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Detection of protease inhibitors in the hemolymph of resistant *Anticarsia gemmatalis* which are inhibitory to the entomopathogenic fungus, *Nomuraea rileyi*

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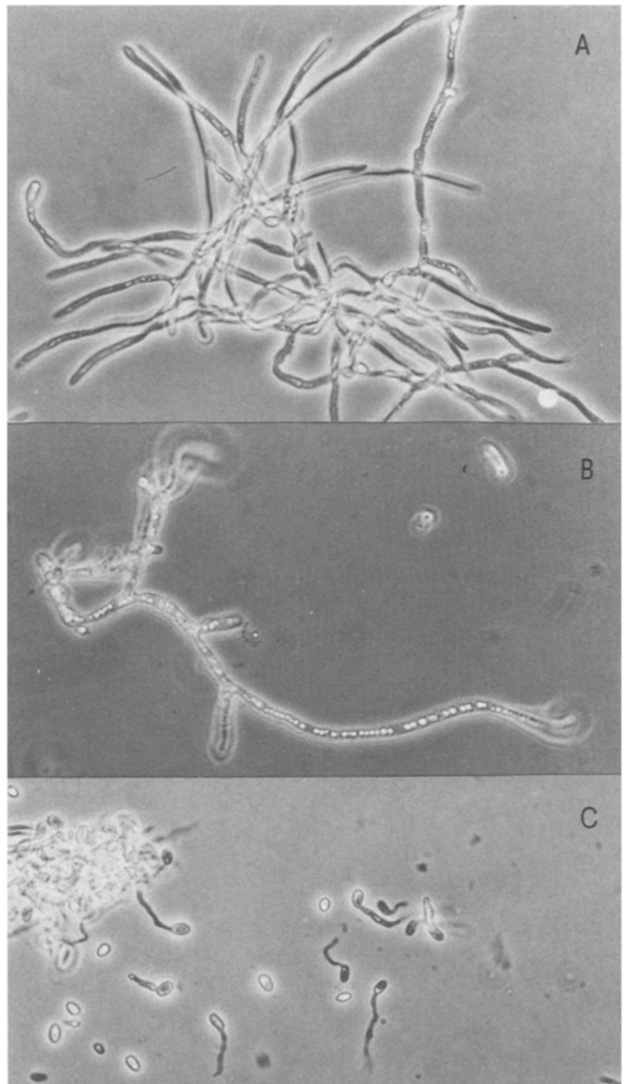
Summary. A complex of protease inhibitor activities has been detected in the hemolymph of the 6th instar *Anticarsia gemmatalis* larvae that are resistant to infection by the fungus *Nomuraea rileyi*. A site-specific serine protease inhibitor extracted from *A. gemmatalis* hemolymph inhibits both the germination of *N. rileyi* conidia and subsequent germ tube development.
Key words. Protease inhibitor; insect immunity; *Anticarsia gemmatalis; Nomuraea rileyi*.

The entomopathogenic fungus, *Nomuraea rileyi*, is recognized as an important naturally occurring biological control agent of a variety of noctuid pest defoliators[1]. This species is comprised of numerous pathotypes having distinct biological properties[2,3]. Many of the *N. rileyi* pathotypes are highly virulent to larvae of the cabbage looper, *Trichoplusia ni*, whereas only a few pathotypes (i.e. F178-6) have been shown to be virulent to the velvetbean caterpillar, *Anticarsia gemmatalis*. As this host insect matures it becomes resistant to those *N. rileyi* pathotypes that are virulent to younger *A. gemmatalis* larvae (1st-4th instar). The mechanisms responsible for the resistance expressed by mature *A. gemmatalis* larvae are poorly understood. Bioassays involving the injection of vegetative cells of various *N. rileyi* pathotypes into the hemocoel of late instar *T. ni* and *A. gemmatalis* produce a differential mortality response similar to that achieved with topical application[4]. These findings suggest that the resistance of late instar *A. gemmatalis* to *N. rileyi* is due in part to an internal defense system.

