# *Glossina brevipalpis* (Diptera: Glossinidae) in South Africa: distribution, endosymbionts and assessment of irradiated male mating proficiency

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## DEDICATION

Dedicated to my parents Dorcus and the late Michael Moyaba may his soul Rest in Peace.

“Psalm 23”

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## ABSTRACT

*Glossina brevipalpis* found in the north-eastern KwaZulu-Natal Province of South Africa represents the southernmost distributional limit of this genus in Africa. This species covers approximately 18 000 km$^2$ where it co-occurs with its congener *G. austeni*. An Area Wide-Integrated Pest Management (AW-IPM) strategy that include Sterile Insect Technique (SIT) component was proposed to establish a tsetse free status in South Africa. Initially, this study was designed to only focus on *G. brevipalpis* however due to the crucial role played by *G. austeni* in the transmission of trypanosome parasites that cause nagana in South Africa it was included and any control effort needs to consist of both species. This study was aimed at investigating the current apparent density (AD) of the two populations. Subsequently, this study also assessed the male mating ability and the multi mating behavior of the *G. brevipalpis*. In addition, the detection and abundance of the symbionts (*Sodalis* and *Spiroplasma*) harboured by wild captured flies colonized flies as well as to determine the prevalence of trypanosome parasites from wild captured flies.

The Apparent Density (AD) of the two tsetse flies were determined using entomological surveys conducted at five protected and four communal farming sites in 2008-2009 and compared to that of 2018-2019. An overall abundance of *G. brevipalpis* was significantly higher ($p < 0.05$) with an increase in AD from 4.02 to 5.33 flies /trap/day in 2008-2009 and 2018-2019 respectively. A significant decrease ($p > 0.01$) in AD from 0.21 in 2008-2009 to 0.14 AD flies/trap/day in 2018-2019 was observed in *G. austeni* populations from protected areas. These results indicated that the current tsetse control program, as applied in the communal farming areas,

doesn’t have a substantial long-term effect on tsetse abundance and suggest that more efficient control measures be implemented in the affected areas of north-eastern KwaZulu-Natal Province of South Africa.

In preparation of the SIT, male mating ability and the female multi mating behavior of *G. brevipalpis* were determined. For the male mating ability, the flies were given a radiation dose of 80 Gy and allowed to first mate with the 3 days old virgin females followed by second mating with fertile non-irradiated males. Both irradiated and non-irradiated males have shown the potential to mate several times and the propensity of mating as well as the ability to transfer sperm does not decline significantly ($p > 0.05$) in subsequent mating events. Irradiated males were able to inseminate several females comparable to that on non-irradiated males. The spermatheca fill in females that mated with non-irradiated and irradiated males did not differ. The multi-mating potential of the male tsetse flies from the colony indicated their suitability to be used for SIT based on their mating performance.

The multi mating behavior of *G. brevipalpis* females that first mated with irradiated and then with fertile males was determined under laboratory conditions. As expected, high numbers of pupae were produced by the females that only mated with the fertile males. Females which were only inseminated by irradiated males were going through recurring cycles of aborted eggs. The results showed that younger females appear to be more receptive to a second mating compared to older females.

The detection of endosymbionts DNA with species-specific PCR primers were determined. *Sodalis* were abundant in colonized *G. brevipalpis* and relatively low in

field collected flies and was not detected in both colonized and field collected *G. austeni*. *Spiroplasma* DNA was detected from 37% colonized *G. brevipalpis* and 30% colonized *G. austeni*. Trypanosome DNA was detected in 17.4% of field collected tsetse assayed with a high prevalence of *Trypanosoma congolense* and *T. theileri*.

The initiative to eliminate AAT and tsetse in South Africa need to be supported by accurate information of geographic distribution of tsetse populations as well as information on which *Trypanosoma* species are prevalent. Such baseline information is crucial when selecting most cost-effective strategy of intervention of the priority areas. This study highlighted that effective control measures are essential in affected areas of north-eastern-KwaZulu-Natal Province. Secondly, this study demonstrated that ARC-OVR tsetse colony flies are suitable to be used in tsetse eradication campaigns in South Africa. Lastly, this study could play a vital role in the decision making on the environmentally friendly tsetse control method that can be implemented in southern Africa.

**Key words:** *Glossina brevipalpis*, *Glossina austeni*, ARC-OVR tsetse colony, Apparent Density, mating potential, endosymbionts, trypanosome parasites.

## OPSOMMING

Die *Glossina brevipalpis* (Diptera: Glossinidae) populasie wat in die noordoostelike deel van die KwaZulu-Natal Provinsie aangetref word verteenwoordig die mees suidelike voorkoms van die genus in Afrika. Te same met *Glossina austeni*, wat saam met *G. brevipalpis* in die gebied voorkom, beslaan die twee spesies ‘n area van 18 000 km$^2$. Dit was voorgestel dat ‘n area wye geïntegreerde plaag beheer program, met 'n steriele insek tegniek (SIT) komponent, gebruik kan word om Suid-Afrika tsetse vry te verklaar. Alhoewel die huidige studie aanvanklik ontwerp was om slegs op *G. brevipalpis* te konsentreer, is *G. austeni* ingesluit in die studie. *Glossina austeni* speel ‘n belangrike rol in die oordrag van trypanosome parasiete wat nagana veroorsaak en dat ‘n beheerprogram, om suksesvol te kan wees, beide spesies sal moet insluit. Die doel van die studie was om die huidige Oënskynlike Digtheid (OD) van die twee spesies te bepaal. Verder is die parings vermoë en die vermoë van *G. brevipalpis* mannetjies om meer as een maal te paar bepaal. Bykomstig is die teenwoordigheid en volopheid van die simbionte (*Sodalis* en *Spiroplasma*) in die veld- en kolonie vlieë bepaal. Die volopheid van trypanosome parasiete soos aangetref in die veld versamelde vlieë is ook bepaal.

Die OD van die twee spesies soos bepaal deur entomologiese opnames wat in vyf bewaar areas en vier gemeenskaplike plase in 2008-2009 uitgevoer is, is vergelyk met vangste wat in 2018-2019 gedoen is. Die algemene volopheid van *G. brevipalpis* het aansienlik ($p < 0.05$) verhoog met ‘n toename in die OD van 4.02 vlieë/val/dag in 2008-2009 tot 5.33 vlieë/val/dag in 2018-2019. ‘n Aansienlike verhoging ($p > 0.01$) in die OD, van 0.21 vlieë/val/dag in 2008-2009 tot 0.14 OD vlieë/val/dag in 2018-2019

is by die *G. austeni* populasies in die bewaar areas aangetref. Die resultate is ‘n aanduiding dat die huidige tsetse beheer program, soos toegepas op die plase, geen doeltreffende langtermyn effek op tsetse volopheid het nie en dat meer effektiewe beheermaatreëls benodig sal word om die vlieë in die besmette areas in die noordooste van die KwaZulu-Natal Provinsie in Suid-Afrika te beheer.

Ter voorbereiding vir die implementering van ‘n SIT, is die parings-vermoë van mannetjies die veelvuldige parings gedrag van wyfie *G. brevipalpis* bepaal. Vir die bepaling van die parings-vermoë van die mannetjies is die vlieë bestraal met ‘n dosis van 80 Gy en gepaar met 3-dae oue wyfies wat nog nie voorheen gepaar het nie. Daarna is die gepaarde wyfies met vrugbare mannetjies wat nie bestraal was nie gepaar. Beide die bestraalde en onbestraalde mannetjies het verskeie kere gepaar en die geneigdheid tot paring so wel as die vermoë om sperms oor te dra het nie betekenisvol ($p > 0.05$) verminder in opeenvolgende parings nie. Bestraalde en onbestraalde mannetjies het wyfies in vergelykbare getalle bevrug. Spermateka vul in wyfies wat met bestraalde en onbestraalde mannetjies gepaar het, het ook nie verskil nie. Die veelvoudige parings vermoë van kolonie mannetjies dui daarop dat hulle, soos aangedui op parings gedrag, in ‘n SIT gebruik kan word.

Die veelvoudige parings gedrag van *G. brevipalpis* wyfies wat eers met bestraalde en daarna met vrugbare onbestraalde mannetjies gepaar het, is in die laboratorium ondersoek. Soos te wagte het die wyfies wat met vrugbare mannetjies gepaar het hoë getalle papies geproduseer. Wyfies wat slegs met bestraalde mannetjies gepaar het, het deur herhalende siklusse van geaborteerde eiers gegaan. Daar is aangetoon dat jonger wyfies meer gewillig as ouer wyfies is om meermale te paar.

Die teenwoordigheid van endosimbiont DNS is met spesies-spesifieke PKR opspoorders bepaal. Daar is gevind dat *Sodalis* volop was in die gekoloniseerde *G. brevipalpis* en relatief laag in die veld-versamelde vlieë. *Sodalis* was afwesig in beide die gekoloniseerde en veld-versamelde *G. austeni. Spiroplasma* DNS was teenwoordig in 37% en 30% van die gekoloniseerde *G. brevipalpis* en *G. austeni* respektiewelik*. Trypanosoma* DNS, en hoofsaaklik die van *Trypanosoma congolense* en *T. theileri*, was teenwoordig in 17.4% van die veld versamelde vlieë.

Enige inisiatief om nagana in Suid-Afrika te elimineer moet deur akkurate inligting van die vlieë se geografiese verspreiding en inligting oor die *Trypanosoma* spesies wat voorkom ondersteun word. Die basiese inligting sal krities wees voordat daar besluit kan word op die mees koste effektiewe strategie en sal ook die belangrikste areas identifiseer waar beheer toegepas moet word. Die huidige studie aksentueer dat effektiewe beheer maatreëls noodsaaklik is in die noordoostelike deel van die KwaZulu-Natal Provinsie. Die studie dui ook aan dat die vlieë in die LNR-OVR tsetse kolonie geskik sal wees om in ‘n tsetse uitwissings program in Suid-Afrika te gebruik. Die studie kan ‘n belangrike rol speel in die besluit om ‘n omgewings vriendelike tsetse beheer program in suidelike Afrika te implementeer.

**Sleutel woorde:** *Glossina brevipalpis*, *Glossina austeni*, ARC-OVR tsetse kolonie, oënskynlike Digtheid, parings vermoë, endosimbiont, *Trypanosoma* parasiete.

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# CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW

## 1.1 Blood Feeding Insects as Disease Vectors

Hematophagy has extensively evolved in the Animal Kingdom especially so among invertebrates. Vertebrate blood, normally rich in nutritious proteins and lipids, represent an abundant food source (Lehane, 2005). As such it forms part of the diet of several classes of animals incorporating Insecta (insects), Arachnida (ticks), Hirudinea (leeches), and vertebrates such as Pisces (lampreys, candirus), Aves (vampire finches, hooded mockingbirds), Amphibia (vampire flying frog (*Rhacophorus vampyrus*) as well as Mammalia (vampire bats). It even forms part of the diet in some human societies, e.g., the Maasai tradition of drinking raw blood, cooked blood, and blood-milk mixtures and several variants blood sausages, considered a delicatessen worldwide (Lehane, 2005).

Hematophagy has evolved multiple times independently amongst the Diptera and as such open the opportunity for the transmission of pathogens between vertebrate hosts (Lehane, 2005). At present, an impressive list of pathogens are transmitted by blood-feeding insects and among them are the causative agents for several important medical diseases such as malaria, sleeping sickness, leishmaniasis, river blindness, elephantiasis, yellow fever and dengue, and veterinary diseases such as nagana, surra, souma, bluetongue, African horse sickness and Rift Valley fever (Lehane, 2005). Of approximately 14000 species of hematophagous insects, from five orders: Diptera, Hemiptera, Lepidoptera, Phthiraptera and Siphonaptera Diptera,

(Adams, 1999), 300 to 400 regularly attract attention and among them are tsetse flies from the order Diptera.

## 1.2 Tsetse flies

Tsetse flies, males and females, solely depends on blood for survival and the completion of their life cycle. Tsetse flies resort under the genus *Glossina* Wiedemann, the sole genus in the family Glossinidae (Mutika *et al.,* 2019), and are the key representatives of the dipteran clade Calyptratae of which the majority of species are blood feeders and of biomedical importance (Solano *et al.,* 2015). The genus consists of 31 species with subspecies, grouped into three subgenera: *Austenina* (Fusca group), *Nemorhina* (Palpalis group) and *Glossina* (Morsitans group) (Rogers & Robinson, 2004). Tsetse flies, apart from a small fly belt in Saudi Arabia (Elsen *et al*., 1990), are restricted to the African continent where they are widely distributed from the southern borders of the Sahara to the north-eastern parts of South Africa

(Figure 1.1).

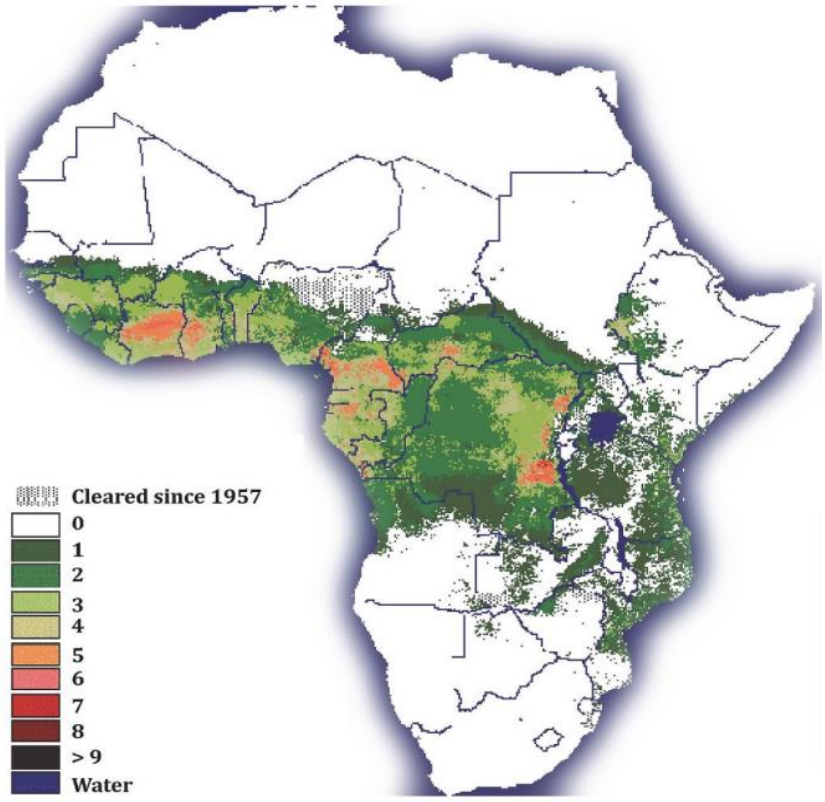


**Figure 1.1: Tsetse fly distribution in sub-Saharan Africa. Figure legend correspond to the colours as highlighted in the fiqure** (Kariithi *et al.,* 2013).

### 1.2.1 Reproduction and Life cycle

The reproduction of tsetse flies is through adenotrophic viviparity. During larvagenesis maternal milk gland secretions produced by the female provide the developing offspring with nourishment as well as distinct endosymbiotic bacteria (Abd-Alla *et al*., 2013). On completion of larvagenesis the female deposits a fully develop fourth instar larva in an appropriate microhabitat in the soil where it pupariates in (Krafsur, 2009). The duration of adult development at 25°C is about 27 days (Figure 1.2). Their uniqueness among most other insects in reproduction as well as their peculiar life cycle has important implications for control or eradication efforts (Vreysen, 2001).

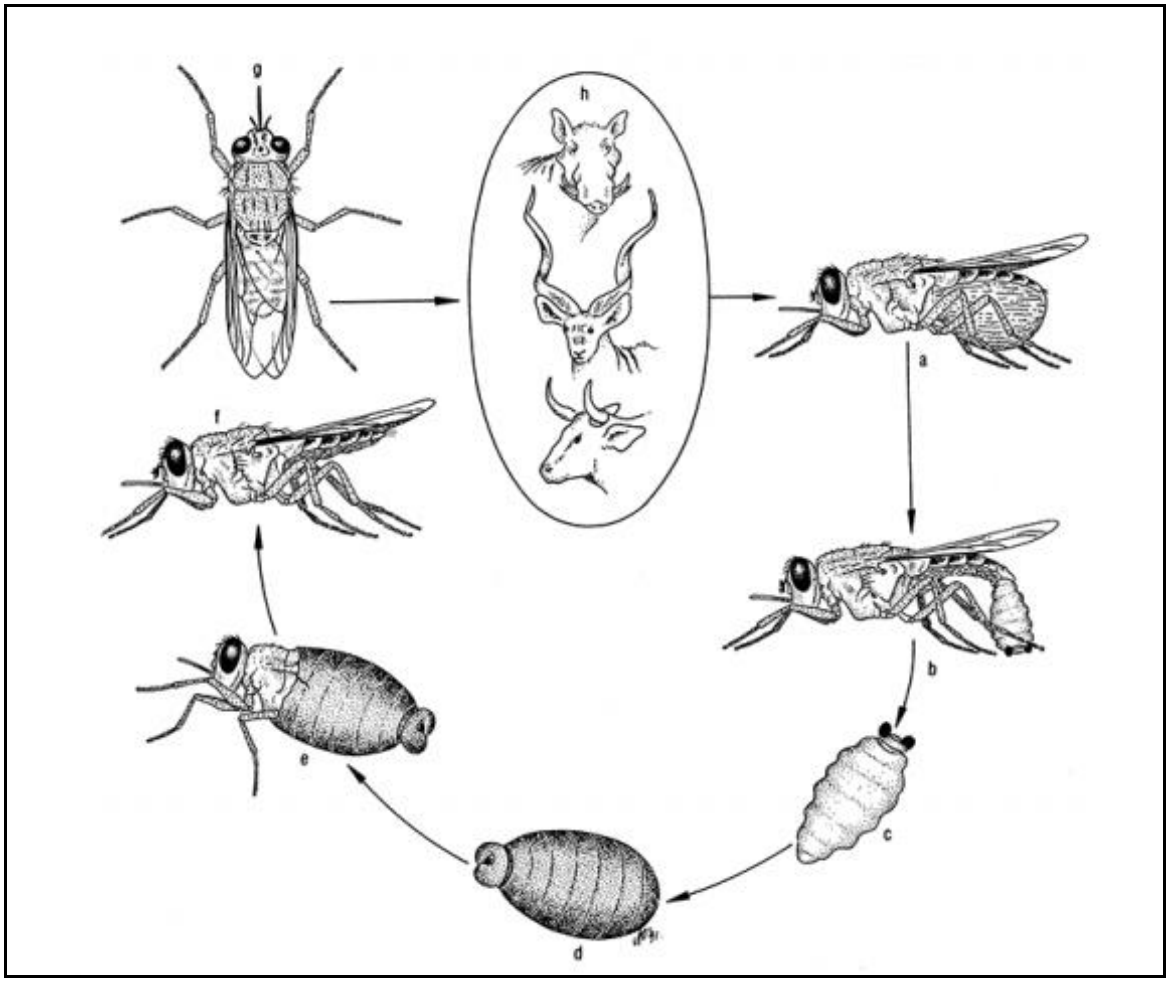


**Figure 1.2: Life cycle of a tsetse fly from larva to adult and their various animal hosts** (Phelps & Lovemore, 2004).

In nature, mating of tsetse flies usually takes place near to or on host animals. During mating the male settles on the back of the female, and claspers at the posterior end of the male abdomen grip the end of the female abdomen. This position is maintained for up to two hours after which the male releases his grip and flies away. Male tsetse flies such as in *Glossina morsitans* have been shown to mate several times with more than one female (http://www.fao.org/3/p5178e/P5178E04.htm). Older males are better able to mate successfully than young ones (de Beer *et al*., 2015). During mating, a ball of sperm is deposited into the vulva in a spermatophore. During the next few hours, the sperm

migrate from the spermatophore via the spermathecal ducts into the spermathecae. Sperm remain active in the spermathecae for the rest of the female's life (http://www.fao.org/3/p5178e/P5178E04.htm).

Mating in the females commence within 3 to 5 days after adult emergence and sperm are stored in the spermathecae until fertilization (Benoit *et al*., 2015). Larviposition in young females occur within at least 16 days and a minimum time of 25 days for offspring production (Krafsur, 2009) (Figure 1.2). Females mature only one egg at a time (Hargrove & Williams, 1998). Under natural conditions females only mate once in their lifetime; however, polyandry in wild populations of *Glossina fuscipes fuscipes* has been recorded under laboratory conditions (Bonomi *et al.,* 2011). In nature of *Glossina* an individual females can produce 8 to 10 progeny during their reproductive lives (Benoit *et al*., 2015).

Adult tsetse flies have a longer lifespan than most other insects, which partly compensate for their slow reproduction rate (Solano *et al*., 2015). They are typical *K* strategists, which differentiate them from most insect species which usually are *r* strategists with relatively large number of offspring and short lifespans (Leak, 1998).

### 1.2.2 Tsetse flies as vectors of trypanosomiasis

In 1895, David Bruce, working in KwaZulu-Natal (KZN), South Africa, showed that tsetse flies transmit the unicellular trypanosome parasite that cause nagana in animals and eight years later the trypanosome parasite that cause African trypanosomes to humans (Bruce, 1895). In Africa, the endemicity of tsetse-transmitted trypanosomiasis cover an estimated geographical area of approximately

10 million $km^2$, coinciding with the geographical distribution of the tsetse vector which covers the tropical area extending from the latitude 15° N to 30° S (Abenga, 2014).

Trypanosome parasites successively infect two hosts during its lifetime, i.e., digenetic parasites, the first being the insect vector and the second most often a mammal (Geiger *et al*., 2015). After feeding on a trypanosome infected mammal, the ingested trypanosome reaches the midgut of the fly, where it differentiates from the blood stream form (Figure 1.3). During the migration of the parasite from the gut to the salivary glands, it differentiates into several forms like short stumpy trypanomastigotes which is present in the mammal into the early procyclic form (Geiger *et al*., 2015).

Cyclical transmission is a process whereby the parasite, undergo some development before the infective stage can be transmitted to a subsequent host (Phelps & Lovemore, 2004). The different developmental stages of the various species of the trypanosome parasites are found in different parts of the tsetse host (Figure 1.3), allows for the identification of species depending on the site in the vectors body where it is found (Esterhuizen, 2007). In *Trypanosoma vivax* the development cycle is normally completed within 5 to 14 days and is restricted to the mouthparts of the fly. Whereas *T. brucei* and *T. congolense* develop up to the mesocyclic stage in the midgut of the fly (Figure 1.3). The process continues to move into the endoperitrophic space via the oesophagus to the proboscis and salivary glands, respectively. In *T. brucei* the development takes 17 to 45 days and 7 to 40 days in *T. congolense*. Only the metacyclic form is infective for mammals and is transmitted

from the fly's saliva into the bloodstream of subsequent mammalian hosts during the ingestion of a subsequent blood meal (Geiger *et al*., 2015).

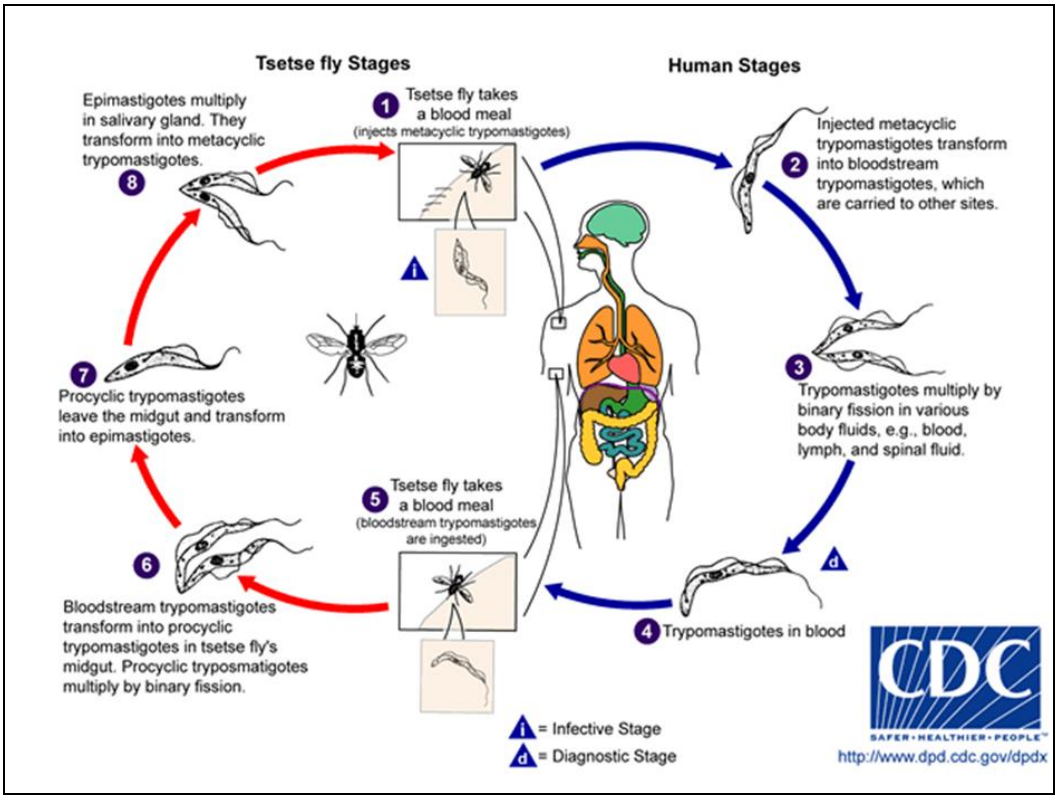


**Figure 1.3: The lifecycle of trypanosomes parasite in the definitive (tsetse) and intermediate host (human).**

**(https://www.cdc.gov/parasites/sleepingsickness/biology.html)**

Transmission is either through saliva (Salivarian) or faecal by contamination (Stercorarian) through the wound caused by the vectors biting (Hoare, 1966; Abenga, 2014). *Trypanosoma brucei* belong to the Salivaria group and in particular the subgenus *Trypanozoon* (Abenga, 2014). Salivarians resort under the subgenera *Duttonella*, *Trypanozoon*, *Nannomonas* or *Pycnomonas*. The first three subgenera contain the species that causes the most cases of trypanosomiasis in humans and livestock. *Trypanosoma brucei rhodesiense* causes acute infections and zoonotics in East Africa whilst *T. b. gambiense* causes chronic infections in West and Central Africa (Abd-Alla *et al*., 2013). The major pathogens in cattle and other ruminants,

which cause nagana, are *T. vivax* and *T. congolense* (Abd-Alla *et al*., 2013). In domestic pigs (*Sus domesticus*) *Trypanosoma simiae* is more involved in high mortality, whereas *T. b. brucei* infects all livestock.

### 1.3 Economic impact of trypanosomiasis

Trypanosomiasis affects the public health and socio-economic development throughout sub-Saharan Africa. An estimated 70 million people are at risk of contracting Human African trypanosomiasis (HAT), while the economic losses on African Animal trypanosomiasis (AAT) are measured in billions of dollars annually (Cecchi *et al*., 2015). In humans the tools for disease control are limited and infections are most often fatal if left untreated. The impact of trypanosomiasis in Africa is multidimensional as it affects human health, production of livestock, agricultural production (lack of draught animals and manure), rural socio-economic development, national economies (import and exportation of animals) and the environment (increased application of insecticides) (Abd-Alla *et al*., 2013).

While nagana causes severe symptoms in domesticated animals it causes only mild infections with no clinical symptoms in wild animals converting them into reservoir hosts (Taioe, 2013). Clinical symptoms of infected livestock include anaemia, lethargy, abortions, and eventual mortality (Esterhuizen *et al*., 2005). Despite the severity of the disease the control for trypanosomosis in Africa remains under studied.

