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Sex separation of tsetse fly pupae using near-infrared spectroscopy

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Abstract

Implementation of the sterile insect technique for tsetse (*Glossina* spp.) requires that only sterile male insects be released; thus, at some stage of the fly production process the females have to be removed. A further constraint in the use of the sterile insect technique for tsetse is that the females are needed for colony production and hence, a non-destructive method of sex separation is required. In most tsetse sterile insect technique programmes thus far, females have been eliminated from the released material by hand-separation of chilled adults. Using near-infrared (NIR) spectroscopy, significant differences have been found between the spectra for the pupae of male and female *G. pallidipes* Austen. Significantly, the differences appear to be maximized 4–5 days before emergence of the adults. Tsetse fly pupae up to five days before emergence can be sexed with accuracies that generally range from 80 to 100%. This system, when refined, will enable effective separation of male and female pupae to be carried out, with emerged females being returned to the colony and males being irradiated and released. If separation can be achieved five days before emergence, this will also enable irradiated male pupae to be shipped to other destinations as required. Other Diptera were evaluated using this system but had lower classification accuracies of 50–74%. This may be due to the difference in reproductive physiology between these different fly groups.

Keywords: Sexing, *Glossina pallidipes*, Glossinidae, near-infrared spectroscopy, sterile insect technique, *Musca*, *Cochliomyia*, *Stomoxys*

Introduction

Tsetse flies *Glossina* spp. (Diptera: Glossinidae) are important as the vectors of African trypanosomes, which cause sleeping sickness in humans and nagana, often a fatal disease, in livestock. About 50,000 human deaths are reported annually (WHO, 2002), but less than 10% of the population is under surveillance and actual deaths are estimated at 300,000 with 60 million people at risk (WHO, 1998). Only about 40,000 cases are diagnosed and treated, and if left untreated, the fatality rate is 100%. Approximately three million cattle deaths occur annually, causing a direct annual loss of about $1.5 billion. The disease threatens about fifty million cattle. Indirect losses due to abortion, milk loss, reduced growth, and loss of animal traction for cultivation are estimated at over $4.5 billion annually (Rickwood, 2001).

Tsetse flies can be controlled by aerial application of insecticides, but environmental concerns have led to the use

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of alternative control means such as traps (FAO, 1992; Leak, 1998), insecticide-treated targets, i.e. screens (Green, 1994), pour-on treatment of cattle, and the sterile insect technique (Knipling, 1955). The sterile insect technique has been used successfully to eradicate or suppress the New World screwworm *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae), the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), and other pests (Hendrichs *et al*., 2002). The sterile insect technique involves the mass rearing and release of large numbers of sterilized insects, and the regular introduction of sterilized males can effectively suppress populations and under certain circumstances lead to eradication. The feasibility of using the sterile insect technique for tsetse eradication has been demonstrated on the island of Zanzibar (Vreysen *et al*., 2000).

A contributing factor to the efficiency of the sterile insect technique is the release of only sterile males. It is observed in various species that the simultaneous release of both sexes is less effective than the release of males only, as there may be a tendency towards assortative mating (Robinson *et al*., 1999; Rendón *et al*., 2004). In species where females are vectors of disease or inflict painful bites, it is important to minimize the number of females released.

The reproductive rate of tsetse is very low, one female producing around nine pupae over a period of 100 days. Because both sexes feed exclusively on blood, both can transmit disease. Separation of females is essential so they can be retained to maintain the colony and to reduce the risk of disease transmission, as sterilized female tsetse survive in the field longer than sterilized males and have a much greater potential to act as vectors. In the sterile insect technique in Zanzibar, separation of females from males was done by hand-sexing of chilled adults before release and returning the females to the colony. The males destined to be sterilized and released received one blood meal containing a trypanocidal drug (isometamidium chloride, Samorin®). In sexing systems used for other pests or vector insects, chromosome translocation strains and conditional lethal genes have been used that ensure female insects can be selectively killed (for example by heat or insecticides) at a particular developmental stage before release. Because tsetse have a very low reproductive potential, colony fertility cannot be compromised and chromosomal translocations, which are associated with reduced fertility (Curtis, 1969) cannot be used. Therefore, for tsetse a non-destructive sexing method is required that does not impact on the productivity of the colony and which can be incorporated into a mass rearing system. In tsetse flies, females emerge from the pupae first and by manipulating temperature conditions, sex separation based on the control of temperature and timing of adult emergence is possible (Opiyo *et al*., 1999, 2000), potentially giving better than 99% efficiency. This system, however, is very sensitive to changes in pupal incubation and emergence conditions and only separates the sexes at the end of the pupal developmental period. Separation at the pupal stage a few days before emergence would be preferable as it would leave more time to transport irradiated male pupae to release sites. However, so far, no sexual dimorphism observable before emergence of a batch of pupae begins has been detected.

Near-infrared spectroscopy is commonly used to measure characteristics of biological materials. While most commonly applied to measuring characteristics of bulk materials such as grain (Williams & Norris, 2001), it has also found applications for measuring characteristics of single-grain kernels (Delwiche, 1993; Dowell, 1998) and then adapted for measuring characteristics such as age, species, insects inside grain or parasitization of single insects (Dowell *et al*., 1998, 1999, 2000; Baker *et al*., 1999; Perez-Mendoza *et al*., 2002).

