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FACTORS AFFECTING MEMBRANE FEEDING OF *ANOPHELES STEPHENSI*

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ABSTRACT. The impact of optimal temperature, osmotic pressure, and diet viscosity on the number of mosquitoes (*Anopheles stephensi*) feeding through a membrane, and on the size of the blood meal, was evaluated. An increase in diet viscosity decreased the number of imbibing mosquitoes, reduced the size of the ingested meal, and resulted in a slower rate of weight loss after feeding. The possible effect of these factors on the vectorial efficiency of mosquitoes is discussed.

INTRODUCTION

The impact of chemical and physical factors on the acquisition of food by membrane-fed mosquitoes has been reviewed by Galun (1975) and Friend and Smith (1977). The feeding of culicine mosquitoes and many other bloodsucking insects through membranes requires the use of warm solutions containing sodium chloride, adenine nucleotides, and often sodium bicarbonate. Environmental conditions and diet composition required for the successful artificial feeding of mosquitoes have been studied mainly for *Aedes* and to a lesser extent for *Anopheles.* The requirements for successful membrane feeding seem to differ markedly between *Aedes* mosquitoes, which need adenine nucleotides to engorge, and *Anopheles,* which can also engorge without adenine nucleotides (Galun et al. 1985).

Experimental feeding with defined tissue culture media has not been successful (Hosoi 1959, Mason et al. 1965). This failure has probably discouraged scientists from using such a model for the demonstration of nutritional and other physiological needs of adult mosquitoes and the requirements of pathogens for their intra-mosquito development (Galun 1979).

Reports on the effect of various diets on feeding mosquitoes *via* membranes may explain the differences in blood ingestion of mosquitoes from ill vs. healthy mammals (Rossignol et al. 1984, 1985).

Information on the effect of environmental factors and the physical composition of the diet is limited, whereas the importance of pH, osmotic pressure, temperature, and viscosity of a diet offered through membranes, for the feeding behavior of mosquitoes, was shown for the genera *Aedes* and *Culex* (Hosoi 1959; Galun 1967, 1975; Galun et al. 1985).

Our interest in the developmental interaction between *Anopheles stephensi* Liston and *Plasmodium berghi* in the mosquito led us to attempt to feed this mosquito *via* membranes using techniques proposed for other mosquitoes. Little success was achieved. The large variations in membrane feeding conditions required by various mosquito genera and even species, and the dearth of information on the artificial feeding of *An. stephensi,* induced us to undertake this study.

In this report we analyze the feeding responses of *An. stephensi* fed different diets offered through a membrane under various environmental conditions. The results could aid in improving the techniques of artificial feeding of mosquitoes and the concomitant study of mosquito physiology and vectorial efficacy. The information reported in this publication has been used previously by the authors (Samish 1990, Samish et al. 1991).

MATERIALS AND METHODS

Anopheles stephensi mosquitoes were reared at 28°C under a 12:12 h L:D photoperiod. Larvae were grown under noncrowded conditions (300 eggs in 700 ml of water in a 15 × 28-cm pan) and fed ground-up rodent laboratory chow. Adults were kept at 80–90% RH and provided with wet cotton padding and sugar cubes. The mosquitoes were fed on Syrian golden hamsters. Pupae were kept in cages for 24 h and the age of the adults was defined, with day zero being the time of pupae removal. Forty to fifty 4- to 6-day-old females were placed in 350-ml transparent plastic cups, with open tops and 2 holes in the sides (10 mm diam) covered with nylon netting. Cotton padding soaked in 10% sucrose solution was placed on top of each cup. The padding was removed 20 h before the start of membrane feeding, when up to 6 membrane feeders (Rutledge et al. 1964) were used simultaneously. Each membrane, made of goldbeater's skin (Baudruche) (Long and Long, Belleville, NJ),

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had a feeding area of 12.6 cm^2 and was covered with 3–4 ml of diet solution.

