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REPORT NUMBER 1

# VECTORS AND NATURAL RESERVOIRS OF OROPOUCHE VIRUS IN THE AMAZON REGION, (U)

FINAL REPORT

Donald R. Roberts[1], Francisco de P. Pinheiro[2], A. Lynn Hoch[1], James W. LeDuc[1], Norman E. Peterson[1], Marco Antonio Vasconcelos Santos[2], and Karl A. Western[3] ([1]The Walter Reed Army Institute of Research, Washington, D.C., [2]the Evandro Chagas Institute, Belém, Brazil, and [3]the Pan American Health Organization, Washington, D.C.).

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In (CV) 1977 a program of field and laboratory studies was initiated to study Oropouche virus in the Amazon region of Brazil. This virus is a frequent cause of urban epidemics of a febrile disease in this region and Culicoides paraensis has been incriminated as the vector. This research program includes (1) surveillance of forest vertebrates and invertebrates near Santarem, Brazil to detect the natural reservoirs and vector(s); (2) studies on the biology and population dynamics of C. paraensis in the urban environments of Belem, Brazil,

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(3) laboratory studies at the Evandro Chagas Institute to evaluate vector potential of various hematophagous insects in transmission tests, and (4) efforts to colonize the various potential vector species with emphasis on C. paraensis.

The C.paraensis have efficiently transmitted virus in the laboratory. The other candidate vector Culex quinquefasciatus were not efficient vectors under laboratory conditions. Observations have been made on the biology of the midges in the field and laboratory. The field surveillance program was established and antibodies to the virus have been found in one monkey.

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## ABSTRACT

In CY 1977 an integrated program of field and laboratory studies was initiated to study the ecology of Oropouche virus in the Amazon region of Brazil. The virus is a frequent cause of urban epidemics of a febrile disease in this region and Culicoides paraensis have been incriminated as the urban vectors. In addition, serological surveys of wild animals indicate a natural cycle of transmission in the Amazonian forest. Consequently, studies were designed to obtain information on the dynamics of Oropouche transmission in both the urban and forest environments.

This research program includes (1) surveillance of forest vertebrates and invertebrates near Santarém, Brazil to detect the natural reservoir(s) and vector(s), (2) studies on the biology and population dynamics of C. paraensis in the urban environments of Belém, Brazil, (3) laboratory studies at the Evandro Chagas Institute to evaluate vector potential of various hematophagous insects in transmission tests, and (4) efforts to colonize the varous potential vector species with emphasis on C. paraensis.

Program results during CY 1977 were to document the ability of C. paraensis to biologically transmit Oropouche virus to laboratory animals and its inability to mechanically transmit the virus. Culex quinquefasciatus, another candidate vector, were shown unable to transmit Oropouche virus mechanically. They are capable of inefficient biological transmission.

A distinct preference for host-seeking near human habitation was documented for female C. paraensis. The females were observed to engorge on human hosts in 1.6 minutes (mean time), with about 90% repleting within 2.5 minutes. The mean blood meal size was 0.0523 mg which represents an 88% increase in total body weight. Decaying banana stalks were found to be the preferred breeding sites.

Laboratory reared specimens of C. paraensis feed on humans preferential to other laboratory animals. Egg deposition begins 2-3 days after the blood meal and are deposited singly as the female traverses a wet surface. They do not normally oviposite on open water surfaces. Approximately 48-86 eggs are deposited per female per gonothophic cycle. Additional observations are reported on egg viability, embryogenesis, larval rearing media, pupation and adult emergence. These Culicoides midges have been maintained up through the 3rd generation in the laboratory.

The program of mammal trapping, hunting and mist netting was initiated at the Curuá Una collecting site, Santarém, Brazil, 15 June 1977. During the first 3 months of the collecting program, a total of 458 mammals were collected. The most common mammals trapped were Proechimys

guyannensis and P. longicaudatus; Carolla brevicaudata was the most commonly mist netted bat, followed by Sturnira lilium. Hunting was not very productive in the Curuã Una area, as only 9 specimens were collected. A bird collecting program was initiated on 12 September 1977.

The serum of a monkey, Callicebus torquatus, was the only serum tested which contained antibodies to Oropouche virus (titer 1:20). It also contained antibody to WEE, Mayaro, Mucambo and Utinga viruses. The sera of 25 of 29 (86%) spiny rats, Proechimys guyannensis and P. longicaudatus contained antibody to at least one of the other viruses tested. None of the sera tested contained antibodies to Araguari, EEE, yellow fever, Ilhéus, Sp H34675, Marituba, Caraparú, Catú, Guaroa and Tocaiuma viruses. Antibody to Icoaraci and Itaporanga viruses were found most frequently in the mammal blood.