Recently, it has been proposed that protease inhibitors, detected in hemolymph samples from various invertebrates, play a defensive role against invading microorganisms[5-12]. Entomopathogenic fungi, such as *N. rileyi*, are believed to require a complex of hydrolases (protease, lipase, chitinase) for both penetration and colonization of host insect tissues. Inhibition of these hydrolases could abort the infection process and confer resistance to insect species possessing such protease inhibitory activities. The relative numbers and specific activities of hydrolase inhibitors detected in invertebrates is variable. In the silkworm, *Bombyx mori*, a series of inhibitors ranging from 7000 to 60,000 daltons, which have either antitrypsin or antichymotrypsin activities, have been isolated and reported to be active against commercially available fungal proteases[13-15]. Low molecular weight inhibitors detected in waxmoth larvae, *Galleria mellonella*, were reported to be active against a toxic protease produced by the entomopathogenic fungus, *Metarhizium anisopliae*[5-7]. Hall and Soderhall[16,17] extracted a protease inhibitor from both blood cells and from the cuticle of the crayfish, *Astacus astacus*, which inhibits the protease activity of the fungal entomopathogen *Aphanomyces astaci*. Recently, high molecular weight protease inhibitors possessing properties similar to the α-macroglobulins have been detected in the hemolymph of Crustaceae[18].

In this paper we demonstrate the presence of a protease inhibitor complex present in resistant late instar *A. gemmatalis* larvae that is not detected in the early instars of *A. gemmatalis* and *T. ni* larvae. Furthermore, we show that chromatographic fractions of *A. gemmatalis* hemolymph containing protease inhibitory activity are deleterious to the growth and development of the fungus *N. rileyi*.



Light micrographs of preparations of germinating *Nomuraeae rileyi* conidia that were been incubated in (A) one half strength SMY broth (× 1700); (B) SMY broth cell-free *Anticarsia gemmatalis* hemolymph (1:1 concentrations, × 1600) and (C) SMY broth: insect protease inhibitor (15.2 inhibitor units/µl, × 1600).

Table 1. Relative levels of protease inhibitor activity in hemolymph samples of *Anticarsia gemmatalis* and *Trichoplusia ni* larvae[a]

Sample[b]	n	Degree of inhibition (inhibitor units/µl hemolymph)
Control		
4th instar *A. gemmatalis*	5	0.5 (0.4–0.6)
4th instar *A. gemmatalis* (molting)	5	NI
6th instar *A. gemmatalis*	6	9.0 (6.9–11.3)
prepupal *A. gemmatalis*	5	NI
4th instar *T. ni*	5	NI
6th instar *T. ni*	6	NI
Injected		
6th instar *A. gemmatalis*	6	15.1 (9.6–23.8)
6th instar *T. ni*	6	NI

[a] Each sample represents a 1-ml pooled hemolymph sample. [b] One inhibitor unit is equivalent to a decrease of 0.01 in absorbance of inhibitor-trypsin-BAPNA mixture as compared to control trypsin-BAPNA mixtures. Values in parentheses represent range of activity among various samples assayed. NI, No inhibition.

Materials and methods. Preparation of insect hemolymph samples. Eggs of *A. gemmatalis* and *T. ni* used in these experiments originated from permanent colonies maintained at the Insect Attractants and Basic Biology Laboratory, USDA, Gainesville, Florida. Larvae were reared on an artificial diet at 26 °C under a 16 h:8 h light-dark photoperiod. Hemolymph was collected from last instar larvae by puncturing them at the base of the second proleg and allowing extruded hemolymph to collect in chilled microcentrifuge tubes containing several crystals of phenylthiourea to prevent melanization. 1-ml aliquots (15–25 larvae/aliquot) were centrifuged at 13,000 × g for 5 min to remove hemocytes. Supernates were pooled and stored at −70 °C until needed. In order to determine if protease inhibitory activity was inducible, additional cohorts of newly molted 6th instar larvae were challenged with vegetative cells of *N. rileyi*. A dosage of 30,000 hyphal body cells/insect was injected into the hemocoel of these larvae. Hemolymph samples were collected 48 h postinjection, processed, and frozen at −70 °C.

Enzyme inhibition assays. The relative titer and specificity of protease inhibitors present in hemolymph samples and chromatographic fractions were determined using various commercially available proteases (Sigma) and chromogenic substrates (Sigma). Antitrypsin activity was measured using TPCK-treated trypsin in combination with BAPNA (N-benzoyl-L-arginine-p-nitroanilide)[20]. In our studies the inhibitor samples (total volume 50 µl) were preincubated in a 50-µl trypsin solution (1 mg/ml) prior to dilution with 50 mM tris-HCl (pH 8.0) buffer. The reaction mixtures (inhibitor-trypsin-BAPNA) were incubated 10 min at 37 °C before the reaction was stopped by the addition of acetic acid. Samples were immediately read at 405 nm. The ability of hemolymph samples to inhibit chymotrypsin, subtilisin, protease, *Rhizopus* protease, *Aspergillus* protease, pepsin, and trypsin activities were measured using azocoll or hide azure blue substrates[17].

