

Male stable fly (*Stomoxys calcitrans*) response to CO₂ changes with age: evidence from wind tunnel experiments and field collections

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Received 21 January 2008; Accepted 29 April 2008

ABSTRACT: Male stable flies require at least one or more blood meals to reach sexual maturity and are often caught in CO₂-baited traps. We tested the hypothesis that young male stable flies (one to three days, one blood feeding session) would be more responsive to CO₂ bait than older male stable flies by monitoring the upwind movement of different-aged male stable flies exposed to CO₂ using a wind tunnel. The proportion of males moving upwind toward CO₂ decreased with age (days), from 49% for males ≤ 3 days old, to 4.5% for males > 3 days old. To further test this, we conducted daily sampling of stable fly populations at a beef farm using a CO₂-baited cloth trap. We found that days on which a high proportion of males were caught, females were predominantly from early developmental stages, indicating that proportionately more males were caught from field populations made up of younger cohorts. These results were consistent with the wind tunnel experiment patterns. *Journal of Vector Ecology* 33 (2): 247-254. 2008.

Keyword Index: Stable flies, wind tunnel, CO₂, Nzi trap, Muscidae.

INTRODUCTION

Stable flies are blood-feeding synanthropic pests that can potentially act as vectors of food-borne diseases of humans (Mramba et al. 2007) and cause significant weight loss in cattle (Campbell et al. 2001, Cantigui 1997) and reduced milk yields (Mullens et al. 2006). Consequently, monitoring stable fly populations is an important part of their management (Gerry et al. 2007). Stable fly populations are commonly sampled using sticky panel traps (e.g., Beresford and Sutcliffe 2006, Kaufman et al. 2005) and CO₂-baited cloth traps or Nzi traps (e.g., Mihok 2002). The analysis of such trap catch data depends upon understanding the behavior of stable flies that are associated with different trapping protocols (Muirhead-Thomson 1991, Beresford and Sutcliffe 2008).

We examine the response of different-aged male stable flies to air-borne CO₂, an important chemical attractant used by biting flies to find their bloodmeal hosts (Sutcliffe 1987, Schreck et al. 1975, Hoy 1970, DeFoliart and Morris 1967). Unlike female stable flies that feed on blood several times throughout each gonotrophic cycle (Chia et al. 1982), male stable flies require at least one blood meal to reach sexual maturity (Anderson 1978), and generally mate after three or four blood meals (Morrison et al. 1982). We test the hypothesis that male stable flies respond differently to CO₂ based on their age, in a manner consistent with their different blood meal requirements for sexual maturity. If this is correct, we would expect that only the younger males would respond to CO₂ by moving upwind, behavior consistent with searching for a host.

As a further test of this hypothesis, we examined field collections of stable flies obtained at beef farms using a CO₂-baited Nzi trap. The emergence of separate cohorts of stable flies over the summer (Lysyk 1993) can produce daily

trap catches of different-aged females¹. We reasoned that the male portion of daily trap catches would decline as their cohort aged, and that this would be manifested by males being caught predominantly in association with younger females, rather than with later stage or parous females.

METHODS AND MATERIALS

Rearing

Stable flies were obtained from the stock colony maintained at Trent University (25°C [±5°C] at 50% relative humidity, and 16:8 L:D photoperiod) (Sutcliffe et al. 1993). Daily cohorts of colony adults of known age (plus or minus 1.5 h) were established by placing pupae in a succession of small, screened cages. Adults that emerged in the cage between 09:00 and 12:00 each day were considered a daily cohort. Daily cohorts were given *ad libitum* access to 0.2 M sucrose in water from the time of emergence. These were given the opportunity to blood feed (citrate bovine blood from a local abattoir on an absorbent cloth) every second day for 30 min starting at 12:00, two days after emergence (flies three days old had one blood meal and flies four days old had two blood meals, etc). Flies that did not blood feed during the 30 min were culled from the cohort. Flies were not blood-fed on the day of the experiment.