## 1.4 Tsetse and trypanosomiasis control methods

The management of trypanosomiasis relies on the effective control of the insect vector (tsetse), or the parasite or combination of both (Delespaux *et al*., 2008). The control strategies of sleeping sickness are focused on the patients. Effective control is commonly interrupted by a lack of vaccines and a limited drug toolbox, some of which produces harmful side effect (Simarro *et al*., 2008; Solano *et al*., 2015). The situation has worsened on the emergence of the resistant trypanosome strains, which is boosted by the current drug treatments (Geiger *et al*., 2015).

A range of vector control tools, each with certain advantages and limitations, are available (Kariithi *et al.,* 2013). Effective historical control methods, such as removal of suitable vegetation of the tsetse fly and the culling of the host game animals have become environmentally unacceptable (Dransfield *et al*., 1991). The use of trypanotolerant cattle in areas of low to medium trypanosomosis challenge had given a certain degree of resistance to AAT (d'Ieteren *et al*., 1998). However, due to their smaller size, lower milk and meat productivity as well as lack of strength to provide adequate power resulted that these trypanotolerant cattle are not favoured by livestock owners (d'Ieteren *et al*., 1998; Vreysen, 2001).

The extensive long-term use of broad-spectrum insecticides to control insect pests of agricultural importance resulted in resistance, increased residues in food and contamination of natural resources and the environment (Vreysen *et al.,* 2006). Greater awareness of the increasing impact of chemicals on the environment results to a decline in the number of agents available for livestock pest management. Reliance on only a few active ingredients may exaggerate problems with insecticide

resistance. The chemical control of insect vectors has been unsuccessful due to the emergence of insecticide resistance in the vector populations as well as the unacceptable ecological impact thereof (Askoy, 2003).

### 1.4.1 Sequential aerosol technique (SAT)

This method aims to kill all adult flies in each spraying cycle by direct contact with the insecticide mist and to kill emerging flies before the reproductive stage (Vreysen, 2001). Ultra-low volumes of non-residual insecticide are sprayed from 10 m to 15 m above the tree canopy. Usually between five and six subsequent spraying cycles, 16 to 18 days apart, adequately reduce the population (Vreysen *et al*., 2013). This technique is effective for area wide tsetse suppression in dense humid forest ecosystems or eradication in open savannah type ecosystem when modern GPS-guided navigation or spray system is used. The SAT, in combination with other control tactics, was successfully used in Botswana, Kenya, South Africa, Uganda, Zambia, and Zimbabwe for the control of tsetse flies. In Botswana 7 180 $km^2$ and 8 722 $km^2$ of the Okavango delta respectively were treated with deltamethrin applied at dose rates of 0.26 g/ha during 2001 and 2002 (Vreysen *et al*., 2013). The accompanying environmental study indicated that non target aquatic and terrestrial invertebrates recovered well while tsetse may have been eliminated from the area (Vreysen *et al*., 2013). This operation proved SAT to be a rapid, efficient and cost-effective method for tsetse eradication in the open savannah areas of east and southern Africa with minimal negative residual environmental impact. Recently SAT was used to suppress *Glossina tachinoides* and *G. palpalis gambiensis* populations with more than 99% in a savannah area of about 6 745 $km^2$ in the upper west region of Ghana (Vreysen *et al*., 2013).

### 1.4.2 Targets and traps

Although targets and traps have been successfully used to suppress populations below the transmission threshold in sleeping sickness foci eradication has never been achieved with this technique against riverine tsetse species in West Africa (Vreysen *et al*., 2013). Esterhuizen *et al*. (2006) indicated differences in the effectiveness of odour-baited insecticide-treated targets for the control of *Glossina austeni* and *G. brevipalpis* in South Africa. While targets deployed at a density of eight per km² can suppress *G. austeni* this density was ineffective for the control of the co-occurring *G. brevipalpis* (Esterhuizen *et al*., 2006). However, for the suppression of *G. austeni* on Unguja Island, Zanzibar, a 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). Tiny targets, based on the same principles as the large savannah-type cloth targets, despite being eight times smaller (0.25 m x 0.5 m), apparently have the same killing efficiency as the bigger (1 m x 1.5 m) traditional targets (Esterhuizen *et al.,* 2006). Less cloth is required to make the tiny targets and less insecticide is needed for impregnation, making them more economical and easier to deploy over large areas (Esterhuizen *et al*., 2006; Shaw *et al*., 2015).

### 1.4.3 Live-bait technique

In this technique livestock is being treated with an insecticide thereby exposing the blood feeding tsetse to a lethal dose of insecticide when they feed on the animal (Vreysen *et al.,* 2013). The impact of this method on the environment can be reduced by treating only the legs of animals, i.e., the preferred feeding site of tsetse

flies (Vale *et al*., 2015). The success of the live-bait technique depends on a high feeding rate on the treated host as well as that of a sufficient proportion of the livestock population being treated with a lethal dose of insecticide (Vreysen *et al.,* 2013). The competitive advantage of this technique is that it usually consists of easy pour-on formulations which are rapid with no requirements of sophisticated equipment required. Insecticide treated cattle or livestock bait can be regarded as the simplest and cheapest available tsetse control technology (Barrett, 1997). This method was successfully used in Zimbabwe against *Glossina pallidipes*, in Burkina Faso against *G. morsitans submorsitans* and *G. p. gambiensis* and against *G. f. fuscipes* and *G. pallidipes* in Ethiopia (Vreysen *et al.,* 2013). Currently the nagana management strategy in South Africa relies on the long-term vector suppression using the live-bait technique and *ad hoc* treatment of cattle with trypanocidal drugs (de Beer *et al.,* 2021). This treatment involves a wide spectrum of insecticide, such as the pyrethroid cyhalothrin ($C_{23}H_{19}ClF_3NO_3$) and has been adopted since 2015 (de Beer *et al.,* 2021).

### 1.4.4 Genetic based control

#### 1.4.4.1 Sterile insect technique (SIT)

The increasing impact of chemicals on the environment led to a growing demand for sustainable environment friendly pest control approaches and methods to address the rising concerns about food safety, food quality and biodiversity (Vreysen *et al.,* 2006). These concerns resulted in the development and acceptance of the concept of an integrated pest management (IPM) approach (Vreysen *et al.,* 2006). The integration of different control tactics into IPM systems can be done in ways that greatly facilitate the achievement of the goals either of field-by-field pest

management, or of area wide integrated pest management (AW-IPM), which is the management of the total pest population within a delimited area (Vreysen *et al*., 2007).

In the 1940's, E.F. Knipling, working with New World screwworm *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae), develop the concept of using insects to control themselves (i.e., autocidal control) by reducing their reproductive capacity. During the 1950's there was a breakthrough in this concept when it was shown that male and female of the screwworm could be rendered sterile, without excessive loss in their lifespan and competitiveness, by exposing them to X-rays (Vreysen *et al*., 2006). Knippling (1955) shown that the sustained, sequential and systematic release of sterile males could eliminate a target insect population within a few generations (Vreysen *et al*., 2006). The feasibility of the sterile insect technique (SIT) was confirmed in 1954 with the implementation thereof on the island of Curaçao, close to Venezuela (Hendrichs & Robinson, 2009). Release of sterile screwworms as part of an AW-IPM approach eradicated this pest from North and Central America. Currently, a permanent "sterile fly barrier" consisting of the aerial release of 40 million sterile flies per week in eastern Panama protects Central and North America from reinvasion.

In addition to the success obtained with screwworm SIT was successfully used, in combination with other control tactics, to eradicate or contain pest populations of Diptera, Coleoptera and Lepidoptera. In California, the containment of the pink bollworm *Pectinophora gossypiella* was achieved and control of the Mediterranean fruit fly *Ceratitis capitata* in Guatamala / Mexico (Lindquist *et al*., 1999; Wyss, 2000).

Further successes are the suppression of codling moth (*Cydia pomonella*) in Canada (Bloem *et al*., 2005) and *Cydia capitata* in South Africa, Israel and Jordan (Kariithi *et al.,* 2013).

During the 1970s and 1980s feasibility studies were conducted for using SIT against populations of *G. m. submorsitans* in Zimbabwe and Tanzania, *G. tachinoides* in Chad, *G. p. gambiensis* in Burkina Faso and *Glossina palpalis palpalis* in an agro pastoral area of Latifa in Nigeria (Takken *et al.,* 1986; de Beer *et al*., 2017). Although all four targeted populations were temporally eradicated from the controlled zones it was not conducted area wide and the pest free status of these areas was lost due to reinvasion from neighbouring areas (Klassen & Curtis, 2005). The current eradication campaign of a *G. p. gambiensis* population in the Niayes, Senegal integrates SIT into an AW-IPM programme (Bouyer & Guerrini, 2010; Dicko *et al*., 2014). Suppression of tsetse fly populations was achieved using long term insecticide-impregnated traps/targets and pour-on for cattle which included a mixture of repellents and insecticide and SIT was subsequently implemented in the targeted area to eliminate the remaining relic fly pockets (Ciss *et al.,* 2019).

The SIT, as conceived in the 1930's, has become one of the most effective tools to be incorporated into AW-IPM programmes (Vreysen & Hendrichs, 2005). Since the SIT is species-specific it is considered an environmentally friendly control method (Knipling, 1959, sited in Bourtzis *et al*., 2016). It is a biologically based control method with many advantages and minimum disruption to the environment.

#### 1.4.4.2 Symbiotic-based control methods

Symbiotic associations with bacterial microbes supplement the blood diet, which lacks many vitamins and coenzymes, in tsetse flies (Abd-Alla *et al.,* 2013).The microbiota in the insect's gut affects their host in many ways, ranging from nutritional contributions to the insect diet, protecting them from pathogenic bacteria and the production of semiochemicals (Lindh & Lehane, 2011). In tsetse flies, symbiotic microbes play a vital role due to their significant influence on the biology of the fly, their reproduction, immunity, elicitation of phenotypes and potential effects on their vector competence for trypanosomes (Demirbas-Uzel *et al*., 2018). An understanding of the interaction between symbionts and the parasite in the tsetse host may facilitate the development of flies that are refractory to trypanosome infection by modifying their symbionts. Paratransgenesis is a genetic based method based on modifying the symbiotic organism of the insect vector with recombinant technologies to express effector molecules that potentially block the development of the pathogen (Demirbas-Uzel *et al*., 2018).

##### 1.4.4.2.1 Symbiotic bacteria associated with tsetse flies

Tsetse flies harbour four main symbiotic bacteria (Ouedraogo *et al.,* 2018) with each playing different roles. These are *Wigglesworthia glossinidia*, an obligate symbiotic bacterium, secondary endosymbiont *Sodalis glossinidius*, the reproductive symbiont *Wolbachia pipientis* (Weiss *et al*., 2006; Doudoumis *et al*., 2012) and *Spiroplasma* as recently found in *Glossina fuscipes* and *G. tachinoides* (Ouedraogo *et al.,* 2018; Schneider *et al.,* 2019).

#### 1.4.4.2.1.1 *Wigglesworthia*

All tsetse species and individuals harbour the enteric symbiont *Wigglesworthia* species (Enterobacteriaceae) to supplement their diet with the essential nutrients, such as vitamins which are absent from the tsetse's blood diet (Doudoumis *et al*., 2013; Benoit *et al*., 2015). In tsetse flies, this bacterium mainly resides within the cells bacteriocytes of a specialised organ, the bacteriome, in the anterior midgut. It consists of a highly streamlined 700-Kb genome (Weiss *et al*., 2006). The absence of this symbiont in female flies results in sterility (Doudoumis *et al*., 2013). Amongst its nutritional role *Wigglesworthia* influences the host immune maturation process during the larval stages (Doudoumis *et al*., 2013). In the absence of *Wigglesworthia* the progeny of the flies is immunocompromised and infertile (Benoit *et al*., 2015).

#### 1.4.4.2.1.2 *Sodalis*

This second symbiotic bacteria in natural tsetse fly populations are heterogeneous and also harboured by flies in insectary colonies (Abd-Alla *et al*., 2013). The *Sodalis* genome is about 4.5 Mb in size and unlike *Wigglesworthia* had never shown a reduction in size and as such more in line with free-living enteric microbes (Abd-Alla *et al*., 2013). *Sodalis* contains pseudo genes more especially in the pathways unlikely to be active in the restricted nutritional ecology of its host biology. It is found in the midgut of tsetse, coinciding with the site where trypanosome maturation takes place. These interactions between the symbionts and trypanosome may provide an avenue for control of the disease (Wamwiri *et al*., 2013).

#### 1.4.4.2.1.3 *Wolbachia*

The third symbiont, *Wolbachia pipientis,* an obligatory intracellular symbiont, is a maternally transmitted bacterium (Doudoumus *et al*., 2013). *Wolbachia* was

described by Hertig and Wolbach in 1920s as a microorganism infecting the ovaries of female mosquitoes belonging to the *Culex pipiens* L. complex (Diptera; Culicidae), hence the name *W. pipientis* (Hertig & Wolbach, 1924; Hertig, 1936). A survey of arthropods revealed *Wolbachia* to be abundant in insects, isopods and mites (Bourtzis, 2007). The bacteria, transmitted through the egg cytoplasm, cause infection in the reproductive tissues of arthropods where it alters the reproduction in their host. This bacterium is associated with the Cytoplasmic Incompatibility (CI), parthenogenesis, feminization of genetic males as well as male mortality (Werren & Windsor, 2000). The simplest form of CI is expressed as the embryonic lethality when the infected male is crossed with sterile female. *Wolbachia* induced CI is proposed as a control tool of vector populations and diseases (Abd-Alla *et al*., 2013). It has the ability to produce or release specific bio-molecules that leads to the embryonic death which also interfere with the development and establishment of trypanosomes in tsetse flies (Kanté *et al.,* 2018). In contrast, the phenomenon occurs when an infected male mates with an uninfected male which lead to the degeneration of the future embryo (Geiger *et al.,* 2015)

#### 1.4.4.2.1.4 *Spiroplasma*

*Spiroplasma* is a genus of wall less bacteria belonging to the class Mollicutes which are associated with plants and arthropods (Doudoumis *et al.,* 2017). *Spiroplasma* has the capacity to live intracellularly in a variety of tissues and systematically in the haemolymph (Doudoumis *et al.,* 2017). Several species of *Spiroplasma* are associated with the reproductive alterations such as male mortality in some arthropods (Doudoumis *et al.,* 2017; Schneider *et al.,* 2019). *Spiroplasma* has developed a wide range of symbiotic associations, producing diverse effects on

insect evolution, ecology, reproduction and sex determination (Doudoumis *et al.,* 2017). *Spiroplasma* can be pathogenic in plants, insects and crustaceans and has been found to be capable of protection against the nematode in *Drosophila neotestacea*, fungi in the pea aphid (*Acyrthosiphon pisum*) and a parasitoid wasp in *Drosophila hydei* (Doudoumis *et al.,* 2017). Functions of *Spiroplasma* in tsetse host remains largely unknown (Schneider *et al.,* 2019).

## 1.5 Occurrence of tsetse and nagana in north-eastern KwaZulu-Natal

### 1.5.1 Tsetse distribution

In South Africa, the tsetse infected zone is restricted to an area of 18 000 $km^2$ in the north-east of Kwazulu-Natal Province (KZN) and represent the southernmost distributional limit of this genus in Africa (Motloang *et al*., 2012; de Beer *et al*., 2016). This population continues into the Matutuine District in the Maputo Province in southern Mozambique (Esterhuizen *et al*., 2005; de Beer *et al.,* 2016). Livestock production and agricultural development in these areas are hindered by the presence of these flies as vectors of trypanosomes and causes considerable stress to farmers (Motloang *et al.*, 2012). Tsetse infested areas in KZN are mainly used for communal farming and are inhabited by 426 000 humans, 300 000 heads of cattle and approximately 130 000 small ruminants (Kappmeier *et al.,* 1998; Esterhuizen *et al*., 2005).

The first record of tsetse flies in north-eastern KZN dates back to 1836 when the fly's disease was reported to strike the cattle herds of the early pioneers and the diseases was referred to as Animal trypanosomiasis (Fuller, 1923). Animal African trypanosomiasis (AAT) is endemic in the rural areas of north-eastern KZN Province

in South Africa with an estimated 300 000 cattle being at risk of exposure to the disease (Kappmeier *et al.,* 1998; Kappmeier Green *et al.,* 2007). The distribution of AAT in the area is closely correlated with the distribution of the tsetse fly vectors.

Historically four species of tsetse flies, *Glossina m. morsitans*, *G. pallidipes*, *G. brevipalpis*, and *G. austeni* were present in South Africa (Kappmeier *et al*., 1998). *Glossina m. morsitans* was the only species encountered in the most northern part of the country and its distribution extended towards Mozambique and Eswatini (Fuller, 1923). The other three species, *G. pallidipes*, *G. brevipalpis* and *G. austeni,* were restricted to north-eastern parts of the KZN Province (Fuller, 1923). In 1897, *G. m. morsitans* was eradicated from South Africa during the rinderpest epizootic of 1896-1897, followed by the *G. pallidipes* which was eradicated in 1950 after the South African government undertook campaigns which involved the aerial spraying to control the populations (Du Toit, 1954). *Glossina pallidipes* was the second most predominant species. Based on its abundance, it was considered the most important vector of AAT (Fuller & Mossop, 1929). *Glossina brevipalpis* and *G. austeni*, which were confined to smaller areas associated with dense vegetation, were not considered as important vectors of AAT. The sheer abundance of *G. pallidipes* was illustrated by the large numbers of flies being trapped e.g., in 1932 two million flies were collected within a month after deployment of 1 000 Harris traps in north-eastern KZN and nearly eight million flies over that entire year (Harris, 1932) (Figure 1.4). The presence of large numbers of tsetse flies, and especially *G. pallidipes*, in north-eastern KZN resulted in severe outbreaks of nagana between 1942 and 1946 (Du Toit, 1954). This led to an insecticide-based spraying programme with DDT (Dichlorodiphenyltrichloroethane) and HCH (Hexachlorocyclohexane) and by the

early 1950s *G. pallidipes* was eradicated from KZN (Du Toit, 1954; de Beer *et al.,* 2016).

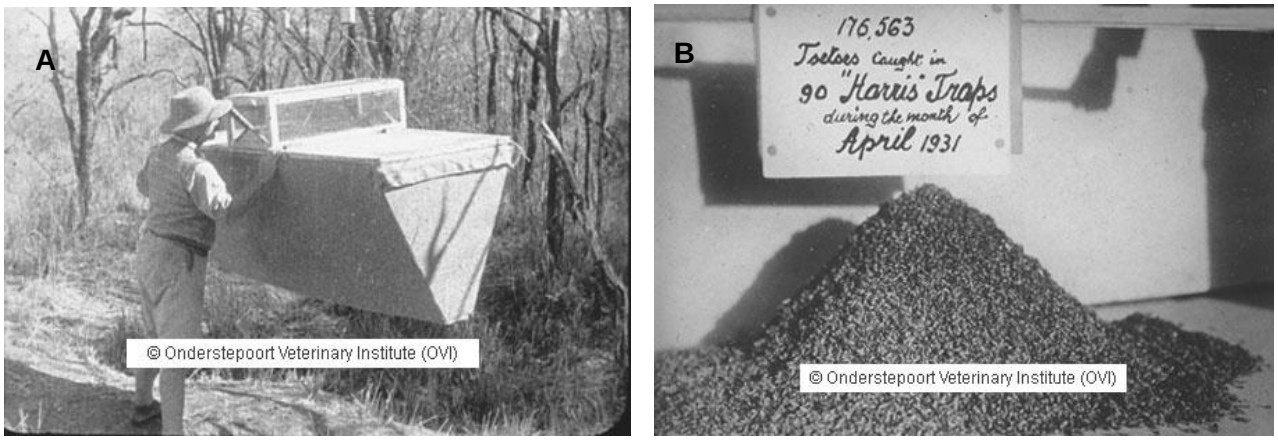


**Figure 1.4: A: The Harris tsetse trap used in north-eastern KwaZulu-Natal, South Africa for the collection of tsetse flies and B: the large numbers of flies collected with this trap (Source: https://repository.up.ac.za/handle/2263/4801).**

No control was implemented in areas where *G. brevipalpis* and *G. austeni* were found in the absence of *G. pallidipes* and consequently they remained present in north-eastern KZN till to date (Figure 1.5) (Kappmeier *et al.,* 1998; de Beer *et al.,* 2016). From 1955 onwards, only sporadic cases of nagana were recorded in the area (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).

Currently only *G. brevipalpis* and *G. austeni* remains in the north-eastern parts of KZN (de Beer *et al*., 2016). Both these species feed on cattle (de Beer *et al.,* 2021) and have been shown to be involved in the transmission of *T. congolense* and *T.*

*vivax* (Van den Bossche *et al*., 2006; Mamabolo *et al*., 2009; Motloang *et al*., 2012; 2014) with *T. congolense* being the most abundant tsetse transmitted *Trypanosoma* species in South Africa (Mamabolo *et al*., 2009). Limited vector competence studies indicated *G. austeni* to be a more competent vector for the transmission of *Trypanosoma* parasites in South Africa (Motloang *et al*., 2012).

In addition to cattle the host range for *G. brevipalpis* includes larger games animals such as hippopotamus (*Hippopotamus amphibious*), African elephant (*Loxodonta Africana*) and buffalo (*Syncerus caffer*) (Wetzel & Glasgow, 1956; Moloo, 1993; Clausen *et al*., 1998). In contrast, *G. austeni* prefer smaller game e.g., bush pig (*Potamochoerus larvatus*) and duikers (*Cephalophus* sp.) (Wetzel & Glasgow, 1956; Moloo, 1993; Clausen *et al*., 1998).

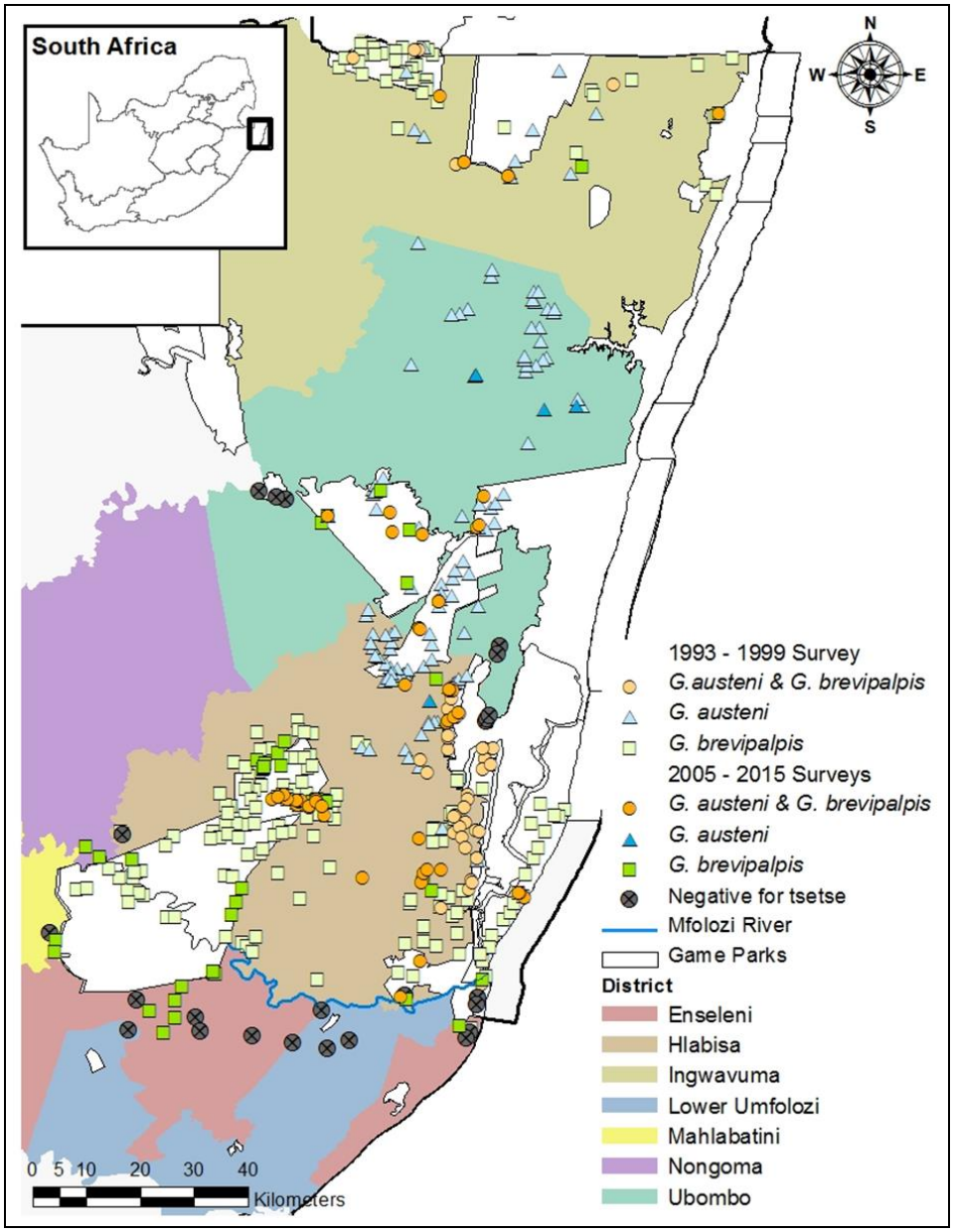


**Figure 1.5: Map showing north-eastern KwaZulu-Natal and localities of magisterial districts, major game parks, lakes and major rivers** (de Beer, 2016)**.**

### 1.5.2 Tsetse control campaigns in north-eastern KwaZulu-Natal

The first tsetse campaign in South Africa started in 1929 and lasted until 1930 following a large outbreak of trypanosomiasis (Kappmeier *et al*., 1998). Almost 27 000 wild animals in and around the Umfolozi Game Reserve were killed in this eradication campaign. In addition, the approximately 12 000 Harris traps (H-trap)

(Figure 1.4) deployed in the area appeared to control this outbreak. There was a decline in the disease prevalence and the destruction of game was halted between 1930 and 1939. However, in 1942 and 1946 large numbers of tsetse in north-eastern KZN once again resulted in a severe outbreak of nagana (Kappmeier *et al*., 1998). More than 60 000 cattle died in the Hluhluwe and Mkuze areas in the summer months. Together with bush clearing to create a bush free barrier, another campaign was started in conjunction with a game eradication whereby, more than 13 000 animals were killed (Kappmeier *et al*., 1998). During this campaign non-target host species such as zebra and wildebeest were killed, whereas host species such as rhinoceros and hippopotamus were left out (Kappmeier *et al*., 1998). Tsetse flies were, however, not eliminated by these culling operations.

After the Second World War in 1945 newly develop synthetic insecticides such as DDT and benzene hexachloride (BHC) were introduced in South Africa and used at the permanent breeding areas of tsetse flies in Mkuze, Hluhluwe Game Reserve and Umfolozi (Kappmeier *et al*., 1998). These insecticides were applied in a form of smoke for DDT or from the ground and thermal aerosol from the air for BHC (Kappmeier *et al*., 1998). The campaign which ended in 1952 was historically the first time that DDT and BHC were used on an extensive scale for the control of nagana (Kappmeier *et al*., 1998).

