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UNITED STATES GOVERNMENT

Memorandum

ACTION MEMORANDUM

DATE: July 25, 1977

TO : AA/DS, Marjorie S. Belcher

FROM : TA/H, Lee M. Howard, M.D.

SUBJECT: Small Research Project entitled "Control of Anopheles balabacensis by Larviciding."

Problem and Rationale:
In the narrow hilly eastern and northern borders of Bangladesh as well as in the Chittagong Hill tracts malaria has remained epidemic or holoendemic in spite of malaria control efforts. The major vector of malaria in these areas is Anopheles balabacensis which breeds in jungle areas, avoids lethal contact with DDT coated walls and is, in general, an outdoor biter. The problem is how to control malaria in areas where A. balabacensis is the major vector of malaria.

There are number of reasons to support research on the problem of A. balabacensis control. First, malaria persists in the jungle areas in spite of twice yearly spraying of houses due to this vector; second, the area is a source of reinfection for the central lowlands of Bangladesh which are practically freed from malaria; and third, these areas are the last unexploited resource for development by the population of Bangladesh.

The investigator, Dr. Rosenberg, spent the years 1974 to mid-1976 in Bangladesh in the A. balabacensis areas carrying out research on control of this vector. His investigations lead to the conclusion that control of this vector might be accomplished by a simple larviciding methodology in the dry season at the limited breeding sites of the vector mosquito.

The Anopheles balabacensis range includes northeast India, Burma, South China, Thailand, Laos, Cambodia, Vietnam, large portions of Indonesia and Palawan in the Philippines. The development of a suitable larviciding technique for this mosquito vector in Bangladesh would have wide implications in the Asian area.

Research Proposed: The purpose of this research is to develop and test techniques of a larviciding procedure for the control of An. balabacensis in jungle areas. The basic research on the biology of the vector has been reached as to how control may be accomplished. The past research activities on this project were carried out principally through the Bangladesh Malaria

Control Program staff. The Government of Bangladesh has approved of this research work and has agreed to provide support to the effort in the form of manpower and logistics. It is planned that a variety of relatively inert, non-toxic, and specific larvicides which have been extensively marketed and tested for years will be used. Product used in the project will be those approved by the EPA and used for mosquito control work in the United States i.e. Abate, oils, Fenthion. There are no adverse enviromental implications for the research project in the experimental area.

The research work will be carried out at the Chaklapungee Tea Estate in Sylhet District which was site of Dr. Rosenberg's previous research study.

Research Objectives and Outputs: The work will entail development of a scientific conclusion into a possible mosquito control methodology which may be use in a wide variety of Asian situations. The basic objectives are:

1) to continue study of the malaria vector, *Anopheles balabacensis*, in the research area at Chaklapungee through surveys of the breeding sites, mosquito population census, mosquito collections, and malaria case detection in the population.
2) to carry out mosquito control on *A. balabacensis* during the period from 1 April through 31 October 1978.
3) to evaluate the results of the mosquito control methodology through studies relating to bite counts, reduction human malaria incidence and numbers of biting mosquito which are infected, with malaria parasites.

The outputs will consist of (1) increased basic knowledge of this important vector of malaria; (2) completed studies on the practicality of using the proposed larviciding methodolgy against *An. balabacensis*; (3) a final report on the research work which will be use to other malaria control programs in Asia.

Plans to link Research and to Utilize Results: If the larviciding methodology is found to be effective, the techniques can be provided to other Asian malaria control programs, the World Health Organization, and private and University researchers in this field. It will be written up for technical journals and discussed with health officials at the country level, with AID officers and the World Health Organization. Malaria control workers all over the world as well as in Asia will be advised of the technique.

Management Consideration: TA/H approximates the expenditure of 5 man days to develop the project and 8 man days to initiate information dissemination and utilization programs.

Research Design and Methods: The attached proposal (Pgs. 17-22) contains the proposed study plan. The estimated budget is to be found on Pages 22-25. The resume of the investigator (Appendix I) and references related to this research are included in the proposal.

Reviews of the Proposal: The proposal has been reviewed by Mr. Larry Cowper, TA/H/VBD, Mr. Ed. Smith, Ta/H/VBD, and Dr. John Scanlon, University of Texas at Houston.

Each of these people are technically qualified in vector-borne diseases including malaria and are aware of the operational, management and technical implications of the proposal. It is their judgement after reviewing the proposal that the small research to be carried out would be of importance, is focused on practical field problem and, if successful, would have a major effect on the on-going control efforts against *A. balabacensis* in Asia.

Recommendation: Therefore, it is recommended that:

1. That you approve the use of Small Research Project funds in the amount of $14,540 for the work outlined above and described in detail in the attached proposal.
2. That the contract for these studies be awarded to Dr. Ronald Rosenberg without consideration of other sources.

Approved: Caroline D. McGraw

Disapproved: ____________

Date: Jan. 25, 1978

Clearances:

TA/PPU, Robert Simpson RBS/1/9

ASIA/BIS, Joan Coe JCoe

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Control of *Anopheles balabacensis* by Larviciding

Ronald Rosenberg, Sc. D. candidate

N. P. Maheswary, Senior Entomologist,
Malaria Control Program of Bangladesh

submitted 9 June 1977 valid to 31 December 1978

duration of project: 1 March 1978 - 28 February 1979

signature of Principal Co-Investigator ______________ Milan Trpis, Ph. D.

signature of Principal Investigator ______________ Ronald Rosenberg

signature of Department Chairman (for contracting organization) ______________ F. B. Bang, M. D.

Human Experimentation Plan for this proposal was approved for renewal by the Johns Hopkins Committee on Human Volunteers on 1 June 1977.

This proposal has been submitted in part for funding to the Malaria Section, Southeast Asia Regional Office, World Health Organization on 20 January 1977.

Unpublished original research is included herein for purposes of proposal evaluation only; any other use of these data requires specific permission of the principal investigator.

APPROVED:

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The Problem and Relevance of its Solution to AID's Goals

In the large, central deltaic area of Bangladesh, where rice is grown, a low malaria endemicity was maintained by the mosquito Anopheles philippinensis until extensive DDT spraying, sponsored in part by AID, began in 1962. Endemic malaria subsequently disappeared from this area but low population levels of philippinensis, which also feed on cattle, persist. The narrow hilly eastern and northern borders of the country and the Chittagong Hill tracts are forested and here malaria has remained epidemic or holoendemic (Fig. 1); the major vector is the jungle mosquito Anopheles balabacensis. This belt of endemic malaria, a relatively small area, is worrisome for three reasons: First, malaria persists here in spite of twice yearly house sprayings; second, the area is a source of reinfection for the central lowlands now that most DDT coverage there has ceased (since amalgamation of most of the Malaria Eradication Program with the Rural Health Scheme in 1976); and third, because for Bangladesh's burgeoning population, these forested areas are the last unexploited resource, growing numbers of low-landers with no immunity to malaria go there yearly for work, farming and tourism (in the Fall of 1975 a falciparum malaria epidemic killed nearly 2,100 such non-immunes in one Thana of Ramgarh subdivision alone).

The problem, then, is how to control malaria in the hills. The strategy of malaria control here has necessarily emphasized, even depended on, controlling the mosquito vectors; but the tactic of spraying house interiors and eaves with DDT has failed to interrupt transmission in balabacensis areas because balabacensis avoids lethal contact with DDT coated walls.

In 1974, we joined a team from the National Malaria Control Program (MCP) in an effort to define the malaria problem on the border and to explore new ways of eliminating it by controlling balabacensis. We conducted most of our research at Chaklapungee Tea Estate in Sylhet District and propose to investigate control methods at the same site. Our study, which included the first longitudinal malaria survey ever done in the East India-Burma region, has revealed a number of peculiar attributes of balabacensis, some of which have a direct influence on its vectorial capacity. One of our findings may be of particular practical importance since it suggests that at least in a monsoon climate larval control measures may be effective.