Wind tunnel

We used the Sediment Transport Wind Tunnel at Trent University Geography Department. This wind tunnel is 0.71 m high, 0.76 m wide, with a working section 12.5 m long. It is constructed of 2 cm plywood, with acrylic windows along

¹Beresford, D.V. 2006. Dispersal and Population Dynamics of *Stomoxys calcitrans* (Diptera: Muscidae) in South-Central Ontario. PhD thesis. Trent University, Peterborough, Ontario Canada.

the side to view the operation. The fan pulls air through the tunnel and can be set for various wind speeds. At the upwind end, CO₂ was released at 1 liter/min. The fan was set at 0.5 m/s, which produced a measured wind speed on an anemometer (Turbo Meter[®], Davis Instruments, Hayward, CA) of 0.38 m/s at 5 to 11 cm height in the center of the wind tunnel where flies were released.

Experiment design

Male stable flies of various ages (see Figure 1 for ages in days and numbers) were colored with powdered dust for identification purposes within the wind tunnel. Flies were first anaesthetized with CO₂ and then dusted with color pigment obtained from Day Glo[®] Color Corporation. The flies were counted and placed in a 500 ml beaker at the center of the wind tunnel and allowed to revive. The few flies that did not leave the beaker were removed from the initial count. The location of flies within the wind tunnel was recorded every 30 min. At the end of each two-h trial, the final locations were identified and flies categorized as either upwind or downwind. The experiments were conducted for two hours in order to maximize the opportunities for all flies to be exposed to CO₂. All wind tunnel experiments were run from 12:00 to 14:00. There were three experiments: Feb. 28, Jul. 10, and Aug. 2, 2000, with the room temperatures at 18, 20, and 23°C, respectively.

Analysis

A logistic regression was used to determine if the proportion of males attracted to CO₂ decreased with age, with age in days as the independent variable and counts upwind or downwind as the dependent variable. Parameter estimates were obtained by least squares method.

Comparison to field data

Field-sampled stable flies came from a beef farm near Warsaw Ontario, 10 km north of Trent University, from 1998 to 2001. This farm consisted of 34.4 ha of pasture with two small wooded areas. Daily samples of stable flies were collected using CO₂-baited blue (phthalogen blue) and black-cloth covered Nzi traps (Mihok 2002). CO₂ was released from a compressed gas tank placed at the east edge of the trap at 1 liter/min from a plastic tube, the end of which was placed at the entrance to the trap. The Nzi traps was set up in the middle of a small rise of land in an open pasture 40 m south of the barnyard with the trap opening oriented south. Cattle passed the trap area each day, which was surrounded by an electric fence at a radius of 5 m from the trap center. Vegetation within the electric fence was maintained at 10 cm height or less to give flies approaching the trap free access to the trap entrance.

The trap was run daily, starting in May of each year prior to the establishment of local stable fly populations in early to mid-June. Sampling at each site ended in the fall after three successive weeks of zero catch. Because stable flies are diurnal (Harley 1965), CO₂ was released from 07:00 to 19:00 each day. Thus, each day's catch represents 12 trapping hours (plus or minus 30 min). At the end of the

daily trapping day, flies within and on the trap were removed with a handheld vacuum cleaner modified with a screened transparent plastic tube on the opening of the unit. Samples were then briefly chilled on ice prior to processing or, when large collections made immediate processing impossible, they were stored in a refrigerator at approximately 4°C and processed the following morning.

Data were collected on the sex and number of flies, and females were dissected for insemination and physiological age-grading in 0.9% saline. Spermathecae were examined for the presence of sperm. Ovarian follicles were assessed for their developmental stage according to the qualitative criteria of Moobola and Cupp (1978) and Scholl (1980) (length:width ratio and proportion of follicle occupied by oocyte). The first three ovarian stages used by Moobola and Cupp occur during the first gonotrophic cycle (i.e., nulliparous, N, I, II) while the three later ovarian stages (III, IV, V gravid) repeat over a female's lifetime, once per gonotrophic cycle, i.e., nulliparous III, IV, V, parous III, IV, V. In order to differentiate by parity level, we assigned follicles according to an age-grading continuum from 1 to 12 reproductive stages. Our categories are 1 to 6 nulliparous; 7, 8, 9, uniparous; 10, 11, 12, multiparous; with stages 6, 9, and 12 being gravid stages, respectively (Moobola and Cupp's stage V). Parity was assessed in the course of ovarian dissections based on the presence or absence of follicular relics (Scholl 1980). It was possible to discriminate between nulliparous (no follicular relics), uniparous (one follicular relic), and multiparous (more than one relic or relics appearing as yellow bodies) individuals in this way. Males were not dissected. Note that while a correspondence between age and physiological state exists (Lysyk and Krafusur 1993), the lack of a blood meal can delay physiological development (Jones et al. 1998). Our use of age of colony males as an indicator of physiological state was relevant because the males were fed on a regular basis.

