Bloodmeal digestion by strains of *Anopheles stephensi* Liston (Diptera: Culicidae) of differing susceptibility to *Plasmodium falciparum*

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SUMMARY

Blood digestion was studied in strains of *Anopheles stephensi* which had been genetically selected for either refractoriness or susceptibility to infection by *Plasmodium falciparum*. Females of the refractory Pb3-9a strain ingested more blood than selected (Sda-500) and unselected (Punjab) susceptible females and began to degrade the haemoglobin soon after feeding. In susceptible females, haemoglobin degradation started only after a significant post-feeding lag period. Total protein content of the midgut after the bloodmeal was correspondingly higher for refractory than for susceptible females, but absolute and relative rates of protein degradation were not significantly different between the different mosquito strains. Bloodmeal induction of midgut trypsin activity and the maximal trypsin activity were the same for the different strains. The residual aminopeptidase activity and its relative post-feeding activity (enzyme units per midgut) were significantly higher in refractory females. However, when converting to specific aminopeptidase activity, no differences between strains were evident. The results indicate that both the early initiation of haemoglobin degradation and higher aminopeptidase activity in the Pb3-9a refractory strain are important in the limitation of parasite development within the mosquito midgut, whereas trypsin plays no role in this process.

Key words: *Anopheles stephensi*, digestion, susceptibility, *Plasmodium falciparum*.

INTRODUCTION

Trypsin and aminopeptidases are the major proteolytic enzymes involved in blood digestion by female mosquitoes, and are produced by the midgut cells in direct response to blood feeding (Briegel & Lea, 1975; Graf & Briegel, 1982). In *Aedes aegypti*, proteases have been shown to damage ookinetes of *Plasmodium gallinaceum* (Gass, 1977; Gass & Yeates, 1979) but in these studies, digestion was induced prior to infection, thereby not reflecting the true situation. Normally both protease synthesis (Gooding, 1966) and parasite development (Nijhout, 1979; Sinden, 1984) are simultaneously initiated by the arrival of an infective blood meal in the mosquito midgut.

Mosquito strains refractory to *Plasmodium* transmission may have potential within long-term integrated malaria control strategies (Curtis, 1968; Whitten, 1971) and also provide investigators with useful models to examine the interruption of mosquito-stages of the malaria life-cycle. Variability in susceptibility of the mosquito vector to the parasite has a genetic basis, the selection of which has resulted in mosquito strains which are either more refractory or more susceptible to infection by the malaria parasite (Kilama & Craig, 1969; Huff, 1930; Collins *et al.* 1986; Van der Kaay & Boorsma, 1977; Frizzi, Rinaldi & Bianchi, 1975; Ward, 1963). Until recently, differences in susceptibility in mosquitoes have been selected only for simian, avian or rodent malarias but since infection of mosquitoes by *Plasmodium falciparum* has become routine (Ponnudurai *et al.* 1982), genetic selection of *Anopheles stephensi* for differing degrees of susceptibility to this human malaria has been achieved (Feldmann & Ponnudurai, 1989).

These unselected (Punjab, Kasur and Hutchpot), and selected refractory (Pb3-9a) and susceptible (Sda-500) strains of *An. stephensi* were used in the present study.

In Pb3-9a, the frequency of *P. falciparum* oocyst development is 20–100 lower than in the original Punjab strain. Parasite development in Pb3-9a is somehow blocked in the midgut lumen as no degenerated or retarded oocysts have been observed in the epithelium (Feldmann & Ponnudurai, 1989). The present study examines the digestive physiology of these strains to elucidate its importance in confering susceptibility or refractoriness. The first indications of differences in bloodmeal digestion among *An. stephensi* strains were observed by A. M. F. and E. S. of the Insect Genetics Department at ITAL in Wageningen. Using different techniques, these observations were subsequently confirmed at the STI in Basel by P. F. B. and E S.

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MATERIALS AND METHODS

The experiments were performed in two different laboratories, each with its own techniques. In Wageningen, haemoglobin degradation and trypsin activity were studied in whole body homogenates of 5- to 6-day-old females. In Basel the same assays plus assays for total protein degradation and aminopeptidase were performed on midgut homogenates of 2- to 4-day-old females. In this way a counter check on possible differences between strains was achieved.

