Blood digestion in the mosquito, *Anopheles stephensi*: the effects of *Plasmodium yoelii nigeriensis* on midgut enzyme activities

N. JAHAN[1]†, P. T. DOCHERTY[2]‡, P. F. BILLINGSLEY[2]* *and* H. HURD[1]

[1] *Centre for Applied Entomology and Parasitology, Department of Biological Sciences, Keele University, Staffordshire ST5 5BG*
[2] *Department of Zoology, University of Aberdeen, Tillydrone Avenue, Aberdeen AB24 2TZ*

(*Received 6 May 1999; revised 29 June 1999; accepted 29 June 1999*)

SUMMARY

Midgut proteases contribute to the success or failure of *Plasmodium* infection of the mosquito. This paper examines the reciprocal effect of *Plasmodium yoelii nigeriensis* on midgut trypsin, chymotrypsin, aminopeptidase and carboxypeptidase in the mosquito *Anopheles stephensi*. The total protein ingested and the rate of protein digestion were unaffected by the parasite, but more protein was ingested at the first than the second bloodmeal. All peptidases were unaffected by the presence of the parasite during the first gonotrophic cycle, when ookinetes were penetrating the midgut. In the second gonotrophic cycle, trypsin and chymotrypsin were unaffected by growing oocysts, but aminopeptidase activity was reduced in the midguts of infected mosquitoes. Chymotrypsin activity was depressed and aminopeptidase activity elevated during the second gonotrophic cycle. *Plasmodium* infection has a negligible effect on bloodmeal digestion and does not limit the availability of the protein for egg production. The significance of changes in aminopeptidase activity when oocysts are present is discussed.

Key words: *Plasmodium*, *Anopheles stephensi*, trypsin, chymotrypsin, aminopeptidase, bloodmeal.

INTRODUCTION

The sporogonic cycle of *Plasmodium* spp. is associated intimately with the mosquito midgut. During this period the mosquito synthesizes and secretes into the midgut an array of digestive enzymes. The mechanisms that underlie successful parasite development are poorly understood. Some mosquito factors are essential for parasite development (Gwadz 1994; Sinden *et al*. 1996; Billingsley & Sinden, 1997; Billker *et al*. 1998; Shahabuddin *et al*. 1998) while others, initiated by blood feeding or the parasite, reduce parasite survival (Shahabudin *et al*. 1998). Invasion of the midgut initiates several responses in vectors, including increased mRNAs encoding defence molecules (Richman *et al*. 1997; Luckhart *et al*. 1998). Other responses contribute to refractoriness to malaria transmission and include activation of a phenoloxidase cascade (Collins *et al*. 1986) and non-specific esterases (Vernick & Collins, 1989).

Mosquito digestive enzymes can also affect vector competence. Immature ookinetes are susceptible to protease digestion while mature forms are relatively resistant (Gass & Yeates, 1979). The proteases also deactivate complement and macrophages in the midgut, both of which kill parasites (Grotendorst & Carter, 1987) and digest haemoglobin which is required for ookinete maturation. Midgut proteases can also enhance infection by activating parasite prochitinase to chitinase, thereby ensuring invasion of the peritrophic matrix (Shahabudin, 1998; Shahabuddin, Cocianich & Zieler, 1998), or reduce infection when elevated in selected strains (Feldmann, Billingsley & Savelkoul, 1989). Thus, the post-feeding kinetics of enzyme activity affect parasite development and invasion success at a number of levels (Billingsley & Sinden, 1997). However, little is known of any reciprocal effect of the parasite upon mosquito proteases. Here we examine whether the presence of *Plasmodium yoelii nigeriensis* can affect the post-feeding activity of four digestive peptidases and the efficiency of bloodmeal digestion in the mosquito, *Anopheles stephensi* during two gonotrophic cycles coinciding with ookinete invasion of the midgut wall and maturation of the oocyst.

* Corresponding author: Department of Zoology, University of Aberdeen, Tillydrone Avenue, Aberdeen AB24 2TZ, UK. Tel: +44 1224 272 882. Fax: +44 1224 272 396. E-mail: p.billingsley@abdn.ac.uk
† Current address: Lahore College for Women, Lahore, Pakistan.
‡ Current address: Medical Entomology Section, Laboratory for Parasitic Diseases, NIAID/NIH, Bethesda, MD 20892-0425, USA.