Preliminary results are available from the entomological surveillance program at this time. Since these studies were initiated during the onset of the dry season (May-September), the populations of most species were low. The largest numbers of insects, collected to date, were from the Culicinae and Phlebotominae groups; however, all hematophagous insects are being processed for virus isolation.

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## FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee on the Guide for Maboratory Animal Resources, National Academy of Sciences - National Research Council.

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## INTRODUCTION

During CY 1977 an integrated program of field and laboratory studies was initiated to study the ecology of Oropouche virus in the Amazon region of Brazil. This on-going research is conducted in the Virology Section of the Evandro Chagas Institute, Belēm, Brazil and is staffed by 2 researchers from the Walter Reed Army Institute of Research, Washington, D.C. one consultant from the Pan American Health Organization and members of the Evandro Chagas Institute.

## PROBLEM

The problem for this research program is to elucidate the ecology of Oropouche virus in the Amazon region. Specific research objectives are:

1. To establish a field program for studying the ecology of Oropouche virus.

2. To study the general biology and taxonomy of suspected vectors and vertebrate reservoirs in the sylvatic environment.

3. To monitor the seasonal abundance and study the biology of a recognized vector (Culicoides paraensis) in the urban environs of Belēm, Pará, Brazil.

4. To colonize and conduct laboratory studies on the infection threshold of C. paraensis and to test the vector potential of other suspect vector species.

5. To study virological and serological methods to facilitate the processing and characterization of Oropouche outbreaks.

The long-term goals for this research are to:

1. Understand the ecology of Oropouche virus during epidemic and interepidemic periods.

2. Identify the means of ingress of Oropouche virus into the urban areas.

3. Upgrade the techniques of working with this specific virus.

4. Identify and assess the feasibility of controlling those factors in the urban environment that contribute to high attack rates of Oropouche virus.

BACKGROUND

Oropouche virus was first isolated from a human in the Melajo Forest, Trinidad in 1955[1] and has been found by CF (complement fixation) test to be a member of the Simbu group of arboviruses[2]. The virus was first discovered in Brazil by members of the Evandro Chagas Institute in 1960[3]. It was subsequently recognized as a public health problem in the Amazon basin due to its occurrence in urban epidemics.

Known epidemics of Oropouche virus have been documented twice in Belēm, Parā, Brazil, and once each in the Bragança area, Itupiranga, the Santarēm area and Belterra, Parā, Brazil[3]. The attack rate within human populations during epidemic periods has been documented to be as high as 40%[4]. Much of the available information on Oropouche virus occurrence and epidemic characteristics has resulted from studies conducted by Dr. Francisco Pinheiro, Chief of the Virology Section at the Evandro Chagas Institute.

Oropouche virus has been isolated from Mansonia mosquitoes in Trinidad, W. I., from Aedes serratus and Culex quinquefasciatus in or near Belēm, Brazil, and recently from C. paraensis in the area of Santarēm, Brazil[3]. Oropouche virus has been shown to replicate, following intrathoracic inoculation; in Aedes scapularis, Aedes serratus, Culex quinquefasciatus and Psorophora ferox[1]. Culicoides paraensis was incriminated epidemiologically as a vector in urban areas during field studies during 1975[3]

In Trinidad a high percentage of Capuchin (Cebus) and howler (Alouatta) monkeys were found to have neutralizing antibodies. The Evandro Chagas Institute in Belēm has found neutralizing antibodies in the Amazonian woodcreeper (Xyphorhynchus spixii), black-faced antthrust (Formicarius analis), black-spotted bare-eye (Phlegopsis nigro maculata), silver-breaked tanager (Ramphocelus carbo), and white-beaker fire-eye (Pyriglena leuconota). A domestic duck and a pigeon were found to have antibodies during an epidemic in Belēm, and a monkey and a kinkajou had antibodies in another epidemic in the state od Parā.

During the Bragança, Parā epidemic of 1967, sera from 40 monkeys and 2 kinkajous were tested for neutralizing antibodies. Both kinkajous and 1 monkey were seropositive for Oropouche virus[5]. Sera from 775 other animals (birds, mammals and reptiles) were also tested; but were found to be negative[6].

