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Abstract

Substrates composed of hay residues, dung, and urine accumulate around winter hay feeding sites in cattle pastures, providing developmental habitats for stable flies. The objective of this study was to relate physiochemical and microbial properties of these substrates to the presence or absence of stable fly larvae. Properties included pH, temperature, moisture, ammonium concentration, electrical conductivity, and numbers of coliform, fecal coliform, *Escherichia coli*, and *Enterococcus* bacteria. Each physiochemical sample was classified as a function of belonging to one of the three 2-m concentric zones radiating from the feeder as well as presence or absence of larvae. In total, 538 samples were collected from 13 sites during 2005–2011. Stable fly larvae were most likely to be found in moist, slightly alkaline substrates with high levels of ammonium and low temperature. The probability of larvae being present in a sample was the highest when the moisture content was 347% relative to dry weight and the average pH was 8.4. Larvae were recovered within all zones, with a nonsignificant, but slightly higher, percentage of samples containing larvae taken 2–4 m from the center. All methods used to enumerate bacteria, except total coliform, indicated decreasing concentrations in hay bale residue throughout the summer. In addition to the environmental parameters, cumulative degree day 10°C had a significant effect on the probability of observing stable fly larvae in a sample, indicating that unidentified seasonal effects also influenced immature stable fly populations.

Key words: *Stomoxys calcitrans*, larval substrate, pest management, coliform

Stable flies are blood-feeding pests that primarily attack cattle, but will feed opportunistically on most animals and humans. Stable flies feed daily by abrading skin with sharp prestomal labellar teeth and imbibing from the resulting pool of blood (Elzinga and Broce 1986). Hosts respond physically (Mullens et al. 2006) and physiologically (Schwinghammer et al. 1986) to the painful bites, reducing weight gains and performance (Taylor et al. 2012a).

Elimination of larval substrates through sanitation is the most effective management practice for stable flies (Thomas et al. 1996) but is challenging to implement due to cost and the diversity of substrates supporting larval development. Stable fly larvae typically develop in accumulations of fermenting vegetation such as silage (Williams et al. 1980), animal bedding, and debris around hay feeders (Broce et al. 2005), feed bunks, and fence lines (Meyer and Petersen 1983). Occasionally, stable flies will develop in more transient substrates such as accumulated seaweed (Simmons and Dove 1941), grass clippings (Ware 1966), and sewage biosolids (Doud et al. 2012). While several studies have examined the spatial distribution of larvae within common developmental substrates (i.e., confinement lots; Meyer and Petersen 1983, Skoda et al. 1991), characterization of biotic and abiotic attributes of the substrate has received little attention (Talley et al. 2009, Wienhold and Taylor 2012). Quantifying physical and biological parameters associated with larval development may reveal potential targets for management practices.

This study had three interrelated goals: first, to evaluate the relationships among physiochemical properties of larval substrates; second, to evaluate changes in substrate properties relative to time and season; and third, to characterize the relationships between physical and biological properties of potential stable fly developmental substrates and the observed presence or absence of larvae. In Nebraska, as forage becomes limited for pastured cattle in the fall, hay is often provided in stationary feeders. Hay residues accumulate around the feeders through the winter and combine with urine and manure, producing a major source of stable flies in the spring (Broce et al. 2005, Talley et al. 2009, Taylor and Berkebile 2011). We monitored physical factors believed to affect larval development (residue depth, moisture content, and temperature) as well as pH, electrical

conductivity (EC), and ammonium concentration of hay residue. Because bacteria play an important role in stable fly development (Lysyk et al. 1999, Romero et al. 2006) and stable flies are able to retain pathogenic bacteria into adulthood (Rochon et al. 2004, 2005), microbial activity in the substrate was also assessed.

Materials and Methods

Investigations were conducted at the University of Nebraska Agricultural Research and Development Center (ARDC) located near Ithaca, NE. The ARDC comprises ~4,000 ha, half of which is designated for row crop research, while the other half is maintained as pasture. The center supports up to 3,500 animals including swine and beef cattle. Approximately 400 Angus, Husker Red, and Husker Black hybrids are maintained in three pastured herds.