Anopheles balabacensis range includes northeastern India, Burma, south China Thailand, Laos, Cambodia, Vietnam, Borneo, Java, Sumatra, and Palawan. In all these areas it is a major vector of malaria, often chloroquine-resistant Plasmodium falciparum. Previous observations on balabacensis breeding were done in Borneo and Malaysia which lie in the equatorial rain forest belt south of 10° latitude, where rain falls equitably throughout the year. Early observers thought it impractical to control the larvae there because breeding took place in large numbers of small surface water accumulations per acre. We have found, however, that in the monsoon forest, that region between 10° and 28° latitude North, where we worked, that breeding takes place in many fewer breeding places per acre, that breeding places have sharply defined characteristics which facilitate their discovery, that the normal flight range of balabacensis is less than 3/4 mile, and that consequently it should be possible to control the larval stages in a 1 mile defensive buffer zone around a village at risk by focal chemical or oil treatment, by removing the shade from around breeding places, or by draining and filling.

There are certain advantages to testing such a program of control in Bangladesh.

First, a 1 or 2 year trial can be run on the same site the longitudinal study was and can thus be evaluated for efficacy by comparison with 2 years pre-test conditions. Second, there is a well trained, led, and motivated team of entomologists in the Bangladesh National Malaria Control Program who worked on the longitudinal study and are competent to carry out the similar control trial with benefit of only periodic consultation from abroad. Third, a larviciding program is inexpensive, labor intensive, and can in large part be organized and carried out by village leadership, thereby compensating for the recent decrease in trained lower level malaria personnel. Additional reliance on insecticides will be small since control of one larval focus is equivalent to control of scores of adult foci (houses). In many cases, topographic alteration will prove as effective as use of insecticide or oil surfactant. Fourth, despite the potential danger of reintroduction of malaria to the lowlands, malaria is now a relatively minor problem in Bangladesh: 31,247 positive slides examined in 1975 from mostly passive case detection. However, new control strategies for balabacensis tested in Bangladesh will likely be applicable to areas in northeast India, Burma, south China, Thailand and Indochina: all of which have the typical dry winter, monsoon climate, highland soils with low clay content and high water infiltration rates, and problems with drug resistant malaria transmitted by balabacensis unaffected by conventional house sprayings. To many of these countries an inexpensive community manned control program should have great economical appeal. For others, such as southeastern Thailand where malaria is rife, houses have no walls to be sprayed, and balabacensis breeds in water-filled gem mines, larval control seems a logical possible solution.

In the long perspective, control of malaria in the hills will have the beneficial effect of allowing fuller exploitation of forest resources, opening large tracts to cultivation, and safeguarding the non-endemicity of the lowlands. As the forest disappears, so will Anopheles balabacensis. There are, however, possibly serious ramifications of any type of malaria control in the hills: displacement of native tribes (e.g. Chakma, Mogh) by immigrating Bengalees and consequent social unrest; destruction of primary jungle and its wildlife; increased soil erosion; and population increase due to lowered infant mortality. Most countries faced with similar difficulties resulting from the elimination of a disease threat, choose to ameliorate, if not to solve, their problems through legislation rather than forego improved health and prosperity.

A more immediate concern is that the semi-efficacious immunity to malaria naturally acquired by people at constant risk is short lived, losing potency exponentially after antigen challenge has ceased. Approximately two years in a non-endemic area is sufficient time to deplete the host antibody level to one of minimal protection. Therefore any successful control program must be established for the long term, with provision for surveillance and drug distribution procedures to be left intact after disappearance of an endemic focus. A community freed of malaria and then allowed to be reinfected is in far greater danger than it was before control efforts were begun and in the absence of speedy therapy high levels of morbidity and mortality can be expected until immunity is reestablished. For this reason, the trial village at Chaklapungee has never been removed from the context of the National Malaria Control Program. As additional precautions an area malaria supervisor was appointed in a neighboring village by the MCP in order to tighten surveillance procedures during periods when the malaria research team was not in residence, both he and the tea garden Dispensor are provided with ample stores be malarious, and the test population themselves have been successfully instructed of where to go for treatment and blood smears when ill.

The pollutant effects of larviciding the breeding places of balabacensis are conjectural. They would seem to be, particularly on our limited study site, negligible. First, a variety of relatively inert, non-toxic, and specific larvicides have been extensively marketed and tested for years. Those under consideration for the future trial -- Fenthion, Abate, Paris Green, kerosine oil, and Dursban -- are among the safest. Second, the breeding places to be treated are temporary, often artificial, and with a small biota of invertebrates; tadpoles are the only vertebrate inhabitants. Third, the pools are unused in any way by man and do not run off into his drinking supply. Domestic and feral animals must, however, occasionally drink from them. Fourth, the nature of this control proposal is to treat a few specific pools only within a 1 mile radius of a village at risk. These are an infinitesimally small fraction of the total number of such pools lying outside the borders of control. Obviously, different terrains different from Sylhet not only may dictate modifications of the basic larviciding proposal tested, but may be affected in different ways. We stress again that one eventual advantage of our approach is the possibility of draining or filling sites as a "clean" alternative to chemical treatment.

Scientific Aspects

The objective is to test the hypothesis that control of the larval stages of Anopheles balabacensis will control malaria transmitted by that mosquito in Bangladesh. The formulation of a new approach is required by the inefficacy of DDT interior treatment to interrupt transmission; figure 3 clearly shows that in our test area infectivity continued to rise after spraying. Inherent in our hypothesis are the assumptions that the breeding places of balabacensis in Sylhet are peculiar and few per unit area, that we are certain we can find them all in a given area, that the effective lifetime flight range is limited to less than 1 mile from the site of emergence, and that weekly chemical treatment of breeding places is physically feasible. The hypothesis and its antecedents are based on facts established by us during 1975-77 in the same site proposed for control. As will be discussed in the following description of this completed work, the likelihood of successfully interrupting transmission is high. Should we prove this basic hypothesis in the limited area proposed, more extensive trials to determine alternate procedures, cost effectiveness, and feasibility for other regions would be warranted.

Literature Review

Less is known about the natural history of Anopheles balabacensis than of most other important vectors of malaria. There are several reasons for this paucity: the mosquito's jungle habitat is remote and uncomfortable to work in, its resting habits are elusive, and its taxonomic status was, until 20 years ago, confused. Most important, however, has been the belated recognition that balabacensis is an important vector. During the first half of this century, until the introduction of DDT, it was overshadowed by the more easily studied Anopheles minimus, whose habitat it overlaps. Furthermore, the increasing invasion since the Second World War of Asian forests by homesteaders has increased the breeding habitat of balabacensis while simultaneously putting an ever increasing population at risk to malaria, of which balabacensis is an enormously potent vector.

The first published report of balabacensis (then called leucosphyrus) as a vector is from northeastern India by Clark and Choudhury in 1941. Next came reports by Macan (1948) and by Kuitert and Hitchcock (1948) of malaria transmitted by it to Allied troops in Burma. At about the same time, Mac Arthur (1948, 1951), studying malaria in northern Borneo, concluded that contrary to prevailing opinion, balabacensis, not An. maculatus, was the vector in the interior valleys. He determined that the mosquito was breeding under heavy shade in small seepage pools at the bases of hills, but, because of inappropriate technique, was frustrated in his attempt to find infective adults. Nevertheless, by careful epidemiological work, he was able to correlate the presence of balabacensis breeding with hyper-endemic villages, thereby implicating it as vector. MacArthur tried to prevent larval development by dusting breeding places with Paris Green (this was prior to the advent of DDT). This approach failed, apparently because rainfall and seepage flow pushed the insecticide scum aside. He had more success in an attempt to prevent breeding by clearing the shade from around the breeding area. As measured against control areas where the shade was left intact, larval densities in unshaded pools decreased.