Mosquito maintenance

In Wageningen, mosquito larvae were reared at a density of 300–400 larvae/tray (36 cm × 24 cm). Each larval tray was filled with 4·5 l of temperature-conditioned tapwater and 250 ml of aquarium water. Three drops of Liquifry(R) (no. 1, Interpet Ltd) were added to each tray on the first day of rearing, then larvae were fed twice a day with Tetra-Mikromin(R) from the third day onward. The amount of larval food was adjusted to the daily needs of the developing larvae. Air and water temperatures were maintained at 29 °C and 27 ± 1 °C respectively. Mosquitoes were exposed to a 12/12 h light–dark cycle with 45 min dawn and dusk simulations. The development time from egg to adult ranged from 9 to 11 days. Adults had permanent access to a filter-paper wick placed in a 10 ml bottle filled with 5 % (w/v) fructose, and the relative humidity was maintained at 75–80 %. The 5- to 6-day-old females were routinely fed upon an anaesthetized mouse laid on the holding cage.

The rearing in Basel differed in the following respects. Eggs were placed in tap water containing a small amount of ascorbic acid and larvae were fed sterile ground hamster food. Adults were maintained on 10 % (w/v) sucrose and on warm defibrinated pig blood presented through a stretched Parafilm(R) membrane.

Mosquito strains

The Kasur strain originated from 40 females, field collected in a cattle shed in the Kasur district of the Pakistan Punjab province in 1981. The Punjab and Sind strains were obtained in 1982 from Dr A. Sakai at the former Pakistan Medical Research Centre, Lahore, and are a mixture of laboratory strains originating from different geographic locations of the Pakistan Punjab and Sind provinces respectively. The Hutchpot strain is a mixture of *An. stephensi* strains, field collected in 1984 from various geographic locations in the Pakistan Punjab, and of both the Punjab and Sind strains. Sda-500 and Pb3-9a have been obtained through genetic selection from females of the Sind and Punjab strains respectively, which were exposed to highly infective *in vitro*-reared *P. falciparum* gametocytes (Feldmann & Ponnudurai, 1989). Sda-500 has a 2-fold increased susceptibility and Pb3-9a has a 20- to 100-fold reduced susceptibility to this parasite in comparison with the unselected Punjab and Kasur strains, as determined by counting the number of mature oocysts on the midgut wall.

Preparation of whole-body homogenates

Five-day-old adult females were deprived of fructose for 12 h then allowed to feed on an anaesthetized mouse for 10 min. All unfed or partially fed females were removed from the cage. Two batches of 5–10 females each were collected and stored frozen at −20 °C in Eppendorf tubes at the indicated intervals following the end time point of the bloodmeal. For the assays, samples were thawed and homogenized in 200 µl of 0·1 M Tris(Tris[hydroxymethyl]aminomethane) buffer, pH 8·0, containing 0·02 M $CaCl_2$, using an ice-cooled Eppendorf homogenizer. The mixture was transferred to a 3 ml Kontron(R)-cellulose centrifuge tube, overlaid with 2·5 ml of paraffin oil and centrifuged for 60 min at 60000 ***g***. The clear supernatant was removed from below the oil with a Pasteur pipette and was used immediately for the assays.

Preparation of midgut homogenates

Fully engorged 2- to 4-day-old females were separated and used for subsequent experiments. For dissection, individuals were immobilized on ice and midguts pulled into a drop of *Aedes* Ringer solution (Hayes, 1953). After removal of all non-midgut tissue, whole midguts were stored 2/1·5 ml Eppendorf tube at −20 °C for later use. For enzyme assays, each pair of midguts was homogenized on ice in 1·0 ml of 0·15 M NaCl, in an ice-cooled glass/Teflon Potter-Elvejheim homogenizer, then centrifuged at 10000 ***g*** for 10 min at 4 °C. Supernatants were assayed for aminopeptidase and trypsin activities, and for haemoglobin and protein contents. Samples could be stored for several months at −80 °C with no significant loss of activity (Billingsley, 1989).

