ELECTROPHORETIC ENZYME COMPARISON OF WILD AND LABORATORY TSETSE FLY POPULATIONS OF *GLOSSINA PALLIDIPES* AND *G. BREVIPALPIS* (DIPTERA: GLOSSINIDAE)

by

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University or institution.

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Date: 9. 10. 1990

This thesis has been submitted for examination with our approval as University supervisors

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DEDICATION.

To my mother Mrs. Merenia Bujune and Uncle D.B. Barisigara.

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LIST OF ABBREVIATIONS.

Alkph	=	Alkaline phosphatase locus
AO	=	Aldehyde oxidase
Ao	=	Aldehyde oxidase locus
Apk	=	Arginine phosphokinase locus
D	=	Genetic distance
EDTA	=	Ethylene diaminetetraacetic acid
EST	=	Esterase
Est	=	Esterase locus
G6pd	=	Glucose-6-phosphate dehydrogenase locus
GPI	=	Glucose phosphate isomerase
H	=	Heterozygosity
$\bar{H}$	=	Mean heterozygosity
HEX	=	Hexokinase
Hex	=	Hexokinase locus
I	=	Nei's identity
$\bar{I}$	=	Nei's mean identity
ICIPE	=	International Centre of Insect Physiology and Ecology
ILRAD	=	International Laboratory for Research on Animal Diseases
KETRI	=	Kenya Trypanosomiasis Research Institute
Lap	=	Leucine aminopeptidase
MDH	=	Malate dehydrogenase
Mdh	=	Malate dehydrogenase locus
ME	=	Malic Enzyme

Me	=	Malic enzyme locus
MPI	=	Mannose-6-phosphate isomerase
NADD	=	Nicotinamide adenine dinucleotide dehydrogenase
Nadd	=	Nicotinamide adenine dinucleotide dehydrogenase locus
P	=	Proportion of polymorphic loci
PMS	=	Phenazine methosulphate
PGM	=	Phosphoglucomutase enzyme
Pgm	=	Phosphoglucomutase locus
SIT	=	Sterile Insect Technique
TC	=	Tris citrate
TEB	=	Tris EDTA Boric Acid
TM	=	Tris maleate
To	=	Tetrazolium oxidase locus
TRIS	=	Tris-Hydroxymethyl aminomethanol
XDH	=	Xanthine dehydrogenase
Xdh	=	Xanthine dehydrogenase locus
Xo	=	Xanthine oxidase locus

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ABSTRACT.

An electrophoretic comparison of 11 randomly selected enzyme systems was made between two laboratory colonies of Glossina pallidipes and one of G. brevipalpis with their corresponding ancestral wild populations. The samples were compared with regard to the amount of genetic variation within each population. The genetic variability in each population was measured by calculating the proportion of polymorphic loci(P), and the average heterozygosity per locus. Other parameters considered for comparison were:- allele frequency data, the number of alleles per locus and effective number of genotypes at each locus. To quantify the extent to which the colony flies carried electromorphs similar to those found in natural tsetse, the identity statistics, (Nei, 1972) and the standard distance between field and colony flies were calculated. As a partial test of the genetic interpretation of the observed variations, the electrophoretic patterns of the polymorphic loci were examined for their goodness of fit with regard to expectations based on Hardy Weinberg equilibrium model.

Of the 11 enzyme systems, only nine were consistently scorable. The important observations of these investigations are that the field flies were genetically approximately the same as those of the laboratory samples. There was no difference as regards the percentage of polymorphic loci. The difference in allozyme frequencies were not statistically significant. The identity values between field and laboratory samples were high and genetic distances small, showing that the laboratory stocks were still closely related to their ancestral wild populations. It was concluded that colonization has had no significant change on genetic variations within the colony flies.

CHAPTER ONE

GENERAL INTRODUCTION AND LITERATURE REVIEW.

1.1 TAXONOMY OF TSETSE FLIES.

Tsetse is a word of the Tswana tribe of Botswana for insects of the genus Glossina. The genus has been included in the family Muscidae and placed in subfamily Glossinidae (Newstead et al., 1924; Roberts et al., 1979). However, Haeselbarth et al. (1966) and Jordan and Itard (1977) place the genus in a monogeneric family Glossinidae, a view accepted by most taxonomists. The whole question has been thoroughly examined and discussed by Pollock (1971) and Jordan (1974). According to these authors, there are 22 named species and 8 subspecies under the genus Glossina.

Three subgenera or species groups are generally recognized, based mainly on morphology and supported by ecological data. The fusca group, also called subgenus Austenina, the morsitans group (Subgenus: Glossina (s.str.) and the palpalis group (subgenus Nemorhina). The 22 species and the limited number of recognized subspecies are listed in Table 1.1.

Table 1.1 The Genus Glossina (Jordan, 1974)

The morsitans group (subgenus Glossina s.str.)

Glossina longipalpis Wiedemann 1930

G.pallidipes Austen 1903

G.morsitans morsitans Westwood 1850

G.morsitans centralis Machado 1970

G.morsitans submorsitans Newstead 1910

G.swynnertoni Austen 1923

G.austeni Newstead 1912

The palpalis group (Subgenus Nemorhina).

Glossina palpalis palpalis (Robineau-Desvoidy) 1830

G.palpalis gambiensis Vanderplank 1949

G.fuscipes fuscipes Newstead 1910

G.fuscipes martini Zumpt 1933

G.fuscipes quanzensis Pires 1948

G.tachinoides Westwood 1850

G.pallicera pallicera Bigot 1891

G.pallicera newsteadi Austen 1920

G.caliginea Austen 1911

The fusca group (subgenus Austenina).

Glossina nigrofusca nigrofusca Newstead 1910

G.nigrofusca hopkinsi van Emden 1944

G.fusca fusca Walker 1849

G.fusca congolensis Newstead and Evans 1921

G.fusca fuscipleuris Austen 1911

Table 1.1 The Genus Glossina (Jordan, 1974) - continued

G.haningtoni Newstead and Evans 1922

G.schwetzi Newstead and Evans 1921

G.tabaniformis Westwood 1850

G.nashi Potts 1955

G.medicorum Austen 1911

G.vanhoofi Henrard 1952

G.severini Newstead 1913

G.brevipalpis Newstead 1910

G.longipennis Corti 1895

1.2 DISTRIBUTION OF TSETSE FLIES.

Tsetse flies are restricted to tropical Africa; approximately between latitude 15°N and 20° S (Potts, 1973). They cover about 11 million km^2 of the continent. Their Northern limit corresponds closely to the Southern edges of the Sahara and the Somali deserts (Jordan, 1986). The limits are determined by climate and vegetation. Where rainfall is high, seasonal low temperatures are the limiting factor. Below 16°C flies are inactive and their growth is retarded (Bursell and Taylor, 1980). This factor limits fly belts in Southern East Africa and at high altitudes. Within this large tropical African region, different groups are restricted to definite ecological zones.

1.2.1 Distribution of the morsitans groups.

The morsitans group (subgenus Glossina s. str.) typically inhabit the savannah regions of Africa. They are restricted to areas sufficiently wooded to provide resting sites. In wet areas, the flies roam widely over the woodland but in drier parts of their range, they are restricted to the mesophytic vegetation of the water courses particularly during severe dry seasons (Nash, 1937). Glossina morsitans s.l. is the most important species of Glossina. It infests an enormous area of Africa (Appendix 1.1). Glossina morsitans morsitans is encountered from Zimbabwe and Mozambique in the South to Tanzania in the North. Glossina m. centralis extends Northwards from Botswana and Angola into Southern Uganda and G. m. submorsitans has an East-West distribution from Ethiopia to Senegal. Glossina pallidipes on the other hand is found only in East Africa (Appendix 1.2). It occupies thicket and forest edge vegetation. Glossina austeni occupies secondary scrub thicket and islands of forest along the East African coast from Mozambique to Somalia (Jordan, 1986) (Appendix 1.3).

1.2.2 Distribution of the palpalis group.

The palpalis group of tsetse is limited to waterside communities. They are particularly restricted to the two blocks of the African lowland Equatorial forests. However some species extend far out through the humid, into the drier savannahs along rivers and streams. Glossina palpalis s.l. and Glossina fuscipes s.l., are the most economically important species of the group. They occur throughout the lowland rain forests and extend well into the drier savannahs of the continent (Jordan, 1986).

1.2.3. Distribution of the fusca group.

Much less is known about the distribution and ecology of the fusca group. This is partly because none is of major economic importance. Furthermore, many are difficult to detect because they never feed on man and are not attracted to his presence (Jordan, 1986). What is clear is that, with the exception of G. longipennis and G. brevipalpis, members of the fusca group are typically found in dense forests (WHO, 1988). These (Glossina brevipalpis and G. longipennis) occur only in East Africa, the former being associated with islands of forest often along water courses (Appendix 1.4) and the latter in arid habitats.

1.2.4. Distribution of tsetse flies in Kenya.

In Kenya, tsetse flies occur in isolated localities ranging from sea level to approximately 6,000 feet above sea level. They occupy vegetation varying from coastral dry Commiphora/Acacia grasslands to evergreen riverine forests (National Atlas of Kenya, 1970). But the exact distribution of the tsetse in the country is not known (Mungai, pers. comm.). What is available, is a series of isolated reports by different workers in different parts of the country. This is partly because the distribution of tsetse flies in Kenya is not static. In some areas, a recession has occurred due to factors such as damage to bush habitat by fires, overgrazing and cultivation. Advances of tsetse flies outside their previous known areas have also been recorded (National Atlas of Kenya, loc. cit).

However, it is estimated that tsetse flies cover well over 1/4 of total Kenyan land surface, about 138,000Km^2 (National Atlas of Kenya, loc. cit.). The well known foci are: Nkuruman, Lambwe Valley, Rusinga Islands, Galana, Mwalewa, Shimba Hills, Kibwezi and Sigor (Appendix 1.5). The fly species common in the country include: G. pallidipes, G. austeni and G. swynnertoni of the morsitans group; G. fuscipes fuscipes of the palpalis group, G. longipennis, G. brevipalpis and G. fuscipleuris of the fusca group.

1.3 ECONOMIC IMPORTANCE OF TSETSE FLIES.

Members of the genus Glossina are known to be both of great medical and economic importance in tropical Africa. This is mainly because they are vectors of both human and animal African trypanosomiasis; commonly known as sleeping sickness and nagana respectively. A severely disabling and highly fatal disease, trypanosomiasis has been and still is a major obstacle to the development of Africa. The disease covers about 10 million km^2 of the continent (Roberts et al., 1979). There are two trypanosome species causing African sleeping sickness: Trypanosoma brucei gambiense and T. brucei rhodesiense. These parasites are morphologically indistinguishable but produce different clinical syndromes in man and have different epidemiology. The main tsetse-borne trypanosomes causing nagana are Trypanosoma brucei, T. congolense and T. vivax. Trypanosoma uniforme is less important but both T. simiae and T. suis are deadly to domestic pigs. Furthermore, within the genus Glossina, different species have been reported to show different capabilities of transmitting trypanosomiasis.

1.3.1 Sleeping sickness tsetse vectors.

Probably any species of tsetse fly can transmit sleeping sickness in man and indeed all but three (G. severini, G. schwetzi, G. nashi) have either been shown to be capable of such transmission in the laboratory, or have been found infected in nature. However, relatively few species are natural vectors, because many of them rarely if ever feed on man. The major vectors of the Gambian sleeping sickness in West Africa are G. palpalis s.l. and G. tachinoides. On the other hand Glossina fuscipes s.l. is the most important vector in East and Central Africa. The most important vectors of Rhodesian sleeping sickness are G. morsitans s.l., G. swynnertoni, G. pallidipes and G. fuscipes s.l.

1.3.2. The sleeping sickness problem.

Sleeping sickness was a much greater problem in Africa in the past than it is today. It has been estimated that between 1896 and 1906, more than 500,000 people died in the Congo Basin. In some villages 30-50% of the population was infected (Ford, 1971). An epidemic in Uganda between 1902 and 1908 took 200,000 human lives. The population of the affected area was 300,000 people before the epidemic, mortality consequently being 67% of the entire population (Ford, loc. cit.). Duggan (1962) cites a case where an entire tribe, the Rukuba of Northern Nigeria was virtually brought to extinction between 1931 and 1952.

Today, according to Jordan (1986), 10 thousand cases of sleeping sickness are actually reported yearly. A report by WHO/FAO (1979) shows that Zaire was most affected with 33,617

reported cases. This was followed by Tanzania (3,406), Nigeria (2,884), Central African Republic (1,847), Mali (1,631) and Zambia (1,518). The reported cases in the same period in Kenya and Uganda were 140 and 696 respectively. Cases continued to rise in Uganda, reaching a climax of 7,445 in 1981, but fell again to 794 cases in 1983. The increase was due to breakdown of law and order in the country (Okoth, 1985).

In Kenya, sleeping sickness the (Rhodesian type) is limited to a well known 300km^2 of Lambwe Valley, but there is evidence that the disease transmission has escaped the Valley to the nearby Lake Victoria shores (Turner, 1986). Over 200 cases were reported between 1980 and 1984 with several deaths. Currently both ICIPE and KETRI are involved in the disease control in Lambwe Valley. The aim is to lower levels of disease endemicity through tsetse control operations combined with routine surveillance and treatment of cases. Prophylactic measures are taken in case of livestock. There is an obligation to maintain the services if this is to succeed. This commitment entails recurrent financial expenditure both on tsetse control and trypanocidal drug costs.

1.3.3. Nagana tsetse vectors.

Whereas only certain Glossina species in relatively restricted areas of their distribution are infected with trypanosomes that cause sleeping sickness in man, all Glossina species wherever they occur are almost invariably infected with trypanosomes that cause nagana (Jordan, 1974, 1986). Infection rates of 10-15% and 5% in morsitans and palpalis groups respectively are typical. The risk of domestic livestock

contracting nagana is therefore enormously greater than that of man contracting sleeping sickness. Six species of tsetse flies are of outstanding veterinary importance. These are Glossina palpalis s.l., G. fuscipes s.l.. and G. tachinoides of the palpalis group, G. morsitans s.l., G. swynnertoni, and G. pallidipes of the morsitans group. A number of additional species are also of some importance in nagana transmission. These are G. austeni, G. longipennis, G. vanhoofi and G. brevipalpis.

1.3.4. The nagana problem.

The importance of nagana is to a large extent indirect. African savannahs have a vast potential for domestic livestock production but the potential cannot be exploited, particularly where morsitans group of tsetse is present (Jordan, 1986). This results in starvation, particularly among pastoral tribes, resulting from cattle losses due to the disease. In most parts of the continent, cattle are restricted to more arid areas where tsetse cannot survive such as Maasai and Fulani areas of East and West Africa respectively. This is because most of the ample pasturages are avoided due to the vector. This results in insufficient milk and meat production, the much needed animal proteins for the ever increasing human population on the continent.

In Kenya, like anywhere else in the tsetse zone, nagana is more widespread than sleeping sickness. Large numbers of domestic animals die annually and a lot of the limited funds are spent on trypanosomal drugs (Wilde et al., 1983). The most affected districts are Isiolo, Kitui, Kilifi, Kwale, Lamu, Narok, Kajiado, Laikipia and Nyanza.

In spite of the aforesaid, some authors argue that tsetse and trypanosomes should be regarded the most stalwart guardians of the African ecosystem and its magnificient wild fauna. "But for them, vast areas of the continent would turn into a desert through overgrazing and excessive human agricultural practices" (Desowitz, 1976). In view of the importance of trypanosomiasis in man and his livestock as highlighted here, several attempts have been made towards controlling tsetse flies.