The standard feeding solution consisted of 0.15 M NaCl and 0.01 M $NaHCO_3$ or Leibovitz L15-B medium (Leibovitz 1963, Galun et al. 1985, Munderloh and Kurtti 1985) with 0.01 M $NaHCO_3$. The osmotic pressure was adjusted to 300 ± 5 milliosmol (mOsm) by adding water or mannitol. The freezing point depression was established with a micro-osmometer (Precision Systems, Sudbury, MA). In viscosity trials we used the human blood expanders dextran (*ca.* 150,000 mol. wt.) or polyvinylpyrrolidone (PVP) (360,000 mol. wt.). The pH of the solutions was adjusted to 7.3 with 0.2 M NaOH or 0.2 M HCl. In studies of the effect of diet pH on the engorging response, the pH of all feeding solutions was first lowered to pH 6.2 with H_2SO_4 in order to reduce fluctuations due to the instability of bicarbonate. The solutions were prewarmed and kept at 37°C for 30 min during feeding. Before each experiment, 10 unfed female mosquitoes were weighed for subsequent calculation of the net meal weight. Immediately after feeding, the mosquitoes were anesthetized with CO_2 and fully engorged mosquitoes were counted and weighed. Part of the ingested meal that was discharged during feeding and before anesthetization (Briegel and Rozzonico 1985) caused an underestimation of the volume of the blood meal actually ingested.

Forty to fifty female mosquitoes were placed in each membrane feeder. The exact number was counted after feeding. Each experiment was repeated 4 times unless stated otherwise. In each experiment, one feeder with saline and bicarbonate served as a control.

Preliminary experiments showed considerable variation in the number of mosquitoes feeding on identical solutions on different days; therefore, the results are reported mostly as an average of the percentage of the values obtained in each experiment.

In each experiment (up to 6 feeders), the results of each feeder were expressed relative to the control of the same experiment or to optimal conditions (i.e., the maximum percentage of feeding females), whichever was relevant. The average of these relative values of 4 or more repeated experiments is therefore presented as a percentage.

Statistical analysis was performed by linear or parabolic regression. Overall means were compared by the independent sample *t*-test or by analysis of variance (one-way or two-way). Simultaneous comparison of means was carried out by Duncan's Multiple Range Test.

RESULTS

The age of female mosquitoes affected the percentage of individuals feeding. When 4 batches (4 × 50 females of each age) of mosquitoes aged 3, 4, 5, or 8–10 days were offered 0.15 M NaCl with 0.01 M $NaHCO_3$, the mean percentages (±SD) of feeding mosquitoes were 15.0 ± 6.3, 38.0 ± 9.1, 56.0 ± 14.3, and 55.0 ± 12.7%, respectively.

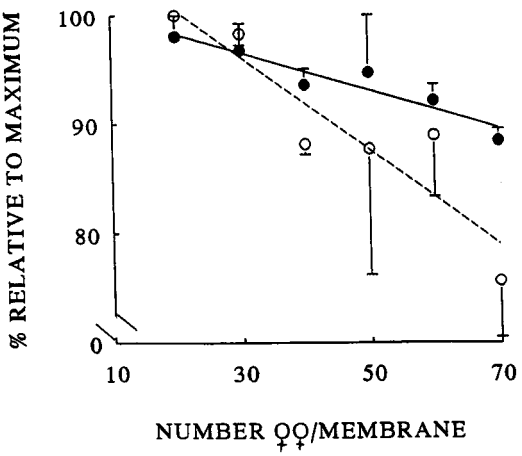

Fig. 1. Effect of crowding on engorgement of mosquitoes (●——●) and on meal weight (○— —○), expressed as the percentage of mosquitoes engorged from the maximum quantity fed in each experiment. Diet consisted of 0.15 M NaCl + 0.01 M $NaHCO_3$.

The percentage of mosquitoes from 14 different batches that fed on saline with sodium bicarbonate solution (control) varied considerably, averaging 58.9 ± 21.5%. The correlation between the percentage of mosquitoes feeding and the weight of the ingested meal was not significant ($r = 0.22$, $P > 0.10$).

