FACTORS AFFECTING THE REPRODUCTIVE PERFORMANCE AND EFFECTS OF CERTAIN INSECT GROWTH REGULATORS ON REPRODUCTION IN *GLOSSINA FUSCIPES FUSCIPES* NEWSTEAD (DIPTERA: GLOSSINIDAE)

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DECLARATION

This thesis is my original work except where acknowledged, and has not been presented in any other University for examination.

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DEDICATION

To my parents, John and Hellen Ager, for their sacrifice and dedication to see me go this far.

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ABSTRACT

The reproductive behaviour of *Glossina fuscipes fuscipes* was studied with the aim of determining factors that influence its laboratory colonization. Further work was designed to determine the effects of certain insect growth regulators (IGRs) on reproduction and metamorphosis with a view to assess the possibility of their use in managing tsetse populations.

Receptivity was highest ($F= 2.40$, $p< 0.05$, $n= 473$) in young females aged one to two days, older females being less receptive. Males from four days of age inseminated females but mating was more successful ($F= 4.55$, $p< 0.01$, $n= 246$) in older ones (eight to 12 days). Fecundity of females from Rusinga Island, Kenya was low ($F= 143$, $p< 0.0001$, $n= 331$) compared to that of an established laboratory population from Seibersdorf, Austria which originated from Central African Republic (C.A.R). Fecundity of females from the Rusinga population was affected by abortion, slow growth of follicles and egg retention in inseminated females. First filial generation females produced low weight pupae compared to those produced by parental females ($F= 136.9$, $p< 0.001$, $n= 397$). Laboratory reproductive performance was also affected by low adult emergence rates ($\chi^2 = 70.02$, $p< 0.001$, $df = 1$) of the F2 generation compared to that of the F1. Other reproductive abnormalities, e.g. equality in size of the two most developed follicles, fusion of follicles and abortions, were observed both in laboratory and wild populations. Abortion rates were significantly higher in the Rusinga population ($\chi^2 = 104.2$, $p< 0.001$, $n=1331$) than in the C.A.R population.

Topical treatment of females with the insect growth regulators (IGRs) pyriproxifen, W-328 and precocene had no effect on fecundity or pupariation. However, adult emergence was inhibited. Pupariation failed in 1.7% (n= 235) of the larvae produced by ketoconazole treated females. Juvenile hormone replacement in precocene treated females resulted in some emergence in the first reproductive cycle in pyriproxifen-treated females but not in W-328-treated ones. Treated males transferred sterilizing effects of JH analogues to females with which they mated, resulting in inhibition of emergence. The frequency of adults without any tergites was higher ($\chi^2 = 8.70$, $p < 0.01$, n=360) in W-328 treated females than in pyriproxifen treated ones. Treatment of puparia of varying developmental stages with the two JH analogues inhibited emergence in puparia below five days post-larviposition. Those over five days showed high emergence rates irrespective of dose. Where emergence was inhibited, histological studies indicated failure of formation of the adult abdominal cuticle.

Poor reproductive performance of the Rusinga population may be due to lack of adaptation to laboratory conditions and supplementation of existing colonies with wild females or puparia needs to be continued until a stable colony is attained. Although a small proportion of larvae produced by ketoconazole-treated females did not pupariate, the fact that some went through normal development suggests that ketoconazole may not be effective in inhibiting synthesis of ecdysteroids in *Glossina*. Since the JH analogues have been shown to disrupt development in this species, field studies, which have shown promising results with the *morsitans* group, need to be initiated for the *palpalis* group as well to determine their response in the field.

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Mass rearing of laboratory insects is important for research and in control programmes. For *Glossina* species, sufficient numbers of sexually competitive individuals are necessary to effect control programmes such as the sterile insect technique (SIT) and the lethal insecticide technique (LIT). Mass rearing requires that females have high reproductive performance in the laboratory, evaluated through fecundity, offspring size, and survival (Gooding *et al.*, 1997). Several species of *Glossina* have been reared since the first successful colonization of *Glossina morsitans morsitans* Westwood (Azevedo & Pinhao, 1964). Colonization of *G. austeni* Newstead was similarly achieved by maintaining females *in vivo* on rabbits (Nash *et al.*, 1966). Successful rearing of *G. f. fuscipes* that originated from Central Africa Republic has been achieved through *in vitro* feeding, on a diet consisting of 75% bovine and 25% porcine blood (Gooding *et al.*, 1997). In Kenya, the Rusinga population of *G. fuscipes fuscipes* Newstead was reared under semi-natural conditions (Mwangelwa, 1990) with limited success. The colony was not self-sustaining and required wild female supplementation. Similar attempts have been made in Kenya at the International Livestock Research Institute (ILRI) and the International Center for Insect Physiology and Ecology (ICIPE), but without success. In all cases, unknown factors affected reproductive performance of the colonies, leading to population crash. There is need to assess factors affecting the various reproductive parameters under laboratory conditions, with a view to establish self-sustaining colonies.

The present study on reproductive performance of *G. f. fuscipes* was designed to identify factors that affect successful laboratory rearing of the species. Overcoming such factors would result in increased fecundity and survival as well as large offspring sizes, which in turn would lead to regular supplies of tsetse.

Over the years, various methods of tsetse control have been tried, the conventional one being the use of insecticides. However, insecticides have the disadvantage of being environmentally detrimental and costly. Recently, research on the use of insect growth regulators (IGRs) as a way of controlling tsetse has been the focus of many scientists. Certain IGRs like juvenile hormone analogues (JHAs) sterilize females, while males can transfer sterility to females with which they mate (Langley *et al.*, 1990; Hargrove & Langley, 1990, 1993). Sterility is effected through interference with development of the fly within the puparium, culminating in inhibition of emergence (Langley & Pimley, 1986; Langley *et al.*, 1988). Precocene sterilizes female offspring by causing retardation of oocyte maturation (Samaranayaka-Ramasamy & Chaudhury, 1981), while the ecdysteroid biosynthesis inhibitor ketoconazole suppresses oocyte maturation in the cricket, *Gryllus bimaculatus* de Geer (Ensifera: Gryllidae), hence reducing fecundity (Hoffmann *et al.*, 1996).

Studies on effects of IGRs on *Glossina* have not focused on *G. f. fuscipes*, yet it is of economic importance in the eastern Africa region. The present study tested the sensitivity of *G. f. fuscipes* to pyriproxifen, W-328 and ketoconazole, their potential in disrupting reproduction, and hence their possible use in controlling this species. My study also assessed the morphogenetic effects of the IGRs on *Glossina*.

In the following review I have described, with particular emphasis on *G. f. fuscipes*, the distribution and taxonomy of tsetse flies, their economic importance, and the historical and conventional control methods. Also described are the mating behaviour of *Glossina*, rearing

methods, and effects of insect growth regulators (IGRs) on cyclorrhaphous flies. Finally, the effect of photoperiod on eclosion behaviour is considered.

1.2 Literature Review

1.2.1 Distribution of *G. f. fuscipes*

The distribution of tsetse flies, *Glossina* species, was until recently, reported to be confined to tropical Africa (Buxton, 1955; Mulligan, 1970; Service, 1980, Katondo, 1984). This is mostly in areas with agricultural activities, covering approximately 11 million km². The area is delimited by latitudes 14°N and 29°S (Kuzoe, 1993). However, the occurrence of *G. f. fuscipes* and *Glossina morsitans submorsitans* Newstead in south-western Saudi Arabia was recently reported (Elsen *et al.*, 1990) (Fig. 1.1). Such a distribution is exceptional because tsetse limits are known to be determined by climate, palaeoecology, feeding habits and vegetation (Buxton, 1955; Mulligan, 1970), with areas devoid of trees being free of the insect. The proximity of the Jizan area of Saudi Arabia to the Red Sea may provide the humid conditions and vegetation requirements for *G. f. fuscipes*. Adaptation to new environments may give rise to new disease foci and compound management programmes.

1.2.2 Identification

Tsetse flies are generally identified by the rigid and forwardly projecting proboscis, and the characteristic wing venation that has a closed hatchet-like cell between veins four and five, hence the hatchet cell. Also, the two wings are folded scissors-like when the flies are at rest, and extend beyond the end of the abdomen (Kettle, 1990). The antenna has an elongated third segment like other cyclorrhapha, but is distinguishable by the feathered rays of the arista, the rays being present only on its dorsal side.

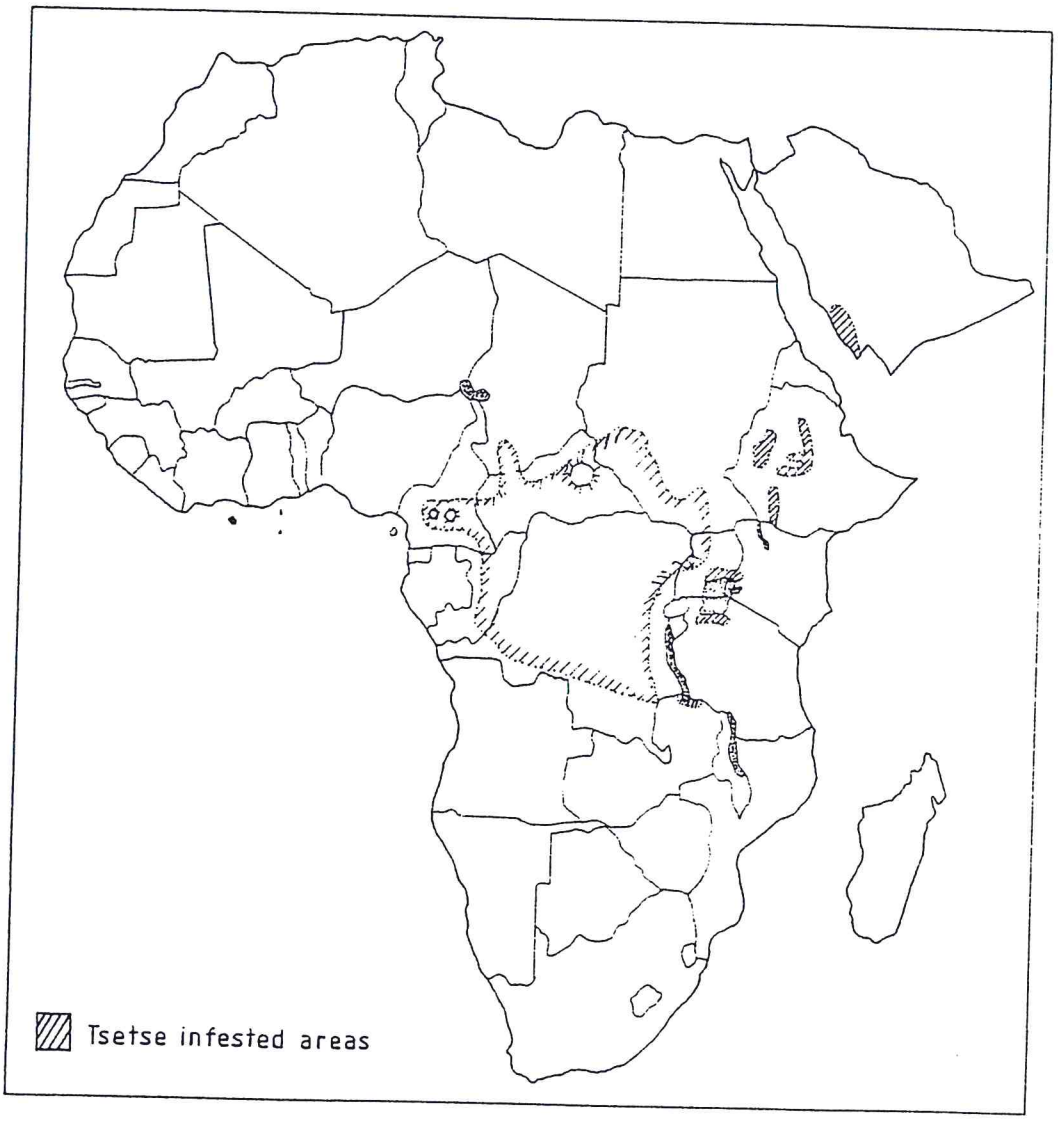


Fig. 1.1: The distribution of *G. f. fuscipes*.

1.2.3 Taxonomy

Tsetse flies belong to the monogeneric family, the Glossinidae. The genus *Glossina* has 31 taxa, comprising 23 species and 14 subspecies (Moloo, 1993). These flies are sub-divided into three groups: the *fusca*, the *palpalis* and the *morsitans* groups (Table 1.1). The groups are generally identified by the external genital armature of both sexes and habitat preferences (Jordan, 1993). For instance, in the *palpalis* group females have six genital plates while in males, a thin membrane that is deeply divided medially connects the superior claspers.

Glossina f. fuscipes belongs to the *palpalis* group of tsetse flies, which contains five species and seven subspecies. Like other members of the group, *G. f. fuscipes* is found in lowland forests and river systems associated with some of the great lakes of East Africa (Jordan, 1993). It also occurs in lake shores and rivers draining to the Atlantic Ocean, Mediterraenian Sea and Indian Ocean (Jordan, 1986). The species is widespread in eastern and central Africa, but is also found in Cote d'Ivoire (West Africa), Zambia (southern Africa), and Saudi Arabia (Moloo, 1993). In Kenya, *G. f. fuscipes* inhabits thickets along the shores of Lake Victoria (Fig.1.1). Members of this group are the major vectors of human African trypanosomiasis.

1.2.4 Economic Importance

Tsetse flies transmit the protozoan parasite, *Trypanosoma,* which causes human African trypanosomiasis (sleeping sickness) and nagana in cattle. FAO reports indicate that over 50 million people, 50 million heads of cattle, and tens of millions of small ruminants are at a risk of being infected with trypanosomes transmitted by the tsetse flies. According to Cattand (1995), 20 - 25,000 new cases of sleeping sickness are reported each year. When cattle are infected, both milk and meat production are reduced (Jordan, 1986).

Table 1.1: *Glossina* species and subspecies in the *fu*
(From Moloo, 1993).

Fusca	*Palpalis*
G. fusca fusca Walker 1849	*G. palpalis palpalis*Rob
G. f. congolensis Newst. &	*G. p. gambiensis* Vand.
Evans 1921	*G. fuscipes fuscipes* Ne
G. tabaniforms West.1850	*G. f. martini* Zumpt 19
G. longipennis Corti 1895	*G. f. quanzensis* Pires 1
G. brevipalpis Newst.1910	*G. pallicera pallicera* E
G. nigrofusca nigrofusa Newst.	*G. pallicera newstead*
1910	*G. caliginea* Austen 19
G. n. hopkinsi van Emden 1944	*G. tachinoides* Westwo
G. *fuscipleuris* Austen 1911	
G. medicorum Austen 1911	
G. frezili Gouteux 1987	
G. severini Newstead 1913	
G. schwetzi Newst. & Evans 1921	
G. haningtoni Newst. & Evans 1922	
G. vangoofi Henrard 1952	
G. nashi Potts 1955	

Direct losses from tsetse transmitted bovine trypa
1.2 billion per annum. Investment in tsetse and T
estimated at US$ 200 million annually (ICIPE, 1
reduction in agricultural output since where the c

conditions are also suitable for tsetse flies. The disease limits the development of mixed arable and livestock farming. Besides, cattle are used for draught power for small scale farming in most Sub-Saharan African countries (TDR, 1996), a practice which cannot be carried out effectively in trypanosomiasis-endemic areas. Indirect losses in livestock potential and crop production due to tsetse flies are estimated to cost US $4 billion annually. This estimate excludes loss due to human potential when they keep off tsetse infested areas (TDR, 1996) or when they get incapacitated by the disease. Thus economic growth can be seriously hampered by the presence of tsetse flies in a region.

1.2.5 Control Methods

Tsetse control in Africa started in the early 1900s (Dransfield & Brightwell, 1992). Various methods have been tried, such as the killing of large numbers of wild animals (Jordan, 1986). This resulted in extermination of wild game, and was a threat to species diversity. Later, only host species were culled, as a way of starving the tsetse flies. However, this proved ineffective since the flies switched over to non-host animals such as reptiles (Dransfield & Brightwell, 1992). Clearing of vegetation was introduced but led to indiscriminate destruction and environmental degradation. Besides, when the cleared areas were used for agricultural practices, tsetse flies migrated and occupied the newly created habitats such as banana and cocoa plantations, and even *Lantana camara* L. bushes in peridomestic areas (Okoth, 1986). Man and his domesticated animals thus came into closer contact with the tsetse fly.

Insecticides have also been used in the fight to control tsetse flies (Baldry, 1971; Gao *et al.*, 1990; Kupper, 1988; Turner, 1984). Although no resistance has been shown, the use of conventional area-wide control methods through aerial spraying of insecticides has demerits since insecticides are relatively expensive, are ineffective against tsetse fly populations in

densely vegetated areas, and are environmentally harmful. Besides, small numbers of flies may survive in sprayed areas and re-establish the population (Turner & Brightwell, 1986). Even areas that are completely cleared of flies may have flies immigrating from neighbouring areas.

The use of traps to sample and control tsetse dates back to 1910 (Maldonido, cited by Buxton, 1955). Different *Glossina* species are trapped by specific trap types *G. f. fuscipes* being readily trapped using the biconical trap (Challier & Laviessiere, 1973). For other *Glossina* species, however, traps are more effective when baited with attractants derived from hosts (Challier, 1977).

The sterile insect technique (SIT) has shown promising results in a number of *Glossina* species. Application of deltamethrin followed by the release of sterile males made it possible to suppress a population of *G. p. gambiensis* in the Volta Noire tributaries of Upper Volta (Cuisance *et al.*, 1978; Van der Vloedt *et al.*, 1980). The release of irradiated pupae was also tried to eradicate a population of *G. m. morsitans* in Tanga, Tanzania (Helle, 1978; Dame & Williamson, 1979), while use of a combination of traps, insecticide-impregnated targets and the SIT eradicated *G. palpalis* in a 300 km² area of central Nigeria (Takken *et al.*, 1986). The SIT has a lot of potential in the control of flies in that it is not environmentally detrimental and is specific against the target pest. Its success depends on the fact that once a female mates with a sterile male, she is unlikely to produce offspring for the rest of her life.

Attempts have been made at biological control, parasitoids being more promising than other agents (Laird, 1977). More recently, the use of insect growth regulators (IGRs) has gained importance in tsetse control. IGRs have been described as third generation pesticides (Williams, 1967) because they are selective in their action and have no undesirable effects on man, wildlife, or the environment. They characteristically interfere with growth and development, without directly killing the target pest (Howard & Hall, 1995). The compounds

can be used to suppress insect populations due to their inhibitory effects on metamorphosis and the derangement of embryogenesis (Staal, 1975).

This method has merits over the SIT, which depends on sterile sperm being transferred to a female. This implies that the SIT is ineffective in case of an unsuccessful mating. In the use of IGRs, transfer of sterility can occur even in an unsuccessful copulation, provided that a male has had sexual contact with a female. Thus, males have the potential to transfer sterility to a large number of females in a population. Success of both the SIT and use of IGRs, however, depend largely on the regular supply of sufficient and sexually competitive males from a mass rearing facility.

1.2.6 Reproductive Systems and Mating Behaviour in *Glossina*

1.2.6.1 The Male Reproductive System

The male reproductive system in *Glossina* consists of paired reddish-brown testes, and paired accessory reproductive glands, both of which empty into the ejaculatory duct (Buxton, 1955). In *G. f. fuscipes*, the accessory reproductive gland is not of uniform thickness as in other species of *Glossina*, but has a localized bulge about the apical part of the gland (Fig. 1.2) (Pollock, 1974). The diffuse secretion from these glands, which is believed to take part in protection of sperm during transfer to the female, is practically absent in this species.

1.2.6.2 The Female Reproductive System

The female system consists of two ovaries, each containing two ovarioles (Fig. 1.3) (Saunders, 1960a; 1960b). The ovaries open into a short, broad, common oviduct that widens to form a uterus (Buxton, 1955). Opening into the dorsal anterior end of the uterus is a pair of small spherical reddish-brown structures called the spermathecae, which store sperm. The female accessory reproductive glands are modified to form uterine or milk glands. These consist of a

series of tubules that ramify throughout the female abdomen, the tubules finally joining to form a common duct. The gland synthesizes secretion that flows through the common duct to the uterus, where it is used in nourishing the developing larva. The uterus opens into the vagina, which leads to the outside.

1.2.6.3 Mating Behaviour in *Glossina*

Mating behaviour of *G. f. fuscipes* has not been studied to the same extent as other species of *Glossina*. For successful mating to occur, females have to be sexually receptive, and this should be at a time when male and female reproductive systems are also mature, so that insemination and fertilization are effected (Langley, 1977). Sexual receptivity is the readiness or willingness of a female to copulate, and has been linked to age at mating. In most *Glossina* species, mating takes place while the females are relatively young. In *G. palpalis* females are most receptive between five and eight days after emergence, after which receptivity declines. Different ages of peak receptivity have been reported for *G. pallidipes*, ranging from nine to 14 days (Vanderplank, 1947; Odhiambo, 1968; Rogers, 1972; Davies-Cole, 1990) while receptivity usually occurs one to three days after emergence in *G. austeni*, *G. m. morsitans* and *G. palpalis* in the laboratory (Nash *et al.*, 1968, 1971; Saunders & Dodd, 1972; Jordan, 1974).

The age at mating of male tsetse is another important factor in colony establishment. *Glossina pallidipes* males have been reported to be potent when nine to 13 days old (Rogers, 1972; Davies-Cole, 1990). The age at mating of male tsetse was found to have no effect on reproductive performance of females with which they mated (Rogers, 1972; Jaenson, 1979a). The males used in the study, however, were relatively old, ranging from seven to 13 days. Males of the Ugandan population of *G. f. fuscipes* (referred to as *G. palpalis*) were fully potent

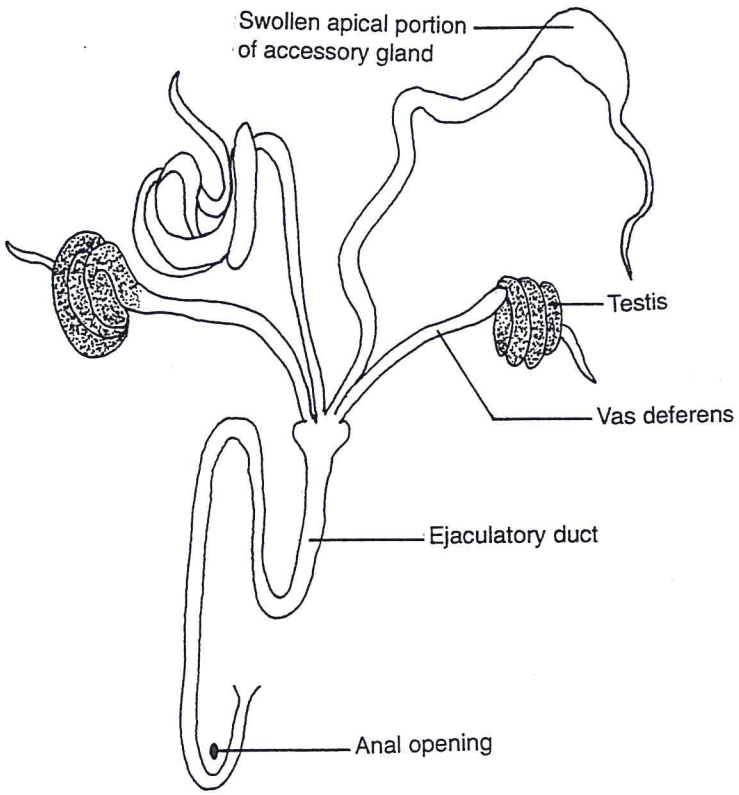


Fig. 1.2: The reproductive organs of male *G. f. fuscipes*.

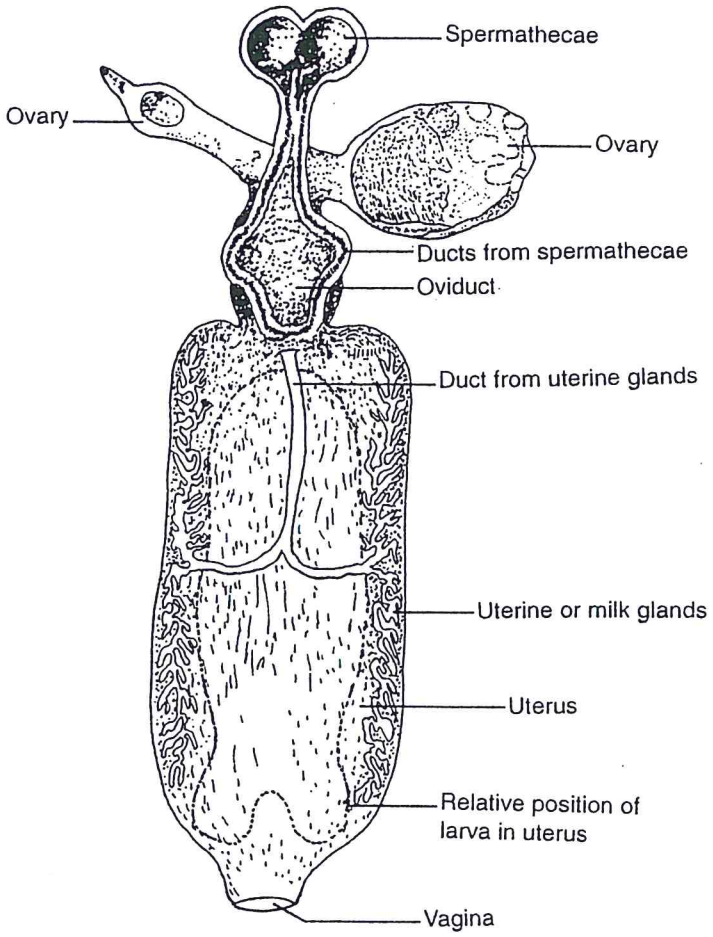


Fig. 1.3: The reproductive organs of female *Glossina* (Modified from Kettle, 1990).

when six days old (Mellanby, 1936), but there is no information on the reproductive performance of males at different ages. In the same study, newly emerged males less than 24 hours old mated newly emerged females. However, there was no report as to whether these males transmit viable spermatozoa during mating. Males of *G. palpalis* must be seven to eight days before their virility can approach that of older males (Nash, 1955). Age at mating for males therefore differ between species, necessitating separate species studies.

There are conflicting views as to whether female *Glossina* mate before or after a blood meal, with several workers suggesting that this occurs only after a blood meal (Langley, 1977; Tobe & Langley, 1978; Jordan, 1974). For instance, unfed female *G. austeni and G. morsitans* resist mating attempts by sexually aggressive males (Tobe & Langley, 1978). In other studies, the state of feeding was found to have no effect on mating in *G. pallidipes* (Odhiambo, 1968) or wild *G. m. morsitans* (Okiwelu, 1977a). In the Ugandan population of *G. f. fuscipes,* mating occurs between newly emerged unfed male and female flies in the laboratory emergence cages (Mellanby, 1936). A similar observation was made by Foster (1976) on *G. austeni* and *G. m. morsitans*.

Repeated (multiple) mating is important in tsetse reproduction since females that mate more than once store more sperms and show higher fecundity than those that mate once (Jaenson, 1979b; Rogers, 1972), without abortions occurring (Mellanby, 1936). Females that mate with sterile males would need a second mating for reproductive success (Jaenson, 1979b). However, in these experiments females were maintained together with males and it is not certain whether they accepted sperm more than once under such circumstances.

In the days following a blood meal, receptivity declines with age, and older virgin and mated females are rarely receptive even to sexually aggressive males (Tobe & Langley, 1978; Langley, 1977; Gillot & Langley, 1981). The factors responsible for refractoriness in mated

female *G. m. morsitans* are the relative amount of semen in the spermathecae (Dame & Ford, 1968) and a combination of male accessory reproductive gland (ARG) secretion and mechanical stimulation (Gillot & Langley, 1981). In *G. pallidipes*, however, only mechanical stimulation alters receptivity in subsequent matings (Davies-Cole, 1990).

Duration of the copulatory act is a factor that should be taken into consideration in studying colony performance of a species. Copulation duration acts as a trigger of the first ovulation (Saunders & Dodd, 1972; Chaudhury & Dhadialla, 1976) and may affect the degree of insemination, which correspondingly, would affect reproductive success (Rogers, 1972; Tobe & Langley, 1978). In *G. palpalis* copulation should last for at least 30 minutes for inseminations to be achieved (Mellanby & Mellanby, 1937) while in *G. morsitans*, the minimum duration required is one to 1.5 h (Saunders & Dodd, 1972). Duration of copulation has been found to be correlated with age of the female tsetse (Jaenson, 1979b). Copulation may also take place without sperm being transferred, hence contributing to reproductive loss (Madubunyi, 1975).

Following successful copulation, an egg is ovulated after about 7-9 days post-emergence, at 25°C (Saunders & Dodd, 1972). The ovulated egg lies in the uterus with the micropyle lying close to the spermathecal duct opening (Jackson, 1949; Roberts, 1973a; Langley, 1977). Fertilization of the ovulated egg then takes place with sperm stored in the spermathecae, embryonic development follows and lasts three to four days (Jackson, 1949; Roberts, 1973b).

Tsetse flies have a very low reproductive capacity. During its three to four month life span, a female produces on average five to seven offspring (Feldmann, 1993). The flies reproduce through adenotrophic viviparity, where a developing larva is nurtured by the female through nourishment from the 'milk glands'. In the laboratory, a single third instar larva is produced every nine days, at 25°C (Denlinger & Ma, 1974; Langley, 1977; Tobe & Langley,

1978). Meanwhile, another mature egg gets ovulated, and the cycle is repeated. The deposited larva pupariates in about one to two h (Zdarek & Denlinger, 1991) and after about 30 days of intrapuparial development at 25°C, a young adult *Glossina* emerges (Langley, 1977).

Studies on mating behaviour of *Glossina* in different populations of the same species have shown differences. For instance, *G. pallidipes* from Kibwezi forest (Kenya) has longer copulation duration than those originating from Lambwe valley (Jaenson, 1978). Differences in copulation duration, pupal weight, age at first larviposition and duration of interlarval periods were also reported for two populations of *G. pallidipes* originating from Nguruman in the Rift Valley Province and Mwalewa forest in the Kenya coast (Van Etten, 1981). The key factor affecting the colony from Mwalewa forest was premating mortality while flies from Nguruman were affected by loss due to non- production of pupae. Success in the rearing of the Ugandan population of *G. pallidipes* and failure to do so for the Zimbabwean one (Langley, 1989) would also imply differences in their reproductive biology. In Seibersdorf, Austria, a colony of *G. f. fuscipes* originating from Central African Republic has been established since 1986 (Feldmann, 1993). Attempts to colonize *G. f. fuscipes* from Kenya have faced bottlenecks, probably due to low fecundity or high mortalities.

1.2.7 Laboratory Rearing of *Glossina* Species

1.2.7.1 Early Colonization Attempts

Laboratory colonization of *Glossina* has not been successful in many countries, particularly in Africa. This is especially so for *G. f. fuscipes*, and is partly due to problems in attaining the right conditions for the species, and may also be partly due to certain behavioural characteristics of the species, which require investigation.