MATERIALS AND METHODS

Mosquito maintenance and infection

Anopheles stephensi (Dubai) Liston were maintained at 26±1 °C, 80% relative humidity and 12:12 h

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light/dark cycle. Adults were fed *ad libitum* 10% glucose with antibiotics (2·8 ml/l of a stock solution, of 10000 units penicillin and 10 mg streptomycin/ml 0·9% sodium chloride). Larvae were reared under standardized conditions to produce adult females of similar size (Jahan & Hurd, 1997). Male CD mice were infected with *Plasmodium yoelii nigeriensis* following one serial passage of infected blood from mice infected from cryopreserved stocks (Hogg & Hurd, 1995*a*). Parasitaemia and gametocytaemia were monitored in stained thin and fresh thick blood films respectively at 24–30 h post-infection.

Experimental protocol

Experiments were designed to observe the effect upon protein digestion and enzyme activity of invading ookinetes during the first gonotrophic cycle (GC1) and of growing oocysts during the second cycle (GC2). Two groups of nulliparous females (6 days post-emergence) were starved for 12–18 h then fed upon a non-infected mouse or infected (gametocytaemic, exflagellating) mouse 24–30 h post-infection. The packed-cell volumes (PCV) of control and infected mice were measured immediately prior to feeding mosquitoes. A subgroup of engorged females fed on the infected mouse were checked for oocysts 7 days post-blood-feeding. The wing length of each mosquito was measured (Jahan & Hurd, 1997) and those outside the range $3{\cdot}3 \pm 1$ mm were discarded. Fully engorged females were separated randomly into polystyrene pots (11·5 cm diameter), 24 per pot, and supplied with glucose solution containing 0·05% para-aminobenzoic acid (Peters & Ramkaran, 1980) until dissection. Additionally, mosquitoes were fed as above and groups (infected or control) of engorged females maintained as above. On day 7 post-blood-feed (PBF) both groups of mosquitoes were fed on the same uninfected mouse and fully engorged females were immediately separated into pots as above. Mosquitoes were sampled at 0 or 4, 12, 24, 36, 48 or 60 h PBF, anaesthetized on ice and midguts dissected into a drop of phosphate-buffered saline (PBS), within 2 h of chilling. Midguts were stored in a minimum volume of PBS at −20 °C until homogenization.

Preparation of midgut homogenates

Five (unfed), two (4 and 48–60 h PBF) or one (8–36 h PBF) midguts were homogenized in 200 μl of 0·15 M NaCl in a tight-fitting Teflon-glass homogenizer and centrifuged at 10000 ***g*** for 20 min at 4 °C. Supernatants were stored at −80 °C. Pellets were re-homogenized in 0·15 M NaCl/1% Triton–X 100, centrifuged at 10000 ***g*** for 20 min at 4 °C and these supernatants stored at −80 °C.

Trypsin, chymotrypsin and aminopeptidase assays

For trypsin assays *N*-benzoyl-DL-arginine-*p*-nitroanilide (BApNA) was dissolved in dimethylfluoride (DMF), while chymotrypsin (*N*-succinyl-ala-ala-*p*-nitroanilide) and aminopeptidase (L-leucine *p*-nitroanilide) substrates were dissolved in dimethyl sulfoxide (Me_2SO). Substrate buffers contained 2 mM substrate, 7% DMF or 3% Me_2SO in 100 mM Tris–HCl, pH 8·0. Triplicate 10 μl samples of midgut homogenate supernatants were added to 100 μl of substrate buffer in a microwell plate. The change in absorbance at 405 nm was monitored at 30 °C for 20 min in an Anthos III microplate reader. Kinetic rate was calculated by linear regression (Deltasoft programme, Biometallics Inc., Princeton, NJ) and enzyme activity calculated using an extinction coefficient of 8800 mM/cm (Erlanger, Kokowsky & Cohen, 1961). One enzyme unit is the activity required to hydrolyse 1 μmol substrate per min.

Carboxypeptidase

Twenty microlitres of supernatant were incubated with 20 mM Z–Gly–Phe in veronal buffer (35 mM Na-barbitone, 35 mM CH_3COONa, pH 5·5–9·5) at 37 °C for 1 h. The reaction was stopped by boiling. Released phenylalanine was detected by adding 300 μl L-amino acid oxidase reagent (10 mg *o*-dianiside-HCl, 6 mg amino acid oxidase, 2 mg peroxidase type II, 100 ml of 250 mM Tris–HCl, pH 8·0) and the absorbance was read at 405 nm (Nicholson & Kim, 1975). Enzyme activity (μM substrate hydrolysed per min) was calculated by reference to a standard curve of the optical density of phenylalanine.

