AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D. C. 20523

BIBLIOGRAPHIC INPUT SHEET

FOR AID USE ONLY

1. SUBJECT CLASSIFICATION

A. PRIMARY: Agriculture

B. SECONDARY: Insects and Pests

2. TITLE AND SUBTITLE

Storage and sexual separation of Glossina morsitans morsitans Westwood puparia

3. AUTHOR(S)

Birkenmeyer, D.R.; Dame, D.A.

4. DOCUMENT DATE	5. NUMBER OF PAGES	6. ARC NUMBER
1974	6 p.	ARC

7. REFERENCE ORGANIZATION NAME AND ADDRESS

Agricultural Research Service, United States Department of Agriculture, Tsetse Research Unit, University College of Rhodesia, Salisbury, Rhodesia

8. SUPPLEMENTARY NOTES (Sponsoring Organization, Publishers, Availability)

(In Annals of tropical medicine and parasitology, v.69, no.3, p.399-405)

9. ABSTRACT

An investigation of techniques by which puparial sexual separation can be achieved and of the feasibility of stockpiling Glossina morsitans morsitans Westwood by storing puparia for extended periods at temperatures near the threshold of metabolic activity. Maintenance of unknown-age tsetse puparia at 4°C for six days substantially reduced emergence; normal emergence occurred when puparia were held at temperatures of 12°, 14°, or 16°C for two, three, or four weeks, respectively. In-storage eclosion of adults either was suppressed completely at the temperatures used or was slight. Weight, puparial duration, and density of puparia were examined in an attempt to detect sexual differences in puparia. There appeared to be no usable correlation between pupal weight and sex, but the difference between the puparial duration of each sex offers a mechanism for retaining 81% of the females produced and releasing 84% of the males. The flotation of unknown or known-age puparia in methanol-water solutions of varying specific gravities demonstrated some differences in the density of male and female puparia. However, these differences were not great or consistent enough to provide an efficient system of separating puparia by age or sex.

10. CONTROL NUMBER

PN-AAB-635

11. PRICE OF DOCUMENT

12. DESCRIPTORS

Larvae
Test facilities
Tsetse flies

13. PROJECT NUMBER

14. CONTRACT NUMBER

PASA RA 1-00 Res.

15. TYPE OF DOCUMENT

AID 590-1 (4-74)

Annals of Tropical Medicine and Parasitology, 1975, Vol. 69, No. 3

Tsetse fly

Storage and sexual separation of *Glossina morsitans morsitans* Westwood puparia*

By D. R. BIRKENMEYER† and D. A. DAME‡

Agricultural Research Service, United States Department of Agriculture, Tsetse Research Unit, University College of Rhodesia, Salisbury, Rhodesia

Received 14 October 1974

The possibility of controlling tsetse flies (*Glossina* spp.) by the release of sterilized males was suggested by Simpson (1958) and Knipling (1963). The difficulties encountered in such a release programme were discussed by Dame and Schmidt (1970). Of particular interest was the shortened lifespan of released males that had previously emerged in the laboratory from field-collected puparia. Subsequent studies by Dame *et al.* (1968) and by Langley (1970) suggested that this early death is caused by a delay in flight muscle development in 80% of the adults that had been confined before field release. The problem could be avoided by treating puparia in the laboratory and allowing the adults to emerge in the field. However, a large-scale control programme involving treated tsetse puparia for field releases would necessitate the separation of males from females during the puparial stage.

In sterility programmes conducted with several other insect species, sterile females have been released along with the sterile males. However, the release of female tsetse flies would be detrimental for two reasons: the sterilization procedure does not inhibit the ability of males to harbor and transmit trypanosomes (Dame and Mackenzie 1968); the same is true of the females, their release could result in a higher incidence of trypanosomiasis in the test area. Secondly, the relatively low reproductive potential of female tsetse necessitates their retention for rearing purposes.

