Medical and Veterinary Entomology

Medical and Veterinary Entomology (2010) **24**, 32–37

Age-grading the biting midge *Culicoides sonorensis* using near-infrared spectroscopy

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Abstract. Age-grading of insects is important in the control and monitoring of both insect populations and vector-borne diseases. Microscopy and morphological techniques exist to age-grade most blood-feeding flies, but these techniques are laborious, often destructive to the insects, and slow. Near-infrared spectroscopy (NIRS) can be automated and is a non-destructive technique for age-grading. We applied NIRS techniques to age-grade females of the biting midge, *Culicoides sonorensis* Wirth & Jones (Diptera: Ceratopogonidae), the vector of bluetongue and other arboviruses in North America. Female flies of five known age cohorts (1, 3, 6, 9 and 12 days post-emergence) from three laboratory colonies were used. The data indicate that NIRS can be used to differentiate age groups of *C. sonorensis*.

Key words. *Culicoides*, age-grading, bluetongue, near-infrared spectroscopy.

Blood-feeding species of Ceratopogonidae, commonly known as biting midges, no-see-ums or sandflies, are annoying pests and transmit arboviruses, malarial parasites and filarial nematodes to humans, domestic animals and wildlife (Borkent, 2005). In North America, *Culicoides sonorensis* (Diptera: Ceratopogonidae) is the most economically important ceratopogonid because it transmits bluetongue and epizootic haemorrhagic disease viruses (BTV and EHDV) (Foster *et al*., 1963, 1977) and several other arboviruses (Borkent, 2005). In addition, *C. sonorensis* is an experimental vector of exotic viruses including Akabane and African horse sickness viruses (Boorman *et al*., 1975; Jennings & Mellor, 1989). *Culicoides sonorensis* is a member of the *Culicoides variipennis* species complex that was recently revised by Holbrook *et al*. (2000). The *C. variipennis* species complex includes *Culicoides occidentalis* Wirth & Jones, *C. sonorensis* and *C. variipennis* (Coquillett).

Age-grading of female *C. sonorensis* is significant for vector monitoring, control, arbovirus surveillance and for gaining insight into the age distribution of field populations of *Culicoides*. In order to transmit BTV or EHDV, female midges must feed on a viraemic host, become infected, and survive the extrinsic incubation period before they can transmit virus to a new host. The extrinsic incubation period for transmission of BTV or EHDV in *C. sonorensis* lies in the range of approximately 5–26 days depending on the virus strain and environmental temperatures (Whittmann *et al*., 2002). The targeted control of biting midges would be most significant if flies are older and potentially feed on viraemic animals.

Currently, age-grading of *Culicoides* is based on microscopy. Several techniques exist to age-grade females of *Culicoides* spp. and to determine if they are parous or nulliparous. These techniques include microscopic dissection of the ovaries or observations of a red pigment in parous females (Dyce, 1969). This study discusses the use of near-infrared spectroscopy (NIRS) to age-grade females of *C. sonorensis.* This technique has been used to differentiate species of Lepidoptera (Jia *et al*., 2007), to determine the sex of tsetse pupae (Dowell *et al*., 2005) and to detect insect pests in stored grains (Dowell *et al*.,

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1998). It has also been used to age-grade stored-product beetles (Perez-Mendoza *et al*., 2004) and houseflies (Perez-Mendoza *et al*., 2002), but it has not been tested on insects as small as *Culicoides* or tested on independent test sets. NIRS can be automated and a large number of insects can be processed quickly. The technology is non-destructive and insects can be tested by polymerase chain reaction (PCR) or virus isolation after age-grading.