Protein assays

Individual midguts were homogenized in 0·15 M NaCl and protein contained in the midgut was determined using a Coommassie Blue binding assay (Bio-Rad) according to manufacturer's instructions. Bovine serum albumen (BSA) was used as a standard.

Analysis of results

Mean E.U./gut for each time-point were compared between groups for significance using a two-way ANOVA and multiple means were compared using Tukey's test (MINITAB Inc., State College, PA).

RESULTS

Parasitaemia and PCV

The PCV of the infected mice (43–44%) was always slightly reduced compared to that of the control,

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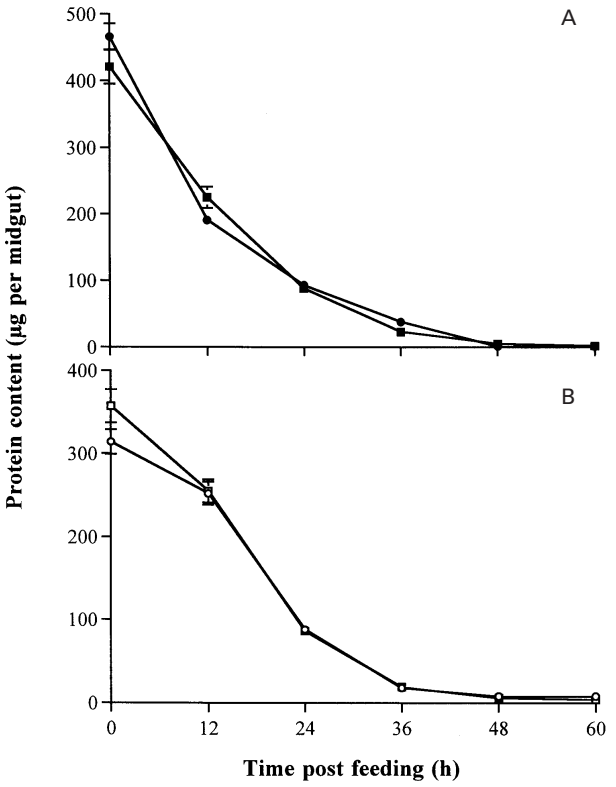

Fig. 1. Total protein content of the midgut of uninfected (○, ●) and infected (□, ■) *Anopheles stephensi* during digestion of a bloodmeal during the first (A, ●, ■) or second (B, ○, □) gonotrophic cycles. Each point represents the mean ± S.E. of 10–12 mosquitoes.

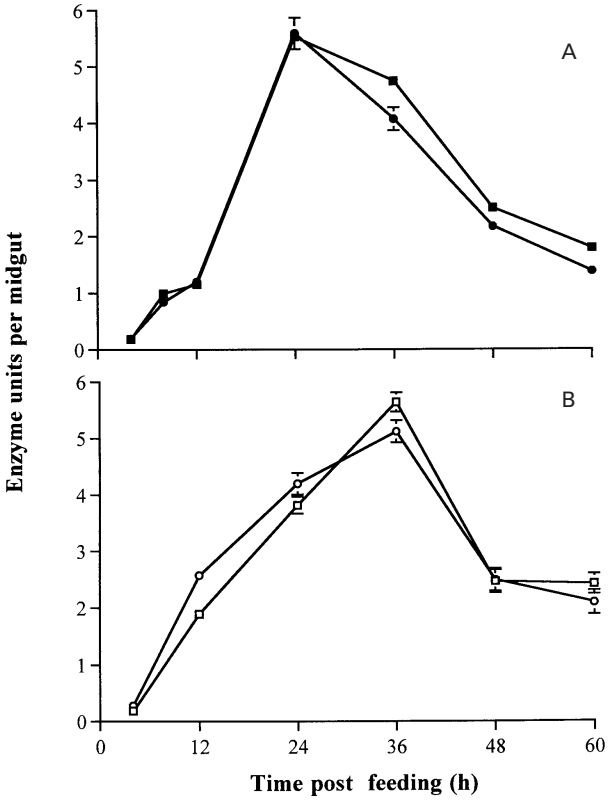

Fig. 2. Soluble trypsin activity in the midgut of uninfected and infected *Anopheles stephensi* during digestion of a bloodmeal during the first (A) or second (B) gonotrophic cycles. Each point represents the mean ± S.E. of 4–6 mosquitoes. For legend see Fig. 1.

uninfected mice (45–46 %). Total parasitaemia ranged from 10 to 15 % and all blood samples from infected mice contained exflagellating gametocytes (density not estimated) at the time of blood-feeding. The prevalence of infection in mosquitoes was 100 % and the density of infection 50–150 oocysts per mosquito in each experiment.

