

Antibody Immunoglobulin G (IgG) Response to *Alouattamyia baeri* (Diptera: Cuterebridae) Parasitism of Howler Monkeys, *Alouatta palliata*, in Panama

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ABSTRACT Larval bot fly burdens and the presence of immunoglobulin G (IgG) antibodies to larval antigens of *Alouattamyia baeri* (Shannon & Greene) were determined in howler monkeys, *Alouatta palliata* authority, from Barro Colorado Island, Panama, during July and August of 1991 and 1992. Monkeys produced antibodies (IgG) to both 1st- and 3rd-instar proteins of the monkey bot as measured by an enzyme immunoassay. The response to 1st-instar antigen was correlated with number of bots for the 1991 data and for pooled data from 1991 and 1992. No correlation was observed for the response to 3rd instar antigen. First-instar extracts were composed of 9 major proteins as visualized by SDS-PAGE. Bands at 17, 25, and 32 kDa were positive in Western blots. Third-instar extracts contained at least 13 major bands, with those at 120 and 130 kDa reactive in immunoblots. The immune response to *A. baeri* may be involved in limiting larval bot numbers.

KEY WORDS Panama, *Alouattamyia baeri*, howler monkeys, immune response, *Alouatta palliata*

HOST-PARASITE INTERACTIONS IN myiasis vary both in degree and intensity (Baron and Colwell 1990). Extensive tissue invasion, lasting several months, is observed in common cattle grub *Hypoderma lineatum* (Villers), northern cattle grubs, and *H. bovis* (L.) infestations; whereas, sheep bot fly *Oestrus ovis*, L., infestations involve only superficial tissue invasions. The degree of tissue damage can be small, as in cattle grubs and sheep bots, or massive, as with infestations with screw-worms, *Cochliomyia hominivorax* (Coquerel), that routinely result in death of the host. Cuterebrid larvae generally have a relatively brief host association, undergoing a short internal migration followed by completion of development at subdermal sites (Catts 1982). This short host association generally is viewed as benign, causing no significant reduction in host fitness (Catts 1982).

Howler monkeys, *Alouatta palliata*, on Barro Colorado Island, Panama, are parasitized regularly by larvae of the cuterebrid *Alouattamyia baeri* (Shannon & Greene) resulting in significant host mortality (Milton 1996). This mortality results from a synergistic effect among the age, physical condition, and fat reserves of parasitized individuals, the size of the larval burden, and increasing dietary stress as the rainy season progresses (Mil-

ton 1996). The lack of growth of the isolated monkey population is postulated to result from primary and secondary effects of bot fly parasitism. Howler monkey mortality is correlated strongly with the prevalence and intensity of bot fly infestation (Milton 1996). This observation challenges the dogma that host-parasite relationships evolve toward a benign state (Holmes 1983), where the prudent parasite limits its impact on the host. However, because the host must maintain some defense to limit the parasite and the parasite needs to maintain sufficient virulence to overcome the host defense, there is a cost associated with the relationship that is often overlooked. In the howler monkey-bot fly system the development of immune responses (both humoral and cellular) and the production of the warbles are energetic costs to the host (see Keymer and Read 1991) that may influence fitness.

The number of bot flies infesting monkeys appears limited (Milton 1996). Mature monkeys may acquire resistance to bot fly infestation through multiple exposures, which limits the size of parasite burdens and reduces mortality in this host age class. Acquired resistance to myiasis occurs in other host-parasite systems (Baron and Colwell 1990); cattle grub survival diminishes in cattle with each successive exposure (Baron and Weintraub 1987). Protective effector mechanisms of acquired resistance to myiasis-producing arthropods are not well understood. For example, the evidence for a protective role of anti-*Hypoderma* spp. antibody is

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weak Pruett and Barrett 1983, Colwell and Baron 1990, whereas evidence for a protective role of cell-mediated immunity in *Hypoderma* spp. infestations is somewhat stronger (Baron and Weintraub 1987, Baron and Colwell 1991). However, immunization with *Hypoderma* spp. proteins does stimulate protection that is defined by the intensity of both the humoral and cellular responses (Baron and Colwell 1991). Therefore, the antibody response should not be overlooked.