Materials and methods

Insects

Maintenance of three continuous colonies of *C. sonorensis* named VanRyan (VR), Ausman (AU) and AK at the U.S. Department of Agriculture (USDA) Arthropod-Borne Animal Diseases Research Laboratory was described by Hunt (1994). The VR colony originated from California, AU from Colorado and AK from Idaho. We tested five distinct ages of female midges. Adult midges were collected from each colony as they emerged from pupae 12, 9, 6 and 3 days prior to testing. Midges were maintained on a 10% sugar water diet. One-day-old midges from each colony were collected as they emerged on the day of testing. On the day of testing live midges were removed from their holding cages with aspirators and were killed by fumigation with 99% triethylamine for 15 min prior to testing. At least 50 female midges were sorted onto a Spectralon diffuse reflectance plate (Labsphere, North Sutton, NH, U.S.A.) for testing. Males were excluded because they are not significant as vectors of disease.

Voucher specimens from each colony of *C. sonorensis* were deposited in the University of Wyoming Insect Museum.

NIR spectra collection

An NIR QualitySpec Pro spectrometer (ASD, Inc., Boulder, CO, U.S.A.) was used to collect all spectra. The spectrometer measures absorbance from 350 nm to 2500 nm using silicon and indium-gallium-arsenide sensors. A 3-mm diameter bifurcated fibre-optic probe was positioned 2 mm above a 2.5-cm diameter Spectralon reflectance standard. The probe had about 50 illumination fibres and five reflectance fibres. A Mikropack HL-2000 halogen light source (Mikropack GmbH, Ostfildern, Germany) was used for illumination. The spectrometer was optimized and a baseline collected using RS^3 software (Version 3.1) from ASD, Inc.

At least 20 females were positioned on the 7.6-cm diameter Spectralon plate for scanning and the probe was positioned 2 mm above this plate. The insects were manually positioned under the probe and spectrum collected for each insect. Positioning insects under the probe and collecting spectra required less than 1 min per insect.

For data analysis, spectra were converted using ASD ViewSpecPro to a format that could be imported into GRAMS (Thermo Scientific, Salem, NH, U.S.A.). Partial least squares (PLS) regression was used to analyse the spectra and to develop calibration models. Perez-Mendoza *et al*. (2004) give a description of this statistical technique for analysing NIR spectra and classifying test sets. The accuracy of the classification models was determined by the standard error of cross-validation (SECV) using a one-sample-out procedure (Williams, 2001), and by predicting independent test sets. The number of regression factors used in the PLS prediction models depended on the realizing of no significant additional decrease in the predicted residual sum of squares. Least significant differences (LSDs) were calculated for each age group.

Results

The results from the PLS cross-validation showed that the age of insects in the three colonies could be predicted with r^2 values of >0.70 (Table 1). Cross-validation results showed significant differences ($P < 0.05$; LSD $\approx$ 0.60 days) between all age groups. Generally the 1-, 3- and 6-day-old insects were predicted by NIRS to be older than their actual age, and the 12-day-old insects were predicted to be younger than their actual age. However, the LSDs showed that the average predicted age of each age group was significantly different from that of adjacent age groups. Although the average ages were significantly different in the cross-validation, there was overlap between age groups resulting in some misclassifications. Figure 1 shows that although there was overlap between adjacent age groups, young (1- and 3-day-old) insects overlapped very little with older (9- and 12-day-old) insects when analysed using a

Table 1. Actual and predicted midge age when using a cross-validation (CV) with the specified number of partial least squares regression factors (F) and when using the VR colony as the calibration set to predict the colonies.

	VR		AU		AK	
Age, days	CV ($r^2 = 0.77$, 7F)	VR prediction	CV ($r^2 = 0.77$, 10F)	VR prediction	CV ($r^2 = 0.71$, 10F)	VR prediction
1	1.8	1.7	1.3	2.2	2.0	2.1
3	3.4	3.1	3.9	5.5	4.0	5.4
6	7.3	7.3	6.9	7.8	6.6	7.7
9	9.3	9.3	8.5	9.4	8.7	9.4
12	10.0	10.0	10.1	9.6	10.0	9.8
LSD	0.63	0.63	0.63	0.64	0.60; 1.0*	0.62; 1.3*
n	47	47	48	48	52; 28*	52; 28*

*LSD for the AK 1- vs. 3-day-old midges. There were only 28 1-day-old midges and thus the LSD (least significant difference) was calculated based on unequal replicates for this comparison.

