion. For a start, the small herds of sentinel pigs routinely used by AQIS in the
wet season for JEV surveillance in northern CYP (Ritchie et al., 2007) could also be examined for Trypanosoma evansi infection.

10.6. Vector surveillance

Interventions to control surra transmission by tabanids must be complemented with some measures of vector abundance and host-biting rates, which rely on unbiased methods for sampling adult horse flies. The Nzi trap baited with octenol and/or CO2 is probably the best trap available for sampling Australian tabanid species (Muzari et al., 2010), and it is still fairly effective even without odour attractants (van Hennekeller et al., 2008). Although trappability of different local tabanids varied among species in the current study, the trap proved to be a reliable means of detecting the presence of the species in the study area. Collections from the traps were very good indicators of P. silvester activity around a live animal, gave a reasonable indication for T. pallipennis and T. townsvilli but were unable to reflect the activity levels of T. dorsobimaculatus, while numbers from other species were too low to allow similar assessment.

Development of the trap to better represent activity of other species may not be an immediate priority, but an unbiased trapping device would be an immensely valuable tool in developing a better understanding of spatial and seasonal patterns of tabanids. Improvements may need a better understanding of the visual and olfactory responses of species, which is likely to take time to develop. One potential area for exploration in this regard is to analyse and synthesize the key chemical components of odour plumes emanating from macropods (evidently a favoured host by local tabanid species) and use them as attractants on traps. For best results, surveillance should be conducted in wooded areas which are the preferred habitat of most tabanid species.

A supplementary surveillance method, depending on circumstances, is the use of well trained bait-animals walking about with teams of people who are good at using hand-nets to capture alighting flies (Phelps and Vale, 1976). Similar ‘bait-ox surveys’ have been effective in tsetse control operations, especially for species such as Glossina morsitans morsitans whose response to stationary traps is known to be relatively poor (Gibson and Torr, 1999).
Electrocuting nets are only feasible as a research tool, and should be used more in teasing out the various stages of tabanid host-oriented behaviour, such as long-range olfactory responses, visual and short-range responses. They have always caught many more insects than conventional traps when used for sampling tsetse flies (Dransfield and Brightwell, 2001), stable flies (Foil and Younger, 2006), mosquitoes (Torr et al., 2008) and tabanids (Mohamed-Ahmed et al., 2007) and this was demonstrated in this study (Chapter 8; Muzari et al., 2010b). However, the cost and logistical requirements of E-nets would never justify their use in routine surveys.

10.7. Conclusion

This project substantially enhances our understanding of the ecology and blood-feeding behaviour of some important Australian tabanid species and the relative risks of surra infection among some of the major potential hosts in northern Australia. By evaluating the relative vectorial capacity of different species, the findings provide a firm foundation for more specific research on the biology of the most potent vectors like T. pallipennis. They also form a basis for better disease preparedness through the development of rational prevention and management strategies based on scientific evidence. We can now explore practical ways to integrate the available intervention options into current livestock management practices by Australian farmers. The massive risk that macropod populations face from a surra incursion has now been confirmed, and researchers need to seriously search for solutions to protect the precious marsupials of Australia before it’s too late.
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**APPENDIX**

[Copies of research papers from this Doctoral study published in peer-reviewed journals]
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