In 1956, Donald Colless published a brief but seminal paper on the "leucosphyrus" group of Anopheles which is even today easier to amend than add to significantly. Foremost, Colless determined that the "Anopheles leucosphyrus" being incriminated with increasing frequency as a malaria vector in Southeast Asia was in fact Anopheles balabacensis, Baisas 1936. This taxonomic clarification was essential to further research because balabacensis is only one of a large group of sibling species that are closely similar in appearance but very dissimilar in habits and vectorial capacity. Continuing MacArthur's work in north Borneo, he suggested that human and domestic animal activity on the village-forest edge increased the breeding places for what is basically a rare forest mosquito, thereby increasing malaria. He thought that natural forest breeding places were of limited importance in transmission. He discovered that elimination of shade did gradually reduce breeding in the now sunlit focus, but that the bullocks that had sheltered under these trees, and whose hoofprints were a major source of breeding, simply moved to adjacent shade and formed new breeding places. Colless first observed that DDT residue greatly reduced the period balabacensis would rest on a treated wall, larger catches of balabacensis during periods of early moon rise, increased longevity during the wettest season, dispersed daylight resting on low vegetation, a flight range of less than 800 yards, and a marked preference for human blood.

Since 1956, there have been frequent and accelerating reports of balabacensis as a vector of P. falciparum and P. vivax in Burma (Khin-Maung-Kyi, 1970, 1974), Cambodia (Eyles et al., 1963, 1964), Indonesia (Verdrager et al., 1975), Vietnam (Powell et al,, 1963), Malay Archipeligo (Montgomery and Eyles, 1963), and Thailand (Young et al., 1963; Scanlon and Sandhinand, 1965). The evidently copatric range of balabacensis and chloroquine resistant falciparum in Asia has been frequently noted (Verdrager et al., 1975, Scanlon et al., 1967), but whether this relationship is causal or coincidental has been neither tested nor resolved. Numerous biting catches, some quite elaborate, have been done; most notable is the 4 year study by Ismail (1974, 1975) and his colleagues in northern Thailand. Besides the human biting catches, which all show about the same pattern of a single, sharp nightly peak sometime between 2100 and 0200 hours, considerable effort has successfully proved that the leucosphyrus group are natural vectors of simian malaria among Asian monkeys (Cheong et al., 1965) and occasionally from monkey to man (Chin et al., 1965).

Although a number of investigators have been puzzled by aspects of balabacensis behavior that are related to its breeding habits, such as the disappearance of the mosquito from biting catches during the dry monsoon forest winter (Ismail, 1974), only one significant experiment has been done on breeding since those of Colless. This study, by Rajapakska (1971) demonstrated that balabacensis eggs remained viable on moist filter paper in covered petrie dishes for up to a month without hatching. He reported that after addition of water to these stranded eggs, an additional 2 days were needed for hatching to occur, thereby implying no advantage to a temporary pool breeder. As will be discussed later, we have tested Rajapakska's claim in the field with somewhat different results, which necessitate a much different interpretation.

Summary of Unpublished Work: Sylhet, Bangladesh 1975-1977

It is here necessary to discuss some findings from as yet unpublished recent work on balabacensis breeding done by us in Bangladesh. Both the experimental design of that investigation and the discoveries consequent of it are basic to the proposal made here.

As a collaborative effort of the Malaria Control Program and Johns Hopkins University, a field station was continuously maintained at Chaklapungee Tea Estate, Sylhet District, from March 1975 to November 1976. The site of our epidemiological and entomological study was Labor Line #1 (figure 2). This village, chosen for its relatively high balabacensis densities and malaria endemicity, is a cluster of 39 bamboo and thatch houses inhabited by 180 garden workers and their families and isolated from any other human habitation by 1 mile. All the houses in the village had been sprayed twice yearly with 2 gm DDT per sq. mile from 1966 to April 1975 as part of normal Malaria Eradication Program procedure.

Parasitology

Initially the entire population was censused. This census, which lists every individual by house, name, family relation, sex, and age, also recorded in each succeeding month his episodes of fever and chills, spleen size (for children) drug history, births, deaths, and nights spent away from the labor line. Every month a thick and thin blood film from nearly every resident was taken and supplemented quarterly by Indirect Hemagglutination Antibody titers for _falciparum_ and _vivax._ Those found positive were treated immediately with chloroquine, and, in the case of _vivax,_ with primaquine at conventional doses: 25 mg chloroquine phosphate/kg body weight to a maximum 1500 mg on a 3 day schedule and 15 mg primaquine/day for 14 days. Briefly, the parasitological survey revealed a semi-immune population holoendemic for malaria in the species ratio of approximately 6 cases of _falciparum_ to 4 of _vivax._ They acquired their infections while asleep within their houses. The first cases of chloroquine resistant _Plasmodium falciparum_ in Bangladesh were discovered by us in this village population (Rosenberg & Maheswary; Rosenberg et al.). Figure 3 gives a graphic representation of the monthly combined malaria prevalence.

During the period March, 1975 to November, 1976, 87.8% of the stabile population of 189 people were demonstrably positive for _falciparum_ for at least one month, 72.5% for _vivax,_ and 94.7% for one or the other. Those positive for _falciparum_ averaged 3.76 cases for the 21 months, and those positive for _vivax_ averaged 2.64 cases. This extremely high incidence is not surprising in light of the high potency of the vector. _Anopheles balabacensis_ was the most numerous biting anopheline and was, with the exception of one infective _An. annularis_, the only vector discovered in 2 years (Table 1). The 2 year gross infectivity rate for _balabacensis_ was 3.74%, one of the highest natural transmission rates for malaria recorded from anywhere in the world. As can be seen from Table 2, in the month of peak infectivity, August, 1976, nearly 5 infective bites were received by each man per week.

Breeding Dynamics and Biology of the Vector

For the study of breeding dynamics and dispersion, the site of the biting catches, Labor Line #1, was taken as the center of a series of concentric circles (Figure 2). The area of the largest of these circles, with a radius of two miles (12.56 sq. mi.), was thoroughly explored, much of it traversed on 50 yd. parallels, during both the wet and dry seasons, all surface water mapped and sampled, and all sources ever found positive for balabacensis checked at least once every three days. We are confident that no site of surface water in this area has escaped our attention. Additionally, we have made six extensive collections of water from tree holes (mostly less than 10' high), bamboo stumps and internodes, and various other natural and artificial container habitats. An. balabacensis has been found only in pools.

Anopheles balabacensis' adaptations to life in temporary pools is extensive and intriguing. A summary of our observations and experiments are:

• Fourth instar larvae are capable of surviving in nature on moist soil devoid of any standing water for at least 72 hours.

• Larvae are actively motile out of the water, progressing forward on a random path by persistltic motion, usually leave a puddle when there is no longer enough water to float in and in this way sometimes make their way successfully to nearby puddles. This seems a particularly useful survival mechanism since common breeding sites are waterfilled hoof prints around a small pool. Another effect of overland movement is to throw a thin film of mud over the advancing larva. An. balabacensis larvae suffer less from ant predation than any other species studied and possibly this mud coat confers some protection when coming to rest after an unsuccessful quest. Larvae have been found alive in nature as long as 96 hours after being stranded.

• Eggs survive in nature on moist earth for at least 15 days. At 15 days, 60% of eggs recovered were viable, hatching within 4 hours of adding water. Some data imply that balabacensis may oviposit directly on wet mud. Numbers of fully embryonated eggs resting on a muddy depression that is suddenly flooded by a monsoon shower explain both how it is that we have recovered adult balabacensis in emergence nets only five days after a previously drained pool was flooded, and the unusual synchrony between total numbers biting and percentage parous seen in the biting catches. Results of the biting catch for August, 1975 illustrate this latter phenomenon (Figure 4). An increase in biting density generally coincided with an increase in parous mosquitoes, and these increases came at regular intervals, about every 10 days. This phenomena can, we think, be best explained by assuming large numbers of stranded viable embryonated eggs hatched by the same flooding subsequently emerging, feeding, ovipositing and seeking a second blood meal as a group. Because of the inevitable compounding of irregularities, the cycle was seen to be considerably damped after several generations of uninterrupted breeding, fitting well with Lotka's (1945) equations for animal cycles.

