Mating behaviour and competitiveness of
male *Glossina brevipalpis* and *Glossina austeni*
in relation to biological and operational attributes for use in the
Sterile Insect Technique

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DECLARATION

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PREFACE

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Ethical considerations

Materials used in the study posed no health risk to researchers and no vertebrate animals were harmed. Permission to do research in terms of Section 20 of the animal diseases act of, 1984 (ACT no. 35 of 1984) has been granted for tsetse fly collection and colony maintenance, Ref 12/11/1/1/9 and 12/11/1/1. The study was done as part of a project on National Assets (000773) at the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI) in collaboration with the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture under the coordinated research project (CRP) 12618/R0/RBF and research project 17753/R0 as well as the Department of Technical Cooperation of the IAEA under project RAF 5069.

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I leave u with this very important question to consider while reading this work, as it was first pondered by Lewis Carrol's Mad Hatter: Why is a Raven like a Writing desk?

ABSTRACT

In South Africa, African Animal Trypanosomosis (AAT), caused by *Trypanosomae* parasites transmitted by *Glossina brevipalpis* and *Glossina austeni* (Diptera: Glossinidae), is restricted to the north east of KwaZulu-Natal Province with an estimated 250 000 cattle being at risk. For the control of these flies an area-wide integrated pest management (AW-IPM) strategy with a sterile insect technique (SIT) component has been proposed.

Accurate knowledge of the distribution of target populations is fundamental to the success of any control programme. In the present study tsetse fly distribution was determined with odour baited H traps and cattle screened using the buffy coat analyses to produce updated tsetse fly distribution, abundance and trypanosome prevalence maps for north eastern KwaZulu-Natal. *Glossina brevipalpis* and *G. austeni* were collected in areas where they had previously not been captured. Vegetation and temperature was shown to influence their distribution and abundance. The fact that no significant correlation between tsetse fly abundance and nagana prevalence could be established underlines the complex interactions between these two entities. This was epitomised by the fact that despite large differences in the apparent densities of *G. austeni* and *G. brevipalpis*, overall trypanosome prevalence was similar in all districts in north eastern KwaZulu-Natal. This indicated that both species can play a role in transmission of AAT and need to be controlled.

The *G. brevipalpis* and *G. austeni* populations of north eastern KwaZulu-Natal extends into southern Mozambique (both species) and Swaziland (*G. austeni*). Morphometrical analyses showed an absence of any significant barriers to gene flow between the various KwaZulu-Natal populations as well as between the South African populations and those of the two neighbouring countries. Tsetse fly control in a localised area will therefore be subjected to reinvasion from uncontrolled areas. An area-wide approach, *i.e.* against the entire tsetse fly population of South Africa, southern Mozambique and Swaziland will therefore be essential.

The maintenance of colonised *G. brevipalpis* and *G. austeni* at the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI), necessitate a high quality blood source. For the potential improvement of the current rearing diet various anticoagulants, phagostimulants and blood sources were evaluated and production assessed using standardised 30-day bioassays. Defibrinated bovine blood was found to be the most suitable. Anticoagulants such as sodium citrate, a combination of citrate and sodium acid, phosphate dextrose adenine and citric acid can be used to simplify blood collection. While *G. brevipalpis* preferred bovine to porcine blood, *G. austeni* preferred a mixture of equal parts bovine and porcine blood. The phagostimulants adenosine triphosphate, as well as tri-posphates of inosine, and the mono-posphates of guanosine

and cytosine improved production in both species. Attempts to colonise the local KwaZulu-Natal strain of *G. brevipalpis* failed due to a reluctance of field flies to feed on the artificial feeding system.

In preparation for the SIT component the irradiation sensitivity of colonised *G. brevipalpis* and *G. austeni* when treated as adults and late-stage pupa was determined. A dose of 40 Gy induced 97% sterility in *G. brevipalpis* males when irradiated as late-stage pupae and 80 Gy induced a 99% sterility in flies irradiated as adults. Higher doses were required for *G. austeni*, with 80 Gy and 100 Gy inducing higher than 97% sterility in females that mated with males treated as adults or late-stage pupae.

As colonised and irradiated males must be able to compete with their wild counterparts the mating performance of the colonised *G. brevipalpis* and *G. austeni* was determined under near natural conditions in walk-in field cages. Although the mating latency for both species was shorter, their mating performance did not differ significantly between mornings and afternoons. For both species mating frequency was significantly higher in nine-day-old males compared to six- or three-day-old males. Age did not affect the males' ability to transfer sperm, their mating duration or mating latency. There was no significant difference in mating performance of sterile and fertile males.

This study indicated that AAT and tsetse flies are abundant in KwaZulu-Natal and tsetse fly presence seems to be a dynamic process that is influenced by a number of environmental factors. The earlier proposed AW-IPM strategy with a SIT component, although still applicable, will need to be adapted to incorporate the new distributions records. Initial results indicate that the colonies at the ARC-OVI will be suitable for programmes that have a SIT component.

Keywords:
Glossina brevipalpis, Glossina austeni, distribution, Trypanosomosis, morphometrics, colonies, radiation sensitivity, mating performance

UITTREKSEL

In Suid-Afrika is Afrika Trypanosomiase van diere (ATD), wat deur *Trypanosomae* parasiete veroorsaak word en deur *Glossina brevipalpis* en *Glossina austeni* (Diptera: Glossinidae), oorgedra word, beperk tot die noordoostelike KwaZulu-Natal Provinsie. Na beraming is sowat 250 000 beeste tans blootgestel aan die siekte. 'n Area-wye geïntegreerde plaagbestuur (AW-IPB) strategie met 'n steriele insek tegniek (SIT) komponent word vir die beheer van die vlieë voorgestel.

Die sukses van 'n beheerprogram sal afhang van akkurate inligting oor waar tsetsevlieë voorkom. In die huidige studie is H tipe valle saam met geurlokaas gebruik om tsetsevlieë te versamel. Trypanosomiase infeksiesyfers in beeste is ook gemonitor. Die inligting is gebruik om bestaande kaarte van tsetsevlieg verspreiding, volopheid en trypanosomiase voorkomsyfer in diere op te dateer. *Glossina brevipalpis* en *G. austeni* is versamel in gebiede waar hulle voorheen afwesig was. Daar is gevind dat plantegroei en temperatuur die verspreiding en volopheid van tsetsevlieë beïnvloed. Die feit dat geen betekenisvolle korrelasie tussen vlieg getalle en ATD voorkomsyfer bepaal kon word nie beklemtoon die komplekse wisselwerking tussen hierdie twee entiteite. Dit word beklemtoon deur die waarneming dat, ten spyte van groot verskille in die oënskynlike digthede van *G. austeni* en *G. brevipalpis*, die algehele trypanosomiase voorkomsyfers tussen die distrikte in die noordoostelike KwaZulu-Natal nie verskil het nie. Dit dui aan dat beide spesies 'n rol kan speel in die oordrag van die siekte en dus beheer sal moet word.

Die verspreiding van die tsetsevliegbevolking wat in die noordooste van KwaZulu-Natal voorkom, strek tot in Swaziland en die suide van Mosambiek. Morfometriese ontledings toon 'n afwesigheid van betekenisvolle grense aan en dat dat inteling tussen die verskillende KwaZulu-Natal bevolkings asook tussen die Suid-Afrikaanse bevolking en dié van die twee buurlande voorkom. Tsetsevlieg beheer in 'n gelokaliseerde afgebakende area sal dus onderworpe wees aan herbesmetting vanaf die onbeheerde aangrensende gebiede. 'n Area-wye benadering, dit wil sê teen die hele tsetsevliegbevolking van Suid-Afrika, Swaziland en die suide van Mosambiek sal dus noodsaaklik wees.

Die instandhouding van die kolonies van *G. brevipalpis* en *G. austeni* by die Landbounavorsingsraad-Onderstepoort Veeartsenykunde-Instituuut (LNR-OVI) vereis 'n bloedvooraad van hoë gehalte. Vir die moontlike opgradering van die huidige dieet, is 'n aantal antistolmiddels, voedingstimulante en bloedbronne geëvalueer deur produksie met 'n gestandardiseerde 30-dag biologiese keuringsproses te bepaal. Gedefibriniseerde beesbloed was die mees geskikste. Antistolmiddels soos natriumsitraat, 'n kombinasie van sitraat en natriumsuur, fosfaat-dekstrose-adenien en sitroensuur kan gebruik word om

bloed versameling te vergemaklik. Terwyl *G. brevipalpis* bees- bo varkbloed verkies, verkies *G. austeni* 'n mengsel van gelyke dele bees- en varkbloed. Die voedingstimulante adenosientrifosfaat, asook tri-fosfate van inosien, en die mono-fosfaat van guanosien en sitosien het verbeterde produksie in beide spesies tot gevolg gehad. Pogings om die plaaslike KwaZulu-Natal *G. brevipalpis* te koloniseer was onsuksesvol as gevolg van 'n onwilligheid van veldvlieë om op die kunsmatige voedingstelsel te voer.

As 'n voorvereiste vir SIT was die bestraling-sensitiwiteit van *G. brevipalpis* en *G. austeni* volwassenes en laat-stadium papies bepaal. 'n Dosis van 40 Gy het 'n 97% steriliteit in *G. brevipalpis* tot gevolg gehad wanneer laat-stadium papies bestraal is, en 80 Gy 'n 99% steriliteit as volwasse vlieë bestraal is. *Glossina austeni* het hoër dosisse vereis, 80 Gy en 100 Gy veroorsaak 'n hoër as 97% steriliteit in wyfies wat met mannetjies wat as volwassenes of laat-stadium papies bestraal is, gepaar het.

Gekoloniseerdes bestraalde mannetjies moet in staat wees om met met hul veld eweknieë te kan meeding. Die parings gedrag van gekoloniseerde *G. brevipalpis* en *G. austeni* was onder bykans natuurlike veldtoestande in instap-veldhokke bepaal. Alhoewel die tydsverloop voor paring het vir beide spesies korter was, was daar nie betekenisvolle verskille in hulle paring-prestasie soos in die oggend of middag bepaal nie. Vir beide spesies was die paring-frekwensie vir 9-dae-oue mannetjies aansienlik hoër as dié van 6 of 3-dae-oue mannetjies. Ouderdom het geen invloed op die vermoë van die mannetjies om sperm oor te dra nie of tydverloop voor paring gehad nie. Daar was geen beduidende verskil in paring-prestasie van steriele en vrugbare mannetjies nie.

Die huidige studie dui aan dat ATD en tsetsevlieë algemeen in KwaZulu-Natal voorkom en dat tsetsevlieg teenwoordigheid 'n dinamiese proses is wat deur omgewingsfaktore beïnvloed word. Die voorgestelde AW-IPB met 'n SIT komponent, alhoewel steeds van toepassing, sal aangepas moet word om die ogedateerde data te inkorporeer. Voorlopige resultate dui aan dat die kolonies by die LNR-OVI geskik sal wees vir gebruik in SIT.

Sleutelwoorde:
Glossina brevipalpis, Glossina austeni, verspreiding, trypanosomiase, morfometrie, kolonies, bestraling sensitiwiteit, paring-mededingendheid

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Chapter 1

Introduction

1.1   Literature review

1.1.1   The history of the tsetse fly and Trypanosomosis

Blood feeding habits of insects, and therefore the potential for pathogen transmission, evolved between 200 to 150 to million years ago (MYA) (Grimaldi & Engel, 2005; Mans, 2011). It has been estimated that Salivarian trypanosomes (including African trypanosomes) became established as gut parasites of some insects around 380 MYA (Steverding, 2008). Krafsur (2009) suggested that the tsetse fly origins predate continental separation in the cretaceous by more than 100 MYA. He based his findings on the discovery of a sister group of the modern tsetse fly in the Florissant shale of Colorado dating to 35 MYA (Grimaldi, 1992; Krafsur, 2009) as well as a *Glossina*-like fossil from the Oligocene strata in Germany (Grimaldi & Engel, 2005). These findings suggest a near worldwide distribution of tsetse flies 30 to 40 MYA and indicate that trypanosomes have been transmitted by tsetse flies to mammals for more than 35 million years.

Throughout history, from Ancient Egyptian times, through the Middle Ages up to early modern times diseases very similar to Human African Trypanosomosis (HAT) and African Animal Trypanosomosis (AAT) have been recorded (Steverding, 2008). The first medical report on HAT was published by John Aktins in 1734, but the nature of the illness was, however, still unknown (Cox, 2004). More than a century later, in 1852, David Livingston, after observing tsetse fly biting activity on cattle, suggested that the bites of these flies might be the cause of AAT (Steverding, 2008). It was, however only in 1895 that David Bruce showed that *Trypanosoma brucei* caused AAT. Six years later, in 1901, Robert Michael observed trypanosomes in human blood (Forde, 1902; cited in Steverding, 2008).

In 1903 David Bruce showed that tsetse flies were transmitting HAT. Although he initially believed that only mechanical transmission was involved, he changed his view when Friedrich Karl Klein demonstrated the cyclical transmission of *T. brucei* in tsetse flies (Steverding, 2008). Today, more than a 100 years after this crucial discovery, African trypanosomes still have a devastating effect on humans and animals in sub-Saharan Africa.

1.1.2   Economic impact of Trypanosomosis

Africa has a surface area of 30.2 million km^2 (including adjacent islands) covering 20.4% of the Earth's land area and 6% of its total surface, and is, after Asia, the second-largest continent (Sayre, 1999). With 1.1 billion inhabitants Africa is again, after Asia, the second-

most-populous continent. In 2013 its inhabitants accounted for about 15% of the world's human population (Gudmastad, 2013). Africa is the poorest and most underdeveloped continent and in 2005 it was estimated that 80.5% of the population in sub-Saharan Africa was living on an income of less than USD 2.50 a day (SESRIC, 2007). Sub-Saharan Africa is considered to be the least successful in reducing poverty. In 2005 half of Africa's population was living in poverty (<USD 1.25 per day). It has been estimated that the average poor person in sub-Saharan Africa survive on USD 0.70 per day, and that he or she was poorer in 2003 than in 1973 (Economic report on Africa, 2004). Although a number of factors may influence the development and economical growth in Africa, tsetse flies have been mentioned as one of the fundamental contributing detrimental factors (Feldmann *et al.*, 2005; Alsan, 2015).

Tsetse flies, considered as the sole cyclical vectors of African trypanosomes, infest about 10 million km² of sub-Saharan Africa (Leak, 1999; Rayaisse *et al.*, 2011). These trypanosomae parasites cause Human African Trypanosomosis (HAT), also known as sleeping sickness and African Animal Trypanosomosis (AAT) or nagana (Leak, 1999; Vreysen *et al.*, 2013).

Sleeping sickness occur in two forms, an acute form, caused by *Trypanosoma brucei rhodesiense*, mainly present in East Africa and a chronic form, caused by *Trypanosoma brucei gambiense,* in West Africa. Both forms are fatal if left untreated and have an impact of 1.59 M DALYs (disability adjusted life years) (Esterhuizen *et al.,* 2011). In sub-Saharan Africa, the disease is endemic in 36 of 48 countries with 60 million of 400 million inhabitants being at risk. In 1997 about 450 000 people were infected with sleeping sickness (Barrett, 2006). This number was reduced to 70 000 cases per year in 2000 (Simarro, 2006; Aksoy, 2011). Since 2000, as the result of intensified surveillance and treatment campaigns in combination with vector control (Courtin *et al.,* 2015; Tirados *et al.,* 2015), the number of cases have declined by 73% (WHO, 2014).

African Animal Trypanosomosis (AAT) is considered by many agricultural and veterinary economists as the single greatest constraint to increased livestock production in sub-Saharan Africa (Vreysen *et al.*, 2013). The direct annual production losses in cattle are estimated between USD 600 and 1200 million (Hursey & Slingenbergh, 1995; Vreysen *et al.*, 2013). The overall annual lost potential in livestock and crop production can be as high as USD 4750 million (Budd, 1999; Vreysen *et al.*, 2013). A second important consideration is that tsetse flies prevent the integration of crop farming and livestock keeping, which is crucial to the development of sustainable agricultural systems (Feldmann & Hendrichs, 1995; Vreysen *et al.*, 2013).

Human African Trypanosomosis (HAT) is absent in South Africa and AAT is restricted to the uMkhanyakude District Municipality in the north east of the KwaZulu-Natal Province. This is a rural district with approximately 573 000 (Statistics South Africa, 2016) inhabitants and is considered to be one of the most deprived districts in South Africa. Unemployment rates are high, access to piped water and electricity low. The area is characterised by many female-headed households with high numbers of children and low education levels. The majority of the farmers are communal farmers that follow a free roaming grazing practice. It is estimated that 350 000 cattle are at risk of AAT in the area (Kappmeier *et al.,*1998; Kappmeier Green *et al.*, 2007).

1.1.3   Tsetse fly systematics, distribution and biology

Tsetse flies are Diptera classed in the family Glossinidae, which consists of only one genus *i.e. Glossina* Wiedemann 1830. The genus is divided into three subgenera namely *Austenina, Nemorhina* and *Glossina* that correspond to the *Fusca, Palpalis* and *Morsitans* species groups respectively (Leak, 1999; Krafsur, 2009). Based on habitat preferences these groups are also referred to as Forest (*Fusca*)*,* Riverine (*Palpalis*) and Savannah (*Morsitans*) flies. There are 31 recognised species and subspecies at present (Leak, 1999). Although the family is considered to be restricted to Africa (Moloo, 1993) (Fig. 1.1), they have recently been recorded in south-west Arabia (Elsen *et al.*, 1990) as well as in Gizar in Saudi Arabia (Phelps & Lovemore, 2004).

Tsetse flies' feeding behaviour and reproductive biology make them not only unusual but also highly successful (Krafsur, 2009). Differing from most insects that have a r-reproductive strategy, tsetse flies are typical K-strategists. They are obligatorily haematophagous and both males and females feed on blood. They reproduce by adenotrophic viviparity, and one larva at a time is being nourished in utero by a secretion from the uterine gland (Saunders & Dodd, 1972; Tobe & Langley, 1978; Benoit *et al.,* 2015). The adult and larval stages are depended on the same source of food *i.e.* vertebrate blood.

There are three instars while the larva matures in the female fly. After 7 to 12 days, depending on environmental temperatures, a mature larva is deposited in the soil were it pupates within approximately four hours. Adult development approximately takes between 27 to 40 days and is temperature dependant. Females are inseminated within their first week of adulthood and can larviposite their first larvae at the earliest, 16 days after emergence. The minimum time needed to produce two offspring is about 25 days, generation time is *c.* 43 days at 25 °C (Krafsur, 2009). Characteristically for K-strategists the high adult survival rate, which typically exceeds 97% per day (Rogers & Radolph, 1984a, b, 1985), compensates for their slow reproduction rate.
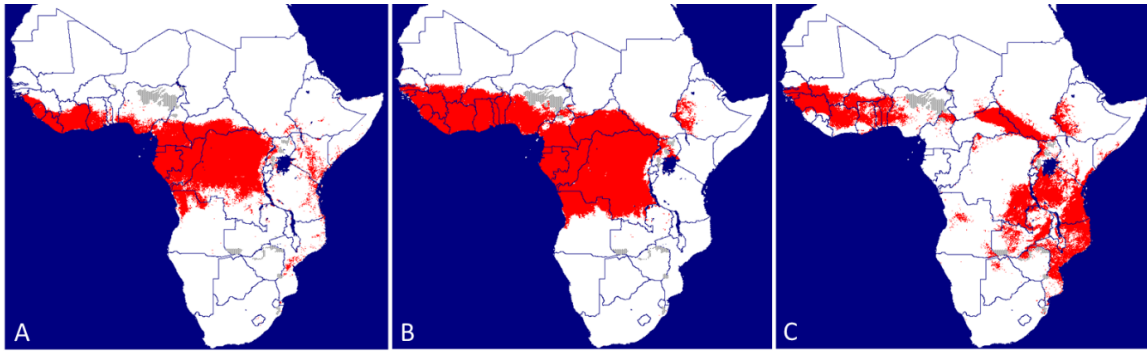
Fig. 1.1. Predicted distribution (in red) of *Fusca* (*Austenina*) (A)*, Palpalis* (*Nemorhina*) (B) and *Morsitans* (*Glossina*) (C) species groups in Africa (Wint & Rogers, 2000).
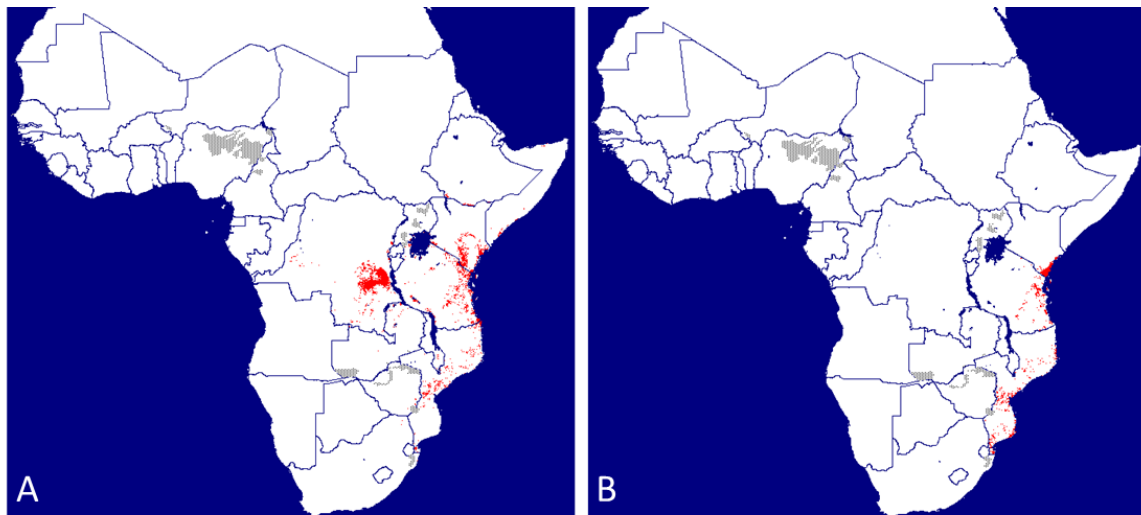
Fig.1.2. Predicted distribution (in red) of *Glossina brevipalpis* (A) and *Glossina austeni* (B) in Africa (Wint & Rogers, 2000).

1.1.4 African Trypanosomosis management

There are several tools available in the African Trypanosomosis management toolbox. Control can be focused on the *Trypanosome* parasite or on the tsetse fly vector or on a combination of both parasite and vector. More options are available for vector control than for the control of the *Trypanosome* parasite. If the most applicable strategy for a specific area or situation is not selected the effective management of Trypanosomosis will remain problematic.

1.1.4.1 Disease control

There is no available preventive vaccine against African Trypanosomosis and treatment depends on continuous dosage with trypanocidal drugs. Drugs used for nagana control

include therapeutic Diminazene (Berenil®) or the prophylactic Isometamidium (Samorin® and Trypamidium®) (Kappmeier Green, 2002). Ethidium bromide (Homidium®) has also been commonly used since the 1950s to treat Trypanosomosis in cattle. The continuous use of these drugs can increase the risk of drug management errors, such as under dosing or excessive use, that can lead to the development of trypanosome resistance. Drug resistance has been reported in 11 of the 36 AAT endemic countries (Leak, 1999).

Another means of disease control is through the promotion of trypanotolerant livestock in tsetse fly infested areas. These trypanotolerant breeds can to a certain degree control the intensity, prevalence and duration of parasitism and thereby limiting the pathological effect (Murray *et al.,* 1982). A shortcoming of this approach is that trypanotolerance is an innate characteristic under genetic control. A limiting factor is that trypanotolerant livestock only comprise approximately 5% of the current cattle population in Africa (Dolan, 1998). These breeds are usually smaller animals that produce less milk and meat and have limited draught power.

1.1.4.2   Vector control

Because of the limited options for controlling the parasite, *e.g.* no vaccines and drug resistance, vector control remains to date the most effective and economical means of Trypanosomosis management. A number of measures, which are continuously being improved, are available for the control of tsetse flies.

▪ **Earlier control**
  o **Bush clearing**

The removal or alteration of suitable tsetse fly habitat is one of the oldest forms of tsetse fly control (Du Toit, 1954). Discriminative bush clearing has been used successfully in West and East Africa (Leak, 1999). This method has however, become environmentally unacceptable and is no longer practised. The expansion of the human populations had a very similar effect in many instances. It is evident in rural Africa, and even in South Africa, that due to the ever increasing need for agricultural land, more and more of the tsetse fly habitat outside of protected areas is becoming unsuitable for tsetse fly survival (Leak, 1999).

  o **Host animal reduction**

The culling of wild animals in South Africa from 1872 to 1888 resulted in a gradual disappearance of tsetse flies from two-thirds of the infested area. This epitomized the close relationship between tsetse fly presence and densities of wild animal populations. Similarly,

in 1897 a severe rinderpest pandemic in southern Africa decimated large numbers of animals in the north eastern parts of South Africa which led to the disappearance of *Glossina morsitans morsitans* from the area until today (Rossiter, 2004). The decisive elimination of wild animals for the control of tsetse fly started in 1914 (Jack, 1914; Leak, 1999). This method was used in many southern African countries (Leak, 1999) and between 1946 and 1950, more than 138 000 wild animals were culled in Zululand (north eastern KwaZulu-Natal) alone as part of a tsetse fly control programme (Du Toit, 1954). This practise has not only become environmentally unacceptable, but tsetse fly feeding habits that differ between tsetse groups can shift from wild to domestic animals and the removal of all wild potential tsetse hosts can be challenging as they can be elusive and difficult to eliminate (Leak, 1999).

- **Chemical control**

The chemical warfare on tsetse flies started in 1945 when DDT (1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane) became available. Compounds that have commonly been used for tsetse fly control are organochlorines (*e.g.* DDT, dieldrin and endosulfan), pyrethroids (*e.g.* deltamethrin, alpha-cypermethrin, natural pyrethrum) and avermectins (ivermectin) (Leak, 1999). Insecticide spraying can be divided into ground and aerial spraying. Ground spraying with residual insecticides was the principal method used from 1950 to the 1970's (Leak, 1999). The chemicals were, depending on the selectiveness and accessibility of the area, dispensed to tsetse fly resting sites from knapsacks, mist blowers and unimogs. Aerial spraying was developed for a more effective control of tsetse flies over larger areas using fixed-wing aircraft and helicopters. In many instances, a combination of ground and aerial spraying was used to apply residual as well as non-residual insecticides (Du Toit, 1954; Hursey & Allsopp, 1984).

The campaign to eradicate *G. pallidipes* from Zululand between 1945 and 1952, was the first successful widespread use of insecticides. Novel synthetic insecticides such as DDT and HCH (hexachlorobenzene) were used, in residual aerial spraying campaigns together with trapping and bush clearing resulting in a zone free of *G. pallidipes* in north eastern KwaZulu-Natal (Du Toit & Kluge, 1949; Du Toit, 1954).

Glossina m. centralis was eradicated from the Okavango Delta and Kwando-Linyanti system in Botswana using aerial spraying of non-residual doses of deltamethrin (Kgori *et al.,* 2006). Two successive spraying operations, with deltamethrin applied at night using turbo thrust fixed-wing aircraft, were implemented in the Okavango Delta in 2001 and 2002 (Allsopp & Phillemon-Motsu, 2002; Kgori *et al.,* 2006; 2009). A target barrier was erected between successive operations to prevent fly reinvasion into the cleared areas. In 2006 the

Kwando-Linanti systems were similarly treated (Kurugundla, 2012). As insecticides are deemed to be harmful to the environment an impact assessment study was conducted in the Okavango Delta. However, according to this study the non-residual insecticide aerial spraying did not inflict serious harm to terrestrial or aquatic invertebrates (Kurugundla *et al.,* 2012). This campaign indicated that it is possible to eliminate *G. m. centralis* over relatively large areas. The sequential aerosol technique is, however, not always suitable for eradication of a tsetse fly population as was demonstrated in Ghana for the riverine tsetse species (Adam *et al.*, 2013).

▪ **Bait technologies**

Instead of the large scale indiscriminative treatment of an area, insecticides can be used with selective methods such as traps, targets and animals. Bait technology combines visual and olfactory cues to attract, capture or kill tsetse flies (Vale, 1974; 1993). Tsetse flies are attracted to the blue/black colour combination of the trap, or animal, and when the fly alights on the treated cloth or animal, it absorbs the insecticide through its tarsi and dies. This strategy has been used in the past 30 years in many tsetse fly control campaigns, either on its own or as part of integrated pest management strategies. Although bait technologies can be labour intensive it is relatively cheap and simple to implement and is probably most useful as a long term suppression strategy.

Much time and efforts have been invested in the development and refinement of the tsetse fly traps and targets (Lindh *et al.,* 2009) and various designs are available for specific tsetse species. Kuzoe & Schofielld (2005) provide a comprehensive review on advances in this field and emphasise that traps and targets are species specific and that all traps are not equally effective for all species. Various traps and targets are available to be used against specific species in specific environments (Kuzoe & Schofielld, 2005). It is therefore important that the most appropriate trap or target be selected for monitoring or control.

Glossina austeni and *Glossina brevipalpis* exist sympatrically in certain areas in South Africa. These two species are not equally attracted to the H trap or the sticky XT trap (cross-shaped targets) (Kappmeier, 2000). Trapping efficiency can be improved by baiting the traps with odours. While this seems effective for *G. brevipalpis* it has no effect on *G. austeni* (Kappmeier & Nevill, 1999a). Esterhuizen *et al.* (2006) indicated differences in the effectiveness of odour-baited insecticide-treated targets (Kappmeier & Nevill, 1999b). While targets deployed at a density of eight per km² can suppress *G. austeni* this density will be ineffective for the control of *G. brevipalpis* (Esterhuizen *et al.,* 2006). However, for the suppression of the same species, *G. austeni,* on Unguja Island, Zanzibar a much higher density of 50 targets per km² was needed (Vreysen *et al.*, 2000).

Current vector control in HAT endemic areas is mainly with the newly developed tiny targets (Rayaisse *et al.*, 2011; Torr *et al.,* 2011). These tiny targets, based on the same principles as the large savannah-type cloth targets, are eight times smaller (0.25 m x 0.5 m) than the traditional targets (1 m x 1.5 m), and apparently have the same killing efficiency for *Glossina fuscipes* as the bigger ones, however, this point has been much debated (Bouyer *et al.*, 2013a). Less cloth is required to make the tiny targets and less insecticide is needed for impregnation, making these targets more economical and easier to deploy over large areas in tropical Africa (Esterhuizen *et al.,* 2006; Shaw *et al.,* 2015).

The live-bait technology involves the application of insecticides on domestic animals through dipping, spraying or pour-ons. This will only be effective if a large proportion of the tsetse fly population is feeding on domestic rather than wild animals (Leak, 1999). The existing extensive dipping network in KwaZulu-Natal that was established mainly for tick control was used in combination with insecticide-treated targets and animal drug treatment to control an outbreak of nagana in 1990 (Bagnall, 1993; Kappmeier *et al.,* 1998). A disadvantage of using live animals as baits is that it may not prevent initial trypanosome transmission, as the flies can feed and potentially transmit the parasite before dying.

A method that can be used to reduce cost in live-bait technology is the restricted application of insecticides to only those areas on the animal where the majority of the tsetse flies land and feed (Vale, 2003; Esterhuizen, 2007). In terms of cost saving, restricted application of insecticides can achieve a reduction of up to 80% in chemical usage (Vale, 2003). This technique was shown to be promising for control in communal farming areas where livestock constitutes a major source of food for the tsetse fly population (Bouyer *et al.*, 2007; Torr *et al.,* 2007; Bouyer *et al.*, 2009; 2011). Continuous research is needed to improve insecticide applications on animals (Ndeledje *et al.*, 2013; Muhanguzi *et al.*, 2014) and to reduce these disadvantages.

- **Biological control**

A range of natural enemies of tsetse flies have been recorded, *e.g.* hymenopteran and dipteran parasites (Fiedler, 1954; Fiedler *et al.,* 1954; Leak, 1999), parasitic mites, helminth parasites (Poinar *et al.*, 1981) and fungi (Kaaya, 1989). Although some hymenopteran and dipteran parasites and fungi had a marginal detrimental effect on tsetse fly populations (Leak, 1999) they were not as successful as anticipated in reducing them.

- **Insect growth regulators and juvenile hormone**

Both these techniques exploit as a weakness, the complex and low reproductive potential of tsetse flies (Leak, 1999). Insect growth regulators interfere with chitin synthesis thereby

preventing successful reproduction, whereas juvenile hormones disrupt the reproductive cycle resulting in abortions; both methods essentially render the females sterile (Leak, 1999). Laboratory experiments showed that the growth regulator difubenzuron (DFB; Dimilin) was effective for *G. m. morsitans* (Jordan *et al.*, 1979). A limitation of the method is the need to apply sufficient quantities of the chemicals to sterilize flies throughout their reproductive lives (Leak, 1999).

The field effectiveness of the juvenile hormone pyripoxyfen was proven (Hargrove & Langley, 1993). A potential disadvantage of these techniques is that they have a delayed effect on the population as these do not kill the fly immediately. This delayed effect can, however, also be seen as an advantage, since the fly itself will transmit the bio agent within the population thereby amplifying the impact of the control (Bouyer & Lefrançois, 2014).

- **Genetic control methods**
  o **Symbiont-based methods**

Sophisticated symbiotic associations between tsetse flies and at least three endosymbiotic bacteria*, Wigglesworthia, Sodalis* and *Wolbachia* have been documented (Doudoumis *et al.*, 2013; Snyder & Rio, 2013; Bourtzis *et al.,* 2016). The presence of different strains of *Wolbachia* in different tsetse fly populations may lead to incompatible genetic crossings between these groups resulting in embryonic death of the fly. This cytoplasmic incompatibility (CI) (Bourtzis, 2007) can thus be used to control tsetse flies (Alam *et al.,* 2011). *Wolbachia*-induced CI is known as the incompatible insect technique (IIT) and it has been suggested for use in parallel with the sterile insect technique (SIT) or alone, as a driving system to replace the population with a strain that has a desirable genotype (e.g. resistant to trypanosome infections) (Bourtzis, 2007; McGraw & O'Neill, 2013; Bourtzis *et al.*, 2016). Investigations into the use of the genetically modified symbiont *Sodalis* so that they carry a gene that expresses anti-trypanosomal nanobodies, and as making the tsetse fly refractory to trypanosome infections are underway (De Vooght *et al.*, 2014; Bourtzis *et al.*, 2016), tsetse flies that harbour the recombinant *Sodalis* can vertically transmit them to their offspring and this will enable the spread of vector incompetence within the tsetse fly population. *Wigglesworthia glossinidia* is critical for tsetse fly reproduction and plays a role in progeny development (Benoit *et al.,* 2015). When *Wigglesworthia* are absent in tsetse flies their progeny is immunocompromised and infertile (Benoit *et al.,* 2015) and this might lead to newly developed control methods.

o **Sterile insect technique**

Although the sterilising effect of radiation on insects was already known in the 1930s (Runner, 1916; Muller, 1927), it was only 20 years later in the 1950s that the potential of this technique for the control of insects was realised (Baumhover, 2001; 2002). The sterile insect technique (SIT) involves the sterilisation with radiation of males from the mass-rearing of the target species (Robinson, 2005) followed by the release of these males in sufficient numbers to outcompete their wild counterparts (Klassen & Curtis, 2005; Bourtzis *et al.,* 2016). The mating of these sterile males with wild fertile females results in no progeny, which leads to a population reduction and in some cases the eradication of the target population (Bourtzis *et al.,* 2016).

For maximum effectiveness the sterile males must outnumber the fertile males and the SIT may therefore be less cost-effective if the target population is large. Many conventional control methods (*e.g.* insecticide spraying) are both cost and operationally effective when the target population is high. The application of conventional control methods followed by SIT may lead to eradication (Fig. 1.3) (Feldmann & Hendrichs, 2001). In contrast to conventional control tactics, such as insecticide spraying that are very effective at high insect population densities but less effective at low population densities, the SIT will become more effective as the targeted insect population decreases (Fig. 1.3).
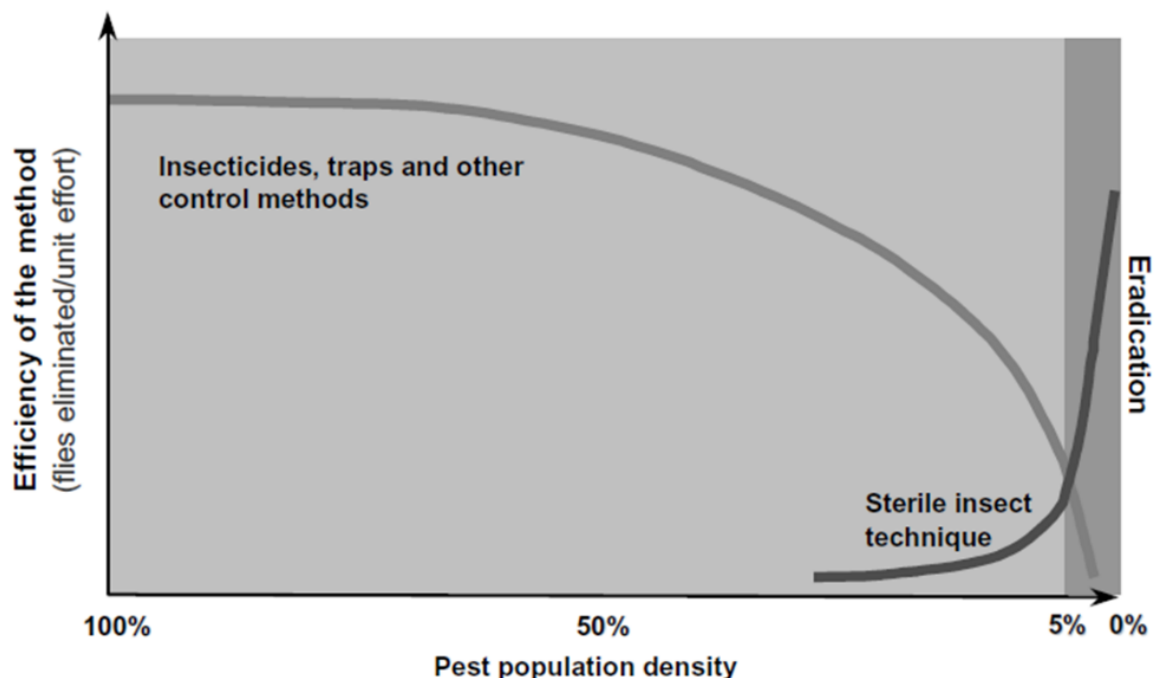
Fig.1.3. The efficiency of a conventional control approaches in combination with the sterile insect technique in relation to target population densities (Feldmann & Hendrichs, 2001).

The slow reproduction rate in tsetse flies will contribute to the susses of the SIT. The feasibility of the SIT for the eradication of entire populations has been demonstrated on several occasions (Vreysen *et al.,* 2000; Feldmann *et al.,* 2005; Vreysen *et al.,* 2013). The elimination of *G. austeni* from the island of Unguja, Zanzibar, is probably the most remarkable. The last *G. austeni* individual was collected in September 1996, and the last *T. vivax* recorded in 1998 (Dyck *et al.,* 2000). The SIT is species-specific and is considered an environmentally friendly control method by most authors (Knipling, 1959, sited in Bourtzis *et al.,* 2016).

1.2   Study justification

After an epidemiological silence of nearly 33 years, a severe outbreak of nagana in north eastern KwaZulu-Natal in 1990, showed the devastating socio-economic impact of tsetse-transmitted nagana on these largely rural communities of South Africa (Bagnall, 1993). These outbreaks led to the re-establishment of tsetse flies and trypanosome research in South Africa.

Surveys conducted between 1993 and 1999, indicated two species, *G. brevipalpis* and *G. austeni,* to be present in an area of 16 000 km^2 in the north eastern part of the KwaZulu-Natal Province (Kappmeier Green, 2002). The infested area stretched from St Lucia (-28.499639, 32.395194) in the south to the border of Mozambique (-26.8692, 32.8342) in the north and from the coast in the east up to the Hluhluwe-Imfolozi Game Reserve (-28.33416, 31.691222) in the west (Kappmeier Green, 2002).

The *G. brevipalpis* belt in Africa starts in Ethiopia in East Africa from where it extends southwards to Somalia, Uganda, Kenya, Rwanda, Burundi and Tanzania (Fig. 1.2 A). In southern Africa *G. brevipalpis* is present in Malawi, Zambia, Zimbabwe and the northern and central part of Mozambique (Moloo, 1993) (Fig. 1.2 A). *Glossina austeni* is found, in lower numbers than *G. brevipalpis* in East Africa from Somalia in the north, extending south into Kenya, Tanzania, Zimbabwe and the northern and central parts of Mozambique (Fig. 1.2 B) (Moloo, 1993).

These South African tsetse fly populations, extending to southern Mozambique (both species) and Swaziland (*G. austeni*) represent their most southern distribution (Saini & Simarro, 2008; Sigauque *et al.,* 2000) they are expected to be geographically isolated from the main tsetse fly belt.

Initial studies facilitated the development of an area-wide integrated pest management (AW-IPM) strategy that include a sterile insect technique (SIT) component to establish a tsetse fly free South Africa (Kappmeier Green *et al.*, 2007). Using the tsetse fly presence and abundance data, as determined with odour baited XT sticky traps, this AW-

IPM strategy suggested the division of the infested area into four zones from south to north with the successive implementation of four phases (pre-suppression, suppression, SIT and post-eradication) in each zone following the rolling carpet principle (Kappmeier Green *et al.*, 2007).

Determining the accurate geographic distribution and abundance of the tsetse fly population to be eradicated will be vital for the success of not only the strategy as proposed by Kappmeier Green *et al.* (2007) but for any proposed control effort. The area that needs to be treated will directly affect the outcome, sustainability and cost of any proposed control campaign.

A basic requirement for determining the accurate distribution of a population is the availability of efficient sampling devices. The development of the H trap and its accompanying artificial odour system, which was shown to be more effective than the odour baited XT sticky traps for the sampling of *G. brevipalpis* and *G. austeni* (Kappmeier & Nevill, 1999a; Kappmeier, 2000), necessitated a re-assessment of the tsetse fly distribution in the area. The initial surveys were conducted between 1993 and 1999 and the available data may not be a true reflection of the current situation. A "probability of presence" model (Hendrickx, 2002; Hendrickx *et al.,* 2003) predicts a wider geographic distribution range for both *G. brevipalpis* and *G. austeni* than had been indicated by the 1993 to 1999 survey data (Hendrickx, 2002) and this needs to be validated.

The degree of geographic isolation of a population targeted for control will determine to what extent reinvasion form neighbouring populations play a role in the success of any proposed control programme. Kappmeier Green (2002) stated that the tsetse fly infected area could be divided into four zones. In Zone I, in the south, only *G. brevipalpis* were present, in zone II and III mainly *G. austeni* were present while zone IV contained both species. This apparent fragmentation suggested that these populations might be geographical isolated from each other and that reinvasion from neighbouring areas may be minimal. Before designing an appropriate control strategy for the area it needs to be determine if the South African populations are geographically isolated from those in southern Mozambique and Swaziland.

As part of the proposed strategy for an area-wide control campaign with a SIT component, laboratory colonies of both South African tsetse species were established at the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI), Pretoria, in 2002. These colonies of *G. brevipalpis* and *G. austeni* were respectively established using seed material from the Tsetse and Trypanosomiasis Research Institute (TTRI) (now named Vector & Vector-Borne Diseases Research Institute) Tanga, Tanzania and the Entomology Unit of the Food and Agriculture Organization (FAO)/International Atomic

Energy Agency (IAEA) Laboratories in Seibersdorf, Austria (now called the FAO/IAEA Insect Pest Control Laboratory). The development of tsetse fly rearing capabilities, with the capacity to produce high quality males that would be comparable and competitive with their wild counterparts will be essential in the SIT component of an area-wide control campaign (Parker, 2005). These colonies need to be maintained at sustainable and economical acceptable levels.

The SIT involves the sterilisation of males with radiation obtained from a mass-rearing facility of the target species (Bakri *et al.*, 2005; Robinson, 2005). It is essential to determine the optimal radiation dose to induce the necessary level of sterility in the colonised males. A too low dose may lead to the release of fertile males in the area while a too high dose will negatively impact on their competitiveness (Bakri *et al.*, 2005). The optimal age of the adult flies or the pupa for irradiation needs to be determined as well as any detrimental effect of radiation on the fitness and mating capability of these males. The sterilised males must be able to outcompete their fertile counterparts (Parker, 2005; Vreysen *et al.*, 2011).

1.3 Aim

The main aim of this study was to contribute towards the development of the proposed AW-IPM strategy. The more specific aims were (1) to determine the mating behaviour and performance of colonised irradiated *G. brevipalpis* and *G. austeni* flies in relation to biological and operational attributes for use in SIT, (2) to update the maps of tsetse fly abundance and trypanosome prevalence, and (3) to assess the degree of isolation between the different populations of the two species.

1.4 Objectives

To achieve these aims, the following objectives were defined.

- The existing distribution maps of tsetse fly abundance and Trypanosomosis prevalence in South Africa were updated. The distribution and abundance of tsetse flies were discussed in terms of environmental factors such as vegetation and climate. Lastly the correlation between tsetse fly apparent densities and infection prevalence of the disease in livestock was investigated.
- The applicabilty geometric morphometrics to determine the extent of potential genetic isolation between the various populations of *G. brevipalpis* and *G. austeni* present in South Africa were assessed. Subsequently the South African populations were compared with flies collected in neighbouring southern Mozambique and Swaziland.

These three wild populations was also be compared with laboratory colonies maintained at the ARC-OVI. Geometric morphometrics was used to assess seasonal and sexual dimorphism differences.

▪ To optimise the blood diet provided to the colonies maintained at the ARC-OVI and to accecc the effect of anticoagulants, phagostimulants and hosts on the nutritional value of the blood. Attempts was made to establish a KwaZulu-Natal strain colony of *G. brevipalpis.*

▪ The radiation dose needed to sterilise *G. brevipalpis* and *G. austeni* adults and pupae was determined.

▪ The mating behaviour and performance of colonised *G. brevipalpis* and *G. austeni* under near natural conditions using walk-in field cages were assessed.