Extraction of protease inhibitors from A. gemmatalis hemolymph. Pooled hemolymph samples (15 ml) collected from control 6th instar *A. gemmatalis* larvae were fractionated with increasing concentrations of saturated $(NH_4)_2SO_4$. The resulting fractions (0–30%, 30–60%, > 60% cuts) were dialyzed overnight against 50 mM tris-HCl (pH 8.0) and concentrated with aquacide. The 30–60% cut, which was found to contain > 98% of the protease inhibitor activity, was centrifuged at 112,000 × g for 1 h to remove precipitates. Resulting supernates were applied (5-ml aliquots) to a DEAE-Sephacryl (Pharmacia) jacketed column (0.9 × 22.5 cm) preequilibrated with 50 mM tris-HCl (pH 7.8). The column was washed sequentially with 75 ml of 50 mM tris-HCl (pH 7.8) followed by a 130 ml 0.0–0.5 M NaCl gradient in 50 mM tris-HCl buffer (pH 7.8). Collected fractions (4 ml) were assayed directly for antitrypsin activity using BAPNA as the indicator substrate. Fractions containing antitrypsin activity were pooled and dialyzed against 50 mM tris-HCl (pH 8.0) containing 1 mM EDTA and 0.1 M NaCl. This fraction concentrated in aquacide was chromatographed on a jacketed column (1.6 cm × 50 cm) of Biogel 1.5A. Fractions containing protease inhibitor activity, as detected with trypsin-BAPNA assay, were pooled, dialyzed against 50 mM tris-HCl (pH 8.0), concentrated with aquacide and frozen at −70 °C. Levels of protein in samples were determined[20].

Propagation and assay of fungal cell preparations. The *N. rileyi* F174-6 strain (ATCC 52631) was propagated under in vitro conditions on Sabouraud maltose agar + 2% yeast extract and under in vivo conditions in host *T. ni* larvae. Yeast-like hyphal body cells were scraped off inoculated plates, washed twice in H_2O and diluted to a concentration of 3.0×10^5 cells/ml. Fungal suspensions were used to inoculate 6th instar *A. gemmatialis* and *T. ni* larvae (10 µl/larvae).

Conidial preparations were harvested from sporulating cadavers of *N. rileyi* infected *T. ni* larvae. Conidia were suspended in SMY broth and diluted to a final concentration of 1×10^6 conidia/ml broth. 50-µl aliquots of this conidial suspension were placed in wells of a 96 well microtiter plate. Dilutions of Biogel 1.5 A protease inhibitor fraction were prepared in distilled H_2O and added (50 µl) to the wells containing conidial suspensions. At 24 and 48 h postinoculation the wells were examined with an inverted phase contrast microscope and percent conidial germination recorded.

A second series of assays were performed to determine the effect of the *A. gemmatalis* protease inhibitor on germ tube growth and development. In these studies a series of microtiter wells were inoculated with FL74-6 conidia and incubated at 26 °C to allow for normal germ tube formation. At 36-h postinoculation (> 70% germination) the wells containing germinating conidia were inoculated with dilutions of the protease inhibitor. At 24 and 48 h postchallenge these wells were examined with inverted phase contrast microscope. Representative samples were removed from wells and photographed under phase contrast microscopy using Kodak Pan-X film.

Results. Levels of proteinase inhibitor activity, detected with trypsin-BAPNA, were highest in the cell free hemolymph samples drawn from mid-6th instar *A. gemmatalis* larvae (table 1). Hemolymph from mid-4th instar larvae contained low levels of protease inhibitor whereas both molting 4th instar larvae and prepupae did not contain detectable levels of inhibitory activity. Injection of *N. rileyi* vegetative cells into early 6th instar *A. gemmatalis* larvae induced significantly higher levels of protease inhibitor activity. Hemolymph samples extracted from either control (noninjected) or injected *T. ni* larvae contained no detectable protease inhibitor activity. Hemolymph samples taken from both control and injected *A. gemmatalis* larvae were capable of inhibiting a wide range of commercial proteases (table 2). Pepsin

Table 2. Inhibition of different proteolytic enzymes by native and *N. rileyi* challenged *A. gemmatalis* hemolymph samples

