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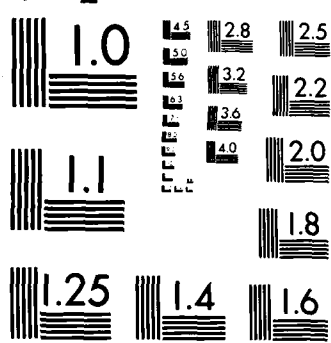
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REPORT NUMBER 1

REGULATION OF SALIVARY OUTPUT BY MOSQUITOES

ANNUAL PROGRESS REPORT 1981-82

Andrew Spielman
Philippe A. Rossignol

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19. ABSTRACT (Continue on reverse if necessary and identify by block number) A major scientific advance was registered during the present reporting period when we discovered that the saliva of mosquitoes mainly functions during blood-feeding by preventing platelets from aggregating. Salivary-ablated mosquitoes probe extensively, but without feeding. The salivary enzyme mainly responsible for this antihemostatic effect was identified as an apyrase, which may partially be conserved during sugar-feeding, but selectively released when hosts are probed. Resynthesis, however, is rapid. Chemosensory factors regulate salivary secretion. Salivarian pathogens, such as malaria sporozoites, impair the ability of the salivary glands to secrete apyrase, thereby increasing the ability of infected mosquitoes to transmit the infection. In addition, we discovered that these salivarian pathogens require a day or more to pass from the salivary acini to the lumen of the duct, and this suggests that the extrinsic incubation period of these organisms may be longer than previously thought. Taken together, these discoveries have greatly enhanced our understanding of the epidemiology of arboviral and other vector-borne infection.					
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SUMMARY

In order to ascribe a blood-feeding function to the saliva of mosquitoes, we determined whether this secretion may limit the initial probing phase of biting behavior. Indeed, probing of hosts was prolonged when the salivary ducts were severed, but this prolongation was absent when mosquitoes fed on heparinized blood contained beneath membranes. Once feeding began, duct-ablated and duct-intact mosquitoes fed similarly. In vitro, turbidimetric assays demonstrated that saliva inhibits aggregation of platelets. ADP was hydrolyzed by saliva, and this apyrase activity explains, in part, the observed effect upon platelets. We conclude that the saliva of mosquitoes functions by facilitating location of blood vessels.

Mosquito saliva aids in dissolving solid sugars, and in taking a bloodmeal. Salivary apyrase mediates host hemostasis during blood feeding by mosquitoes, but appears unnecessary for sugar feeding, since apyrase activity is absent in male mosquitoes. We determined whether or not female Aedes aegypti may selectively deplete apyrase levels during a sugar meal. Apyrase activity remains constant in salivary glands of sugar fed mosquitoes, but is depressed immediately following blood feeding. Apyrase is rapidly resynthesized, however, suggesting that sugar fed mosquitoes may retain the ability to blood feed by controlling the loss of apyrase activity to a level equal that of the rate of resynthesis.

Salivation by mosquitoes may be directly regulated by chemical, thermal, or tactile factors, or indirectly by factors inherent to the feeding process. Accordingly, we determined whether or not chemosensation may modulate salivation in Aedes aegypti. Exposure of mouthparts to 2M

sucrose stimulates salivation, a response that did not vary with ambient temperature or age of mosquito. Lower concentrations were nonstimulatory. The concentration of ATP optimal for inducing salivation in serum-exposed mosquitoes was 0.001M, identical with that found to stimulate ingestion of serum.

Because malaria sporozoites destroy segments of the salivary glands of vector mosquitoes, we determined whether the probing phase of feeding behavior may be impaired. Indeed, non-infected mosquitoes located blood-vessels more rapidly than did either sporozoite-infected or saliva-deprived mosquitoes. Salivary apyrase activity is reduced following maturation of sporozoites. Apyrase activity, normally, is confined to those regions invaded by sporozoites. Sporozoite-infected and non-infected mosquitoes produced equal volumes of saliva. We conclude that sporozoite infection impairs the vector's ability to locate blood vessels by affecting the quality of salivary product and thereby increasing potentially infective host contacts.

Estimates of extrinsic incubation period and sporozoite rates are made by dissection of mosquito salivary glands. Such calculations ignore possible barriers to sporozoite migration out of the salivary glands. We analyzed the time course of sporozoite output in Aedes aegypti infected with Plasmodium gallinaceum. While virtually all experimental mosquitoes were infected upon salivary gland dissection, only about 80% ejected sporozoites upon oil simulation. Furthermore, at least one day separated appearance of sporozoites in salivary glands from appearance in saliva. This previously

unmeasured aspect of vector competence may skew estimates of basic reproduction rate for malaria.

FOREWARD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsements or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. [NIH] 78-23, Revised 1978).



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REPORTS

I. Role of mosquito saliva in blood vessel location

Although the saliva of mosquitoes provides the all-important vehicle for transmission of pathogens to vertebrate hosts, its pharmacological function in blood feeding has not conclusively been established. Without doubt, it serves as a solubilizer for solid sugar (Eliason, 1963), a role that would contribute to ingestion of honeydew in nature. Less persuasive evidence ascribes some anticoagulant activity which may assist blood ingestion (Yorke and Macfie, 1924; Metcalf, 1945; Hudson, 1964), but blood ingestion proceeds normally in mosquitoes surgically deprived of saliva (Hudson et al., 1964; Mellink and van den Bovenkamp, 1981; Rossignol and Spielman, 1982).

Observations on movements of mosquito mouthparts within living tissue provide a basis for assigning salivary function. After penetrating skin, mosquitoes thrust their stylets back and forth for several seconds before locating a blood vessel and beginning to feed. During this probing phase, mosquitoes salivate copiously (Griffiths and Gordon, 1952), an activity that is difficult to interpret in terms of blood-ingestion. Such extravascular secretion could not inhibit coagulation of ingested blood, and this creates a paradox.

It may be that saliva of mosquitoes mainly contributes to vessel location, a hypothesis that has not yet been examined. Accordingly, we determined whether saliva influences the ability of mosquitoes to locate blood vessels, thus facilitating the early probing phase of biting behavior.

A. Materials and Methods

A strain of Aedes aegypti, originating from material collected in 1969 on Grand Bahama Island, was used throughout this work.

Disodium salts of ADP from equine muscle (99% pure) were obtained from Sigma Chemical Co. (U.S.A.). Collagen (2%) suspension in saline (Collagen type II, Sigma) was prepared from homogenized bovine achilles tendon and centrifuged at 1,000 g for 5 minutes. The supernatant was stored at 4°C. All other chemicals were of analytical grade. Distilled and deionized water was used throughout.

To obtain platelets, blood (9 ml) from a healthy human volunteer was collected in plastic syringes containing 1 ml of 3.8% sodium citrate. Blood was centrifuged at 200 g for 15 minutes. The resulting platelet-rich plasma was collected and used within 5 hours.

Rearing of mosquitoes and surgical procedures used were those described by Rossignol and Spielman (1982).

Measurement of probing time was accomplished in the following manner. Guinea pigs were anesthetized with Nembutal and Ketamine administered intraperitoneally. We presented individual mosquitoes to guinea pigs in small (1.5 x 3 x 6 cm) cages covered with nylon net (8 mesh) and recorded duration of time between stylet penetration and the appearance of blood in the gut as observed through the thin abdominal pleura. When mosquitoes withdrew their mouthparts before taking blood, timing began anew following subsequent penetration. Mosquitoes were observed until 420 seconds had

elapsed, but those which ceased probing before this time were discarded, provided that no blood was ingested. Each insect was used only once.

For artificial feeding, insects were exposed to a feeder containing a warm diet (150 mM NaCl, 1 mM ATP, and phenol red added for color) and covered by a Boudrouche membrane.

In all cases, ducted and sham-operated insects were observed alternately in order to minimize possible bias.

Saliva was collected in oil in a device modified from that of Rossignol and Spielman (1982). Mosquitoes were chilled on wet ice for 20 seconds and their legs and wings removed. Using a dissecting microscope, the stylets were exposed and placed in a section of PVC tubing (I.D. 0.28 mm) filled with ca 0.5 ul of mineral oil. As described for Calliphora (Hansen Bay, 1978), we observed that serotonin induces salivation by Aedes aegypti (unpublished). Thus, 0.1 ul of a serotonin solution (20 mM) in saline was injected into the hemocoel. Mosquitoes were then allowed to salivate for 10-15 minutes and the tubes were emptied into 1.5 ml conical plastic tubes. Samples obtained from 25 mosquitoes were pooled and stored at -70°C until use. Before assay, 25 ul of 0.15 M NaCl was added per sample (1 ul for each mosquito). Following shaking, the tube was centrifuged for 1 min at 10,000g. The resulting water layer was used in the assays.

