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ARTICLE

Chronological Age-Grading of House Flies by Using Near-Infrared Spectroscopy

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ABSTRACT The sensitivity and accuracy of near-infrared spectroscopy (NIRS) was compared with that of the pteridine fluorescence technique for estimating the chronological age of house flies, *Musca domestica* (L.). Although results with both techniques were significantly correlated with fly age, confidence limits on predicted ages generally were smaller with NIRS. Young flies could be readily differentiated from old flies by using NIRS. Age predictions using the pteridine method are dependent upon size, sex, and temperature at which adult flies are exposed. In contrast, those factors do not need to be determined for age-grading using NIRS. Classification accuracy using the NIRS method was similar for whole flies, fresh heads, dried heads, and ethanol-preserved heads. The NIRS method was also suitable for predicting age of stable flies, *Stomoxys calcitrans* (L.), and face flies, *Musca autumnalis* De Geer. NIRS has several advantages over the measurement of pteridine levels for age-grading field-collected flies, including speed and portability of instrumentation, and not needing to determine sex, size, and temperatures to which adult flies were exposed.

KEY WORDS *Musca autumnalis*, *Musca domestica*, *Stomoxys calcitrans*, age-grading, near-infrared spectroscopy, pteridine

CHRONOLOGICAL AND PHYSIOLOGICAL age structures of an insect population have a significant impact on the dynamics of that population, including its rate of growth, behavior of its individuals, and effectiveness of control programs. Among dipteran vectors of pathogens, the age structure of a given population can affect the epidemiology of transmitted diseases. Whereas chronological age is a "unidirectional and irreversible course of events" leading the organism from birth to death and is related intimately to senescence (Collatz and Sohal 1986), physiological age relates to physiological maturation, has cyclical attributes, and is related to behavior. Age structures of insect populations have been determined mainly for Diptera and some Coleoptera. Methods for determining chronological and physiological age in insects of medical and veterinary importance, especially mosquitoes, other nematocerous disease vectors, and cyclorrhaphous higher Diptera, have been reviewed by Black and Moore (1996) and Hayes and Wall (1999). The majority of age-grading techniques are based on changes in the insect reproductive system, cuticular deterioration, or somatic changes (Hayes and Wall 1999).

With muscoid flies, physiological age determination has been accomplished by examining the state of ovarian development and parity status. In contrast, chronological age determination has been more difficult to attain with a high degree of accuracy, although various methods have been employed. For example, wing fray, or the progressive deterioration of fly wings, was used by Jackson (1946) to estimate age in the tse-tse fly *Glossina morsitans morsitans* Westwood. However, this method is a relatively imprecise technique for individual insects, and wing damage is clearly a direct result not of longevity, but of factors such as wing movement, predator attack, or abrasion from the habitat (Hayes and Wall 1999). Also, counting daily growth layers of cuticle in the thoracic apodemes was used to estimate age of sheep blowflies with limited success (Tyndale-Biscoe and Kitching 1974).

Searching for a more reliable method for assessing chronological age, Mail et al. (1983) discovered that the level of fluorescent pteridine pigments in heads of stable flies, *Stomoxys calcitrans* (L.), increased linearly as age and head size increased. Thus, by measuring the total fluorescence of head extracts, fly age could be predicted. Lysyk and Krafsur (1993) reported that the rate of pteridine accumulation was a function of temperature, suggesting that for this method to be accu-

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rately applied to field-collected flies, the effects of temperature on the accumulation rate had to be considered and the temperatures to which the flies had been exposed needed to be known. The pteridine method of age determination has been used in studies with other flies, including tse-tse flies (Lehane and Mail 1985); face flies, *Musca autumnalis* De Geer (Krafsur et al. 1995); blow flies, *Lucilia sericata* Meigen (Wall et al. 1991); horn flies, *Hematobia irritans* (L.) (Krafsur et al. 1992); house flies, *Musca domestica* L. (McIntyre and Gooding 1995); Mediterranean fruit flies, *Ceratitis capitata* (Weidemann) (Camin et al. 1991); and Mexican fruit flies, *Anastrepha ludens* (Loew) (Tomic-Carruthers et al. 1996). Although the pteridine fluorescence technique is relatively simple, it is time consuming and requires several laboratory instruments that cannot readily be taken to the field. Thus, a rapid, simple method of chronological age-grading that has the potential to be applied in field situations would be of considerable value.

We investigated the potential of near-infrared spectroscopy (NIRS) to determine the chronological age of several economically important flies that have veterinary and medical significance. As insects age, both chemical and physical changes occur (Sohal 1985), and these changes may significantly influence NIR absorption characteristics. All organic matter is composed of constituents possessing functional groups of atoms that absorb in the near-infrared region. These groupings include –CH-, -OH-, -NH-, and other groupings (Murray and Williams 1990). Near-infrared spectroscopy has been applied successfully to a number of entomological research problems including development of a rapid, automated NIR-based method for the in-kernel detection of hidden insects in wheat (Dowell et al. 1998); identification of several coleopteran species (Dowell et al. 1999); detection of parasitized weevils in wheat kernels (Baker et al. 1999); and detection of parasitized puparia of the house fly (Dowell et al. 2000). The ability to automate rapid NIR scanning and the potential development of hand-held NIR scanners appropriately calibrated for field use are two reasons for the interest in this technology and its application to these types of problems. If such a technology could be used to age-grade insects, it would have an impact on population analysis of insects of both agricultural and medical-veterinary importance.