In 7 experiments (*ca.* 350 females/time interval), mosquitoes feeding at 0900, 1300, or 1800 h constituted mean feeding percentages of 56.0 ± 13.6, 55.0 ± 8.9, and 58.0 ± 11.1%, respectively. These values indicate that the time of day had no significant effect on food intake.

When the density of female mosquitoes was increased from 20 to 70 per cup, the average percentage of engorged females dropped significantly ($P < 0.01$), although the variation among experiments was great, particularly at the higher densities. For each increase of 10 females, engorgement relative to the maximum decreased by 1.7%. Similarly, the meal weight decreased significantly ($P < 0.01$) with an increase in density: for each increase of 10 females, the percentage of maximum meal weight decreased by 4.3% (Fig. 1).

The highest percentage of engorged females was obtained with a diet temperature of 35–38°C; only 16% of the mosquitoes engorged at 23–26°C (Fig. 2).

The optimal pH of a diet consisting of phys-

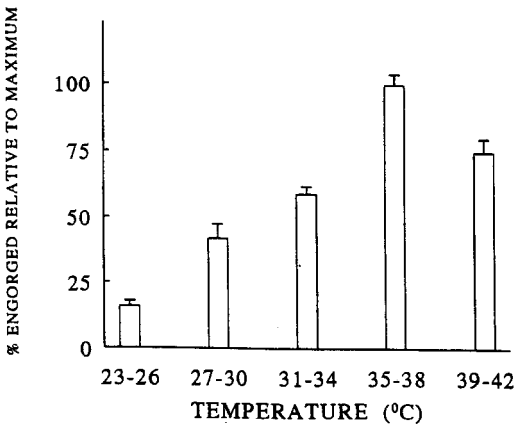

Fig. 2. Effect of diet temperature on engorgement of mosquitoes, expressed as the percentage of mosquitoes engorged from the maximum quantity fed in each experiment. Diet consisted of L15-B with 0.01 M $NaHCO_3$. Maximum quantity fed in the 4 experiments varied from 66 to 94% (*ca.* 200 females/column).

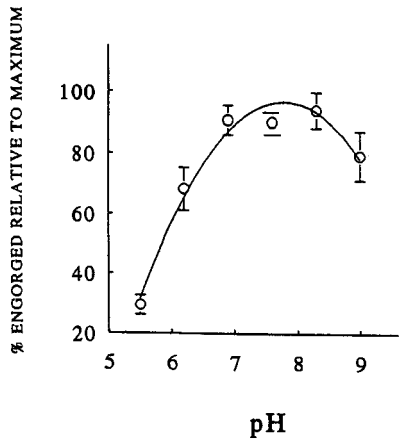

Fig. 3. Effect of diet pH on engorgement of mosquitoes, expressed as the percentage of mosquitoes engorged from the maximum quantity fed in each experiment. Diet consisted of 0.15 M NaCl + 0.01 M $NaHCO_3$. Maximum quantity fed in the 6 experiments varied from 74 to 91% (*ca.* 150 females/point).

iological saline with 10 mM sodium bicarbonate varied between 6.9 and 8.3 (Fig. 3). The pH of the diet above the membrane increased by 0.4 units during the 30-min feeding.

The effect of osmotic pressure of the diet on engorgement of the mosquitoes was studied with 3 different types of diets (Fig. 4). The L15-B medium was diluted with distilled water to an osmotic pressure of 200 mOsm; the osmotic pressure was increased to 450 mOsm by the addition of mannitol. The most intensive feeding was obtained at 300 mOsm. The level dropped by 39% at 200 mOsm and by 37% at 450 mOsm (Fig. 4).

When the osmolarity of water with 10 mM sodium bicarbonate was elevated gradually to nearly 300 mOsm by adding NaCl or mannitol, the percentage of feeding individuals rose linearly for the NaCl solution ($P < 0.0001$) and showed a parabolic increase for mannitol ($P < 0.01$) and for L15 with mannitol ($P < 0.001$) (Fig. 4).

Figure 4 shows the osmotic pressure at the start of the feeding period. During a feeding period of 30 min, the osmotic pressure of the diet increased by 5–15% mainly due to evaporation through the membrane.