A turbidimetric method for measuring platelet aggregation was used (Born and Cross, 1963), employing siliconized glass cuvettes. The transmittance of the suspension at 550 nm was recorded during continuous stirring at 37°C.

Apyrase activity was assayed by measuring the release of inorganic phosphate from ADP and ATP (Fiske and Subbarow, 1925). One milliunit of activity is defined as the amount of enzyme that releases one nanomole of inorganic phosphate per minute from a defined standard medium held at 30°C.

B. Results

First we determined whether saliva serves to shorten probing time and whether probing aids in locating blood vessels. Duct-sectioned (saliva-deprived) and neck-pierced mosquitoes were placed on the shaved back or ear of an anesthetized guinea pig. We recorded the duration of time between initial penetration of skin and appearance of blood in the midgut, as gauged visually under 3x magnification. Both on the ear and on the back of a guinea pig, probing time of saliva-deprived mosquitoes was prolonged (Figure 1a). In addition, mosquitoes feeding on sparsely vascularized skin (on the back) probed longer than did mosquitoes feeding on the ear. These experiments demonstrate the usefulness of our measure of probing time in describing the duration of blood vessel location, and prove that presence of saliva reduces duration of probing.

Because saliva seems most important when mosquitoes attempt to feed on sparsely vascularized skin, it would follow that saliva might not facilitate feeding from a pool of medium. Accordingly, we measured probing time of duct-sectioned and neck-pierced mosquitoes placed over a membrane-covered feeding apparatus containing a dyed solution of normal saline and 1 mM ATP (Figure 1b). Probing time was brief and nearly identical in both treatment

groups, thereby supporting the hypothesis that saliva is important to mosquitoes mainly when blood vessels are sparse.

In seeking an explanation for this finding, we suggested that saliva may assist mosquitoes in placing their mouthparts by promoting hematoma formation around lacerated blood vessels. Platelet aggregation provides the main limit for such hemorrhage (Mustard and Packham, 1977). Accordingly, we sought evidence that saliva of mosquitoes may inhibit platelet aggregation. The effect of mosquito saliva on ADP and on collagen-triggered platelet aggregation was assayed in turbidimetric in vitro tests employing citrated platelet-rich human plasma. Saliva collected for 10 minutes from a single insect was sufficient to inhibit platelet aggregation induced by either ADP or collagen in 100 μ l of platelet-rich plasma (Figure 2). Similar results were obtained with homogenized salivary glands. These results demonstrate, for the first time, that mosquito saliva inhibits platelet aggregation, a discovery that supports our hypothesized anti-hemostasis mechanism.

ADP is one of the main physiological factors triggering platelet aggregation (Mustard and Packham, 1977; Vargaftig et al., 1981), and apyrases (enzymes that break ATP and ADP to AMP and orthophosphate) antagonize ADP stimulation of that process. Accordingly, we sought evidence of apyrase activity in mosquito saliva. Both ATP and ADP were hydrolysed by saliva (Figure 3), demonstrating apyrase activity in saliva. preliminary biochemical characterization demonstrated a divalent cation requirement for hydrolysis of both ATP and ADP ($\text{Ca} > \text{mg} = \text{Sr}$) and an optimum pH plateau for both substrates at pH 8.5-10. Activity was inactivated rapidly in saline, but stabilized in the presence of albumin (1 mg/ml) or detergent (Triton X-

100, 0.003%). No hydrolysis was detected when AMP, glycerol-3P, p-nitro-phenyl-phosphate, or pyrophosphate was used as substrate. This enzymatic activity is consistent with inhibition of platelet aggregation.

C. Discussion

These experiments demonstrate that mosquito saliva reduces the duration of probing prerequisite to blood vessel location. We have shown that saliva inhibits platelet aggregation and have identified apyrase activity in saliva that may fulfill such a function. We suggest that saliva may promote hematoma formation around punctured blood vessels, thereby facilitating location of such vessels.

A previous report suggests that duct-sectioned mosquitoes experience difficulty in penetrating skin (Mellink and van den Bovenhamp, 1981), an effect that progressively disappears as mosquitoes repeatedly feed at the same site. These observations were attributed to gradual recovery from surgical trauma rather than to salivary deprivation. In contrast, our duct-sectioned mosquitoes appeared to be unimpaired because neck-pierced and duct-ablated mosquitoes similarly probed membrane-covered solutions.

The kissing bug, Rhodnius prolixus, possesses complex anti-hemostasis machinery (Ribeiro and Sarkis, 1982; Ribeiro and Garcia, 1981). These studies proposed a role for saliva in "positioning the maxillae inside blood vessels" where saliva induces formation of hematomas, thus shortening initiation of engorgement. Similarity of salivary function between kissing bugs and mosquitoes suggests a common strategy wherein saliva promotes location of blood vessels by inhibiting hemostasis. Such a function may be common to other vessel feeders. Indeed, Glossina saliva has factors which

inhibit platelet aggregation (Mant and Parker, 1981), suggesting that probing may be prolonged in saliva-deprived tsetse flies. Indeed, previous studies have shown that salivariectomized Glossina fed with difficulty (Lester and Lloyd, 1928).

Mosquitoes must rapidly engorge on their hosts, and a slow-feeding mosquito may not complete engorgement before irritating its host (Gillet, 1967). Thus, brief periods of host contact increase survival. Anti-hemostatic components of saliva play such a role by facilitating blood vessel location, thus shortening the duration of vector-host contact.

II. Apyrase activity in mosquito salivary glands after blood of sugar feeding

Mosquito saliva serves at least two different functions. First, it dissolves solid sugars (Eliason, 1963) that are necessary for nutrition. Second, it inhibits host hemostasis. This function is performed through inhibition of platelet aggregation and is mediated by an apyrase activity (Report I). Apyrase activity is absent in male mosquitoes (Report II) and thus appears to function solely in blood feeding.

This double function of saliva raises the possibility that a mosquito may selectively deplete apyrase levels following a sugar meal. Such a mosquito would suffer impaired ability to feed on blood. Accordingly, we determined whether apyrase activity was depleted during sugar feeding. We also measured the rate of apyrase activity resynthesis following its loss.

A. Materials and Methods

A Grand Bahama Island strain of Aedes aegypti, derived from material collected in 1969, was used throughout this work. All reagents were

obtained from Sigma Chemical Company (USA). Apyrase activity was assayed as previously described (Ribeiro and Garcia, 1981). One unit of enzyme activity was defined as the amount of enzyme that released 1 μ mol of orthophosphate per minute at 30°C. The Coomassie Blue technique of Spector (1978) was used for protein determination employing egg white lysozyme as a standard.

B. Results

First, we queried whether apyrase was lost following both sugar and blood meals. We deprived mosquitoes of sugar and water overnight and exposed them to dyed solid sucrose for 90 minutes. We then assayed their crops and salivary glands for apyrase activity as well as protein content. Similarly treated mosquitoes were fed on blood and assayed for midgut and salivary gland apyrase activity. Sugar fed mosquitoes contain apyrase activity in the crop, but without loss from the salivary glands (Table 1). On the other hand, blood fed mosquitoes lose apyrase activity from the salivary glands, part of which is displaced to the midgut. Specific activity indicates that apyrase activity is lost preferentially to other proteins. Thus, a meal of solid sugar paradoxically results in apyrase loss in saliva, but not from the glands.

Then, we determined whether apyrase activity was resynthesized rapidly enough to explain this difference. We allowed mosquitoes to feed on a guinea pig and compared resulting apyrase activity and protein content immediately following a blood meal and again 2 hours later. Our results (Table 2) indicate that specific apyrase activity is rapidly resynthesized following its loss.

Because ingestion of solid sugars took longer than that of blood, we determined whether apyrase activity was lost following ingestion of dissolved sugar. We exposed mosquitoes to solutions of 0.2 M and 2 M food-dyed sucrose for 10 minutes. We then assayed the crops and salivary glands of engorged mosquitoes. Our results (Table 3) indicate that little apyrase is lost following ingestion of dissolved sugar.

C. Discussion

Our experiments demonstrate that apyrase activity is lost following a meal of solid sugar, but that apyrase activity is lost following a meal of solid sugar, but that apyrase activity can specifically be resynthesized. Thus, by controlling loss of apyrase activity during sugar feeding to a level equal to resynthesis, mosquitoes preserve their ability to feed on vertebrate hosts.

III. Chemosensory Regulation of Salivation in Mosquitoes

External stimuli that regulate the quantity of saliva produced by insects remain largely unknown. Especially in the case of hematophagous insects, the small size of the salivary stylets hinders attempts to observe salivation. Tsetse salivate following mouthpart contact with such warm surfaces as a glass slide (Burtt, 1946) and mosquitoes salivate when their probosces are immersed in oil (Hurlbut, 1966; Rossignol and Spielman, 1982). An impressive body of literature is available on internal regulation of secretory activity in blowflies (Berridge, 1973; Hansen bay, 1978), but external stimuli are ignored. One promising technique for recording salivary activity of mosquitoes has been developed (Kashin and Wakeley, 1966) but not used to identify stimuli. It may be that salivation

by mosquitoes is directly regulated by chemical, thermal or tactile factors, or even that regulation is indirect, dependent upon some internal component of the feeding process. Accordingly, we determined whether certain phagostimulants may also stimulate salivation.