In laboratory studies reported herein, we compared the effectiveness of NIRS with that of the pteridine method for determination of chronological age in adult houseflies that were exposed to different temperatures and flies that were stored using different preservation methods before assessing age. The NIRS method also was applied to age-grading of house flies with different head capsule sizes and to adults from cultures of face flies and stable flies.

Materials and Methods

Rearing Methods. Flies for the tests were from cultures maintained in the Department of Entomology at Kansas State University. House fly adults were from a > 20-yr-old strain. Flies were reared at 26 ± 2°C, 60% RH, and a photoperiod of 16:8 (L:D) h in a wheat bran and high protein feed supplement (Calf Manna, Manna Pro, St Louis, MO) medium. Face fly adults were from a 6-yr-old strain originally collected in Riley County, KS, and were reared at 25 ± 2°C, 70 ± 10% RH, and a photoperiod of 18:6 (L:D) h on fresh dung obtained from bulls fed a low energy maintenance diet (Broce et al. 1988). Stable fly adults were from a 9-yr-old strain from Riley County, KS, and were reared at 25 ± 2°C, 70 ± 10% RH, and a photoperiod of 18:6 (L:D) h in a vermiculite, wheat bran, and fish meal medium (McPheron and Broce 1996).

NIRS Data Collection. A near-infrared spectrometer (DA7000, Perten Instruments, Springfield, IL) was used to collect spectra from single whole insects and heads that were placed in a V-shaped trough. An 8-mm-diameter fiber-optic probe illuminated the insect, and a 2-mm-diameter fiber-optic probe carried reflected energy from the insect to the spectrometer. Dowell et al. (1999) gave a complete description of the spectra collection equipment and analysis procedure. All insects were placed with their ventral surface facing the reflectance probe, whereas single heads were placed face down to scan the posterior part of the head capsule. Eight spectra were collected from single insects, averaged, and stored in <1 s.

Measuring Pteridine Levels. Pteridine levels from individual fly heads were determined using a method modified from Mail et al. (1983) and Moon and Krafsur (1995). Before homogenization, head capsule widths of individual fly heads were determined using a binocular microscope equipped with an ocular micrometer. Individual fly heads were homogenized for 20 s in a 1.5-ml Kontes homogenizing tube (Kontes Glass, Vineland, NJ) containing 100 μl of degassed 50 mM Tris-Cl pH 8.6 buffer using an Eberbach Con-Torque Power Unit (model 7265, Eberbach, Ann Arbor, MI) equipped with a Kontes homogenizing pestle. Fly head homogenates were centrifuged at 2,000 g for 5 min. Aliquots (20 μl) of the fly head supernatants were added to 180 μl of the Tris buffer in a 96-well black microplate, and fluorescence was determined with a Fluoroskan Ascent FL fluorometer (Labsystems, Franklin, MA). Excitation and emission wavelengths were 357 and 450 nm, respectively. Several pteridines contribute to the total fluorescence measured in dipteran head capsules (Tomic-Carruthers et al. 1996). In our study, the amount of pteridine extracted from each fly head was calculated by using the average of three pterin (Aldrich, Milwaukee, WI) standard curve equations. Pteridine fluorescence was calculated in terms of pterin equivalents and expressed as relative head capsule fluorescence (RHCF), which is pmol of pterin per head divided by head capsule width (millimeters).

Whole Flies Versus Heads. This study was conducted to determine whether NIRS could be used for age-grading whole flies, fresh heads, or dried heads. One- and 10-d-old female house flies ($n = 50$ for each age group) reared at 26°C, 60% RH, and a photoperiod of 16:8 (L:D) h were killed with dichlorvos vapor and

scanned. Subsequently, heads were removed and scanned. Each head then was dried for 2 d over molecular sieves containing CaSO4 and rescanned. Flies of different ages were collected and scanned on the same day.

Preservation Method. An experiment was conducted to determine whether preservation method affected the ability of NIRS to age-grade flies. Three cohorts of female house flies were reared so that, on a given day, the ages of adult flies were 1, 7, and 11 d. Flies were reared at 26°C, 60% RH, and a photoperiod of 16:8 (L:D) h, and each age group consisted of 200 flies. Flies were killed with dichlorvos vapor, and heads were removed and scanned fresh. Then, 70 heads of each age group were placed in a desiccator containing CaSO4 desiccant and 120 were placed in 70% ethanol; pteridine levels were measured in the remaining 10 heads. At 2-wk intervals for 8 wk, NIR spectra were collected from all dried heads and 30 ethanol-preserved heads and pteridine levels were measured in 10 dried heads and 10 ethanol-preserved heads from each age group.

Temperature. This study was conducted to determine the effect of adult exposure to different temperatures on accuracy of age-grading using the NIRS method. Temperature is known to affect accuracy of age-grading using the pteridine method (Lysyk and Krafsur 1993). Cohorts of newly emerged mixed sex adult house flies, which were reared at 26°C, 60% RH, and a photoperiod of 16:8 (L:D) h, were maintained at 20, 25, and 30°C. All adults were supplied with powdered egg and sugar separately ad lib. On days 1, 4, 8, 12, 16, 20, 24, and 28 after emergence, batches of 100 males and 100 females were collected at 20°C and killed with dichlorvos vapor. Due to reduced longevity at 25 and 30°C, we only collected adults from days 1, 4, 8, 12, 16, and 20 at 25°C and from days 1, 4, 8, and 12 at 30°C after adult emergence. Heads were removed, transferred to individual sample tubes that contained several crystals of CaSO4, and stored in an environmental chamber at 25°C in the dark until heads from flies at all of the different ages were collected. One hundred heads from each sex, age, and temperature combination were scanned with NIRS. Eighteen fly heads from each sex, age, and temperature combination were also assayed for pteridine content.