The purpose of this study was initiated to determine if bot fly infestation stimulates an immune response that limits the level of infestation in previously infested monkeys. We determined if antibody production to specific *A. baeri* proteins occurred and examined the relationship between antibody levels and bot fly larval burdens. The major antigenic components of 1st and 3rd instars also were examined.

Materials and Methods

Study Site. Larval and blood collections were made on Barro Colorado Island, a 1,500-km² nature preserve in the Republic of Panama. Detailed descriptions of the island and its flora and fauna are available (Leigh et al. 1982, Hubbell and Foster 1990). Climate in this region is characterized by a rainy season lasting 7 mo (May–November), followed by a 4- to 6-wk transition season (mid-December to mid-January), and a 3-mo (mid-January to mid-April) dry season. Temperature and rainfall may affect bot fly abundance at some locales (Mourier and Banegas 1970, Bergstrom 1992, Brigada et al. 1992, Vieira 1993).

Data Collection. In July and August 1991 and 1992, 47 and 24 howler monkeys, respectively, were captured using tranquilizing darts. Monkeys were sexed and weighed and bot fly larvae were collected, counted, and their developmental stage and locations on the host recorded. Monkeys were classified using the following age and sex classes (Milton 1996): ≥ 5 yr of age = adult male or female, 1–5 yr of age = juvenile, < 1 yr = infant. Live larvae were collected for rearing and blood samples were taken. Serum was separated and frozen (-20°C) before transport to Canada for further analysis.

Antigens. Crude extracts of 1st- and 3rd-instar *A. baeri* were prepared from field-collected and laboratory-reared material. Insufficient 2nd instars were obtained to conduct assays. Larvae were homogenized with a Polytron tissue homogenizer (Brinkmann, Rexdale, ON) with 0.02 M phosphate buffered saline, pH 7.1, used as buffer. The homogenates were centrifuged at $80,000 \times g$ at 4°C for 30 min, and the supernatants were collected and filter-sterilized through a 0.22- μm Millex filter (Millipore, Bedford, MA). The protein concentration of the 2 antigen preparations was determined using the Bradford (1976) technique.

Enzyme-Immunoassay. Animal sera were evaluated for antibody to monkey bot fly 1st- and 3rd-instar extracts using a microplate enzyme-immunoassay (EIA) (Colwell and Baron 1990) with the following modifications. Antigens were diluted in coating buffer (0.05 M Tris, 2.0 mM EDTA, pH 8.0) and 100 μl was placed into each well (0.36 μg per well 1st instar, 2.19 μg per well 3rd instar) of 96-well Maxisorp immunoplates (Gibco, Burlington, ON). After overnight incubation at 4°C , plates were washed once with PBS containing 0.5% Tween 20. Plates then were blocked by addition of 1% goat's milk in distilled water to each well for 2 h. Serum from monkeys was diluted 1:250 in PBS-Tween and added to duplicate wells. After 72 h of incubation with shaking, the plates were rinsed 4 times with PBS-Tween. Rabbit antimouse immunoglobulin G (IgG) (Organon Teknika, Scarborough, ON), conjugated to horseradish peroxidase was added to each well (1:1,000 dilution in PBS-Tween). Plates were incubated for 24 h with shaking followed by another rinse cycle. Substrate (0.2 M ABTS) in 0.05 M citrate, pH 4.0, was added to each well and incubated for 5, 15, and 60 min. Absorbance was measured at 410 nm with a V-max EIA plate reader (Molecular Devices, Menlo Park, CA). Readings taken during exponential phase of color development (15 min) were used for final determination of antibody levels. A hyperimmune antiserum was included on each plate as a standard. Negative control serum was included in 8 of the wells. The standard deviation of absorbance for the negative control serum was calculated for each plate. Mean absorbance in the paired serum samples was calculated and adjusted by subtracting twice the standard deviation of absorbance of the negative control serum. The adjusted absorbance values, which are representative of antibody level (Colwell and Baron 1990), were used in all further analyses.