Haemoglobin assays

The absorbance at 412 nm in whole-body homogenates was read in the same sample mixture as used for the trypsin assays, but before the substrate (Na-*p*-toluolsulphonyl-L-arginine methyl ester; TAME) had been added to the reaction mixture. The absorbance was directly proportional to the amount of haemoglobin present (Briegel, Lea & Klowden, 1979; Coluzzi, Concetti & Ascoli, 1982).

For haemoglobin determinations in midgut homogenate supernatants, 25 µl and 75 µl aliquots were

taken from each sample and made up to 0·5 ml with 0·15 M NaCl containing 0·875 mg/ml soybean trypsin inhibitor (Sigma) to prevent further degradation during the assay. The absorbance at 412 nm was measured against saline containing trypsin inhibitor.

Protein assay

A precipitation step is required to separate proteins from interfering compounds (e.g. peptides, uric acid) which are present in mosquito midguts during, and as a result of, bloodmeal digestion (Houseman & Downe, 1986). Therefore proteins in the midgut homogenate supernatants were precipitated using an excess of 72% trichloroacetic acid (TCA), and protein assays using the Folin-Ciocalteau reagent (Sigma) were performed on the precipitates resuspended in distilled water (Peterson, 1977). Bovine serum albumin (Fraction V, Sigma) was used as a standard and duplicate assays were made on different sized aliquots from each sample.

Trypsin assays

Whole-body homogenates: 25 µl of homogenate and 2·225 ml of buffer (0·05 M Tris containing 0·02 M $CaCl_2$ at pH 8·0) were equilibrated in a quartz cuvette for 1 min at 30 °C in a Shimadzu spectrophotometer. After addition of 250 µl of TAME (3·8 mg/ml in H_2O) the rate of increase in absorbance at 247 nm was read during the linear phase for at least 5 min against a reference cuvette containing TAME and buffer only (Gass, 1977).

Trypsin activity in midgut homogenate supernatants was assayed using the substrate benzoyl alanine *p*-nitroanilide (BApNA), 40 mg/ml in dimethylsulphoxide (Me_2SO) substituted for leucine *p*-nitroanilide (LpNA) in the aminopeptidase assay described below (Huang, 1971; Houseman & Downe, 1986).

Aminopeptidase assay

A vol. of 1·5 ml of 0·05 M Tris–HCl buffer (pH 8·0 at 30 °C) and 40 µl of LpNA (40 mg/ml in Me_2SO), were equilibrated at 30 °C for 5–10 min. Midgut homogenate (10–25 µl) was added, and the reaction continued for 30–90 min at 30 °C, then stopped by addition of 0·5 ml of 30% (v/v) acetic acid (Billingsley, 1989). The absorbance at 410 nm (Erlanger, Kokowsky & Cohen, 1969) was determined in duplicate samples. Duplicate control tubes containing substrate and buffer were incubated (to allow for spontaneous breakdown of substrate), and homogenate added after the acetic acid (to allow for absorbance inherent in the sample).

Treatment of results

Trypsin activity in whole-blood homogenates was expressed as µmol TAME hydrolysed/min/mosquito, using an extinction coefficient of 0·409 $cm^2/\mu mol$ (Gass, 1977). Activities against BApNA and LpNA were calculated as enzyme units (EU) per midgut, where 1 EU was the amount of enzyme required to hydrolyse 1 ng of substrate using an extinction coefficient of 8·8 mM (Erlanger *et al.* 1969). Specific activities were calculated separately for each sample, expressed as EU/µg protein. Differences between curves were tested using the two-tailed Wilcoxon's signed rank test and between means at single time-points using the Student's *t*-test (Campbell, 1974) on individual samples.

Haemoglobin degradation was expressed as the percentage of absorption at a given time post-bloodmeal relative to the extinction at $T = 0$. The haemoglobin content of the midgut does not always decrease immediately after feeding, and this delay in reduction of haemoglobin content is termed the lag phase. After the lag phase, the haemoglobin content decreases approximately linearly. To estimate this lag phase a mathematical model was fitted to the data using non-linear regression analysis, the fitted function being:

$$h = a - b(t - l) \quad \text{if} \quad t > l,$$

h = haemoglobin content; $a = h$ on $t = 0$; b = regression coefficient; t = time after bloodmeal in hours; l = lag phase in hours.

This model allows the direct numerical comparison of the lag phases for all strains when fed on both blood sources.