1.4. CONTROL OF TSETSE FLIES.

The use of chemical pesticides, destruction of game animals - the source of food for the vectors - and habitat destruction are the methods commonly used to control tsetse flies. However, traps and insecticide impregnated screens today are increasingly being used. Attention has also been devoted to various novel techniques that seem less hazardous to the environment. The use of insect growth regulators, pathogenic viruses, deliberate use of predators, synthetic pheromones combined with insecticides, may all become important in future. In the forefront, however, are the genetic methods, particularly the use of sterile male tsetse flies. Other genetic control strategies that have been attempted include: chromosomal translocations, hybrid sterility and cytoplasmic incompatibility. All these methods rely on mass production of tsetse flies in the laboratory and releasing them among wild populations in the field so that mating with normal insects will either not result in an offspring or lead to reduced fitness such as failure to adapt properly to environment.

1.4.1. The sterile male insect technique.

A lot of funds have been directed to evaluating the sterile male insect technique. The method involves introduction of sterilized males into a wild tsetse population of the same species. The sterile males will suppress the fecundity of the wild flies and in the long run may lead to eradication. The method is based on the fact that tsetse females mate only once and preserve male sperms whether fertile or sterile throughout their life span. Even if they mated twice, which they rarely do, sperms from both matings, sterile and possibly non-sterile males, compete for fertilization of eggs (Jordan, 1986). The prerequisite of the success of the technique is thus dependent on laboratory breeding and sterilization of large numbers of male tsetse and their subsequent release into wild population.

Large scale breeding and sterilization of males has been made possible from experience gained at Bobo Dioulasso, Burkina Faso. Breeding and sterilization of males has been done with G. palpalis gambiensis, G. tachinoides and G. morsitans submorsitans (WHO, 1988). The technique has been tried on a small scale on an island in Lake Kariba, Zimbabwe with G. morsitans morsitans. It achieved 98% control. Two relatively larger trials have been conducted. In Burkina Faso, G. palpalis gambiensis was used (Politzar and Cuisance 1982). In Tanzania, the trial was with G. morsitans morsitans (Williamson et al., 1983). Both achieved over 90% control. Evaluation is still in progress (Jordan, 1986), but results so far show that the technique is able to suppress and in isolated habitats may

even achieve eradication of the vector. A large scale tsetse eradication programme involving the SIT has also been started in Nigeria. It is anticipated that by the end of this project (Biological Control of Tsetse by the Sterile Insect Technique - BICOT) sufficient information will have become available to apply the SIT on a practical basis to eradicate tsetse flies in other parts of Africa (Offori, 1980).

1.4.2. Hybrid sterility.

It has been reported that isolated distribution of tsetse flies in ecologically widely differing localities favours development of behavioural and genetic differences within species (Langley et al., 1984). In the long run, this may lead to speciation or just some form of partial genetic reproductive isolation. This favours certain genetic control procedures. For example, Curtis (1972) showed that the release of G. morsitans morsitans males might be an effective method of controlling other subspecies of the morsitans group. This is because the resulting crosses are sterile. In the same way, G. morsitans s.l. released in the habitat of G. swynnertoni might lead to a considerable reduction in numbers of the latter species (Vanderplank, 1947).

1.5 EFFECTS OF LABORATORY COLONIZATION OF TSETSE FLIES.

The promising genetic control methods of tsetse flies require production of tsetse flies in specialized insectaries and their release in massive numbers to achieve control

(Jordan, 1986). Because of their low fecundity, it takes a long period to produce the required numbers. The insectary in which they are reared is a highly specialized environment specially designed for maximum efficiency of production. Many conditions are maintained to attain a high reproductive and survival rate. Such insects are therefore shielded from environmental pressures of various kinds.

Wild insects on the other hand, live in environments that vary in time and space. They are faced with a much higher selection pressure than the laboratory stock. It has been suggested that organisms subjected to large fluctuations in environmental conditions require more genetic variabity than organisms that live in a more stable environment (Selander and Kaufman, 1973; Joslyn, 1984). Moreover, as a result of founder effect, a form of sampling error, only part of the wild gene pool is actually incorporated into the laboratory stock. This is because the genetic composition of the laboratory stock comes from a limited genetic variation present in the original founders, normally a small number of field flies. It is advisable to establish colonies using as many flies as can be collected from the wild population but this is often not the case.

Furthermore, the genetic constitution of a sample chosen for laboratory colonization can be dramatically affected by the timing of sampling (MacDonald, 1976). In many insects, the activity times might vary among segments of the population, reflecting different genotypes at a given period. This problem can be avoided by collecting the founder members during all discernable periods of daily activity, but this is rarely done.

Moreover, for various reasons, the field sampling techniques used to obtain the founder members have seldom been based on knowledge regarding the ecological genetics of a species concerned. In nature, species consist of genetically unified yet heterogeneous populations (Joslyn, 1984). Each of such local populations is adapted through natural selection to a specific local environment within the broader species habitat. Normally genotypic variations differ from population to population (MacDonald, 1976). If insects intended to establish a laboratory colony came from only one of such populations, part of the species genetic pool would not be represented. Initially, therefore, insects from a variety of geographical areas should be sampled and mixed in the laboratory. This is rarely put into consideration when establishing most colonies.

In addition, a majority of workers believe that species can vary genetically along an ecological gradient (Carson, 1967; Lewontin, 1957; MacDonald, 1976). It is also known that within such ecological gradient, central populations are more subject to density dependent regulations than marginal populations (Haldane, 1956). Because of such findings, laboratory populations might have a gene pool that radically departs from that of wild populations. Furthermore, populations in seasonally variable environments may also periodically be reduced to a very small gene pool. The field populations might also be dramatically reduced through man's efforts to control the pest. A population that passes through such period of reduced numbers will lose some of its alleles

and will take long to recover to the original level of heterozygosity (Ferguson, 1980). This will further widen the gap between the genetic composition of the wild and laboratory populations.

Nevertheless, even if variability was initially similar in the laboratory to that in the field populations, decay of variability is still much more rapid in laboratory colonies because of inbreeding (Joslyn, 1984). Inbreeding, which is inevitable in laboratory colonies, causes genetic decay as heterozygotes decrease and homozygotes increase, a condition known as Wahlund's effect (Boller, 1972; Bush and Neck, 1976; MacDonald, 1976). These homozygous individuals often exhibit harmful traits (Mackauer, 1976). In natural populations however, Wahlund's effect is controlled through gene flow. In the laboratory, effects of inbreeding can be reduced through periodic infusion of fresh field materials, a process often ignored, though necessitated by inbreeding depression. However, maintaining separate laboratory strains under unique conditions and crossing them systematically to increase variability, can also solve the problem but is often ignored too.

Furthermore, selection operates continuously during establishment and maintenance of a colony. During the initial days of a colony for example, directional selection filters out insects that cannot adjust to laboratory conditions. This contributes greatly to genetic decay and accounts for the high mortality of newly introduced field material (Joslyn, 1984). In addition, the periodic removal of large numbers of

individuals from the breeding colony leads to fluctuations in colony size, bottlenecks and concomitant random losses of genotypes. A solution to this is to rejuvenate the gene pool of the surviving colony with wild insects. A colony should be started with large numbers of founders and should not decline below this number if sufficient heterogeneity is to be maintained (Huettel, 1976). This is often ignored too. Drift, the most important process influencing gene frequencies, is very pronounced in colonies (Falconer, 1981; Joslyn, 1984). If gene frequencies are followed in colonies, they are seen to change in an erratic manner from generation to generation. Maintenance of high effective population size however reduces effects of both random drift and inbreeding (Joslyn, 1984).

Inspite of all the aforementioned, most studies of tsetse flies are based on stocks that have been maintained in laboratories for many generations. To provide a source of insects of reasonable standards, it is important that the colonized insects should be as closely akin as possible to wild insects and fully viable (Huettel, 1976; Joslyn, 1984: Mackauer, 1976). Both kind and range of genetic variability should be retained by these colonies if they are to be true representatives of field populations.

1.6 EVIDENCE FOR GENETIC VARIATIONS WITHIN TSETSE FLIES.

The possibility of genetic variations within species of tsetse flies was first suggested by Baldry (1969). Earlier however, Harley (1965) had reported intraspecific differences

in activity patterns of a number of field populations of Glossina pallidipes. This was later confirmed by a number of other workers (Van Jaensen, 1981; Vale, 1981; Van Etten, 1982b). Moreover, Van Etten (1982a) showed that one out of three populations of G. pallidipes in Lambwe valley contained higher fat reserves than the other two. The difference was significant and his conclusion was that it was genetically controlled. Furthermore, a comparison of the performance of laboratory colonies of G. pallidipes from two allopatric populations in Kenya, revealed differences in reproductive rate, puparial weight, duration of copulation and showed polymorphism for a number of their enzymes (Van Etten, 1981). Also Jaenson (1978) reported a genetic basis for difference in copulation time between flies of allopatric populations.

Intraspecific differences in reproductive rate of tsetse flies have been reported by Jordan and Itard (1977). They noted differences in reproductive rates between two laboratory colonies of G. morsitans from Zimbabwe and Tanzania. This was accompanied by evidence of structural chromosomal differences including inversions on some of the "X" chromosomes of the Tanzania flies. The two strains, however, were able to interbreed in the laboratory. Chromosomal differences have also been observed between two laboratory stocks of G. pallidipes from Zimbabwe and Uganda (Maudlin, 1970). According to Andrewartha and Birch (1974), such chromosomal polymorphisms are indicative of genetic plasticity within animal species.

Intraspecific differences of surface fine structure of Glossina eggs have also been recorded (Hillen and Southern,

1979). Furthermore, studies of protozoan life cycles within their vectors have demonstrated differences in infection rates, which in part reflect genetic differences (MacDonald, 1976; Maudlin, 1976). According to Harley and Wilson (1968), Maudlin (1980) and Jordan (1986), tsetse flies also differ in their response to infection. Within tsetse species, some individuals are more readily infected than others, a trait that is genetically determined.

These and similar observations led Langley et al. (1984) to suggest that behavioural and genetic differences, between separated populations of tsetse flies, may lead to subspeciation; a factor of great importance in genetic control of tsetse.

1.7 USE OF GEL ELECTROPHORESIS.

Landmark publications on the use of electrophoresis in studying insect population genetics include those of Hubby and Lewontin (1966) and Ayala et al. (1972). Although tsetse flies were among the first to be recognized as vectors of disease causing organisms and among the vectors genetic control methods were first directed (Service, 1978; Curtis and Langley, 1982), their genetic studies have lagged far behind those of other vectors (Gooding, 1984). Very little work had been done on the tsetse genetics until the last two decades. This is probably because the tsetse have been readily colonized for only three decades. When compared with other insects, maintenance of its

colonies is relatively expensive. The flies have a low reproductive rate, too.

But since Geest and Kawooya (1975) extended use of gel electrophoresis to tsetse flies, substantial work has been done on tsetse genetics. Most available information comes from studies of the morsitans group. Glossina m. morsitans and to a lesser extent G. pallidipes are the most extensively studied. Little information exists on the palpalis group and nothing has been done on the fusca group. Geest et al. (1978) was the first to attempt description of genetic variation in the tsetse by starch gel electrophoresis. Since then, the technique has helped to demonstrate polymorphism in many populations of tsetse flies (Rolseth and Gooding, 1978; Gooding, 1981, 1982, 1984, 1989; Gooding and Rolseth, 1978, 1979, 1982; Gooding and Jordan, 1986; Van Etten, 1982c: Agatsuma and Otieno, 1988).

Behavioural and ecological genetics have also been covered (Dame et al., 1975; Vale et al., 1976; Maudlin, 1980; Jordan, 1980). But still much remains to be done before complete understanding of tsetse population genetics is achieved. Unfortunately, very little information is available on the genetic comparison of field and laboratory populations. Even then, what is available shows fairly contradictory results.

1.7.1. Intrataxon comparison of tsetse enzymes.

When Geest and Kawooya (1975) observed genetic polymorphism in their G. morsitans ICIPE colony which was drawn from a long standing colony at Maisons Alfort, France, they concluded that variation in field collected flies must be a lot

more. A higher degree of heterozygosity was observed at the malic enzyme locus in an outbred stock of the same species (G.morsitans morsitans) in comparison with a more inbred colony (Geest et al., 1978). This was in line with the earlier conclusion (Geest and Kawooya, 1975). But observations at the lap-3 locus showed that three laboratory colonies were almost identical to field flies (Geest et al., 1978). Comparing enzyme phenotypic frequencies of Gooding's colony and those of another larger and older colony from which his had originated $3^{1}/_{2}$ years earlier, Gooding and Rolseth (1978) showed that the two colonies differed significantly in their phenotypic frequencies. Gooding's colony, smaller and younger, showed higher average heterozygosity contrary to expectations. Because similar differences were not observed in males, the differences in females were attributed to sampling error. Furthermore, although no variations were observed at the Arginine phosphokinase (Apk) locus in natural populations of G. morsitans morsitans, two alleles were noted in a relatively small laboratory colony (Gooding and Rolseth, 1979).

In an attempt to verify effects of inbreeding in tsetse flies, Jordan et al. (1970) initiated a small colony of G. austeni with 10 males and 10 females per generation. The colony continously degenerated and finally died out after 16 generations. It was concluded that this was at least partly caused by deleterious effects of inbreeding. However, intensive inbreeding of G. morsitans morsitans for forty generations did not result in significant change in female longevity, puparial weight, eclosion rate, and sex ratio, the

characters closely linked with colony fitness (Jordan, 1980). But when this same highly inbred colony was studied with polyacrylamide gel analysis, by generation 26 all flies studied were homozygous for malic and alkaline phosphatase (Jordan, 1980). Thirty-one out of thirty-two flies were also homozygous at the Lap locus. By generation 40, all flies examined were also homozygous at both Ao and Xo loci. The parental colony flies were polymorphic at all these loci (Gooding, 1984). The inbred colony had no doubt become homozygous over many generations.

But there was considerably more genetic variation in laboratory strains of G. morsitans submorsitans than in corresponding enzymes in natural populations (Gooding, 1981). Variations in laboratory populations of G. palpalis gambiensis were lower in the laboratory strains than in natural populations. In the same study, however, results showed as much heterozygosity in laboratory stocks of G. tachinoides as in the wild caught flies of the same species.

Studies of enzyme polymorphism in natural populations of G. pallidipes in Kenya revealed that the mean heterozygosity in the populations ranged from 2.4-5.5% (Van Etten, 1982c). This was rather low when compared to values of 12.2 $\pm$ 5.4% observed in laboratory colonies of the same species originating from Uganda (Gooding, 1982). The differences might have been due to a lower resolving power of starch gel used by Van Etten (1982c) compared to polyacrylamide gel analysis used by Gooding (1982).

Despite nineteen years (130) generations as laboratory colony, G. morsitans morsitans strain had 5 polymorphic loci

just as did the field collected flies (Gooding and Jordan, 1986). There was no significant difference as regards average heterozygosity per locus or average effective number of alleles per locus. These two values, however, were slightly greater in field flies. Some rare alleles at each of To, Est and Alkph, loci were found in field collected flies but absent in laboratory strains. Laboratory flies too had two alleles each on Est-2 and G-6-pd loci that were absent in field flies. The sample sizes per locus varied from 108 to 168 and 40 to 140 respectively for the field and laboratory populations. The differences were attributed to genetic drift in the laboratory strain and sampling error in field populations.

But observations of three polymorphic loci out of eleven enzymes studied showed that G. morsitans centralis from a main fly belt in Zambia had significantly higher mean heterozygosity per locus and allele frequencies than those from a nearby small fly pocket (Gooding, 1989). The differences were attributed to both drift and founder effect in the isolated pocket. The results support the theory about inbreeding effects in small isolated populations (Hilburn et al., 1984).

1.8 BEHAVIOURAL AND ECOLOGICAL STUDIES.

1.8.1 Intrataxon comparison of behavioural and Ecological tsetse genetics.

The controversy reflected in tsetse enzyme studies is not well stressed in ecological and behavioural reports. For instance, females from a G. morsitans morsitans laboratory

colony (a mixed population, colonized six to eighteen generations) showed just slight differences in fecundity and survival rate from the wild caught flies (Jordan et al., 1970). Furthermore, results indicated that laboratory reared males of the same species (G. morsitans morsitans) did not differ in survival, dispersal and recapture rates from wild flies (Dame et al., 1975). Moreover refuge seeking response, ability to sense and respond to host odour and ability of laboratory males to locate a mate did not undergo any detectable degeneration, compared to field flies, in spite of 8 years of colonization. In addition, no differences could be found in the rate at which wings frayed in both populations suggesting that the released insects, the laboratory and field populations, did not differ in their level of activity. This lack of differences led to the conclusion that laboratory flies were genetically compatible to the corresponding field populations (Vale et al., 1976).