A. Materials and Methods

Salivation by non-mated female Aedes aegypti was measured using a recording microfluorometer. Mosquitoes were cold narcotized, sodium fluorescein (10 mg/ml) was injected intrahemocoelically, their probosces were placed (intact) in an optically clear (0.1 x 0.1 mm) capillary tube (microcell, Vitro Dynamics Inc., Rockaway, N.J.) and the assembly mounted on a cold stage placed on a microscope stage. Various concentrations of sucrose and of ATP were contained in the capillary tubes. The temperature of the cold-stage was raised incrementally, and intensity of ejected fluorescence was recorded. In each treatment, 15 mosquitoes were used. Fluorescence was recorded solely at the tip of the salivary stylet, visible through the proboscis sheath.

B. Results

In a preliminary experiment we exposed the probosces of mosquitoes to graded concentrations of sucrose and measured resulting intensity of fluorescence over the tip of the salivary stylet. Marked response was recorded solely at 2 M concentrations (Fig. 4a). Lesser concentrations generally failed to elicit increased fluorescence over the salivary stylet. Those mosquitoes that salivated following sucrose stimulation generally produced fluorescence measuring about 25 times background fluorescence (Fig. 4b). Ejection of fluorescence commenced when ambient temperature reached

15°C. Temperature itself did to serve as the primary stimulus for salivation because mosquitoes exposed to 0.1 or 1 M sucrose generally did not respond at any temperature. We concluded that exposure of mosquito mouthparts to sucrose stimulates salivation.

In the next experiment, we determined whether age of mosquitoes affects the quantity of saliva that they produce. Thus, mosquitoes aged 3, 9 or 15 days were exposed to graded concentrations of sucrose. Mosquitoes did not eject fluorescein when exposed to 1 M sucrose (Fig. 5a), but about half did respond when exposed to 2 M or 3 M sucrose (Fig. 6a, 7a). Mosquitoes of all ages responded similarly. Thus, we confirmed that exposure of mouthparts to sucrose stimulates salivation and established that age (within limits) does not affect this response.

We then determined whether blood-related phagostimulants similarly stimulate salivation. The mouthparts of mosquitoes were exposed to undiluted human serum, to 10% serum in phosphate buffered serum (PBS) and to diluted serum containing graded concentrations of dissolved adenosine triphosphate (ATP). Maximum ejection of fluorescent material was recorded when 0.001 M ATP was dissolved in serum (Fig. 8a). Other dilutions were essentially non-stimulatory. We concluded that mosquitoes salivate when exposed to certain mixtures of nucleotide and serum.

C. Discussion

These observations indicated, for the first time, that chemosensation modulates salivation in mosquitoes. Ejection of salivary tracer was dependent upon concentration of stimulant.

It is interesting that much greater concentrations of sucrose were required to simulate ejection of saliva than ingestion of solution. About 0.1 M sucrose provides the threshold for ingestion (Feir et al., 1961; Owen, 1963), but 20 times that concentration is prerequisite for salivation. This suggests that ingestion and ejection are stimulated by different processes. Salivation would not be stimulated secondarily by ingestion of food.

The concentrations of ATP that seems most stimulatory for salivation is identical to that stimulating ingestion of serum (Mason et al., 1965). This mixture simulates normal blood feeding in that the meal flows directly to the midgut and not to the crop as in sugar feeding.

IV. Malaria-infected mosquitoes: sporozoite-induced pathology increases probing time

The "interests" of malaria parasites and mosquito vectors conflict in that prolonged probing of the host favors transmission. Thus, malaria transmission would be enhanced if sporozoites impaired salivary function. Interestingly, malaria sporozoites invade only the distal regions of mosquito salivary acini (Sterling et al., 1973), regions that contain cells specific to female mosquitoes (Janzen and Wright, 1971). Perhaps destruction of these cells would prolong probing. Accordingly, we determined whether malaria sporozoites may specifically impair salivary function without lowering the volume of saliva delivered.

A. Materials and Methods

Mosquitoes (Aedes aegypti) rearing procedures and surgical intervention techniques have been described previously (Rossignol and Spielman, 1982). Plasmodium gallinaceum parasites were in White Rock chicks. Mosquitoes were

used 16 days (1 week after extrinsic incubation period) after blood-feeding on infected or non-infected chicks.

Apyrase activity was measured by colorimetric detection of orthophosphate release (Ribeiro and Garcia, 1981). Mosquito saliva was collected in oil-filled, siliclad coated microcells (Vitro Dynamics, Rockaway, N.J.).

B. Results

First, we determined whether Aedes aegypti mosquitoes infected with Plasmodium gallinaceum as described in Rossignol and Spielman, 1982, had impaired ability to probe. We compared probing time (interval between insertion of stylets and appearance of blood in the midgut) of sporozoite-infected, saliva-deprived (Rossignol and Spielman, 1982), and sham-operated mosquitoes exposed to the shaved back of an anesthetized guinea pig. Indeed, probing time of sporozoite-infected mosquitoes was greater than that of non-infected (sham-operated) mosquitoes, but less than that of saliva-deprived mosquitoes (Figure 9).

We then devised a measure of salivary pathology produced by malaria sporozoites based on detection of apyrase activity in the salivary glands of mosquitoes. Activity in sporozoite-infected mosquitoes was compared to that in non-infected mosquitoes. Apyrase activity of sporozoite-infected mosquitoes was only about a third of that of non-infected mosquitoes (Table 4). Indeed, sporozoites destroy salivary function.

The distribution of apyrase activity within the salivary glands was then identified. Because sporozoites reside solely throughout the apical (posterior) portions of the glands, basal and apical portions were compared.

Apyrase activity was confined to the apical tissues (Table 5). Male glands are devoid of such tissues and lack apyrase activity. These results correlate morphological and biochemical lesions.

Finally, we determined whether sporozoite infection reduced volume of saliva delivered to a host. Using oil-filled, siliclad-coated microslides, we measured volume of saliva ingested by infected and by non-infected mosquitoes. It was apparent that sporozoite-infection did not reduce volume of saliva delivered (Table 6).

C. Discussion

These experiments demonstrate, for the first time, that malaria parasites produce pathology in vectors that contributes to transmission. On the one hand, quantity of saliva delivered remains unaffected. But, quality, as measured by apyrase activity, is affected. This biochemical lesion is consistent with prolonged probing via an effect upon platelet aggregation (Ribeiro et al., MS). In this manner, sporozoites preserve their vehicle for delivery to vertebrate hosts while prolonging the period that permits delivery.

Similarly modified behavior characterizes other vector-pathogen-host relationships. Plague bacilli induce an embolus in the fore-gut of the flea vector that prevents ingestion (Cavanaugh, 1971). Such "blocked" fleas regurgitate into their hosts while vainly attempting to feed, thereby enhancing transmission (Wheeler and Douglas, 1941). Similarly, trypanosomes impair sensory function and blood flow in the food channel of tsetse flies (Jenni et al., 1980); such flies probe more frequently before engorgement. In addition, virus-infected mosquitoes take longer to feed (Grimstad, 1980).

Although no mechanism has been described for this observation. It may be that similar modification of behavior may be a frequent adaptation on the part of vector-borne pathogens.

Although these modifications directly affect vector competence, indirectly increased vectorial capacity may result as well. In this manner, infected vectors will attain more host-contacts than do non-infected vectors. Present conceptual models for malaria transmission omit these parasite-determined variables (MacDonald, 1957).

V. Delayed Appearance in Saliva of Malaria Sporozoites

The timetable of sporozoite output is an important aspect of malaria transmission. MacDonald's (1957) model for the basic reproduction role of Plasmodium depends largely on two parameters, namely extrinsic incubation period and sporozoite rate. Extrinsic incubation period is the period of time from a mosquito's uptake of an infective bloodmeal to its ability to infect a vertebrate host. Sporozoite rate represents the proportion of mosquitoes in a population that is infective. In practice, estimates of both parameters depend on appearance of sporozoites in dissected salivary glands, thus ignoring possible barriers to sporozoite migration within the glands.

We therefore devised techniques to estimate the time sporozoites take to pass from the substance of the gland into saliva; and the number of sporozoites in saliva.

A. Materials and Methods

In our work, we studied Plasmodium gallinaceum transmission by Aedes aegypti. To determine presence and estimate the number of sporozoites

ejected in saliva, we inserted the mouthparts of mosquitoes into mineral oil contained in an optically clear rectangular capillary tubes (Microcells, Vitro Dynamics, Rockaway, N.J.) (Figure 10). We allowed such mosquitoes to salivate for one minute, pulled back the tube slightly and let the mosquito salivate for another minute, and so on. Thus we obtained puddles of saliva containing sporozoites representing successive minutes of salivation. We could then determine the number of sporozoites in each puddle.