RESULTS

Haemoglobin degradation

Mouse blood, whole mosquito homogenates. There were clear differences between mosquito strains with regard to the onset (lag phase) and subsequent rate of haemoglobin degradation. Unselected Punjab and selected refractory Pb3-9a mosquitoes exhibited a continuous degradation of haemoglobin, whereas selected susceptible Sda-500 mosquitoes did not permanently significantly reduce haemoglobin until 20 h after feeding (Fig. 1A). The results of a single, representative experiment in which the whole sequence of observations for each of the three mosquito strains was performed are given in Fig. 1A. These results correspond with other experiments (Table 1) showing that haemoglobin degradation is consistently delayed in Sda-500 females. The marked fluctuations, i.e. the decrease to about 70% after 4 h and a return to 100% at 18 h (Fig. 1A, Sda-500),

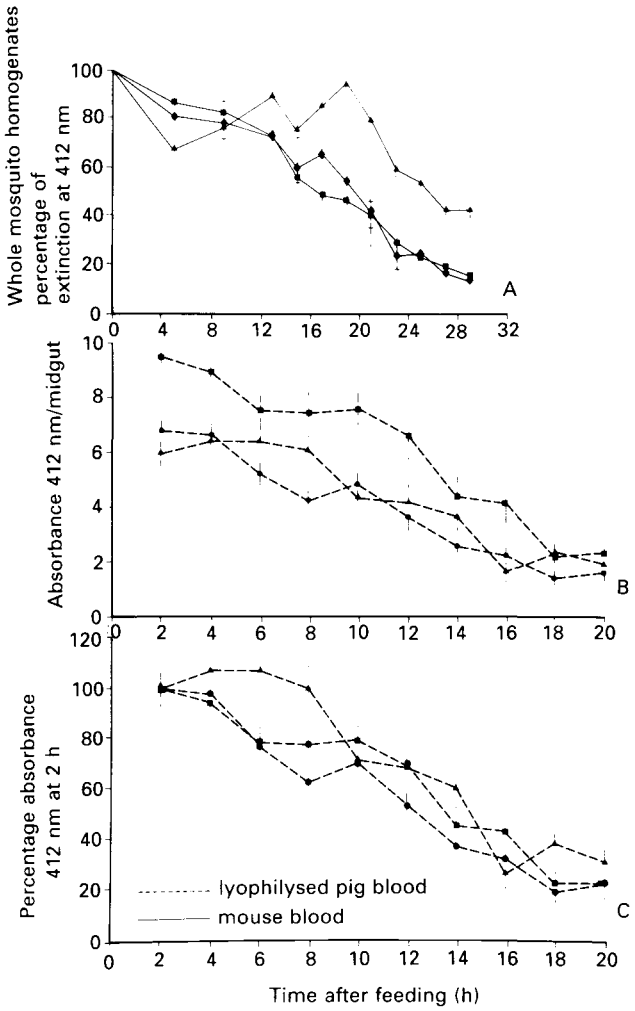

Fig. 1. Haemoglobin degradation in *Anopheles stephensi* strains Kasur (A, ◆) and Punjab (B and C, ●) both having normal susceptibility to *Plasmodium falciparum*. Sda-500 (▲) and Pb3-9a (■) were selected for high and low susceptibility respectively to *P. falciparum*. (A) Haemoglobin degradation in whole body homogenates of mouse-blood-fed mosquitoes: absorbance at 412 nm is given as the percentage of that at $T = 0$ *h*. (B) Haemoglobin degradation in midgut homogenates of pig-blood-fed mosquitoes. (C) Haemoglobin degradation as in B; absorbance is given as the percentage of that at $T = 2$ h.

was not observed in other experiments (results not shown) and is therefore attributed to experimental variability.

Pig blood, midgut homogenates. Significantly larger blood meals were ingested by Pb3-9a mosquitoes than by other strains ($P = 0{\cdot}005$, $n = 6$). Subsequently the midgut haemoglobin content remained higher in Pb3-9a during the first 18 h of digestion (Fig. 1B). As with the mouse-fed mosquitoes, the Punjab and Pb3-9a strains exhibited a short lag phase in the reduction of midgut haemoglobin content (Fig. 1B). In selected susceptible Sda-500 mosquitoes, however, there was a lengthened post-feeding lag-phase in haemoglobin degradation of approximately 8 h (Fig. 1C).

