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COMPARATIVE DEVELOPMENT OF *BRUGIA PAHANGI* AND VARIATION IN ACID HYDROLASE ENZYME TITERS IN *AEDES AEGYPTI*

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ABSTRACT. The development of the filarial nematode *Brugia pahangi* was monitored and compared in susceptible (BLACK EYE) and refractory (ROCK) strains of *Aedes aegypti*. Simultaneously, the activities of acid phosphatase, β-glucuronidase, α-glucosidase, and *N*-acetyl-β-glucosaminidase were measured. Three- to five-day-old females of both strains were fed on infected and uninfected clawed jirds (*Meriones unguiculatus*) then dissected or homogenized at 2 h, at 24-h intervals for 5 days, and at 8 and 10 days after treatment. Enzyme activities were assayed by a fluorometric procedure. The susceptible strain maintained an 80% infection and 18.6 larvae/mosquito over the 10-day period. In contrast, the refractory strain was initially 33% infected and had a mean of 4.9 larvae/mosquito and this decreased to 20% by 3 days, and to 3% with a mean of 0.33 larvae/mosquito at 10 days. Significantly higher acid phosphatase and β-glucuronidase activities were observed in the refractory strain at specific time intervals after infection. α-Glucosidase and *N*-acetyl-β-glucosaminidase were highly variable among strains and according to infection status. Analysis of the results of this study suggests that certain acid hydrolase enzymes could be involved in the elimination of *B. pahangi* in refractory strains of *Ae. aegypti* and could be used to monitor biochemical changes in response to filarial nematode infections in certain mosquito populations.

KEY WORDS *Aedes aegypti*, acid hydrolases, *Brugia pahangi*, filarid development

INTRODUCTION

Previous studies with *Aedes aegypti* (L.) reported genetic differences in a variety of traits, including susceptibility to vertebrate pathogens (Macdonald 1962, Craig and Hickey 1967, Kilama and Craig 1969, Munstermann and Craig 1979, Thathy et al. 1994). For filariasis, Macdonald (1962) showed that a sex-linked recessive gene named f^m (filarial susceptibility, *Brugia malayi*) controlled susceptibility in this mosquito. Subsequently, Macdonald and Ramachandran (1965) established that the f^m gene controlled development of the human parasites *Wuchereria bancrofti* (Cobbold) and *Brugia malayi* (Brug), as well as the animal filarid *Brugia pahangi* (Buckley and Edeson). Research in our laboratory has focused on the biochemical genetic basis of filarial susceptibility in *Ae. aegypti*. Both *B. pahangi* and *B. malayi* were used as the filarial nematode models, and the clawed jird (*Meriones unguiculatus*) served as the vertebrate host (Ash and Riley 1970a, 1970b; Ash 1972).

Changes have been found to occur in the titers of certain acid hydrolase enzymes in *Ae. aegypti* in response to filarial nematode infections (Schirf and Rodriguez, unpublished data). Others have proposed that certain hydrolase enzymes afford a humoral or cell-mediated response to immunologic challenge (Soderhall and Smith 1986, Stoffolano 1986). In addition, specific experiments have shown variation in acid hydrolase titers in development life stages of different strains of both *Ae. aegypti* and *Aedes togoi* (Theobald) (Nasr-Schirf et al. 1989, Rodriguez et al. 1998). Earlier studies also demonstrated differential development of filarids in various mosquitoes (Lawrence and Pester 1961, Eslinger 1962, Schacher 1962, Ewert 1965, Rodriguez et al. 1984). The present study sought to compare development of *B. pahangi* and activity differences of four acid hydrolase enzymes post-infection in 2 specific strains of *Ae. aegypti*. Both strains were tested with both *B. pahangi* and *B. malayi* infections and the BLACK EYE strain was shown to be 95% susceptible (Rodriguez 1973), whereas the ROCK strain was refractory to infective larvae or L_3 development (Rodriguez and Craig 1973). The acid hydrolases included acid phosphatase (ACP, orthophosphoric monoester phosphohydrolase), β-glucuronidase (β-GlcUr, β-D-glucuronidase glucuronosohydrolase), α-glucosidase (α-Glc, α-D-glucoside glucohydrolase), and *N*-acetyl-β-glucosaminidase (β-GlcNAc, 2-acetaminido-2-deoxy-acetylaminodeoxy-glucohydrolase).