According to Kidwell (1983), crosses between long standing laboratory populations with field flies could result in hybrid dysgenesis (sterility in F_1 females). Gooding and Jordan (1986) tested this trait in tsetse flies by crossing flies from a nineteen year old colony (130 generations) with field flies from which the colony had originated. No evidence of dysgenesis was noted, showing that the flies were still identical. Moreover, although Brady (1972) concluded that human odour was attractive and certainly not repellant to laboratory reared G. morsitans morsitans, a trait genetically controlled, Vale et al. (1976) did not find any difference between wild and laboratory reared tsetse flies in this respect

1.9 OBJECTIVES AND HYPOTHESIS.

The aim of this study was to examine genetic variations between laboratory and field populations of Glossina pallidipes and G. brevipalpis with the following specific objectives.

1. To electrophoretically examine genetic variations in two laboratory colonies of Glossina pallidipes and one of G. brevipalpis.

2) To examine electrophoretic genetic variations in three natural populations from which the laboratory colonies were obtained.

3) To compare and contrast the genetic variations in the laboratory colonies and the populations from which they were drawn in order to assess the extent to which the laboratory stocks are representative of the tsetse in the field.

The hypothesis under test is that the laboratory colonies are subpopulations of the species they belong to, with similar gene frequencies to the wild populations.

CHAPTER TWO

MATERIALS AND METHODS.

2.1 STUDY AREAS.

The project areas included three tsetse foci in Kenya. Glossina brevipalpis wild flies were collected from Kibwezi Forest in March 1989. Two wild populations of G. pallidipes were sampled from Lambwe Valley and Shimba Hills in February and March 1989 respectively. The corresponding laboratory populations, G. brevipalpis and Shimba Hills G. pallidipes are kept at ILRAD. The Lambwe Valley G. pallidipes colony is kept at ICIPE field station, Mbita Point.

2.1.1 Kibwezi Forest.

The forest is about 200km south of Nairobi, 2^{o} 27' S, 37^{o} 55' E (Van Etten, 1982c). The natural vegetation of Kibwezi consists largely of deciduous woodland thicket (Fenner, 1982). However, patches of bush woodland and bush thicket occur. In the least disturbed, the vegetation consists of three strata of woody plants. Below 3 metres, a thick layer of dense and much branched shrubs exist. Acacia seyal is one of the commonest species. Commiphora africana and Acacia tortilis are commonest species in the strata 3 to 10 and above 10 metres respectively.

Annual rainfall is markedly seasonal. 80% of the annual total falls in two wet seasons, November to December and March to April. The mean annual rainfall from 1969 to 1978 was

489.7mm. The rain is extremely erratic. The mean monthly temperatures show an annual cycle that closely parallels that of rainfall. Temperatures are high during the wet season but low in the dry season. Unlike rainfall however, there is little variation in the temperature cycle from year to year.

The present animal species in Kibwezi include hartebeeste, Grant's gazelle, bushbuck, buffalo, warthog, bush pigs (Owaga, 1984). Most of the big mammals such as elephants, waterbuck and zebra have disappeared through poaching and habitat destruction. Smaller species such as bush squirrels, unstriped ground squirrel, genet cat, dwarf mongoose, bush baby, dik-dik, baboon and black faced vervet monkey are still common.

2.1.2 The Lambwe Valley.

The Lambwe Valley is located in South Nyanza district Western Kenya. It is approximately within latitude 30^{o} 30' and 0^{o} 45'S and longitude 34^{o} 10' and 34^{o} 20' E (Allsopp and Baldry, 1972). It is a South Westerly extension of the Kavirondo fault trough lying between Kaniamwia escarpment to the East and the Gwasi massif to the West. Its floor slopes gently from an altitude of 1280m to 1190m at the shores of the Kavirondo Gulf. Olambwe river meanders the whole length of the valley but carries water only seasonally.

The vegetation of the valley is the tropical woodland savannah. Three distinct types of vegetation can be recognized. The climax community is in areas not much influenced by man. The areas include the crest of Gwasi

escarpment and along the river. Grewia similis with canopies 5-7m high is most dominant. Clumps of Acacia seyal woodland are also common. Between blocks of this type of vegetation are extensive grassland associations. Recently, 500ha of Kaniamwia escarpment have been forested with coniferous trees and the tsetse flies have colonized the plantation.

The Lambwe Valley, which lies close to the Equator, tends to be hot and relatively humid. Extremes of temperatures and humidity are seldom experienced due to the occurrence of onshore breezes from Lake Victoria. Monthly mean temperatures vary from 20^{o}C in June to 34^{o}C in January with a mean of 26.5^{o}C. Rainfall follows a typical bimodal season with peaks in March-May and October-December (Allsopp and Baldry, 1972). The mean annual rainfall is approximately 1270 to 1525mm. Prolonged dry periods are common.

Animal populations in the Valley, which has been recently gazetted as the Ruma National Park, include bushbuck, buffalo, bush pig, Bohar reedbuck, Defassa waterbuck, grey duiker, hyaena, impala, Jackson's hartebeeste, leopard, oribi, roan antelope and topi (Otieno, 1985). Recently zebra and ostrich have been introduced. The animals roam the fenced floor of the Valley.

2.1.3 The Shimba Hills.

The Shimba Hills of coast province, 4^{o} 15'S 39^{o} 25' E (Van Etten, 1982c), have got a lot in common with Lambwe Valley of Western Kenya. Like Lambwe Valley, the Shimba Hills vegetation is mainly extensive blocks of dense thickets,

separated by grassland. The interface of the two plant communities is sharply defined. But the Shimba Hills lack scattered clumps of Acacia seyal woodland which is common in Lambwe Valley. Shimba Hills has also recently been forested with coniferous plantation and the plantation has also been colonized by tsetse flies.

The Shimba Hills has a low altitude and is close to a large water body, the Indian Ocean. It therefore does not experience extremes of temperature. Its humidity is always high due to occurrence of onshore breezes. Monthly temperatures vary a little from 27^{o}C (National Atlas of Kenya, 1970). It experiences a long rainy season. The year starts with a dry period and remains dry until March when rainfall gradually increases. A fairly rapid increase occurs through April and builds up to maximum in May. It then decreases steadily. During December, a more rapid decrease is experienced leading to rainfall minimum in January and February.

Shimba Hills is also a Game Reserve. The large fauna species of Shimba Hills is the same as Lambwe Valley except for the absence of elephants in Lambwe Valley. The preferred animals, the bushbuck, bush pigs and buffaloes, exist in both places.

2.2 FIELD TSETSE POPULATIONS.

2.2.1 Lambwe Valley Glossina pallidipes.

The existence of G. pallidipes in Lambwe Valley was known as early as 1910 (Ford, 1971). To date, there have been

five attempts to control or eradicate the vector. Between 1968 and 1971, intensive control activities were conducted jointly by Food and Agriculture Organization (FAO), the World Health Organization (WHO) and the Kenyan Ministries of Health and Agriculture. But when the control activities were relaxed, the tsetse population recovered to the earlier size and even extended their range from the Valley to a new habitat, the 500 hectare coniferous forest (Turner, 1986).

The 1981 campaign was an outright eradication attempt (Otieno, 1985). Sequential aerial applications of endosulfan aerosol, supplemented by ground spraying of residual dieldrin and bush clearance, reduced pallidipes by 99.9% (Turner, 1986; Otieno, 1985). But in the principal habitat the thicket, the tsetse population grew at a maximum rate recovering fully to its prespray level in about 12 months. The 1983 aerial spraying had no noticeable impact on the fly density (Otieno, loc. cit.). Further emergency control measures have been undertaken since 1984, but the fly is still entrenched. The Lambwe Valley environment has no major seasonal habitat changes. This, combined with all year round availability of food to the vector, are conducive to the tsetse survival.

Today, ICIPE and KETRI are continuing the control measures using traps (ICIPE) and insecticide impregnated screens (KETRI) but with little success. Integrated tsetse control measures, using the sterile insect concept in addition to the above measures recommends itself in an isolated tsetse habitat like the Lambwe Valley (Turner, 1986).

2.2.2 Other Field Populations.

Very little information is available on both G. pallidipes population in the Shimba Hills and G. brevipalpis in Kibwezi. What is clear, however, is that these populations are well established in their respective habitats and man's efforts to control them have been and still are mainly limited to their habitat fringes (Mungai, pers. comm.).

2.3 LABORATORY TSETSE POPULATIONS.

2.3.1 Lambwe Valley Glossina pallidipes colony.

The Lambwe Valley G. pallidipes colony used in this study is kept at ICIPE, Mbita Point Field Station along the shores of Lake Victoria. It was started on 17th January, 1983, with 185 flies all collected from the thicket. The flies were trapped using Challier traps. They were not sexed and therefore there is no information regarding the sex of the parental stock (Banda, pers. comm.). Sophisticated equipment for controlling environment are not used at Mbita Point tsetse insectary. The floor and lower wall (one metre from the floor) of the insectary are cemented, but the rest of the building is grass thatched. A 0.3 metre space is left between the roof and wall plate for ventilation. It is lined with steel wire to prevent fly escape. The space allows free flow of air from the surrounding environment. Therefore, the insectary environment simulates the surrounding natural environmental conditions (Ochieng et al., 1987). There is sufficient light during the day. Its temperatures like that of the surrounding area, fluctuate between a minimum of 20^{o}C in June and a maximum of 34^{o}C in January. The mean temperature is 26.5^{o}C (Ochieng et al., 1987).

The colony performance in terms of survival, puparia production and weight of puparia, demonstrates that it is clearly a viable colony. The colony had increased to 2800 breeding females by January 1986. But it suffered a setback later in that year when food used to feed rabbits on which flies fed was contaminated. This was quickly corrected and tsetse mortality restored to original level. A regular supply of colony flies to scientists for experimental purposes began in the year (1986). Today, the mean female stock is 4,412 flies. The mean daily female mortality is 1.5%.

The flies are fed Monday to Friday on rabbit ears according to Nash et al. (1966). They are kept in groups of 20 flies in polyvinyl cages measuring 18x8x4cm. Since the start of this colony, there has not been any infusion of field flies. The flies used in this study were collected from the colony in March 1989 and were over 10 days old.

2.3.2 Shimba Hills Glossina pallidipes colony.

The colony kept at ILRAD, Nairobi was begun on 20th October, 1986, with 32 males and 88 females (Moloo, pers. comm.). The flies were caught using biconical traps. Females were kept in groups of twenty in Geigy cages with only two males per mating period. They were kept in an experimental holding room maintained at 25$\pm$1^{o}C and 80% relative humidity. Larviposited pupae were kept in a colony-holding room in wooden emergency boxes. Up to the death of the last wild female fly on 3rd January, 1987, a total of 119 pupae had been larviposited. To date, the flies are maintained by feeding on

rabbits 5 days per week. Although the size of the colony has varied, it is currently maintained at about 3,200 pregnant females. The daily female mortality is 0.08% and there is an average natality of 0.30 per female per week. There has not been any replenishment of stock from the wild. The flies that were used in this study were collected from the colony in April, 1989. They were 10 days old.

2.3.3 Kibwezi Glossina brevipalpis Colony.

The colony also kept at ILRAD, Nairobi was started in mid-1982, with 181 wild females caught in Kibwezi forest using a moving landrover catching method (Brightwell et al., 1987). They were kept in a climate controlled room at a temperature of $25\pm0.5^{o}C$ and 80-85% relative humidity. They were fed on ears of lop-eared rabbits daily except Sundays. A total of 531 pupae were produced by the field caught flies. The tsetse that emerged from the pupae formed the parental stock of the colony. The number of mated females had increased to 1000 in 1983, 4000 in 1984 and 5238 in 1985. The mean survival by day eighty post emergence was first 34.4% at the beginning, but has now increased to 52.5% (Moloo and Kutuza, 1988). There has not been any replenishment of the stock from the wild since the colony began. Currently, the colony is kept at 1500 pregnant females and provides adequate surplus for research workers at ILRAD and elsewhere. The flies used in this experiment were collected from the colony when they were between 10 and 12 days old.

2.3.4 Glossina austeni Colony.

This colony is also kept at ILRAD, Nairobi. It was started in 1980, but the sex and number of flies that started the colony is not recorded. The parental stock was from another colony kept at Bristol University. The colony at Bristol University was started in 1961 with field collected flies from Zanzibar, Tanzania. The colony was initiated with 49,000 field collected pupae and over a period of 3 years (Nash et al., 1968). Therefore its initial gene pool was great but there has not been any further infusion of the colony with field flies since 1966. The daughter colony at ILRAD is also entirely self supporting with no infusion from either its parental colony at Bristol University or its grand-parental population in the field in Zanzibar (Moloo, pers. comm.). The colony is maintained at 25^{o}C and 80% relative humidity. It is fed Monday to Friday on rabbit ears.

2.4 METHODOLOGY AND EQUIPMENT

2.4.1 FLY COLLECTION.

All field collected flies with the exception of Glossina brevipalpis were caught using either biconical traps (Challier et al., 1987) or Nguruman traps (Brightwell et al., 1987) and at times both. G. brevipalpis were trapped using the moving Landrover method (Bursell, 1961) supplemented by the hand net method. Since most G. pallidipes populations show a bimodal activity pattern (Turner, 1987), traps were set in mornings and collected at 6.00 p.m. Trapping of G. brevipalpis started from 6.00 a.m. to 11.30 a.m. and then 4.00 p.m. to 7.00 p.m., the

period they appeared to be most active. In all localities, trapping continued for a period of one week. A profile covering a variety of habitats was sampled and insects from one locality mixed in one container. A minimum of 100 flies of each population under study was collected, though sampling aimed at higher numbers.

2.4.2. TRANSPORTATION OF SPECIMENS.

Each day the samples were transported to my field stations in small cages lined with damp, dark cloth. The wet cloth ensured high humidity required by the flies. The tsetse were kept alive for at least 24 hours to digest their blood meal. Freshly taken meals interfere with zymograms by yielding extra bands (Geest and Kawooya, 1975). Tenerals and female flies at late pregnancy yield aberrant patterns and were discarded (Geest and Kawooya, 1975). The remaining flies were fixed by freezing in liquid Nitrogen and brought to the genetics laboratory at the National Museums of Kenya, Nairobi. They were stored in a deep freezer at temperatures ranging from -75^{o} to -86^{o}C until electrophoretic analysis was done. In some cases the analysis was after 10 months.

2.4.3 SAMPLE ANALYSIS.

Analysis of samples was done using protein separation technique by strach gel electrophoresis. Electrophoresis is the movement of charged particles under the influence of an electric field (Ferguson, 1980). The technique relies on the fact that identically charged proteins migrate the same

distance while differently charged proteins usually migrate different distances. Isoenzymes were used in this study. Isoenzymes are either in a single individual or different members of the same species. They have similar but not necessarily identical enzyme properties (Vesell, 1968).

Detailed electrophoretic and staining procedures have been described by a number of authors; (Brewer, 1970; Shaw and Prasad, 1970; Selander et al; 1971; Harris and Hopkinson, 1977). Preparations to separate and stain isoenzymes in this work follows those described by Harris and Hopkinson (1977) but with some necessary modifications according to Pieter (pers. comm.).

The starch gels were prepared by heating 32 grams of partially hydrolysed starch in 250 ml of a suitable buffer (Appendix, 2.1 and 2.3). They were heated in a toughened glass conical flask as described by Harris and Hopkinson (1977). The hot starch was then degassed until it was bubble free, and quickly poured into a mound and the gel stored overnight in a refrigerator at $4^{o}C$.