Modelling of degradation kinetics. Data from these and other feeding trials were used in the described model to estimate the lag phase and the subsequent haemoglobin degradation rate (regression). Of 5 mosquito strains, 3 were fed on both blood sources (Table 1). In fitted data, unselected (Punjab, Kasur and Hutchpot) and selected susceptible (Sda-500) strains exhibited extended lag periods which exceeded 19 h in the highly susceptible (Hutchpot and Sda-500) strains (Table 1). Conversely, the lag phase in the selected refractory strain (Pb3-9a) was consistently reduced to less than 2·79 h (Table 1). These trends in the lag phase modifications were more pronounced in whole mosquito homogenates after mouse feeding than in midgut homogenates of pig-blood-fed mosquitoes.

The regression varied considerably within and between strains (Table 1) but, in all cases, regression was higher in assays performed on midgut rather than whole-body homogenates. There was no correlation between the regression coefficient and either the level of susceptibility or the lag phase.

Protein intake and degradation

The TCA-precipitable protein content of the dissected midguts was measured every 2 h, from 2 to 20 h after a meal of lyophilysed pig's blood taken by the Punjab, Pb3-9a and Sda-500 strains (Fig. 2A). The refractory Pb3-9a mosquitoes ingested significantly higher amounts of protein than the other two strains ($P = 0{\cdot}005$, $n = 6$), and the midgut protein content in Pb3-9a mosquitoes was always significantly higher up to 18 h after feeding ($P = 0{\cdot}005$, $n = 56$). At 20 h, mosquitoes from all three strains retained similar quantities of midgut protein. There were no significant differences in the protein ingested and protein degradation rates between the Sda-500 and the Punjab strain. When the protein degradation rate was calculated as a percentage of the protein present at 2 h after feeding, there were no measurable differences between the three strains (Fig. 2B). In all the protein estimations, no lag phase equivalent to that in the haemoglobin degradation could be found (*cf.* Figs 1C and 2B).

Trypsin activity

Trypsin activity was determined in homogenates of both whole mosquitoes (mouse-blood fed) and midguts (pig-blood fed) by the TAME or BApNA assays respectively. No differences were observed between the various mosquito strains in the onset and subsequent post-feeding increase in activity (Fig. 3) when calculated as either relative (Fig. 3A, B) or specific (Fig. 3C) activities.

Table 1. Estimated values for the length of the lag phase (± S.E.) in hours and regression (change/h in extinction at 412 nm × 1000) (±S.E.) resulting from non-linear regression analysis of the rate of blood digestion by *Anopheles stephensi* strains of varying susceptibility to *Plasmodium falciparum*

Strain	Relative susceptibility	Lag phase	Regression	*N*	Blood source	Homogenate
Pb3-9a	L	0·01 ± 4·36	5·23 ± 0·49	19	Mouse	w.b.
		2·16 ± 1·56	4·36 ± 0·24	23	Mouse	w.b.
		0·13 ± 6·04	3·48 ± 0·44	22	Mouse	w.b.
		2·79 ± 1·75	43·80 ± 3·92	32	Pig	m.g.
		2·60 ± 2·63	48·32 ± 5·21	36	Pig	m.g.
Punjab	M	5·27 ± 0·32	6·19 ± 0·64	11	Mouse	w.b.
		1·96 ± 1·66	31·83 ± 2·07	54	Pig	m.g.
Kasur	M	9·61 ± 2·92	4·30 ± 0·96	19	Mouse	w.b.
		10·98 ± 1·76	5·24 ± 0·66	22	Mouse	w.b.
Hutchpot	H	19·04 ± 1·00	12·57 ± 2·35	12	Mouse	w.b.
Sda-500	H	16·16 ± 2·95	7·09 ± 2·80	12	Mouse	w.b.
		19·22 ± 1·69	5·98 ± 2·03	24	Mouse	w.b.
		6·04 ± 0·45	57·87 ± 7·99	24	Pig	m.g.
		8·94 ± 1·33	35·70 ± 4·77	33	Pig	m.g.