Separation of some species of *Glossina* is possible by comparison of overall puparial size or by the distance between the polypneustic lobes (Buxton, 1955). However, investigators of pre-emergence sexing of tsetse have had relatively little success in detecting morphological differences. For example, differences in linear dimensions and external puparial morphology (Phelps *et al.*, 1967) could not be used. Azevedo and Pinhão (1968) were unsuccessful when they tried sexual preparation of tsetse puparia by historadiographic methods.

The present work was undertaken to further investigate techniques by which puparial sexual separation could be achieved and also to determine the feasibility of stockpiling *Glossina morsitans morsitans* Westwood by storing puparia for extended periods at temperatures near the threshold of metabolic activity.

* This research was conducted with funds provided to ARS by the Agency for International Development under PASA RA(AJ)1–00.

† Present address: Metabolism and Radiation Research Laboratory, ARS, USDA, State University Station, Fargo, North Dakota 58102.

‡ Present address: Insects Affecting Man Research Laboratory, ARS, USDA, P.O. Box 14565, Gainesville, Florida 32604.

Office of Agriculture
Bureau for Technical Assistance
Agency for International Development
Washington, D. C. 20523

METHODS AND RESULTS

The unknown-age puparia used in our studies were collected by hand in the Zambezi Valley near Kariba, Rhodesia (16°32′ S., 28°48′ E.). The known-age puparia used in experimentation were derived from two sources: the Salisbury tsetse colony, described by Dame and Ford (1966), and the progeny of field-collected puparia. Puparia were held until eclosion to ascertain sex, but, no attempt was made to evaluate subsequent survival or the reproductive potential of the adults. All puparia, whether field- or laboratory-derived, were maintained at a temperature of 26 ± 1°C and a relative humidity of 70 ± 5% unless otherwise indicated. Deformed and partially emerged flies were considered non-viable and were discounted in all calculations.

In this work the word puparium refers to the puparial shell and its contents and the term puparial period refers to all stages of development passed within the puparial shell from the time it takes on the physical appearance of a puparium to adult eclosion.

Storage of Puparia

A series of experiments were conducted with field-collected unknown-age puparia held in an incubator at various temperatures in an attempt to temporarily delay eclosion. No effort was made to regulate humidity during the incubation period since Bursell (1958) showed that puparia are tolerant of a wide range of humidity.

Initially, several batches, each of at least 100 puparia, were held in an incubator at 3°C for two or four weeks and then were moved to the standard holding conditions of the laboratory. From 0%–8% of these puparia survived. In another test, the holding temperature was gradually reduced to 3°C by incubating puparia for 24 hours at 15°C, holding them at 10°C for 24 hours, at 5°C for a further 24 hours, and finally reducing the temperature to 3°C for two weeks. When these puparia were returned to standard laboratory conditions, only 8% emerged.

A further series of storage tests was initiated to determine what temperatures above 3°C might produce satisfactory survival. Groups of 66–180 puparia were incubated at a temperature of 4°–16°C for 1–84 days; then they were brought to standard conditions and held for eclosion. Concurrently, weekly control batches of 66–100 puparia were held at standard laboratory conditions until emergence. The results, expressed as a percentage of the control emergence, are presented in Table I. Since age at incubation was not known, the data do not

TABLE I

Emergence of *G. morsitans* adults from field-collected puparia* held at various temperatures and then returned to 26 ± 1°C (expressed as a percentage of control emergence)

Starting temperature	Percent emergence after storage for indicated no. of days at starting temperature										
	1	2	3	4	6	7	14	21	28	56	84
4°C	96	88	79	75	62	—	—	—	—	—	—
6°C	109	92	117	100	88	—	—	—	—	—	—
8°C	100	96	96	90	99	—	—	—	—	—	—
10°C	—	86	—	90	86†	—	—	—	—	—	—
12°C	—	133	—	118	122†	100	93	77	56	—	—
14°C	—	104	—	97	96	85†	88†	90†	75†	9†	8
16°C	—	—	—	—	100	107	100	99	97	—	—