MATERIALS AND METHODS

The strains of *Ae. aegypti* used were obtained from the Vector Biology Laboratory, University of Notre Dame (Notre Dame, IN). Rodriguez (1973) and Rodriguez and Craig (1973) reported their history and place of origin. The clawed jirds infected with *B. pahangi* were obtained from John McCall (School of Veterinary Medicine, University of Georgia, Athens, GA). Mosquitoes were reared according to methods described by Craig and VandeHey (1962) and Thompson and Rodriguez (1979) in a walk-in environmental chamber (Environator Calumet Scientific, Inc., Elk Grove, Village, IL) at 27 ± 1°C and 85% relative humidity. All female mosquito infections were established in

a reach-in incubator (Scientific Systems, Baton Rouge, LA) using similar temperature and humidity, and infection techniques as reported by Rodriguez (1973).

In preparation for infections, 700 3- to 5-day-old female mosquitoes of either strain were starved for 24 h then fed simultaneously on 2 jirds having a mean microfilarial density of 491/20 mm^3 of blood. The same jirds were used in each of 4 separate replicates. Also, only fully engorged females (175) per strain and replicate were selected. A 2nd group of 200 females of similar age and strain were bloodfed on 2 uninfected jirds (Tumbelbrook Farm Inc., West Brookfield, MA). To minimize desiccation, 2 paper cups (5-oz) containing moistened cotton balls and lined with paper toweling were placed in each of the cages.

In the course of 4 replicates, 40 mosquitoes per strain were dissected 2 h after infection. Seven other comparable groups of 60 mosquitoes were dissected at 24-h intervals over a period of 5, 8, and 10 days postinfection. Samples for the enzyme assays were taken at similar time intervals postinfection or normal bloodfeedings. Overall, 18 females per enzyme, strain, and infected or uninfected groups were tested. Moreover, 2 replicates per strain, group, and experiment with a random pool of 6 females per sample were used.

Mosquitoes were dissected under a stereoscopic microscope in *Aedes* physiologic saline (Hayes 1953) containing 1 or 2 drops of dilute Giemsa stain and analyzed under a binocular compound microscope. During dissection, each female was anesthetized with chloroform and separated into head, thorax, and abdomen using dissecting needles. Individual parts were then carefully teased apart and the number of microfilariae and subsequently active developing larvae (days 1–10) per female mosquito, strain, and replicate were recorded. In preparation for enzyme assays, mosquito samples were homogenized in 0.5 ml of 0.25 M sucrose with a Potter-Elvejham homogenizer. The homogenates were then centrifuged (Microcentrifuge, Eppendorf Model 5414, Fisher Scientific, Springfield, NJ) at 14,000 rpm at 4°C for 5 min and the supernatant was collected.

Fluorometric assays (Turner Model 112 fluorometer, Sequoia-Turner Corp., Mountain View, CA) were used to determine the activities of the 4 enzymes with 4-methylumbelliferone as the standard (Guilbault 1973). Also, these assays are based on enzymatic hydrolysis of unfluorescent 4-methylumbelliferone (4-MU)-conjugated substrates to produce 4-methylumbelliferone that is highly fluorescent at an alkaline pH (Rodriguez et al. 1998). Appropriate fluorometric substrates were purchased from Sigma Chemical Co. (St. Louis, MO).

Acid phosphatase was analyzed according to Robinson and Glew (1980) using 4-MU phosphate as the substrate. β-Glucuronidase was tested according to Mead et al. (1955), but modified with

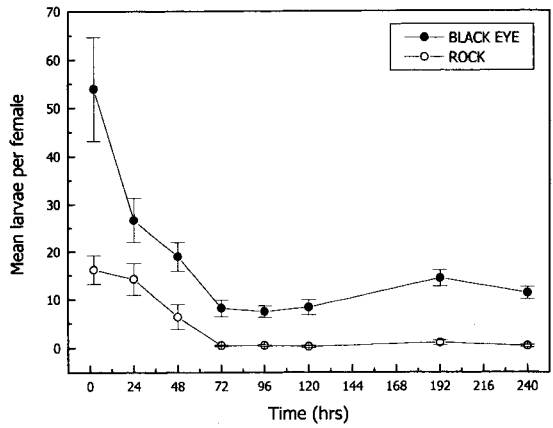

Fig. 1. Variation in mean larvae (±SEM) of *Brugia pahangi* infections in female susceptible BLACK EYE (BE) and refractory ROCK (RK) strains of *Aedes aegypti* at various time intervals. Open circles represent ROCK (RK) strain infections; closed circles are BLACK EYE (BE) infected strains.

