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Sex-specific and blood meal-induced proteins of *Anopheles gambiae* midguts: analysis by two-dimensional gel electrophoresis

GI Prévot*^{1,4}, C Laurent-Winter², F Rodhain¹ and C Bourgoïn*^{1,3}

Address: ¹Ecologie des Systèmes Vectoriels, Institut Pasteur, 25 Rue du Dr Roux, 75724 Paris Cedex15, France, ²Laboratoire de Chimie Structurale des Macromolécules, Institut Pasteur, 25 Rue du Dr Roux, 75724 Paris Cedex15, France, ³Biologie et Génétique du Paludisme, Institut Pasteur, 25 Rue du Dr Roux, 75724 Paris Cedex15, France and ⁴Present address: Université des Antilles et de la Guyane Campus Saint Denis BP 792, 97337 CAYENNE CEDEX

Email: GI Prévot* - ghislaine.prevot-linguet@guyane.univ-ag.fr; C Laurent-Winter - chwinter@pasteur.fr; F Rodhain - frodhain@pasteur.fr; C Bourgoïn* - cabourg@pasteur.fr

* Corresponding authors

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Abstract

Background: *Anopheles gambiae* is the main vector of *Plasmodium falciparum* in Africa. The mosquito midgut constitutes a barrier that the parasite must cross if it is to develop and be transmitted. Despite the central role of the mosquito midgut in the host/parasite interaction, little is known about its protein composition. Characterisation of *An. gambiae* midgut proteins may identify the proteins that render *An. gambiae* receptive to the malaria parasite.

Methods: We carried out two-dimensional gel electrophoresis of *An. gambiae* midgut proteins and compared protein profiles for midguts from males, sugar-fed females and females fed on human blood.

Results: Very few differences were detected between male and female mosquitoes for the approximately 375 silver-stained proteins. Male midguts contained ten proteins not detected in sugar-fed or blood-fed females, which are therefore probably involved in male-specific functions; conversely, female midguts contained twenty-three proteins absent from male midguts. Eight of these proteins were specific to sugar-fed females, and another ten, to blood-fed females.

Conclusion: Mass spectrometry analysis of the proteins found only in blood-fed female midguts, together with data from the recent sequencing of the *An. gambiae* genome, should make it possible to determine the role of these proteins in blood digestion or parasite receptivity.

Background

Anopheles gambiae is the main vector of the human malaria parasite, *Plasmodium falciparum*, in Africa. The number of cases of malaria, and their severity, have increased, leading to an increase in the social and economic burden of this disease [1,2]. The development of drug resistance in parasites and insecticide resistance in mosquitoes has contributed to this situation. Malaria incidence could be reduced by controlling parasite transmission by the

mosquito. The sporogonic development of *Plasmodium*, from gamete to oocyst formation, takes place in the lumen and epithelium of the mosquito midgut. Mosquito-specific factors probably determine the outcome of this sporogonic development. Indeed, xanthurenic acid, produced by the mosquito, is important for the exflagellation of parasite microgametes [3,4]. Trypsin, produced in the mosquito's digestive tract, probably activates parasite chitinase(s), facilitating the passage of the parasite

through the peritrophic matrix surrounding the parasite-containing blood meal in the mosquito [5,6]. Recent studies have shown that early sporogonic stages of *Plasmodium* parasite modulate the mosquito midgut immune response [7–9]. This suggests that certain immune molecules could be used to inhibit the development of *Plasmodium* in transgenic mosquitoes [10,11]. These studies, and others, were based on analyses of mRNA production; very little has been published concerning proteome analysis for mosquito midguts [7–9,12,13].