In the early attempts to colonize *Glossina*, success was achieved only partially on a few occasions, and with a lot of difficulty (Lumsden & Saunders, 1966; Wetzel, 1977). Attempts to produce self-sustaining colonies were made by Willet (1953) in Tanzania, with partial success on *G. swynnertoni*. Colonization of *G. palpalis* (actually *G. f. quanzensis*) was initially successful (Evens, 1954) but the colony soon died out. Similar attempts were made for *G. palpalis* (Nash *et al.*, 1958), *G. m. submorsitans* (MacDonald, 1960; Dame, reported in Wetzel, 1977), *G. p. gambiensis* (Challier, 1970; Laveissiere, 1973; Clair, 1975), and *G. tachinoides* (Gruvel, 1970; Gruvel & Balis, 1966). Itard (1971) also succeeded in rearing *G. f. fuscipes* from Central African Republic (C.A.R.). Most of the colonies were not self-sustaining and had to be supplemented with wild adults or puparia. The first successful colony was attained with *G. m. morsitans* at the Lisbon Institute of Tropical Medicine (Azevedo & Pinhao, 1964), leading to many fully self-sustaining colonies in other regions.

1.2.7.2 Laboratory Hosts of Tsetse

Animal hosts that have been used to maintain tsetse *in vivo* include Guinea pigs, sheep (Nash *et al.*, 1968; Van der Vloedt, 1982), goats and rabbits (Curtis & Jordan, 1970; Jordan & Curtis, 1968a, 1968b; Nash *et al.*, 1966; Williamson *et al.*, 1983). Nash *et al.* (1966) were the first to use rabbits' ears for feeding tsetse. Their results showed that the full reproductive potential of *G. austeni* was reached and the insects were not physically inferior to those found in nature. The productivity of flies fed on lop-eared rabbits was also found to be better than any previously obtained for *Glossina* using other methods (Jordan & Curtis, 1968a, 1968b; Curtis & Jordan, 1970). It was concluded that the improvement was due to excellent nutrition of the flies. Also, flies fed on rabbits had a substantially constant fecundity, compared to goat fed flies, in which fecundity declined at an earlier age (Curtis & Jordan, 1970; Davies-Cole *et al.*, 1994; Jordan & Curtis, 1968a). In countries where *in vivo* feeding is still being used,

rabbits are widely used for maintaining tsetse colonies. Reproductive performance also depends on species since female *G. austeni* performed better than *G. morsitans* when both were fed on rabbits' ears (Nash, 1968).

1.2.7.3 Feeding Regimen

The frequency of feeding tsetse in a colony may influence their productivity. In most cases, colonies of *Glossina* have been maintained by feeding them six days a week (Rogers, 1972; Jaenson, 1978; Van Etten, 1981). However, Leegwater van der Linden (1981) observed no differences in larval production of *G. pallidipes* fed four days per week instead of daily feeding. Some tsetse colonies may be fed three times a week without jeopardizing survival and pupal production (Madubunyi, 1988). The mass rearing facility for *G. f. fuscipes* in Seibersdorf, Vienna employs a 5-day feeding regimen (Gooding *et al.*, 1997). Due to differences in the behaviour of tsetse, it is important to study each population separately, to establish optimal feeding requirements. For instance, flies in a colony may be consuming small amounts of blood at a single feed and therefore require more frequent meals than those that take large blood meals.

1.2.7.4 Environmental Conditions

Holding conditions for tsetse flies as well as incubation conditions for pupae also have to reflect natural demands. For example, a relative humidity of 85% at 24°C is adequate for *G. f. fuscipes* (Gooding *et al.*, 1997).

1.2.8 The Effects of Juvenile Hormone Analogues and Ketoconazole on Insect Reproduction

The endocrine control of reproduction in insects has been extensively studied. The factors that have been shown to regulate reproduction are neurohormones from the central nervous system, juvenile hormones (JH) secreted by the corpus allatum (CA), and ecdysteroids from the follicle cells surrounding the oocytes (Engelman, 1970, 1983; Koeppe *et al.*, 1985).

In most insects, juvenile hormones produced by the CA play important roles both in the female and male reproductive systems. In females of the house fly *Musca domestica* L., juvenile hormone induces maturation of pre-vitellogenic eggs, in response to neurosecretory hormone (Adams, 1981). It also controls synthesis of vitellogenin by the fat body, controls entry of vitellogenin into oocytes and stimulates deposition and uptake of vitellogenin by the developing oocytes (Strambi *et al.*, 1997). In the males, JH speeds up the development of the accessory reproductive glands as well as synthesis of the gland proteins (Monsma *et al.*, 1990; Couche *et al.*, 1985; Davey, 1985; Leopold, 1976; Regis *et al.*, 1985). In *G. austeni*, JH has been shown to take part in oocyte maturation and accessory gland function (Ejezie & Davey, 1976). Allatectomy of female *G. morsitans* early in adult life leads to delayed production of one larva and to the arrested development of all subsequent oocytes before vitellogenesis (Endo *et al.*, 1979), implying JH involvement in vitellogenin uptake.

However, it is apparent that response to allatectomy varies with species. Surgical allatectomy of female *Achaeta domesticus* L. results in lack of ovarian growth while in *Gryllus bimaculatus* de Geer, the operation results in reduced fecundity but not total suppression of ovarian growth (Orshan & Pener, 1991; Hoffmann *et al.*, 1996). In the tsetse fly *G. m. morsitans*, the operation did not affect the number or size of pupae produced by the operated females (Langley & Pimley 1986). Similarly, females of *G. m. morsitans* treated

with precocene undergo normal reproductive cycles, but their female offspring become sterile (Samaranayaka-Ramasamy & Chaudhury, 1981). Since vitellogenesis occurs in the absence of JH in both cases, it is apparent that JH does not have an all-encompassing control over oogenesis and ovulation, and probably acts in conjunction with other compounds to control vitellogenesis.

There is possible involvement of ecdysone in vitellogenin production in females in which allatectomy does not completely suppress oocyte maturation (Hoffmann *et al.*, 1996). Determination of haemolymph ecdysteroid titres in female *G. f. fuscipes* shows three peaks: (i) the first occurs during vitellogenesis of the first oocyte, (ii) the second during vitellogenesis of the second oocyte and post-embryonic development of the first ovulated oocyte, and (iii) the third occurs just before parturition (Roberts *et al.*, 1986). This suggests that the hormone could have a role to play in vitellogenesis and parturition in this species. In *M. domestica*, 20-hydroxyecdysone has been shown to stimulate vitellogenin production (Adams *et al.*, 1985). The endocrine control of reproduction in *Glossina* is still unclear, and further investigation is neccessary to find out whether JH or ecdysteroids have a role to play in vitellogenesis in these species.

Following the discovery that juvenile hormones could be used to interrupt developmental processes in insects (Williams, 1956), several thousands of compounds with JH activity have been synthesized. Such compounds are referred to as juvenoids, bioanalogues or juvenile hormones analogues (JHA). When administered to insects, these juvenoids elicit hormonal effects or disrupt the normal physiological processes in insects (Sehnal, 1983). The discovery of the "paper factor" and its effects on *Pyrrhocoris* (Slama & Williams, 1965) led to the search for more juvenile hormone analogues. These analogues have accelerated studies leading to an understanding of the effects of natural JH in insects, its mode of action and characterization of the binding sites.

Sterility transfer was demonstrated on *Pyrrocoris apterus* L. (Heteroptera: Pyrrocoridae) (Masner *et al.*, 1968) in an experiment in which treated males transferred juvenile hormone analogues during mating to females that were not treated, leading to their sterility. Female insects could also be directly sterilized by treatment with the analogue. Such a possibility has been tried on tsetse flies both in the laboratory and field (Langley & Pimley, 1986; Anon, 1988; Langley *et al.*, 1988; Hargrove & Langley, 1990). In the laboratory, male *G. m. morsitans* treated with pyriproxifen transferred sterility to females with which they mated (Langley *et al.*, 1988). Field trials (Anon, 1988; Hargrove & Langley, 1990) demonstrate that pyriproxifen significantly reduces emergence rates from *G. m. morsitans* and *G. pallidipes* pupae below control levels.

Doses of juvenile hormone analogue topically applied to the ventral abdominal surfaces of pregnant *G. m. morsitans* females induced abortions (Denlinger, 1975; Meidell, 1982). However, in subsequent experiments, Langley and Pimley (1986) were unable to effect abortions in *G. m. morsitans*, using a similar approach and reported that even acetone (a solvent) alone was sufficient to cause abortions. Treatment of female *G. m. morsitans* with benzylphenols causes degeneration of oocytes, ovarian atrophy and reduction in fecundity (Langley *et al.*, 1982). The compounds vary in severity of effects, some of them reducing fecundity to zero and are dose-dependent. Treatment of males with the compounds did not affect their fertility, except that the most potent compound affected male survival. Topical application of pyriproxifen to *G. m. morsitans* adults does not affect the reproductive process when applied to mated females (Langley *et al.*, 1988), the females depositing normal puparia. However, adults fail to emerge from the puparia, development being arrested by day 22.

Glossina fuscipes fuscipes is one of the tsetse species targeted for control using biological methods in the eastern Africa regional tsetse programme. It is therefore important

to study the effects of JH analogues on reproduction in this species in order to pave way for prospects of using such IGRs for its control.

1.2.9 The Effects of Juvenile Hormone Analogues on Insect Metamorphosis

Metamorphosis in cyclorrhaphous Diptera involves pupariation, the formation of a hard ovoid puparium derived from the third instar larval cuticle, inside which pupation takes place (Denlinger & Zdarek, 1991a). After pupation, other developmental stages described by Zdarek and Denlinger (1993) and Denlinger and Zdarek (1994) follow.

Juvenile hormones are involved in regulating the development of insect juvenile stages. The morphogenetic effects of JH and JH analogues on insects was realized as early as 1956 by Williams (1956) who first isolated natural JH from adult *Hyalophora* males. He observed that JH could be absorbed through the insect cuticle and inhibits metamorphosis, and could therefore be used as a pesticide. This led to the search for more compounds with JH activity, and to studies on the effects of JH and its analogues on insects (Sehnal, 1983).

Studies on morphogenetic effects of JH analogues have been carried out on cyclorrhaphous Dipterans other than *Glossina*. Treatment of young pupae of *Sarcophaga bullata* Parker with JH analogues causes the formation of pupal-adult intermediates (Srivastava & Gilbert, 1969) while in *Drosophila melanogaster* Meigen, the treatment either reduces adult emergence or completely inhibits eclosion (Madhavan, 1973). The juvenoids have been shown to cause inhibition of eclosion and various morphological effects in several Dipteran families, different species showing varied effects and sensitivities (Sehnal & Zdarek, 1976). The morphogenetic effects include lack of pigmentation and sclerotization, and failure of differentiation and development of setae and of rotation of male genitalia.

In *Glossina*, differentiation of new adult tissues is probably inhibited by JH. When JH analogues are applied to *G. morsitans* pupae, pharate adult development is disrupted or halted

(Langley & Pimley, 1986; Langley *et al.*, 1988), emergence of adults being adversely affected. It results in a high proportion of abnormal adults that die soon after emergence. Pyriproxifen treatment of zero to two day-old pupae results in arrestment of adult development but a proportion of adults emerge if the treatment is applied to two to four day-old puparia (Langley *et al.*, 1988). It has not been established whether the proportion of flies that emerge is fertile.

1.2.10 Eclosion in Cyclorrhaphous Diptera

Insects undergo many aspects of development in their life cycle. One such aspect is the emergence or eclosion of flies from their puparia, which determines reproductivity of a given species and hence has applications in mass rearing facilities. It is also necessary to know the particular time of the day when peak emergence occurs.

Eclosion in most insects is controlled by rhythms so that it occurs at a certain time of the day or night. A clear rhythm of activity is apparent only in a population of mixed developmental ages (Saunders, 1982). In most insects, adult eclosion usually occurs at a particular time of the day or night and for many Dipterans, peak activity is at dawn (Saunders, 1982). In these insects, the environmental cue driving the eclosion rhythm is photoperiod. In *G. morsitans*, peak emergence is in mid-afternoon (Bursell, 1959; Dean *et al.*, 1968), and thermoperiod, rather than photoperiod, drives the eclosion rhythm (Phelps & Jackson, 1971; Zdarek & Denlinger, 1995). The latter workers demonstrated that eclosion peaks followed artificially generated thermocycles whatever the photoregime employed.

In tsetse, eclosion is limited to a temperature range of 18-32°C and in *G. m. morsitans*, it takes place at 25°C (Langley, 1977). Females generally complete development and emerge earlier than males (Denlinger & Zdarek, 1991a; Harley, 1968; Phelps & Burrows, 1969), making it possible to separate the sexes easily (Zdarek & Denlinger, 1995). At 24°C, intrapuparial life lasts for 37-42 days in *G. brevipalpis*, 33 days in *G. morsitans* (Zdarek &

Denlinger, 1993), while at 23°C it lasts for 37-39.5 days in *G. f. fuscipes* (Harley, 1968). Females have a shorter intrapuparial period compared to males, indicating that the rate of development differs between the sexes. Many tsetse fly species produce an increased number of weak emerged flies when the peak of female emergence occurs before day 32 post-larviposition (Gooding *et al.*, 1997). For some tsetse species, a major problem in colonization attempts is pre-mating mortality (Van Etten, 1981). This could be as a result of weak emerged female flies.

1.3 Justification

Self-sustaining colonies of economically important *Glossina* species are important in providing standard fly materials for toxicological, physiological and behavioural studies. Such studies would help develop effective insecticide formulations and improve attractive devices for tsetse control. The colonies also provide quality insects for release in tsetse programmes such as the SIT. However, success in colonization attempts has not been achieved with *G. f. fuscipes*. Attempts to rear this species usually end up in the extermination of the colonies before the third generation. *Glossina f. fuscipes* differs from other species of *Glossina* that have been colonized in its habitat requirements and feeding habits, factors that could make its colonization difficult. Besides, this review has shown that tsetse flies inhabiting different geographic locations differ in behaviour, hence the need to study the Kenyan population of *G. f. fuscipes*.

The conventional method of controlling tsetse has been through spraying with insecticides. However, insecticides are costly and are environmentally detrimental. Besides, they are ineffective in densely vegetated areas. A promising method of control lies in the use of biological methods such as the SIT, particularly the use of IGRs. Insect growth regulators

are species specific and sterilize females, thus curtailing species propagation. Although research on use of IGRs to control tsetse has shown promise with *G. m. morsitans* and *G. pallidipes* (Hargrove & Langley, 1990; 1993), such studies on the *palpalis* group are lacking. This work was initiated to study the factors that affect the laboratory reproductive performance and effects of juvenile hormone analogues and ketoconazole on the Rusinga population of *G. f. fuscipes*.

1.4 Objectives of the Study

1.4.1 General Objectives

1. To determine factors affecting the laboratory reproductive performance of *G. f. fuscipes*

2. To determine the effects of juvenile hormone analogues and ketoconazole on reproduction and metamorphosis.

1.4.2 Specific Objectives

1. To assess the effects of age on receptivity, insemination and fecundity in a laboratory population of *G. f. fuscipes* from Rusinga, Kenya and from Seibersdorf, Vienna (ex-Central African Republic).

2. To assess for multiple mating and its effect on insemination and fecundity in two populations of *G. f. fuscipes*

3. To determine the effect of blood meals on puparial weights in the two populations.

4. To study eclosion behaviour in *G. f. fuscipes*.

5. To determine the effects of JH analogues and ketoconazole on reproduction in *G. f. fuscipes*.

6. To determine the morphogenetic effects of JH analogues in this species

7. To determine the effects of JH analogues on fecundity and fertility of flies emerging from treated puparia.

CHAPTER TWO

GENERAL MATERIAL AND METHODS

2.1 Capture and Maintenance of Tsetse Flies

Wild tsetse flies were collected from Rusinga Island, Suba District, using biconical traps (Challier & Laveissiere, 1973) (Plate 2.1) and sorted by sex. Between 15 - 20 females were kept together in PVC cages measuring 18 x 8 x 5 cm while males were discarded. The wild females were transported to ICIPE laboratories, at Duduville, Nairobi, where they were maintained in an insectary at 24 ± 1^{0}C, 75-85% RH and 12 h photophase. The flies were fed *in vivo* rabbit ears according to the method described by Nash *et al.* (1966) (Plates 2.2 & 2.3). Females collected were inseminated and continued larviposition in captivity. The pupae produced were kept in emergence cages, under the same conditions as the adults. Upon emergence, the flies were sexed and kept separately in PVC cages measuring 10 x 8 x 4.5 cm. Flies were considered to be one day old on the day of collection, and were maintained until experimentation.

Puparia of *G. f. fuscipes* that originated from Central African Republic (C.A.R.) were kindly supplied by Dr. Elizabeth Opiyo of the Entomology Unit, International Atomic Energy Agency (I.A.E.A), Seibersdorf, Vienna. Upon reception, the puparia were kept in emergence cages as described above, i.e. under similar conditions to the wild population.

Plate 2.1: The biconical trap (Challier & Laveissiere, 1973) which was used in capture of tsetse flies.

Plate 2.2: Tsetse flies in PVC cages feeding from ears of rabbits.

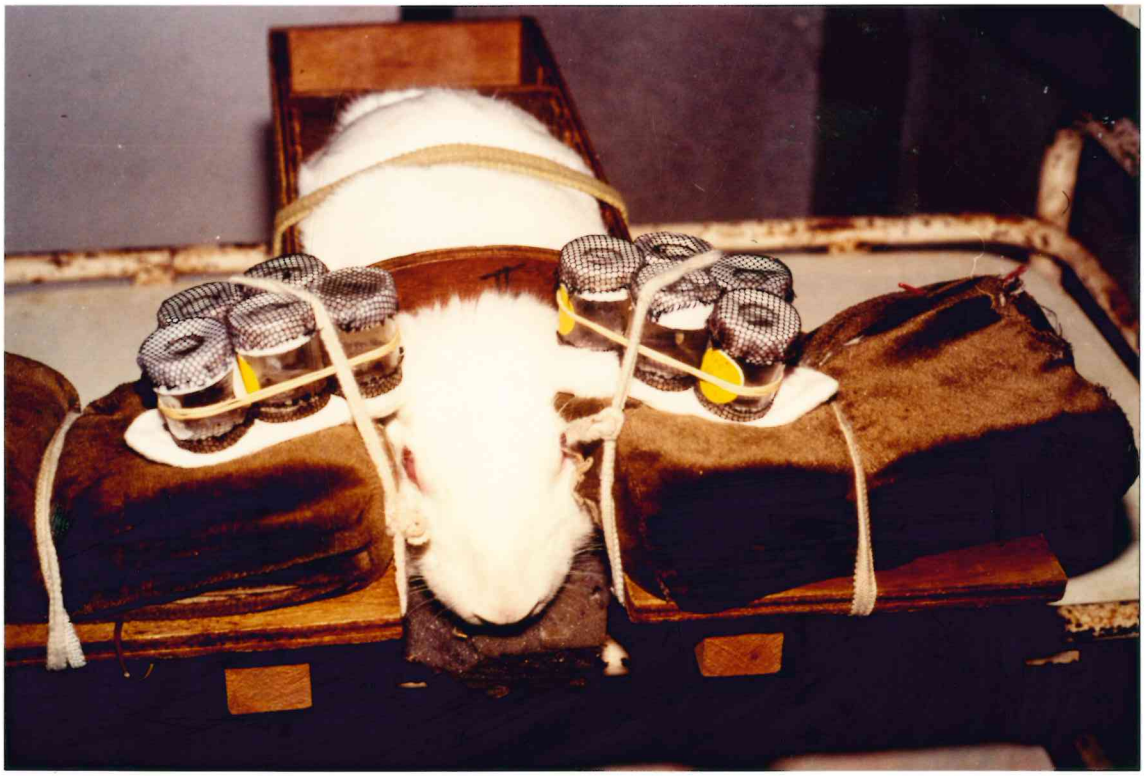


Plate 2.3: Tsetse flies in single mating vials feeding from ears of rabbits.

2.2 Mating Experiments

In some experiments, female tsetse flies were kept singly in transparent plastic vials measuring 4 x 3 cm. A male was then introduced into the female cage and receptivity of the female noted. The frequency of mating strikes at 10, 20, and 30 min; success of mating; and duration of copulation were recorded for each pair. In other experiments, groups of 10 females were kept in PVC holding cages and an equal number of males introduced. Such group mating was employed where males or females were to be treated with insect growth regulators.

2.3 Dissections

Flies for dissections were at first immobilized at 4°C, euthanized by a gentle squeeze of the thorax, and wings and legs removed. Under a dissecting microscope, the fly was placed on its ventral side on a microscope slide, and held at the thorax with a pair of forceps. Two small slits were made at the posterior end of the abdomen about the second last segment. The reproductive organs were then pulled out in physiological saline, using a second pair of forceps. Measurements were made on the ovarioles or uterine content using an eye-piece micrometer.

2.4 Determination of Degree of Insemination

The spermathecae (sperm sacs) of female tsetse were examined for the presence of spermatozoa. According to Mellanby (1936), when the spermathecae are empty, they appear transparent but when they contain sperm, the entire spermatheca becomes opaque, depending on the degree of insemination. A 4-tier grading of the spermathecal contents was used. Each spermatheca could be ¼ full (0.25), ½ full (0.5), ¾ full (0.75) or full (1.00) (Nash, 1955). If both spermathecae are full, the spermathecal value (SV) would be 1.00 + 1.00 = 2.00. If one

is full, the other empty, the SV would be 1.00 + 0 = 1.0. If one was 1/4 full, the other half-full, the SV would be 0.25 + 0.5 = 0.75, etc. The degrees of insemination were categorized into six classes (Pinhao & Gracio, 1973).

2.5 Defination of Terminologies Used

Sexual receptivity: This is the readiness of a female to mate, at 24^0 C and 80% RH, with a sexually mature male. Receptive females opened their wings, or allowed a male to open their wings with their mid-legs, and were generally stationary.

Mating / Copulation: The interlocking of male and female genitalia, culminating in the male jerking phase.

Mating strike: This is the landing of the male onto the female fly, with the intention of mating. This could be committed or uncommitted.

Mating attempt: This is the trial by the male fly to get access to the female genitalia, using its aedeagus. This could be successful if the female is receptive or abortive if the female is refractory.

Duration of copulation: This is the period between the engagement of male and female genitalia, until they disengage at the end of mating or copulation.

Age at mating: This is the age at which females are most receptive, or at which most males mate with, and inseminate females.

Age at first larviposition: This is the interval between the emergence of an adult female fly and deposition of the first larva.

Emergence / Eclosion: These two terms were used interchangeably to mean the breaking open of the puparium wall and the expansion of body and wings.

Puparium / Pupa: Puparium refers to the puparial wall including its contents while pupa refers to the contents only.

CHAPTER THREE

A COMPARATIVE STUDY OF FACTORS AFFECTING LABORATORY REPRODUCTIVE PERFORMANCE IN TWO POPULATIONS OF *GLOSSINA FUSCIPES FUSCIPES*

3.1 Introduction

Attempts made at the ICIPE, Nairobi, to colonize *G. f. fuscipes* have not been successful, the colonies collapsing before reaching self-propagating status. This happens despite regular supplementation of the existing colonies with wild females, or puparia.

The probability that adequate insemination rates will be achieved among mated females generally depends on receptivity and completion of the copulatory act (Ringo, 1996). In *Glossina*, these factors are in turn dependent on the age at which mating occurs. Age also determines the levels of fecundity reached by female tsetse (Rogers, 1972; Tobe & Langley, 1978), with old *G. pallidipes* females having higher fecundity than young ones. It is therefore necessary that the precise age at mating be known for each species and sex in order to enhance rearing success.

Presently, there is no laboratory in the African continent that has a self-sustaining colony of *G. f. fuscipes*. This study was conducted with the aim of determining the factors that affect the laboratory reproductive performance of this species. In addition, some aspects of the mating behaviour of the Rusinga Island population of *G. f. fuscipes* were compared with that from Central African Republic.

3.2 Study Objectives

The present study was designed to:

1. Assess the effects of age on receptivity in a laboratory population of *G. f. fuscipes* from Rusinga, Kenya and from Seibersdorf, Vienna (ex-Central African Republic).
2. Determine the relationship between receptivity and follicular development.
3. Assess any effects of age on:
   (i) size of male accessory reproductive glands (ARG).
   (ii) insemination
   (iii) copulation duration, and
   (iv) fecundity.
4. To assess for multiple mating and its effect on insemination and fecundity in two populations of *G. f. fuscipes*
5. To determine the effect of blood meals on puparial weights in the two populations.

3.3 Material and Methods

3.3.1 Effect of Age on Receptivity

Single matings were carried out as described under section 2.2. Female flies that were one, two, four, six, eight, ten or twelve days old were each put in labeled single mating vials. One unmated male fly, aged 10 days was introduced into each of the vials. The number of mating strikes at 10, 20 and 30 min were recorded. Also noted were female receptivity or refractoriness and the duration of copulation. Receptivity was evaluated within a 30-minute observation period, although mating outside this period was also noted. The pairs of flies that did not copulate within 30 minutes were enclosed together for five days, or until they larviposited. If no larva was deposited within 22 days post-emergence, the female was

withdrawn and dissected to assess the degree of insemination. This was an indirect way of determining receptivity outside the 30-minute observation period. The experiment was repeated with males, in which flies aged two, four, six, eight, 10 and 12 days post-emergence were paired with two day-old females.

3.3.2 Measurement of Egg Follicles

Virgin females aged one to 12 days were dissected in order to follow the development of the egg follicles. Fifteen females in each age group were dissected, as outlined in section 2.3. The egg follicles were then measured under a dissecting microscope fitted with an eye-piece micrometer.

3.3.3 The Male Accessory Reproductive Glands (ARG)

The male reproductive system of the Rusinga population of *G. f. fuscipes* was dissected out by making two incisions on both sides of the second-last abdominal segment, and pulling out the reproductive tract. Care was taken not to damage the male ARG, since such damage was found to affect the success of observing the apical body. The dissection was carried out as given by Pollock (1974), except that after transferring the reproductive system to distilled water, no cut was made on the ARG. The male ARG was dissected out in 0.9% sodium chloride (Analytical Rasayan, Fine-Chem. Ltd.). After removing fat bodies, the system was transferred to phosphate-citrate buffer (pH 2.2). The set-up was examined through a microscope and after the gland contents had clarified (2-3 min), the system was transferred into distilled water. The apical body becomes opaque and the gland bursts open, ejecting it. Many preparations were, however, spoilt at this stage when the secretions flowed towards the natural exit of the gland instead. Breaking open the gland by puncturing it also deforms the apical body, which then fails to eject intact. The widths of the male ARG and apical body

were measured under a dissecting microscope, using an eyep
measurements were made at the swollen apical section of the

3.3.4 Effect of Age at Mating on Degree of Insemination

Flies were dissected as detailed in section 2.3, and the
determined as described in section 2.4. Because of shortage
were dissected post-mortem. In cases where females failed t
were dissected to determine their mean spermathecal values
experimental period, all females were dissected and their MS
of males at mating was determined by estimates of MSV in f

3.3.5 Effect of Age on Copulation Duration

Copulation duration was recorded for each mating pa
(section 3.3.1).

3.3.6 Effect of Age at Mating on Fecundity

Females from the experiment described in section 3.
separated from males. While still in their mating vials, they
petri-dishes (Plate 3.1), which had a hole in the top cover to
female flies were maintained as described under section 2.1.
45-day period. Female fecundity was calculated as the actua
initial female divided by the theoretical maximum number o
female could have produced in the given time interval. The
and any death that occurred was recorded. Only females tha

Plate 3.1: The set-up used in fecundity studies.

pregnancy cycle were taken into consideration. Also recorded were age at first larviposition, interlarval periods and puparial weights.

3.3.7 Effect of Blood Meal Index on Puparial Weights

Single mating vials were weighed empty then with a single teneral female aged one day. The female was then fed and re-weighed immediately. Females were mated when two days old with males aged 10 days, and thereafter maintained in the insectary as described under section 2.1 for 45 days. They were fed *in vivo* on rabbit blood every other day, but those that failed to feed were offered a meal daily until they fed. On each feeding occasion, the weighing procedure outlined above was followed, and any failure to feed recorded. Larvipositions were recorded and the pupae weighed within 24 hours of larviposition. The blood meal index was calculated as the weight of blood consumed divided by the weight of the fly before feeding and expressed as a percentage. For each population, twenty females were used.

3.3.8 Effect of Multiple Mating on Degree of Insemination

Females were first mated to males as outlined in section 3.3.1. In cases where a female mated successfully, the male was removed from the mating cage and another unmated male introduced. The couple was visually observed for five consecutive days, red light being used in the scotophase. Females were then dissected and their degree of insemination (MSV) determined. In the second trial, mated females were separated from males and left to larviposit. After the first larviposition, an unmated male was introduced into the female vial and left until the second larviposition following which the females were dissected and their

MSV determined. The MSV was compared with those of a group of females that were mated and immediately separated from males.

3.3.9 Effect of Multiple Mating on Fecundity

Females were mated as in the above experiment (section 3.3.8) and maintained as described in section 2.1 for 45 days after which fecundity was evaluated (section 3.3.6).

The experiments in sections 3.3.1 to 3.3.9 (except section 3.3.3) were carried out using both the Rusinga and the Central African populations of *G. f. fuscipes*. This enabled me to assess the reproductive performance of different populations of the same species under laboratory conditions.

3.3.10 Statistical Analyses

Data on age and population effects on receptivity, insemination and fecundity were analyzed using the General Linear Models (GLM) procedure (SAS, 1985). Percent receptivities were arcsine-transformed while copulation durations were log-transformed before being subjected to the GLM procedure. Spearman's rank correlation was used to determine relationships between ovarian growth and receptivity, and between blood meal index and puparial weights. The Chi-square test of independence was applied to determine effect of population and age on mortality, abortion frequency and effect of age on mating strikes.