Protein digestion

Nulliparous mosquitoes ingested the same size bloodmeals from uninfected or infected mice (Fig. 1A). Similarly, the presence of oocysts did not affect protein ingestion at the second bloodmeal (Fig. 1B). However, for both groups of mosquitoes, the amount of protein ingested during the second bloodmeal was significantly reduced ($P = 0{\cdot}0001$). In the first 48 h PBF, bloodmeals were digested more rapidly (approx. 9·8 μg protein digested/h) during GC1 (Fig. 1A) than GC2 (approx. 7·1 μg protein digested/h) (Fig. 1B), but in each case, > 98 % of the bloodmeal was digested by 60 h PBF. Infection had no significant effect on the protein content of the midgut at any time-point PBF in either cycle (GC1, $F = 0{\cdot}43$; D.F. = 1, 125; $P = 0{\cdot}514$ or GC2 $F = 1{\cdot}41$; D.F. = 1, 127; $P = 0{\cdot}238$).

Trypsin

Trypsin was active only in soluble fractions. Activity during GC1 increased gradually from 4 to 12 h then rapidly from 12 to 24 h, before gradually declining from 24 to 36 h and rapidly declining from 36 to 60 h PBF (Fig. 2A). No significant differences between infected and control females were observed ($F = 2{\cdot}30$; D.F. = 1, 64; $P = 0{\cdot}134$). At 60 h, 24·7 % and 32·5 % of the maximum activity was found in uninfected and infected mosquitoes respectively. The peak trypsin activity during GC2 was slightly delayed to 36 h PBF in both groups, but the overall pattern and peak activities were similar to those during GC1 (Fig. 2B). There were no significant differences between infected and control females ($F = 0{\cdot}29$; D.F. = 1, 57; $P = 0{\cdot}589$).

Chymotrypsin

Chymotrypsin activity was always restricted to the soluble midgut fraction and some activity was present in unfed mosquitoes. During GC1 activity increased rapidly from 4 to 12 h, then gradually to a peak at 24 h PBF after which activity declined in an almost linear fashion (Fig. 3A). Activity was un-

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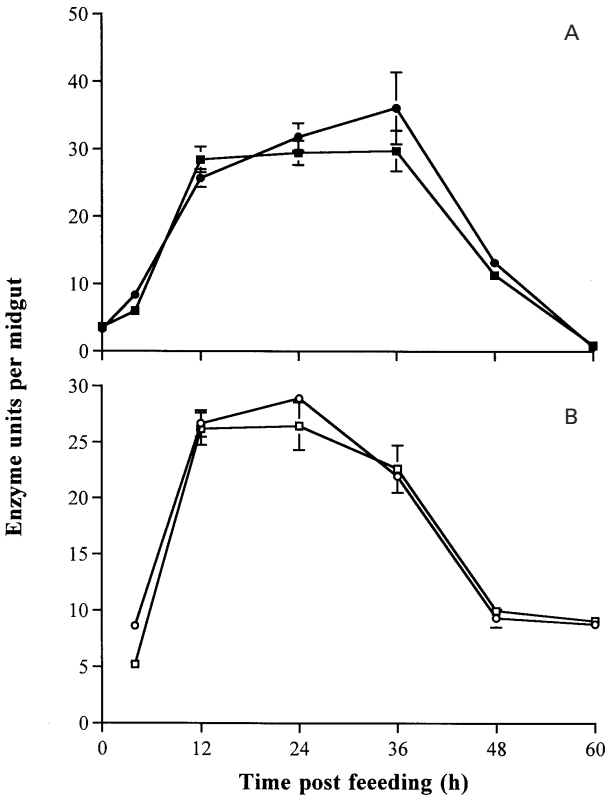