0.25 mM acetate buffer at pH 5.0 and 4-MU-β-glucuronide as the substrate. α-Glucosidase was assayed based on procedures developed by Robinson (1956) with 250 mM acetate buffer at pH 5.5. Determinations for β-GlcNAc were adapted from Leaback and Walker (1961), but modified using 40 mM citrate-phosphate buffer at pH 4.4 and 4-MU-*N*-acetyl-β-glucosaminide as the substrate. Also, 10% Triton-X-100 was added to all samples before incubation, and reactions were stopped with ammonium hydroxide-glycine buffer, pH 10.5. Total protein for both strains was estimated according to Bradford (1976) with a commercially available Coomassie concentrate (Bio-Rad, Richmond, CA). Bovine serum albumin was used as the standard. The specific enzyme activities are reported as picomoles 4-MU released/min/mg protein.

Data were analyzed with an IBM/CMS system using SAS subprograms (SAS Institute 1985). Analysis of variance and Duncan's multiple range tests were used to test for significant differences in enzyme activities among strains and time intervals. Student's 2-tailed *t*-tests were conducted to test for significance differences between groups in each interval of filarial nematode development (Sokal and Rohlf 1995).

RESULTS

Differential filarial nematode development

The susceptible BLACK EYE strain maintained an 80% infection and 18.6 larvae/mosquito over the 10-day period, whereas the refractory ROCK strain was initially 33% susceptible with a mean of 4.9 larvae/mosquito, but this declined to 20% by 3 days, and was 3% with 0.33 larvae/mosquito at 10 days (Figs. 1 and 2). Two-sample *t*-tests (Sokal and

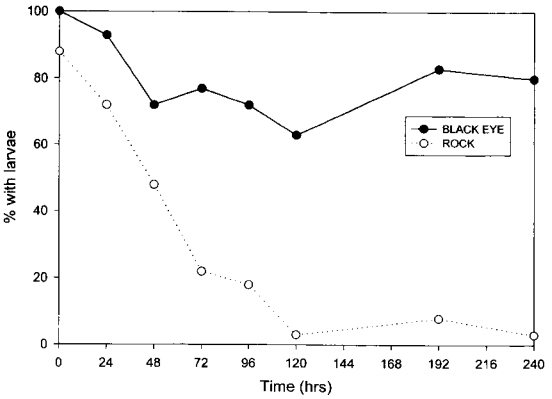

Fig. 2. Variation in the percentage of females infected with *Brugia pahangi* in susceptible BLACK EYE (●) and refractory ROCK (○) strains of *Aedes aegypti* at various time intervals.

Rohlf 1995) indicated that these changes were highly significant for the BLACK EYE strain between the 2-h and 3-day intervals ($t = 4.198$, $P < 0.001$), but not statistically significant when the 3-day and 5-day ($t = 0.073$, $P < 0.05$) or the 5-day and 10-day ($t = 1.465$, $P < 0.05$). For the ROCK strain, similar comparisons between the 2-h and 3-day intervals were significant ($t = 5.221$, $P < 0.001$). However, comparisons between the 3-day and 5-day intervals ($t = 0.678$, $P < 0.05$) or 5-day and 10-day intervals ($t = 0.307$, $P < 0.05$) were not different when the α level is equal to 99% ($P > 0.01$). The mean number of larvae per female in BLACK EYE was greater at each of the time intervals when compared to ROCK. Moreover, these differences were statistically significant (e.g., $t = 3.372$, $P < 0.01$; at 2 h; $t = 5.738$, $P < 0.001$ at 3 days; $t = 5.073$, $P < 0.001$ at 5 days; $t = 8.488$, $P < 0.001$ at 10 days).