3.4 Results

3.4.1 Effect of Age on Receptivity

In the Rusinga population, female receptivity was high from day one (60 ± 1.45%) post-emergence and peaked by day two (71 ± 6.6%) (Fig. 3.1a). Receptivity then declined gradually with age to 22 ± 4.3% by day 12. In contrast, female receptivity in the

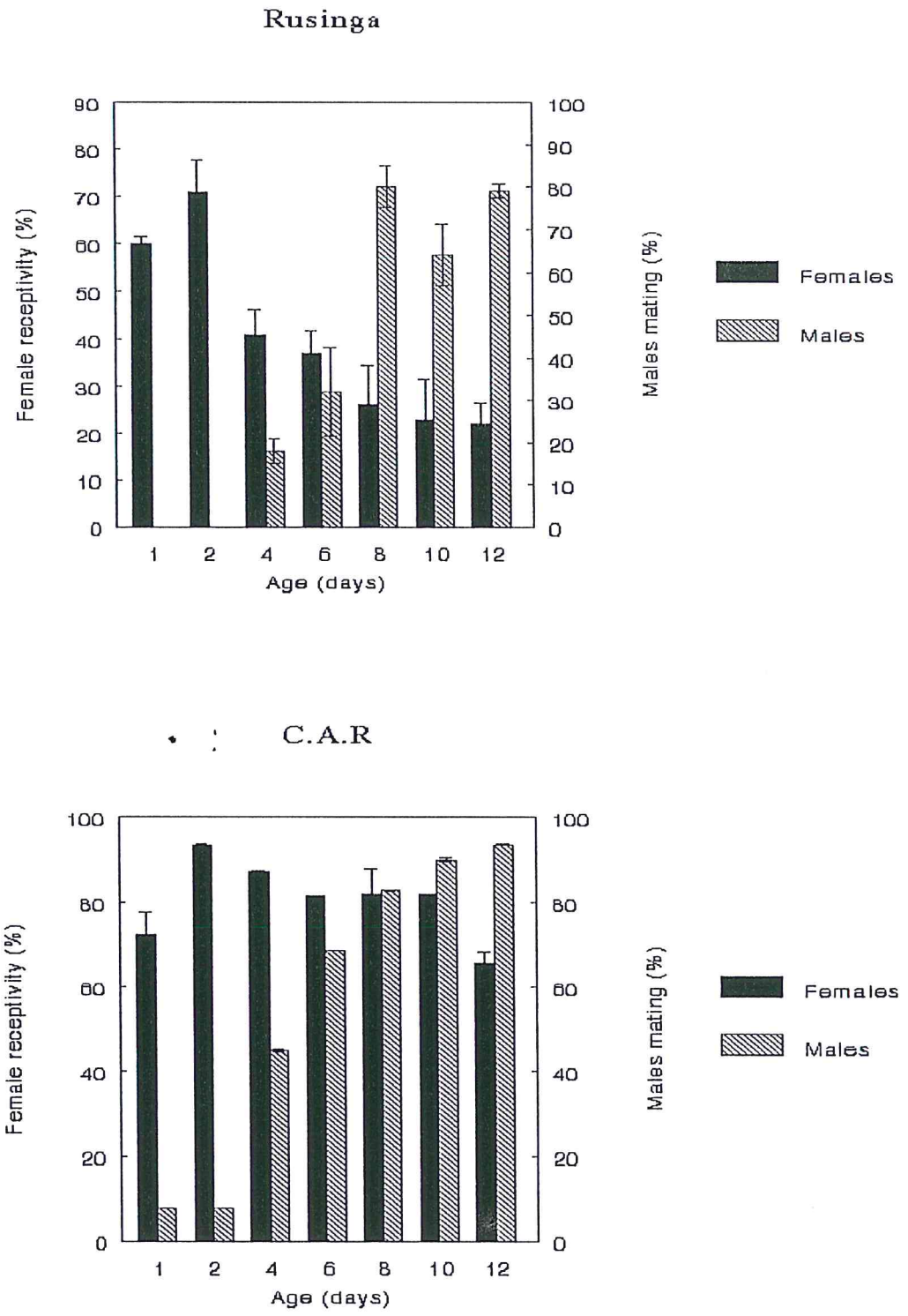


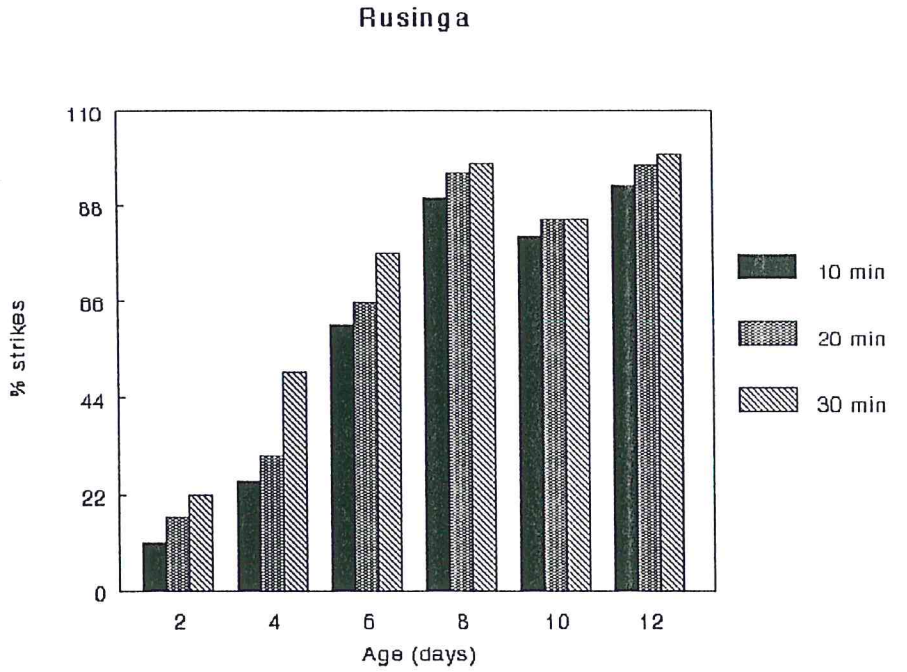
Fig. 3.1: Effect of age at mating (days) on % receptivity ($\bar{X} \pm$ SEM) and % mating success of males ($\bar{X} \pm$ SEM) in the Rusinga and C.A.R populations of *G. f. fuscipes*.

C.A.R. population remained high at all the ages (Fig. 3.1b). The first mating strike occurred immediately a sexually mature male was introduced into a female's vial. Where the female was receptive, this resulted in successful copulation while several strikes occurred where the female was refractory.

Males from the Rusinga population were more successful (F= 4.55, p= 0.007, n= 246) at mating between eight and 12 days post-emergence but failed to mate when less than four days old (Fig. 3.1a). However, they showed mating strikes from day two the frequency of which increased with age (Fig. 3.2), leading to greater success in copulation. In the C.A.R. population, six day-old males mated as well as older ones (Fig. 3.1b).

3.4.2 Follicular Growth

The length of the inside right follicle (designated A1) increased from one day old females to a maximum (1.3 ± 0.07mm) in 10 day-old Rusinga females (Fig. 3.3a). Follicular growth was not regular, with some age groups having a similar mean size. The ovarian dissections revealed that in about 4% (n= 325) of the females, the right and left follicles were more or less of similar size. This abnormality was observed in both virgin and parous females. Other ovarian abnormalities included slow growth of follicles (4.9 %, n= 325) and a few cases where the left follicles developed first instead of the right follicles (0.28 %, n =325). Egg retention (failure of ovulation) occurred in 0.9 % (n= 325) of the females from Rusinga Island. Growth of follicles in females of the C.A.R. population of *G. f. fuscipes* reached a peak (1.4 ± 0.08mm) in eight day-old virgin females then declined (Fig. 3.3b). Follicular length was inversely correlated with receptivity (R= -0.98, p= 0.0001, df= 6) (Fig. 3.4).



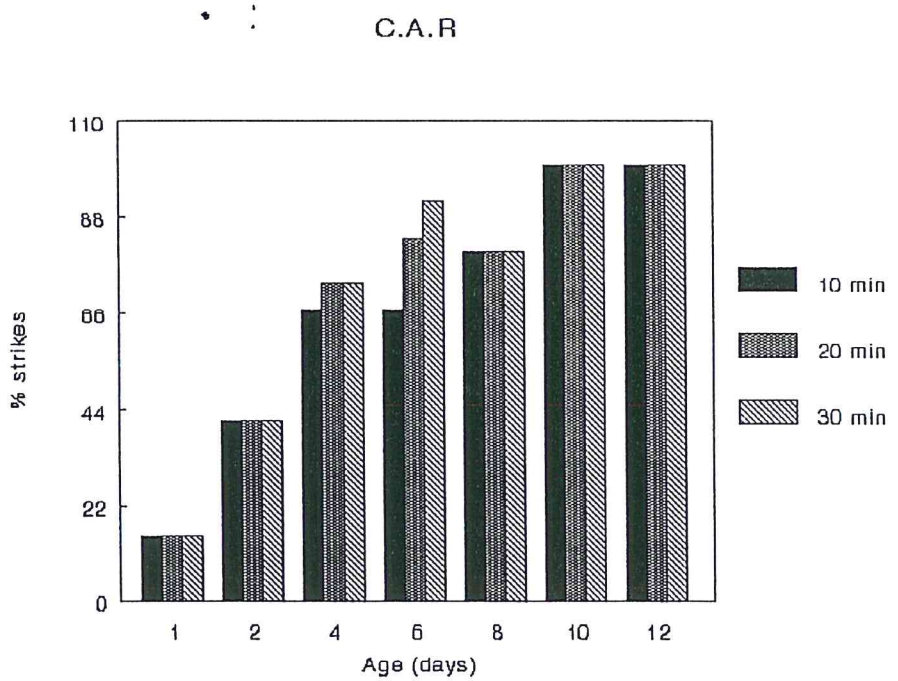


Fig. 3.2: Effect of age of male *G. f. fuscipes* on % strikes in the first 10, 20 and 30 min of introduction into female vials.

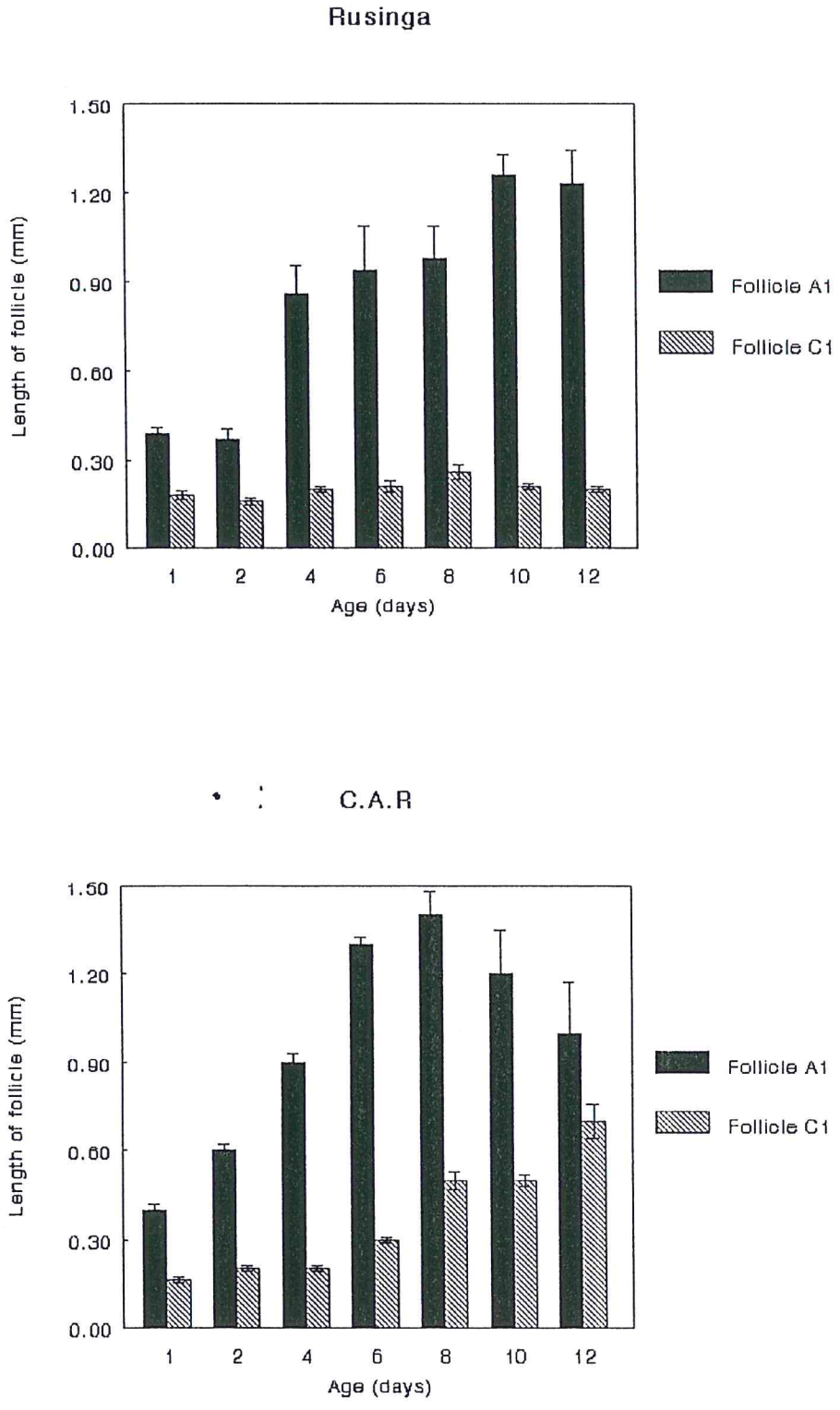


Fig. 3.3: Follicular growth rate ($\bar{X} \pm$ SEM) in virgin *G. f. fuscipes* females from Rusinga and C.A.R populations.

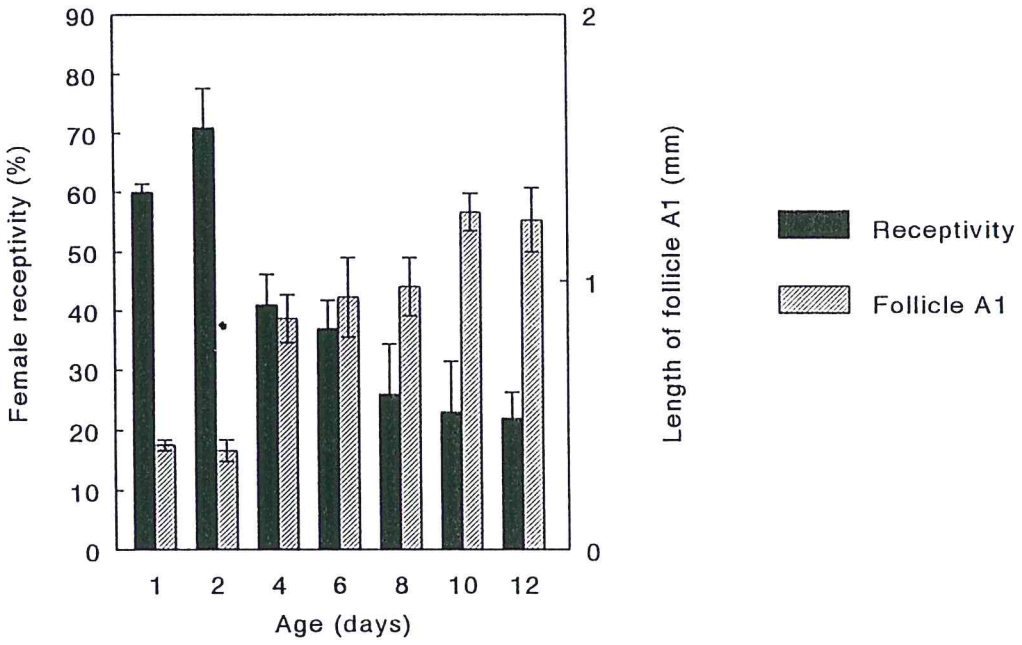


Fig. 3.4: Effect of age on % receptivity ($\bar{X}$ ± SEM) and length of follicle A1 (mm ± SEM) in female *G. f. fuscipes*.

3.4.3 Growth of the Male ARG and Apical Secretion

The ARG increased in size from one to 14 day-old males, after which it remained constant (Fig. 3.5). The apical body in males aged between one and four days post-eclosion could not be measured, but increased in width from five to 18 day-olds, after which it remained constant. The apical body diameter was significantly higher ($F= 4.45$, $p= 0.0003$, $n= 82$) in males over eight days compared to younger ones.

3.4.4 Effect of Age at Mating on the Degree of Insemination

Mean spermathecal values did not differ in Rusinga females aged between one and 10 days ($F= 0.86$, $p = 0.48$, $n= 152$) (Table 3.1). However, females mated when 12 days old contained less sperm in their spermathecae ($F= 13.6$, $p= 0.0001$, $n= 173$) compared to younger females. In the C.A.R. population, the mean MSV was 1.39 ± 0.08 and was independent of age within the 12 days period.

The mean spermathecal values of females mated by males of varying ages were not different in the Rusinga ($F= 0.29$, $p= 0.89$, $n= 125$) and C.A.R. ($F= 0.62$, $p= 0.65$, $n= 104$) populations (Table 3.1). The degree of insemination of females between the two populations was not statistically different ($F= 0.77$, $p= 0.38$, $n= 338$). Females mated at two days post-emergence had the highest score in MSV class IV (MSV=2) while those mated at 12 days post-emergence had the least score of females in this class (Figure 3.6).

3.4.5 Effect of Age at Mating on Copulation Duration

The mean duration of copulation in females of the Rusinga population was 49.4 ± 1.24 min while in the C.A.R. population, it was 44.4 ± 1.17 min. Age at mating had no effect on duration of copulation in males ($F= 1.21$, $p= 0.311$, $n= 128$) but had a significant

Table 3.1: Mean spermathecal values (± S.E.M) of populations of *G. f. fuscipes* from Rusinga and C.A.R. in relation to age at mating. Values in parentheses are sample sizes (n).

Age at mating (days)	Mean spermathecal value (± S.E.M)			
	Rusinga population		C.A.R population	
	♀	♂	♀	♂
1	1.36 ± 0.13 (20)	-	1.08 ± 0.14 (24)	-
2	1.42 ± 0.12 (36)	-	1.62 ± 0.10 (26)	-
4	1.65± 0.12 (18)	1.25 ± 0.22 (8)	1.23 ± 0.15 (26)	1.22 ± 0.17 (16)
6	1.49 ± 0.15 (23)	1.45 ± 0.15 (28)	1.27 ± 0.15 (21)	1.44 ± 0.15 (24)
8	1.28 ± 0.17 (16)	1.59 ± 0.12 (35)	1.50 ± 0.14 (27)	1.54 ± 0.09 (24)
10	1.54 ± 0.10 (39)	1.42 ± 0.12 (36)	1.52 ± 0.16 (28)	1.64 ± 0.08 (24)
12	0.10 ± 0.09 (21)*	1.49 ± 0.14 (18)	1.37 ± 0.22 (13)	1.34 ± 0.17 (16)

Asterisk (*) denotes significantly lower MSV value

effect in females (F=9.76, p = 0.0001, n=162). Females aged one to two days spent significantly longer time in copula than older ones (Table 3.2). In the C.A.R. population, neither female age at mating (F= 1.47, p= 0.204, n=120) nor age of males (F= 0.32, p= 0.862, n= 96) with which the females mated had effect on duration of copulation. The population of females from Rusinga exhibited higher copulation duration than those of C.A.R. population (F= 8.08, p= 0.005, n= 282).

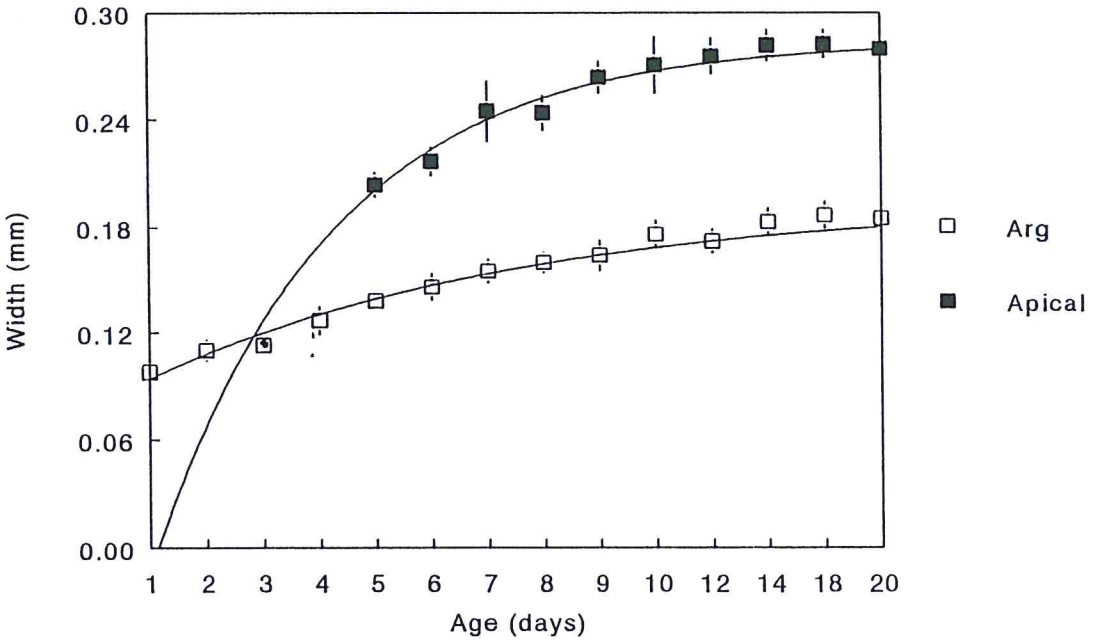


Fig. 3.5: Increase in width ($\bar{X} \pm$ SEM) of ARG (□---□) and apical body (■---■) with age in male *G. f. fuscipes*.

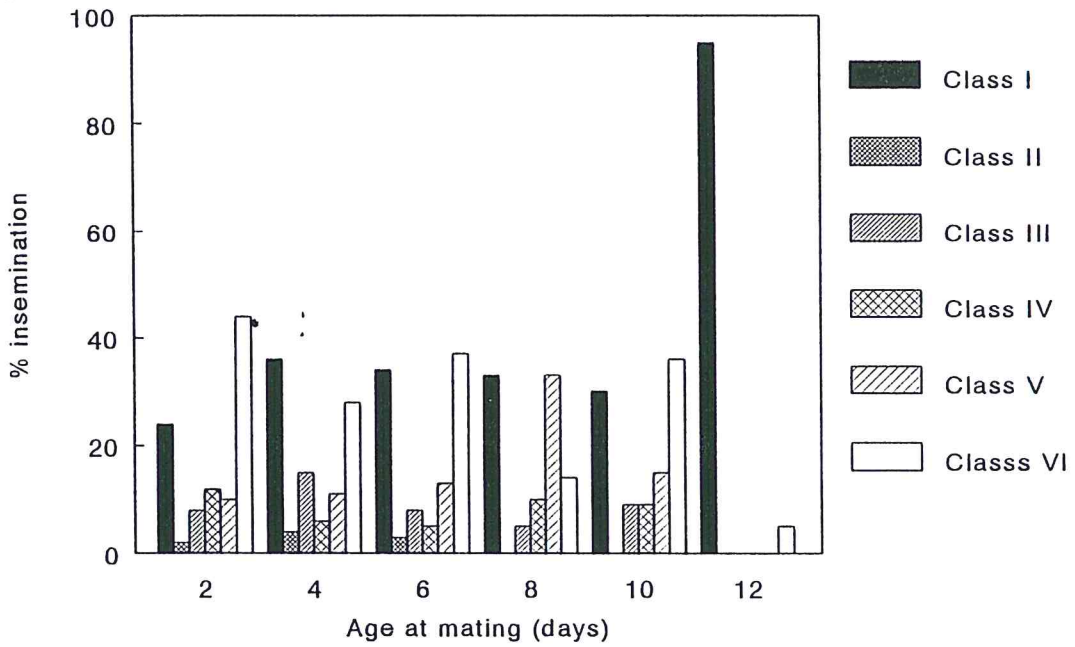


Fig. 3.6: Insemination rates in relation to age in *G. f. fuscipes* females.
(Class I, MSV = 0; Class II, MSV ≤ 0.50 > 0; Class III, MSV > 0.5 <1.00;
Class IV, MSV> 1.00 < 1.50; Class V, MSV > 1.50 < 2.00;
Class VI, MSV = 2.00).

Table 3.2: Mean durations (± S.E.M) of copulation in Rusinga Island and Central Africa Republic (C.A.R.) populations of *G. f. fuscipes* in relation to age at mating. Values in parentheses are sample sizes (n).

Age at mating (days)	Copulation Duration (Min ± S.E.M)			
	Rusinga population		C.A.R population	
	♀	♂	♀	♂
1	65.3 ± 1.05 (18) [a]	-	52.0 ± 1.14 (15)	-
2	64.3 ± 1.06 (37) [a]	-	43.2 ± 1.08 (20)	-
4	53.4 ± 1.07 (18) [ab]	77.0 ± 1.16 (10)	59.3 ± 1.12 (15)	58.1± 1.10 (14)
6	50.4 ± 1.10 (24) [ab]	68.4 ± 1.05 (28)	39.5 ± 1.10 (18)	58.6 ± 1.09 (20)
8	44.1 ± 1.10 (16) [ab]	58.1 ± 1.06 (35)	38.9 ± 1.08 (20)	55.4 ± 1.05 (22)
10	38.3 ± 1.06 (39) [b]	64.5 ± 1.05 (37)	44.0 ± 1.10 (22)	48.7 ± 1.12 (22)
12	36.9 ± 1.11 (10) [b]	59.0 ± 1.08 (18)	40.3 ± 1.11 (10)	53.3 ± 1.05 (18)

Different letters along columns denote significantly different means.

3.4.6 Multiple Mating

In both the Rusinga and C.A.R. populations, mated females were not receptive to subsequent mating attempts by unmated males for up to five days, despite repeated strikes. However, cases of repeated mating were observed where a male that had just completed mating immediately mounted the same female. This occurred in 3.6% (n= 169) of matings involving two day-old females and 2.8% (n= 71) of those involving eight day-old ones. Mean spermathecal values of females from C.A.R population that were maintained together with males and given chance to remate (1.35 ± 0.04) or enclosed with unmated males after the first

larviposition (1.45 ± 0.06 units), were statistically similar to those of females separated from males immediately after mating (1.45 ± 0.07units). Similarly, in the Rusinga population the degree of insemination in females maintained together with males (1.46 ± 0.22) did not differ from those of females separated from males immediately after mating (1.42 ± 0.12).

3.4.7 Effect of Age at Mating on Fecundity

Percent larviposition in the Rusinga population was low, age having a significant effect on percent larviposition ($\chi^2 = 12.6$, p= 0.05, n= 140). Females mated at eight days post-emergence showed lower larviposition rate than those mated when two ($\chi^2 = 5.1$, p= 0.02, n= 49) or 10 ($\chi^2 = 8.3$, p= 0.004, n= 42) days post-emergence (Fig. 3.7). Percent larviposition in females mated at one, two, four, six, and 10 days post larviposition were similar. Ovarian dissections showed that females were inseminated and had regular ovulations, but aborted eggs while others (0.9%) failed to ovulate. This was in sharp contrast to the C.A.R. population where age at mating had no influence on percent larviposition, with high proportions of females larvipositing at all the age groups. Maximum larviposition (93%) was attained in females mated when 10 days old and a minimum in one day-old females.

The C.A.R. population showed higher mean fecundity (F= 143, p= 0.0001, n= 331) compared to the Rusinga one, age having no effect on mean fecundity, interlarval periods or pupal weights (Table 3.3). Pupal weights ranged from 24-46 mg, the average weight being 34.4 ± 0.39 mg. On average females larviposited when about 20 days old, with those mated when two days old larvipositing earlier (F= 25.8, p= 0.0001, n= 191) than those mated when above 8 days post-emergence (Table 3.4). It was observed that some females (3.1%, n= 191) larviposited only once in the whole period of the experiment. When such females were dissected at the end of the experiment, they were found to be uninseminated. The mean age at

first larviposition in the Rusinga females was 24 ± 1.33 days. The mean fecundity was 0.32 ± 0.05 pupae per female per 11 days, interlarval periods and pupal weights being independent of age. Pupal weights ranged from 14-32 mg, with a mean of 20.8 ± 0.31 mg.

Fecundity of females mated by four day-old males from the C. A. R. population was lower ($F= 3.7$, $p= 0.007$, $n= 98$) than for those mated by older males (Table 3.5). From six days however, fecundity was independent of age in both populations.

3.4.8 Abortion Rates

Frequency of abortion in both the Rusinga and C.A.R populations was not influenced by age at which the female tsetse were mated (Table 3.6). Abortion rates were, however, higher in the Rusinga population ($\chi^2 = 104.2$, $p= 0.001$, $n= 1331$).

3.4.9 Effect of Blood Meal Index on Puparial Weights

The mean blood meal index of females of the Rusinga population was lower ($F= 6.21$, $p= 0.02$, $n= 39$) than that of the C.A.R. population (Table 3.7). Puparia formed from larvae deposited by females of the C.A.R population also weighed more ($F= 172$, $p= 0.0001$, $n= 33$) than those deposited by females from Rusinga island. There was positive correlation between the amount of blood meal taken and weights of puparia ($R = 0.35$, $p= 0.05$, $n=33$).

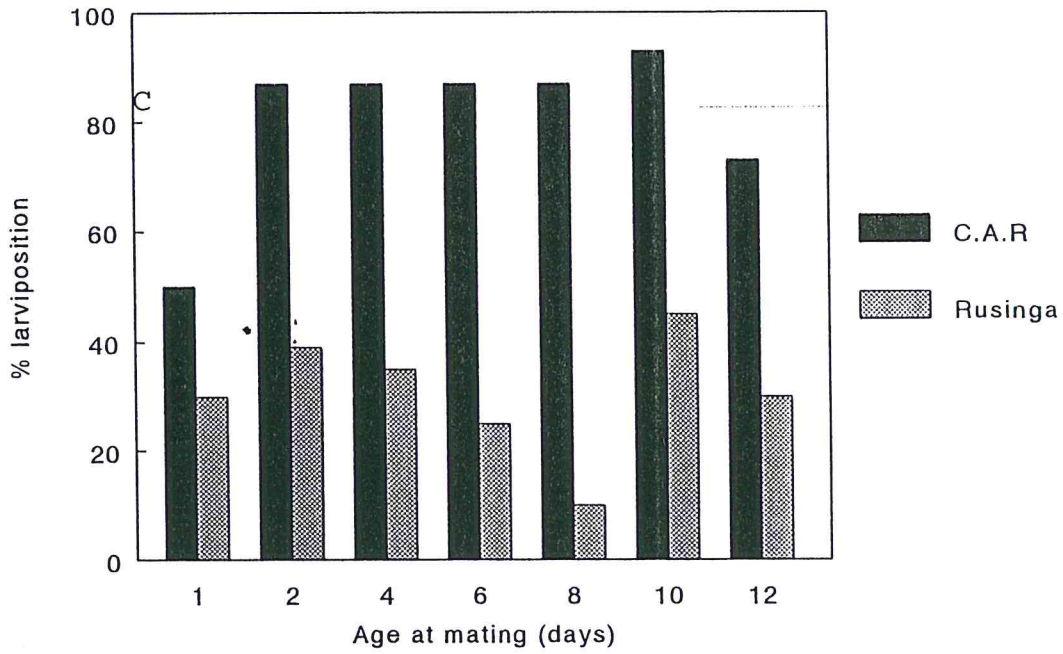


Fig. 3.7: Comparison of larviposition rates in the Rusinga and C.A.R populations of *G. f. fuscipes* in relation to age at mating.

Table 3.3: Effect of age at mating on the reproductive performance of *G. f. fuscipes* females from C.A.R.