Near-infrared spectroscopy can be used to measure insect characteristics because all biological materials absorb near-infrared radiation based on their unique chemical compositions. Molecules comprised of C, H, N and O have unique vibration frequencies that absorb near-infrared radiation at specific wavelengths that correspond to these vibration frequencies, or overtones of these frequencies (Miller, 2001). Thus, the amount of protein, carbohydrate, water, etc. in biological materials affects the near-infrared spectra. It was thought that male and female tsetse pupae would have sufficiently different chemical compositions to have measurably different near-infrared spectra. In higher Diptera, the puparium is the hardened cuticle of the last larval instar that encases the pupa. The objective of this research was to measure near-infrared spectra of tsetse fly puparia and determine if the spectra could be used to automatically identify the sex and accordingly, sort the pupae. The infrared light may penetrate the puparium to the enclosed pupa, or to the pharate adult within a late stage pupa. For simplicity the term pupa is used in this paper, but this should be understood to include the puparium or pharate adult as appropriate.

Materials and methods

Three general tests were performed to determine how well near-infrared spectroscopy could be used to sex tsetse flies. These consisted of: (i) preliminary tests conducted at the United States Department of Agriculture, Agriculture Research Service, Grain Marketing and Production Research Center, Engineering Research Unit, Manhattan, Kansas, USA, using two different spectrometers and manually positioned pupae; (ii) tests at the Food and Agriculture Organization/International Atomic Energy Agency, Agriculture and Biotechnology Laboratory, Seibersdorf, Austria, using an automated near-infrared spectrometer in semi-automated mode; and (iii) automated sorting of bulk samples in Seibersdorf, Austria.

Fly samples

A culture of *Glossina pallidipes* Austen (Diptera: Glossinidae) was reared at the Entomology Unit, Food and Agriculture Organization/International Atomic Energy Agency, Agriculture and Biotechnology Laboratory, Seibersdorf, Austria, under standard conditions (Gooding *et al*., 1997). The larviposition day was recorded for each sample set. For tests conducted in Manhattan, Kansas, *G. pallidipes* pupae from three larviposition days were shipped for scanning. Pupae were held at 25°C and 65% RH after scanning until emergence. After all flies had emerged, they were killed by freezing and sent to the Centers for Disease Control and Prevention, Entomology Branch, Atlanta, Georgia, USA, for sex determination. One hundred pupae from each of the three larviposition days were scanned from 1–23 days before the first adults began to emerge. In all tests, no further scanning was done after the first adult emerged.

For tests conducted in Seibersdorf, Austria, *G. pallidipes* pupae were held in the pupal incubation room at 24°C until required for near-infrared scanning. Following scanning, individual pupae were incubated at 26.5°C until adult emergence. The adult emergence date was recorded and the flies were sexed. The pupae containing flies that had not emerged were dissected and their sex determined. The reason for the fly not emerging was noted (larva failed to complete pupation, died early in pupal development, or mature but not emerged). Comparisons of scanned pupae to pupae incubated without scanning showed no significant increase in mortality due to the near-infrared scanning procedure (data not shown). Pupae from five larviposition days were scanned 1–12 days before adult emergence.

Near-infrared instrumentation

Initial spectra were collected with a DA7000 spectrometer (Perten Instruments, Springfield, Illinois, USA). This spectrometer measures absorbance from 400–1700 nm using a silicon sensor that measures absorbance from 400–1100 and an indium-gallium-arsenide sensor that measures absorbance from 950–1700 nm. The sensors overlap in the 950–1100 nm range and only the absorbance measured by the indium-gallium-arsenide sensor is used in this region. Chemical functional groups have first and second absorption overtones in the 950–1700 nm region, with the weaker third overtones in the 700–950 nm region (Miller, 2001). The 400–700 nm region contains colour information. Pupae were manually placed into a V-shaped trough (12 mm long, 10 mm wide, 5 mm deep) and illuminated with white light. A fibre-optic bundle transmitted the reflected energy to the spectrometer. Pupae were manually placed in the sample tray and spectra collected at a rate of several pupae per minute. Dowell *et al*. (1999) gives a complete description of this instrumentation.

Additional spectra were collected from an automated single kernel near-infrared instrument that collects spectra and sorts wheat kernels at a rate of about 1 s^{-1} (fig. 1). *Glossina pallidipes* pupae are similar in diameter and length to wheat kernels and were fed through the unmodified automated system. The feeding device consists of a nearly vertical 15-cm diameter wheel with eight evenly spaced 0.7-mm vacuum ports located 0.6 cm from the edge of the wheel. The feeding device picks up one pupa at a time and deposits it into the viewing trough that is about the same dimensions as described above for the DA7000. The pupa is illuminated and the reflected energy is transmitted to a spectrometer that uses an indium-gallium-arsenide sensor and measures absorbance from 950–1700 nm. The trough is rotated by a solenoid and drops the pupa through a series of gates that can either direct the specimen back into the feeding bin, or sort the pupa into one of four bins based on a predefined calibration.