In 1990 yet another outbreak of trypanosomiasis was diagnosed in cattle at dip tanks close to the Hluhluwe-iMfolozi Park. This outbreak was caused by *T. congolense* and *T. vivax*. Livestock that exhibited clinical signs were treated with Berenil® (Kappmeier *et al*., 1998). It was not possible to estimate the actual mortality due to a

severe drought that occurs simultaneously with this outbreak. At least 61 (46%) of 132 dip tanks were infected in areas between the Umfolozi River and the Mozambique border and it was realised then, that this outbreak was widespread (Kappmeier *et al*., 1998). An adapted cattle-dipping regime, in combination with animal treatments with trypanocidal drugs, brought the outbreak under control (Kappmeier *et al.,* 1998). In 1994, the prevalence of nagana was surveyed again at 59 of the dip tanks and the highest prevalence determined was between 10% and 15% (Kappmeier *et al*., 1998). The last extensive survey was done from 2005 to 2009, blood samples from 1034 cattle at 25 dip tanks were microscopically examined. The results showed as in the 1990 outbreak that trypanosome infection was widespread with infected cattle found at 21 (84.0%) of the 25 dip tanks surveyed and the trypanosome prevalence ranged from 3% to 20% (de Beer *et al.,* 2016). Currently the nagana management strategy in South Africa relies on the long-term vector suppression using the live-bait technique and the ad hoc treatments of animals with trypanocidal drugs. This cattle-dipping regime with a wide-spectrum insecticide, i.e. the pyrethroid cyhalothrin ($C_{23}H_{19}ClF_3NO_3$) for suppressing the tsetse populations has been adopted since 2015 (personal communication, Department of Agriculture, KwaZulu-Natal, South Africa).

### 1.5.3 Colonies

The implementation of the SIT necessitates breeding facilities to produce sufficient numbers of the target insect for sterilization and subsequent field release of the males (Feldmann *et al*., 2005; Vreysen *et al*., 2006). The SIT will only be effective if the target population density is low (Feldmann & Hendrichs, 2001; Vreysen *et al*., 2013). Maximum effectiveness results when sterile males outnumber the fertile

males and therefore become less cost effective if the target population is large (Feldmann & Hendrichs, 2001). This is in contradiction with conventional control methods such as insecticide spraying, which are cost and operationally effective when the target population is high.

Laboratory colonies are instrumental for sophisticated vector control strategies such as SIT (Feldmann *et al*., 2005), transgenic (Alphey, 2014) and symbiont-based control methods (Atyame *et al*., 2011). The first successful attempt to maintain tsetse flies in the laboratory was achieved with *G. m. submorsitans* (Roubaud, 1917). These flies were collected in Senegal and maintained for three years at the Pasteur Institute in Paris, France (Roubaud, 1917). However, this colony consisted of only 32 flies (Ward, 1970). The next species to be colonised with varying degrees of success, over a period of 30 years, was *Glossina palpalis* in Belgium and England (Mellanby & Mellanby, 1937; Rodhain & Van Hoof, 1944). The sustainability of large colonies only become feasible with the development of blood feeding station which simultaneously hold four guinea pigs and eight cages of flies for feeding (Geigy, 1948). Professor Geigy, using several holders, succeed to feed as many as 2 000 flies over 3 to 4 hours between 1945 and 1948 (Ward, 1970). He, however, depend on repeated pupal introduction to sustain the colony.

*Glossina austeni* was colonised at the Tsetse Research Laboratory, School of Veterinary Medicine, University of Bristol, Langford, England from pupae collected on Unguja Island, Zanzibar. The emerged flies were fed on the ears of lop-eared rabbits (Nash *et al*., 1966a). Laboratory protocols on emergence, fertilization, adult and pupae maintenance, fly feeding and host suitability were developed (Nash *et al*.,

1966a, b, c; 1967; 1968; Jordan *et al*., 1966; 1967; 1968; Curtis & Jordan, 1968). This colony become self-sustaining in 1966 and produced 88 000 pupae in 1967 (Nash *et al*., 1968).

A colony of *G. 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 fed on the ears of lop-eared rabbits under controlled laboratory conditions (25°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).

As part of a proposed strategy for an AW control campaign with a SIT component, laboratory colonies of both South African tsetse species were established at the Agricultural Research Council-Onderstepoort Veterinary Research (ARC-OVR), Pretoria, in 2002. These colonies of *G. brevipalpis* and *G. austeni* were respectively established using seed material from the 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 will be competitive with their wild counterparts will be essential in the SIT component of an AW control campaign (Parker, 2005). These colonies need to be maintained at sustainable and economical acceptable levels.

## 1.6 Study justification

Continuous outbreaks of AAT in KZN and southern Mozambique still have a significant impact on the economic development in these rural areas. As such, the sustainable control of tsetse flies as the vectors of trypanosome parasites remains a priority throughout its distribution area. Increasing concerns and public opposition against chemical control led to the development of several alternative environmentally friendly control methods. These include strategies such as biological control using natural enemies (Leak, 1999), insect growth regulators (Jordan *et al*., 1979), juvenile hormones (Hargrove & Langley, 1993) and genetic control methods. Genetic control includes symbiotic-based methods and the SIT.

Selecting the most appropriate control methods can be challenging. Tsetse suppression needs to be done continuously as populations may recover or reinvade from untreated neighbouring regions (Hargrove, 2003). Kaba *et al*. (2012) suggested that eradication of selected tsetse populations may be more sustainable and economical than long-term suppression. Genetic control methods are therefore, an important consideration, when the aim is to eradicate a tsetse population. Genetic control methods depend on several prerequisites (Dyck *et al*., 2005) with biological quality and the sexual competitiveness of the insects used amongst the most important ones (Vreysen *et al*., 2007).

A comprehensive knowledge of the target tsetse populations will be essential for successful implementation of vector control. The biological quality of the colonised flies to be used in any genetic control method will be decisive in the success of the method. The mating potential of males and multiple mating behavior of the female *G.*

*brevipalpis* colonised at the ARC-OVR need to be evaluated. Furthermore, the symbiont fauna of *G. brevipalpis* and *G. austeni* collected from the north-eastern KZN need to be determined and compared with symbiont fauna of the colonised *G. brevipalpis* and *G. austeni* that will be used for SIT.

## 1.7 Aims

The aims of the current study were to determine the potential impact of the current nagana control methods on tsetse fly abundance in KZN. Secondly as part of the potential implementation of a proposed AW control campaign with a SIT component the aim was to assess the irradiated mating proficiency of colonised *G. brevipalpis* and lastly to compare the symbiont fauna of colonised *G. brevipalpis* and *G. austeni* with that of field-collected flies.

## 1.8 Objectives

To address the main aim of this study, the following objectives were set:

1. To compare the impact of the current nagana control measures on *G. brevipalpis* and *G. austeni* abundance in the affected area.
2. To assess the male mating ability of *G. brevipalpis* under laboratory conditions and the influence of irradiation on male mating proficiency.
3. To determine multiple mating behavior of *G. brevipalpis* females with irradiated and non-irradiated males under laboratory conditions.
4. To examine the abundance and association of endosymbionts *Sodalis* and *Spiroplasma* and trypanosome infections in *G. brevipalpis* and *G. austeni*.

# CHAPTER 2

# THE IMPACT OF NAGANA CONTROL MEASURES ON TSETSE ABUNDANCE IN COMMUNAL FARMING AND PROTECTED AREAS

## 2.1 Introduction

After an epidemiological silence of more than three decades a severe outbreak of nagana once again occurred in north-eastern KwaZulu-Natal Province (KZN) in 1990. 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 ($C_{19}H_{23}N_3$), an acaricide, to the pyrethroid cyhalothrin ($C_{23}H_{19}ClF_3NO_3$), a board spectrum insecticide (Hall & Fischer, 1984) for two years (de Beer *et al.,* 2021). The adopted cattle-dipping regime, in combination with animal treatments with trypanocidal drugs, brought the outbreak under control (Kappmeier *et al.,* 1998).

This unanticipated 1990 outbreak indicated that nagana and the vectors of the disease still play an important role in animal health in the north-eastern KZN. A subsequent extensive tsetse fly survey was conducted between 1993 and 1999 with odour-baited sticky XT traps (cross-shaped targets) to assess tsetse abundance and distribution in an area of approximately 12 000 $km^2$ (Kappmeier & Nevill, 1999; Kappmeier Green & Venter, 2007). This survey confirmed that *Glossina pallidipes* was still absent in the area and that *G. brevipalpis* was present in a southern and a northern band, commonly associated with game reserves and other protected areas

(Hendrickx, 2002; Hendrickx *et al.,* 2003). The survey indicated that although *G. austeni* was continuously distributed from south to north and that its distribution did not extend as far west as that of *G. brevipalpis*. *Glossina austeni* was also common in communal farming areas (Nevill *et al*., 1993; Kappmeier Green, 2002).

The 1993-1999 sticky trap data were incorporated in the development of a probability of presence model (Hendrickx, 2002; Hendrickx *et al*., 2003). This model, based on climatic and environmental variables; predicted a wider geographical distribution range for both *G. brevipalpis* and *G. austeni* than what was indicated by the available survey data (Hendrickx, 2002). Subsequent tsetse research led to the development of a more efficient trap, the H-trap (Figure 2.2), for the collection of both *G. brevipalpis* and *G. austeni* (Kappmeier, 2000). Based on the results of the distribution survey and prediction model these newly develop H-traps were used in a transect study, conducted between 2005 to 2015, to determine the distribution of *G. austeni* to the west of the Hluhluwe Dam (de Beer, 2016) and to assess fly presence and densities at dip tanks (Mamabolo *et al*., 2009; Gillingwater *et al*., 2010; Motloang *et al*., 2012; Ntantiso *et al*., 2014, de Beer *et al*., 2016).

The acquired data allowed for an update of tsetse distribution and abundance maps in the area (de Beer *et al*., 2016; de Beer *et al.*, 2019). During the surveys conducted between 2005 and 2015, low numbers of *G. brevipalpis* were encountered in at least five sites in a central area in the tsetse infested belt located east of the Mkuze Game Reserve and north of the southern previously defined distribution band (de Beer, 2016; de Beer *et al*., 2016). These positive trap sites were used to update the prediction model of Hendrickx *et al*., 2003. The updated model indicated that *G.*

*brevipalpis* was also present in the central and southern parts of Ubombo District, where it was previously not encountered (de Beer, 2016; de Beer *et al*., 2016). Furthermore, this survey indicated that the two tsetse species were present close to the coastal areas which were apparently not sampled in the 1950s by du Toit (1954) (Kappmeier, 2002; de Beer *et al.,* 2016).

A transect study was carried out along the Hluhluwe River, starting at the Hluhluwe Dam and progressing to the west into Hluhluwe-iMfolozi Park, to determine the presence of *G. austeni* in the park to validate the distribution model of Hendricks *et al*. (2003). All traps along the selected transect captured *G. austeni* albeit at relatively low apparent densities (0.02 flies/trap/day) (de Beer, 2016). The presence of *G. austeni* along with this transect confirmed the accuracy of the *G. austeni* distribution prediction model (Hendrickx *et al*., 2003). The transect study confirmed the presence of *G. brevipalpis* inside and outside the Hluhluwe-iMfolozi Park (Kappmeier Green, 2002). *Glossina austeni* are primarily restricted to dense vegetation and do not disperse far from it (Esterhuizen *et al.*, 2005; Esterhuizen, 2007). Bush clearing in the communal farming areas has increased considerably (Kappmeier *et al*., 1998) and it is likely that game reserves and protected areas will become more important in sustaining tsetse populations in the future.

These studies indicated that AAT and tsetse flies are abundant in KZN and that tsetse fly presence, and especially tsetse abundance, to be a dynamic process that is influenced by several environmental and ecological factors (de Beer, 2016). The factors that influence the presence and abundance of *G. austeni* and *G. brevipalpis*

may, include temperature, humidity, vegetation type and host presence (de Beer, 2016; de Beer *et al.,* 2021).

Initiatives to eliminate or control trypanosomiasis should be supported by contemporary and accurate information of the geographic distribution of tsetse flies (Cecchi *et al*., 2015). The strategic level of albeit at large and small scale, relatively coarse cartographic representations to provide information on the occurrence and abundance of the fly's populations and the number of species present in an area. The possible geographical isolation of the flies population will have important implications in selecting the most cost-effective strategy of intervention of the priority areas (Cecchi *et al*., 2015).

Periodic screening of cattle at dip tanks in the tsetse-infested area 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). From 2015 onwards a cattle-dipping regime (Bourn *et al.,* 2005) has been utilised for vector control (personal communication Dr. L. Ntantiso, DARD - Veterinary Services Department KZN). The current live-bait control program in north-eastern KZN Province started in 2015 and coincided with a severe drought in the area. This control program seems to be successful as there are currently no reported nagana cases in the area (personal communication Dr. L. Ntantiso, DARD - Veterinary Services Department KZN).

Despite the apparent success of the current live-bait control program no tsetse abundance data is available to verify the success thereof. No tsetse data is available since 2015 and the effects of the control program and drought couple to environmental changes in the area on the abundance of *G. austeni* and *G. brevipalpis* abundance are unknown.

The aim of the present study was to determine the effect of the current control program on tsetse abundance during the past 10 years. To achieve this aim tsetse Apparent Density (AD) as determined in 2008-2009 was compared to the AD as determined in 2018-2019 at nine sites. Five of these sites were in protective areas and a further four were in communal farming areas. The live-bait control program only targets the tsetse populations outside of the protective areas as cattle are prevented from entering these areas. No tsetse control is done in the protected areas.

## 2.2. Materials and methods

### 2.2.1 Study area

Distribution of tsetse flies is limited to the north-eastern KZN Province of South Africa in the uMkhanyakude District. This region mainly consists of Game Reserves and conservation areas surrounded by communal and commercial farming areas, state forests and commercial plantations of pine and eucalyptus. Based on comparable survey data, four sites from the communal farming areas and five sites from the protected areas were selected for comparison (Figure 2.1).

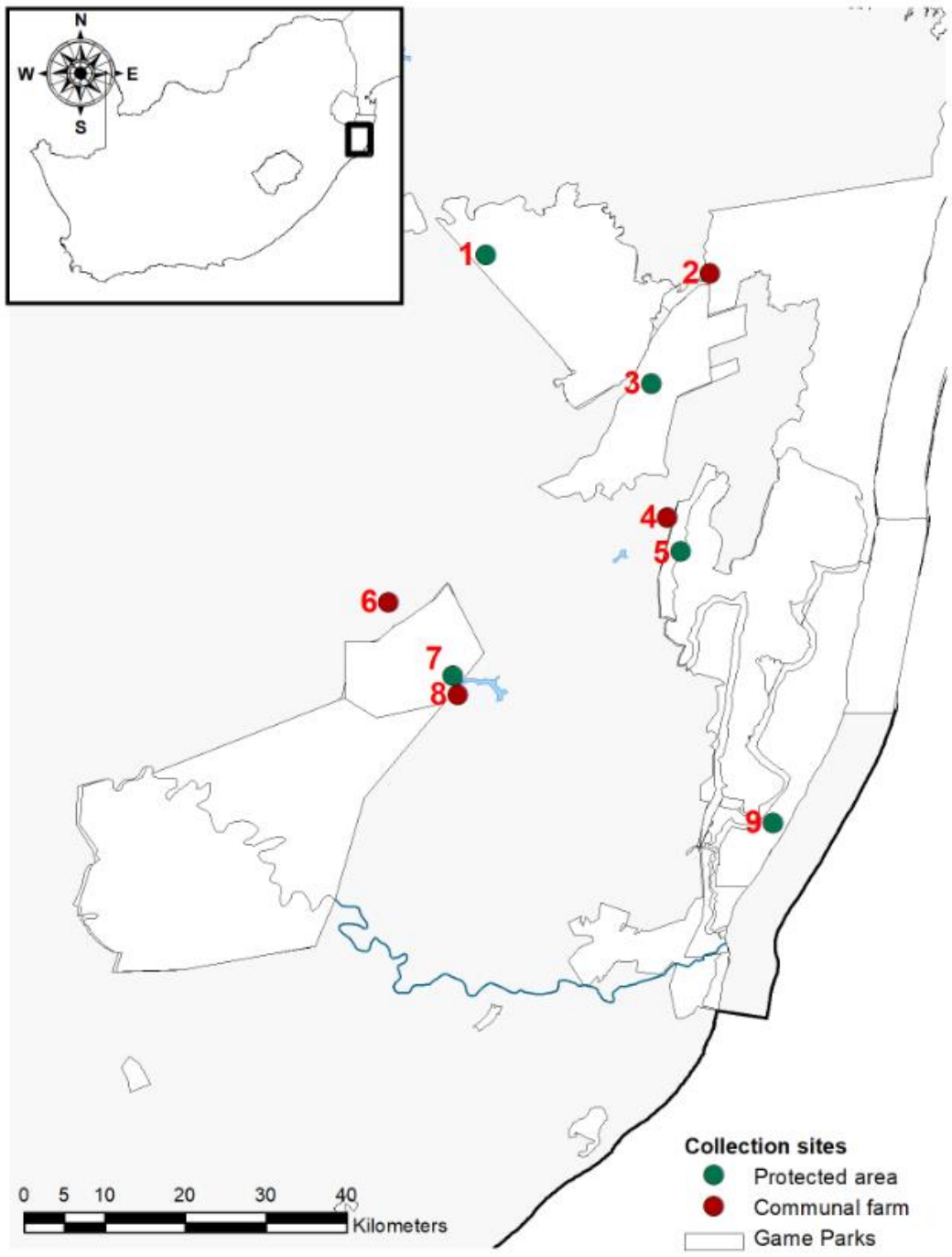


**Figure 2.1: Sites from communal land and protected areas where H-trap collections of tsetse flies were done in 2008-2009 and 2018-2019 in north-eastern KwaZulu-Natal (1: Mkuze; 2: Lower Mkuze; 3: Phinda; 4: Kuleni; 5: False Bay; 6: Ekuphinidisweni; 7: Hluhluwe-iMfolozi Park; 8: Mvutshini; 9: Eastern Shores).**

A variety of game animals is available as hosts in the study areas where both *G. austeni* and *G. brevipalpis* occur. These include hippopotamus (*Hippopotamus amphibious*), bushbuck (*Tragelaphus scriptus*), nyala (*Tragelaphus angassi*), red duiker (*Cephalophus natalensis*), reedbuck (*Redunca arundinum*), bush pig (*Potamochoerus porcus*) and warthog (*Phacochoerus aethiopicus*) (Esterhuizen *et al.,* 2005).

The area has an annual rainfall of about 900 mm, mainly occurring during the hot seasons from September/October to March/April. Records from the South African Weather Bureau showed the mean maximum annual temperatures of 24°C to 30°C with the mean annual minimum of 12°C to 22°C with the monthly mean relative humidity at 50-100%. The main land-cover classes in the area are savannah woodland, herbaceous savannah, shrub savannah, dense dry forest, gallery forest, tree plantations, agricultural areas, urban areas, swamps, water bodies and bare ground (Bouyer & Guerrini, 2010, de Beer *et al.,* 2021).

### 2.2.2 Sampling of entomological data

Tsetse collection data from September 2008 to January 2009 were compared to data collected from October 2018 to January 2019. Tsetse flies were collected with odour-baited H-traps (Figure 2.2) at five and four sites located in protected areas and the communal farming areas respectively (Figure 2.1). To enhance trapping for *G. brevipalpis* the traps were baited with 1-octen-3-ol and 4-methylphenol at a ratio of 1:8 that 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 (Figure 2.2). 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 (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 for the period of four months and the number of each species collected were sexed and counted.

**Figure 2.2: Odour-baited H-trap for the collection of *Glossina brevipalpis* and *G. austeni* in north-eastern KwaZulu-Natal, South Africa. Note the presence of the sachets and acetone bottle at the entrance of the trap.**

### 2.2.3 Statistical analysis

The relative abundance of tsetse flies sampled in 2008-2009 and 2018-2019 was expressed as apparent density (AD) i.e., the number of flies collected per trap per day (de Beer *et al.*, 2016). One-way analysis of variance (ANOVA) was used to differentiate the means in AD between the tsetse collected in 2008-2009 and 2018-2019. The data was not normally distributed and the non-parametric ANOVA, Kruskal-Wallis Test with a Dunn post-test were used at 95% significance. GraphPad Instat (version 3.00, 2003) was used for statistical analysis.

## 2.3 Results

Nine sites, four in communal farming and five in protected areas, were sampled in 2008-2009 and 2018-2019 for *G. brevipalpis* and *G. austeni* (Figure 2.1). A total of 46 H-traps collected 17 909 (AD = 4.02 flies/trap/day) *G. brevipalpis* and 895 (AD = 0.21 flies/trap/day) *G. austeni* in 2008-2009. Whereas in 2018-2019, 33 traps collected 16 692 (AD = 5.33 flies/trap/day) *G. brevipalpis* and 450 (AD = 0.14 flies/trap/day) *G. austeni* from the nine sites.

### 2.3.1 Apparent Density of *Glossina brevipalpis*

For *G. brevipalpis* there was an overall increase in the AD from 4.02 flies/trap/day in 2008-2009 to 5.33 flies/trap/day in 2018-2019. This increase was mainly driven by the largest statistically significant ($p < 0.01$) increase found at Phinda, a private game farm. At Phinda the AD increased from 0.05 flies/trap/day in 2008-2009 to 9.83 flies/trap/day in 2018-2019, i.e., a 215.2 factor of increase (Table 2.1, Figure 2.3). At Mkuze, a protected area, no *G. brevipalpis* were collected in 2008-2009 and an AD of 3.60 flies/trap/day was found in 2018-2019 (Table 2.1, Figure 2.3). The only

significant ($p < 0.01$) reduction in AD of *G. brevipalpis* was found at False Bay, a protected area. At this site, the AD of *G. brevipalpis* decrease from 0.83 flies/trap/day in 2008-2009 to 0.06 flies/trap/day in 2018-2019, i.e., a decrease factor of 0.92 (Table 2.1, Figure 2.3). The slight in increase in *G. brevipalpis* ADs from 2008-2009 to 2018-2019 at all the sites in the communal farming areas was not statistically significant ($p > 0.06$) (Table 2.1).

The AD of *G. brevipalpis* collected in the protected areas in 2008-2009 and 2018-2019 was 7.26 flies/trap/day and 8.36 flies/trap/day respectively (Figure 2.4). The ADs in the protected areas were significantly higher than that of 1.34 flies/trap/day for *G. brevipalpis* in 2008-2009 in ($p < 0.05$) and 1.69 flies/trap/day in 2018-2019 ($p < 0.01$) from the communal farming areas.

In the protected areas, the AD of male and female *G. brevipalpis* in 2008-2009 was the highest in Hluhluwe-iMfolozi Park at 6.99 males/trap/day and 8.68 females/trap/day respectively. The lowest AD for *G. brevipalpis* was recorded at Phinda Private Game reserve with the AD recorded at 0.02 males/trap/day and 0.03 females/trap/day (Table 2.1). During 2018-2019, the AD for *G. brevipalpis* males and females was again highest in the Hluhluwe-iMfolozi Park at 6.99 males/trap/day and 8.68 females/trap/day. It was the lowest in False Bay at 0.02 males/trap/day and 0.04 females/trap/day respectively (Table 2.1).

The AD for *G. brevipalpis* in the communal areas in 2008-2009 was highest at Mvhutshini at 1.00 males/trap/day and 0.87 females/trap/day. Kuleni had the lowest ADs at 0.02 males/trap/day and 0.08 females/trap/day. Mvhutshini had the highest

AD in 2018-2019 as well with 1.24 males/trap/day and 1.34 females/trap/day whilst Lower Mkuze had the lowest at 0.06 males/trap/day and 0.08 females/trap/day in 2008-2009 and again in the lowest in 2018-2019 at 0.06 for males and 0.09 for females.

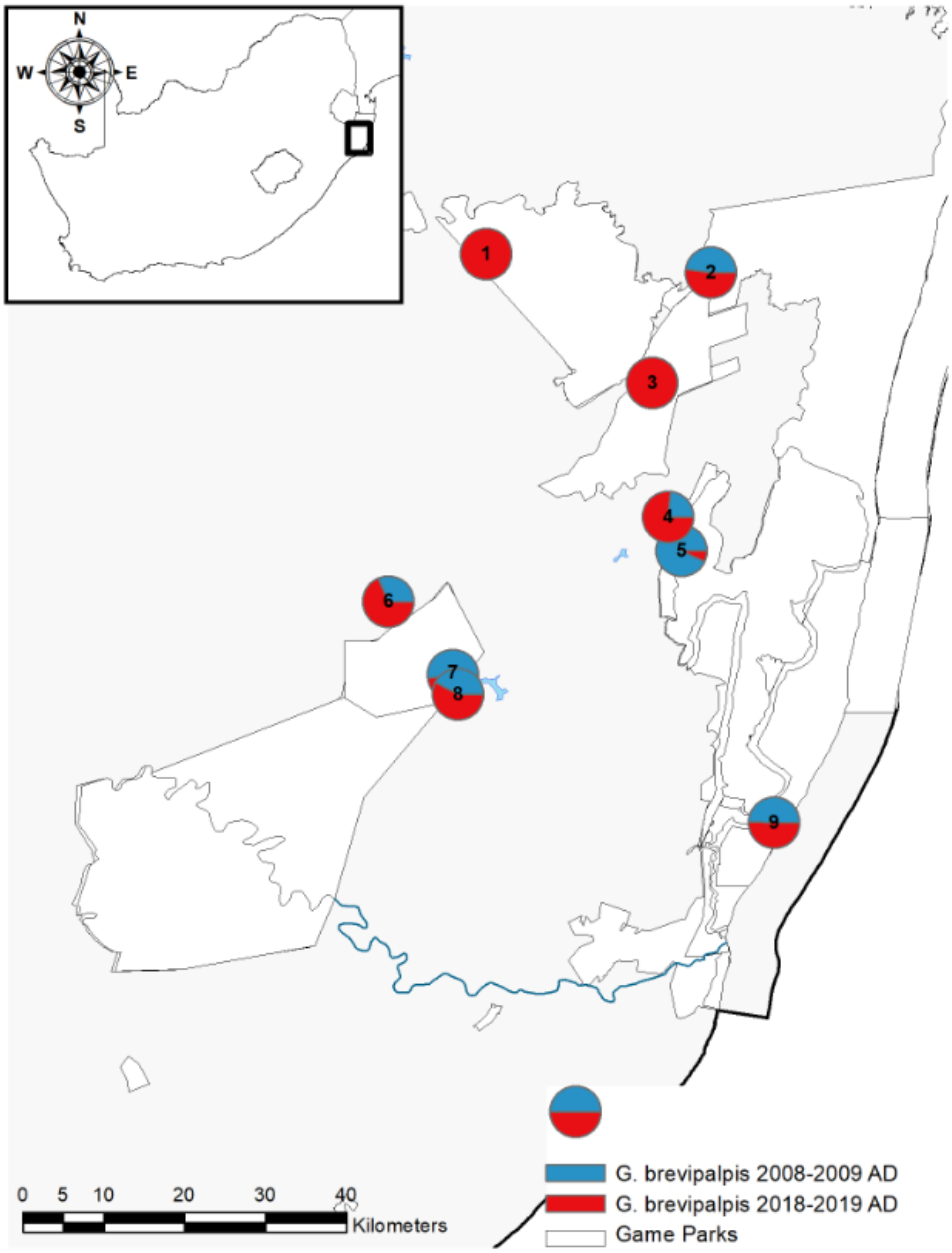


**Figure 2.3: The change in AD from 2008-2009 to 2018-2019 of *Glossina brevipalpis* found in the protected areas and communal areas at various geographical locations in north-eastern KwaZulu-Natal (1: Mkuze; 2: Lower Mkuze; 3: Phinda; 4: Kuleni; 5: False Bay; 6: Ekupinidisweni; 7: Hluhluwe-iMfolozi Park; 8: Mvutshini; 9: Eastern Shores).**

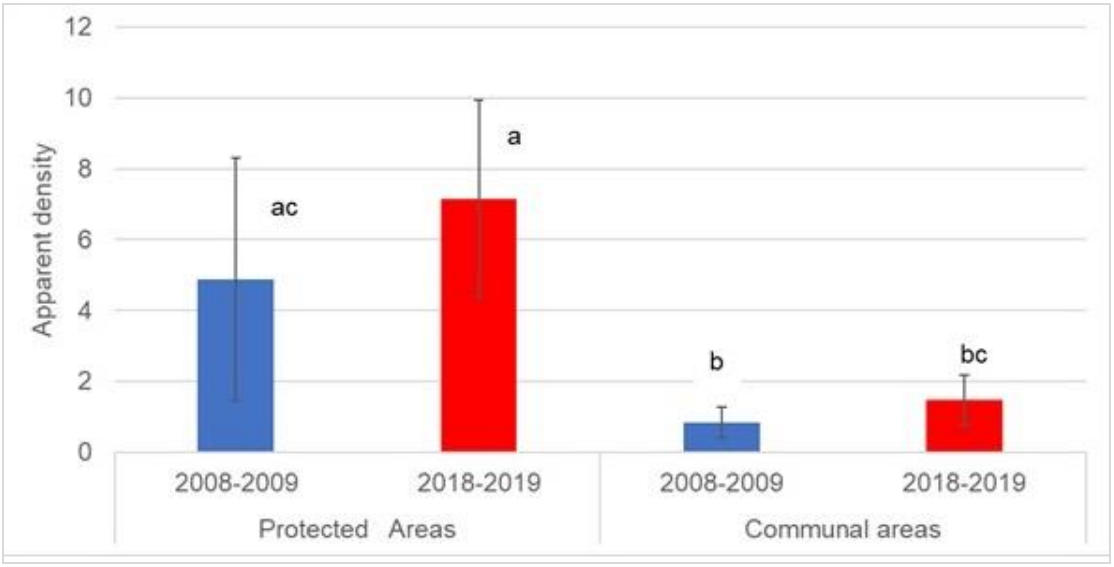


**Figure 2.4: Mean apparent density (AD) and standard deviation of *Glossina brevipalpis* collected during 2008-2009 and 2018-2019 from protected areas and communal farms and in KwaZulu-Natal. Different alphabetical letters indicate statistically significant ($p < 0.05$) differences in AD.**

### 2.3.2 Apparent Density of *Glossina austeni*

For *G. austeni* there was a significant decrease ($p < 0.01$) in the AD from 0.21 flies/trap/day in 2008-2009 to 0.14 flies/trap/day in 2018-2019 and this decrease was mainly in the protected areas (Table 2.2, Figure 2.5 & 2.6). In the protected areas e.g., Phinda and False Bay the AD of *G. austeni* decrease from 0.94 flies/trap/day to 0.05 flies/trap/day, a 0.95 reduction, and from 0.59 flies/trap/day to 0.46 flies/trap/day, 0.23 reduction, respectively (Table 2.2, Figure 2.5). In Hluhluwe-iMfolozi Park, *G. austeni* were present in 2008-2009 at an AD of 0.01 flies/trap/day but it was absent in 2018-2019 (Table 2.2, Figure 2.5). There was an increase in *G. austeni* AD at two sites, Eastern Shores and Mkuze, in the protected areas (Table 2.2). At Mkuze, *G. austeni* was absent in 2008-2009 and was collected at an AD of 0.05 flies/trap/day in 2018-2019 (Table 2.2). At Eastern Shores the AD of *G. austeni*

increased from 0.23 flies/trap/day in 2008-2009 to 0.36 flies/trap/day in 2018-2019, however this increase was not significant ($p > 0.05$) (Table 2.2, Figure 2.5).