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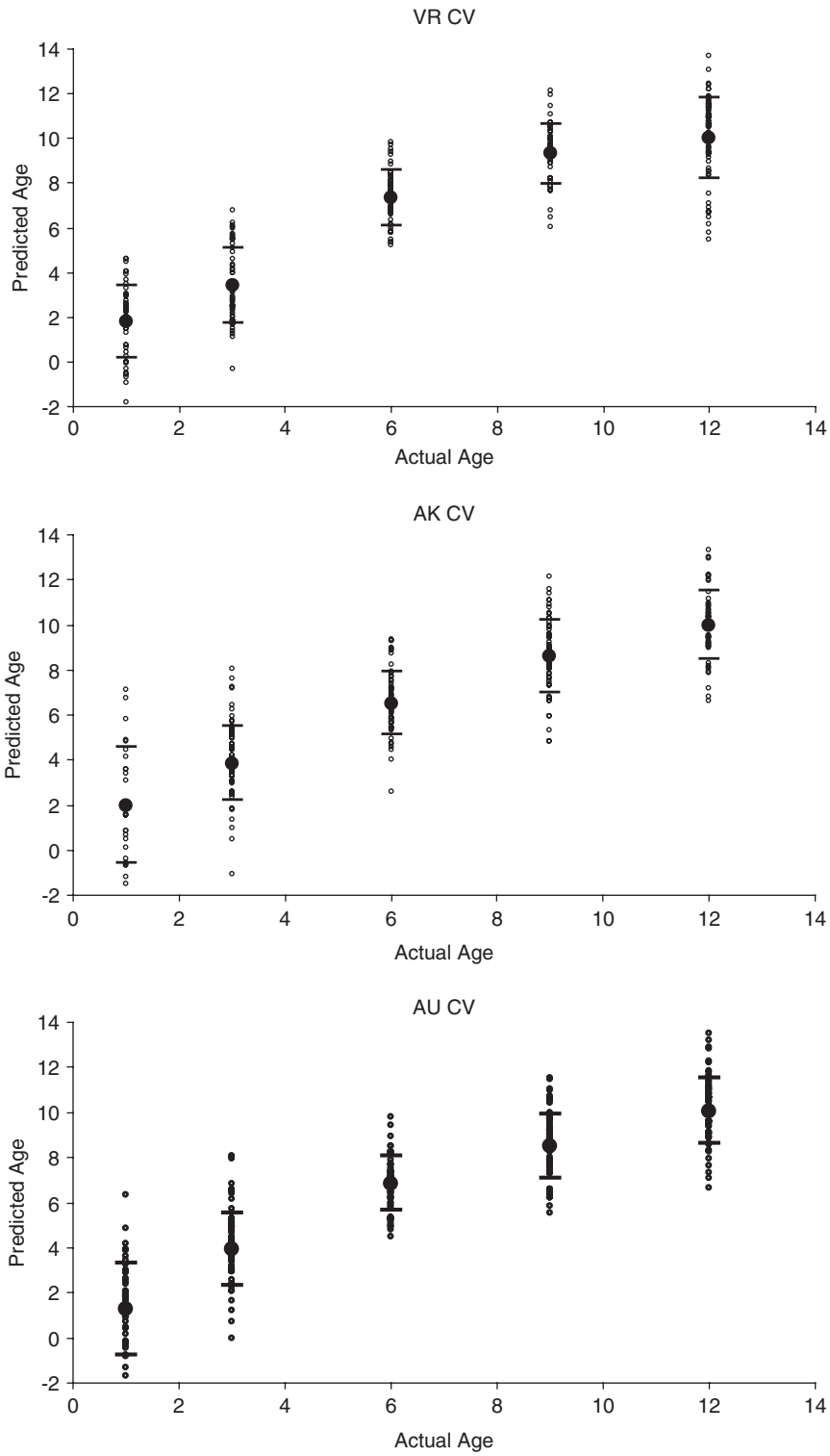

Fig. 1. Actual vs. predicted age of midges by colony (VR, AK, AU) when using a cross-validation (CV). Averages and values for ±1 standard deviation are marked on the plots.