This report includes results from two studies. In the first, physical parameters and larval abundance in hay residue were measured weekly April–October at nine hay feeding sites in 2005–2009. At each site, a golf hole cutter, 11.43 cm in diameter, was used to remove cores from three zones of hay residue. The area within a 2-m radius from the edge of the feeder was referenced as zone 1, while the 2- to 4- and 4- to 6-m annuli were referred to as zones 2 and 3, respectively. One sample per zone per site was collected each week. Sampling location within each zone was randomized. At each sampling site, depth of the hay–manure residue was measured, and temperature measurement was taken 4 cm below the surface with an Oakton Temp-300 dual input data-logging thermocouple thermometer (Oakton Instruments, Vernon Hills, IL) before core removal. Core samples were transported to the laboratory where stable fly larvae and pupae were removed and counted. Approximately 16 g of substrate was added to 80 ml of distilled water, stirred for 1 h, and allowed to rest for 30 min before pH, EC (mS), and ammonium content (ppm) were measured with an Oakton pH/ion 2100 meter (Oakton Instruments, Vernon Hills, IL). A second subsample was weighed, dried for 48 h at 80°C, and reweighed (Gardner 1986). Percent moisture content was calculated as $((W_{wet} - W_{dry}) / W_{dry}) \times 100$ to give percent moisture relative to dry weight of the substrate sample. Measured in this way, there is a linear relationship between percent moisture and actual water content within the substrate sample, allowing for improved statistical analysis.

For the second study, substrate samples from zone 2 of four hay feeding sites were collected once per week April–July and once every other week in March, August, and September of 2011. Samples were subdivided for microbial and physical analyses. Microbial analysis included enumeration of total coliforms, *Escherichia coli*, and *Enterococcus* with the Quanti-tray (IDEXX Laboratories, Westbrook, ME) system following the manufacturer's instructions. Bacterial concentration was estimated using the "most probable number" technique (MPN; Oblinger et al. 1975) and recorded as MPN per mg of substrate. In addition, samples were plated onto MacConkey agar, trypticase soy agar (TSA), and mEntero agar (Romero et al. 2006) with an Eddy Jet Spiral Plater (Neu-Tec Group Inc., Farmingdale, NY; Gilchrist et al. 1973). Samples were incubated at 37°C for 24 h and results recorded as colony-forming units (CFU) per mg of substrate.

Physical parameters that were measured included temperature, water content, EC, total carbon (C), and total nitrogen (N). Temperature of the substrate 10 cm below the surface was recorded. Water content was determined by weighing, oven drying (105°C), and reweighing samples with moisture content determined as indicated above. Laboratory $EC_{1:1}$ was determined in 1:1 water:substrate slurry using a conductivity meter for $EC_{1:1}$ and a glass electrode for pH (Smith and Doran 1996). Total C (g kg^{-1}) and total N (mg kg^{-1}) were measured by dry combustion (EA1112 Flash NC Elemental analyzer, Thermo Finnegan Scientific Inc., Waltham, MA) using air-dried, ground samples.

Weather data were acquired from the MEADTURFFARM station of the High Plains Regional Climate Center (http://www.hprcc.unl.edu/). Degree days were calculated from daily maximum–minimum temperatures using sine-wave integration (Allen 1976).

Statistical Analyses

Principle components analysis (PCA; SAS 9.3) was used to evaluate covariance of the six physical properties—residue depth, EC, ammonium concentration, pH, moisture content, and temperature.