In the communal farming areas the most significant ($p < 0.01$) reductions occurred in Lower Mkuze where the AD of *G. austeni* decrease from 0.39 flies/trap/day in 2008-2009 to 0.22 flies/trap/day, a 0.49 reduction, in 2018-2019 and at Mvutshini where *G. austeni* were present in 2008-2009 at an AD of 0.01 flies/trap/day and absent in 2018-2019 (Table 2.2, Figure 2.5). No *G. austeni* were collected at Ekuphindisweni during 2008-2009 as well as 2018-2019 (Table 2.2). The AD of 0.34 flies/trap/day of *G. austeni* in the protected areas was significantly ($p < 0.01$) higher in 2008-2009 than that of 0.09 flies/trap/day in the communal farming areas. This same trend was not observed for the AD collected in 2018-2019 from the protected areas and the communal farming areas (Figure 2.6). In 2008-2009 the AD of *G. austeni* was the highest in Phinda at 0.12 males/trap/day and 0.82 females/trap/day (Table 2.2). Followed by False Bay at 0.07 males/trap/day and 0.52 females/trap/day (Table 2.2). The lowest AD for *G. austeni* was recorded at Hluhluwe-iMfolozi Park at 0.01 females/trap/day and the absence of males in 2008-2009. No *G. austeni* were collected from Mkuze in 2008-2009 (Table 2.2). During 2018-2019, the highest AD for *G. austeni* males and females was recorded at False Bay at 0.05 males/trap/day and 0.41 females/trap/day (Table 2.2). No *G. austeni* were recorded from Hluhluwe-iMfolozi Park in 2018-2019 (Table 2.2).

The AD of *G. austeni* in the communal areas was highest in Lower Mkuze at 0.03 males/trap/day and 0.39 females/trap/day. Mvutshini had the lowest AD at 0.01 females/trap/day and the absence of males. In 2018-2019, Kuleni had the highest AD for males at 0.04 flies/trap/day.

**Table 2.1 *Glossina brevipalpis* apparent density (AD) as determine with odour-baited H-traps during 2008-2009 and 2018-2019 from communal and protected areas in the KwaZulu-Natal Province, South Africa**

| | 2008 – 2009 | | | | | | | | | 2018 - 2019 | | | | | | | | | Change in AD 2008/2009 - 2018/2019 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Total collected | | | Apparent density (AD) | | | | | | Total collected | | | Apparent density (AD) | | | | | |
| Tsetse fly sample site | Start – End date | No of traps | No of trap days | ♂ | ♀ | Total | ♂ | ♀ | Total | Start – End date | No of traps | No of trap days | ♂ | ♀ | Total | ♂ | ♀ | Total | ♂ | ♀ | Total |
| **Protected areas** | | | | | | | | | | | | | | | | | | | | | |
| False Bay | 23/09 – 20/01 | 4 | 392 | 136 | 191 | 327 | 0.35 | 0.49 | 0.83 | 2/10 – 28/01 | 3 | 285 | 6 | 12 | 18 | 0.02 | 0.04 | 0.06 | 0.06 | 0.08 | 0.07* |
| Eastern Shores | 26/09 – 23/01 | 3 | 294 | 1296 | 1004 | 2300 | 4.41 | 3.41 | 7.82 | 2/10 – 28/01 | 3 | 285 | 1354 | 903 | 2257 | 4.75 | 3.17 | 7.92 | 1.08 | 0.93 | 1.01 |
| Mkuze | 10/10 – 23/01 | 2 | 168 | 0 | 0 | 0 | 0.00 | 0.00 | 0.00 | 3/10 – 29/01 | 3 | 285 | 496 | 529 | 1025 | 1.74 | 1.86 | 3.60 | nd | nd | nd |
| Hluhluwe-iMfolozi Park | 23/09 – 20/01 | 9 | 765 | 5346 | 6642 | 11988 | 6.99 | 8.68 | 15.67 | 2/10 – 28/01 | 6 | 570 | 3824 | 4375 | 8199 | 6.71 | 7.68 | 14.38 | 0.96 | 0.88 | 0.92 |
| Phinda | 30/09 – 27/01 | 4 | 396 | 8 | 10 | 18 | 0.02 | 0.03 | 0.05 | 2/10 – 28/01 | 3 | 285 | 1276 | 1525 | 2801 | 4.48 | 5.35 | 9.83 | 221.76 | 211.86 | 216.26* |
| **Communal farms** | | | | | | | | | | | | | | | | | | | | | |
| Lower Mkuze | 30/09 – 27/01 | 3 | 297 | 18 | 23 | 41 | 0.06 | 0.08 | 0.14 | 3/10 – 29/01 | 3 | 285 | 18 | 25 | 43 | 0.06 | 0.09 | 0.15 | 0.99 | 1.16 | 1.09 |
| Mvutshini | 25/09 – 02/02 | 12 | 1308 | 1305 | 1140 | 2445 | 1.00 | 0.87 | 1.87 | 3/10 – 29/01 | 6 | 570 | 705 | 762 | 1467 | 1.24 | 1.34 | 2.57 | 1.24 | 1.54 | 1.37 |
| Ekuphinidisweni | 28/09 – 25/01 | 6 | 588 | 241 | 523 | 764 | 0.41 | 0.89 | 1.30 | 4/10 – 30/01 | 3 | 279 | 239 | 547 | 786 | 0.86 | 1.96 | 2.82 | 2.10 | 2.20 | 2.17 |
| Kuleni | 26/09 – 20/01 | 3 | 252 | 5 | 21 | 26 | 0.02 | 0.08 | 0.10 | 4/10 – 30/01 | 3 | 285 | 39 | 57 | 96 | 0.14 | 0.20 | 0.34 | 7.06 | 2.40 | 3.30 |

**An * indicate a significant change in AD**

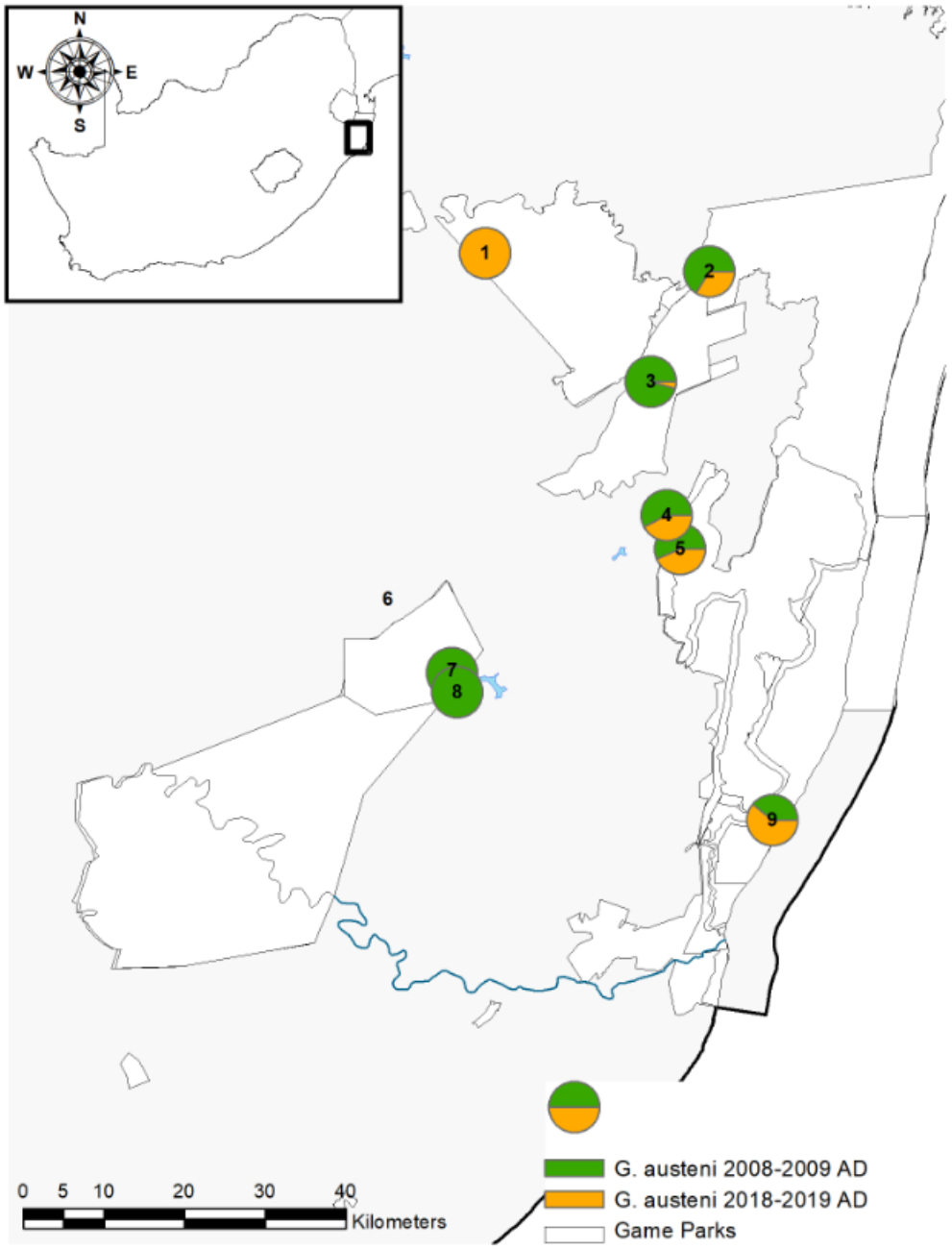


**Figure 2.5: The change in AD from 2008-2009 to 2018-2019 of *Glossina austeni* found in the protected areas and communal areas at various geographical locations in north-eastern KwaZulu-Natal (1: Mkuze; 2: Lower Mkuze; 3: Phinda; 4: Kuleni; 5: False Bay; 6: Ekuphinidisweni; 7: Hluhluwe-iMfolozi Park; 8: Mvutshini; 9: Eastern Shores).**

**Table 2.2: *Glossina austeni* apparent density as determine with odour-baited H-traps during 2008-2009 and 2018-2019 from communal and protected areas in KwaZulu-Natal Province, South Africa**

| | 2008 – 2009 | | | | | | | | | 2018 - 2019 | | | | | | | | | | Change in AD 2008/2009 - 2018/2019 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Total collected | | | Apparent density (AD) | | | | | | Total collected | | | Apparent density (AD) | | | | | | | |
| Tsetse fly sample site | Start – End date | No of traps | No of trap days | ♂ | ♀ | Total | ♂ | ♀ | Total | Start – End date | No of traps | No of trap days | ♂ | ♀ | Total | ♂ | ♀ | Total | ♂ | ♀ | Total | |
| **Protected areas** | | | | | | | | | | | | | | | | | | | | | | |
| False Bay | 23/09 – 20/01 | 4 | 392 | 29 | 204 | 233 | 0.07 | 0.52 | 0.59 | 2/10 – 28/01 | 3 | 285 | 15 | 116 | 131 | 0.05 | 0.41 | 0.46 | 0.68 | 0.79 | 0.77* | |
| Eastern Shores | 26/09 – 23/01 | 3 | 294 | 13 | 56 | 69 | 0.04 | 0.19 | 0.23 | 2/10 – 28/01 | 3 | 285 | 29 | 74 | 103 | 0.10 | 0.26 | 0.36 | 2.26 | 1.37 | 1.53 | |
| Mkuze | 10/10 – 23/01 | 2 | 168 | 0 | 0 | 0 | 0.00 | 0.00 | 0.00 | 3/10 – 29/01 | 3 | 285 | 14 | 63 | 77 | 0.05 | 0.22 | 0.27 | nd | nd | nd | |
| Hluhluwe-iMfolozi Park | 23/09 – 20/01 | 9 | 765 | 2 | 9 | 11 | < 0.01 | 0.01 | 0.01 | 2/10 – 28/01 | 6 | 570 | 0 | 0 | 0 | 0.00 | 0.00 | 0.00 | nd | nd | nd | |
| Phinda | 30/09 – 27/01 | 4 | 396 | 49 | 323 | 372 | 0.12 | 0.82 | 0.94 | 2/10 – 28/01 | 3 | 285 | 1 | 12 | 13 | < 0.01 | 0.04 | 0.05 | nd | 0.05 | 0.05* | |
| **Communal farms** | | | | | | | | | | | | | | | | | | | | | | |
| Lower Mkuze | 30/09 – 27/01 | 3 | 297 | 10 | 116 | 126 | 0.03 | 0.39 | 0.42 | 3/10 – 29/01 | 3 | 285 | 7 | 55 | 62 | 0.02 | 0.19 | 0.22 | 0.59 | 0.49 | 0.52* | |
| Mvutshini | 25/09 – 02/02 | 12 | 1308 | 1 | 7 | 8 | < 0.01 | 0.01 | 0.01 | 3/10 – 29/01 | 6 | 570 | 0 | 0 | 0 | 0.00 | 0.00 | 0.00 | nd | nd | nd | |
| Ekuphinidisweni | 28/09 – 25/01 | 6 | 588 | 0 | 0 | 0 | 0.00 | 0.00 | 0.00 | 4/10 – 30/01 | 3 | 279 | 0 | 0 | 0 | 0.00 | 0.00 | 0.00 | nd | nd | nd | |
| Kuleni | 26/09 – 20/01 | 3 | 252 | 11 | 65 | 76 | 0.04 | 0.26 | 0.30 | 4/10 – 30/01 | 3 | 285 | 13 | 51 | 64 | 0.05 | 0.18 | 0.22 | 1.15 | 0.70 | 0.73 | |

**An * indicate a significant change in AD**

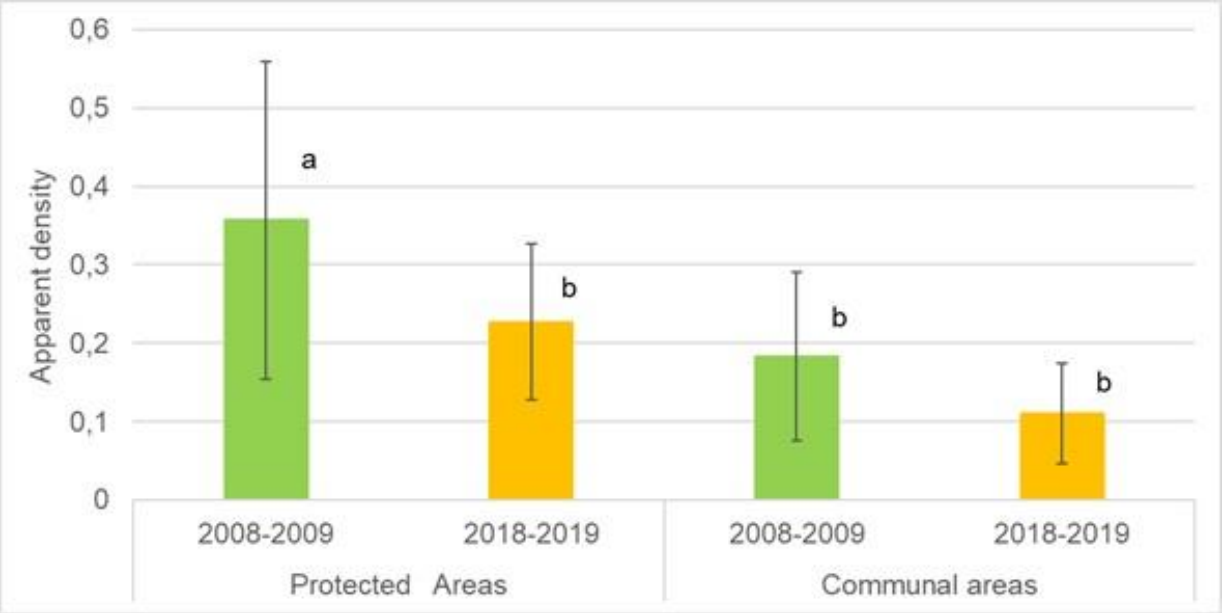


**Figure 2.6: Mean apparent density and standard deviation of *Glossina austeni* collected during 2008-2009 and 2018-2019 from protected areas and communal farms and in KwaZulu-Natal. Different alphabetical letters indicate statistically significant differences in AD.**

## 2.4 Discussion

The aim of the study was to determine the effect of the current control program on tsetse abundance during the past 10 years. Tsetse AD from nine sampled sites of 2008-2009, were compared to the AD of nine sampled sites of 2018-2019. From the nine sites sampled, five were in protective areas and four from communal farming lands. The current live-bait control program only targets the tsetse populations outside the protective areas as the cattle are prevented from entering the protected areas. No tsetse fly control is done in the protected areas. Based on the collections from 2008-2009 and 2018-2019, there were no significant increase or decrease in AD of *G. brevipalpis* collected from both communal farmlands as well as in protected game reserves. Although the abundance of *G. austeni* decreased in both the

protected game reserves and communal lands only the decrease in the protected areas were statistically significant ($p < 0.01$).

Overall, the abundance of *G. brevipalpis* was higher than that of *G. austeni* during both 2008-2009 and 2018-2019 surveys. Similar observations were reported by Esterhuizen *et al.,* (2005), who stated that *G. brevipalpis* had a higher Index of Apparent Abundance (IAA) compared to that of *G. austeni.* This disparity in AD between *G. brevipalpis* and *G. austeni* may partly be resulting from the intrinsic biases of the trapping system as indicated by de Beer (2016).

The survival of tsetse flies depends on the availability of suitable habitats and a high abundance of appropriate vertebrate hosts (Ducheyne *et al.,* 2009). This may explain the higher AD of both species in the protected areas compared to that in communal farming areas. Similar observations were made in Malawi, where tsetse flies were confined to protected areas and as a result the abundance of tsetse flies reduced in human settlements and consequently a reduction in the prevalence of the disease (Ducheyne *et al.,* 2009).

Deforestation for human settlement and agricultural practices has led to a decrease in suitable habitats for *G. austeni* in communal areas where this species was previously sampled. It suggests that *G. austeni* has a more restricted distribution compared to its congener. Similarly, in a study by Esterhuizen *et al*. (2005), most *G. austeni* individuals were caught in or near areas dominated by indigenous forest patches. de Beer *et al*. (2016) postulated that in the future, the populations of *G. austeni* will be sustained by game reserves and protected areas due to the

availability of suitable habitats. In the current study, *G. austeni* was sampled in 2008-2009 in Mvutshini where it was absent in 2018-2019. This could be ascribed to the removal of suitable tsetse fly habitats in the areas for farming and human settlement. Habitat fragmentation has been known to lead to major declines in wild tsetse populations (Ducheyne *et al*., 2009). It must, however, be considered that even low fly densities may still sustain potentially lethal diseases in livestock (Ntantiso, 2014). When planning tsetse and trypanosomiasis control strategies, communal farm areas are the main priority since livestock are more exposed to diseases due to lack of grazing land and as a result, animals are forced to graze near protected areas. Such interfaces between livestock and wild animals, places livestock in a higher probability of being infected with animal trypanosomes.

*Glossina brevipalpis* has been demonstrated to be a vector of *T. congolense* and *T. vivax* (Harley, 1965; 1966; Moloo *et al*. 1988; Moloo, 1992). It has been reported that *G. brevipalpis* inhabits the evergreen thickets associated with ground water and forests islands in the savannah (Ford, 1972). This species consequently come into contact with domestic animals, and feeds on cattle (Weitz, 1963). In the current study, the two communal areas adjacent to the Hluhluwe-iMfolozi Park, namely, Mvutshini and Ekuphinidisweni had recorded the highest AD of *G. brevipalpis* during both years of sampling. Ntantiso *et al.* (2014) reported a higher AD of *G. brevipalpis* at the communal dip tanks which are lying towards the northern side of Hluhluwe-iMfolozi Park (Ekuphinidisweni) and the southern side of the park at Ocilwane. The 1990 outbreak of nagana was reported at dip tanks in the vicinity of the Hluhluwe-iMfolozi Park and the infections were attributed to *T. congolense* and *T. vivax* (Motloang *et al*., 2012).

The AD of males and females as determined in protected areas and communal areas did not differ significantly. Higher numbers of females than males of *G. brevipalpis* and *G. austeni* were observed in the protected areas and the communal areas. Leak (1999) had reported that in unbiased samples, females comprise about 70% to 80% of the total mean population. The distribution of tsetse flies and its abundance is a result of the interaction between tsetse population with biotic factors which regulate their population size and their distribution boundaries (Rogers & Randolph, 1985). It has been observed that the association between tsetse distribution and cattle has important repercussions for the control of animal trypanosomiasis (Van Den Bossche & De Deken, 2002; Esterhuizen *et al.*, 2005). An understanding of the natural dynamics of tsetse populations in the field is critical in determining the most appropriate control strategies for the various tsetse fly species encountered (Rogers & Randolph, 1986; Echodu *et al*., 2011).

The current study showed that the overall AD of *G. brevipalpis* did not change significantly from 2008-2009 to 2018-2019 in both the protected and communal areas. This can, however, differ between sites. E.g., at one (False Bay) of the five sites in the protected areas there was significant decrease in AD while there was a significant increase at another (Phinda). The higher AD of *G. brevipalpis* obtained in 2018-2019 at the four sites in communal farming areas did not differ significantly from that in 2008-2009. Overall, there were a significant decrease in the AD of *G. austeni* as determined in 2008-2009 and 2018-2019 in the protected areas. The lower overall numbers collected in 2018-2019 in the communal farming areas, however, did not differ significantly from that collected in 2008-2009. Similar results were found for *G. brevipalpis* with a variation between sampled sites. In the

protected areas there was a significant decrease at three of the five sampled sites and the observed increase in two remining sites was not significant. A significant decrease in *G. austeni* was only found at one of the four sites in the communal farming areas.

The results indicate that the current control program as apply in the communal farming areas may not have a significant long-term effect on tsetse abundance in the wider area and that several factors can play a role in regulating tsetse abundance. E.g., the drought in the area could have play a role in the reduction of the AD of *G. austeni* found in the protected areas. Although both *G. austeni* and *G. brevipalpis* have been shown to be involved in the transmission of *T. congolense* and *T. vivax* in South Africa *G. austeni* is consider the more competent vector for the transmission of *Trypanosoma* parasites (Motloang *et al*., 2012; 2014). The apparent reduction in *G. austeni* numbers could as such have contributed to the success of the current control program.

## 2.5 Conclusion

The variation change found between sites within both the protected and communal sites highlighted the dynamic nature of tsetse populations. Although this study was limited to a relatively small portion of the tsetse infected area in the KZN Province some populations stay stable over a period of ten years while there were significant increases or decreases in other nearby populations. It shows that several factors can contribute to tsetse abundance in an area and that the apparent success of the current control program may be restricted to certain sites. It furthermore highlighted the implementation of a sustainable long-term effective control program for the area.

Despite the higher vector competence of *G. austeni,* the higher abundance and wide distribution of *G. brevipalpis* emphasised that this species must not be excluded from potential control efforts.

# CHAPTER 3

# MALE MATING ABILITY OF *GLOSSINA BREVIPALPIS* UNDER LABORATORY CONDITIONS

## 3.1 Introduction

Leading from the successful elimination of *Glossina austeni* from the island of Unguja, Zanzibar, (Dyck *et al*., 2000) an area wide integrated pest management (AW-IPM) with a sterile insect technique (SIT) component was proposed to eradicate tsetse flies in South Africa (Kappmeier Green *et al.,* 2007). The incorporation of the SIT into an AW-IPM strategy can only be successful if the sterilized released males are of the high biological quality (Mutika *et al*., 2001; Sow *et al*., 2012). The released sterilized males must be able to locate, form mating pairs and transfer sperm to wild virgin females at the same or higher frequency than their natural counterparts (Vreysen, 2005; Sow *et al*., 2012; de Beer *et al.,* 2015).