We compared daily trap catches of 1998 to 2001 by plotting the proportion of catch that was male against: 1) the average stage of the females that were caught; 2) the proportion of uninseminated females; and 3) for 2001 only (the year with the largest total catch), the proportion of females that were in various stages. Data from days with fewer than ten stable flies were not included in this last analysis. Critical *p* values for these correlations were corrected using a sequential Bonferroni method (Sokal and Rohlf 1997).

All statistical analyses were done using either STATISTICA 4.5 (StatSoft[®]) or an EXCEL Spreadsheet.

RESULTS

About half (24 of 49, or 49%) of the male stable flies three days or younger traveled upwind, whereas only four of 88 of the males (4.5%) older than three days were observed to go upwind (one 10-day-old male and three five-day-old males) (Figure 1). The probability of a male stable fly moving upwind toward the CO₂ source decreased with age in days (logistic regression *R* = 0.481, 23.1% variance explained).

Table 1. Logistic regression of age vs proportion upwind from release site in the wind tunnel experiments after 0.5, 1, 1.5, and 2 h. The model used was $y = [\exp(a+bx)]/[1+\exp(a+bx)]$, where y = proportion travelling upwind, x = age in days.

Time (h)	Model parameters								
	a	SE	$t_{(135)}$	p	b	SE	$t_{(135)}$	p	R
0.5	0.700	0.623	1.12	0.26	-0.669	0.216	-3.09	0.002	0.40
1	0.710	0.484	1.47	0.14	-0.559	0.144	-3.88	<0.001	0.42
1.5	0.768	0.452	1.70	0.09	-0.503	0.123	-4.09	<0.001	0.43
2	1.411	0.489	2.88	0.005	-0.585	0.130	-4.49	<0.001	0.48

There was a significant regression of age of male stable flies vs proportion found upwind after 2 h, but not after 0.5, 1, or 1.5 h (Table 1, Figure 1).

The Nzi trap catches varied each year, from 115 in 2000, to 1,041 in 2001 (Table 2). Based on the field collections at the CO₂-baited Nzi trap, generally fewer males were caught among physiologically advanced female cohorts than among early stage female cohorts. That is, the proportion of each day's catch that was male was positively correlated with the mean physiological stage of females each day in 1999 and 2001, but not in either 1998 or 2000 (Figure 2). The proportion of males in each day's catch was also correlated with the proportion of females that were unseminated in 2000 and 2001, but not 1998 or 1999 (Table 2).

The proportion of males in the 2001 daily catch data was analyzed with respect to specific female stages. The strongest correlation was between males and stage 1 and 2 females (proportions, $R^2 = 0.40$), followed by a negative correlation between males and multiparous females ($R^2 = 0.35$) (sequential Bonferroni corrected alpha levels of 0.05) (Table 3, Figure 3).

DISCUSSION

Field studies of *Stomoxys* spp. indicate that both sexes are similarly attracted to CO₂ baited traps (Torr et al. 2006, Schofield 1998, Mihok and Clausen 1996), with little difference between the sexes in catch rates. However, stable fly sex ratios can change throughout the summer. Black and Krafur (1985) reported proportions of males as weekly averages of sticky card trap catches, with traps

collected three to five times each week. They found that the proportion of males caught was not connected to fly numbers, although higher proportions of males tended to follow peak populations (Black and Krafur 1985). The general trend was lower catches of 56% to 70% males at the beginning of the summer, increasing from 70% to 90% after mid-July. Mihok et al. (1996) caught fewer female stable flies at Vavoua traps in spring than fall (19.4%, $N = 31$ vs 43%, $N = 460$) in Kenya. Neither of these studies reported daily changes in sex ratios or related these to female demographics.