Fig. 3. Soluble chymotrypsin activity in the midgut of uninfected and infected *Anopheles stephensi* during digestion of a bloodmeal during the first (A) or second (B) gonotrophic cycles. Each point represents the mean ± S.E. of 4–6 mosquitoes. For legend see Fig. 1.

affected by infection during GC1 ($F=2{\cdot}12$; D.F. = 1, 60; $P=1{\cdot}51$). A similar pattern occurred during GC2 (Fig. 3B) and there was no significant difference between treatments at any time ($F=1{\cdot}43$; D.F. = 1, 51; $P=0{\cdot}24$; Tukey's test $P>0{\cdot}05$). At 60 h after the second bloodmeal, 30·4 % and 34·3 % of the maximum activity was present in uninfected and infected mosquitoes, respectively.

Aminopeptidase

Aminopeptidase was active in both soluble and membrane fractions. Approximately 20·7 % of the maximum soluble activity was present in nulliparous (sugar fed) females at 6 days post-emergence. During GC1, soluble activity gradually increased from 4 h PBF to peak at 24 h (Fig. 4A). There was no significant difference due to infection at any time during GC1 ($F=0{\cdot}89$; D.F. = 1, 48; $P=0{\cdot}35$) although peak activity was depressed in infected mosquitoes. In GC2, soluble aminopeptidase activity followed a similar post-feeding activity pattern, but was reduced in infected females up to 24 h post-feeding ($F=9{\cdot}32$; D.F. = 1·50; $P=0{\cdot}004$) and peak activity was delayed (Fig. 4B).

Membrane-associated aminopeptidase activity showed less clear post-feeding trends. During GC1, activity peaked slightly at 12 PBF then declined gradually until 60 h in both control and infected groups (Fig. 4C). Activity was significantly increased in infected mosquitoes at 4 h PBF (Tukey's test $P>0{\cdot}05$) but not at any other time. During GC2, the membrane-associated aminopeptidase activity in uninfected mosquitoes showed a clear peak at 24 h PBF, then a subsequent decline to 36 h (Fig. 4D). Activity during GC2 was higher than during GC1 at all times after 4 h PBF ($F=159{\cdot}5$; D.F. = 1; $P=0{\cdot}0001$). The presence of oocysts was associated with a significant reduction in activity at 24 h and a significant elevation at 60 h, but there was no peak activity (Fig. 4D).

Carboxypeptidase

Carboxypeptidase was active only in the soluble supernatants. Activity increased during GC1 to peak at 24 h PBF then declined to baseline levels at 60 h (Fig. 5). There was no difference in carboxypeptidase activities between infected and uninfected mosquitoes during GC1. Activity was not assayed after the second bloodmeal.

DISCUSSION

Mosquitoes respond to ookinete penetration of the midgut epithelium by up-regulation of molecules associated with the immune response (Richman *et al.* 1996; Dimopoulos *et al.* 1997), resorption of developing follicles (Carwardine & Hurd, 1997), and an increase in yolk protein content of the haemolymph (Jahan & Hurd, 1998). At later stages in the sporogonic cycle similar changes in vitellogenesis again result in reduced fecundity (Hogg & Hurd, 1995*b*; Jahan & Hurd, 1997, 1998; Ahmed *et al.* 1999), blood-feeding behaviour is altered (Anderson, Koella & Hurd, 1999), or a phenoloxidase response may be triggered (Collins *et al.* 1986). However, parasite-modulated modifications to the vector physiology are generally poorly understood (Maier, Becker-Feldman & Seitz, 1987). Blood proteins are digested by midgut proteases (Billingsley & Hecker, 1991) and the resultant amino acids used to synthesize vitellogenin. Thus infection-induced changes in protein intake or the activity of digestive enzymes could affect egg production. Furthermore, an alteration in the post-feeding dynamics of midgut protease activities could alter the susceptibility of the mosquito to *Plasmodium* infection (Sinden *et al.* 1996; Ramasamy *et al.* 1996).