Spatial and temporal variation of physical properties was evaluated with general linear mixed models (Proc Glimmix, SAS 9.3) (SAS 2011). Hay ring site was treated as a random effect. Weekly collections from each zone within each site were treated as repeated measures. Temporal comparisons were made by assigning observations to 100 cumulative degree day 10°C (cDD_{10}) bins beginning with 1 January of each year. In the first analysis, year, cDD_{10} bin, and zone were considered discrete fixed effects. Interactions of zone with year and cDD_{10} bin were included in the models. The lognormal distribution was used for all of the properties. Because several of the properties exhibited underdispersion, the Kenward–Roger method for determining denominator degrees of freedom was used. Tukey's adjustment was used when comparing multiple means. When a significant effect of cDD_{10} bin was observed, a second analysis with cDD_{10} and cDD_{10}^2 as continuous variables was conducted to determine if the variables response to time was linear or curvilinear. Temporal variation in bacterial counts was evaluated similarly.

Logistic regression was used to evaluate the probability of observing stable fly larvae in a sample relative to temporal and physical properties of that sample as well as components of the principle components analysis. As for the previous analyses, hay ring site was treated as a random effect, and weekly collections from each zone within each site were treated as repeated measures. To determine if responses were linear or curvilinear, the property-squared term was included in each model initially. If insignificant, the squared term was removed from the model, and the response was considered to be linear. All results were considered significant when $P \leq 0.05$.

Results

Principal component and correlation analyses indicated that depth, EC, pH, and ammonia were positively correlated (Fig. 1; Table 1). Temperature was negatively correlated with moisture. The first two components of the PCA accounted for 59% of the variation. The first principle component was correlated with ammonia (0.49), EC (0.55), and moisture levels (0.47). The second principle component correlated primarily with temperature (0.95).

All parameters except pH and total coliforms varied temporally (Table 2). Depth, EC, ammonia, and moisture levels in the substrate decreased from spring to fall (Table 3; Fig. 2). Temperature of the substrate peaked at 1,086 cDD_{10} (~31 July) and then decreased for the remainder of the year. pH did not vary significantly during the collection period, with a mean value of 8.0 (Table 3). All bacterial counts other than total coliforms decreased during the season. *Enterococcus*, MacConkey agar, and TSA counts declined linearly (Table 3; Fig. 3). *Escherichia coli* counts declined curvilinearly with

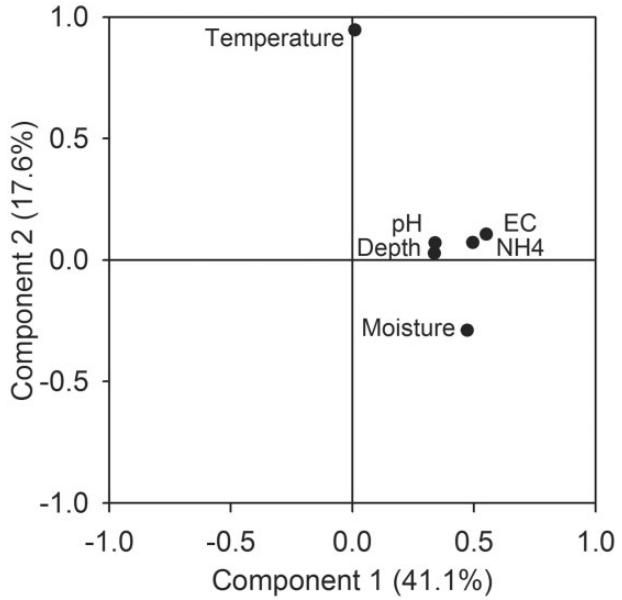

Fig. 1. Principle component analysis load plot with temperature, pH, depth, EC, ammonium, and moisture.

Table 1. Correlation matrix for physical properties of substrates

	Depth	EC	Ammonium	pH	Moisture
EC	0.30*				
Ammonium	0.19*	0.75*			
Ph	0.15*	0.33*	0.19*		
Moisture	0.38*	0.43*	0.38*	0.31*	
Temperature	0.07	0.06	0.02	0.05	−0.12*

*$P \leq 0.05$

a minima of 4.2 MPN/mg at 1,386 cDD_{10} (~23 August). The probability of observing stable fly larvae in samples was highest in May and then decreased to <0.05 with ~1,600 cDD_{10} (~11 September, Fig. 2).