* Groups of 66–180 puparia per replicate.
† Mean of 2–3 replicates.

show total duration at the puparial stage. Emergence remained near normal in batches held six days at temperatures of 6°, 8°, or 10°C; those puparia held at 4°C for the same period suffered a substantial reduction in eclosion. Adult eclosion was better than 50% after four weeks of puparial incubation at 12°C and except for one fly, in-storage emergence was suppressed completely at this temperature. However, storage for longer than two weeks reduced emergence below acceptable levels. At 14°C, the mean puparial survival ranged from 85%–90% during the first three weeks and dropped to 75% or less after four weeks; in-storage emergence was less than 3% during the first three weeks, but increased to 15%–100% thereafter. Emergence was normal after four weeks of incubation at 16°C; however, 18% and 21% emergence occurred during the third and fourth week of storage, respectively. Increasing the temperature during puparial incubation by 2°C each week did not appear to substantially delay eclosion or improve survival.

Sexual Separation of Puparia

Puparial duration and weight. Puparial duration of male and female tsetse and their weight distribution at the onset of pupation were studied to determine whether a useful sexing technique could be found. To obtain known-age puparia, we examined collection trays (Dame and Ford, 1966) daily and numbered, weighed, and held the collected puparia individually in vials. The vials were then observed daily for adult emergence, and the data on eclosion and sex of the adult were recorded. The results are presented in Table II.

TABLE II

Emergence of known-age *G. morsitans* adults depicting relationship of puparial duration and weight to sex

Puparial duration (days)	Cumulative daily emergence				Puparial weight (mg)	Emergence over 32-day period			
	Males eclosed		Females eclosed			Males eclosed		Females eclosed	
	No.	%	No.	%		No.	%	No.	%
<21	5	0·9	3	0·5	<18	3	3·8	7	9·0
21	5	0·9	5	0·8	18–19	8	10·0	3	3·8
22	6	1·0	7	1·2	20–21	12	15·0	13	16·7
23	9	1·5	37	6·1	22–23	8	10·0	15	19·2
24	14	2·4	204	33·7	24–25	12	15·0	7	9·0
25	47	8·0	466	76·9	26–27	15	18·8	8	10·3
26	230	39·2	563	92·9	28–29	12	15·0	13	16·6
27	469	79·9	603	99·5	30–31	5	6·2	9	11·5
28	541	92·2	606	100·0	32	3	3·7	2	2·6
29	584	99·5	—	—	>32	2	2·5	1	1·3
30	586	99·8	—	—		—		—	
>30	587	100·0	—	—					
Total	587	—	606	—	Total	80	—	78	—

Of a total of 212 puparia that were weighed, only 80 males and 78 females successfully emerged. This percentage (75%) represented a much lower adult emergence than we were obtaining with puparia held normally during this period (93%); the reduction probably was the result of the additional handling during the weighings. We found no correlation between puparial weight and sex that would be useful in separating the majority of males from females. However, there was an important difference in the developmental periods of the

587 males and 606 females whose time of eclosion was monitored. For example, at the stage when 77% of the females had eclosed (day 25), only 8% of the males had eclosed. On the following day, 93% of the females and 39% of the males had emerged.

Density of puparia. Since adult females are normally larger than males (though the ranges of puparial weights overlap almost completely), we felt that the density of females might differ sufficiently from that of males as to provide adequate separation by flotation methods. Known- or unknown-age puparia were placed in different methanol-water solutions daily to find the concentration at which flotation occurred. The unknown-age puparia (100 per concentration) were immersed each day in a solution of fixed specific gravity and those floating were removed and held for observation of eclosion; the puparia that sank were held until the next day and again immersed. The procedure was repeated daily until all puparia had either floated once or emerged. Likewise, known-age puparia were immersed daily in a

TABLE III

Sexual preparation of *G. morsitans* by daily floatation of unknown-age puparia (mean of two replicates of 100 puparia per concentration)