In preparation of a proposed AW-IPM with a SIT component, laboratory colonies of *Glossina brevipalpis* were established at the Agricultural Research Council-Onderstepoort Veterinary Research (ARC-OVR) in 2002. The colonised *G. brevipalpis* were shown to be highly sensitive to irradiation and that a dose of 40 Gy and 80 Gy induced 97% and 99% sterility respectively in untreated females that mated with males irradiated as adults (de Beer *et al.,* 2017). Assessment of the mating performance of *G. brevipalpis* males, irradiated with a dose of 80 Gy, showed that they were able to compete successfully with fertile untreated males from the colony for virgin untreated females in walk-in field cage experiments (de Beer *et al*., 2017). These results indicated that the colonised *G. brevipalpis* have the ability to be used in a SIT program for South Africa (de Beer *et al*., 2017). Leading from the

encouraging results obtained on mating performance, the mating ability of *G. brevipalpis*, the aim of the present study, is of great interest.

Under laboratory conditions, male tsetse flies can successfully inseminate a female fly every 2 to 3 days and up to 6 to 10 times (Pollock, 1970; Jordan, 1972). Although copulation may take place soon after emergence, they are only fully fertile a few days after emergence and it was found that older males are better able to mate successfully than very young ones (de Beer *et al*., 2015). Although male tsetse flies may mate more than once under natural conditions, spermatogenesis only occurs during the pupal period and males emerge from the pupae with an entire complement of sperm (Solano *et al*., 2010; Vreysen *et al*., 2013).

Although feeding is at similar intervals for both males and females, males may have greater energy reserves for flight since they do not nourish larvae (Bursell & Taylor, 1980). Generally, the mean life span of various species of male tsetse flies (*Glossina morsitans, G. swynnertoni, G. palpalis* and *G. tachinoides*) in the wild is approximately four weeks and for females it is at least eight weeks (Jackson, 1949), this may, however, vary depending on the environmental conditions.

Multiple male mating ability can play a decisive role in the success of the SIT. In the present study, the mating ability and behavior of laboratory reared *G. brevipalpis* irradiated and non-irradiated males was compared under laboratory conditions. The parameters compared between irradiated and non-irradiated males included the evaluation of the propensity of mating (PM), mating latency, mating duration and spermathecae fill in mated females. Understanding the mating behavior between

irradiated and non-irradiated males of *G. brevipalpis* may contribute to enhancing knowledge on the success of tsetse population control programmes through AW-IPM and SIT as well as the improvement of tsetse fly mass rearing programmes.

The objective of the study was to assess the male mating ability of *G. brevipalpis* under laboratory conditions and to ascertain the influence of gamma cell irradiation on male mating proficiency.

## 3.2 Materials and methods

### 3.2.1 Tsetse colony flies

All experiments were conducted using *G. brevipalpis* from the ARC-OVR tsetse colony (Figure 3.1). The colony was maintained at 24°C to 25°C, with a relative humidity of 70% to 80% and a 12 hour sub-dued / indirect light and 12-hour dark cycle (FAO/IAEA standard operating procedures, 2006). The flies were maintained on defibrinated irradiated bovine blood using an artificial *in vitro* membrane feeding system (Feldmann, 1994; FAO/IAEA standard operating procedures, 2006). Experiments to compare the male mating ability of irradiated and non-irradiated laboratory reared male *G. brevipalpis* was conducted over nine weeks from January to March 2018.

**Figure 3.1: *Glossina brevipalpis* colony at the ARC-OVR.**

Pupae was collected at 24-hour intervals to ensure that the emerged flies were of comparable ages. The flies were sexed and transferred to standard holding cages (Ø 20 cm) (Figure 3.2) immediately after emergence to ensure an unmated status. Subsequently, all emerged flies were fed for 5 minutes on defibrinated irradiated bovine blood using the artificial *in vitro* membrane feeding system as routinely used for colony maintenance.

Ninety male flies were irradiated five days after emergence with a dose of 80 Gy using a $^{137}$Cs source (Gammacell 40 S/N50) at a dose rate of 0.69 Gy / minute in air. It was previously shown that 80 Gy induced 99% sterility in untreated females that mated with males irradiated as adults (de Beer *et al.,* 2017). To ensure sexual

maturity of the males the mating ability experiments was conducted with 7-day old males. These males were given the opportunity to mate with 3-day old virgin females at standard colony conditions.

### 3.2.2 Male mating ability

The mating ability of the irradiated males was compared to that of fertile males (non-irradiated) in standard holding cages (Ø 20 cm) (Figure 3.2). Thirty irradiated males were mated with 30 virgin females giving a male to female ratio of 1:1 for two replicates. Concurrently 30 non-irradiated males were mated with 30 virgin females in a separate cage as a control.

**Figure 3.2: Standard holding cage (Ø 20˚cm) with blood fed *Glossina brevipalpis*.**

The female flies was introduced 5 minutes before males in the cages. Mating pairs were removed as soon as they started to copulate. Custom made glass tubes were used to transfer the mating pairs to small vials (Ø 2.5cm and 6cm high). To quantify male mating ability mating duration, propensity of mating (PM), mating latency as well as spermathecael fill were recorded.

Propensity of mating (PM) was defined as the overall proportions of released females that mated (Mutika *et al*., 2001; de Beer *et al.,* 2017). Mating duration was calculated as the time when the pairs separated minus the time they started to mate, whilst mating latency was expressed as the time elapsed from the release of males up until initiation of a given mating.

After two hours all flies that did not form mating pairs were removed from the experimental holding cage and returned to the colony. All males that mated during the first event were given the opportunity to mate again the following day with 3-day old virgin females maintaining a 1:1 sex ratio. This procedure was repeated until mating ceased or the male fly died.

### 3.2.3 Evaluation of the Spermathecae fill

The mated females were dissected 24 hours after mating to allow time for the sperm to migrate to the spermathecae. The females were immobilised at 4˚C and dissected in a 0.9% NaCl solution to prevent drying out of the specimen. Dissections were carried out under a dissecting microscope as follows: the wings were removed using fine forceps (152.4 mm) whereafter the fly was positioned dorsally. While holding the thorax with the forceps, the last abdominal segment was gently removed to reveal

the reproductive organs. The reproductive organs were removed and transferred to a light microscope slide and the spermathecae examined for the presence of sperm. Spermathecael fill was recorded as either empty (0), quarter-full (0.25), half-full (0.5) or full (1) (Nash, 1955). These values were used to calculate the mean spermathecael value (MSV) for each replicate.

### 3.2.4 Statistical analysis

All statistical calculations were done using GraphPadInstat (version 3.00, 2003). To determine the significance of the observations made between the females that mated with irradiated and non-irradiated males, 1-tailed ANOVA at 95% confidence level was conducted. The data was not normally distributed and Wilcoxon test was performed. Linear regression analysis was conducted to determine the correlation between observations.

## 3.3. Results

Seven days old irradiated males were given opportunity to subsequently mate with 3 days old virgin females in a standard holding cage until the last male cease to form a coupling. The males continue to mate with the females for at least 16 successive mating events (Figure 3.3). Time intervals between mating events were 24 hours. Since only males that mated in a first mating event were used in a subsequent evaluation the potential mating pairs decline over time (Figure 3.3). The linear reduction in the potential mating pairs was similar between the two irradiated groups and the control non-irradiated group ($R^2$=0.99; $p < 0.01$; *df* =1; *F*=639.36) (Figure 3.3). In the non-irradiated group and irradiated group mating continue for at least 16 (a single male) and 15 events (four males) respectively.

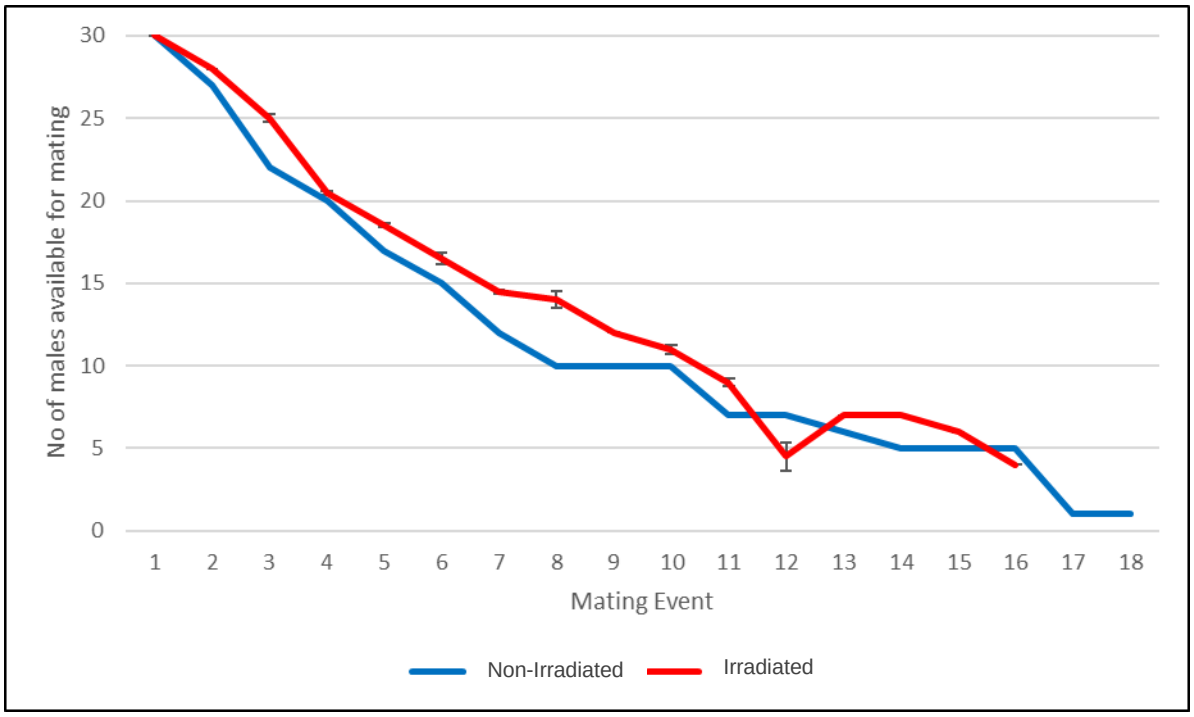


**Figure 3.3: Reduction in the potential number of mating pairs in non-irradiated (control) and mean ±SD of two replicates of irradiated *Glossina brevipalpis* over successive mating events. The interval between mating events was 24 hours. Group size = 30.**

### 3.3.1 Mating latency

Mating latency, the time elapsed from the release of the males up to initiation of mating, in the non-irradiated control group varied between 0 minutes and 130 minutes (Figure 3.4). The extraordinary long mating latency of 130 minutes observed in the 15$^{th}$ mating event was for a single mating pair (Figure 3.4). In the irradiated groups the mating latency varied between 5 minutes and 32 minutes (Figure 3.4). Except for two mating events (9$^{th}$ and 16$^{th}$) mating latency was consistently higher in the irradiated flies compare to that of non-irradiated flies (Figure 3.4). The overall mean mating latency for the non-irradiated flies was at 12.56 ± 31.43 minutes and differed significantly ($p < 0.01$) from that of 16.10 ± 7.05 minutes from the irradiated

flies. The mean overall difference between the non-irradiated and irradiated males was at 3.54 minutes.

The weak upwards and downwards trends in mating latency in the non-irradiated ($R^2$=0.14) and irradiated ($R^2$=0.51) groups respectively did not correlate with the observed data, indicating that mating latency was not significantly affected as the number of mating events increased (Figure 3.4).

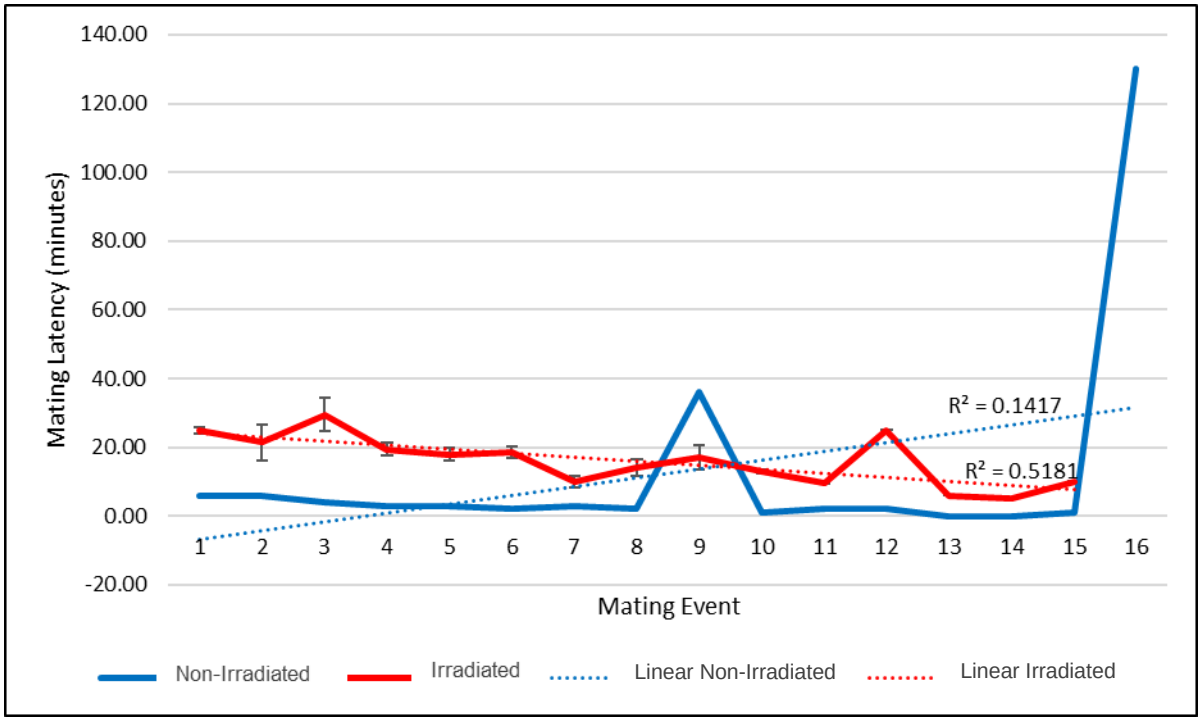


**Figure 3.4: Variation in the mating latency over 16 successive mating events as observed in irradiated and non-irradiated *Glossina brevipalpis* males from ARC-OVR colony. The interval between mating events was 24 hours.**

### 3.3.2 Propensity of mating (PM)

The variation in the propensity of mating (PM) between irradiated and non-irradiates *G. brevipalpis* males for 16 successive mating events can be seen in Figure 3.5. Except for a single mating event ($11^{th}$) the mean PM was above 0.7 in all mating

events (Figure 3.5). In the non-irradiated control group, the PM varied between 1.00 and 0.70 and between 0.49 and 1.00 in the irradiated groups (Figure 3.5). The overall mean PM for the non-irradiated control group (0.90 ± 0.09) did not differ significantly ($p < 0.01$) from that of the irradiated groups (0.86 ± 0.13).

Despite a reduction in the number of potential mating pairs in both the control and irradiated groups no reduction in PM could be observed and it was still relatively high (1.00) at 16$^{th}$ mating event (Figure 3.5). The weak upwards ($R^2$=0.16) and downwards ($R^2$=0.07) trends in non-irradiated and irradiated groups respectively did not correlate with the observed data supporting the notion that PM was not significantly affected as the mating events increased (Figure 3.5).

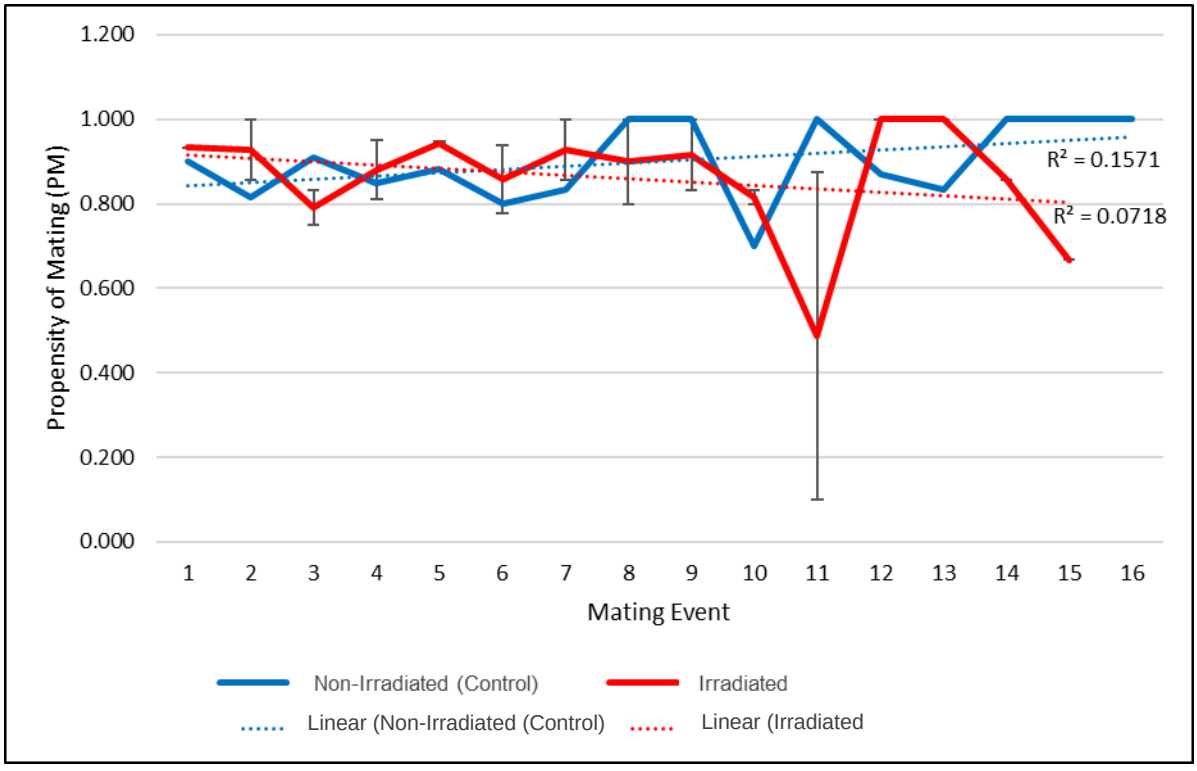


**Figure 3.5: Variation in the Propensity of Mating (PM) over 16 successive mating events in irradiated and non-irradiated *Glossina brevipalpis* males from ARC-OVR colony. The interval between mating events was 24 hours.**

### 3.3.3 Mating duration

The mean mating duration ranged from 68 minutes to 136 minutes in the non-irradiated control group (Figure 3.6). In the irradiated groups the mean mating duration ranged from 65 minutes to 119 minutes (Figure 3.6). All mating duration events exceeded 60 minutes. The overall longer mean mating duration for the non-irradiated group was 101.81 ± 20.41 minutes, differed significantly ($p < 001$) from that of the irradiated group at 90.27 ± 12.53 minutes. Overall mating duration was at 11.54 minutes shorter in the irradiated flies compare to that of non-irradiated flies.

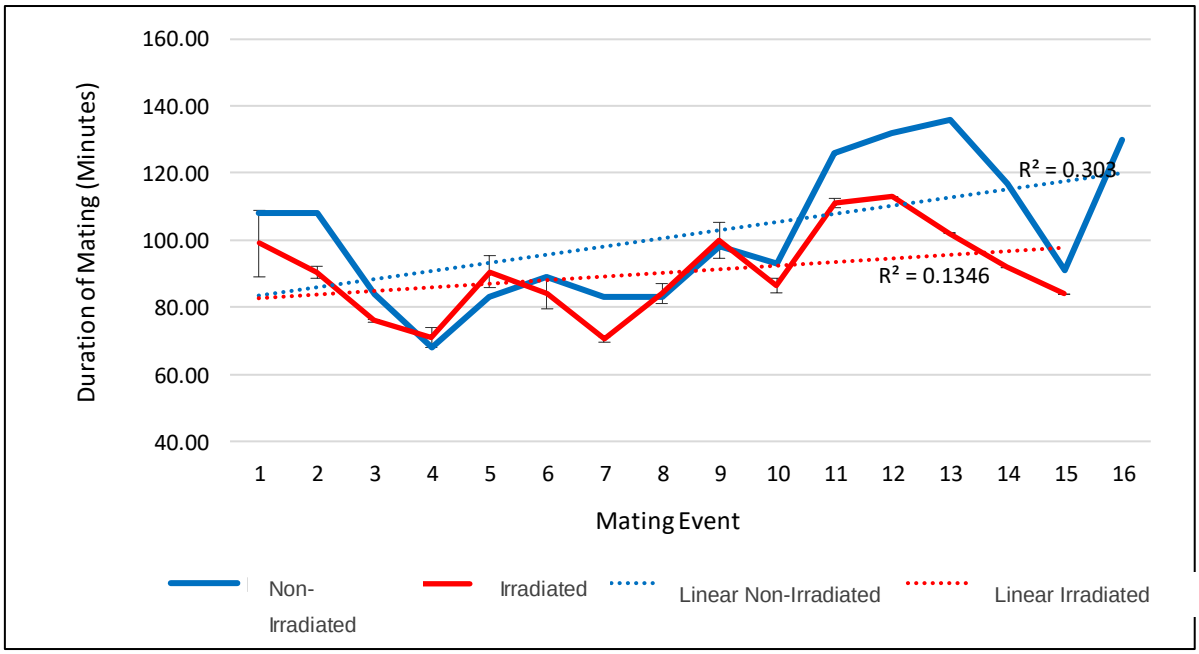


**Figure 3.6: Variation in Duration of Mating over 16 successive mating events in irradiated and non-irradiated *Glossina brevipalpis* males from the ARC-OVR colony. The interval between mating events was 24 hours.**

The slight upwards trend in both the non-irradiated ($R^2$=0.30) and irradiated ($R^2$=0.13) groups did not correlates with the observed data (Figure 3.6), indicate that

duration of mating was not significantly affected as the number of mating events increased.

### 3.3.4 Spermathecael fills in mated females

The mean spermathecael fill in females that mated with non-irradiated males ranged from 0.80 (3rd mating event) to 0.08 (16th mating event). In females that mated with the irradiated males the mean spermathecael fill ranged from 0.73 (2nd mating event) to 0.08 (15th mating event). Mean overall spermathecael fill, 0.48 ± 0.18 in females that mated with the non-irradiated males did not differ significantly ($p < 0.01$) from that of 0.41 ± 0.20 of females that mated with irradiated males.

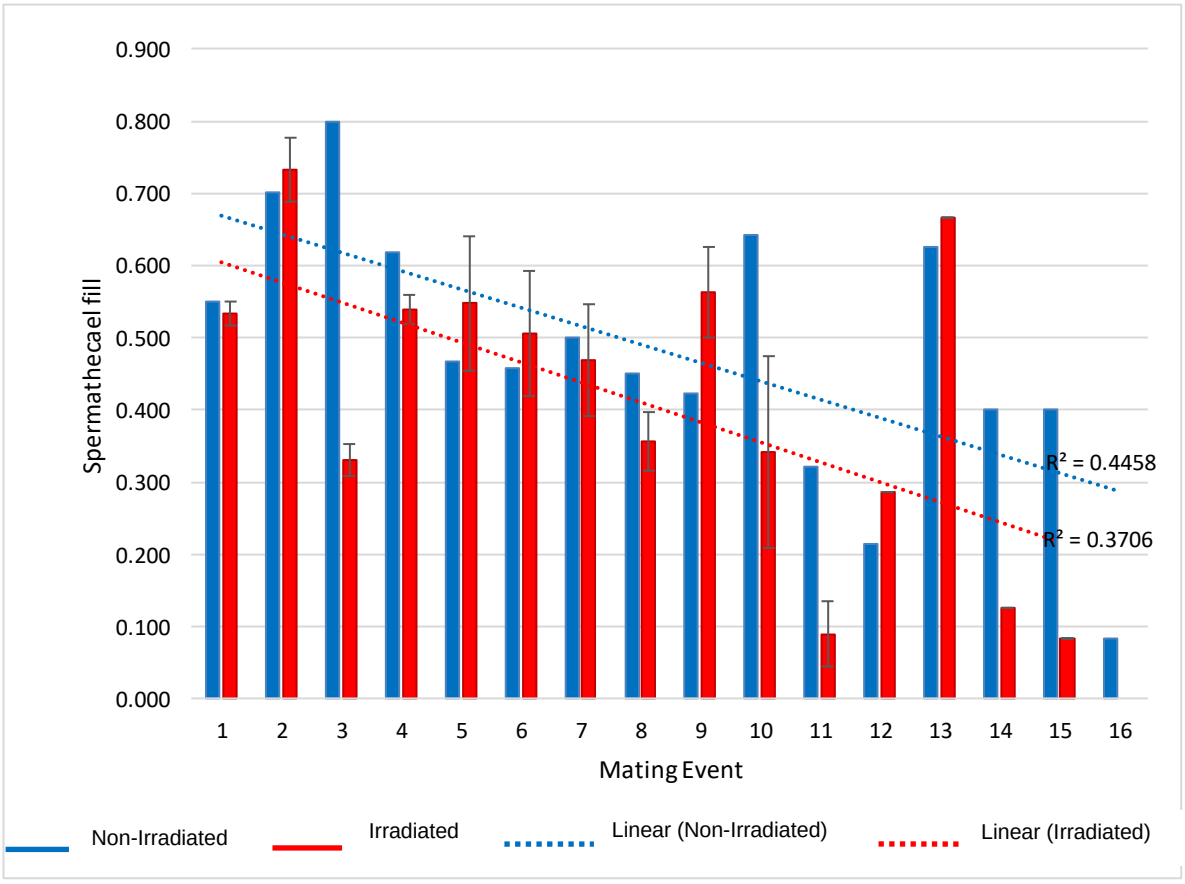


**Figure 3.7: Spermathecael fills as observed in *Glossina brevipalpis* females that mated with irradiated and non-irradiated males. The interval between mating events was 24 hours.**

The downward trend in spermathecael fill found in both the non-irradiated ($R^2$=0.46) and irradiated ($R^2$=0.37) groups did not correlate with the observed data (Figure 3.7), indicating that spermathecael fill did not decline significantly as mating events increased.

## 3.4 Discussion

The study reports on the influence of irradiation on the multiple-mating ability of colonised *G. brevipalpis* males. Groups of irradiated and non-irradiated 7-day old males were given the opportunity to mate with 3-day old virgin females under standard colony conditions. The PM in both the irradiated and non-irradiated groups were relatively high and in agreement with results obtained by Van der Vloedt *et al.* (1978) who reported that 7- to 8-day old males of *G. morsitans morsitans* formed mating pairs within 10 minutes after being introduced to females.

To determine how many times *G. brevipalpis* males will mate successfully irradiated and non-irradiated males were given daily mating opportunities to mate until mating cease or the death of the males. Because only males that mate in a previous mating event were subjected to subsequent matings there was a decline in the number of mating pairs over the duration of the experiment. This linear decline in the number of mating pairs over the duration of the experiment was not influenced by radiation. Despite a similar reduction in the number of mating pairs in both the control and irradiated groups there was no significant reduction in the PM with subsequent matings and the PM was still relatively high at the end of the experiment. The lack of a significant difference in the PM as obtained for irradiated and non-irradiated males indicates that irradiation did not influence the PM.

In the current experiment the last male to form a mating pair was 22 days old and mate 16 times. A potential short coming of the present study is, due to the reduction in mating pairs over time, the relatively small number of mating pairs available after the 16$^{th}$ mating event. Considering that the PM was constantly high over time it is uncertain if mating would have continued, after 16 mating events, if greater numbers of flies were available. The mean life span of male tsetse flies in the wild is approximately four weeks (Jackson, 1949) and the current results appear to indicate that they can mate up to 16 times (or more) during their lifetime.