Regarding samples, a single adult fly was macerated in about 0.3 ml of tissue buffer (Appendix 2.1). The hydrogenate was spun down and centrifuged at about 3000 rounds per minute for 5 minutes. The supernatant of each fly was absorbed into three one thickness chromatography papers of whatman No. 3. Each wick was carefully inserted into a gel slot cut abot 6 cm from the anodal end of each gel. Therefore, each fly provided enough sample material for 3 gels. Each gel was loaded with 20 samples. To ensure consistency in designating banding

patterns, 4 G. austeni samples (laboratory colony) were included on each gel. After every 5 samples of a particular population under study, a mobility control (marker) G. austeni, was inserted. A mobility control is a sample repeated more than once on every gel, whose function is to act as a known mobility guideline for interpretation comparisons. It is useful for both intra- and inter-gel standardization.

Each loaded gel was transferred to trays containing appropriate tray buffers (Appendix 2.2). Three buffer systems were used to characterize eleven enzymes. Malic enzyme (ME), Malate dehydrogenase (MDH) and Phosphoglucomutase (PGM) were characterized by Tris maleate buffer pH 7.4. A discontinous Tris citrate pH 6.3/6.7 buffer of Selander et al. (1971) with necessary modifications was used to characterize Glucose phosphate isomerase (GPI), Hexokinase (HEX), Isocitrate dehydrogenase (IDH) and Nicotinamide adenine dinucleotide dehydrogenase (NADD). The remaining four enzymes, Aldehyde oxidase (AO), Esterase (EST), Xanthine dehydrogenase (XDH) and Mannose-6-phosphate isomerase (MPI) were characterized on TEB pH 9.1.

Electric power was provided by a stabilized Ephortec voltage source working off the mains. TM 7.4 gels run at 125 volts (78mA). The Tris citrate 6.3/6.7 and TEB 9.1 gels were run at 350 volts (35 mA) and 400 volts (40 mA) respectively. The gels were run for 20 minutes and then sample wicks removed. Thereafter, the TM 7.4 gels were run for 5 hours. The Tris citrate 6.3/6.7 and TEB 9.1 gels were run for $2^{1}/_{2}$ and 4 hours respectively. All gel temperatures were maintained at $2^{o}C$ during the experiment.

After electrophoresis, the gels were sliced horizontally into four slices using a slicing wire. The cut surface of each slice was stained and characterized for a particular enzyme. Except for EST, the staining mixture of each enzyme (Appendix 2.4) was always added and mixed in molten agar and Tris buffer (10 ml of each). The solution was carefully poured over the cut surface of each gel. This brings reagents into intimate contact with the enzyme being stained. It also prevents diffusion of bands since the agar hardens as it cools. For EST however, the gels were immersed into the staining mixture contained in a staining bowl.

Apart from GPI, all stained gels were incubated at $37^{o}C$ until they were scorable. GPI developed fast and needed no incubation. It developed slowly in a darkened box at room temperature. The incubation period varied from enzyme to enzyme. The relative mobility values were determined using distances measured from the sample origin on the gel to the centre of the enzyme band. For standardization, the scored distances were compared to the measured distances of the commonest band of the marker.

CHAPTER THREE

RESULTS

3.1 GENERAL DESCRIPTION OF RESULTS

Although the eleven enzyme systems were examined, GPI was consistently too weak to score while MPI showed no activity. The remaining nine enzyme systems were consistently scorable and one could interpret their banding patterns in terms of genotypes and allele frequencies. All the six population samples (4 of G. pallidipes and 2 of G. brevipalpis) showed the same enzyme banding patterns in both field and laboratory populations. However their mobility was usually different in the two species showing the two species have different isoenzymes.

3.1.1 Non-varying enzymes.

The simplest banding pattern observed was the presence of a single band in each individual of a population, in the same position relative to the marker. This pattern was observed in ME, AO, HEX NADD and XDH. The pattern is interpreted as indicating that each such enzyme system is controlled by a single gene locus and that in each population there is a single allele. Alternatively, any allelic variations that do exist in these enzymes were beyond the resolving power of the sampling technique used in these investigations. It would be necessary to employ a different

electrophoretic method to screen the same enzymes in order to confirm or refute their apparent monomorphism.

The exact number of loci controlling IDH was uncertain. This is because the enzyme was consistently producing double-banded but non-varying patterns (Figs. 3.1 and 3.4; plate 3.2). This may indicate that we are dealing here with a genotype under the control of a single gene or that two loci are involved. Since there was no variation in the position or number of the bands, a definitive conclusion about the number of alleles and genes involved is rather difficult.

3.1.2 The Esterase locus.

Three alleles were observed at the Est locus. In all cases, one allele was present at a very high frequency (0.952 or greater), so that this locus did not qualify as being polymorphic. (In this study, a locus was designated as polymorphic when the frequency of the commonest allele was equal to or less than 0.95). In most cases, there were two other alleles, one responsible for a band faster than the common one and the other one for a slower band. No genetic model was offered for this enzyme because the frequencies of the rare alleles were too low to determine if the observed frequencies of the genotypes agreed with those predicted for a population in Hardy-Weinberg equilibrium. Details of the frequencies of these three alleles in various populations are given below in subsections:- 3.2, 3.3 and 3.4.

3.1.3 The phosphoglucomutase locus.

Four different electrophoretic patterns were observed at the Pgm locus: slow, medium and fast migrating bands and a double-banded pattern commonly involving the fast and slow bands. This double-banded pattern, which was interpreted as heterozygous, was never seen in males (Figs. 3.2 and 3.5; and plates 3.1, 3.4 and 3.5). It is therefore concluded that the locus controlling Pgm is X-linked and has three alleles. The allele frequencies in various samples were calculated directly from the data shown in Tables 3.1, 3.5 and 3.9, and are given in Tables 3.2, 3.6 and 3.10 for both the laboratory and field samples. They were used to calculate the expected numbers of each genotype. The chi square test was used to determine if the populations were in Hardy-Weinberg equilibrium and the results are shown in the relevant sections below.

3.1.4 The malate dehydrogenase locus.

For Mdh, three genotypes were observed, all of them single band homozygotes. No heterozygotes were scored but the enzyme is highly variable (Figs. 3.3 and 3.6). Allele frequencies at this locus are shown in Tables 3.4, 3.8 and 3.12. There was no agreement with HWE frequencies because of the lack of heterozygotes (see discussion). No differences in banding patterns were observed between males and females. However in the absence of heterozygotes, it was not possible to interpret the genetics of this enzyme.

3.1.5 The glucose phosphate isomerase locus.

The activity at the Gpi locus was observed but there was too much overlap to permit accurate scoring. The zymograms were also often streaky, making it hard to identify individual bands. The results for this locus have therefore not been tabulated.

3.1.6 Comparison methods.

Field samples were compared with their corresponding laboratory samples with regard to the amount of genetic variation within each population. Genetic variability in each population was measured by calculating the proportion of polymorphic loci (P). It was also quantified by calculating heterozygosity for each locus (H), using the formula $H = 1 - Eq_i^2$, (Gooding, 1981), where q_i is the frequency of the i^{th} allele at a locus. The average heterozygosity ($\bar{H}$) was calculated by dividing the total heterozygosity for all loci by the total number of scorable loci.

Other parameters examined were the allele frequency data, the number of alleles per locus and the effective number of genotypes at each locus. To quantify the extent to which the colony flies carried electromorphs similar to those found in wild tsetse, the identity statistic (I) of Nei (1972) was calculated for all possible pair-wise comparisons using the formula:

$$\bar{I} = (Eq_{ix})\ (Eq_{iy})/(Eq_{ix}^2)\ (Eq_{iy}^2)^{1/2}$$ (Ferguson, 1980)

where q_{ix} and q_{iy} are allele frequencies of the i^{th} allele at a locus in the field population (x) and laboratory population (y) respectively. A mean was taken over all the scorable loci examined to get the average identity (I). The standard genetic distance (D) between laboratory and field samples was calculated using the formula

$$D = \ln \bar{I} \qquad \text{(Ferguson, 1980)}$$

It should be noted that in this study no single pair matings were carried out to test the genetic interpretation of the observed variation. This was not possible due to time limit and difficulties in maintaining tsetse flies, particularly G. pallidipes. The observed allozyme variation is inferred from previous workers who carried out breeding tests to establish the genetic basis of the enzyme systems concerned (Gooding and Rolseth, 1979; Gooding, 1982; Van Etten, 1982c; Geest et al., 1978). In addition, electrophoretic patterns were examined for their goodness of fit with regard to expectations based on the Hardy-Weinberg equilibrium (HWE) model. In the case of small expected frequencies, genotypes were lumped together until the expected numbers were big enough to suit the chi-square test. The degrees of freedom (d.f.) were calculated according to the formula

$$\text{d.f.} = {}^{1}/_{2}(n^2-n) \text{ (Ferguson, 1980)}$$

where n is the number of alleles observed. The contingency chi-square test of homogeneity was used to test for significant

differences in genotype frequencies between the field and laboratory populations.

3.2 COMPARISON OF FIELD AND LABORATORY POPULATIONS OF *GLOSSINA PALLIDIPES* FROM LAMBWE VALLEY.

3.2.1 Scorable enzymes.

For this comparison, IDH, ME, AO, HEX, MDH, NADD, EST, XDH and PGM were scored. Only two (*Pgm* and *Mdh*) were polymorphic in both laboratory and field samples. At the *Est* locus, as described earlier, more than one allele was present but the commonest had frequencies of 0.960 (field) and 0.952 (laboratory), so that this locus was not polymorphic on the criterion adapted in this study. One slow rare allele existed in the field population but has been replaced by another rare but faster allele in the laboratory population. *Idh* and all the monomorphic loci were similar in the two populations

3.2.2 The phosphoglucomutase locus.

Pgm was very variable in both populations, providing a good tool for analysing genetic differences between laboratory and field populations. In both, there was the sexual difference in electrophoretic patterns described earlier. A fast migrating and a slow migrating band, together with a two banded heterozygote corresponding to the two slow and fast bands, were observed in females of both samples. Only single bands corresponding to the females' fast and or slow bands were observed in males from the field sample. A third rare medium migrating band was observed in 3 out of 49 males from the laboratory sample (Table 3.1).

Chi-square values for goodness of fit to the Hardy-Weinberg expectations were calculated. Data from total examined (males + females) were in good fit with the Hardy-Weinberg expectations in both populations (X^2=0.624, 1df, P>0.05 and X^2=1.526 ,1df P>0.05 in laboratory and field samples respectively) (Table 3.3). Furthermore, considering data from female populations separately, there was good agreement between observed genotype numbers and the expectations of HWE in both laboratory and field populations (X^2=1.382, 3df, P>0.05 and X^2=1.156, 3df, P>0.05 respectively). The observed and expected heterozygosity in female samples at this locus also showed no significant difference (Table 3.3).

The contingency chi-square test of homogeneity showed no significant difference in genotype frequencies between laboratory and field male samples (X^2=0.166, 5df, P<0.05). There was also no heterogeneity when female samples were considered separately (X^2=2.243, 5df, P>0.05).

3.2.3 The malate dehydrogenase locus.

Mdh had 3 bands of homozygotes in both samples. No heterozygotes were noted. Although the commonest gene electromorph was the medium migrating band in both populations, its frequency varied in the two, being 0.910 in the field population compared to 0.584 in the laboratory stock (Table 3.4). Two others controlling the fast migrating band and the slow migrating band were very rare in the field population. They were observed at a frequency of 0.045 each but their

frequencies in the laboratory were 0.260 and 0.156 for the fast and slow migrating bands respectively. The contingency chi-square test of homogeneity was significant suggesting that the two were from different populations (X^2=21.727, 5df, P<0.05).

3.2.4 Nei's mean identity, heterozygosity and genetic distance.

Calculating Nei's mean identity ($\bar{I}$) and the genetic distance (D) is one method of addressing the question of how similar or how different two populations are. These were calculated for all possible pairs of the two populations using allele frequency data for all loci scored. But because of uncertainty of the number of loci and alleles involved in Mdh electromorphs, its data was not used. The values found for Nei's mean identity was 0.9992 and 0.0008 for the genetic distance. The mean heterozygosity ($\bar{H}$) for the eight loci excluding Mdh was 0.0689 in the field but 0.0714 in the laboratory sample. This shows that genetic variation in the ICIPE Laboratory colony is still high despite the 27 generations under colonization.

TABLE 3.1: Presumptive genotype numbers at the *Pgm* locus in laboratory and field flies of Lambwe Valley *G. pallidipes*

Population	Number Examined	Genotypes:					
		aa	ab	ac	bb	bc	cc
Laboratory females	28	7	11	0	10	0	0
Laboratory males	49	10	0	0	36	0	3
Total laboratory	77	17	11	0	46	0	3
Field females	57	8	31	0	18	0	0
Field males	10	1	0	0	9	0	0
Total field	67	9	31	0	27	0	0

TABLE 3.2: Allelic frequencies and heterozygosity at the Pgm locus calculated from total examined (laboratory and field) samples of Lambwe Valley G. pallidipes

PGM	Field	Laboratory
Pgm^a	0.387	0.333
Pgm^b	0.613	0.638
Pgm^c	0.000	0.029
H	0.475	0.481

$H = [1 - (\text{freq a})^2 + (\text{freq b})^2 + (\text{freq c})^2]$

TABLE 3.3: Observed and expected equilibrium genotype numbers at the Pgm locus in total examined (Males and females) of Lambwe Valley G. pallidipes samples

Population	Number Examined	Genotypes:					
		aa	ab	ac	bb	bc	cc
Laboratory total observed	77	17	11	0*	46*	0*	3*
Laboratory total expected		19.444	11.911	0.533	42.667	1.021	1.423
Field total observed	67	9	31		27		
Field total expected		12.412	27.047		27.541		

Laboratory $X^2 = 0.624$ 1df $P>0.05$
Field $X^2 = 1.526$ 1df $P>0.05$
* Combined to calculate chi-square.

TABLE 3.4: Observed electromorph frequencies at the Mdh locus in laboratory and field populations of Lambwe Valley G. pallidipes

	F I E L D		L A B O R A T O R Y	
Mdh Electromorphs	No.observed	Frequency of the Electromorphs	No. observed	Frequency of the Electromorphs
Fast band	3	0.045	20	0.260
Medium band	61	0.910	45	0.584
Slow band	3	0.045	12	0.156

$X^2 = 21.727$, 5df, $P<0.05$

3.3 COMPARISON OF FIELD AND LABORATORY POPULATIONS OF *GLOSSINA PALLIDIPES* FROM SHIMBA HILLS.

3.3.1. Scorable enzymes.

The scorable enzymes for this comparison were the same as those scored for the Lambwe Valley *G. pallidipes* except that the *Est* locus was not examined. Again, only two enzymes PGM and MDH were polymorphic in the two populations. No difference in mobility was observed in *Nadd*, *Idh* and the rest of the monomorphic loci between the two populations. The mobility of these loci was the same as that described for the Lambwe Valley *G. pallidipes* populations.

3.3.2. The phosphoglucomutase locus.

Pgm was again highly variable in the two samples and was thus a useful tool for analysing the differences between the field and laboratory populations. Five genotypes and three alleles were scored for the field population as opposed to three genotypes and two alleles in the laboratory population (Table 3.5 and 3.6). The commonest genotypes and alleles were the same in the two samples. Calculation of the Chi-square value for goodness of fit to Hardy-Weinberg equilibrium showed that the observed genotypes were in good agreement with Hardy-Weinberg expectations (X^2=0.046, 1df, P>0.05 and X^2=0.116, 1df, P>0.05) for field and laboratory samples respectively (Table 3.7). The female samples' data when considered separately also agreed with HWE (X^2=0.973, 1df, P>0.05 and X^2=2.112, 1df, P>0.05) for field and laboratory

samples respectively. There was good agreement between the number of heterozygotes observed and expected (Table 3.7). The contingency chi-square test of homogeneity at the locus for females showed that the samples originated from the same population (X^2=5.916, 3df, P>0.05).