Depth, ammonia, pH, moisture, and temperature did not vary among the three radial zones of the hay feeding sites although all except pH and temperature tended to be highest in the middle zone (Table 2; Fig. 2). EC was higher in the middle zone than in the outer zone. The probability of larvae being present in a sample did not differ among zones although tended to be higher in the middle zone than in the outer zone.

Logistic regression validated the significance of the first two components of the PCA in predicting larval absence or presence. Other principle components were insignificant and eliminated from further analyses. When cDD_{10} was included with the first two principle components in logistic regression, the second component was insignificant and eliminated from the model (Table 4). When the first principle component and cDD_{10} were included, both terms were significant, indicating a significant seasonal effect after accounting for the effects of substrate quality as measured by the physiochemical parameters.

The probability of stable fly larvae being in a sample increased with depth throughout the range of depths (2–31 cm) observed in this study and decreased throughout the range of temperatures (Table 5; Fig. 4). The probability of collecting larvae was highest in samples with an EC value of 3.1, 216 ppm ammonia, and 347% moisture.

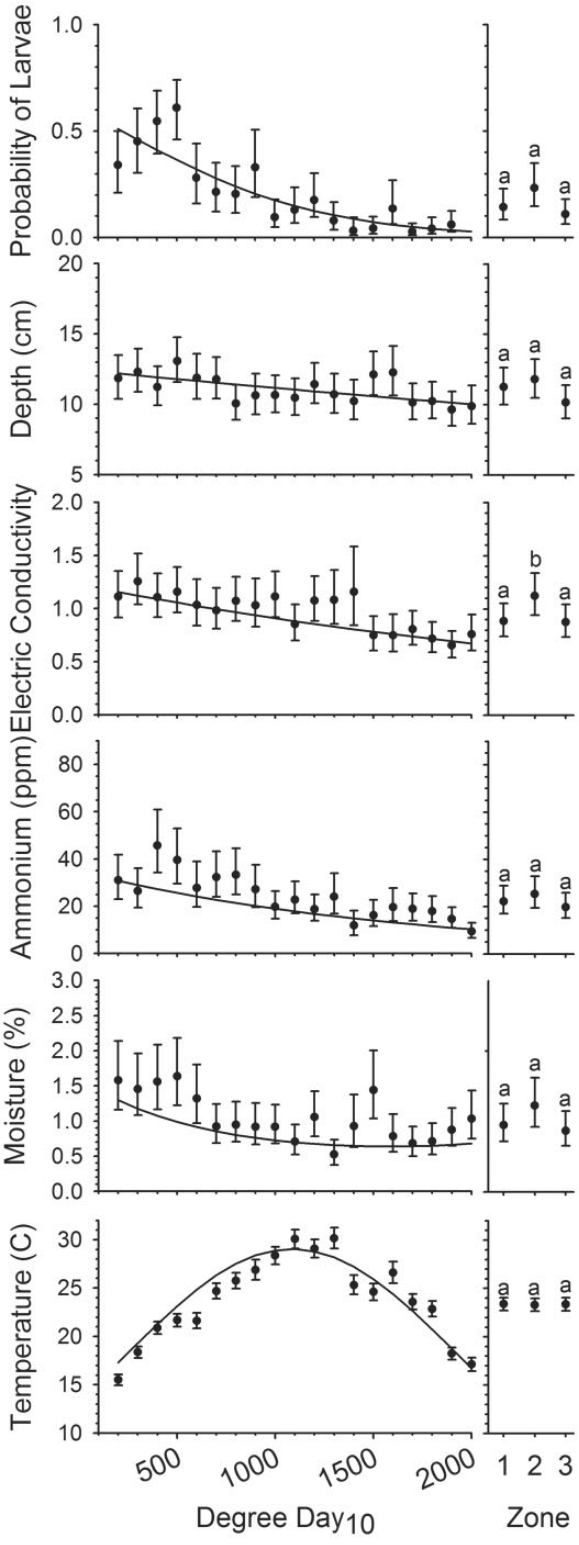

Fig. 2. Least squared means of the probability of collecting stable fly larvae and physical properties of hay residue in relation to 100 cDD_{10} bins.