Age at mating (days)	n	Age at 1st larviposition (days)	Mean ± S.E.M Fecundity	Interlarval period (days)	Pupal wt (mg)
1	16	20 ± 0.96^{ab}	0.66 ± 0.12	11 ± 0.93	33.1 ± 0.30
2	28	18 ± 0.43^{a}	0.80 ± 0.06	11 ± 0.75	33.7 ± 0.83
4	30	18 ± 0.20^{ab}	0.78 ± 0.07	9 ± 0.07	34.7 ± 0.64
6	29	20 ± 0.56^{ab}	0.72 ± 0.06	10 ± 0.43	35.4 ± 0.30
8	30	20 ± 0.40^{ab}	0.75 ± 0.07	10 ± 0.30	35.2 ± 0.52
10	30	21 ± 0.51^{b}	0.80 ± 0.06	10 ± 0.43	35.5 ± 0.61
12	28	22 ± 0.29^{b}	0.71 ± 0.07	10 ± 0.15	33.3 ± 0.72

Different letters along columns denote significantly different mean age at first larviposition.

Table 3.4: Effect of age at mating on the reproductive performance of *G. f. fuscipes* females from Rusinga Island. (2m= 2 day-old females given chance to re-mate)

Age at mating (days)	n	Age at 1st larviposition (days)	Mean ± S.E.M		
			Fecundity	Interlarval period (days)	Pupal wt (mg)
1	12	21 ± 0.75[a]	0.21 ± 0.09	14 ± 2.50	21.3 ± 0.53
2	31	22 ± 0.98[a]	0.40 ± 0.06	11 ± 0.66	20.5 ± 0.74
2m	32	21 ± 0.94[a]	0.47 ± 0.06	10 ± 0.56	22.4 ± 0.81
4	20	24 ± 3.24[ab]	0.32 ± 0.07	10 ± 1.13	21.5 ± 1.53
6	25	27 ± 2.09[ab]	0.30 ± 0.08	11 ± 0.97	19.4 ± 1.03
8	18	20 ± 0.50[a]	0.15 ± 0.07	9 ± 0.00	23.5 ± 0.55
10	24	26 ± 1.77[ab]	0.38 ± 0.07	10 ± 0.86	21.3 ± 0.47
12	10	29 ± 1.64[b]	0.23 ± 0.10	12 ± 1.44	21.5 ± 0.57

Different letters along columns denote significantly different mean age at first larviposition.

Table 3.5: Effect of male age on fecundity ($\bar{X}$ ± S.E.M) of females. Values in parentheses are sample sizes (n).

Age at mating (days)	Fecundity	
	C.A.R	Rusinga
4	0.40 ± 0.10 (10)*	-
6	0.68 ± 0.07 (22)	0.14 ± 0.04 (23)
8	0.81 ± 0.08 (24)	0.20 ± 0.06 (32)
10	0.75 ± 0.08 (24)	0.40 ± 0.06 (31)
12	0.88 ± 0.04 (18)	0.30 ± 0.06 (26)

Asterisk (*) denotes significantly lower fecundity along column.

Table 3.6: Suspected abortion rates (%) in the Rusinga and C.A.R. populations of *G. f. fuscipes*. Figures in parentheses denote sample size (n).

Age at mating (days)	Abortion rate (%)	
	Rusinga	C.A.R.
1	20.8 (48)	1.6 (64)
2	16.9 (124)	3.6 (112)
4	17.5 (80)	0.8 (120)
6	15.0 (100)	3.5 (116)
8	23.6 (72)	1.7 (120)
10	13.5 (96)	1.7 (120)
12	30.0 (40)	1.8 (112)

Table 3.7: Effect of blood meal ($\bar{X}$ ± S.E.M) on puparial weights (mg ± S.E.M). Means given are mean of means. Figures in parentheses denote sample sizes (n).

Population	Mean blood meal index ($\bar{X}$ ± S.E.M)	Mean puparial weight (mg ± S.E.M)
Rusinga	47.3 ± 4.20 (19)	18.2 ± 0.85 (16)
C.A.R	61.7 ± 3.94 (20)[b]	32.5 ± 0.69 (17)[b]

Different letters along columns denote significantly different means.

3.4.10 Percent Mortality

Post-mating mortality was higher in females from Rusinga ($\chi^2 = 63.2$, p= 0.001, n= 680) than from C.A.R. population (Table 3.8). In the C.A.R population, age had no significant effect on mortality while females from the Rusinga population mated two days post-eclosion experienced the highest post-mating mortality ($\chi^2 = 15.1$, p= 0.02, n= 473).

Table 3.8: Percent mortalities in the Rusinga and C.A.R. populations of *G. f. fuscipes*. Figures in parentheses denote sample sizes (n).

Age at mating (days)	Mortality (%)			
	Rusinga	C.A.R.	χ^2	p
1	17.9 (28)[a]	11.1 (27)	0.5	0.5
2	45.0 (80)[b]	3.3 (30)	17.0	0.001**
4	33.8 (74) [ab]	0 (30)	13.3	0.001**
6	20.9 (68) [a]	3.3 (30)	4.8	0.03 *
8	26.8 (71) [a]	0 (30)	9.9	0.002*
10	32.7 (110) [ab]	3.2 (31)	10.9	0.001**
12	36.6 (41) [ab]	6.7 (30)	8.5	0.004*

Significantly different mortalities are denoted by different letters along columns (p= 0.02) and by asterisks (*) across rows.

3.5 Discussion

The present study has shown that females from the Rusinga population of *G. f. fuscipes* are most receptive two days after emergence, while male sexual virility peaks eight days post emergence under laboratory conditions (Figs. 3.1a & 3.2a). This means that by the time males are sexually mature, females of the same age have lost receptivity. However, in natural environments there are always older mature males, and females are mated quite early in life (Okiwelu, 1977a; Tobe & Langley, 1978). In contrast, females from the C.A.R. population exhibited high receptivity at all age levels (Figs. 3.1b & 3.2b). This is of significance in mass rearing since the wide range of receptivity suggests that high insemination rates are achieved

and hence fecundity. Receptivity in *G. morsitans*, *G. austeni* and *G. palpalis* females is highest one to three days after emergence (Tobe & Langley, 1978), a range which falls within that of the Rusinga population of *G. f. fuscipes*. In a population of *G. f. fuscipes* from Uganda, females mated most willingly between five and eight days post-emergence (Mellanby, 1936). The present findings suggest that the population of *G. f. fuscipes* from Uganda differs from the Kenyan one in sexual receptivity. The findings could also reflect ecological differences between the two populations, since behaviour is known to be environmentally labile. The present observation contrasts with that in *G. pallidipes*, where receptivity occurs between nine and 13 days post-emergence (Davies-Cole, 1990). In this species the females are reluctant to mate and do so only after enclosure with males for several hours.

Ovarian development in the Rusinga population of *G. f. fuscipes* was found to be intermittent and growth did not progress steadily with age as in the C.A.R. population (Fig. 3.3). Lack of proper growth of follicles probably affects reproductive performance in the Rusinga population since the slow follicular growth leads to delayed larvipositions. Growth was maximal 10 days after emergence while in the C.A.R population this occurred by day eight (Fig. 3.3). In both populations, receptivity declined as follicle size increased (Fig. 3.4) suggesting that females with mature follicles are unwilling to mate. The decline in follicle length in the C.A.R population indicates that ovulations occurred in some of the older virgin females. My study has shown that in *G. f. fuscipes*, females mate early in life and since sperms are stored in the spermathecae (Tobe & Langley, 1978), they can fertilize eggs once ovulation occurs. Early mating also occurs in *G. austeni*, *G. morsitans* and *G. palpalis*. In *G. pallidipes*, however, the females are more receptive once the follicle matures, and receptivity increases with maturity of the follicle (Davies-Cole & Chaudhury, 1990), so that mating occurs only once the follicle is about to be ovulated.

It is rare to get the two most developed follicles being of the same size, or having the left follicle developing first. The latter abnormality has been reported in *G. pallidipes* from the Lambwe area of western Kenya (Turner & Snow, 1984) and was attributed to temperature effects causing reversal in ovarian development (Mohammed-Ahmed *et al.*, 1994). There were instances where ovulation failed to occur in inseminated females from the Rusinga population (section 3.4.2). Harley (1968) attributed this effect to high constant temperatures of 28°C and above. This effect most likely acts through inhibition at the brain or the corpus allatum-corpus cardiacum complex level (Chaudhury & Dhadialla, 1976). These investigators showed that removal of this complex before or after mating inhibited ovulation. Since in the present study such extremes of temperatures were not employed, it is possible that inactivity due to confinement of the flies caused such inhibition of the brain or the corpus allatum-corpus cardiacum complex.

Sexual competence in male *G. f. fuscipes* from the two populations was high from eight days post-emergence, but younger males over three days old could also mate and inseminate females (Figs.3.1a & b). At the mass rearing facility at Seibersdorf, Austria, males mate when seven days old and above (Gooding *et al.*, 1997) while in the Ugandan population, males are fully competent six days after emergence (Mellanby, 1936). In this respect, the age at mating for males is similar in all populations of *G. f. fuscipes* studied to date. Although newly emerged male *G. f. fuscipes* have been observed to mate with young females, this is a rare event. The females mated by such males were not inseminated. Also, the percentage of males aged one day that made mating strikes was low. Despite the fact that spermatogenesis is complete by the time the males emerge (Itard, 1970), such young males cannot effectively transfer spermatozoa. Sperm are transferred to the female through the agency of a spermatophore (Pollock, 1970), the male ARG providing the precursors that go into spermatophore synthesis (Pollock, 1974). Examination of the apical bodies of male ARG

showed that apical secretion is well developed only in males that are sexually mature (above six days old). This is supported by the measurements of ARG widths, which showed significant increases from four days (Fig. 3.5) and commencement of mating in four day-old males of the C.A.R population (Fig. 3.1b). Those from the Rusinga population showed mating success from six days (Fig. 3.1a), perhaps pointing to delayed development of the apical secretion in the population. Although small amounts of this secretion could be observed in three day-old males, their measurements could not be made presumably because they were underdeveloped. Size of the apical secretion reflects the state of sexual maturity and mating status of male tsetse (Pollock, 1974) and the fact that it was readily measured in males over six days old shows that the males were sexually competent. The apical body may therefore be used in estimating age and hence sexual competence of male tsetse, based on an exponential factor (Fig. 3.5).

Pollock (1974) did not succeed in his attempts to separate the apical body from the male ARG of *G. f. fuscipes*. This may have been caused by dissection of young, sexually immature males that did not have well-developed apical secretions. This would imply that the pattern of development of the apical secretion in *G. austeni* and *G. morsitans* on one hand, and *G. f. fuscipes* on the other are different, since apical bodies of young males in the former species can be observed and measured.

The degrees of insemination were high on average and were similar across all the ages of females tested (Table 3.1). However, females from Rusinga Island mated when 12 days showed significantly lower degrees of insemination, with 95 % of the females being in MSV Class I (Fig. 3.6). This was probably caused by low receptivity of the females. This concurrs with similar observations on *G. pallidipes* where relatively old, less receptive females showed lower degrees of insemination (Davies-Cole & Chaudhury, 1990). Similarly, degrees of insemination showed no relation to age of males at mating (Table 3.1). This suggests that

once males attain sexual competence, they transfer nearly similar quantities of sperms. This supports similar findings on G. pallidipes, where males aged between two and 30 days showed similar degrees of insemination (Jaenson, 1979b).

Correlation analysis showed that there was no relationship (R= 0.15, p= 0.16, n=83) between degree of insemination and fecundity, slightly inseminated females larvipositing as well as highly inseminated ones. It seems that very little sperm is utilized during fertilization (Mellanby and Mellanby, 1937).

The degrees of insemination of females that were given a chance to re-mate were not statistically different from those that were separated from males immediately after mating (section 3.4.6). This suggests that females of *G. f. fuscipes* do not normally re-mate or accept sperms from a second mating. Physical observations carried out in this study involving mated females and virgin males showed that after the first mating, females were refractory irrespective of whether the male was introduced soon after the first mating or after the first larviposition. This supports the findings (Mellanby and Mellanby, 1937) that females do not deplete sperm in their lifetime and a second mating is therefore unnecessary. Cases of re-mating observed, where a male that had just completed copulation immediately mounted the same female occurred mostly in young females aged two days, none of which survived. This may account for the high post-mating mortality recorded in two day-old females. Such matings are of no significance in improving reproductive performance since males require a lapse period of 12-24 hours before the ARG secretion is replenished (Pollock, 1974) and males that mate immediately after the initial mating are unlikely to transfer any spermatozoa. Moreover, such matings have a negative effect on performance since survival is reduced. Repeated mating could not be observed after a time lag. Receptivity is not cyclical in *Glossina* and once a female becomes refractory, it rarely engages in further matings. Once a follicle is

ovulated, the next one in the ovulation sequence starts developing and the latter is ovulated between one and two h after parturition (Denlinger & Ma, 1974).

The mean duration of copulation of one and two day-old females of the Rusinga population were significantly higher than those of 10 and 12 day-old ones (Table 3.2). Duration of copulation is critical in triggering ovulation of the first mature oocyte in female tsetse (Jaenson, 1979a). Since young females took a longer time in copula, it is likely that they require stronger stimuli to trigger ovulation of the first oocyte than do older females. Similar observations have been reported on *G. pallidipes* females (Jaenson, 1979a). The mean duration of copulation of the Rusinga population were significantly higher than that of the C.A.R population. This shows that the two populations differ in mating behaviour. Similar observations were made on the mating behaviour of two allopatric populations of *G. pallidipes* (Jaenson, 1978; Van Etten, 1981). In the present study, the minimum duration of copulation was 20 minutes, which is lower than that reported for the Ugandan population of the same species (Mellanby, 1936). In the Ugandan population, sperm transfers occurred only in matings lasting for at least 30 minutes. On the other hand, females of *G. m. morsitans* mated for less than 60 minutes were never inseminated (Dame & Ford, 1968), and mating could last up to between six and eight hours (Davies-Cole, 1990). Copulation duration in *G. pallidipes* lasts for 24 minutes on average. *Glossina f. fuscipes*, like *G. pallidipes*, seems to have adapted to short mating durations which may have the advantage of reducing chances of predation (Drummond, 1984).

Age at mating had no effect on fecundity of females in both the C.A.R and Rusinga populations (Tables 3.3 and 3.4). However, the females the C.A.R population showed higher fecundity than the Rusinga one at all the ages tested (Fig. 3.7). Females given an opportunity to re-mate showed similar fecundity to those not given such an opportunity (Table 3.4). As it has been pointed out (section 3.4.6), probably females did not engage in repeated matings.

Females from the C.A.R population mated by males aged four days exhibited lower fecundity than those mated by older ones (Table 3.5). From six days, however, age at mating of males had no effect on fecundity. In the Rusinga population, none of the females mated by four day-old males larviposited. Fertility of males older than four days was similar within all the ages tested. As with age at mating of females, fecundity of females mated by the Rusinga Island males was lower than that of those mated by the C.A.R males. At the age of four days, male tsetse are still in the process of sexual maturity and may not have sufficiently inseminated the females. From six days however, most males were able to mate, suggesting that most had attained sexual maturity and therefore inseminated females optimally. This finding concurs with that of Rogers (1972) in which male *G. pallidipes* aged 13 days showed higher fertility than younger males. It suggests that sexual maturity in male *G. f. fuscipes* occurs earlier than in *G. pallidipes.*

Fecundity in the Rusinga Island population was affected mostly by abortion, which was higher than in the C.A.R population (Table 3.6). This was exhibited through missed pregnancy cycles, where eggs were aborted. Aborted eggs were not readily recovered, and so abortions were inferred from the gaps in larvipositions (Madubunyi, 1975). Aborted larvae were, however, recovered from the female vials. The abortions may be explained by the feeding performance of females in the two populations. The mean blood meal index of females of the C.A.R population was higher than that for the Rusinga population, and was positively correlated with puparial weights (Table 3.7). The fact that weights of pupae produced by the females from the C.A.R population were higher than those produced by females from Rusinga suggests that larvae of the latter were not getting sufficient nutriments. Insufficient blood meals induce abortions (Mellanby, 1937; Saunders, 1972) and the low mean blood meal index for females of the Rusinga population may explain the high incidence of abortions. The Rusinga population of *G. f. fuscipes* is poor at feeding, and those that feed

seem inefficient in converting the blood meal into larval food. The proportion of blood meal that is converted into fat is lower in artificially inactive compared active flies, and may lead to laboratory reproductive underperformance in the former (Loder *et al.*, 1998).

The high rates of abortion, especially of eggs, may have also been due to failure of fertilization to occur, so that eggs were extruded. Roberts (1973a) suggested that a valve-like opening into the spermathecal duct, which opens and closes, regulates sperm supply from the spermathecae. Failure of this valve to open would stop the release of sperms, fertilizations would fail, and hence abortions of eggs would occur.

Three percent of the females from the C.A.R population larviposited once but on dissection were found to be uninseminated. This would suggest that very little sperm was passed to the female during mating, and may not have been stored in the spermathecae. The sperms may have been present in the uterus during the first ovulation, and fertilized only one oocyte. However, this raises the question of the length of time sperms can stay in the uterus and still remain viable. Alternatively, such females may have larviposited without the oocytes being fertilized. This form of reproduction in which eggs undergo full development in the absence of fertilization is called parthenogenesis. It was not established, however, whether such offspring are viable. Okoth (1986) also observed larviposition in the absence of insemination in two out of a sample of 23 virgin *G. p. gambiense* females. It appears that if parthenogenesis occurs in *Glossina*, it is a rare event that could have negative implications for the sterile insect technique. Facultative parthenogenesis has been reported in normal bisexually reproducing species (Richard & Davies, 1977).

Post mating mortality was higher in two day-old females from the Rusinga population compared to other ages tested and higher in the Rusinga Island population than in the C.A.R one (Table 3.8). This could have arisen due to weak emerged adults that survived to the day of mating but could not withstand mating rigour and soon succumbed to death. Studies

carried out on *G. pallidipes* showed higher mortality at mating in three day-old females compared to older ones (Rogers, 1972). Young and recently fed females may be taken advantage of by males (Vanderplank, 1947), probably due to their inability to resist male mating attempts. Also, females that engaged in repeated matings, mostly those mated when two days old, did not survive to the first larviposition.

My study has shown that the main factors affecting laboratory rearing of the Rusinga population of *G. f. fuscipes* are abortion and low pupal weights, both of which are due to poor nutritional states of the flies. An enhancement of feeding performance, probably through *in vitro* feeding and use of feeding stimulants could reduce abortion rates and raise puparial weights. Colonization of tsetse flies is a long process since survival declines with each generation, until a stable colony is attained (Gooding *et al.*, 1997; Van Etten, 1981) and consequently, regular supplementation of the existing colonies with wild puparia or females is necessary.

CHAPTER FOUR

ECLOSION BEHAVIOUR OF *GLOSSINA FUSCIPES FUSCIPES*

4.1 Introduction

There is a dearth of information on eclosion behaviour of *G. f. fuscipes* yet such information is important in mass rearing. Modern automated methods of rearing are being developed that involve knowledge of the time when each sex of a fly species emerges under given conditions, for programming of the collection of emerging flies. Equally important is information on the quality of a colony, particularly offspring size and emergence rates. Many tsetse fly species produce an increased number of weak emerged flies when the peak of female emergence occurs before day 32 post-larviposition (Gooding *et al.*, 1997). For some species, a major problem in colonization attempts is pre-mating mortality (Van Etten, 1981). This mortality could be as a result of weak emerged female flies that cannot feed and so die soon after emergence. The productivity of any colony depends on the number of reproducing females, and a high female: male ratio would contribute to high colony performance. The present study aims at elucidating factors that affect eclosion in *G. f. fuscipes*.

4.2 Study Objectives

This study was designed to:

(a) Examine the influence of photoperiod on eclosion times.

(b) Determine emergence rates under laboratory conditions.

(c) Determine male and female developmental periods.

(d) Determine sex ratios at emergence.

4.3 Material and Methods

Batches of mated female flies were kept under laboratory conditions at 25 ± 1.0°C, R.H. 80-85% and LD 12:12. Pupae produced by these females were kept in plastic petri-dishes and put in standard emergence cages under similar conditions as adults. Once eclosion began, the cages were checked every two hours for emerging flies. Records were made of time of emergence, sex of the fly, and total number of flies emerged. When no more flies emerged, the empty puparial shells as well as the non-emerged flies were counted to determine the emergence rates. The photoregime was then changed to LD 8:16 and the same procedure repeated.

4.4 Statistical Analyses

Data on number of flies emerging and the duration of male or female development were analyzed using the General Linear Models (GLM) procedure while percent emergences were arcsine-transformed before being similarly analyzed. Sex ratios were analyzed using χ^2 test of independence.

4.5 Results

4.5.1 Effect of Photoperiod on Emergence Peaks

At a photoperiodic regime of LD 12:12, emergence peaked between 1000-1200 h in both males and females of the Rusinga ($F= 16.7$, $p= 0.0001$, $df = 11$) and C.A.R populations ($F= 11.9$, $p= 0.0001$, $df = 11$) (Figs. 4.1a & b). Small numbers of flies emerged between 1400 - 1800 h and 0400 - 0800 h. No flies emerged between 1800- 0400 h in both populations. At shorter photoperiods the eclosion peak shifted to between 0600 and 0800 h, emergence beginning from 0200 h (Fig. 4.2). There was no emergence from 2000 - 0200 h.

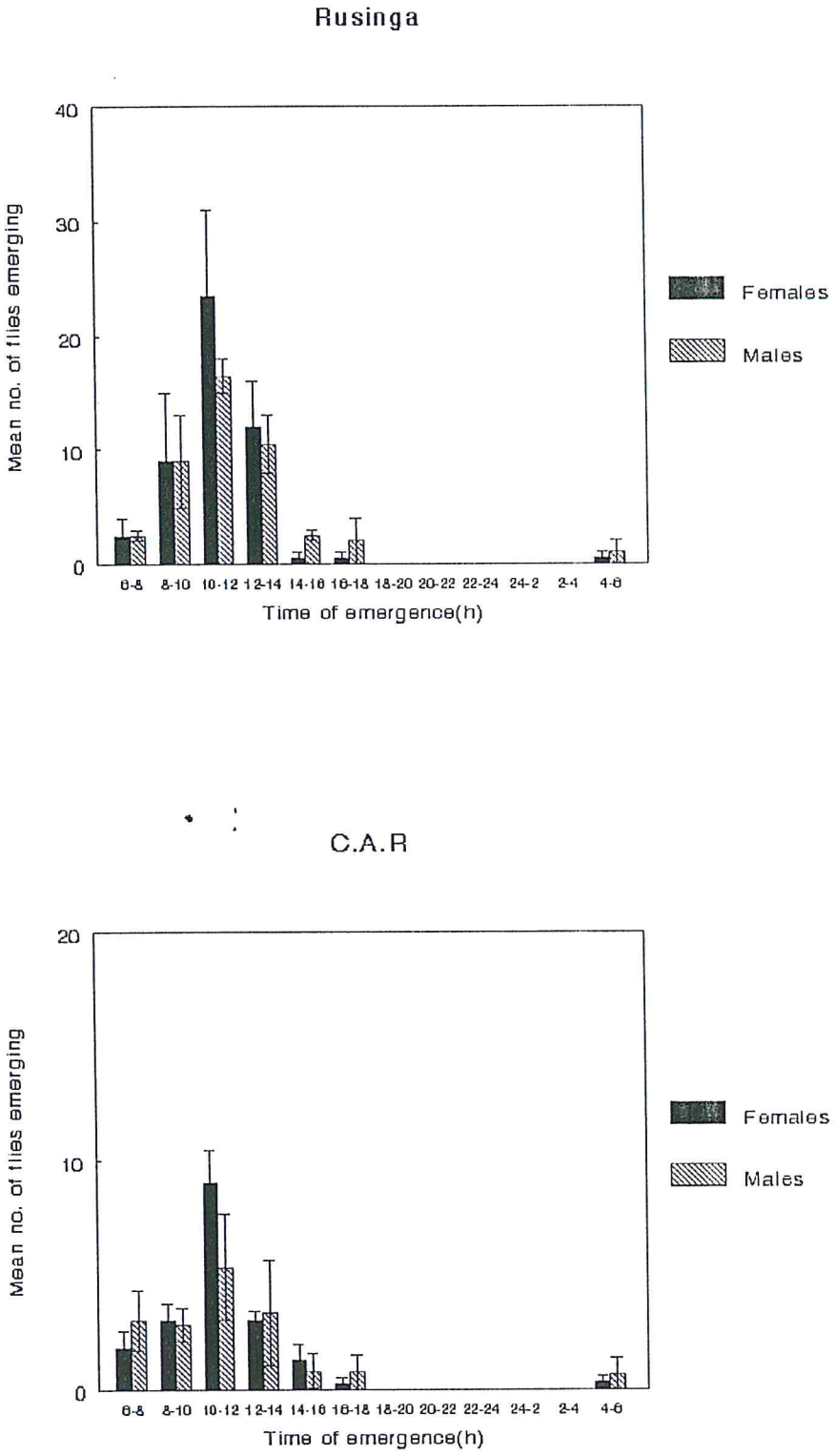


Fig. 4.1: Mean number of flies ($\bar{X}$ ± S.E.M) emerging at a photoregime of L D: 12: 12, 25°C in the Rusinga and C.A.R populations of *G. f. fuscipes*.

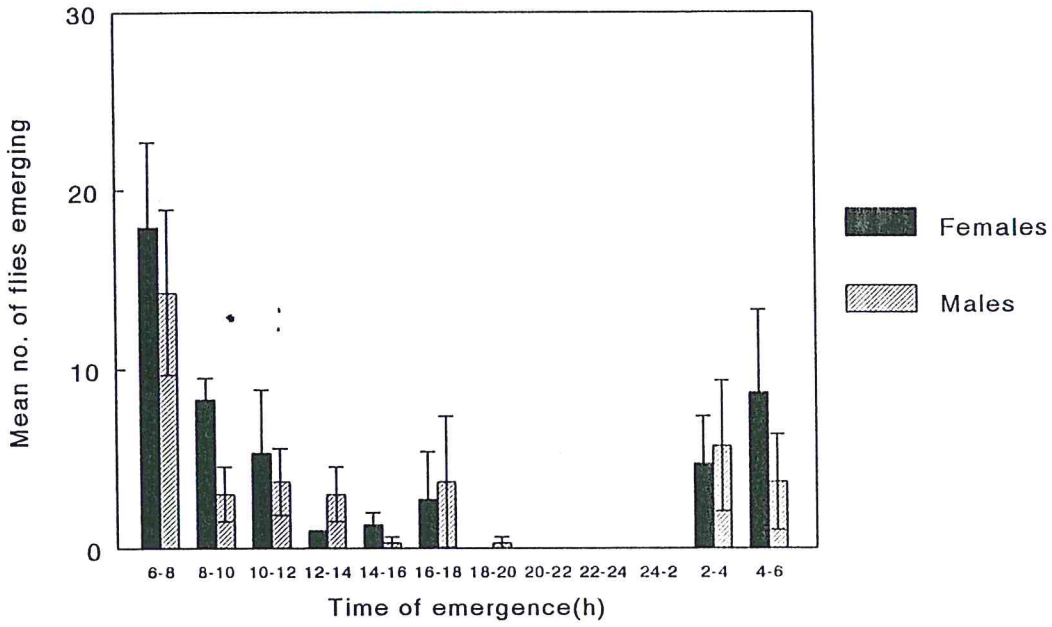


Fig. 4.2: Mean number of flies ($\bar{X} \pm$ S.E.M) emerging at photoregime of 8:16, 25°C in the C.A.R population of *G. f. fuscipes*.

4.5.2 Emergence Rates

Emergence rate from puparia deposited by F1 generation females from the Rusinga population (33.5 ± 1.1%) was significantly lower (F= 70.0, p= 0.0002, df = 1) than that for puparia produced by wild females (80 ± 1.8%). Dissections of some the non-emerged puparia showed that some died at the larval stage while in some the adult within the puparium developed normally but failed to emerge. In the C.A.R population, the mean emergence rate of 89 ± 2.8 was similar to that of Rusinga population.

4.5.3 Duration of Development

Female development in the Rusinga population took 32.1 ± 0.3 days, while it took 35 ± 0.5 days in males. In the C.A.R population, development in females took 30.4 ± 0.2 days and in males, 32 ± 0.1 days. Peak female emergence in the Rusinga population occurred 33 days post-larviposition (Fig. 4.3a) while in the C.A.R population, peak emergence for females was 32 days post-larviposition (Fig. 4.3b)

4.5.4 Sex Ratios

Female to male ratio in the F1 Rusinga population was 1: 1.2, the ratios being similar (χ^2= 3.3, p = 0.07, n = 486). Sex ratio in the F2 generation (1.4: 1) were also similar (χ^2= 2.8, p= 0.09, n= 127). In the C.A.R population, the ratio was 1.3:1, females being significantly more (χ^2= 10.3, p= 0.001, n= 624) than males. Female ratio was significantly higher (χ^2= 12.1, p= 0.0005, n= 1110) in the C.A.R than the Rusinga population.

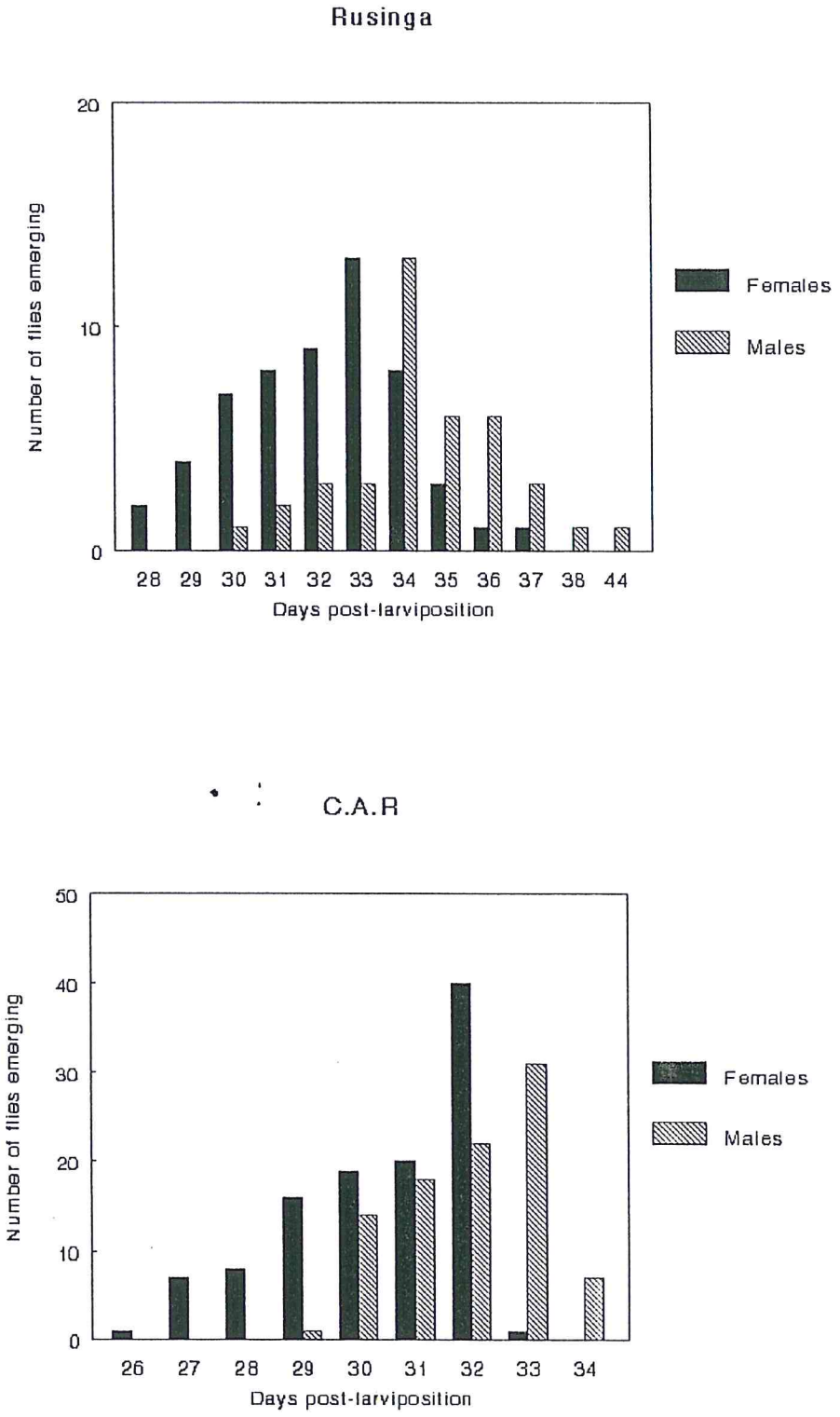


Fig. 4.3: Number of flies emerging from puparia in relation to days post-larviposition in the Rusinga and C.A.R populations of *G. f. fuscipes*.