3.3.3. The malate dehydrogenase locus.

Again, Mdh had 3 bands of homozygotes in both samples, and no heterozygotes were observed. The electromorph controlling the medium band was the commonest, its frequency being 0.729 and 0.810 in the field and laboratory samples respectively (Table 3.8). To test for homogeneity among the two samples, the contingency chi square test was used. No heterogeneity could be detected (X^2=1.306, 5df, P>0.05).

3.3.4 Nei's identity, genetic distance and heterozygosity.

The Nei's mean genetic identity, genetic distance and mean heterozygosity, were calculated for all possible pairs of the two samples using allele frequency data. But again Mdh data was not used. The value for Nei's mean Identity was 0.9996 and that of genetic distance was 0.00036. The values for average heterozygosity were 0.03 and 0.023 in field and laboratory samples respectively.

TABLE 3.5: Presumptive genotype numbers at *Pgm* locus in laboratory and field flies of Shimba Hills *G. pallidipes*

Population	Number Examined	Genotypes:					
		aa	ab	ac	bb	bc	cc
Laboratory females	21	1	5	0	15	0	0
Laboratory males	38	0	0	0	38	0	0
Total laboratory	59	1	5	0	53	0	0
Field females	45	1	4	0	33	7	0
Field males	50	2	0	0	47	0	1
Total field	95	3	4	0	80	7	1

TABLE 3.6: Allelic frequencies and heterozygosity at the Pgm locus calculated from total examined (laboratory and field) samples of Shimba Hills G. pallidipes

Pgm	Field	Laboratory
Pgm^a	0.057	0.0875
Pgm^b	0.886	0.9125
Pgm^c	0.057	0.000
H	0.2085	0.1597

$$H = [1 - (\text{freq a})^2 + (\text{freq b})^2 + (\text{freq c})^2]$$

TABLE 3.7: Observed and expected equilibrium genotype numbers at the Pgm locus in total examined (males and females) of Shimba Hills G. pallidipes populations

Population	Number Examined	Genotypes:					
		aa	ab	ac	bb	bc	cc
Laboratory total observed	59	1*	5*		53		
Laboratory total expected		3.486	3.353		52.161		
Field total observed	95	3#	4#	0*	80	7*	1*
Field total expected		3.004	4.555	0.294	79.588	4.555	3.041

laboratory $X^2 = 0.116$, 1df, $P>0.05$
Field $X^2 = 0.046$, 1df, $P>0.05$
*, # Combined to calculate chi-square

TABLE 3.8: Observed electromorph frequencies at the Mdh locus in laboratory and field samples of Shimba Hills G. pallidipes

	F I E L D		L A B O R A T O R Y	
Mdh Electromorphs	No. observed	Frequency of the Electromorphs	No. observed	Frequency of the Electromorphs
Fast band	14	0.146	6	0.103
Medium band	70	0.729	47	0.810
Slow band	12	0.125	5	0.086

$X^2 = 1.306$, 5df, $P>0.05$

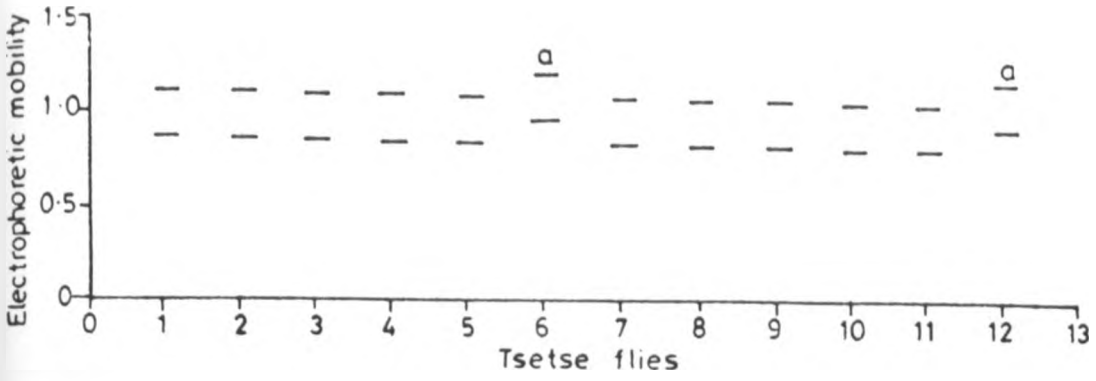

Fig. 3·1 Diagrammatic representation of electrophoretic pattern of isocitric dehydrogenase (IDH) found in populations of G. pallidipes. The vertical scale denotes mobility level of various bands. O = origin. The numbers 1-5 then 7-11 show the two non varying bands observed in G. pallidipes. a = G. austeni (control).

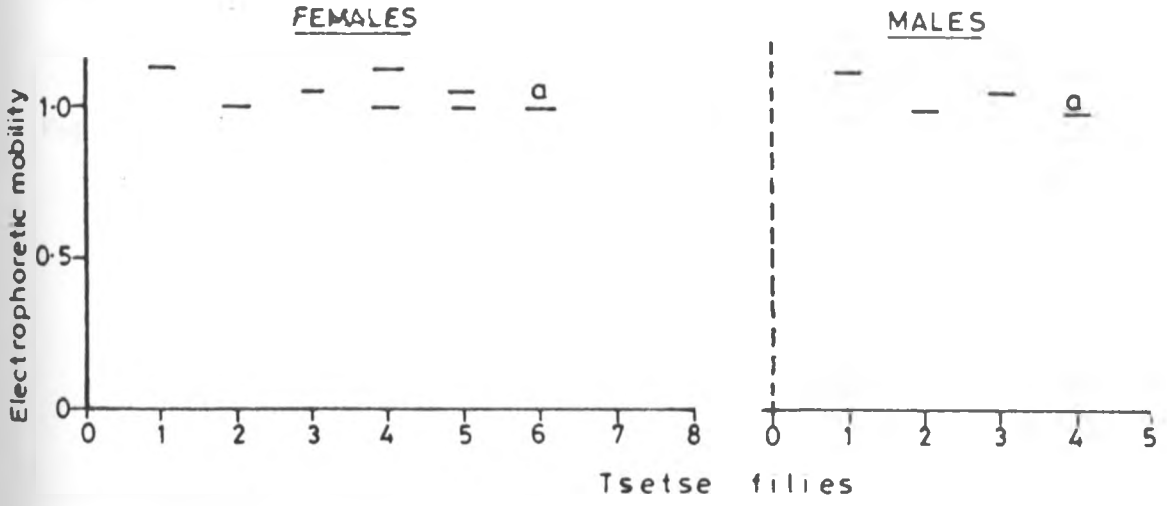

Fig. 3·2: Diagrammatic representation of electrophoretic pattern of phosphoglucomutase (PGM) found in populations of G. pallidipes. The vertical scale denotes mobility level of various bands. O= origin. The numbers show different genotypes as follows. 1. P_{gm}-a / P_{gm}-a, 2. P_{gm}-b / P_{gm}-b, 3. P_{gm}-c / P_{gm}-c, 4. P_{gm}-a / P_{gm}-b, 5. P_{gm}-b / P_{gm}-c, a = G. austeni (control)

/

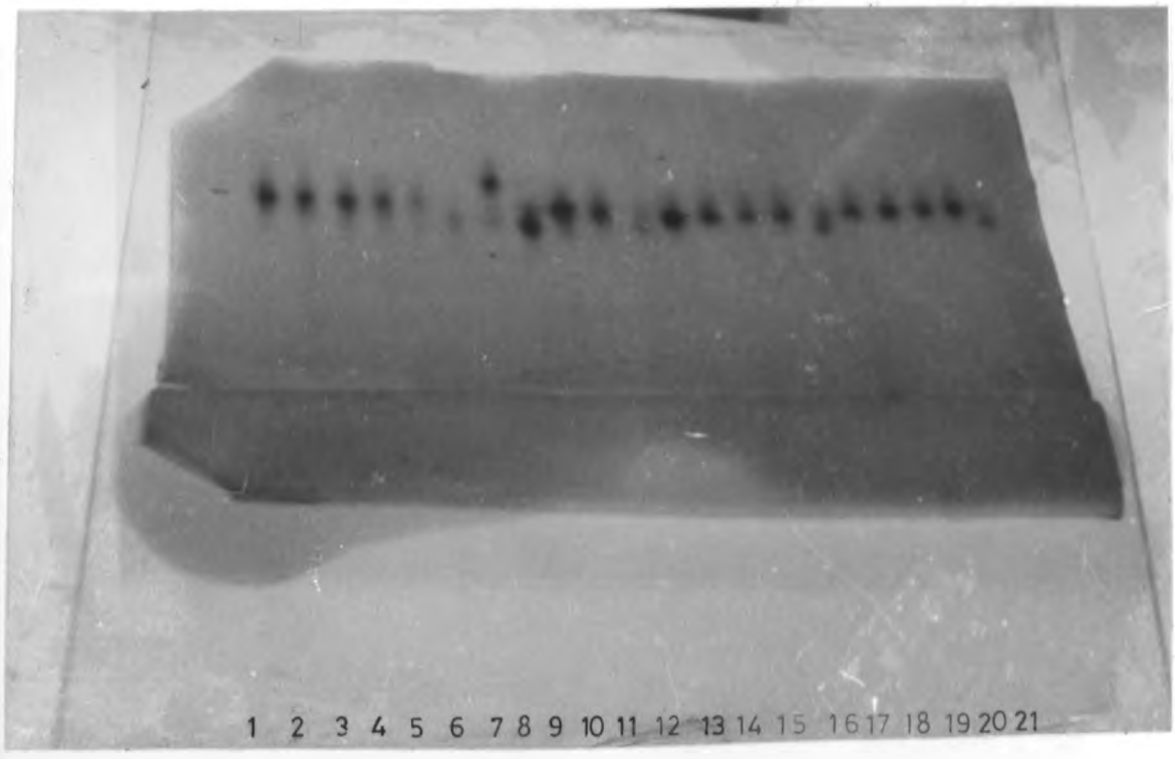

Plate: 3.1 G. pallidipes Pgm locus. Lambwe Valley laboratory population (males only). The fast migrating band was rare. Only single bands were observed. Left to right, 1-5, 7-10, 12-15 and 17-20 = G. pallidipes. 6, 11, 16 and 21 = G. austeni (control).

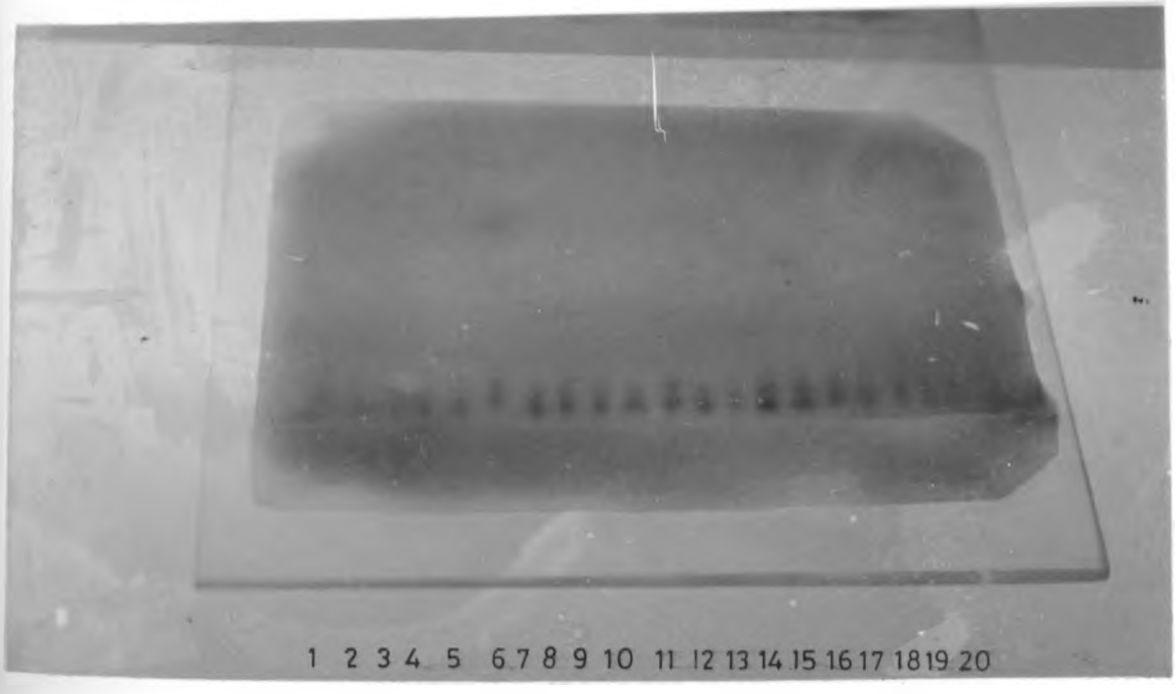

Plate: 3.2 G. pallidipes Idh locus/loci. Shimba Hills field population (mixed sex). Left to right, the numbers 1-5, 7-10, 12-15 and 17-20 = G. pallidipes. 6, 11 and 16 = G. austeni (control). The first three and the last two are not clear. Idh showed a double banded but non-varying pattern.

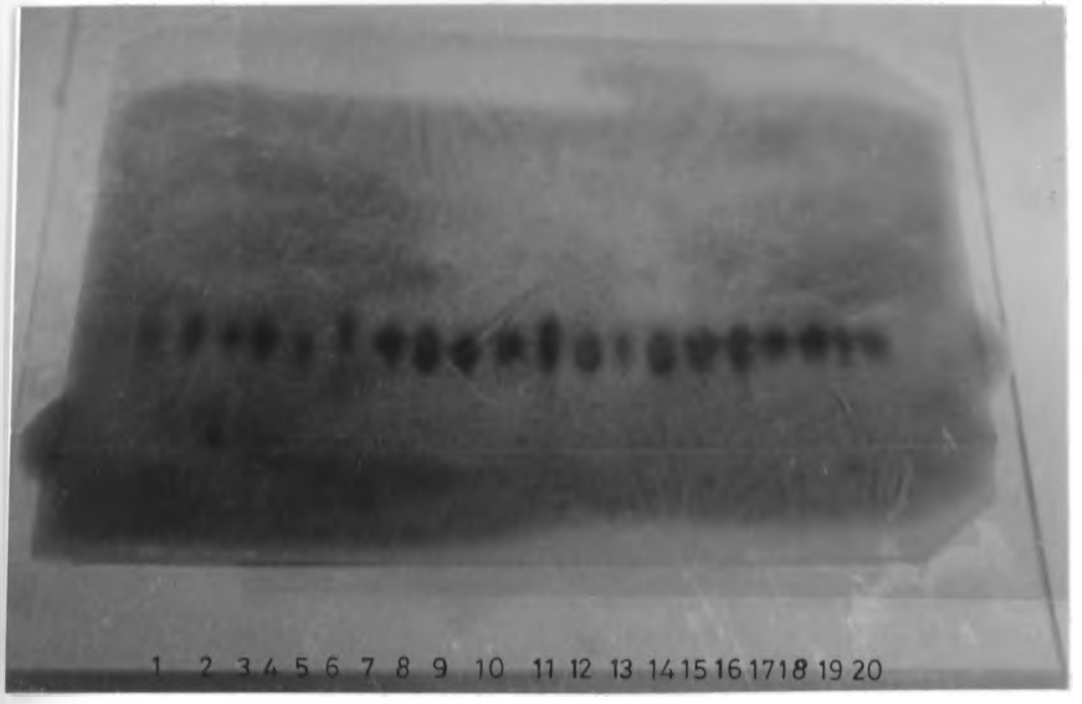

Plate: 3.3 G. pallidipes Ao locus. Lambwe Valley laboratory population (mixed sex). Left to right, 1-5, 7-10 and 12-20 = G. pallidipes. 6 and 11 = G. austeni (control).