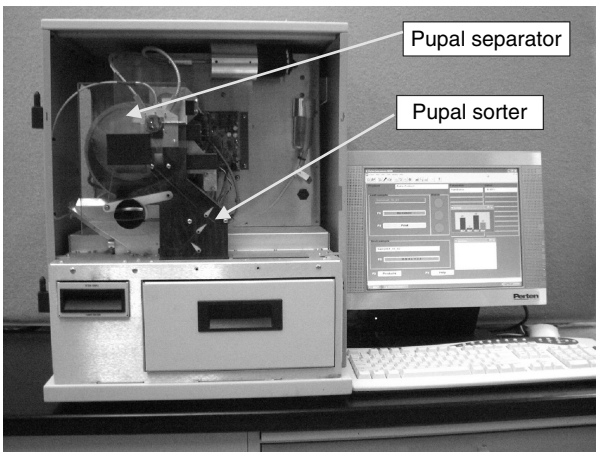

Fig. 1. Automated single-kernel near-infrared sorting system for scanning and sorting single fly pupae.

After scanning with either near-infrared instrument, each pupa was placed in a compartmentalized pillbox so that, after emergence, the fly sex could be matched with its specific near-infrared spectrum. For most tests, the pupa was automatically positioned in the trough, but the spectrometer was manually triggered after ensuring the pupa was correctly positioned below the reflectance fibre. This was done to minimize any placement error effects on spectra.

Automated bulk sorting tests

A calibration developed from the pupae scanned in Manhattan (August 1 larviposition day, one day before adult emergence, $n = 79$, four partial least squares regression factors) was used in Seibersdorf to automatically bulk-sort pupae 4–6 days before emergence into one of four bins. Different rejection thresholds were used to affect the proportion of the pupae that were males in bin four.

Tests with other species

This near-infrared technique was also used in sexing pupae of the house fly, *Musca domestica* Linnaeus (Diptera: Muscidae), face fly, *M. autumnalis* DeGeer (Diptera: Muscidae), secondary screwworm, *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae), primary screwworm fly, *C. hominivorax* (Coquerel) (Diptera: Calliphoridae), and stable fly, *Stomoxys calcitrans* (Linnaeus) (Diptera: Muscidae). House flies, face flies, stable flies and secondary screwworms were obtained as one-day-old pupae from colonies maintained in the Department of Entomology, Kansas State University. House flies and stable flies were reared on wheat bran-based media (McPheron & Broce, 1996; Perez-Mendoza *et al*., 2002); whereas face flies were reared on fresh dung from bulls on a low energy, maintenance diet (Broce *et al*., 1988). Secondary screwworms were reared on a bovine blood-based gelled diet (Harris *et al*., 1984). Primary screwworm pupae were obtained from the USDA-ARS Midwest Livestock Insects Laboratory. Pupae were placed in individual containers after scanning when they were 1–4 days before emerging. Pupae were held under laboratory conditions until all flies had emerged, after which the sex of the fly was determined.

Data analysis

All spectra were analysed by examining average spectra from each larviposition day, and by analysing spectra by partial least squares regression using Grams software (Galactic Industries, 1996). The average spectra from each larviposition day showed only if gross changes in spectra occurred as pupae matured, whereas the partial least

squares regression analysis helped determine subtle differences between spectra of males and females that were not obvious in the raw spectra. All spectra were mean centred before analysis. For calibrations, the female pupae were assigned a value of 100 and male pupae a value of 200 for the bulk sorting tests, and a value of 1 or 2 for all other tests. The values of 100 and 200 were used because the automated classification software for bulk samples only accommodated whole numbers. To determine the classification accuracy, a pupa with a predicted value of 150 (or 1.5) or greater was predicted as male, and a value of less than 150 (or 1.5) was predicted as female.

The number of factors used in the partial least squares regression calibrations was determined from cross-validation plots and by examining beta coefficients. The cross-validation was performed by removing one spectrum, developing a calibration from the remaining spectra, and then predicting the spectrum that was removed. That spectrum was then placed back in the calibration set, and another spectrum was removed and predicted from a calibration developed from the remaining spectra. The process was repeated until all spectra had been predicted in this manner. The calibration used all wavelengths and consisted of beta coefficients that were analogous to a calibration equation. There was a beta coefficient for each wavelength and the larger the absolute value of the beta coefficient, the more that wavelength contributed to the calibration model. Thus, examining the beta coefficients indicated what wavelength regions were important for determining the sex of the fly within the pupa. The calibration that resulted in the lowest predicted residual sum of squares with beta coefficients that were interpretable was then selected. If too many factors are chosen for the calibration model, the corresponding beta coefficients cannot be interpreted, the data can be over-fitted, and the model performs poorly on data not included in the calibration. Cross-validation results were almost identical to results achieved from developing a calibration and predicting all samples used in that calibration.

Initial tests in Manhattan, Kansas, utilized blind samples where all samples were scanned and then shipped to the Centers for Disease Control, but the sex of only 90% of the pupae were reported and used for a calibration. The remaining 10% were then predicted from this calibration.

Results

Preliminary tests

Classification accuracies for pupae of *G. pallidipes* shipped to Manhattan, Kansas, ranged from 100% for pupae scanned one day before emergence to 78% for pupae 23 days before emergence (table 1). Similar results were achieved with the DA7000 spectrometer that measures absorbance from 400–1700 nm as achieved with the single kernel near-infrared spectrometer that measures absorbance from 950–1700 nm (table 1). No colour difference between the pupae of male and female flies was expected or observed. Pupae that were one and six days before emergence were classified by models developed using DA700 wavelength ranges of 400–1700 nm, 400–1100 nm, and 950–1700 nm. Classification accuracies were 75, 75, and 83% (for six factors), respectively, for the three wavelength ranges. Thus, only the spectrometer with the indium-gallium-arsenide sensor (950–1700 nm) was used in subsequent tests.