• We have compiled life tables for the various instars of balabacensis in nature. Eclosion to adult averages slightly less than 6 days, requiring approximately 24 hrs. for each instar. The delayed development of some progeny from the same hatch, which has commonly been reported from laboratories, is not observed in nature. Most newly emerged males and females rest on vegetation or preferably wet pond walls around the breeding site. Of the 157 of those we have examined, more than 90% of the males have completely rotated terminalia, and all nulliparous females have been uninseminated. Since our laboratory observations indicate that balabacensis terminalia make a 180° rotation in 12-14 hours, this suggests that at the time of dispersal, all are sexually mature and that it is then, over the breeding site, that mating takes place.

• An. balabacensis 4th instar larvae are carnivorous. They are active scavengers of detritus and of dead and dying larvae and adult mosquitoes, both on the surface and on the bottom. They also prey on healthy 1st and 2nd instar mosquito larvae of other species (and possibly their own) as well as on various nematodes. Table 3 summarizes several experiments done to quantify their predatory behavior, using An. annularis and An. barbirostris as comparisons. An. balabacensis is only the third Anopheles and the first important malaria vector for which predatory behavior has been observed. Most anopheline larvae are passive feeders, and the significance of scavenging behavior may be to better exploit a limited environment and support rapid development.

• We have found as many as five species of mosquito larvae cohabiting in a puddle with balabacensis; and, as can be expected, the number of larvae of each species present before draining diminishes after draining proportionally to the length of time the pool stands without water. Although it is usually the least populous of the species represented before draining, balabacensis larvae are inevitably the only ones still alive after 48 hours. Should the water stand in a pool for a few weeks without draining, the predatory Culex (Lutzia) species often appear with devistating effect. It was common in our emergence trap studies to see relatively large and heretofore stabile populations of balabacensis so decimated by the introduction of Lutzia that all further emergence of balabacensis ceased, even though the continued deposition of balabacensis eggs could be demonstrated. Twenty-four hours complete drainage, however, would result in the complete destruction of the Lutzia larvae but none of the balabacensis eggs and only some of the larvae. It would seem than that not only is balabacensis well adapted to life in temporary pools, but that it derives certain definite benefits from their temporary nature, of which freedom from Lutzia predation is probably only one.

We have found that the nightly biting cycle of balabacensis is influenced by time of moonrise. During the periods of First Quarter and Full Moon, biting peaks both outdoors and indoors at 2100 hours with a second, smaller peak at 2400 hours. During the periods Last Quarter and New Moon, however, albeit the biting cycle curve is nearly identical to that of early moonrise, it is delayed by two hours, the first peak occurring at 2300, the second at 0100 hours. These findings throw doubt on the significance that has occasionally been attached by some investigators to "differences" in biting cycles from one region to another. Figure 5 graphically portrays this phenomena for June and July 1976, when we did four consecutive weeks all-night biting catches.

The effective lifetime flight range of balabacensis adults away from their breeding place appears to be less than 3/4 mile. The evidence for this is indirect: when the closest breeding to the village is 3/4 mile or more away, biting counts drop drastically. Numerous biting catches done by us in the forest and in other villages of known distances from breeding sites as well as the observations of

Colless and others support this deduction. Although considerable breeding was occurring 2 miles from the biting catch during the dry season, for instance, only 3 balabacensis were captured in 140 man-nights of catching during November, 1975 through March, 1976.

Parous females have been found resting around breeding sites only in the presence of newly emerged adults: none have been gravid, 2 of 8 examined have been infective. On six occasions, we have by chance collected parous females biting on man in the jungle as late as 1130 hours, always near a breeding place. This is well after local people have entered the jungle to work, and suggests minor daylight transmission in the forest.

We have discovered that balabacensis survives the winter by breeding in the rare perennial springs and streams found in deep forest. Within our 12-1/2 sq. mi. area of study there is only one such "primary breeding site": a series of springs that give rise to a small stream that marks the boundary between India and Bangladesh. It is located in dense jungle and is the only place where we consistently find monkeys (Presbytis obscuris). Anopheles balabacensis has been found in still portions of the stream itself and more frequently in stabile, black-colored seepage pools along its sides.

After the rains begin in May, the mosquito disperses outwards to take advantage of two types of breeding places that hold water only during the monsoon: one type is in turbulence pits at the heads of the sandy gulleys that traverse the valleys where deep pools of rainwater runoff can accumulate; and the second is in the compacted topsoil of foot paths where shallow puddles are formed. In our area there are only these two types of "secondary" breeding places -- their nature being determined by the hilly topography and the low clay content of the soil. The hills limit the area where water can accumulate and increase run off, while the low clay content (12-25%) of the friable sandy loam found throughout the tea growing region of Sylhet is responsible for rapid infiltration (or vertical drainage) by standing water. Infiltration rates, as measured by the Cylinder Infiltrometer Method (USDA, 1956), averaged 6-1/4" per 24 hours for sandy gully bottom. Consequently, to allow for uninterrupted development from stranded, embryonated eggs to adults would require a depression capable of holding at least 1 meter of water. In the case of heavily traveled footpaths that cross natural depressions, sufficient compaction of the low clay content to form a pan nearly impermeable to water may occur. In these cases infiltration and evaporation rates combined average 1/2"/day, thereby requiring accumulations of only 3" to allow complete development. Furthermore, such puddles benefit from rainfalls of less than 1/2" that would not appreciably lengthen the life of gulley breeding places.

While the location of breeding sites is strictly limited to specific topographic features, the numbers are not totally static. Gulley heads are, for instance, susceptible to filling in by sand after heavy rains if the upstream drainage area has been recently cleared of vegetation. Increased traffic may form new sites on foot paths, but if too heavy it appears to disrupt breeding. In 1975 there were a total of 7 secondary breeding places within a mile radius of the biting catch site; in 1976 one of these sites no longer supported breeding while one new site was monitored, again totaling 7.

We conjecture then the following seasonal dispersion pattern: During the dry winter season, small numbers of balabacensis actively breed in widely spaced primary sources in the forest, obtaining their blood meals from primates (most often monkeys) that also find these water sources attractive. Anopheles balabacensis seldom travels more than 3/4 mile from these winter breeding places and those that do stray farther will be unlikely to reproduce because of the scarcity of surface water. At the beginning of the monsoon a number of secondary breeding places will become available. Those nearest the primary source will be first utilized, and, considering the rapid development of balabacensis immatures, farther secondary sources will be seeded quickly along the circumferences of incrementally larger concentric circles whose center is the primary source. Secondaries closest to human settlement will support the heaviest breeding, while otherwise suitable sites in the forest that are not within this 3/4 mile diameter will be rarely utilized for want of suitable hosts (the monkey population in this area is a migratory troop of 15-20). It must be kept in mind that balabacensis is a relatively rare forest mosquito that reaches dangerously high densities only around sites of human activity at the forest edge. It is this phenomena that makes the concept of treating only a limited area around a village at risk logical and feasible.

Experimental Design and Methods

The design and methods, with exception of larviciding procedures, are essentially the same as those used in the base line study of 1975-77.

In March, 1978 the one mile radius area will be resurveyed for balabacensis breeding places, the population census will be updated, and monthly blood film and mosquito collections recommenced. The biting catches in 1978 will be limited to 7 consecutive days per month and during the April-October transmission season only. All biting catches will be done in the unsprayed house already constructed on the labor line. Except for window trap provisions and interior screen baffles under the eaves to minimize escape of missed feeding mosquitoes, the house is of the same material and dimensions as those inhabited by the garden workers. The interior of this house will not be sprayed with DDT, although the rest of the houses on the labor line will receive a single 2 gm/sq m coat in April. Biting catches will, for the sake of uniformity and comparison, begin at the full moon each month.