While CO₂ is used by stable flies for finding hosts (Gatehouse and Lewis 1973, Gillies 1980, Vale 1980), it was conceivable that male stable flies might also respond positively to CO₂ for purposes other than as a source of a blood meal, a behavior seen in some Diptera. For example, male mosquitoes (*Culicoides variipennis sonorensis*) have been caught with CO₂-baited traps, which was interpreted as males using CO₂ to locate females for mating (Gerry and Mullens 1998).

This does not appear to be the case with male stable flies. Our wind tunnel results – that it was largely only the younger males of two to three days old (about half) which travelled upwind toward the CO₂ source, whereas most males older than three days flew downwind – supports the hypothesis that only young males in need of a blood meal to reach sexual maturity fly toward CO₂. Curiously, a small proportion of older males also flew upwind. It would be interesting to determine what, if any, proportion of males in wild populations continue to blood feed after reaching sexual maturity. Our assumption (the null hypothesis)

Table 2. Summary of trap data each year and correlations of proportion of males in daily trap catch (males/total per day) vs proportion of females that were unseminated (unseminated/females per day). N is the number of days included in the analysis, data from trap catches less than five were excluded.

Year	Number trapped			Correlations					
	Males	Females	Unseminated	R^2	t	p	N	Constant	Slope
1998	316	278	103	0.08	1.84	0.07	43	0.063	0.57
1999	252	329	123	0.07	1.72	0.09	42	0.243	0.292
2000	115	158	36	0.37	2.43	0.04	12	-0.175	1.038
2001	1041	1155	569	0.16	3.27	0.002	58	0.233	0.636

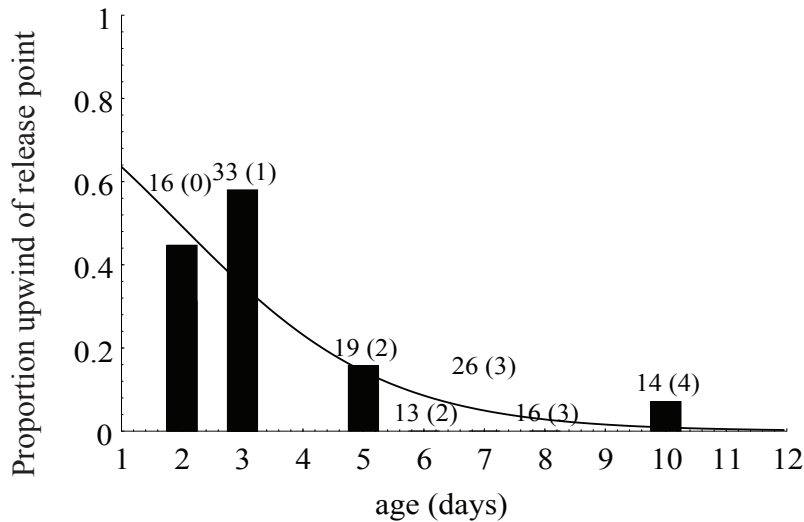


Figure 1. Logistic regression of age of male stable flies vs the proportion flying upwind toward CO₂ within the Trent University wind tunnel. The numbers over the bars are the number within each age group, with the number of blood meals in brackets.