Discussion

Concentric zones of hay residue extending from the center of bale feeders were first conceptualized by Talley et al. (2009) based on visual assessments of varying hay to manure ratios. In our study, the distinction between zones was not as obvious. Similar to Wienhold

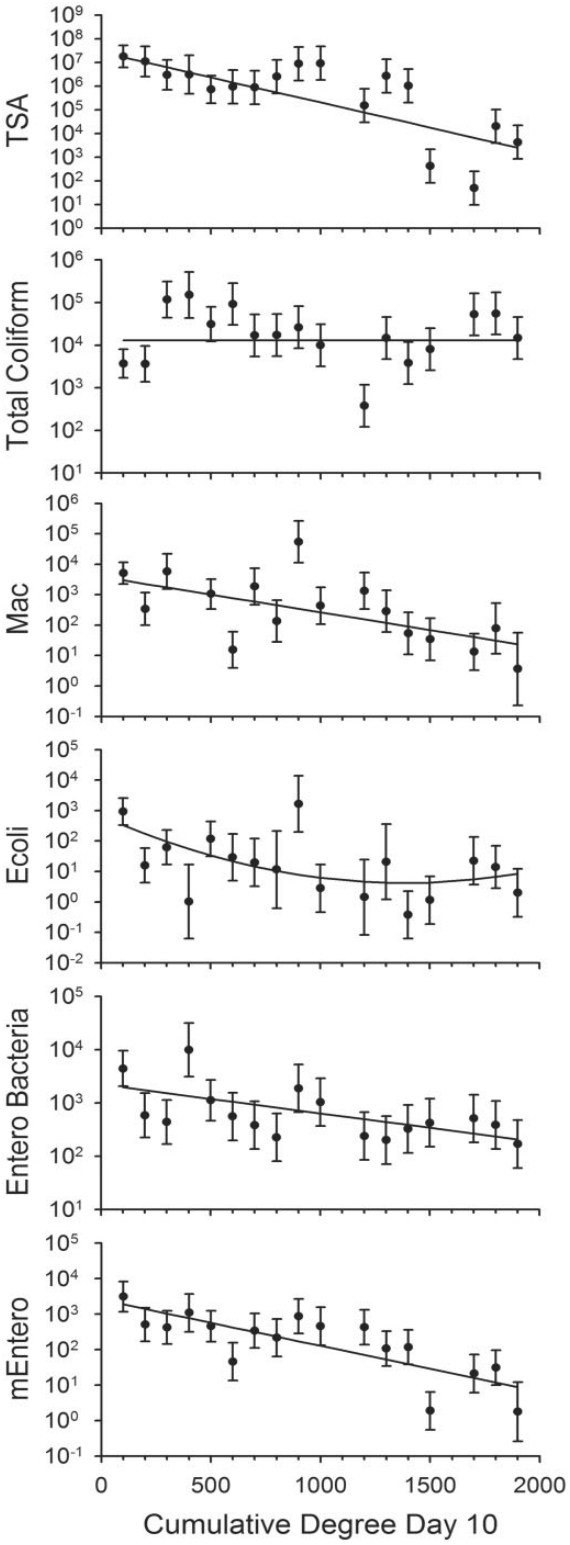

Fig. 3. Least squared means of bacterial CFU (MacConkey agar [Mac], trypticase soy agar [TSA], mEntero agar) or MPN (total coliforms, *E. coli, Enterococcus* [Entero Bacteria]) per mg of substrate in relation to 100 cDD_{10} bins.

and Taylor (2012), annuli were somewhat apparent, though irregular. Larvae were recovered within all zones, with a slightly higher, though nonsignificant, percentage of samples containing larvae taken 2–4 m from the center.