Methanol concentration (%)	Specific gravity	Cumulative % floating		Mean puparial duration following flotation (days)		Mean total emergence of both sexes (%)
		Males	Females	♂	♀	
40	0·94	100	100	15·8	11·3	53·0
45	0·93	98	88	11·8	9·1	55·5
50	0·92	98	93	11·8	8·9	64·5
55	0·91	80	62	8·9	5·3	56·0
60	0·90	66	32	6·5	7·6	65·5
Control						53·0

TABLE IV

Sexual separation of *G. morsitans* by flotation of known-age puparia*

Puparial age and flotation day	Males			Females		
	Puparia dipped (no.)	Mean methanol concentration producing flotation (%)	(S.G.)†	Puparia dipped (no.)	Mean methanol concentration producing flotation (%)	(S.G.)†
1	31	6·3	(0·99)	35	8·0	(0·99)
2	18	24·4	(0·97)	30	27·3	(0·97)
3	23	48·5	(0·92)	23	41·7	(0·94)
4	29	58·4	(0·90)	18	52·8	(0·91)
5	33	62·1	(0·90)	32	58·4	(0·90)
6	28	63·6	(0·89)	46	61·4	(0·90)
7	29	62·9	(0·89)	34	60·7	(0·90)
8	17	65·0	(0·89)	20	64·2	(0·89)
9	4	63·8	(0·89)	8	56·9	(0·91)
10	2	65·0	(0·89)	2	57·5	(0·90)

* Although 120 puparia were tested, calculations were made from only those insects which subsequently emerged, e.g. 47 ♂ and 48 ♀.
† (S.G.) specific gravity was extrapolated from values presented in Table III.

series of solutions ranging (15 equal steps) from 0%–70% methanol. The highest concentration of methanol at which each puparium floated was recorded daily. The changes in density with increasing age observed in this manner were subsequently related to sex when eclosion occurred. Although a total of 120 puparia of known age were eventually tested, only those males (47) and females (48) which emerged were used for computation purposes. The specific gravity of the solutions was determined by replicated weighings of 10 ml of water and of each methanol solution. Control groups were dipped in water for 60 seconds and held until emergence.

Density decreased appreciably as the puparial age increased, with the density of female puparia decreasing more rapidly with age than males (Tables III and IV). Also, puparia of an unknown age appeared to float at an earlier age than did puparia of known age, a difference which could be related to the first type being field collected and the latter of laboratory origin. The mean puparial durations of unknown-age puparia following flotation (Table III) suggest that flotation might effectively separate puparia of different ages. However, after further inspection of the raw data we found that the emergence periods of the individual puparia covered a very wide time range, e.g. 1–25 days elapsed before the emergence of females floated in 60% methanol. Thus, the differences observed were not sufficiently great or consistent to provide a high degree of accuracy in separation.

DISCUSSION

The ability to stockpile tsetse puparia for extended periods could be of benefit in a sterile male release programme because fewer and larger field releases would be possible. Table I shows good survival for puparia incubated from one to six days at 6–10°C, but storage at 4°C for only two days reduced emergence. Bursell (1960) noted similar poor survival of adult tsetse held as puparia for five days at 6°C and suggested that the threshold for development may be 4°C because energy reserves (fat content) would likely be inadequate for development to proceed at such a low temperature.

Bursell (1960) also stressed the importance of sufficient energy to enable the imago to eclose and sustain flight activity for sufficient time to obtain a bloodmeal. He found that the longer the puparial stage persisted, the less likely the teneral fly was to have the energy reserve necessary to find a suitable host animal. Thus, he suggested that low temperatures decreased the daily rate of fat consumption while increasing the total consumption (because of the increased puparial period)—a fact later confirmed by Phelps (1973). The present studies (Table I) indicate that puparia held at 12°, 14°, or 16°C for periods of two, three or four weeks, respectively, had sufficient energy reserves for eclosion. However, longer periods at these temperatures reduced eclosion below acceptable levels. Using predicted rates of development for males, Phelps and Burrows (1969) assigned implied puparial durations of 192, 131, and 92 days for puparia held constantly at 12°, 14°, or 16°C, respectively. Our studies indicate that such extensive holding periods would be detrimental at temperatures lower than 14°C. Unfortunately, survival data are lacking for puparia stored longer than four weeks at 16°C. However, Phelps (1973), using known-age laboratory-reared material, noted that over 50% of puparia incubated continuously at 16°C failed to yield adults.