Enzyme[a]	Relative units/µl hemolymph[b,c,d] Control	Challenged
Trypsin	2.1 (1.6–2.6)	1.9 (1.3– 2.6)
Chymotrypsin	1.5 (0.9–1.7)	1.6 (0.6– 2.0)
Aspergillus protease	0.2 (0.1–0.3)	1.6 (0.6– 2.6)
Rhizopus protease	5.6 (1.6–7.8)	10.0 (9.7–12.3)
Protease K	1.2 (1.1–1.2)	0.9 (0.8– 1.0)
Pepsin	6.3 (3.2–7.6)	10.4 (9.6–11.1)
Subtilisin	1.1 (0.4–2.0)	0.7 (0.5– 1.2)
Collagenase	NI	NI
Papain	NI	NI

[a] All enzymes except for papain were analyzed using azocoll as substrate. Hide azure blue was used as a substrate for papain. [b] One inhibitor unit is equivalent to a decrease of 0.01 in the absorbance of inhibitor-enzyme substrate mixtures. Values in parenthesis represent range of activity detected among 3 replicates. [c] Control samples represent pooled hemolymph samples (1–2 ml) collected from uninjected larvae. Challenged samples were collected from *N. rileyi* injected larvae 48 h post injection. [d] NI, no inhibition.

Table 3. Summary of the extraction of the major protease inhibitor detected in control *A. gemmatalis* hemolymph

Step	Total protein (mg)	Total units[a]	Specific activity (unit/mg)	Recovery %	Purification factor
Hemolymph	1350	1.09×10^5	81	100	1
30–60% $(NH_4)_2SO_4$	552	1.09×10^5	198	100	2.2
112,000 × g supernatant	525	7.50×10^4	143	69	1.8
DEAE-Sephacryl	12	6.40×10^4	5500	59	67.9
Biogel 1.5 A	1.75	3.04×10^4	17,370	28	214.4

[a] One inhibitory units is equivalent to a decrease of 0.01 in the absorbance of inhibitor-trypsin-BAPNA-substrate mixture as compared to control buffer-trypsin-BAPNA mixture.

and *Rhizopus* protease were the most sensitive to inhibition by *A. gemmatalis* hemolymph samples. Trypsin, chymotrypsin, protease K, and subtilisin were also inhibited, whereas collagenase and papain were not inhibited by *A. gemmatalis* hemolymph. Injection of *N. rileyi* into *A. gemmatalis* larvae induced increased inhibitory activity against pepsin and *Rhizopus* protease but did not induce increased inhibition of the other proteases.

A combination of precipitation, centrifugation, ion exchange, and gel permeation methods were used to extract and concentrate protease inhibitory activity from cell free hemolymph preparations (table 3). Total inhibitory activity in the hemolymph, as estimated with the trypsin-BAPNA assay, was recovered in the 30–60 $(NH_4)_2SO_4$ cut. Clarification of the 30–60% $(NH_4)_2SO_4$ cut by centrifugation (112,000 × g) resulted in a 31% loss of protease inhibitor activity. The supernate, containing the greater part of the antitrypsin activity, effectively inhibited chymotrypsin, pepsin, a protease K activity against an azocoll substrate. However, the inhibitory activity against subtilisin, *Aspergillus* protease, and *Rhizopus* protease present in the parental hemolymph samples was not detected in the 112,000 × g supernate. This fraction, eluted from the DEAE-Sephacryl column between 0.20 and 0.25 M NaCl, inhibited both trypsin and chymotrypsin but lost activity against both pepsin and protease K activity. Partitioning the protease inhibitor from the DEAE-Sephacryl step on a Biogel 1.5A column resulted in a 3.3-fold purification of the inhibitor, which remained active against only trypsin and chymotrypsin.

Both unfractionated control *A. gemmatalis* and the extracted protease inhibitor fraction suppressed the germination of *N. rileyi* conidia (table 4). Whole hemolymph diluted 1:1 or 1:4 with SMY broth inhibited conidial germination throughout the 48-h test period. At a 1:20 dilution partial inhibition was noted. The Biogel 1.5A fraction, containing only antitrypsin and antichymotrypsin activity, was capable of suppressing conidial germination at the two highest concentrations assayed, whereas the lowest concentration did not inhibit conidial germination. The influence of hemolymph and extracted protease inhibitor samples on germ tube development was evaluated by microscopic examinations. When compared to control wells that contained germinated conidia in ½ strength SMY (fig. A), both hemolymph and protease inhibitor fractions caused observable affects on germ tube development. Whole hemolymph samples diluted 1:1 with SMY induced germ tubes to become filled with lipid droplets (fig. B). No noticeable inhibition of germ tube or wall lysis was noticed with the hemolymph-germ tube mixtures. The protease inhibitor extract at the highest concentration assayed inhibited germ tube development causing noticeable vacuolization and detectable lysis of the cell wall at the apex of germ tube (fig. C). At lower concentrations tested, the protease inhibitor caused no detectable effects on the developing germ tubes.