Table 2. Results of regression analyses for the relationships between physical properties of the substrate and annual cDD_{10} as a class variable and feeding site zone from which the sample was collected

	Cumulative DD_{10}			Zone		
	F	df	*P*	*F*	df	*P*
Larvae	3.95	17, 469	<0.01	2.86	2, 469	0.06
Depth	1.72	18, 372	0.03	2.86	2, 80	0.06
EC	2.87	18, 347	<0.01	4.55	2, 137	0.01
Ammonium	3.68	18, 325	<0.01	1.16	2, 89	0.32
pH	1.04	18, 361	0.42	0.62	2, 124	0.54
Moisture	3.78	18, 364	<0.01	2.95	2, 84	0.07
Temperature	26.69	18, 372	<0.01	0.01	2, 57	0.99
C:N	3.98	15, 41	<0.01			
E. coli	3.96	16, 24	<0.01			
Entero	1.52	16, 44	<0.01			
MAC	3.21	15, 32	<0.01			
mEntero	3.00	16, 36	<0.01			
TSA	4.86	16, 44	<0.01			
Total coliform	3.71	16, 44	0.17			

In general, stable fly larvae were recovered from moist, slightly alkaline substrate with relatively high levels of ammonium and an average temperature of 23°C. Moisture content averaged >200% of dry weight, or 45% of wet weight, when calculated as $((W_{wet} - W_{dry}) / W_{wet}) \times 100$. This supports other field-based investigations that have collectively reported a range of 19–71% (Rasmussen and Campbell 1981, Talley et al. 2009). House fly larvae, which develop in similar substrates, may survive with moisture contents as low as 4.7%, albeit survival is reduced (Hogsette 1996). The obvious function of adequate moisture is to prevent larval desiccation, but equally important is the pivotal role of distributing nutrients and microorganisms (Ranjard and Richaume 2001). Stable fly larvae are equipped with a pair of Keilin's organs on each of their three thoracic segments (Friesen et al. 2015). Keilin's organs are trichoid sensilla that serve as hygroreceptors (Hafez 1950), indicating that larvae can detect and presumably orient with respect to moisture levels.

The average observed temperature correlates with life history models predicting greatest larval survival at 20–25°C (Lysyk 1998, Gilles et al. 2005). The range of temperatures associated with development was broad, 10–42°C, although few larvae were observed at either extreme.

pH values were relatively stable at 8.0 despite fluctuations in moisture levels and ammonium concentrations, indicating the presence of a strong buffering system. Consequently, the effect of pH on larval development was not obvious. Reported values of substrate pH correlated with larval development vary widely, from slightly alkaline (Rasmussen and Campbell 1981) to neutral (McPheron and Broce 1996, Talley et al. 2009) and acidic (Gilles et al. 2005). Although acidification of larval substrates can reduce house fly survival (Calvo et al. 2010), a similar tactic may or may not be effective for stable fly control.

Accumulating evidence continues to highlight the importance of ammonium in stable fly development and behavior. Larvae were collected in hay residue with high ammonium concentrations. Additionally, ammonia is one of the main metabolic products of bacteria supporting larval development and is an attractant for gravid stable flies (Romero et al. 2006).

Concentrations of fecal coliforms, *E. coli*, and *Enterococcus* in hay residues decreased throughout the summer months, likely due to

Table 3. Results of regression analyses for the relationships between substrate properties and annual cDD_{10}