As the early phase of *Plasmodium* sporogonic development occurs at the same time as blood-meal digestion, the physiology and biochemistry of this process have been extensively studied [14–18]. Indeed, several digestive enzymes secreted within the midgut lumen have been characterised [17,19–21]. However, very few studies have focused on characterisation of the proteins of the mosquito midgut epithelium. Using monoclonal antibodies, Lal *et al.* [22] recently identified a set of midgut proteins that may be involved in *Plasmodium* development. Ghosh *et al.* [23] screened a phage display library and selected a peptide that recognised midgut protein(s), as yet unidentified. The production of this peptide in transgenic *Anopheles stephensi* mosquitoes reduced the development of *Plasmodium berghei* oocysts on the mosquito midgut wall [24].

We analysed the midgut protein profile of female *An. gambiae* by two-dimensional (2-D) gel electrophoresis. Midguts were isolated 19 h after feeding on uninfected human blood. This time course corresponds to the early phase of ookinete interaction with midgut cells in mosquitoes fed on the blood of gametocyte carriers. We compared the profile obtained with those from the midguts of males and females not fed on blood. We identified a set of proteins that were specifically produced and regulated in females following blood ingestion. The recently determined sequence of the *An. gambiae* genome could be used in the analysis of these proteins and their potential functions, and transgenesis may provide a new tool for studying the involvement of mosquito proteins in *Plasmodium* sporogonic development.

Methods

Mosquito rearing and blood-feeding

An. gambiae strain G3 was reared at 26°C, in conditions of 80% relative humidity and a 12/12 h light/dark cycle. For mass rearing, female mosquitoes were allowed to feed on the blood of an anaesthetised rabbit. The blood-fed females used in this analysis were first starved for 12 h and then fed on uninfected human blood, using a membrane-feeding device [25]. Unfed or partially fed females were discarded.

Midgut preparation

All dissections were performed on ice, in PBSI (phosphate-buffered saline containing 1 mM EDTA and 1 mg/ml Pefabloc®). Midguts were dissected from 4-day-old sugar-fed male and female mosquitoes. Midguts were isolated from blood-fed mosquitoes 19 hours after blood-feeding. Midguts from blood-fed mosquitoes were opened by a longitudinal incision and thoroughly rinsed in ice-cold PBSI to remove all traces of peritrophic matrix and gut contents. Dissected midguts were stored at -80°C until processing.

Protein extraction

Proteins were extracted as previously described [26]. Briefly, sixty midguts were placed in 150 µl of extraction buffer (0.6% sodium-dodecyl sulphate (SDS), 0.2% β-mercaptoethanol, 10 mM Tris-HCl pH 8.0) supplemented with a cocktail of protease inhibitors (1 µg/ml antipain; 1 µg/ml aprotinin; 1 mM EDTA; 100 µM TPCK; 1 µg/ml leupeptin; 1 mg/ml Pefabloc®; 1 µg/ml pepstatin (Boehringer Mannheim). Samples were homogenized with a Wheaton-33® Potter-Elvehjem tissue grinder, transferred to an Eppendorf tube and boiled for 4 minutes. The samples were centrifuged for 10 minutes at 9,000 g, 4°C, and the supernatant was transferred to a fresh tube. Proteins were concentrated and SDS and β-mercaptoethanol eliminated by precipitation with acetone as follows: 1.5% 1 N NaOH and 9 volumes of ice-cold acetone were added to each protein sample. Samples were incubated for 3 hours or overnight at -20°C and centrifuged for 10 minutes at 9,000 g, 4°C. Pellets were dried for 1 hour under vacuum, using a "speed vac" (Savant) and resuspended in either distilled water, for protein determination, or sample buffer (9.95 M urea; 4% NP-40; 2% ampholines; 100 mM dithiothreitol), for 2-D electrophoresis. For each extract, proteins were precipitated from two separate aliquots: a 30 µl aliquot for protein determination, and a 120 µl aliquot for 2-D electrophoresis.

Protein determination

Protein concentrations were determined in duplicate, using the Lowry-SDS method and microtitre plates [27,28]. Bovine serum albumin was used as a standard (1 to 20 µg). Absorbance at 630 nm was measured with a Biotech EL 311 spectrophotometer.