Inoculations of Oropouche virus into 2-toed and 3-toed sloths at the Evandro Chagas Institute resulted in detectable viremias from 3 to 9 days. Two inoculated squirrel monkeys (Saimiri sciureus) had viremias for 4 and 8 days. A marmoset (Saguinus) had a viremia for 5 days and died on the 6th day. A field rodent (Zygodontomys), laboratory mice, white

rats, chickens, doves, and 3 wood creepers (Xiphorhynchus spixii) were tested with little or no response. Three howler monkeys showed a low viraemia for 1 to 6 days. Many arboreal birds and mammals remain to be tested. The only wild caught animal from which virus was isolated was a 3-toed sloth (Bradypus tridactylus), collected 150 km southeast of Belém.

Although the public health importance of Oropouche virus had been recognized for years, the vector was unknown until the current studies were undertaken. The "maruim", Culicoides paraensis, has been shown, within reasonable limits, to be a vector of Oropouche virus to humans during epidemics. This finding is particularly significant since Oropouche virus is the first disease of public health importance shown to be transmitted by Culicoides midges.

## APPROACH TO THE PROBLEM

The approach for this research program will be presented under the headings of 1) forest vector-reservoir surveillance; 2) urban vector-ecology studies and 3) laboratory studies.

I. Forest Vector-Reservoir Surveillance: Entomological and animal surveillance programs are conducted in the Santarém area. Based on previous surveillance data, the operational hypothesis is that the vectors and reservoir hosts will be found in the tree canopy.

   a. The first task of vertebrate surveillance is to locate an area where Oropouche virus is or has been active as revealed by virus isolation or serological tests on animal specimens. The approach and methods of this program are to:

      1. Collect mammals and birds by trapping, hunting and mist netting.

      2. Collect from each animal whole blood, serum and organ specimens to process for Oropouche virus isolation and serology.

      3. Preserve organ specimens in 10% formalin for pathological examination.

      4. Gather habitat preference and population data on the animals suspected of being reservoirs for Oropouche virus.

      5. Preserve animal study skins to be curated at the Instituto

Evandro Chagas.

6. Maintain sentinel hamsters in the trap areas to increase the potential of detecting Oropouche virus transmission.

The animal surveillance team requires at least 3 weeks per site and is relatively nomadic until signs of Oropouche activity in an area are documented. Once Oropouche activity is identified, the entomology team will work in the area to seek naturally infected insects.

b. The entomology program is a comprehensive surveillance effort limited to the Santarēm region. One team of collectors conducts routine collections at 2-4 sites (1 site/team/week). Various types of collections are made to obtain specimens for virus isolations and biological information on potential vectors.

Where needed, rearing of specimens for taxonomic studies is undertaken at the field sites.

II. Urban-Vector Ecology Studies: The entomological studies in and near the city of Belēm, Parā, Brazil are designed to study the seasonal abundance and detailed biology of the suspect urban vector, C. paraensis. Detailed objectives are to:

1. Study the seasonal abundance (to be correlated w/ rainfall etc).

2. Quantify the endo/exophilic tendencies.

3. Study host preferences by special studies and blood meal identification.

4. Find and describe the breeding sites.

5. Find and describe the resting sites.

6. Collect live specimens for colonization attempts.

7. Test repellents against Culicoides paraensis and other anthropophilic Diptera.

One team of collectors makes systematic collections at two urban sites which have been selected for routine surveillance. Collection methods are standardized so comparisons can be made between sites and meteorological data will be recorded continuously.

The team in Belēm also assists in evaluating new collection techniques designed specifically for Culicoides spp.

III. Laboratory Studies: The laboratory phase of this program includes a large number of activities. A partial list of activities is as follows:

a. Virology:

1. Virus isolation: Blood samples and suspensions of mammal viscera are inoculated intracerebrally into suckling mice for virus isolation attempts. Alternatively, isolation attempts will be made in cultures of Vero cells. This system might also be adapted for isolation attempts with hematophagous insects.

2. Serology: All the vertebrate sera are screened for HI antibodies to Oropouche virus. The HI positive sera are confirmed by neutralization tests in Vero cells.

3. Identification of virus isolates: Isolates are identified with the complement fixation test using mouse hyperimmune ascitic fluid, made against selected viruses found in the Amazon region.

b. Entomology and Animal Ecology: Activities in the entomology and animal ecology programs will consist of:

1. Enumerating and identifying field collected specimens.

2. Collating collections with ecological and environmental data.

3. Taxonomic studies on specimens collected.

4. Colonizing suspect vectors for Oropouche virus transmission studies.

5. Conducting transmission studies and colonizing insects species for study.

6. Biological studies in the laboratory, viz., adult longevity studies, blood meal quantification, etc, of suspected vectors.