Man biting catches are made by 2 men armed with flashlights and aspirators, seated on stools facing each other inside the house and a second pair similarly equipped seated outdoors 15' away under a 6' x 6' tarpaulin. Catching teams work from a clock set at 1800 hours for that day's sunset, as determined from the 1978 Nautical Almanac for 20 degrees north latitude. Two teams of 4 men are used each night: one works from 1800 to midnight, the second from midnight until 0600. Indoor and outdoor teams are rotated on the hour. A night's biting catch consists of 12 episodes of 45 minutes uninterrupted catching each. Each episode begins on the hour and all mosquitoes resting on or biting either member of the pairs are collected. The legs, feet, arms and heads of the collectors are left bare. Each station uses 2 paper cup cages per episode: one for culicine mosquitoes collected and one for anopheline. In this way 48 separate cages are filled and accumulated during each night's catch. Cages are labeled for hour and station and stored in humidified buckets until the next morning when all <u>Anopheles</u> are identified and ovaries, spermathaeca, midguts and salivary glands are dissected out and examined by a team of two technicians for parity, fertilization, infection and infectivity, respectively. Findings are recorded for individual mosquitoes in a format developed by us for the base line study. The 15 minutes of each catching hour not devoted to catching is used to record, on site, previous hours rainfall, wind direction and velocity, percentage cloud cover, and wet and dry bulb temperatures. (This data is also collected every 4 hours during non-collection periods.) Also during this intermission window traps and any light traps operating elsewhere in the village are emptied. This parasitological monitoring activity will require the full collection team's living on site for 2 weeks per month.

The mosquito control phase of the experiment will require the twice weekly activity of two men -- one to spray and one to supervise -- from 1 April through 31 October 1978. On the same 2 days of every week (e.g. Mondays and Thursdays) this team will visit each of the breeding places marked on the map. The area of their responsibility will be bounded by the 1 mile radius, or 3.14 square miles. Just prior to each treatment each breeding site will be examined and survivorship from the previous treatment will be determined by dipping and sieving. The number and identity of any eggs, larvae, or pupae found will be recorded and samples preserved. Should a site be drained, it will be so marked, but because of the viability of stranded eggs, the exposed mud bottom will nevertheless be sprayed in anticipation of the next rain and hatching. Twice weekly applications may be excessive, however, since the object of the study is to determine if transmission can be broken by interrupting breeding in only a few pools, the certainty provided by such overkill is necessary.

Evaluation:

Efficacy of the larval control program should affect all the following changes: reduction in numbers of total biting balabacensis, reduction of percentage biting which are infected, infective and nulliparous, and reduction in human malaria incidence. All of these indices are directly responsive to increased larval mortality, and, with the exception of human incidence, monthly biting catches and dissections are immediatel sensitive indicators. Reduction of incidence will be monitored in this study by gradual reduction in monthly prevalence. Because of delayed prepatent periods in semi immunes, chloroquine resistant falciparum, and the sporodic appearance in the peripheral blood of parasites in chronic infections, case detection and radical therapy are often delayed, causing this indicator to lag behind the others.

Facilities, Resources and Personnel

Field operations will all be carried out at Chaklapungee Tea Estate, Chunaraghe Police Station, Habiganj Thana, Sylhet District. Planning of day-to-day specifics, logistics, and field operations will be the responsibility of the MCP Senior Entomologist, N. P. Maheswary, who is headquartered in Dacca, but will reside with the catching team at Chaklapungee during field operations. Mr. Maheswary collaborated on the original study as well as on this protocol, and his future duties will be similar to what they have been. He has been with the malaria program since its inception in 1962; is a graduate of Presidency College, Calcutta, of the University of Dacca, and of the University of Florida, from which he obtained a M. S. in entomology while on an A. I. D. fellowship. His position as head of all entomological research and evaluation for MCP gives him sufficient institutional latitide to make necessary on-the-sp changes in operations or personnel with approval needed from only the MCP Director himself.

Field operations require:

1) Approximately 15 lower level employees of MCP with the designations of "Entomology technicians" and "Insect Collectors", including those named in appendix

Most of these men, who are stationed in various parts of the country when not at Chaklapungee, have been active in the research from the start, and represent those chosen over 2 years for exemplary work and intelligence. One of these men whose home is near Chaklapungee will act as larvicide spray supervisor.

2) Initial staining and examination of blood films is done the day after collection at the Thana MCP office at Habiganj, about 20 miles distant. Results of this examination are used for immediate therapy. All slides are then rechecked at the National Headquarters after return to Dacca. In Dacca, problems of parasite identification can be referred to the Senior Parasitologist.

3) Housing for the staff, field laboratory, and insectary will be provided by the management of the Tea Garden. Medical advice is rendered when needed by the resident physician of a neighboring British-owned garden.

4) Transport, a land Rover and driver, will be provided by MCP. In the past, when mechanical breakdowns or seizure by the military have threatened delays, a Johns Hopkins vehicle was used. This will not be available in the future and the possibility of assuring a back-up vehicle from another source, possibly by rental from an agency such as the AID mission, is important.

5) Statistical analysis and interpretation will be done at the Johns Hopkins School of Hygiene and Public Health by R. Rosenberg, who will also act as overseas consultant on the project. His duties will include one 4-6 week visit to Bangladesh in March, 1978 to set up the trial and to see that it functions properly, and again in October, 1978 to collect the data and discuss the findings with MCP. His curriculum vitae is in Appendix I.

Previous Funding:

From March, 1975 to October, 1977, approximately half the costs of research were borne by funds allocated by NIH grant RO7-AI-10048 to the Johns Hopkins International Center for Medical Research. The remaining half were from MCP. Additional funds in the form of a stipend to R. Rosenberg were from NIH Training Grant in Parasitology and Medical Entomology, 5TO1-AI-100020. Because the JHU-ICMR will, beginning in 1977, be devoted only to diarrheal and malnutritional diseases, replacement of the research funds are being sought from AID.

Approval of the Bangladesh Government:

Preliminary approval was given to the proposal by the advisor to the President for Health (Appendix II) and the basic program, operating under initial formal agreement with the government and supported by Johns Hopkins funds, is still in progress and will continue to 31 October 1977.

Budgetary Estimates:

The estimated total funds required from AID for the one year program proposed is $ 14, 540. 00. Only funding for one year, 1 January - 31 December 1978, is estimated since the nature of any subsequent work will be dependent on the degree and type of success of the initial year.

Cost sharing:

It is expected that the Bangladesh Malaria Control Program will provide adequate trained personnel (as listed below), their normal base salaries; a vehicle for transport in and to and from the field station; facilities, parts and mechanics for major vehicle repairs; examination of blood films in Habiganj and Dacca; and backpack spray applic and other minor equipment used in their normal routine (e. g. chloroquine, primaquine stools, aspirators). From the allocated AID funds will be payed the normal gazetted per diem for every man below the rank of "entomologist" plus an additional 10 taka/day hardship and food expense; all petrol, motor oil, tire repair costs; all ferry and train fares; all costs of expendible items needed (insecticide, torchlight batteries, tarpaulins, wire and cloth netting, etc.); all specially required non-expendible equipment (e. g. light traps); all necessary medical and drug expense of the subject population other than chloroquine and primaquine (e. g. Fansidar, antibiotics, emerg hospitalization); and all costs of day labor and refurbishment needed at the field sta A limited emergency fund will be available for transport rental and other unforeseen exceptional needs.