These results will contribute to the understanding of the ecology and behaviour of *G. brevipalpis* and *G. austeni* not only in South Africa but in all areas in Africa where these two species are found. Information generated in this study will enable us to refine the proposed control strategy and determine if southern Mozambique and Swaziland need to be included in the proposed strategy. As very little research is conducted elsewhere on these two species specifically, the data generated can be applied to all areas where *G. brevipalpis* and *G. austeni* are present. It must be emphasised that in Africa, a sustainable alleviation or if possible the removal of HAT and AAT should be the aim irrespectively of the control method.

Chapter 2

Tsetse fly distribution and Animal Trypanosomosis prevalence[1]

2.1   Introduction

The discovery that *Trypanosoma brucei* was the cause of African animal Trypanosomosis (AAT), also known as nagana, can be dated back more than a century when it was recorded for the first time in north eastern KwaZulu-Natal (formerly Zululand), South Africa, in the 1880's (Bruce, 1895; Bagnall, 1993; Steverding, 2008). In 1895, Sir David Bruce stated that game animals were the reservoir hosts of the causative trypanosome species and that these protozoan parasites were transmitted between their mammalian hosts by tsetse flies (Diptera: Glossinidae) (Bruce, 1895). Of the four species, *Glossina morsitans morsitans, Glossina pallidipes, Glossina brevipalpis,* and *Glossina austeni* (*Glossina brandoni*) that have historically been recorded in South Africa, *G. m. morsitans* was the only one that was encountered in the most northern part of the country (Fuller, 1923). *Glossina brandoni*, which has its type locality in north eastern KwaZulu-Natal, is currently considered as a synonym of *G. austeni* (Phelps & Lovemore, 2004)*.* The other three species, *i.e.*, *G. pallidipes*, *G. brevipalpis* and *G. austeni* were restricted to north eastern KwaZulu-Natal Province in South Africa (Fig. 2.1) (Fuller, 1923). *Glossina pallidipes* was the predominant species. Based on its abundance, it was considered the most important vector of AAT at that time (Fuller & Mossop, 1929). *Glossina brevipalpis* and *G. austeni,* which were confined to smaller areas with mostly dense vegetation, were not considered important vectors of AAT. The sheer abundance of *G. pallidipes* was illustrated by the large numbers of flies being trapped on certain occasions, *i.e.* in 1932, two million flies were collected within a month after deployment of 1000 Harris traps in north eastern KwaZulu-Natal and nearly eight million flies over that entire year (Harris, 1932). The presence of large numbers of tsetse flies, and especially *G. pallidipes,* in north eastern KwaZulu-Natal resulted in severe outbreaks of nagana between 1942 and 1946 (Du Toit, 1954). This led to an insecticide-based spraying programme with DDT and HCH and by 1953 *G. pallidipes* was eradicated from KwaZulu-Natal (Du Toit, 1954).

The programme succeeded because it integrated several control tactics (aerial and ground spraying, trapping and bush clearing) and proved to be sustainable as the entire

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[1] Partially published as:
De Beer, C.J., Venter, G.J., Kappmeier Green, K., Esterhuizen, J., De Klerk, D.G., Ntshangase, J., Vreysen, M.J.B., Pienaar, R., Motloang, M., Ntantiso, L. & Latif, A.A. (2016) An update of the tsetse fly (Diptera: Glossinidae) distribution and African animal trypanosomosis prevalence in north-eastern KwaZulu-Natal, South Africa. *Onderstepoort Journal of Veterinary Research*, **83 (1),** a1172.

population was targeted and KwaZulu-Natal is today still free of *G. pallidipes*. This represents one of the first tsetse fly control programmes that implemented area-wide integrated pest management (AW-IPM) principles successfully (Klassen, 2005; Vreysen *et al.*, 2007). An additional benefit from this campaign was the apparent removal of *G. brevipalpis* from the Hluhluwe-iMfolozi Park (Du Toit, 1954).
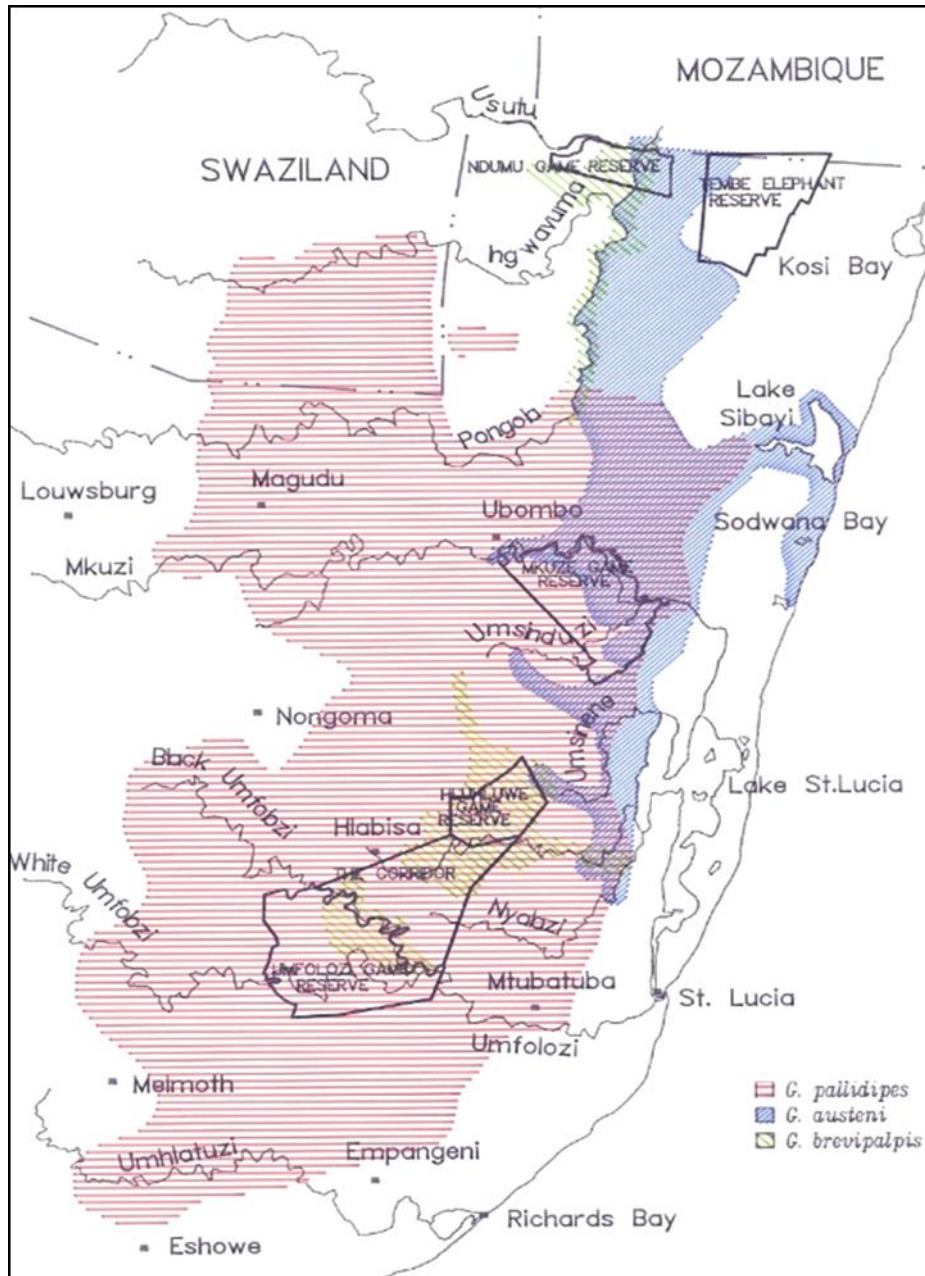
Fig. 2.1. The distribution of *Glossina pallidipes, Glossina brevipalpis* and *Glossina austeni* in KwaZulu-Natal prior to 1953 (Du Toit, 1954).

In areas where *G. brevipalpis* and *G. austeni* were found in the absence of *G. pallidipes* (Fig. 2.1)*,* no control was implemented, and consequently, these two species

remained present in north eastern KwaZulu-Natal (Kappmeier *et al.,* 1998). From 1955 onwards, only sporadic cases of nagana were recorded there (Bagnall, 1993) and it was assumed that agricultural developmental changes such as the establishment of commercial pine and eucalyptus plantations and bush clearing for livestock production had rendered this area unsuitable for tsetse flies (Kappmeier *et al.,*1998).

In 1990, a severe outbreak of nagana once again occurred in north eastern KwaZulu-Natal and cattle mortalities during this outbreak were exacerbated by the co-occurrence of a severe drought (Kappmeier *et al.,*1998; Emslie, 2005). Emergency control measures, utilising the extensive dipping network used for tick control, were implemented (Kappmeier *et al.,*1998). As part of the control measures the active ingredient of the routinely used dipping agent, was changed from amitraz, an acaricide, to the pyrethroid cyhalothrin, a wider spectrum insecticide (Hall & Fischer, 1984) for two years (Bagnall, 1993). Whereas the pyrethroid cyhalothrin is very effective at controlling both ticks and tsetse flies (Bagnall, 1993), the efficacy of amitraz is limited to ticks and is considered ineffective against dipteran flies (Hall & Fischer, 1984). The adopted cattle-dipping regime in combination with animal treatments using trypanocidal drugs brought the disease outbreak under control (Kappmeier *et al.*, 1998).

The 1990 outbreaks illustrated the fact that nagana had remained a serious debilitating problem in north eastern KwaZulu-Natal, with mixed infections of *Trypanosoma congolense* and *Trypanosoma vivax* (Bagnall, 1993). Periodic screening of cattle at dip tanks in the tsetse-infested area indicated that *T. congolense* was the dominant species (Van den Bossche *et al.,* 2006; Mamabolo *et al.,* 2009; Motloang *et al.,* 2012; 2014) with *T. vivax* being less abundant (Mamabolo *et al.,* 2009). Recently *Trypanosoma theileri* and *T. brucei* were detected based on the alignment of 18S rRNA gene sequences which were compared to trypanosome sequences available at the National Centre for Biotechnology Information (NCBI) (Taioe, 2014). Two sub-genotypes of *T. congolense,* the Savannah and Kilifi types, and mixed infections (Gillingwater *et al.*, 2010) of these were identified with PCR in the area (Mamabolo *et al.,* 2009). These surveys showed that trypanosome infections in cattle at dip tanks close to the Hluhluwe-iMfolozi Park were higher than at dip tanks further away from this Game Reserve (Van den Bossche *et al.,* 2006; Motloang *et al.,* 2014; Ntantiso *et al.,* 2014).

Long-term management of nagana by dipping and/or curative treatment of infected cattle with trypanocidal drugs is neither cost-effective nor sustainable (Bagnall, 1994; Shaw, 2009) and it became evident that a long-term solution to the nagana problem in South Africa needed to be investigated. Initial surveys confirmed the absence of *G. pallidipes* and

showed that *G. brevipalpis* and *G. austeni* were still the only species present in KwaZulu-Natal (Kappmeier Green, 2002).

Between 1993 and 1999, an extensive tsetse fly survey was conducted in an area of approximately 12 000 km^2 using odour baited sticky XT traps (cross-shaped targets) to assess the distribution of each species (Kappmeier & Nevill, 1999a; Kappmeier Green & Venter, 2007) (Fig. 2.2). This survey indicated that *G. brevipalpis* was present in a southern and a northern band, commonly associated with game reserves and other protected areas (Fig. 2.2). The distribution of *G. austeni* was continuous from south to north but did not extend as far west as that of *G. brevipalpis. Glossina austeni* was also very common in communal farming areas (Fig. 2.2) (Nevill, 1993; Kappmeier Green, 2002). The 1993-1999 survey did not establish the southernmost distribution limit of *G. brevipalpis* and *G. austeni*, as the sampling frame was only designed to determine broad distribution limits. It was, however, suggested that the southernmost limit of the tsetse fly distribution in South Africa, and therefore Africa, could roughly be regarded as the southernmost extent of the Umfolozi River (Kappmeier Green, 2002). The 1993-1999, surveys also showed that *G. brevipalpis* and to a lesser extent *G. austeni*, were present close to the coast (Fig. 2.2) in areas not sampled in the 1950's (Du Toit, 1954).

The 1993 to 1999 sticky trap data were used to develop a probability of presence model (Hendrickx, 2002; Hendrickx *et al.,* 2003). This model, based on environmental and climate variables, predicted a wider geographical distribution range for both *G. brevipalpis* and *G. austeni* than what was indicated by the survey data (Hendrickx, 2002). However, Hendrickx *et al.* (2003) suggested that the model may have overestimated tsetse fly distribution.

Determination of the accurate distribution of the target insect, as well as the interaction between the insect and the causative agent it transmits, is vital for the successful implementation of any proposed control campaign, and will directly affect its outcome, sustainability and cost (Vreysen *et al.,* 2007; Shaw, 2009). Additional ongoing efforts to generate continental as well as national Atlases of tsetse flies and AAT (Cecchi *et al.,* 2014; 2015) will benefit from continuously updated data sets.

The development of an improved trap for *G. brevipalpis* and *G. austeni* (Kappmeier, 2000) and an enhanced artificial odour system for *G. brevipalpis* (Kappmeier & Nevill, 1999a), allowed a more effective sampling of both species in South Africa.

Although the 1993 to 1999 surveys of Kappmeier Green *et al.* (2007) provided accurate data on the distribution and abundance of the two fly species, these maps may be outdated. Additionally the model presented by Hendrickx (2002), predicting a wider distribution range for both species, reinforced the need to verify the distribution and validate

the model. An update of the geographical distribution and abundance of tsetse flies as well as Trypanosomosis prevalence in KwaZulu-Natal was considered a prerequisite for future planning of control efforts.
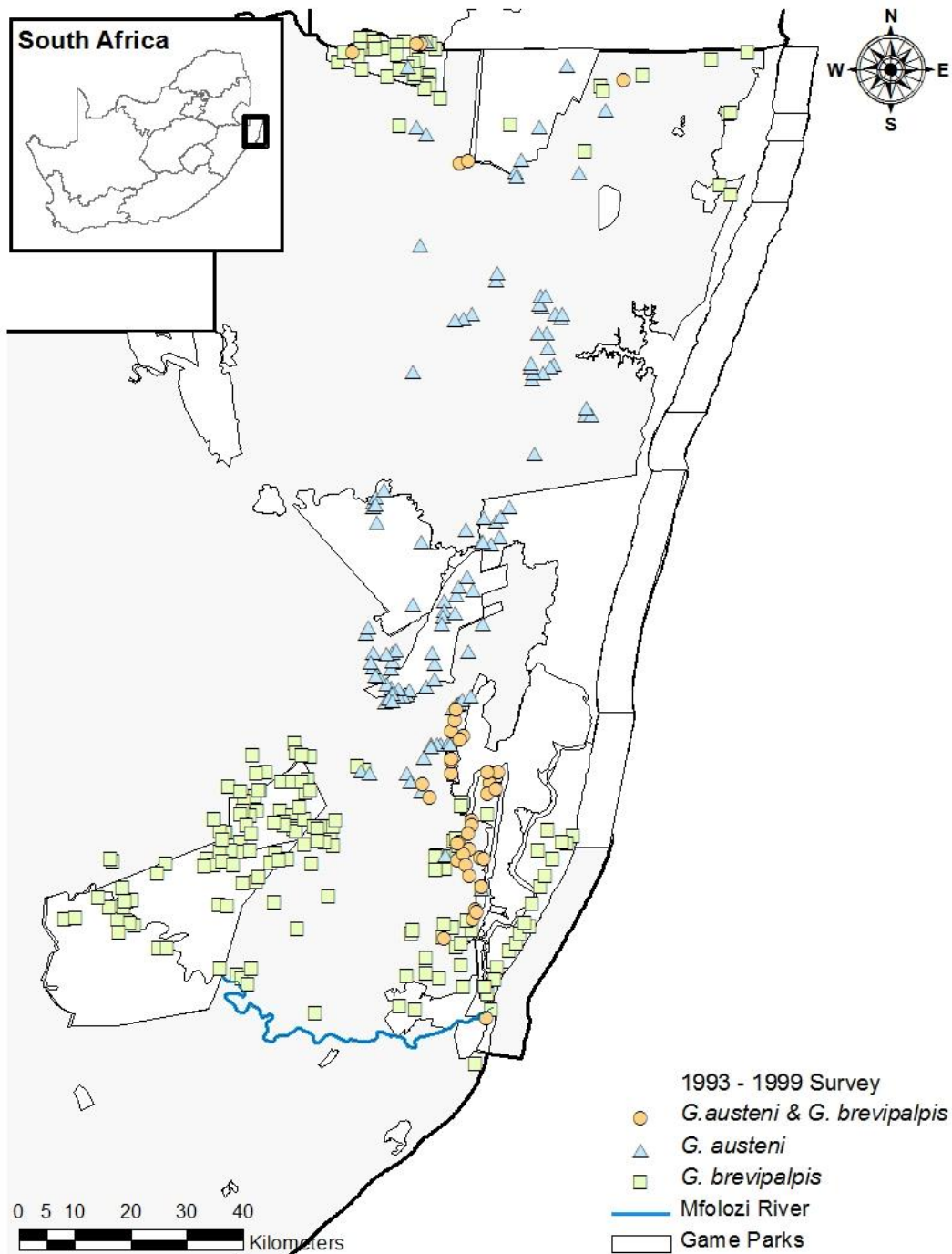
Fig. 2.2. Distribution of *Glossina brevipalpis* and *Glossina austeni* based on a survey carried out from 1993 to 1999 with odour baited XT sticky traps. Only positive trap catches are indicated.

The objective of the present study was to update the distribution maps of tsetse flies and Trypanosomosis prevalence in South Africa. Additionally, the distribution and

abundance of tsetse flies in the area were assessed in terms of some additional environmental factors such as vegetation and climate. Correlation between tsetse fly apparent densities and infection prevalence of the disease in livestock were investigated.

2.2   Materials and methods

2.2.1   Study area

The tsetse fly infested area (± 16 000 km^2) in South Africa is confined to the north eastern part of the KwaZulu-Natal Province. The area stretches approximately from the Umfolozi River (-28.5204, 32.3123) in the south to the border of Mozambique (-26.8692, 32.8342) in the north, and from the Indian Ocean coast in the east up to the west of the Hluhluwe-iMfolozi Park (-28.33416, 31.691222) (Fig. 2.2) (Kappmeier Green, 2002). Although some commercial cattle farms are present, it is predominantly a subsistence cattle-farming area with numerous communal farms interspersed with a number of protected areas. According to the local State Vetenarian there are an estimated 250 000 cattle and 130 000 small ruminants in the area and cattle are treated with trypanocides on an ad hoc basis.

The protected areas consist of provincial and private game parks and reserves. The area surrounding the extensive fresh water lake system, iSimangaliso Wetland Park, was proclaimed as South Africa's first world heritage site in 1999. These areas contain a wide variety of game animals, such as birds, rodents and small primates (*e.g. Cercopithecus mitis*) but also large numbers of bigger mammals that are potential hosts for tsetse flies. The main game animals in the area are buffalo (*Syncerus caffer*), hippopotamus (*Hippopotamus amphibius*), white rhinoceros (*Ceratotherium simum*), elephant (*Loxodonta africana*), bush pig (*Potamochoerus porcus*) and warthog (*Phacochoerus aethiopicus*). Antelopes in the area include species such as red duiker (*Cephalophus natalensis*), blue duiker (*Philantomba monticola*), impala (*Aepyceros melampus*), reedbuck (*Redunca arundinum*), blue wildebeest (*Connochaetes taurinus*), waterbuck (*Kobus ellipsiprymnus*), bushbuck (*Tragelaphus scriptus*), nyala (*Tragelaphus angasii*) and kudu (*Tragelaphus strepsiceros*). The target area contains a number of state forests, mostly pine and eucalyptus plantations, and commercial sugarcane farms.

The climate is subtropical with the average minimum temperatures ranging from 10.52 ± 1.26 °C in July (winter) to 21.71 ± 0.67 °C in January (summer). The average maximum ranges from 24.65 ± 0.77 °C in July to 30.86 ± 1.38 °C in January. The average minimum relative humidity ranges from 39.92 ± 8.23% in July to 62.04 ± 6.39% in October whereas the average maximum ranges from 92.23 ± 3.73% in June to 94.94 ± 3.13% in February. The area receives an average maximum of 132.92 ± 92.95 mm of rain in January, and most of the precipitation is received in the hot season from October to March. However,

it can also rain in the cold dry season from April to September with a minimum average 9.27 ± 9.68 mm of rain in July. On average, rainfall is higher on the coast compared to the interior.

2.2.2   Tsetse fly survey

For the main part of the survey, tsetse flies were collected at 18 sites located in four magisterial districts: Ingwavuma, Ubombo, Hlabisa and the northern part of Enseleni (Fig. 2.4). A total of 77 odour-baited H traps (Kappmeier & Nevill, 1999b; Kappmeier, 2000) were deployed at these 18 sites from April 2005 to April 2009. The number of trap days ranged from 723 at Ocilwane (northern Enseleni) to 1661 days at False Bay Park (eastern Hlabisa district). Two supplementary surveys were carried out in Hlabisa and the northern parts of Enseleni from April to May 2012 (an average of 26 ± 1.62 days) and from April to June 2015 (an average of 63 ± 1.36 days), mainly to determine more accurately the southernmost occurrence of the tsetse fly populations.

The traps were baited with odours to enhance trapping of *G. brevipalpis* (Kappmeier & Nevill, 1999a). These baits consisted of 1-octen-3-ol and 4-methylphenol at a ratio of 1:8 that were released at 4.4 mg/h and 7.6 mg/h, respectively. The chemicals were dispensed from seven heat-sealed sachets (7 cm x 9 cm) made of low-density polyethylene sleeves (wall thickness 150 microns) placed near the entrance of the trap. A 300 mL brown glass bottle that dispensed acetone through a 6 mm hole in the lid at a rate of ca. 350 mg/h was placed next to the H trap (Esterhuizen, 2007; Kappmeier Green, 2002). Flies were collected in a 20% ethanol solution to which an antiseptic, Savlon® (Johnson & Johnson, Pharmedica Laboratories (Pty) Ltd. Rattray Road, East London, South Africa) (0.4 mL/L) and formalin (0.4 mL/L) had been added to preserve the sampled flies as well as to combat ant and spider predation. Traps were emptied and serviced every 14 days. The number of each species collected over this period was counted and results expressed as apparent density (AD), *i.e.* the number of flies per trap per day.

Data from several independent surveys, done over a couple of years, was incorporated in this study.

2.2.3   Environmental factors affecting tsetse fly distribution
2.2.3.1   Vegetation
An updated vegetation map for the infested area (north eastern KwaZulu-Natal, southern Mozambique and Swaziland) was developed in 2010 (Bouyer & Guerrini, 2010). The main land-cover classes that were considered relevant for the presence or absence of *G. brevipalpis* and *G. austeni* were indicated on the map as savannah woodland,

herbaceous savannah, shrub savannah, dense dry forest, gallery forest, tree plantations, crops (agricultural areas), urban areas, swamps, water bodies and bare ground (Bouyer & Guerrini, 2010). In the present study, this vegetation map was combined with satellite images (Google earth) of the area to assess the potential correlation between vegetation and tsetse fly distribution and/or abundance. To enable comparison with the vegetation data produced in 2010, tsetse fly apparent densities as obtained from the surveys implemented between July 2008 and August 2009 were correlated to the different vegetation classes found at each trapping site.

2.2.3.2 Climate

Climate data obtained from seven permanent weather stations (Fig. 2.3), maintained by the Agricultural Research Council-Institute for Soil, Climate and Water (ARC-ISCW), were used to develop climate maps of the study area. Tsetse fly apparent densities as obtained from five of the sampling sites (Kosi Bay, Mkuzi, Kuleni, Bushlands and St Lucia) located within 8 km from one of these seven weather stations (Fig. 2.3), were used to determine correlations between tsetse fly AD and certain climate variables. Similar to the vegetation comparisons, climate data and tsetse fly AD from July 2008 to August 2009 were used.
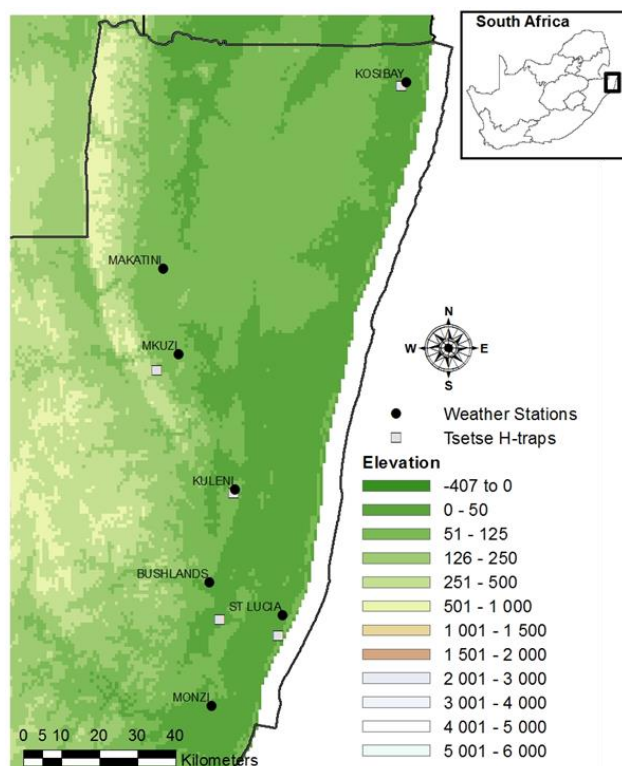
Fig. 2.3. Location of the weather stations in relation to the tsetse H traps used for determining potential relationships between tsetse fly apparent density (AD) and climate variables in north eastern KwaZulu-Natal.

2.2.4   Trypanosomosis survey

A large number of communal dip tanks, constructed, maintained and operated by the Provincial Department of Veterinary Services, are routinely used for tick control and are evenly distributed throughout the tsetse-infested area. The tick control policy, as prescribed by the Department of Veterinary Services, consists of weekly dipping of cattle in the summer and fortnightly dipping during winter. Cattle in the communal farming areas as well as on the commercial farm Boomerang roam freely. The trypanosome infection prevalence in cattle was determined at 25 dip tanks from November 2005 to November 2007. All animals that congregated at a particular dip tank were considered as one herd as they grazed together and were managed using the same animal husbandry practice (Emslie, 2005; Ntantiso *et al.,* 2014). Herd size was variable and ranged from 13 to 6411 animals, and the number of cattle owners at each dip tank was likewise very variable and ranged between five and 296. Cattle were screened on the day they were scheduled for routine dipping. These herds were divided into groups of 30 to 40 cattle and two to three animals in each group were, after consent from the owner, randomly sampled. Data from several independent surveys (published and unpublished), done over a couple of years, was incorporated in this study.

Blood was collected from the tail or jugular vein of adult animals using 10 mL vacutainer tubes containing the anticoagulant EDTA (BD Vacutainer®; BD, Plymouth, UK). The blood was transferred to micro haematocrit centrifuge capillary tubes (Marienfeld-Superior, Lauda-Königshofen, Germany) that were sealed with Cristaseal (Hawksley) and centrifuged in a haematocrit centrifuge for 5 minutes at 9000 rpm. The buffy coat of each specimen was extruded onto a microscope slide, under a cover slip and examined for motile trypanosomes using a compound microscope at 40 times magnification (Paris *et al.*, 1982). Trypanosomosis prevalence at each dip tank was expressed as the number of cattle with positive trypanosomes in their buffy coat expressed as a proportion of the number of cattle screened.

2.2.5   Statistical analysis

All data were analysed using the statistical software GraphPadInstat (version 3.00, 2003). Tsetse fly relative abundance was expressed as the apparent density (AD) of each species, *i.e.* the number of flies collected per trap per day. For comparison of the relative abundance between *G. brevipalpis* and *G. austeni* populations, a paired test was used. Data was not normally distributed and the nonparametric Wilcoxon matched pairs test was used. To evaluate the AD within each tsetse species population a one-way analysis of variance (ANOVA) was used to differentiate between the mean tsetse fly AD. The data was not

normally distributed so a nonparametric method (Kruskal-Wallis test) was used. Additionally, Dunn's multiple comparison tests were used if the P value < 0.05.

Proportional differences in trypanosome infection prevalence, expressed as the number of cattle that had positive trypanosomes in their buffy coat as a proportion of the number of cattle screened, were determined with Chi-square (χ^2) analysis with the Yate's continuity correction.

Linear regression analysis was carried out on trypanosome infection prevalence and tsetse fly AD, as well as infection prevalence and distances from game reserves. All statistical tests were done at the 5% significance level.

Maps were developed in ArcGIS10.1. For the trypanosome infection prevalence as well as the climate data an inverse distance weighted interpolation method was used with a power 2 function and a variable search radius setting at 10 points.

2.3   Results

2.3.1   Tsetse fly distribution and apparent density (AD)

2.3.1.1   Apparent density (AD)

The 77 H traps deployed at 18 sites located in the four magisterial districts *i.e.* Ingwavuma, Ubombo, Hlabisa and Enseleni, collected 216 449 *G. brevipalpis* (AD = 2.64 flies/trap/day) and 17 097 *G. austeni* (AD = 0.21 flies/trap/day) between 1 April 2005 and 31 August 2009 (Table 2.1; Fig. 2.4 A, B).

While both tsetse fly species were collected in the three northerly districts, only *G. brevipalpis* was trapped (with six H traps) at Ocilwane in the northern part of the southerly Enseleni district (Table 2.1; Fig. 2.4 A). The overall AD of *G. brevipalpis* (2.64 flies/trap/day) was significantly higher (P < 0.01) than that of *G. austeni* (0.21 flies/trap/day). Comparison for the three northerly districts indicated that the AD of *G. brevipalpis* was significantly higher than that of *G. austeni* in the Ingwavuma (P = 0.01) and Hlabisa (P < 0.01) districts (Table 2.1; Fig. 2.4 A, B). The AD of *G. brevipalpis* (0.03 flies/trap/day) in the Ubombo district was significantly lower than that of *G. austeni* (0.33 flies/trap/day) (P < 0.01) (Table 2.1).

Glossina brevipalpis was most abundant in the Hlabisa district (3.76 flies/trap/day), and the AD was significantly higher (P < 0.01) than that of the Ubombo district (0.03 flies/trap/day); an area where previously no *G. brevipalpis* had been collected (Kappmeier Green, 2002) (Table 2.1; Fig. 2.4 A).

Significantly higher (P = 0.03) numbers of *G. austeni* (AD = 0.33 flies/trap/day) were collected in the Ubombo district as compared to the Hlabisa (0.02 flies/trap/day) and the Ingwavuma districts (0.04 flies/trap/day) (Table 2.1). No *G. austeni* was trapped at Ocilwane

in the Enseleni district (Table 2.1; Fig. 2.4 B). The trapping data indicated that the Monzi forest in the Hlabisa district was the most southerly distribution of *G. austeni* in KwaZulu-Natal (and Africa).

The greatest variations in ADs between sites within the same magisterial district (Table 2.1) were observed in the Hlabisa district for both *G. brevipalpis* and *G. austeni* ($P < 0.01$ for both species). The AD of *G. brevipalpis* in the Hluhluwe-iMfolozi Park (10.74 flies/trap/day) was significantly higher than that seen at Kuleni (0.17 flies/trap/day). There were also significant differences in AD of the *G. brevipalpis* populations of St Lucia (7.25 flies/trap/day) and Kuleni (0.17 flies/trap/day). *Glossina austeni* was most abundant at Boomerang (0.78 flies/trap/day), and this AD was significantly higher than that of 0.02 flies/trap/day obtained in the Hluhluwe-iMfolozi Park. In the Ubombo district the AD of *G. brevipalpis* at False Bay Park (1.55 flies/trap/day) was significantly higher ($P = 0.04$) than that at Phinda (0.06 flies/trap/day) and Lower Mkhuze (0.06 flies/trap/day). The AD of *G. austeni* was significantly higher at False Bay Park (0.60 flies/trap/day) ($P < 0.01$) in the Ubombo district as compared with the AD in Mkhuze (0.01 flies/trap/day). Finally, in the Ingwavuma district there were no significant differences in relative abundances between individual sites for both *G. brevipalpis* ($P = 0.11$) and *G. austeni* ($P = 0.11$).

2.3.1.2 Tsetse fly distribution

The updated distribution of *G. brevipalpis* and *G. austeni*, taking only absence and presence into account, as determined with H traps during the last 10 years, from 2005 to 2015, is presented in Fig. 2.5.

The most obvious differences in the present survey results, compared to those of the odour baited XT sticky traps (Kappmeier Green, 2002) (Fig. 2.2), were that *G. brevipalpis* was collected in the Ubombo district and *G. austeni* in the Hluhluwe-iMfolozi Park (Fig. 2.5). Additionally, *G. brevipalpis* was collected at sites south of the Umfolozi River previously considered the southernmost distribution of tsetse flies in South Africa (Kappmeier Green, 2002). This is an indication that the most southern limit of *G. brevipalpis* is still not clearly defined. The present distribution data is, however, in agreement with the prediction maps of Hendrickx *et al.* (2002).

Table 2.1. Tsetse H trap collections of *Glossina brevipalpis* and *Glossina austeni* from April 2005 to April 2009 at 18 sites in north eastern KwaZulu-Natal.

No. on map*	Tsetse fly sample site	Number of traps	Start date	End date	Total no. of trap days	G. brevipalpis		G. austeni	
						Total collected	Apparent density	Total collected	Apparent density
Ingwavuma									
1	Ndumu	1	14-06-2006	26-08-2009	1169	6358	5.44	210	0.18
2	Tembe	4	14-06-2006	26-08-2009	4606	1529	0.33	135	0.03
3	Pelani	1	14-06-2006	26-08-2009	1169	11	0.01	0	0.00
4	Kosi Bay	2	19-04-2006	26-08-2009	2262	444	0.20	1	<0.01
Total/District		**8**	**14-06-2006**	**26-08-2009**	**9206**	**8342**	**0.91**	**346**	**0.04**
Ubombo									
5	Tshongwe	2	04-07-2006	25-08-2009	2275	0	0.00	7	<0.01
6	Mbazwana	2	14-06-2006	11-08-2009	1750	0	0.00	263	0.15
7	Lower Mkhuze	3	14-06-2006	25-08-2009	3504	204	0.06	2022	0.58
8	Mkhuze	2	25-10-2006	21-08-2009	2062	1	<0.01	23	0.01
9	Phinda	4	04-07-2006	25-08-2009	4550	261	0.06	2284	0.50
Total/District		**13**	**14-06-2005**	**25-08-2009**	**14 141**	**466**	**0.03**	**4599**	**0.33**
Hlabisa									
10	Kuleni	3	01-01-2006	21-08-2009	3984	697	0.17	856	0.21
11	False Bay Park	4	01-04-2005	18-08-2009	6400	9894	1.55	3849	0.60
12	Ekuphinidisweni	6	01-05-2006	23-08-2009	5371	8529	1.59	0	0.00
13	Hluhluwe-iMfolozi Park	15	01-11-2005	19-08-2009	11 201	120 313	10.74	277	0.02
14	Mvutshini	12	01-05-2005	18-08-2009	15 239	32451	2.13	65	<0.01
15	Hlambanyathi	1	01-03-2006	31-08-2009	1279	28	0.02	1	<0.01
16	Boomerang	6	01-04-2005	03-08-2009	8199	11 627	1.42	6424	0.78
17	St Lucia	3	23-10-2006	21-08-2009	3099	22 470	7.25	680	0.22
Total/District		**50**	**01-04-2005**	**31-08-2009**	**54 772**	**206 009**	**3.76**	**12 152**	**0.22**
Enseleni									
18	Ocilwane	6	01-11-2007	24-08-2009	3882	1632	0.42	0	0.00
Total/District		**6**	**01-11-2007**	**24-08-2009**	**3882**	**1632**	**0.42**	**0**	**0.00**
Total/Study area		**77**	**01-04-2005**	**31-08-2009**	**82 001**	**216 449**	**2.64**	**17 097**	**0.21**

*The number on map refers to Fig. 2.4.
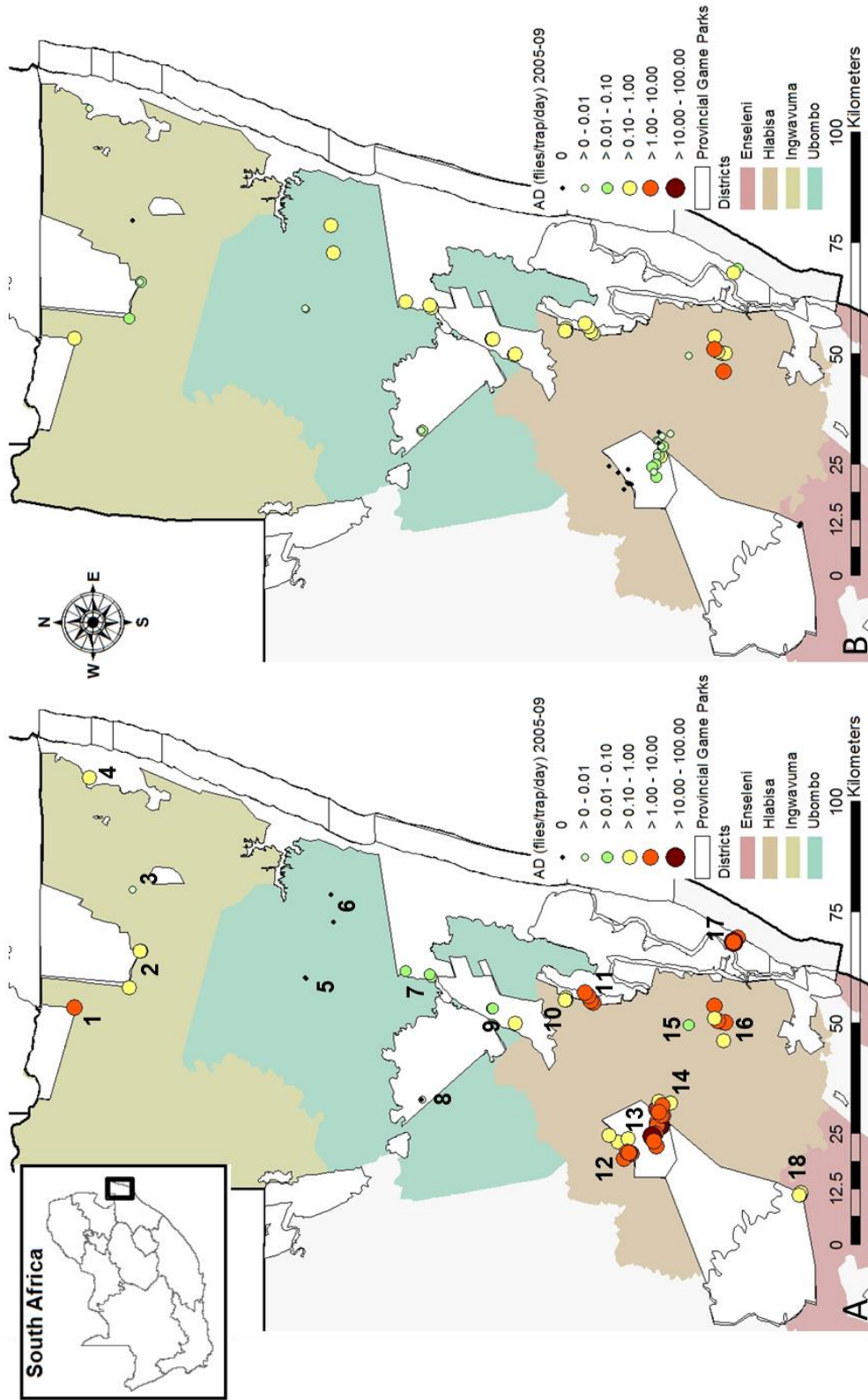
Fig. 2.4. Tsetse fly apparent density (AD: flies/trap/day) for *Glossina brevipalpis* (A) and *Glossina austeni* (B) from April 2005 to August 2009.
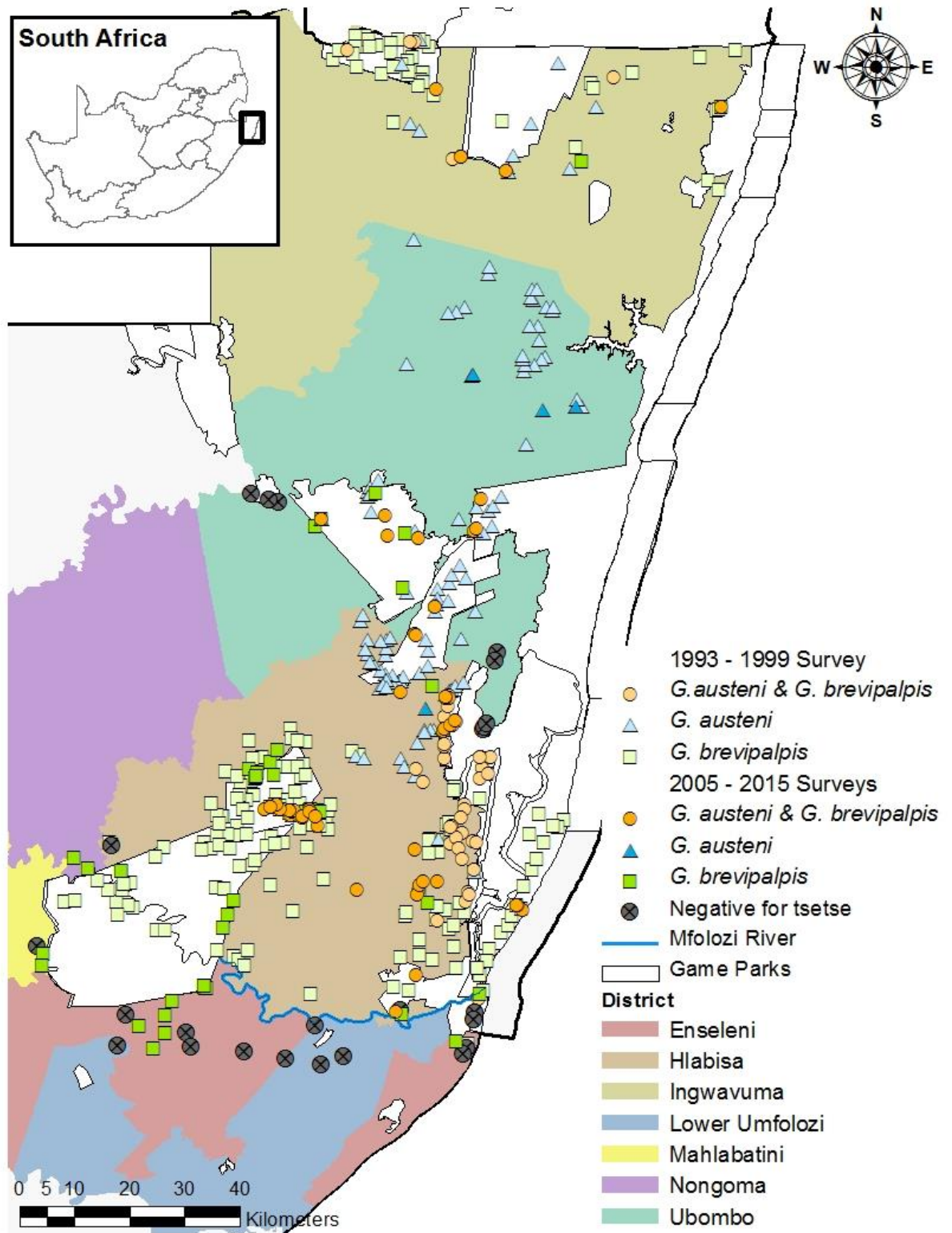
Fig. 2.5. Updated distribution of *Glossina brevipalpis* and *Glossina austeni* as determined with odour baited XT sticky traps (1993 - 1999) and odour baited H traps (2005 – 2015) surveys.

2.3.2   Environmental factors affecting tsetse fly distribution

2.3.2.1   Vegetation

Fly collections made with a subset of H traps, from July 2008 to August 2009, were used to correlate tsetse fly AD with vegetation classes at each trapping site. In these subsets 75 traps collected 73 078 *G. brevipalpis* and 4011 *G. austeni* over one-year of sampling.

The highest ADs for both *G. brevipalpis* (4.60 flies/trap/day) and *G. austeni* (0.32 flies/trap/day) were found in the dense dry forest vegetation class. The second highest AD of *G. brevipalpis* was found in the shrub savannah (2.14 flies/trap/day) followed by the savannah woodland (1.99 flies/trap/day) and the herbaceous savannah (1.57 flies/trap/day). The AD of *G. brevipalpis* was the lowest in the swamp (0.93 flies/trap/day) class. The ADs of *G. brevipalpis* were significantly higher ($P = 0.05$) for the dense dry forest than the savannah woodland classes.

The AD for *G. austeni* was significantly different ($P = 0.02$) between the five vegetation classes, particularly between the dense dry forest (0.32 flies/trap/day) with was higher than the shrub savannah (0.07 flies/trap/day). After the dense dry forest, *G. austeni* was most abundant in the swamps (AD = 0.29 flies/trap/day), followed by the savannah woodlands (0.13) and the shrub savannah (0.07). The lowest AD (0.001 flies/trap/day) was recorded in the herbaceous savannah.

These AD results seem to indicate that *G. austeni* is less adaptable to different vegetation types and that this fly was mainly restricted to denser vegetation *e.g.* dry forest. This difference in adaptation to vegetation classes of *G. brevipalpis* and *G. austeni* can be illustrated by the results obtained at four sites.

At Pelani in the north, located 4 km from the Ndumo Game Reserve, tsetse fly abundance was low and only *G. brevipalpis* (AD = 0.02 flies/trap/day) was sampled (Fig. 2.6 A). Dense dry forest was not very abundant in the area. The vegetation mainly consisted of swampy areas with shrub savannah, herbaceous savannah and bare ground, with patches of savannah woodlands (Fig. 2.6 A).

Mbazwana (Fig. 2.6 B) was not near any game areas and its vegetation consisted mainly of savannah woodlands and shrub savannah with patches of dry forest and herbaceous savannah. Dense dry forest was, however, more abundant than at Pelani. Only *G. austeni* was collected with an AD of 0.07 flies/trap/day.