7. Experimenting with various designs for insect traps.

The first 4 activities are applicable to the animal ecology program.

RESULTS

Field Surveillance. Site Description. A program of conducting systematic collections of insects, birds and mammals was begun in a study area located 44 km south and 40 km east of Santarém, Pará, at the Curuá Una Hydroelectric Plant (latitude 2°50' south and longitude 54°22' west). Animals are collected on both sides of the Curuá Una river which flows from west to east and is approximately 100 m wide. The general topography of the area ranges from flatland to steep slopes. The river is 47 m above sea level and the hills rise to approximately 100 m. The forest is classified as tropical semi-evergreen seasonal forest[7]. The soil along the river is sandy, and ranges from a reddish-brown sandy clay to clay-sand. The $A_1$ horizon (humus layer) is 1 to 2 cm thick and contains many fine hair-like roots. The $A_0$ horizon (litter layer) is to 4 to 7 cm in thickness, and is composed of fallen leaves and twigs. Moss cover on fallen trees is also common. A shrub layer of thin woody plants, 0.5-3 m tall, is present, but a machete is not required for walking in the forest. Small trees and palms, to 10 m in height, make up an open canopy. Larger trees, from 15 to 20 m tall, are common, and emergents, to 30 m in height, are scattered throughout. Epiphytes are not common, but small vines and lianas are plentiful. Some of the smaller trees have stilt roots, and buttressing is evident on a few of the emergents.

The program of mammal trapping, hunting, and mist netting was initiated at the Curuá Una collection site 15 June 1977. During the first 3 months of the collecting program, a total of 458 mammals were collected. The most common mammals trapped were Proechimys guyannensis and P. longicaudatus; Carolla brevicaudata was the most commonly mist netted bat, followed by Sturnira lilium. Hunting was not very productive in the Curuá Una area, as only 9 specimens were collected. A bird collecting program was initiated on 12 September 1977.

The serum of a Monkey, Callicebus torquatus, was the only serum tested which contained antibodies to Oropouche virus (titer 1:20). It also contained antibody to WEE, Mayaro, Mucambo and Utinga viruses. The sera of 25 of 29 (86%) spiny rats, Proechimys guyannensis and P. longicaudatus contained antibody to at least one of the other viruses tested. None of the sera tested contained antibodies to Araguari, EEE, yellow fever, Ilhéus, Sp H34675, Marituba, Caraparú, Catú, Guaroa, or Tocaiuma viruses. Antibody to Icoaraci and Itaporanga viruses were found most frequently in the mammal blood.

The whole blood and organ specimens collected from the mammals are currently being processed for virus isolation in the virus laboratory at the Evandro Chagas Institute, and results are not yet available. The whole blood smears taken from 472 animals have been examined and sorted, although the suspected positive slides have not, as yet, been comfirmed. Preliminary results indicate that no anthrax has been found, while microfilariae have been tentatively identified on 59 slides, and trypanosomes on 17 slides.

Entomological surveillance was conducted to monitor virus activity among blood feeding insects and to obtain basic entomological information concerning vector ecology. Entomological information is being gathered by using multiple collecting techniques (animal bait traps, light traps, shannon traps, suction traps and man biting collections) to capture medically important insects. Insect captures are being conducted during both the day and night, and at both ground and canopy levels. When sufficient data are available for selecting promising species as vectors of Oropouche virus, the species will be evaluated in the laboratory to delineate vector competence.

Preliminary results are available from the surveillance program at this time. Since these studies were initiated during the onset of the dry season (May-September), the populations of most species were low. The largest numbers of insects, collected to date, were from the Culicinae and Phlebotominae groups; however, all hematophagous insects are being processed for virus isolation.

Laboratory Transmission Studies. Species representing 2 families of hematophagous insects (Culicidae and Ceratopogonidae) have been tested in the laboratory for virus transmission. Culex quinquefasciatus were reared under laboratory conditions (26.5°C ± 1.95% RH and 12/12 light/dark photo period). The colony was established in 1975 from mosquito collections conducted in Belēm, Parā, Brazil and this colony supplied the mosquitoes used in the transmission studies. Since a colony of C. paraensis has not been established, all adult Culicoides used in the studies were collected from an agriculture experiment station near Belēm. Mosquitoes and midges were maintained on a 10% sugar solution. Oropouche virus, strain Br An 19991, was employed in the tests and young hamsters (23-25 days of age) were used as the donor and recipient vertebrate hosts in all transmission experiments.