Detailed Budget[6]

a) Salaries:

Spray laborer	64 days at $1.50/day	$ 96.00
Daily paid labor, misc.		50.00

b) Consultants: 00

c) Per diem and local travel:

N. P. Maheswary[1]		00
R. Rosenberg	90 days at $30/day	2,700.00
A. K. Das, chief technician	112 days at $2/day	224.00
14 men, biting catch team[2]	112 days at $1.75/day/man	2,744.00
Spray supervisor	64 days at $1.50/day	96.00

d) Travel.

international, R. Rosenberg		
Baltimore-Dacca, return; March 1978		2,000.00
Baltimore-Dacca, return; October 1978		2,000.00
local		
gasoline[3]	\$2.50/gallon at 50 gallons/month	1,000.00
ferry	\$2.00/month	16.00
motor oil and tires	\$5.00/month	40.00
misc. repair, parts		250.00
emergency vehicle rental		300.00

e) Non-expendible equipment

flashlights	6 at \$5.00 each	30.00
motorcycle batteries	2 at \$20.00 each	40.00
fluourescent light trap and battery		123.00
misc. (e.g. mignifying glasses, enamel pans)		250.00

f) Expendible supplies

dry cell flash light batteries	10 dozen at \$5/dozen	50.00
wheat and grinding[4]	\$15/month	120.00
site preparation (e.g. experimental house repair)		50.00
misc. (e.g. paper cups, rubber bands, netting)		150.00

g) Publication costs

typing	20 hours at \$5/hour	100.00
reprints		25.00

h) Shipping, postage, and customs	300.00
i) Indirect costs[5]	1,786.00
TOTAL	$14,540.00

Notes:

1. The per diem for Mr. Maheswary and other personnel at the rank of entomologist or above will be borne by MCP.
2. Biting catch team consists of 12 men in the MCP categories "entomology technician" and "insect collector", one driver, and one cook-sweeper.
3. Current price is about $2.00/gallon but an increase is anticipated.
4. A small quantity of flour is given to each person contributing a monthly blood specimen.
5. Indirect costs to Johns Hopkins School of Hygiene and Public Health for "sponsored training off campus" are 14% of sub total at (h), $12,754.00.
6. Budget for eight months.

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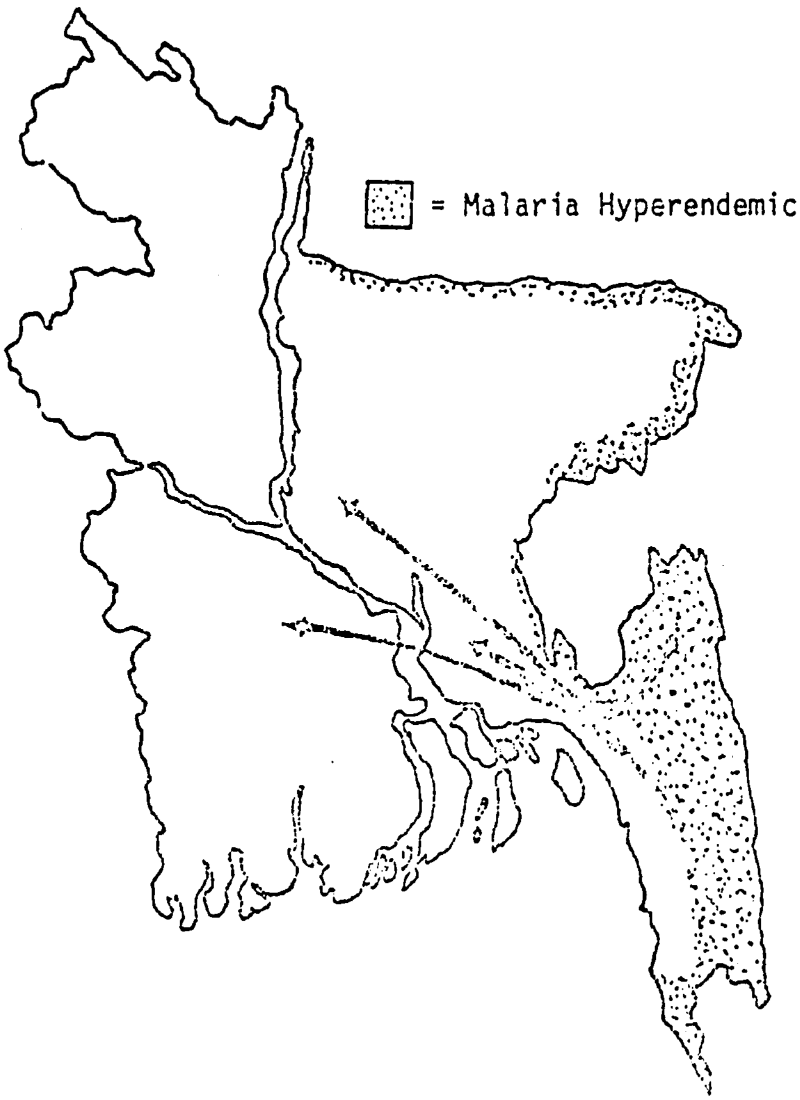

Figure 1

HUMAN MIGRATION IN BANGLADESH

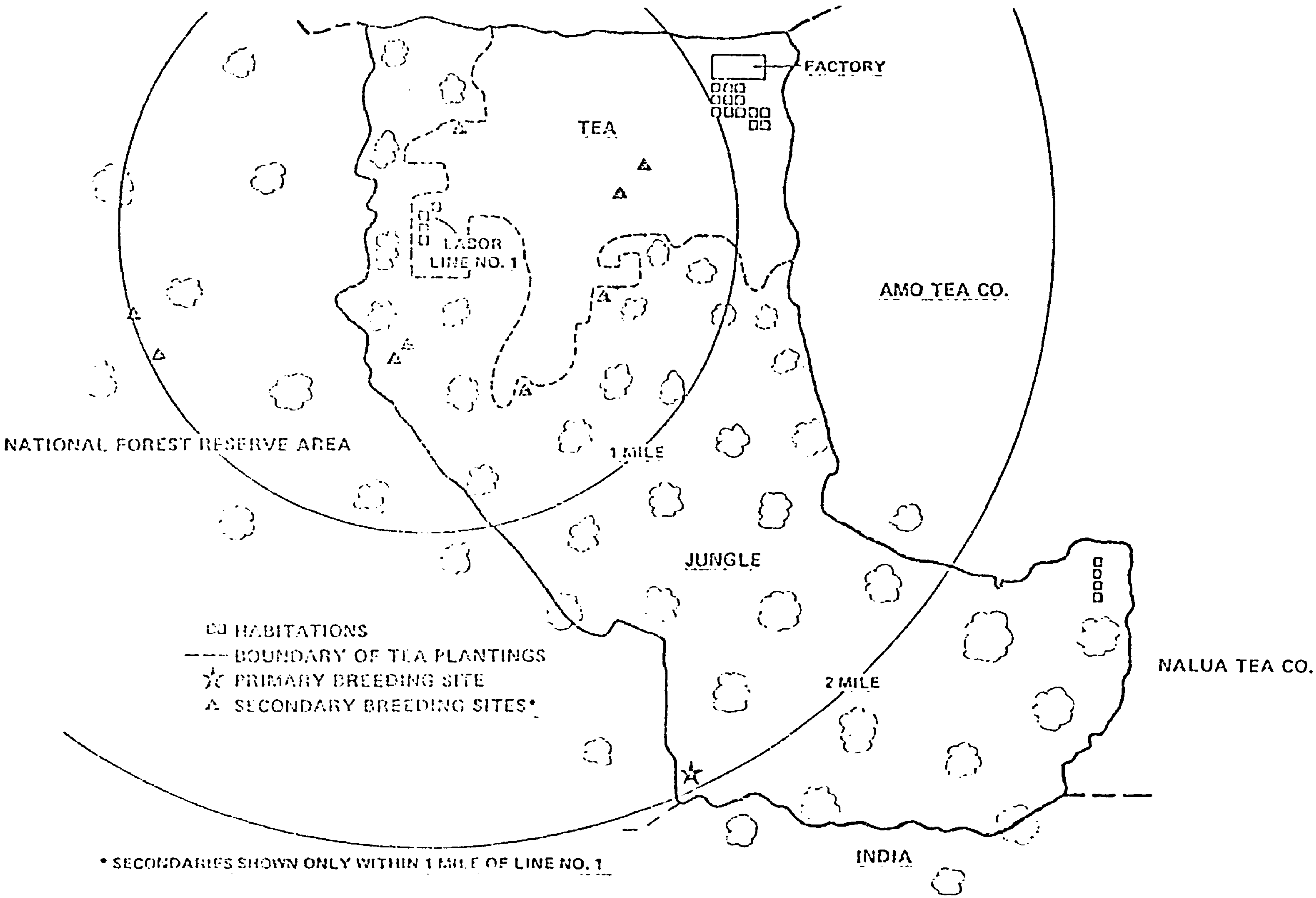

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Figure 2: MAP OF CHAKLAPUNJI TEA ESTATE SHOWING BREEDING SITES OF *An. balabacensis*