The third site, False Bay Park (Fig. 2.6 C) is located inside the iSimangaliso Wetland Park. Vegetation at this site consisted mainly of savannah woodland with large patches of dense dry forests. Both *G. b*revipalpis (average AD = 1.03; SD = 0.56 flies/trap/day) and *G. austeni* (average AD = 0.53; SD = 0.16 flies/trap/day) were collected and their ADs where similar and relatively high.

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The most southern site, Hluhluwe-iMfolozi Park, also in a protected area, contained mainly shrub savannah, with patches of savannah woodland, dense dry forest and herbaceous savannah. Here the average AD (11.14, SD = 8.42 flies/trap/day) of *G. brevipalpis* was much higher than the average AD (0.01, SD = 0.01 flies/trap/day) of *G. austeni*.

2.3.2.2   Climate

Climate data from the weather stations and tsetse fly ADs, obtained during the July 2008 to August 2009 surveys, at Kosi Bay, Mkuzi, Kuleni, Bushlands, and St Lucia, are presented in Table 2.2. A total of 16 traps collected 15 978 *G. brevipalpis* and 1345 *G. austeni* over the one-year period at these five sites.

The highest average maximum temperature (27.5 °C ± 4.2) as well as the lowest average minimum temperature (15.6 °C ± 4.5) during this period were recorded at Mkuzi located 54 km from the coast (Table 2.2). At the coastal sites, variations in both the average temperature and relative humidity were less pronounced (Table 2.2). At the coastal sites of Bushlands and St Lucia that had more moderate temperature ranges (Fig. 2.7 A, B; Table 2.2) and higher relative humidity (Fig. 2.7 C, D; Table 2.2), ADs for both *G. brevipalpis* and *G. austeni* were high. In contrast, at Mkuzi a site characterised by more extreme variations between the average maximum (Fig. 2.7 A) and minimum (Fig. 2.7 B) temperatures and a lower relative humidity (Fig. 2.7 C, D) and rainfall (Fig. 2.7 E), relative abundance of *G. brevipalpis* (average AD = 0.001, SD = 0.009 flies/trap/day) was very low (Table 2.2). The relationships between tsetse fly AD and average evaporation (Fig. 2.7 F), radiation (Fig. 2.7 G) and wind speed (Fig. 2.7 H) were less obvious.

Table 2.2. Weather station data and tsetse fly apparent density (July 2008 to August 2009).

	Kosi Bay	Mkuzi	Kuleni	Bushlands	St Lucia
Av. Maximum Temperature °C	25.67	27.46	26.82	26.75	26.45
(SD)	(3.15)	(4.20)	(4.04)	(3.52)	(3.52)
Av. Minimum Temperature °C	17.44	15.56	17.03	16.35	17.08
(SD)	(3.86)	(4.50)	(3.82)	(3.74)	(4.30)
Av. Maximum Relative Humidity %	94.47	85.65	89.16	91.12	99.47
(SD)	(4.16)	(10.81)	(4.05)	(4.42)	(1.71)
Av. Minimum Relative Humidity %	61.79	37.68	49.52	51.07	69.03
(SD)	(12.31)	(17.63)	(14.69)	(14.22)	(13.09)
Av. Rainfall mm	2.01	0.64	2.17	1.50	2.31
(SD)	(9.27)	(3.51)	(10.66)	(4.98)	(6.54)
Av. Relative Evapotranspiration mm	3.05	2.89	3.36	2.94	2.89
(SD)	(1.10)	(2.59)	(1.38)	(1.54)	(1.31)
Av. Hourly Wind Speed m/s	2.69	0.84	1.93	2.78	2.26
(SD)	(0.91)	(0.35)	(0.54)	(2.22)	(1.17)
Av. Radiation MJ/m^2	14.47	13.07	16.66	13.52	15.83
(SD)	(8.75)	(5.20)	(6.40)	(7.45)	(6.71)
Av. Apparent Density *Glossina brevipalpis* (SD)	0.14 (0.20)	0.001 (0.009)	0.10 (0.11)	2.23 (2.31)	10.87 (8.07)
Av. Apparent Density *Glossina austeni* (SD)	0	0	0.27 (0.27)	0.41 (0.41)	0.40 (0.46)
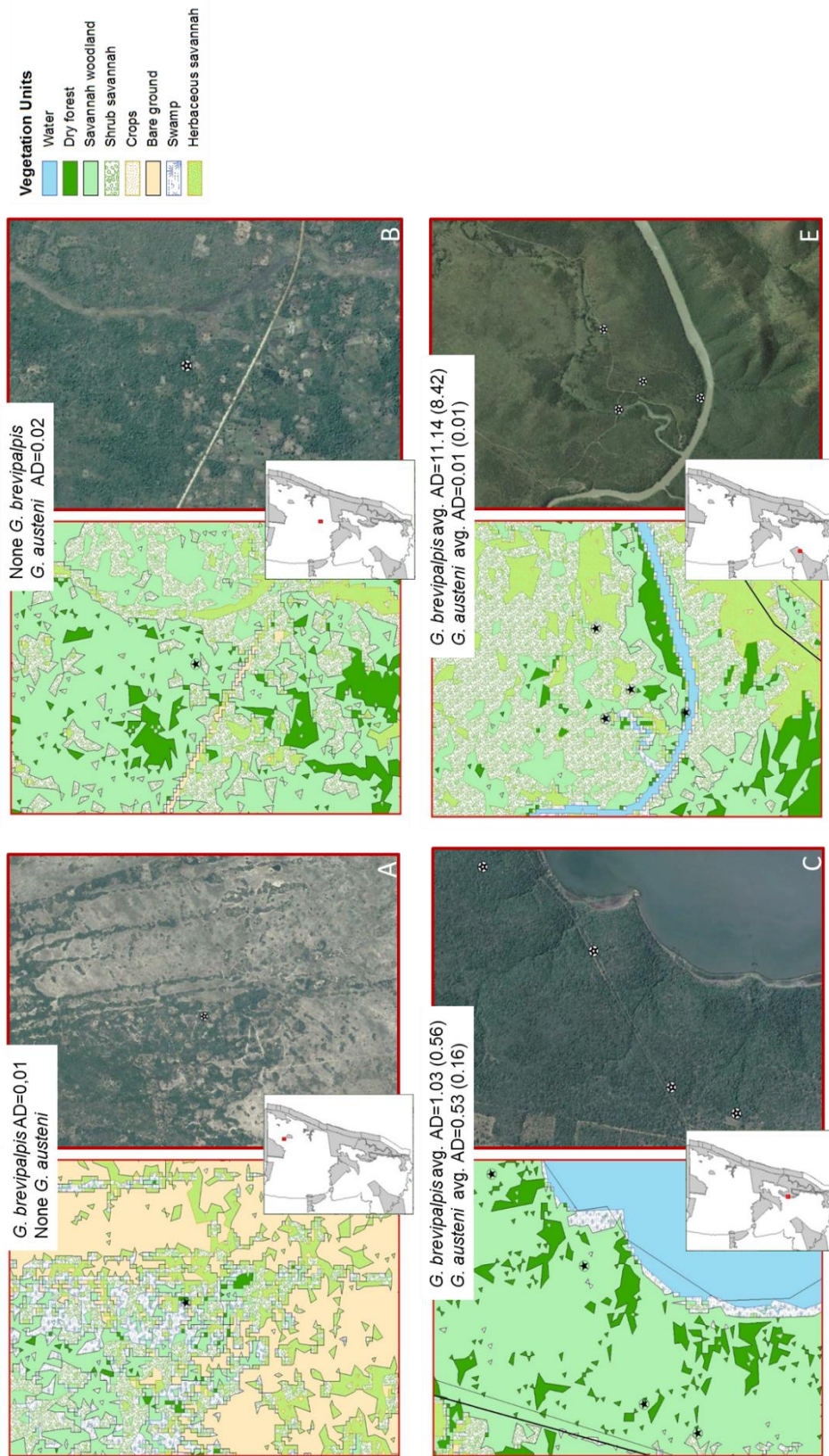
Fig. 2.6. Vegetation class (Bouyer & Guerrini, 2010) maps and satellite images of four tsetse fly collection sites in north eastern KwaZulu-Natal. A: Pelani, B: Mbazwana, C: False Bay Park, E: Hluhluwe-iMfolozi Park (Google earth, 2013a, b, c; 2014). The average AD (SD) for each species, expressed as flies/trap/day are indicated for each site.

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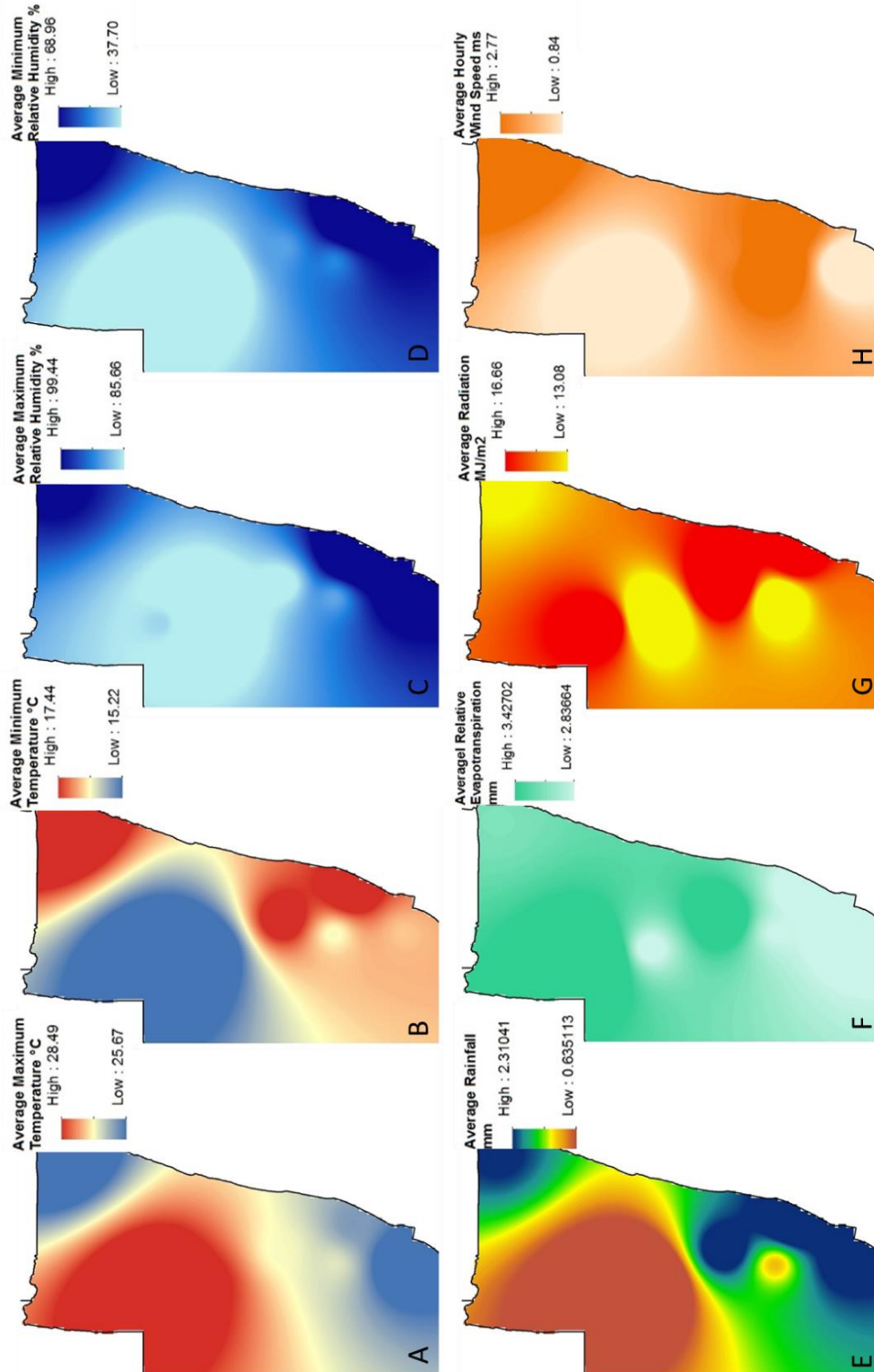
Fig. 2.7. Inverse distance weighted interpolation of environmental data collected at weather stations in north eastern KwaZulu-Natal from July 2008 to August 2009 (A: Av. Maximum Temperature (°C), B: Av. Minimum Temperature (°C), C: Av. Maximum Relative Humidity (%), D: Av. Minimum Relative Humidity (%), E: Av. Rainfall (mm), F: Av. Relative Evapotranspiration (mm), G: Av. Radiation (MJ/m²) and H: Av. Hourly Wind Speed (m/s)).

2.3.3 Tsetse fly and Trypanosomosis association

2.3.3.1 Trypanosome infection at dip tanks

Blood samples collected from 1034 cattle at 25 dip tanks in the tsetse-infested area were examined microscopically for trypanosomes (Table 2.2). The number of cattle screened per dip tank ranged from 27 at Ekuphindisweni to 54 at Boomerang (Table 2.2).

Data of trypanosome prevalence (Table 2.2) were, as for the tsetse fly data, grouped per magisterial district (Table 2.1). The results showed that trypanosome infection was widespread in north eastern KwaZulu-Natal with infected cattle found at 21 (84.0%) of the 25 dip tanks surveyed (Table 2.2; Fig. 2.8). Trypanosome prevalence ranged from 20% in the Ubombo district to 3% for the Enseleni district and did not differ significantly ($P = 0.05$, $\chi^2 = 7.91$, d.f. = 3) between the four magisterial districts. Notwithstanding the absence of significant differences between the four districts, the proportional representation of positive animals at 25 individual dip tanks differed significantly ($P < 0.01$, $\chi^2 = 173.30$, d.f. = 24). Differences in the proportion of positive animals per dip tank were also significant within the districts of Hlabisa ($P < 0.01$, $\chi^2 = 51.462$, d.f. = 6), Ingwavuma ($P < 0.01$, $\chi^2 = 59.078$, d.f. = 9) and Ubombo ($P < 0.01$, $\chi^2 = 51.211$, d.f. = 6). These proportional differences in infection prevalence between individual dip tanks seemed to depend on the distance of the dip tank from a protected area or game reserve.

The highest prevalence of 48% was recorded at the Mseleni dip tank situated next to the northern parts of iSimangaliso Wetland Park in the Ubombo district (Table 2.2). Similarly, trypanosome prevalence in cattle was high at dip tanks close to protected areas and/or game parks (Fig. 2.6). Especially in the Hlabisa district, a positive linear regression ($r^2 = 0.62$, $P = 0.04$) was evident between trypanosome prevalence and the distance between the dip tank and the protected areas/game parks. This same trend, although no statistically significant (Ingwavuma: $r^2 = 0.22$, $P = 0.17$ and Ubombo: $r^2 = 0.19$, $P = 0.41$) was seen in the other two districts. The correlation between infection prevalence and distance from the game parks was further epitomised by the absence of infected animals at the two dip tanks (Ntabayengwe and Makhathini) located more than 20 km from a game park (Table 2.2).

2.3.3.2 Tsetse fly abundance and Trypanosomosis infection rate correlations

The trypanosome prevalence in cattle, determined with the buffy coat technique was correlated with the tsetse fly relative abundance, as determined with odour-baited H traps. Only dip tanks (N = 18) that had H traps deployed within a 12 km radius were selected for the regression analyses (Table 2.2). No linear correlation was found between trypanosome prevalence and the relative abundance of either *G. brevipalpis* ($r^2 = 0.01$, $P = 0.68$),

G. austeni ($r^2 = 0.05$, *P* = 0.40) or the total tsetse catches ($r^2 = 0.04$, *P* = 0.43). However, at some of the dip tanks with high trypanosome prevalence in the cattle, *e.g.* Mseleni (48%) and Mkhumbikazana (37%) in the Ubombo district, the AD for *G. austeni* was relatively high in the absence of *G. brevipalpi*s (Fig. 2.4 A, B). In contrast, at the Ekuphindisweni dip tank in Hlabisa where screened cattle showed a similarly high infection prevalence (37%), the relative abundance of *G. brevipalpis* was high, in the absence of *G. austeni* (Fig. 2.4 A, B). The relative abundance of both *G. brevipalpis* and *G. austeni* was relatively high at the dip tank on the commercial farm Boomerang located west of and next to the southern extent of the iSimangaliso Wetland Park and was also where the highest trypanosome prevalence (44%) in the Hlabisa district was recorded.
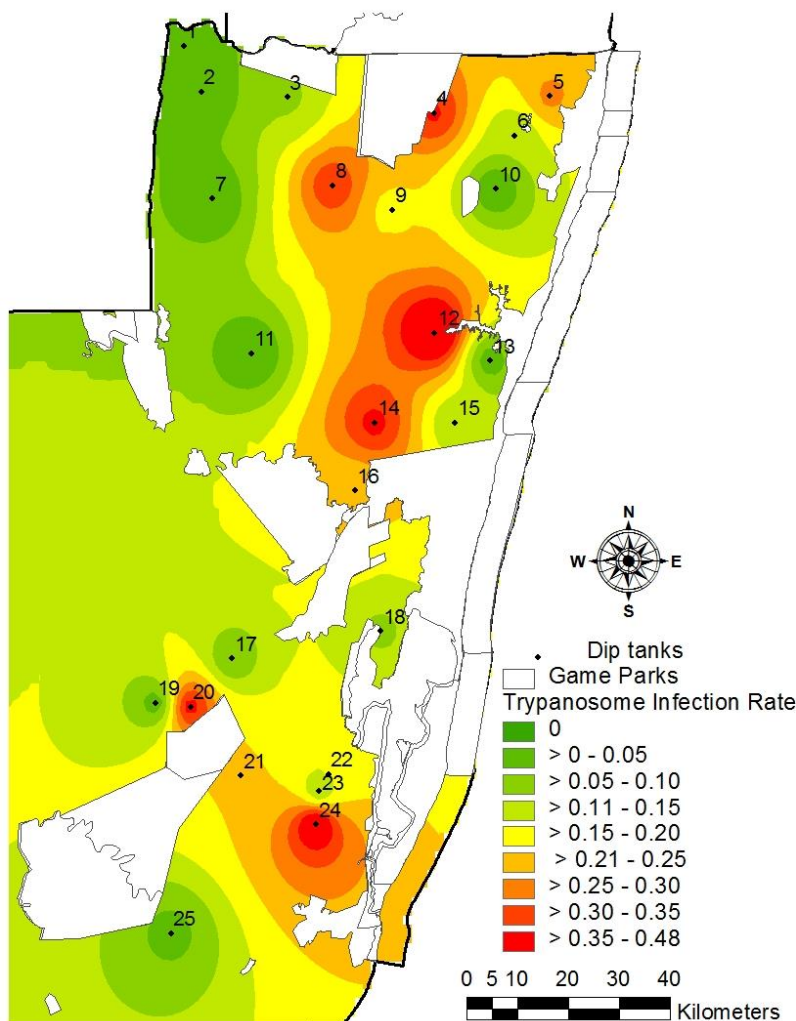
Fig. 2.8. Inverse distance weighted interpolation of trypanosome infection rate at dip tanks in north eastern KwaZulu-Natal (1: Ekuhlehleni, 2: Khume, 3: Ndumo, 4: Manzibomvu, 5: Nhlanjwana, 6: Thengani, 7: Ntabayengwe, 8: Ngwenyambili, 9: Phelendaba, 10: Pongola, 11: Makhathini, 12: Mseleni, 13: Mpini, 14: Mkhumbikazane, 15: Mbazwana, 16: Zineshe, 17: Khipha, 18: Nibela, 19: Nthwati, 20: Ekuphindisweni, 21: Mvutshini, 22: Qakweni, 23: Mahlambanyathi, 24: Boomerang, 25: Ocilwane).

Table 2.3. Trypanosome infection rates in cattle from November 2005 to November 2009 in north eastern KwaZulu-Natal. The blood was collected from cattle at dip tanks and the infection rate was determined by the buffy coat technique.

No. on map*	Dip tank	Dip tank sample date	Distance from Game Park (km)	Buffy coat examination		Trypanosome infection prevalence
				Positive	Negative	
Ingwavuma						
1	Ekuhlehleni	25-05-2007	11.6	1	49	0.02
2	Khume	01-06-2007	9.4	0	30	0.00
3	*Ndumo	30-05-2007	3.4	4	39	0.09
4	*Manzibomvu	05-09-2007	0.3	18	32	0.36
5	*Nhlanjwana	31-05-2007	2.6	11	31	0.26
6	Thengani	17-05-2007	2.1	4	35	0.10
7	Ntabayengwe	29-05-2007	26.9	0	38	0.00
8	*Ngwenyambili	14-07-2006	7.5	11	21	0.34
9	*Phelendaba	15-05-2007	8.2	7	31	0.18
10	*Pongola	06-08-2007	3.3	0	30	0.00
Total/District				**56**	**336**	**0.14**
Ubombo						
11	Makhathini	06-07-2007	20.2	0	32	0.00
12	*Mseleni	05-08-2007	1.9	22	24	0.48
13	Mpini	05-11-2007	2	1	41	0.02
14	*Mkhumbikazare	05-10-2007	7	18	31	0.37
15	*Mbazwana	05-07-2007	4.3	4	34	0.11
16	*Zineshe	06-04-2007	2.2	9	33	0.21
17	*Nibela	27-01-2007	3	3	30	0.09
Total/District				**57**	**225**	**0.20**
Hlabisa						
18	Khipha	23-11-2006	7.3	2	34	0.06
19	*Nthwati	15-02-2006	6	1	38	0.03
20	*Ekuphindisweni	25-10-2006	3	10	17	0.37
21	*Mvutshini	21-05-2007	3	7	27	0.21
22	*Qakweni	15-08-2006	7.4	8	83	0.09
23	*Mahlambanyati	30-11-2005	9.6	5	45	0.10
24	*Boomerang	18-08-2006	9.5	24	30	0.44
Total/District				**57**	**274**	**0.17**
Enseleni						
25	*Ocilwane	09-07-2007	4.3	1	28	0.03
Total/District				**1**	**28**	**0.03**
Total/Study area				**171**	**863**	**0.17**

*Dip tanks used for trypanosome infection and tsetse fly numbers regression analysis. The number on map refers to Fig. 2.8.

2.4   Discussion

Baseline data on tsetse fly distribution, relative abundance and species composition, as well as the severity of the Trypanosomosis problem are essential in developing an appropriate cost effective, sustainable area-wide control strategy (Vreysen 2005; Leak *et al.*, 2008; Shaw, 2009). Previous surveys, using odour baited XT sticky traps, showed that *G. brevipalpis* was restricted to two distinct bands in the north and south of the area in KwaZulu-Natal (Kappmeier Green, 2002) (Figs. 2.2 & 2.5). These two bands, except for a few patches predicted centrally, were also more or less defined in the distribution prediction model for this species (Hendrickx *et al.*, 2003; Kappmeier Green *et al.,* 2007). In the present survey, the H trap sampled low numbers of *G. brevipalpis* in at least five sites in the central area east of the Mkuzi Game Reserve and north of the southernmost previously defined distribution band (Kappmeier Green, 2002) (Fig. 2.5). Therefore, these positive catches could partly be used to validate the prediction model of Hendrickx (Hendrickx *et al.*, 2003) showing that *G. brevipalpis* is also present in the central and southern parts of the Ubombo district, where it was previously not found. Whether this distribution is indeed patchy or continuous still needs to be verified.

During the 1993-1999 surveys (Kappmeier Green, 2002), *G. austeni* was sampled at the Hluhluwe Dam east of Hluhluwe-iMfolozi Park (Figs. 2.2 & 2.5). During the present surveys all traps inside the Hluhluwe-iMfolozi Park adjacent to the Hluhluwe Dam found *G. austeni* at very low AD (0.02 flies/trap/day) (Table 2.1; Fig. 2.4 B). The presence of *G. austeni* along this transect confirmed the accuracy of the *G. austeni* distribution prediction model of Hendrickx *et al.* (2003). *Glossina brevipalpis* was also trapped, but this was expected considering that it was previously collected in and outside the Hluhluwe-iMfolozi Park (Kappmeier Green, 2002). Taking into account that *G. austeni* is primarily restricted to and does not disperse far from dense vegetation (Esterhuizen *et al.*, 2005; Esterhuizen, 2007), and that bush clearing in the communal farming areas has increased considerably (Kappmeier *et al.*, 1998), it is likely that game reserves and protected areas will become more important in sustaining *G. austeni* populations in the future.

The H traps seemed to be more effective than the XT sticky trap as both species were collected in areas previously considered negative. However, caution is required in interpreting trap efficiencies if traps are not evaluated simultaneously. In addition, land use and the vegetation cover might have changed considerably over a period of ten years, which could have significantly influenced the distribution and abundance of tsetse flies.

The 1993 to 1999 survey indicated the southernmost extent of the Umfolozi River (- 28.5204, 32.3123) as the most southerly distribution of *G. brevipalpis* (Kappmeier Green, 2002). *Glossina austeni* was not trapped south of the St Lucia estuary, where the Umfolozi

River enters the lake (Kappmeier Green, 2002). The tsetse fly prediction model of Hendrickx *et al.* (2003) suggested a very low probability of occurrence for *G. austeni* south of the Umfolozi River, but a much higher probability of *G. brevipalpis* occurring. During the survey conducted in 2015 *G. brevipalpis* was collected at a communal farm 10 km south of the southern border of the Hluhluwe-iMfolozi Park - an area which had not previously been surveyed (Fig. 2.5). This indicates that the most southern limit of *G. brevipalpis* remain undefined. Available data indicates the southern limit for *G. austeni* to be the Umfolozi River, as was previously assumed (Kappmeier Green, 2002). Similarly, the most western extent of the distribution of each species still needs to be verified. Available trap catches indicate that the western distribution limit of *G. austeni* was -28.10357, 32.08377 (in the Hluhluwe-iMfolozi Park), whereas *G. brevipalpis* was collected much further west at -28.50192, 31.881, *i.e.* west of the Hluhluwe-iMfolozi Park (Fig. 2.5).

In agreement with previous studies, vegetation and climate do play a noticeable role in determining the distribution and abundance of both *G. brevipalpis* and *G. austeni* (Kappmeier Green, 2002; Esterhuizen *et al.*, 2005; Esterhuizen, 2007). Preliminary data presented in the current study indicates that vegetation and climate cannot always fully explain the distribution patterns of both species. This indicates that factors such as differential fly mobility as discussed for *G. brevipalpis* and *G. austeni* by Esterhuizen (2007) or host preference and availability may also play an important role. Despite the presence of suitable vegetation and climate in most parts of the area high relative abundance of *G. brevipalpis* was closely linked to the vicinity of protected game areas, where important hosts of *G. brevipalpis* such as hippopotamus, elephant and buffalo were present (Wetzel & Glasgow, 1956; Moloo, 1993; Clausen *et al.,* 1998). In contrast, *G. austeni,* albeit only in low numbers, was also found in areas were only small game such as bush pig and duikers, which are known hosts for this species (Wetzel & Glasgow, 1956; Moloo, 1993; Clausen *et al.*, 1998), were present. Notwithstanding the apparent host preferences of these two species, it was shown that both will also feed on cattle (Moloo, 1993) and that cattle will probably be able to sustain tsetse fly populations in the absence of game.

It is, however, clear that the presence and abundance of both species will largely depend on environmental conditions in the area. Changes in the climate, agricultural practises and land use can have a significant and rapid impact on tsetse fly abundance in the area. To manage the Trypanosomosis problem successfully it will be essential to constantly monitor tsetse fly burdens at selected sites. This will be of special importance before the implementation of any proposed area-wide control campaign.

Trap catches have been used to update the tsetse fly distribution prediction models for both *G. brevipalpis* and *G. austeni* (Hendrickx, 2007). Since only data up to 2009 are

presented, ongoing and future surveys will have to be processed and incorporated into these distribution maps and models. The available trap data have partly validated this prediction model, emphasizing that the value of these models as tools for planning tsetse fly and Trypanosomosis intervention practices should not be underestimated. Furthermore, the new distribution data will need to be taken into account in the tsetse fly control strategy proposed by Kappmeier Green *et al.* (2007), which needs to be modified accordingly. Notwithstanding the fragmentary nature of the data, consolidating data from several independent studies, trypanosome infections in cattle were recorded at 21 of the 25 dip tanks which clearly showed that the disease was still abundant and highly prevalent in north eastern KwaZulu-Natal at the time of these surveys (Fig. 2.8). A potential shortcoming of the trypanosome survey may have been that infections were not identified to species level. It was, however, previously indicated that *T. congolense* is the dominant species in the area (Van den Bossche *et al.*, 2006; Mamabolo *et al.,* 2009; Motloang *et al.,* 2012).

The data on the spatial distribution of the trypanosome infection in north eastern KwaZulu-Natal indicated that the disease was widespread in cattle. This encompasses the areas close to the Mozambique border in the north to a dip tank close to the Umfolozi River in the south, covering the entire length of the tsetse fly infested area of north eastern KwaZulu-Natal (Fig. 2.8). In general, higher infection rates were found in dip tanks located near game reserves and protected natural areas, an observation also made by Van den Bossche *et al.* (2006) and Ntantiso *et al.* (2014) for dip tanks near the Hluhluwe-iMfolozi Park. This game-livestock-tsetse fly interface poses a high risk for cattle becoming infected not only for the Hluhluwe-iMfolozi Park (Ntantiso *et al.,* 2014), but possibly throughout north eastern KwaZulu-Natal.

In 1994, the highest prevalence of Trypanosomosis was recorded in the Ubombo district (Kappmeier *et al.,* 1998). Although in the present study the dip tank (Mseleni) with highest incidence of cattle infection (48%) was also found in this district, the overall trypanosome prevalence for the district was not significantly different from that of the Ingwavuma and Hlabisa districts confirming that Trypanosomosis remains a problem throughout the whole area.

The apparent absence of a significant linear correlation between trypanosome prevalence and the relative abundance of tsetse flies in north eastern KwaZulu-Natal may indicate that a number of factors, such as the tsetse fly and Trypanosomosis monitoring regimes used (a trap placement radius of 12 km could be too large), could influence the outcome of such a regression. This can also partly be attributed to the co-existence of the two tsetse species, each with a different vectorial capacity and/or competence. Previous studies indicated that the vector competence of *G. austeni* for *T. congolense*, the most

abundant *Trypanosoma* species in north eastern KwaZulu-Natal, was significantly higher than that of *G. brevipalpis* (Motloang *et al.,* 2012). Our data seems to corroborate this greater vector competence of *G. austeni* in view of the relatively high trypanosome prevalence recorded in cattle at the Mseleni dip tank where only *G. austeni* flies were sampled (Mbazwana AD = 0.15 flies/trap/day). However, cattle at the Ekuphindisweni dip tank showed a similar high trypanosome prevalence, but *G. austeni* was not sampled and; only *G. brevipalpis* was trapped in relatively high numbers (Ekuphindisweni AD = 1.59 flies/trap/day) indicating that *G. brevipalpis* was most likely responsible for transmission in this area. In general, high apparent densities of *G. brevipalpis* in the vicinity of dip tanks resulted in trypanosome infection rates that were as high as those found at dip tanks where *G. austeni* was present but at low apparent densities. Hendrickx *et al.* (2003) also noted that low *G. austeni* densities coincided with areas of higher Trypanosomosis prevalence in the northern communal areas. However, the very high ADs of *G. brevipalpis* (on average 12.6 times higher than that of *G. austeni*) and the much lower trap catches of *G. austeni* might be resulting from the intrinsic biases of the trapping system. It is known that *G. austeni* responds relatively poorly to traps (Kappmeier, 2000) and is not attracted to any of the odours (Kappmeier & Nevill, 1999a; Kappmeier Green, 2002) used to bait the traps. Essentially, mark-release-recapture studies will be needed to confirm the low efficacy of the sampling tool used for *G. austeni*.

If the relative abundance of *G. brevipalpis* is indeed a reflection of actual population densities, it can be hypothesised that the high trypanosome infection prevalence in certain areas might be the result of the greater densities of *G. brevipalpis* despite their lower vector competence. The relative low vector competence of *G. brevipalpis* previously found in South Africa has probably resulted in an underestimation of the importance of *G. brevipalpis* in the epidemiology of nagana in north eastern KwaZulu-Natal. Vector competence studies in the laboratory have shown that the susceptibility of *G. brevipalpis* for *T. congolense* can be as high as 12.3% (Moloo *et al.*, 1998). In Uganda it was shown that the infection rates in field collected *G. brevipalpis* could be 2.6% (Harley 1967a, b; Moloo *et al.*, 1980). In addition, trypanosome infection rates in tsetse flies are not constant and can change over time and between populations. In Uganda infection rates varied considerably between seasons and with the age of the *G. brevipalpis* population (Harley 1966; 1967a, b).

Both *G. brevipalpis* and *G. austeni* will readily feed on cattle (Moloo, 1993; Clausen *et al.,* 1998) and can therefore play a role in the transmission of Trypanosomosis in KwaZulu-Natal. Similar average trypanosome infection rates in the three northerly districts, even though the relative abundance and distribution of *G. austeni* and *G. brevipalpis* were clearly different, seem to support this hypothesis.

Chapter 3

Comparison of geometric morphometric markers between tsetse populations of South Africa, southern Mozambique and Swaziland

3.1   Introduction

Due to the debilitating impact of African trypanosomoses, effective management of tsetse flies is gaining increased importance throughout its distribution area in Africa. It has been suggested that eradication of selected tsetse fly populations may in some cases be of greater economic benefit than long-term suppression (Kaba *et al.*, 2012). Tsetse fly suppression needs to be done continuously, because tsetse fly populations may recover if suppression is stopped, due to surviving flies (Hargrove, 2003; Patterson & Schofield, 2005). Knowledge of the structure of the targeted tsetse fly population and the size of the geographical area that needs to be covered is very important, as this will determine the most suitable control strategy (Patterson & Schofield, 2005). The degree of geographic and genetic isolation of the targeted population is extremely important as it will indicate the reinvasion potential from neighbouring populations into the target area (Solano *et al.*, 2010).

Eradication will only be feasible and sustainable if the targeted population represents a geographically well-defined or genetically isolated population, or if a rolling carpet approach is implemented (Hendrichs *et al.,* 2005). The rolling-carpet or wave-principle approach is dynamic as the basic operational phases (pre-intervention, population reduction, release of sterile insects and maintenance of pest status) are carried out simultaneously in a phased manner (Hendrichs *et al.,* 2005).

An eradication strategy was successfully implemented on several geographically isolated islands off the coast of Africa *e.g. Glossina pallidipes* was eradicated on São Tomé and Príncipe in 1914 (Leak, 1999) and *Glossina austeni* was eradicated from Unguja Island, Zanzibar between 1993 and 1997 (Vreysen *et al.*, 2000). An isolated population of *G. pallidipes* was successfully eradicated in South Africa and southern Mozambique after the implemantaion of an eradication strategy from 1945 to 1951 (Du Toit, 1954). It was also reported that *Glossina tachinoides, Glossina palpalis palpalis* and *Glossina morsitans submorsitans* were eradicated from an area of over 200 000 km^2 in northern Nigeria in 1955-1978 (Davies & Blasdale, 1960) using a rolling carpet approach, anecdotal data indicating that these riverine species might have reinvaded this, however, is not confirmed.

On mainland Africa, determining the geographic limits of tsetse fly populations and the degree of genetic interactions between them remains challenging, even more so if abundance is low. The difficulty in determining the most southerly distribution of

Glossina brevipalpis and *G. austeni* in South Africa as discussed in Chapter 2 serves as an example.

In South Africa tsetse flies are distributed from the Enseleni district (*G. brevipalpis)* or the Hlabisa district (*G. austeni*) to the border with Mozambique (Fig. 2.4), where they extend into the Matutuine district of Maputo province in southern Mozambique (Fig. 3.1) (Sigauque *et al.*, 2000; Mulandane, 2013). In southern Mozambique both these species are continuously distributed up to the northern border of the Reserva Especial de Maputo and no genetically isolated pockets could be identified (Mulandane, 2013). In the absence of *G. brevipalpis, G. austeni* flies were collected in the Mlawula Nature Reserve in Swaziland close to the Mozambique border (Fig. 3.1) (Saini & Simarro, 2008).

Previous surveys in South Africa showed *G. brevipalpis* to be restricted to two distinct bands in the north and south of north eastern KwaZulu-Natal (Du Toit, 1954; Kappmeier Green, 2002). As indicated in Chapter 2, low numbers of *G. brevipalpis* were recently found in at least five sites between these two defined bands. It has now become evident that *G. brevipalpis,* as well as *G. austeni*, is distributed more or less continuously throughout the area. However, due to the relative low abundance of *G. brevipalpis* in the Ubombo district (Table 2.1; Fig. 2.5 A) it might well be that the two pockets found in the north and south of the tsetse fly infected area could be both geographically and genetically isolated. The very low abundance of *G. austeni* and their relatively sedentary behaviour as well as their association with specific vegetation types (Chapter 2) that have a patchy distribution in the target area (Esterhuizen, 2007) may favour the creation of genetically isolated localised populations. Despite the fact that *G. austeni*, and perhaps also *G. brevipalpis*, are distributed throughout north eastern KwaZulu-Natal, populations are restricted to pockets of dense vegetation (Esterhuizen, 2007). It has been shown that habitat fragmentation creates conditions to which tsetse fly populations respond physiologically and demographically thereby affecting tsetse-trypanosome interactions and hence influencing Trypanosomosis risk (Mweempwa *et al.*, 2015).

Molecular and/or morphometric markers can be used to determine possible gene flow between subpopulations as an indirect measure of fly dispersal (Solano *et al.*, 2000; Gooding & Krafsur, 2005; Camara *et al.*, 2006; Bouyer *et al.*, 2007; 2009; Solano *et al.*, 2009). Geometric wing morphometry has proven to be a reliable and inexpensive alternative to the more expensive techniques of DNA-sequence analysis (Patterson & Schofield, 2005; Kaba *et al.*, 2012). Using geometric wing morphometry as well as microsatellite markers, the *Glossina palpalis gambiensis* populations that inhabit the Loos Islands 5 km off the coast of mainland Guinea, were shown to be genetically isolated from two sites in mainland Guinea (Camara *et al.*, 2006). Another example is the

G. p. gambiensis population of the Niayes in Senegal that was shown to be isolated from the main tsetse fly belt 120 km away in the eastern part of the country (Solano *et al.*, 2010).

Geometric morphometrics is a simple and cost effective tool that can easily be used in any laboratory to study tsetse fly population structure (Patterson & Schofield, 2005). Some authors , *e.g.* Solano *et al.* (2010) have indicated that genetic analysis is more reliable than morphometrics, as the correlation between these two techniques is only 0.5 and that morphometric analysis is probably influenced by environmental factors. It is hypothesised that morphometrics can be used as an easy first level of screening for possible population fragmentation as no specialised equipment is needed for this analysis. In the present study, the applicability of phenetic (geometric morphometrics) markers to differentiate between the two tsetse species found in South Africa was determined. Geometric morphometrics were subsequently used to assess the extent of the potential genetic isolation between the different populations of *G. brevipalpis* and *G. austeni*. The possible influence of seasonal variation on the reliability of geometric morphometrics was also determined. In addition, the South African populations were compared with flies collected in the neighbouring Mozambique and Swaziland. Lastly, these wild populations were compared with laboratory colonies maintained at the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI), established from seed material from Tanzania and Kenya respectively. The level of genetic isolation, indicated by the phonetic markers, between these different populations within South Africa and between the three countries will indicate the invasive potential of flies from neighbouring areas into the controlled or tsetse fly free areas and will facilitate in selecting the most appropriate control strategy.

3.2   Materials and methods

3.2.1   Study sites and fly collection

Glossina brevipalpis and *G. austeni* were collected from July 2008 to September 2009 at 10 sites in South Africa and at one site each in southern Mozambique and Swaziland (Fig. 3.1) with odour-baited H traps as described in Chapter 2. In Swaziland the site (# 1) was in the Mlawula Nature Reserve (Fig. 3.1) and the vegetation consists mainly of shrubs and herbaceous savannah with very small patches of dry forest and savannah woodland (Bouyer & Guerrini, 2010). In southern Mozambique, the site (# 2) was in the Reserva Especial de Maputo (Fig. 3.1) where the vegetation was mainly dry forest and shrub savannah with small patches of swamp and herbaceous savannah (Bouyer & Guerrini, 2010). Sites three to twelve were in South Africa (Fig. 3.1) and are described in detail in Chapter 2. At three of these sites *G. brevipalpis* (Ndumu (# 3) and St Lucia (# 12)) and *G. austeni* (Phinda (# 8) and St Lucia (# 12)) was collected monthly to assess the effect of

seasonal variation on geometric morphometrics results. Flies collected from June to August were grouped as the winter collection, from September to November as spring, from December to February as summer and from March to May as an autumn collection.
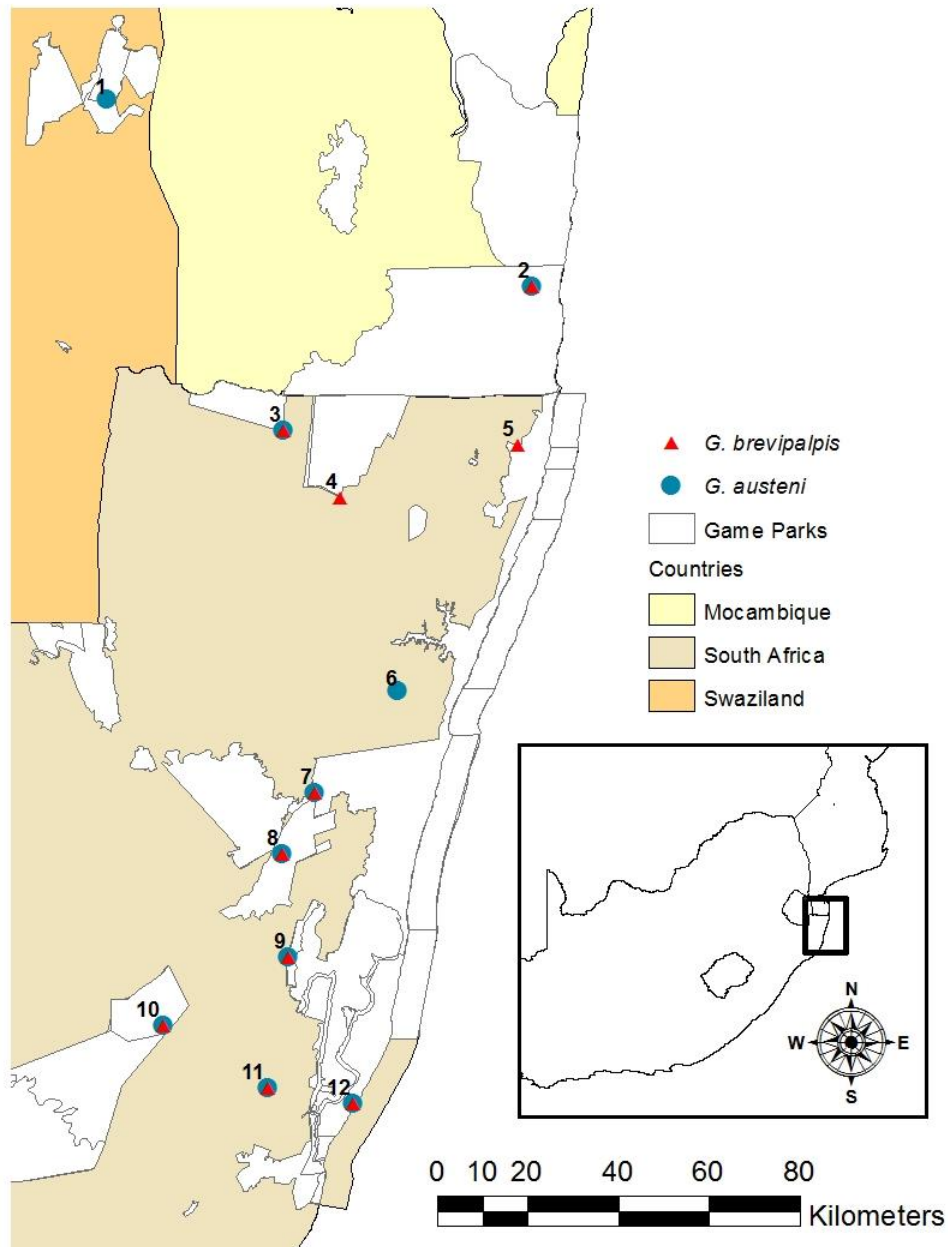
Fig. 3.1. Sites where *Glossina brevipalpis* and *Glossina austeni* were collected with odour-baited H traps for comparative geometric morphometrics in Swaziland (1: Mlawula Nature Reserve), Mozambique (2: Reserva Especial de Maputo) and South Africa (3: Ndumu; 4: Tembe, 5: Kosi Bay, 6: Mbazana, 7: Lower Mkhuze, 8: Phinda, 9: False Bay, 10: Hluhluwe-iMfolozi Park, 11: Boomerang, 12: St Lucia).

The geographical distances between sites ranged from 228 km (Mlawula Nature Reserve and St Lucia) to 35 km (Lower Mkhuze and Phinda) (Fig. 3.1). Geographic barriers can promote genetic isolation, e.g. the Lebombo Mountain range between Swaziland and Mozambique and Lake St Lucia between the sites at St Lucia and Boomerang (Fig. 3.1).

The study area has a subtropical climate as described in Chapter 2. Most of the rain is received in the hot season from October to March. However, it also rains during the cold, dry season from April to September. The coastal areas receive on average more rain than the interior.

Flies from laboratory colonies of *G. brevipalpis* and *G. austeni* were compared to field collected flies. The colony flies were maintained under standard colony conditions (23-24 °C, 75-80% RH and subdued/indirect lighting, 12 h light/12 h dark) (Feldmann, 1994a; FAO/IAEA standard operating procedures, 2006).

3.2.2 Morphometric analysis

For morphometrics analysis tsetse fly wings were removed and dry mounted between two microscope slides (Patterson & Schofield, 2005). The wings were photographed using a Dino X Lite Digital Microscope.