A slight increase in longevity was recorded for *G. austeni* males that were irradiated as pupae three days before emergence (de Beer, 2016). The observation that radiation increase the average lifespan of males was also documented for *G. morsitans* pupae irradiated either in air (Dean & Wortham, 1969) or nitrogen (Curtis & Langley, 1972). Although many reasons for this increase in lifespan have been suggested it is most likely that a range of radiation induced repair mechanisms are involved (Calabrese, 2013).

In the current study a single *G. brevipalpis* male was able to inseminate up to 16 females under laboratory conditions. Previous studies indicated that a single male tsetse fly can inseminate several females, e.g., an individual *G. austeni* male can inseminate between 9 and 15 females (Jordan, 1972). Additionally, it was shown that, the minimum mean number of females inseminated per male varied between 4.9 and 5.6 in groups of *G. m. morsitans* males that were successively exposed to groups of virgin females (Dame & Ford, 1968; Jordan, 1972). Jordan (1972)

however, pointed out that the actual number of matings varied among males as some males were left with each successive batch of females for a period that ranged between three to seven days. In the current experiment the interval between mating events was restricted to 24 hours. To determine the number of matings over the lifespan of a male tsetse fly, it will be necessary to also evaluate shorter and longer time intervals between mating events in future studies.

The mean duration of mating ranged between 101.81 minutes for non-irradiated males and 90.27 minutes for irradiated males. The duration of mating was longer than that of *G. pallidipes* as the 30 to 69 minutes reported by Wall and Langley (1993) or that of 60 minutes and 90 minutes reported by Saunders & Dodd (1972). On average the irradiated males took 3.54 minutes longer to form mating pairs and mated 11.54 minutes shorter than the non-irradiated males. These results agree with that of de Beer (2016) who also found that 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). Although differences were found in mating duration and latency between irradiated and non-irradiated flies it did not decrease or increase significantly over time in both groups as mating events increase. Any delay in initial mating by the irradiated males can lead to a reduction of their competitiveness and need to be reduced (de Beer, 2016).

The presented data showed that both irradiated and non-irradiated males were able to produce and transfer spermatophore to females and that mean spermathecael fill in females that mated with the non-irradiated males did not differ statistically from

that of females that mated with irradiated males. Differences as found in mating duration and latency therefore did not seem to influence the volume of sperm transfer. In the current experiment there was no significant decline in spermathecael fill following subsequent mating events. These observations were contrary to those of Saunders & Dodd, (1972), who reported that males of *Glossina morsitans orientalis* become aspermic due repeated mating despite being given ample time for copulation as no sperm was transferred post voluntary separation. These authors reported that males became aspermic after the 7$^{th}$ mating event since they failed to pass sperm to females. This finding was confirmed by the lack of deposited spermathecae in females and a complete failure to produce larvae (Saunders & Dodd, 1972). The empty spermathecae from the last female to mate in the current study might have been caused by depletion of sperm from the male after repeated mating events.

## 3.5 Conclusion

Operational success in an AW-IPM programmes that include an SIT component depends on the biological quality of the sterile males released in the targeted area (Calkins & Parker, 2005; Vreysen *et al*., 2007). Previous studies indicated that the colonised *G. brevipalpis* at the ARC-OVR to be highly sensitive to irradiation if irradiated as adults and in addition these flies were able to compete successfully with fertile untreated colony males for virgin untreated females in walk-in field cage experiments as in the study by (de Beer *et al*., 2017). The current data furthermore indicated that irradiation did not influence the male mating potential of colonised *G. brevipalpis*. Both irradiated and non-irradiated males have shown the ability to mate several times and that PM as well the ability to transfer sperm does not decline

significantly in subsequent mating events. Irradiated males will be able to inseminate several females comparable to that of non-irradiated males. This is beneficial as it can reduce the fecundity of wild females and also reduce or collapse *G. brevipalpis* populations in the wild.

# CHAPTER 4

# MULTIPLE MATING BEHAVIOR OF *GLOSSINA BREVIPALPIS* FEMALES MATED WITH IRRADIATED AND NON-IRRADIATED MALES UNDER LABORATORY CONDITIONS

## 4.1 Introduction

Multiple mating behaviors have been observed in several species of tsetse females (Dean *et al.*, 1969). Dame and Ford (1968) demonstrated that female *Glossina morsitans* can be inseminated by sperm transferred from two or more males. Squire (1951) reported that *Glossina palpalis* females may mate several times under confined laboratory conditions. Work done by Dame and Ford (1966) with tepa-sterilized males *G. morsitans* indicated a reduction in fertility in relation to ratios of sterile to fertile females as well as mating time. Although multiple mating in tsetse females have been confirmed in the laboratory the significance of this phenomenon to the competitiveness for mating of the sterile versus fertile males is not clear. Although a multi mating has been recorded for colonized male *Glossina brevipalpis* (Chapter 3), it can be noted that a single fertilization is sufficient to produce offspring for the entire life of the female (Buxton, 1955) and that multiple mating may be uncommon in wild tsetse populations (Dean *et al.*, 1968a).

It has been shown that females post their first mating, even in the absence of sperm transfer, can be resistant to successive mating events (Dean *et al.*, 1968a). This is either due to the mechanical stimulation or physiological activity of hormones released by males prior to sperm transfer (Dean *et al.*, 1968a). Craig (1967)

discovered a component in the seminal fluid of *Aedes aegypti* that inhibit insemination from subsequent mating events. However, this inhibition mechanism was not obviously noticeable in *Glossina austeni* since the re-mating events were only partially suppressed (Dean *et al.*, 1968b). Additionally, Mellanby (1936) reported that age plays a role in mating success based on an observed decline in insemination in 8-day old virgin *Glossina fuscipes* females in the laboratory.

Induced sterility by irradiated males mated with females can be expressed as, females that do not produce pupae throughout their entire life and females that produce reduced numbers of pupae after they have mated with sterile males (Taze *et al.,* 1977). This may be due to the chromosomic damaged caused by the gamma rays in the spermatozoa of males (Taze *et al.,* 1977). Sterility induced in female tsetse flies, declines when the number of subsequent matings with fertile males increase (Taze *et al.*, 1977). It was found that 75% of sperm from the first mating, irrespectively if it was from a sterile or fertile male, are used for fertilization (Curtis, 1968). A reduction in the length of ovulation cycles of females that mated with sterile male *G. austeni* was observed (Curtis, 1968).

Studies on *G. brevipalpis* indicated that sterility of 99% was achieved in females that mated with males that were irradiated as adult or late-stage pupae with a dose of 80 Gy (de Beer *et al*., 2017). This is a relative low dose compared to that of 120 Gy needed to sterilize *G. tachinoides* males (de Beer *et al*., 2017). *Glossina brevipalpis* is therefore suspected to be highly sensitive to irradiation (Vreysen *et al.,* 1996; de Beer *et al*., 2017). It was also shown that *G. brevipalpis* males irradiated with a dose of 80 Gy can compete successfully with non-irradiated males for females in walk-in

field cage experiments, indicating that the dose of 80 Gy did not affect the mating ability of sterilized colony males (de Beer *et al*., 2017).

From the above it is clear that multiple mating behavior is not uncommon in tsetse females and that it may affect the success of the sterile insect technique (SIT) if implemented. Information on multiple mating behavior of *G. brevipalpis* females, considered as vectors of Animal African Trypanosomiasis (AAT) in southern Africa, is not available. The current study therefore aimed to evaluate the multiple mating behavior in female *G. brevipalpis* when given the opportunity to mate with 7-day old irradiated and non-irradiated *G. brevipalpis* males under laboratory conditions.

## 4.2 Materials and methods

### 4.2.1 Colony tsetse flies

To evaluate the multiple mating behaviors of *G. brevipalpis* females, females were given the opportunity to form multiple mating pairs. These evaluations were done from July to December 2018 with laboratory reared *G. brevipalpis* at the ARC-OVR. The origin and holding conditions (Feldmann, 1994; FAO/IAEA standard operating procedures 2006) of the colony flies are described in detail in Chapter 3. The flies were maintained on defibrinated bovine blood using an artificial *in vitro* membrane feeding system. In the current evaluations the flies were not fed on the day of mating to enhance mating activity. The males were irradiated with a dose of 80 Gy using a $^{137}$Cs source (Gammacell 40 S/N50) at a dose rate of 0.69 Gy/min in air five days after emergence.

### 4.2.2 Multiple mating behavior

Thirty 3-day old virgin females were mated with 30 7-day old irradiated males at a 1:1 ratio. Concurrently 30, 7-day old non-irradiated males were mated to 30 virgin females in a separate cage as a control. During each replicate male and female flies was kept together for three hours in large (50 cm x 50 cm x 50 cm) laboratory-mating cages (Figure 4.1). The cage made up of a metal frame covered with a polyester netting and a cloth bottom. In addition, a sleeve is placed in, the front part of the cage attached along the three edges by Velcro to allow access to the interior of the cage. The females were released into the mating cage 5 minutes before the males. All mating pairs were collected individually into small vials as described in Chapter 3 section 3.2.

**Figure 4.1: Large laboratory mating cages (50 cm x 50 cm x 50 cm) with mating *Glossina brevipalpis.***

All evaluations were conducted under standard colony holding conditions, (23°C to 24°C, 75-80% RH and subdued/indirect lighting) and feeding regime (Feldmann, 1994; FAO/IAEA standard operating procedures 2006).

Females that formed mating pairs during the initial mating event were given the opportunity to mate, again at a ratio of 1:1 and were observed after 24 hours (Group 1), 48 hours (Group 2) or 72 hours (Group 3) with 7-day old fertile non-irradiated males. Four replicates were done for each treatment with, control groups of females mated with non-irradiated fertile males being included for each treatment. The mating protocol, as described for the first mating event, was followed. The females were subsequently grouped into females that mated only once with irradiated males and females that mated twice, once with irradiated and once with fertile males.

Female survival and productivity were recorded daily. All pupae produced were counted on the day of deposition. After 30 days, all surviving females were dissected as described in Chapter 3 to determine their insemination rate (Feldmann, 1994; FAO/IAEA standard operating procedures, 2006). The spermatecae were removed and the fill was microscopically examined and scored as either, empty (0), quarter full (0.25), half (0.5), three quarters (0.75) or full (1) (Nash, 1955).

### 4.2.3 Statistical analysis

Propensity of mating (PM) was defined as the overall proportion of released females that had mated (Mutika *et al.*, 2001). Fecundity was expressed as the number of pupae produced per mature female per day (Curtis, 1968). Mature female days were calculated for each treatment by totaling the number of flies alive each day, starting

on day 18 after emergence until the end of the experiment on day 30 (Curtis, 1968). The production relative to control is a percentage value determined by dividing the number of pupae produced per treatment and divided this by the number of pupae produced for the control (Vreysen *et al.,* 1996). Induced sterility was determined by subtracting the production relative to control with a 100.

Data were analyzed using the statistical software GraphPadInstat (version 3.00, 2003). For comparison of the fecundity between multiple mated *G. brevipalpis* females, a one-way analysis of variance (ANOVA) was used and Tukey's test was applied. Data was normally distributed, and a Paired t test was used. All statistical tests were done at the 5% significance level.

## 4.3 Results

Propensity of mating (PM), fecundity, mature female days, production relative to control and induced sterility in the females for the three experimental groups and two control groups are presented in Table 4.1.

### 4.3.1 Group 1: Re-mated after 24 hours

The propensity of mating (PM) of females of the control group (n = 60) and the group first mated with irradiated males (n = 118) were both 1.00 as all the flies formed mating pairs immediately after the introduction of males into the cage with the females (Table 4.1). When the mated females were given the opportunity to mate for a second time with fertile males after 24 hours, less than half of them mate and the PM declined to 0.49 (Table 4.1). All females that mated survived up to 30 days with

no mortality observed. The 60 females that mated only with fertile males (control) produced the highest number of pupae (n = 68) with no egg abortions observed (Table 4.1). The 118 females that mated only with the irradiated males showed and increased in the number of aborted eggs (n = 60) and produced only 11 pupae (Table 4.1). For the females that mated with fertile males 24 hours after they have mated with irradiated males the pupal production (n = 48) and egg abortions (n = 14) were respectively higher and lower than for females that only mated with the irradiated males (Table 4.1).

Dissection of the surviving females 30 days after insemination showed that there was no significant difference ($p < 0.05$) between the mean spermatecae fill in the females that only mated with fertile males (0.49 ± 0.18), with irradiated males (0.44 ± 0.22) or those that mated twice with both irradiated and fertile males (0.54 ± 0.23) (Table 4.1).

The highest mean fecundity, 0.09 ± 0.01, was observed in the females that mated only with the fertile males (Figure 4.2). The mean fecundity, 0.01 ± 0.00, was significantly ($p < 0.01$) lower in the females that mated only with the irradiated males (Table 4.1). However, in the females that first mated with the irradiated males and 24 hours later with fertile males the mean fecundity (0.06 ± 0.00) was not significantly ($p < 0.01$) different to that of females that mated only with the fertile males (Table 4.1). The mean production relative to control and induced sterility for the females that first mated with irradiated males and 24 hours later with fertile males was 43.99 ± 11.69% and 56.01 ± 11.69% respectively (Table 4.1; Figure 4.3).

**Table 4.1: Production of multiple mated *Glossina brevipalpis* females in the laboratory first mated with irradiated (80 Gy) followed by mating fertile males**

| | Possible pairs | Actual mated | Propensity of mating (PM) | No. of mature females | Mean survival rate of females ±SD% | Total no. pupae produced | Total no. of aborted eggs | Insemination rate % | Mean spermatecae fill ±SD | Mean fecundity ±SD | Mean production relative to control ±SD% | Mean induced sterility ±SD % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Group 1: Second mating 24 hours after first** | | | | | | | | | | | | |
| Fertile Control | 60 | 60 | 1.00 | 780 | 100.00 ±0.00 | 68 | 0 | 100 | 0.49 ±0.18**a*** | 0.09 ±0.01**a** | | |
| Mated with irradiated males | 118 | 118 | 1.00 | 754 | 100.00 ±0.00 | 11 | 60 | 100 | 0.44 ±0.22**a** | 0.01 ±0.01**b** | | |
| Re-mated with fertile males | 118 | 58 | 0.49 | 754 | 100.00 ±0.00 | 48 | 14 | 100 | 0.54 ±0.23**a** | 0.06 ±0.01**a** | 43. 99 ±11.69 | 56.01 ±11.69**a** |
| **Group 2: Second mating 48 hours after first** | | | | | | | | | | | | |
| Fertile Control | 60 | 60 | 1.00 | 535 | 87.12 ±3.79 | 60 | 0 | 100 | 0.42 ±0.23**a** | 0.11 ±0.01**a** | | |
| Mated with irradiated males | 120 | 120 | 1.00 | 897 | 85.69 ±6.82 | 7 | 109 | 100 | 0.38 ±0.23**a** | 0.01 ±0.01**b** | | |
| Re-mated with fertile males | 120 | 40 | 0.33 | 481 | 92.92 ±4.15 | 25 | 44 | 100 | 0.39 ±0.17**a** | 0.05 ±0.02**c** | 26.79 ±10.50 | 73.21 ±10.50**b** |
| **Group 3: Second mating 72 hours after first** | | | | | | | | | | | | |
| Fertile Control | 60 | 60 | 1.00 | 676 | 87.68 ±1.97 | 82 | 0 | 100 | 0.43 ±0.23**a** | 0.12 ±0.01**a** | | |
| Mated with irradiated males | 115 | 115 | 1.00 | 1248 | 87.61 ±5.15 | 8 | 121 | 100 | 0.29 ±0.11**b** | 0.01 ±0.01**b** | | |
| Re-mated with fertile males | 115 | 10 | 0.09 | 73 | 58.33 ±25.00 | 4 | 7 | 100 | 0.25 ±0.00**b** | 0.06 ±0.07**c** | 7.58 ±6.00 | 92.42 ±6.00**c** |

*** Different alphabetical letters indicate statistically difference within groups (Mean spermathecae fill and Mean fecundity) or between groups (Mean induced sterility).**

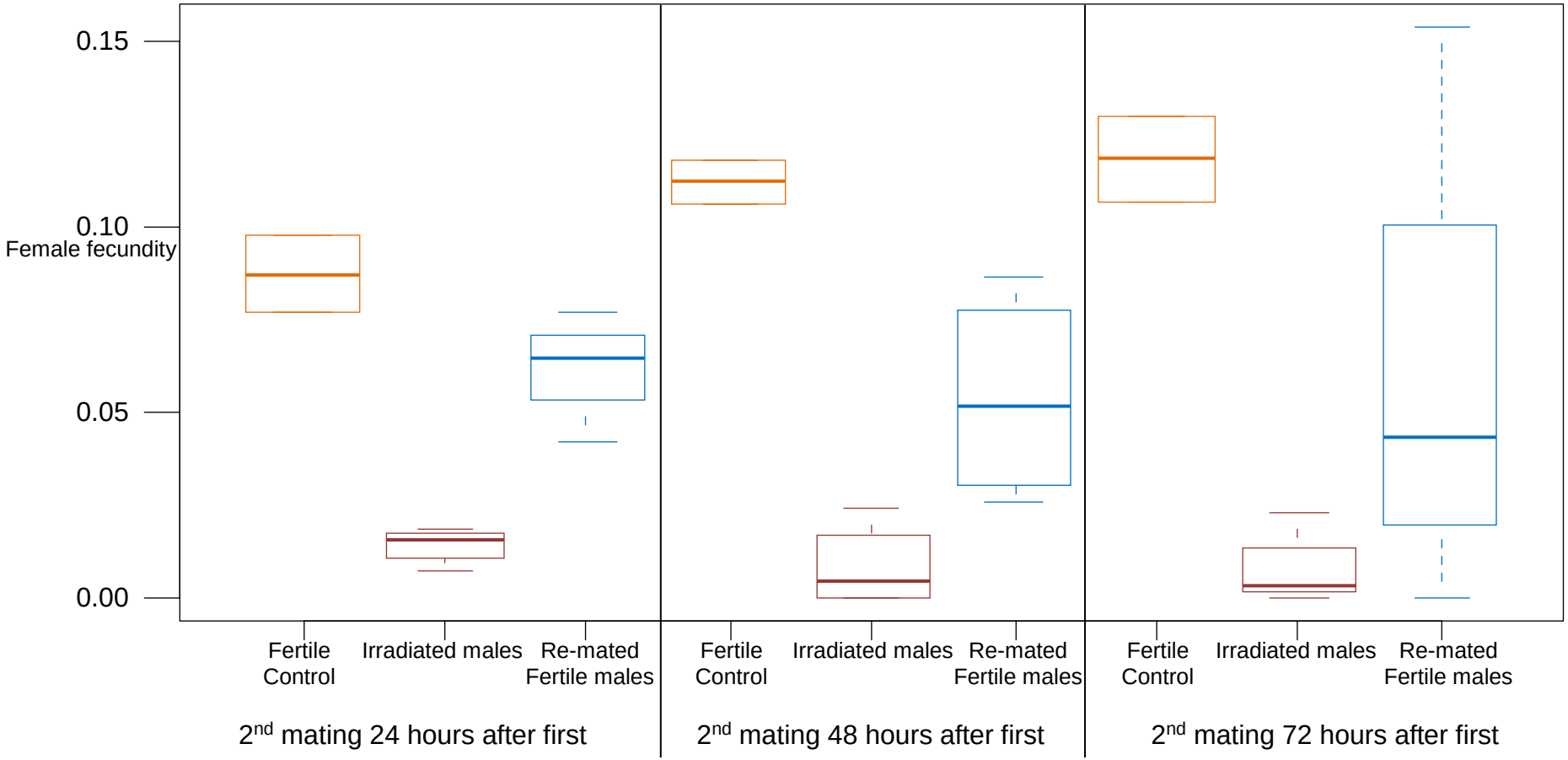


**Figure 4.2: Fecundity of *Glossina brevipalpis* females mated with fertile males (control), irradiated (80 Gy) males and re-mated with fertile males after 24, 48 and 72 hours. Each box shows the group median separating the 25th and 75th quartiles; capped bars indicate maximum and minimum values**

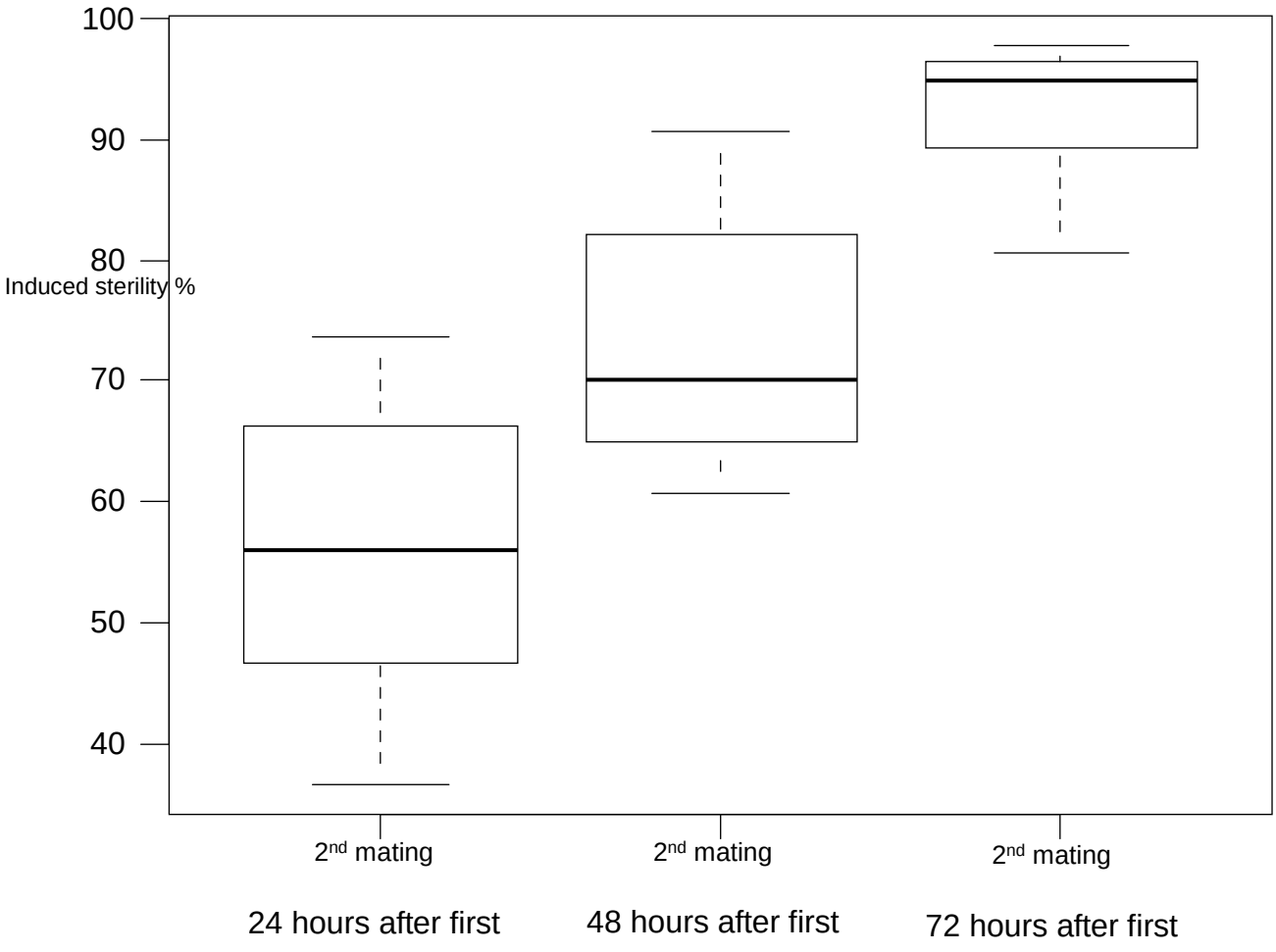


**Figure 4.3: Induced sterility of *Glossina brevipalpis* females mated first with irradiated (80 Gy) males and re-mated with fertile males after 24, 48 and 72 hours. Each box shows the group median separating the 25th and 75th quartiles; capped bars indicate maximum and minimum values.**

### 4.3.2 Group 2: Re-mated 48 hours

In the second group the females were given the opportunity to mate with fertile females 48 hours after the first mating with irradiated males. Similar to Group 1 the PM was 1.00 as all the females that were mated with fertile (control) males (n = 60) and irradiated males (n = 120) formed mating pairs (Table 4.1). During the re-mating with the fertile

males 48 hours after the first mating the PM decline to 0.33 (Table 4.1). The mean survival rate of the females at day 30 after mating was 87.12 ± 3.79% for the control females, 85.69 ± 6.82% for females that mated only with the irradiated males and 92.92 ± 4.15% for the females re-mated with the fertile males (Table 4.1). The females that mated only with fertile males (control) once again produced the highest number of pupae (n = 60) with no abortions observed (Table 4.1). As for Group1 the females that mated only with the irradiated males showed an increase in egg abortion (n = 109) and, with only seven pupae produced, a large reduction in pupal production (Table 4.1). As for Group 1 the females that mated with both irradiated and fertile males the pupal production (n = 25) and egg abortions (n = 44) were respectively higher and lower than the females that only mated with the irradiated males (Table 4.1). The 120 females that re-mated 48 hours after mating with irradiated males and then with fertile males produced less pupae (n = 25) than the 118 females that re-mate after 24 hours (n = 48) (Table 4.1).

As in Group 1 all females dissected after 30 days were inseminated (Table 4.1). There was no significant ($p < 0.05$) difference between the mean spermatecael fill in the females that mated only with fertile males (0.42 ± 0.23), only with irradiated males (0.38 ± 0.23) or those that mated once with irradiated males and once with fertile males (0.39 ± 0.17) (Table 4.1).

The highest mean fecundity of 0.11 ± 0.01, was recorded for the females that only mated with the fertile males (Figure 4.2). The mean fecundity was significantly ($p < 0.01$) lower in the females (0.01 ± 0.01) that mated only with the irradiated males (Table 4.1).

The lower mean fecundity of 0.05 ± 0.02 for the females that mated with the irradiated males and 48 hours later with the fertile males was significantly ($p < 0.05$) different to that of females that mated with fertile males at 0.11 ± 0.01 (Table 4.1). It was, however, significantly higher than that of 0.01 ± 0.01 for females that mated only with irradiated males. The mean production relative to control and induced sterility for the females that mated first with irradiated males and then 48 hour later with fertile males was 26.79 ± 10.50 and 73.21 ± 10.50% respectively (Figure 4.3).

### 4.3.3 Group 3: Re-mated 72 hours

In the third evaluation females mated with irradiated males were given the opportunity to re-mate with fertile females 72 hours after the first mating. Again, the PM of the females that mated once with fertile males and once with irradiated males was 1.00 (Table 4.1). The PM of the females that re-mated with fertile males after 72 hours was reduced to 0.09, i.e., being the lowest PM in all the re-mating groups evaluated. The mean survival rate after 30 days for females that mated with the fertile and irradiated males only was 87.68 ± 1.97 and 87.61 ± 5.15, respectively. However, for the females that mated with both fertile and irradiated males the survival rate was only 58.3 ± 25.00 (Table 4.1). The females that only mated with fertile males again produced the highest number of pupae (n = 82) with no egg abortions observed. With only eight pupae produced, there was a large reduction in pupal production accompanied by an increase in egg abortion (n = 121) for the females that mated only with the irradiated males (Table 4.1). The 10 females that mated with both irradiated and fertile males produced four pupae with a relatively high proportion (n = 7) of aborted eggs (Table 4.1). The number of pupae (n = 4) produced by females that re-mated 72 hours after the first mating was much lower

than the number of pupae produced by females that re-mated 24 hours (n = 48) and 48 hours (n = 25) after first mating (Table 4.1).