3.4 COMPARISON OF FIELD AND LABORATORY POPULATIONS OF G. BREVIPALPIS FROM KIBWEZI FOREST

3.4.1 Scorable Enzymes.

The enzymes scored for this comparison were the same as those scored for G. pallidipes of Lambwe Valley but the Hex locus was too weak on gels and not scored. The banding patterns were also the same as G. pallidipes but with fixed loci differences in mobility between species on most of the scored loci. Idh and the rest of the monomorphic loci were similar in the two populations, in that each showed a single band in each individual fly, in the same position relative to the marker.

3.4.2 The Esterase locus.

On the Est locus there was a slow, very rare allele in the field population that was absent in the laboratory strain. The allele was observed at a frequency of 0.042. The common allele was scored at a frequency of 0.958 and 1.00 in the field and laboratory populations respectively.

3.4.3 The Phosphoglucomutase locus.

The Pgm locus was again highly variable in both populations and thus a good tool for analysing the differences between field and laboratory samples (plates 3.4 and 3.5). Four genotypes and 3 alleles were observed in the field samples while 5 genotypes and 3 alleles were scored for the laboratory samples (Tables 3.9 and 3.10). However, the two commonest genotypes were the same in both. In the laboratory samples,

the chi-square value for goodness of fit to Hardy-Weinberg expectations was in good agreement for total examined (X^2=0.810, 3df, P>0.05). However the difference between observed and expected was significant in the field samples (X^2=9.357, 3df, P<0.05) (Table 3.11). Analysis of female samples separately showed that they agreed with HWE (X^2=4.621, 3df, P>0.05 and X^2=4.958, 3df, P>0.05) for field and laboratory females respectively. There was also good agreement between observed and expected heterozygosity. The difference between female genotype frequencies, in the laboratory and field samples were significant (X^2=6.840, 2df, P<0.05, lumping ac, bc, cc genotypes together) but not in the males (X^2=2.856, 2df, P>0.05).

3.4.4. The Malate dehydrogenase locus.

Mdh again had 3 genotypes in both samples. Unlike with G. pallidipes populations, the commonest electromorph was the fast band. It was scored at a frequency of 0.699 and 0.681 in laboratory and field samples respectively (Table 3.12). The medium band was scored at a frequency of 0.173 and 0.181 while the slow band was scored at a frequency of 0.123 and 0.138 in laboratory and field samples respectively. The contingent Chi-square test of homogeneity showed no difference between the two sample populations (X^2=0.118, 5df, P>0.05).

3.4.5 Nei's mean Identity, heterozygosity and genetic distance.

The Nei's mean genetic identity between the laboratory and field samples was 0.9995. It was calculated using allele

frequency data of the 7 enzymes scored excluding Mdh. The value for genetic distance between the two samples was 0.00046. The mean heterozygosity was 0.067 in the field population compared to 0.064 in the laboratory population.

TABLE 3.9: Presumptive genotype numbers at Pgm locus in laboratory and field flies of Kibwezi G. brevipalpis

Population	Number Examined	Genotypes:					
		aa	ab	ac	bb	bc	cc
Laboratory females	51	27	8	15	0	0	1
Laboratory males	47	30	0	0	13	0	4
Total laboratory	98	57	8	15	13	0	5
Field females	20	12	7	0	0	1	0
Field males	85	56	0	0	15	0	14
Total field	105	68	7	0	15	1	14

TABLE 3.10: Allelic frequencies and heterozygosity at Pgm locus calculated from the total examined in laboratory and field samples of Kibwezi G. brevipalis

Pgm	Field	Laboratory
Pgm^a	0.696	0.718
Pgm^b	0.184	0.141
Pgm^c	0.120	0.141
H	0.467	0.445

$H = [1-(\text{freq a})^2 + (\text{freq b})^2 + (\text{freq c})^2]$

TABLE 3.11 Observed and expected equilibrium genotype numbers at the Pgm locus in total examined (males and females) of Kibwezi G. brevipalpis samples

Population	Number Examined	Genotypes					
		aa	ab	ac	bb	bc	cc
Laboratory observed	98	57	8	15	13*	0*	5
Laboratory total expected		60.052	10.324	10.324	7.637	2.026	7.638
Field total observed	105	68	7	0*	15	1*	14*
Field total expected		68.848	5.123	3.341	16.317	0.883	10.400

Laboratory $X^2 = 9.357$, 3df, $P<0.05$
Field $X^2 = 0.810$, 2df, $P>0.05$
* Combined to calculate chi-square.

TABLE 3.12: Observed electromorph frequencies at the Mdh locus in laboratory and field samples of Kibwezi G. brevipalpis

	F I E L D		L A B O R A T O R Y	
Mdh Electromorphs	No. observed	Frequency of the Electromorphs	No. observed	Frequency of the Electromorphs
Fast band	72	0.692	69	0.711
Medium band	19	0.183	16	0.165
Slow band	13	0.125	12	0.124

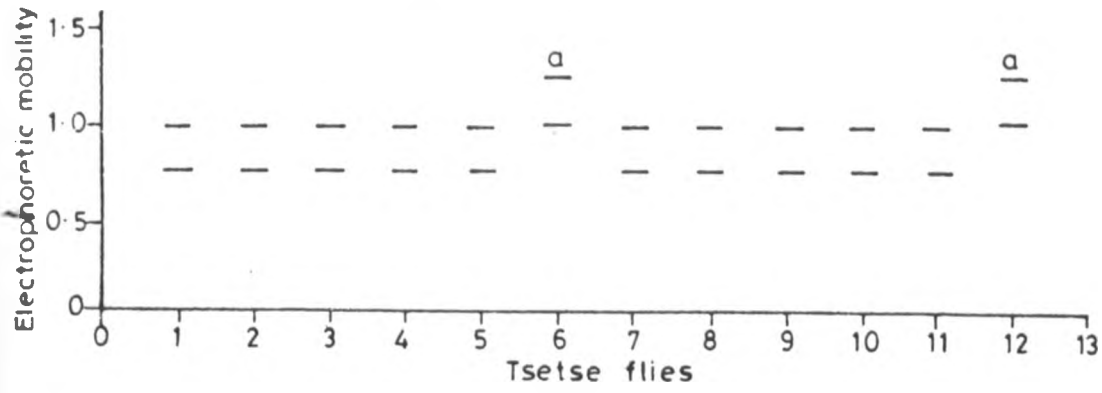

Fig. 3·4 : Diagrammatic representation of electrophoretic pattern of isocitric dehydrogenase (IDH) found in populations of G. brevipalpis. The verticcl scaledenotes mobility level of various bands. O = origin. The numbers 1 - 5 then 7 -11 show the two non varying bands observed in G. brevipalpis. a = G. austeni (control).

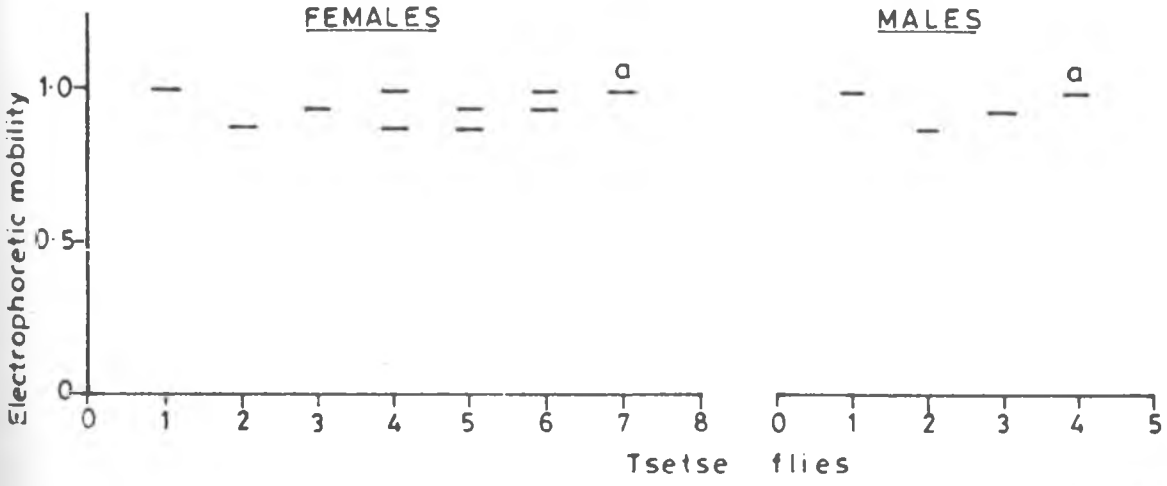

Fig. 3·5 : Diagrammatic representation of electrophoretic patterns of phosphoglucomutase (PGM) found in populations of G. brevipalpis. The vertical scale denotes mobility level of various bands. O = origin. The numbers show different genotypes as follows: 1. P_{gm-a} / P_{gm-a}, 2. P_{gm-b} / P_{gm-b}, 3. P_{gm-c} / P_{gm-c}, 4. P_{gm-a} / P_{gm-b}, 5. P_{gm-b} P_{gm-c}, 6. P_{gm-a} / P_{gm-c}, a = G. austeni (control)

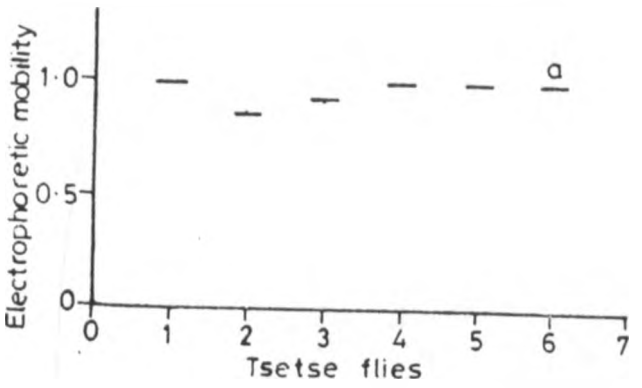

Fig. 3·6 : Diagrammatic representation of electrophoretic pattern of malate dehydrogenase (MDH) found in populations of G. brevipalpis . The vertical scale denotes mobility level of various bands . O = origin . Numbers 1-5 denotes the single band varying in three positions on the locus . a = G. austeni (control)

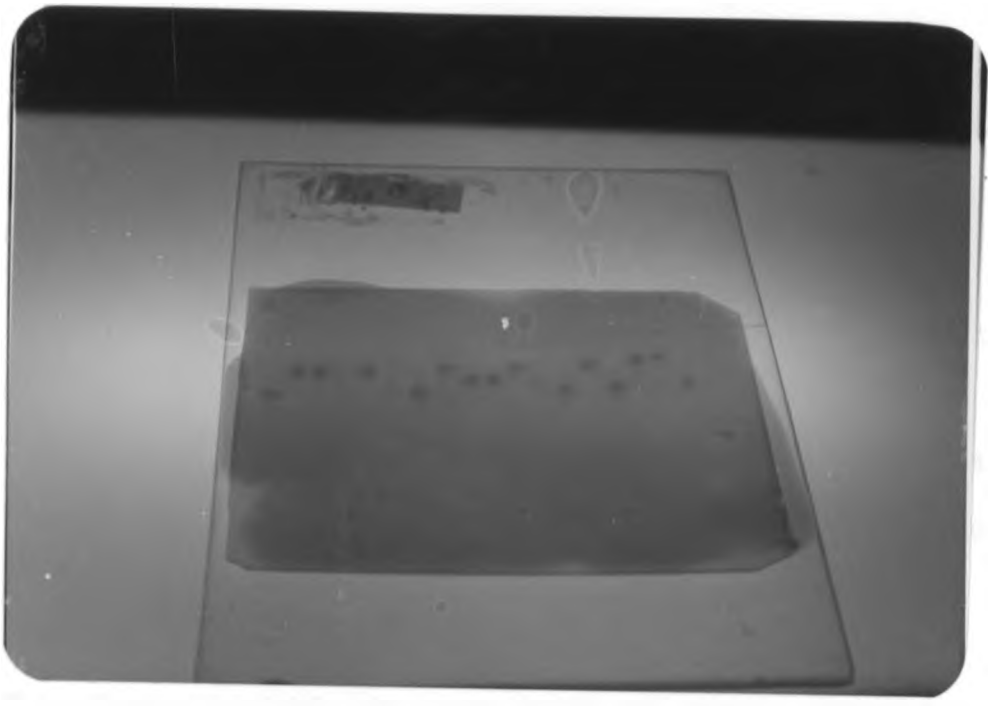

Plate: 3.4 G. brevipalpis Pgm locus. Kibwezi laboratory population (males only). Only single bands were observed in male samples. The medium band was rare.

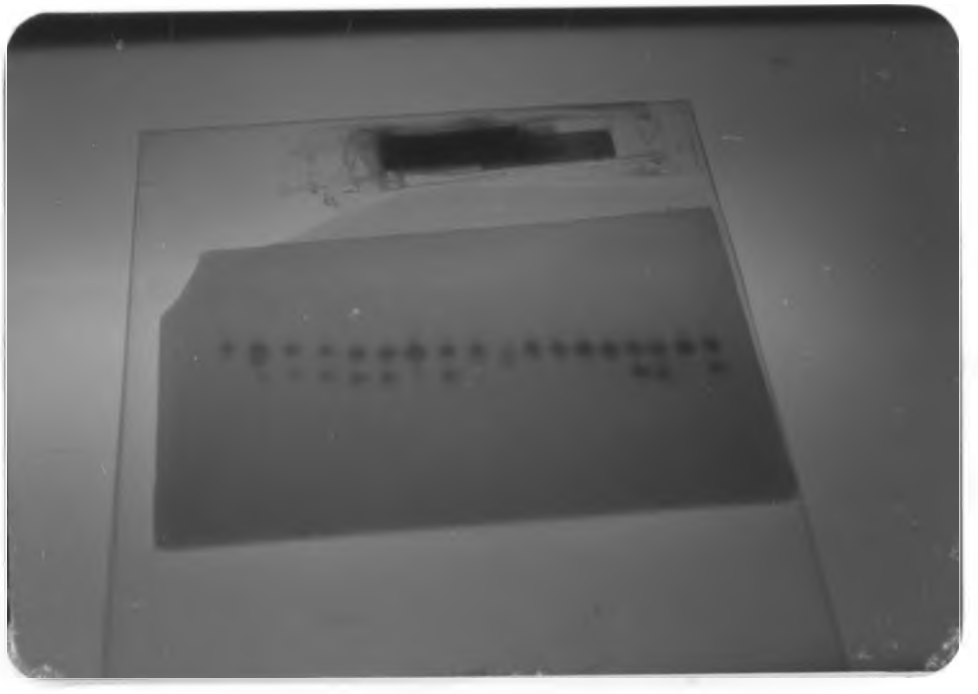

Plate:3.5 G. brevipalpis Pgm locus. Kibwezi field population (females only). Pgm was very variable in females with 2 banded heterozygotes.

CHAPTER FOUR

DISCUSSION AND CONCLUSIONS

4.1 GENERAL DISCUSSION OF SCORED LOCI

4.1.1. Proportion of polymorphic loci.

Twenty-four of the enzyme systems routinely used at the National Museums of Kenya laboratory were screened. The enzymes considered here were those consistently stainable and interpretable. To this extent, the loci were randomly chosen. The proportion of polymorphic loci (*Mdh* and *Pgm*) in both the laboratory and field flies was 22-25% for each population. This figure is within the range of 20-40% usually reported for other organisms (Nei, 1975). It is also in the range that has been observed in most other populations of tsetse flies. This is the first report of its kind for *G. brevipalpis*. But many populations of *G. pallidipes* and many other taxa of the *morsitans* and *palpalis* groups have been studied using electrophoresis. Out of 9 species and subspecies, Gooding (1982) observed P values between 16.6% to 66.6% with a modal value of 33.3%. In studies that involved populations of *G. pallidipes* in Kenya, P values of 27.3% (Van Etten, 1982c) and 15.4.% (Agatsuma and Otieno, 1988) were observed. Most of these figures are within the range of polymorphism observed in tsetse flies under this study.