Head Capsule Size. This study was conducted to determine whether fly head size affected accuracy of NIRS age-grading. Head capsule size is known to affect accuracy of age-grading using the pteridine method (Wall et al. 1991). House flies were reared at two different densities to obtain adults with different body sizes. Eggs laid in a 12-h period, by 1- to 2-wk-old adults, were collected and stirred in distilled water until thoroughly mixed. A volume of either 0.25 or 0.5 ml of eggs was added to 250 g of insect diet. This gave an approximate density of five and 10 eggs/g of diet and consistently produced large or small adults, respectively. After emergence, batches of 0- to 24-h-old adults were maintained at 26°C. Adults were supplied with powdered egg and sugar separately ad libitum. On days 2, 5, 8, 11, and 14 after emergence, groups of 50 males and 50 females from each colony were collected and killed with dichlorvos vapor. Heads were removed and head capsule widths of individual flies were recorded. All heads were transferred to individual sample tubes containing CaSO4 desiccant and stored in an environmental chamber at 25°C in the dark until all heads from the different chronological ages were collected. Then, heads were scanned with NIRS.

Face Flies and Stable Flies. A test was conducted to determine whether NIRS can be used to predict chronological age in other fly species. Female face flies (1 and 13 d old) and stable flies (1 and 14 d old) were tested. All age groups were supplied on the same day. Flies were killed with dichlorvos vapor, and their heads were removed and scanned. Face flies were scanned after 5 d of desiccation using CaSO4. Stable flies were scanned immediately after they were killed.

Statistical Analysis. Paired and multiple-comparison statistical analyses were performed on NIR spectra using partial least squares (PLS) regression (Galactic Industries 1996). The PLS regression takes advantage of the correlation between the spectral data and constituent concentrations. In paired comparisons, all combinations of ages were paired and analyzed. Cross-validation was used to determine classification accuracy. Results are presented as the classification accuracy (%) and the coefficient of determination (R^2) when the optimum number of PLS factors was used. The number of factors selected was based on the reduction in residual sum of squares achieved by adding PLS factors to the calibration models. The R^2 is the proportion of the sum of squares of the predicted age that can be attributed to the known age. The PLS regression reports the importance of wavelengths used in calibrations as beta coefficients. For any given wavelength, the absolute value of the beta coefficient indicates how important that wavelength was for classification. An age prediction is obtained by multiplying the absorbance at each wavelength by the corresponding beta coefficient, and then summing each of those products across all wavelengths. Thus, the beta coefficients represent the calibration model. The number of beta coefficients does not change as the number of factors changes. The spectral region from 550–1,700 nm was analyzed.

The relationship between pteridine concentration and age was determined by linear regression (PROC REG, SAS Institute 1998). The effects of temperature on the slopes of these regressions were determined by using analysis of variance (ANOVA) (PROC ANOVA, SAS Institute 1998). These data provided a measure of the daily change in pteridine concentration as a function of temperature and were used to estimate a temperature threshold for pteridine accumulation (Lysyk and Krafsur 1993). Accumulated degree-days above this threshold at the time of sampling were calculated by using this threshold and the constant temperature at which the adult flies were exposed. The relationship between pteridine concentration and accumulated degree-days was determined by using linear regression. Homogeneity of this relationship was based on

Table 1. Relationship between NIRS-predicted age and actual age in house flies stored using three different preservation methods

Head condition	Equation parameters			95% CL of predicted age (days)
	a ± SE	b ± SE	r^2	
All storage methods combined	1.02 ± 0.10	0.84 ± 0.01	0.83	3.8
Fresh	1.01 ± 0.11	0.84 ± 0.02	0.84	3.6
Dried 8 wk	1.07 ± 0.3	0.83 ± 0.04	0.80	4.4
Stored in ethanol for 8 wks	1.03 ± 0.37	0.83 ± 0.05	0.78	4.7

Relationship is y = a + bx, where y = NIRS-predicted age (days), x = actual age (days), and r^2 is the percentage variation in y accounted for by using a linear model.

the reduction of the error sum of squares of a model containing no temperature effects from the error sum of squares of a full model containing parameters for each temperature.

Based upon the combination of PLS factors and beta coefficients, calibration models were developed with the PLS software for each level of each factor studied (sex, temperature, size, and preservation method). With the pteridine method, calibration models were developed with linear regression (PROC REG, SAS Institute 1998) for each level of each factor studied (sex and temperature). More complex calibration models were developed for the NIRS and pteridine methods by combining across all levels of each factor studied. Finally, a combined calibration model that included all variables studied was developed for each of the two methods. We determined whether several levels of a factor could be combined in one model by using ANOVA (PROC ANOVA, SAS Institute 1998), and by comparing the complex and simpler models by examining R^2 values and confidence limits on predicted ages. We also conducted independent validations of the calibration models by using the calibration model from the temperature experiment to predict age of flies from the size experiment and vice versa.