	Intercept					cDD_{10}					cDD_{10}^2				
	Coef	SE	*t*	df	*P*	Coef	SE	*t*	df	*P*	Coef	SE	*t*	df	*P*
Larvae	0.441	0.427	1.03	13	0.32	-19.9^a	2.72^a	−7.30	152	<0.01			−0.34	169	0.74
Depth	2.522	0.099	25.56	8	<0.01	-1.1^a	0.38^a	−2.99	484	<0.01			0.01	483	0.99
EC	0.204	0.211	0.96	9	0.36	-3.0^a	0.53^a	−5.61	140	<0.01			−1.34	154	0.18
Ammonium	3.550	0.375	9.47	8	<0.01	-6.1^a	0.92^a	−6.56	438	<0.01			−1.10	437	0.27
pH	2.077	0.028	73.50	8	<0.01			−1.39	152	0.165			0.35	166	0.72
Moisture	0.482	0.299	1.61	13	0.13	-11.7^a	3.47^a	−3.38	136	<0.01	0.004^a	0.0017^a	2.22	139	0.03
Temperature	2.589	0.034	75.99	76	<0.01	14.36^a	0.70^a	20.44	133	<0.01	-0.007^a	0.0003^a	−19.98	134	<0.01
C:N	17.721	0.927	19.11	3	<0.01	−0.003	0.001	−4.19	76	<0.01			1.65	75	0.10
E coli	6.526	1.137	5.74	3	0.01	-73.5^a	24.27^a	−3.03	61	<0.01	0.027^a	1.3^a	1.99	61	0.05
Entero	7.703	0.667	11.55	3	<0.01	-12.5^a	6.00^a	−2.09	83	0.04			0.83	82	0.41
MAC	8.363	0.639	13.08	3	<0.01	-26.9^a	6.62^a	−3.99	67	<0.01			−0.92	66	0.36
mEntero	7.837	0.931	8.41	3	<0.01	-29.8^a	7.62^a	−3.90	72	<0.01			−0.41	71	0.68
TSA	17.114	0.945	18.11	3	<0.01	-48.9^a	9.36^a	−5.22	83	<0.01			−1.64	82	0.11
Total coliform	9.462	0.695	13.61	3	<0.01			0.74	83	0.46			−0.61	82	0.54

Coefficients and SEs are presented in log units.
[a] $\times 10^{-4}$.

Table 4. Results of regression analyses for the relationships between the probability of the presence of stable fly larvae in a substrate sample, the first and second principle components, and annual cDD_{10}

	$PC1 + PC2 + cDD_{10}$			$PC1 + cDD_{10}$		
	F	df	*P*	*F*	df	*P*
PC1	62.31	1, 409	<0.01	62.55	1, 410	< 0.01
PC2	2.60	1, 409	0.11			
cDD_{10}	25.60	1, 409	<0.01	31.89	1, 410	<0.01