P. y. nigeriensis infections eventually cause anaemia in mice although during the initial days of infection (parasitaemia of <15 %) this effect is minimal. Anopheline mosquitoes concentrate the bloodmeal during feeding (Vaughan, Hensley & Beier, 1994) and bloodmeal haemoglobin is not reduced when *A. stephensi* feeds upon gametocytaemic mice with a very slightly reduced PCV (Hogg & Hurd, 1995*b*;

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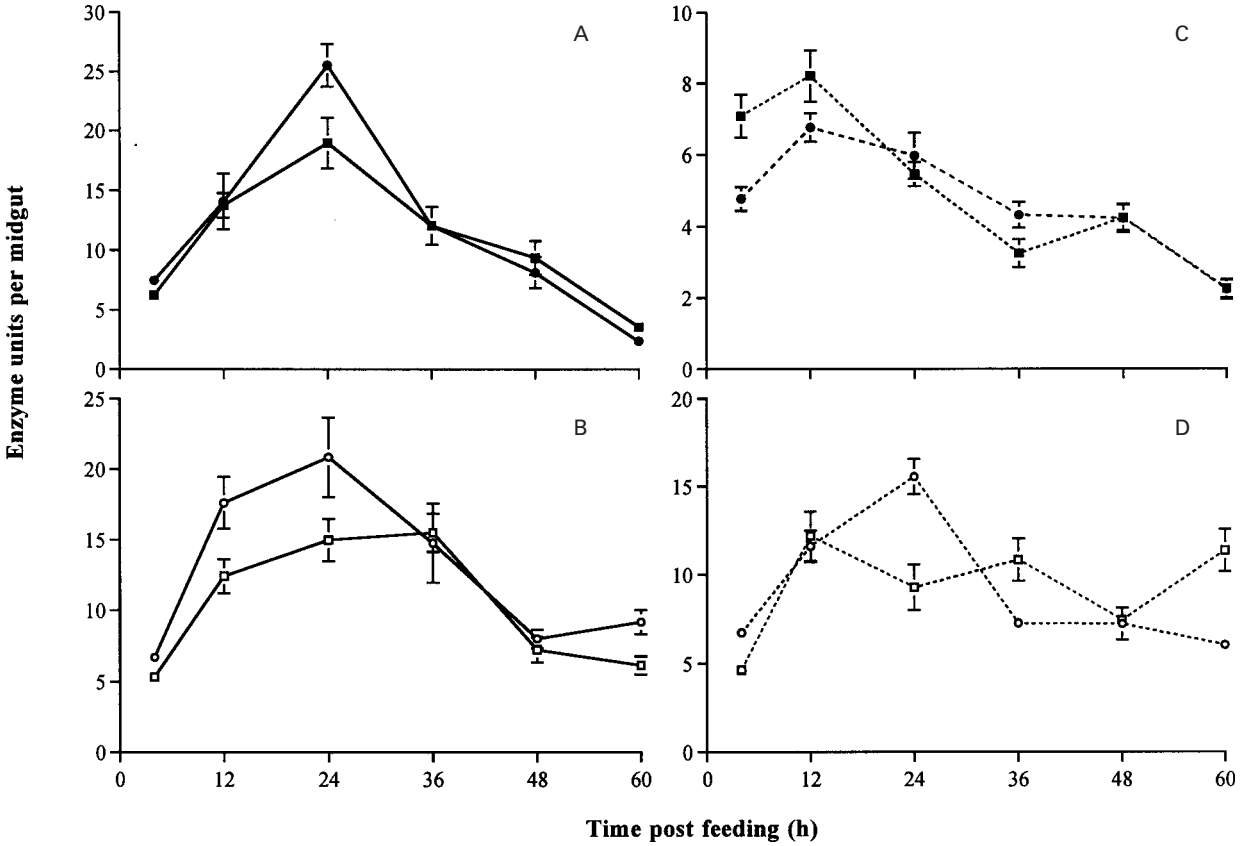

Fig. 4. Soluble (A, B, ——) and membrane-associated (C, D, ----) aminopeptidase activities in the midgut of uninfected and infected *Anopheles stephensi* during digestion of a bloodmeal during the first (A, C) or second (B, D) gonotrophic cycles. Each point represents the mean ± S.E. of 4–6 mosquitoes. For legend see Fig. 1.