Results

Whole Flies Versus Heads. In this test to determine the effectiveness of using either whole flies, fresh heads, or dried heads to predict age, 91.0% of fresh whole flies ($n = 200$, $R^2 = 0.67$), 88.5% of fresh heads ($n = 200$, $R^2 = 0.51$), and 89.3% of dried heads ($n = 197$, $R^2 = 0.52$) were classified correctly as being either

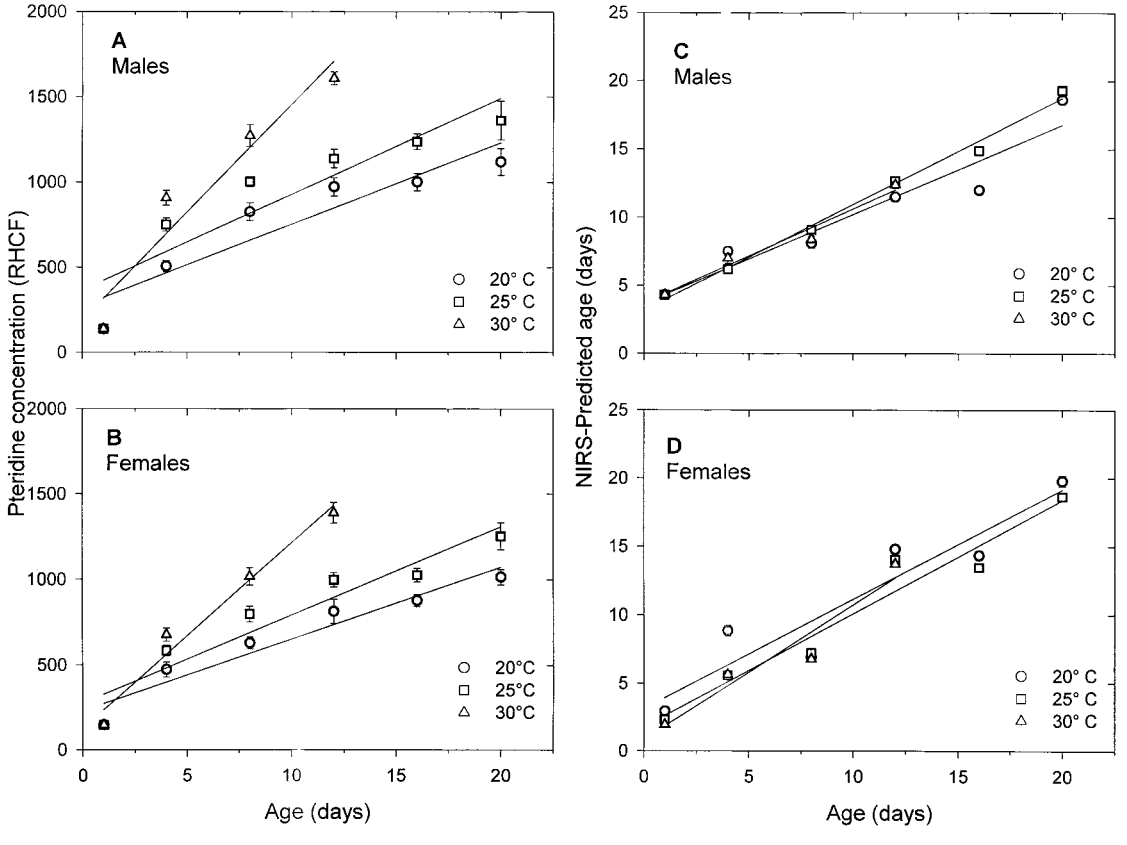

Fig. 1. Relationship between pteridine concentration (RHCF) and age of (A) male and (B) female and between NIRS-predicted age and actual age of (C) male and (D) female house flies at 20, 25, and 30°C. Lines are regression equations from Tables 2 and 3.

Table 2. Relationship between pteridine concentration and fly age under three temperature regimes

Temp, °C	Sex	Equation parameters			95% CL of predicted age (days)
		a ± SE	b ± SE	r^2	
20	F	274.8 ± 30.4	36.1 ± 1.8	0.76	10.1
	M	344.9 ± 40.2	38.4 ± 2.4	0.64	13.3
25	F	276.3 ± 40.2	51.6 ± 3.3	0.72	8.2
	M	366.9 ± 52.1	56.1 ± 4.3	0.62	10.4
30	F	128.7 ± 43.6	108.5 ± 5.8	0.85	3.6
	M	188.9 ± 50.9	126.7 ± 6.8	0.83	3.8

Relationship is y = a + bx, where y = relative head capsule fluorescence [pmol pteridine/head capsule width (mm)], x = actual age (days), and r^2 is the percentage variation in y accounted for by using a linear model.

1 or 10 d old. Thus, either whole flies or heads could be used for age-grading house flies.

Preservation Method. There was a strong relationship between NIRS-predicted age and actual age for all of the preservation methods (fresh: $F = 2{,}085$; df = 2, 605; $P < 0.01$; dry: $F = 232$; df = 2, 110; $P < 0.01$; and ethanol-preserved: $F = 161$; df = 2, 82; $P < 0.01$). Separate regressions were conducted for each preservation method (Table 1); however, the slopes and intercepts of these regressions were homogeneous among the preservation methods ($F = 0.48$; df = 2, 799; $P = 0.62$). Based on confidence limits on predicted ages, the best predictions of ages would be obtained by using fresh heads (Table 1). However, the confidence limits on predicted ages were similar for the three preservation methods and for the combined model. The relationship between NIRS-predicted age and actual age did not change over the 8-wk period (slopes and intercepts homogeneous over time, data not shown).