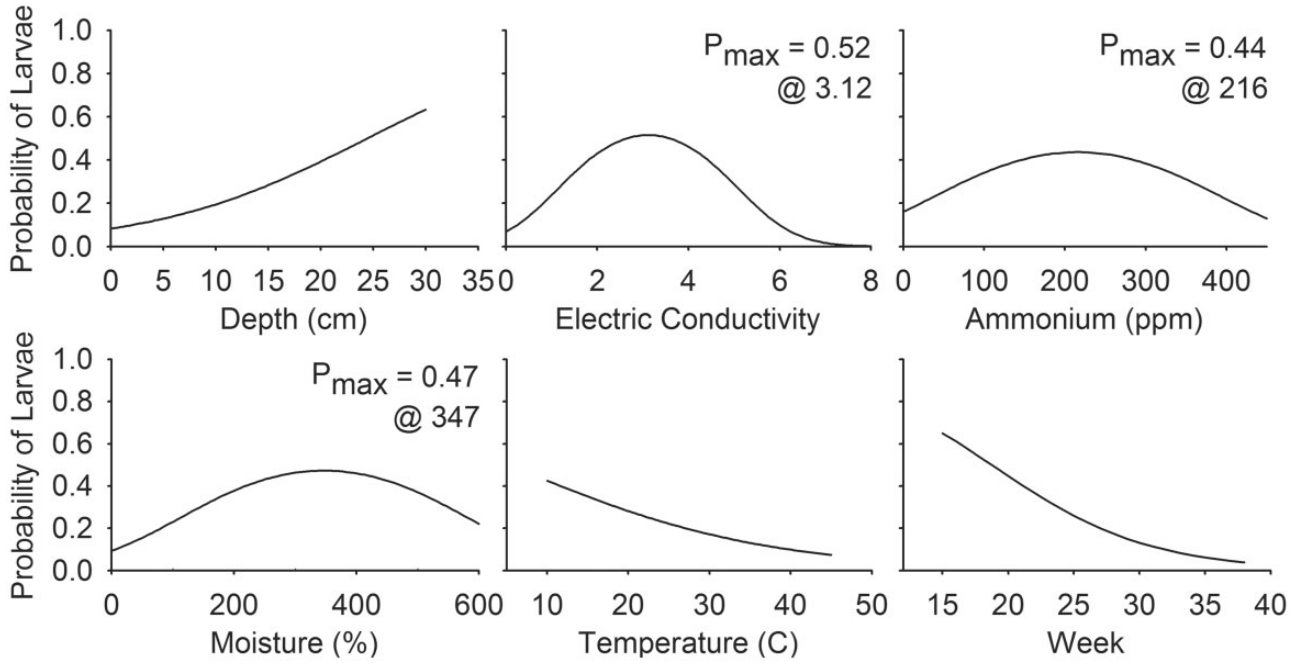

Fig. 4. Logistic regression of the probability of collecting larvae and physical properties of hay residue.

the exclusion of animals, and therefore manure and urine, during the sampling period. Although we mechanically isolated the animals from the hay sites for purposes of the study, once forage is available in the pastures, cattle no longer feed on hay or congregate near hay feeders. This same behavior is to be expected even when feeding sites have not been fenced off. Talley et al. (2009) suggested a similar occurrence for fecal coliforms in hay residue monitored in northeastern Kansas. *Enterococcus* CFU per mg tended to be higher than

Table 5. Results of regression analyses for the relationships between the probability of the presence of stable fly larvae in a substrate sample and physical characteristics of the sample

	Intercept					Term					$Term^2$				
	Coef	SE	*t*	df	*P*	Coef	SE	*t*	df	*P*	Coef	SE	*t*	df	*P*
Depth	−2.40	0.49	−4.93	8	<0.01	0.098	0.026	3.84	484	<0.01			−0.33	483	0.74
EC	−2.60	0.37	−7.11	8	<0.01	1.704	0.337	5.05	468	<0.01	−0.273	0.069	−3.93	468	<0.01
Ammonium	−1.65	0.31	−5.26	8	<0.01	0.013	0.005	2.79	437	<0.01	−0.00003	0.000014	−2.16	437	0.03
pH			−1.25	8	0.25			0.38	474	0.70			0.52	473	0.60
Moisture	−2.27	0.34	−6.63	8	<0.01	1.247	0.311	3.96	473	<0.01	−0.180	0.070	−2.57	473	0.01
Temperature	0.33	0.69	0.48	8	0.64	−0.064	0.025	−2.50	481	0.01			−0.61	480	0.54

Coefficients and SEs are presented in units of logit(p).

MPN as measured by spiral plating and Quanti-Tray, but the trends of declining concentration were similar regardless of methodology.

In addition to substrate quality, an unexplained seasonal effect influences stable fly development. Succession of microbial communities has a strong role in larval development during periods in which gravid females are ovipositing (Romero et al. 2006, Talley et al. 2009, Albuquerque and Zurek 2014). Our results indicate that in Nebraska, there may be a period in which adults are no longer utilizing developmental sources, regardless of substrate quality. In addition to the current analysis, we documented group calf pens at a separate livestock facility with substrate that consistently supports stable fly larval development in early and midsummer. Although the substrate is continuously renewed, stable fly larvae are rarely observed after early August (D.B.T., unpublished data). Concurrent adult surveillance reveals a small population of adults that increases as temperatures cool in late summer and early fall. Yet, even as temperatures decrease and the adult population increases, larvae are not detected in known larval developmental substrates. Further investigations on stable fly developmental habitats and phenology are needed.

When larval developmental sites are known, a recommended management option is elimination through drying. Substrate is spread into a thin layer, thereby accelerating heating and drying of the material and rendering it unsuitable for stable flies (Campbell et al. 2001). If implemented, care should be taken to spread the material thin enough so that the entire depth of the substrate is subject to treatment. Most larvae are collected in the upper 5 cm of hay residue (Taylor and Berkebile 2011). Another effective management option is application of insect growth regulators on the hay residue in late spring (Taylor et al. 2012b, 2014). A single application may reduce the number of emerging adults by 97%.

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