SDS-Polyacrylamide Gel Electrophoresis (SDA-PAGE) and Immunoblotting. First- and 3rd-instar *A. baeri* extracts were separated using SDS-PAGE (8×8 cm, 4–10% T gradient) with the Laemmli buffer system (Laemmli 1970) and a minigel apparatus (EP system, Schleicher and Schuell, Keene, NH). Proteins separated by SDS-PAGE were transferred onto nitrocellulose paper and either visualized using a colloidal gold stain (Bio-Rad, Mississauga, ON) or processed further as immunoblots. Blots were incubated with all monkey sera and a representative run with reactivity characteristic of all monkeys shown in the results.

Definition of Terms and Data Analysis. Following Margolis et al. (1982), prevalence refers to proportion of infected monkeys in any given sample. Mean intensity refers to the number of parasites counted in any given sample divided by the number of infected monkeys in that sample and relative density to the number of parasites counted in any given sample divided by the total number

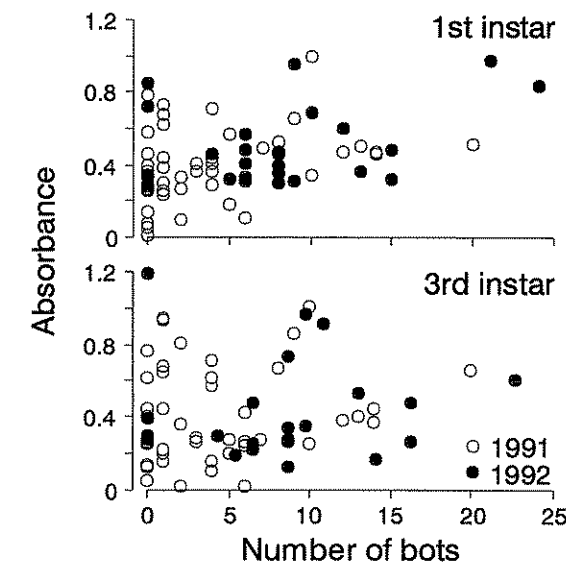
Table 1. Prevalence (percentage \pm SE), relative density, and mean intensity (mean \pm SE) of bot fly larvae in the howler monkey population by class and by sex (if adult) for 1991 and 1992

Year	Class	n	Prevalence ^a	Relative ^b density	n	Mean ^c intensity	Range
1991	Adult ♂	22	55	2.95 \pm 0.90	12	5.42 \pm 1.26	1-14
	Adult ♀	16	88	4.63 \pm 1.16	14	5.29 \pm 1.22	1-14
	Juvenile	7	86	6.43 \pm 2.52	6	7.50 \pm 2.69	1-20
	Infant	2	100	3.50 \pm 1.50	2	3.50 \pm 1.50	2-5
	All	47	82 \pm 9.60	4.06 \pm 0.69	34	5.62 \pm 0.81	1-20
1992	Adult ♂	8	88	9.88 \pm 2.91	7	11.29 \pm 2.92	6-24
	Adult ♀	10	80	7.10 \pm 1.60	8	8.88 \pm 1.36	4-15
	Juvenile	6	83	8.50 \pm 2.06	5	10.20 \pm 1.43	8-15
	All	24	83 \pm 2.33	8.38 \pm 1.25	20	10.05 \pm 1.18	4-24

^a Prevalence, proportion infested \times 100.^b Relative density, bot flies per all monkeys.^c Mean intensity, bot flies per infested monkeys.

of monkeys, infected plus uninfected, in that sample.

Statistical analyses were carried out using the Instat package (GraphPad, San Diego, CA) release 2.0. Prevalence, mean intensity and relative density were examined using the Mann-Whitney *U* test when data for the variable had 2 levels (e.g., male, female) or the Kruskal-Wallis test when data had ≥ 3 levels (e.g., adults, juveniles, infants). To test whether years differed significantly from each other in terms of larval parameters, an analysis of variance (ANOVA) was used to perform the Tukey studentized range test. Correlation coefficients between corrected absorbance as a measure of antibody level and bot fly numbers were calculated for both years combined and for each year separately using both 1st- and 3rd-instar antigen. Sex differences in corrected absorbance values were examined using the Mann-Whitney test.