B. Results

By analyzing the time course of sporozoite output (Figure 11), we observed that appearance of sporozoites in saliva was delayed compared to appearance of sporozoites in conventionally dissected salivary glands. At least one day separated appearance of sporozoites in glands from appearance in saliva.

Furthermore, while all of our experimental mosquitoes obtained salivary infections, only about 80% secreted sporozoites upon oil stimulation (Table 7).

We noted that, overall, twice as many sporozoites were ejected in the first minute as in the second, and again less in the third minute (Table 7).

C. Discussion

We concluded that 1) extrinsic incubation period may be at least one day longer than is conventionally estimated, 2) sporozoite rate may be 20% lower than conventionally estimated, and 3) disproportionate numbers of sporozoites are produced during initial stimulation.

The lining of the salivary duct in A. aegypti appears to constitute a barrier to sporozoite passage. P. gallinaceum sporozoites accumulate slowly

in the duct lumen. It would appear that only those sporozoites already in the lumen are ejected during stimulation. Our measurements record the emptying of the luminal reservoir, which process may correspond to sporozoite output during feeding.

We note that the duct lining of various Anopheles is less sclerotized than that of A. aegypti, but the mechanism of penetration of this lining has not yet been described. This previously unmeasured aspect of vector competence may vary from species to species.

Organ	Mean Apyrase Activity (nM Pi/organ.min.)		
	Non-fed	Sugar-fed	Blood-fed
Pair of Salivary Glands	106.0 (17.0)	107.0 (19.0)	44.0 (12.0)
Midgut	1.1 (0.1)	1.1 (0.1)	26.0 (2.0)
Crop	0.4 (0.1)	37.0 (7.0)	0.8 (0.3)

Protein	Total Protein Content (μ g/pair of glands)		
	Non-fed	Sugar-fed	Blood-fed
Specific Activity	3.3 (0.2)	3.5 (0.2)	2.5 (0.2)
(Apyrase/Protein)	30.6 (3.2)	30.1 (3.6)	14.1 (3.5)

TABLE 1: Mean apyrase activity and total protein content of organs of non-fed, crystalline sugar-fed, and blood-fed mosquitoes. Ten mosquitoes were used for each meal treatment. The same mosquitoes were assayed for apyrase activity and protein content. Standard error in parentheses. Protein content was assayed by the Spector-Coomassie blue technique.

	Time After Meal of Blood (min.)		
	Before Blood Meal	5	90
Apyrase Activity	127.0 (12.0)	28.0 (8.0)	60.0 (14.0)
Protein Content	3.3 (0.3)	2.1 (0.1)	2.5 (0.2)
Specific Activity	39.5 (3.3)	12.2 (2.8)	21.0 (4.0)

TABLE 2: Mean apyrase activity (of 10 each) and total protein content of non-fed and blood-fed mosquitoes. Standard error in parentheses.

Sucrose Concentration	Treatment	Apyrase Activity	
		Pair of Salivary Glands	Crop
0.2 M	Fed	129 (3)	1.6 (0.5)
	Non-fed	126 (21)	0.2 (0.1)
2.0 M	Fed	154 (31)	3.2 (1.2)
	Non-fed	149 (22)	0.1 (0.1)

TABLE 3: Mean apyrase activity (of ten each) of mosquitoes fed on sucrose solutions. Standard error in parentheses.

Substrate	nM Phosphate Released/Gland	
	Sporozoite-infected	Non-infected
	(n = 9)	(n = 10)
ATP	84.7 (15.4)	245.8 (34.0)
ADP	48.8 (9.2)	167.2 (21.5)
ATP/ADP*	1.77 (0.13)	1.45 (0.03)

*Mean of ratios.

TABLE 4 Mean apyrase activity (nM Pi released/gland/minute) in salivary glands of sporozoite-infected and non-infected mosquitoes. Standard error in parentheses.

Sex	Portion of Salivary Gland	Apyrase Activity ($\pm$ S.E.) (n = 10)
Female	Distal	89 $\pm$ 11
	Basal	5 $\pm$ 1
Male	Whole	4 $\pm$ 0.3

TABLE 5 Mean apyrase activity (nM P_i released/gland/minute with ADP substrate) of regions of salivary glands of mosquitoes.

Treatment	Volume of Saliva μm^3 ($\pm$ S.E.)
Malaria-infected	4081 $\pm$ 545
Non-infected	4328 $\pm$ 486

TABLE 6 Mean volume (of 10 mosquitoes each) of mosquito saliva delivered in an oil-filled capillary tube during 2 minutes.

<u>Days After Infection</u>	<u>Mean Sporozoites Ejected in Minutes:</u>		
	<u>1</u>	<u>2</u>	<u>3</u>
9	37	53	104
10	56	61	33
11	37	39	15
12	157	16	4
13	69	25	24
14	68	19	4
15	18	13	7
16	78	3	1
17	74	57	50
18	31	5	0
19	35	37	0
Mean	60	30	22

Table 7. Time course of sporozoite ejection.

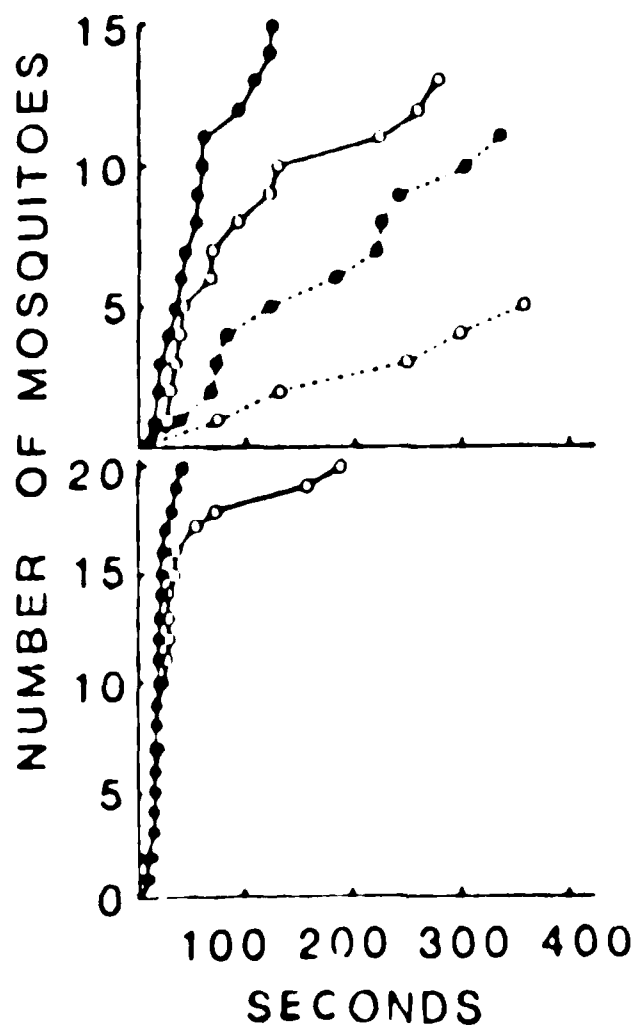


Figure 1a (Top): Duration of probing by neck-pierced (unbroken line) and duct-sectioned (dashed line) mosquitoes exposed to the ear (black circles) or back (white circles) of a guinea pig. Each treatment contained 15 mosquitoes.

Figure 1b (Bottom): Duration of probing by neck-pierced (dark circles) and duct-sectioned (white circles) mosquitoes exposed to a membrane-covered saline solution containing AIP (1 mM). Each treatment contained 20 mosquitoes.