4.6 Discussion

At L D: 12:12 eclosion peak occurred between 1000 and 1200 h while at L D: 8: 16, it occurred between 0600 and 0800 h, emergence beginning at 0400 h (Figs. 4.1& 4.2). Hence, photoperiod had a significant effect on the time at which the emergence peak occurred. No emergence occurred in the scotophase, indicating that developing flies within the puparium may respond to light stimulus. However, when the photoperiod is shortened emergence begins earlier before lights are on, indicating that the flies become programmed for shorter light cycles (Saunders, 1982). This supports findings in *Drosophila*, where eclosion peaks appear before dawn in short periods of less than six and seven hours. Eclosion peaks in *G. m. morsitans* follow thermocycles and are independent of light (Dean *et al.*, 1968; Phelps & Jackson, 1971; Zdarek & Denlinger, 1995). In their experiments with artificially generated thermocycles, Zdarek and Denlinger (1995) showed that the peaks followed bursts of temperature rise. Under laboratory conditions, however, temperature is controlled thermostatically and although this goes on throughout the night, no emergence occurred in the scotophase. Temperature has been shown to affect endogenous circadian rhythms, adult eclosion being temperature dependent (Saunders, 1982). High temperatures shorten the periodicity of eclosion rhythm, since rate of development varies with temperature (Saunders, 1982) and this explains the peaks that followed temperature bursts in *G. m. morsitans*. In *G. m. morsitans*, eclosion peaks occurred between 1400 and 1730 h at LD 12:12 and 25°C. In the present study, the peak occurred between 1000 and 1200 h, when 43% of the flies from the Rusinga population emerged. *Glossina f. fuscipes* and *G. m. morsitans* are two different species occupying different habitats, and it is possible that they have species-specific times of emergence, the former emerging in the morning while the latter emerges in the afternoon.

Emergence rate from puparia produced by the F1 generation females was lower than that for puparia produced in the laboratory by wild females. This may be explained by the

heavier puparia (25 ± 0.21 mg) produced in the laboratory by the wild females compared to those produced by the F1 females (20.8 ± 0.31 mg) ($F= 136.9$, $p= 0.0001$, $n= 397$). Small pupae are less viable since they use up all their fat reserves and die before emergence (Jackson, 1949), lowering emergence rates. Flies that emerge from small puparia are also small and may not survive after emergence since the pupae use most of the fat reserves. Since dissections revealed that the flies developed normally in the puparium it was inability to emerge, perhaps due to weakness that affected emergence, hence affecting reproduction in this population.

Females from the Rusinga population started emerging from puparia 28 days after larviposition, with the peak being 33 days after larviposition (Fig. 4.3a). Males on the other hand, started emerging after 30 days, the peak being 34 days after larviposition. Females complete development faster than males (Zdarek & Denlinger, 1995), emerging earlier. In the C.A.R population, females started emerging after 26 days with a peak at 32 days, while males started emerging after 29 days, peaking at 33 days (Fig. 4.3b). Puparial development was thus slightly faster in the C.A.R. than in the Rusinga population.

Female to male ratio was higher in C.A.R than in the Rusinga population. This could be due to factors that affect males within the puparium, inhibiting their emergence. If this occurs in nature, it would be an advantage to mass rearing since colony success depends on the number of reproducing females (Feldmann, 1993). This variation from the norm was observed in only one sample. In the Rusinga population, the sex ratios were similar.

The experiments carried out in the present study show that in *G. f. fuscipes*, photoperiod controls the pattern of emergence. In the tropics however, the study may not have practical implications since no significant changes in photoperiod are experienced. This study has also shown that reproductive performance of the Rusinga population of *G. f. fuscipes* was affected by low emergence rates of the F2 generation adults while in the C.A.R population, high emergence rates contributed to better performance.

CHAPTER FIVE

A COMPARATIVE STUDY OF FEMALE REPRODUCTIVE CHARACTERISTICS OF WILD AND LABORATORY POPULATIONS OF *GLOSSINA FUSCIPES FUSCIPES*

5.1 Introduction

Certain factors, including reproductive abnormalities, have been reported to affect laboratory rearing of *Glossina* (Harley, 1968; Jaenson, 1979b; Van Etten, 1981). Some of these factors occur in the laboratory and are probably due to environmental conditions. Others, however, occur in nature and if their frequencies are appreciable could contribute to overall population regulation of a species. For instance, egg retention has been reported in wild inseminated females of *G. pallidipes, G. fuscipes* and *G. brevipalpis* from Lugala, Uganda (Harley, 1966; 1967) with higher frequencies occurring in the laboratory. In Kenya, studies of *G. pallidipes* showed that reproductive performance in the Nkuruman and Mwalewa populations were affected largely by premating mortality and low productivity (Van Etten, 1981). The Lambwe valley and coastal populations also had certain ovarian abnormalities, including inhibition of ovulation, irregular sequence in ovarian development and cessation of development of ovaries (Turner & Snow, 1984). Such abnormalities have not been recorded in wild *G. f. fuscipes* females though certain abnormalities affect their reproductive performance in the laboratory. In the present study, I assessed the presence and frequency of female reproductive abnormalities in laboratory and wild *G. f. fuscipes* from Rusinga Island.

5.2 Study Objectives

This study was designed to:

1. Assess for presence of reproductive abnormalities in a wild population of *G. f. fuscipes*
2. Compare the abnormalities in wild and laboratory populations of the species.

5.3 Material and Methods

5.3.1 Capture of Tsetse Flies and Age Grouping of Females

Tsetse flies were sampled from Rusinga Island, Kenya using biconical traps (Challier & Laveissiere, 1973), in the wet (May) and dry (September) seasons of 1996. Collections were made twice daily at 0900 and 1500 h. The flies were transported to the laboratory at Mbita Point Field Station where females were dissected immediately, as given in section 2.4. The female flies were age-grouped using the ovarian method (Saunders, 1962; Challier, 1965). This method relies on the regularity with which eggs are released from the two ovaries (Saunders, 1960a; 1960b; 1962). Each ovary contains two ovarioles, making a total of four ovarioles. The two ovaries ovulate alternately, starting with the right ovary. In nulliparous females (0-10 days post-eclosion), the inside right ovariole is the most developed, followed by the inside left ovariole, then the outside right ovariole, and lastly, the outside left ovariole (Saunders, 1960a; 1960b). Thus if the ovarioles are numbered 1-4 in order of size, the configuration that emerges is 4213 (Challier, 1965) (Fig. 5.1).

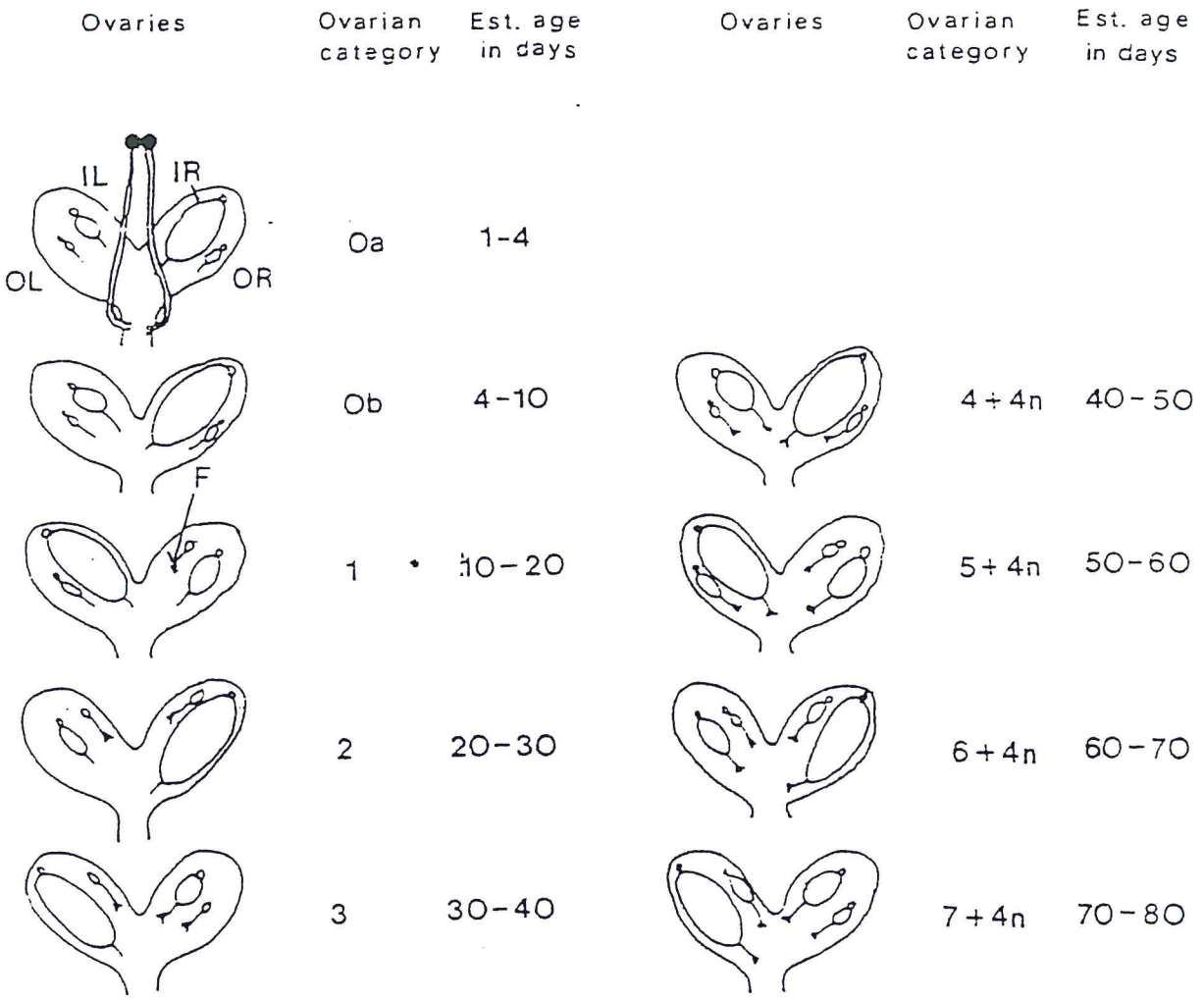


Fig. 5.1: Ovarian changes with age in female *Glossina*. F, follicular relic; IR, inside right follicle (A1); IL, inside left follicle (C1); OR, outside right follicle; OL, outside left follicle. (From Murray *et al.*, 1983).

After the first ovulation (10-20 days post-eclosion), the inside left ovariole is the most developed, and assigning numbers to the egg follicles results in the configuration 3142. After the second ovulation, it becomes 2431 and after the third, 1324. This cycle is repeated, with females that have had four ovulations having the configuration 4213. After each ovulation, the position of the discharged egg in each ovariole is represented by a small residual body called a follicular relic. Through the use of the number and position of such relics, the size and position of the four egg follicles, and the uterine contents, Challier (1965) recognized eight age categories (0-7) which included females up to 80 days post-eclosion. Flies that have had four ovulations and therefore show the configuration 4213 could have had 4, 8, 16, etc. ovulations, and are therefore designated 4 + 4n, where n is the number of ovulation cycles. Similarly, flies in age categories 5, 6, or 7 are designated 5 + 4n, 6 + 4n, 7 + 4n, etc.

5.3.2 Estimation of Abortion Rates.

Abortion rates were determined using the method first devised by Madubunyi (1975) and modified by Turner and Snow (1984). The method takes into account the mean size of the intra-uterine egg and the follicle next in the ovulation sequence (FNOS). Following parturition, the FNOS is mature and its size approaches that of the intra-uterine egg which it shortly becomes. The uterus thus remains empty for about 1-2 h (Denlinger & Ma, 1974) after which the FNOS is ovulated. An abortion, however, can occur before the FNOS is mature (hence its size will be significantly below that of the intra-uterine egg). Consequently, the uterus will remain empty for a longer time. By taking the mean size of the intra-uterine eggs and comparing it to individual sizes of FNOS, it is possible to determine whether a normal parturition or an abortion has occurred in a female fly with an empty uterus. The test for comparison takes into account the mean and the variance of the distribution of intra-uterine egg sizes. The standard normal deviate d, is calculated as:

$d = (X-\mu)/\delta$, where

X is the size of the most developed egg follicle,

μ is the mean intra-uterine egg size

δ is the standard deviation of the intra-uterine eggs

If $|d| \geq 1.96$, it is 95% probable that the value of X lies outside the distribution range, signifying an abortion. For example, if X = 0.8,

$\mu = 1.4$,

and $\delta = 0.298$

$d = (0.8 - 1.4)/0.298$

$|d| = 2.01$

A $|d| > 1.96$ means that the size of the most developed follicle is significantly below the mean size of the intra-uterine egg, and so an abortion must have occurred.

Females in age group 1 were not included in these determinations, since the first eggs produced are smaller than those produced in later age-groups (Challier, 1973).

Measurements were made on the length of intra-uterine eggs, follicle next in the ovulation sequence and the follicle to develop next.

5.3.3 Other Abnormalities

The degree of filling of the spermathecae were also determined for each female fly, as outlined in section 2.5. "Slow growth of follicle" was assigned to cases where, for example, there was a second instar larva in the uterus yet the follicle next in the ovulation sequence had not reached a given mean size. This size was calculated from a sample of females that had a second larva in the uterus. Any abnormalities in the ovaries, uterus or follicle were recorded. Field samples were obtained both in wet and dry seasons.

5.4 Statistical Analyses

Data on intra-uterine eggs and mean spermathecal values were analyzed using GLM procedure while abortion rates were analyzed by the χ^2 test of independence (SAS, 1985).

5.5 Results

In the dry season, parous females constituted 88% (n = 330) of the wild female population while in the wet season they constituted 90% (n= 105). The mean length of the intra-uterine egg in the dry season measured 1.40 ± 0.01 mm while in the wet season, it was 1.60 ± 0.02 mm. The mean length of the intra-uterine egg in laboratory females (1.37 ± 0.05) was similar to that of wild females sampled in the dry season.

5.5.1 Abortion Rates

The major abnormality affecting the two populations was abortion, which occurred in 20% (n= 325) of the laboratory females, 10.3% (n=290) of the wild females sampled in the dry season and 1.4% (n= 73) of those sampled in the wet season (Table 5.1). Frequency of abortion was heterogeneous between females sampled in the dry and wet season (χ^2= 6.1, p= 0.01, n= 363) and between laboratory and wild (χ^2 = 18.8, p= 0.001, n= 688) populations.

5.5.2 Empty Spermathecae

The mean spermathecal value (MSV) of the wild females was high (1.80 ± 0.002), compared to that for the laboratory population (1.0 ± 0.02). All the 330 wild females sampled in the dry season were inseminated to different degrees. In the wet season 2.9% (n= 105) were not inseminated, while 2.3% (n= 88) had larvae in the uterus but were not inseminated. In the

laboratory population, copulation without insemination was recorded in 6% (n= 325) of the females.

Table 5.1: Frequency of reproductive abnormalities (%) in laboratory (n = 325,) and wild (dry season, n = 330; wet season, n= 105) *G. f. fuscipes* from Rusinga Island. (A1= inside right follicle, C1= inside left follicle).

Abnormality	Laboratory females	Wild females	
		Dry season	Wet season
Abortion	20	10.3	1.4
A1= C1	4	0.3	0
Slow growth of follicle	4.9	1.4	0
Empty spermathecae	6	0	2.9
Egg retention	0.9	0	0
Follicular degeneration	0.6	0.3	1.2
Ovarian atrophy	0	0.3	1.2
Fusion of F1 and F2	1.2	2.1	2.9

5.5.3 Other Abnormalities

Other abnormalities found in the wild females included cases where the two most developed follicles were equal (Plate 5.1b). Slow development of follicles and equality in size of F1 and F2 were also observed in virgin laboratory females from Rusinga, at frequencies of 12.6% and

2.4%, respectively. These abnormalities were not observed in the population of *G. f. fuscipes* from C.A.R and during the rainy season. In one case (n = 330)), both ovaries of a female were reduced to small round structures. Ovarian dissections also revealed that in some female flies, one ovariole gets fused to the lower third of the other ovariole (Plate 5.1c). This was observed in both laboratory (1.2%, n= 325) and wild females (2.3%, n= 435 i.e., 2.1%, n= 330 in the dry season; 2.9%, n= 105 in the wet season). Egg retention in inseminated females (Plate 5.1d) was found in laboratory, but not in wild females. Follicular degeneration was observed in both populations.

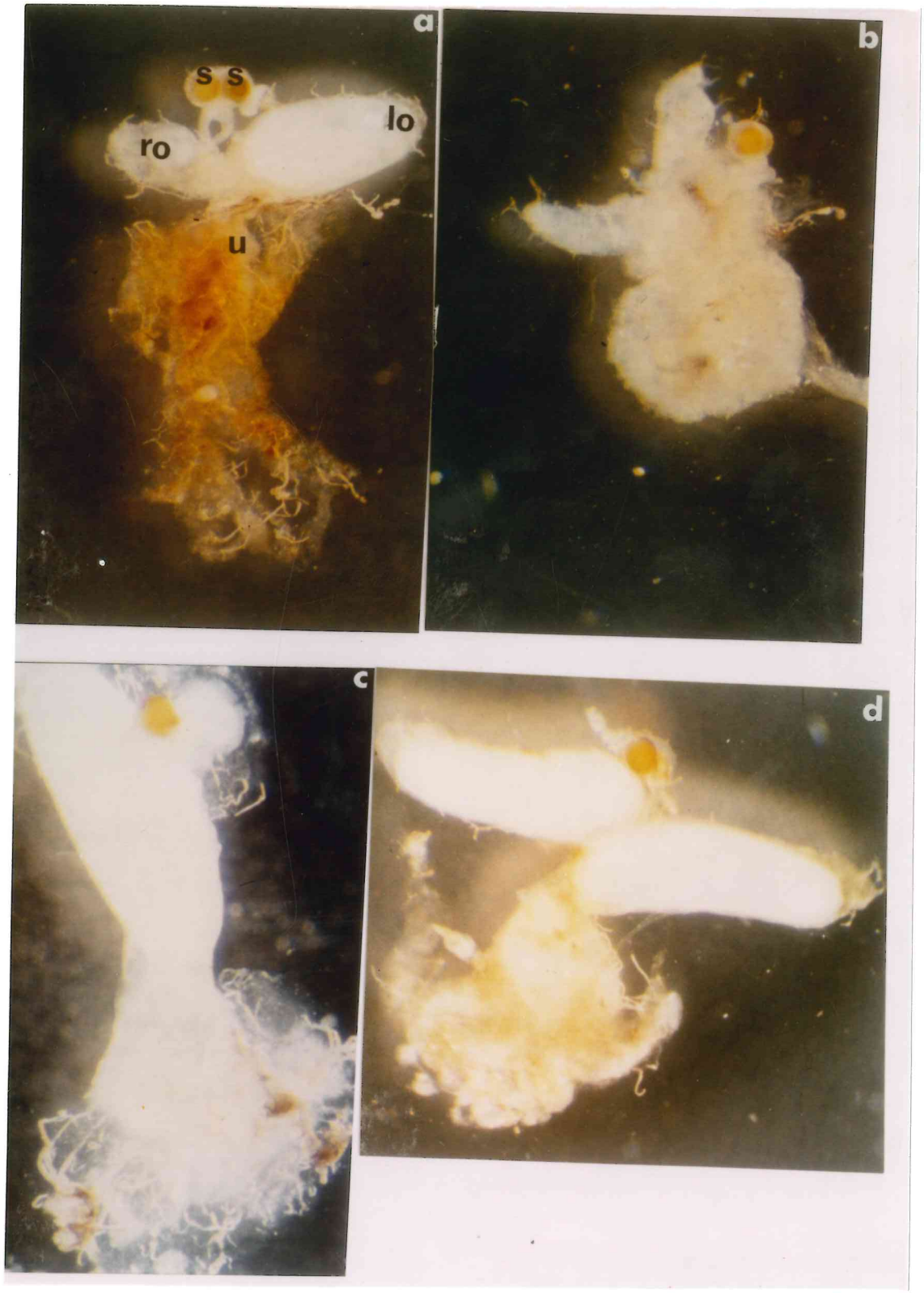


Plate 5.1: Female reproductive systems of laboratory and wild populations of *G. f. fuscipes*. a: normally developing ovaries; b: left ovary of same size as the right one; c: left ovary fused to the right one, d: retained egg follicles. s, spermathecae; ro, right ovary; lo, left ovary; u, uterus. The structures in Plates 5.1b-d are as labeled in a.

5.6 Discussion

The present study has shown that abortion contributes most to reproductive loss both in the wild, particularly during the dry season, and in laboratory populations of *G. f. fuscipes* (Table 5.1). However, it was nearly twice in the laboratory F1 generation females as compared to the wild females during the dry season. This is most likely due to lack of adaptation of the flies to confinement in laboratory cages, which affected either feeding or conversion of the blood meal taken into larval food. This was evidenced by the low weights of pupae produced by the females (Table 3.6). The reproductive abnormality has also been reported to be more frequent in laboratory than in wild populations (Madubunyi, 1975) because laboratory conditions rarely reflect optimal environmental requirements for tsetse flies.

Abortion rate is positively correlated with the time females spend in the traps before collection (Turner & Snow, 1984), probably due to stress. Hourly catches are therefore recommended for samples to be used for estimating abortion rates (Turner & Snow, 1984). In the present study it was not possible to collect hourly samples. Since the flies were sampled from twice-a -day catches, this may have exacerbated abortions.

In the present study, the frequency of suspected abortions was much lower in the rainy season (May) compared to the dry season (September) (Table 5.1), probably due to cooler ambient temperatures. This observation is in agreement with a study on suspected abortion rates in a natural population of *G. m. morsitans* in Zambia (Okiwelu, 1977a) which, in the hot dry season was 11.3% (n= 53) and in the warm rainy season, was 4.37% (n= 164). It is probable that abortion rate increases during the dry season due to harsh environmental conditions and also unavailability of hosts for a blood meal, since insufficient blood meals have been shown to induce abortions (Saunders, 1972). Different rates of abortion that have been reported for various species (Harley, 1966; Jordan, 1962; Madubunyi, 1975, 1978)

probably reflect varied nutritional states, since poor nutrition is a recognized environmental stress (Mellanby, 1937). Abortion has been reported to be the single most important factor contributing to reproductive loss both in laboratory (Ahmed *et al.*, 1995) and wild (Madubunyi, 1975; Okiwelu, 1977a; Turner & Snow, 1984) *Glossina* populations.

Although there was noticeable difference between the mean length of the intra-uterine egg measured in the dry season (1.40 ± 0.01 mm) and that measured in the wet season (1.60 ± 0.02 mm), the difference was statistically insignificant. In *G. m. morsitans*, the intra-uterine eggs are longer in the dry season compared to the wet season (Okiwelu, 1977b).

Slow growth of follicles and equality in follicle size were found both in wild and laboratory populations originating from Rusinga Island, but not in the C.A.R population (Table 5.1, Plate 5.1b). This probably occurs when one follicle ceases to grow, while the follicle next in the ovulation sequence (FNOS) overtakes it in growth. The abnormality, which has been attributed to high temperature (Mohammed-Ahmed *et al.*, 1994), probably contributes to population regulation in wild and laboratory populations of *G. f. fuscipes* from Rusinga.

Fusion of ovarioles occurred in both wild and laboratory populations (Plate 5.1c), but it was five times higher in the wild population (Table 5.1). Although fusion of ovarioles is a deviation from normality, it probably does not affect productivity since females with such ovaries still ovulated and deposited larvae.

Failure of insemination was more frequent in the laboratory than in the wild population, particularly for the sample taken in the dry season where all females were inseminated (Table 5.1). The high proportion of inseminated females in the wild population may be accounted for by the suggestion that high proportions of uninseminated females die within a few days of emergence (Nash & Kernaghan, 1965) and so are underrepresented in trap catches. Such females are weaklings that are not preferred by males. Copulation without

insemination contributed to reproductive loss in the first generation of laboratory females, and was more pronounced in cases where relatively old females were mated (Section 3.4). The fact that all the wild females including non-tenerals that were sampled in this study were inseminated indicates that mating in this species occurs very early in adult life of the female. This is supported by the mating experiments (section 3.3.1) which demonstrated that even unfed females mate successfully. Similar insemination rates occur in wild *G. m. morsitans* and *G. austeni* (Langley, 1977; Okiwelu, 1977a; Tobe & Langley, 1978).

Egg retention in inseminated females was recorded in the laboratory females but not in wild females (Table 5.1, Plate 5.1d), indicating that it is of no importance in population regulation in nature. Harley (1968) observed this abnormality in females kept in extremes of temperature, and attributed it to inactivity of the females in the constant temperature conditions. In the present study, temperature ranged from 23-25°C. Relative inactivity due to confinement in the holding cages apparently interferes with the ovulation process, probably through inhibition of the corpus allatum -corpus cardiacum complex (Chaudhury & Dhadialla, 1976).

The mean length of intra-uterine eggs was considerably greater in the wet than dry season (section 5.5). This probably occurred due to favourable environmental conditions in the wet season. This supports similar observations on a population of *G. m. morsitans* in Zambia (Madubunyi, 1974) but contrasts with those of Okiwelu (1977b) who observed larger eggs in the dry season. The larger egg sizes in the wet season are also probably due to good nutritional states of females, which may be related to movements of hosts (Okiwelu, 1977b). The present study also showed that in 1.5% (n= 435) of the wild females, the ovary was reduced to a club at the end of a stalk. This condition probably arises when vitellogenesis fails to occur, causing cessation in growth of the follicle. Cessation of follicular growth has been reported for a population of *G. pallidipes* from Lambwe valley (Turner & Snow, 1984), but

seems to be rare. On the other hand, it is probable that the area in which trapping of the females was done had been sprayed, so that this was an effect of the insecticides. Certain insecticides have been reported to have juvenile hormone analogue effects (Ashburner, 1970). This kind of abnormality is reported elsewhere in this study (section 6.4), where females were treated with pyriproxifen.

Of interest was the presence of larvae in the uteri of females whose spermathecae were virtually empty (Table 5.1). Out of three parous females that were not inseminated (n= 88), two contained first instar larvae in their uteri. As it has already been shown (section 3.4), it is possible that whatever sperms were passed to the female during mating remained in the uterus and were not stored in the spermathecae. Such sperms could have fertilized an oocyte, which then proceeded with development. This postulation, however, raises the question of the period of time that sperms can stay in the uterus and still be viable. Moreover, it has been reported that an egg gets fertilized as it passes the opening of the duct leading to the spermathecae (Langley, 1977), hence fertilization of the egg by sperms already present in the uterus is unlikely. This is so because the micropyle faces the distal end of the uterus, which is proximal to the opening of the spermathecal duct. Alternatively, it could be that female tsetse can reproduce through parthenocarpy, the implication of which was discussed in section 3.5.

In conclusion, the study has shown that in *G. f. fuscipes*, the major factor affecting reproduction both in wild and laboratory populations is abortion. Abnormal development of follicles may also contribute to reproductive loss in the laboratory, but only to a small extent in the wild during dry seasons. The study has also shown that larviposition can take place in the absence of insemination but is rare and may not contribute to an appreciable increase in population because of its low incidence. Other abnormalities such as ovarian atrophy and egg retention are of a small magnitude and may not contribute to population regulation while fusion of follicles has no effect on reproduction.

CHAPTER SIX

THE EFFECT OF JH ANALOGUES AND KETOCONAZOLE ON REPRODUCTION AND METAMORPHOSIS IN *GLOSSINA FUSCIPES FUSCIPES*

6.1 Introduction

Fecundity and offspring viability determine the survival of a population, thus compounds that affect these parameters can be used to suppress fly populations. Compounds that have been shown to disrupt reproduction and development in tsetse flies include pyriproxifen (Langley *et al.*, 1988; Hargrove & Langley, 1990, 1993), precocene (Samaranayaka-Ramasamy & Chaudhury, 1981), diflubenzuron (Jordan & Trewern, 1978; Jordan *et al.*, 1979) and benzyl-1, 3-benzodioxoles (Langley *et al.*, 1982) among others. These biological compounds act on the female tsetse by interfering either with development of the adult in the puparium or by inhibiting development of follicles, and are easily applied topically on the female integument.

The process of vitellogenesis is controlled by juvenile hormone in most cyclorrhaphous dipterans (Yin & Stoffolano, 1997) but not in *Glossina*, where surgical allatectomy (Langley & Pimley, 1986) or precocene treatment (Samaranayaka-Ramasamy & Chaudhury, 1981) does not interrupt the reproductive process. A similar situation occurs in the cricket, *Gryllus bimaculatus* de Geer where allatectomized females continue to lay eggs but treatment with the ecdysteroid biosynthesis inhibitor ketoconazole significantly reduces egg production (Orshan & Pener, 1991; Hoffman *et al.*, 1996). Thus, ecdysteroids may account for the portion of eggs produced by allatectomized females. Female tsetse were therefore first allatectomized by precocene treatment while others were treated with

ketoconazole in order to assess effects on vitellogenesis and reproduction. In a second experiment, precocene-treated females were treated with ketoconazole and the juvenile hormone (JH) analogues W-328 and pyriproxifen separately, in order to assess effects on fecundity.

Pyriproxifen treatment of female *G. m. morsitans* does not affect their ability to larviposit normal larvae, but adult development within the puparia is inhibited at the red eye and pigmented seta stage (Langley *et al.*, 1988). In addition, emergence is inhibited in 75% of puparia treated between two and four days post-larviposition. Two JH analogues, pyriproxifen and W-328, were studied in relation to sensitivity, fecundity and fertility of *G. f fuscipes*. Also investigated were morphogenetic effects of the IGRs following treatment of adults and puparia.