**Fig. 1.** Distribution of corrected absorbance values to 1st- and 3rd-instar antigen versus number of bot flies for combined 1991 and 1992 data.

Results

Larval Burdens. A summary of larval infestation data is shown in Table 1. Prevalence was similar for both years (82 \pm 9.6 versus 83 \pm 2.3%); however, there were significant differences in relative density and mean intensity. The combined relative density was significantly lower in 1991 than in 1992 (Mann-Whitney *U* statistic = 317, *U'* = 811; *P* = 0.003). Similarly, the combined mean intensity in 1991 was lower than that observed in 1992 (Mann-Whitney *U* statistic = 161, *U'* = 479; *P* = 0.003).

These differences were attributed to variation in relative density among age classes (ANOVA, *F* = 2.39, *df* = 71, *P* = 0.037), although there were no significant differences among age classes for mean intensity (ANOVA, *F* = 1.95, *df* = 53, *P* = 0.092). Relative density of larval populations in adult males in 1991 (2.95 \pm 0.90) was significantly less than that observed for adult males in 1992 (9.88 \pm 2.91) (Tukey Studentized range test, *P* \leq 0.05).

Although differences occurred within years for prevalence, relative density, and mean intensity (Table 1), we found no significant differences in these infestation parameters between different age classes of monkeys. For example, there was no difference in relative density between males and females in either 1991 (Mann-Whitney *U* statistic = 123.5, *U'* = 228.5, *P* = 0.124) or 1992 (Mann-Whitney *U* statistic = 35.5, *U'* = 44.5, *P* = 0.697). This was also the case when we compared relative density in adult males, adult females, juveniles, and infants in 1991 (Kruskal-Wallis statistic *KW* = 4.29, *P* = 0.231) and in adult males, adult females and juveniles in 1992 (Kruskal-Wallis statistic *KW* = 0.299, *P* = 0.861).

Antibody Response. Monkeys responded to both 1st and 3rd instar antigens (Fig. 1). Correlation between corrected absorbance using 1st-instar antigen and bot fly numbers was highly significant for 1991 and 1992 data combined (*r* = 0.37, *n* = 71, *P* \leq 0.001) and for 1991 data alone (*r* = 0.39, *n* = 47, *P* \leq 0.006). Correlations for 1992 data were not significant (*r* = 0.27, *n* = 24, *P* = 0.19).

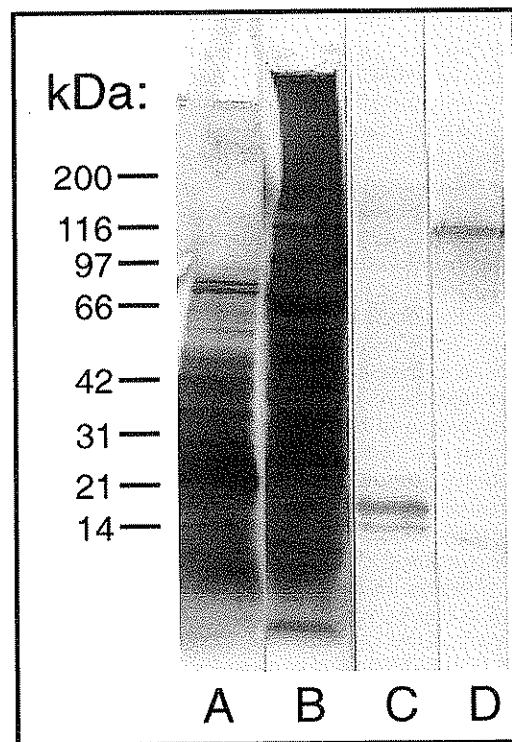


Fig. 2. Extracts of 1st- and 3rd-instar *A. baeri* subjected to SDS-PAGE, transfer to nitrocellulose and colloidal gold staining, lane A and B, respectively. Western blots of 1st- and 3rd-instar proteins, lanes C and D, respectively.

