Microsporidiosis (Microsporidia: Culicosporidae) Alters Blood-Feeding Responses and DEET Repellency in *Aedes aegypti* (Diptera: Culicidae)

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**ABSTRACT**  Infection of *Aedes aegypti* (L.) (Diptera: Culicidae) with *Edhazardia aedis* (Microsporidia: Culicosporidae) reduced mean human host attraction and landing/probing rates in female mosquitoes by 53 and 62%, respectively, compared with rates in microsporidia-free females. Infection with *E. aedis* reduced the average weight of unfed female mosquitoes by 4%, caused them to imbibe 23% less blood, and to lay 30% fewer eggs than healthy females. In contrast, *E. aedis*-infected mosquitoes required 20% more time (>1 h) than healthy females to bite skin treated with 15% DEET. Statistically significant morbidity in *E. aedis*-infected females was indicated by reductions in host attraction and landing/probing responses, the mass of unfed and blood-engorged females, and fecundity, and by increased DEET repellency.

**KEY WORDS**  *Edhazardia aedis*, attraction, landing/probing, blood feeding, fecundity, repellent protection time

Microsporidia are intracellular parasites. They infect most animal groups, including humans (Chancin-Bonilla et al. 2006), and they are common in mosquitoes, of which >100 host species worldwide are known (Becnel 1994). Mosquitoes become infected with the spores of the parasite, which can be ingested by the larva to germinate in the gut (horizontal transmission), or when the sporoplasm enters a developing egg (transovarial/vertical transmission).

In *Edhazardia aedis* (Kudo 1930) (Microspora: Culicosporidae) (Becnel et al. 1989), different spore types are associated with each mode of transmission, and two generations of *Aedes aegypti* (L.) (Diptera: Culicidae) are required to complete the parasite life cycle. The parental generation commences with ingestion of uninucleate spores by the mosquito larva. Surviving (binucleate spore-containing) female mosquitoes deposit infected eggs into new habitats where the death of filial generation larvae releases uninucleate spores that infect the subsequent generation of *Ae. aegypti*.

Unlike other microsporidia, such as *Amblyospora californica* (Andreadis and Hall 1979), *E. aedis* does not require an intermediate copepod host to complete its life cycle (Becnel 1992). This fact, combined with the capacity of infected female mosquitoes to spread the parasite to new habitats, has provided the impetus for recent efforts to develop *E. aedis* as a biological control agent for *Ae. aegypti* (Becnel 1990, Becnel and Johnson 1993, Becnel et al. 1995). As a result, there is a substantive knowledge of *E. aedis* natural history, host range, and specificity (Becnel et al. 1989, Becnel 1992, Becnel and Johnson 1993, Andreadis 1994), and we understand much of the impact of the parasite on host mosquito biology (Becnel and Johnson 1993; Becnel et al. 1995; Agnew and Koella 1997, 1999; Koella and Agnew 1999; Koella and Offenberg 1999).

However, the effects of *E. aedis* infection on the behavior of *Ae. aegypti*, including how diseased mosquitoes respond to repellents (such as DEET) are poorly understood. Moreover, the nature of parasite-induced variations in host attraction and blood-feeding–related behaviors in mosquitoes has been studied in only a few instances, and then with infectious disease agents of humans, animals, or both, not entomopathogens (Grimstad et al. 1980; Rosignol et al. 1984, 1986; Li et al. 1992; Putnam and Scott 1995).

The question thus remains as to whether sick/diseased mosquitoes manifest differences in their host-seeking– and blood-feeding–related behaviors, compared with healthy mosquitoes, and the extent to which such differences impact the effectiveness of topical repellents such as DEET. In this regard, host attraction and landing/probing rates, body mass and bloodmeal size, fecundity, and repellent protection time are important parameters to understand, because their variation can affect the risk of disease agent transmission to humans and animals, neutralize the potential benefits to vector abatement achieved from

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a pathogen-based biological control program for *Ae. aegypti*, or both.