Table 4. Germination of *Nomuraea rileyi* (F174-6) in the presence of *A. gemmatalis* protease inhibitor

Inhibitor sample	Inhibitory activity (units/final volume)	Percentage germination[b,c] 24 h	48 h
Buffer control (0)	0	32–39	60–71
Hemolymph (5 µl)	36	0	13–20
(25 µl)	180	0	0
(50 µl)	360	0	0
Biogel 1.5 A (5 µl)	152	28–35	68–76
(25 µl)	760	0	25–32
(50 µl)	1520	0	< 1.0

[a] One inhibitor unit is equivalent to a decrease of 0.01 in the absorbance in inhibitor-trypsin-BAPNA mixture as compared to control trypsin-BAPNA solution. [b] Minimum of 100 conidia examined for each treatment. Values given represent the range in 2 trials. [c] Dilutions of test solutions were mixed with 5×10^4 conidia in 50 µl SMY broth[5], adjusted with buffer to 100 µl and incubated in wells of microtiter plates at 26°C.

Discussion. Previous research on *Anticarsia gemmatalis* has suggested that its resistance to infection by *Nomuraea rileyi* is in part due to factors associated with the hemolymph. Comparative analysis of hemolymph extracted from the susceptible host *T. ni* and the resistant host *A. gemmatalis* has revealed distinct differences. For example, whole blood and cell-free hemolymph samples of *A. gemmatalis* (4th, 6th instar larvae) contain 10-fold less phenoloxidase activity than similar samples extracted from *T. ni* larvae (Boucias, unpublished). The addition of *A. gemmatalis* hemolymph to *T. ni* hemolymph results in reduced phenoloxidase activity suggesting the presence of a hemolymph-borne inhibitor of the prophenoloxidase cascade. Secondly *A. gemmatalis* hemolymph contains a heteroagglutinin activity that is induced by hemocoelic injection of *N. rileyi* yet is absent from susceptible *T. ni* larvae[21]. Recent in vitro assays have shown that this heteroagglutinin adsorbs readily to the cell walls of various entomopathogenic fungi including *Nomuraea rileyi, Beauveria bassiana* and *Metarhizium anisopliae*.

In this paper, we have detected relatively high levels of protease inhibitory activity in the hemolymph of sixth instar *A. gemmatalis* larvae. Combined ion exchange and gel permeation chromatography of cell free hemolymph resulted in the extraction of a site specific serine protease inhibitor. Challenging *A. gemmatalis* with hemocoelic injection of *N. rileyi* resulted in a stimulation of inhibitory activity against trypsin when assayed with the BAPNA substrate. However, when assayed against trypsin in combination with azocoll no stimulation in inhibitory activity was noted. Interestingly, injection of *N. rileyi* did increase the inhibitory activity against pepsin, *Rhizopus* protease and the *Aspergillus* protease. No inhibitory activity was noted in the hemolymph samples extracted from either 4th instar in the prepupal stage of *A. gemmatalis* or from late instar *T. ni larvae*.

Potentially the protease inhibitory activity detected in *A. gemmatalis* could be responsible for the inhibition of phenoloxidase activity. However, the hemolymph of 4th instar *A. gemmatalis*, which contains levels of phenoloxidase comparable to those of 6th instar larvae, possesses significantly less protease inhibitor activity. Additionally, adsorption experiments involving *A. gemmatalis* hemolymph and various substrates (Sepharose 4B, erythrocytes), which result in the removal of host agglutinin activity and the phenoloxidase inhibitor, do not result in the adsorption of the serine protease inhibitor activity. Other inhibitors present in hemolymph and not associated with the major serine protease inhibitor detected in our assay system may be responsible for the inhibition of phenoloxidase.