6.2 Study Objectives

This study was designed to:

1. Investigate the effects of pyriproxifen, W-328 and ketoconazole on reproduction in *G. f. fuscipes*.

2. Determine the morphogenetic effects of the IGRs on adult development in *G. f. fuscipes*

3. Determine the critical stage at which adult development cannot be disrupted by JH analogues.

4. Determine the fertility and fecundity of flies emerging from puparia treated with JH analogues.

6.3 Material and Methods

6.3.1 Effect of JH Analogues on Female *G. f. fuscipes*

The Juvenile hormone analogues Pyriproxifen (2-[1-methyl-2- (4-phenoxyphenoxy) ethoxy] pyridine) (Sumitomo) and W-328 (Institute of Organic Chemistry and Biochemistry, Prague) were used in this study. The analogues were dissolved in acetone and serially diluted into 0.001, 0.01, 0.1, 1, 2, 5, 10, 50, 100, and 1000 µg\ µl dosages. Female tsetse flies were mated when two or three days old and placed in each of the treatment groups per analogue or control group. Treatment was done after 24 h. Before treatment, the animals were exposed to cold anaesthesia (4°C) for 2-3 min and an analogue topically applied on the abdomen ventrally. Survival of flies was affected by the anaesthesia and so in subsequent treatments they were held between thumb and index finger during topical applications. In control experiments, adults were treated with acetone.

Experimental flies were then maintained under insectary conditions as described in section 2.1, observed daily, and any mortality noted. Survival was calculated as the number of females alive on the final day of the experiment and expressed as a percentage of the number that would have been alive had no mortality occurred. Fecundity was evaluated as given under section 3.2.3.

Puparia formed from larvae deposited by the females were kept in vials under similar conditions to adults. Adult development in *Glossina* takes approximately 30 days (Langley, 1977). After this period, the puparia were observed further for 10 days before dissection (Bursell, 1959) of puparia of non-emerged adults to determine the morphogenetic effects of the analogues on adult development.

6.3.2 Effect of Precocene Treatment on Females

Instead of surgical allatectomy, female *G. f. fuscipes* were topically treated with precocene II (6,7- Dimethoxy-2, 2-dimethyl (-3- chromene) (Sigma) two h post emergence. This treatment was preferred to avoid the high mortalities that arise from surgical operations, and particularly since tsetse flies were in short supply.

One group of 10 females was topically treated on the thorax with 5µg precocene II once while another was given precocene treatment after every nine days for the first three reproductive cycles. The treatments were replicated twice. The females were fed after 24 h following which they were mated. Fecundity and survival were calculated as given in section 6.3.1. Puparia of non-emerged adults were dissected to determine morphogenetic effects of precocene.

6.3.3 Effect of JH Replacement in Precocene-treated Females

In this experiment, the juvenile hormone analogues were applied to 10 females that had been topically treated with precocene. The females were fed after 24 h following which they were mated. Fecundity and survival were calculated as given in section 6.3.1. The treatment was replicated twice. Non-emerged adults had their puparia dissected to determine morphogenetic effects of the treatment.

6.3.4 Effect of Ketoconazole Treatment on Females

Females tsetse were topically treated with doses of ketoconazole ((+)-cis-1-acetyl-4-[4-[[2, 4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1, 3-dioxolan-4-yl]-methoxy] phenyl] piperazine) (Janssen Research Foundation), ranging from 0.01 µg to 10 µg. This was applied to the thoracic or abdominal area on the first, fifth and 12th day post emergence. Another group of females was treated with 5µg precocene II and 10µg ketoconazole, and the effects on

reproduction investigated. Fecundity was assessed after 45 days, and the puparia formed from the larvae deposited by the treated and control females allowed to go through the development period. At the end of this period, any adult that did not emerge had the puparium dissected to determine the effects of the insect growth regulator on development.

6.3.5 Effects of JH Analogues on Males

Male flies were treated with the JH analogues as above (section 6.3.1) on day seven post-eclosion and paired with two day-old females on day eight. After mating, the females were maintained as earlier described (section 2.1) until larviposition. Puparia of non-emerged adults were dissected to determine the morphogenetic effects of the analogues.

6.3.6 Effect of JH Analogues on Puparia

Puparia deposited by female *G. f. fuscipes* were collected each morning, between 0800 and 0900 h, at which time they were considered to be one day-old. Puparia aged between one and 10 days were treated with between 0.1 and 1000 µg pyriproxifen or W-328 in 1 or 2 µl acetone. Control puparia were treated with acetone only. The puparia were kept under similar conditions to adult flies (Section 2.1) until the expected date of emergence. The emergence rate was then determined and any abnormalities in flies recorded. Those that failed to emerge were dissected to determine the morphogenetic effects of the analogues on development.

6.3.7 Direct Application of JH Analogues to Pupae or Pharate Adults

A small section of the puparium wall was carefully broken in puparia aged five, 10 and 20 days, using gentle pressure from a needle. The analogues were then applied to the pupa or pharate adult, and the opening sealed with wax, using a cauter. Control puparia were treated

with acetone only. The puparia were kept under similar conditions to adult flies (Section 2.1) until the expected date of emergence. The emergence rate was then determined and any abnormalities in flies recorded.

6.3.8 Classification of Morphogenetic Effects

Following dissections of puparia that failed to emerge, the morphological effects of the IGRs were classified and scored depending on degree of the effects (Appendix 1). The scores were used to compare effects of the IGRs.

6.3.9 Effect of JH Analogues on Fertility and Fecundity of Flies Emerging from Treated Puparia

Flies emerging from the above experiment (section 6.3.6) were sexed and kept in holding cages. Females were mated with normal males from untreated puparia, and their fecundity determined (section 3.2.3). Fertility of males was determined indirectly by mating them with normal females and similarly determining fecundity.

6.3.10 Histological Studies

The adult flies were removed from the puparium and fixed in alcoholic Bouin's solution. Sections were cut at 7 μm and stained with hematoxilin and eosin. They were then mounted in DPX and examined under a light microscope.

6.3.11 Statistical Analyses

Effects of IGRs on age at first larviposition, interlarval periods, puparial weights and fecundity were determined by the GLM procedure (SAS, 1985) while effects on abortion and tergite development were analyzed by χ^2 test of independence (SAS, 1985).

6.4 Results

6.4.1 Effects of the Insect Growth Regulators on Females

6.4.1.1 Pyriproxifen

Pyriproxifen had no effect on age at first larviposition, interlarval periods, puparial weights or fecundity, irrespective of dose or time of application during the pregnancy cycle in both C.A.R (Table 6.1) and Rusinga (Table 6.2) populations of *G. f. fuscipes*. The puparia produced appeared physically normal, with similar mean weights at all dose levels and stage of pregnancy.

The Rusinga population of *G. f. fuscipes* had lower fecundity ($F=174.7$, $p= 0.0001$, $n= 291$) compared to the C.A.R population. Survival was affected by dosage, with no survivors at a dose of 1000 µg (Fig. 6.1). Two females from the Rusinga population from a total of 51 females that were dissected had both ovaries reduced in size, appearing like small clubs at the end of a long stalk.

Emergence occurred in 4.3% (n= 47) (Table 6.3) of the puparia from females that were treated with 0.001 µg pyriproxifen while those from acetone treated females showed 86.4% (n= 66) emergence.

6.4.1.2 W-328

Females that were treated with this JH analogue larviposited, with no difference in the number of pupae or puparial weight from those of controls. At the end of the development period, 1.6% (n= 64) adults emerged from puparia of females treated with 0.001 µg W-328 while 2.6% (n= 75) emerged from those produced by females treated with 0.01 µg W-328 (Table 6.3). In addition, 2.8% (n= 36) adults emerged from puparia from females treated with 10 µg W-328.

Table 6.1: Effects of various doses of IGRs on reproductive performance of the C.A.R population of *G. f. fuscipes*. (Pyri. = pyriproxifen, Keto. = ketoconazole, Prec. = precocene, c2= second pregnancy cycle).

IGR	n	Dose (µg)	Age at 1st larviposition	Interlarval Period	Puparial weight	Fecundity	Survival (%)
W-328	20	0.001	18.4 ± 0.45	11.1 ± 0.53	31.6 ± 1.53	0.90 ± 0.10	100
W-328	20	0.01	18.8 ± 0.93	10.6 ± 0.66	31.9 ± 1.24	0.90 ± 0.05	100
W-328	27	0.1	17.8 ± 0.40	9.4 ± 0.16	31.0 ± 0.53	0.80 ± 0.09	96
W-328	20	1.0	18.1 ± 0.63	9.7 ± 0.22	30.6 ± 0.91	0.91 ± 0.10	90
W-328	15	10	18.2 ± 0.85	10.4 ± 1.01	31.1 ± 0.76	0.73 ± 0.13	80
W-328	20	100	17.9 ± 0.67	9.5 ± 0.17	30.4 ± 0.53	0.77 ± 0.08	90
W-328	20	1000	18.0 ± 0.42	9.7 ± 0.12	30.6 ± 1.05	0.70 ± 0.03	100
Pyri.	14	0.001	18.0 ± 0.79	9.9 ± 0.13	31.8 ± 0.78	0.83 ±0.07	100
Pyri.	15	0.01	18.9 ± 0.26	9.5 ± 0.25	30.5 ± 0.60	0.90 ± 0.08	100
Pyri.	15	0.02	18.3 ± 0.44	10.3 ± 0.22	31.9 ± 0.40	0.82 ± 0.02	100
Pyri.	20	0.05	18.9 ± 0.36	10.2 ± 0.26	31.6 ± 0.63	0.92 ± 0.08	100
Pyri.	20	0.5	18.0 ± 0.28	10.1 ± 0.24	31.5 ± 0.77	0.94 ± 0.05	90
Pyri.	20	1.0	19.5 ± 0.30	9.6 ± 0.15	31.4 ± 0.78	0.89 ± 0.04	90
Pyri.	20	2.0	18.6 ± 0.24	10.2 ± 0.44	31.0 ± 0.68	0.90 ± 0.10	95
Pyri.	20	5.0	18.2 ± 0.31	10.3 ± 0.33	32.6 ± 0.58	0.92 ± 0.08	85
Pyri.	20	100	18.3 ±1.32	9.8 ± 0.28	31.5 ± 0.78	0.38 ± 0.18	70
Pyri.	20	1000	–	–	–	–	0
Pyri.	15	0.01c2	18.1 ± 0.60	9.7 ± 0.21	31.6 ± 0.70	0.81 ± 0.21	80
Pyri.	15	1.0c2	18.1 ± 0.16	10.0 ± 0.15	33.6 ± 0.49	0.83 ± 0.08	93
Keto.	18	0.01	19.5 ± 0.54	9.6 ± 0.30	32.4 ± 0.51	0.81 ± 0.20	94
Keto.	20	0.1	20.0 ± 0.34	10.5 ± 0.78	30.8 ± 0.87	0.81 ± 0.06	95
Keto.	20	1.0	20.3 ± 1.27	9.9 ± 0.54	32.7 ± 0.70	0.81 ± 0.06	80
Keto.	20	10	20.5 ± 0.95	9.8 ± 0.11	32.2 ± 0.87	0.88 ± 0.00	90
Precocene	20	5.0	18.0 ± 0.37	10.8 ± 1.17	30.5 ± 0.56	0.78 ± 0.22	90
Prec. (9 days)	20		18.0 ± 0.52	10.2 ± 0.66	32.1 ± 0.92	0.83 ± 0.11	85
Prec. & keto.	20		19.2 ± 1.14	9.5 ± 0.14	31.1 ± 0.84	0.53 ± 0.13	80
Prec. & W-328	15		18.8 ± 0.49	9.7 ± 0.17	30.2 ± 1.58	0.88 ± 0.03	100
Prec. & Pyri.	20		20.3 ± 0.73	9.8 ± 0.24	30.8 ± 0.73	0.89 ± 0.11	100
Acetone	19		18.1 ± 0.30	10 ± 0.21	32.3 ± 0.49	0.92 ± 0.03	100

Table 6.2: Reproductive performance of the Rusinga population of *G. f. fuscipes*, measured over 45 days following topical treatment on day three or four of adult life with pyriproxifen in 1µl of acetone.

Dose (µg)	n	(Mean ± S.E.M) Fecundity	Puparial wt. (mg)	Survival (%)
1.0	15	0.63 ± 0.07	22.7± 0.89	100
2.0	20	0.33 ± 0.13	20.9 ± 0.94	75
5.0	13	0.31 ± 0.10	21.4 ± 0.87	69.2
10.0	12	0.38 ± 0.10	23.5 ± 0.92	83.3
50	22	0.38 ± 0.09	24.5 ± 1.19	63.6
100	16	0.17 ± 0.03	22.7 ± 0.85	44
Acetone	14	0.48 ± 0.06	22.6 ± 0.66	78.6

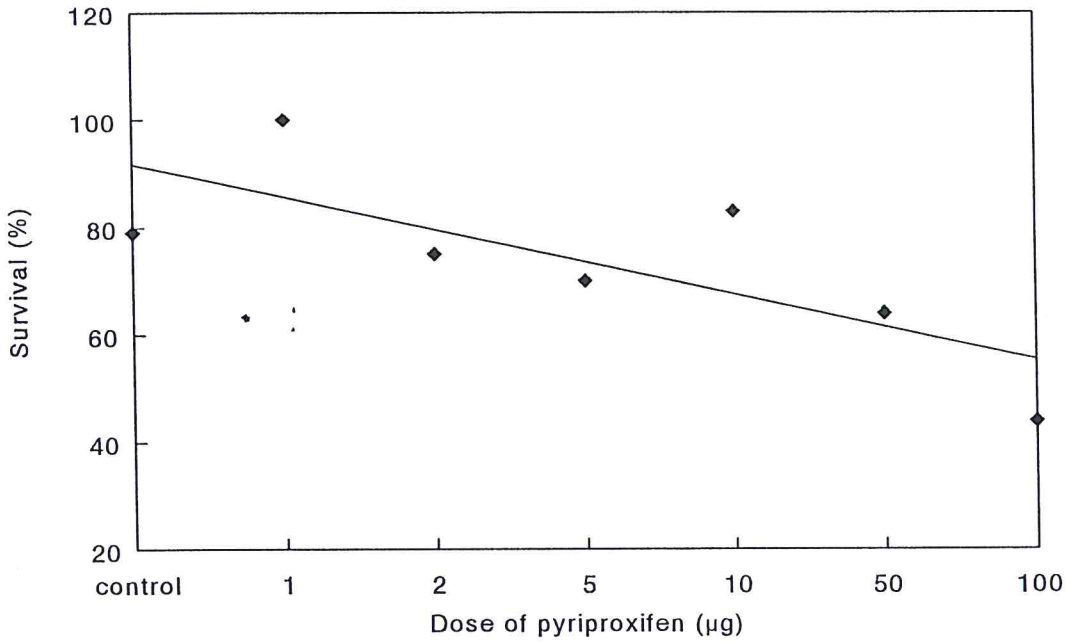


Fig. 6.1: Effect of dose of pyriproxifen (µg/µl) on survival when applied on female *G. f. fuscipes* (Rusinga population) on day three post-emergence.

6.4.1.3 Precocene

Similar observations were made on fecundity when the females were treated with precocene, but emergence rate was 19% (n = 58) in puparia from females treated with precocene once and 16.7% (n= 48) in those produced by females treated with precocene every nine days (Table 6.3). The emergents were only from the second, third, and fourth reproductive cycles, with none of the puparia produced in the first cycle emerging.

6.4.1.4 Ketoconazole

The females larviposited normal larvae, but at a dose of 0.1 µg, 6.6% (n= 61) of the larvae did not pupariate, but remained soft and tanned black. There was 10% (n= 58) emergence at a dose of 0.01 µg, and 4.9% (n= 61) emergence at a dose of 0.1 µg (Table 6.3).

6.4.1.5 Precocene and Ketoconazole

Females given this treatment larviposited, the larvae pupariated and at the end of the developmental period, 18.9% (n = 37) of the adults emerged. Upon dissection of puparia of non-emerged adults, 5.4% were found to be normally formed but failed to emerge.

6.4.1.6 Precocene and Pyriproxifen

Females treated with precocene and JH analogue larviposited normally. Only 2.8% (n= 71) of the adults emerged (Table 6.3).

6.4.1.7 Precocene and W-328

The females in this experiment also larviposited, but no adults eclosed (Table 6.3).

Table 6.3: Effect of IGRs on emergence rates in *G. f. fuscipes* following topical application on females which were later mated.

IGR	Dose	n	Emergence (%)
Pyriproxifen	0.001	47	4.3
Pyriproxifen	0.01	80	0
Pyriproxifen	0.02	68	0
Pyriproxifen	0.05	55	0
Pyriproxifen	0.5	64	0
Pyriproxifen	1.0	68	1.5
Pyriproxifen	2.0	54	0
Pyriproxifen	5.0	63	0
Pyriproxifen	100	28	0
W-328	0.001	64	1.6
W-328	0.01	75	2.6
W-328	0.1	84	0
W-328	1.0	66	0
W-328	10	36	2.8
W-328	100	56	0
W-328	1000	58	0
Ketoconazole	0.01	58	10
Ketoconazole	0.1	61	4.9
Ketoconazole	1.0	48	0
Ketoconazole	10	68	0
Precocene once	5	58	19
Precocene every nine days	5	48	16.7
Precocene & ketoconazole	_	37	18.9
Precocene & pyriproxifen	_	71	2.8
Precocene & W-328	_	68	0
Acetone	_	66	86.4

6.4.2. Effect of Pyriproxifen and W-328 on Males

Application of pyriproxifen and W-328 on males that were then mated to females resulted in production of larvae which pupariated normally. Fecundity of the females was high, puparial weights heavy and independent of dosage (Table 6.4). Female survival was not

severely affected and varied from 67-100%. However, adults failed to eclose from the puparia.

6.4.3 Morphogenetic Effects of the Insect Growth Regulators

6.4.3.1 Pyriproxifen

In the puparia formed from larvae deposited by pyriproxifen-treated females and those produced when treated males were mated to normal females, development was arrested at the reddish-brown eye pigmentation and pigmentation of bristles stage. The most common morphogenetic effect was failure of tergite formation (Table 6.5), adult abdomens having flabby, transparent and moist integuments. The abdomen, proboscis and mid-dorsum of the thorax were unpigmented (Plates 6.1a-c). Also, the adults had abdominal hairs pointing in various directions instead of posteriorly. One out of the 162 puparia had an adult that appeared morphologically normal, but failed to emerge. There were a few instances where the eyes were unevenly pigmented, with sections or whole of an eye having yellow pigments (Plate 6.2). Some of the offspring (4.2%, n= 162) died at the larval stage (Score 9, Table 6.5).

6.4.3.2 W-328

As in the case of pyriproxifen treatment, there were high percentages of adults in which tergites did not form (Table 6.5). The abdomen, proboscis and thorax were unpigmented and the abdominal integuments remained as soft, transparent and flabby structures. Growth of microchaetae was also affected whereby tergal areas were covered with sparsely distributed hairs. Microchaetae in the mid-dorsum of the thorax were sparse and short compared to control flies. Treatment of females with same dose of the analogue produced varied degrees of tergite formation in the same reproductive cycle (Plates 6.1a-f). In some instances, adults removed from puparia showed red eye pigmentation (Plate 6.3).

Table 6.4: Fertility of males measured over 45 days, following topical treatment with pyriproxifen and W-328, as assessed from fecundity of females with which they mated.

Compound	Dose (µg)	n	(Mean ± S.E.M) Fecundity	Puparial wt. (mg)	Survival (%)
Pyriproxifen	1.0	30	0.77 ± 0.05	33.1± 0.87	67
Pyriproxifen	10.0	30	0.77 ± 0.09	32.6 ± 0.65	70
Pyriproxifen	100	30	0.68 ± 0.08	30.2 ± 0.43	70
W-328	0.1	20	0.87 ± 0.05	31.5 ± 0.71	100
W-328	1.0	20	0.85 ± 0.06	30.4 ± 0.70	100
W-328	10	20	0.85 ± 0.08	31.0 ± 0.56	95
Acetone	-	19	0.92 ± 0.03	32.3 ± 0.49	100

On the other hand, adults from puparia formed from larvae deposited by control females were developed with reddish-brown eyes, and the whole body pigmented. They had well-developed tergites with abdominal hairs pointing posteriorly and dry integuments, which were closely adhered to the puparial wall (Plate 6.4). The frequency of adults without tergites (scores 7 & 8) were similar at doses of 0.001 and 0.01 µg but increased significantly (χ^2 = 13.6, p= 0.001, n= 82) from 0.1 µg, except at a dose of 10 µg where fewer adults were affected. All tergites formed in 27% (n= 30) and 15% (n= 41) of adults treated with 0.001 and 0.01 µg W-328 respectively, but microchaetae were sparse. Tergites also developed in 18% of

adults treated with 10 µg W-328. The frequency of adults in which tergites failed to form due to W-328 treatment were more ($\chi^2 = 8.7$, p= 0.003, n=360) than those due to pyriproxifen.

Table 6.5: Effect of different doses of IGRs on development in *G. f. fuscipes* following topical treatment of females. (Pyri= pyriproxifen, keto.= ketoconazole, preco= precocene).

IGR	Dose (µg)	n	Scores									
			0	1	2	3	4	5	6	7	8	9
Pyri.	0.001	43	4	0	0	0	2	28	26	33	2	5
Pyri.	0.01	41	0	0	0	0	0	0	7	85	0	7
Pyri.	0.02	16	0	0	0	0	6	19	19	50	0	6
Pyri.	0.05	10	0	0	0	0	0	10	20	50	0	20
Pyri.	0.5	15	0	0	0	0	0	0	33	67	0	0
Pyri.	1.0	27	4	0	0	0	0	11	33	44	0	7
Pyri.	2.0	29	0	0	0	0	0	7	31	41	0	10
Pyri.	5.0	28	0	0	0	0	0	4	36	54	4	7
Pyri.	10	18	0	0	0	0	0	0	22	56	0	22
Pyri.	100	15	0	0	0	0	0	0	13	66	0	20
W-328	0.001	30	0	0	0	0	0	27	20	43	0	10
W-328	0.01	41	2	0	0	0	0	15	29	44	0	12
W-328	0.1	52	0	0	0	0	0	0	14	83	0	4
W-328	1.0	26	0	0	0	0	0	0	4	92	0	4
W-328	10	22	5	0	0	0	0	18	5	64	5	9
W-328	100	10	0	0	0	0	0	0	0	90	0	10
W-328	1000	31	0	0	0	0	0	0	0	90	3	7
Keto.	0.01	52	10	0	0	0	0	23	15	46	0	4
Keto.	0.1	41	5	2	0	0	7	22	22	34	0	7
Keto.	1.0	35	0	0	0	0	0	11	6	77	3	3
Keto.	10	62	0	0	0	0	0	11	13	73	0	3
Preco.	5	58	19	0	0	0	10	16	10	40	0	5
Preco. every 9 days		48	17	0	0	0	6	2	6	69	0	0
Preco. & keto		29	19	0	0	0	7	10	21	21	0	17
Preco. & pyri.		35	3	0	0	0	0	20	26	49	0	3
Preco. & W-328		33	0	0	0	0	0	0	46	55	0	0
Acetone		66	86	3	0	0	11	0	0	0	0	0

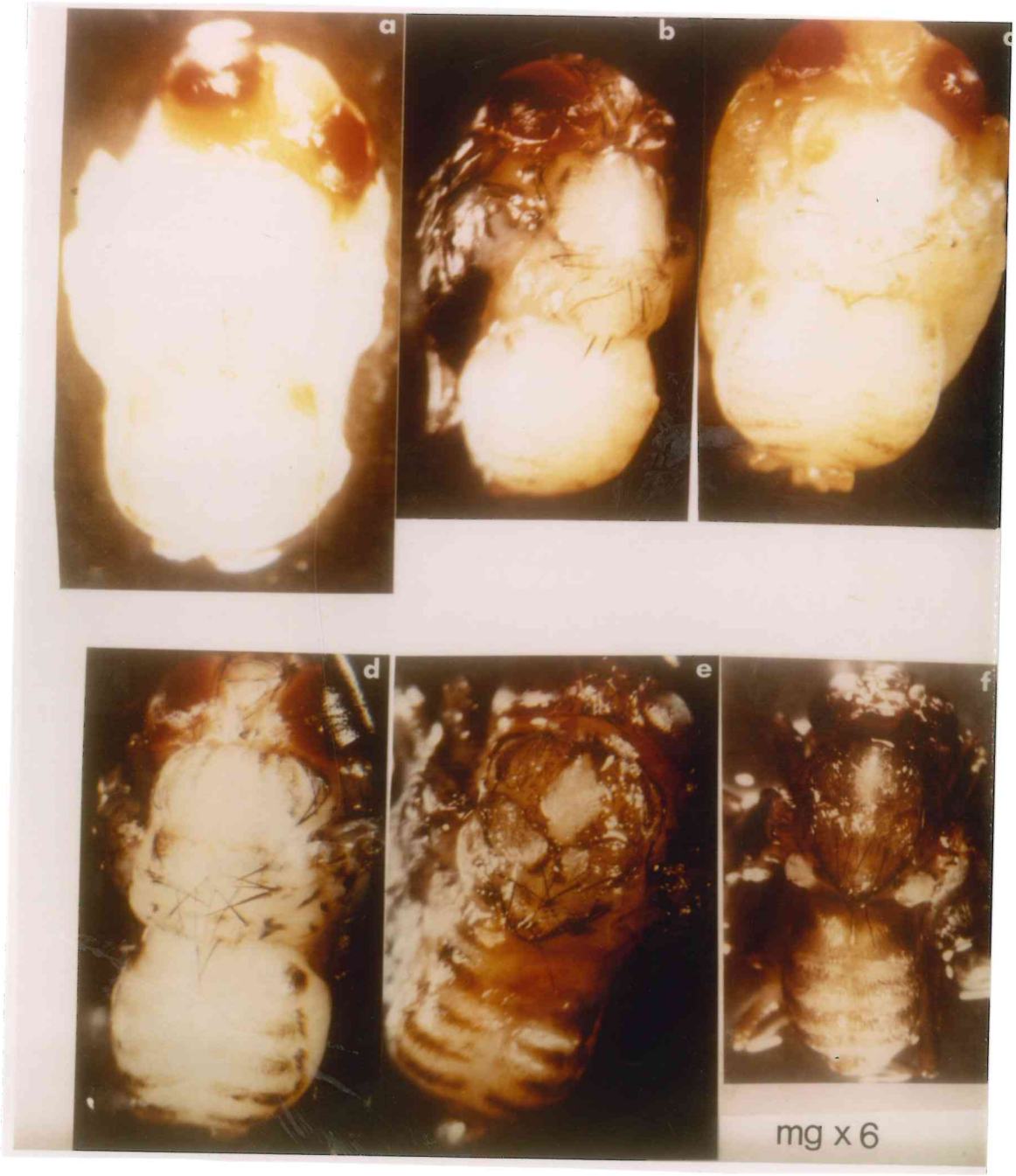


Plate 6.1: Morphogenetic effects of IGRs on adult development in *G. f. fuscipes*.

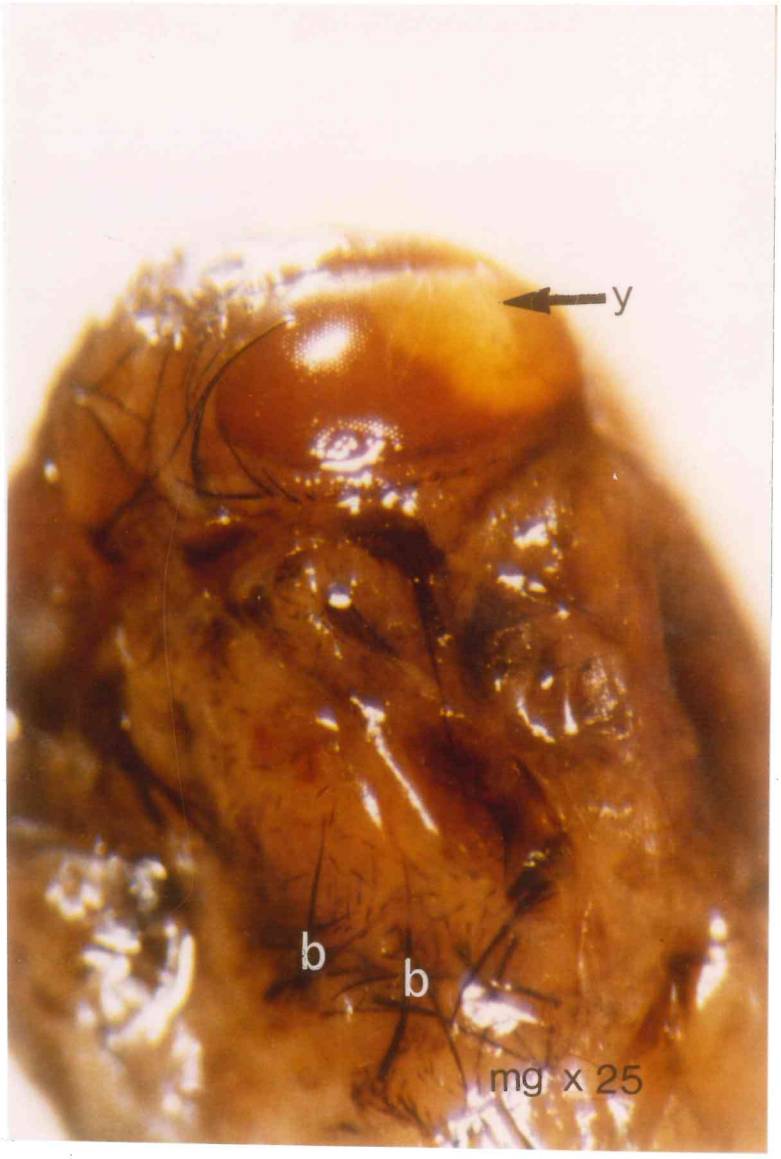


Plate 6.2: Incompletely developed adult with one eye pigmented yellow (y). b, bristles.