**Materials and Methods**

**Mosquitoes.** The *Ae. aegypti* used in this study were from a laboratory colony maintained (27°C, 80% RH, and a photoperiod of 14:10 [L:D] h) at the USDA–ARS Center for Medical, Agricultural, and Veterinary Entomology, Gainesville, FL. Larvae were reared according to the technique described by Gerberg et al. (1994). Adults emerged directly into screened stock cages, and they were provided continuous access to 10% sucrose/water solution. Bloodmeals (for colony maintenance) consisted of defibrinated bovine blood warmed to 36–44°C and presented to mosquitoes (in stock cages) in a lamb gut membrane.

**Infection of Mosquitoes with *E. aedis***. The Thailand isolate of *E. aedis* has been maintained since 1981 in laboratory colonies of *Ae. aegypti* (Orlando strain) as described by Hembree and Ryan (1982). Larvae from infected eggs were reared at 27 ± 1°C for 7–9 d, after which patenty infected larvae (detected against a black background) were collected and triturated in a glass tissue grinder. Particulate matter was removed by forcing the extract through cotton packed inside a 50-ml syringe. The resultant spore suspension was washed and centrifuged three times, stored at 20°C, and used within 24 h.

Cohorts of healthy and *E. aedis*-infected adult mosquitoes were reared using equivalent procedures but in separate physical locations within the insectary. To infect mosquitoes, 1,000 third instars of *Ae. aegypti* (Orlando strain) as described by Hembree and Ryan (1982). Larvae from infected eggs were reared to 27 ± 1°C were exposed to 1 × 10^4 spores per larva in 500 ml of water for 2 h (spore concentrations were determined with a hemocytometer before exposure). The same number of larvae without spores was used as controls. Both groups of larvae were transferred to separate trays (50 by 40 cm) each containing 3 liters of water. Three grams of liver: yeast powder (3:1) was added to each tray, and 2 g was added daily thereafter. Pupae from healthy and infected groups were placed in separate cages (45 by 45 by 45 cm) for emergence. Adults were provided 10% sucrose solution in saturated cotton balls. Before and after each test, adult specimens from the healthy and infected groups were crushed on glass slides and examined using phase-contrast microscopy to verify the status of the test populations as either 0 or 100% infected (healthy or infected, respectively) (Becnel and Johnson 1993).

**Host Attraction.** Host attraction in 7- and 14-d-old female mosquitoes was measured using a triple-cage, dual-port olfactometer (Posey et al. 1998). A single test made up the comparison of healthy with infected mosquitoes, and it was replicated four times each (n = 8) with 7- and 14-d-old female mosquitoes.

**Blood Engorgement and Fecundity.** Blood engorgement was quantified according to mass (milligrams) of the engorged female. This was done by individually measuring and comparing the pre- and postblood engorgement masses (to the nearest 0.001 mg) of 25 healthy and 25 infected mosquitoes that were allowed to blood feed to repletion on a restrained chicken. Blood feeding was accomplished by holding the 1.7-mm mesh gauze-covered open end of a plastic specimen cup (5 cm in diameter by 4 cm in height; 78.4 cm^3), with the female mosquito inside, against the exposed skin of a chicken. Once feeding commenced it was allowed to continue until the mosquito voluntarily withdrew its mouthparts from the host’s skin. Females were fasted for 12 h before their prefeeding mass was determined, and then they were narcotized with CO2 immediately after blood engorgement to determine the postfeeding mass. Tests were replicated three times each (n = 150) for 7- and 14-d-old females.