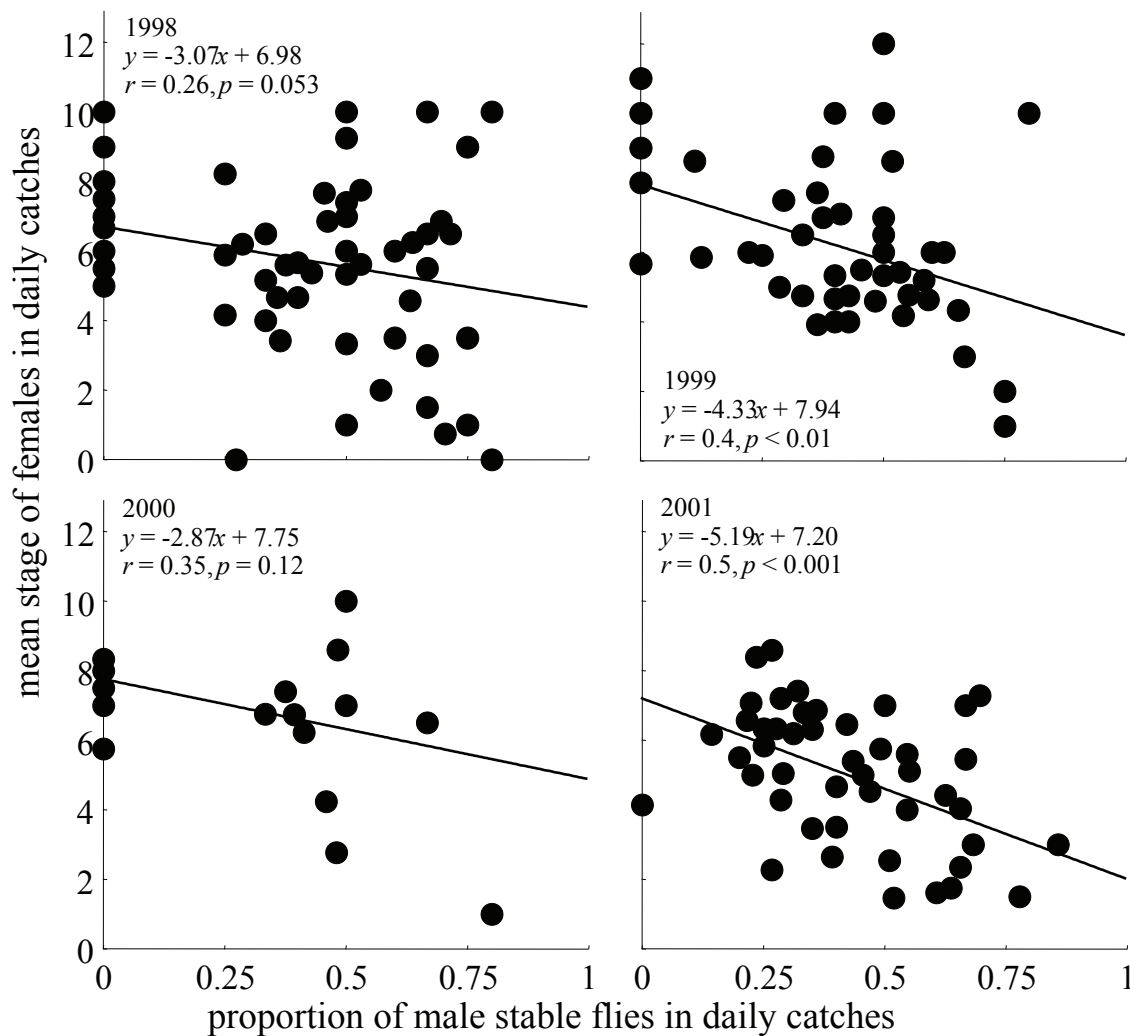


Figure 2. Correlations of the daily proportion of males caught with the mean stage of females caught at the CO₂-baited Nzi trap, 1998 to 2001, at the Warsaw farm site.