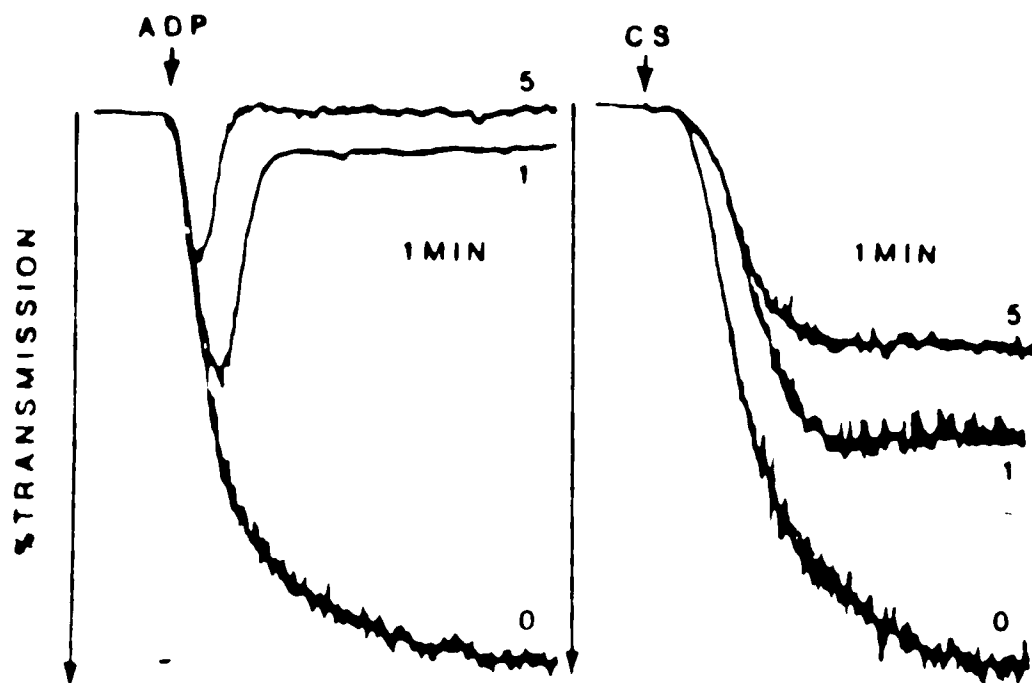


Figure 2: Aggregometer tracings of ADP- (left) and collagen-Induced (right) platelet aggregation of 100 μ l human citrated platelet-rich plasma in the presence of *A. aegypti* saliva. Inhibition results from the addition of saliva harvested from 1 or from 5 mosquitoes as indicated.

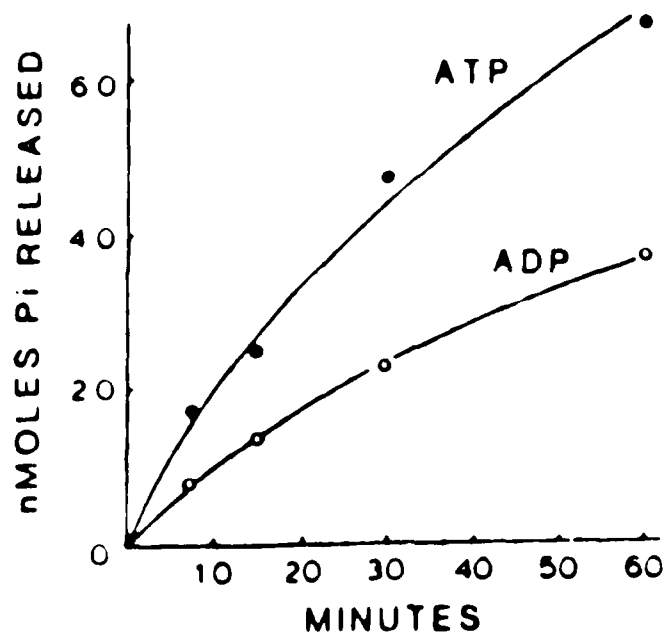


Figure 3: Orthophosphate released from ATP or ADP by the saliva secreted by a single mosquito. Reaction media contained 100 mM NaCl, 4 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, and 50 mM Hepes buffer pH 7.5.