The test set consisting of seven to nine unknowns from each larviposition day was predicted with accuracies similar to the cross-validation results (78–100% accurate). A calibration was developed (nine factors) from all pupae that were 1–3 days before emergence and used to predict all pupae from all larviposition days. Again, the classification accuracies were high for pupae 1–6 days before emergence (85–97% correct), with classification accuracies dropping to 64–75% for pupae up to 23 days before emergence.

These tests showed that good classifications could be achieved from 1–6 days before emergence using a spectrometer with a wavelength range of 950–1700 nm. While these results showed that near-infrared spectra could be used to determine the sex of a fly, these tests were limited to 300 pupae. Additional tests were conducted to further define how well the near-infrared system could determine a fly's sex, and if the flies could be automatically sexed and sorted.

Table 1. Accuracy of sex-separation of *Glossina pallidipes* using a DA7000 spectrometer (400–1700 nm) and a single kernel near-infrared spectrometer (950–1700 nm). Tests conducted in Manhattan, Kansas.

Larviposition day	Approx days before emergence when scanned	No. of pupae scanned[1]	DA7000 spectrometer		Single kernel near-infrared spectrometer		Prediction (all pupae)[3] (%)
			Cross-validation[2] (%)	Test set[2] (%)	Cross-validation[2] (%)	Test set[2] (%)	
August 1	1	100	96	100	97	100	97
August 10	3	100	90	89	88	78	87
August 20	6	100	88	100	88	100	85
August 10	10	–	84	78	84	78	75
August 20	16	–	86	78	82	78	72
August 20	23	–	85	78	81	78	64

[1] For a given larviposition day, the same 100 pupae were scanned on each spectrometer. About 10% of the pupae did not emerge and were not included in cross-validation, test, or prediction results.
[2] Each cross-validation consisted of about 90 pupae that were used to develop a calibration (9 partial least squares regression factors) to predict the 7 pupae in the August 1 test set, and 9 pupae in the other sets.
[3] This prediction equation was developed using all pupae from 1 and 3 days before emergence and scanned on the DA7000 (9 partial least squares regression factors).

Table 2. Accuracy of sexing pupae of *Glossina pallidipes* using a single kernel near-infrared spectrometer (950–1700 nm). Tests conducted in Seibersdorf, Austria.

Larviposition day	Approx. days before emergence when scanned	No. of pupae scanned	Cross-validation results		Prediction results[1] (%)	Test set results[2] (%)
			Accuracy (%)	No. factors		
December 17	1	120	86.0	6	93.2	
December 19	1	120	92.2	6	88.5	85.7
December 19	2	120	84.0	6	83.1	83.3
December 19	3	120	94.6	6	45.1	
December 21	4	120	93.7	4	59.9	
December 21	5	120	97.3	4	93.8	86.4
December 21	6	120	67.4	4	66.2	
December 25	7	60	64.8	4	51.9	
December 25	8	120	51.1	4	49.7	
December 25	9	60	42.1	4	61.4	
December 31	12	120	66.7	5	45.4	

[1] Fly sex predicted from a calibration developed from all flies 1–5 days before emergence (7 partial least squares regression factors, $n = 675$).
[2] Fly sex predicted from a calibration developed from 80% of flies 1, 2 and 5 days before emergence (7 partial least squares regression factors). The test set consists of the remaining 20%.

Classification results

Cross-validation classification results for subsequent tests conducted in Seibersdorf, Austria, gave results that ranged from about 84 to 97% for pupae 1–5 days before emergence (table 2). Classification accuracy dropped to less than 70% for pupae 6–12 days before emergence. These results were very similar to those achieved in the previous tests and confirmed the ability of near-infrared technology to determine the sex of tsetse fly pupae 1–5 days before emergence.

A calibration was developed from about 80% ($n = 322$, 7 factors) of flies one, two, and five days from emergence, then the remaining 20% ($n = 77$) were predicted. The sex of 87% of the 77 flies in the test set was correctly predicted by the calibration (table 2). These same 77 flies were correctly sexed in the cross-validation with an accuracy of 86% (data not shown). When a calibration was developed using flies from 1–5 days before emergence, and then all flies predicted, classification accuracies were similar to the cross-validation results except those 3–4 days from emergence (table 2). These results show that a single calibration that includes samples with different ages of pupae can give good classification results for some samples with different maturity levels, but some age groups are predicted poorly. The best prediction results were achieved when using calibrations for specific pupal age levels.

Effect of pupal maturity levels on spectra

Average spectra were examined to determine how they might be changing as a function of pupa1 age in an attempt to understand why classification accuracies by the cross-validation improved 3–4 days before emergence, but these same pupae were predicted poorly by the calibration that included several maturity groups. The average spectra for male and female tsetse flies changed as the pupae matured from six days to one day before emergence (fig. 2). The male and female spectra at six days before emergence were very similar in magnitude and shape. Male and female average spectra for 7–12 days before emergence were also similar (data not shown). At five days before emergence, the female spectra began to show a higher absorbance in the 950–1400 nm region, and a change in the shape of the spectra around 1100–1200 nm. This continued to four days before emergence. At three days before emergence, the male spectra showed a change, thus, at 4–5 days before emergence, females and males were easily separated based on their absorbance in the 1100–1400 nm region.