4.1.2 The esterase locus.

Both Van Etten, (1982c) and Agatsuma and Otieno (1988) included leucine aminopeptidase (*Lap*) and *Est* in their investigations of *G. pallidipes* populations. But, while Van

Etten (1982c) reported polymorphism at the two loci, Agatsuma and Otieno (1988) did not observe variation on either enzyme. The Est enzyme in the investigations reported here cannot be described as polymorphic either, since the frequency of the commonest allele was above 0.95. The discrepancy in the reports may be due to different electrophoretic methods used or due to small numbers used in the present study and in the study by Agatsuma and Otieno (1988) compared to those used by Van Etten (1982c).

4.1.3 The aldehyde oxidase locus.

Similarly, aldehyde oxidase (AO) has been demonstrated previously as being controlled by a single locus with three alleles, whose heterozygous phenotype in G. pallidipes is expressed with two bands (Van Etten, 1982c). Although polymorphism was noted at this locus in seven out of nine taxa analysed by Gooding (1982), it was not reported at the locus in G. fuscipes fuscipes and G. pallidipes. Polymorphism at this locus was not observed in the present work in either G. pallidipes (plate 3.3) or G. brevipalpis. The different electrophoretic methods used might explain this discrepancy. Single pair mating experiments have given genetic support to polymorphism at the locus in G. morsitans (Geest and Kawooya, 1975; Geest et al., 1978; Rolseth and Gooding, 1978). The experiment should be extended to G. pallidipes to support the genetic involvement in the reported variations on the locus.

4.1.4 The malate dehydrogenase locus.

Discrepancies have also been observed on the Mdh

locus. While Agatsuma and Otieno (1988) did not report any variations on the locus in G. pallidipes, results in this work demonstrated a single band varying in three positions on the locus in both G. pallidipes and G. brevipalpis (Figs. 3.3 and 3.6. Mdh was first reported as being polymorphic in G.m. morsitans by Geest and Kawooya (1975), and controlled by at least one and at most four loci. This information, however, was withdrawn by Geest et al. (1978) as being an error but no further information about the enzyme was given. Gooding (1981) noted polymorphism on the locus in all three species (G. tachinoides, G.m. submorsitans and G.p. gambiensis). Each fly had one or three bands indicating a single locus with three alleles. But Mdh in 9 taxa of tsetse flies was later reported as being under the control of two loci, a polymorphic and monomorphic locus (Gooding, 1982, Gooding and Rolseth, 1982). The Mdh reported upon in this study may not be the same Mdh reported by Agatsuma and Otieno (1988).

Lack of heterozygous phenotypes at the locus in this case could be due to low viability of heterozygotes, although such a situation is unstable and is expected to lead to a loss of polymorphism (Mayr, 1970). Nevertheless it seems heterozygosity for this enzyme is indeed very low although it showed a high level of variation in this work. Gooding (1981) reported 4 heterozygous phenotypes after examination of 71 G.m. submorsitans. Nine and six heterozygotes were observed after examining 424 and 468 flies of G.p. gambiensis and G. tachinoides respectively.

Breeding experiments have ascertained genetic involvement in the Mdh variations in G.m. morsitans (Gooding,

1981). A similar experiment should be done with G. pallidipes and G. brevipalpis and much larger samples of each examined to elucidate the genetics of this locus.

It is hard in the studies described here to interpret the position of Mdh locus in the absence of heterozygotes but in G.m. submorsitans, G.p. gambiensis and G. tachinoides, it is located on autosomes (Gooding, 1981). For each enzyme that has been assigned to either X or the autosomes, the assignment has been the same in all taxa (Gooding, 1984). Therefore, it is likely that the locus or loci controlling Mdh in these taxa is/are autosomal. In both laboratory and field samples, three genotype patterns were scored. Tables 3.4, 3.8 and 3.12 show the frequencies of various electromorphs at the Mdh locus. The commonest electromorph in all the G. pallidipes populations was that controlling the medium migrating band. However, it seems that the Lambwe Valley colony flies have elevated frequencies of the otherwise rare fast and slow electromorphs. Both these electromorphs in Lambwe Valley field population were observed at a frequency of 0.045 each but had increased to 0.260 and 0.156 for the fast and slow electromorphs respectively in the laboratory colony. The commonest electromorph for G. brevipalpis populations on the other hand is that controlling the fast band and no major differences in electromorph frequencies were found in G. brevipalpis field and laboratory populations. Except for the Lambwe Valley populations, the contingency chi-square test of homogeneity detected no heterogeneity between laboratory and field samples. The increased frequency of the rare electromorphs in the Lambwe

Valley laboratory stock was the primary contributor to the significant difference observed in the Lambwe Valley samples.

4.1.5 The phosphoglucomutase locus.

Results from this work have demonstrated that the locus controlling Pgm may be under X chromosome control. Sex linkage in G. pallidipes has also been reported by Agatsuma and Otieno (1988). Observations from this work have shown that this is true for G. brevipalpis too. However it is not surprising, since, according to Gooding (1984), there is uniformity of enzyme linkage in tsetse flies from taxa to taxa. The assignment of this enzyme to linkage on the X chromosome has been possible due to presence of heterozygous individuals in females but not in males (Figs. 3.2 and 3.5. Heterozygous males can only occur if the locus is on the autosome but heterozygous females will occur when the locus is on either. This criterion was used to assign Arginine phosphokinase (Apk) (Gooding and Rolseth, 1979 and glucose-6-phosphate dehydrogenase (G6pd) (Gooding and Rolseth, 1982) to the X chromosome of G.m. morsitans and breeding experiments have confirmed the location.

Tables 3.2, 3.6 and 3.10 show the frequencies of various alleles at the Pgm locus in the six populations. Except for G. brevipalpis, the commonest allele was the same in all G. pallidipes populations and controls the slow band. In the Lambwe Valley field and Shimba Hills laboratory samples, only two alleles were observed. A third rare allele was scored in Lambwe Valley (laboratory) and Shimba Hills (field) flies.

It was observed at frequencies of 0.029 and 0.057 in the Lambwe Valley and Shimba Hills samples respectively. While three genotype patterns were noticed in the Lambwe Valley (field) and Shimba Hills (laboratory) samples, four and five genotypes were expressed in laboratory and field samples of Lambwe Valley and Shimba Hills respectively. The two alleles Pgm^a and Pgm^b observed in the Lambwe Valley field flies presumably correspond directly to Pgm^a and Pgm^b observed by Agatsuma and Otieno (1988) in Lambwe Valley field flies. In both cases, the results show that the allele Pgm^b was the commonest. Agatsuma and Otieno (1988) reported the allele at a frequency of 0.563 as compared to 0.613 in this work. In G. brevipalpis, three alleles, Pgm^a Pgm^b and Pgm^c were demonstrated in both field and laboratory samples. The mobility of the alleles, however, was different from that observed in G. pallidipes (Figs. 3.2 and 3.5). Unlike in G. pallidipes, the allele controlling the fast band was commonest in both field and laboratory G. brevipalpis samples and both populations showed five genotypes at this locus.

Using the chi-square test of homogeneity to examine whether colonization has had significant changes in the genotype frequencies of colony flies, the test detected differences between laboratory and field samples of G. brevipalpis. However, no heterogeneity could be detected in male samples (Chapter 3). The significant differences observed when female samples were examined may therefore reflect sampling errors particularly so since the sample size was small. Only 20 field females, for example, were examined. If the observed differences were true and due to effects of

colonization, similar differences would also be observed in male samples. For the G. pallidipes populations, no differences were observed between field and laboratory samples. This shows that colonization resulted in no significant changes in these populations. There is therefore little evidence for significant gene frequency changes in the laboratory colonies. Except for the laboratory G. brevipalpis samples, the total examined were in good fit with Hardy-Weinberg expectations (Tables 3.3, 3.7 and 3.11) showing that the enzyme variations were genetically controlled and that equilibrium conditions were satisfied.

4.1.6 The remaining loci.

My observations on the banding patterns of Me, Idh, Xdh and Hex were in line with results previously reported in G. pallidipes (Van Etten, 1982c; Agatsuma and Otieno, 1988), that all these loci are monomorphic although these results need to be confirmed by formal genetic crosses. This is the first report concerning the genetics of NADD in tsetse flies. This enzyme has been grouped together with Me, Xdh, Hex and Idh as monomorphic loci. Due to lack of variations on the loci, conclusions concerning their locations is difficult to make. No differences between field and laboratory samples were observed at the loci.

4.2 GENETIC IDENTITIES, DISTANCES AND HETEROZYGOSITIES.

4.2.1 Identities and distances.

In each case, the field populations were extremely similar to the laboratory populations. Pair identity values of

0.9995 and 0.9996 were observed for G. brevipalpis and G. pallidipes (Shimba Hills) respectively. The Lambwe Valley field population shares an identity value of 0.9992 with the laboratory stock. The D value between G. brevipalpis and G. pallidipes (Shimba Hills) pair was 0.00046 and 0.00036 respectively. The D value was 0.0008 for Lambwe Valley G. pallidipes pair.

The identity values calculated in this work between laboratory and field populations are high and actually fall in the range usually observed between populations of the same species. The lowest "I" value found between different populations of tsetse in the same species was 0.9986 and the highest was 0.9997 (Gooding, 1981). In the same work, Gooding (loc. cit.) observed identity values of 0.4055 (between G.p. gambiensis and G.m. submorsitans) and 0.4085 (between G.m. submorsitans and G. tachinoides). The identity value found between the more closely related G. tachinoides and G.p. gambiensis was 0.8014.

Nei's mean identity values between each pair (laboratory versus field) are all larger than the identity value of 0.9794, the value found between Lambwe Valley and Shimba Hills field G. pallidipes populations in the present studies. This shows the laboratory stocks are still very closely related to their ancestral populations. The genetic distance calculated in this work between Lambwe Valley and Shimba Hills field G. pallidipes populations was 0.0206. This is larger than the genetic distance of 0.00154 calculated

between Lambwe Valley and Nguruman field G. pallidipes populations (Agatsuma and Otieno, 1988). However this is not surprising because maps with the distribution of G. pallidipes (Ford and Katondo, 1978) suggest that, in Kenya, two major fly belts are distinguished. The Eastern belt includes the Shimba Hills population, but both the Nguruman and Lambwe Valley G. pallidipes populations are included in the Western belt. The two fly belts are separated by ecological barriers. These findings have also been supported by Van Etten (1981, 1982c).

4.2.2 Average heterozygosities.

Except for the Lambwe Valley populations, average heterozygosity in field samples was slightly higher than in laboratory populations. The $\bar{H}$ value was 0.03 in field compared to 0.023 in the laboratory population of Shimba Hills G. pallidipes. H values for G. brevipalpis were 0.067 in field compared to 0.064 in laboratory samples. But for the Lambwe Valley G. pallidipes, the $\bar{H}$ value was 0.0714 in the laboratory population compared to 0.0689 in the natural flies. The amount of heterozygosity retained in the Lambwe Valley colony as compared to its ancestral field population differed from expected on theoretical grounds. The theoretical expectations are that the inbreeding characteristics of even large colonies will eventually reduce the level of heterozygosity below that observed in natural populations (Hilburn et al., 1984). Although the differences are small, this is what was observed in other pairs investigated. But after approximately 27 generations in captivity, the Lambwe Valley colony showed $\bar{H}$ value higher than its ancestral natural population.

This, perhaps, reflects the history the Lambwe Valley natural population has gone through. The parental colony flies were collected from Lambwe Valley in January 1983 when the fly population had just recovered from the 1981 eradication attempt (Turner, 1986). Later that year (1983), another eradication attempt was done by aerial spraying using pyrethrum (Otieno, 1985). Even then, the Lambwe Valley field flies analysed in this report were collected during the time KETRI and ICIPE had intensified their tsetse control activities in the Valley using insecticide impregnated screens (KETRI) and both the Nguruman and biconical traps (ICIPE). The population size had been highly reduced (personal observation). It therefore, seems reasonable to attribute the low heterozygosity in Lambwe Valley field samples to bottleneck effects they have experienced before and at the time wild tsetse flies were collected.

At this point however, it is important to remember that the ICIPE G. pallidipes colony unlike other laboratory colonies under study is kept under conditions that simulate those of the surrounding climate (Chapter 2). The natural conditions at Mbita point where the colony is kept are not very different from those of Lambwe Valley where the parental stock was collected in January 1983. This point might have a role to play in the high genetic variation retained in the Lambwe Valley Laboratory colony. To elucidate this point however, it will be necessary to carry out further investigations.

The $\bar{H}$ values obtained in these investigations are similar to the degree of heterozygosity reported previously in tsetse fly populations. Few natural populations of tsetse

flies have been studied but in three species, G. tachinoides, G.p. gambiensis and G.m. submorsitans, the average heterozygosity varied from 0.0146 to 0.0431 (Gooding, 1981). In 9 taxa of tsetse flies examined, $\bar{H}$ values varying from 0.05 to 0.21 were observed (Gooding, 1982). The average heterozygosity was 0.088 and 0.092 for the Nguruman and the Lambwe Valley G. pallidipes populations respectively (Agatsuma and Otieno, 1988). The values reported here are also within the range of $\bar{H}$ found in natural populations of other organisms, for example 0.134 in 57 species of invertebrates and 0.06 in 68 species of vertebrates (Dobzhansky et al., 1977).

4.3 SUMMARY AND CONCLUSIONS.

4.3.1 EXPECTED RESULTS.

One would have expected that as a result of sampling error, genetic drift or even selection, genetic variations in colony flies would definitely be less than in natural populations. Several examples to this effect have been reported. For example, there was a decrease in heterozygosity of Phormia regina (Berlocher and Friedman, 1981) and Cochliomyia hominivorax (Bush et al., 1976) after colonization for 1 and 15 generations respectively. Extensively inbred G.m. morsitans colony flies were all homozygous for ME and LAP enzymes while the same enzymes showed a high degree of heterozygosity in a more outbred colony of the same species (Gooding, 1984).

4.3.2 OBSERVED RESULTS.

The important observations of these investigations, however, are that laboratory flies are genetically approximately the same as those of the field. The differences in the allozyme frequencies are not statistically significant and not consistent. For example, at the Pgm locus, while more genotypes were observed for the Shimba Hills (wild) G. pallidipes than its corresponding laboratory population, the reverse was true for the Lambwe Valley (G. pallidipes). Assuming five generations per year (in fact, generations vary from 5 to 7 yearly) G. brevipalpis has been in captivity for 28 generations while G. pallidipes colonies have been colonized for 7 and 27 generations for the Shimba Hills and Lambwe Valley colonies respectively. Yet the colony flies did not differ from field samples in the percentage of polymorphism. Even within the monomorphic loci, there was no fixed locus difference. Nei's mean identity values between each pair were very high, showing that the laboratory and field samples were extremely similar. Although the number of genotypes was slightly higher in G. pallidipes (Lambwe) and G. brevipalpis (laboratory samples) than the corresponding field populations, the differences were small and not statistically significant in the female samples. The differences in the mean heterozygosities were also small.

It appears therefore, that colonization has had no significant change on genetic variations within the colony flies. The small differences observed probably reflect

sampling error, particularly so, since only a few samples and loci were analysed. It may be that with greater numbers of generations, some divergence and drift might occur, although Burri (1956) demonstrated significant drift after only 10 generations in Drosophila. Probably the large population sizes of the colonies help to prevent drift.

4.3.3. SUMMARY OF SIMILAR EARLIER OBSERVATIONS.

This low level of genetic difference between field and laboratory populations, is not surprising considering reports from the previous authors. For example, adult flies from G.m. morsitans colonies of 6 to 48 generations showed no difference in fecundity and survival rate from the wild caught flies (Jordan et al., 1970). Recaptures from artificial refugees, odour attractants and mobile baits indicated no clear differences between native G.m. morsitans and two laboratory reared groups in respect of: body size, amount of fat present at emergence, survival, dispersal, availability to a range of baits, diet, speed of taking a first meal, wing damage, and insemination rate (Vale et al., 1976). An intensively inbred G.m. morsitans colony for 40 generations did not show significant changes in female longevity, puparial weight, female fecundity, emergence rate and sex ratio (Jordan, 1980). Despite approximately 130 generations as a laboratory colony, G.m. morsitans maintained at the Tsetse Research Laboratory University of Bristol was not significantly different from field collected flies with respect to mean heterozygosity per locus, average number of alleles per locus and number of polymorphic loci for the 12 enzyme systems analysed (Gooding and Jordan, 1986).