Hydrolase enzyme titer variation

The mean specific activities of the 4 acid hydrolases at different time intervals after uninfected bloodfeedings in both strains are given in Figs. 3–6. These figures also compare the mean specific activities for the same 4 enzymes at various time intervals postinfection with *B. pahangi.*

Significantly higher ACP and β-GlcUr titers were observed in the refractory ROCK strain at specific time intervals after infection. For example, ACP levels were greatest at 2 and 8 days postinfection with *B. pahangi.* β-Glucuronidase activities increased at 1, 2, and 8 days after infection, as well as at 2 h and 3 and 4 days in uninfected groups. Some significant titers were obtained with α-Glc, but β-GlcNAc gave variable results. Higher ACP (Fig. 3) and β-GlcUr (Fig. 4) activities were observed in the refractory strain at specific time intervals postinfection. Acid phosphatase showed significantly higher activities (22.78–72.44 pmoles/min/mg) 2–8 days postinfection, with highest levels (72.44) at day 2 (Fig. 3; $F = 10.95$, $P < 0.0001$).

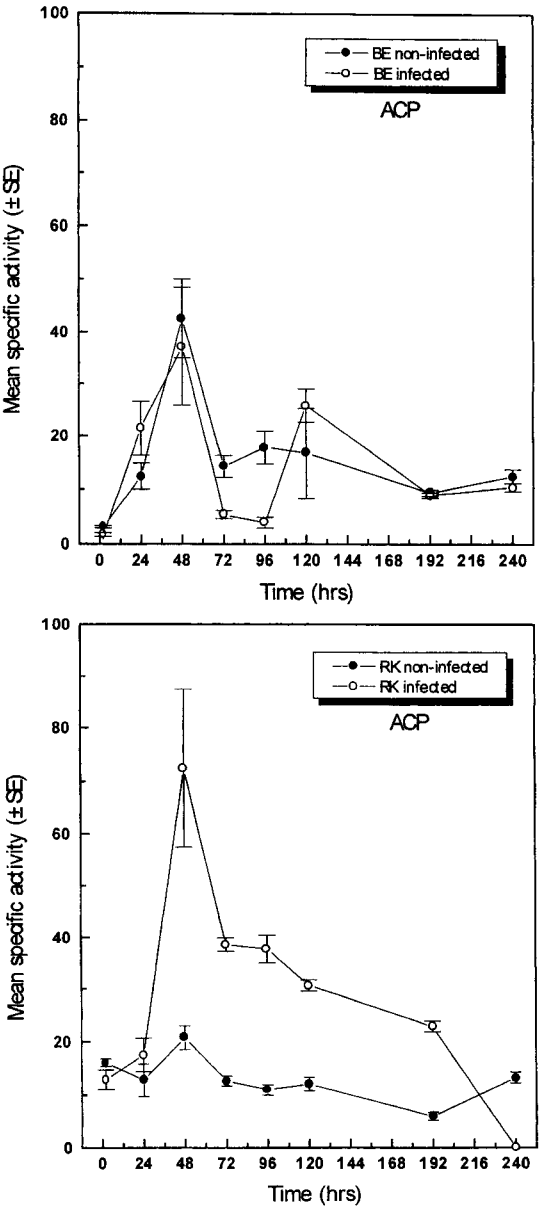

Fig. 3. Mean specific activity (±SEM) of acid phosphatase (ACP) in uninfected (●) and infected (○) strains of *Aedes aegypti* at various time intervals. Top graph shows susceptible BLACK EYE (BE) strain; bottom graph indicates ROCK (RK) strain.

β-Glucuronidase activity was variable with more pronounced levels at 2 h (mean = 0.72 pmoles), and 3 days (mean = 0.60 pmoles) and 4 days (mean = 0.45 pmoles) after a normal blood meal in the refractory strain (Fig. 4; $F = 59.10$, $P = 0.0001$). A significantly high level of β-GlcUr was also ob-