**DEET Repellency.** Repellent treatment consisted of 15% DEET mixed in ethanol and applied evenly to the forearm of a volunteer between the elbow and the wrist at the rate of 1 ml of repellent solution to 650 cm^2 of skin surface. Testing commenced 30 min after re-
pellent application when the volunteer placed his or her arm (treated with repellent) into one of two (randomly selected) test cages containing either 100 healthy or 100 infected Ae. aegypti females. The arm was observed for 3 min for any mosquitoes that landed and attempted to feed (probed the skin). The same procedure was used for the remaining cages. Observations were repeated every 30 min until a confirmed bite was recorded. A confirmed bite occurred when more than one mosquito attempted to feed in the same observation period or when one mosquito attempted to feed in a given observation period followed by a second feeding attempt (by any mosquito) in the subsequent observation period. Complete protection time (CPT) was the number of minutes elapsed between the time of repellent application and the time of the first attempted bite (i.e., that preceding the confirmatory bite). We also calculated repellency in terms of time to the fifth bite (after which each test was ended). Tests were replicated four times each (n = 8) with 7- and 14-d-old females.

Data Collection and Analysis. Host attraction, landing/probing, body mass, blood-engorged mass, fecundity, and CPT responses by Ae. aegypti females were observed based on a split-plot design (Cochran and Cox 1957) with infection status (healthy [h], infected [i]) as whole plots and mosquito age (7 and 14 d postemergence) as subplots. Percentage responses were transformed by arcsine before analysis to minimize heteroscedasticity. Differences between treatment means in each response variable category were compared using t’ (Steel and Torrie 1980) at P = 0.05. Standard errors appropriate to the comparison of main-plot means ([h_i + h_i] versus [i_i + i_i] females), subplot means ([i_i + h_i] versus [i_i + h_i] females), or any two treatment means (h_i versus h_i, h_i versus i_i, h_i versus i_i, i_i versus i_i, h_i versus i_i, i_i versus i_i) were calculated as a weighted average of the whole-plot and subplot experimental errors (Cochran and Cox 1957). Frequency distributions of fecundity responses for each treatment group were compared by assigning a percentile rank to each egg count datum and using analysis of variance (ANOVA) procedures (PROC GLM, SAS Institute 2003) to compare ranks among treatment groups.

The same individual was used in all tests requiring a human subject. Written informed consent was obtained from this individual in accordance with protocol IRB-01 445-96 as approved by the University of Florida, Health Sciences Center, Institutional Review Board for Human subjects. Animal use protocols were reviewed and approved (project A057) by the University of Florida, Institutional Animal Care and Use Committee, Gainesville, FL.

Results

Host Attraction. Healthy (h_i + h_i) females were attracted to host odor at twice the rate (38.8 ± 7.1%) of infected (i_i + i_i) females (18.9 ± 4.1%) (F1,3 = 11.1; P < 0.05). Fourteen-day-old (h_i + i_i) females were attracted at a 20% higher rate (32.7 ± 6.4%) (P > 0.05) than 7-d-old (h_i + i_i) females (25.1 ± 7.0%). Age and age × infection status effects were not significant. On average, 2.6 times more h_i females than i_i females responded to host odor (P < 0.05) (Table 1), but other mean comparisons were not significant.

Landing/Probing. Mean landing/probing responses of healthy Ae. aegypti females (8.9 ± 2.6%) exceeded those for infected females (3.4 ± 2.0%) by 2.6 times (F1,3 = 39.9; P < 0.01), but they were comparable (P > 0.05) when grouped by mosquito age (h_i + i_i, 7.1 ± 4.3%; h_i + h_i, 5.3 ± 2.7%). Age and age × infection status effects were not significant. Differences in the mean response between h_i and i_i, h_i and i_i, i_i and i_i, and h_i and i_i (Table 1) were significant (P < 0.05). In general, healthy females landed/probed 3 times more often than infected females with the largest difference in this regard (44%) between h_i and i_i females.

Blood Engorgement and Fecundity. Age was the only significant source of variation (F1,3 = 9.87; P < 0.05) for unfed female mass, with 14-d-old females weighing 0.156 mg less, on average, than their 7-d-old counterparts (1.92 ± 0.08 and 2.12 ± 0.08 mg, respectively). Age and age × infection status effects were not significant. Only the unfed mass of h_i and i_i females (Table 2) differed significantly (P < 0.05).