The predicted residual error sum of squares for pupae 1–5 days from emergence ranged from 3 to 6, whereas the predicted residual error sum of squares for pupae 6–12 days from emergence ranged from 10 to 14. The predicted residual error sum of squares value is the sum of the squared difference between the predicted and actual values. Thus, the prediction error was much greater for pupae that were more than five days from emergence than for pupae that were more mature.

While the absolute value of the beta coefficients predicting a fly's sex at one and five days before emergence showed common peaks around 950, 1050, 1390, 1450, and 1600 nm, the most notable differences between *G. pallidipes* one and five days before emergence occurred around 1200 nm and 1520 nm (fig. 3). Thus, the physical changes that occurred during the final five days before emergence that differentiate male and female flies affected the absorbance of near-infrared radiation in the 1200 and 1520 nm regions.

Beta-coefficients for calibrations developed for the pupae that were 6–12 days from emergence were noisy and difficult to interpret (data not shown). Thus, there appeared to be very little difference in male and female spectra at 6–12 days before emergence, which explains the poor classification accuracies for these age groups.

The sorting accuracy for males and female *G. pallidipes* was changed by modifying the rejection threshold. Values created from a cross-validation of 399 pupae that were one, two and five days from emergence showed that as the rejection threshold increased, the ratio of males to females in the flies to be released increased (table 3). For example, at a rejection threshold of 1.44, the ratio of males to females in the pupae to be irradiated and released was 80:20. At this

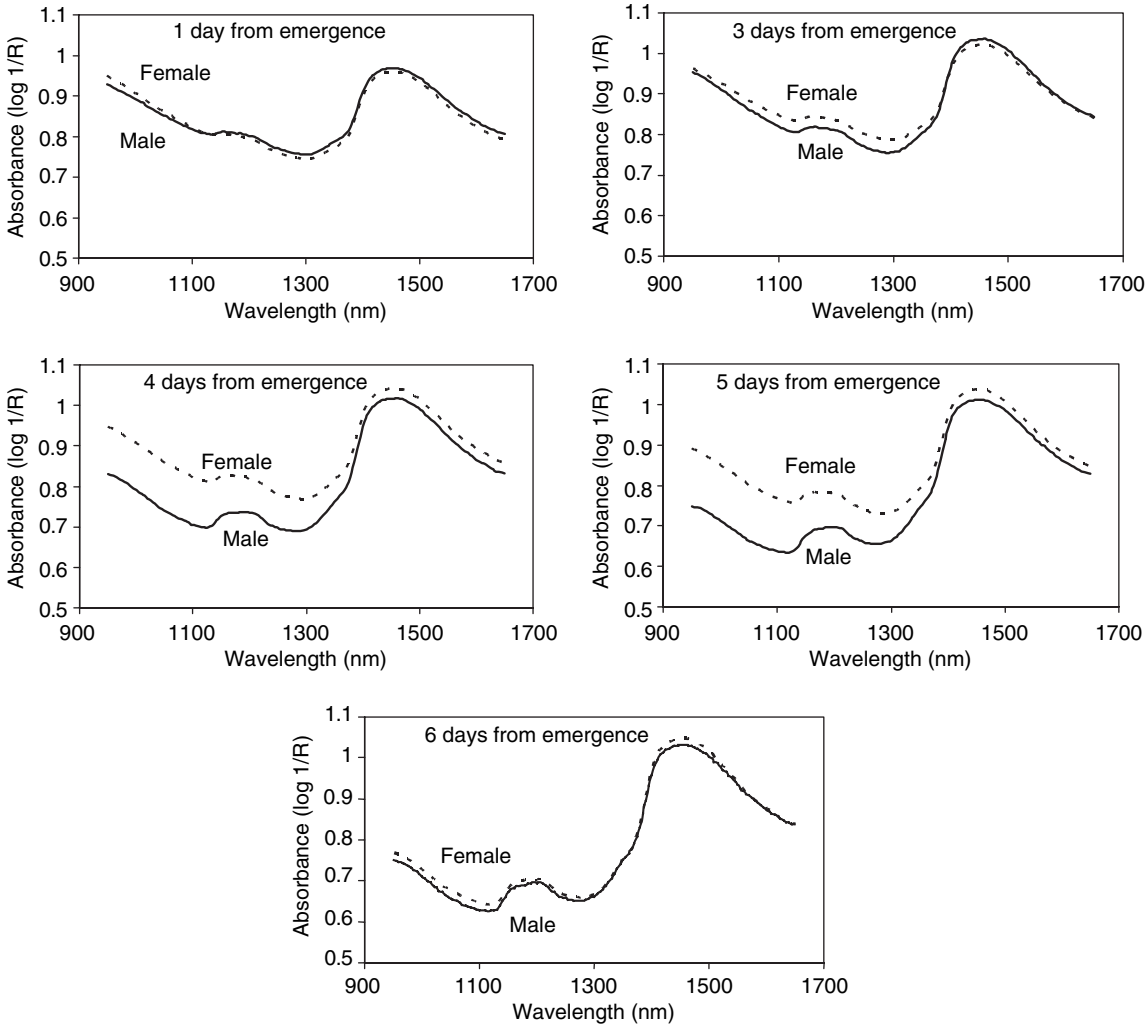

Fig. 2. Near-infrared spectra of *Glossina pallidipes* pupae from 1–6 days before emergence of adults.