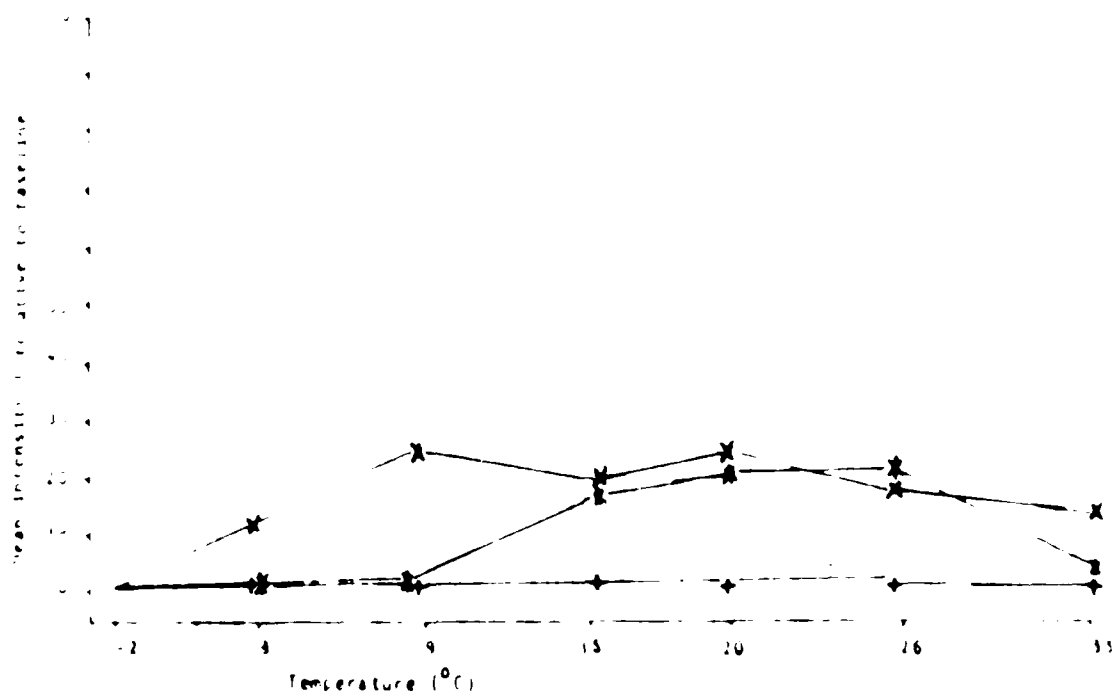
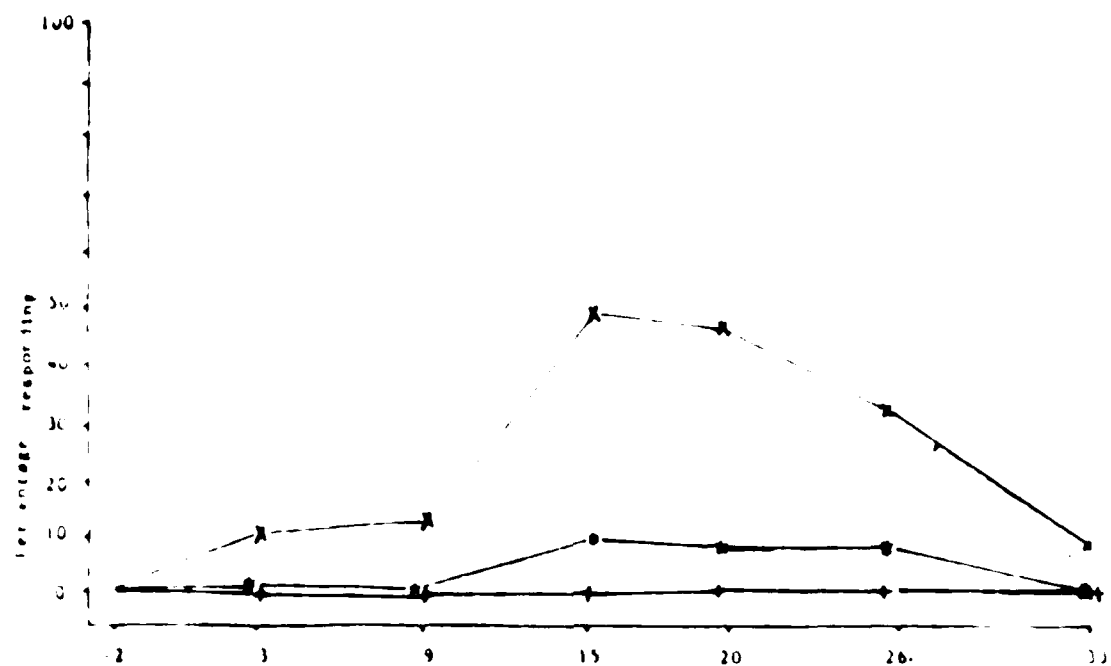
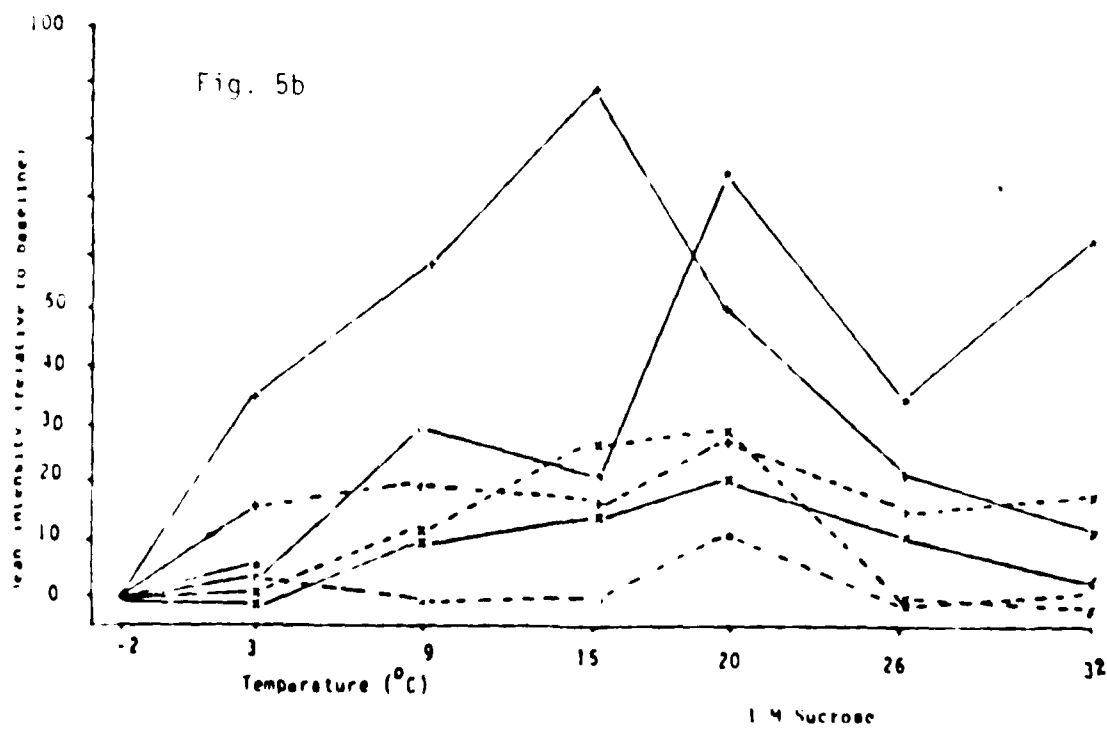
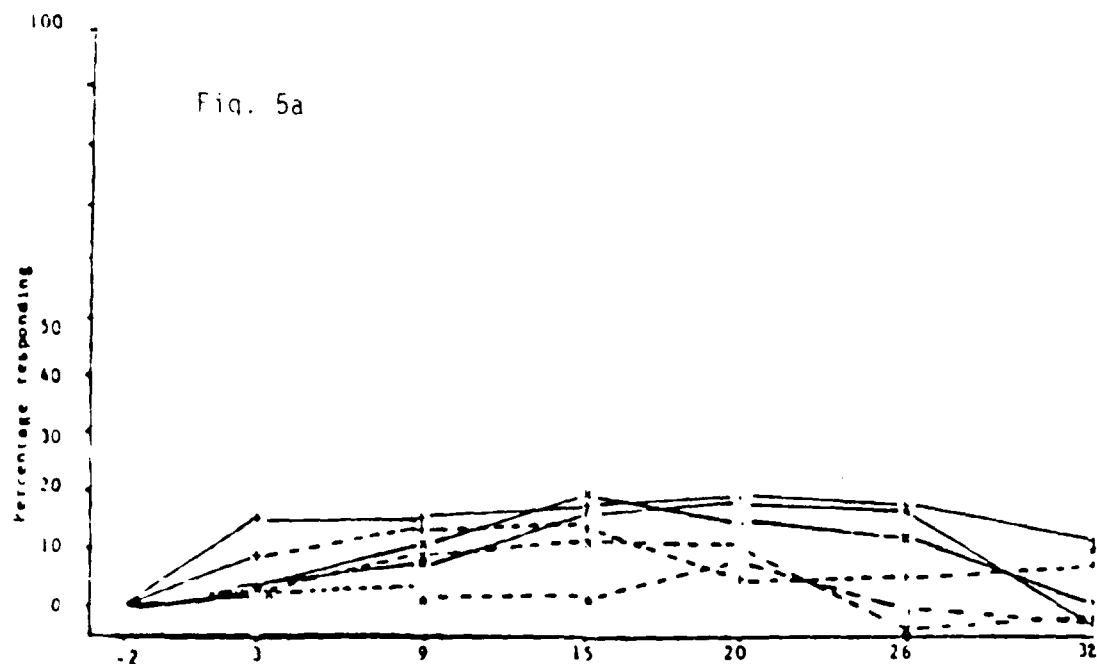
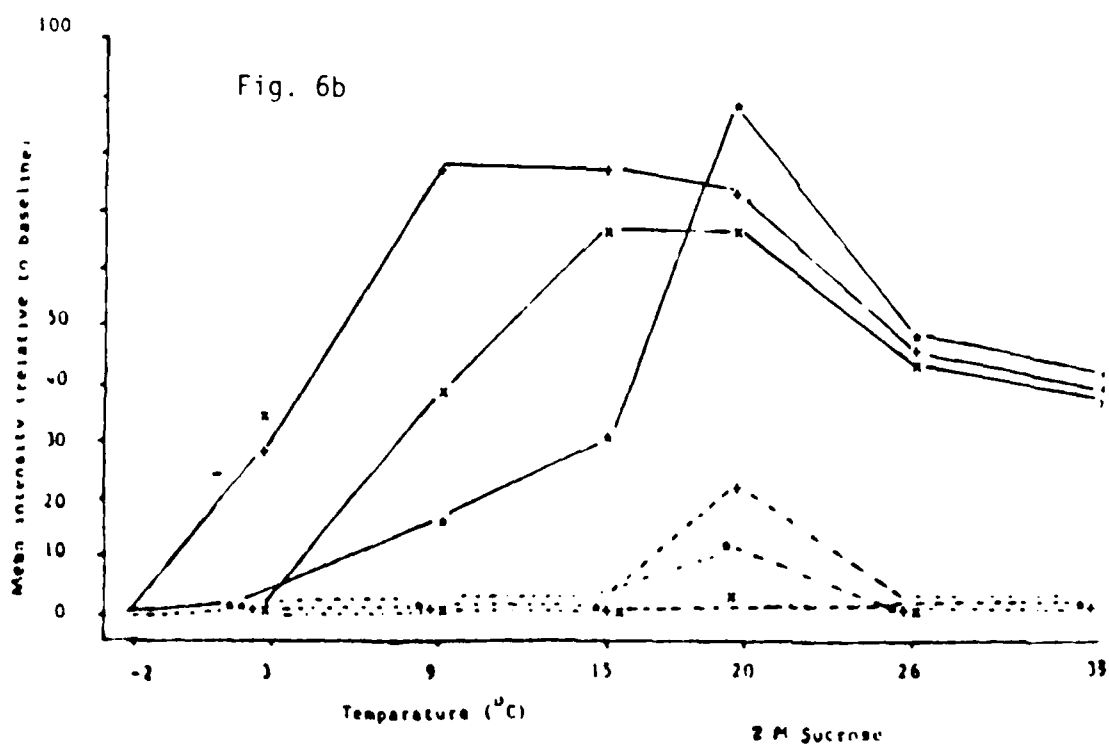
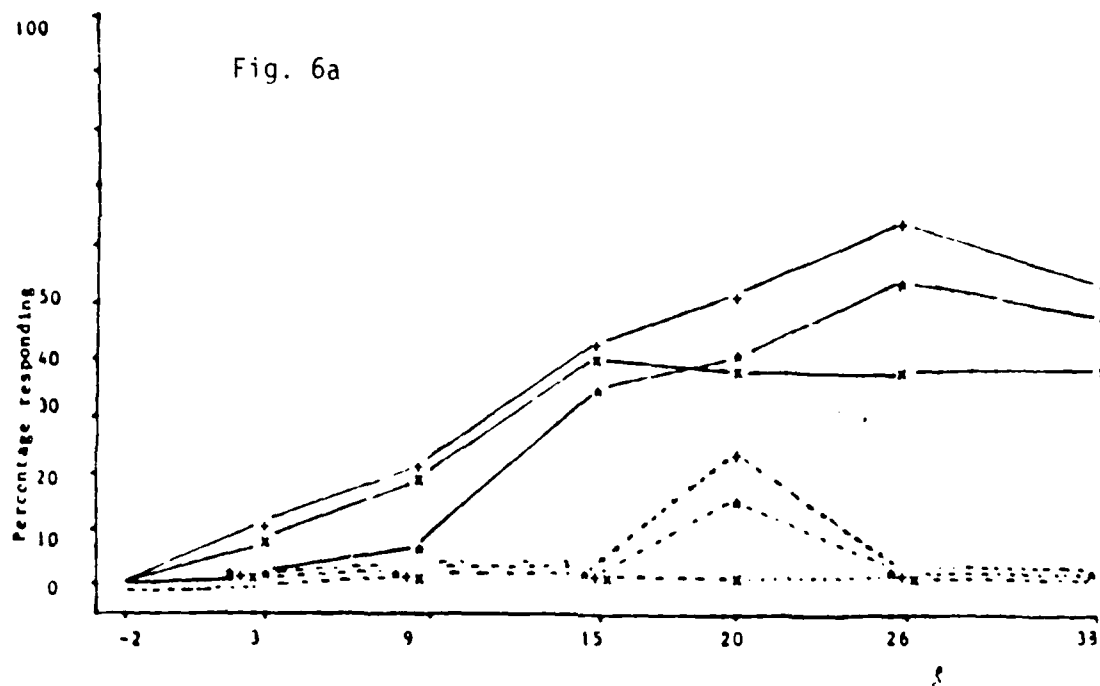


Figure 1. Subjective responses to thermal stimuli at different concentrations of a substance.