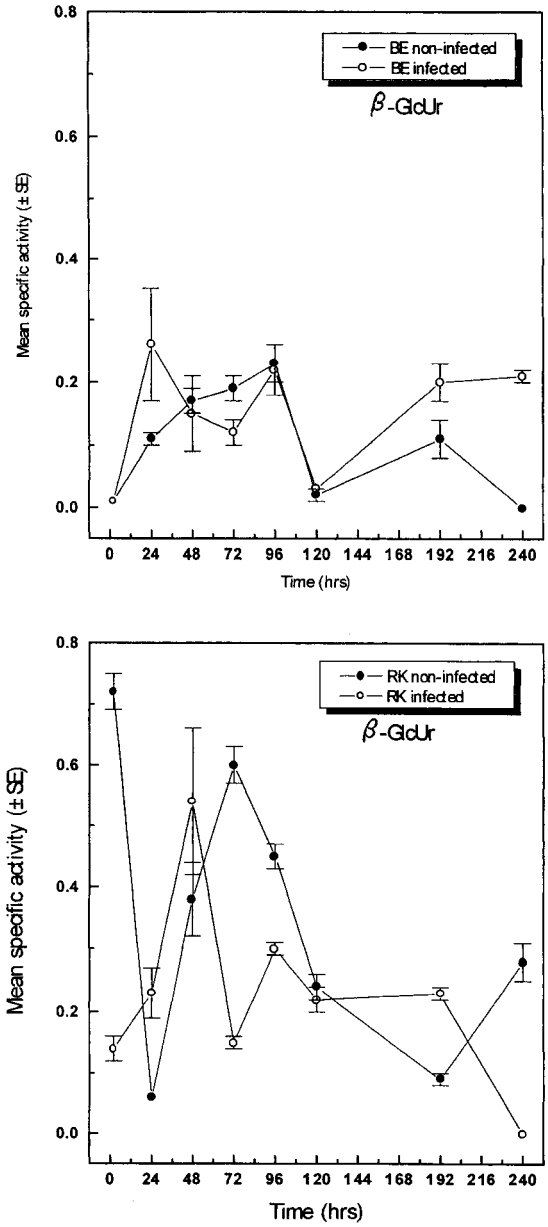

Fig. 4. Mean specific activity (±SEM) of β-glucuronidase (β-GlcUr) in uninfected (●) and infected (○) strains of *Aedes aegypti* at various time intervals. Top graph shows susceptible BLACK EYE (BE) strain; bottom graph indicates refractory ROCK (RK) strain.

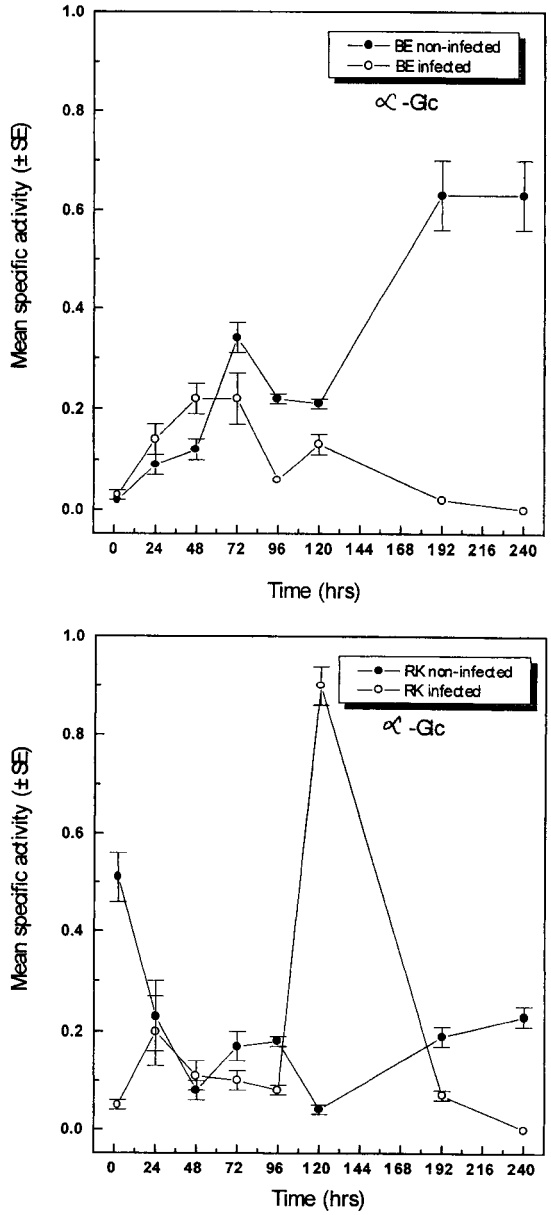

Fig. 5. Mean specific activity (±SEM) of α-glucosidase (α-Glc) in uninfected (●) and infected (○) strains of *Aedes aegypti* at various time intervals. Top graph shows susceptible BLACK EYE (BE) strain; bottom graph indicates refractory ROCK (RK) strain.

tained in the same refractory strain 2 days postinfection (mean = 0.54 pmoles; F = 7.20, $P < 0.0001$).