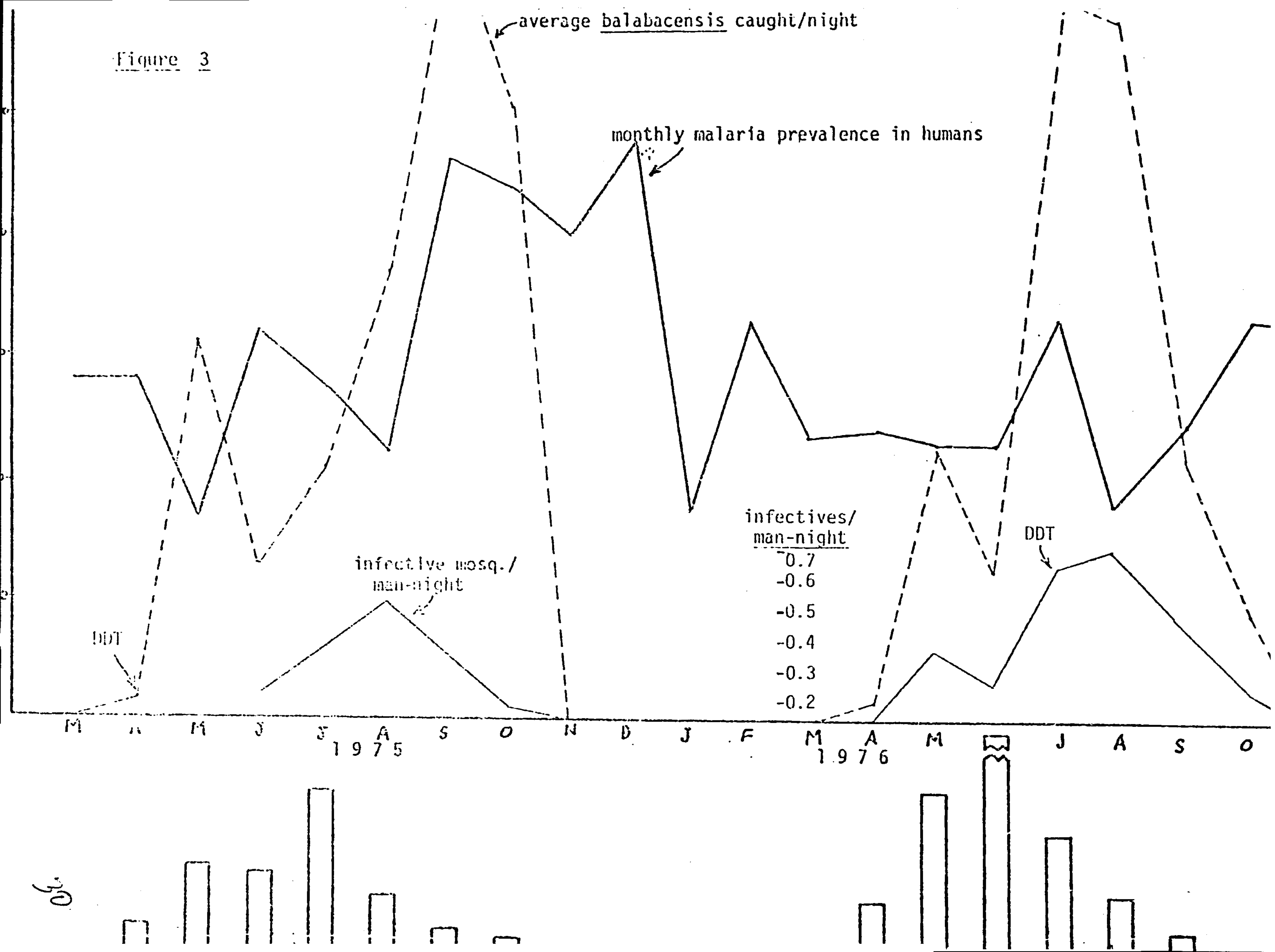


Figure 3
average balabacensis caught/night
monthly malaria prevalence in humans
infectives/
man-night
0.7
-0.6
-0.5
-0.4
-0.3
-0.2
infective mosq./
man-night
DDT
DDT
M
A
M
J
J
A
S
O
N
D
J
F
M
A
M
J
J
A
S
O
1 9 7 5
1 9 7 6

Table 1:

Parous Anopheline Gland Dissections

(17 month's totals)

Species	Number Glands Dissected	Number Glands Positive	Percent Infective
aconitus	12		
annularis	73	1	1.29
balabacensis	2446	130	5.31
barbirostris group	1		
hyrcanus group	26		
jamesii	3		
jeyporiensis c.	20		
karwari	30		
kochi	2		
maculatus	67		
minimus	4		
philippinensis	176		
tesselatus	15		
vagus	36		

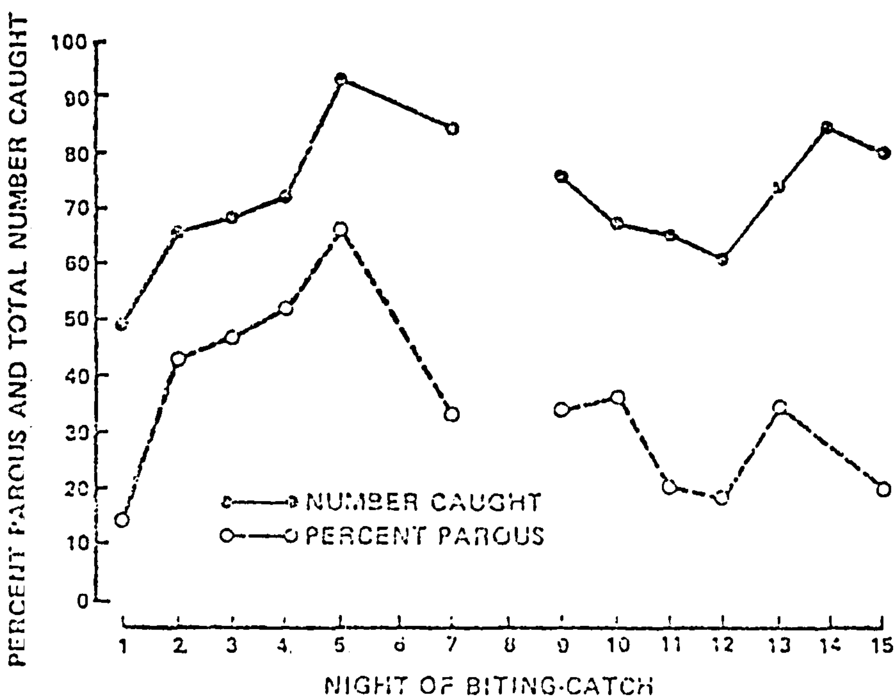
Table 2:

Monthly Infectivity Rates: Man-biting *Anopheles balabacensis*

Month & Year	Total caught	% parous	% of total infective	% of parous infective
1975				
March	0*	0	0	0
April	8*	-	-	-
May	94*	-	-	-
June	90	70.3	2.70	3.85
July	294	71.9	7.54	9.15
August	525	74.6	6.67	8.94
September	911	56.6	1.73	3.06
October	703	40.2	0.43	1.07
November	0	0	0	0
December	0	0	0	0
1976				
January	1*	0	0	0
February	0*	0	0	0
March	1*	0	0	0
April	26	65.4	3.80	6.00
May	330	82.4	4.90	5.88
June	94	54.2	4.25	7.84
July	957	69.3	3.76	6.24
August	811	66.1	4.90	7.5
September	154*	73.4	7.12	9.7
October	129	66.7	6.20	9.3
November	0	0	0	0
TOTALS	5,128	61.8	3.74	6.05

results of only seven all-night biting catches

Figure 4:



NIGHTLY BITING CATCH AND PERCENT PAROUS, *An. balabacensis.* AUGUST, 1975

TABLE: 3

Predation by *An. balabacensis* larvae

No. of live first instar *An. vagus* larvae surviving in presence of 5 fourth instar* larvae of:

Hours	*balabacensis*	*barbirostris*	*annularis*
0	20	20	20
4	11.2	20	20
8	9.0	20	20
18	8.0	20	19.25
24	6.7	20	19.25
48	2.7	20	19.0

Average of 4 trials; each was a set of the three 4th instar species done simultaneously under identical conditions in 3 1½" diameter stendor dishes half filled with water.