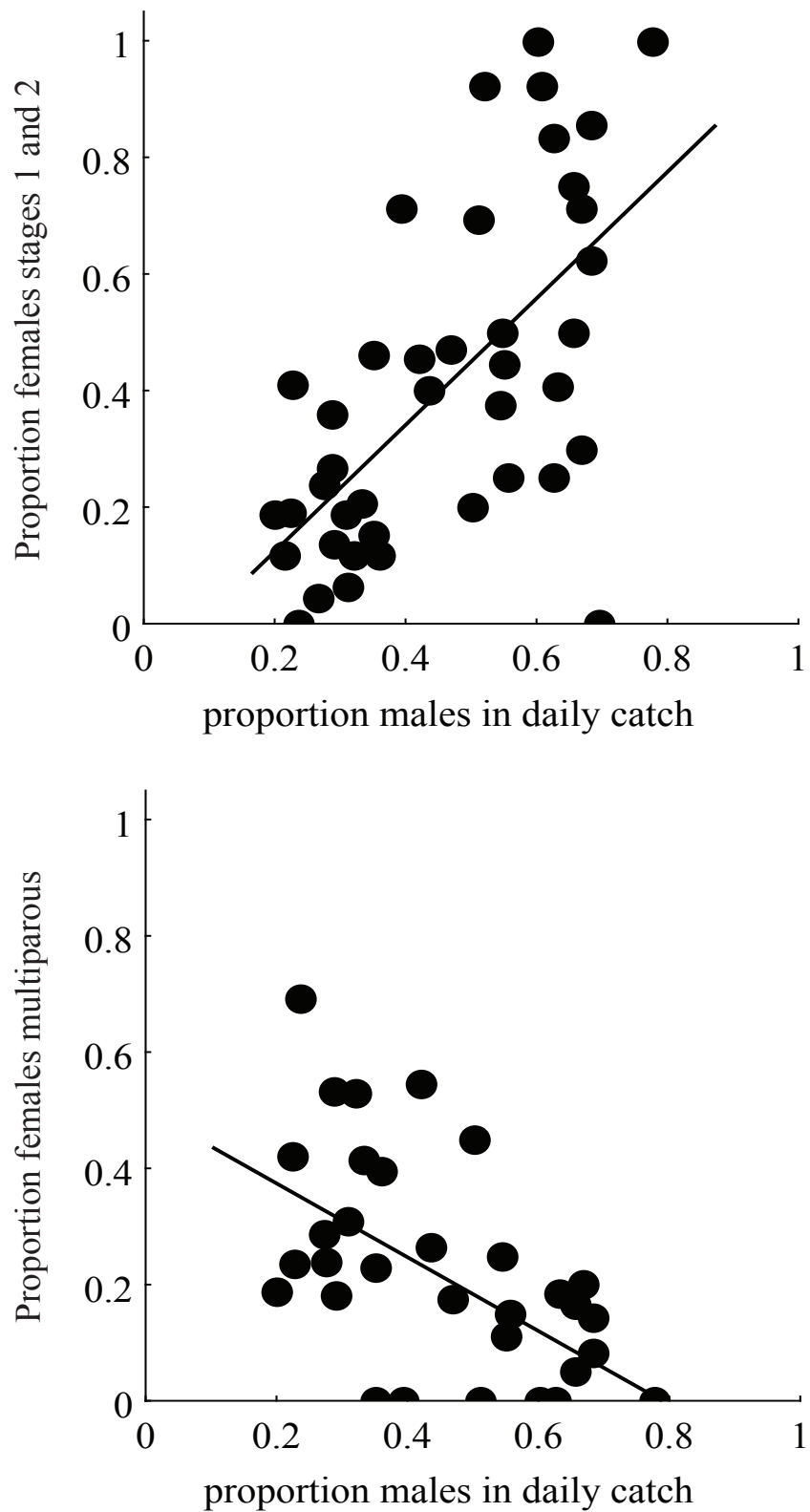


Figure 3. Correlations using daily trap catch data caught with a CO₂-baited Nzi trap at the Warsaw farm site, 2001. Proportion of stable flies that were male in daily catches were plotted against: proportion of female stable flies in stages 1 and 2 (upper), and proportion of females that were multiparous (lower). Only days with more than ten females were included.

Table 3. Summary of correlations between proportions of males in daily trap catches vs proportion of females in each category, year 2001. $N = 44$, the number of days included in the analysis. Days with trap catches of ten or fewer females were excluded from the analysis. Note that females in stages 1, 2, and 3 are unseminated, and stages 6, 9, and 12 are gravid.

Parity	Stages	R^2	t	p	Constant	Slope
nulliparous	1	0.13	2.54	0.015	-0.008	0.858
nulliparous	1,2	0.40	5.32*	<0.0001	-0.093	1.088
nulliparous	2,3	0.09	1.98	0.054	0.082	0.266
nulliparous	3,4	0.12	2.38	0.022	0.261	-0.244
nulliparous	4,5	0.05	1.54	0.131	0.156	-0.115
nulliparous	5,6	0.01	0.77	0.446	0.008	0.026
nulliparous	uninsem	0.35	4.76*	<0.0001	0.072	0.985
nulliparous	insem	0.06	1.64	0.109	0.102	-0.115
nulliparous	1 to 6	0.34	4.63*	<0.0001	0.175	0.870
uniparous	7 to 9	0.06	1.66	0.103	0.325	-0.236
multiparous	10 to 12	0.35	4.80*	<0.0001	0.500	-0.633