Firstly, a comparison between the two South African species was done by comparing the right wings of female *G. brevipalpis* and *G. austeni*. Secondly, the potential seasonal effects and sexual dimorphism were investigated using the right and left wings of both sexes. Finally, to assess the degree of isolation of the different populations, only the right wings of female flies were used.

Nine landmarks (Cartesian coordinates) were defined by vein intersections (Fig. 3.2) and digitised using the COO program of the CLIC software package (Dujardin *et al.*, 2010). All further analyses on the Cartesian coordinates were done using the MorphoJ integrated software package (Klingenberg, 2011).
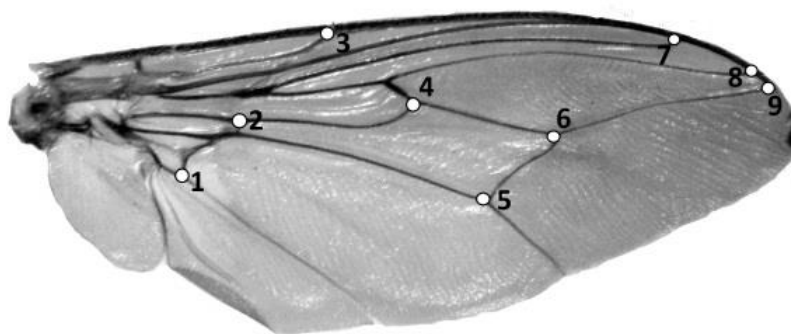
Fig. 3.2. *Glossina austeni* wing indicating the nine landmarks as defined by vein intersections.

The Cartesian coordinates were subjected to a generalized Procrustes analysis (Rohlf, 1996), and variations in wing shape (partial warps) were determined by Procrustes superposition through generalized least squares (Rohlf, 1999). Centroid size was determined by the square root of the sum of the squared distances of all landmarks from the centroid (Bookstein, 1991). A frequently used insect body size estimator is the length of the wing along its largest axis. Furthermore, the relationship between centroid size and the traditional wing length showed good correlation (Dujardin, 2011). Centroid size was thus used as an estimator of tsetse fly body size. Centroid size was analysed using the statistical software GraphPadInstat (version 3.00, 2003). To differentiate between the centroid size of the two species an unpaired test was done with a Welch correction that provided a two-tailed P-value. Additionally, ANOVA was used to assess significant differences between the means of the centroid size (P values < 0.05 were considered significant) of the wings from flies collected seasonally at the various sites in the study area. As data were normally distributed standard (parametric) methods were used, Tukey's test was applied. Principal component analyses of the shape variables were done using MorphoJ (Klingenberg, 2011) and provided 14 partial warps. Multivariate regression of partial warps on size was used to estimate the residual allometry, and the statistical significance estimated by 10 000-runs permutation tests (Klingenberg, 2011). Differences in wing shape were determined by Canonical shape dissimilarity. Linear regression was used to determine both Mahalanobis distances and geographic distances. The Mahalanobis distances (Mahalanobis,1936) were examined for significance by the permutation test (10 000 runs).

3.3   Results

3.3.1   Species differentiation

The right wings of 345 female *G. brevipalpis* and 346 female *G. austeni* (Table 3.3) were analysed for size and shape differences. There was a significant difference (P < 0.01) between the average wing centroid size of *G. austeni* (924 ± 31) and *G. brevipalpis* (1537 ± 47) (Table 3.3), the latter being much larger (Figs. 3.3, 3.4, 3.7 & 3.10). The first discriminant factor (shape components) accounted for 80% of the variance which clearly indicated a separation of the shape of the wings of *G. brevipalpis* and *G. austeni* (Fig. 3.3).

This provided sufficient evidence that geometric wing morphometry would differentiate between two species one of which belonged to the fusca group (*G. brevipalpis)* and the other to the morsitans group (*G. austeni*). The multivariate regression of the first relative warp against centroid size (Fig. 3.3) (100 000 permutation rounds) was significant (P = 0.03), which indicates that there is an allometric effect (*i.e.* the size influences the

shape in a significant way) when comparing the wing morphometric of *G. brevipalpis* and *G. austeni.*
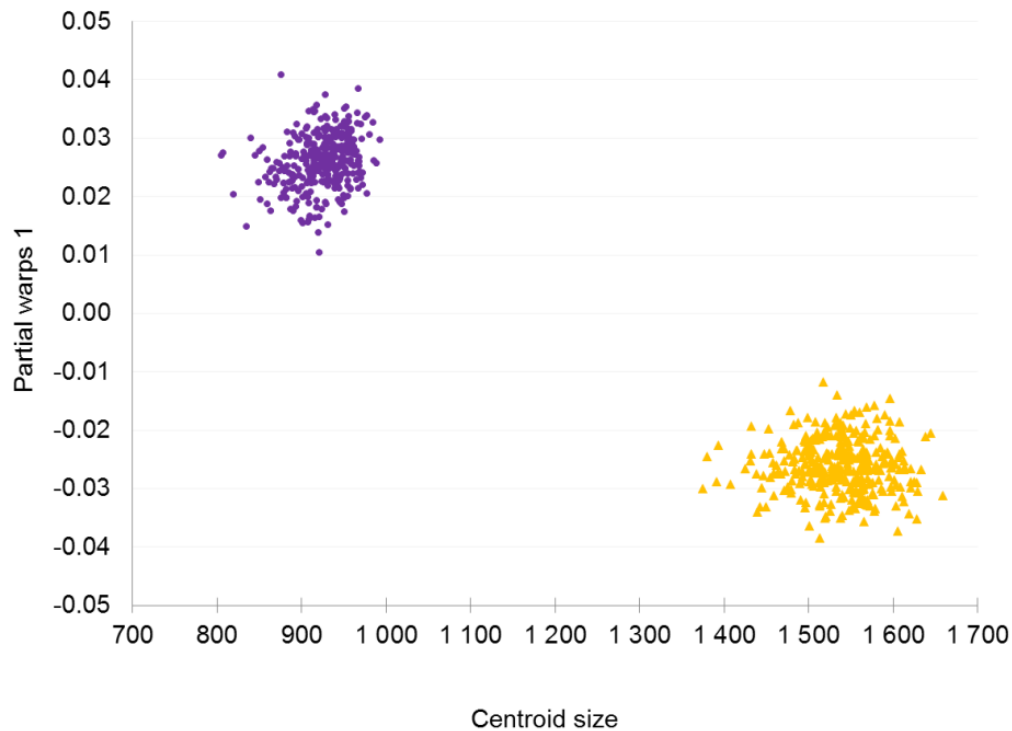
Fig. 3.3. Multivariate regression of the first partial warp (derived from the shape of the wing) on centroid size of the right wings of female *Glossina brevipalpis* (yellow triangles) and *Glossina austeni* (purple circles). The first partial warp represents 80% of the total discrimination.

3.3.2 Seasonal effects and sexual dimorphism

Seasonal variation and sexual dimorphism in centroid size and shape of the two species was determined at Ndumu, Phinda and St Lucia. *Glossina brevipalpis* was collected from Nduma and St Lucia and *G. austeni* from Phinda and St Lucia. The wings were evaluated separately for each species, site and sex. The allometric effect was removed so that size and shape could be analysed separately. Table 3.1 summarises the collection dates as well as the number of female/male and right/left wings analysed.

3.3.2.1 Centroid size variation

A seasonal effect on the wing centroid size of males and females of both species was observed. At Ndumu, on average the smallest centroid size for males were collected in spring (1346 ± 50) and the largest (1409 ± 23) in autumn. There were significant differences

in the average centroid size of *G. brevipalpis* males collected in winter (1380 ± 37) versus those collected in spring (1346 ± 50) ($P < 0.01$) and autumn (1409 ± 23) ($P < 0.05$). Significant differences were also found in the average centroid size for males collected in spring (1346 ± 50) versus summer (1367 ± 35) ($P < 0.05$) and autumn (1409 ± 23) ($P < 0.01$). The average male centroid size was also different between summer (1367 ± 35) versus autumn (1409 ± 23) ($P < 0.01$) (Fig. 3.4 A).

The centroid size of *G. brevipalpis* females at Ndumu also differed seasonally. *Glossina brevipalpis* females with the smallest average centroid size were collected in the spring (1461 ± 80) and the largest (1541 ± 43) in autumn. The average centroid size for females collected in spring were significantly smaller from those collected in all other seasons ($P < 0.01$ in all cases) (Fig. 3.4 A).

At the second *G. brevipalpis* collection site, St Lucia, the males with the smallest average wing centroid size were from spring (1362 ± 46) and the largest (1449 ± 66) from winter (Table 3.1; Fig. 3.4 A). There were also significant differences in the centroid size of males collected in winter (1449 ± 66) versus spring (1362 ± 46), summer (1388 ± 31) and autumn (1397 ± 28) ($P < 0.01$ in all cases) as well as spring versus summer ($P < 0.05$) and spring versus autumn ($P < 0.01$) (Fig. 3.4 A).

For the *G. brevipalpis* females collected at St Lucia the average female *G. brevipalpis* wing centroid size ranged from 1514 ± 64 in spring to 1585 ± 43 in autumn (Table 3.1; Fig. 3.4 A). There were significant differences in the average centroid size between autumn (1586 ± 43) versus winter (1558 ± 64) ($P < 0.05$), spring (1514 ± 64) and summer (1539 ± 49) ($P < 0.01$), as well as winter versus spring ($P < 0.01$) (Fig. 3.4 A).

A similar seasonal effect on the wing centroid size was observed for *G. austeni*. At Phinda the average wing centroid size for *G. austeni* males ranged from 860 ± 20 in autumn to 892 ± 19 in winter and the males collected in winter was significantly smaller from those of summer ($P = 0.01$) (Table 3.1; Fig. 3.4 B).

For *G. austeni* females the average wing centroid size ranged from 911 ± 29 in autumn to 941 ± 21 in spring and was significantly different in females collected in winter (939 ± 25) versus summer (919 ± 21) and autumn (911 ± 29) also spring (941 ± 21) versus summer and autumn ($P < 0.01$ in all cases) (Table 3.1; Fig. 3.4 B).

At the second *G. austeni* collection site, St Lucia, the male wing centroid size ranged from 850 ± 16 in spring to 885 ± 28 in winter. (Table 3.1; Fig. 3.4 B). There were significant differences in the average wing centroid size of males collected in winter (885 ± 28) versus spring (850 ± 16) as well as those of spring versus autumn (860 ± 20) ($P < 0.05$ in both cases).

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The female wing centroid size ranged between 932 ± 20 in summer and 946 ± 29 in winter and there were also significant differences in the centroid size of females collected in winter (946 ± 29) compared with summer (932 ± 20) and autumn (933 ± 26) ($P < 0.05$) (Fig. 3.4 B).

There was a strong sexual dimorphism for both *G. brevipalpis* and *G. austeni*. The average wing centroid size of males was significantly smaller than that of females in all seasons for both species ($P < 0.01$) (Fig. 3.4 A, B).

The average centroid size of male *G. brevipalpis* ranged from 1346 ± 50 at Ndumu in the interior to 1449 ± 65 for males at St Lucia on the coast (Table 3.1). Except for autumn the average centroid size of males from Ndumu were on average smaller than the males from St Lucia, especially in winter ($P < 0.01$). Similarly, the average centroid size of females from Ndumu (1461 ± 80) were on average significantly smaller than those from St Lucia (1586 ± 43) ($P < 0.01$) except in summer (Table 3.1; Fig. 3.4 A).

These trends were not observed for *G. austeni* and for both males ($P = 0.10$) and females ($P = 0.09$). There were no significant size differences in the average centroid size between flies of Phinda and St Lucia. The average wing centroid size of males from St Lucia (850 ± 16) was slightly smaller than that of those collected at Phinda (892 ± 19) (Table 3.1; Fig. 3.4 B). The *G. austeni* females collected at St Lucia (942 ± 29) were on average marginally bigger than those of Phinda especially in autumn (911 ± 29) (Table 3.1; Fig. 3.4 B).

For both species, the variation in centroid size between localities was similar to the seasonal variation in centroid size at each of the sites (Table 3.1, Fig. 3.4 B).

Table 3.1. Summary of number of *Glossina brevipalpis* and *Glossina austeni* wings analysed in the seasonal and sexual dimorphism geometric morphometric analysis. Multivariate regression of partial warps on size, statistical significance estimated by 10 000-runs permutation tests.

	Collection Date	No. of wings analysed				Shape component variation (%)				Average Centroid Size (± SD)		Multivariate regression (10 000 runs, *P* value)	
		Female		Male		Female		Male					
		Left	Right	Left	Right	Canonical Variate 1	Canonical Variate 2	Canonical Variate 1	Canonical Variate 2	Female	Male	Female	Male
G. brevipalpis													
Ndumu													
Winter	Jul'08 - Aug'08	30	30	30	30					1532 (42)	1380 (37)		
Spring	Oct'08	30	30	30	30	76	14	74	16	1461 (80)	1346 (50)	0.004	<0.001
Summer	Dec'08 - Jan'09	30	30	19	20					1539 (30)	1367 (35)		
Autumn	Mar'09	10	10	40	40					1541 (43)	1409 (23)		
St Lucia													
Winter	Jul'08	30	30	30	29					1558 (64)	1449 (66)		
Spring	Aug'08	28	29	30	30	75	18	64	25	1514 (64)	1362 (46)	0.008	0.061
Summer	Dec'08 - Feb'09	30	29	30	30					1539 (49)	1388 (31)		
Autumn	Apr'09	29	29	30	30					1586 (43)	1397 (28)		
G. austeni													
Phinda													
Winter	Jul'08 - Aug'08	30	30	9	8					939 (25)	892 (19)		
Spring	Sep'08 - Nov'08	30	30	29	29	64	27	55	28	941 (21)	872 (37)	0.006	0.091
Summer	Dec'08 - Feb'09	21	20	20	20					919 (21)	864 (23)		
Autumn	Mar'09	9	9	3	3					911 (29)	860 (20)		
St Lucia													
Winter	Jun'09 - Aug'09	30	30	30	30					946 (29)	885 (28)		
Spring	Sep'08 - Nov'08	30	30	4	4	69	17	66	29	938 (21)	850 (16)	0.625	<0.001
Summer	Dec'08 - Feb'09	29	30	9	9					932 (20)	870 (12)		
Autumn	Mar'09 - May'09	31	30	31	31					933 (26)	883 (35)		
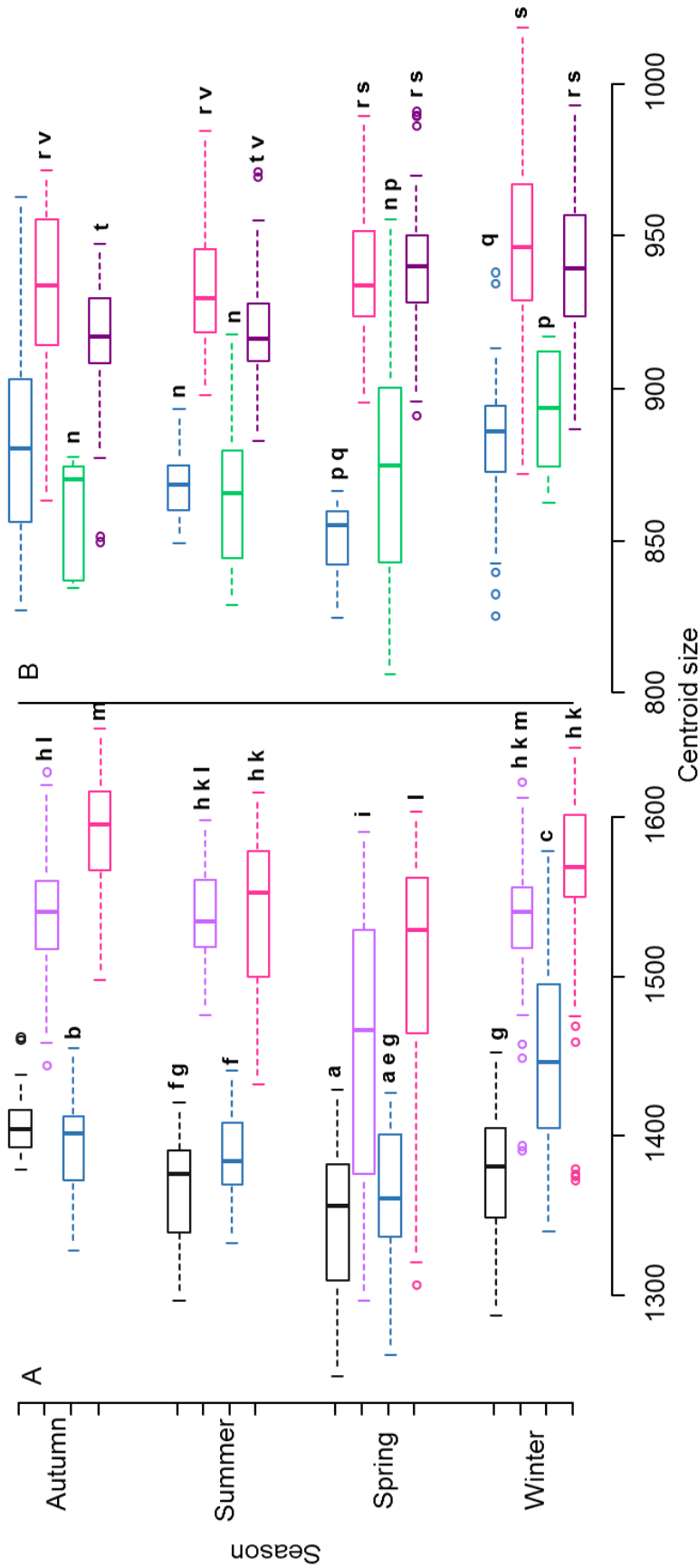
Fig. 3.4. Centroid size variations of the wings of male and female *Glossina brevipalpis* (A) and *Glossina austeni* (B) according to season. Each box shows the group median separating the 25th and 75th quartiles, capped bars indicate maximum and minimum values, circles indicating the outliers. (**Black:** Ndumu *G. brevipalpis* males; **Light purple:** Ndumu *G. brevipalpis* females; **Dark blue:** St Lucia *G. austeni* (A) or *G. brevipalpis* (B) males; **Dark pink:** St Lucia *G. brevipalpis* (A) or *G. austeni* (B) females;**Green:** Phinda *G. austeni* males; **Dark purple:** Phinda *G. austeni* females). Boxes followed by a different letter indicate that the sizes were significantly different at the 5% level.

3.3.2.2   Shape variation

The first two discriminant factors (shape components) in all the analyses (species, site and sex) accounted for more than 83% of the variance (Table 3.1). The multivariate regression of the first relative warp against centroid size (100 000 permutation rounds) was significant (Table 3.1) in all the analyses except for *G. brevipalpis* males ($P = 0.06$) and *G. austeni* females ($P = 0.62$) collected at St Lucia as well as *G. austeni* males ($P = 0.09$) from Phinda (Table 3.1). This indicates an allometric effect which could be due to seasonal influences but the overall low numbers of *G. brevipalpis* and *G. austeni* collected in selected seasons (Table 3.1) could have amplified the allometric effect.

Although there was no significant shape separation between sites as well as season for both species some tendencies were observed. The first discriminant factor for *G. brevipalpis* at Ndumu for both males (Fig. 3.5 C) and females (Fig. 3.5 A) indicated a shape change between flies collected in autumn versus spring; this trend was absent in the *G. brevipalpis* collected from St Lucia (Fig. 3.5 B, D). Similar shape changes of the first discriminant factor between flies collected in autumn versus spring were also observed for *G. austeni* males (Fig. 3.6 C) and females (Fig. 3.6 A) from Phinda as well as for males (Fig. 3.6 B) from St Lucia. Furthermore, a second shape change of the first discriminant factor of *G. austeni* males collected at Phinda in winter versus autumn was observed (Fig. 3.6 C) but the more pronounced autumn separation at Phinda was most likely due to the very low sample size (Table 3.1). No shape separations of both the first and second discriminant factor were observed for *G. austeni* females collected at St Lucia (Fig. 3.6 B).

Comparisons of spatial versus temporal shape differences at these three sites indicated that Mahalanobis distances for *G. brevipalpis* wings were similar or greater between seasons as compared with between sites (Table 3.2). The Mahalanobis distances between Ndumu and St Lucia was 1.28 (Table 3.2). At Ndumu the Mahalanobis distances between seasons ranged from 1.01 (summer versus autumn) to 2.67 (spring versus autumn) (Table 3.2). At St Lucia it ranged from 0.70 (summer versus autumn) to 1.88 (spring versus autumn). The Procrustes distances showed the same pattern (Table 3.2). For *G. austeni* similar results were seen, but, to a lesser extent. Mahalanobis distances were greater between seasons *e.g.* winter versus autumn (1.90), spring versus autumn (2.24) at Phinda and winter versus spring (1.82), spring versus autumn (1.76) at St Lucia than between Phinda versus St Lucia (1.47). The Procrustes distances had similar results (Table 3.2).
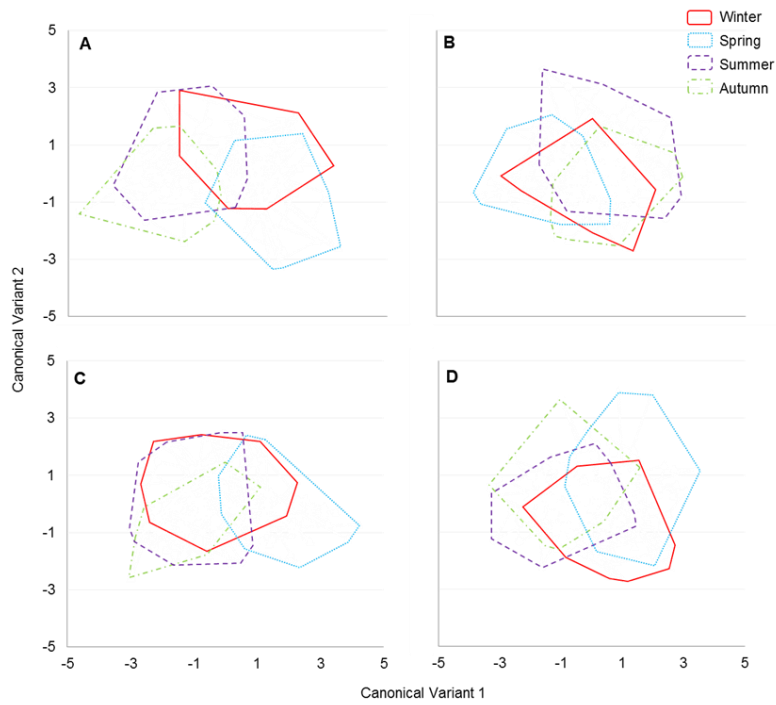
Fig. 3.5. The distribution of *Glossina brevipalpis* female (A, B) and male's (C, D) wing shape in the morhospace defined by the first two Canonical variants, flies were collected from Ndumu (A, C) and St Lucia (B, D).
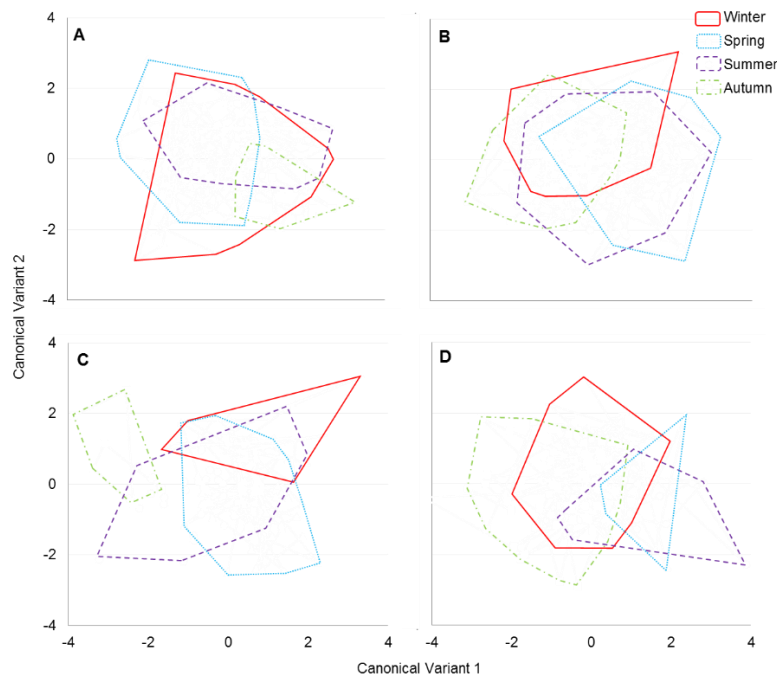
Fig. 3.6. The distribution of *Glossina austeni* female (A, B) and male's (C, D) wing shape in the morhospace defined by the first two Canonical variants, flies were collected from Phinda (A, C) and St Lucia (B, D).

Table 3.2. Summary of the Mahalanobis and Procrustes distances for the spatial and temporal wing shape changes in *Glossina brevipalpis* and *Glossina austeni.*

	Shape component variation (%)		Mahalanobis distances (P < 0.01)	Procrustes distances
	Canonical Variate 2	Canonical Variate 2		
G. brevipalpis				
Spatial				
Ndumu vs. St Lucia	100	0	1.28	0.004
Temporal				
Ndumu				
Winter vs. Spring			1.31	0.005
Winter vs. Summer			1.46	0.005
Winter vs. Autumn	78	17	2.20	0.007
Spring vs. Summer			2.13	0.009
Spring vs. Autumn			2.67	0.010
Summer vs. Autumn			1.01	0.003
St Lucia				
Winter vs. Spring			1.41	0.005
Winter vs. Summer			1.13	0.005
Winter vs. Autumn	74	18	1.14	0.004
Spring vs. Summer			1.82	0.008
Spring vs. Autumn			1.88	0.007
Summer vs. Autumn			0.70	0.003
G. austeni				
Spatial				
Phinda vs. St Lucia	100	0	1.47	0.005
Temporal				
Phinda				
Winter vs. Spring			0.94	0.003
Winter vs. Summer			1.02	0.004
Winter vs. Autumn	65	21	1.90	0.009
Spring vs. Summer			1.11	0.004
Spring vs. Autumn			2.24	0.010
Summer vs. Autumn			1.63	0.008
St Lucia				
Winter vs. Spring			1.82	0.006
Winter vs. Summer			1.31	0.005
Winter vs. Autumn	70	18	0.89	0.004
Spring vs. Summer			1.13	0.002
Spring vs. Autumn			1.76	0.005
Summer vs. Autumn			1.22	0.004

3.3.3 Population isolation

Right wing size and shape of females for both species were analysed to assess the level of genetic isolation between populations in South Africa as well as between them and those populations of southern Mozambique and the *G. austeni* population in Swaziland. Table 3.3 summarises the sites and dates that flies were collected and the number of female right wings analysed. The allometric effect was removed so that size and shape could be analysed independently.

3.3.3.1 Centroid size

The average female wing centroid size for *G. brevipalpis* ranged from 1512 ± 35 collected in Hluhluwe-iMfolozi Park to 1568 ± 35 from St Lucia (Table 3.3; Fig. 3.7 A). Significant differences were found between the populations of the Hluhluwe-iMfolozi Park (1512 ± 35) and southern Mozambique (1550 ± 69) ($P < 0.01$), Ndumu (1544 ± 38) ($P < 0.05$), Kosi Bay (1549 ± 41) ($P < 0.05$) and St Lucia (1568 ± 35) ($P < 0.01$) (Fig. 3.7 A). Significant

differences were also seen between the *G. brevipalpis* population collected in southern Mozambique (1550 ± 69) and those of Boomerang (1518 ± 45) ($P < 0.05$), Tembe (1520 ± 40) versus St Lucia (1568 ± 35) ($P < 0.01$) and Boomerang versus St Lucia ($P < 0.01$) (Fig. 3.7 A).

The average female wing centroid size of *G. austeni* ranged from 892 ± 37 collected in Swaziland to 940 ± 52 from St Lucia (Table 3.3; Fig. 3.7 A). The average wing centroid size for flies from Swaziland (892 ± 37) was significantly smaller ($P < 0.01$) from that of the flies from southern Mozambique as well as the flies from South Africa (Fig. 3.7 B). The only other significant difference ($P < 0.01$) in wing centroid size was observed between female *G. austeni* from Phinda (916 ± 26) and St Lucia (941 ± 52) (Table 3.3; Fig. 3.7 B).

3.3.3.2   Shape variation

The two species were evaluated separately and the first two discriminant factors (shape components) accounted for 71% and 69% of the variance for *G. brevipalpis* and *G. austeni*, respectively.

These discriminant factors indicated that there was no clear shape separation between the *G. brevipalpis* collected from the sites in South Africa and southern Mozambique (Fig. 3.8 A). The multivariate regression of the first relative warp against centroid size (100 000 permutation rounds) was also not significant ($P = 0.30$) indicating that there was no residual allometry.

In contrast the multivariate regression was significant ($P < 0.01$) for *G. austeni.* The same no shape separation was observed for *G. austeni* collected from the different sites in South Africa as well as the *G. austeni* collected from southern Mozambique (Fig. 3.8 B). The only separation within the first discriminant factors was between *G. austeni* from Swaziland and St Lucia. These two sites were 228 km apart and were the two most geographically distant sites (Fig. 3.1). A linear regression of Mahalanobis distances derived from shape variation and geographical distances for both *G. brevipalpis* ($r^2 = 0.51$, $P < 0.01$) (Fig. 3.9 A) and *G. austeni* ($r^2 = 0.65$, $P < 0.01$) (Fig. 3.9 B) was significant, which suggests a process of genetic isolation by geographic distance.
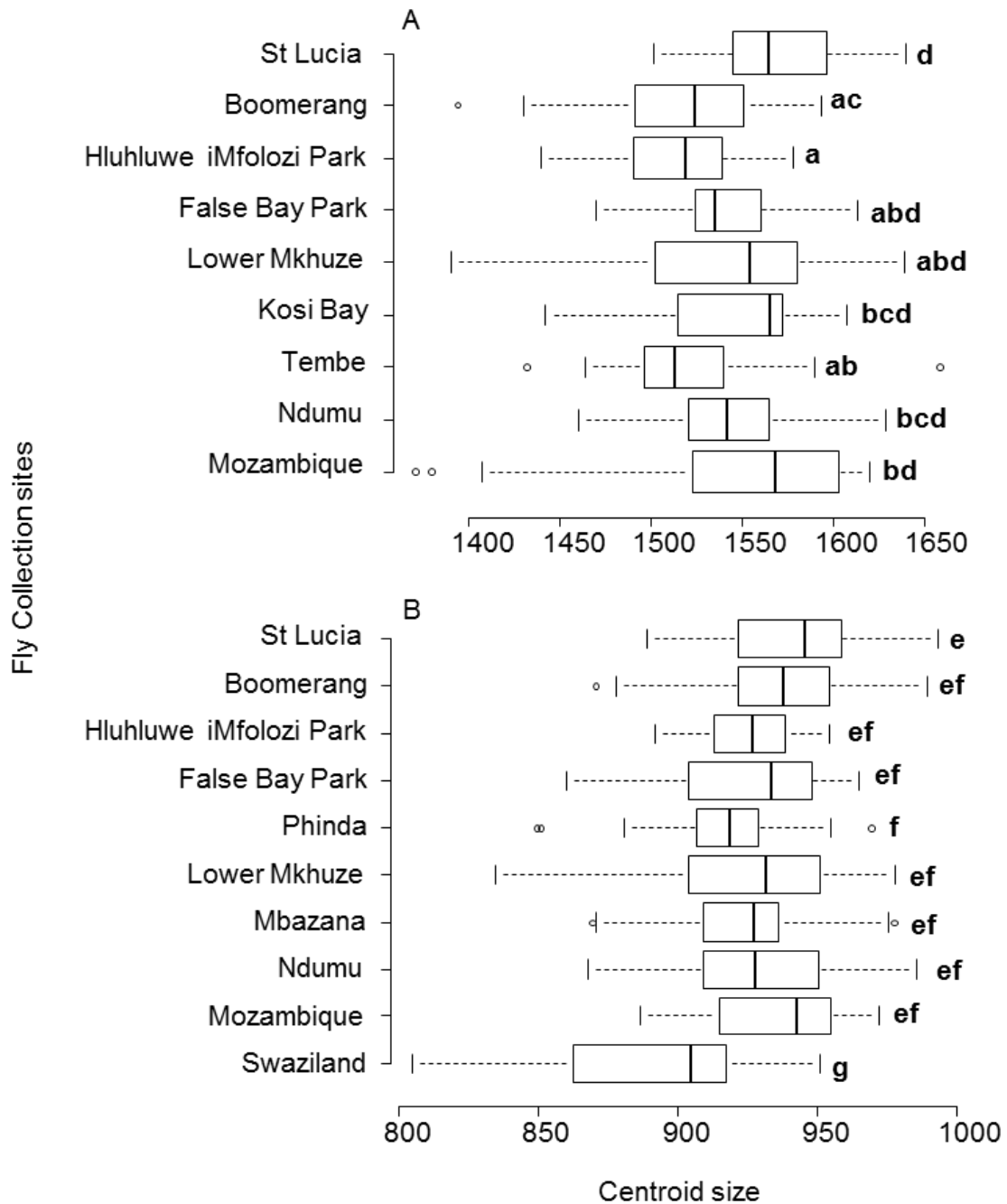
Fig. 3.7. Centroid size variations of the right wings of female *Glossina brevipalpis* (A) and *Glossina austeni* (B) according to localities. Each box shows the group median separating the 25th and 75th quartiles, capped bars indicate maximum and minimum values, circles indicating the outliers. Boxes followed by a different letter indicate that the sizes were significantly different at the 5% level.

Table 3.3. Summary of *Glossina brevipalpis* and *Glossina austeni* wings used in the phenetic geometric morphometric analysis.

	Date	No. female right wings used	Shape component variation (%)		Average Centroid Size (± SD)	Multivariate regression (10 000 runs, *P* value)
			Canonical Variate 1	Canonical Variate 2		
G. brevipalpis		**345**				
Mozambique						
Reserva Especial de Maputo	Aug '09 – Sep '09	36			1550 (±69)	
South Africa						
Ndumo	Nov '08 – Mar '09	40			1544 (± 38)	
Tembe	Sep '08 – Oct '08	40			1520 (± 40)	
Kosi Bay	Jul '08 – Mar '09	31	47	24	1549 (±41)	0.303
Lower Mkhuze	Sep '08 – Apr '09	40			1542 (± 56)	
False Bay Park	Nov '08 – Mar '09	37			1540 (± 33)	
Hluhluwe-iMfolozi Park	Nov '08 – Mar '09	41			1512 (± 35)	
Boomerang	Nov '08 – Mar '09	40			1518 (± 45)	
St Lucia	Feb '09 – Mar '09	40			1568 (± 35)	
G. austeni		**346**				
Swaziland						
Mlawula Nature Reserve	Apr '09	40			892 (± 37)	
Mozambique						
Reserva Especial de Maputo	Aug '09 – Sep '09	14			934 (± 28)	
South Africa						
Ndumo	Sep '08 – Mar '09	40			929 (±28)	
Mbazana	Aug '08 – Mar '09	40	52	17	925 (±25)	0.001
Lower Mkhuze	Aug '08 – Mar '09	40			926 (± 33)	
Phinda	Nov '08 – Mar '09	40			916 (± 26)	
False Bay Park	Jan '09 – Mar '09	40			924 (± 30)	
Hluhluwe-iMfolozi Park	Nov '08 – Mar '09	13			925 (± 19)	
Boomerang	Nov '08 – Feb '09	39			935 (± 26)	
St Lucia	Mar '09 – Apr '09	40			940 (± 52)	
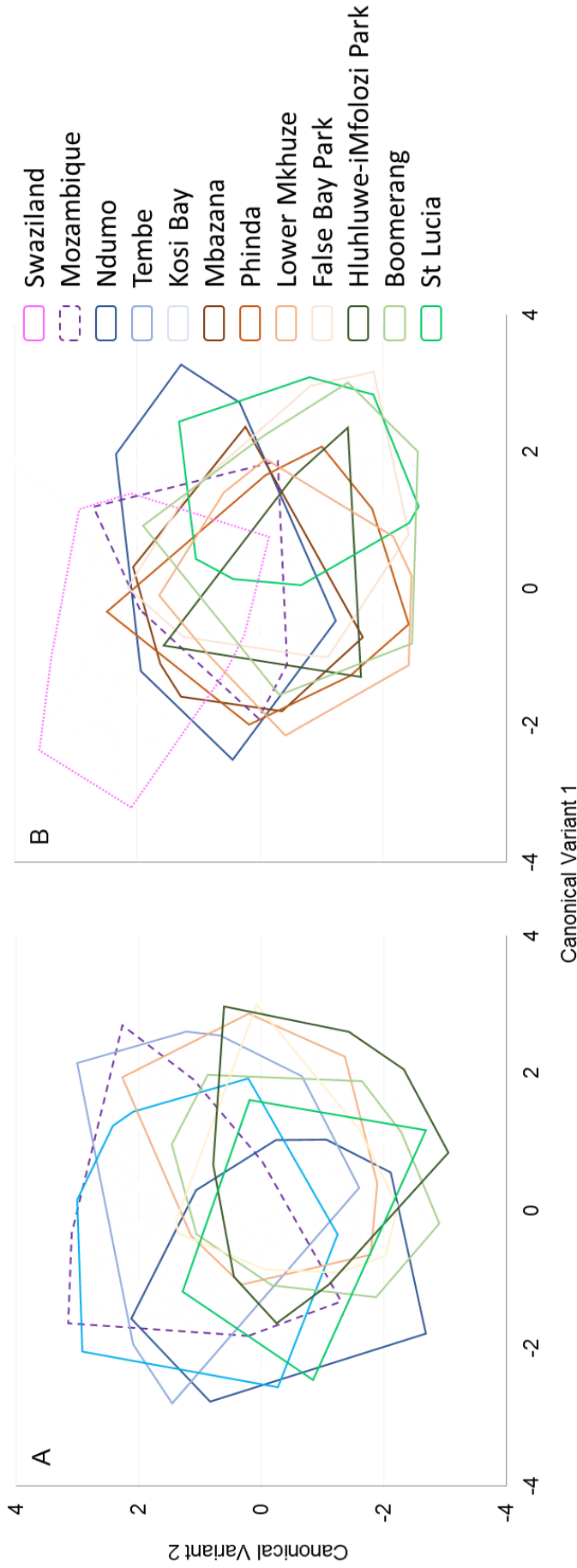
Fig. 3.8. The distribution of *Glossina brevipalpis* (A) and *Glossina austeni* (B) female right wing shape in the morhospace defined by the first two Canonical variants.
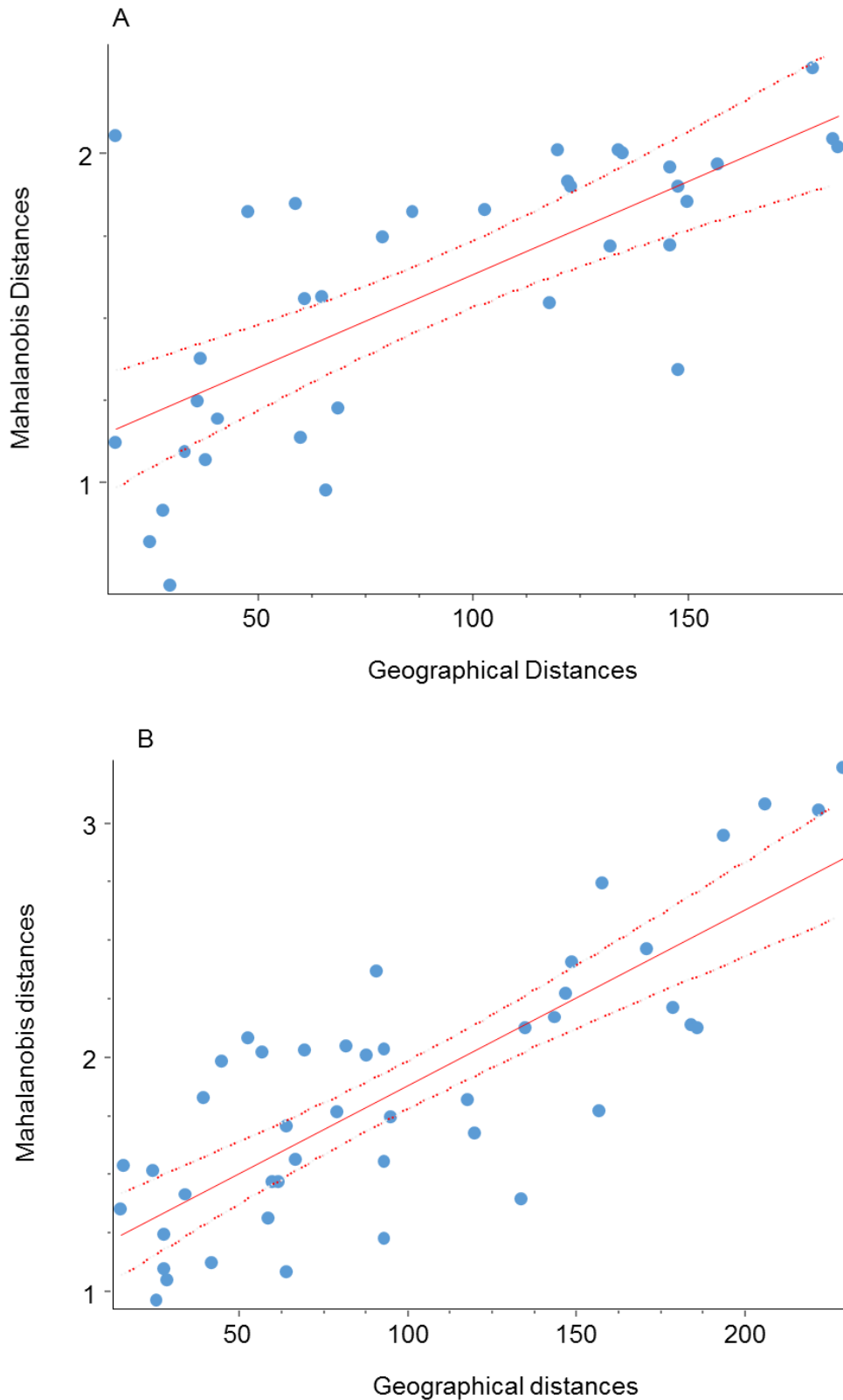
Fig. 3.9. Linear regression of the Mahalanobis distances of *Glossina brevipalpis* (A) and *Glossina austeni* (B) compared with geographic distances (km) between their collection sites (Solid red line represents the linear regression, broken red line the 95% confidence interval).

3.3.4   Field flies compared with colony flies

Female *G. brevipalpis* (St Lucia (40), Lower Mkuze (40), Mozambique (36)) and *G. austeni* (St Lucia (40), Lower Mkuze (40), Mozambique (14), Swaziland (40)) from the study area (Table 3.3) and colonised *G. brevipalpis* (34) and *G. austeni* (39) wings were analysed for size and shape differences.

Colonised female wing centroid size of *G. brevipalpis* ($P < 0.01$) and *G. austeni* ($P < 0.01$) were significantly smaller from those of field collected flies (Fig. 3.10 A, B). The colony flies were on average much smaller than the field collected flies (Fig. 3.10 B).

The first two discriminant factors (shape components) accounted for 73% and 76% of the variance for *G. brevipalpis* and *G. austeni,* respectively. The multivariate regression of the first relative warp against centroid size (100 000 permutation rounds) was not significant for *G. brevipalpis* ($P = 0.08$) and was significant for *G. austeni* ($P = 0.03$).