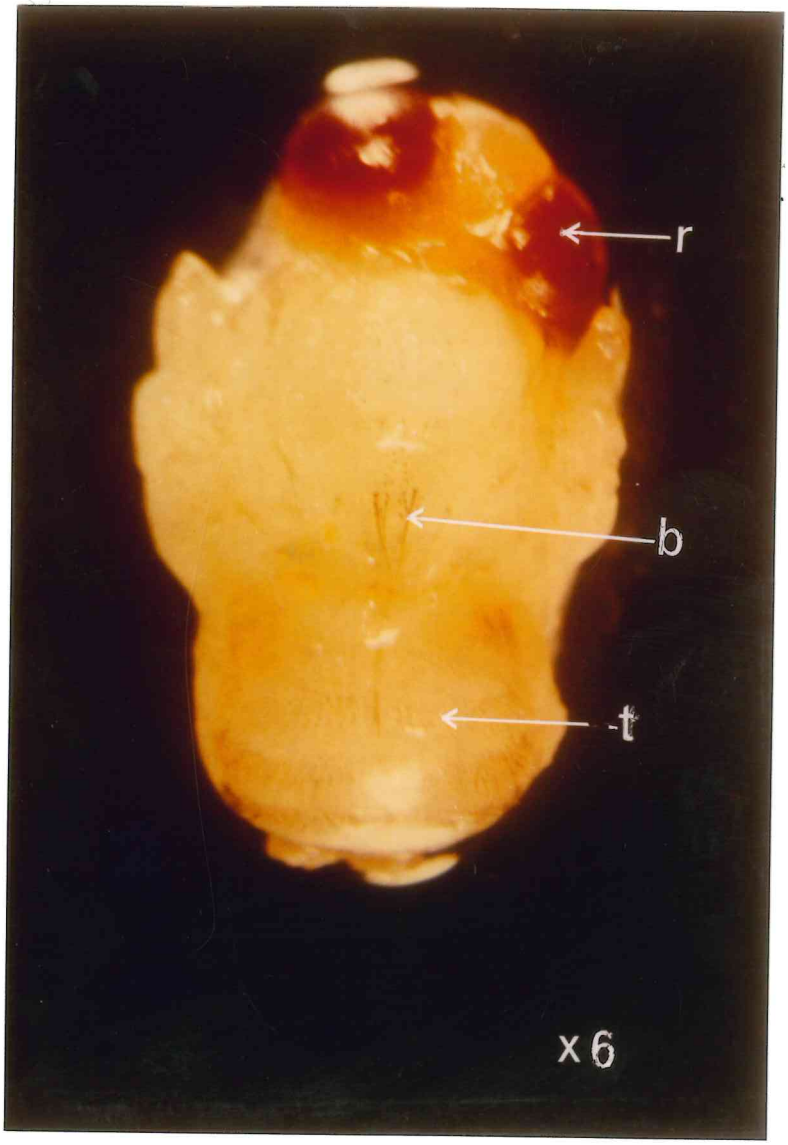


Plate 6.3: Incompletely developed adult with eyes pigmented red (r). Pigmentation of bristles (b) and microchetae on tergal areas (t) was affected in this adult by treatment with IGRs (compare with Plate 6.2).

Plate 6.4: Normally developed adult from control experiments. t, tergite. One wing was removed to expose all abdominal tergites.

6.4.3.3 Precocene

Precocene interfered with tergite development in 40% of the offspring where females were treated with precocene once and in 69% where females were treated with precocene every nine days. There were varying degrees of tergite development (Table 6.5) and only adults produced in the first reproductive cycle lacked pigmentation of the abdominal cuticle.

6.4.3.4 Precocene and Pyriproxifen

This treatment affected the adults to varying degrees. It was found that 49% (n=35) of the adults had no abdominal tergites, 26% had a few tergites, while 20% had all the tergites formed as narrow bands, with sparsely distributed microchaetae on the tergites, but not on the intersegmental membranes (Plate 6.1e). The effects of the treatments were more pronounced in the first reproductive cycle than in later cycles where development of tergites and microchaetae improved.

6.4.3.5 Precocene and W-328

Here, 55% (n=33) of the adults had no abdominal tergites, whereas 46% had from one to two posterior tergites formed.

6.4.3.6 Ketoconazole

This compound also affected the development of the adults in the puparium. As in the other cases, tergite development was most affected, with 58% (n=190) of adults having no tergites, but only sparsely distributed microchaetae. The proportion of adults with undeveloped tergites increased from 1 μg (Table 6.5). Eyes were either pigmented orange or

reddish-brown, while the abdomens were unpigmented. Bristle sockets on the head and thorax appeared enlarged compared to controls.

6.4.3.7 Precocene and Ketoconazole

With this treatment, 21% (n= 29) of the adults had no tergites in the abdominal area and a similar percentage had just a few tergites formed. Some 7% of the adults had all tergites formed, but did not eclose.

In all the treatments, non-emerged adults from different females treated with a similar dose of IGR exhibited varied degrees of tergite formation. Histological sections taken in the abdominal area showed that the adult epidermis was thin, with groups of histoblasts aggregated in some areas of the epidermis (Plate 6.5), which lacked cuticle. Microchaetae were undeveloped compared to controls (Plate 6.6).

6.4.4 Effects of Pyriproxifen and W-328 when Applied to Puparia

6.4.4.1 Effects on Eclosion

In the C.A.R population of *G. f. fuscipes*, the effect of pyriproxifen on emergence rates was age-dependent. Age had a significant effect on emergence rates, with emergence from puparia treated before five days post-larviposition being lower ($\chi^2 = 16.7$, p= 0.0008, n= 121) than in older ones. Generally, emergence was inhibited in puparia aged between one to four days post larviposition, with the exception of one emergent in four day-old puparia at each of the doses of 10 µg and 1000 µg (Table 6.6). Eclosion was suppressed to 26% (n= 65) in five day-old puparia. However, 53 % (N = 17) of these emergents were deformed (Fig. 6.2). There were high rates of emergence in older pupae, ranging from 53-93% (Figure 6.3), although a small proportion of these were also deformed. Dose of pyriproxifen had no

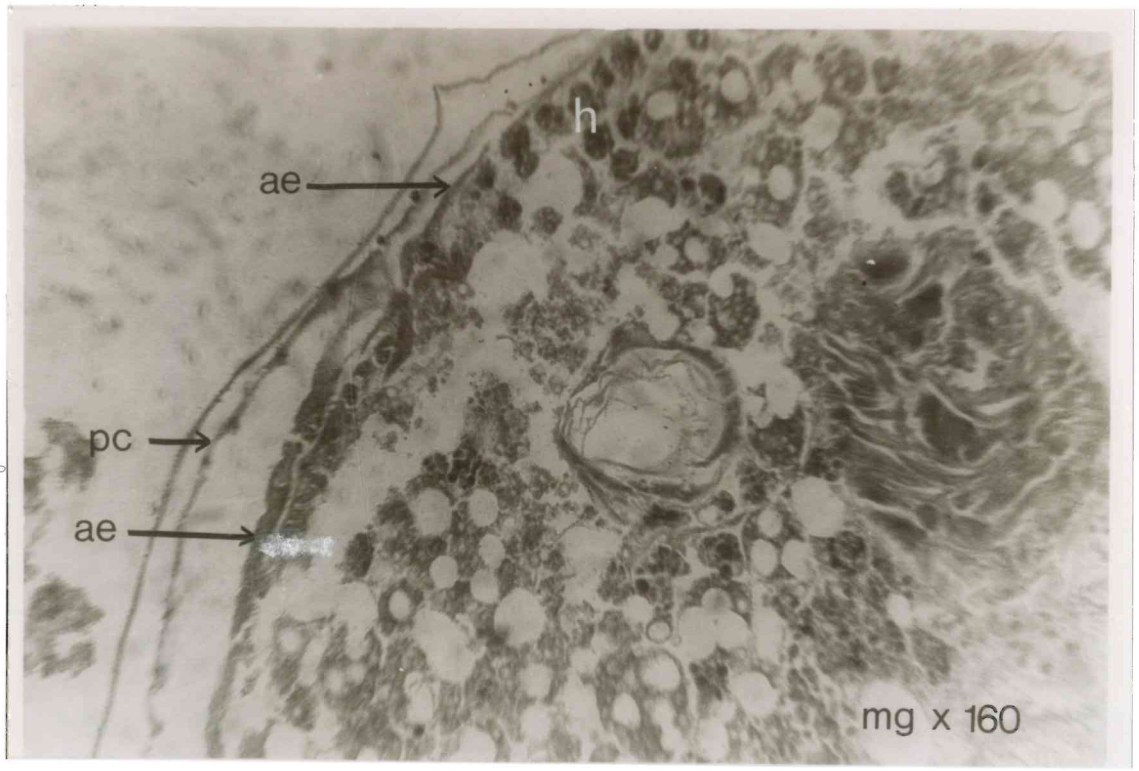


Plate 6.5: Histological cross section taken in the region of the abdomen of adult *G. f. fuscipes* that failed to emerge following treatment of females or young puparia with IGRs. Note the underdeveloped epidermis (ae), lacking cuticle or microchetae. The epidermal cells (h) are undifferentiated. ae, adult epidermis; pc, pupal cuticle; ac, adult cuticle

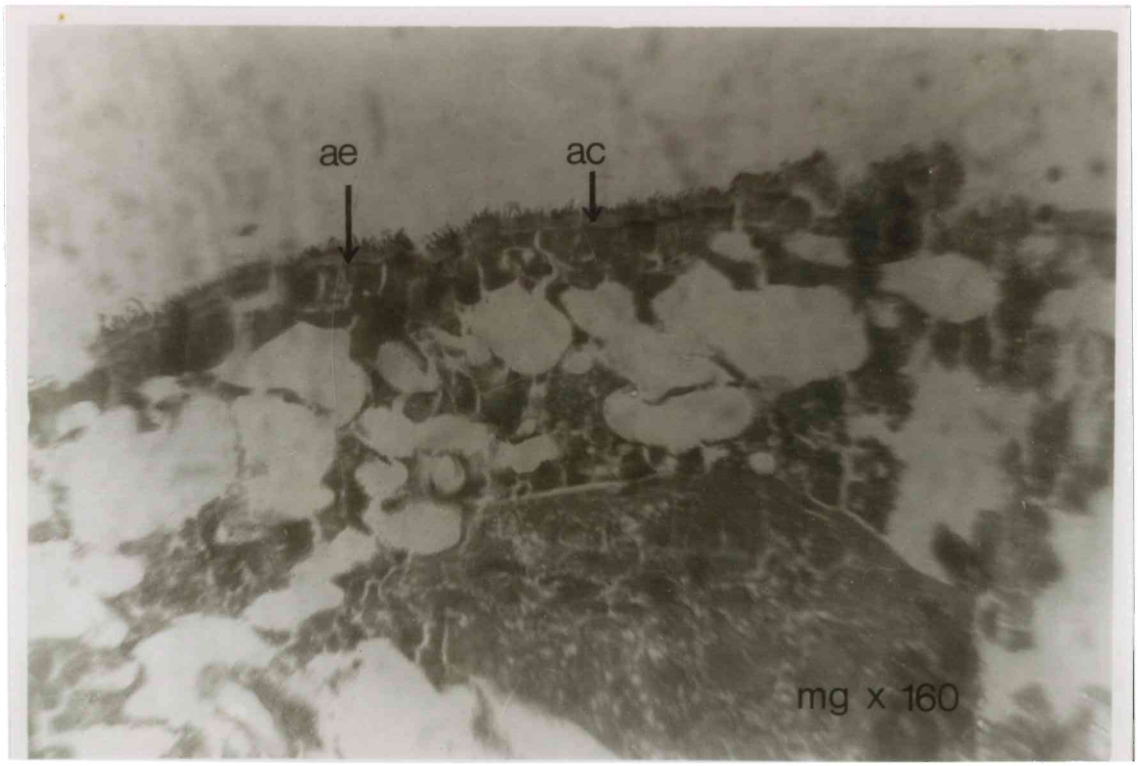


Plate 6.6: Histological cross section taken in the region of the abdomen of adult *G. f. fuscipes* from control experiments. The epidermis (ae) was developed with cuticle (cu) that had microchetae.

significant effect on emergence rates. A similar trend in emergence rates occurred in treatments with W-328 (Fig. 6.4), with puparia treated at one to four days failing to emerge. The emergence rate in puparia treated at five days was 22.7% (n= 59) while the overall emergence rate was 50.9% (n= 478). This was lower ($\chi^2 = 14.4$, p= 0.001, n= 753) than the rate in pyriproxifen-treated puparia.

Similar effects were observed in the Rusinga population of *G. f. fuscipes* (Fig. 6.5), although in 10 day-old puparia, doses of 10 and 100 µg inhibited eclosion in 50% (n= 20) of the puparia.

6.4.4.2 Morphological Effects of Pyriproxifen on Adults that Emerged

Of all the adults that emerged (65%, N=275), 25.7% (N=179) were abnormal. The least and most common morphological effect of pyriproxifen on emerged adults was a reduction in size of either the pre-abdomen, post-abdomen or the whole abdomen. This reduction involved the width, or both width and length of the abdomen, due to lack of expansion of the abdominal cuticle. These abnormalities affected both males and females. In two cases (n= 179), females had the post-abdomen reduced in width, they fed successfully, but blood only collected in the pre-abdomen. The flies eventually died in the same state, probably due to inability to digest the blood meal successfully. In another case where a female abdomen was reduced in length, the female took a blood meal but could not mate successfully, for blood started oozing from the genital area when the male inserted its aedeagus resulting in death. Wing crumpling, or crumpled and criss-crossed wings also affected the emerged flies and in some of the males that emerged, the aedeagus was either straight or bent sideways, rendering them incapable of successful copulation. With W-328, the most common effect was lack of expansion of the abdomen while 0.8% (n= 243) of the flies that emerged had orange-pigmented abdomens.

Table 6.6: Effect of age of puparia on the frequency of morphological effects produced by the topical application of pyriproxifen. Effects 0-3 represent emerged flies.

		Scores									
Age post-larviposition	n	0	1	2	3	4	5	6	7	8	9
1	35	0	0	0	0	0	0	29	66	3	3
2	40	0	0	0	0	0	10	45	50	0	5
4	73	3	0	0	0	3	16	21	60	0	0
5	62	11	5	0	13	52	13	7	0	0	0
7	55	58	6	4	11	16	4	2	0	0	0
8	71	59	11	0	10	7	3	0	0	0	0
9	20	70	5	5	10	10	0	0	0	0	0
10	71	75	6	1	3	13	3	0	0	0	0
Control	30	80	7	0	0	13	0	0	0	0	0

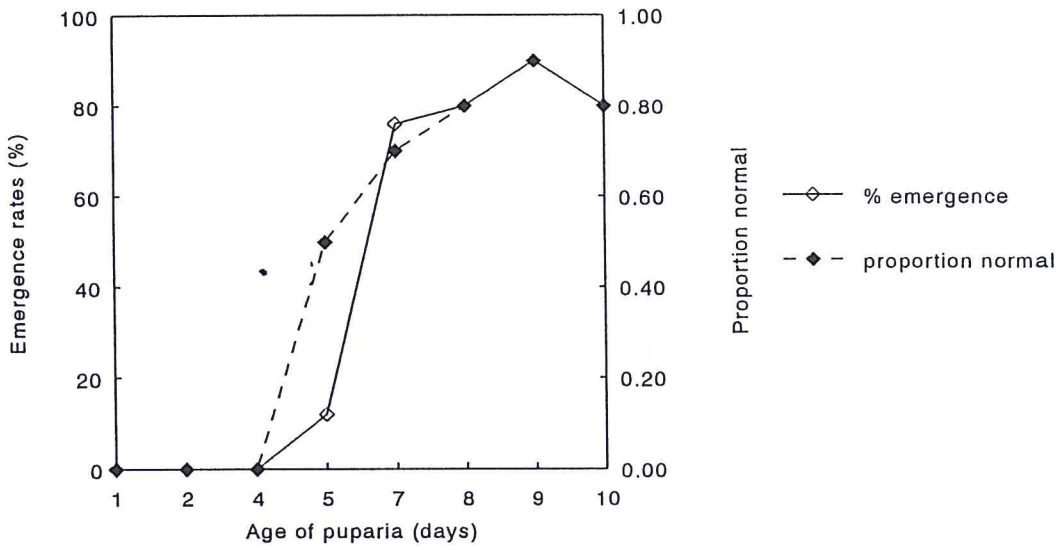


Fig. 6.2: Effects on emergence rates and proportion of normal adults of topical application of pyriproxifen in acetone on puparia of different ages.

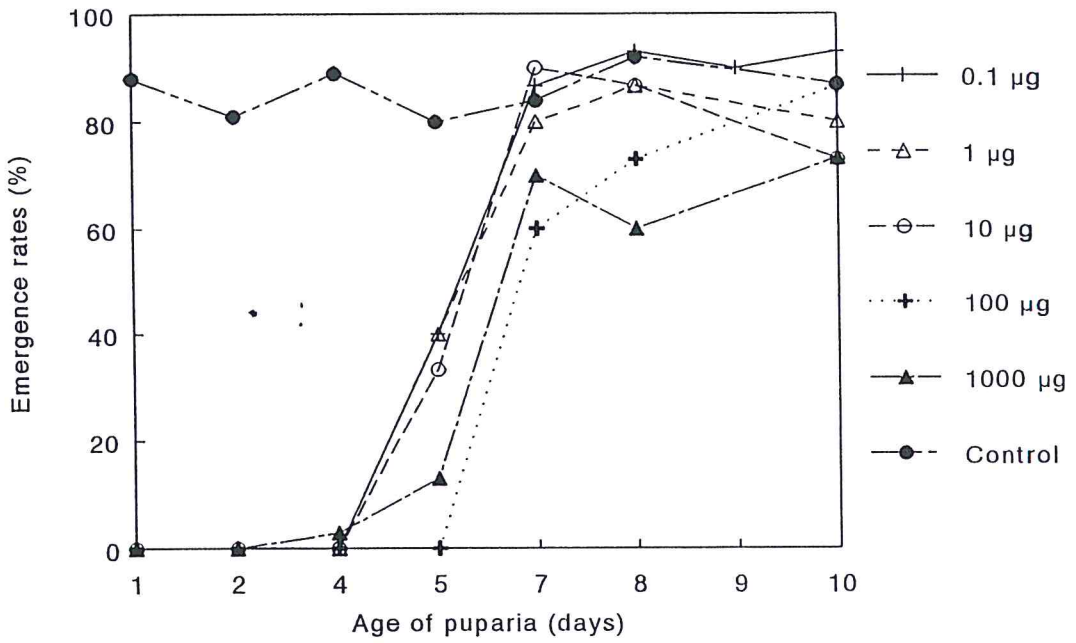


Fig. 6.3: Effect on emergence rates of various doses of pyriproxifen topically applied on puparia of different ages from the C.A.R population of *G. f. fuscipes*.

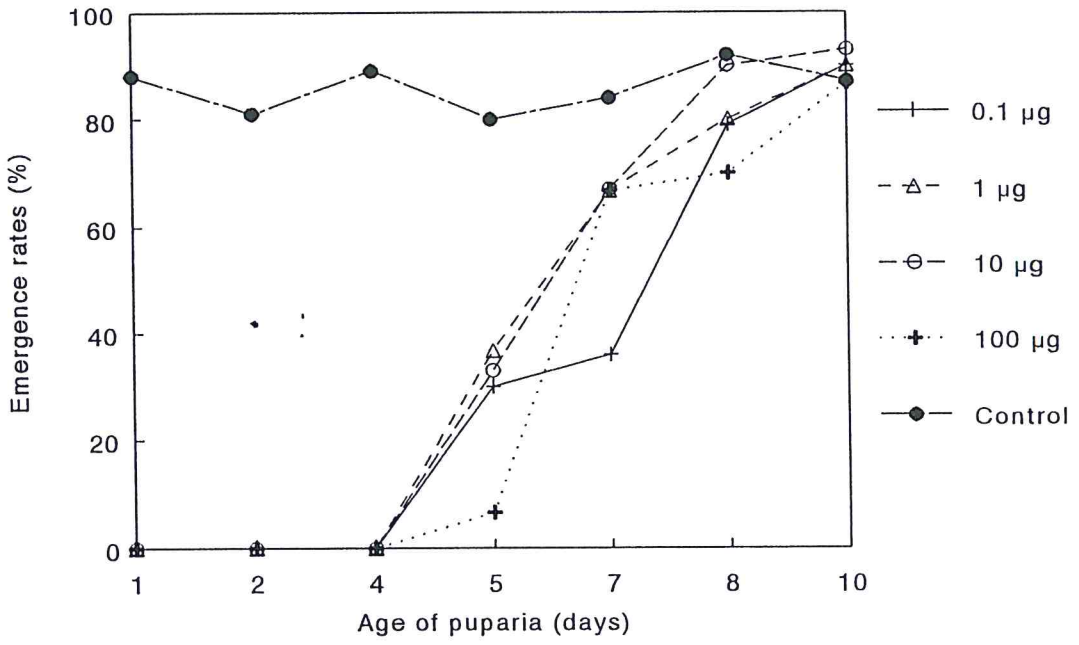


Fig. 6.4: Effect on emergence rates of various doses of W-328 topically applied on puparia of different ages from the C.A.R population of *G. f. fuscipes*..

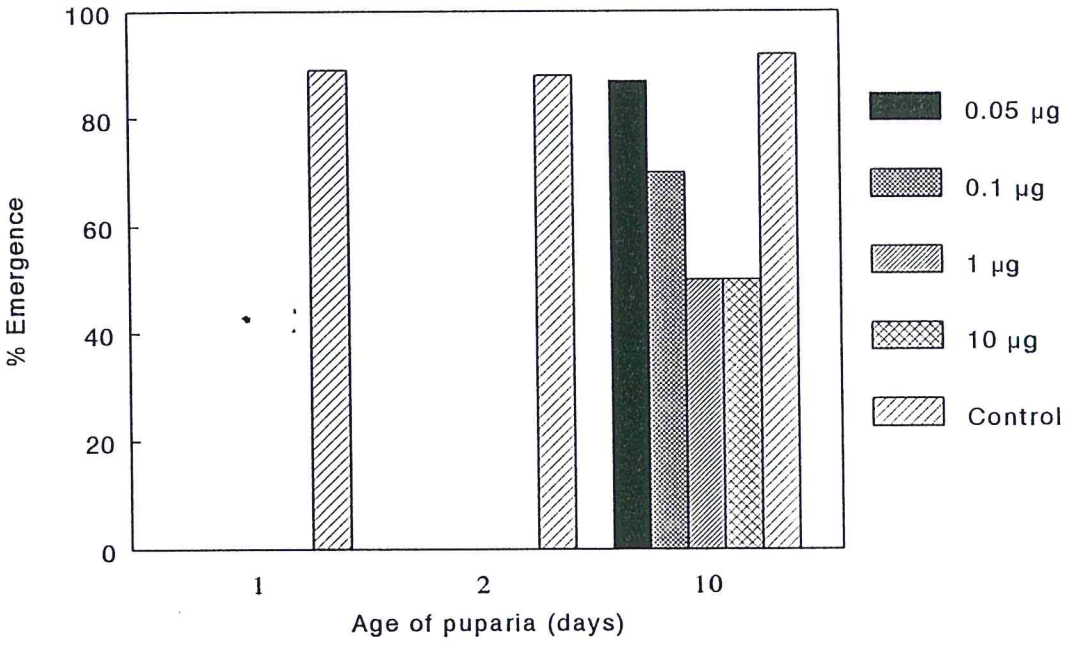


Fig. 6.5: Effect on emergence rates of various doses of pyriproxifen topically applied on puparia of different ages from the Rusinga Island population of *G. f. fuscipes*.

6.4.4.3 Morphological Effects of Pyriproxifen on Adults that Failed to Emerge

Although there were high emergence rates in the older pupae, dissections of the puparia that failed to eclose showed that eye pigmentation was not uniform in some of them. In one case, part of the femur was not pigmented. However, the abdominal cuticle was sclerotized. Where there was complete inhibition of eclosion as in the young puparia, the effects were similar to the ones already described (section 6.4.3) (Table 6.5). The pharate adults formed had unsclerotized abdomens, with reduced hairs or no hair at all on the abdomen compared to a control group. The abdomen, proboscis and mid dorsum of the mesothorax was also non-pigmented. The abdomen was also moist and flabby, showing failure of reabsorption of moulting fluid. Similar observations were made with W-328.

6.4.5 Effect of Applying Pyriproxifen or W-328 Directly on Pupae or Pharate Adults

No emergence occurred in the puparia treated with the analogues at between one to 10 days post-larviposition. On dissection of the puparia aged five days, only a white or yellowish mass was found sticking to the inner wall of the puparium. In some, the outline of adult appendages were barely recognizable while in others, development was arrested at stage II or III (Appendix 2). In pupae treated at 10 days adult development proceeded, but emergence was inhibited. The adults showed similar effects to those produced when the analogue is applied on mated females (section 6.4.1). The eyes also had a red coloration (Plate 6.3), instead of the reddish-brown pigmentation observed in the other cases, and abdomens were sclerotized in others. Varying levels of emergence occurred when 20 day-old puparia were treated (Fig. 6.6).

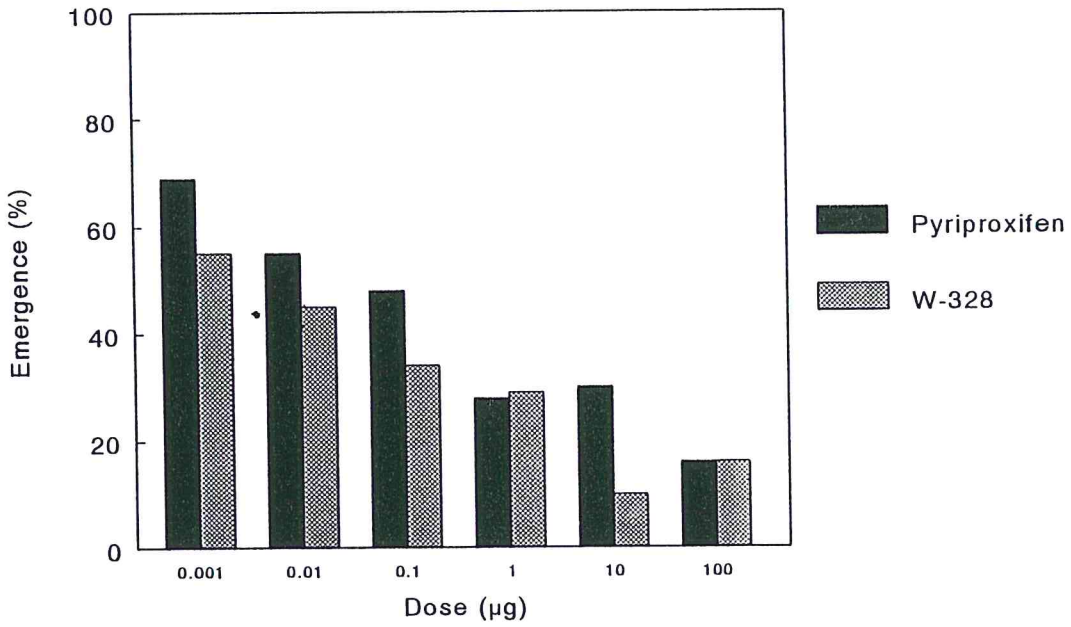


Fig. 6.6: Effect on emergence rates of various doses of pyriproxifen and W-328 directly applied on the C.A.R population of *G. f. fuscipes* pharate adults aged 20 days.

6.4.6 The Critical Stage of Metamorphosis

The critical stage in development when JH analogues are applied to puparia was found to be five days. However, this stage extended to 20 days when the analogue was directly applied to the pupa or pharate adult.

6.4.7 Effects of Pyriproxifen on the Fecundity of Females Emerging from Treated Puparia

Females emerged from treated pupae mated and larviposited (Table 6.7).

Table 6.7: Fecundity of females following treatment of puparia with pyriproxifen. Figures in parentheses are sample sizes (n).

Age of puparia (days)	Dose of pyriproxifen (μg)			
	Control	0.1	1.0	10
7	0.9 (6)	1.0 (4)	0.3 (2)	1.0 (6)
8	0.9 (6)	1.0 (3)	0.5 (3)	0.5 (3)
10	1.0 (6)	0.5 (4)	0.3 (5)	0.8 (4)

Fecundity of the females was independent of dosage and age at which puparia were treated. Post-mating mortality was high at doses of 100 and 1000 μg.

6.4.8 Effects of Pyriproxifen on Fertility of Males Emerging from Treated Puparia

Males that emerged from the treated pupae also mated successfully, and were fertile, as indicated by the fecundity of females with which they mated (Table 6.8).

Table 6.8: Fertility of males following treatment of puparia with pyriproxifen. Figures in parentheses represent sample sizes (n)

Age of puparia		Dose of pyriproxifen (μg)			
(days)	Control	0.1	1.0	10	100
7	0.9 (4)	0.3 (3)	0.3 (3)	0.75 (3)	0.3 (3)
8	—	1.0 (4)	0.4 (3)	0.75 (3)	0.0 (3)
10	—	0.4 (4)	0.4 (3)	—	0.5 (4)

Fecundity in the control experiments was higher than in both cases where male and female flies emerged from treated pupae.

Also, fecundity of adults that emerged from puparia treated at five days post-larviposition is not given, because males and females that emerged were weak and died before mating or larviposition. Generally, fecundity of the females that emerged from treated puparia was low compared to the controls. However, there was no relationship between age at which puparia were treated, or dose of pyriproxifen and fecundity.

6.5 Discussion

6.5.1 Effects of JH Analogues on Reproduction and Metamorphosis

Females treated with the JH analogues, pyriproxifen and W-328, larviposited normally and the larvae formed were able to pupariate (Tables 6.1 & 6.2). However, emergence was inhibited in most of the puparia (Table 6.3). Similarly, males that were treated with the analogues mated and inseminated untreated females which larviposited (Table 6.4), indicating that the analogues do not interfere with inseminating ability of males. However, adults failed to emerge from puparia formed from larvae deposited by the females. It is therefore clear that the analogues do not interfere with reproductive processes or pupariation. This supports work by Langley and Pimley (1986) and Langley *et al.* (1988) on *G. m. morsitans*. Tsetse flies reproduce through adenotrophic viviparity, in which a larva is nurtured in the uterus through secretion from the milk glands (Langley, 1977). The gland receives lipids through haemolymph (Moloo, 1976; Langley & Bursell, 1980; Langley *et al.*, 1981) and the JH analogues absorbed through the abdominal wall are most probably transferred through haemolymph to the milk gland. Thus as the developing larva receives its nourishment from its mother, it also receives doses of the analogues. These analogues apparently have no effects on vitellogenesis or pupariation. This is in agreement with observations on *D. melanogaster* where continuous exposure of larvae to JHAs has little effect on puparium formation (Riddiford & Ashburner, 1991). The reduced survival following treatment of Rusinga Island females with 100 and 1000 µg (Fig. 6.1) could be due to toxicity of the high doses applied.

The incompletely developed adults showed varying degrees of inhibition of tergite development (Table 6.5, Plate 6.1) and the integuments were soft, flabby and moist. Microchaetae were sparsely developed on the abdomen, which was non-pigmented (Plate 6.1a-d. During metamorphosis, the cells that make pupal epidermis on the abdomen are replaced by abdominal histoblasts (Bhaskaran, 1972; Madhavan & Schneiderman, 1977).

Inhibition of tergite development implies that the analogues interfered with differentiation of abdominal histoblasts, the cells that go into formation of the adult epidermis (Roseland & Schneiderman, 1979). The occurrence of moist integuments implies that the analogues interfered with resorption of moulting fluid, which is undertaken by the epidermis after its formation (Richards & Davies, 1977). However, since the epidermis failed to form normally, moulting fluid could not be resorbed. Similarly, pigmentation of abdominal integuments may have failed due to underdevelopment of the epidermis, which is responsible for pigmentation (Riddiford, 1994). The flaccid integuments may have formed due to inability of the abdominal histoblasts to differentiate to epidermal cells, which make adult cuticle (Riddiford, 1994). The untimely presence of juvenile hormones or its analogues suppresses the synthesis of adult cuticular proteins (Gnatzy & Romer, 1984), which normally interact with chitin to produce the adult cuticle (Riddiford, 1994).