Our data indicate that the percentage of *An. stephensi* that became engorged on solutions with various concentrations of NaCl (0.01 M sodium bicarbonate adjusted with mannitol to an osmotic pressure of 300 mOsm) differed considerably. Optimal feeding started when 25–50% (i.e., *ca.* 0.04–0.08 M NaCl) of the osmotic pressure was due to the addition of NaCl, whereas any additional increase in NaCl failed to affect the percentage of engorged mosquitoes (Fig. 5). In 2 cases approximately 50% of the mosquitoes became engorged in the absence of NaCl. The percentage of mosquitoes feeding on solutions of 0.01 M sodium bicarbonate, becoming isotonic with only NaCl or only mannitol, averaged 69.9 ± 4.5 and 34.4 ± 8.5%, respectively, in 6 trials.

At higher diet viscosities, fewer mosquitoes became engorged and they consumed less. The higher the viscosity, the lower the rate of feeding, concurrent with a subsequent slowed rate of weight loss of the engorged mosquitoes (Figs. 6, 7, and Table 1).

The decrease in the rate of feeding, relative to the maximum, was uniform for all 3 media—4.3% for each of the dextran concentrations used ($P < 0.0001$) (Fig. 6A). The overall level did not differ for the different media. The rate of weight reduction was also uniform for the various media—2.2% of the maximum for each of the dextran concentrations used ($P < 0.0001$). The overall weight reduction for PVP in L15 was significantly less than for dextran in NaCl ($P < 0.05$) or for dextran in L15 ($P < 0.01$) (Fig. 6B).

The average weight of a meal consumed by one female mosquito feeding on a hamster for between 5 and 30 min was 2.25 ± 0.12 mg. This value is significantly higher ($P < 0.01$) than that obtained after the mosquitoes fed on 7% dextran (1.50 ± 0.08 mg). When the diet was more viscous (14% dextran), the weight of the meal engorged by the mosquitoes was far less (0.60 ± 0.05 mg, $P < 0.01$). The average weight of a meal of each of the 3 diets did not increase when the meal was interrupted after 5, 10, 15, or 30 min (Table 1).

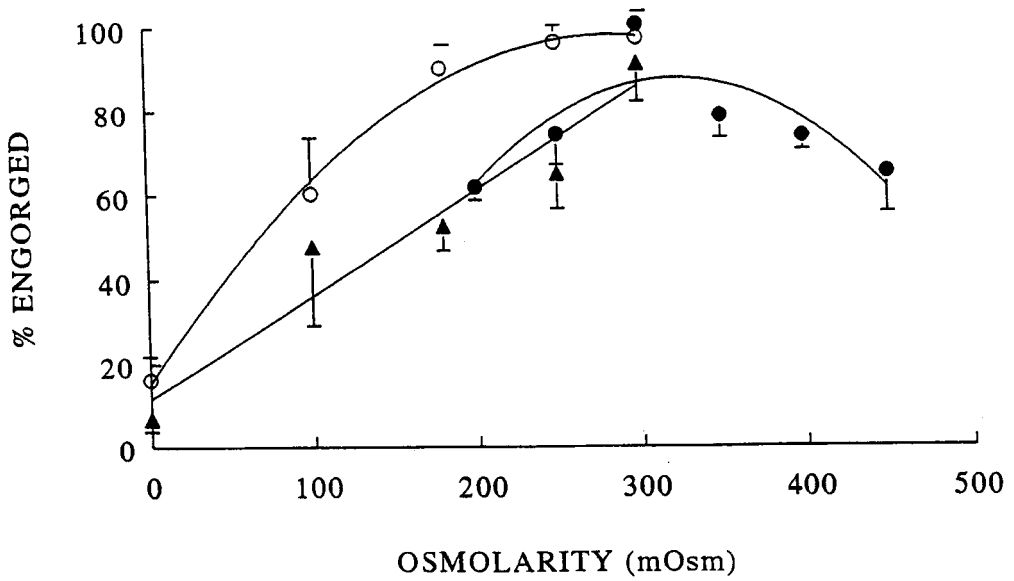

Fig. 4. Effect of diet osmolarity (in milliosmols, mOsm) on engorgement of mosquitoes, expressed as the percentage of mosquitoes engorged from the maximum quantity fed in each experiment. Diet consisted of NaCl (▲———▲) (*ca.* 200 females/point), mannitol (○———○) (100 females), or L15 with mannitol (●———●) (200 females), all with 0.01 M $NaHCO_3$. Maximum fed in the 6 experiments varied from 77 to 96%.