Chemical analyses showed negligible pteridine fluorescence in heads stored in 70% ethanol for 2 wk. In contrast, there was no significant change in the pteridine content in dried heads held for 8 wk over desiccant (data not shown).

Temperature. *Effects on Pteridine Concentration.* The relationships between pteridine concentration and age were significantly different among temperatures ($F = 106.9$; df = 2, 612; $P < 0.01$) and sexes ($F = 59.3$; df = 1, 612; $P < 0.01$) (Fig. 1 A and B). The interactions for age by temperature ($F = 11.3$; df = 8, 612; $P < 0.01$) and age by sex ($F = 2.84$; df = 7, 612; $P < 0.01$) also were significant. The rate of pteridine accumulation was greater at 30°C than at 20 or 25°C. Separate regressions between RHCF and age were conducted for each temperature and sex to predict age by using the pteridine method (Table 2).

Slopes of the regressions in Table 2 provide a measure of the change in RHCF per day at a given temperature. The relationship between slopes and temperature (T) for male flies was

$$\Delta\text{RHCF} = -147.19 + 8.84(\text{T}) \quad [1]$$

($F = 8.41$; df = 1, 1; $P = 0.21$; $R^2 = 0.89$) and for females was

$$\Delta\text{RHCF} = -115.62 + 7.24(\text{T}) \quad [2]$$

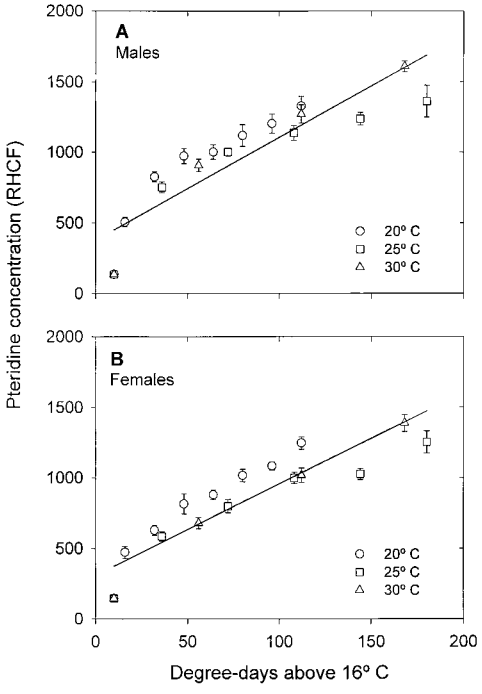

Fig. 2. Relationship between pteridine concentration (RHCF) and degree-days above 16°C for (A) male and (B) female house flies at 20, 25, and 30°C. Lines are from equations 3 and 4 in the text.

($F = 9.11$; df = 1, 1; $P = 0.20$; $R^2 = 0.90$). According to Lysyk and Krafsur (1993), the threshold for pteridine accumulation can be calculated from these equations as $-a/b$, where a and b are the intercepts and slopes, respectively, of the equations. With our laboratory conditions, the temperature thresholds were calculated to be 16 and 16.6°C for female and male house flies, respectively. We used a threshold of 16°C for both sexes.

The relationship between pteridine concentration and accumulated degree-days above 16°C (DD16) for males was

$$\text{RHCF} = 377.1 + 7.28*\text{DD}_{16} \quad [3]$$

($F = 548.3$; df = 1, 322; $P < 0.01$; $R^2 = 0.63$) and for females was

$$\text{RHCF} = 309.5 + 6.46*\text{DD}_{16} \quad [4]$$

($F = 609.4$; df = 1, 286; $P < 0.01$; $R^2 = 0.68$). Slopes of the relationships were homogeneous among temperatures for both males ($F = 0.10$; df = 4, 323; $P = 0.75$) and females ($F = 2.67$; df = 4, 287; $P = 0.10$). Males accumulated pteridines faster than females (Fig. 2 A and B). Confidence limits of the predicted ages were smaller and R^2 values were higher for the models developed using data from flies maintained at 30°C than for those models developed using data from the flies maintained at 20 or 25°C (Table 2).

Effects on NIRS-Predicted Age. The relationship between NIRS-predicted age and actual age was signif-

Table 3. Relationship between NIRS-predicted age and actual age in house flies under three temperature regimes

Temp, °C	Sex	Equation parameters			95% CL of predicted age (days)
		a ± SE	b ± SE	r^2	
20	F	3.02 ± 0.26	0.78 ± 0.02	0.77	5.4
	M	3.28 ± 0.25	0.70 ± 0.02	0.73	6.2
25	F	1.77 ± 0.20	0.83 ± 0.02	0.82	3.9
	M	3.00 ± 0.24	0.82 ± 0.02	0.73	4.7
30	F	1.66 ± 0.18	0.74 ± 0.02	0.72	4.3
	M	3.71 ± 0.29	0.70 ± 0.04	0.64	4.5

Relationship is y = a + bx, where y = NIRS-predicted age (days), x = actual age (days), and r^2 is the percentage variation in y accounted for by using a linear model.