As for first two groups all surviving females that were dissected after 30 days were inseminated. There was, however, a significant ($p < 0.01$) difference between the mean spermatecae fill in the females that mated with fertile males only (0.43 ± 0.23), with both the females that mated with the irradiated males only (0.29 ± 0.11) and that mated twice with both irradiated and fertile males (0.25 ± 0.00).

As was found for Groups 1 and 2 the mean fecundity, 0.12 ± 0.01, recorded for the females that only mated with the fertile males was again the highest (Figure 4.2). The lowest mean fecundity of 0.01 ± 0.01 in the females that mated only with the irradiated males was significantly different ($p < 0.01$) from that of females that mated with fertile males at 0.12 ± 0.01 (Table 4.1). The mean fecundity of 0.06 ± 0.07 obtained for females that re-mate with fertile males was significantly higher than that of 0.01 ± 0.01 for females that only mated with irradiated males (Table 4.1).

The mean production relative to control and induced sterility for the females that mated first with irradiated males and then 72 hour later with fertile males was 7.58 ± 6.00 and 92.42 ± 6.00% respectively, the highest of all the re-mated groups (Figure 4.3). Comparisons of the three groups indicate that the observed induced sterility in females that were mated first with irradiated males and then given an opportunity to re-mate 24 hours (56.01 ± 11.69), 48 hours (73.21 ± 10.50) and 72 hours (92.42 ± 6.00) after first mating differ significantly ($p < 0.01$) (Table 4.1; Figure 4.3). It indicates that induced sterility may significantly increase as re-mating time increase (Table 4.1).

## 4.4 Discussion

In this study, multiple mating behavior of *G. brevipalpis* females that first mated with irradiated and then with fertile males was determined under laboratory conditions. Females successfully formed mating pairs immediately after their first introduction to both irradiated and non-irradiated males. Similar observations were made by Van der Vloedt *et al.* (1978), where they recorded 100% of pairs formed immediately when *G. p. palpalis* females were introduced to either irradiated or non-irradiated males. The same can be said about irradiated females where Vreysen and Van der Vloedt (1992), documented between 80-90% mating response when irradiated female *G. austeni* were introduced for the first time to non-irradiated males and showed no significant preference between the two groups. It must be taken into consideration that the current evaluations were done under relative confined laboratory conditions. The density of the flies in the cages was 480 flies $m^3$. It remains to be determined to what extent the PM and female multi mating behavior will be influenced by fly density and sex ratio as found under field conditions.

Re-mating in *G. brevipalpis* under laboratory conditions agree with previous studies indicated multiple mating in laboratory reared *G. p. palpalis* (Squire, 1951; Jordan, 1958). Studies on *G. pallidipes* females revealed that only 45% of mated females were-inseminated and only 9% of inseminated females re-mated (Wall & Langley, 1993). As expected, high numbers of pupae were produced by the females that only mated with fertile males in the current study. Females which were only inseminated by irradiated males were going through recurring unsuccessful cycles with a high rate of observed egg abortions.

In agreement with Curtis (1968) females that re-mate, sperms from the first mating were used more frequently than from the second mating since more egg abortions were observed in the case where the females only mated with the fertile males. Dissections 30 days after mating showed that all the females were inseminated for all the re-mating groups and that there was no significant difference in the mean spermatecae fill of the females that mated once with irradiated male compared to the females that re-mated (Table 4.2).

In the females that mated first with irradiated males followed by the opportunity to re-mate with fertile males 24, 48 and 72 hours later showed a re-mating of 49%, 33% and 9% respectively (Table 4.1). As the females in the first mating was 3-days old and the females from the re-mating of 24, 48 and 72 hours were between 4, 5 and 6 days old respectively, imply that the probability of re-mating were higher in younger females flies than older flies. This was similar to observations made by Nash *et al.* (1971) where they recorded maximum insemination rates in 3-day old female *G. m. morsitans* when mated to 7-day old males as compared to older females. In contrast Curtis (1968) observed no decline in female receptivity in *G. austeni* when they could mate for the first time at 3-days old and to re-mate a day later.

After the 72 hour treatment, females showed less interest to the second mating at 9% compared to those observed after 24 hours at 49%. Vreysen and Van der Vloedt (1992) indicated that there was no female receptivity to male on day 15 in *G. austeni* when offered several mating opportunities but only accepted male on the second, third and fourth mating occasions. It is therefore postulated that the 72 hour treatment females had reached the status requiring no further insemination or they had lost their vigor for re-mating or even appear to become refractory to further mating. It is therefore,

suggested that under controlled conditions, sexual aggressiveness declines in female *G. brevipalpis* as they age.

In the current study the induced sterility observed in females that were first mated with irradiated (80 Gy) males and then given the opportunity to re-mate 24- (56.01 ± 11.69), 48- (73.21 ± 10.50) and 72 hours (92.42 ± 6.00) after the first mating with fertile males, significantly ($p < 0.01$) increased as re-mating time increase (Figure 4.3). de Beer *at al.* (2017) demonstrated that a radiation dose of between 80 Gy and 100 Gy induced a sterility of 95% in *G. brevipalpis* females that mated with irradiated males. This induced sterility of 95% is similar to the induced sterility of 93% which was observed for the females in the re-mated group 3 (Second mating 72 hours after first), which indicate that the effect of a re-mating 72 hours later might not have a significant negative effect in the SIT control strategy.

## 4.5 Conclusion

The present study demonstrated multiple mating behavior of colonized *G. brevipalpis* females under laboratory conditions. Younger females seem to be more receptive to a second mating compared to older fem ales. Re-mating is possible with females becomes more refractory to the second mating overtime, and it is likely that the sperm from the first mating will be used first as proved by the induced sterility from irradiated males. This may become even more relevant if considering that in Chapter 3, it was shown that both irradiated and non-irradiated males have the ability to mate several times and that PM as well the ability to transfer sperm does not decline significantly in subsequent mating events.

# CHAPTER 5

# ENDOSYMBIONTS (*SODALIS* AND *SPIROPLASMA*) AND TRYPANOSOME ABUNDANCE IN *GLOSSINA BREVIPALPIS* AND *GLOSSINA AUSTENI* IN SOUTH AFRICA

## 5.1 Introduction

Endosymbiotic bacteria are a common feature among insects. While some endosymbiotic bacteria may form mutualistic relationships with their hosts others affect various biological functions in the host (Eleftherianos *et al.,* 2013). Endosymbionts are transmitted either vertically or horizontally (Eleftherianos *et al.,* 2013). Vertically transmitted symbionts, or primary endosymbionts, are transmitted from mother to offspring and provide the host insect with nutritional compounds essential for its survival and development (Eleftherianos *et al.,* 2013).

Of more than 23 species of bacteria identified from various tsetse species, three, *Wigglesworthia glossinidia, Sodalis glossinidius* and *Wolbachia*, are regularly isolated from these flies (Rio *et al*., 2004; Lindh & Lehane, 2011; Doudomius *et al.,* 2017). *Wigglesworthia glossinidia* supplement the tsetse diet with essential vitamins that are absent in the blood of the vertebrate host (Eleftherianos *et al.,* 2013; Geiger *et al.,* 2018). Phylogenetic analysis has shown that the association between the tsetse and the symbiont *Wigglesworthia*, have evolved more than 50 to 80 million years ago (Cheng *et al.,* 1999; Askoy & Weiss, 2007; Pais *et al.,* 2008). *Wigglesworthia* was shown to be essential for tsetse fecundity as the elimination thereof with antibiotics result in sterility in females (Pais *et al.,* 2008).

A second frequently isolated tsetse symbiont, the facultative mutualist *Sodalis,* is found in the intra and extracellular cavity of the midgut (Dale & Welburn, 2001; Eleftherianos

*et al.,* 2013). *Sodalis* can be detected in the hymolymph as well as the milk gland tissues (Askoy & Weiss, 2007; Pais *et al.,* 2008) (Figure 5.1). The elimination of *Sodalis,* with the metabolic inhibitor strepto-zotoicin, has been associated with decreased longevity in the progeny (Pais *et al.,* 2008; Wang *et al.,* 2013). Although *Sodalis* do not influence the flies' fecundity, infected flies may become more resistant to trypanosome infections (Wang *et al.,* 2013). This symbiont is usually found at high abundance in insectary-reared tsetse flies (Lindh & Lehane, 2011; Geiger *et al.,* 2018). Lastly, it can be exploited to express foreign gene products in the midgut of tsetse, thus affecting parasite viability (Cheng *et al.*, 1999; Askoy & Weiss, 2007).

Tsetse populations can harbour a third microbe from the genus *Wolbachia* (Pais *et al.,* 2008). *Wolbachia* represents a genus of obligate intracellular alpha-Proteobacteria which are maternally transmitted and infects many arthropods and filarial nematodes (Ouedraogo *et al.,* 2018). The crucial role this symbiont plays in biological, ecological as well as in the evolutionary processes, attracted interest for the development of novel and environmentally friendly control strategies for various insect pests and disease vectors (Doudoumis *et al.,* 2012). In tsetse species, the prevalence and distribution of *Wolbachia* species range from intracellular infection of only germ line tissue to persistent in other various tissue cells (Pais *et al.,* 2008) (Figure 5.1). The elimination of *Wolbachia* infections with antibiotics resulted in host sterility (Pais *et al.,* 2008). This symbiont has been implicated in the cytoplasmic incompatibility of tsetse and has been found in ovarian tissues (Cheng *et al.,* 2000; Dale & Welburn, 2001; Demirbas-Uzel *et al*., 2018). In wild tsetse populations, *Wolbachia* has been reported to vary in abundance between 0 and 100% depending on the species involved (Askoy & Weiss, 2007; Demirbas-Uzel *et al.,* 2018). One report stated the prevalence of *Wolbachia* to be

zero in South African *Glossina brevipalpis* and 100% in *G. austeni* (Askoy & Weiss, 2007).

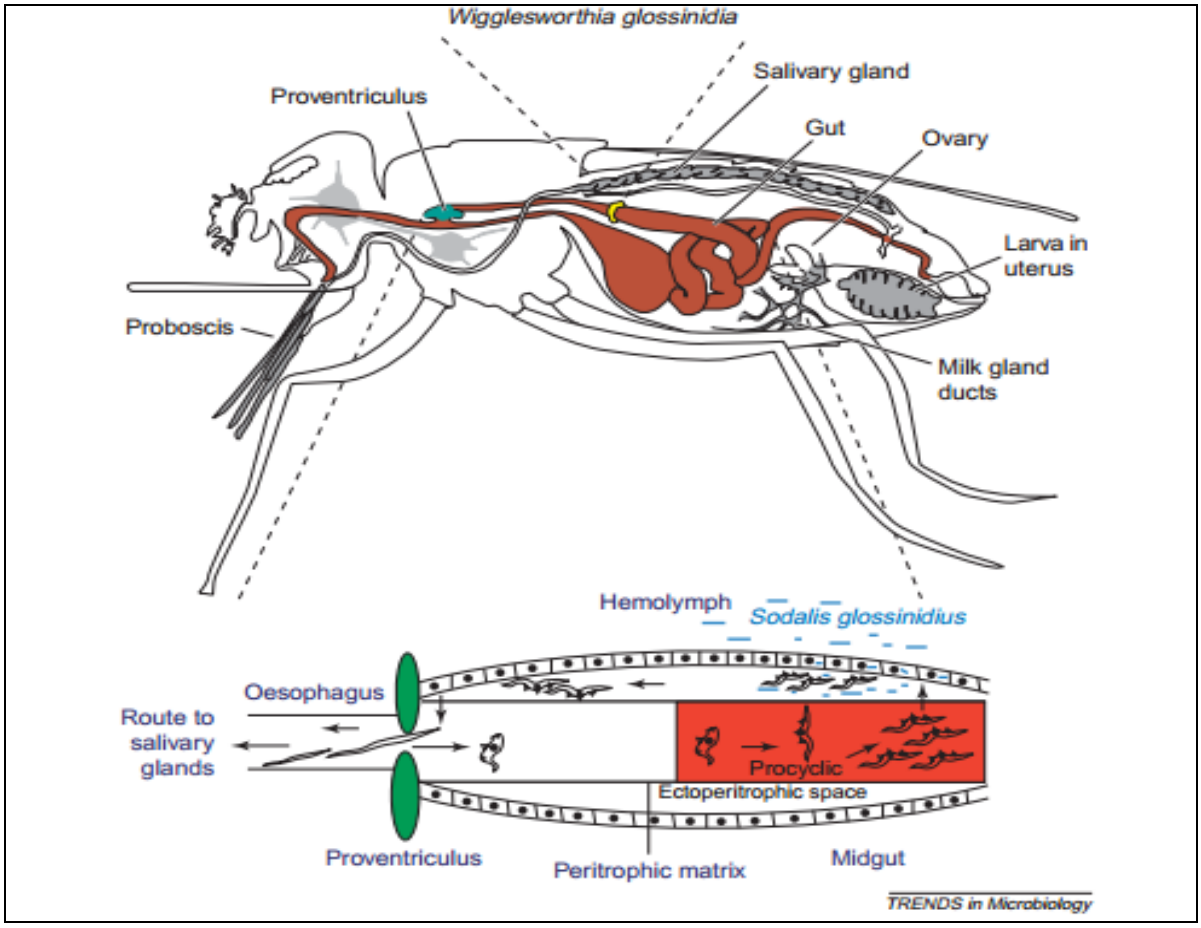


**Figure 5.1: The digestive and internal reproductive systems of female tsetse (Rio *et al.,* 2004). *Wolbachia* reside exclusively within reproductive tissues, while *Sodalis* and *Wigglesworthia* are associated with digestive tissues and hemolymph (the latter only being inhabited by *Sodalis*).**

Recently a novel endosymbiont, *Spiroplasma,* was identified in *Glossina fuscipes fuscipes* and *G. tachinoides* (Demirbas-Uzel *et al.,* 2018). *Spiroplasma* consists of a genus of wall free motile, gram-positive bacteria which are associated with both the intracellular and extracellular cavities in a variety of arthropods (Demirbas-Uzel *et al.,* 2018). *Spiroplasma* can be pathogenic in plants, insects and crustaceans and has been

found to be capable of protection against the nematode infections in *Drosophila neotestacea*, against fungal infections in pea aphids (*Acyrthosiphon pisum*) and against parasitoid wasps in *Drosophila hydei* (Doudoumis *et al.,* 2017). However, the role of this bacterium in tsetse remains unknown (Schneider *et al.,* 2019). Phylogenetic analysis has shown that *Spiroplasma* species which infect field and laboratory populations of *G. f. fuscipes* were closely related to *Citri-Chrysopicola-Mirum* (*Spiroplasma insolitum*) which was isolated from laboratory reared flies of the same species (Schneider *et al.,* 2019).

From the above it is clear that endosymbionts can have diverse ecological and evolutionary roles in their hosts. They affect various aspects of ecological interactions ranging from nutrition to defence as well as the reproductive system which can influence population structure, reproductive isolation and speciation in the host (Moran *et al*., 2008; Kuchler *et al*., 2010). In some instances, endosymbionts can protect its host against co-infections with parasites (Doudoumis *et al.,* 2017).

Nagana is caused by the related parasites *Trypanosoma brucei brucei*, *Trypanosoma congolense* and *Trypanosoma vivax* biologically transmitted by various species of tsetse (Chapter 1). These trypanosomes, except for *T. vivax,* establish within the midgut of tsetse before moving into either the salivary glands or proboscis where they mature into the mammalian infective stage (Van den Abbeele *et al.,* 1999) (Figure 5.1). Outbreaks of nagana in South Africa are attributed to *T. congolense* and *T. vivax* (Motloang *et al*., 2012; de Beer *et al*., 2016).

The outcome of eliminating a pathogen from the infected insect vector prior to the potential transmission to a susceptible vertebrate host, reflects the presence of a

dynamic active and passive immune barriers that function locally within the insect's gut as well as systemically in the haemocoel (Weiss *et al.,* 2019). An understanding of the interactions between the symbionts and the pathogen occurring within tsetse hosts may facilitate the development of tsetse refractory to trypanosome infection by manipulating the symbionts (Demirbas-Uzel *et al.,* 2018). Paratransgenesis is a novel genetic based method whereby the symbionts of an insect vectors are modified with recombinant technologies to express effector molecules that can potentially block the development of a pathogen (Demirbas-Uzel *et al.,* 2018). As such it has been postulated that the manipulation of the tsetse endosymbionts interactions may present the next avenue to disease and vector control (Wamwiri *et al.,* 2013).

*Glossina brevipalpis* and *G. austeni* are considered the sole vectors of nagana in the north-eastern KwaZulu-Natal (KZN) Province of South Africa. Information on the presence and abundance of symbionts in South African populations of these two tsetse species are relatively limited. The current study investigates the presence and abundance of the endosymbionts *Sodalis* and *Spiroplasma* and the *Trypanosoma* species in South African wild caught populations as well as the endosymbionts in laboratory reared *G. brevipalpis* and *G. austeni*. The comparative presence and abundance of endosymbionts in wild and colony reared flies may play a role in the incorporation of colony reared flies in a sterile insect technique (SIT) in a control program of nagana in South Africa.

## 5.2 Materials and methods

### 5.2.1 Field collected tsetse flies

Field collections of tsetse flies for endosymbiont and *Trypanosoma* detection were done between October 2018 and March 2019. For endosymbiont detection *G. brevipalpis*

were collected from Phinda game reserve and *G. austeni* were caught from False Bay and Eastern Shores (Figure 4.1). For trypanosome detection *G. brevipalpis* were collected from the protected areas at Phinda, Eastern Shores and Hluhluwe-iMfolozi Park and *G. austeni* from the communal area at Lower Mkuze, and protected areas at the Eastern Shores and False Bay (Figure 2.1). The flies were collected with odour-baited H-traps as described in Chapter 2 and stored in 95% ethanol until analysed.

### 5.2.2 Colony tsetse

The endosymbiont component of *G. brevipalpis* and *G. austeni,* from the laboratory colonies at the ARC-OVR, was determined and compared with that of the field collected flies. The origin and holding conditions of these colonies are described in detail in Chapter 3 (Feldmann, 1994; FAO/IAEA standard operating procedures 2006).

### 5.2.3 DNA extraction from whole fly

Prior to DNA extraction, the flies were removed from the ethanol used for storage, rinsed twice with double distilled water ($ddH_2O$) and their legs removed for genetic population studies. The flies were air dried and placed in a 1.5 ml Eppendorf® tube. Total genomic DNA was extracted from individual whole flies using the DNeasy Blood and tissue kit (QIAGEN, Germany) following the manufacturer's protocol. A sterile blue micro pestle (Kontes, Vinelan, NY) was inserted into the Eppendorf tube with the fly, frozen with liquid nitrogen, and pulverised with the pestle to a fine powder. Subsequently 200 µl of ATL buffer solution and 20 µl of Proteinase-k were added to the powder obtained per individual tube and homogenised using a micropipette and incubated overnight at 55°C in a water bath. The following morning 410 µl of AL buffer was added per sample tube and vortexed.

A DNeasy 96-well plate was placed on top of a 96-well S-block and 610 µl of supernatant transferred from each Eppendorf into the 96-well DNeasy plate using a micropipette using a new tip for each sample. After the transfer of all samples the plate was sealed with an Air Pore adhesive tape sheet (QIAGEN, Germany) and centrifuged at 5 400 revolutions per minute (rpm) for 5 minutes. Thereafter, the samples were washed initially with 500 µl of AW1 buffer, sealed with a new Air Pore and centrifuged again for 5 minutes at 5 400 rpm. This process was repeated with 500 µl of buffer AW2. Lastly, the DNA was eluted with 200 µl of AE buffer and sealed with a new Air Pore and centrifuged at 5 400 rpm for 5 minutes. The DNA concentration was measured with a Nano-drop spectrophotometer and the 96-well plate containing the extracted DNA stored at -20°C until used.

### 5.2.4 Detection of endosymbionts by Polymerase Chain Reaction (PCR)

A total of 350 flies were used for the detection of endosymbionts. Hundred *G. brevipalpis* were from the colony at the ARC-OVR and 100 from Phinda. For *G. austeni,* 50 flies each were used from the colony, Eastern Shores and False Bay.

Each PCR reaction for DNA amplification of different endosymbionts was prepared in a 25 µl final reaction volume which included: a 1.1 x 22.5 pre-aliquoted 96 well PCR plate with master mix was used, a 1 µl (10 µM each) of primers used both forward and reverse for each symbiont, 1.5 µl of DNA. The PCR conditions for each reaction as well as their references are stipulated in Table 5.1. In all PCR reactions, molecular grade water was used as a negative control. Samples that tested positive in trial experiments were used as positive controls for the different endosymbionts. This was because species-specific primers were used for the amplification of various endosymbionts and

as a result, positive amplifications were determined by a bright band at the corresponding band size on a 1 500 bp ladder on the 2% agarose gel.

### 5.2.5 Detection of *Trypanosoma* species by ITS-PCR

A total of 437 flies from the field were used for the detection of *Trypanosoma* species. *Glossina brevipalpis* was represented by 250 samples (30 from Eastern Shores, 170 Phinda and 50 from Hluhluwe-iMfolozi Park) whilst for *G. austeni* 187 flies were used (77 from False-Bay Park, 57 Eastern Shores and 53 Lower Mkuze Park). Universal Internal transcribed spacer-1 (ITS-1) (Ouedraogo *et al.,* 2018) primers were used for the detection of various *Trypanosoma* species (Table 5.1). The PCR reaction was prepared as described above. The PCR conditions are summarized in Table 5.1.

**Table 5.1: Primers and PCR conditions used for the detection of endosymbionts and trypanosomes in field collected and colonised *Glossina brevipalpis* and *Glossina austeni***

| Name / Reference | Primer Sequence 5'-3' | Gene region | PCR conditions and cycles | Product size (bp) |
|---|---|---|---|---|
| ***Sodalis*** **SODFLIC (Toh *et al*., 2006)** | GCAGTTTCAGGATACCC GGCGGAAAATGGTATAG | *fliC* | 95°C for 5 min, 95°C for 30 s, 52.5°C for 30 s, 72°C for 30 s, 34 cycles followed by 72°C for 12 min | 508 |
| ***Spiroplasma*** **63F+TKSS (Mateos *et al.,* 2006)** | GCCTAATACATGCAAGTCGAAC TAGCCGTGGCTTTCTGGTAA | *16rRNA* | 94°C for 2 min, 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, 40 cycles followed by 72°C for 5 min | 455 |
| ***Trypanosome*** **ITS1-CF and ITS1-BR (Ouedraogo *et al.,* 2018)** | CCGGAAGTTCACCGATATTG TTGCTGCGTTCTTCAACGAA | *ITS1 of rDNA* | 95°C for 5 min, 95°C for 30 s, 58°C for 30 s, 72°C for 30 s, 34 cycles followed by 72°C for 10 min | 250 to 710 |

### 5.2.6 Gel electrophoresis

To visualize the amplified DNA, PCR products were exposed to 2% agarose gel electrophoresis. The 2% agarose gel was prepared with 100 mL 1X TAE buffer (40 mM

Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0) and 2 g of agarose powder. The agarose gel was stained with a low range SYBR® Green dye and a 1 500 bp ladder was used to determine the corresponding band size of the amplified DNA and visualized under UV-light (Figure 5.2).

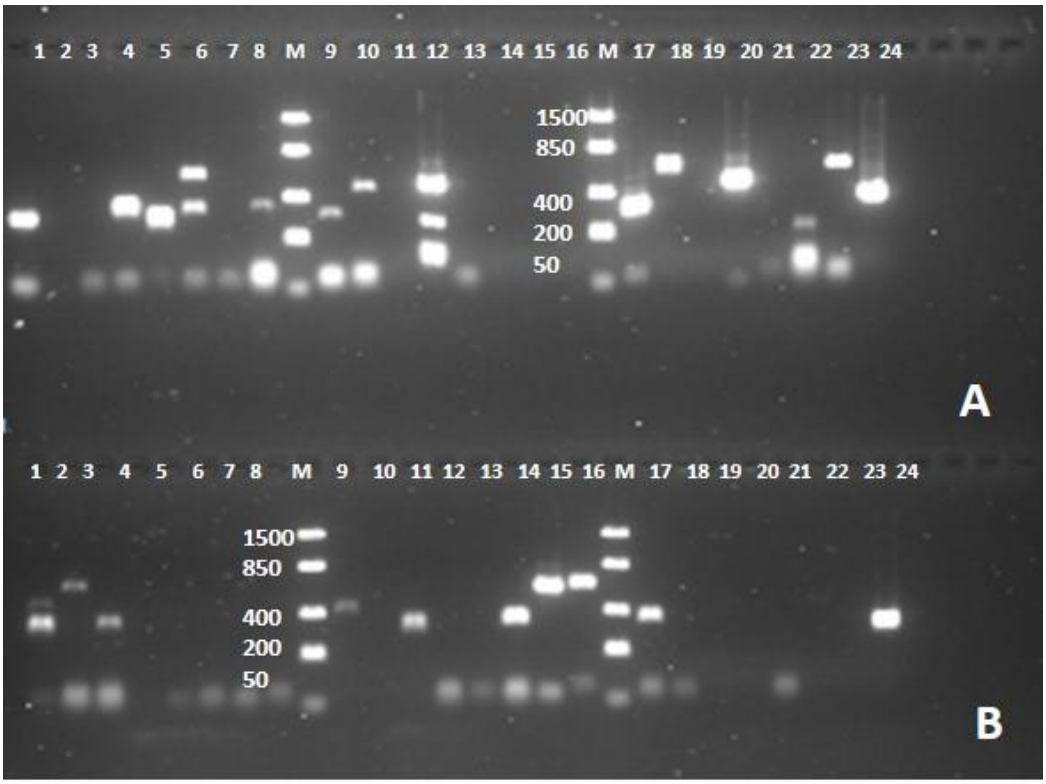


**Figure 5.2: Gel electrophoresis of DNA samples amplified with ITS-1 PCR showing various trypanosome species from tsetse collected in north-eastern KwaZulu-Natal. Gel-A Lane 1 and Gel-B Lane 24 = positive controls. Gel-A Lane 2 and Gel-B Lane 22 = negative controls. Gel-B M = 1 Kb DNA ladder. Gel-A Lanes 4, 6, 8, 17 & 24 = samples that tested positive for *Trypanosoma theileri*. Lanes 5 & 9 = samples that tested positive for *Trypanozoon.* Lanes 6, 10, 18, 20 & 23 = samples that tested positive for *Trypanosoma congolense.* Lane 22 = a sample that tested positive for *Trypanosoma vivax* and Lane 6 = a co-infection of *T. theileri* and *T. congolense.* Gel-B Lanes 1, 3, 9 & 17 = samples that tested positive for *T. theileri.* Lanes 2, 14 & 15 = samples that tested positive for *T. congolense.***

### 5.2.7 Statistical analysis

The prevalence of the detected symbionts and trypanosome parasites infections found in the tsetse were expressed in terms of parasite infection rate and symbiont occurrence rate and expressed as percentages. Fisher's Exact Test was applied to differentiate

between proportions of infected *G. austeni* and *G. brevipalpis* as was found at different sites. Testing was conducted at the 95% level of significance. GraphPad Instat (version 3.00, 2003) was used to for statistical analysis.