Hamsters were inoculated intracerebrally (IC) with 0.1 ml of undiluted hamster serum containing Oropouche (Br An 19991) virus and 24 hrs later 0.1 ml of blood was obtained for virus titration in Vero cells. After bleeding, the hamsters were exposed to test populations of insects.

Virus Isolation and Identification. Individual insects included in the tests were processed for virus isolation in Vero cells. To identify the isolates, a 1:100 dilution of the infected fluid was mixed with equal amounts of Oropouche hyperimmune mouse ascitic fluid, incubated for 1 hour at 37°C and assayed for infectivity. These tests were performed in microtiter plates to which Vero cells were added after incubation of the virus and virus-serum mixtures. The test control series consisted of infected fluids without additions of the Oropouche virus hyperimmune mouse ascitic fluids. The tests were routinely read 3-4 days post-inoculation or when the virus control showed a 3-4 + CPE.

Culex quinquefasciatus. Mechanical Transmission. Mosquitoes were fed on

a viremic hamster ($10^{7.0}$ $SMLD_{50}$/0.02 ml of blood) during the dark phase of the photo period. Feeding mosquitoes were removed prior to full engorgement and transferred to a 2nd holding cage. Following the transfer, a susceptible hamster was exposed and the mosquitoes were allowed to feed to repletion. The hamster was removed and observed for 3 weeks for signs if illness. No evidence of Oropouche virus infection was detected in the recipient hamster.

Biological Transmission. Three separate attempts were made to demonstrate biological transmission of Oropouche virus by Cx. quinquefasciatus. In the initial attempt, mosquitoes were fed on a hamster circulating $10^{7.0}$ $SMLD_{50}$/0.02 ml of Oropouche virus. Engorged mosquitoes were removed and held until tested for virus transmission. Susceptible hamsters were exposed to the Cx. quinquefasciatus population on days 0, 1, 5, 10 and 17 post-infectious blood meal. Several mosquitoes were removed on each of these days and assayed, individually, for virus. Oropouche virus was recovered from 1 of 5 mosquitoes individually assayed on day 1. Virus was not recovered from any of the other mosquitoes tested; nor was virus successfully transmitted to susceptible hamsters.

In the 2nd experiment, 2 groups of mosquitoes were fed on viremic hamsters. The 1st group was fed on a hamster circulating $10^{8.2}$ $SMLD_{50}$/0.02 ml of Oropouche virus, and the 2nd group on a hamster circulating $10^{8.0}$ $SMLD_{50}$/0.02 ml. Mosquitoes were then allowed to feed on susceptible hamsters at various periods post infection. Insects from this study were not assayed for virus isolation. A considerable number of mosquitoes fed on each of the susceptible hamsters exposed on days 2, 4, 6, 8, 10, 12, 14 and 16. One hamster, fed upon on day 8, seroconverted to Oropouche virus.

In the final experiment with Cx. quinquefasciatus, 3 day old mosquitoes were fed upon a viremic hamster circulating $10^{8.0}$ $SMLD_{50}$/0.02 ml of Oropouche virus and 4 day old mosquitoes were fed on a hamster circulating $10^{7.8}$ $SMLD_{50}$/0.02 ml of virus. Mosquitoes exposed to viremic hamsters were allowed to engorge on non-infected hamsters on days 15, 16 and 21.

Oropouche virus was recovered from 1 hamster exposed to infected mosquitoes on day 21. Thirty mosquitoes were assayed individually from this group and only a single, non-engorged individual was found positive for Oropouche virus. On day 22, 15 mosquitoes (10 engorged, 5 non-engorged) were assayed individually for Oropouche virus. No mosquitoes were positive for virus and none of the hamsters exposed to this subgroup of mosquitoes exhibited signs of virus infections.

Culicoides paraensis. Mechanical Transmission. Techniques used to demonstrate mechanical transmission with C. paraensis were similar to those used with Cx. quinquefasciatus. The resulting data indicated that C. paraensis do not transmit Oropouche virus mechanically under the test conditions. This was substantiated by interrupted blood feeding of midges on infected hamsters with a high viremia ($10^{7.0}$ $SMLD_{50}$/0.02 ml) and the

refeeding of 19 and 28 midges on susceptible hamsters at 1½ hrs, respectively. Oropouche virus was not detected in test hamsters during the 3 week laboratory observation period.