Correlations between bot fly numbers and antibody response using 3rd-instar antigen for both years combined was not significant ($r = 0.14$, $n = 71$, $P = 0.24$). Neither were correlations significant for 1991 ($r = 0.19$, $n = 47$, $P = 0.18$) nor 1992 ($r = 0.03$, $n = 24$, $P = 0.87$) data when analyzed separately.

We found no differences in the mean antibody response of males versus females. The mean corrected absorbance of females (0.457 ± 0.04) and males (0.455 ± 0.04) to 1st-instar antigen was not significantly different (Mann-Whitney U statistic = 358.5, $U' = 366.5$, $P = 0.952$). The response to 3rd-instar antigen in females (0.479 ± 0.06) and males (0.376 ± 0.04) was also similar (Mann-Whitney U statistic = 319, $U' = 406$; $P = 0.4557$).

Antibodies apparently persist after all bot flies have left the host, because the 17 monkeys with no bot flies had mean corrected absorbance values of 0.364 ± 0.06 to 1st-instar antigen and 0.357 ± 0.07 to 3rd-instar antigen. All monkeys in this group were adults with a mean age of 8.9 ± 1.0 yr.

SDS-PAGE. The analytical SDS-PAGE conditions used in this study allowed us to demonstrate that *A. baeri* 1st- and 3rd-instar larval extracts were composed of a number of proteins (Fig. 2). The

1st-instar extract consisted of 9 major protein bands ranging in molecular size from 17 to 105 kDa, with the most intense bands appearing at 25 and 32 kDa. The 3rd-instar extract was more complex and consisted of at least 13 major bands ranging from 11 to 130 kDa. Most intense staining was associated with bands at 20, 24, 52, and 76 kDa.

Immunoblotting. Western blots revealed the antigenic properties of each larval extract (Fig. 2). Three protein bands at 17, 25, and 32 kDa were positive by this technique in the 1st-instar extract. Only 2 bands at 120 and 130 kDa were positive in 3rd-instar extracts. This reactivity was observed with all monkey sera, including those obtained from monkeys without evidence of active infestations. Therefore, only a representative immunoblot is displayed.

Discussion

Yearly variation in mean intensity and relative density of monkey bot fly populations can be attributed to differences in the relative density of bot fly infestation in the male population. Milton (1996) also found yearly differences in prevalence and mean intensity, in a more extensive study. Our data did not show any significant differences in prevalence, mean intensity and relative density between age classes within any one year, thereby indicating no apparent selectivity of bot flies for any one age class. The lack of significance observed in this study within years is probably a reflection of the small sample size in some age classes (e.g., infants and juveniles). Milton (1996) found consistent and significant differences between infants in the 3 parameters relative to the other classes and attributed the differences to as yet unknown characteristics of particular age classes.

We have shown that howler monkeys exposed to natural infestations of *A. baeri* produce antibodies that are directed at both 1st- and 3rd-instar larval proteins. There does not appear to be any difference in the level of antibody response between males and females as the corrected absorbances in these 2 groups are similar. All noninfested adult monkeys sampled in this study responded to 1st- and 3rd-instar antigens, indicating that humoral antibodies persist after bots leave their hosts although the duration of the response could not be ascertained. Many of these monkeys also exhibited scars where bot fly larvae recently had exited. The fact that monkeys may be infested as many as 3 times per year (Milton 1996) indicated that they were under significant antigenic pressure that could maintain high antibody titers to bot fly antigens throughout their adult life. Maintenance of this response may be a significant energy cost to bot fly infested monkeys. Previous studies have shown that antibody titers to *Hypoderma* spp. decrease once grubs have emerged (Pruett et al. 1987) particularly in animals experiencing their 1st infestation. However, calves harboring heavier in-

festations of this parasite were shown to have persisting antibodies (Colwell and Baron 1990).