An average of 82% of the mosquitoes became engorged when exposed to 7% dextran for various time intervals, but only 56.8% ($P < 0.01$) became engorged when exposed to 14% dextran. When offered live hamsters, a significantly higher percentage (95.5%, $P < 0.01$) fed.

The time of exposure to each of the 3 diets had a marked effect on the number of feeding mosquitoes (Table 1). Mosquitoes given a chance to feed for only 5 min fed significantly less ($P < 0.05$) than those offered food for 15 or 30 min. This phenomenon was most marked when they were offered the diet with 14% dextran.

A change in viscosity of the diet affected the loss in weight after a meal. Two hours after gorging on a diet of 10% dextran in saline the mosquitoes had lost only 5% of their weight, whereas 20 h after feeding they had lost *ca.* 25%

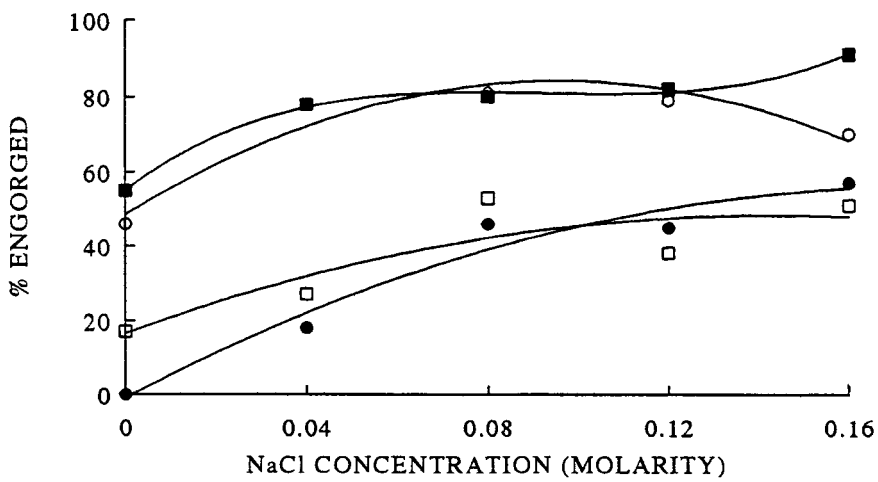

Fig. 5. Effect of NaCl concentration in diet solutions with 0.01 M $NaHCO_3$ on engorgement of mosquitoes. Diet osmolarity was adjusted with mannitol to 300 milliosmol. (Each type of symbol represents one experiment, *ca.* 50 females/point.)