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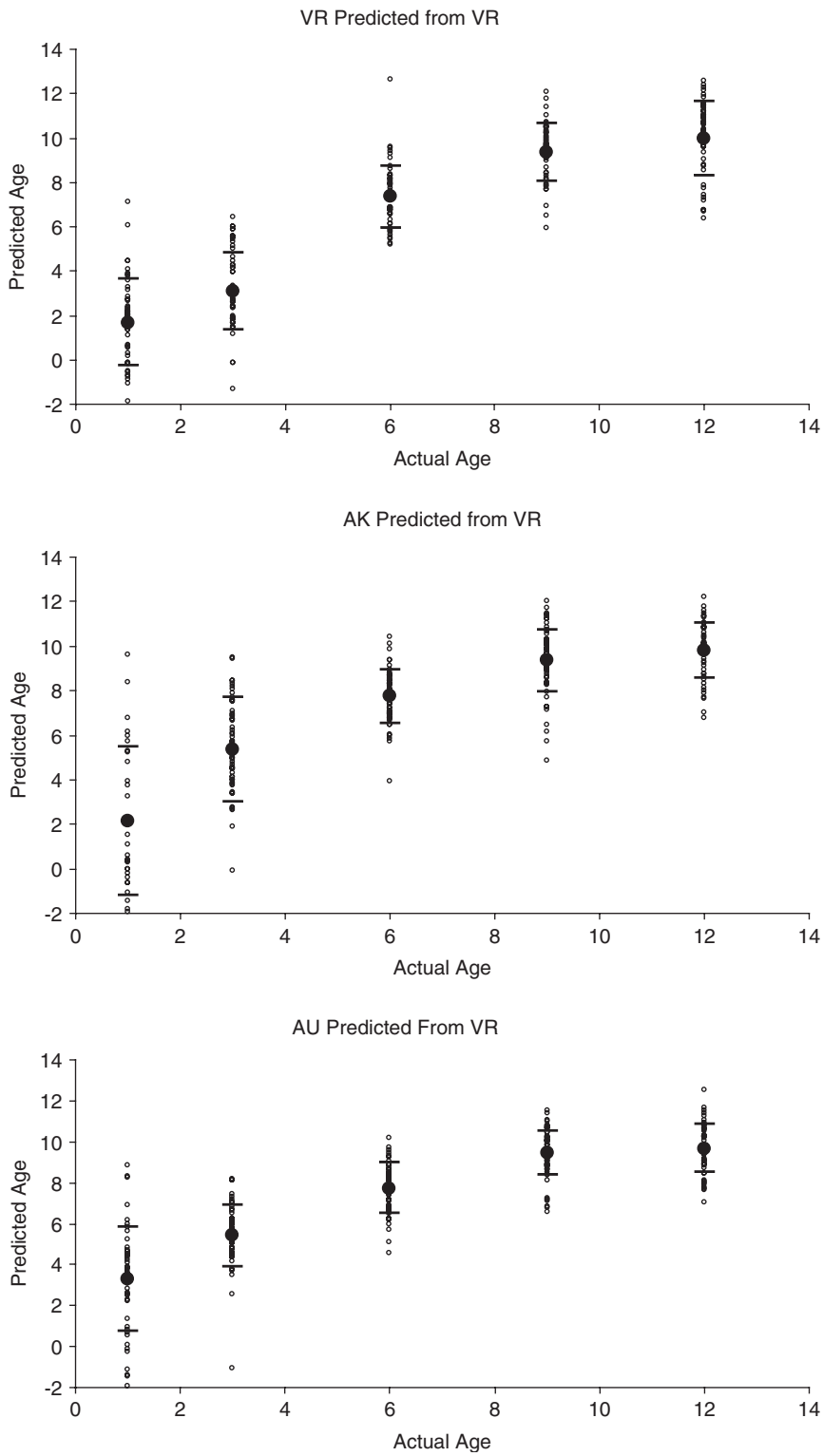