Appendix I

PERSONNEL

Proposed MCP personnel for the larvicide field trial, Chaklapungee, Sylhet

Name		Post	Time devoted to project during 8 mo
N. P. Maheswary	Dacca	Senior Entomologist	50%
Aniel K. Das	Dacca	Chief Entomology Technician	50
Shamsuddin Ahmed	Habiganj	Entomology Technician	50
Shajahan Ahmed	Comilla	" "	50
Serajul Islam	Dacca	" "	50
Milon K. Dey	Rangamati	" "	50
Abdul Mannan	Cox's Bazaar	" "	50
Subash Roy	Mymonsingh	" "	50
Raziuddin Ahmed	Dacca	" "	50
Milon P. Kor	Cox's Bazaar	Insect Collector	50
Ali Akbar	Comilla	" "	50
Ramproshad	Ramganga T. E.	Malaria Supervisor	25

No specific recommendations are made for the remaining positions of 1 insect collector, 1 driver, and 1 cook.

CURRICULUM VITAE

Ronald M. Rosenberg

Personal Data

Date of birth: [redacted]
Place of birth:
Citizenship: United States
Marital status: Single

Education

1965-1969 — A. B., Zoology, Washington University, St. Louis

1972 - date — Sc.D. Candidate, entomology and parasitology, Department of Pathobiology, The Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland
Advisers: 1972-73 - G. A. Schad, Ph.D.
1973-74 - F. B. Bang, M. D.
1974-date - M. Trpis, Ph.D.

Honors:

National Merit Scholar, 1965
Phi Beta Kappa

Recent Employment

1969 - 1972 - Research Officer for Soils and Horticulture, Tonga Department of Agriculture, Nuku'alofa, Tonga (Peace Corps)

1973 - 1975 - Research Assistant, Department of Pathobiology, Johns Hopkins University School of Hygiene and Public Health, and Johns Hopkins International Center for Medical Research, Dacca, Bangladesh.

Publications

Rosenberg, R., Alam, A. K. M. J., Alamgir S. M., Brown, K. H. 1977. Chloroquine resistant Falciparum malaria in Dacca: the first 2 case reports from Bangladesh. Bangladesh Medical Journal (in press).

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Rosenberg, R., Maheswary, N. S. Epidemiology of chloroquine resistant *P. falciparum* in a Bangladesh tea garden: Part II Breeding dynamics of *Anopheles balabacensis* (in preparation).

Rosenberg, R. Predation by *Anopheles balabacensis* larvae and its possible evolutionary significance (in preparation)

__________ Terrestial motility by *Anopheles balabacensis* larvae: its importance as survival mechanism (in preparation)

__________ A new species of *Toxorhynchites* from Bangladesh. Mosquito Systematics (in preparation).

Peyton, E. L. and Rosenberg, R. A revised checklist of the mosquitos of Bangladesh. Mosquito Systematics (in preparation).

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__________ 1971. A reconnaissance soil survey of Niua Foou Island. Department of Agriculture, Tonga. (38 pp. & maps)

PROCEEDING OF THE MEETING HELD ON 7/8/75 AT 9 A.M. IN THE OFFICE OF THE HON'BLE ADVISOR, MINISTRY OF HEALTH, LABOUR AND SOCIAL WELFARE

The following were present:

1. Col. (Retd.) M. M. Haque,
   Member, Advisory Council to the President
2. Mr. J. Toner,
   Chief, USAID Mission, Dacca.
3. Dr. Sung Soo Han,
   WHO Representative to Bangladesh, Dacca.
4. Dr. M. Sathianathan,
   WHO., Dacca
5. Dr. Ibnoe Soontarman,
   WHO., Dacca.
6. Mr. R. Rosenberg,
   Johns Hopkins University, Dacca.
7. Dr. A. M. Mustaqul Huq,
   Director of Health Services (Preventive),
8. Dr. Mahboober Rahman,
   Project Director,
   Malaria Eradication Programme.
9. Mr. N. P. Maheswary,
   Senior Entomologist,
   MEP Bangladesh.

The following decisions were taken;

1. The Malaria Eradication Programme to implement the six month action programme for intensive anti-malaria efforts already developed.

2. To persue efforts in making the DDT Factory in Chittagong operational.

3. To persue efforts in getting 200 to 300 metric tons of DDT from Pakistan.

4. To initiate negotiations with USAID to obtain additional requirements for anti-malaria programme pending the joint

5. Dr. Han, WHO Representative stressed the importance of considering the malaria situation as a national programme. He emphasised the need to maintain the present malaria structure till the appraisal of malaria situation by a joint review team in October, 1976 is completed and concrete recommendations are made. The six month emergency action plan will be implemented by the present malaria structure.

This was accepted.

6. Routine Malaria Eradication activities will be carried out in those areas where malaria is not a problem i.e. the white areas in the map enclosed. In the areas where malaria is now considered as a problem i.e. the read and blue areas in the map, the following additional measures will be carried out.

6.1. Drug:

The drug Chloroquine/Amodiaquine will be made available in all the villages and all the fever cases will be given radical treatment with the drugs. If the cases got fever again or if the first follow-up of the cases is positive, the patient will be given a second course of Chloroquine/Amodiaquine. If P.falciparum resistance is identified or if second follow-up is positive, then the alte nate drug Fansidar will be used. The use of Fansidar will be limited only to problem cases to avoid development of resistance to this drug.

6.2. Insecticide:

When DDT becomes available the spraying will be carried out along the 5 mile areas starting from the western border of the red (hyper endemic) areas and the spraying will be proceeded towards the eastern side when DDT becomes more available. In every limited areas where Anopheles balabacensis is a problem and is feared that DDT may not be fully effective, supplementary measures will be considered.

6.3.But before using larvicide in a general way it will be necessary to study its efficiency in limited areas. The use of ULV malathion may be tried in a very limited areas ~~--the use of ULV~~

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School of Hygiene & Public Health
The Johns Hopkins University
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Baltimore, Maryland 21205 U. S. A.

Control of *Anopheles balabacensis* by Larviciding

Ronald Rosenberg, Sc. D. candidate
N. P. Maheswary, Senior Entomologist,
Malaria Control Program of Bangladesh

submitted 9 June 1977 valid to 31 December 1978

duration of project: 1 March 1978 - 28 February 1979

signature of Principal Co-Investigator ______ Milan Trpis, Ph. D.

signature of Principal Investigator ______ Ronald Rosenberg

signature of Department Chairman (for contracting organization) ______ F. B. Bang, M. D.

Human Experimentation Plan for this proposal was approved for renewal by the Johns Hopkins Committee on Human Volunteers on 1 June 1977.

This proposal has been submitted in part for funding to the Malaria Section, Southeast Asia Regional Office, World Health Organization on 20 January 1977.

Unpublished original research is included herein for purposes of proposal evaluation only; any other use of these data requires specific permission of the principal investigator.

APPROVED:

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