icantly different among temperatures ($F = 7.43$; df = 2, 3,491; $P < 0.01$) and sexes ($F = 10.7$; df = 1, 3,491; $P < 0.01$) (Fig. 1 C and D). The interactions for age by temperature ($F = 12.1$; df = 8, 3,491; $P < 0.01$) and age by sex ($F = 15.5$; df = 7, 3,491; $P < 0.01$) were also significant. Therefore, separate regressions were fit for each sex and temperature (Table 3). However, the slopes of the regressions in Table 3 were homogeneous among temperatures for both males ($F = 4.41$; df = 4, 1,779; $P = 0.07$) and females ($F = 0.24$; df = 4, 1,712; $P = 0.62$). Confidence limits of the NIRS-predicted ages were smaller for the models developed using data from flies maintained at 25 or 30°C than for the model developed using data from the flies maintained at 20°C (Table 3). Confidence limits were smaller with NIRS than with the pteridine method, except at 30°C (Tables 2 and 3), and confidence limits were more homogeneous across all temperatures with the NIRS method than with the pteridine method. R^2 values also indicated that predictions were better and more homogeneous with NIRS than with the pteridine method, except at 30°C, where R^2 values were higher for the pteridine method.

Greater NIR absorbance was observed in younger insects (Fig. 3). Difference spectra (not shown) indicated that the largest differences among spectra occurred at 1380 and 1700 nm.

Head Capsule Size. Head capsule widths of house flies reared at different densities varied significantly for males [2.3 ± 0.15 versus 1.8 ± 0.16 mm for low and high densities, respectively ($F = 2,693$; df = 1, 430; $P < 0.01$)] and females [2.4 ± 0.09 versus 1.9 ± 0.17 mm for low and high densities, respectively ($F = 4,433$; df = 1, 448; $P < 0.01$)]. ANOVA indicated that head capsule size had a significant effect on NIRS-predicted age ($F = 8.4$; df = 1, 803; $P = 0.01$). However, sex had no significant effect ($F = 0.3$; df = 1, 803; $P = 0.60$). The interactions for age by size ($F = 10.5$; df = 4, 803; $P < 0.01$) and age by sex ($F = 4.0$; df = 4, 803; $P < 0.01$) were significant, whereas the interaction for size by sex ($F = 0.9$; df = 1, 803; $P = 0.35$) was not significant. Therefore, separate regressions between NIRS-predicted age and actual age were conducted for each size and sex (Table 4). The slopes of these relationships were heterogeneous ($F = 3.6$; df = 4, 803; $P < 0.01$). Confidence limits of the NIRS-predicted ages and R^2 values were also heterogeneous among the different models (Table 4).

Accuracy of Combined Calibration Models. In the previous sections, we developed calibration models for each level of each factor studied. In this section, we develop calibration models combining data for all levels of each factor studied to determine whether one needs to know sex, head capsule size, or temperature at which flies were maintained to determine their chronological age using NIRS.

Temperature Study. For flies maintained at different temperatures, confidence limits on predicted ages generally were smaller and more homogeneous using the NIRS method (±4.4–7.3 d) than with the pteridine method (±4.0–17.5 d) when we combined across levels of temperature and/or sex (Table 5). Confidence limits for the pteridine method were smaller than for the NIRS method only at 30°C. R^2 values also were higher with NIRS than with the pteridine method, except at 30°C, where R^2 values were similar (Table 5). Thus, one can predict the chronological age of a house fly with confidence limits of ±1 wk using the NIRS method without knowing sex or the temperature at which the fly was exposed. Knowing the sex or the temperature at which the flies were exposed can decrease confidence limits to 3.9–6.2 d, depending upon the sex and the temperature (Table 3).

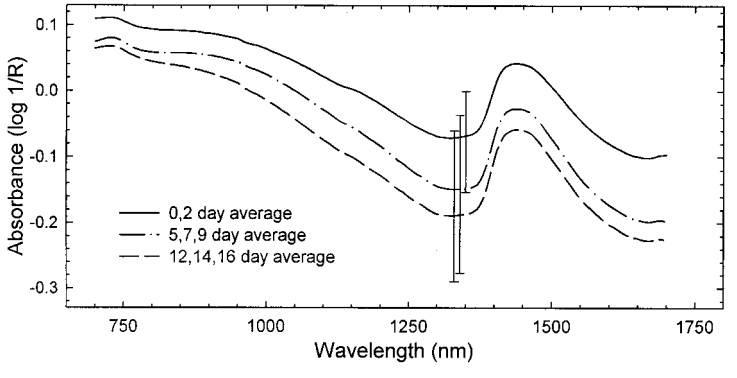

Fig. 3. Average NIR absorption spectra for heads of young (0, 2 d), medium-aged (5, 7, 9 d), and old (12, 14, 15 d) house flies. Error bars show an example of variation (2 SE) in absorbance for the three age groups at one wavelength.

Table 4. Relationship between NIRS-predicted age and actual age in large and small house flies

Fly sex and size	Equation parameters			95% CL of predicted age (days)
	a ± SE	b ± SE	r^2	
Large ♂'s	2.53 ± 0.32	0.68 ± 0.04	0.65	5.8
Large ♀'s	3.13 ± 0.36	0.61 ± 0.04	0.55	7.4
Small ♂'s	2.28 ± 0.26	0.75 ± 0.03	0.77	4.5
Small ♀'s	3.14 ± 0.28	0.63 ± 0.03	0.65	6.1

Relationship is y = a + bx, where y = NIR-predicted age (days), x = actual age (days), and r^2 is the percentage variation in y accounted for by using a linear model.