Biological Transmission. The 1st preliminary test for biological transmission of Oropouche virus by C. paraensis resulted in the death of 4 hamsters which had been exposed to C. paraensis that had fed on a viremic hamster (with a viremia of $10^{7.0}$SMLD$_{50}$/0.02 ml of blood). Unfortunately, samples were not taken from these animals since they died before blood could be obtained. Therefore, it could only be assumed that Oropouche virus was the cause of death of the test animals. However, sub-samples of the Culicoides used for this test, taken at various intervals after the infectious blood meal, demonstrated that 46% (17/37) of the midges were positive for Oropouche virus.

A 2nd study was conducted to demonstrate biological transmission of the virus. Virus was isolated from 2 hamsters which had been exposed to C. paraensis after 8 and 9 days extrinsic incubation. In addition to the positive transmission of Oropouche virus by C. paraensis, it was interesting that only 1 blood feeding, on day 9, from an infected midge resulted in successful transmission of Oropouche virus. Attempts were also made to isolate virus from individual specimens of C. paraensis. Although fifty per cent of the specimens removed immediately after engorgement on the hamster, containing $10^{6.0}$SMLD$_{50}$/0.02 ml of virus, were positive for Oropouche virus, all subsequent attempts at virus isolation were negative.

The 3rd and 4th experiments reconfirmed biological transmission of Oropouche virus by C. paraensis. It was noted that virus was transmitted to the hamsters by a single bite each and confirmed the observations made in the 2nd test.

When comparing the isolation of Oropouche virus in the 2 experiments (numbers 3 and 4) it was noted that virus was not recovered from any of the Culicoides midges in the 3rd experiment, while 20.5% (26/127) of the C. paraensis tested in the 4th experiment were positive for Oropouche virus.

Special Field Studies With Culicoides paraensis. In 1976 efforts were made to find areas producing high populations of C. paraensis near Belém to obtain sufficient numbers of midges to conduct Oropouche transmission and preliminary colonization studies. A location meeting the experimental requirements was found at an agriculture experimental research station (CEPLAC - Executive Commission Plan of Agriculture and Cocoa) located on the periphery of Belém, Pará, Brazil. This area was being utilized to investigate cocoa and banana tree production and had been established for a number of years.

Dense populations of biting midges were found near the buildings and it was speculated that the breeding sites would also be found in the area.

Therefore, a transect study was conducted to establish the spatial distribution of biting activity, and surveillance for breeding sites was initiated to find the preferred breeding habitats.

Transect Studies. Midge biting activity near one of the house was intense; therefore, this site was choosen as the focus for the transects. A team of 2 collectors with mouth aspirators were positioned at 4 locations separated by 20 m along the transect. Biting midges were aspirated from the collectors exposed legs for a collecting period of 15 minutes. The Culicoides collected by each team were counted and the 2-man teams were then rotated to another collecting point along the same transect to repeat the collection. A series of 5 transects were conducted. Female C. paraensis were found concentrated within a 20 m radius from the house. The number of C. paraensis recorded at this site was considerably higher than those recorded for the next station, which was located 20 m away. It seems reasonable to speculate that the midges are attracted to this site due to human activity near the house and/or exhibit a house-seeking behavior. In either case, their close association with human activity and/or houses would be an important behavioral pattern for disease transmission.

Breeding Site Surveillance. Emergence trap studies are being conducted to determine the preferred breeding sites for C. paraensis. Even though the present data are incomplete for seasonal breeding habitat preferences, some observations have been made. The breeding habitat which has produced the greatest number of Culicoides species is decaying cocoa pods; however, Culicoides paraensis only accounted for about 1% of the emerging midges collected from this habitat. C. paraensis also emerge from micro-habitats associated with the banana trees. Cut banana tree stalks, decaying on the ground, appear to be the largest producer of C. paraensis during this season of the year. Culicoides paraensis were not collected from the leaf litter and marshy lowland habitats, although these habitats were routinely sampled.

Seasonal Abundance. Collections for C. paraensis are being conducted near houses in 2 districts of Belém, Brazil. Captures are conducted for 4 consecutive 30 min. intervals, from 1400-1600 hrs., at each house for 2 consecutive days on alternate weeks. Two collectors are employed for each capture and they aspirate the biting midges only from their exposed legs. This study was initiated in July, 1977 and will be continued for a full 12 to 18 month period.