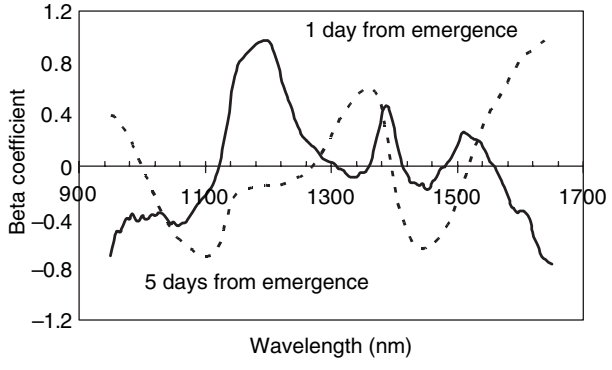

Fig. 3. Beta coefficients for partial-least-squares regression predictions for *Glossina pallidipes* one (—) and five (---) days before emergence of adults (four partial least squares regression factors). The absolute values of the coefficients indicate the importance of wavelengths for determining fly sex.

rejection threshold, 86.9% of all males will be available to be irradiated and released, and 80.8% of all females will be returned to the rearing colony.

Automated sorting results

Tests to sort bulk samples using the preliminary calibration developed in Manhattan, Kansas, with 79 samples showed the system could automatically scan and sort single pupae at a rate of about one pupa s^{-1} with an accuracy similar to that achieved when running the system in semi-automated mode. Depending on the sorting criteria used, the proportion of males among the flies available to be irradiated ranged from 70 to 100% (data not shown). While 79 samples were certainly not enough to develop a robust calibration, it did indicate the effectiveness of a calibration developed on a different set of samples many months earlier for sorting pupae, and the ability of the system to automatically scan and sort pupae.

Table 3. Effect of the sorting threshold on the ability to separate male from female *Glossina pallidipes* pupae. The calibration was developed from pupae 1, 2 and 5 days before emergence (n = 399, 7 partial least squares regression factors).

Sorting threshold	Ratio of males to females in irradiated and released pupae	% of all reared males in irradiated and released pupae	% of all reared females returned to the colony
0.92	50:50	100	8.6
1.06	55:45	100	25.5
1.17	60:40	98.4	40.4
1.24	65:35	97.4	52.4
1.33	70:30	95.3	63.0
1.40	75:25	92.7	72.1
1.44	80:20	86.9	80.8
1.52	85:15	79.6	87.5
1.61	90:10	70.2	93.8
1.70	95:5	57.1	97.6
1.93	100:0	24.6	100

Size effects

Pupae of *G. pallidipes* were divided into two groups using diverging rollers in tests to determine if pupal size affected classification accuracy. The size ranges were 2.96–3.23 mm and 3.23–3.41 mm diameter and the corresponding average weights of the two size categories were 31.1 mg (n = 60) and 33.9 mg (n = 58). Flies were about one day from emergence. There appeared to be no effect of pupal size on near-infrared classification accuracy with the smaller pupae classified with 87.5% accuracy and the larger with 89% accuracy. Mechanical sizing did not affect the sex ratio since those in the smaller size category were comprised of 27 females and 33 males, and those in the larger size category were comprised of 27 females and 31 males.

Tests with other species

This near-infrared technique was able to differentiate between the sexes of pupae of other muscids with accuracies of 51–74% (table 4). *Cochliomyia hominivorax* had the highest classification accuracy of these other species with a correct classification rate of 74%. *Musca domestica* had a correct classification rate of 63%, whereas *M. autumnalis*, *C. macellaria*, and *Stomoxys calcitrans* had poor classification rates of about 50%.

Table 4. Accuracy of sexing other fly species using near-infrared spectroscopy.

Species	Approx. days before emergence when scanned	No. of pupae scanned	Cross-validation accuracy (%)
Musca domestica	4	203	62.8
M. autumnalis	2	139	52.7
Cochliomyia macellaria	4	154	50.7
C. hominivorax	1	177	74.0
Stomoxys calcitrans	4	161	53.5

Discussion

Glossina pallidipes pupae can be sexed up to five days before emergence with accuracies that generally ranged from 80 to 100%. However, the other Diptera tested were classified with lower accuracy. The reason for this lower classification accuracy when compared to *G. pallidipes* flies might be based on the differences in reproductive physiology between these different fly groups. There does not appear to be a major gross sexual dimorphism in pupae of these muscid species, as the follicles are still undifferentiated and without yolk (pre-vitellogenic) in teneral females and spermatogenesis does not commence until after emergence in males. In contrast, the reproductive systems of tsetse pupae differ by sex as spermatogenesis occurs during the pupal stage and is complete by the time of adult emergence (Leak, 1998) and yolk deposition (vitellogenesis) is initiated about seven days before emergence (Saunders, 1961). The observed fluctuations in near-infrared efficiency in sexing tsetse pupae (peaking at 4–5 days pre-emergence) might be due to these differences of the reproductive systems. Improved classifications may result for all species tested if spectra were collected using a spectrometer that measures absorbance at wavelengths above 1700 nm.