Figs. 5, 6, 7. Percent (a) and intensity of response (b) of mosquitoes salivating after response to graded concentrations of sucrose (—) or water (----) (10x increase in fluorescence or greater.) Each point represents 15 mosquitoes.

- = 3 days old
- ♦ = 9 days old
- x = 13 days old





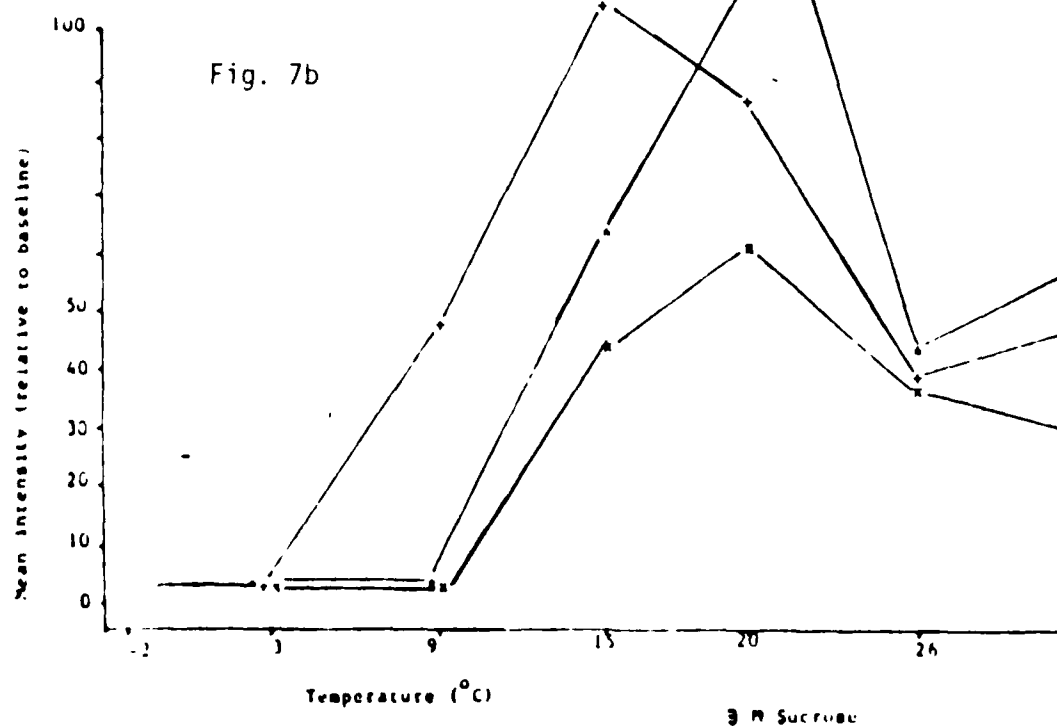
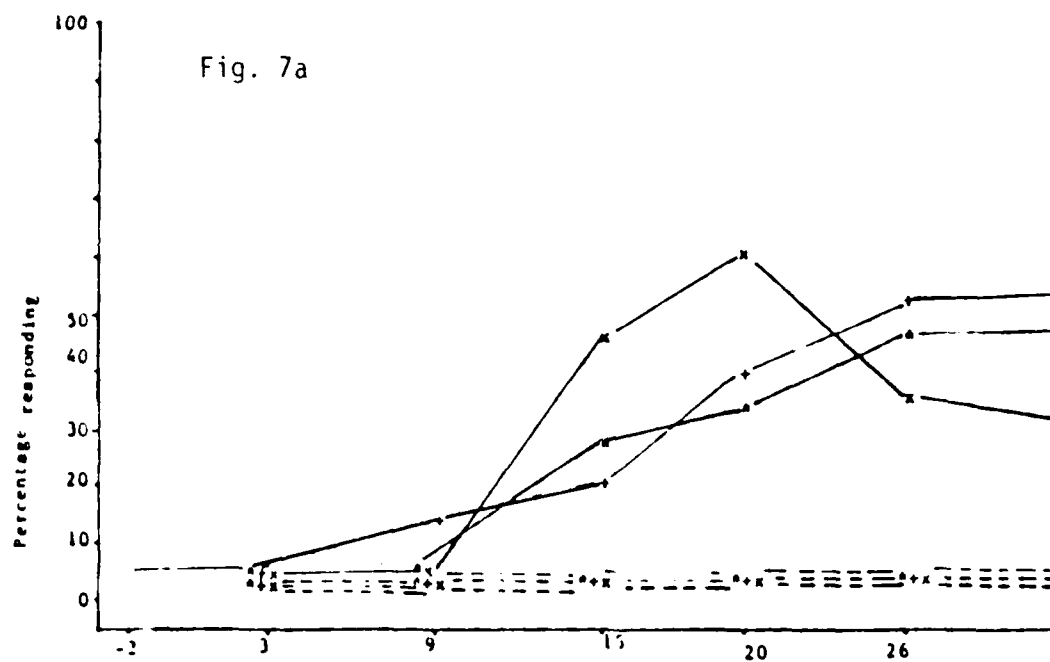
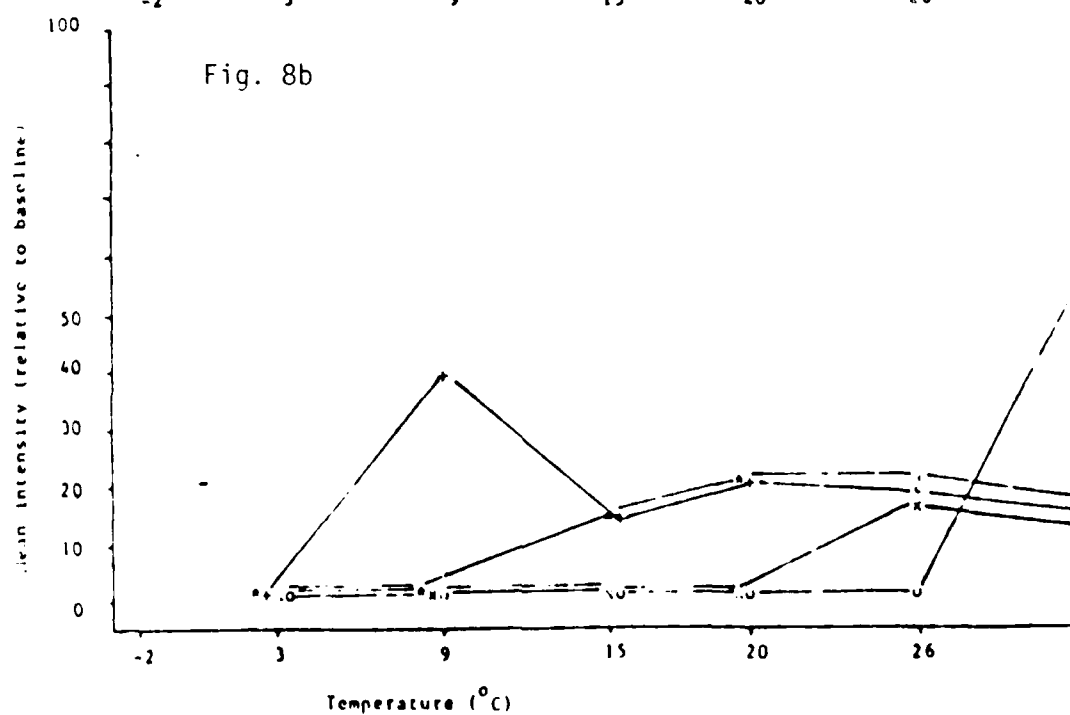
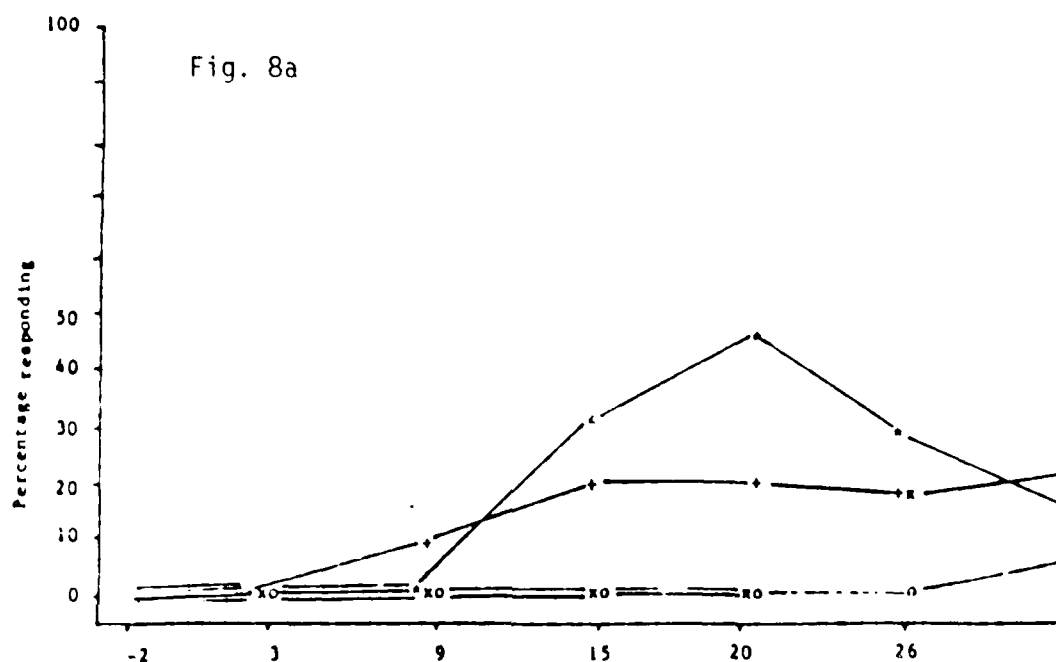