Size of Blood Meal and Engorgement Time. The study was conducted by allowing individuals of field populations of C. paraensis to feed at liberty on the exposed legs of 4 collectors. When a midge was observed to alight and begin to feed, a small glass tube was placed over the feeding midge. Following the voluntary withdrawal from the host, the glass vial was stoppered. The engorgement time was recorded for each specimen. Engorged midges were killed by chloroform fumes and grouped into units of 10 for weight measurements. Similar capture techniques were used with midges that were not permitted to probe and feed. All specimens were weighed with a Cahn model 4400 Electro balance R.

Considerable variation was found in individual engorgement times of C. paraensis. The minimal and maximal time recorded during our studies were 0.5 and 5.00 minutes, respectively. The mean engorgement time was about 1.6 minutes, with approximately 90% of midges repleting within 2.5 minutes.

The weight differences of non-engorged and engorged C. paraensis indicate that body weight increases by approximately 88% due to blood and tissue fluid ingestion. The mean blood meal weight was found to be 0.0523 mg.

Colonization of Culicoides paraensis. Biting midges (C. paraensis) used to initiate colonization studies were obtained from an experimental agriculture experiment station (CEPLAC), Belēm, Parā, Brazil. Due to the availability of various breeding sites in this area, Culicoides paraensis could be collected through the year; however, highest population densities seemed to be found during the rainy season (November-May). Specimens for laboratory studies were collected by man-biting captures.

Young hamsters (21-23 day old) were the principal blood source used in the colonization efforts. Oviposition containers were constructed from 50 ml wide mouth beakers and filter paper. The filter paper was saturated with water from decaying cocoa pods.

Larvae were reared in transparent plastic containers (L-26 cm, W-22 cm, D-11 cm). These chambers were fitted with removable lids perforated with a center hole (7 cm) for placement of collecting chambers for emerging adults and small lateral holes for aeriation tubes. Emergence chambers for adult midges were fabricated from pint plastic containers with inverted funnel inserts and fine mesh screen tops. Small cotton pads saturated with a 10% sugar solution were placed on the fine mesh screen tops to provide a carbohydrate source for newly emerging adults. Since C. paraensis adults are positive phototrophic, the rearing chambers were covered with black opaque plastic to exclude light from the transparent sides of the containers. Thus, the only light in the rearing container was through the emergence chamber into which the adults would enter and be trapped. Adults are removed from the emergence containers on a routine schedule and maintained in holding cages at 26.5°C and 95% + RH. When necessary, $CO_2$ gas is used to anesthetize adults for counting, transfer, etc.

Presently, the principal larval media is being formulated from processed rice hulls and routinely supplemented with material from cocoa pods and colonized nematodes (Anguillula silusiae). These nematodes are easily cultured in the laboratory on yeast and wetted oat-meal. Rice hulls are added to a rearing container to a depth lf 1 cm, then wetted with distilled water to a depth of 3-4 cm. The larval medium is prepared 2-3 days before midge eggs and/or larvae are introduced into it to provide time for the natural multiplication of bacteria, protozoa, nematodes and algae, which are food substances of Culicoides larvae.

Laboratory Blood Sources for Culicoides paraensis. Blood meal ingestion studies have shown that Culicoides midges will imbibe similar quantities of blood when allowed to engorge on man or hamsters. Other animals used in preliminary blood donor attempts were rabbits, young chickens and white laboratory mice. Midges were observed to feed on all of these animals; however, laboratory reared midges show a definite preference for human host as opposed to young hamsters.

Oviposition of C. paraensis. Blood fed C. paraensis begin laying eggs 2-3 days after blood ingestion. Eggs are oviposited singly as the female traverses a wet substrate. Present studies indicate that gravid females do not normally deposit eggs on the water surface. Approximately 48 to 86 eggs are deposited per adult female per gonotrophic cycle.

Egg Viability and Embryogenesis. Within 24 hrs. after oviposition a random sample of the eggs was removed and evaluated for egg hatchability. The greatest number of eggs hatched 3-4 days post-oviposition and approximately 85% of the eggs sampled were viable.

Larval Rearing Media. Six formulations of media have been or are being evaluated for larval development and adult emergence. The different media consisted of wood chips, hay infusion, leaf-litter infusion, cocoa pods, banana stalk infusion and rice hull infusion. The only rearing formulation which failed to support larval development and adult emergence was the forest leaf litter infusion. A few adults were reared in the wood chip infusion, but this was unsatisfactory due to fungal growths on the chips and unknown chemicals associated with the wood. Natural breeding substrates associated with banana trees and cocoa pods are presently being evaluated as potential laboratory rearing mediums. Midge rearing data from these materials indicate that sufficient food substances are available for development of the immature stages.