Differences in the mean mass of blood engorged healthy (h_i + h_i) (2.86 ± 0.22 mg) and infected (i_i + i_i) females (2.22 ± 0.29 mg) were significant (F1,2 = 8.28; P < 0.05) but not for 7- (2.12 ± 0.08 mg) and 14-d-old (h_i + i_i) females (1.96 ± 0.21 mg). Age and age × infection status effects were not significant. Differences in the mean mass of h_i and i_i, h_i and i_i, h_i and i_i, and i_i and i_i blood-engorged females (Table 2) were significant (P < 0.05), including a 35% disparity in blood-engorged mass between i_i and h_i females.

Infection status but not mosquito age, or the interaction of age with infection status, significantly (F1,3 = 81.94; P < 0.005) influenced differences in mean fecundity. Healthy (h_i + h_i) females developed 53% more eggs (102.6 ± 4.9) than their infected (i_i + i_i) counterparts (71.6 ± 8.9 eggs), whereas variation in

| Table 1. Mean percentage of attraction and landing/probing responses of 7- and 14-d-old healthy female Ae. aegypti and female mosquitoes infected with E. aedis to human arm odor in an olfactometer in 3 min (attraction) or after access for 30 s to exposed human forearm skin in a 40-cm cubic screened cage (landing/probing) |
|---|---|---|
| Response | Age | Mean ± SE by status* |
|          |     | Healthy (h) | Infected (i) |
| Host attraction | 7   | 33.7 ± 12.6 | 16.8 ± 4.6 |
|                | 14  | 43.9 ± 7.5  | 21.4 ± 7.4 |
| Landing/probing | 7   | 7.3 ± 0.8   | 3.2 ± 0.8  |
|                | 14  | 10.5 ± 1.2  | 3.6 ± 1.3  |

*Data subjected to arcsine transformation before analysis.
† Differences in mean landing/probing ≥25.6% [F(1,0.05); SE = 3.9%] are significant; n = 8.
‡ Differences in mean host attraction ≥33.3% [F(1,0.05); SE = 1.2%] are significant; n = 8.

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mean fecundity between age groups (7 d old, 87.1 ±
3.0 eggs; 14 d old, 88.0 ± 2.8 eggs) was not significant.
Differences in fecundity were significant (P < 0.05) for all
treatment group mean comparisons (Table 2) except h7 versus h14 and i7 versus i14.

There was a significant difference (F3,180 = 24.88; P < 0.001) in the frequency distribution of the
ranked fecundity responses among treatment
groups. The mean ranks for i7 (35.7th percentile)
and i14 females (39.5th percentile) differed signifi-
cantly (P < 0.05) from those for h7 (62.6th per-
centile) and h14 (58.9th percentile) females, but not
between 7- and 14-d-old mosquitoes within healthy
or infected groups. Observed 50th percentiles of
fecundity for h7, i7, h14, and i14 females were,
respectively, 108, 82, 104, and 84 eggs.

DEET Repellency. Differences in the mean CPT to
first bite were significant (F1,3,14 = 22.99; P < 0.05) for
healthy versus infected females, but not for means
separated according to mosquito age (7 versus 14 d
old). Age and age × infection status effects were not
significant. Mean comparisons for h7 and i7; h7 and i14;
h14 and i14; and i7 and i14 (Table 3) were significant
(P < 0.05), each differing by >56.8 min. On average,
15% DEET prevented infected females (i7 + i14) from
biting 68 ± 38 min longer than healthy (h7 + h14)
females.

Differences in the mean CPT to fifth bite between
healthy and infected females were significant (F1,3 = 11.31; F < 0.05) but not for other means comparisons.
The CPT to fifth bite for healthy (h7 + h14) mosquitoes
averaged 67 ± 39 min less than for infected (i7 + i14)
mosquitoes. There was no significant interaction be-
tween age and infection status with respect to repel-
lation protection time.