α-Glucosidase gave significantly higher levels in the refractory uninfected group at 2 h (Fig. 5; mean = 0.51; F = 17.35, $P < 0.0001$). However, the infected refractory mosquitoes had a more pronounced level of α-Glc at day 5 (mean = 0.90 pmoles; F = 90.47, $P < 0.0001$). High α-Glc activity (0.63 pmoles) was also observed in the susceptible BLACK EYE strain, but this level of the enzyme only occurred at days 8 and 10 in uninfected populations (Fig. 5; F = 40.38, $P < 0.0001$).

N-acetyl-β-glucosaminidase also gave variable results, with the highest level in refractory populations 1 day (mean = 31.53 pmoles) after a uninfected or normal blood meal (Fig. 6; F = 12.81,

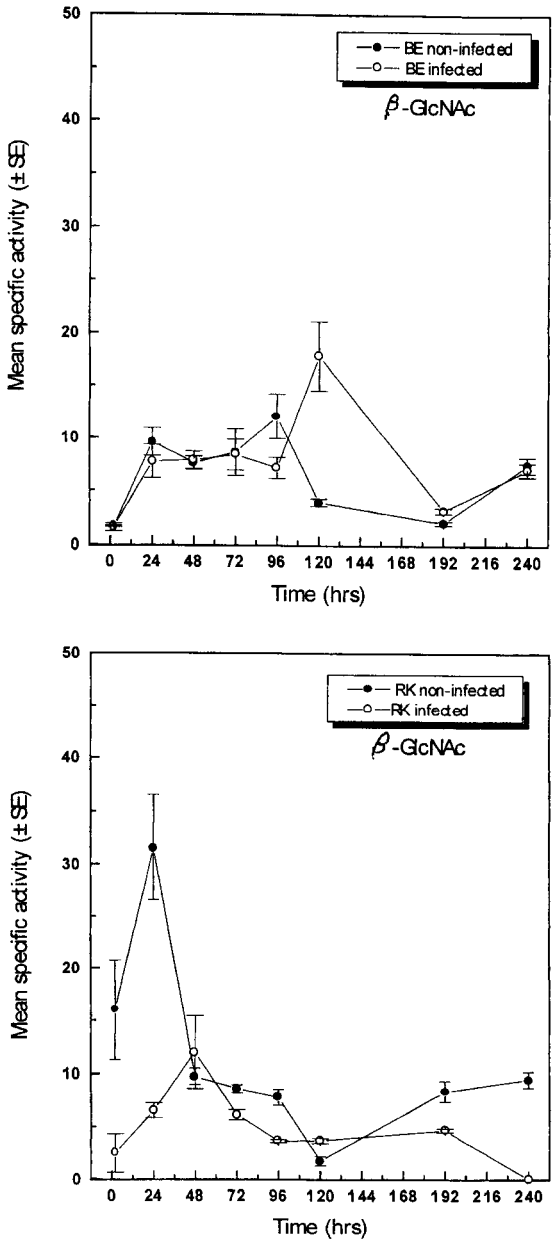

Fig. 6. Mean specific activity (±SEM) of *N*-acetyl-β-glucosaminidase (β-GlcNAc) in uninfected (●) and infected (○) strains of *Aedes aegypti* at various time intervals. Top graph shows susceptible BLACK EYE (BE) strain; bottom graph indicates refractory ROCK (RK) strain.

$P < 0.0001$). High levels of this acid hydrolase also were obtained in infected refractory populations (mean = 11.99 pmoles) at 2 days and in susceptible ones (mean = 17.79 pmoles) at 5 days postinfection (Fig. 6; $t = 1.176$, $P > 0.05$ and $t = 4.278$, $P > 0.01$, respectively).

DISCUSSION

Various investigators (Edeson et al. 1960, Esslinger 1962, Schacher 1962, Ewert 1965, Schacher and Khalil 1965) have studied the progressive development of *B. pahangi* and other filarids in natural and experimental laboratory mosquito vectors. However, relatively few studies have been published in the area of invertebrate lysosomal enzymes and their role in response to parasitic infections (Cheng et al. 1977, Cheng and Garrabrant 1977, Cheng and Dougherty 1989).