Pupation and Adult Emergence. Mature larvae will pupate when submerged in aqueous medium or placed on a saturated substrate. Immature midge pupae lack buoyancy and are incapable of swimming movements such as observed with pupae of mosquitoes. Lateral movements are accomplished by a series of abdomen-tail thrusts. Within 12-18 hrs after the 4th instar larvae pupate, the pupae can ascend to the water surface by means of a physical buoyancy that is believed to be from a gaseous accumulation in the ventral cephalothorax. When disturbed, the pupae cannot sound (submerge). However, when displaced from the water surface the pupa will quickly float to the surface and initiate lateral abdominal movements to properly reorient the respiratory horns with the water-air interface. The pupae stay at the water surface prior to adult emergence approximately 48 to 72 hrs. The adult escapes the pupal exuviae by a longitudinal split along the anterior-dorsal cephalothorax. Preliminary data on pupation time indicate that the pupal stage of C. paraensis lasts from 2 to 5 days at 26.5 ± 1°C.

Laboratory Colonization. Preliminary observations on adult midges obtained by laboratory colonization efforts are as follows:

1. Sex ratio - A 1:1 sex ratio has been obtained under insectary conditions for the $F_2$ generation (based on a count of 715 specimens).

2. Mating - Mating of C. paraensis has been observed in laboratory holding cages. Successful fertilization has also been demonstrated by finding sperm bundles in spermethecae dissected from laboratory reared females.

3. Oviposition - Eggs oviposited by female midges reared in the laboratory are being evaluated for viability; however, present observations indicate that a high percentage of the eggs are infertile.

Size of Blood Meal. Weights for non-engorged and blood engorged C. paraensis in the laboratory revealed a 74% body weight increase due to blood and tissue fluid ingestion. The mean body weight of a non-engorged midge was 0.588 mg and the mean blood meal weight was 0.435 mg.

Adult Longevity Under Laboratory Conditions. Field collected C. paraensis of unknown ages were maintained under laboratory conditions (26.5°C, 95% RH) to determine their lenght of survival. Saturated cotton pads (10% sugar solution) and blood donor hamsters were used to maintain the Culicoides until 100% mortality was recorded. Dead midges were removed daily and enumerated.

Preliminary results indicate that mortality of the study population was low for the first 5 days. Fifty per cent mortality was recorded at 10-11 days and 100% mortality was noted 6 days later.

## DISCUSSION

Culicoides paraensis have been incriminated in epidemiological studies and laboratory transmission tests as the vector of Oropouche virus in urban areas.

The infection threshold of this virus in C. paraensis remains to be defined. However, available data indicate that at the on-set of symptoms of Oropouche virus infection in humans there is a virus titer similar to that in hamsters (ablout $10^7$ $SMLD_{50}$), employed in the laboratory transmission tests. This indicates that humans are the amplifying hosts in urban areas and circulate enough virus for infecting populations of C. paraensis. The other potential vector in urban areas, Cx. quinquefasciatus has demonstrated marginal ability to be infected and transmit the virus in the laboratory.

More precise efforts are being made to define the susceptibility of C. paraensis to Oropouche virus. Since these midges are difficult to work with, under controlled conditions, additional experimental techniques must be developed and many parameters must be defined, e.g., to what extent will they feed on tissue fluid opposed to peripheral blood. Obviously, an insect with a mean body weight of 0.0586 mg is not easy to evaluate for blood engorgement, which is necessary information for infection threshold studies. The resultant techniques and studies methods from this research should have broad application to studies with Culicoides midges as vectors of other viruses and studies in other geographical areas. Surveillance for natural reservoirs and vectors in forest environments was initiated in mid-CY 77 and will be continued in CY 78. This program is designed to detect an area where Oropouche virus is active. In addition to collections of wild animals, sentinel hamsters are being employed for virus detection and sentinel birds may be employed at some time in the future. These efforts are being augmented by viremia tests in several potential vertebrate reservoirs, e.g. members of the Formicariidae.

Oropouche virus is of considerable public health importance in the Amazon basin and is the first virus that causes human epidemics transmitted by biting midges. The vector, C. paraensis, has been shown to be day-active, endophilic and be most abundant in the peridomiciliary environments. These characteristics, by analogy, portray this species as the "Aedes aegypti" of the biting-midge family and warrants in-depth studies to elucidate the ecology and dynamics of the reservoir-vector-human relationships in both urban and rural settings.

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