## 5.3 Results

### 5.3.1 Symbiont prevalence in colony and field collected *G. brevipalpis* and *G. austeni*

The prevalence of the symbionts as found in colony and wild collected *G. brevipalpis* and *G. austeni* is shown in Table 5.2**.**

**Table 5.2: Prevalence of *Spiroplasma* and *Sodalis* in colony and field collected *Glossina brevipalpis* and *Glossina austeni* in north-eastern KwaZulu-Natal, South Africa.**

| Location | No of flies tested | *Spiroplasma* (%) | *Sodalis* (%) |
|---|---|---|---|
| *G. brevipalpis* | | | |
| Colony | 100 | 37 (37.0) | 96 (96.0) |
| Phinda | 100 | 0 (0) | 4 (4.0) |
| *G. austeni* | | | |
| Colony | 50 | 15 (30.0) | 0 (0) |
| False Bay | 50 | 33 (66.0) | 0 (0) |
| Eastern Shores | 50 | 0 (0) | 0 (0) |

*Sodalis* was found to be highly abundant in the colonised *G. brevipalpis.* at 96% and was least detected in specimens collected from Phinda private game reserve at 4%. In contradiction, *Sodalis* was not detected in 50 colonised *G. austeni* assayed and it was also absent in 50 *G. austeni* assayed from False Bay and Eastern Shores respectively (Table 5.2).

*Spiroplasma* infection in the colonised *G. brevipalpis* (37%) was significantly lower (P < 0.01) compared to that of *Sodalis* (96%) (Table 5.2). Although *Spiroplasma* was detected in 37% of colonised *G. brevipalpis* it was absent from all samples collected from Phinda (n=100), indicating a significantly higher (p < 0.01) infection prevalence in the colonised flies*. Spiroplasma* infection in the colonised *G. brevipalpis* (37%) and *G. austeni* (30%) did not differ significantly (p < 0.01). The higher infection prevalence detected in *G. austeni* (66%) collected in False Bay was significantly (p > 0.06) different from that detected in colonised *G. austeni* (30%) or those collected at Eastern Shores (0%) (Table 5.2).

### 5.3.2 Trypanosome prevalence in field collected *Glossina brevipalpis* and *G. austeni*

At least four *Trypanosoma* species were detected from 437 *G. brevipalpis* and *G. austeni* collected in north-eastern KZN between October 2018 and March 2019. Generally, one or more *Trypanosoma* species were detected in 76 (17.4%) of 437 field collected tsetse tested (Table 5.3). The overall infection prevalence was significantly higher (p < 0.01) in *G. austeni* (25.1%) compared to *G. brevipalpis* (11.6%). Lower Mkuze had the highest infection prevalence of 43.4% detected from *G. austeni* compared to those collected from other sites (Table 5.3). The highest infection prevalence of 43.3%, detected in *G. brevipalpis* collected at Eastern Shores, was not statistically different (p < 0.01) from those collected from Hluhluwe-iMfolozi Park (32.0%), and Phinda Private Game reserve (0%) (Table 5.3). Remarkably, no trypanosome infections were detected in flies collected from Phinda private reserve.

With an overall prevalence of 8.2% *Trypanosoma theileri* was the most prevalent species detected (Table 5.3). Its higher overall infection prevalence in *G. austeni*

(12.3%) was significantly different ($p < 0.05$) to that of 5.2% found in *G. brevipalpis*. It was absent in 187 *G. austeni* specimens collected at False Bay and its prevalence did not differ significantly from than of *T. congolense,* the second most abundant species at Lower Mkuze ($p < 0.01$) and Eastern Shores ($p < 0.05$). For *G. brevipalpis* the highest prevalence of *T. theileri* was recorded at Eastern Shores (Table 5.3). The higher infection prevalence of 36.7% recorded in *G. brevipalpis* at Eastern Shores was significantly ($p < 0.01$) different from that of 4.0% recorded at Hluhluwe-iMfolozi Park.

The overall higher infection prevalence of the pathogenetic *T. congolense* in *G. austeni* (8.0%) was significantly different ($p < 0.01$) from that recorded in *G. brevipalpis* (2.4%) (Table 5.3). The highest abundance of *T. congolense* recorded in *G. austeni* at Eastern Shores was only significantly different ($p < 0.05$) from that recorded at False Bay. The infection prevalence as found in Eastern Shores, 12.3%, and Lower Mkuze (11.3%) did not differ significantly ($p < 0.01$). In *G. brevipalpis, T. congolense* was recorded in 6 (12.0%) of 50 specimens assayed (Table 5.3).

A second pathogenetic species, *T. vivax,* was recorded in low prevalence in both *G. austeni* and *G. brevipalpis* (Table 5.3). The higher infection prevalence of 1.1% found in *G. austeni* did not differ significantly from that of 0.8% found in *G. brevipalpis* ($p < 0.01$). While *T. vivax* was not detected in 57 *G. austeni* assayed from Eastern Shores it was detected in 1 of 30 (3.3%) *G. brevipalpis* from this site (Table 5.3).

**Table 5.3: Prevalence of trypanosome infection in field collected *Glossina austeni* and *Glossina brevipalpis* with the co-infections of trypanosome species in north-eastern KwaZulu-Natal**

| Location | No of flies tested | No of flies tested positive for the presence of *Trypanosoma* species (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Tspp* | Tc | Tv | Tz | Tt | TcTv | TcTt |
| ***Glossina austeni*** | | | | | | | | |
| Lower Mkuze | 53 | 23 (43.4) | 6 (11.3) | 2 (3.8) | 2 (3.8) | 13 (24.5) | 1 (1.9) | 1 (1.9) |
| Eastern Shores | 57 | 22 (38.6) | 7 (12.3) | 0 (0.0) | 6 (10.5) | 10 (17.5) | 0 (0.0) | 1 (1.8) |
| False Bay Park | 77 | 2 (2.6) | 2 (2.6) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| **Total: *G. austeni*** | 187 | 47 (25.1) | 15 (8.0) | 2 (1.1) | 8 (4.3) | 23 (12.3) | 1 (0.5) | 2 (1.1) |
| ***Glossina brevipalpis*** | | | | | | | | |
| Phinda | 170 | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Eastern Shores | 30 | 13 (43.3) | 0 (0.0) | 1 (3.3) | 1 (3.3) | 11 (36.7) | 0 (0.0) | 0 (0.0) |
| Hluhluwe-iMfolozi Park | 50 | 16 (32.0) | 6 (12.0) | 1 (2.0) | 7 (14.0) | 2 (4.0) | 0 (0.0) | 0 (0.0) |
| **Total: *G. brevipalpis*** | 250 | 29 (11.6) | 6 (2.4) | 2 (0.8) | 8 (3.2) | 13 (5.2) | 0 (0.0) | 0 (0.0) |
| **Overall Total: *G. austeni* + *G. brevipalpis*** | 437 | 76 (17.4) | 21 (4.8) | 4 (0.9) | 16 (3.7) | 36 (8.2) | 1 (0.2) | 2 (0.5) |

*Tspp = *Trypanosoma* species; Tc = *Trypanosma congolense*; Tv = *Trypanosoma vivax* = Tz *Trypanozoon* sp.; Tt = *Trypanosoma theileri*

*Trypanosoma* species belonging to the subgenus *Trypanozoon* was detected in both *G. austeni* and *G. brevipalpis* (Table 5.3). The higher infection prevalence of 4.3% found in *G. austeni* did not differ significantly ($p < 0.05$) from that of 3.2% found in *G. brevipalpis* (Table 5.3). Likewise, the higher infection prevalence, 10.5%, found in *G. austeni* collected at Eastern Shores did not differ significantly ($p < 0.05$) from that of 3.8% found in *G. austeni* collected at Lower Mkuze. The higher infection prevalence of 14.0% found in *G. brevipalpis* collected at Hluhluwe-iMfolozi Park did not differ significantly ($p < 0.05$)

from that of 3.3% in *G. brevipalpis* collected at Eastern Shores (Table 5.3). The infection prevalence found in *G. austeni* (10.5%) and *G. brevipalpis* (3.3%) collected in Eastern Shores also did not differ significantly ($p < 0.01$).

Co-infections with *T. congolense* with either *T. vivax* (1.9%) or *T. theileri* (1.9%), as seen by multiple bands on the agarose gel (Figure 5.2), were recorded in single specimens of *G. austeni* collected at Lower Mkuze (Table 5.3). Co-infections with *T. congolense* and *T. theileri* were recorded in *G. austeni* collected in Eastern Shores (Table 5.3). No co-infections between *Trypanozoon* species and other trypanosomes were detected.

## 5.4 Discussion

In the current study, PCR with species-specific primers was used to detect endosymbionts DNA (*Sodalis* and *Spiroplasma*) from colonised and field collected north-eastern KZN populations of *G. austeni* and *G. brevipalpis*. In addition, the presence of trypanosome DNA, using ITS-1 universal primers, from *G. austeni* and *G. brevipalpis* collected in KZN was determined. The findings of this study were based on the expected band size which were observed on the 2% agarose gel and should be noted that positive samples were not sequenced. The author suggests that the positive samples be further explored for possible different strains of the bacteria as well as that of the parasite.

While *Sodalis* was found to be highly abundant in the colonised *G. brevipalpis* it was relatively low abundant in field collected specimens. It could not be detected in 50 colonised and 100 field collected *G. austeni.* In agreement with the results obtained for *G. brevipalpis, Sodalis* is usually found to be highly abundant in insectary-reared tsetse

(Lindh & Lehane, 2011; Geiger *et al.,* 2018). Lindh and Lehane (2011) have reported that, the abundance of *Sodalis* in tsetse flies varies between species e.g., in Liberia *S. glossinidius* was detected in 85% of *Glossina nigrofusca,* 31% of *Glossina pallicera* and 9.3% of *G. p. palpalis* tested. The low prevalence, <2%, or absence of *Sodalis* may be due to the fact that genetically distant species of *Sodalis* are known to be present in some wild captured tsetse (Wang *et al.,* 2013).

*Spiroplasma* DNA was detected from colonised *G. brevipalpis* and *G. austeni* at 37% and 30% respectively. While it was absent in 100 field collected *G. brevipalpis* its prevalence ranged from 0% (Eastern Shores) to 66% (False Bay) in *G. austeni*. Variations in prevalence of *Spiroplasma* is unexceptional as this bacterium has previously been reported to occur in variable abundances in wild and laboratory reared *G. f. fuscipes* from Uganda (Schneider *et al.*, 2019). Additionally, it has been reported that *Spiroplasma* infections may have a significant negative impact on trypanosome transmission efficiency by the host tsetse. It is therefore suggested that this bacterium be further explored, especially so in colonized flies designated to be used in a Sterile Insect Technique (SIT), to potentially inhibit the infection of trypanosome parasites in the released flies.

Trypanosomal DNA was detected in 17.4% of field collected tsetse assayed with a relative high prevalence of *T. congolense* and *T. theileri*. These results agree with that of Taioe (2013) who reported that the most dominant species in bovine serum from the study area were *T. congolense* at 57% followed by *T. theileri* at 43%. This finding was validated by DNA sequencing and BLASTn analysis on the results sequenced for the positive amplicons were both *T. congolense* and *T. theileri* showed strong identity scores from 90% to 98% (Taioe, 2013). Mamabolo *et al*. (2009) also reported *T.*

*congolense* to be prevalent and widespread in most bovines sampled at dip tank in the Hlabisa municipality in the north-eastern parts of KZN. The present results, indicating a higher infection prevalence of *T. congolense* in *G. austeni* compared to that in *G. brevipalpis* agree with that of Motloang *et al*. (2012) who reported a 1% prevalence of *T. congolense* in *G. brevipalpis* midgut compared to an 8% infection from the midgut and salivary glands of *G. austeni*.

The most abundant *Trypanosoma* species detected in the present survey was the non-pathogenic *T. theileri. Trypanosoma theileri* resorts in the subgenus Megatrypanum of the section Stercoraria (Stevens & Brisse, 2004) along with other non-pathogenic trypanosome species of ruminants such as *Trypanosoma melophagium* (sheep), *Trypanosoma theodor* (goats), *Trypanosoma cervi* (deer) and other trypanosome species of wild ruminants (Fisher *et al.,* 2013; Ganyukova *et al.,* 2018; Suganuma *et al.,* 2019). *Trypanosoma theileri* was discovered by Theiler in 1902 in South Africa as common parasite of bovines (Suganuma *et al.,* 2019). It is a relatively large trypanosome parasite and are mainly found in domestic and wild ruminants. It has a cosmopolitan distribution in domestic cattle and is commonly found in African antelopes (Stevens & Brisse, 2004). The main mechanical vector of this species is considered to be horse flies (Diptera, Tabanidae) (Hoare, 1972; Stevens & Brisse, 2004; Taioe *et al.,* 2017). In the present study the trypanosomes species were differentiated by their ITS-1 size. The ITS-1 region of trypanosomes varies in size (Desquesnes *et al.,* 2001; Desquesnes & Davila, 2002), however some exceptions are known e.g., closely related members of the subgenus *Trypanozoon*. Furthermore, some of the sizes are close to each other in the case of *Trypanosoma godfreyi*, *T. simiae* and *T. theileri* which can lead to uncertainty and potential misidentification. In this case sequencing of PCR products would need to be done to confirm. However, the findings by Mnkandla (2022)

while comparing the diagnostic techniques for the detection of trypanosomes in the northern KZN, discovered that some of the *T. brucei* parasite DNA (66.7%) were found to be identified as *T. theileri* by the *T. theileri* cathepsin L (CATL) qPCR assay. Its detection limit of the *T*. *theileri* cathepsin L-like (CATL) qPCR assay was $10^2$ copies /reaction which correspond to a cut-off quantification cycle (Ct) value of 37.9. The ITS qPCR only detected *T. congolense* and *T. brucei* with no mixed infections observed in the cattle tested (Mnkandla *et al.,* 2022).

Another subgenus of *Trypanosome* which was found in near equal numbers in both species and relativity widespread in the area was *Trypanozoon. Trypanozoon* is the most homogeneous subgenus group of salivarian trypanosomes. There are at least three recognised species contained in this subgenus, i.e., *T. brucei*, *Trypanosoma evansi* and *Trypanosoma equiperdum* (Wen *et al.,* 2016). Although these three species are morphologically indistinguishable, even using some molecular methods, they exhibit distinct epidemiological, pathological and genetic characteristics (Wen *et al.,* 2016). Taioe (2013) reported on the presence of *T. brucei* species which was further supported by DNA sequencing and Basic Local Alignment Search Tool (BLASTn) analysis. Since some of the species in this subgenus can cause severe disease in humans, e.g., *T. brucei* cause human sleeping sickness, and other animals, e.g., *T. evansi* causes a form of surra in horse, the apparent presence of this subgenus in the area will require further investigations as there are no publications, other than that of Taioe (2013) and Taioe *et al.,* 2017, that reports on the presence of *T. brucei* or *Trypanozoon* species in South Africa.

In the current study, the pathogenetic *T. vivax* DNA was detected in 3.8% of the *G. austeni* sampled at Lower Mkuze. In *G. brevipalpis* it was detected in 3.3% and 2.0% in

collections made at Eastern Shores and Hluhluwe-iMfolozi Park, respectively. These findings agree with those of Mamabolo *et al.* (2009) where *T. vivax* was found to be the second most dominant trypanosome species in samples collected north-eastern KZN. In contradiction Taioe (2013) did not report any *T. vivax.*

Limited vector competence studies indicated *G. austeni* to be the more competent vector of *Trypanosoma* parasites in the area (Motloang *et al*., 2012; 2014). Interesting enough is that the absence of *Sodalis* in colony and field collected flies exhibit the susceptibility of trypanosome by *G. austeni* compared to that of *G. brevipalpis* with the high abundance of *Sodalis* from the colony flies.

The relative low vector competence of *G. brevipalpis,* compared to that of *G. austeni,* as found in previous studies in South Africa may have resulted in an underestimation of the importance of this species in the epidemiology of nagana in north-eastern KZN (de Beer *et al.,* 2016). Vector competence studies 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 for *T. congolense* in field collected *G. brevipalpis* collected in field could be 2.6% (Harley 1967a, b; Moloo *et al.*, 1980).

Co-infections of abundant trypanosome species were observed in relatively low abundance in *G. austeni.* Co-infections can occur when a tsetse fed on infected multiple hosts or the fly was infected with a certain trypanosome species and fed on an infected animal host infected with a different species. These results correspond with those of Mamabolo *et al*. (2010) who found mixed infections of *T. congolense* and Kilifi type *T. congolense* in the midgut and proboscis of *G. brevipalpis.* It is yet to be determined if a

vector once infected with two or more strains will be able to transmit both strains simultaneously and with the same efficiency?

## 5.5 Conclusion

The current study indicated that the endosymbionts *Sodalis* and *Spiroplasma* are present in the sampled flies and that their abundance can vary significantly between species and populations in the same different sampling sites.. Field infections rates seemed to vary from that of colonised flies. It is worth noting that the current results depend on the PCR amplification conditions employed in this study. And it should be highlighted that the *Spiroplasma* was recently found in *G. f. fuscipes* and *G. tachinoides*. So far, the role of this bacterium in tsetse is largely unknown and the detection thereof in *G. austeni* and *G. brevipalpis* needs to be explored by sequencing of the positive samples, since this fly species belong to the *morsitans* (savannah) and *fusca* (forest) group respectively. The implication of these findings on the efficiency of a potential SIT component as part of a potential control program in South Africa needs to be determined.

Furthermore, it was indicated that several *Trypanosoma* species are found at variable prevalence in the sampled area and in both tsetse species. Despite a reported decline in nagana in cattle in the area (personal communication Dr. L. Ntantiso, DARD - Veterinary Services Department KZN) the two pathogenic *Trypanosoma* species, *T. congolense* and *T. vivax*, were still relative abundant in vectors in the area. The study highlighted the dynamics of the epidemiology of nagana and the fact that trypanosome infection rates and endosymbiont abundance in tsetse are not constant and can change over time and between populations. E.g., In Uganda, infection rates varied considerably

with the different seasons and with the age of the *G. brevipalpis* population (Harley 1966, 1967a, b).

However, this study was not able to establish a correlation between endosymbionts and trypanosome infections from the sampled flies because different samples were used for each objective. As such, more analysis to establish a correlation between endosymbionts and trypanosome infections in wild South African tsetse is required. Furthermore, DNA sequencing should be conducted on the detected trypanosome species for accurate identification as it is reported that the non-pathogenic parasite *T. theileri* occurs globally and can sometime confuse the diagnostic techniques used and the pathogenicity of these detected trypanosome species needs to be further evaluated.

# CHAPTER 6

# CONCLUSIONS AND RECOMMENDATIONS

## Conclusions

The present study was designed to compare tsetse abundance and apparent density (AD) from samples collected from 2008-2009 and 2018-2019. This data confirmed the perceived higher abundance of *G. brevipalpis* in the area. The higher AD of tsetse flies in protected areas found in this study, highlighted the role of protected areas and Game Reserves in maintaining high populations of tsetse flies with the possibility of reinvasion from uncontrolled protected areas into adjoining communal farming areas. Over the last decade various forms of wildlife-oriented land use, e.g., game farms and bush meat trading have increasingly become prominent features in KZN (Spierenburg & Brooks, 2014). This expansion of protected areas in combination with an apparent reluctance to eradicate tsetse flies in these areas (Armstrong & Blackmore, 2017) increases the probability of migration into the adjoining farming areas and increases the threat of nagana transmission in livestock.

Based on comparisons of tsetse abundance, as determined in 2008-2009 to that of 2018-2019, there was no change in the AD of *G. brevipalpis* in neither the communal farmlands nor the protected areas. Although the AD of *G. austeni* decreased in both the communal farmlands and protected areas. To sustain the long-term control of nagana in the area the current live-bait control program will need to be supported with alternative tsetse control initiatives. An area wide integrated pest management (AW-IPM) with a sterile insect technique (SIT) component was proposed to eradicate this southernmost pocket of tsetse flies in southern Africa (Kappmeier Green *et al.,* 2007).

Observation from the current data on male mating ability has indicated that both irradiated and non-irradiated males have the ability to mate several times and that propensity of mating, as well the ability to transfer sperm, do not decline significantly in subsequent mating events. Irradiated males were able to inseminate several females at a rate comparable to that of non-irradiated males. The mean life span of male tsetse flies in the wild is approximately four weeks (Jackson, 1949), and the current results indicated that they could mate, and transfer sperm, up to 16 times (or more), in confined conditions in the laboratory. The multiple mating ability of irradiated males, comparable to that of non-irradiated males, will be beneficial in a SIT program as it is capable of reducing the fecundity of females in the wild which may lead to the collapse wild populations.

In addition, the mating ability of irradiated males and the multiple mating behavior of female *G. brevipalpis* under laboratory conditions were determined. Generated data was in agreement with the results of de Beer *et al.* (2017), where it was reported that, the highest number of pupae were produced by females that only mated with fertile males while females inseminated by irradiated males exhibit high abortion rates. Fecundity as found in females that first mated with irradiated and then with fertile males was higher than that of females that mated only with irradiated males, but lower than that of females that only mated with fertile males. These observations are similar to those of Curtis (1968) that demonstrated that, *G. austeni* females that re-mate, the sperms from the first mating are used more frequently than that from subsequent mating events. The results indicated that the release of sterile males may still reduce female fecundity despite the apparent multi mating behavior exhibit by tsetse females.

An under-laying risk of applying the SIT in an endemic area is that the sterile males may disperse widely and potentially transmit trypanosomes for several weeks after their release. The presence of certain endosymbionts e.g., *Sodalis,* can render tsetse flies to be resistant to trypanosome infections (Wang *et al.,* 2013; Demirbas-Uzel *et al*., 2018). Information on the endosymbiont component of South Africa's tsetse populations is limited. The current study, focusing on the detection of *Sodalis* and *Spiroplasma* in colonised and field collected tsetse flies, indicated that there is variation in the abundance of symbionts between colonized and field tsetse flies and between different species as well as different fly populations. The implication of this variation on the efficiency of a potential SIT component as part of a potential control program in South Africa still requires to be explored further.

The present study indicated that several species of *Trypanosoma* can be found at variable abundancies in the area and in both tsetse species. Despite an apparent decline in nagana in cattle in the area the two pathogenic *Trypanosom*e species, *T. congolense* and *T. vivax*, were still relative abundant in the area. In line with previous studies *T. congolense* was the more abundant of the two species (Mamabolo *et al*., 2009) and that *G. austeni* exhibits a higher infection prevalence than *G. brevipalpis* (Motloang *et al*., 2012).

The implication of the relative high infection prevalence of non-pathogenic species *T. theileri,* the most abundant *Trypanosoma* species in the current survey, as found in *G. brevipalpis* and *G. austeni* will need further investigations. Although mixed *Trypanosoma* infections were found in the present survey, the influence thereof on vector competence remains unknown.

The association between endosymbionts and trypanosomes could not be established in the present study. This was due to the relatively small sample size and because different samples were used in determining endosymbionts and trypanosomes abundance. It is worthwhile to note that the higher vector competence of *G. austeni* as reported by Motloang *et al*. (2012), may be linked to the apparent absence of *Sodalis* in this species as suggested by Wang *et al.* (2013). The study highlighted the dynamics of the epidemiology of nagana and the fact that trypanosome infection rates and endosymbiont abundance in tsetse can change over time and between populations.

**Recommendations**

The determination of the accurate distribution of the targeted tsetse fly species, the interaction between the insect and the causative agent it transmits, and the factors that will influence these interactions, will be vital for the implementation of any proposed control campaign, and will directly affect its outcome, sustainability and cost (Vreysen *et al*., 2007; Shaw, 2009). The improvement of tsetse control strategies will be beneficial in a changing environment of the African continent. The following can be considered as future research aspects:

1) Considering the dynamic nature of tsetse abundance and distribution tsetse fly abundance needs to be monitored constantly at selected sites in the area to predict the risk of nagana. Ongoing efforts to generate national Atlases of tsetse flies and AAT (Cecchi *et al*., 2014; 2015) will benefit from continuously updated data sets. The availability of habitat suitability maps will be advantageous to develop an appropriate management strategy. Habitat suitability maps will help to focus future entomological surveys. These, distribution maps need to be supported by knowledge of the biology and vector competence data of the tsetse

species and populations involved. More communal areas and protected lands should be sampled to accurately confirm the current observations as the current study did not sample the entire known distribution of these tsetse flies.

2) The study indicated that, despite the apparent perceived success of the current control program, tsetse abundance and *Trypanosoma* infections are high in field collected flies in the area. *Trypanosoma* in tsetse flies and livestock need to be monitored on a regular basis. Furthermore, DNA sequencing should be conducted on the detected *Trypanosoma* species for accurate identification as well as the pathogenicity of these detected trypanosome species needs to be evaluated.

3) Male multiple mating ability and multiple mating behavior of *G. brevipalpis* females as demonstrated under laboratory conditions need to be repeated under field or semi field conditions, e.g., in field cages to determine to what extent the relative confined conditions in the laboratory have contributed to this behavior and what would be the situation in an open space which will represent the field environment.

4) An understanding of the interactions between all the known symbionts and the pathogens occurring within tsetse hosts may facilitate the development of tsetse refractory to trypanosome infection by manipulating the symbionts (Demirbas-Uzel *et al.,* 2018). As such, more analysis to establish an association between endosymbionts and trypanosome infections in wild South African tsetse is required.

5) It is furthermore unclear to what extent *Wolbachia* infections may influence fecundity, parthenogenesis, sex ratios and/or mate discrimination traits in South African tsetse populations. These traits form the basis of this bacteria utility in vector control as observed in other *Wolbachia*-infected arthropods (Hofmann *et al*., 2015, Werren *et al*., 2008).

The findings of the present study will contribute to the improvement of the proposed AW-IPM tsetse fly elimination strategy for southern Africa. The results can furthermore be used in the decision making of control in other regions in Africa where these two species may act as vectors of *Trypanosoma* parasites.

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## CONGRESS PROCEEDINGS

Full delegate on two chapters of my MSc at the10th Biennial SA Veterinary & Paraveterinary (SAVA) Congress 2019 which was held at the Emperors Palace, Kempton Park from 15-18 July 2019, p.32. **(Title: ASSESSING THE MATING PROFICIENCY OF GLOSSINA BREVIPALPIS UNDER LABORATORY CONDITIONS)**.

**Abstract:** A single bite of an infected tsetse fly may transmit the unicellular parasites that causes African Human Trypanosomiasis (Sleeping sickness) and Animal African Trypanosomiasis (Nagana). Sterile Insect Technique (SIT) has been proposed for the control of tsetse in South Africa. The implementation of a SIT relies on the release of high quality sterile males that can compete with their wild counterparts. Research on quality control aspects and mating behaviour of colonised *Glossina brevipalpis* in relation to its potential to be used in SIT are lacking. The multiple mating behaviour of females mated with irradiated males and then re-mated with untreated males and the mating potential of irradiated compared to untreated males were determined. A Caesium Gamma cell, providing 0.69 Gy/min, was used to administrate a dose of 80 Gy to sterilise *G. brevipalpis* males. In the multiple mating experiment, 96% (115) of the females mated with the irradiated males during their first mating. Of these 115 mated females, 63 mated again the following day with fertile males, i.e., a re-mating rate of 55%. Induced sterility varied from 45% to 66% between replicas. There was no reduction in the mating rate or mating potential of the males throughout the duration of the experiment. The highest number of matings was 23 and sperm was transferred every time. In the absence of any correlation between mating rate, spermatecae fill and mating event, it is recommended that individual flies should be monitored to assess their mating potential until mating attempts by male insects ceased.

## LIST OF PUBLICATIONS CO-AUTHOR

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