Esslinger (1962) reported the behavior of *B. pahangi* during the 1st hour postinfection in *Anopheles quadrimaculatus.* Schacher (1962) published detailed information and specific morphologic changes of *B. pahangi* larvae in both *An. quadrimaculatus* Say and *Armigeres obturbans* (Wolper). Comparative migration and development of *Brugia patei* in *Mansonia uniformis* (Theobald) (Lawrence and Pester 1961) and *B. pahangi* (Ewert 1965) in various mosquitoes also have been demonstrated.

Cheng and Garrabrant (1977) described the encapsulation of *Schistosoma* mother sporocytes in the gastropod *Biomphalaria glabrata.* Granulomas surrounding the intact sporocytes were produced. This was followed by disintegration of the parasite with a significant increase in levels of ACP 2–3 days postinfection. Other experiments gave increased levels of lysozyme in the serum and phagocytes of *B. glabrata* challenged with bacterial infections (Cheng et al. 1977). A subsequent study with schistosome infections in snails of the same species also provided evidence for the destruction of sporocysts associated with elevated levels of ACP and aminopeptidase enzymes (Cheng and Dougherty 1989). For mosquitoes and other arthropods, hydrolases or lysosomelike enzymes have been proposed as a humoral or cell-mediated response to immunologic challenge upon nematode infections (Soderhall and Smith 1986, Stoffolano 1986).

Marked differences were found in the progressive development of *B. pahangi* larvae in susceptible and refractory strains of *Ae. aegypti.* Significantly higher levels of ACP and β-GlcUr enzymes were also observed in the refractory ROCK strain associated with specific time intervals after *B. pahangi* infections. Other acid hydrolases, α-Glc, and β-GlcNAc gave variable titers. Both strains initially showed high-end infections (BLACK EYE = 100%; ROCK = 88%), which were followed by a marked decrease in susceptibility and mean number of larvae 3 days postinfection (BLACK EYE = 77%, mean larvae = 8.10; ROCK = 22%, mean larvae = 0.40). In addition, the susceptible strain maintained a higher infection (80%) and a significantly greater mean number of larvae (mean = 18.61). However, the refractory mosquito strain gave substantial and significant decreases in filarids

by 3 days. At 5 and 10 days postinfection most of the filarids were eliminated (Figs. 1 and 2).

Acid phosphatase levels were most pronounced at 2, 3, 4, and 8 days postinfection in the refractory strain (Fig. 3). β-Glucuronidase had a significantly higher or almost 4-fold increased activity in the refractory strain 2 days postinfection (Fig. 4). Other related experiments completed in our laboratory with *B. malayi* using the same susceptible (BLACK EYE) and refractory (ROCK) strains of *Ae. aegypti* have given similar results. *Brugia malayi* infections were eliminated from the refractory vector host 8 days postinfection. Significant differences in development also occurred 2–3 days postinfection, with the refractory strain showing a much greater decrease in both susceptibility and the mean number of larvae (Rodriguez et al. 1984). Follow-up experiments using refractory ROCK females gave pronounced levels of ACP 4 days postinfection with *B. malayi.* β-Glucuronidase titers also were higher in infected populations of refractory mosquitoes at 3 and 5 days postinfection (Schirf and Rodriguez, unpublished data).

Analysis of the results of this study suggested that ACP and β-GlcUr could be involved in the destruction or elimination of *B. pahangi* filarids in refractory strains of *Ae. aegypti* once humoral or hemocytic encapsulation responses occur. A previous study with *B. pahangi* has already given some encapsulation responses to developing larvae (Morrison and Rodriguez, unpublished data), and other investigators propose hemocytic encapsulation and intracellular melanization as an immune response of insects to nematodes (Salt 1970, Forton et al. 1985, Stoffolano 1986). In addition, elevated enzyme titers were more pronounced 2–8 days postinfection. Also, these and other acid hydrolases could well be used as effective measures to monitor biochemical changes, in certain mosquito populations, in response to filarial nematode infections. Currently, further studies are being pursued to substantiate possible specific genetic causes. Other techniques, including ultrastructural analyses and protein characterizations, are being developed to test for important enzyme differences. Normal, bloodfed, infected, and chemical mutagen-altered filarial-susceptible or -refractory strains of both *Ae. aegypti* and *Ae. togoi* mosquitoes will be analyzed.

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