(*N*, number of samples; blood-source = type of blood fed to female mosquitoes; homogenate = whole bodies (w.b.) or midgut (m.g.); L, low; M, medium; H, high.)

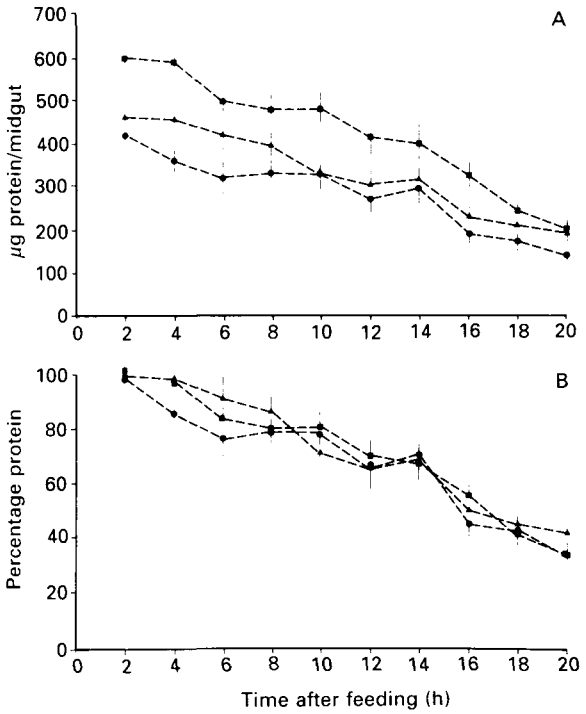

Fig. 2. (A) Course of protein content in midgut homogenates of pig blood-fed females of *Anopheles stephensi* strains Punjab (●), Pb3-9a (■) and Sda-500 (▲) (see legend of Fig. 1). (B) Percentage protein degradation.

Aminopeptidase activity

Aminopeptidase activity against LpNA was determined before and 2-20 h after feeding pig blood to Punjab, Pb3-9a and Sda-500 strains. Relative activity (EU/midgut) was always significantly higher in Pb3-9a mosquitoes (Fig. 4A) ($P = 0{\cdot}005$, $n = 61$) than in Punjab and Sda-500 females, the relative activities of which were similar. The post-feeding induction and increase in aminopeptidase followed the same pattern in all strains. When converted to specific activities (EU/μg protein) (Fig. 4B), there were no discernable differences between the three strains.

DISCUSSION

This study confirms the previously determined characteristics of digestion in *An. stephensi* (Berner, Rudin & Hecker, 1983; Billingsley, 1989), demonstrating that protease activity cycles remain similar in comparative studies, even though different techniques have been utilized. In *Ae. aegypti*, trypsin activity is restricted to the midgut, so comparisons between whole body and midgut homogenates are feasible for this enzyme (Felix *et al.* 1989; Kunz, 1978). Protein and haemoglobincyanide assays (Briegel *et al.* 1979; Houseman & Downe, 1986) have been used to determine bloodmeal size and digestion in mosquitoes, but the present results indicate that these assays are not interchangeable as a lag phase was observed only in the haemoglobin assay. Use of only one technique may therefore not necessarily provide a true indication of midgut digestive events.

In the unselected Punjab strain, both protein and haemoglobin degradation curves followed the same pattern, whereas the selected strains exhibited dissociation of protein digestion and haemoglobin degradation, such that the immediate post-feeding lag phase in the latter process was either increased (susceptible) or decreased (refractory). Such temporal dissociation of decreases in protein and hae-