Size Study. Confidence limits on predicted ages for flies with different head capsule sizes varied from 6.2 to 7.4 d when we combined across levels of size and/or sex (Table 5). Thus, one can predict the chronological age of a house fly with confidence limits of ±1 wk using the NIRS method without knowing the sex or head capsule size of the fly. Knowing the sex or head capsule size might reduce the confidence limits to 4.5–7.4 d, depending upon the sex and head capsule size.

Combined Data from Temperature and Size Studies. When we combined across all levels of temperature and size, or across all levels of sex, size, and temperature, confidence limits on predicted ages were about ±1 wk (Table 5). Thus, we can use the calibration model that combines data from the size and temperature experiments to predict the chronological age of house flies of unknown sex, size, or temperature at which flies were exposed with accuracy of ±1 wk. Confidence limits of NIRS-predicted age with the combined calibration models were almost always higher than the confidence limits of the calibration models developed for a single level of a factor. This was expected because the calibration models developed for the single level of a factor were optimized for that one level.

The beta coefficient plot from this combined calibration model indicated that absorption regions corresponding to CH functional groups contributed most to classifications (Fig. 4). Wavelengths corresponding to the first, second, and third CH absorption overtones of CH3, CH2, and CH groups and first combination CH absorption overtones (850–950, 1,125–1,225, 1,350–1,450, and 1,650–1,700 nm) are likely responsible for the ability to differentiate young and old flies using NIRS (Murray and Williams 1990).

Independent Validation of Combined Calibration Model. A combined calibration model that included all data from the size study predicted age [young (≤6-d-old) or old (>6-d-old)] of flies exposed to different temperatures with 83% accuracy ($n = 881$, $R^2 = 0.74$). A combined calibration model that included all data from the temperature study predicted age [young (≤6 d old) or old (>6 d old)] of flies with different head capsule sizes with 80% accuracy ($n = 1{,}954$, $R^2 = 0.83$). Thus, a calibration model that was developed using data for flies exposed to 26°C accurately predicted age of flies exposed to 20–30°C, and a model that was developed using data for flies of mixed unknown head capsule sizes accurately predicted age of very small and very large flies.

Face Flies and Stable Flies. Accuracies of classifications between young (≤3 d old) and old (≥7 d old) stable flies and face flies were 93% ($n = 259$, $R^2 = 0.65$) and 89% ($n = 255$, $R^2 = 0.58$), respectively.

Discussion

Confidence limits on predictions obtained by using the NIRS method generally resulted in more accurate estimations of age than did the method of measuring pteridine levels. NIRS can be used to determine chronological age of house flies within ±1 wk without knowing the sex, head capsule size, or temperatures at which the flies have been exposed. The pteridine method exceeded this level of accuracy only for flies of known sex and head capsule size reared at 30°C. If sex and field temperatures are determined, then confidence limits on age predictions using the NIRS method can be reduced to 3.9–6.2 d. NIRS can be successfully used to classify house flies as young or old

Table 5. Confidence limits and R^2 values for ages of house flies predicted by using the near-infrared spectroscopy (NIRS) or the pteridine methods

Experiment	Data included in model	NIRS predictions		Pteridine predictions	
		95% Conf. Limit	r^2	95% Conf. limit	r^2
Temperature	All sexes and temperatures	7.3	0.80	16.5	0.47
	Males, all temperatures	6.6	0.82	17.5	0.44
	Females, all temperatures	6.0	0.84	14.3	0.55
	Both sexes, 20°C	6.3	0.77	12.2	0.68
	Both sexes, 25°C	4.6	0.79	9.8	0.64
	Both sexes, 30°C	4.4	0.78	4.0	0.81
Size	All sexes and sizes	7.1	0.56	—	—
	Large males and females	7.0	0.57	—	—
	Small males and females	6.7	0.61	—	—
	Males all sizes	6.2	0.63	—	—
	Females all sizes	7.4	0.54	—	—
Combined	All sexes, temperatures, and sizes	7.1	0.79	—	—
	Males, all temperatures and sizes	7.3	0.78	—	—
	Females, all temperatures and sizes	6.9	0.80	—	—

—, The pteridine method was not used in the experiment to determine whether head capsule size affects ability of NIRS to age-grade flies.

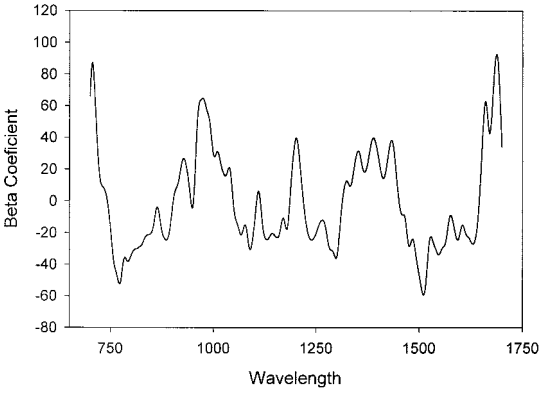

Fig. 4. Beta coefficients resulting from partial least squares calibrations to age-grade house flies with NIR spectra obtained from the combined calibration model.

when whole insects, fresh heads, or preserved heads are used, although we recommend developing a separate calibration for each preservation method to improve classification accuracy. The pteridine method is not useful for age-grading ethanol-preserved flies.