For *G. brevipalpis* the first discriminant factor indicated a shape separation between colony and field flies (Fig. 3.11 A). The same shape separation between colony and field flies was also seen in *G. austeni,* however, this was more pronounced. There was also a separation indicated by the second discriminant factor between colonised *G. austeni* and flies from Swaziland (Fig. 3.11 B).
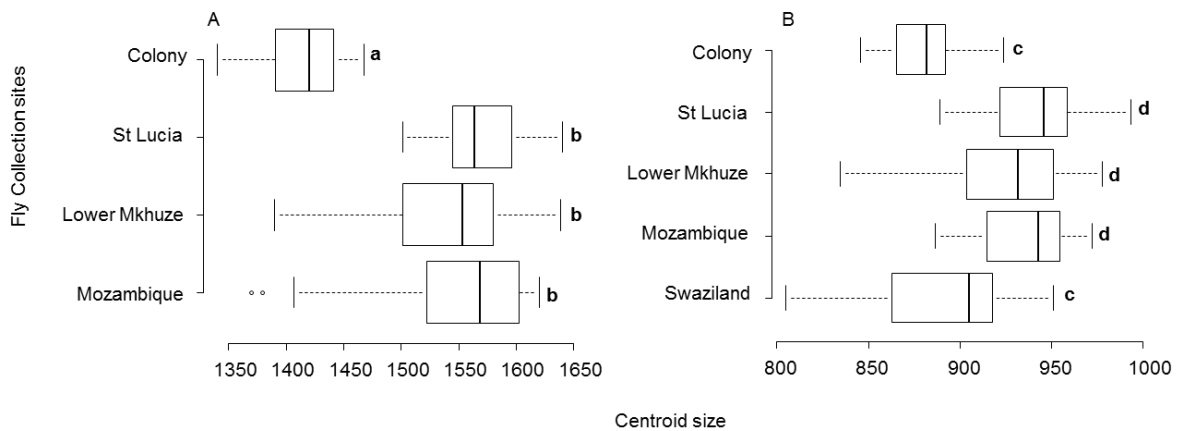
Fig. 3.10. Variations in centroid size of the right wings of female *Glossina brevipalpis* (A) and *Glossina austeni* (B) according to localities, as well as colony reared flies. Each box shows the group median separating the 25th and 75th quartiles, capped bars indicate maximum and minimum values, circles indicating the outliers. Boxes followed by a different letter indicate that the sizes were significantly different at the 5% level.
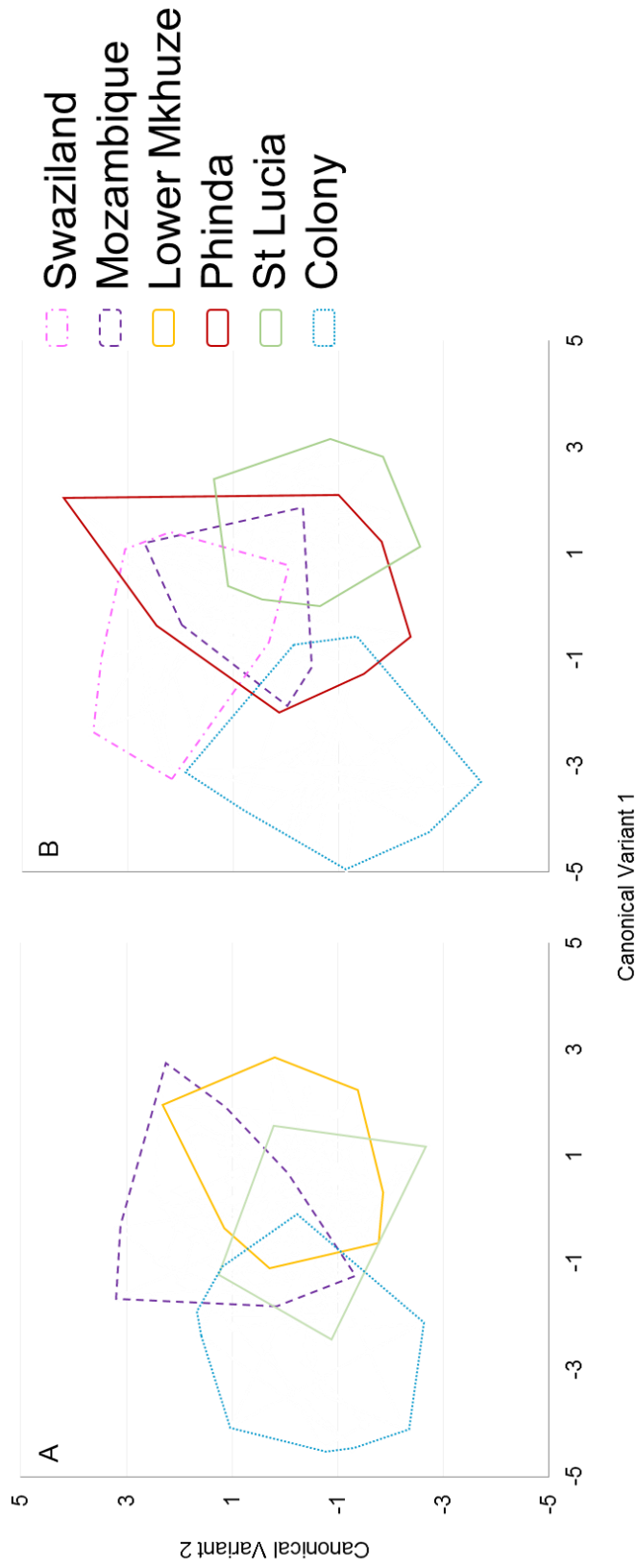
Fig. 3.11. The distribution of *Glossina brevipalpis* (A) and *Glossina austeni* (B) female right wing shape in the morhospace defined the first two Canonical variants, flies were collected from varies sites as well as colony reared.

3.4 Discussion

We demonstrated that geometric analysis could successfully distinguish between *G. brevipalpis* and *G. austeni* at a species level as also revealed by results presented by Patterson & Schofield (2005) for *G. morsitans s.l.*, *G. pallidipes* and *G. swynnertoni* in the morsitans group, *G. p. gambiensis*, *G. fuscipes* and *G. tachinoides* in the palpalis group and *G. brevipalpis* in the fusca group.

Patterson & Schofield (2005) stated that wing shape apparently represents a relatively neutral trait that is not heavily modulated by ecological adaptation or environmental constraints. It is, however, evident from this study that environmental factors such as humidity and temperature can influence both shape and size to a certan extend of *G. brevipalpis* and *G. austeni* (Fig. 3.4; Table 3.2). In tsetse flies most of the pre-imago development is relatively protected from external influences as the larvae develops *in utero* (females are mobile and can avoid unfavourable environments) which to a certain extent buffers pre-imago against factors that can lead to morphometric variations (Glasgow, 1961). The effect of humidity and temperature becomes more evident when pupae are in the soil. Glasgow (1961) showed that higher temperatures tend to result in smaller individuals, while Déjardin & Maillot (1964) indicated that increased humidity tends to result in larger individuals. This "temperature-size rule" (Atkinson, 1994) can be considered as an adaptive strategy that allows insects to optimize their fitness in a changing seasonal environment (Angilletta *et al.*, 2004; Kingsolver & Huey, 2008; Clemmensen & Hahn, 2015).

Seasonal climatic effects were shown to influence the size (as determined by the length of the "cutting edge" of the "hatchet cell" and the thorax) of *G. palpalis palpalis* in forested areas of the Ivory Coast (Sane *et al.,* 2000). These authors indicated that the size of an individual during collection is directly related to the ecological conditions in which the "mother" lived, giving an indication of the possible physiological state of the population about two months prior to collection (Sane *et al.,* 2000). These authors collected larger individuals in the wet season and smaller ones in the dry season, therefore larger individuals in the wet season could reflect a population that has a good physiological start in the dry season (Sane *et al.,* 2000).

Similar to the results obtained by Sane *et al.* (2000) seasonal climatic effects in size (as determined by wing centroid size) were seen for both *G. brevipalpis* and *G. austeni* collected in north eastern KwaZulu-Natal. The *G. brevipalpis* collected at Ndumu, located in the interior of the study area, were on average smaller than those collected from St Lucia on the coast. Temperature and humidity fluctuations were more pronounced at Ndumu than at St Lucia. The temperature at Ndumu ranged from 27 °C in the hot months to 15.5 °C in the colder months, and the relative humidity from 80% in the rainy to 50% in the dry season (Chapter 2). Whereas the temperature at St Lucia ranged from 26 °C to 17 °C and the

relative humidity from 99% to 68% (Chapter 2). Additionally, for both *G. brevipalpis* and *G. austeni* males and females, larger individuals were collected at the end of the hot season in autumn. This could reflect that populations located at sites with less pronounced fluctuations in environmental conditions, together with those at the end of a more favourable season (*i.e.* a hot, wet summer), would be in a better physiological condition than individuals collected in both areas and seasons where they were subjected to increased environmental stress.

Sexual dimorphism is well-known in insects and sex-related differences can occur in various organs such as eyes (Lau *et al.,* 2007), legs (Tseng & Rowe, 1999) head capsules (Wilkinson & Dodson, 1997) and wings (Ribak *et al.,* 2009; Tejeda *et al.,* 2014, Virginio *et al.,* 2015). For tsetse flies sexual size dimorphism was previously reported for *G. palpalis gambiensis* (Camara *et al.*, 2006; Bouyer *et al.*, 2007). An obvious sexual size dimorphism with females being larger than males for both *G. brevipalpis* and *G. austeni* was reported. This size difference between males and females was more pronounced in summer than in winter, indicating that environmental conditions can also play a role in sexual dimorphism. The evolution of sexual dimorphism in tsetse flies might be more influenced by natural selection than sexual selection.

An objective of the present study was to assess if geometric morphometric markers can be used to determine whether the various tsetse fly populations in South Africa were genetically isolated, and whether the South African populations were isolated or not from those in southern Mozambique or Swaziland. This will have important consequences for the selection and development of control strategies. Despite the apparent size differences, as mentioned above, morphometric markers indicated that there were no significant differences in wing shape between the various tsetse fly populations in South African (SA), southern Mozambique (SM) or Swaziland. Based on wing shape variations the *G. austeni* from Swaziland seem to be more isolated from those in the main SA-SM tsetse fly belt. This may indicate that a more reliable method such as genetic analysis will be needed to confirm these results. Preliminary molecular work with mitochondrial DNA also indicated the *G. austeni* from Swaziland to be more isolated from the main SA-SM tsetse fly belt but that gene flow between these population does exist (Koekemoer, ARC-OVI, unpublished data). Based on our current results the Swaziland population can, however, not be treated as an isolated population. Clear positive linear relationships between Mahalanobis distances and geographical distances were seen in both species. This could indicate that it is a slow on-going process for these populations to have less genetic exchanges due to restricted tsetse fly dispersal (Kaba *et al.*, 2012).

Our data indicate the absence of any significant barriers to gene flow between the three countries and the whole population should therefore be considered as a homogenous one and treated as such.

This means that localised control efforts against tsetse flies in a given area will be subjected to reinvasion from uncontrolled sites. There is therefore a need for an area-wide approach, *i.e.* the control effort should be directed against the entire tsetse fly population of South Africa, southern Mozambique and Swaziland. This would entail that the entire area should be controlled simultaneously or by a continuous progression of interventions, using barriers of impregnated traps and/or targets between sites following the rolling carpet principle (Vreysen *et al.,* 2007). There is in addition the need to confirm that the South African, southern Mozambique and Swaziland population is isolated from the main tsetse fly belt north of Maputo (it is assumed that this belt starts approximately 500 km further north in central Mozambique south of the Save River) (Dias, 1961; Mulandane, 2013).

A strategy based on area-wide integrated pest management (AW-IPM) principles that include a SIT component was proposed to create a sustainable tsetse fly free area in South Africa (Kappmeier Green *et al.,* 2007; Vreysen *et al.*, 2007). The proposed strategy included the suppression of the *G. brevipalpis* and *G. austeni* populations with the sequential aerosol technique (Kappmeier Green *et al.,* 2007) followed by the release of sterile males (Vreysen *et al.*, 2000) to eradicate all potential relic pockets (Kappmeier Green *et al.*, 2007). The two colonies that are being maintained at the ARC-OVI will need to be up-scaled to be able to produce sufficient sterile flies for the campaign. However, our study has indicated that the colony flies are different in shape and smaller in size compared with wild flies in KwaZulu-Natal, southern Mozambique and Swaziland. As this may lead to concerns about the sterile insect's quality as well as their competitiveness, field studies are required to further investigate these aspects.

Chapter 4

Collection, processing and host source of the rearing diet for colonised tsetse flies[2]

4.1   Introduction

Studying insects in the wild, although essential and rewarding, presents a wide range of logistical, financial and sometimes even ethical challenges. This becomes more relevant if these insects are involved in pathogen transmission. Laboratory colonies allow research to be done on pathogen-free insects of a known age and biological background. Insect colonies are valuable assets that bring highly equipped laboratories together with insects that can only be found in remote areas. A good example of this is reflected in tsetse fly research. Tsetse flies are only present in Africa south of the Sahara (Moloo, 1993). Although most tsetse populations are found in rural areas, a large amount of research has been done on tsetse flies as a result of colonisation. This ranges from basic research on the life cycle and pathogenic role of tsetse flies (Ward, 1970) to advanced genome sequencing projects (International *Glossina* Genome Initiative, 2014). Furthermore, laboratory-adapted colonies were instrumental in the development of sophisticated vector control strategies such as the sterile insect technique (SIT) for tsetse flies (Feldmann *et al.*, 2005) and other vectors (Oliva *et al.*, 2012), as well as the implementation of transgenic (Alphey, 2014) and symbiont-based (Atyame *et al.,* 2011) control methods for mosquitoes.

The first successful attempts to maintain tsetse flies in the laboratory were reported in 1917 when E. Roubaud succeeded in maintaining *Glossina morsitans submorsitans* collected in Senegal in culture for three years at the Pasteur Institute in Paris, France (Roubaud, 1917). This colony, however, consisted of only 32 flies (Ward, 1970). *Glossina palpalis* was the next species to be colonised at laboratories in Belgium and England with varying degrees of success for the next 30 years (Mellanby & Mellanby, 1937; Rodhain & Van Hoof, 1944; Ward, 1970).

The possibility of larger self-sustainable colonies only became feasible with the development of a blood feeding station that could simultaneously hold four guinea pigs and eight cages of flies for feeding (Geigy, 1948). Despite the fact that 2000 flies could be fed in 3 to 4 hours with this system (Geigy, 1948; Ward, 1970) the colony proved not to be self-sustainable. The first self-sustainable colony was established from 43 *Glossina morsitans* collected in Govura, Mozambique and maintained at the Escola Nacional de Saúde

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Pública e de Medicina Tropical, Lisbon, Portugal in 1959 (De Azevedo & Da Pinhão, 1964; Ward, 1970). This colony was kept in a controlled environmental chamber at 26 °C and 70% RH with a 12-hr light/dark photoperiod. These flies were fed on the shaved flanks of guinea pigs (De Azevedo & Pinhão, 1964; Ward, 1970).

The colonisation of *Glossina austeni* at the Tsetse Research Laboratory, School of Veterinary Medicine, University of Bristol, Langford, England was another noteworthy contribution towards the large-scale rearing of tsetse flies. This colony was established from pupae collected on Unguja Island, Zanzibar and the emerged flies were fed on the ears of lop-eared rabbits (Nash *et al.*, 1966a). This laboratory subsequently developed protocols on emergence, fertilization, adult and pupae maintenance, fly feeding and host suitability (Nash *et al.*, 1966a, b, c; 1967; 1968; Jordan *et al.*, 1966; 1967; 1968; Curtis & Jordan, 1968). This colony was self-sustaining by 1966 and in 1967 produced 88 000 pupae (Nash *et al.*, 1968).

By 1970, a variety of biological studies had been conducted on colonised tsetse species including but not limited to *Glossina tachinoides, G. austeni*, *G. morsitans* and *G. m. submorsitans* around the world (Ward, 1970). A colony of *Glossina brevipalpis* was established from 181 females collected in mid-1982 in the Kibwezi Forest, Kenya (Moloo & Kutuza, 1988). Adult flies were collected using the moving vehicle method (Bursell, 1961) and were fed on the ears of lop-eared rabbits under controlled laboratory conditions (25 ± 0.5 °C and 80-85% RH) at the Tsetse Vector Laboratory of the International Laboratory for Research on Animal Diseases (ILRAD) (Moloo & Kutuza, 1988). The number of mature females in this colony increased from 1000 in mid-1983, to 4000 in mid-1984 and to 5000 by December 1984 (Moloo & Kutuza, 1988).

One of the main obstacles in tsetse fly colonisation is the supply of high quality blood meals. Tsetse flies are obligate blood feeders and they reproduce by adenotrophic viviparity, making adult and larval stages dependent on the same source of food and insufficient nutrition will lead to abortions (Mellanby, 1937; Ward, 1970). A high quality food source is essential for the growth and sustainability of tsetse fly colonies (Feldmann, 1994a). Increased abortion rates and reduced productivity of pupae resulting from an inadequate diet will hamper colony growth, which is already inherently low due to the slow reproductive cycle of tsetse flies. The maintenance of live animals for tsetse fly feeding will be costly, labour intensive and come with a set of ethical considerations. The development of *in vitro* feeding techniques became essential as the need for mass-rearing of the fly for experimental work, as well as for the possible use in programmes that had an SIT component became more pressing (Mews *et al.*, 1976). Many attempts were made to feed tsetse flies *in vitro* through membranes (Roubaud, 1917; Lester & Loyd, 1928; Rogers, 1971; Langley, 1972; Bauer & Wetzel, 1976) and laboratories in Europe, shifted their *G. m.*

morsitans colonies from animal to *in vitro* feeding (Mews & Ruhm, 1971; Nash *et al.*, 1971; Mews *et al.*, 1977; Wetzel & Luger, 1978). *In vitro* feeding also proved to be suitable for the establishment of colonies from field collected flies and a *G. austeni* colony was established from pupae collected on Zanzibar in 1986 at the Insect Pest Control Laboratory (IPCL) (formerly Entomology Unit) in Seibersdorf, Austria and maintained on an *in vitro* feeding system using bovine blood (Opiyo *et al.*, 2000).

In any tsetse fly mass-rearing facility, the logistics of obtaining sterile, high quality blood remains challenging. An added complication is the variation in nutritional value of the collected blood which is influenced by genetic, environmental, chemical and physical factors of the host animal (Kabayo *et al.*, 1988). Other factors which play a significant role include chemicals and microbiological contaminants which the blood was exposed to during collection, handling and storage (Kabayo *et al.*, 1988). To maintain viable healthy tsetse fly colonies, it is important that standard blood collection procedures are strictly adhered to, as this will ensure the continuous supply of a product that has the same quality as fresh blood collected directly from donor animals.

In 2002, laboratory colonies of *G. brevipalpis* and *G. austeni* were established at the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI) in Pretoria, South Africa using seed material from the Tsetse and Trypanosomiasis Research Institute (TTRI) (now named Vector & Vector-Borne Diseases Research Institute) Tanga, Tanzania and the Entomology Unit of the Food and Agriculture Organization (FAO)/International Atomic Energy Agency (IAEA) Laboratories in Seibersdorf, Austria (now called the FAO/IAEA Insect Pest Control Laboratory (IPCL)) respectively. The original *G. brevipalpis* colony was initiated in mid-1982 from material collected in the Kibwezi Forest, Kenya as described above (Moloo & Kutuza, 1988). The original *G. austeni* colony was established at the TTRI in September 1982 from pupae collected in the Jozani Forest, Unguja Island of Zanzibar. This colony was initially maintained on rabbits before an *in vitro* feeding system was introduced in 1988 (Tarimo *et al.*, 1988).

In view of the importance of providing high quality blood to tsetse colonies, optimisation of blood collection procedures and the development and validation of methods that may improve colony performance should be part of any colony maintenance program. The process of collecting blood at the abattoir could be simplified by using anticoagulants as a surrogate for mechanical stirring. We therefore tested the effect of mixing various anticoagulants on the nutritional value of the blood.

Ii is known that the host preference of *G. brevipalpis* and *G. austeni* in nature may include, in addition to bovines, smaller mammals such as bush pigs (Moloo, 1993). Porcine- and bovine blood, and various combinations thereof, were therefore evaluated as rearing diets for these two species. Finally, it is also known that phagostimulants may enhance

blood intake of female tsetse flies and hence, increase productivity. We therefore also tested the effects of some phagostimulants on the performance of the two colonies.

4.2   Materials and methods

4.2.1   Tsetse fly colonies

The *G. brevipalpis* and *G. austeni* colony flies were maintained under standard rearing conditions of 23-24 °C, 75-80% RH and subdued/indirect lighting with a 12 h light/12 h dark photoperiod (Feldmann, 1994a; FAO/IAEA standard operating procedures 2006). Since the establishment of these colonies at the ARC-OVI, they have been maintained on defibrinated bovine blood, collected from slaughtered cows at an abattoir, using an artificial *in vitro* membrane feeding system (Wetzel & Luger, 1978; Feldmann, 1994a; FAO/IAEA standard operating procedures, 2006). In 2015 the *G. brevipalpis* colony consisted of 13 000 reproducing females that produced 7000 pupae weekly and the *G. austeni* colony had a size of 16 000 reproducing females that produced 5000 pupae weekly. The total colony size including both males and females of both species was 37 000 flies at the end of 2015.

4.2.2   Blood collection and processing

4.2.2.1   Porcine and bovine blood collected from commercial abattoirs for routine colony maintenance

The two tsetse colonies at the ARC-OVI are currently maintained on cattle blood collected at Morgan (Pty)Ltd abattoir (-26.2545, 28.4299), situated outside the tsetse fly and nagana infected area, where cattle are slaughtered for human consumption. Although the veterinary history of these animals is unknown, all animals slaughtered are accompanied by a health certificate stating they are healthy and of good quality. This abattoir does not slaughter for disease control purposes.

Collection of blood was carried out as follows: cattle were stunned, then suspended by their hind legs and their throat slit. The swath of blood was directed into a bucket and transferred to 40 L containers where it was defibrinated for 10-15 minutes using a custom-made stainless steel electric paddle stirrer. The clotted fibrin was removed by hand and the blood pooled in a 500 L container after which it was divided into 5 L canisters and stored at -20 °C (FAO/IAEA standard operating procedures, 2006).

Porcine blood was obtained from pigs with a known veterinary history, kept at the Agricultural Research Council-Animal Improvement Institute for breeding and experimental purposes. The pigs were slaughtered at the Bon Accord Abattoir in Irene, south of Pretoria. Animals were stunned, the jugular vein exposed and severed, and the blood collected in a 3 L sterilized glass jar containing glass beads, and defibrinated by agitating for 10 to 15 minutes before it was dispensed into 0.3 L containers and stored at -20 °C.

The bovine and porcine blood collected from the abattoirs was irradiated when frozen with 2 kGy at Synergy Heath, a commercial irradiation facility and stored in 5 L (bovine) or 0.3 L (porcine) containers at -20 °C until used. All collected blood was tested for bacterial contamination before being used for tsetse feeding. Contaminated blood samples were appropriately discarded (FAO/IAEA standard operating procedures, 2006).

4.2.2.2   Bovine blood collected with a closed sterile system

As the veterinary history of the cattle slaughtered at the commercial abattoir was not known, cattle from a closed quarantine herd with a known veterinary background was also used for blood collection. For this purpose, animals were bled from the jugular vein using a trocar and cannula. Blood was drained directly (in a closed sterile system) into a sterilized 3 L glass jar containing one of the following anticoagulants; acid citrate dextrose (ACD), sodium citrate, a combination of citric acid and sodium citrate, citrate phosphate dextrose adenine (CPDA) or citric acid. For the evaluation of the phagostimulants defibrinated blood was used. Blood was collected in 3 L glass jars containing only glass beads and agitated for 10 to 15 minutes. The blood collected with anticoagulants and defibrination were decanted into 20 mL containers and stored at -20 °C.

4.2.3   Assessment of suitability of blood source as maintenance diet

Bovine and porcine blood and mixtures thereof were evaluated as diets for the routine maintenance of *G. brevipalpis* and *G. austeni* colonies. In a first set of experiments, productivity of both species was assessed when fed daily on a single blood source (*i.e.* bovine or porcine) or in the following mixed combinations (75% bovine / 25% porcine, 50% bovine / 50% porcine, and 25% bovine / 75% porcine). Blood that was collected for colony maintenance form the commercial abattoirs were used.

In a second set of experiments, productivity of both species was assessed when fed daily on a single blood source that was alternated between days according to the following schedule:

- One day bovine followed by four days of porcine blood,
- Three days bovine followed by three days of porcine blood,
- Four days bovine followed by one day of porcine blood.

For this set of experiments bovine blood that was collected using the closed sterile system and porcine blood from the abattoir system was used.

4.2.4   Phagostimulation

The nucleotides adenosine triphosphate (ATP), inosine triposphates (ITP), guanosine monophosphate (GMP) and cytosine monophosphate (CMP) were the phagostimulants evaluated. An amount of 0.055 g of each compound was diluted in 1 mL of distilled water and the dilutions were kept at -20 °C and used within three days. Only 0.02 mL of each solution (concentration of 10^{-3} M) was used for 20 mL of bovine blood collected with the closed sterile system, as described above.

4.2.5   Bioassay

Female fly survival (percentage alive on day 18 and 30 post eclosion respectively), fecundity (number of pupae per mature female at day 18) and pupal size are three essential parameters routinely used for assessing colony performance (Wetzel & Luger, 1978). Fecundity was determined by the number of pupae produced per mature female day (Curtis, 1968). Mature female days were calculated for each treatment by adding the number of flies alive each day, starting on day 18 after emergence until the end of the experiment on day 30 (Curtis, 1968).

These parameters are used in a formula that calculates a Quality Factor (QF) of the blood as a comprehensive indicator for colony production (Feldmann, 1994a; FAO/IAEA standard operating procedures, 2006).

Blood quality was evaluated using a standardised bioassay (FAO/IAEA standard operating procedures, 2006) whereby productivity of flies that were fed daily on the selected diet for 30 days using an artificial membrane feeding system was assessed. This standardised bioassay is also routinely used for quality control on the blood used in the colony. That enabled the QF of the selected diet to be calculated (Feldmann 1994b; FAO/IAEA standard operating procedures, 2006). A QF above 1 is considered acceptable and indicates that the blood diet is suitable for colony maintenance (Feldmann, 1994a; b).

The protocol of the bioassay consisted of mating 30 three-day-old females with 30 eight-day-old males at a 1 : 1 ratio. Males and females were kept together for four days under standard colony conditions (23-24 °C, 75-80% RH and subdued/indirect lighting). After removing the male flies, female survival, pupae production and abortions (*i.e.* expelled eggs and immature larval stages) were monitored daily for 30 days (Feldmann, 1994a; FAO/IAEA standard operating procedures, 2006).

All pupae produced were mechanically sorted into one of five class sizes (A - E). The sorter was calibrated according to the standards established by the FAO/IAEA. For *G. austeni* the measurements ranged between 2.3 mm (A) and 3.0 mm (E), and for *G. brevipalpis* between 3.5 mm (A) and 4.3 mm (E). The weight (mg) of the different pupal

size classes were A (<16), B (16– <19), C (19– <21), D (21– <23), E (≥23) for *G. austeni* and A (<56), B (56– <68), C (68– <76), D (76– <84), E (≥84) for *G. brevipalpis*.

After 30 days, all surviving females were dissected to determine their reproductive status (presence/absence of egg/larvae in the uterus and spermatecae fill). All bioassays were replicated four times.

The QFs were calculated using the formula (Feldmann, 1994a; FAO/IAEA standard operating procedures, 2006):

$$QF = \frac{FS30 + PT + 0.3(PB) + 0.4(PC) + 0.5(PD) + 0.6(PE) + 0.3(E\&I) + 0.6(II\&III) - 0.2(PA) - 0.5(AB) - BO}{FS30 + FS18}$$

Where:

QF	Quality factor
FS18	Female flies surviving on day 18
FS30	Female flies surviving to day 30
PA	No. of size class A pupae
PB	No. of size class B pupae
PC	No. of size class C pupae
PD	No. of size class D pupae
PE	No. of size class E pupae
PT	Total pupae
AB	Abortions
E&I	Eggs or first instar larvae *in utero*
II&III	Second or third instar larvae *in utero*
BO	Blockage of the oviducts or other reproductive abnormality

4.2.6   The colonisation of tsetse flies

The *G. brevipalpis* and *G. austeni* colonies at the ARC-OVI have an East-African origin, and come from a different geographical population than those in north eastern KwaZulu-Natal. Morphometric differences between the local KwaZulu-Natal and the ARC-OVI colony's flies have been recorded (Chapter 3). Before the initiation of an operational programme that includes the release of sterile males, it is essential to establish colonies of the local strains to study their biology, mating competitiveness in field cages and in the target area as well as Trypanosomosis/fly interactions. These aspects need to be compared to those of the available established colonies with an East-African origin. This information will be required for the development of an appropriate control strategy to manage the nagana problem in north eastern KwaZulu-Natal.

Flies for potential colonization were collected from the western shores of the iSimangaliso Wetland Park, Phinda and Hluhluwe-iMfolozi Park (Fig. 2.5). These areas were previously shown to harbour large populations of *G. brevipalpis* and *G. austeni* (Chapter 2). Flies were collected from 11 August 2014 to 25 April 2015 with 34 odour baited H traps (Kappmeier & Nevill, 1999a; Kappmeier, 2000). Traps were emptied twice daily and the flies transferred from the collection bottles to fly holding cages. Flies were fed on the day of collection on abattoir collected bovine blood (as described 4.2.2.1) using an artificial membrane feeding system. The day after collection male and female flies were separated. Collected flies were kept at the KwaZulu-Natal Tsetse Research Station under standard colony conditions (23-24 °C, 75-80% RH and subdued/indirect lighting) (Feldmann, 1994a; FAO/IAEA standard operating procedures, 2006) for up to 30 days before transportation in Styrofoam boxes (with wet tissue paper) to the colony facility at the ARC-OVI.

4.2.7   Statistical analysis

Data was analysed using the statistical programs GraphPadInstat (version 3.00, 2003) and VSN International (2012). The experiments were designed as a randomised block design in four blocks. Differences in survival rate and QF values between treatments were evaluated with an analysis of variance (ANOVA). The data was shown to be normally distributed with homogeneous treatment variances. Treatment means were separated using Fishers' protected t-test least significant difference (LSD) at the 5% level of significance (Snedecor & Cochran, 1980), if the F-probability from the ANOVA was significant at 5%.

4.3   Results

4.3.1   Anticoagulants in the rearing diet

The anticoagulant study indicated that survival of both *G. brevipalpis* females (98%) and *G. austeni* females (95%) was the highest for flies that had fed on defibrinated blood (Table 4.1).

The survival rate of *G. brevipalpis* females that had fed on blood collected with ACD (93%) ($P = 0.14$) and sodium citrate (95%) ($P = 0.50$) was not significantly different from that of defibrinated blood (98%). However, survival of *G. brevipalpis* fed on blood collected with the citric sodium combination (88%, $P = 0.01$), CPDA (84%, $P < 0.01$) and citric acid (80%, $P < 0.01$) was significantly lower than that of flies fed on defibrinated blood (Table 4.1).

Survival of *G. austeni* females that were fed on blood collected with ACD (86%), citric sodium combination (85%), citric acid (85%), sodium citrate (68%) and CPDA (80%) was

significantly lower ($P < 0.02$ in all cases) than that of flies fed on the defibrinated blood (95%) (Table 4.1).

The highest fecundity was observed for *G. brevipalpis* females fed on blood collected with the citric sodium combination (0.059) and citric acid (0.061) both was above that of the defibrinated blood and these flies also produced the highest percentage of large pupae (Class C > 68 mg), *i.e.* 87% and 93%, respectively (Table 4.1). *Glossina brevipalpis* females that fed on blood collected with ACD had the lowest fecundity (0.047) and produced the smallest pupae.

The fecundity of *G. austeni* fed on defibrinated blood as well as blood collected with CPDA and citric acid was above 0.060, and those that had fed on CPDA produced the largest pupae (88% > 19 mg). The lowest fecundity of 0.049 was observed for *G. austeni* fed on blood collected with sodium citrate. The smallest pupa was produced by females that fed on blood collected with ACD (Table 4.1).

Insemination rate of all surviving females on day 30 was higher than 95% and the spermatecae fill was above 0.5 for most of the female flies of both species, irrespective of the treatment (Table 4.1).

In the current comparison, the bioassay for both *G. brevipalpis* and *G. austeni* that had been fed blood collected with the anticoagulants as well as the defibrinated blood gave a QF above 1, except for *G. brevipalpis* that fed on blood collected with ACD (0.93) (Table 4.1; Fig. 4.1).

For the *G. brevipalpis* there was no significant difference ($P = 0.14$) in the QF values between defibrinated blood and the tested anticoagulants. For *G. austeni* a significant difference ($P = 0.05$) was seen between the defibrinated blood (1.31) and that collected with ACD (1.16) and sodium citrate (1.03) (Fig. 4.1).
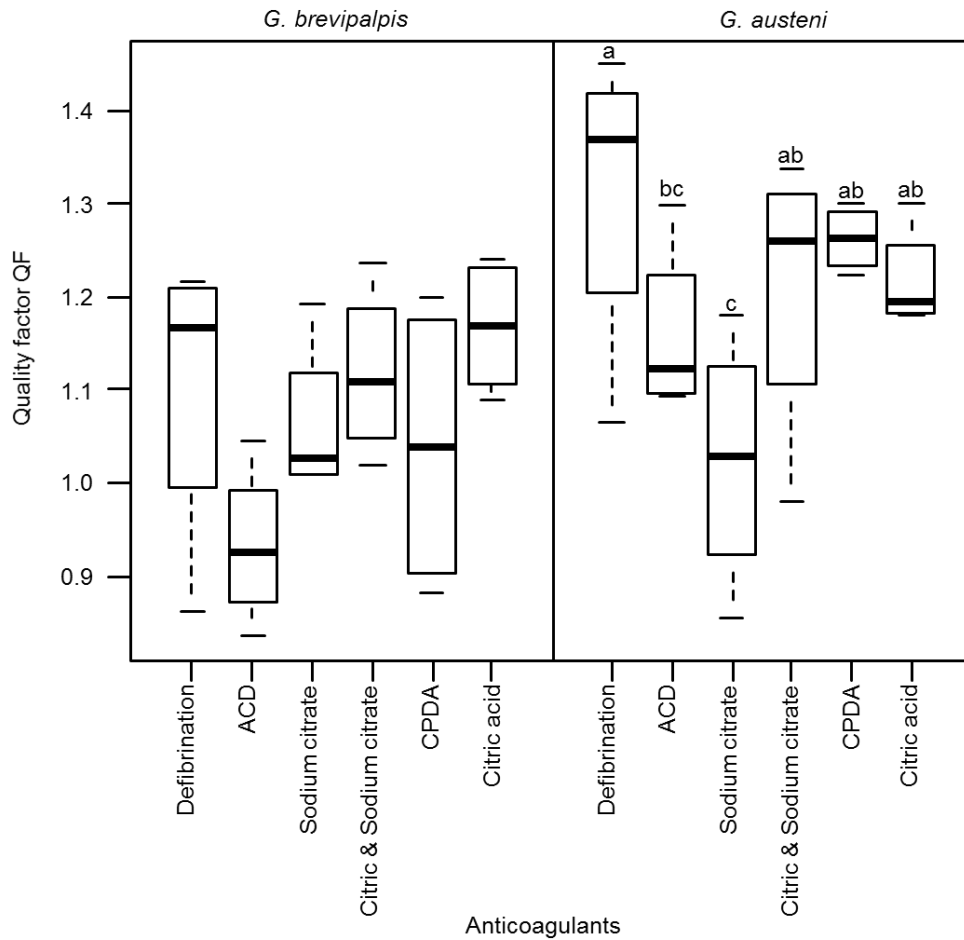
Fig. 4.1. Quality Factor (QF) values for the blood collected with anticoagulants as obtained in the bioassay for *Glossina brevipalpis* and *Glossina austeni.* Each box shows the group median separating the 25th and 75th quartiles, capped bars indicate maximum and minimum values. Boxes denoted by a different letter indicate that the QF values were significantly different for each species at the 5% level.

Table 4.1. Anticoagulants tested for their potential use in blood collection, as opposed to defribination, for both *Glossina brevipalpis* and *Glossina austeni* rearing diets. Numbers followed by an * indicate significant differences between the anticoagulant and the defribinated blood for each species at the 5% level.

	No. of mature Females		Pupae produced	Fecundity	Pupal size classes					Quality factor (QF)	Uterus					Insemination	Spermatheca fill			
	Day 18	Day 30			A	B	C	D	E		Recently ovulated egg	Empty due to abortion	Viable instar larvae I	II	III		0.25	0.5	0.75	1
G. brevipalpis																				
Defibrination	117	117	80	0.053	4	6	14	21	35	1.11	13	31	29	20	24	1.00	7	49	49	12
Acid Citrate Dextrose	118	111	71	0.047	11	24	21	9	6	0.93	23	36	33	11	8	1.00	5	42	56	8
Sodium citrate	116	114	82	0.055	15	16	18	23	10	1.07	30	26	25	22	10	0.95	15	42	46	4
Citric & Sodium Citrate	109	106*	83	0.059	1	5	17	32	28	1.12	6	33	48	12	5	1.00	0	60	39	6
Phosphate Dextrose Adenine	108	101*	68	0.050	3	7	15	21	22	1.05	21	24	31	16	9	0.98	12	52	35	2
Citric acid	98	96*	77	0.061	4	4	14	24	31	1.17	6	24	42	14	9	1.00	0	48	43	4
G. austeni																				
Defibrination	115	114	100	0.067	12	14	28	31	15	1.31	27	7	8	32	39	0.97	16	58	29	5
Acid Citrate Dextrose	111	103*	82	0.059	13	18	22	22	7	1.16	28	11	14	19	31	1.00	27	37	35	4
Sodium citrate	98	81*	57	0.049	9	10	22	11	5	1.03	23	12	3	17	25	0.99	25	30	16	8
Citric & Sodium Citrate	115	102*	88	0.062	6	6	24	34	18	1.22	25	9	10	28	23	0.98	9	60	29	2
Phosphate Dextrose Adenine	108	96*	86	0.065	4	6	25	34	17	1.26	15	9	19	28	24	0.97	7	61	18	7
Citric acid	106	102*	86	0.063	8	8	24	35	10	1.22	15	16	23	25	22	0.99	18	50	26	5

4.3.2 Blood source for maintenance diet

Two feeding regimes, *i.e.* mixtures of bovine and porcine blood in different proportions or only bovine- or porcine blood in different combinations of days in a six-day cycle, were tested.

The comparison of mixtures of bovine and porcine blood in different proportions showed that the survival rate of both *G. brevipalpis* (83%) and *G. austeni* (72%) was the highest for the 50% / 50% combination (Table 4.2). Feeding flies only with bovine (control) blood reduced the overall survival rate to 53% and 61% for *G. brevipalpis* and *G. austeni*, respectively (Table 4.2). The 25% bovine / 75% porcine combination gave the lowest survival rate, 35% for *G. brevipalpis* and 32% for *G. austeni.* The survival rate of *G. brevipalpis* that fed on 100% bovine (control) (53%) blood was significantly different from flies that had fed on all the combination diets ($P < 0.01$) as well as the 100% porcine (80%) one ($P < 0.01$) (Table 4.2). The survival rates of *G. austeni* fed on the 100% porcine (78%) and the 25% bovine / 75% porcine (32%) combination was significantly different ($P < 0.01$) compared to flies that had fed on the 100% bovine (61%) diet (Table 4.2).

The overall fecundity was very low with the highest fecundity recorded for *G. brevipalpis* that had fed on the 50% / 50% combination diet (0.039) and *G. austeni* that had fed on the 75% bovine / 25% porcine combination (0.050) and the 50% / 50% combination (0.052). The highest percentage of large pupae (size class C and above) was produced by the *G. brevipalpis* fed blood in the 75% bovine / 25% porcine combination (87%), and this was also the case for *G. austeni* (60%) (Table 4.2).

Insemination rate was for both species and for all treatments ≥ 0.88 and the spermathecal fill was in most cases between 0.5 and 0.75 (Table 4.2).

All the QF values for the premix combination feeding regimes for *G. brevipalpis* were below 1, except for flies that fed on the 100% bovine (control) (1.11) diet (Table 4.2; Fig. 4.2). The QF values for the *G. brevipalpis* flies fed on the 100% porcine (0.73), 25% bovine / 75% porcine combination (0.54) and 75% bovine / 25% porcine combination (0.70) were significantly different ($P < 0.01$) from the flies that had fed on the 100% bovine diet (1.11) (Fig. 4.2). The QF values for *G. austeni* that fed on the 100% bovine (control) (0.84) diet were significantly higher ($P < 0.01$) than those that fed on the 25% bovine / 75% porcine combination (0.56) diet. In this specific evaluation the QF values obtained for both *G. brevipalpis* and *G. austeni* with the bovine blood control were lower than normal which may indicate that the overall quality of the bovine as well as porcine blood used in this trial was low.

During the second feeding regime evaluation, blood from a single host was offered to the flies in different sequences during a six-day cycle. This evaluation was not done concurrently with the first and a different blood batch was used.

Survival of the *G. brevipalpis* females ranged from 98% for those fed on the bovine (control) as well as only the porcine blood, but survival was reduced to 88% for flies fed on the bovine (3 days) – porcine (3 days) combination (Table 4.2). The survival rate for *G. brevipalpis* that fed on the bovine (3 days) – porcine (3 days) (88%) combination ($P < 0.01$) were significantly lower than that compared to those fed only on bovine blood (97%).

Glossina austeni females had a slightly lower survival rate ranging from 83% for flies that had fed on bovine blood only to 73% for flies fed on the porcine diet. There were no significant differences in survival rate of the *G. austeni* fed on bovine blood (83%) compared with any of the other combinations including the porcine diet (73%) (Table 4.2).

Fecundity ranged from 0.067 for the *G. brevipalpis* that had fed on the bovine (3 days) – porcine (3 days) combination to 0.057 for flies that had fed on the porcine only diet. *Glossina brevipalpis* females that fed on pure porcine produced the smallest pupae (Table 4.2), whereas the largest pupae were produced by flies fed on the bovine (4 days) – porcine (1 day) combination. These data seem to indicate a relationship between the use of bovine blood and the size (larger) of the *G. brevipalpis* pupae.

Glossina austeni females that had fed on the bovine (4 days) – porcine (1 day) combination produced the highest number of pupae with a fecundity of 0.061 and those fed on the bovine (3 days) – porcine (3 days) produced the least (0.055). The *G. austeni* females that were offered blood in combination diets produced larger pupae than those fed on a single source diet (Table 4.2).

The insemination rates for both species were above 0.95 and in the majority the spermatecae fill was in the 0.5 or 0.75 class (Table 4.2). All the combination diets in this second feeding regime resulted in QF values above 1 for both species (Table 4.2; Fig. 4.2), with no significant differences between treatments. The highest QF value for *G. brevipalpis* (1.21) was obtained when using the bovine (3 days) – porcine (3 days) combination while the bovine (1 day) – porcine (4 days) gave the highest QF for *G. austeni* (1.21).
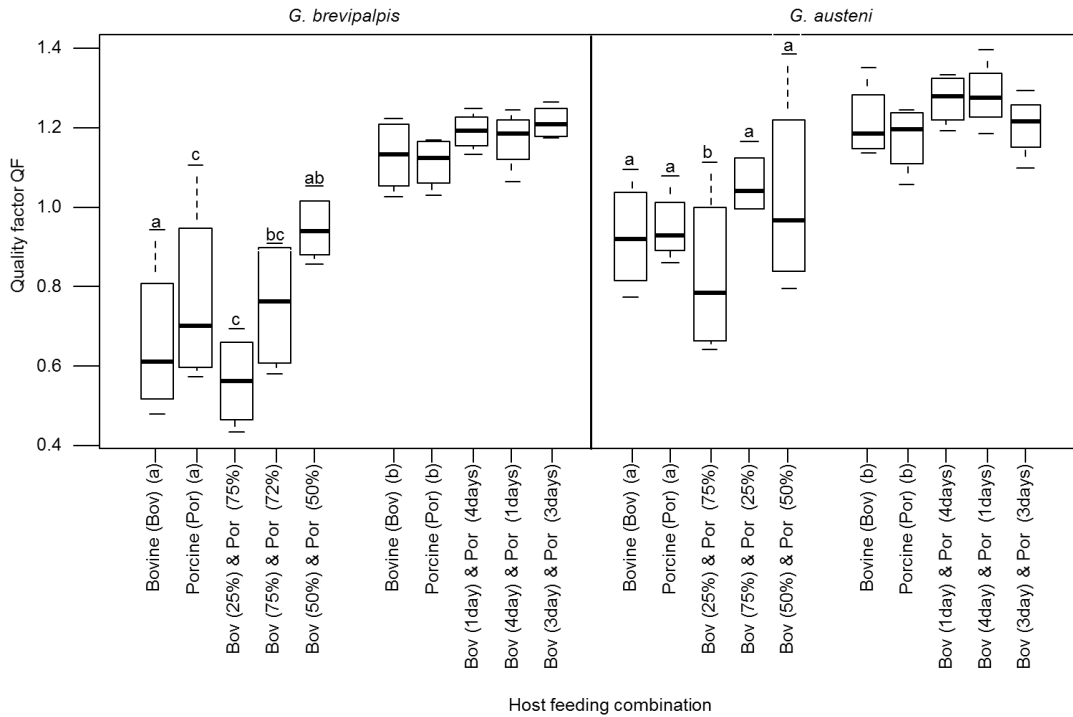
Fig. 4.2. Quality Factor (QF) values for different combinations of bovine/porcine blood diets obtained using the standard bioassay for *Glossina brevipalpis* and *Glossina austeni*. Each box shows the group median separating the 25th and 75th quartiles, capped bars indicate maximum and minimum values. Boxes denoted by a different letter indicate that the QF values were significantly different for each species at the 5% level.

Table 4.2. Bovine/porcine blood combinations tested for their potential use as rearing diet for *Glossina brevipalpis* and *Glossina austeni*. Numbers followed by an * indicate a significant difference between the bovine blood (control) and the various combinations for each species and each group at the 5% level.

	No. of mature Females		Pupae produced	Fecundity	Pupal size classes					Quality factor (QF)	Uterus					Insemination	Spermatheca fill			
											Recently ovulated egg	Empty due to abortion	Viable instar larvae							
	Day 18	Day 30			A	B	C	D	E				I	II	III		0.25	0.5	0.75	1
Premix combination diet																				
G. brevipalpis																				
Bovine (bov)	80	63	20	0.020	2	2	5	5	6	1.11	22	18	9	5	9	0.98	10	31	20	1
Porcine (por)	112	96*	41	0.031	7	5	13	8	8	0.73	15	24	30	16	3	1.00	0	36	54	6
25%bov/75%por	60	38*	22	0.035	4	2	7	6	3	0.54	10	17	3	3	5	0.88	3	13	20	1
75%bov/25%por	100	85*	42	0.032	1	4	9	13	11	0.79	27	29	16	7	3	0.95	19	20	39	3
50%bov/50%por	113	99*	55	0.039	8	8	14	19	6	0.95	11	14	17	36	8	0.96	18	24	43	1
G. austeni																				
Bovine (bov)	83	73	36	0.035	6	15	9	5	1	0.84	27	20	9	11	6	0.97	22	33	23	3
Porcine (por)	105	93*	56	0.040	29	9	15	2	1	0.86	44	17	15	10	4	1.00	20	31	37	2
25%bov/75%por	60	38*	13	0.022	5	2	3	2	1	0.56	10	17	2	4	5	0.88	3	13	20	1
75%bov/25%por	96	85	58	0.050	13	10	17	13	5	0.93	27	28	5	18	3	0.95	19	20	39	3
50%bov/50%por	105	86	63	0.052	17	10	15	14	7	0.99	14	12	31	20	7	0.96	18	24	43	1
Alternating diet																				
G. brevipalpis																				
Bovine (bov)	118	117	91	0.060	5	6	16	34	30	1.13	30	35	24	23	5	1.00	0	57	52	8
Porcine (por)	118	118	88	0.057	6	14	19	27	22	1.11	43	24	28	7	7	0.99	5	62	43	6
bov(1#)-por(4#)	118	112	95	0.063	5	8	26	24	32	1.19	50	9	16	7	7	0.95	11	48	23	7
bov(4#)-por(1#)	117	116	92	0.061	5	5	24	26	32	1.17	37	16	28	12	6	0.96	26	43	28	3
bov(3#)-Por(3#)	112	105*	95	0.067	6	9	21	26	33	1.21	35	15	20	16	3	0.95	11	37	29	4
G. austeni																				
Bovine (bov)	104	99	79	0.059	8	13	18	16	24	1.17	48	11	19	16	5	0.97	14	49	25	8
Porcine (por)	88	87	66	0.058	9	12	24	11	10	1.08	36	22	15	9	5	1.00	11	49	22	5
bov(1#)-por(4#)	93	91	72	0.060	2	6	12	26	26	1.21	50	8	16	8	7	0.95	11	48	23	7
bov(4#)-por(1#)	104	103	82	0.061	2	8	18	22	32	1.19	37	16	28	9	6	0.96	26	43	28	3
bov (3#)-por(3#)	95	94	68	0.055	4	7	12	21	24	1.13	35	15	19	16	3	0.95	11	37	29	4

Days fed on specific host blood

4.3.3   Evaluation of phagostimulants to improve production in the tsetse fly colonies

Bovine blood was spiked with phagostimulants to assess potential stimulated feeding responses that could increase overall colony productivity. *Glossina brevipalpis* that fed on the bovine (control) diet had a survival rate of 94% which was similar to those that fed on blood with ITP (Table 4.3). All *G. brevipalpis* that fed on a diet with other phagostimulants had a lower survival rate. The diet with ITP as well as with GMP improved the survival rate (94% and 92% respectively) of *G. austeni* as compared with flies fed on the bovine (control) diet only (72%) (Table 4.3). The *G. brevipalpis* (76%) as well as the *G. austeni* (49%) flies fed on a diet with ATP had the lowest survival rate which was significantly lower ($P < 0.01$) from the survival obtained for flies fed on the bovine blood (Table 4.3).