Extracts of various larval instars have been used to monitor the antibody response of animals to myiasis-producing flies. First-instar larval extracts of *H. lineatum* and *H. bovis* (Boulard 1975, Robertson 1980) have proved useful in detecting antibody to this parasite and have been employed in large scale serological surveys of this parasite (Sinclair et al. 1991). Similarly, extracts of 1st-instar *Cuterebra fontinella* (Clark) have been used to detect antibodies to *Cuterebra fontinella* (Clark) (Pruett and Barrett 1983). Both 1st- and 2nd-instar larval extracts were useful in detecting antibody to *O. ovis* in sheep (Bautista-Garfias et al. 1988). No direct correlation has been observed between degree of infestation and the concentration of circulating antibody for myiasis-producing ectoparasites (Baron and Colwell 1990). However, the relationship among mean number of grubs per animal, variance, and proportion of uninfested calves as determined by EIA has been used successfully as the basis for a sampling model of cattle grub abundance (Lysyk et al. 1991). *A. baeri* infestations in howler monkeys exhibit a correlation with antibody concentrations in some instances, however, the validity of this relationship is not clearly established.

Protective immunity induced by exposure to parasites has been described for a variety of ectoparasitic arthropod-host interactions. Three proteinases isolated from *H. lineatum* are antigenic to the infested bovine (Pruett et al. 1988) and stimulate development of serum antibodies (Pruett et al. 1987, Colwell and Baron 1990) as well as a cellular response (Baron and Weintraub 1987). Development of acquired resistance in cattle after exposure to 3 consecutive infestations significantly diminishes survival of *H. lineatum* (Baron and Weintraub 1987). Vaccination with these proteins provides protection to cattle against infestation with this parasite (Pruett et al. 1989, Baron and Colwell 1991).

Sheep have been shown to acquire resistance to *Lucillia cuprina* (Wiedemann) (Watts et al. 1979); however, this resistance only is seen in some sheep, and only when infestations are given at 2- and 4-wk intervals (Sandeman et al. 1991). Monkeys are exposed to up to 3 infestations of *A. baeri* per year (Milton 1996), which indicates that relatively larger bot fly populations should be evident in some members of the monkey population. However, Milton (1990) did not find this to be the case, because mean intensity data on >5,000 monkeys showed no individual with >24 larvae and the great majority with <10. She postulated that an active immune response may limit the number of larvae that can establish infestations. Our data established the presence of an antibody response directed toward larval monkey bot fly antigens; however, the protective ability of this humoral response, as well as cellular immunity remains to be established. Antigen-specific proliferation of

lymphocytes derived from *H. lineatum*-infested cattle has been shown to vary with specific phases of the infestation and correlates positively with host resistance (Baron and Weintraub 1987). Challenge of older monkeys that have experienced several infestations and that have high titers to larval antigens could provide some evidence to support a role for a protective response associated with the development of acquired resistance. The fact that bot fly numbers are correlated with antibody response to 1st-instar proteins in some instances indicates that the immune response may be directed at new infestations. However, the lack of consistency in this response, does not allow us to conclude that there is a clear correlation between antibody response and bot fly numbers.

Protective immunity following natural exposure is often incomplete despite the presence of antibodies. Arthropod salivary proteins may impact negatively on the development of a protective immune response (Baron and Colwell 1991). Two of the proteins isolated from *H. lineatum* salivary gland extracts, which are involved in protection, can curtail the immune response (Baron and Colwell 1991). Two of the proteins isolated from *H. lineatum* salivary gland extracts, which are involved in protection, can curtail the immune response of cattle (Boulard and Bencharif 1984, Baron 1990, Pruett 1993). This immunomodulation has been suggested to play a role in maintaining the life cycle in young animals (Baron 1990). An effective antibody response to *A. baeri* also may be subject to immunoregulatory components.

In conclusion, we have been able to demonstrate that monkeys do develop antibodies to *A. baeri* and that the response is correlated with larval burden in some instances. The response, particularly to 1st-instar antigens, also may limit establishment of new infestations, however, this requires further investigation.

Acknowledgments

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