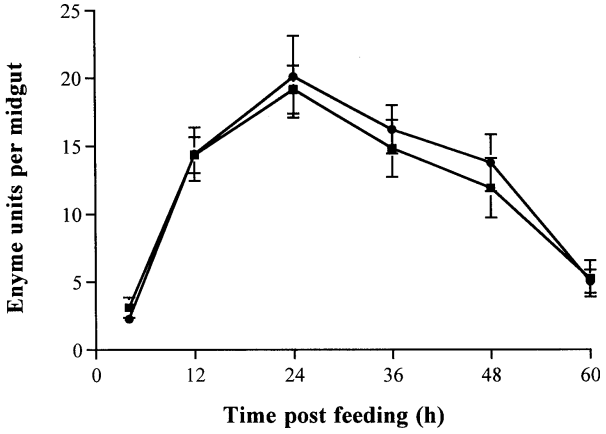

Fig. 5. Soluble carboxypeptidase activity in the midgut of uninfected and infected *Anopheles stephensi* during digestion of a bloodmeal during the first gonotrophic cycle. Each point represents the mean ± S.E. of 5 mosquitoes. For legend see Fig. 1.

Taylor & Hurd, personal observations). Here, the amount of protein ingested is similarly unaffected by infection at the ookinete or oocyst stages.

The time and peak height of enzyme activities differ according to mosquito species. Peaks of trypsin activity occur between 18 h (*A. albimanus*; Horler & Briegel, 1995) and 30 h (*A. stephensi*; Billingsley & Hecker, 1991). In this study, all enzymes peaked at 24–36 h PBF. *Aedes aegypti* and *Culex fatigans* fed on chick blood infected with *P. gallinaceum* showed a higher activity compared to those fed upon a normal chick (Gooding, 1966). Conversely, *A. stephensi* which, in contrast to culicines, will concentrate the blood during feeding, imbibed less protein from infected rather than uninfected chickens (Rudin, Billingsley & Saladin, 1991). Subsequent trypsin activity was reduced in mosquitoes with parasites in the bloodmeal only after 12 h PBF, suggesting that only early trypsin was stimulated (Felix *et al.* 1991). In the present study, enzyme activities and post-feeding protein digestion rates were largely unaffected by the infective blood feed, even though ookinetes were burrowing through the midgut from about 12 h PBF onwards (Vaughan *et al.* 1994). Therefore *P. y. nigeriensis*-induced fecundity reduction during the first gonotrophic cycle is not caused by quantitative changes in bloodmeal protein or digestive enzyme activities.

The transient, but significant, changes in aminopeptidase activity observed during the second cycle may have occurred as a result of oocyst-induced changes in midgut cell activity or as a result of damage caused by migrating ookinetes, even though this event occurred several days earlier. While a specific midgut cell type is preferentially invaded by

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P. gallinaceum ookinetes (Shahabuddin & Pimenta, 1998) and a variety of cell types exist in the mosquito midgut (Veenstra *et al*. 1995), whether there is cell-specific production of proteases and whether these cells are affected directly by the parasite remains unknown. Aminopeptidase activity is higher in a strain of *A. stephensi* selected for refractoriness to *P. falciparum* (Feldmann *et al*. 1990), and the parasite may confer some reciprocal down-regulation of aminopeptidase in order to enhance infection. As insect digestive enzymes are usually secretogogue regulated (Lehane, Müller & Crisanti, 1996), the parasite may affect a rate-limiting step within an aminopeptidase-specific secretogogue pathway. Aminopeptidase is the only membrane-associated protease of those studied (Billingsley, 1990), and in *Manduca* is the receptor for *Bacillus thuringiensis* Cry1Ac endotoxin (Cooper *et al*. 1998).

Although parasites had minimal effect on enzyme activities *per se*, consistent differences were observed between mosquitoes at each gonotrophic cycle. Compared to those in GC1, mosquitoes offered a second meal ingested 20–40 % less protein, produced less chymotrypsin and more aminopeptidase. The decrease in protein may be attributable to reduced capacity for ingestion as all other synthetic organs would occupy more space in the body cavity. Indeed, mosquitoes previously provided with a saline meal containing latex particles will ingest similar amounts of protein to those receiving their first bloodmeal (Billingsley & Rudin, 1992). If secretogogue mechanisms are enzyme specific (Briegel & Lea, 1975), any change in one enzyme could alter the digested peptide components within the lumen and thereby the secretogogue of other primary hydrolases. Thus, if one enzyme, namely aminopeptidase is up-regulated by the products of trypsin digestion, then it would be under-produced when the trypsin: chymotrypsin ratio is increased.

We wish to acknowledge Pam Taylor for assistance with maintenance of malaria infections and the Government of Pakistan for funding to N.J. through a COT award. P.F.B. was funded for part of this work by the Royal Society and P.T.D. by a BBSRC–CASE award with Zeneca.

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