Fig. 2. Actual vs. predicted age of midges when age is predicted using a calibration developed from the VR colony. Averages and values for ±1 standard deviation are marked on the plots.

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cross-validation. There was considerable overlap between the 6-day-old insects and the younger and older groups, but 6-day-old insects were more likely to overlap with the older insects.

When the VR insects were used to develop a calibration and predict the age of AU and AK insects, classifications were similar. Figure 2 shows that plots of the predicted ages of the three colonies were very similar to the cross-validation results shown in Fig. 1. This shows that the cross-validation models did not over-fit the data and that models developed from one set of insects can be used to predict the ages of independent sets.

Table 2 shows the proportion of insects that would be classed into different age ranges when using either a cross-validation or a calibration developed from the VR colony to predict the age of all colonies. The predicted age of the VR colony is very similar to the VR cross-validation results because the prediction calibration was developed from this colony. However, the AU results show that, for example, 44% of the 1-day-old insects were predicted into the 2–4.5-day-old age range, and 6% were predicted into the 7.5–10.5-day-old age range. This is by contrast with the cross-validation results for the AU colony, in which 67% of the 1-day-old insects were predicted to be <2 days old and no insects were predicted to belong to the 7.5–10.5-day-old range. Although the results from using the VR colony as the calibration set were generally not as good as the cross-validation results, Table 2 shows that a very small percentage of young insects are classed as old (<7.5 days) and very few older insects are classed as young (<4.5 days), whether a calibration or a cross-validation is used to predict age. Table 2 also shows that 6-day-old insects are more likely to be predicted into their correct age range or into an older group.

The regression coefficients for the cross-validation for each colony were very similar to one another (Fig. 3). The visible region (<700 nm) was excluded from the analysis because no colour differences between colonies were expected. The region above 2300 nm was also excluded because of excessive noise in the spectra that reduced classification accuracy. The wavelength regions that are common to all analyses occur around 800, 1000, 1200, 1380, 1670, 1720 and 1865 nm. The regions around 1200, 1380, 1670 and 1720 nm correspond to C-H absorption overtones (Shenk *et al.*, 1992). Dowell *et al.* (1999) reported that similar absorption regions were important for classifying insect species because of the unique absorption

Table 2. Proportion of insects predicted in different age ranges using a cross-validation (CV) or when using a calibration developed from the VR colony.

	Actual age, days	Predicted age range, days				
		<2	2–4.5	4.5–7.5	7.5–10.5	<10.5
VR predicted	1	0.52	0.44	0.04		
	3	0.31	0.26	0.43		
	6			0.53	0.45	0.02
	9			0.06	0.78	0.16
	12			0.13	0.40	0.47
VR CV	1	0.49	0.47	0.04		
	3	0.25	0.47	0.27		
	6			0.52	0.48	
	9			0.06	0.80	0.14
	12			0.12	0.39	0.49
AU predicted	1	0.25	0.44	0.25	0.06	
	3	0.02	0.17	0.77	0.05	
	6			0.40	0.60	
	9			0.10	0.73	0.17
	12			0.02	0.72	0.26
AU CV	1	0.67	0.29	0.04		
	3	0.06	0.58	0.32	0.03	
	6		0.02	0.70	0.28	
	9			0.23	0.65	0.12
	12			0.05	0.44	0.51
AK predicted	1	0.57	0.13	0.23	0.07	
	3	0.05	0.26	0.52	0.18	
	6		0.02	0.42	0.56	
	9			0.11	0.71	0.18
	12			0.04	0.67	0.29
AK CV	1	0.57	0.25	0.18		
	3	0.10	0.52	0.34	0.03	
	6		0.05	0.73	0.22	
	9			0.21	0.67	0.12
	12			0.06	0.60	0.35