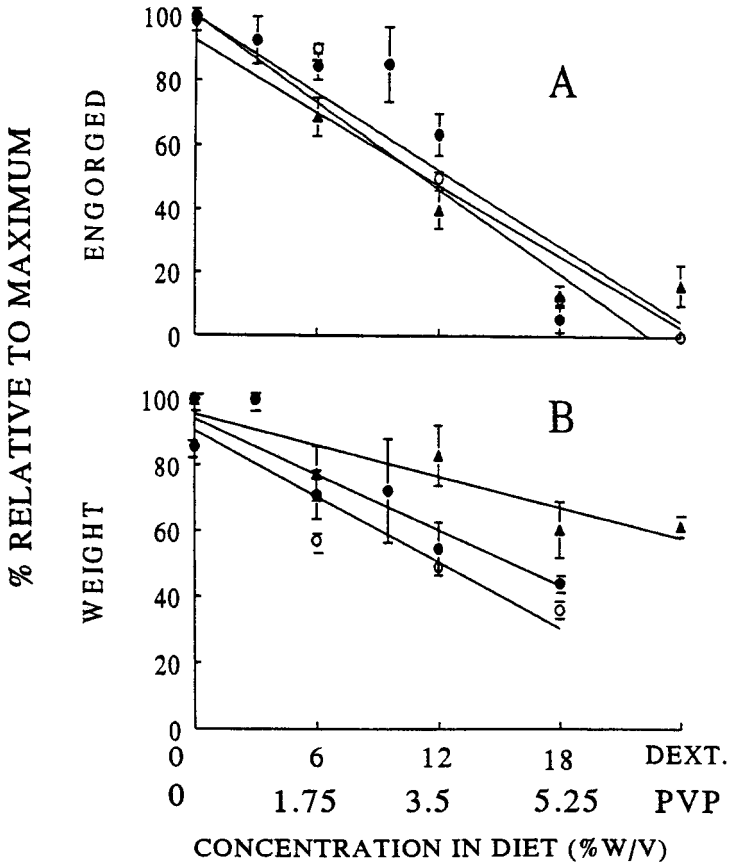

Fig. 6. Effect of diet viscosity on the engorgement of mosquitoes (A) and on their weight (B), expressed as the percentage of mosquitoes engorged or their weight from the maximum in each experiment. Diet consisted of dextran in 0.15 M NaCl (●———●) (*ca.* 100 females/point), dextran in L15 (○———○) (*ca.* 50 females/point), and PVP in L15 (▲———▲) (*ca.* 100 females/point), all with 0.01 M $NaHCO_3$. Maximum quantity fed in the 5 experiments varied from 69 to 98%.

(P = 0.01) (Fig. 7). However, after feeding on a dextran-free diet they had lost *ca.* 20% of their weight after 2 h and over 50% of their weight within 20 h (Fig. 7). After feeding on blood, the mosquitoes lost 14–16% of their weight after 2 h and 32–38% of their weight after 20 h. The rate of weight loss 20 h after feeding was significantly greater than that 2 h after feeding (P = 0.01) (Fig. 7).

DISCUSSION

Our results on the effect of crowding on mosquito feeding agree with those obtained by Edman et al. (1975), who reported on artificially interrupted blood meals in *Culex nigripalpus* Theobald. Apparently, crowding interfered with mosquito feeding and caused many of those disturbed not to return to complete their meal. A similar effect was reported when mosquitoes were allowed to feed on restrained or actively defensive rabbits (Klowden and Lea 1979, Waage and Nondo 1982).

Anopheles stephensi feeding on an artificial diet through a membrane preferred a pH similar to that of mammalian blood, which in most cases is 7.3–7.5. The optimal pH of an artificial diet for *Aedes aegypti* (Linn.) was reported to be 8–9 (Galun 1967).

A change in the osmotic pressure of a diet required considerable changes in its composition. These changes may have affected the willingness of mosquitoes to feed not only because of differences in osmotic pressure but also because of phagostimulatory or phagosuppressive influences of the compounds. Therefore, the osmotic pressure of the diets was changed by means of 3 different compounds (NaCl, mannitol, or tissue culture media). Diets containing any of these 3 compounds gave similar results