The fecundity of *G. brevipalpis* females that fed on bovine blood or blood mixed with ITP was the highest 0.056 (Table 4.3). *Glossina brevipalpis* females fed on pure bovine blood produced the largest (98% in the C class and above) pupae (Table 4.3), whereas flies that were fed blood containing ATP produced the smallest pupae (86% in the C class and above). *Glossina austeni* females fed on blood containing ATP, ITP and GMP produced the most as well as the largest pupae (Table 4.3).

The insemination rate for *G. brevipalpis* ranged from 0.87 for flies fed on the bovine (control) diet to 0.94 for flies fed on the diet with GMP. The spermatecae fill of *G. brevipalpis* females was low for all the diets with the majority of the femalesa spermatecae filling in the 0.25 or 0.5 class range. The *G. austeni* females had an insemination rate of > 0.98 for all the diets and the majority of the females had a spermatecae fill of between 0.5 and 0.75 (Table 4.3).

All the phagostimulant-treated as well as the bovine (control) diets resulted in QF values of above one for *G. brevipalpis* (Table 4.3; Fig. 4.3) and there was no significant differences ($P = 0.64$).For *G. austeni*, the QF values of the diet spiked with CMP (0.98) was below one (Table 4.3; Fig. 4.3). The highest QF value was obtained with the blood mixed with ATP (1.18) and this was significant different ($P < 0.01$) from that of the bovine (control) diet (1.06).

4.3.4   The colonisation of tsetse flies collected from north eastern KwaZulu-Natal

A total of 16 569 *G. brevipalpis* and 198 *G. austeni* were collected from 11 August 2014 to 25 April 2015 with 34 odour baited H traps. For *G. brevipalpis* the flies were both males (7356) and females (9213) of different ages. For *G. austeni* the majority were female. All collected flies were transferred to the insectary and the sexes separated. Flies were fed on bovine blood using an artificial membrane. Very few flies fed on this system and large numbers of deaths were observed. At the end of the collection period the flies were

transported to ARC-OVI tsetse fly colony. A 58% mortality rate was observed for *G. brevipalpis* after collection and transport to the ARC-OVI.
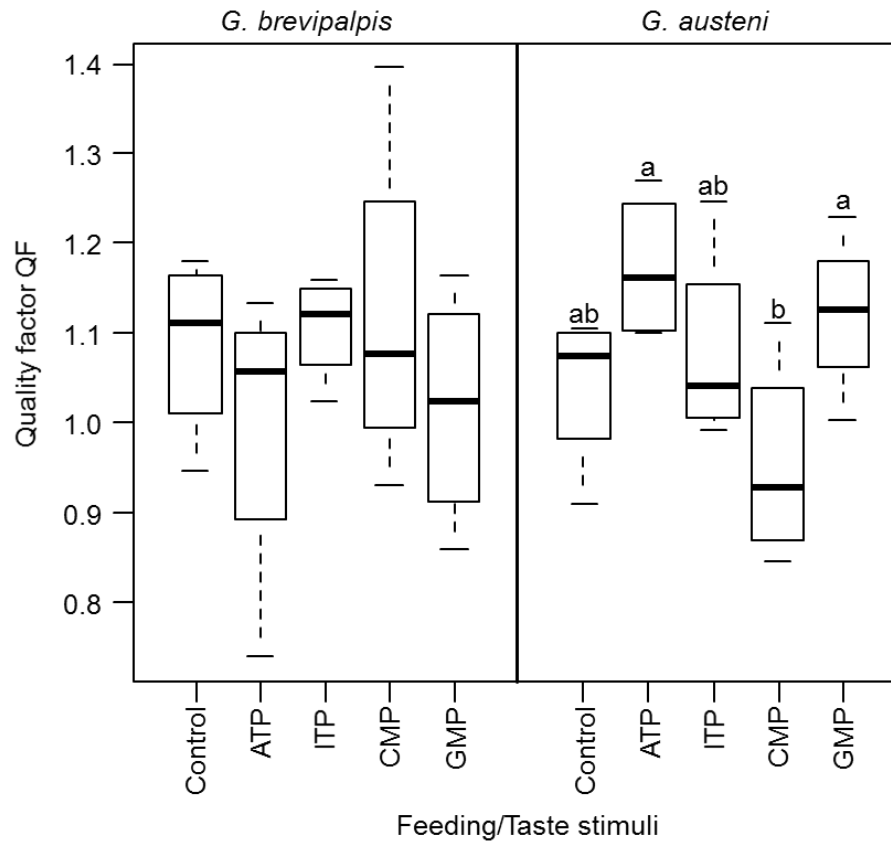
Fig. 4.3. Quality Factor (QF) values for blood mixed with different phagostimulants as obtained in the standard bioassay for *Glossina brevipalpis* and *Glossina austeni*. Each box shows the group median separating the 25th and 75th quartiles, capped bars indicate maximum and minimum values, boxes denoted by a different letter indicate that the QF values were significantly different for each species at the 5% level.

Table 4.3. Blood mixed with different phagostimulats tested for their potential use as rearing diet for *Glossina brevipalpis* and *Glossina austeni.*

	No. of mature Females		Pupae produced	Fecundity	Pupal size classes					Quality factor (QF)	Uterus					Insemination	Spermatheca fill			
											Recently ovulated egg	Empty due to abortion	Viable instar larvae							
	Day 18	Day 30			A	B	C	D	E				I	II	III		0.25	0.5	0.75	1
G. brevipalpis																				
Control	115	113	83	0.056	1	1	20	23	38	1.09	38	35	22	7	11	0.87	39	17	8	3
ATP	92	91	57	0.048	2	6	16	21	12	1.02	26	21	9	13	9	0.87	22	14	13	0
ITP	116	113	84	0.056	3	3	19	21	38	1.11	47	28	20	10	6	0.89	42	25	6	4
CMP	109	107	75	0.054	0	6	20	24	25	1.10	48	21	26	6	5	0.93	41	17	6	3
GMP	116	110	65	0.044	1	5	10	22	27	1.10	44	21	21	18	3	0.94	50	17	14	2
G. austeni																				
Control	96	86	66	0.057	9	17	20	17	3	1.06	39	10	20	4	7	0.99	11	38	30	6
ATP	71	59	56	0.067	3	7	20	23	3	1.18	22	7	14	13	0	0.98	9	22	22	4
ITP	103	94	74	0.058	7	8	29	21	9	1.08	42	10	25	6	4	0.99	5	57	21	4
CMP	89	75	52	0.049	16	9	19	7	1	0.98	41	5	17	6	6	0.99	8	40	21	4
GMP	103	96	72	0.056	6	4	27	20	15	1.14	50	5	18	13	4	1.00	6	60	21	7

The 9213 field-collected *G. brevipalpis* females produced 3722 pupae (40.4% pupation rate) out of which 1216 flies emerged (32.7% emergence rate); 768 were females and 448 males. These F_1 flies subsequently produced 891 pupae (73.3%) from which 288 flies emerged (32.3%), *i.e.* 167 females and 121 males. The F_2 flies produced 162 pupae (56.3%), however, only five flies emerged.

The use of porcine blood to improve survival and pupae production of *G. brevipalpis* was also assessed. Flies were collected from the field and fed a 100% bovine, 100% porcine and a 50% bovine 50% porcine blood meals. Twenty females were used in each of the five replicates. High initial mortalities were seen for all three diets, *i.e.*, on day seven, 50% of flies fed on the porcine diet had died, while on day eight, 50% of flies fed on the bovine diet died and on day nine, all flies fed on the combination diet were dead. The flies fed on the bovine and the combination diets did significantly better ($P < 0.01$) than the flies that fed on the porcine diet. Additionally, flies that fed on the combination diet produced more pupae (138) than flies on the bovine diet (111) and lastly flies that fed on porcine blood produced significantly ($P = 0.03$) fewer pupae (29). A higher percentage of flies died of starvation in the porcine (77%) and combination (71%) diet trials compared with the bovine diet (48%).

4.4   Discussion

A high quality rearing diet (blood) is essential for growth and sustainability of tsetse fly colonies (Feldmann, 1994b). The extent of the influence of chemicals and microbiological contaminants as well as genetic, environmental, chemical and physical factors on the quality of the diet can differ between colonies and species.

Sterile, freshly frozen, defibrinated or heparinized blood used with a silicon membrane is considered the most efficient way of maintaining tsetse fly colonies (Bauer & Wetzel, 1976; Gooding *et al.*, 1997). In the present study, defibrinated bovine blood was the most suitable diet for both *G. brevipalpis* and *G. austeni* colonies. Additionally, it was shown that bovine blood collected with the anticoagulants sodium citrate, citric sodium combination, CPDA and citric acid were suitable for both *G. brevipalpis* and *G. austeni* feeding. While ACD did not impacted negatively on the maintenance of *G. austeni* it was not suitable for the rearing of *G. brevipalpis*. Although the addition of anticoagulants will simplify collection and make the process more sterile, it is expensive and will increase costs for large-scale operations. As these anticoagulants did not significantly improve the blood quality and colony productivity it would be more economical to use defibrinated blood in tsetse fly mass-rearing facilities.

Both *G. brevipalps* and *G. austeni* are known to feed on bovine and porcine hosts in the wild (Moloo, 1993; Clausen *et al.*, 1998). Porcine blood has been used successfully for the *in vitro* feeding of colonies of *G. morsitans* and *G. austeni* (Gee, 1977; Clausen *et al.*, 1998). Combinations of fresh/frozen bovine blood and reconstituted lyophilized porcine blood have been used for a *G. austeni* colony in Tanzania (Tarimo *et al.*, 1988). Combinations of bovine and porcine blood as supplements in the synthetic diets gave survival rates between 70% and 90% for *Glossina palpalis palpalis* (Kabayo & Taher, 1986). In the present study feeding on a 50% / 50% combination of defibrinated bovine and porcine blood or feeding on either bovine or porcine on alternating days improved the overall *G. austeni* pupae production, and would be useful to accelerate colony growth. This is not surprising as DeLoach & Spates (1991) discovered that porcine erythrocytes contain phosphatidycholine wheras bovine erythrocytes do not, choline is considered an essential dietary requirement for insects. Although bovine blood seemed to be more appropriate for a *G. brevipalpis* colony, feeding a single source on alternating days did improve productivity. Using porcine blood for the ARC-OVI colonies to boost production in times of low performance would be beneficial.

Observed differences in the quality of the blood collected from bovines at commercial abattoirs and the closed quarantined herd that effect the production of the tsetse flies clearly indicates that the quality of the blood used in the colony is very important.

Adding phagostimulants to the blood diet at the concentrations used in the present study did not improve colony productivity as indicated by the obtained QF values. Taste receptors of tsetse flies, which assist in ingestion of blood, are stimulated by adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) (Galun & Margalit, 1970). ATP at a concentration of 10^{-3} M has been used to promote fly engorgement and thus improve colony production (Mews *et al.*, 1976; Galun, 1988; Galun & Kabayo, 1988). ATP is an expensive component to add to the maintenance diet (Galun, 1988), especially in mass-rearing but it has been shown that *G. tachinoides* are more sensitive to ATP and that even a low dose will enhance feeding (Galun, 1988). The simultaneous use of ATP and sodium bicarbonate will synergistically enhance feeding; however, the use of a suitable feeding membrane is also important (Galun, 1988). Other nucleotides such as AMP, ADP, as well as mono- and tri-phosphates of inosine (IMP, ITP), guanosine (GMP, GTP) and cytosine (CMP, CTP), all at a concentration of 10^{-4} M are also effective phagostimulants for *G. brevipalpis* and *G. austeni* (De Beer *et al.*, 2012). Increasing the concentration of ITP, CMP and GMP to 10^{-3} M improved production of *G. brevipalpis* more than the addition of ATP alone. However, ATP and ITP at a 10^{-3} M concentration did not improved the production of *G. austeni*. These phagostimulants remain

expensive and is recommended as a tool in colonies where flies struggle to adapt to artificial feeding *i.e.* at the onset of colonization of wild flies.

Most tsetse fly colonisation was initially done on animals (rabbits, goats, guinea pigs) before migrated to *in vitro* feeding (Mews & Ruhm, 1971; Nash *et al.*, 1971; 1972; Mews *et al.*, 1977; Wetzel & Luger, 1978; Mutika *et al.*, 2013). Colonising field-collected tsetse flies on *in vitro* feeding is challenging, due to the initial high mortalities, it was, however, successful for *G. austeni* (Opiyo *et al.*, 2000) and *G. palpalis gambiensis* (Momar Seck, ISRA-LNERV, personal communication). An advantage is that an early selection is made for field flies that will feed on the artificial system and this will be beneficial for further colonization. However, the high initial mortality necessitates starting with large numbers as was the case with *G. brevipalpis.* A different feeding approach seems to be required for *G. austeni* as none fed on the membrane system. Because of the low initial *G. austeni* numbers there should be a system in place that will reduce the mortality in collected flies.

The failure to colonise *G. brevipalpis* from KwaZulu-Natal was mainly due to three reasons. Firstly, the field flies were reluctant to feed on blood using an *in vitro* feeding system. Although a combination of bovine and porcine blood can be used to increase survival and pupae production, this combined blood diet did not increase the feeding response and could not reduce the initial mortalities. High mortalities were mainly due to flies not feeding and starvation. Phagostimulants mixed with the combined blood meal might improve the initial feeding response; however, this still needs to be evaluated. A second contributing factor was the high mortality during transport from the field to the ARC-OVI rearing facility. Transporting pupae rather than adults might solve this problem. The third and most significant problem was the very low adult emergence rate. The reasons for this very low emergence are not clear, but sub-optimal environmental conditions at which the pupa were kept as well as the use of a blood meal of low quality might be contributing factors. Further effort is required to improve fly emergence. Nash *et al.* (1968) developed a pupae maintenance system where pupae were collected in dry sand trays suspended over wet sand to increase humidity. The benefits of keeping the *G. brevipalis* (KwaZulu-Natal strain) pupae in such a management system need to be investigated.

The mass-rearing of tsetse flies remains challenging, especially so if more than one species is involved. The optimal rearing diet may differ between colonies and tsetse species and might need to be customised for each production unit. Decisions on the most suitable rearing diet will not only depend on the biological requirements of the flies involved but will also be influenced by the availably of a suitable blood source on a continuous and economic basis. Quality control and research on factors to optimise the diet needs to be done continuously.

Chapter 5

Evaluation of radiation sensitivity of tsetse males

5.1   Introduction

The idea of using tsetse flies to control tsetse flies was conceived by F.L. Vanderplank and colleagues in the 1930s and 1940s. Several studies (Corson, 1932; Potts, 1944; Vanderplank, 1944; 1947; 1948) demonstrated that laboratory crosses between *Glossina morsitans* and *Glossina swynnertoni* produced offspring with low fertility. These hybrid males were sterile and the females partially so (Vanderplank, 1947). Vanderplank (1944) suggesting that this induced sterility, by crossing two closely related species, could be used to control populations, especially as random matings between these two species had been recorded (Jackson, 1945).

The successful implementation of this control technique (hybrid sterility) was demonstrated in an arid area in Tanzania where only *G. swynnertoni* was found. For a period of seven months, from August 1944 to February 1945, 101 000 fertile *G. morsitans* were released in this area from adults that had emerged from field collected pupae. The size of the target area was 26 km^2 and geographically isolated from other tsetse fly infested areas with a barrier of approximately 19 km (Klassen & Curtis, 2005). After the release of these *G. morsitans* adults, the numbers of *G. swynnertoni* declined drastically and continued doing so into 1946 (Vanderplank, 1947). This release of *G. morsitans* virtually eliminated *G. swynnertoni* from the area. Since the released *G. morsitans* were not able to become established in this arid habitat their numbers also declined. The area was returned to the local inhabitants who, then, cleared the bush to eliminate any tsetse fly habitat (Klassen & Curtis, 2005). This was one of the first examples showing that sterility, induced when different species are hybridized, can be used for tsetse fly control (Robinson, 2005).

Independent of Vanderplank, E.F. Knipling suggested in the 1930s that sterile males, although he was unable to sterilise them, could be used to reduce or eradicate wild populations of pest insects (Lindquist, 1955; Klassen & Curtis, 2005). Although it was already known before the 1930s that X-rays and ionizing radiation could induce sterility in insects (Runner, 1916; Muller 1927), it was only in the 1950s that Knipling became aware of the detrimental biological effects of radiation (Muller, 1950) and its potential for insect control (Baumhover, 2001; 2002). This led to the most successful area-wide integrated pest management (AW-IPM) programme, integrating a Sterile Insect Technique (SIT) component, that was implemented over 50 years, *i.e.* the eradication of the New World screwworm *Cochliomyia hominivorax* (Diptera: Calliphoridae) from the southern USA,

Mexico and Central America to Panama (Van der Vloedt & Klassen, 1991; Vargas-Terán *et al.,* 2005). Presently the SIT is used for the control of a variety of insects of agricultural, veterinary and medical importance (Dyck *et al.*, 2005).

After the successful control of *G. swynnertoni* in Tanzania (Vanderplank, 1947; Klassen & Curtis, 2005), the SIT was used in feasibility studies (chemical or radiation sterilization) for the control of *Glossina morsitans morsitans* in Zimbabwe and Tanzania, *Glossina tachinoides* in Chad and *Glossina palpalis gambiensis* in Burkina Faso (Dame & Schmidt, 1970; Cuisance & Itard, 1973; Dame *et al.,* 1975; 1981; Van der Vloedt *et al.*, 1980; Williamson *et al.,* 1983a; Klassen & Curtis, 2005). The first eradication campaign that integrated the use of radiation-sterilized adults with other suppression methods such as insecticide impregnated targets was implemented in Burkina Faso in the 1980's against *Glossina morsitans submorsitans*, *G. p. gambiensis* and *G. tachinoides* (Politzar & Cuisance, 1984). *Glossina palpalis palpalis* was simultaneously targeted in Nigeria and populations of all these four species were eradicated from the target areas (Takken *et al.*, 1986). These control strategies were, however, not following area-wide IPM principles, and their pest free status was lost due to reinvasion (Klassen & Curtis, 2005).

The most successful AW-IPM programme, with a SIT component to eradicate tsetse flies, was implemented on Unguja Island, Zanzibar, in the 1990's (Vreysen *et al.,* 2000). Suppression of *Glossina austeni* by means of insecticide-treated screens and cattle was started in 1988 and from August 1994 to December 1997, 8.5 million sterile male flies were released on the iland (Vreysen *et al.,* 2000). This was also the first aerial releases of sterile tsetse males. The last wild tsetse fly was collected on the island in September 1996 and to date Unguja Island is still free of tsetse flies and Trypanosomosis.

The current eradication campaign of a *G. p. gambiensis* population in the Niayes, Senegal also integrates SIT into an AW-IPM programme (Bouyer *et al.,* 2010; Dicko *et al.,* 2014). The Niayes area was divided into three zones for control. Suppression with insecticide treated targets and cattle started in the first zone in December 2010 and sterile males were released as of March 2012, and the last wild female was collected in August 2012 (Dicko *et al.,* 2014). In the second zone suppression started in November 2012 and sterile males were released in March 2014. During 2016 sterile males were still being released in zone two with suppression underway in the third zone (Dicko *et al.,* 2014).

To be successful, the released sterile males must be able to compete with local wild males (Vreysen *et al.,* 2011). In this chapter the focus will be on the influence of radiation on the productivity of the insect. The effect of gamma radiation on the reproduction and competitiveness of several tsetse species has been investigated. Radiation doses ranging from 50 Gy for *Glossina brevipalpis* up to 170 Gy for *G. tachinoides* has been reported to

induce acceptable or complete sterility in these two species (Itard, 1968; Vreysen *et al.,* 1996). A dose of 110 Gy to 120 Gy has been reported sufficient to induce sterility in a number of species, *i.e. G. austeni* (Curtis, 1968), *G. tachinoides* (Offori & Clock, 1975), *G. p. palpalis* (Van der Vloedt *et al.*, 1978), *Glossina pallidipes* (Opiyo, 2001) and *G. p. gambiensis* (Sow *et al.*, 2012).

The *G. brevipalpis* and *G. austeni* colonies at the Agricultural Research Council – Onderstepoort Veterinary Institute (ARC-OVI) in Pretoria, South Africa have been in culture for longer than 30 years (Chapter 4). Very little is known on the radiation sensitivity of *G. brevipalpis* adults and there is no information on irradiation of pupae. The radiation sensitivity of *G. austeni* has been investigated previously, however, the sensitivity of these long colonised tsetse flies was re-evaluated.

5.2   Materials and methods

5.2.1   Colony tsetse flies

For radiation sensitivity studies of adults and pupae of *G. brevipalpis* and *G. austeni*, the flies were derived from the laboratory colonies housed at the ARC-OVI*.* The origin and holding conditions (Feldmann, 1994a; FAO/IAEA standard operating procedures 2006) of these colonies are described in detail in Chapter 4.

Adult males of *G. brevipalpis* and *G. austeni* were irradiated four days after emergence. For pupae irradiation they were collected from the colonies in 24 hour increments to synchronise adult emergence. The pupae were irradiated on three specific times, *i.e.*, three (group 1), five (group 2) or seven (group 3) days before expected emergence. The *G. brevipalpis* were irradiated on day 41 (group 1), 39 (group 2) or 37 (group 3) of pupation and the *G. austeni* on day 36 (group 1), 34 (group 2) or 32 (group 3) of pupation.

5.2.2   Radiation evaluation procedures

Adult males and pupae were given a radiation dose of either 40 Gy, 80 Gy, 100 Gy, 120 Gy or 140 Gy using a Caesium Gammacell providing a dose rate of 0.69 Gy/min. To determine their reproductive success, six-day-old males from all treatments (pupae and adults) were mated with three-day-old virgin females at a 1 : 2 male (N = 15) : female (N = 30) ratio. All treatments were repeated three to four times. Males and females were kept together for four days in standard holding cages under colony conditions (23-24 °C, 75-80% RH and sub-dued/indirect lighting) (Feldmann, 1994a; FAO/IAEA standard operating procedures, 2006). The experimental flies were fed daily on bovine blood using an artificial membrane.

The blood was collected with a closed sterile system as described in Chapter 4 (Feldmann, 1994a; FAO/IAEA standard operating procedures, 2006).

Male and female survival was monitored daily and female pupae production recorded. Fecundity was determined by the number of pupae produced per mature female day (Curtis, 1968). Mature female days were calculated for each treatment by adding the number of flies alive each day, starting on day 18 after emergence until the end of the experiment on day 60 (Curtis, 1968).

All pupae produced were mechanically sorted into five distinct size classes as described in Chapter 4. Adult emergence rate was also recorded. Abortions of eggs and immature larval stages were monitored daily. After 60 days all surviving females were dissected to determine their reproductive status, insemination rate and spermatecae fill (Feldmann, 1994a; FAO/IAEA standard operating procedures, 2006). The spermatecae were removed and the fill microscopically scored as either, empty (0), quarter full (0.25), half (0.5), three quarters (0.75) or full (1) (Nash, 1955). Male mortality was monitored until all the males had died.

5.2.3   Statistical analysis

Data were analysed using the statistical software GraphPadInstat (version 3.00, 2003). Proportional differences in adult emergence rates were determined with Chi-square (x^2) analysis with the Yate's continuity correction. Linear and multiple regression analyses were carried out on fecundity as well as male survival in relation to radiation dose. All tests were done at the 5% significance level.

5.3   Results

5.3.1   Adult emergence rate

Glossina brevipalpis females irradiated as pupae three days before expected emergence (group 1), started emerging between day 37 and 38 after larviposition, continued for two days and peaked on day 39. The males from this group started to emerge between day 40 and 41, peaked from day 42 to 43 and no emergence was seen after day 44 post larviposition. Total adult emergence varied from four days for the non-irradiated pupae and pupae irradiated with 40 Gy to seven days for pupae treated with 120 Gy. Although the dose did not affect the day emergence started it extend this period markedly.

This same trend was observed for pupae irradiated five (group 2) and seven days (group 3) before emergence. The females from group 2 pupae started to emerge between day 38 and 39 and peaked at 39 and 40 post larviposition. The males started to emerge between day 41 and 43, peaked on day 43 and no more flies emerged after 49 days. The

total adult emergence period ranged from seven days for the un-irradiated pupae and those irradiated with 40 Gy to 10 days for pupae done with 140 Gy. The females from group 3 pupae started to emerge between day 39 and 41 and peaked on day 42. The males started on day 43, peaked on day 45, and emergence stopped on day 47 post larviposition. The total adult emergence period varied from six days for un-irradiated pupae to eight days for pupae irradiated with 140 Gy. The differences seen in the emergence period between pupae irradiated three, five and seven days before emergence was within the normal variation seen in the controls (non-irradiated pupae) of each group.

Adult emergence from the *G. brevipalpis* pupae that were treated with the five doses was compared with that of the controls for each of the three radiation groups (Table 5.1). Adult emergence from the *G. brevipalpis* pupae (group 1) that had been treated with a dose of 100 Gy or higher was significantly lower (100 Gy: $P < 0.01$, $\chi^2 = 20.14$, d.f. = 1; 120 Gy: $P < 0.01$, $\chi^2 = 29.07$, d.f. = 1; 140 Gy: $P < 0.01$, $\chi^2 = 25.62$, d.f. = 1) as compared to emergence of the control group (95.2%) (Table 5.1). For group 2 pupae, adult emergence from pupae irradiated with a dose of 40 Gy ($P = 0.03$, $\chi^2 = 4.69$, d.f. = 1), 80 Gy ($P = 0.03$, $\chi^2 = 4.69$, d.f. = 1) and 120 Gy ($P < 0.01$, $\chi^2 = 13.29$, d.f. = 1) was significantly lower from that of the controls (77.3%). For group 3 pupae, adult emergence from pupae that had been irradiated with a dose of 140 Gy was significantly higher ($P = 0.04$, $\chi^2 = 4.12$, d.f. = 1) from that of the control ones (69.6%) (Table 5.1). On average the male to female ratio of *G. brevipalpis* that emerged from all three pupal treatment groups and the control pupae was 1 : 1 and did not differ significantly between treatments (Table 5.1).

Table 5.1. Comparison of emergence rates of adult *Glossina brevipalpis* from pupae irradiated with different doses and on different days before expected emergence.

Radiation dose (Gy)	No. pupae	Males (%)	Females (%)	Total (%)	Male / Female ratio
Pupae irradiated 3 days before emergence (group 1)					
Control	167	88 (55.4)	71 (44.7)	159 (95.2)	1 : 0.8
40	167	88 (54.7)	73 (45.3)	161 (96.4)	1 : 0.8
80	167	74 (49.7)	75 (50.3)	149 (89.2)	1 : 1
100	167	63 (48.5)	67 (51.5)	130 (77.8)*	1 : 1.1
120	167	47 (38.5)	75 (61.5)	122 (73.1)*	1 : 1.6
140	167	72 (57.6)	53 (42.4)	125 (74.9)*	1 : 0.7
Pupae irradiated 5 days before emergence (group 2)					
Control	132	65 (63.7)	37 (36.3)	102 (77.3)	1 : 0.6
40	132	57 (67.1)	28 (32.9)	85 (64.4)*	1 : 0.5
80	132	59 (69.4)	26 (30.6)	85 (64.4)*	1 : 0.5
100	132	59 (60.8)	38 (39.2)	97 (73.5)	1 : 0.6
120	132	32 (43.8)	41 (56.2)	73 (55.3)*	1 : 1.3
140	132	50 (54.4)	42 (45.7)	92 (69.7)	1 : 0.8
Pupae irradiated 7 days before emergence (group 3)					
Control	112	34 (43.6)	44 (56.4)	78 (69.6)	1 : 1.3
40	112	38 (52.8)	34 (47.2)	72 (64.3)	1 : 0.9
80	112	44 (55.0)	36 (45.0)	80 (71.4)	1 : 0.8
100	112	44 (54.3)	37 (45.7)	81 (72.3)	1 : 0.8
120	112	47 (57.3)	35 (42.7)	82 (73.2)	1 : 0.7
140	112	55 (59.8)	37 (40.2)	92 (82.1)*	1 : 0.7

*Emergence rate statistical significant different from the control group at the 5% level.

On average adult emergence started five days (± 1.55) earlier in *G. austeni* than in *G. brevipalpis*. Different to *G. brevipalpis* dose rate did affect the commencement day for the total duration of the emergence period for all of the pupae groups. Female emergence from group 1 pupae irradiated three days before expected emergence started between day 33 and 34 post larviposition and peaked on day 37. Male emergence in this group commenced between day 36 and 37, peaked on day 38 and stopped on day 40 post larviposition. A similar pattern was seen in the pupae irradiated five (group 2) and seven (group 3) days before expected emergence. The females started to emerge between day 33 and 35 for group 2 pupae and between day 31 and 35 post larviposition for group 3 pupae. Female emergence from group 3 pupae peaked between day 36 and 38 and for group 2 pupae on day 36 post larvipositon. The male flies started to emerge from group 2 and 3 pupae between day 36 and 38 and between days 36 and 37, respectively. Adult male emergence peaked on day 39 for both pupae groups 2 and 3 and no more emergence was seen in both groups 41 days post larviposition.

The emergence rate of the *G. austeni* pupae irradiated with five doses was also compared separately with those of the untreated controls for each treatment group (Table 5.2).

Table 5.2. Comparison of emergence rates of *Glossina austeni* pupae irradiated with different doses and on different days before expected emergence.

Radiation dose (Gy)	No. pupae	Males (%)	Females (%)	Total (%)	Female / Male Ratio
Pupae irradiated 3 days before emergence (group 1)					
Control	106	43 (49.4)	44 (50.6)	87 (82.1)	1 : 1
40	103	47 (52.8)	42 (47.2)	89 (86.4)	1 : 0.9
80	103	51 (58.0)	37 (42.1)	88 (85.4)	1 : 0.7
100	103	53 (52.5)	48 (47.5)	101 (98.1)*	1 : 1.1
120	103	55 (55.6)	44 (44.4)	99 (96.1)*	1 : 0.8
140	103	47 (51.1)	45 (48.9)	92 (89.3)	1 : 1
Pupae irradiated 5 days before emergence (group 2)					
Control	112	49 (62.0)	30 (38.0)	79 (70.5)	1 : 0.6
40	110	60 (53.5)	50 (46.5)	110 (100.0)*	1 : 0.8
80	110	60 (58.0)	47 (42.1)	107 (85.4)*	1 : 0.8
100	110	55 (52.5)	47 (47.5)	102 (98.1)*	1 : 0.9
120	110	59 (55.6)	46 (44.4)	105 (96.1)*	1 : 0.8
140	110	45 (51.1)	57 (48.9)	102 (89.3)*	1 : 1.3
Pupae irradiated 7 days before emergence (group 3)					
Control	152	75 (54.4)	63 (45.7)	138 (90.8)	1 : 0.8
40	149	61 (49.2)	63 (50.8)	124 (83.2)	1 : 1
80	149	68 (52.7)	61 (47.3)	129 (86.6)	1 : 0.9
100	149	82 (60.3)	54 (39.7)	136 (91.3)	1 : 0.7
120	149	67 (51.9)	62 (48.1)	129 (86.6)	1 : 0.9
140	149	78 (56.5)	60 (43.5)	138 (92.6)	1 : 0.8

* Emergence rate statistical significant different from the control group at the 5% level.

Adult emergence from group 1 pupae (irradiated three days before emergence) that had been treated with a dose of 100 Gy ($P < 0.01$, $\chi^2 = 13.05$, d.f. = 1) and 120 Gy ($P < 0.01$,

$\chi^2 = 9.13$, d.f. = 1) was significantly greater as compared with that of the controls (82.1%) (Table 5.2). Adult emergence from group 2 pupae (irradiated five days before emergence) was significantly greater than that of the controls (70.5%) (40 Gy: $P < 0.01$, $\chi^2 = 35.78$, d.f. = 1; 80 Gy: $P < 0.01$, $\chi^2 = 27.27$, d.f. = 1; 100 Gy: $P < 0.01$, $\chi^2 = 16.71$, d.f. = 1; 120 Gy: $P < 0.01$, $\chi^2 = 22.57$, d.f. = 1; 140 Gy: $P < 0.01$, $\chi^2 = 16.701$, d.f. = 1) (Table 5.2). Adult emergence from group 3 pupae was similar for all doses as compared with the untreated control group. In all treatment groups *G. austeni* emerged in equal female to male ratios, with the exception of the pupae irradiated with 140 Gy in group 2 ($P = 0.02$, $\chi^2 = 5.02$, d.f. = 1) (Table 5.2).

5.3.2   Reproduction in females mated with males irradiated as adults or pupae

Of the 2820 *G. brevipalpis* females (30 for each treatment replicate) at the onset of the experiments, 2668 survived to day 18 post emergence. The survival of mature *G. brevipalpis* females in all individual experiments exceeded 95.9% on day 18, except in the group 3 pupae (irradiated seven days before emergence) where mature female survival was lower (88.8%). The survival rate decrease at the end of the experiment (day 60) for all treatmnets.

Of the 2730 *G. austeni* females (30 for each treatment replicate) at the onset of the experiments, 2449 survived to day 18 post emergence. Although the survival for the mature females was lower than that of *G. brevipalpis* on day 18, it still exceeded 88.6%. The survival rate for *G. austeni* females also decreases at the end of the experiment (day 60) for all treatments.

As the radiation dose increased, fecundity (number of pupae produced per mature female) for both species decreased (Table 5.3 & 5.4). A negative linear regression was found between fecundity of the untreated female *G. brevipalpis* that had mated with adult males treated at the five radiation levels ($r^2 = 0.616$, $P < 0.01$), or that had been irradiated as pupae in all the pupae groups ($r^2 = 0.57$, $P = 0.01$). A similar negative linear regression was found between fecundity of untreated female *G. austeni* and radiation dose administered to their male mates as adults ($r^2 = 0.81$, $P < 0.01$) and as group 1 ($r^2 = 0.72$, $P < 0.01$), group 2 ($r^2 = 0.73$, $P < 0.01$) and group3 ($r^2 = 0.71$, $P < 0.01$), pupae. This showed fecundity to be dose dependent. The number of pupae produced by untreated female *G. brevipalpis* that had mated with males treated as adults with 40 Gy (number of pupae = 16) and 80 Gy (N = 2) was 6.7% and 0.8%, respectively as compared with the untreated controls (N = 240). The pupal production (Table 5.3) relative to the control group for the females mated with *G. brevipalpis* males irradiated with 40 Gy as pupae was 1.4% for group 1, 1.9% for group 2 and 2.9% for group 3 pupae. A radiation dose of 40 Gy and 80 Gy was

thus sufficient to induce 93% and 99% sterility, respectively in *G. brevipalpis* females when the males had been treated as adults. Induced sterility was 97% or higher when the males had been irradiated with 40 Gy for the different groups of pupae.

A dose of 40 Gy induced only 61.5% sterility in female *G. austeni* that had mated with males irradiated as adults. In females mated with males emerging from pupae irradiated three and five days before emergence the sterility was 61.6%, whereas it was 77.3% when the pupae were treated seven days before emergence. Higher doses of 80 Gy and 100 Gy induced 97% to 99% sterility in females that mated with males treated as adults or pupae. The pupal production (Table 5.4) relative to the controls for females mated with males irradiated as adults with a dose of 80 Gy was 2.6% and for males treated as pupae it was 2.2% in group 1, 3.1% in group 2 and 1.8% in group 3 pupae. Using a dose of 100 Gy the pupal production (Table 5.4) relative to the controls was 1.3%, 2.5% and 2.2% for females mated with males irradiated as pupae of group 1, 2 and 3, respectively. No pupae were produced by females that mated with males treated as adults with a dose of 100 Gy.

The number of eggs aborted during the 60 days experimental period was lower for both species in females that mated with non-irradiated males than in females mated with any of the males in the experimental groups (Table 5.3 & 5.4). For both species and all treatment groups the majority of the pupae produced were in or above the pupal size class C (Table 5.3 & 5.4). The male to female ratio that emerged from pupae produced by females mated with irradiated males were similar for both species and equally distributed (1 : 1 ratio) (Table 5.3 & 5.4).

Table 5.3. Production of *Glossina brevipalpis* females mated with males irradiated with different doses at different developmental stages.

Radiation dose (Gy)	Replications	No. of mature females (day 18)	No. of aborted eggs	No. pupae produced	Pupal size classes (mg)					Fecundity[#]	% Emergence / % females
					A (%) <56	B (%) 56-<68	C (%) 68-<76	D (%) 76-<84	E (%) >84		
Irradiated Male Adults											
Control	4	119	36	240	7 (2.9)	18 (7.5)	60 (25.0)	85 (35.4)	79 (29.2)	0.046	92.9 / 49.3
40	4	116	341	16	2 (12.5)	1 (6.3)	6 (37.5)	3 (18.8)	4 (25.0)	0.003	93.8 / 46.7
80	4	114	326	2	0	1 (50.0)	0	1 (50.0)	0	0	2 / 2 / 1 / 2
100	4	119	402	0	0	0	0	0	0	0	
120	4	112	287	0	0	0	0	0	0	0	
140	4	119	307	0	0	0	0	0	0	0	
Pupae irradiated 3 days before emergence (group 1)											
Control	4	114	20	294	15 (6.2)	45 (18.7)	87 (36.1)	68 (28.2)	26 (10.8)	0.060	67.2 / 61.4
40	4	116	248	4	0	0	2 (100)	0	0	0.001	3 /4 /2 / 4
80	4	116	218	0	0	0	0	0	0	0	
100	4	109	187	1	1 (100)	0	0	0	0	<0.001	0 / 1 / 0 / 1
120	4	119	240	1	0	0	0	1 (100)	0	<0.001	0 / 1 / 0 / 1
140	3	88	152	0	0	0	0	0	0	0	
Pupae irradiated 5 days before emergence (group 2)											
Control	4	115	13	269	25 (9.3)	59 (21.9)	92 (34.1)	83 (30.7)	11 (4.1)	0.057	86.3 / 58.2
40	4	115	230	5	1 (20)	2 (40)	1 (20)	1 (20)	0	0.001	80.0 / 25.0
80	4	118	252	0	0	0	0	0	0	0	
100	4	113	232	3	3 (100)	0	0	0	0	0.001	66.7 / 50.0
120	4	116	223	1	0	1 (100)	0	0	0	<0.001	0 / 1 / 0 / 1
140	4	117	221	1	0	0	0	0	0	<0.001	0 / 1 / 0 / 1
Pupae irradiated 7 days before emergence (group 3)											
Control	4	116	10	343	24 (7.8)	64 (20.7)	109 (35.3)	90 (29.1)	22 (71.2)	0.058	91.8 / 52.9
40	4	106	181	10	1 (11.1)	3 (33.3)	6 (55.6)	0	0	0.023	30.0 / 66.7
80	4	111	176	0	0	0	0	0	0	<0.001	
100	4	109	174	0	0	0	0	0	0	0	
120	4	96	150	2	0	1 (50)	1 (50)	0	0	0.001	0 / 0 / 0 / 0
140	3	75	118	2	0	0	2 (100)	0	0	<0.001	0 / 0 / 0 / 0

[#] Number of pupa produced per the mature female day

Table 5.4. Production of *Glossina austeni* females mated with males irradiated with different doses at different developmental stages.

Radiation dose (Gy)	Replications	No. of mature females (day 18)	No. of aborted eggs	No. pupae produced	A (%) <16	B (%) 16-<19	C (%) 19-<21	D (%) 21-<23	E (%) >23	Fecundity#	% Emergence / % females
Irradiated Male Adults											
Control	4	109	40	234	18 (7.7)	17 (7.3)	30 (12.8)	51 (21.8)	118 (50.4)	0.063	93.2 / 55.1
40	4	106	201	90	7 (7.78)	10 (11.1)	11 (12.2)	15 (16.7)	47 (52.2)	0.022	92.2 / 53.0
80	4	107	209	6	2 (33.33)	0	1 (16.67)	1 (16.67)	2 (33.33)	0.001	83.3 / 60.0
100	4	102	210	0	0	0	0	0	0	0	
120	4	109	224	1	0	0	1 (100)	0	0	<0.001	1 / 1 / 0 / 1
140	4	105	225	1	0	0	1 (100)	0	0	<0.001	0 / 1
Pupae irradiated 3 days before emergence (group 1)											
Control	4	97	45	226	4 (1.8)	9 (4.0)	30 (13.3)	82 (23.0)	132 (58.0)	0.064	89.4 / 59.4
40	4	110	261	70	1 (1.4)	2 (2.9)	7 (10.0)	10 (14.3)	50 (71.4)	0.015	87.1 / 54.4
80	4	114	268	5	0	0	0	2 (40.0)	3 (60.0)	0.001	4 / 5 / 1 / 5
100	4	110	280	3	0	0	0	1 (33.33)	2 (66.66)	0.001	2 / 3 / 1 / 3
120	4	111	241	2	0	0	0	0	2 (100)	0.001	2 / 2 / 2 / 2
140	4	107	288	4	0	2 (50.0)	1 (25.0)	0	1 (25.0)	0.001	4 / 4 / 1 / 4
Pupae irradiated 5 days before emergence (group 2)											
Control	3	75	40	159	16 (10.1)	10 (6.3)	40 (28.9)	29 (18.2)	58 (36.5)	0.062	57.9 / 45.7
40	3	78	121	61	2 (3.3)	3 (4.9)	21 (34.4)	18 (29.5)	17 (27.9)	0.021	80.3 / 63.3
80	3	78	156	5	0	0	1 (20.0)	3 (60.0)	1 (20.0)	0.001	3 / 5 / 2 / 5
100	3	87	151	4	1 (25.0)	0	1 (50.0)	0	1 (25.0)	0.001	2 / 4 / 1 / 4
120	3	88	167	0	0	0	0	0	0	0	
140	4	112	178	0	0	0	0	0	0	0	
Pupae irradiated 7 days before emergence (group 3)											
Control	4	99	44	277	11 (40.9)	21 (78.1)	36 (13.4)	62 (23.1)	139 (51.7)	0.070	53.4 / 65.5
40	4	111	168	63	5 (79.4)	7 (11.1)	13 (20.6)	13 (20.6)	25 (39.7)	0.014	69.8 / 65.9
80	4	106	239	5	0	0	1 (20.0)	2 (40.0)	2 (40.0)	0.002	2 / 5 / 1 / 2
100	4	97	200	6	0	0	4 (60.0)	2 (40.0)	0	0.004	3 / 6 / 2 / 3
120	4	118	230	0	0	0	0	0	0	0	
140	4	113	223	2	0	0	0	0	2 (100)	<0.001	2 / 2 / 1 / 2

Number of pupa produced per the mature female day

Table 5.5. Reproductive status of *Glossina brevipalpis* females mated with irradiated males at different developmental stages and radiation levels and dissected after an experimental period of 60 days.

Radiation dose (Gy)	Insemination %	Spermatecae fill				Uterus		Viable instar larvae		
		0.25 (%)	0.5 (%)	0.75 (%)	1 (%)	Recently ovulated egg	Empty due to abortion (abortion rate)	I	II	III
Irradiated Male Adults										
Control	92.2	16 (13.8)	27 (23.3)	60 (51.7)	4 (3.5)	50	17 (0.15)	16	14	16
40	100	10 (8.9)	25 (22.1)	62 (54.9)	16 (14.2)	15	98 (0.87)	0	0	0
80	91.9	23 (20.7)	29 (26.1)	37 (33.3)	13 (11.7)	8	103 (0.93)	0	0	0
100	99.1	5 (4.3)	33 (28.5)	56 (48.3)	21 (18.1)	18	98 (0.84)	0	0	0
120	100	15 (14.0)	36 (33.6)	40 (37.4)	16 (15.0)	19	88 (0.82)	0	0	0
140	100	0	53 (48.2)	46 (41.8)	11 (10.0)	31	79 (0.72)	0	0	0
Pupae irradiated 3 days before emergence (group 1)										
Control	94.6	13 (11.7)	35 (31.5)	57 (51.4)	0	65	23 (0.21)	7	9	7
40	92.7	18 (16.4)	36 (32.7)	48 (43.6)	0	31	76 (0.71)	0	0	0
80	87.6	16 (14.2)	46 (40.7)	33 (29.2)	4 (3.5)	18	95 (0.84)	0	0	0
100	95.3	28 (26.2)	32 (29.9)	41 (38.3)	1 (0.9)	21	86 (0.80)	0	0	0
120	97.4	27 (23.3)	35 (30.2)	51 (44.0)	0	38	78 (0.67)	0	0	0
140	89.9	18 (20.2)	35 (28.1)	35 (39.3)	2 (2.3)	10	76 (0.88)	0	0	0
Pupae irradiated 5 days before emergence (group 2)										
Control	96.9	5 (5.2)	22 (22.9)	63 (65.6)	3 (3.1)	29	12 (0.13)	6	15	31
40	95.9	14 (14.4)	33 (34.0)	45 (46.4)	1 (1.0)	26	75 (0.74)	0	0	0
80	94.3	12 (11.4)	41 (39.1)	45 (42.9)	1 (1.0)	23	81 (0.78)	0	0	0
100	94.3	18 (17.1)	18 (17.1)	60 (57.1)	3 (2.9)	20	86 (0.81)	0	0	0
120	97.2	12 (11.1)	34 (31.5)	58 (53.7)	1 (0.9)	28	79 (0.73)	1	0	0
140	96.4	16 (14.6)	44 (40.0)	46 (41.8)	0	22	88 (0.80)	0	0	0
Pupae irradiated 7 days before emergence (group 3)										
Control	97.1	11 (10.5)	26 (24.8)	64 (61.0)	1 (1.0)	49	24 (0.23)	1	14	16
40	100	7 (7.9)	26 (29.2)	55 (61.8)	1 (1.1)	27	61 (0.69)	1	0	0
80	97.98	14 (14.1)	26 (26.3)	56 (56.6)	1 (1.0)	28	71 (0.72)	0	0	0
100	97.67	11 (12.8)	23 (26.7)	50 (58.1)	0	15	69 (0.82)	0	0	0
120	93.98	12 (14.5)	28 (33.7)	38 (45.8)	0	17	66 (0.80)	0	0	0
140	96.88	2 (3.1)	28 (43.8)	32 (50.0)	0	16	48 (0.75)	0	0	0

Table 5.6. Reproductive status of *Glossina austeni* females mated with irradiated males at different developmental stages and radiation levels and dissected after an experimental period of 60 days.