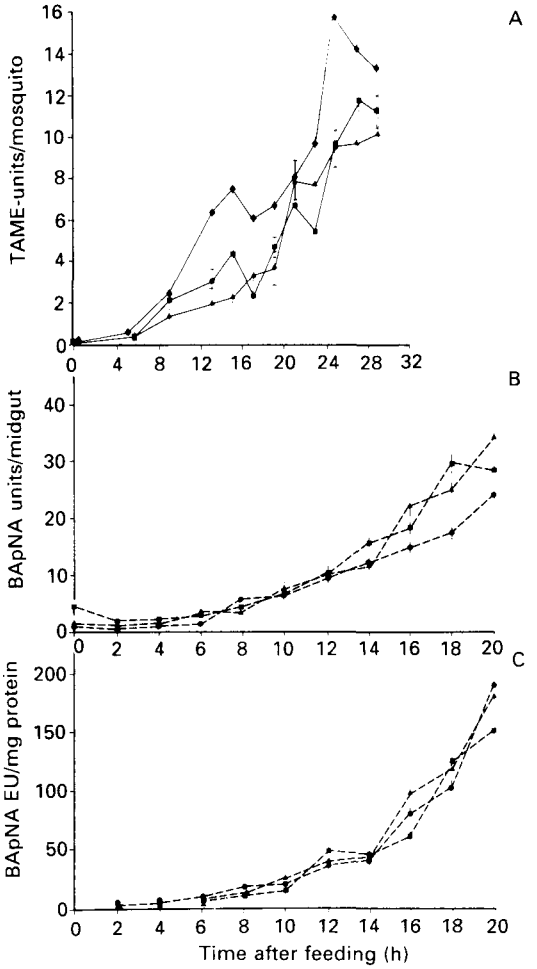

Fig. 3. Course of trypsin activity in *Anopheles stephensi* strains following a bloodmeal (see legend of Fig. 1). (A) Trypsin activity in whole body homogenates of mouse blood-fed mosquitoes (—) of the strains Kasur (◆), Pb3-9a (■) and Sda-500 (▲). (B) Trypsin activity in midgut homogenates of pig blood-fed mosquitoes (.....) of the strains Punjab (●), Pb3-9a and Sda-500. (C) Specific trypsin activity calculated from the data of Figs 2B and 3B.

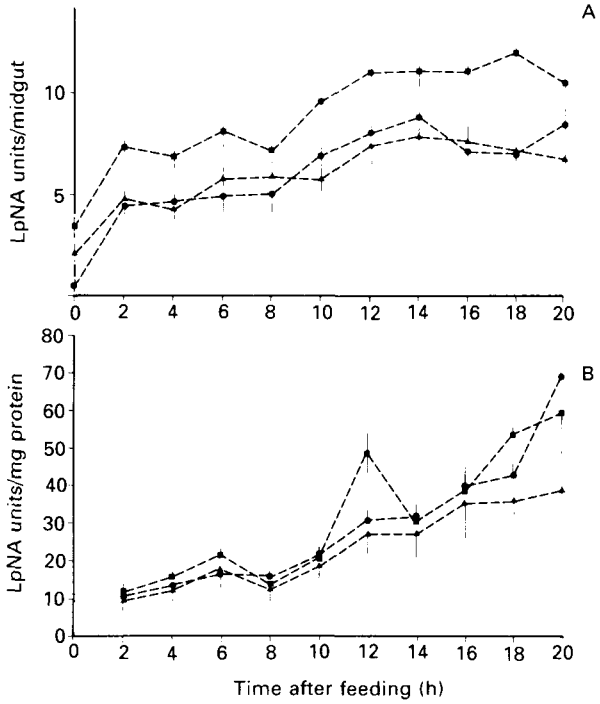

Fig. 4. Course of aminopeptidase activity in midgut homogenates of pig blood-fed females of the *Anopheles stephensi* strains: Punjab (●), Sda-500 (▲) and Pb3-9a (■) (see legend of Fig. 1). (A) Aminopeptidase activity (EU/midgut). (B) Specific aminopeptidase activity, calculated from data of Figs 2B and 4A.

moglobin contents of the midgut has been previously noted in the ticks *Hyalomma anatolicum anatolicum* and *Rhipicephalus appendiculus*, and has been attributed to their ability to synthesize haemin even after blood feeding (Reid & Boid, 1984). Similarly, while induction of digestive enzymes follows the same pattern in all strains, the haemoglobin becomes available as a protease substrate several hours later in susceptible than in refractory females.