*Significant at 5% level after sequential Bonferroni corrections.

in the interpretation of the wind tunnel results is that for the stable flies in the wind tunnel, downwind travel was the default direction, whereas upwind travel required an active response. This may not be the case. In fact, a different null hypothesis is that 50% of male stable flies should be found both upwind and downwind in equal proportions. Interpreting our results under this scenario, it is less the case that the younger flies moved toward the CO_2 , but that the older flies moved away from the CO_2 . However, even if this is so, it does not alter the conclusion with respect to field collections at CO_2 -baited traps. If older male stable flies are repelled by CO_2 , then these will be under-represented in the field collections, as we observed.

This upwind movement of the younger males toward CO_2 provides a possible explanation for the Nzi trap catch data from the beef farm site, in which relatively few males were caught on days in which the female stable flies were found to be physiologically older. It is also interesting that the relationship is not stronger. The proportion of males caught with older stage females suggests that perhaps some proportion of older males may continue to feed on blood, and/or that other factors may be involved.

The only previous evidence from the literature that we could find of possible differences in male response was indirect. Alzogaray and Carlson (2000) rejected the use of male stable flies in their olfactometer tests with CO_2 because their preliminary tests with males produced inconsistent results. Schofield et al. (1997) found that male and female stable flies increased their flight activity in response to CO_2 . Three-day-old male and female stable flies that had received one to two blood meals were used in their experiments. Warnes and Finlayson, (1985a, 1985b) observed that stable flies responded to CO_2 in a wind tunnel with increased flight and found no differences between males and females, using flies which had been fed one blood meal and were two

to three days old.

Is the response of male stable flies to CO_2 an attraction? Previous reports which have described the response of stable flies to CO_2 as attraction have been based on field studies in which attraction effectively means the ability to be trapped. For example, CO_2 was stated to act as an attractant by Hoy (1970), DeFoliart and Morris (1967), Schreck et al. (1975), Gersabeck et al. (1982) and Mohamed-Ahmed and Mihok (1999). However, we recognize that the term "attraction" has a more precise meaning when used in behavioral studies designed to distinguish specific responses. Schofield and Brady (1997) described the upwind movement as anemotaxis and kinesis in response to CO_2 in a wind tunnel, with the response being concentration dependent in both males and females, while other researchers have observed upwind chemotaxis in the presence of CO_2 in wind tunnel experiments (Warnes and Finlayson 1985a, Schofield and Brady 1997).

One possible explanation for the patterns reported in this paper is that males may be using the Nzi traps as an assembly site for mating with females. For example, Mihok et al. observed a high number of males resting on the front and wings of unbaited (2006) and octenol-baited (2007) Nzi traps, and interpreted this as behavior consistent with the Nzi trap acting as a waiting station (Bushman and Patterson 1981), a place from which resting males intercept passing females. Hammer (1941) reported that male and female stable flies used prominent objects such as overhanging branches as assembly sites for mating, and the high collections of stable flies on Alsynite traps has been explained due to the traps being possible assembly sites or waiting stations (Bushman and Patterson 1981). That male behavior may include using the CO_2 -baited Nzi trap as a waiting station is supported somewhat by the high proportion of males being caught with unseminated

females (Table 3). It would be interesting to examine the degree to which males are responding to the baited traps as either waiting stations and/or potential hosts.

Nevertheless, from our research, the differences based on age in male response to CO₂ that we observed in the wind tunnel experiments appears to account for some of the patterns we saw in our field catches. At the beef farm site used in this study, the stable fly population is re-established each year after being killed by cold temperatures each fall (Beresford 2006). The fluctuations in male catches at the CO₂-baited Nzi traps reported in this study argue, at least in part, for using female catch numbers rather than total catch data for measuring population growth rates.

Acknowledgments

We are grateful to Dr. Cheryl McKenna Neuman and the Trent University Geography Department for allowing us to use the wind tunnel. We also thank the two anonymous reviewers for their insightful comments and suggestions. This research was supported in part by a Natural Sciences and Engineering Research Council of Canada (NSERC) Research Grant to JFS and by an NSERC Graduate Fellowship to DVB.

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