Radiation dose (Gy)	Insemination %	Spermatecae fill				Recently ovulated egg	Empty due to abortion (abortion rate)	Viable instar larvae		
		0.25 (%)	0.5 (%)	0.75 (%)	1 (%)			I	II	III
Irradiated Male Adults										
Control	93.9	12 (18.2)	19 (28.8)	29 (43.9)	2 (3.0)	33	16 (0.26)	2	3	8
40	100	13 (15.7)	28 (33.7)	38 (45.8)	4 (4.8)	0	71 (0.84)	0	2	12
80	93.3	12 (14.0)	19 (33.7)	24 (27.9)	15 (17.4)	1	74 (0.99)	0	0	0
100	97.0	13 (19.4)	21 (31.3)	30 (44.8)	1 (1.5)	1	66 (0.99)	0	0	0
120	100	11 (12.4)	38 (42.7)	35 (39.3)	5 (5.6)	7	80 (0.92)	0	0	0
140	100	9 (10.5)	39 (45.4)	31 (36.1)	7 (8.1)	2	84 (0.98)	0	0	0
Pupae irradiated 3 days before emergence (group 1)										
Control	100	12 (18.5)	18 (27.7)	35 (53.9)	0	32	3 (0.05)	11	8	6
40	100	10 (10.8)	40 (43.0)	42 (45.2)	1 (1.1)	37	45 (0.48)	2	4	5
80	99.0	4 (4.0)	33 (32.7)	62 (61.4)	1 (1.0)	17	84 (0.83)	0	0	0
100	100	9 (10.0)	38 (42.2)	43 (47.8)	0	23	67 (0.74)	0	0	0
120	100	16 (12.0)	36 (43.4)	37 (44.6)	0	21	62 (0.75)	0	0	0
140	100	13 (14.0)	47 (49.0)	36 (37.5)	0	14	82 (0.85)	0	0	0
Pupae irradiated 5 days before emergence (group 2)										
Control	100	3 (7.0)	11 (25.6)	27 (62.8)	2 (4.7)	17	12 (0.28)	1	4	9
40	100	0	7 (11.3)	52 (83.9)	3 (4.8)	10	45 (0.73)	0	3	4
80	100	4 (6.0)	8 (11.9)	49 (73.1)	6 (9.0)	12	54 (0.81)	1	0	0
100	100	5 (8.3)	10 (16.7)	42 (70.0)	3 (5.0)	9	51 (0.85)	0	0	0
120	100	2 (2.6)	10 (19.2)	60 (75.7)	2 (2.6)	10	68 (0.87)	0	0	0
140	100	7 (7.0)	26 (26.0)	67 (67.0)	0	7	93 (0.93)	0	0	0
Pupae irradiated 7 days before emergence (group 3)										
Control	98.8	7 (8.4)	28 (33.7)	44 (53.0)	3 (36.1)	42	15 (0.19)	4	8	12
40	97.9	6 (6.5)	29 (31.2)	53 (60.0)	3 (32.3)	17	64 (0.71)	1	5	3
80	100	6 (6.3)	25 (26.3)	62 (65.3)	2 (21.1)	15	81 (0.84)	0	0	0
100	100	4 (4.5)	22 (25.3)	59 (67.8)	2 (23.0)	11	76 (0.87)	0	0	0
120	99.1	5 (4.6)	27 (25.0)	72 (66.7)	3 (27.8)	14	83 (0.85)	1	0	0
140	99.1	9 (8.6)	24 (22.9)	67 (63.8)	4 (38.1)	18	87 (0.83)	0	0	0

5.3.3 **Reproductive status of females inseminated by males irradiated as adult or pupae**

Average insemination of female *G. brevipalpis* (Table 5.5) and *G. austeni* (Table 5.6) flies, that were mated with males irradiated as adults was 98.0% ± 3.55 and 98.1% ± 2.96, respectively, as shown by dissection at the end of the experimental period of 60 days. Average insemination of untreated controls was 92.2% for *G. brevipalpis* and 93.9% for *G. austeni* which clearly showed that treatment of adult males did not affect their ability to transfer sperm to the females. Similar high insemination rates, independent of dose, were seen for both species irradiated as pupae (Table 5.5 & 5.6).

The reproductive status of the females of both species dissected at the end of the experiment at 60 days was markedly different between the females that had mated with non-irradiated males compared with treated males (Table 5.5 & 5.6). For both species the uterus of females mated with males irradiated as adults were, either empty due to abortions or contained a recently ovulated egg. Except *G. austeni* females of the 40 Gy-group, almost none of these females had any viable larvae *in utero* (Table 5.6). The same trend was found for all the pupal groups of both species, irrespective of dose (Table 5.5 & 5.6). In contrast, for both species the uterus of females that had mated with non-irradiated males as well as *G. austeni* females mated with males of the 40 Gy dose contained either a recently ovulated egg or a viable instar larva, and fewer females were found with an empty uterus due to an abortion (Table 5.5 & 5.6). All the females of both species at the end of 60 days appeared to be in good health, however, a build-up of fat bodies was observed in the females mated with males irradiated as adults or pupae.

5.3.4 **Male survival**

The average lifespan of *G. brevipalpis* males was radiation dose dependent with survival decreasing with increasing doses. A relatively strong negative linear regression was found between lifespan and dose rate in males treated as adults (r^2 = 0.67, P < 0.01), males irradiated as group 1 (r^2 = 0.83, P < 0.01), group 2 (r^2 = 0.79, P < 0.01) and group 3 (r^2 = 0.82, P < 0.01) pupae (Fig. 5.1). The age of the *G. brevipalpis* pupae at the time of irradiation affected the lifespan of the adult males. The males irradiated as adults lived the longest followed by males from the group 1 pupae, then group 2, and finally group 3 pupae (Fig. 5.1).

In *G. austeni* this negative linear correlation was only found for the males irradiated as adults (r^2 = 0.72, P < 0.01) and for males treated as pupae seven days before emergence (r^2 = 0.23, P = 0.02) (Fig. 5.2). The age of the pupae at the time of treatment did not affect the lifespan of the adult males as observed with *G. brevipalpis* males.
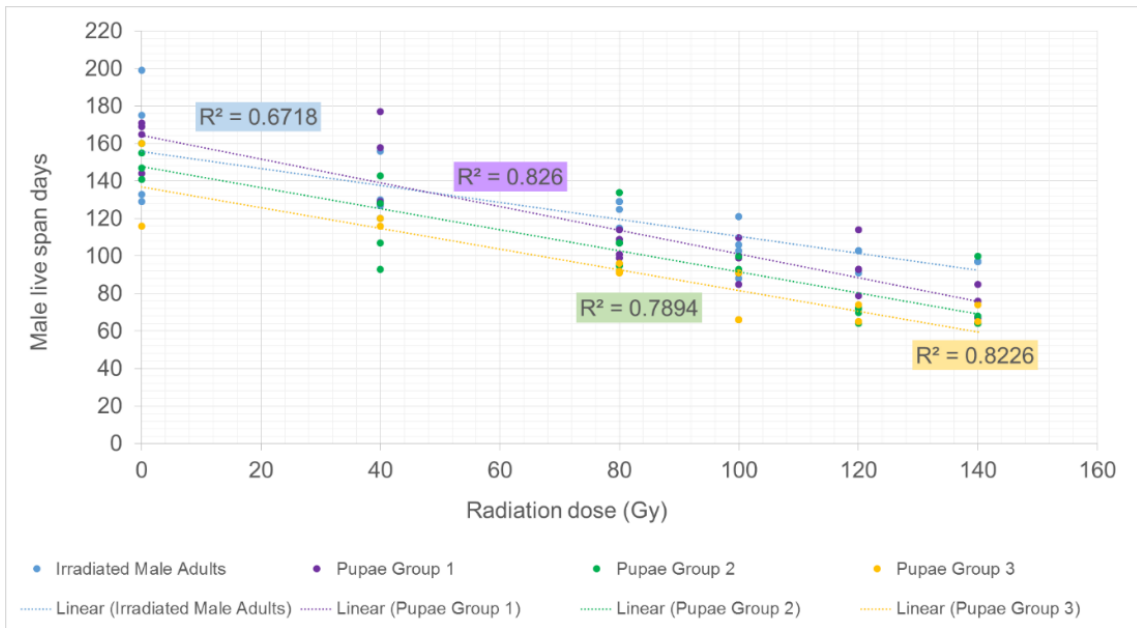
Fig. 5.1. Lifespan of *Glossina brevipalpis* males irradiated either as adults or as pupae three- (group 1), five- (group 2) or seven- (group 3) days before expected emergence.
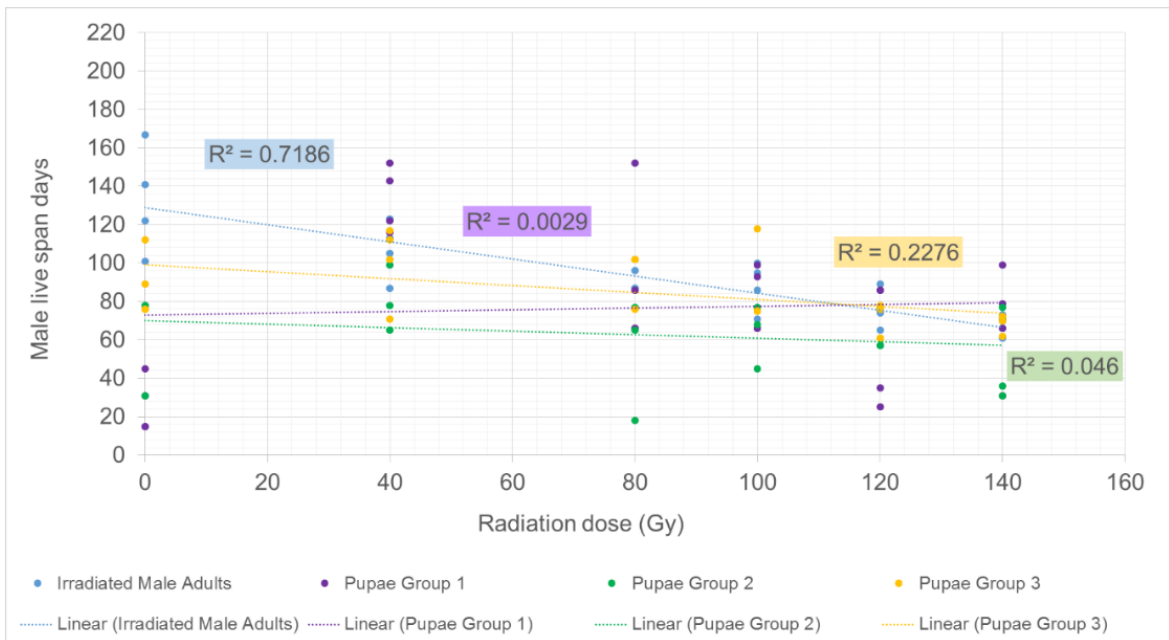
Fig. 5.2. Lifespan of *Glossina austeni* males irradiated either as adults or as pupae three- (group 1), five- (group 2) or seven- (group 3) days before expected emergence.

5.4   Discussion

Selecting an appropriate radiation dose to sterilise insects destined for release in a programme that includes a SIT component is very important, as high radiation doses can have a negative effect on the quality of these insects (Bakri *et al.,* 2005; Calkins & Parker, 2005; Lance & McInnis, 2005). A dose below the optimal will result in insects that are not sufficiently sterile and a too high dose may result in insects that are not competitive with wild flies (Bakri *et al.,* 2005). Factors such as the developmental stage of the insect, its age and the atmosphere used during irradiation can influence dose and the level of sterility achieved (Bakri *et al.,* 2005; Calkins & Parker, 2005).

Similar to other studies (Curtis, 1968; Vreysen *et al.*, 1996), the rate of induced sterility or the proportions of dominant lethal mutations induced in the sperm of *G. brevipalpis* and *G. austeni* when exposed to radiation increased with increasing dose. In the present study a dose of 40 Gy and 80 Gy induced 93% and 99% sterility respectively in *G. brevipalpis* females mated with males irradiated as adults, and a 97% or higher sterility when irradiated as pupae with a dose of 40 Gy. This is comparable with data presented by Vreysen *et al.* (1996), which indicated that a dose of 50 Gy administered to four- to six-day-old males in air induced about 95% sterility. This relatively low dose inducing more than 93% sterility indicates that the sperm of *G. brevipalis* are more sensitive to radiation than the sperm of others species such as *G. fuscipes fuscipes, G. tachinoides* (Vreysen *et al.,* 1996), *G. pallidipes* (Opiyo, 2001), *G. morsitans* (Curtis & Langley, 1972) and *G. austeni* (Curtis, 1969). Although Vreysen *et al.* (1996) suggested that this higher susceptibility might be chromosome related, the main reasons for this difference remain unclear.

Compared with *G. brevipalpis,* higher doses of 80 Gy and 100 Gy were needed to induce more than 97% sterility in *G. austeni* females that mated with males treated either as adults or pupae. The dose rate of 100 Gy or even 80 Gy is lower than that of 120 Gy proposed by Curtis (1970). The same dose (120 Gy) was also used in the successful eradication of *G. austeni* from Unguja Island (Vreysen *et al.*, 2000). Curtis (1969) used doses of 50 Gy, 60 Gy and 70 Gy to produce partially sterile *G. austeni* males and showed that these rates induced dominant lethality in 70-90% of their sperm, which is comparable to our data.

The somewhat longer emergence periods and high variation in emergence rate of irradiated pupae obtained in the present study may be an artefact of the basic rearing conditions in the colony. Due to logistical constrains these two species are kept under the same rearing conditions which may not be the most optimal for both species. Although the longer emergence periods were still within the natural variation observed for non-irradiated pupae these potentially extended pupal periods will have to be taken into consideration in

the implementation of an AW-IPM programme with an SIT component, as this can have time management and operational coast implications. Furthermore, due to the variation in emergence rates obtained for the different irradiation doses and time of irradiation for both species no clear effect of these variables could be established.Our data indicates a relatively higher irradiation susceptibility of *G. brevipalpis* and *G. austeni* pupae as compared to adults, and this was also observed by Van der Vloed & Taher (1978) for *G. p. palpalis*. These authors found that the production rate relative to the control for *G. p. palpalis* irradiated with 80 Gy was 7.1 for the adults and 4.08 for the pupae (Van der Vloedt *et al.,* 1978).

Dissection results revealed a clear abortion pattern for almost all of the *G. brevipalpis* females that mated with males, irrespective of treatment dose and life stage when irradiated. The same pattern was seen for *G. austeni* females that mated with males treated with 80 Gy or higher. The uterus of females that mated with irradiated males contained either an egg or was empty as a result of abortion of the embryo or the immature larva. Van der Vloedt & Barnor (1984) suggested that this observation could be used to monitor the impact of sterile male releases on a natural tsetse fly population. The imbalance between uterus content and the follicle next in ovulation sequence (Vreysen *et al.*, 1996), was indeed used successfully to monitor induced sterility in the wild female *G. austeni* population in the eradication campaign on Unguja Island (Vreysen *et al.,* 2000) and against *G. p. gambiensis* in Niayes Senegal (Bouyer *et al.,* 2010). The natural abortion rate of the population needs to be determined first for comparison with abortion rates during the sterile male release programme. It is clear from the dissection results that this method of assessing reproductive abnormalities as a result of mating of wild females with irradiated males, and hence, as a tool to monitor induced sterility in the targeted population, can, as in *G. austeni* and *G. p. gambiensis,* also be applied to *G. brevipalpis*. In addition, radiation did not affect *G. brevipalpis* and *G. austeni* males' insemination ability whether irradiated as adults or as pupae.

The reduction in average longevity of irradiated males as compared with untreated males is a manifestation of the somatic damage caused by irradiation (Vreysen *et al.,* 2000). This relationship was clearly observed for *G. brevipalpis* and it was furthermore shown that irradiation as pupae reduced adult longevity even more than treatment as adults. This negative linear regression between dose and longevity for *G. austeni* was, however, only observed when adults were irradiated. In contrast, an apparent increase in average longevity was seen when *G. austeni* pupae were treated three days before emergence. This radiation induced increase in average lifespan of males was also documented for *G. morsitans* pupae irradiated in air (Dean & Wortham, 1969) as well as in nitrogen (Curtis

& Langley, 1972). Many reasons for this increase in average lifespan have been suggested and it is most likely that a range of radiation induced repair mechanisms are involved (Calabrese, 2013).

The results of the present study clearly indicate that *G. brevipalpis* adults as well as pupae are highly sensitive to irradiation. It also confirms that the 30-year old colonised *G. austeni* is still susceptible to radiation. The results indicate that *G. brevipalpis* can be treated ether as adult or late pupa (up to seven days before emergence) with a dose of 80 Gy and *G. austeni* as late pupa (also seven days before emergence) with a dose of 100 Gy for use in a tsetse fly control programme with a SIT component. In addition to the small differences in the quality of sterile males when irradiated as adults or pupae, other logistical requirements, *e.g.* distance of mass-rearing facility and radiation source from release site, may also need to be taken into consideration when selecting the most efficient irradiation protocol. Treatment of pupae has its advantages, *i.e.*, large numbers of pupae can be irradiated at a time, the handling and transport of pupae is less cumbersome, and pupae are less fragile than adult flies. The competitiveness of the males irradiated as adults or pupae as compared with untreated males needs to be assessed and taken into account in any proposed radiation protocol.

Chapter 6

Comparative assessment of the mating performance of tsetse males under field cage conditions[3]

6.1   Introduction

The successful implementation of an area-wide integrated pest management (AW-IPM) programme with a sterile insect technique (SIT) component depends on a number of prerequisites (Vreysen *et al.*, 2007) of which the biological quality and sexual competitiveness of the sterile males are amongst the more important ones (Vreysen, 2005). The mass-reared, released sterile males must be able to compete with wild males for mating opportunities with the local virgin females (Vreysen *et al.,* 2011). Releasing low quality sterile males will necessitate higher release rates, requiring more funding that might prolong the duration of the programme and potential failure (Vreysen, 2005).

The age and radiation dose discussed in Chapter 5 can influence the mating success of released sterile males (Liedo *et al.*, 2002; Flores *et al.*, 2014). The competitiveness of *Glossina fuscipes fuscipes, Glossina palpalis palpalis* (Mutika *et al.,* 2001; Abila *et al.,* 2003)*, Glossina palpalis gambiensis* (Van der Vloedt & Barnor, 1984), and *Glossina pallidipes* (Olet *et al.,* 2002) was significantly influenced by the age of the sterilised males. Mutika *et al.* (2001) indicated that a sterilizing radiation dose of 120 Gy did not affect the ability of *G. pallidipes* males to compete with untreated males. He also found that irradiated males that emerged from pupae kept at a low temperature of 15 °C for 24-72 h demonstrated increased competitiveness (Mutika *et al.,* 2001). Determining the optimal mating age of colonised tsetse flies under field conditions as well as their competitiveness after irradiation will be challenging and costly, while the results might also be influenced by several environmental, climatic and ecological parameters which cannot be controlled.

Since 1999, large walk-in field cages have been utilised successfully as a surrogate for open field studies to conduct mating compatibility, mating competitiveness and other behavioural studies on fruit flies, tsetse flies and Lepidoptera (Cayol *et al.,* 1999; Mutika *et al.,* 2001; Vera *et al.,* 2003; Taret *et al.,* 2010). Field cages have been used successfully to determine the optimal mating age for *G. f. fuscipes*, *G. p. palpalis* and *G. p. gambiensis* (Abila *et al.,* 2003).

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[3] Partially published as:
De Beer, C.J., Venter, G.J. & Vreysen, M.J.B. (2015) Determination of the optimal mating age of colonised *Glossina brevipalpis* and *Glossina austeni* using walk-in field cages in South Africa. *Parasites & Vectors*, **8**, 467.

The SIT has never been used or evaluated for *Glossina brevipalpis* and no data are available on the optimal mating age and competitiveness of irradiated sterilised flies. In the successful eradication campaign of *G. austeni* on Unguja Island, Zanzibar (1993-1997), sterile males that were mass-reared at the Vector & Vector-Borne Diseases Research Institute, Tanga, United Republic of Tanzania were released when three to five days old (Vreysen *et al.*, 2000). Notwithstanding the success on Unguja Island, no studies were carried out to assess the optimal mating age of *G. austeni* or the competitiveness of the sterile males.

In Chapter 5 *G. brevipalpis* adults as well as its pupal stages were shown to be highly sensitive to radiation. It was furthermore confirmed that the 30-year-old colonised *G. austeni* at the Agricultural Research Council–Onderstepoort Veterinary Institute (ARC-OVI) are still susceptible to the same level of radiation as previously reported. In the present study, the optimal mating age and mating performance of colonised *G. brevipalpis* and *G. austeni* males irradiated at different levels were determined using walk-in field cages.

6.2   Materials and methods

6.2.1   Colony tsetse flies

The mating performance of colonised *G. brevipalpis* and *G. austeni* housed at the ARC-OVI was determined*.* The origin and holding conditions (Feldmann, 1994a; FAO/IAEA standard operating procedures, 2006) of these colonies are given in Chapter 4.

6.2.2   Walk-in field cage and environmental conditions

Comparative assessment of the mating performance of *G. brevipalpis* and *G. austeni* was conducted separately using walk-in field cages under "near-natural" conditions (Calkins & Webb*,* 1983; Mutika *et al.,* 2001). The cylindrical field cages (Ø 2.9 m x 2.0 m) were made of cream polyester netting with a flat floor and ceiling (Fig. 6.1 A). Black nylon strips, connecting the panels of polyester netting, encircles the top and bottom of the cage where the ceiling and floor meet the sides of the cage (Fig. 6.1 B). A 1.5 m potted weeping boer-bean tree (*Schotia brachypetala*) was placed in the middle of the cage. A zip, also in a black nylon strip, from top to bottom sealed the entrance of the cage. The field cages were deployed in a irrigated small forest, at the ARC-OVI in Pretoria South Africa, of approximately 15 m x 70 m, consisting of a lane of century old Chir pines (*Pinus roxburghii*) on one side and water berry trees (*Syzygium cordatum*) on the other. The forest also contained two large karee trees (*Searsia lancea*) that reduced the natural light intensity and a plentiful undergrowth (below 3 m) of a variety of tree species*; Hyphaene coriacea, Strelitzia nicolai, Ziziphus mucronata, Cussonia spicata, Syringa persica, Ligustrum*

lucidum, Melia azedarach, Dracena aletriformis and Jacaranda species. The shrub and herb foliage layer, below 0.5 m, consisted of *Cyperus rotundus, Asparagus densiflorus, Tradescantia albiflora, Alpinia* species as well as *Hedera helix* growing on the pine trees. The forest floor had a thick carpet of leaf litter and pine needles. Compared to the surroundings, the forest was a cool, humid area with low natural light intensity.

Fig. 6.1. The outside (A) and inside (B) of a cylindrical walk-in field cage, made of panels of polyester netting joined with black nylon strips, deployed in a small forest at the ARC-OVI, Pretoria, South Africa.

Throughout the experiment, temperature and relative humidity were recorded every 10 minutes using a DS1923-F5# Hygrochron iButton data logger. Light intensity at the top and the bottom of the cage and at tree level was recorded every 15 minutes using a Major Tech MT940 light meter.

To determine the time of peak mating activity, optimal mating age and sterile male mating performance, 30 (three-day-old) females of either *G. brevipalpis* or *G. austeni* were released in the middle of the cage 5 minutes before 90 males of the corresponding species were to be released, giving a male to female ratio of 3 : 1. The observer remained inside the cage for the 3-hour duration of the experiment and movements were kept to a minimum. The time of mating was recorded to determine mating latency. The mating pairs were collected individually into small vials, and duration of the mating recorded. Although no direct adverse effect on mating behaviour was seen when the pairs were collected, its potential influence on mating behaviour cannot be ruled out. To minimise this effect mating pairs were similarly collected in all experiments. They were not replaced.

Except for the sterile versus fertile flies experiment, where the females were kept for 60 days, the mated females were immobilised at -5 °C and dissected the following day to determine insemination rate and spermatecae fill (Feldmann, 1994a; FAO/IAEA standard operating procedures 2006). The spermatecae were removed and their fill microscopically scored as either, empty (0), quarter full (0.25), half (0.5), three quarters (0.75) or full (1) (Nash, 1955). Females that did not mate were dissected to confirm virginity. All male flies remaining in the cages at the end of the experiments were collected and returned to the colony. *Glossina brevipalpis* and *G. austeni* were evaluated separately.

6.2.3   Time of peak mating activity

In an initial set of experiments, the time of day at which the flies showed a peak in mating performance was recorded. The performance of nine-day-old males with three-day-old virgin females at a male : female ratio of 3 : 1 was assessed in the morning (9:00 h to 12:00 h) and the afternoon (13:00 h to 16:00 h). The experiment was replicated five times for each species for two weeks in March 2012.

6.2.4   Optimal mating age

The optimal mating age of males was assessed using walk-in field cages. Three-, six- and nine-day-old males (30 of each age) competed for 30 three-day-old virgin females of the same species, giving a sex ratio of 3 : 1 (90 males : 30 females). To distinguish different male age groups, they were marked with a dot of different coloured polymer paint on the notum (Mutika *et al.,* 2001). The males were marked 24 hours before being released. The experiments with *G. brevipalpis* were conducted in the afternoon from 12:00 h to 15:00 h in March 2012 and those with *G. austeni* in the afternoon from 12:00 h to 15:00 h in March 2013.

6.2.5   Sterile versus fertile males

The mating performance of males sterilised with different radiation doses was assessed using walk-in field cages. Based on the results obtained in Chapter 5, *G. brevipalpis* adult males were irradiated four days after emergence with a dose of 40 Gy or 80 Gy and *G. austeni* adult males with 80 Gy or 100 Gy. Two groups of sterile males of each species, irradiated with different doses, and one group of fertile males (30 nine-day-old males in each group) competed for 30 three-day-old virgin females of the same species, giving a sex ratio of 3 : 1 (90 males : 30 females). To differentiate the various male groups, they were marked with a dot of different coloured polymer paint on the notum 24 hours before being released (Mutika *et al.,* 2001). The experiments with *G. brevipalpis* and *G. austeni* were

conducted in the afternoon from 12:00 h to 15:00 h in February 2013 and September 2014, respectively.

The mated females and males were transferred to individual holding cages and kept for 60 days. They were, fed daily on abattoir collected defibrinated bovine blood using an artificial *in vitro* membrane feeding system (Feldmann, 1994a; FAO/IAEA standard operating procedures, 2006).

As a control for the field cage experiments, 15 irradiated males (nine-day-old) were mated with 30 three-day-old virgin females under controlled laboratory conditions. For these control experiments, males and females were kept together for one day in colony cages under standard colony conditions (23-24 °C, 75-80% RH and subdued/indirect lighting) (Feldmann, 1994a; FAO/IAEA standard operating procedures 2006). These experimental flies were fed on bovine blood collected with the closed sterile system using an artificial membrane as described in Chapter 4. Male and female survival, female fecundity and evaluation of pupa produced were determined as described in Chapter 5.

6.2.6 Mating performance indicators

The propensity of mating (PM), relative mating index (RMI) and relative mating performance (RMP) were the mating indices used to compare the mating performance of the males of the various treatments. Propensity of mating (PM) was defined as the overall proportion of released females that had mated. Relative mating index (RMI) was defined as the number of pairs of one treatment group as a proportion of the total number of matings (Mutika *et al.,* 2001). Relative mating performance (RMP) was defined as the difference between the number of matings of two treatments of males as a proportion of the total number of matings (Mutika *et al.,* 2001). In addition, the mating latency time, mating duration, insemination rate and the spermatecae fill of each mated female was determined.

6.2.7 Statistical analysis

Data were analysed using the statistical software GraphPadInstat (version 3.00, 2003). Differences in the overall proportions of peak mating activity were analysed with Chi-square ($\chi2$) analysis with the Yate's continuity correction. The *P* value was two-sided and a relative risk, p1-p2 was determined. Additionally, an unpaired test was used to differentiate between the average mating latency, mating duration and spermatecae fill. Where the data passed the normality test, standard (parametric) methods were used with Welch correction. If the data was not normally distributed a nonparametric method (Mann-Whitney test) was used.

For the optimal mating age and sterile versus fertile comparisons a one-way analysis of variance (ANOVA) was used to differentiate between the relative mating index, average

mating latency, mating duration and spermatecae fill. Where the data passed the normality test, standard (parametric) methods were used and the Tukey's test was applied. If the data was not normally distributed the nonparametric Kruskal-Wallis test was used. All tests were done at the 5% significance level.

6.3   Results

6.3.1   Environmental conditions

All field cage experiments were conducted in summer, March 2012, February to March 2013 and September 2014, outdoors in a small forest at the ARC-OVI.

During the ten replicates (five for each species) conducted in the mornings in March 2012 the mean temperature gradually increased from 21.4 ± 1.4 °C at the onset (9:00 h) to 25.0 ± 2.8 °C at the end (12:00 h) (Fig. 6.2 A). The mean temperature in the field cages during these ten replicates was 24.4 ± 2.4 °C. The increase in temperature was accompanied by a decrease in relative humidity (Fig. 6.2 B) which dropped from an average of 68.0 ± 7.3% to 52.3 ± 13.0%, the mean being 58.6 ± 11.0%.

During the ten replicates (five for each species) in the afternoons the temperature and relative humidity were more stable (Fig. 6.2). The temperature ranged from 27.6 ± 1.3 °C to 29.0 ± 2.0 °C, the mean being 28.6 ± 2.0 °C. The relative humidity ranged from 37.9 ± 7.8% to 46.5 ± 6.8% with a mean of 41.1 ± 4.3%.

The light intensity at the top and bottom of the cage and also at the potted plant was usually higher in the afternoon (433.0 ± 271.6 Lx) than the morning (301.9 ± 94.5 Lx). During the morning the intensity was the highest at the top (351.0 ± 102.7 Lx) of the cage. In the afternoons the difference in light intensity was less pronounced with the top (432.3 ± 155.8 Lx) and bottom (510.2 ± 387.1 Lx).

Comparisons of optimal age and mating performance between sterile and fertile males were conducted in the afternoons from 12:00 h to 15:00 h in March 2012, February to March 2013 and September 2014. The variation in the temperature and relative humidity for these periods is reflected in Fig. 6.3. The lowest mean temperature of 27.0 ± 2.5 °C was recorded in March 2012 (Fig. 6.3). In February and March 2013, the mean temperatures of 29.0 ± 3.0 °C and 28.2 ± 1.8 °C respectively, were on average higher than in March 2012 (Fig. 6.3). The average relative humidity in March 2012 (44.2 ± 13.3%) was similar to that of February 2013 (46.2 ± 12.9%) and March 2013 (43.4 ± 8.7%) (Fig. 6.3). The highest mean temperature of 29.4 ± 2.2 °C was recorded in September 2014 which was also the period with the lowest relative humidity of 21.0 ± 7.1% (Fig. 6.3). September is considered to be the last month of the cold dry season in Pretoria.

The light intensity, ranging from 910.25 ± 351.87 Lx at the top to 653.47 ± 363.67 Lx at the bottom of the cages was the highest in September. This was due to the seasonal change in leaf cover of the trees, being less in the cold dry season. The light intensity ranged from an average of 657.41 ± 280.20 Lx and 604.17 ± 266.54 Lx measured at the tree inside the cage in February and March 2013 to 517.22 ± 390.77 Lx and 440.32 ± 258.21 Lx at the bottom. In March 2012 the bottom (582.29 ± 468.56 Lx) of the cage had on average a higher light intensity than the top (404.96 ± 137.22 Lx) and the lowest taken at the tree (366.07 ± 221.89 Lx), was also the lowest light intensity recorded.
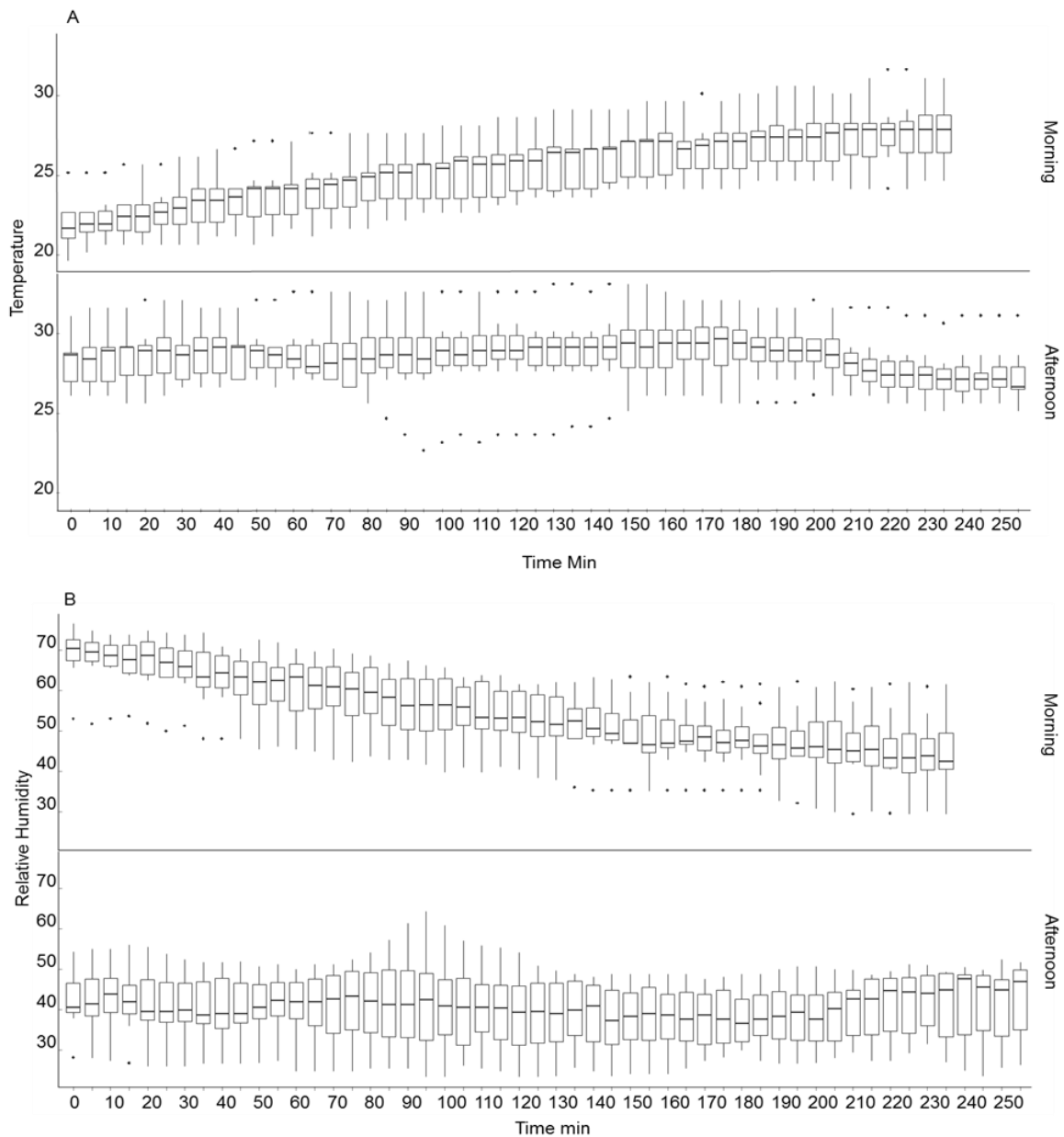
Fig. 6.2. Mean temperature (A) and relative humidity (B) recorded in field cages in the mornings and the afternoons during March 2012. Each box shows the group median

separating the 25th and 75th quartiles, capped bars indicate maximum and minimum values, black dots indicating the outliers.
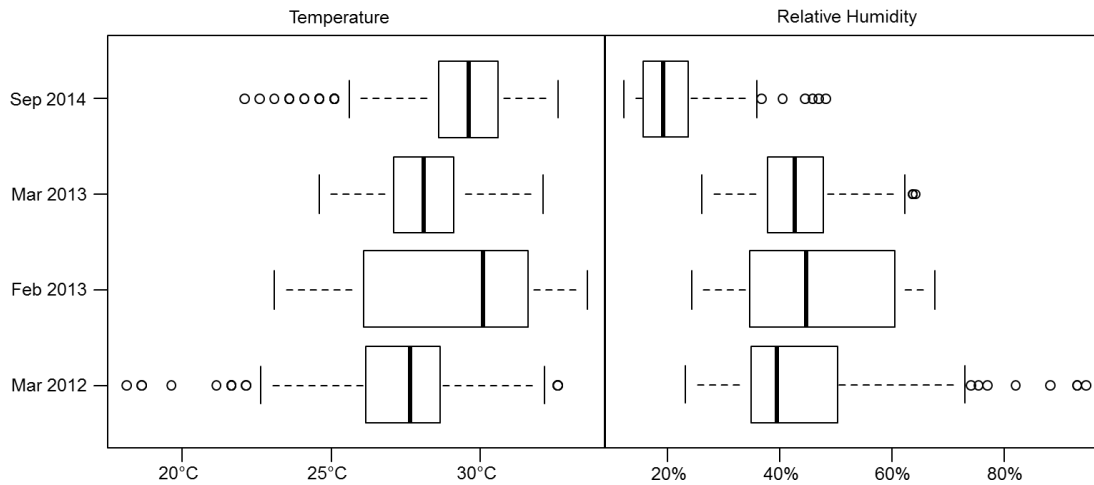
Fig. 6.3. Temperature and relative humidity recorded in the field cage during March 2012, February to March 2013 and September 2014. Each box shows the group median separating the 25th and 75th quartiles, capped bars indicate maximum and minimum values, circles indicating the outliers.

6.3.2   Activity in field cage

After release, males and females of both species dispersed immediately with most of the *G. brevipalpis* (males and females) settling in the top half of the cage and finding a resting site on the black band (Fig. 6.1) that connects the top and the vertical netted panels of the cage. In contrast, male and female *G. austeni* settled mostly in the bottom half of the cage, once again favouring the black band that connects the bottom and the vertical netted panels. No other notable behavioural differences were observed in *G. austeni* and *G. brevipalpis* towards the field cage environment.

For both species, most of the flies, males and females, settled in the more shaded areas and only a few on the tree. Some flies remained immobile after being released until recaptured and did not mate. After male release, there were immediate matings, the overall minimum mating latency time was 2 minutes. Occasionally more than one male tried to mate with the same female. Some attempted matings were met with clear rejection from the female.

6.3.3   Time of peak mating activity

The propensity of mating (PM) in the morning was 0.70 and 0.49 for *G. brevipalpis* and *G. austeni*, respectively. This was not significantly higher than that of 0.66 for *G. brevipalpis* ($P = 0.62$) and 0.59 for *G. austeni* ($P = 0.15$) in the afternoon (Table 6.1). The average

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mating latency was significantly longer in the mornings than in the afternoons for *G. brevipalpis* (*P* = 0.01) and *G. austeni* (*P* < 0.01) (Table 6.1). Fig. 6.4 indicates that for both species, more flies mated in the first hour of the afternoon as compared with the morning.

The *G. brevipalpis* couples mated on average for 174.21 ± 0.06 minutes in the morning and 165.13 ± 0.06 minutes in the afternoon which was not significantly different (*P* = 0.56). For *G. austeni* the average mating duration of 204.50 ± 0.06 minutes in the morning was significantly (*P* < 0.01) longer than the mating duration in the afternoon (138.63 ± 0.04 minutes). The mean spermatecae fill value for *G. brevipalpis* in the morning (0.75 ± 0.20) was slightly lower than in the afternoon (0.86 ± 0.10) (*P* < 0.01), the overall insemination rate was above 99% (Table 6.1). The insemination rate for *G. austeni* was above 94%. The mean spermatecae fill value of *G. austeni* was significantly higher (*P* < 0.01) in the morning (0.80 ± 0.20) than in the afternoon (0.68 ± 0.30) (Table 6.1).
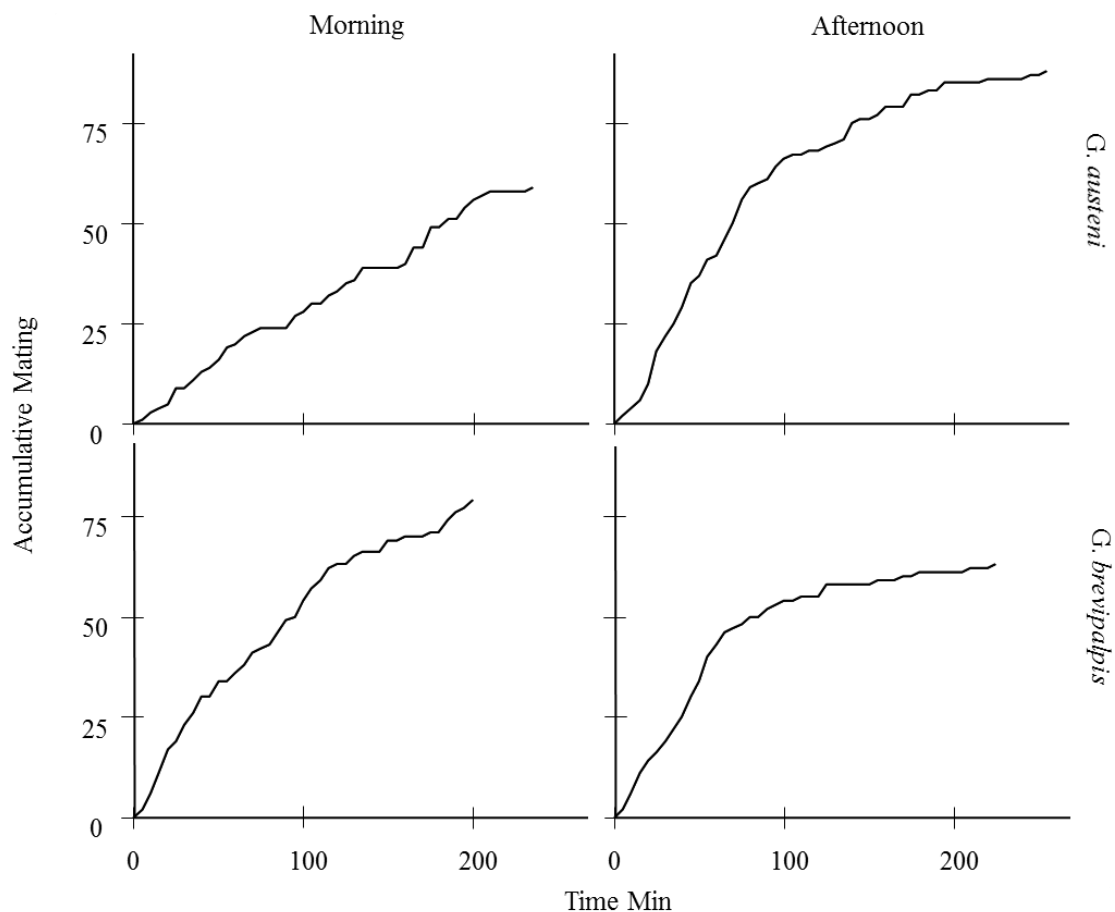
Fig. 6.4. Cumulative mating for *Glossina austeni* and *Glossina brevipalpis* in the morning and in the afternoon.

6.3.4 Optimal mating age

For the optimal mating age assessment, the overall proportions of released females that mated (propensity of mating) was 0.46 for *G. brevipalpis* and 0.43 for *G. austeni* (Table 6.1). The relative mating performance (RMP) for *G. brevipalpis* and *G. austeni* was 0.84 and 0.54, both being in favour of nine-day-old males. The mean relative mating index (Table 6.1) for nine-day-old males (0.68 ± 0.23 for *G. brevipalpis* and 0.54 ± 0.12 for *G. austeni*) was significantly higher than that of the six-day-old (0.25 ± 0.20, *P* < 0.01 for *G. brevipalpis* and 0.30 ± 0.16, *P* < 0.01 for *G. austeni*) and the three-day-old (0.06 ± 0.06, *P* < 0.01 for *G. brevipalpis* and 0.17 ± 0.14, *P* < 0.01 for *G. austeni*) males for both species (Fig. 6.4). The relative mating index (RMI) was not significantly different (*P* > 0.050) between six-day- and three-day-old males for both species (Fig. 6.5).

For *G. brevipalpis* the mean mating latency, ranging from 40.3 ± 0.05 minutes for three-day-old to 56.4 ± 0.03 minutes for six-day-old males was not significantly different (*P* = 0.74) between the age groups (Table 6.1). Similarly mean mating duration ranging from 152.2 ± 0.04 minutes for six-day-old to 193.5 ± 0.04 minutes for nine-day-old males was also not significantly different (*P* = 0.21) between the age groups (Table 6.1). There were, however, significant differences in the mean spermatecae fill between age groups (*P* = 0.01). The mean fill of 0.25 ± 0.30 in three-day-old males was significantly different from that of 0.79 ± 0.30 in six-day- (*P* < 0.01) and 0.74 ± 0.30 in the nine-day-old (*P* < 0.05) males.