The study compared mosquitoes of different ages (2–4 and 5–6 days post-emergence in Basel and Wageningen respectively) provided with different blood sources. While age could account for observed differences in the lag period or other digestion characteristics (Briegel, 1983; Downe & Archer, 1975), it was encouraging that the trends in enzyme cycles, haemoglobin degradation and protein digestion were similar when strains were compared. The estimated lag phases were independent of the blood source in Pb3-9a, while in the Punjab and Sda-500 strains, pig blood feeding resulted in significant lag phase reduction (Table 1). The regression coefficient for post-lag phase haemoglobin degradation was always greatly increased (5- to 13-fold) after feeding pig blood, but without further experimentation it is impossible to determine whether this resulted from dietary or methodological differences. Haemoglobin is probably transferred to other tissues after partial degradation (Wigglesworth, 1943) and both midgut and extra-midgut haemoglobin would be detected in whole-body homogenates. One other contributing factor may be that as the pig blood was lyophilysed, its haemoglobin was no longer contained within erythrocytes and may therefore have been more accessible to immediate degradation by midgut enzymes. Nevertheless, the lag phases were consistent within strains and corresponded very well with the degree of strain susceptibility.

The role of digestive proteinases has been investigated in experimental infectivity of *Ae. aegypti* by *P. gallinaceum* (Gass, 1977; Gass & Yeates, 1979; Yeates & Steiger, 1981). Partially purified digestive proteinases damage ookinetes *in vitro*, and when mosquito digestion was stimulated ahead of infection, artificially high levels of digestive enzymes were correlated with similar damage to immature

ookinetes in the midgut. Conversely, when *P. berghei* ookinetes were fed to *Anopheles atroparvus*, infectivity was increased, particularly in a refractory mosquito line (Janse *et al.* 1985). While these latter results were interpreted as physical obstruction of the microgametes by erythrocytes concentrated in the midgut lumen, thus preventing formation of the zygote, the low level of active digestive proteases and of a fully formed peritrophic membrane shortly after the bloodmeal when the ookinetes penetrated the midgut wall, could have contributed to improved infectivity.

The larger bloodmeal ingested by Pb3-9a females did not induce higher trypsin nor aminopeptidase activities, which had been expected given the secretogogue control of digestive proteases in the midgut (Gooding, 1973; Yang & Davies, 1971; Briegel & Lea, 1975). Before bloodmeal ingestion, residual aminopeptidase activity in the midgut of Pb3-9a females was 1·7 times higher than in susceptible females, and this difference was maintained during the subsequent post-feeding course of activity. This elevated aminopeptidase activity may be one factor rendering females of Pb3-9a refractory to *P. falciparum*. Aminopeptidase is a likely candidate for such involvement as it is active both before and after feeding, and approximately 50% of the activity is associated with the midgut wall (Graf & Briegel, 1982; Billingsley, 1989) through which the ookinete must penetrate. The absence of any differences in trypsin activities between strains indicates that this enzyme probably plays no role in conferring refractoriness or susceptibility. The results further imply that separate physiological control mechanisms may exist for both trypsin and aminopeptidase synthesis and secretion (Billingsley, 1989). This must be considered in future experiments.

In other blood-feeding insects, haemolytic factors are produced to lyse the bloodmeal soon after feeding (Azambuja, Guimaraes & Garcia, 1983). No haemolytic factor has been demonstrated in the mosquito midgut and the prolonged presence of intact erythrocytes in the centre of the midgut lumen suggests that specific haemolysis does not occur. It is possible that the cuticular spines in the cibarium of the mosquito are important in the physical release of soluble erythrocyte factors (Coluzzi *et al.* 1982) and that events taking place in the midgut are therefore pre-determined to a certain degree during the feeding process by non-midgut structures. This could account for the dissociation of haemoglobin degradation and protein digestion without the need for modification of the post-feeding enzymatic digestive events but, under such circumstances, differences between strains should have been lost after feeding the non-cellular lyophilysed blood.

Two properties of the Pb3-9a strain appear to counteract mechanisms that are responsible for suppression of parasite development in the midgut. Blood digestion is important for parasite development because of its dependancy upon an erythrocytic factor which is released during digestion (Behin, 1968; Rosenberg *et al.* 1984), and the larger infective bloodmeal should result in a greater number of oocysts per female (Hovanitz, 1947; Rutledge, Ward & Buckwalter, 1973). These two aspects, each of which should promote parasite development in the refractory stain, have somehow been overcome during strain selection, resulting in suppression of parasite development. Other midgut factors may therefore be important in the establishment of refractoriness or susceptibility. These will be considered in future experiments.

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