Discussion

Known measures of E. aedis-induced morbidity in
Ae. aegypti include wing length (Nasci et al. 1992) and
the intensity of infection, fecundity, and survival
(Hembree and Ryan 1982; Becnel et al. 1989, 1995).
Some of these parameters are linked to the quantity
of horizontally transmitting (uninucleate) spores of E. aedis in the mosquito, and, in turn, with reductions in
ingested bloodmeal volume in infected females as well as diminished blood-feeding success at the population
level (Agnew and Koella 1997; Koella and Agnew
1997).

In the current study, infection of Ae. aegypti with E. aedis reduced mean host attraction and landing/prob-
ing rates in females by 53 and 62%, respectively, com-
pared with rates in microsporidia-free mosquitoes. In-
fection with E. aedis reduced the average weight of
unfed females by 4%, caused them to imbibe 23% less
blood, and to lay 30% fewer eggs than healthy females.
In contrast, infected mosquitoes required 20% more
time than healthy females to bite skin treated with 15%
DEET. Statistically significant morbidity was indi-
cated by variation in host attraction and landing/probing
responses, the mass of unfed and blood-engorged
females, fecundity of E. aedis-infected mosquitoes, and
by differences in DEET repellency.

Few other data are available for comparing the
effects of parasitism in Ae. aegypti on blood-feeding
behavior or repellent efficacy. Rossignol et al. (1986)
observed increased olfactometer responses in
Ae. aegypti infected with Plasmodium gallinaceum and
considered the results to be evidence of a higher biting rate in infected compared with healthy mos-
quitos. Earlier studies by these same workers (Ros-
signol et al. 1984) attributed increased probing
times in sporozoite-infected Ae. aegypti to changes
in mosquito saliva that impaired the female’s ability
to locate blood vessels. In Aedes sierrensis (Ludlow),
infection with Lambornella clarki (Ciliophora: Tet-
rahymenidae) increases the time required for fe-
male mosquitoes to alight on a human hand by
>200% and the time required for probing before
commencement of blood feeding by >130%
(Egerter and Anderson 1989). These workers con-
cluded that inhibition of blood feeding in infected
females was a response to parasite mediation of host
humoral factor(s), a ciliatosis-based physiological
manifestation of morbidity (decreased vigor) in the
mosquito, or both.
Among nonculicid species, tsetse flies infected with salivary trypanosomes probe (mice) more frequently, and feed more voraciously, than uninfected male and female flies (Jenni et al. 1980). However, uncertainty exists regarding the influence of epimastigote colonies in the labrum of infected flies on this behavior or as indirect regulators of the flow rate of ingested blood (Moloo 1983).

Although microsporidia have exploited nearly all tissues of all life stages of the range of insect hosts they infect, affected tissues in mosquitoes comprise the epithelium of the gastric caeca, oenocytes, and the fat body (Becnel and Andreadis 1999). Salivary gland pathology is normally not a consequence of *E. aedis* infection in *Ae. aegypti* (Becnel et al. 1989); thus, it is an unlikely source of variation in the feeding/probing rates of infected females. Similarly, with respect to the involvement of the ovaries in *E. aedis*-infected mosquitoes, the percentage reduction we observed in mean fecundity responses (30%) was less than the 43–69% reductions noted by other workers for *Ae. aegypti* (Hembree and Ryan 1982, Becnel et al. 1995). None of these differences can be resolved by pathology, because *E. aedis* does not measurably damage the ovaries of *Ae. aegypti* (Becnel et al. 1995). Nevertheless, we observed a larger percentage of 7- and 14-d-old blood-fed infected females without stage 5 oöcytes (26 and 21%, respectively), compared with healthy females (4 and 5%, respectively). This phenomenon was manifest as a positively (right)-skewed frequency distribution of rank percentiles for fecundity responses in infected females, and it provided a novel measure of morbidity in *E. aedis*-infected *Ae. aegypti* that may be applicable to the characterization of microsporidiosis in other mosquito species, including *Amblyospora* sp. infections in *Culex salinarius* Coquillett (Andreadis and Hall 1979).