Similarly, for *G. austeni* the mean mating latency (ranging from 84.1 ± 0.04 minutes for six-day- to 103.6 ± 0.05 minutes for three-day-old males) and mean duration of mating (ranging from 126.8 ± 0.04 minutes for three-day- to 144.8 ± 0.06 min for nine-day-old males) was not significantly different (*P* = 0.44, *P* = 0.74) between the age groups (Table 6.1). In contrast to *G. brevipalpis* no significant differences (*P* = 0.37) in the mean spermathecal fill were observed between the different age groups of *G. austeni* (Table 6.1).

For *G. brevipalpis*, the age of the male did affect the insemination rate. The rate for females mated with three-day-old males was only 0.33 compared to 0.96 for six-day-old and 0.94 for nine-day-old males (Table 6.1). For *G. austeni* the insemination rate ranged from 0.80 for three-day-old to 0.96 for six-day-old males (Table 6.1).
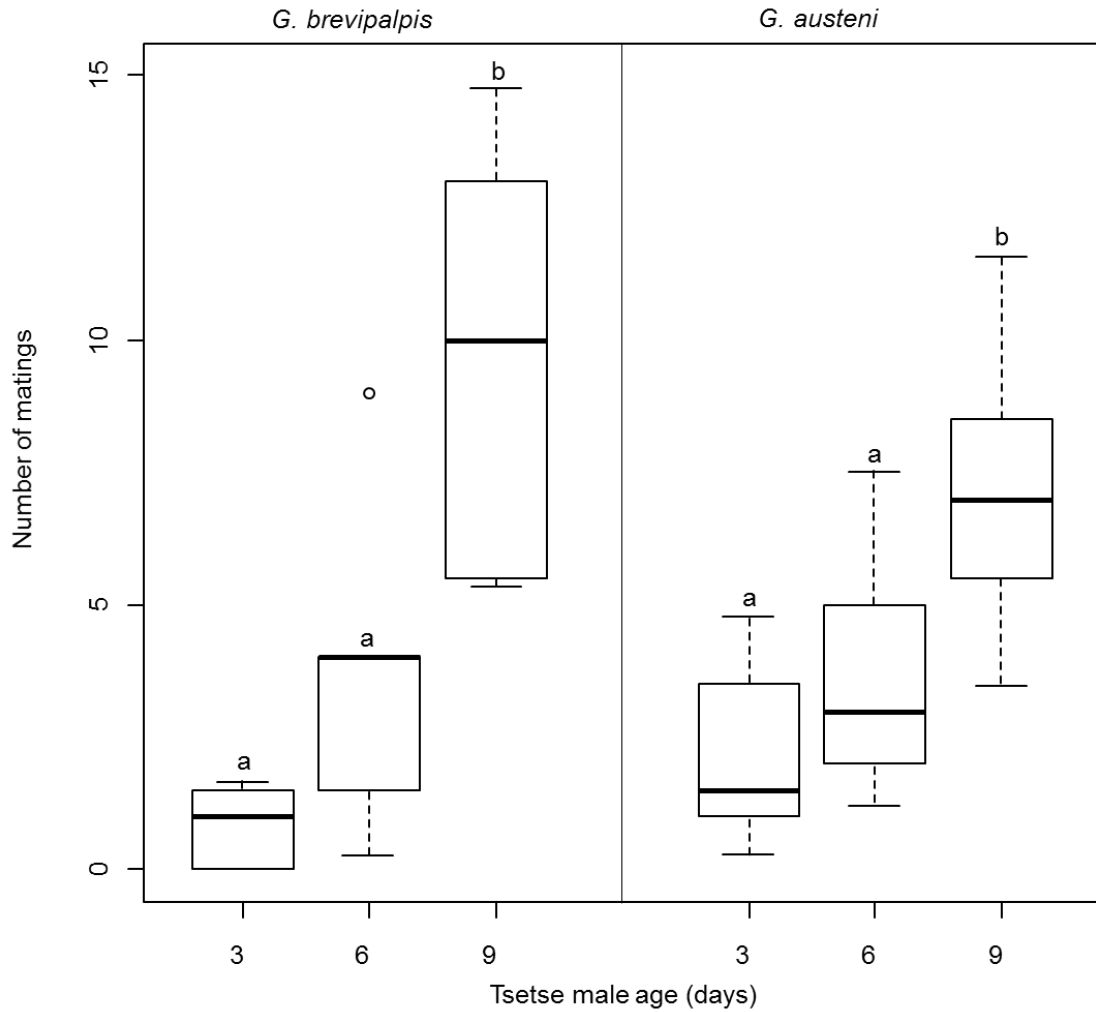
Fig. 6.5. Number of males of different age groups that mated with *Glossina brevipalpis* and *Glossina austeni* females in the field cage. Each box shows the group median separating the 25th and 75th quartiles, capped bars indicate maximum and minimum values, circles indicating the outliers. Boxes with different letters indicate that the numbers were significantly different at the 5% level.

Table 6.1. Mating parameters for *Glossina brevipalpis* and *Glossina austeni* in the field cages for assessing the time of peak mating activity, optimal mating age and the effect of radiation on male competiveness.

	Possible pairs	Actual mated	Overall proportion(PM)	Relative mating index (RMI ±SD)	Mating latency time (min ± SD)	Mating duration (min ± SD)	Mean spermatecae fill value (± SD)	Insemination rate
G. brevipalpis								
Fly activity morning	90	63	0.7	-	73.2 ± 0.04	174.2 ± 0.06	0.75 ± 0.20	1.00
Fly activity afternoon	120	79	0.66	-	47.2 ± 0.03	165.1 ± 0.06	0.86 ± 0.12	0.99
Male age (days)	210	97	0.46	-	54.4 ± 0.03	173.2 ± 0.03	0.72 ± 0.31	0.94
9	-	67	-	0.68 ± 0.23	55.0 ± 0.03	193.5 ± 0.04	0.74 ± 0.28	0.94
6	-	24	-	0.25 ± 0.20	56.4 ± 0.03	152.2 ± 0.04	0.79 ± 0.28	0.96
3	-	6	-	0.06 ± 0.06	40.3 ± 0.05	176.0 ± 0.04	0.25 ± 0.35	0.33
Male sexual status	360	204	0.57	-	66.7 ± 0.04	220.3 ± 0.05	0.59 ± 0.17	0.99
Fertile	-	53	-	0.41 ± 0.13	61.1 ± 0.04	225.1 ± 0.05	0.60 ± 0.17	1.00
Sterile (40 Gy)	-	70	-	0.33 ± 0.19	71.8 ± 0.51	215.8 ± 0.05	0.55 ± 0.15	1.00
Sterile (80 Gy)	-	81	-	0.27 ± 0.12	68.6 ± 0.04	218.6 ± 0.05	0.60 ± 0.18	0.98
G. austeni								
Fly activity morning	120	59	0.49	-	94.3 ± 0.04	204.5 ± 0.06	0.80 ± 0.20	0.98
Fly activity afternoon	150	88	0.59	-	58.4 ± 0.04	138.6 ± 0.04	0.68 ± 0.25	0.94
Male age (days)	360	153	0.43	-	94.3 ± 0.05	137.4 ± 0.05	0.57 ± 0.30	0.93
9	-	83	-	0.54 ± 0.12	97.1 ± 0.05	144.8 ± 0.06	0.61 ± 0.30	0.95
6	-	45	-	0.30 ± 0.16	84.1 ± 0.04	139.2 ± 0.04	0.54 ± 0.25	0.96
3	-	25	-	0.17 ± 0.14	103.6 ± 0.05	126.8 ± 0.04	0.51 ± 0.34	0.80
Male sexual status	360	225	0.63	-	76.8 ± 0.04	149.2 ± 0.04	0.67 ± 0.15	0.99
Fertile	-	72	-	0.33 ± 0.13	81.0 ± 0.04	155.1 ± 0.04	0.64 ± 0.19	0.96
Sterile (80 Gy)	-	77	-	0.35 ± 0.16	79.0 ± 0.49	147.5 ± 0.05	0.72 ± 0.12	1.00
Sterile (100 Gy)	-	76	-	0.32 ± 0.12	69.9 ± 0.04	144.5 ± 0.05	0.65 ± 0.14	1.00

6.3.5   Sterile versus fertile males

The propensity of mating (PM) was 0.57 and 0.63 for *G. brevipalpis* and *G. austeni*, respectively (Table 6.1). A RMI of 0.27 ± 0.12, 0.33 ± 0.19 and 0.41 ± 0.13 was obtained for 80 Gy-, 40 Gy-treated and untreated control *G. brevipalpis* males, respectively and these values were not significantly different (P = 0.08) (Fig. 6.6). Similarly, the RMI of untreated male *G. austeni* (0.33 ± 0.13) and of those irradiated with 100 Gy (0.32 ± 0.12), and 80 Gy (0.35 ± 0.16) was also not significantly different (P = 0.87) (Fig. 6.6).

The RMP of irradiated (40 Gy) to untreated *G. brevipalpis* males was -0.07, which indicates that the mating performance of the 40 Gy-treated and untreated males was almost equal. Similar observations were made for males irradiated with 80 Gy versus untreated males, *i.e.* a RMP of -0.21. The RMP of 80 Gy and 100 Gy-treated *G. austeni* males versus untreated ones was 0.01 (in favour of treated) and -0.03, (in favour of untreated males).

Untreated *G. brevipalpis* males formed mating pairs sooner (on average 61.1 ± 0.04 minutes) than the males irradiated with 40 Gy (71.8 ± 0.51 minutes) and 80 Gy (68.6 ± 0.04 minutes) and they also mated longer (225.1 ± 0.05 minutes) (Table 6.1). The opposite was observed for *G. austeni*: where the irradiated males formed mating pairs sooner (80 Gy: 79.0 ± 0.49 minutes and 100 Gy: 69.9 ± 0.04 minutes) than the untreated males (81.0 ± 0.04 minutes), however, the untreated males mated longer (155.1 ± 0.04 minutes) (Table 6.1).

Mated females and males were collected and placed in individual fly holding cages and kept for 60 days, female survival and pupae production were monitored and surviving females were dissected after 60 days. Ninety percent of the *G. brevipalpis* females that mated with untreated or 80 Gy-irradiated males and 81% of females that mated with 40 Gy-treated males survived to day 60 (Table 6.2). The survival on day 60 of *G. austeni* was 81% and 73% for females that mated respectively with 80 Gy- and 100 Gy- irradiated males and 59% of the females mated with untreated males (Table 6.2).

Females of both species that mated with untreated males produced more pupae (Table 6.2) than those that mated with irradiated males. In parallel, the females of both species that mated with untreated fertile males aborted a lower number of eggs compared to those that had mated with irradiated males (Table 6.2). In both *G. brevipalpis* and *G. austeni* pupae production was dose dependent and fecundity decreased as the radiation dose increased (Table 6.2). A dose of 40 Gy and 80 Gy was sufficient to induce 93% and 98% sterility respectively in *G. brevipalpis*. With *G. austeni* 80 Gy and 100 Gy was sufficient to induce 79% and 89% sterility, respectively.
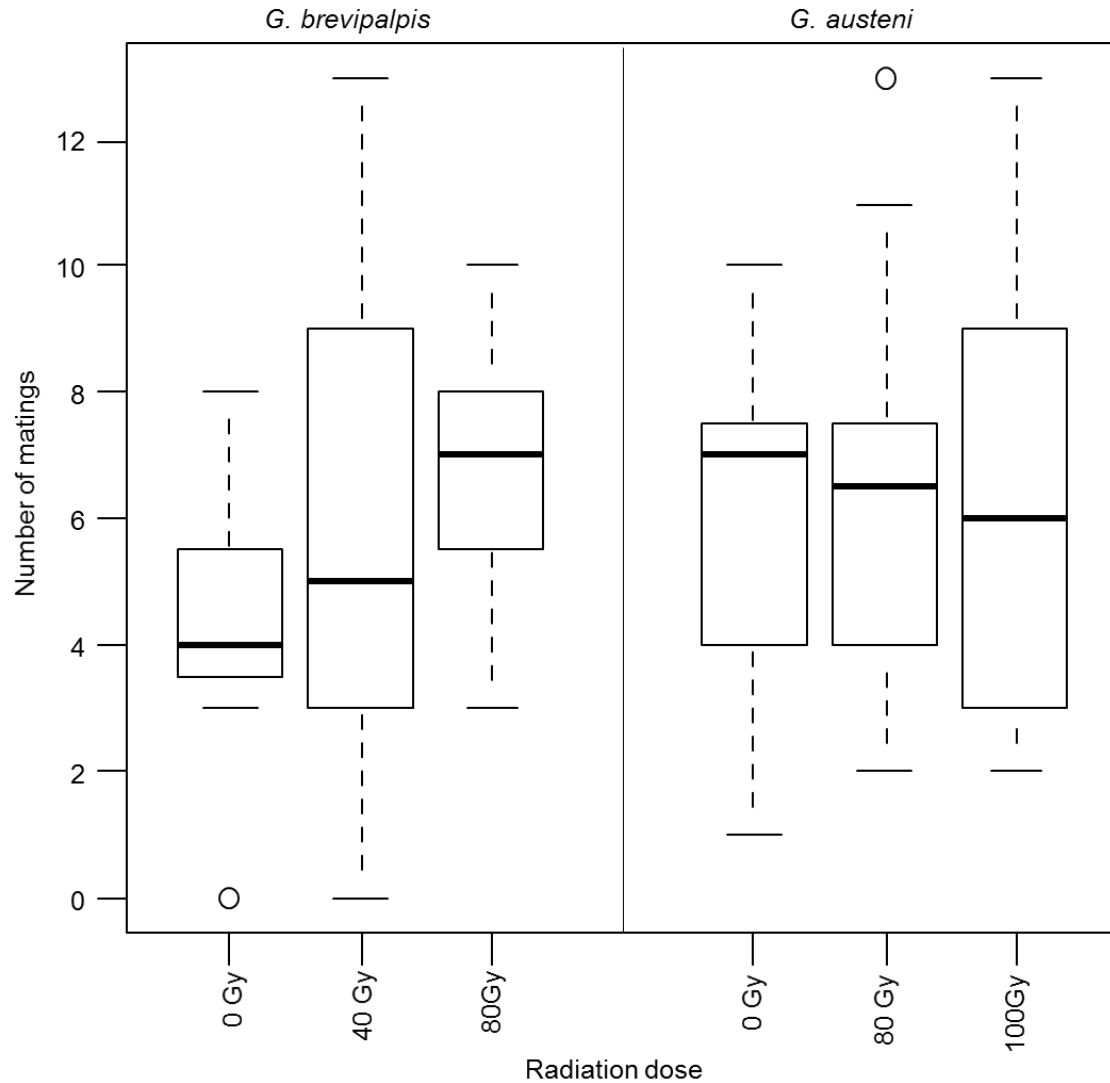
Fig. 6.6. Number of irradiated male (40, 80 or 100 Gy) and untreated male *Glossina brevipalpis* and *Glossina austeni* that mated with untreated females in a field cage. Each box shows the group median separating the 25th and 75th quartiles, capped bars indicate maximum and minimum values, circles indicating the outliers.

The reproductive status of females of both species on the day of dissection indicated a strong difference between those that mated with fertile males compared to irradiated males. In the majority of females of both species that mated with the irradiated males the uterus was either empty due to abortions or contained a recently ovulated egg. Only a few females of both species that had mated with fertile males showed an empty uterus due to abortions and most had either recently ovulated eggs or viable instar larvae in the uterus (Table 6.2). The insemination rate was above 0.98 for *G. brevipalpis* and 0.96 for *G. austeni*, both for females mated with untreated and with irradiated males, which indicated that radiation did not influence the males' ability to transfer sperm.

Table 6.2. Production of *Glossina brevipalpis* and *Glossina austeni* females mated with sterile and fertile males in field cages.

	No. of mature females		No. pupae produced	Fecundity	% Emergence / % females	No. of aborted eggs	Insemination	Uterus					Spermatecae fill %			
								Recently ovulated egg	Empty due to abortion	Viable instar larvae						
										I	II	III	0.25	0.5	0.75	1
	Day 18	Day 60														
G. brevipalpis																
Fertile	74	67	81	0.058	91.5 / 44.7	14	100	27	17	5	6	14	10.1	37.7	52.2	0
Sterile (40 Gy)	65	57	6	0.010	100.0 / 25.0	52	100	19	35	0	0	2	10.7	57.1	32.1	0
Sterile (80 Gy)	53	48	2	0.006	75.0 / 25.0	37	98	8	40	0	0	0	6.3	39.6	52.1	0
G. austeni																
Fertile	76	45	141	0.026	86.4 / 46.2	7	96	8	14	9	9	5	4.4	26.7	68.9	0
Sterile (80 Gy)	77	63	29	0.002	70.8 / 70.8	23	100	16	39	0	0	3	0	19	75.9	5.2
Sterile (100 Gy)	70	53	15	0.001	73.7 / 42.1	24	100	13	39	0	0	0	3.9	29.4	66.7	0

Table 6.3. Production of *Glossina brevipalpis* and *Glossina austeni* females mated with sterile and fertile males under laboratory conditions.

	No. of mature females		No. pupae produced	Fecundity	% Emergence / % females	No. of aborted eggs	Insemination	Uterus					Spermatecae fill %			
								Recently ovulated egg	Empty due to abortion	Viable instar larvae						
										I	II	III	0.25	0.5	0.75	1
	Day 18	Day 60														
G. brevipalpis																
Fertile	112	99	116	0.026	100.0 / 62.9	31	91	34	47	1	5	5	17.9	30.5	42.1	0
Sterile (40 Gy)	113	104	7	0.002	71.4 / 0.0	97	83	8	94	0	1	1	25	27.9	27.9	1.9
Sterile (80 Gy)	108	101	2	<0.001	66.7 / 100.0	80	88	9	62	0	0	0	28	28	32	0
G. austeni																
Fertile	124	83	269	0.065	69.9 / 63.8	52	100	28	30	5	13	4	6.4	42.3	50	1.3
Sterile (80 Gy)	112	87	13	0.003	30.8 / 75.0	182	100	17	67	0	0	1	3.5	40	56.5	0
Sterile (100 Gy)	119	81	5	0.001	60.0 /33.3	210	100	13	68	0	0	0	6.2	39.5	54.3	0

The same linear relationship as seen in Chapter 5 for male life span was also observed as the average life span (119.86 ± 4 7.51 days) of fertile *G. brevipalpis* males was longer than that of irradiated ones, *i.e.* 101.64 ± 36.98 days for males irradiated with 40 Gy and 94.15 ± 28.58 days for the males done with 80 Gy. The average life span of untreated *G. austeni* (72.35 ± 62.63 days) was longer compared to irradiated males (80 Gy: 65.13 ± 34.30 days; 100 Gy: 66.08 ± 34.15 days).

The sterility of irradiated males used in the field cages was assessed by selecting a subsample (N = 15) of males, that had mated with 30 three-day-old virgin females and been maintained under controlled laboratory conditions. Pupae production of females mated with untreated control males is indicated in Table 6.3. In both species irradiation did not affect the males' ability to transfer sperm. *G. brevipalpis* males that had been irradiated with a dose of 40 Gy and 80 Gy induced 94% and 98% sterility in untreated females, respectively. This is similar to sterility observed in flies used in the field cages for dose 40 Gy and somewhat higher than the 80 Gy. Some discrepancies arose when comparing the induced sterility for *G. austeni* females of the field cage experiments with those used in the laboratory. In general, the males of the laboratory experiments induced a higher sterility in untreated females as compared with males used in the field cage experiments. It might, however, have been due to an experimental error in tracking the production of each individual fly. The smaller sample size could also have played a role.

6.4   Discussion

The success of AW-IPM programmes that include a SIT component depends on the capability of the released sterile males to compete with their native counterparts (Calkins & Parker, 2005; Vreysen, 2005). Assessment of the mating competitiveness of the produced, released insects will therefore be a prerequisite before any operational SIT programme can be initiated (Vreysen *et al.*, 2007). Biological attributes such as rate of development, temperature adaptation, circadian rhythm, flight capability, optimal mating age, weight and strain used may affect the biological quality of the produced and released insects (Van der Vloedt & Barnor, 1984; Mutika *et al.,* 2001; Liedo *et al.*, 2002; Olet *et al.,* 2002; Abila *et al.,* 2003). Operational attributes that will contribute to this include collection techniques, handling, radiation, and release methods (Ul Haq *et al.*, 2010; Teal *et al.*, 2013; Flores *et al.*, 2014; Ul Haq *et al.*, 2014). Quantification of the impact of each of these attributes on the released insects' competitiveness is paramount to enable the development of procedures to mitigate any potential negative effects.

In the present study, there was no significant difference in male activity and mating performance in the mornings and afternoons for both species, indicating that field cage experiments could be conducted in either of these two time slots at the ARC-OVI.

Environmental conditions were, however, somewhat more variable in the mornings with a lower average temperature and higher average relative humidity in contrast to the afternoons when conditions were more stable but with on average, higher temperatures and relatively lower humidity. The on average shorter mating latentcy observed in the afternoons might also have been a result of the higher environmentsl temperatures. The afternoon time of 12:00 h to 15:00 h was selected for all other field cage experiments.

The equal mating performance for both *G. brevipalpis* and *G. austeni* in the afternoon and morning seems to conform to the diurnal activity patterns observed for *G. brevipalpis* in South Africa but not for *G. austeni*. Previous studies have indicated a bimodal activity pattern for *G. brevipalpis* with flies being active early in the morning from dawn until after sunrise and then late afternoon (Kappmeier, 2000). *Glossina austeni* on the other hand showed a more pronounced unimodal activity pattern and flies were active from early morning until late afternoon (Kappmeier, 2000). The *G. austeni* data from South Africa was in contrast with that of Owaga *et al.* (1993), who observed two activity peaks, one at 9.00 h to 10.00 h and a second between 14.00 h and 17.00 h, for *G. austeni* in Kenya.

Like most tsetse species, *G. austeni* and *G. brevipalpis* are markedly diurnal and show pronounced periodicity in their activity. Tsetse fly activity patterns are known to be under the control of an endogenous clock but in nature, these rhythms are also influenced by environmental stimuli such as temperature and light (Brady & Crump, 1978). Circadian rhythm of tsetse flies can influence the activity of sterile males in the field, and also their competitiveness. Whereas the differences in activity patterns of *G. austeni* seen in the field might be related to different environmental conditions and stimuli, the differences observed in the circadian rhythm in the laboratory are more difficult to explain. Crump & Brady (1979) reported only one afternoon peak of spontaneous activity of *G. austeni* in the absence of any odours or other stimuli. Owaga *et al.* (1993), however, states that the U-shaped activity pattern observed in the field persisted in the laboratory when flies were maintained under a 12 h light /12 h dark cycle and stable temperature and humidity conditions. The authors therefore concluded that the activity pattern of *G. austeni* was mainly driven by endogenous factors (Owaga *et al.*,1993).

The present study indicated that the age of both *G. austeni* and *G. brevipalpis* males was significantly correlated to their mating performance as shown by the RMI. Nine-day-old males were significantly more successful in securing a female for mating than six- or three-day-old males. These results are in agreement with data obtained for *G. f. fuscipes* and *G. p. palpalis* (Abila *et al.*, 2003). Although older *G. brevipalpis* and *G. austeni* males were more competitive in securing a female in field cages, the age of the males did not influence mating duration or insemination ability. This confirms the data of Malele and Parker (1999) who observed that *G. austeni* males that had mated on the day after

emergence could successfully inseminate females of the same age in small laboratory cages. Our data on optimal mating ages indicate that the propensity of mating for both *G. austeni* and *G. brevipalpis* can potentially be improved by releasing older sterile males. This, however, would necessitate keeping the males longer in the rearing facility, which would increase maintenance and production costs. This protocol would require additional blood meals for the sterile males, more labour to absorb the increased handling needs and larger facilities to stockpile the flies before release.

In the majority of previous control programmes with a SIT component, the sterile males were released at a relatively young age. The sterile male *G. austeni* were 4 to 7 days old when released on Unguja Island, Zanzibar (Vreysen *et al.*, 1999), sterile male *G. p. palpalis* were 3 to 5 days old when released in the Lafia area of Nigeria (Oladunmade *et al.*, 1990), and sterile male *Glossina tachinoides* were 2 to 10 days old when released in a pilot trial in Chad (Cuisance & Itard, 1973). Using younger males will be cost effective in terms of space and labour. These release protocols were driven by mating observations in small laboratory cages that showed mating and insemination was possible in males younger than 5 days (Malele & Parker, 1999). Other researchers used males that were between 5 to 8 days old for various experiments (Van der Vloedt *et al.*, 1978; Van der Vloedt & Barnor, 1984; Vreysen & Van der Vloedt, 1990). In these operational programmes, sterile males were offered at least two blood meals that contained a trypanocidal drug Samorin (12.5 mg/L blood) before release in a programme on Unguja (Vreysen *et al.*, 1999) that significantly reduced the risk of transmitting trypanosomes.

An entirely different sterile male release strategy was used in the programme against *Glossina morsitans morsitans* in the Tanga area, Tanzania, in the 1970's. Here sterile males were released as pupae from fixed release stations and emerging males were consequently teneral and had to find a blood meal right away to build up energy reserves (Williamson *et al.*, 1983a). A drawback of this method was that males were exposed to potential predation before reaching sexual maturity and could also potentially transmit the Trypanosomosis disease. Despite this, the programme was successful and releasing male pupae at a density of 135 pupae/km^2 resulted in a sterile male wild male overflooding ratio of 1.2 : 1 which, despite being low, maintained the indigenous wild fly population at the 80-95% reduction level obtained after the initial insecticide application (Williamson *et al.*, 1983b).

Exposure to radiation may affect the biological quality of the produced released insects (Simmons *et al.*, 2010). This study, however, shows that radiation of up to 80 Gy for *G. brevipalpis* and 100 Gy for *G. austeni* did not affect the ability of sterilised males to compete with fertile males. This is in accordance with field cage evaluations of *G. morsitans* and *G. pallidipes* that also showed that the competitiveness of irradiated males did not differ from that of untreated males (Dean *et al.*, 1968; Mutika *et al.*, 2001). Dean *et al.* (1968)

based their findings of competitiveness on pupal production, however, in this study as well as that of Mutika *et al.* (2001) observing individual flies provided more accurate information on male competitiveness. Untreated fertile *G. brevipalpis* males did form mating pairs sooner and mated for longer than the irradiated males. Although this was not statistically significant, any delay in initial mating by the irradiated males can lead to a potential reduction of their competitiveness and need to be reduced. With *G. austeni* the opposite was seen, irradiated males formed mating pairs sooner and mated for a shorter period than the fertile males. Females that mated with untreated fertile *G. brevipalpis* males did have a larger spermatecae fill than those that mated with irradiated males. This was not confirmed for *G. austeni*.

This study has furthermore shown that field cages can be used to assess the mating performance of *G. brevipalpis* and *G. austeni* with an average propensity of mating above 56%. Although the propensity of mating was lower than that in similar field cages with *G. f. fuscipes* and *G. p. palpalis* (Abila *et al.*, 2003), the values obtained indicate adequate environmental conditions for the evaluations. This relatively high propensity of mating obtained indicated that the potential interference on the mating behaviour of the flies because of the observer presence was minimal. It needs to be pointed out that the field cage experiments were conducted in Pretoria which has a different climate to the tsetse fly infested area in north eastern KwaZulu-Natal. The different environmental conditions might influence the circadian rhythm, the activity patterns of the flies and the propensity of mating.

The results of our mating performance studies using irradiated *G. brevipalpis* males indicate that *G. brevipalpis* will be well suited for use in programmes that have a SIT component. The data obtained for *G. austeni* strengthens the findings of previous studies that indicate that this species can also be used successfully in programmes with a SIT component (Vreysen *et al.*, 1999).

Chapter 7

Concluding remarks and recommendations

After an outbreak of nagana in 1990 in north eastern KwaZulu-Natal an extensive survey of tsetse fly distribution and abundance was conducted with odour baited XT sticky traps during 1993 to 1999. This data was incorporated in an area-wide integrated pest management (AW-IPM) elimination strategy with a sterile insect technique (SIT) component to establish a tsetse fly-free South Africa (Kappmeier Green *et al.,* 2007). A prediction model (Hendrickx, 2002), based on the XT sticky trap data, indicated a wider distribution range for both *Glossina brevipalpis* and *Glosinna austeni* in South Africa. Since this survey Kappmeier & Nevill (1999a) and Kappmeier (2000) have developped a more effective trap, the odour baited H trap, for the collection and monitoring of *G. brevipalpis* and *G. austeni* and the 1993 - 1999 data set may have become outdated. One of the outcomes (Chapter 2) of this study was an updated tsetse fly abundance (Fig. 2.4) and distribution (Fig. 2.5) maps as well as a Trypanosomosis infection rate map (Fig. 2.8).

In the AW-IPM tsetse fly elimination strategy proposed by Kappmeier Green *et al.* (2007), the tsetse fly infested area was divided into four zones from south to north. It was proposed to implement an operational phased approach successively, starting in zone I in the south following a rolling carpet principle (Hendrichs *et al.,* 2005; Kappmeier Green *et al.,* 2007). In each zone, depending on species presence and abundance, initial tsetse population reduction would first be achieved with spraying of non-residual insecticides based on a sequential aerosol technique (SAT) followed by the release of sterile males (Kappmeier Green *et al.,* 2007). The initial tsetse population reduction with SAT was suggested because the available insecticide-impregnated targets were found to be rather inefficient for *G. brevipalpis.*

The most conspicuous change in the tsetse fly distribution as determined in Chapter 2 to that of Kappmeier Green *et al.* (2007) is that *G. brevipalpis* was proven to be present in Zone III and *G. austeni* in Zone I. While the odour baited XT sticky trap data indicated *G. brevipalpis* to be restricted to two distinct bands in the north and south of the area (Kappmeier Green, 2002) (Figs. 2.2 & 2.5) the present data indicate a more continuous distribution for this species. *Glossina brevipalpis* was also collected further south (10 km from the border of Hluhluwe-iMfolozi Park) than previously. The most southerly limit of *G. brevipalpis* therefore remains undefined and similarly the most westerly extent of the tsetse fly distribution still needs to be verified.

This wider tsetse fly distribution as indicated on the updated maps will necessitate modifications of the strategy and zone selection as proposed by Kappmeier Green *et al.*

(2007). The present distribution data indicates that the southern limits of zone I and II have to be expanded to approximately 20 km south of the Hluhluwe-iMfolozi border. The southernmost distribution of *G. brevipalpis* still needs to be confirmed or defined and this will ultimately define the final southern limits of their distribution. Based on the fact that *G. brevipalpis* was present in the Phinda and Mkhuze game reserves it is recommended that the previously defined Zone III must be split into two.

After the eradication of *G. pallidipes* in 1952 the two remaining species in the area, *G. brevipalpis* and *G. austeni,* were considered of lesser importance in the transmission of nagana (Du Toit, 1954). From the Trypanosomosis prevalence data it is clear that nagana is abundant and widespread in north eastern KwaZulu-Natal. The apparent absence of a significant linear correlation between trypanosome prevalence and the relative abundance of the tsetse flies in the present study can partly be attributed to the co-existence of the two species, each with a different vectorial capacity and/or competence. Both these species have been shown to readily feed on cattle (Moloo, 1993; Clausen *et al.*, 1998). Despite a perceived lower vector competence for *G. brevipalpis* (Motloang *et al.,* 2012) our data has indicated that both species can play a role in the transmission of Trypanosomosis in KwaZulu-Natal.

AW-IPM approaches require that the control effort is directed against an entire insect population. It is therefore of paramount importance to assess the degree of isolation of the targeted pest population. Knowledge on gene flow between the target population and adjacent populations can provide the necessary guidance in the decision making process. It was known that the South African tsetse fly populations of *G. brevipalpis* and *G. austeni* extend into southern Mozambique and Swaziland (*G. austeni*). In the present study morphometrical analyses showed that gene flow exists between the three neighbouring countries and that there are no significant barriers (Chapter 3). The proposed AW-IPM tsetse fly eradication strategy should thus be expanded to include southern Mozambique and Swaziland. Zone IV as proposed by Kappmeier Green *et al.* (2007) needs to be extended to include the southern Mozambique tsetse populations. An additional zone, zone V will be needed for Swaziland. It still needs to be determined whether the tsetse populations in southern Mozambique are genetically isolated from the tsetse populations north of Maputo (Fig. 1.2 A).

Any AW-IPM programme that includes on a SIT component will only be successful if the target insect can be mass-reared in adequate numbers, and the sterile males destined for release, are competitive with their native counterparts (Calkins & Parker, 2005; Vreysen, 2005). Assessment of the competitiveness of the produced and released insects is therefore a prerequisite before any operational SIT programme can be launched (Vreysen *et al.,* 2007). Various attributes both biological (*e.g.* rate of development, temperature

adaptation, circadian rhythm, flight capability, optimal mating age, weight) and operational (*e.g.* insect collection techniques, handling, radiation, release technologies) may impact on the quality of the produced and released insects (Simmons *et al.*, 2010). Quantification of the impact of each of these on the released insects' competitiveness is paramount to mitigate potential negative effects.

In Chapter 4, the impact of anticoagulants and phagostimulants that can be added to the blood meal and the effect of source of the blood (bovine, porcine) on tsetse fly production were determined. The colonies of *G. brevipalpis* and *G. austeni* have been maintained at the Agricultural Research Council–Onderstepoort Veterinary Institute (ARC-OVI) on defibrinated irradiated bovine blood that is obtained from a commercial abattoir. The current blood diet was proven to be suitable for the maintenance of these two species.

These colonies would require considerable upscaling to provide sufficient numbers of sterile males for a future SIT component. Since we have two species, with potentially different diet requirements, this can be challenging. Therefore, the diet requirements of the two species were reassessed to provide better protocols that could be used for the upscaling of these colonies. It was found that combinations of bovine and porcine blood or feeding on these blood sources on alternative days improved fecundity in both species, this adjustment in feeding protocols, can therefore be used to boost production in *G. brevipalpis* and *G. austeni* colonies in times of low performance as well as during the upscaling phase.

Any alternation or addition to the rearing diet requirements will need to be reassessed in terms of operational efficiency *e.g.,* although anticoagulants will simplify blood collection and make the process more sterile, the chemicals are expensive and will increase costs in large scale operations.

Furthermore, the current colonies at the ARC-OVI are not from the same strain as the tsetse flies in the proposed control area and it is recommended that mating compatibility of the two strains be determined. For this purpose, colonies of the KwaZulu-Natal strains need to be established. High mortalities and low productivity in the field collected tsetse flies showed that the establishment of colonies will be difficult. The collection of sufficient numbers of field flies in a good physical condition will be essential for colony establishment. In Chapter 3, flies collected at sites with less pronounced fluctuations in environmental conditions together with those at the end of a more favourable season *e.g.* a hot wet summer, were shown to be in a better physical condition and will therefore be more suitable for colonization. Transportation, feeding and pupae maintenance protocols need to be evaluated and adjusted *e.g.* transportation of pupa rather than adults, phagostimulants to improve feeding and maintaining pupae on wet sand.

In Chapter 5, the radiation sensitivity of *G. austeni* and *G. brevipalpis* when treated as pupae or adults was assessed. Treating late stage *G. brevipalpis* pupae with 40 Gy

induced 97% sterility when mated with untreated females and 99% sterility when irradiated with 80 Gy as adults. *Glossina austeni* required higher doses, as 80 Gy and 100 Gy were needed to induce more than 97% sterility in untreated females that mated with males treated either as adults or late stage pupae. Since there were no significant differences in the quality of males irradiated as adults or pupae both stages can be selected for irradiation. There are positive and negative considerations when choosing to irradiate either flies or pupae. As was observed in *G. brevipalpis,* the negative effect of radiation on tsetse fly longevity was more pronounced when pupa, instead of adults, were irradiated. This relationship was less clear in *G. austeni.* Pupae are easier to transport and handle and more pupae can be irradiated at a time (Pagabeleguem *et al.,* 2015). Radiation, however, can delay adult emergence which will negatively impact pupal maintenance time and operational costs. Available sex separation procedures are based on differential development time between males and females and although efficient are not 100% accurate. As a result, there will be some females that are irradiated and released. This is a drawback as the females might live longer and have a higher risk then males for becoming infected with trypanosomes, however, this risk is reduced for both sexes as all flies are fed with trypanocidal drugs before release. The mating competitiveness of males irradiated either as adults or pupae needs to be considered in deciding which stage to irradiate. The use of field cages to assess mating performance and competitiveness of pest insects has gained importance during the last decade. These cages were originally mainly used for testing the mating behaviour of several species of fruit flies, and their use has recently been expanded to include insect groups such as tsetse flies (Mutika *et al.*, 2013) and Lepidoptera (Taret *et al.*, 2010). Walk-in field cages have proved to be good substitutes for field studies, which will be less controlled, more complex, and expensive. Although the data obtained from field cages are good indicators of the behaviour of reared insects, this still need field verification, where the released insects are competing with wild insects and are exposed to a number of varying stimuli.

In Chapter 6, it is indicated that mating performance can be significantly improved in *G. austeni* and *G. brevipalpis* males by using older males. In an operational programme, this would, however, require keeping the males for longer in the rearing facility, with an increase of the maintenance and production costs. This would require more blood meals for the sterile males, more labour to absorb increased handling needs and larger facilities to stockpile the flies before release. In some male thephritid fruit flies, the time needed to reach sexual maturity is significantly reduced if they are exposed to juvenile hormone mimics (Teal *et al.*, 2013). Similarly, the addition of certain supplements (*e.g.* protein) to the diet of the melon fly *Bactrocera cucurbitae* (Ul Haq *et al.*, 2014) or exposure of species such as *Bactrocera carambolae* to methyl eugenol aroma can increase the mating performance

of the males (Ul Haq *et al.*, 2014). Identifying similar factors that can shorten the period before the optimal mating age for *G. austeni* and *G. brevipalpis* is reached will be advantageous.

Colonised nine-day-old males irradiated as adults with 80 Gy for *G. brevipalpis* and 100 Gy for *G. austeni* successfully competed with colonised nine-day-old fertile males for three-day-old colony females. This data indicates that, under the experimental field cage conditions, the irradiated colony flies at the ARC-OVI are suitable to be used for the SIT. However, the mating performance of irradiated colonised *G. brevipalpis* and *G. austeni* males compared to the fertile wild type males still needs to be assessed, preferably under field conditions, in the target area.

The main conclusions from this study can be summarised as follows:

o The updated tsetse fly distribution and Trypanosomosis prevalence maps needs to be taken into consideration in the proposed AW-IPM tsetse fly elimination strategy. Tsetse fly abundance can, however, be dynamic and will rapidly react to changes in environmental conditions.

o Comparisons of the abundance of *G. brevipalpis* and *G. austeni* with trypanosome infection rates in the area indicate that both species can play a role in the epidemiology of this disease.

o Gene flow does occur between the populations of tsetse flies found in South Africa, southern Mozambique and Swaziland.

o The current diet (*i.e* difebrinated bovine blood) and feeding protocols are sufficient for the maintenance of the *G. brevipalpis* and *G. austeni* colonies at the ARC-OVI.

o *Glossina brevipalpis* and *G. austeni* can be irradiated with a dose of 80 Gy and 100 Gy respectively, and can be irradiated either as adults or late stage pupae.

o Irradiation did not affect the mating performance of colonised *G. brevipalpis* and *G. austeni* males.

o Initial results indicate that *G. brevipalpis* to be a good candidate for the use in SIT.

The continuous improvement of tsetse control strategies will be beneficial in a changing environment of the African continent, and the following can be prioritised as future research aspects:

o The southerly and westerly distribution limits of *G. brevipalpis* and *G. austeni* in South Africa need to be defined before the implementation of an AW-IPM programme. Due to its dynamic nature, tsetse fly abundance should be constantly monitored at certain key points in the area.

- o To determine the extent of gene flow between the tsetse fly populations in the northern and southern belts of Mozambique.

- o The efficiency of the current monitoring system is low and need to be improved, especially for *G. austeni.*

- o The vector competence and capacity of *G. brevipalpis* for various trypanosome species need to be determined.

- o The role of mechanical transmission by other biting flies needs to be determined.

- o The role of vegatation, game parks and protected areas in maintaining tsetse fly populations need to be better characterised. The extent to which these species will disperse from and breed outside the parks need to be determined.

- o Assessing suppression methods for the two target species.

- o The *G. brevipalpis* and *G. austeni* colonies at the ARC-OVI will need to be enlarged in a mass-rearing facility of approximately 500 times of its current size before it to be used in an AW-IPM programme with a SIT component. This will necessitate the reassessment of the rearing and operational requirements of the two species involved.

- o The mating compatibility and competiveness between the colonised *G. brevipalpis* and *G. austeni* and the wild type need to be assessed. The degree of compatibility between these strains will determine the need for the establishment of colonies of the KwaZulu-Natal strains of these two species.

- o The mating competitiveness of males irradiated as adults compared to late stage pupae needs to be assessed.

- o Factors that can shorten the time to reach sexual maturity in *G. brevipalpis* and *G. austeni* need to be identified.

- o The mating performance of irradiated colonised *G. brevipalpis* and *G. austeni* males compared to that of fertile wild type males needs to be studied.

The debilitating effect of nagana can still be observed in it severity at the outer fringes of the tsetse distribution belt as in southern Africa. The sustainable control of nagana in the north eastern parts of KwaZulu-Natal will contribute to animal health and will stimulate economic development in this largely underdeveloped area. The findings of this thesis will be used to improve the proposed AW-IPM tsetse fly elimination strategy for southern Africa and can be used in the decision making of control in other regions in Africa where these two species are encountered.

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APPENDIX

Appendix 1. List of conference presentations and publications:

2009:

De Beer, C.J., Venter, G.J. & Latif, A.A. (2009) Colonisation of tsetse flies *Glossina austeni* and *Glossina brevipalpis* for research and development to support an area-wide approach for integrated pest management in northern KwaZulu Natal Province, South Africa. In: *Journal of the South African Veterinary Association. Proceedings of the 37th Annual Congress of the Parasitological Society of Southern Africa.* 1-3 October 2008. Pretoria, South Africa.

De Beer, C.J., Venter, G.J. & Potgieter, F. (2009) Developing quality assurance procedures for the sustainable supply of high quality blood for mass rearing of tsetse fly colonies. In: *Journal of the South African Veterinary Association. Proceedings of the 37th Annual Congress of the Parasitological Society of Southern Africa.* 1-3 October 2008. Pretoria, South Africa.

2010:

De Beer, C.J. & Venter, G.J. (2010) Colonization of tsetse flies as a prerequisite for the use of sterile Insect technique in South Africa. *Proceedings of 18th WiN Global Annual Conference*, 9-14 May 2010, Paradise Hotel Busan, Korea.

2012:

De Beer, C.J. & Venter, G.J. (2012) Evaluation of radiation sensitivity of *Glossina brevipalpis* and *Glossina austeni* (Dipter, Glossinidae). *Proceedings of the 41st Annual Congress of the Parasitological Society of Southern Africa.* 1-3 October 2012. Bloemfontein, South Africa.

De Beer, C.J. & Venter, G.J. (2012) Tsetse flies in the conservation areas of North-Eastern parts of KwaZulu-Natal. *Proceedings of the 41st Annual Congress of the Parasitological Society of Southern Africa.* 1-3 October 2012. Bloemfontein, South Africa.

2014:

De Beer, C.J. & Venter, G.J. (2014) Assessment the factor of age on the mating competitiveness of *Glossina brevipalpis* males under field cage conditions. *Proceedings of the 19th E-SOVE conference*, 13-17 October 2014, Thessaloniki, Greece.

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Ntantiso, L., De Beer, C., Marcotty, T. & Latif, A.A. (2014) Bovine trypanosomosis prevalence at the edge of Hluhluwe-iMfolozi Park, KwaZulu-Natal, South Africa. *Onderstepoort Journal of Veterinary Research*, 81(1), Art. #762, 8.

2015:

De Beer, C.J., Venter, G.J., Motloang, M.A. & Latif, A.A. (2015) Tsetse abundance and nagana prevalence in north eastern Kwa-Zulu Natal. *Proceedings of the joint ESSA and ZSSA Congress.* 12-17 July 2015, Grahamstown, South Africa.

De Beer, C.J., Venter, G.J. & Vreysen, M.J.B. (2015) Determination of the optimal mating age of colonised *Glossina brevipalpis* and *Glossina austeni* using walk-in field cages in South Africa. *Parasites & Vectors*, **8,** 467.

De Beer, C.J., Venter, G.J. & Vreysen, M.J.B. (2015) The competitiveness of sterile male *Glossina brevipalpis* for use in the sterile insect technique (SIT). *Proceedings of the joint ESSA and ZSSA Congress.* 12-17 July 2015, Grahamstown, South Africa.

2016:

De Beer, C.J., Venter, G.J., Kappmeier Green, K., Esterhuizen, J., De Klerk, D.G., Ntshangase, J., Vreysen, M.J.B., Pienaar, R., Motloang, M., Ntantiso, L. & Latif, A.A. (2016) An update of the tsetse fly (Diptera: Glossinidae) distribution and African animal trypanosomosis prevalence in North Eastern KwaZulu-Natal, South Africa. *Onderstepoort Journal of Veterinary Research*, **83 (1),** a1172.

De Beer, C., Venter, G. & Vreysen, M.J.B. (2016) Comparison of geometric morphometric markers between South Africa, southern Mozambique and Swaziland tsetse populations. *Abstracts of the XXV International Congress of Entomology*, 25-30 September 2016, Orlando, United States of America.

Renda, S., De Beer, C.J., Venter, G.J. & Thekisoe, O.M.M. (2016) Evaluation of larviposition site selection of *Glossina brevipalpis*. *Veterinary Parasitology*, **215,** 92-95.

Accepted:

De Beer, C.J., Venter, G.J. & Vreysen, M.J.B. (accepted) Improving the diet for the rearing of *Glossina brevipalpis* Newstead and *Glossina austeni* Newstead: blood source and collection – processing – feeding procedures. *PLoS ONE*.