Phelps (1973) obtained 85% survival from puparia held continuously at 18°C and

calculated (Phelps and Burrows 1969) that puparia held at this temperature would incubate for 66 days before eclosing. Additional tests by Phelps (1973) demonstrated that physiological metabolism at 16°C is apparently sometimes altered by factors other than fat depletion though no attempt was made to define what factors might be involved. The present studies indicate that puparial storage is feasible at certain temperatures for short periods; however, further testing is required to define durations of exposure to 16°–18°C that could provide a relatively lengthy puparial duration without compromising adult survival or quality.

In the present investigations (Table II) puparial weights were not sufficiently different to provide adequate separation of the sexes. There was, however, a difference in developmental rate between the sexes that was discussed by Buxton (1955), and more recently examined by Phelps and Burrows (1969) and Curtis and Langley (1971). Our results showed that after 25 days, approximately 77% of the females had emerged but only 8% of the males; one day later 39% of the males and 93% of the females had emerged. Dame and Ford (1968) reported that males are capable of at least four matings before any reduction in fertility occurs and that the next two matings impart a high level of fertility. Thus, at least 15% of any colony must be male to insure adequate insemination of females; this figure is in agreement with calculations made by Jordan and Curtis (1972). It would be possible to obtain a colony stocked with 15·9% males by collecting emergees through day 25 as three consecutive lots of puparia and collecting the fourth lot of puparia on day 26. With such a procedure, one could sterilize the unemerged puparia (containing 85% of the males) and lose only a small percentage of the females while retaining sufficient fertile males for insemination. Such a technique would provide fertile females for colony reproduction and sterile males for release. This procedure would result in a sacrifice of approximately 19% of the female reproductive potential since that percentage of males would be sterilized in the puparial stage (on day 25 or 26) along with unemerged males.

Narrowing the collection intervals to less than 24 hours or the use of alternative temperatures might produce more dramatic differences between the sexes in puparial duration. However, such refinements in the technique would provide more efficient separation only of the sexes of puparia of known age; the procedure has little to offer in the case of puparia of unknown age.

In view of the reduced effectiveness of adult male tsetse that have been confined before being liberated in nature (Dame and Schmidt, 1970) and the lack of techniques for monitoring flight activity of laboratory-emerged flies to ascertain adequate flight muscle development (Bursell and Kuwengwa, 1972), puparial releases of *G. m. morsitans* will be necessary in sterility programmes. Therefore, further investigation into efficient means of puparial sexual separation is essential to the success of future programmes.

SUMMARY

Maintenance of unknown-age tsetse puparia at 4°C for six days substantially reduced emergence; normal emergence occurred when puparia were held at temperatures of 12°, 14°, or 16°C for two, three or four weeks, respectively. In-storage eclosion of adults was either suppressed completely at the temperatures used or was slight. Weight, puparial duration, and density of puparia were examined in an attempt to detect sexual differences

in puparia. There appeared to be no usable correlation between pupal weight and sex, but the difference between the puparial duration of each sex offers a mechanism for retaining 81% of the females produced and releasing 84% of the males. The flotation of unknown- or known-age puparia in methanol-water solutions of varying specific gravities demonstrated some differences in the density of male and female puparia. However, these differences were not great or consistent enough to provide an efficient system of separating puparia by age or sex.