This NIR-spectroscopy system has only been tested on the one species of tsetse, *G. pallidipes*, so far. Timing of the development of the reproductive system in different tsetse species is similar, and a similar result can be expected for other species. In addition to refining the calibration for *G. pallidipes*, future work will also look at other tsetse species.

Other methods of separating *G. pallidipes* by sex such as hand-sexing chilled adults and manipulating temperature during pupal development (Opiyo *et al*., 1999, 2000) can approach an accuracy of 100%. Hand-sexing using chilling is very labour intensive and although error rates are generally less than 1%, the chilling process itself can be detrimental to the flies. Sex separation by manipulating temperature during pupal development depends on critical timing and temperature control to achieve high accuracy, but in practice under colony conditions in Seibersdorf, Austria, the accuracy is often below 95% (unpublished data). Both these techniques separate sexes at the end of the pupal development period leaving minimal time for handling, irradiating or shipping male pupae before emergence. Although

chromosomal translocations have been studied in tsetse (Curtis, 1969; Curtis *et al*., 1972, 1973), they are associated with an approximately 50% reduction in fertility and selectable genes are not available.

Near-infrared spectroscopy therefore offers the best option for sexing tsetse. If pupal age is accurately known, then a model specific to that age group results in the best classification accuracy. Models that included a range of age groups resulted in lower classification accuracy for some age groups. The near-infrared spectra undergo rapid and significant changes as the flies mature from about six days until emergence. One possible procedure for implementing this technique would be to develop calibrations for each age group and then scan about 50 pupae from a sample that is to be sorted. The approximate physiological age could then be determined from this preliminary data and an appropriate calibration selected by, for example, examining the ratio of the absorbance at 950 and 1600 nm or the slope of the spectra around 1150 nm. A more advisable scenario may be to closely track the insect age and environmental conditions after the larviposition date and select the appropriate calibration for the maturity level of the sample to be sorted.

During future refinement of this technology, it is important to bear in mind the operational realities of using such a pupal separation system. It is probably unrealistic to expect a perfect separation that results in no misclassifications. However, the rejection threshold can be adjusted to either release more males, or return more females to the colony. If the rejection threshold is adjusted so that only males and no contaminating females are included in the cohort to be released, then the cohort returned to the colony will consist of all females and some misclassified males. In the tsetse sterile insect technique, sexing pupae has to serve two purposes. First, it has to facilitate the release of only sterilized males and second, it has to provide pupal material that can be used to stock production cages with flies at a ratio of about one male to four females. The optimum strategy to achieve these two goals using near-infrared spectroscopy still needs to be identified. If pupae can be efficiently sexed five days before emergence, it will also be possible to easily transport male pupae from rearing facilities to distant release sites.

These results demonstrate that efforts to expand the release of sterile tsetse flies should not be limited by the absence of automated sex-separation systems. Our approach of avoiding the difficulties of producing genetic sex separation approaches for various isolates and species also means that sex separation is probably now feasible for numerous tsetse species. The equipment used here has the capacity to sort 3600 pupae per hour, sufficient for programmes operating on the scale envisaged in the next few years. Operating several spectrometers in parallel can increase this capacity.

This research also shows that near-infrared spectroscopy can be used to track changes in the insects as they mature. Further research is needed to explain why the spectra change as the insects mature and the significance of the beta coefficients at specific wavelengths.

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References

Baker, J.E., Dowell, F.E. & Throne, J.E. (1999) Detection of parasitized rice weevils in wheat kernels with near-infrared spectroscopy. *Biological Control* **16**, 88–90.

Broce, A.B., Grodowitz, M.J. & Riley, J.G. (1988) Effect of the ionophore lasalocid on face fly (Diptera: Muscidae) larval survival and physical and chemical parameters of cattle feces. *Journal of the Kansas Entomological Society* **61**, 471–476.

Curtis, C.F. (1969) The production of partially sterile mutants in *Glossina austeni*. *Genetical Research* **13**, 289–301.

Curtis, C.F., Southern, D.I., Pell, P.E. & Craig-Cameron, T.A. (1972) Chromosome translocations in *Glossina austeni*. *Genetical Research* **20**, 101–113.

Curtis, C.F., Langley, P.A., Mews, A.R., Offori, E.D., Southern, D.I. & Bell, P.E. (1973) Sex ratio distortion and semi-sterility in the progeny of irradiated *Glossina morsitans*. *Genetical Research* **21**, 153–165.

Delwiche, S.R. (1993) Measurement of single-kernel wheat hardness using near-infrared transmittance. *Transactions of the American Society of Agricultural Engineers* **36**, 1431–1437.

Dowell, F.E. (1998) Automated color classification of single wheat kernels using visible and near-infrared reflectance. *Cereal Chemistry* **75**, 142–144.

Dowell, F.E., Throne, J.E. & Baker, J.E. (1998) Automated nondestructive detection of internal insect infestation of wheat kernels using near-infrared reflectance spectroscopy. *Journal of Economic Entomology* **91**, 899–904.