4.3.4 CONCLUDING REMARKS.

Although G. pallidipes is one of the most important vectors of nagana and sleeping sickness, no genetic comparison has been made of wild caught and laboratory reared flies of this species. Furthermore, this is the first report of the enzyme electrophoresis in G. brevipalpis. From these results, however, it is apparent that sufficient genetic variability has been retained in the colonies of these two species. One can safely conclude that the laboratory samples are good representatives of the corresponding natural flies.

It seems, therefore, that although too intense inbreeding should be avoided whenever possible, the risk of marked inbreeding depression occuring in average sized colonies is extremely low. Since most established tsetse colonies, including these under study, comprise large numbers of individuals, the inbreeding problem is indeed very negligible. At the time the laboratory samples were collected, the female stock in Lambwe Valley colony was 4,412 flies. This figure was 3,200 and 1,500 for the Shimba Hills G. pallidipes and G. brevipalpis colonies respectively (Chapter 2). Furthermore, because of their low fecundity, most breeding methods of tsetse flies will not tolerate high mortality rates in the colony flies. The mean female mortality rate for example, was 1.5% for the Lambwe Valley G. pallidipes colony. It was 0.08% and 1.06% for G. pallidipes (Shimba Hills) and G. brevipalpis colonies, respectively. The low mortality rates, ensure that most members of the colony will contribute to the breeding population in the next generation, further minimizing effects of selection and drift.

In tsetse flies, polygenic characters such as puparial weights, emergence rates, female longevity, female fecundity, and sex ratio are the characters commonly used to monitor colony performance. Since these characters might have a low heritability, enzyme electrophoresis should be added to the above characters to monitor colony progress (Gooding, 1984). In G. pallidipes and G. brevipalpis, results from this work have shown that there is a large amount of heterozygosity at the Pgm locus in females. Variability at the locus in males is also high (Ch.3). The locus is an excellent marker that can be used to monitor tsetse colony performance.

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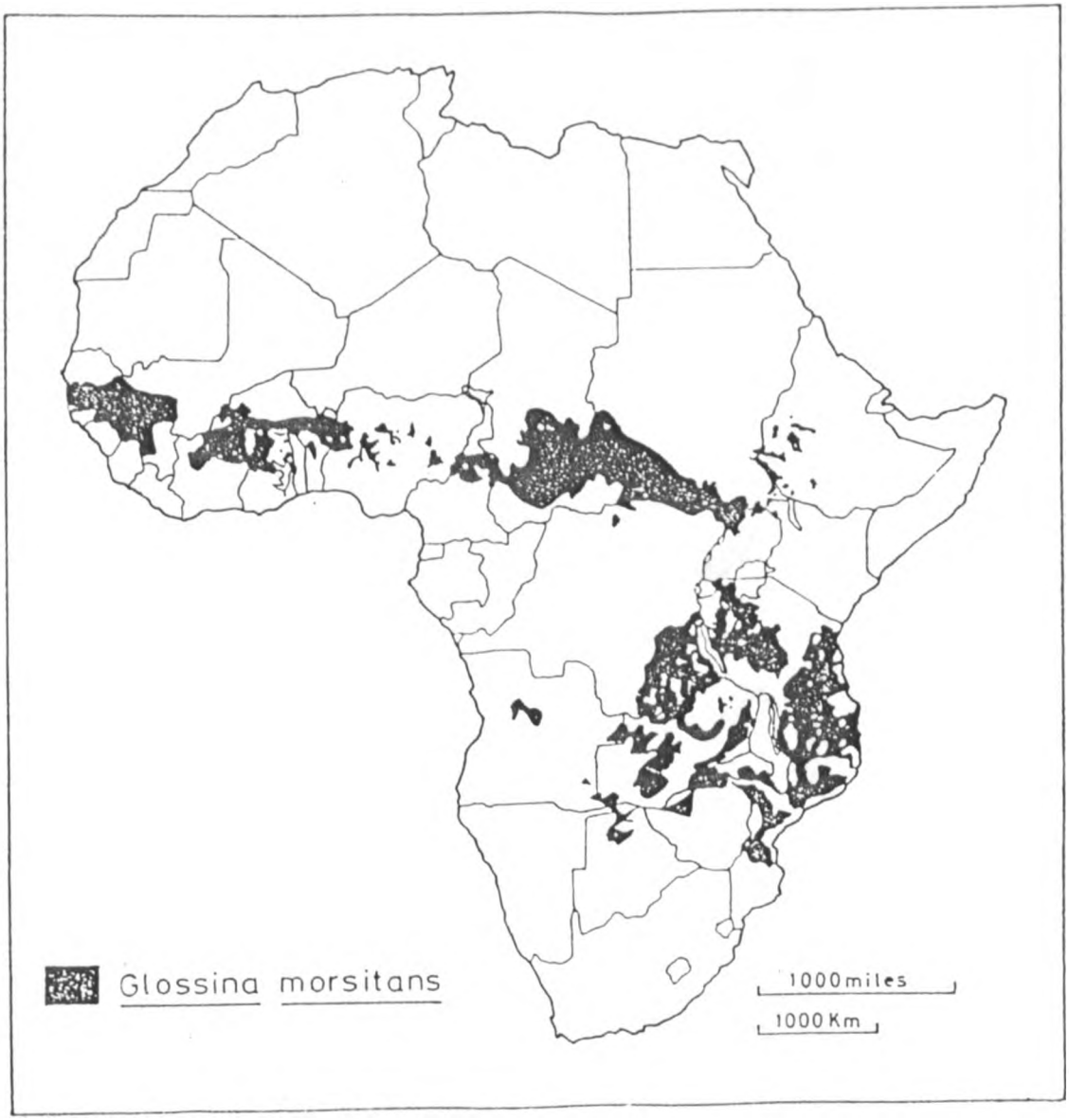
APPENDICES



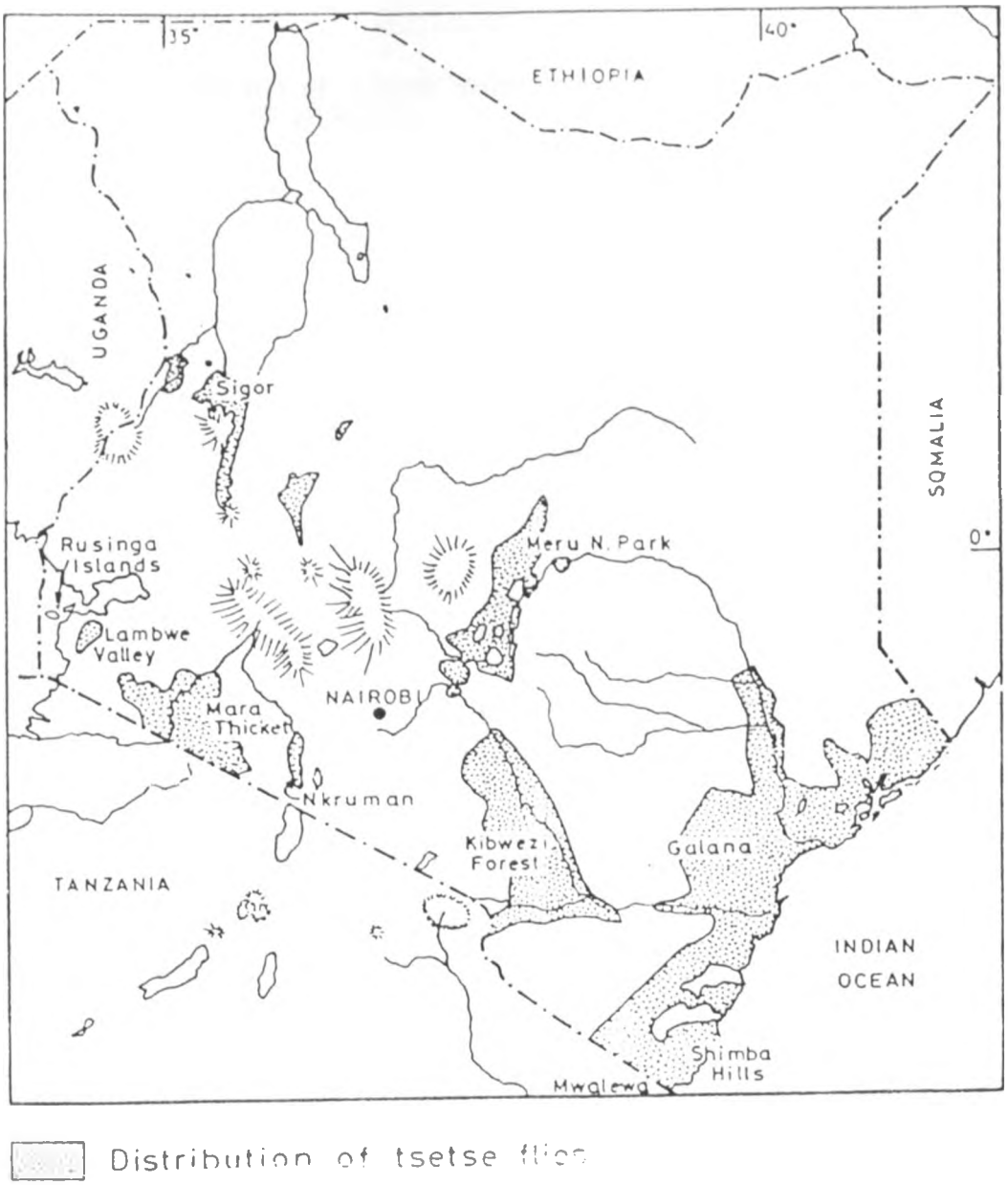
Appendix 1.1 Distribution of Glossina morsitans s.l. in Africa (Pollock, 1982).

Appendix 1.2 Distribution of Glossina pallidipes

in Africa (Pollock, 1982).

Appendix 1.3 Distribution of Glossina austeni in Africa (Pollock, 1982).

Appendix 1.4 Distribution of Glossina brevipalpis in Africa (Pollock, 1982).



Appendix 1.5 Distribution of tsetse flies in Kenya (National Atlas of Kenya, 1970).

Appendix 2.1: Preparation of tissue buffer and stock solutions modified from Harris and Hopkinson (1977)

Buffer and stock solutions	composition	Amount used
Tissue buffer	0.650gm. Tris 0.370gm. EDTA (Ethylene diaminetetraacetic acid) 250ml. Deionized water Adjust pH with HCl to 7. Add 1ml. of NADP stock solution	0.3ml. per fly
NAD (Nicotinamide adenine dinucleotide) stock solution	30mg. NAD powder mixed with 1ml. deionized water.	150 ul
MTT (3-4,5-Dimethiozol-2-yL) -2,5-tetrazolium bromid	40mg. MTT powder in 1ml deionized water.	150 ul
NADP (Nocotinamide adenine dinucleotide phosphate ($NADP-Na_2$).	25mg. ($NADP-Na_2$) powder in 1ml deionized water	150 ul

Appendix 2.2: Preparation of tray and gel buffers modified from Harris and Hopkinson (1977)

Buffer	pH	Use	Composition	Gel Buffer Preparation
Tris maleate (TM)	7.4	Tray buffer	12.1gm tris 11.6gm maleic acid 3.7gm EDTA 2.03gm $MgCl_2$ Add 1 litre deionized water, adjust pH to 7.4 with 1m. NaOH.	1:9 dilution of tray buffer with deionized water (25ml. tray buffer + 225ml. deionized water).
TEB (Tris, EDTA, Boric acid)	9.1	Tray and gel buffers	10.53gm. Tris 0.875gm. Boric acid 0.372gm EDTA 1000ml. deionized water.	Use undiluted tray buffer. 250ml. tray buffer
Tris citrate (TC)	6.3	Tray buffer only	27gm. Tris 18.07gm Citric acid 1 litre deionized water Adjust pH to 6.3 with 1m. NaOH.	-
Tris citrate (TC)	6.7	Gel buffer only	7.74gm Tris 5.04gm. Citric acid 1 litre deionized water Adjust pH to 6.7 with 1m. NaOH.	dilute the gel buffer 1:7 (30ml. gel buffer + 220ml. deionized water

Appendix 2.3: Preparation of developing buffers and substrates modified from Harris and Hopkinson (1977)

Buffer or substrate solution	pH	Use	Composition	Amount used
Esterase buffer	6.1	Esterase developer	6gm. NaH_2PO_4 (Sodium dihydrogen phosphate anhydrous) 1.42gm. Na_2HPO_4 (disodium hydrogen phosphate anhydrous) 1 litre deionized water.	50ml
MDH Substrate	7.0	Substrate for (MDH) + (ME)	13.4gm. L-malic acid 49ml. 2m. Na_2CO_3 pH Adjusted with Na_2CO_3	3ml.
Esterase Substrate solution	-	Substrate for esterase	1gm. Alpha naphyl acetate 100ml. Acetone	1ml.

Appendix 2.4: Staining conditions for the isoenzymes modified from Harris and Hopkinson (1977)

Enzyme	Additional ions.	Coenzymes	linking Enzymes	Substrate	Visualizing Method
PGM	$MgCl_2$ 150 ul	NADP 150 ul	Glucose-6-phosphate dehydrogenase (G-6-P-D) 5 ul	80 mg Glucose 1-phosphate+ Glucose 1-6 diphosphate	MTT 150 ul PMS 2 mg Agar overlay 10ml of 2g/ 100ml solution
ME	$MgCl_2$ 150 ul	NADP 1.25mg	-	L-malate 1m. pH 7 3ml	MTT 150 ul PMS 2 mg Agar overlay 10ml of 2g/ 100ml solution
MDH	-	NAD 150 ul	-	L-malate 1m. 3ml.	MTT 150 ul PMS 2 mg Agar overlay 10ml of 2g/ 100ml solution
IDH	$MgCl_2$ 150 ul	NADP 150 ul	-	Na-isocitric acid 20 mg	MTT 150 ul PMS 2 mg Agar overlay 10ml of 2g/ 100ml solution
HEX	$MgCl_2$ 150 ul	NADP 150 ul ATP 12.5mg	G-6-P-D 5ul	Glucose 1gm	MTT 150 ul PMS 2 mg Agar overlay 10ml of 2g/ 100ml solution
GPI	$MgCl_2$ 150 ul	NADP 150 ul	G-6-P-D 5ul	Fructose 6-phosphate 20 mg	MTT 150 ul PMS 2 mg Agar overlay 10ml of 2g 100ml solution
NADD	-	-	-	NADP 10mg Dichloroindo-phenol 1mg	MTT 5 drops PMS 2 mg Agar overlay 10ml of 2g/ 100ml solution
XDH	-	NAD 150 ul	-	Hypoxanthine 10mg	MTT 150 ul PMS 2 mg Agar overlay 10ml of 2g/ 100ml solution
AO	-	NAD 150 ul	-	Benzaldehyde 0.1 ml EDTA 1mg	MTT 150 ul PMS 2 mg Agar overlay 10ml of 2g/ 100ml solution
MPI	-	NADP 150 ul	G-6-P-D+ phospho-gluconate isomerase 10ul	Mannose 6 phosphate 10mg	MTT 150 ul PMS 2 mg Agar overlay 10ml of 2g/ 100ml solution
EST	-	-	-	NaH_2PO_4 6gm Na_2HPO_4 1.42gm	Fast blue 25mg

NB: Except for EST, the staining mixture of every enzyme was always mixed in molten agar and Tris buffer (10 ml. of each) and poured over a staining plate. For EST, the gels were immersed into the staining mixture contained in a staining bowl.