ACKNOWLEDGMENTS. The authors are grateful for the assistance of H. C. Smith, Experimental Officer, and L. Tsoka, F. Chayambuka, K. Madziwah, and M. Saruchera, Laboratory Assistants, all employed by the Agricultural Research Council of Central Africa.

REFERENCES

AZEVEDO, J. FRAGA DE & PINHÃO, RUI (1968). Studies carried out with the *Glossina morsitans* colony of Lisbon II. Aspects of the tsetse flies appreciated by the soft X-rays. *Anais da Escola Nacional de Saude Publica e de Medicina Tropical*, **2**, 43–50.

BURSELL, E. (1958). Water balance of tsetse pupae. *Philosophical Transactions*, (B) **241**, 179–210.

BURSELL, E. (1960). The effect of temperature on the consumption of fat during pupal development in *Glossina*. *Bulletin of Entomological Research*, **51**, 583–598.

BURSELL, E. & KUWENGWA, T. (1972). The effect of flight on the development of flight musculature in the tsetse fly (*Glossina morsitans*). *Entomologica Experimentalis et Applicata*, **15**, 229–237.

BUXTON, P. A. (1955). *The Natural History of Tsetse Flies*. London School of Hygiene and Tropical Medicine, Memoir 10.

CURTIS, C. F. & LANGLEY, P. A. (1971). The use of chilling and nitrogen in the radiation sterilization of *Glossina morsitans*. (Abstract) *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **65**, 230.

DAME, D. A., BIRKENMEYER, D. R. & BURSELL, E. (1968). Development of the thoracic muscle and flight behavior of *Glossina morsitans orientalis* Vanderplank. *Bulletin of Entomological Research*, **59**, 345–350.

DAME, D. A. & FORD, H. R. (1966). The effect of the chemosterilant tepa on *Glossina morsitans* Westw. *Bulletin of Entomological Research*, **56**, 649–658.

DAME, D. A. & FORD, H. R. (1968). Multiple mating of *Glossina morsitans* Westw. and its potential effect on the sterile male technique. *Bulletin of Entomological Research*, **58**, 213–219.

DAME, D. A. & MACKENZIE, P. K. I. (1968). Transmission of *Trypanosoma congolense* by chemosterilized male *Glossina morsitans*. *Annals of Tropical Medicine and Parasitology*, **62**, 372–374.

DAME, D. A. & SCHMIDT, C. H. (1970). The sterile male technique against tsetse flies, *Glossina spp*. *Bulletin of the Entomological Society of America*, **16**, 24–30.

JORDAN, A. M. & CURTIS, C. F. (1972). Productivity of *Glossina morsitans morsitans* Westwood maintained in the laboratory, with particular reference to the sterile-insect release method. *Bulletin of the World Health Organization*, **46**, 33–38.

KNIPLING, E. F. (1963). Potential role of the sterility principle for tsetse fly eradication. WHO/*Vector Control* **27**, 17 pp.

LANGLEY, P. A. (1970), Post-teneral development of thoracic flight musculature in the tsetse flies, *Glossina austeni* and *G. morsitans*. *Entomologica Experimentalis et Applicata*, **13**, 133–140.

PHELPS, R. J. (1973). The effect of temperature on fat consumption during puparial stages of *Glossina morsitans morsitans* Westw. under laboratory conditions, and its implication in the field. *Bulletin of Entomological Research*, **62**, 423–438.

PHELPS, R. J. & BURROWS, P. M. (1969). Puparial duration in *Glossina morsitans orientalis* under conditions of constant temperature. *Entomologica Experimentalis et Applicata*, **12**, 33–43.

PHELPS, R. J., BURROWS, P. M. & BOYD-CLARK, M. J. (1967). Pre-emergence sexing of *Glossina morsitans orientalis* Vanderplank puparia. *The Rhodesia, Zambia, and Malawi Journal of Agriculture Research*, **5**, 267–271.

SIMPSON, H. R. (1958). The effect of sterilized males on a natural tsetse fly population. *Biometrics* **14**, 159–173.