The extracted protease inhibitor is capable of inhibiting the development of *N. rileyi*. Both conidial germination and germ tube development are adversely affected by this extract. Whole hemolymph samples containing the complete spectrum of protease inhibitory activity were more effective in suppressing co-

nidial germination than the serine protease inhibitory extract. However, when assayed against germinated conidia, the extracted protease inhibitor fraction was capable of inhibiting germ tube development. Within 48 h germ tubes treated with the inhibitor extract began to lyse. Whether or not the host protease inhibitors present in hemolymph accumulate on the walls invading germ tubes and cause a similar mycostatic effect under in vivo conditions remains to be tested.

In conclusion, we have detected a complex of protease inhibitor activities in resistant *A. gemmatalis* larvae that were not detected in either susceptible *T. ni* or molting *A. gemmatalis* larvae. A site-specific serine protease inhibitor was isolated from *A. gemmatalis* hemolymph that was deleterious to both the germination of *N. rileyi* conidia and subsequent germ tube development. The results of these studies indicate that the resistance expressed by last instar *A. gemmatalis* larvae to infection by *N. rileyi* may in part be due to the presence of hemolymph-borne protease inhibitors that act on fungal hydrolases responsible for continued cell wall development.

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Ascorbic acid content of neotropical plant parts available to wild monkeys and bats

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Summary. The ascorbic acid content of foliage available to wild primates and bats in Panama (in transition between wet and dry seasons) was lower than that of temperate zone foliage but higher than that of most fruits and vegetables. Intakes of ascorbic acid (mg/kg b.wt/day) by wild primates and frugivorous bats in Panama are much greater than that of most human populations.
Key words. Ascorbic acid; neotropical plants; vitamin C; primates; anthropoids; bats; monkeys.

L-ascorbic acid plays essential roles in several physiological functions in mammals[1]; one of the most important is hydroxylation of collagen, an essential protein of connective tissue. In deficiency of ascorbic acid, collagen is not laid down correctly and scurvy develops. Many species of mammals synthesize their ascorbate requirements[2,3]. Some, however, are unable to do so and are scurvy-prone because they lack the enzyme L-gulonolactone oxidase (GLO, EC 1.1.3.8), which catalyzes the final step in ascorbate synthesis from glucose[4]. To date 12 species of anthropoid primates[5–9], 45 species of bats[10], and the guinea pig[6–8,11] have been categorized as scurvy-prone on the basis of nutritional studies or assays for GLO or both. Anthropoids are here considered to include the families Cebidae, Callitrichidae, Cercopithecidae, Pongidae, and Hominidae. No species of anthropoid primate or bat yet examined has been found to synthesize L-ascorbic acid; all are thought to require a dependable dietary supply of it[12].

Apparently mutations resulting in the loss of GLO occurred on several occasions in mammalian evolution; these mutations appear to be fixed in several taxa. In man, African green monkey, and guinea pig the gene coding for GLO is not expressed[4], but neither the nature nor the site of the mutation is known. Bats have not been checked for expression of the gene. There is no present evidence that the level of dietary ascorbate directly influenced the mechanisms of mutation or fixation, but certainly a mutation resulting in loss of ability to synthesize ascorbate could not persist or be fixed in a population lacking an adequate and regular dietary supply[4,12].

Anthropoid primates probably evolved in a tropical forest environment[13,14]; even today most tropical anthropoid species are arboreal. Plant foods available to anthropoids for most or all of their evolutionary history have been leaves, fruit and flowers of tropical forest trees and vines[14]. All anthropoids routinely include plant foods in the daily diet and most species are estimated to take $\geq 70\%$ of their annual diet from plants[15]. Likewise, many species of bats secure much of their nutrition from plant materials including flowers, nectar, pollen and fruit[16,17]. Many anthropoid primates and several species of bats tend to focus considerable feeding time on members of the family Moraceae, particularly species of the genus *Ficus*[14,16–20].

It would be expected that the tropical plant foods consumed by bats and wild primates would be relatively rich in ascorbic acid but few data are available. In this paper we report ascorbic acid contents of plant samples collected from a Neotropical forest site inhabited by five anthropoid species (*Alouatta palliata, Ateles geoffroyi, Cebus capucinus, Aotus trivirgatus* and *Saguinus oedipus geoffroyi*) and by a number of species of phyllostomid bats including members of the genera *Artibeus, Carollia, Sturnira* and *Glossophaga*. Because *Ficus* species are of particular importance in the diets of these animals, a special effort was made to analyze leaves and fruits of this genus.

Materials and methods. Plant samples were collected from trees