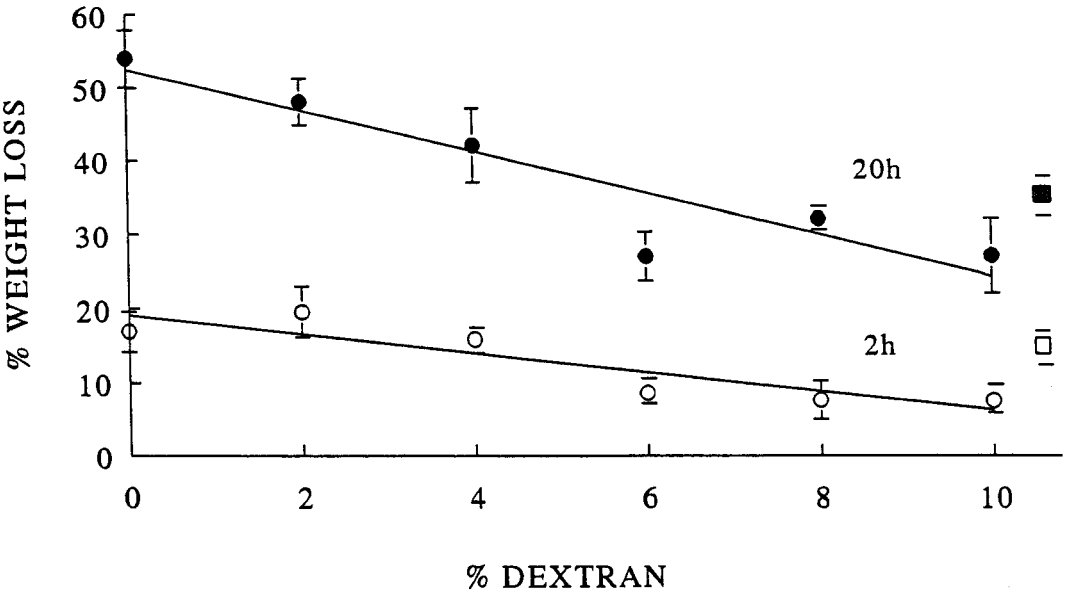

Fig. 7. Effect of diet viscosity on weight reduction of engorged mosquitoes 2 h (○———○) and 20 h (●———●) after feeding. Diet consisted of dextran in 0.15 M NaCl with 0.01 M $NaHCO_3$. Squares represent weight loss 2 h (□) and 20 h (■) after engorgement on healthy hamsters (*ca.* 100 females/point).

(Fig. 4). Previous investigators changed the osmotic pressure of the diets by adding NaCl or sugars, which may have caused undesirable side effects (Hosoi 1959; Galun et al. 1963, 1985). Because their results indicated that Na^+ tended to affect the engorging of mosquitoes, we preferred to change the osmolarity of the diet with mannitol. This sugar did not increase the percentage of engorged mosquitoes above that obtained with added NaCl when both had been offered in isotonic solutions with sodium bicarbonate. Thus, mannitol is not a phagostimulant and does not have any nutritional value for mosquitoes (Galun and Frankel 1957).

The preference of *An. stephensi* for diets isosmotic with blood (Fig. 4) is similar to that reported for *Ae. aegypti* (Hosoi 1959, Galun et al. 1963) and other anophelines (Galun et al. 1985) (Fig. 4). In some trials, as few as *ca.* 14% of the females became engorged when they were offered only water with 0.01 M $NaHCO_3$ (Fig. 4). Galun (1967) reported a reduction in feeding of *Ae. aegypti* when offered KCl, $CaCl_2$, $MgCl_2$, glucose, sucrose, or lactose instead of NaCl in diets containing 10^{-2} M AMP. We obtained similar results when the NaCl in the diet was replaced by mannitol. Maximal feeding was achieved when only 25–50% of the optimal osmolarity was attributed to NaCl, and the additional osmotic pressure was attributed to added mannitol (Fig. 5).

Little attention has been paid to the influence

Table 1. Effect of concentrations of dextran in a diet (0.15 M NaCl + 0.01 M $NaCHO_3$) consumed via a membrane on the percentage of engorged mosquitoes and on their meal weight (mg), as compared with feeding on a host (100 mosquitoes/combination).

Diet	Feeding success	Membrane-offering time (min) 5	10	15	30	% engorged mosquitoes (mean ± SD)
Dextran 7%	% feeding	58.0	89.6	89.4	91.0	82.0 ± 4.9
	(mg meal)	(1.34)	(1.51)	(1.58)	(1.50)	(1.50) ± 0.1
Dextran 14%	% feeding	37.5	45.2	68.7	76.0	56.8 ± 6.4
	(mg meal)	(0.72)	(0.54)	(0.50)	(0.63)	(0.60) ± 0.1
Blood 100% (hamster)	% feeding	89.6	96.7	96.7	99.0	95.5 ± 1.7
	(mg meal)	(2.30)	(2.32)	(2.29)	(2.10)	(2.25) ± 0.1

of diet viscosity on artificial feeding of mosquitoes. In this study it was found that the size of the meal ingested by *Anopheles* via a membrane was inversely related to diet viscosity (Fig. 6B and Table 1). This relationship was also reported for *Rhodnius* (Smith 1979). The viscosity of a meal offered to tsetse flies (*Glossina morsitans*), however, did not affect their weight (Galun 1975).