NIR absorbance decreased in heads of older house flies (data not shown), giving a slightly asymptotic relationship with fly age that was similar to that of pteridines and head capsule fluorescence (Fig. 1 A and B). Although head capsule pteridines probably contribute to the total NIR absorbance, and thus may have contributed to classification by NIRS, there is no evidence that NIRS is just indirectly measuring head capsule pteridine content. For example, when samples of analytical grade pteridine were scanned (data not shown), absorbance peaks for the pure compound did not correspond to all absorbance peaks used for NIRS classification of fly age. Instead, based on beta coefficients, absorbance regions corresponding to CH3, CH2, and CH groups were the most important for NIRS classification of fly age in both individual and combined models. These chemical moieties are common constituents of most insect cuticular and internal lipids. Generally, lipids are composed of hydrocarbons, wax and sterol esters, alcohols, and free fatty acids (Jackson et al. 1974, Blomquist and Dillwith 1985). It is possible that differences in total lipids or lipid composition may be responsible for NIR absorption differences between young and old flies. There is evidence that changes in amount and composition of lipids in some muscoid flies occur during aging. For example, Silhacek et al. (1972) reported an appreciable increase in cuticular hydrocarbon content in male and female house flies between ages 0–5 d. They also showed significant changes in ratios of several n-alkenes in older adults. Mpuru et al. (2001) also found that marked changes occurred in the composition of external lipids as house flies aged. The same pattern was also found in adult fleshflies, *Sarcophaga bullata* Parker, in which the quantity of extracted cuticular lipids in 7-d-old adults was 10–14 times greater than the quantity of extracted lipids from newly emerged adults (Jackson et al. 1974). Sohal (1985) reported that the relative concentrations of linoleic acid in adult house flies decreased with age, while oleic and palmitoleic acids increased. He also noted that the relative degree of lipid unsaturation tended to be relatively greater in younger flies. Although lipids of several dipteran species are known to undergo age-related changes during the adult stage, additional studies are needed to directly link these changes in the house fly to age classification by NIRS. In another study (unpublished data), the authors used NIRS to age-grade adult rice weevils, *Sitophilus oryzae* (L.) with and without cuticular lipids (cuticular lipids removed with a hexane rinse). Results showed that the calibration model generated from insects without lipids was less robust and the degree of correct age classification was lower (≈25%) compared with the model generated from insects with lipids. This suggests that cuticular lipids are not the only chemicals responsible for NIRS classification, but most likely NIRS absorbance is influenced by changes in both external and internal lipids. Other evidence that NIRS is not just indirectly measuring pteridine content of head capsules is that there was no measurable pteridine in ethanol-preserved heads, yet we were able to estimate chronological age of ethanol-preserved heads using NIRS. Further studies relating NIR absorbance to insect chemical composition eventually may show which chemicals are responsible for NIRS classifications and may show why the NIRS method for age grading is not sensitive to temperatures at which flies have been exposed.

The threshold temperature for pteridine accumulation in the house fly, as measured by head capsule fluorescence, was estimated by regression to be ≈16°C for both males and females. This threshold temperature was ≈10°C higher than that reported by Lehane et al. (1986) and Lysyk and Krafsur (1993) for pteridine accumulation in stable flies, a difference that may be related to the different habitat of the two species. At temperatures above the threshold, we found a relationship between the accumulation of pteridine pigments in head capsules and house fly age that was slightly asymptotic. This relationship in rate of pteridine accumulation in older house flies, relative to young flies, was documented previously by McIntyre and Gooding (1995). Because head capsule pteridine accumulation is thought to be a form of storage excretion, these latter authors hypothesized that house flies may have a threshold metabolic rate and that nitrogenous wastes may accumulate as pteridines when metabolism exceeds this basal rate. They also suggest that older house flies may produce decreased amounts of pteridine precursors, which would correspond to the decreased head capsule fluorescence noted in this species. A reduction in rate of pteridine accumulation was also found in older fruit flies (Camin et al. 1991) and face flies (Moon and Krafsur 1995).

Use of NIRS for age-grading flies has several advantages over the pteridine method. The most significant advantages are that age-grading by NIRS is a rapid and nondestructive technique, and that sex, size, and tem-

peratures at which adult flies have been maintained do not need to be determined if these variables have been included in developing the calibration model. In fact, our independent validation studies showed that data for flies exposed to different temperatures do not need to be included in the calibration model. However, we would suggest using a composite sample of both sexes of flies exposed at different temperatures and varying random head capsule sizes to develop a robust calibration model. In addition, the NIRS method may be used for fresh, dried, or ethanol-preserved heads. In contrast, accuracy of age-classification by the pteridine method requires correction for sex, head capsule size, and temperatures to which adult flies have been exposed (McIntyre and Gooding 1995), and is not suitable for use with ethanol-preserved heads. NIRS offers a significant advantage over the pteridine method for age-grading Diptera because this technique lends itself to automation and provides for the potential development of hand-held NIR scanners for field use. We anticipate that portable NIR units will have a significant impact on population analysis of flies of both medical and veterinary importance.

For researchers to use the NIRS technique, a spectrophotometer with a similar wavelength range (minimum 700–1,700 nm) coupled to a fiber optic probe should be obtained and new calibrations specific to that instrument should be developed. Our calibrations are not transferable because calibrations are unique to each instrument. However, PLS beta coefficients obtained with any NIRS instrument should be similar to those shown in Fig. 4. To improve classification accuracy with the NIRS method, we recommend developing a calibration model that includes data from adult males and females of random sizes that have been maintained at two or three different temperatures. This model could be used for age-grading without knowing sex, head capsule size, or field temperatures.

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