Certain steps in the developmental process were inhibited while others proceeded uninterrupted. For instance pupariation and pigmentation of bristles went on unabated (Tables 6.1, 6.2, Plates 6.1d-f, 6.2). Larvae that were produced by treated females were able to go through all the larval moults, transform into a pupa, then to adult in the presence of the JH analogues. This points to the selective action of JH analogues. It shows that the analogues do not interfere with larval tissues or moults, but only affects the expression of certain adult features. Also once commitment to metamorphosis takes place, JH or JH analogues do not interfere with the process.

In some of the adults, adult cuticles formed and were pigmented while in others cuticles were non-pigmented (Plate 6.1). A possible explanation for this variation is that individual flies absorb the analogues at different rates, so that some larvae receive higher doses than others during transfer of the analogues to the eggs or developing larvae (Moloo, 1976; Langley & Bursell, 1980; Langley *et al.*, 1981). It is also worthy to note that pupae of

each day's collection were taken to be a day old, but they were deposited at different times in the day, and therefore were at varying stages of development at the time of JH analogue application in experiments where puparia were treated. This probably contributed to the differences in response to the JHA. This variation is not expected to be great, however, since most larvipositions occur in the mid-afternoon (Zdarek *et al.*, 1992).

Failure of pigmentation of adult cuticle also occurs in *Manduca sexta,* where if JH is absent in the larva at the time of head capsule slippage, the epidermis deposits premelanin granules in the new cuticle. These granules contain prophenoloxidase that is activated later to cause melanization just before ecdysis (Curtis *et al.*, 1984). The synthesis of melanin requires the presence of enzyme dopa decarboxylase (DDC), synthesis of both enzymes being under the control of ecdysteroids (Riddiford, 1994). In the presence of JH, this action of ecdysteroid is interfered with, causing low synthesis of DDC. In *Oncopeltus* and *Rhodnius*, absence of JH is necessary for pigmentation of the adult cuticle to occur (Smith & Nijhout, 1981; Nijhout, 1983). In the absence of JH, ecdysteroids act to initiate metamorphic changes that result in an adult moult. However, in the presence of JH at such critical stages, the insect undergoes moults but the same juvenile genes are expressed in the new moult (Riddiford, 1994). In the present study, presence of the analogues had influence on pigmentation of the integument, not moults (Plate 6.1).

During normal adult development, head and thoracic structures differentiate in advance of the abdominal ones (Slama, 1971; Srivastava & Gilbert, 1969) with the result that JHA effects on head and thoracic structures are less pronounced, compared to abdominal structures. The fact that incompletely formed adults arising from treated females had head and thoracic structures formed (Plates 6.1-6.3) suggests that these are genetically determined and are insensitive to the disruptive effects JHAs. In the present study, formation of thoracic and head structures such as eyes, proboscis, bristles and legs were not affected, but the formation of abdominal

structures was. The only effect of pyriproxifen on head and thoracic structures was on pigmentation of the proboscis, mid-dorsum of the thorax (Plate 6.1) and eyes (Plate 6.2). Bristles were pigmented in nearly all the adults except in 0.5% (n= 805) where bristles lacked pigmentation (Plate 6.3). Under normal circumstances, bristles get pigmented before other structures (Cottrel, 1964). In the case of treatment with W-328, some adults that lacked pigmentation of the abdomen and hair were observed (Plate 6.1a). W-328 thus appears more effective than pyriproxifen in disrupting development.

The eyes were pigmented differentially (Plate 6.2), with parts of one eye having yellow pigments while the other eye was normally pigmented. This suggests that JH analogues do not act on all tissues at the same time or to the same degree. Selective deposition of pigments also occurred in 1.2 % (N = 168) of the adults, where deposition of brown pigments (xanthomatins) was interfered with, resulting in pink eyes (Plate 6.3). This occurred where females were treated with W-328 and in pupae directly treated with either of the analogues. Such interference could be due to effectiveness of W-328 or to high doses received by the pupae. In some adults (1.8%, N = 168), the deposition of red pigments was interfered with, making the eyes to appear brownish. This finding supports similar research on *Drosophila*, in which a racemic mixture of JH caused one eye to be pigmented while the other lacked pigmentation (Madhavan, 1973). The same researcher also observed that JH selectively affected the deposition of brown pigments (xanthommatins), making the eyes appear pink. Possibly, there was localized entry of JH analogues into the pigment cells, with the result that in those cells where JH concentrated, ecdysteroids could not exert their effects and so enzymes for pigment formation were not synthesized. Localized entry of insecticides, JH mimics and JH have been reported in various insects (Lawrence, 1969; Madhavan, 1973; Wigglesworth, 1942, 1961).

Hair development failed in the abdominal region of treated flies, in contrast to controls (Plates 6.4-6.6). Bristles and hairs develop from undifferentiated larval epidermal cells, which then undergo differentiative cell divisions during metamorphosis, to give rise to bristles and hairs (Gnatzy & Romer, 1984). Since the abdominal histoblasts failed to differentiate to epidermal cells, abdominal hairs failed to form (Plate 6.5). The JHA thus probably affected the ability of the abdominal histoblasts to effectively respond to ecdysteroids and differentiate. There was however, an intermediate case in which fewer abdominal hairs were present along lines, representing tergal areas (Plate 6.1). The present findings support work by Madhavan (1973) where JH treatment inhibits formation of adult cuticle in *Drosophila* larvae. For normal adult development to occur, JH must be absent at certain critical stages in metamorphosis. In control experiments, adults that failed to eclose were found to have pigmented integuments (Plate 6.4), suggesting that ecdysteroid action on differentiation of abdominal histoblasts went on successfully.

Application of the JH analogues to puparia of different ages showed inhibition of emergence in young puparia but not in older ones (Table 6.6). In higher Diptera, the third instar larval cuticle transforms into a hardened pupal case that protects the developing pupa (Riddiford, 1994). In young puparia, the pupa is still closely adhered to the puparial wall and may be easily reached by the analogues. However, when pupal apolysis occurs around day five post-larviposition (Denlinger & Zdarek, 1991a), the pupa withdraws from its wall and in addition, the wall probably becomes tougher and impermeable to liquids. Emergence observed in puparia treated at four days with 10 and 1000 µg (Table 6.6) probably arose due to lack of absorption of the analogue through the puparium wall. Feeding resulted in death in 1.7% (n= 179) of the adults that emerged from pyriproxifen-treated puparia. Morphologically such females showed lack of expansion in the abdominal cuticle, which may have been accompanied by metamorphic defects in the digestive tract (Staal, 1975).

Where pyriproxifen was applied directly on pupal cuticle at five days post-larviposition, there was immediate inhibition of development at the pharate pupal stage (section 6.4.5). Formation of adult head and thoracic structures had commenced but proliferation of abdominal histoblasts was not complete. However, application of the analogue to pharate adult cuticle (10 days post-larviposition) inhibits only later stages of development such as pigmentation and sclerotization of the abdomen, as well as adult eclosion (section 6.4.5). This concurs with observations on *Drosophila,* where direct application of JH to pupal cuticle results in 60% of the flies developing to adults but emergence is inhibited in all (Madhavan, 1973). Direct treatment of pharate adults (20 days post-larviposition) resulted in high emergence rates (Fig. 6.6). Reduced effects of the analogues with age arise since various body tissues become gradually insensitive to them (Sehnal, 1983).

When pyriproxifen was applied to puparia of different ages through the puparial wall, there was 65% emergence. Of these emergents, 25.7% had deformities (Fig. 6.2), the abdomen being more affected than other body areas. Sehnal and Zdarek (1976) reported various degrees of abdominal abnormalities in several Dipterans, which included reduction in segments, undersized male genitalia, and holes in the abdominal integument. Madhavan (1973) also reported the occurrence of male genitalia that failed to rotate to their normal positions. Wounds were not observed in the case of *G. f. fuscipes*, but male genitalia failed to form properly. Pyriproxifen thus interfered with formation of the male genitalia.

It is clear that applying JH analogues to puparia is not as effective in blocking adult development as when it is applied directly to the pupa or pharate adult (Figs. 6.3-6.6, Table 6.6). The puparial wall seems fairly effective in controlling entry of harmful substances to the developing adult. The critical stage in adult development then depends on whether the application is made directly to the pupa or through the puparial wall. In the latter case, the critical stage for application is on day four post-larviposition, just before pupal apolysis.The

failure of pupae treated with pyriproxifen to eclose is certainly due to incompetence to complete development in the presence of JH, resulting in the deformities already mentioned. Thus, adults that are unable to form adult abdominal as well as proboscis cuticles cannot survive even if they eclose. For the flies that formed adult cuticles, but were unable to eclose, JH could have interfered with differentiation of muscles, such that they were weak and unable to perform pre-eclosion movements, or eclose (Zdarek & Denlinger, 1992). In the present study, some adults that were manually removed from the puparium were able to walk about, but could not expand the wings to fly. This is likely due to inability of such flies to develop hemocoelic pressure, or due to failure of flight muscles to develop, in the presence of JHA. Adult flies that are manually removed from their puparium die soon afterwards (Madhavan, 1973). The failure to eclose in JH-treated pharate adults was attributed to interference in production of eclosion hormone in the presence of JH. Under normal circumstances, JH is metabolized by degradative enzymes during critical periods in metamorphosis (de Kort & Granger, 1996). Exogenous JH analogue, however, cannot be metabolized by the same enzymes and so they persist throughout the developmental period, resulting in abnormal development.

Although fecundity of females and fertility of males following pyriproxifen treatment was lower than for control set-ups (Tables 6.7 & 6.8), the treatment did not affect ovarian development. In only one case, ovaries of one female from the Rusinga population were found to be atrophied. This abnormality was, however, observed in wild females also, and may not have been due to pyriproxifen treatment. Males were generally weak and some died before inseminating females.

The doses of JH analogues used in the present study were all effective in derailing development in *G. f. fuscipes* when applied to young puparia (one to four days old) (Table 6.6). In the C.A.R. population, the dose effect was not apparent, with high rates of emergence

occurring in puparia aged between seven to 10 days old (Figs. 6.3 & 6.4). In older puparia (10 days post-deposition) of the Rusinga population, doses of 1 and 10 µg inhibited eclosion in 50% of the puparia (Fig. 6.5). Rates of emergence were low at all the doses applied to five day-old puparia (Table 6.6). However, this effect was more marked at doses of 100 and 1000 µg, and was also reflected to a smaller degree in eight day-old puparia. A dose of 1000 µg caused high mortalities both when adults were treated, and in adults that emerged from treated puparia. In *G. m. morsitans*, doses ranging from 0.02 - 2 µg suppress eclosion in 73-75 % of two to four day-old puparia (Langley *et al.*, 1988). The fact that in this study there was 100 % inhibition of eclosion in puparia aged one to four days (Table 6.6) suggests that *G. f. fuscipes* is more sensitive to pyriproxifen treatment than *G. m. morsitans*.

The mechanism by which JH exerts its effects is not known. Riddiford (1994) postulated that it probably acts at the cellular level to inhibit certain tissues so that they do not respond to ecdysone. It is ecdysone that initiates the metamorphic process and so failure to metamorphose in the presence of JH can only be due to blockage of certain steps mediated by ecdysone. The experiments done here demonstrate that pyriproxifen allows growth and proliferation of imaginal discs, but inhibits differentiation of abdominal histoblasts.

6.5.2 Treatment of Females with Ketoconazole

Females treated with ketoconazole larviposited and the larvae pupariated (Table 6.1). This observation implies that ketoconazole is not effective in inhibiting synthesis of ecdysteroids or that ecdysteroids have no role in vitellogenesis in *Glossina*. Upon emergence adults contain some ecdysteroids, which decline by the third day after emergence (Robert *et al.*, 1986) and could thus maintain vitellogenic processes. In addition, yolk deposition in oocytes begins seven days before a female fly emerges from the puparium (Saunders, 1961). Perhaps by the time ketoconazole treatment was given, vitellogenesis was already in process

and the IGR apparently did not interfere with the process. The third peak of ecdysteroid titre in *G. m. morsitans* has been correlated with parturition yet treatment of females with ketoconazole at this stage does not affect it. In the absence of ecdysteroids, the parturition stimulating hormone synthesized from the central nervous system and released in the neurohaemal areas could mediate parturition (Robert *et al.*, 1986). Some larvae (1.7%, n= 235) deposited by females treated with ketoconazole did not pupariate. It is possible that synthesis of ecdysteroids was inhibited in some females, resulting in failure of larvae to pupariate or that ketoconazole caused precocious abortion of larvae. Larvae deposited before commitment to metamorphosis are unable to perform prepupariation behaviour (Denlinger & Zdarek, 1991b). Although adults contain ecdysteroids upon emergence, these would not be sufficient for the entire reproductive cycle and the observation that most larvae produced in the present study pupariated suggests that ketoconazole did not effectively inhibit synthesis of ecdysteroids.

6.5.3 Treatment of Females with Precocene

Females treated with precocene larviposited and the larvae pupariated normally (Table 6.1). Precocene probably has no effect on vitellogenesis since the process begins in females before emergence (Saunders, 1961) and goes on uninterrupted throughout the reproductive life of the female (Samaranayaka-Ramasamy & Chaudhury, 1981). The highest rate of emergence was obtained from puparia formed from larvae deposited by females treated with precocene (Table 6.3), the adults being from the second, third and fourth reproductive cycles. However, some of the adults did not emerge. Precocene did not affect the reproductive capability of treated *G. m. morsitans* females, low rates of emergence also occurring from the puparia. This is in agreement with the findings of Samaranayaka-Ramasamy and Chaudhury (1981) on

G. m. morsitans. In their study, however, emergence occurred in all reproductive cycles, with females from the later cycles having both oocytes retarded in growth. The inhibition of emergence in adults from the first reproductive cycle in the present study arose due to incomplete formation of the abdominal epidermis, the morphogenetic effects being similar to those observed with JH analogues (Plate 6.1 d-f). This would imply that *G. f. fuscipes* is more sensitive in response to the effects of precocene than *G. m. morsitans*.

6.5.4 Treatment of Females with Precocene and Ketoconazole

Application of ketoconazole to precocene treated females did not hinder production of normal larvae, which also pupariated (Table 6.1). At the end of the developmental period, there was 23.3% (n= 29) emergence from the puparia (Table 6.3), suggesting that these two compounds have no influence on reproduction and metamorphosis in this species of *Glossina*.

6.5.5 Juvenile Hormone Replacement

Replacement of juvenile hormone in precocene-treated females with pyriproxifen or W-328 did not have a significant effect in eliminating the disruptive effects of JHA treatment on development. The morphogenetic effects on tergite development were similar to those observed with JH analogues (Plate 6.1c-e). Only two females emerged when pyriproxifen was used in the replacement therapy while no emergence occurred in the case of replacement with W-328 (Table 6.3). This indicates that some recovery could be obtained with the analogues. In *G. m. morsitans*, recovery from sterilizing effects of precocene was obtained by treatment with JH III (Samaranayaka-Ramasamy & Chaudhury, 1981).

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

7.1 Importance of Self-sustaining Colonies

Failure to rear *G. f. fuscipes* successfully in Kenya has led to the need to import puparia from colonies abroad for research. This has shortcomings associated with timing of arrival of the puparia and delays in dispatch of the parcels, leading to losses of adults which emerge before they are kept in suitable insectary conditions. Consequently, such supplies may be insufficient for basic research. Successful self-propagating colonies are a prerequisite for control methods such as the sterile insect technique (SIT) and the lethal insecticide technique (LIT), which require large numbers of sexually competitive flies and for research on novel control methods. Attempts to colonize the Rusinga population of *G. f. fuscipes* has faced bottlenecks, leading to extermination of the colonies. The present study assessed factors that may affect reproductive success of this population in comparison to one from Vienna (ex-C.A.R) that has stabilized to laboratory conditions. In addition compounds which may affect reproduction and development in these flies were investigated for possibility of their use in control programmes.

7.2 Comparative Study of Factors Affecting Reproductive Performance in the Rusinga and C.A.R Populations of *G. f. fuscipes*

Receptivity in the Rusinga population was relatively low compared to that of the C.A.R population (Fig. 3.1). This implies that in the former, insemination rates will be low and hence, fecundity. Receptivity declined with age of females from the Rusinga population

(Fig. 3.1a), meaning that older females are less likely to contribute to colony propagation since chances of having them inseminated are low. This trend of early mating occurs in other members of the *palpalis* group such as *G. palpalis*, as well as in some members of the *morsitans* group such as *G. m. morsitans* (Langley, 1977; Tobe & Langley, 1978). The exception is *G. pallidipes*, in which older females are more receptive than young ones (Davies-Cole, 1990). The high receptivity in the C.A.R population suggests that high insemination rates are attained in this population, leading to high fecundity. In this population, age is not a barrier to insemination since receptivity was high across all age levels. The latter population would be more suitable for mass rearing than the Rusinga one as a small proportion larviposit without insemination (section 3.4.7). Repeated mating was not observed in the present study, despite the maintainance of males with females (section 3.4.6). However, the method employed in assessing repeated mating is not reliable since it could not be established whether a female received sperms from males in subsequent matings. Video recordings, accompanied by genetic labeling studies would provide conclusive evidence on re-mating and paternity. In *G. m. morsitans*, once-mated females produced more progeny than those maintained in the presence of males (Dame & Ford, 1968), indicating that single mating would be sufficient in tsetse reproduction. Insemination rate is a more important consideration in rearing than degree of insemination since females store sperm in their spermathecae for a long time without evidence of depletion (Mellanby & Mellanby, 1937) and as the present study shows, there was no difference in fecundity between females inseminated to different degrees. In the mosquito *A. aegypti*, females are re-inseminated prior to the second gonotrophic cycle (Young & Downe, 1982), implying that the females deplete sperm from previous matings.

The present study has shown that abortion and low puparial weights, which may have been due to poor feeding in the Rusinga population (Table 3.6), affected reproductive performance. Insufficient blood meals have been shown to induce abortions in *G. m. morsitans* (Saunders, 1972). Moreover, female tsetse held under artificial laboratory conditions may not efficiently convert blood meals into fat, leading to poor reproductive performance (Loder *et al.*, 1998). The low puparial weights and the high abortion rates are indicative of nutritional stress (Madubunyi, 1978) since starvation induces abortion (Saunders, 1972). The high rates of mortality (Table 3.8) may have been caused by the emergence of weak flies from the low weight puparia (Jackson, 1949), which also led to low emergence rates (section 4.5.2). The poor feeding may be due to lack of adaptation of flies to insectary conditions. An improvement in feeding performance may lead to less abortions, heavier puparia and increased emergence rates.

Fecundity in the Rusinga population of *G. f. fuscipes* was lower than that for the C. A. R population (Tables 3.4 & 3.5), being affected mainly by abortion (Table 5.1). The present study has also demonstrated that amount of blood meal taken is positively correlated with puparial weights (Table 3.6). Under the same laboratory conditions, females of the C.A.R population consumed significantly more blood and produced heavier pupae than the Rusinga females (Table 3.6). Fecundity was also affected by slow growth of follicles (Table 5.1). These abnormalities were also encountered in wild females. However, abortion was lower in wild than laboratory populations. In wild females, it was higher in the dry season. Insemination rates and degree of insemination of wild population were higher than in the laboratory one (section 5.5.2). Such high rates are probably achieved in the swarms of flies at feeding sites (Jordan, 1993). Larviposition without observable insemination occurred in some females from the C.A.R population, as well as in some wild ones from the Rusinga population (Table 5.1). This phenomenon could have negative implications for the sterile insect

technique, since females that are not inseminated by the sterile males can still propagate populations.

This study has shown that factors that affect laboratory reproductive performance of the Rusinga population of *G. f. fuscipes* are low receptivity, slow growth of follicles, low productivity, high rates of abortion and pupal mortality. Conditions in the experimental room could not have contributed to poor reproductive performance since a population from Seibersdorf (originating from C.A.R.) exhibited higher performances, i.e. high receptivity, productivity and emergence rates. Early mating would be important in this species for high insemination rates to be achieved.

7.3 Eclosion Behaviour of *G. f. fuscipes*

Emergence rates of wild Rusinga flies were high and were comparable with those of the C.A.R population(section 4.5.2). However, emergence from puparia produced by F1 generation of females from the Rusinga population was low and the emergents were weak. Thus pupal mortality affected the performance of the colony. This was caused by the low weight pupae produced by the female F1 generation (Tables 3.4 & 3.6). Eclosion peaks in *G. f. fuscipes* occurred between four and six h after lights switched on at LD 12:12 and 25° C (Fig. 4.1) while in *G. m. morsitans*, the peaks occur between eight and 11h after lights switched on (Phelps & Jackson, 1971; Zdarek & Denlinger , 1995). The difference in eclosion times could be due to species and habitat differences. *Glossina. f. fuscipes* is hygrophytic and the newly emerged flies may require the cool temperatures in the morning hours.

Under natural conditions, a female: male ratio of 1:1 is expected. However, a high female: male ratio would ensure that there are more females that would contribute to colony propagation, particularly in mass rearing. Males often engage in multiple mating and so their

small numbers would not adversely affect colony size. A high female: male ratio occurred in the C.A.R population (section 4.5.4), which also had higher fecundity than the Rusinga population (Tables 3.4 & 3.5). Most experimental matings, however, employ high male: female ratios in order to achieve high fecundity, low abortion rates and higher longevity (Mellanby, 1936; Rogers, 1972). The peak of female emergence in the Rusinga population was 33 days, while it was 32 days for the C.A.R population (fig. 4.3). Thus the emerged weaklings observed in the Rusinga population may not have arisen due an early female peak (Feldmann, 1993), but perhaps due to small pupae. Females emerged earlier than males (Figs. 4.1-4.3), as happens in other species of *Glossina* that have been studied (Denlinger & Zdarek, 1991a; Zdarek & Denlinger, 1995).

7.4 Effects of Insect Growth Regulators on Reproduction and Development in *G. f. fuscipes*.

The JH analogues pyriproxifen and W-328 did not interrupt reproductive processes in *G. f. fuscipes*, but treated females larviposited and the larvae pupariated normally (Tables 6.1 & 6.2). Treatment of females with exogenous analogues in the presence of naturally occurring JH means that there is an excess of JH in the females. Unlike in other insects where JH has been shown to play a role in previtellogenic and vitellogenic processes (Landers & Happ, 1980; Riddiford, 1994) including embryogenesis and metamorphosis, this does not occur in *Glossina*. The same situation applies when females are allatectomized by precocene treatment. It has been shown that in *Glossina* vitellogenesis begins seven days before the female fly emerges, and continues after the first blood meal. Blood meal has been shown to be an important factor for vitellogenesis in anautogenous species (Yin & Stoffolano, 1997) but in *Glossina*, it is difficult to separate nutritional from endocrine factors.

Inhibition of emergence is the only effect of the JH analogues that has been observed in *Glossina* (Langley & Pimley, 1986; Langley *et al.*, 1988). Females transmit sterilizing effects of the analogues to their offspring through the mature oocytes during ovulation (Masner *et al.*, 1968; Samaranayaka-Ramasamy & Chaudhury, 1981) or through larval nutrients. The same phenomenon, first reported in *Pyrrhocorris*, occurs in *G. m. morsitans*. The analogues cause derangements in adult development by preventing imaginal differentiation of cells (Sehnal, 1983) and possibly suppresses the synthesis of cuticular proteins (Gnatzy & Romer, 1984). The present study shows that head and thoracic structures form normally even in offspring from treated females (Plate 6.1), suggesting that the stage of development at which treatment is given has no influence on their formation. It is then apparent that formation of these structures is genetically determined and are insensitive to the analogues regardless of dose or method of application (Willis, 1974). Formation of the abdomen is frequently affected since after pupation, the larval cells are histolyzed and histoblasts spread to form adult epidermis (Roseland & Schneiderman, 1979; Madhavan & Schneiderman, 1977). It is the differentiation of the histoblasts that JH analogues interfere with, resulting in failure to form cuticle (Plate 6.5). The variability in response to the analogues in the development of tergites (Plate 6.1) may be due to factors that favour more absorption of the analogues in the oocytes of one individual or at one pregnancy cycle compared to another. Inhibition of emergence occurred in young puparia but in puparia older than five days, emergence occurred regardless of dose (Table 6.6). High doses after the critical period had no effect on emergence rates (Fig. 6.3 & 6.4) since differentiation of abdominal cells was complete, but have effects on reproduction of the emerged adults (Tables 6.7 & 6.8).

For practical purposes, it is preferable to treat adults with the analogues since adults can be readily trapped in devices that automatically sterilize and releases them. This method

of control has shown promising results with *G. m. morsitans* in Zimbabwe (Hargrove & Langley, 1990) using pyriproxifen. The analogue W-328 has not been tried on tsetse and it will be important to carry out its field assessment.

Treatment of female *G. f. fuscipes* with ketoconazole had no effect on fecundity (Table 6.1), unlike in *G. bimaculatus* in which such treatment significantly reduced fecundity (Hoffmann *et al.*, 1996). Although adults emerge with some level of ecdysteroids that may influence vitellogenesis in treated females (Robert *et al.*, 1986), this could not be sufficient for all the reproductive cycles undergone. Some adults produced by treated females completed development and emerged (Table 6.3), suggesting that the larvae deposited contained sufficient ecdysteriods. Therefore ketoconazole was not effective in inhibiting synthesis ecdysteroids. However, it inhibited development within the puparium in some adults (Table 6.3).

Precocene treatment of females had no influence on the vitellogenic process, but emergence rates were reduced (Table 6.3), with no emergence occurring in the first reproductive cycle. However, some level of emergence occurred in the first reproductive cycle when JH was replaced with pyriproxifen, but not in replacement with W-328 (Table 6.3). W-328 thus seems a better inhibitor of development in *Glossina* than pyriproxifen. In *G. m. morsitans*, low rates of emergence occurred at all cycles, with retarded ovarian growth, but recoveries occurred when JH was replaced with JH III (Samaranayaka-Ramasamy & Chaudhury, 1981).

The JH analogues, pyriproxifen and W-328, have no influence on vitellogenesis and larviposition in *Glossina*, but they affect adult development, with effects of W-328 on tergite development being more than for pyriproxifen (Table 6.5). The JH analogues affected development of epidermis, through their effects on differentiation of abdominal histoblasts (Plate 6.5). In effect the JH analogues inhibited emergence and may therefore be effectively

used in controlling *G. f. fuscipes*. Ketoconazole, however, does not effectively inhibit synthesis of ecdysteroids in *G. f. fuscipes* at the doses that were used in the present study, resulting in normal larviposition and pupariation (Table 6.1).

7.5 Practical Implications and Prospects for Future Work

The present study has shown that reproductive success of the Rusinga population of *G. f. fuscipes* under laboratory conditions is affected by abortion (Table 5.1), low puparial weights (Tables 3.4 & 3.6) and adult mortality (Table 3.8). The first two factors may have been due to poor feeding. Since the C.A.R population showed high reproductive performance under the same conditions (Table 3.5), it is likely that lack of adaptation to confinement under laboratory conditions had a negative influence on feeding. Therefore, with continued supplementation of existing colonies with wild females or puparia and careful monitoring of laboratory conditions, it is possible to obtain a stable colony. Colonization is a long process since strain adaptation to laboratory conditions takes a long time, adult numbers declining with each generation (Feldmann, 1993). However, numbers increase once the colony is stable.

In the course of the study, some females larviposited without evidence of insemination (sections 3.4.7 & 5.5.2). Further work should be carried out to determine whether sperms were actually transferred but remained in the uterus without being stored in the spermathecae, or the females reproduced parthenogenetically. In the latter case, it would be important to determine the ploidy and fertility of such offspring. Parthenogenesis is a phenomenon that would have negative implications for the sterile insect technique. If offspring are fertile, it would imply that females that escape mating by sterile males might still produce offspring. Such females would boost colony size since loss of receptivity with age would not be a limiting factor in their reproduction. It would be important to carry out further studies on

multiple mating using more reliable methods such as genetic markers, e.g. microsatellites to determine paternity and whether the females accept sperms from subsequent matings.

The study has also shown that the JH analogues, pyriproxifen and W-328, can be used to disrupt development of *G. f. fuscipes* (Table 6.3, Plates 6.1-6.3 & 6.5). Pyriproxifen has been tried in the field with *G. m. morsitans* but not with riverine tsetse. It would be important to carry out field studies on *G. f. fuscipes* with pyriproxifen to determine its response in the field. With W-328, it would be necessary in the first place to work out doses that would be effective with the device employed by Hargrove and Langley (1990) before engaging in field assessments.

In this study, ketoconazole showed no influence on vitellogenesis (Table 6.1). It would be interesting to carry out further studies to determine levels of ecdysteroids following topical applications of the IGR. This would provide conclusive evidence as to whether ketoconazole is effective in inhibiting synthesis of ecdysteroids and whether ecdysteroids have any role to play in vetellogenesis and reproduction in general. A similar determination can be made following precocene treatment to determine the level of inhibition of juvenile hormone synthesis exerted by the IGR.

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APPENDIX

Appendix 1 Classification of the morphological effects of IGRs on *Glossina fuscipes fuscipes*. Scores zero to three includes adults which emerged (+) while in scores four to nine, emergence was inhibited (-).

SCORE	DESCRIPTION OF EFFECTS	ABILITY TO EMERGE
0	Normal adults	+
1	Wings crumpled in both males and females, reflecting failure in expansion of wings	+
2	Male genitalia straight, or pointing sideways, reflecting incomplete rotation	+
3	Lack of expansion in cuticle of pre- and/or post-abdomen. Small weak adults with reduced number of abdominal segments	+
4	Epidermis well formed, moulting fluid resorbed.	-
5	All tergites formed as narrow bands; no hairs on segments; moulting fluid resorbed	-
6	Whole abdomen pigmented brown, with long hairs on tergal areas. Head and thorax normally formed, but mid-dorsum with scanty hair; only the last one to three tergites formed	-
7	Abdomen, mid-dorsum and proboscis not pigmented; lack of resorption of moulting fluid; abdomen flabby, with scanty hairs	-
8	Abdominal cuticle of pupal type, with no hairs; moulting fluid not resorbed; head and thorax formed, and both eyes pigmented;	-
9	Dead at pupal stage	-

Appendix 2 Duration of developmental stages attained by tsetse flies.

Stage	Description	Duration (days)	Mean age (days)
I	Contents intimately associated with puparium at all points; the quiescent stage of the third instar larva	1	1
II	Contents bounded by a fine membrane associated with puparial shell only at rectum ; the fourth instar larva	3	2
III	Imaginal buds evaginated and all adult appendages recognizable; the pupa *sensu stricto* and pharate adult. This stage is subdivided on the basis of pigmentation:		
	1. No pigmentation.	7	8
	2. Eyes only pigmented		
	(a) Eyes yellow.	2	12
	(b) Eyes reddish-brown	6	16
	3. Eyes pigmented and bristles black . .	3	21
	4. General body integument pigmented; perceptible first on dorsal surface of abdomen		
	(a) Pupal skin moist and entire . . .	3	24
	(b) Pupal skin dry and adhering to puparial shell	2	26