Fig. 8a Percent of mosquitoes salivating after exposure to various phagostimulants (10x increase in fluorescence or greater). Each point represents 15 mosquitoes.

Fig. 8b Intensity of salivation.

- * = 10% human serum with .001 M ATP
- + = 100% PBS
- x = 10% human serum
- o = 100% human serum

The same experiment included tests of 10% human serum with .01 M ATP as well as .0001 M ATP, but no response was elicited.



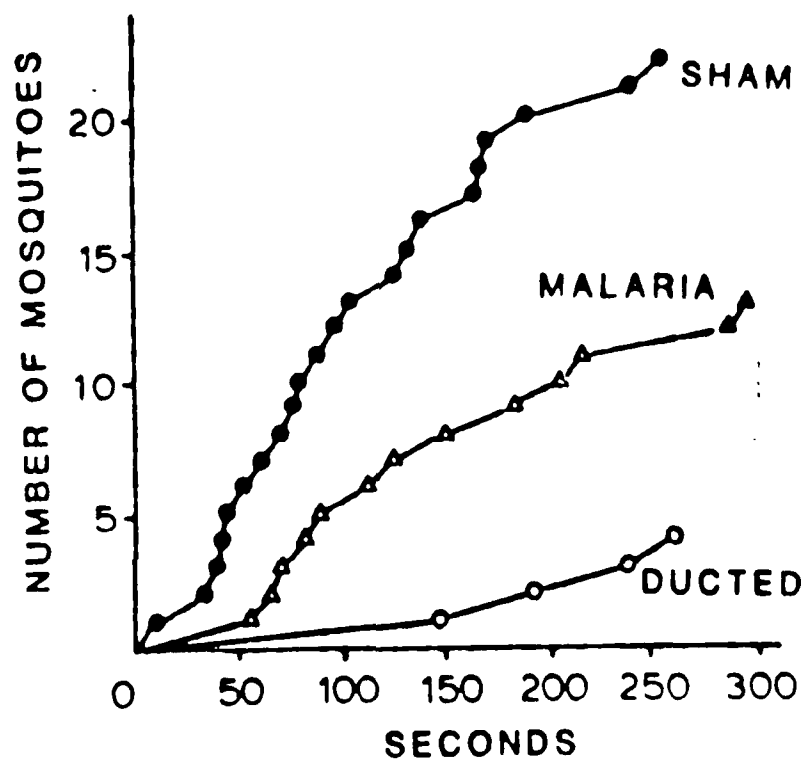


Figure 9 Duration of probing time of saliva-deprived (ducted), malaria-infected (malaria), and neck-pierced (sham) mosquitoes feeding on the back of a guinea pig. Each treatment contained 22 mosquitoes tested up to 300 seconds.

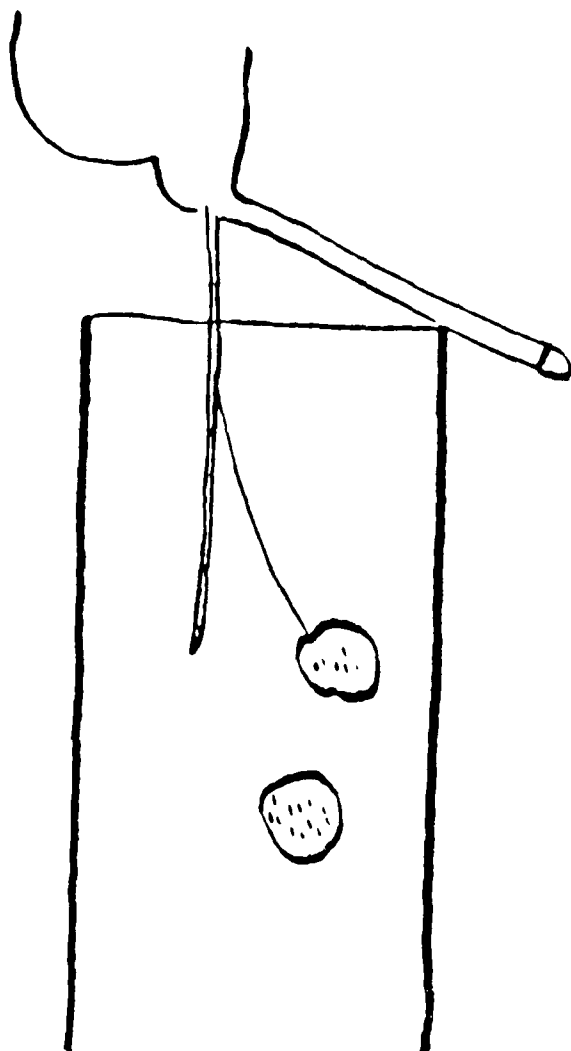


Fig. 10 Diagram of mosquito salivating in an oil-filled rectangular capillary tube. Drops of saliva containing sporozoites are present at the tip of the salivary stylet.

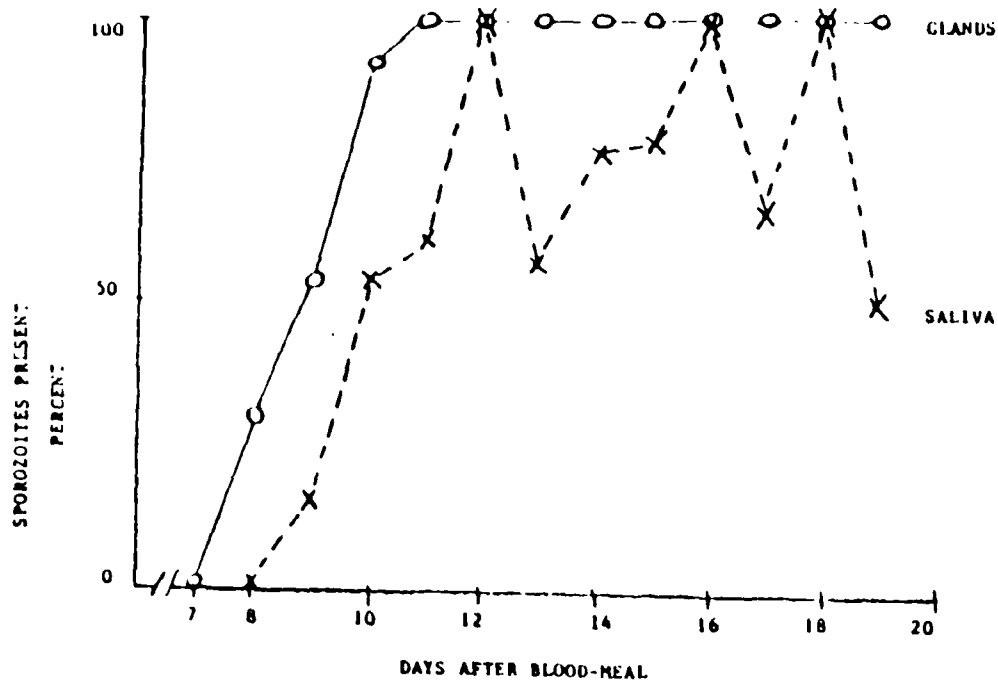


Fig. 11. Time-course of sporozoite appearance in salivary glands and output in saliva. Each point represents 5 to 15 individuals.

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