Dowell, F.E., Throne, J.E., Wang, D. & Baker, J.E. (1999) Identifying stored grain insects using near-infrared spectroscopy. *Journal of Economic Entomology* **92**, 165–169.

Dowell, F.E., Broce, A.B., Xie, F., Throne, J.E. & Baker, J.E. (2000) Detection of parasitized fly puparia by using near-infrared spectroscopy. *Journal of Near Infrared Spectroscopy* **8**, 259–265.

Food and Agriculture Organization (1992) *Training manual for tsetse control personnel. Volume 4. Use of attractive devices for tsetse survey and control*. Food and Agriculture Organization, Rome, Italy.

Galactic Industries (1996) *Grams/32 user's guide, version 4.0*. Galactic Industries Corp., Salem, New Hampshire.

Gooding, R.H., Feldmann, U. & Robinson, A.S. (1997) Care and maintenance of tsetse colonies. pp. 41–55 *in* Crampton, J.M., Beard, C.B. & Louis, C. (*Eds*) *The molecular biology of insect disease vectors: a methods manual*. London, Chapman & Hall Ltd.

Green, C.H. (1994) Bait methods for tsetse fly control. *Advances in Parasitology* **34**, 229–291.

Harris, R.L., Peterson, R.D., Vasquez-Guevara, M.E. & Graham, O.H. (1984) Gelled media for the production of screwworm larvae. *Southwestern Entomologist* **9**, 257–262.

Hendrichs, J., Robinson, A.S., Cayol, J.P. & Enkerlin, W. (2002) Medfly areawide sterile insect technique programmes for prevention, suppression or eradication: the importance of mating behavior studies. *Florida Entomologist* **85**, 1–13.

Knipling, E.F. (1955) Possibilities of insect control or eradication through the use of sexually sterile males. *Journal of Economic Entomology* **48**, 459–462.

Leak, S.G.A. (1998) *Tsetse biology and ecology: their role in the epidemiology and control of trypanosomosis*. 592 pp. Wallingford, Oxon, CABI Publishing.

McPheron, J.L. & Broce, A.B. (1996) Environmental components of pupariation-site selection by the stable fly (Diptera: Muscidae). *Journal of Economic Entomology* **25**, 673–684.

Miller, C.E. (2001) Chemical principles. pp. 19–37 *in* Williams, P.C. & Norris, K. (*Eds*) *Near-infrared technology in the agricultural and food industries*. St Paul, Minnesota, American Association of Cereal Chemists.

Opiyo, E., Luger, D., Nadel, D. & Feldmann, U. (1999) Automation in tsetse mass-rearing process: preliminary observations with *Glossina austeni*. pp. 187–192 *in* International Atomic Energy Agency (*Eds*) *Animal trypanosomosis: vector and disease control using nuclear techniques*. Leiden, Netherlands, Backhuys Publishers.

Opiyo, E., Luger, D. & Robinson, A.S. (2000) New systems for the large-scale production of male tsetse flies (Diptera: Glossinidae). pp. 337–344 *in* Tan, K.H. (*Ed.*) *Area-wide control of fruit flies and other insect pests*. Penerbit Universiti Sains Malaysia, Pulau Pinang, Malaysia.

Perez-Mendoza, J., Dowell, F.E., Broce, A.B., Throne, J.E., Wirtz, R.A., Xie, F., Fabrick, J.A. & Baker, J.E. (2002) Chronological age-grading of house flies by using near-infrared spectroscopy. *Journal of Medical Entomology* **39**, 499–508.

Rendón, P., McInnis, D., Lance, D. & Stewart, J. (2004) Medfly (Diptera: Tephritidae) genetic sexing: large-scale field comparison of males-only and bisexual sterile fly releases in Guatemala. *Journal of Economic Entomology* **97**, 1547–1553.

Rickwood, P. (2001) *Biting the fly. Eradicating one of Africa's most pernicious insect pests*. IAEA Division of Public Information, Vienna, Austria. http://www.iaea.org/About/Policy/GC/GC45/SciProg/sftsetse.html

Robinson, A.S., Franz, G. & Fisher, K. (1999) Genetic sexing strains in the medfly, *Ceratitis capitata*: development, mass rearing and field application. *Trends in Entomology* **2**, 81–104.

Saunders, D.S. (1961) Studies on ovarian development in tsetse flies (*Glossina*, Diptera). *Parasitology* **51**, 545.

Vreysen, M.J.B., Saleh, K.M., Ali, M.Y., Abdulla, A.M., Zhu, Z.-R., Juma, K.G., Dyck, V.A., Msangi, A.R., Mkonyi, P.A. & Feldmann, H.U. (2000) *Glossina austeni* (Diptera: Glossinidae) eradicated on the island of Unguja, Zanzibar, using the sterile insect technique. *Journal of Economic Entomology* **93**, 123–135.

WHO (1998) *Expert Committee on control and surveillance of African trypanosomiasis*, Technical Report Series, No. 881. World Heath Organization, Geneva, Switzerland.

WHO (2002) *The World Health Report. Reducing risks, promoting healthy life*. World Health Organization, Geneva, Switzerland.

Williams, P.C. & Norris, K. (2001) *Near-infrared technology in the agricultural and food industries*. 2nd edn. American Association of Cereal Chemists, St Paul, Minnesota.

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