Infection of *Ae. aegypti* with *E. aedis* influences bloodmeal mass when the latter is estimated on the basis of the intensity (spores per milliliter) of larval infection; thus, a high spore concentration (10^4 spores per ml) results in a 50% reduction in bloodmeal mass, regardless of the age of the larva at exposure (Koella and Agnew 1997). The pathological basis for this effect is unknown; however, differences between healthy and infected female mosquitoes in the amount of ingested blood may reflect the virulence of *E. aedis* infection, including effects more generally defined in the literature as “loss of vigor” (Gaugler and Brooks 1975, Brooks 1988).

The responses of parasite-infected mosquitoes to DEET are known from one study. Robert et al. (1991) tested DEET against *P. falciparum* and *P. berghei*-infected and uninfected *Anopheles stephensi* Liston females (20–24 d-old). They found no statistical differences among the mean responses, although the median effective doses (ED_{50}) observed for *P. falciparum*-infected (3.2 μg/cm^2) and *P. berghei*-infected females (2.3 μg/cm^2) exceeded those for their respective uninfected female counterparts by 170–180% (1.9 and 1.3 μg/cm^2). In addition, in each case, the ED_{50} 95% confidence interval (CI) for the ED_{50} for uninfected females fit inside the 95% CI for infected females. The conclusion of these workers that DEET could be used in the field with equal success against malaria-infected and uninfected *An. stephensi* is consistent with our observations of the responses of *E. aedis*-infected *Ae. aegypti* to DEET, given that mean protection times against the bites of *i_1* and *i_4* females were longer than against *h_2* and *h_4* females when using the same dose of repellent.

A question of theoretical importance in this study is whether the responses of *E. aedis*-infected *Ae. aegypti* are an adaptation to *E. aedis* pathology, or a nonadaptive side effect of *E. aedis* infection, or an increase/decrease in an activity performed before infection (Poulin 1995). Female *Ae. sierrensis* (Ludlow) castrated by *Lambornella clarki* (Ciliophora: Tetrahymenidae) are an example of the first case in that infected mosquitoes respond less to host cues than healthy individuals (Egerter and Anderson 1989) and then expend energy normally used for host seeking to deposit parasite trophonts in mosquito developmental sites (Egerter et al. 1986). The nonadaptive (second) case results in a coincidentally beneficial side effect to the host or parasite. In studies of intradermal probing time of *Ae. aegypti* infected with *P. gallinaceum*, for example, Rossignol et al. (1984) observed the lengthiest responses by infected female mosquitoes. These were attributed to salivary gland pathology (decreased apyrase activity) and interpreted as beneficial to *Plasmodium* because they enhanced the efficiency of transmission (Ribeiro et al. 1985). The third case is illustrated by instances in which researchers were unable to distinguish healthy from parasite-infected mosquito probing times (Li et al. 1992, Paulson et al. 1992, Putnam and Scott 1995), and they concluded the differences in these responses to be an extension of normal mosquito activity.

In *Ae. aegypti*, high mortality in the larval mosquito population caused by *E. aedis* infection favors horizontal transmission of the parasite and its maintenance in the aquatic habitat. Vertical transmission favors dispersal of the parasite among mosquito developmental sites. Both transmission modes are linked to the relative composition of spore types within the mosquito’s body. On this basis, Koella and Agnew (1997) concluded from their study of the relationship between the route of transmission of *E. aedis* and blood-feeding success in *Ae. aegypti*, that although blood-feeding success decreased as the number of uninucleate spores increased, it was not influenced by the number of binucleate spores in the female mosquito’s body. Given this perspective, the impact (virulence) of *E. aedis* infection on the mosquito host seems to decrease during the part of the life cycle when parasite transmission is vertical, thereby favoring *E. aedis* dispersal. However, the results of our study show that benefit(s) to the parasite under these circumstances may be reduced in a manner commensurate with the level of infection-induced morbidity in the mosquito population.
This morbidity is manifest as a significantly diminished capacity for vertebrate host-finding– and blood-feeding–related activity in diseased mosquitoes and is accompanied in same by an increased sensitivity to DEET.

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