According to Maddrell (1963) and Gwadz (1969), termination of a blood meal is related to abdominal stretch receptors. Additional factors may cause termination of a blood meal before maximal stretch is reached, because a diet of high viscosity (Fig. 6B and Table 1) or poorly stimulative phagostimulatory solutions, such as saline with low concentrations of 5′-adenylic acid (Hosoi 1959), will also result in lower consumption. The mechanical stretch of the midgut after a meal was found to influence the occurrence of proteolytic activity in the midgut and to initiate the accumulation of yolk in the ovaries (Samish and Akov 1972, Spielman and Wong 1974, Uchida 1983). The mechanical stretch of the midgut also seems to affect the success of *Plasmodium gallinaceum* ookinetes in penetrating the midgut wall of *Ae. aegypti* (Yasjukevich et al. 1990). Furthermore, *in vitro* infection of mosquitoes showed that a reduction in the rate of discharge of the midgut contents resulted in a concomitant reduction in the discharge of *Dirofilaria immitis* (Ando 1984). That the discharge rate was lower when the viscosity of the diet was increased may be due either directly to diet composition or indirectly to the size of the meal. A higher diet viscosity also affected the amount of liquid consumed. When the meal ingested by *Rhodnius* was smaller, defecation was delayed (Kirk and Schofield 1987).

After incomplete engorgement, mosquitoes will often probe again and some will refeed. This may involve a change of host, making them efficient vectors of many diseases (Edman et al. 1975, Magnarelli 1977, Klowden and Lea 1978, Molyneux and Jeffries 1986).

In a population of healthy mammals, blood viscosity fluctuates widely and is known to be affected by several factors. It has a linear relation to the hematocrit and varies according to sex, blood group, etc. (Dintenfass 1976). The whole blood viscosity of a healthy human population ranges from 3.5 to 5.4 centipoise and that of normal serum from 2 to 4 centipoise. A rise in viscosity of human serum above 10 centipoise is regarded as an abnormal hyperviscosity syndrome (Wintrobe et al. 1981). The viscosity of blood is influenced by various malfunctions of the body. Malaria, for example, changes the hematocrit level and the body temperature, resulting in "sludgy" blood and in changes in kidney function. All of these factors may have a marked effect on blood viscosity (Rigdon 1950, Rand et al. 1964, Sitprija 1970, Dintenfass 1976).

In vitro simulation of *Ae. aegypti* bloodfeeding *in vivo* showed that feeding time increased with an increase in the hematocrit level (Daniel and Kingsolver 1983). Concentration of red blood cells had a major impact on the viscosity of blood (Dintenfass 1976). Our finding that a diet viscosity below that of normal blood improved anopheline engorgement supports the conclusion of Daniel and Kingsolver (1983) that the optimal blood hematocrit for mosquito uptake is close to 0.3 and not 0.4, which is the normal hematocrit level for blood. A low hematocrit level in infected animals may be the reason for faster feeding, resulting in increased feeding success for mosquitoes.

The chemical and physical composition of blood is known to change when animals become ill. A possible correlation between our results with membrane feeding and the vectorial efficiency of mosquitoes fed on diseased animals under natural conditions still has to be demonstrated.

Establishing optima for the various factors that influence the success of *An. stephensi* in feeding *via* membranes led us from the failures in our preliminary tests, based on published trials mainly on other mosquito species, to the performance of successful experiments (Samish 1990, Samish et al. 1991).

The subject of this report has several implications for our understanding of mosquito physiology, i.e., nutritional needs and the effect of imbibed compounds on adult female mosquitoes, the influence of diet composition and viscosity on feeding behavior, and the nutritional needs of the mosquito as well as of pathogens within their vector.

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