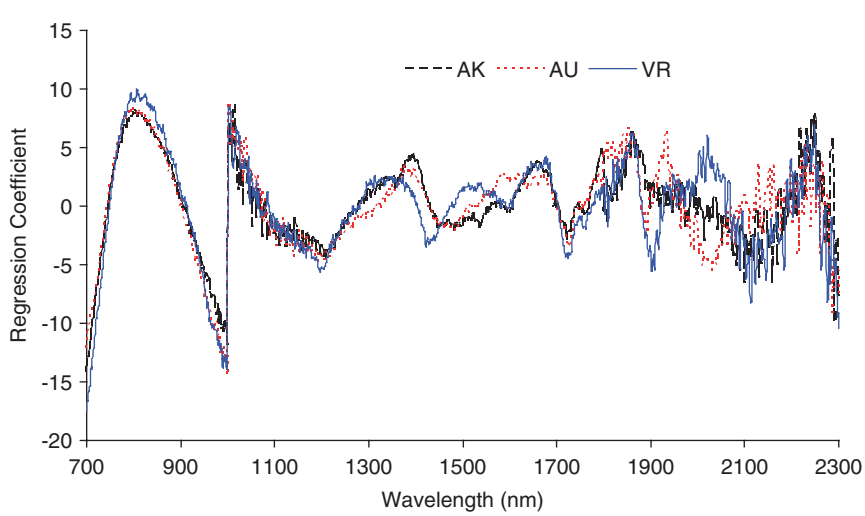

Fig. 3. Regression coefficients from cross-validation models used to predict midge age.

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of their cuticular lipids. Perez-Mendoza *et al*. (2002, 2004) showed similar regression coefficients for age-grading flies and stored-product beetles, but their analysis was limited to <1700 nm.

Alternative means of analysing or pretreating the data were investigated, such as first and second derivatives, discriminate analysis and path length corrections. Various wavelength ranges were also considered, but the best results were achieved when using the 700–2300-nm wavelength range and mean centring.

Dyce (1969) described microscopic techniques to differentiate nulliparous from parous *Culicoides* spp. Using the parous status of insects to measure age, the predominant method, has serious drawbacks. Biting midges can lay eggs more than once and environmental conditions, host availability and abundance can affect the parous status of midges independently of age. Age-grading could be combined with microscopic techniques to determine if midges are parous. The information acquired from age-grading and determining if wild-caught females were parous would allow entomologists to determine the minimum length of time required before insects mate and blood-feed.

Conclusions

Near-infrared spectroscopy can be used to age-grade tiny insects such as *C. sonorensis*. There were some overlaps between age groups; however, the data support the separation of young vs. old age groups. Most female *C. sonorensis* will be unable to transmit arboviruses if they are <6 days old (Whittmann *et al*., 2002). This fast and non-destructive technique can separate the older midges <6 days old from younger midges and could be used to determine how many insects are old enough to be potential vectors. Midges can then be tested for viruses after they have been age-graded, which will save time and resources as younger insects can be eliminated from virus screening. The expansion of NIRS techniques to additional *Culicoides* spp. and field validation of the technology are both needed.

Acknowledgements

We thank L. DeBrey, J. Kempert and W. Yarnell for the maintenance of the colonies of *Culicoides sonorensis* and for their assistance with this project. We also thank E. Maghirang for assistance in analysing data. Trade names and commercial products are mentioned solely for the purpose of providing specific information; this does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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Accepted 31 March 2009

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