Electrophoresis

Two-dimensional gel electrophoresis was performed essentially as described by Garrels [29], with the modifications of Laurent-Winter *et al.* [30]. For the first dimension (isoelectric focusing, IEF), we used gradient gels covering the range pH4 to pH8 (ampholines, Millipore Inc.), which were run for 20,000 Vh. We used 10% acrylamide gels for the second dimension. Gels were silver stained as described by Morrissey [31]. For each sample, 30 µg of

protein was run on the gel and molecular weight markers were applied in a separate slot. For each type of midgut (male, sugar-fed female and blood-fed female), at least three independent sample preparations were used and at least three independent gel analyses performed.

Data analysis

Silver-stained gels were examined by eye, using a light box. Protein extracts were compared in pairs by superimposing the corresponding 2-D gels. Differences in the pattern of spots between two extracts were scored if reproduced in at least three independent experiments. We considered only differences involving the presence or absence of a particular spot but differences in the intensities of spots for proteins with identical migration patterns were also observed.

Spots and their corresponding proteins were classified into three categories: a) constitutive – detected in midgut extracts from both sugar-fed and blood-fed females; b) BDP – detected only in blood-fed mosquitoes; c) SDP – detected only in sugar-fed mosquitoes.

Results and Discussion

Midgut proteins extracted from male and female mosquitoes were analysed by 2-D gel electrophoresis. Gels were silver stained and protein profiles compared in pairs. At least three independent samples were analysed for each physiological condition. A representative example of the 2-D gel patterns observed is presented in Figure 1, with panels A and B corresponding to sugar-fed and blood-fed female mosquitoes, respectively, and panel C, to sugar-fed male mosquitoes. All extracts from male and female mosquitoes gave approximately 375 well-resolved spots. We identified a number of protein spots displaying a specific pattern of expression, with differences either between males and females or between sugar-fed and blood-fed females mosquitoes.

Sex-specific proteins from *An. gambiae* midguts

Comparison of 2-D electrophoresis profiles for male and female mosquitoes (Figure 1) revealed that the midguts of males and females differ by only a few proteins (Figure 2). Ten proteins were identified as specifically produced in male midguts and not in female midguts, whereas 23 proteins were identified as female-specific proteins. Some of these female-specific proteins were produced only after a sugar meal (SDP, for sugar meal-dependent proteins), some were produced only after a blood meal (BDP, for blood-meal-dependent proteins), and some were produced after both types of meal (constitutively expressed). Eight other proteins were common to the midguts of sugar-fed males and females and were absent from the midguts of blood-fed females. We took into account only differences in the presence/absence of protein spots, al-

though some spots, such as spots A and B, also reproducibly displayed differences in intensity (Figure 1, panel A).

Although midgut morphology differs between male and female mosquitoes, the cellular structure of the midgut is almost identical in males and females, consisting of columnar and regenerative cells [32–34]. The morphological differences may reflect specific functions, and may imply differences at the molecular level.

Both male and female mosquitoes feed on nectar. The ten male-specific proteins are therefore more likely to be involved in male-specific functions or structures than in nectar processing, whereas the eight proteins shared by sugar-fed males and females and absent from blood-fed females are probably involved in sugar processing. In contrast, as male mosquitoes never ingest blood, the BDP detected in the midguts of blood-fed females but not in males are presumably involved in blood digestion, a female-specific function.

Cazares-Raga *et al.* [35] analysed the sex-specific proteins and proteases present in the midguts of *Anopheles albimanus* mosquitoes. They detected approximately 150 well-resolved spots and found that the protein profiles of midguts from males and females differed considerably. The overall profile obtained with midguts from *An. albimanus* males was similar to that obtained in this study with midguts from *An. gambiae* males. However, the pattern for *An. albimanus* females mostly included low-molecular weight proteins. In our experimental conditions, profiles of this type were invariably associated with proteolysis of the sample, resulting in the accumulation of proteins in the lower part of the gel.

Identification of midgut proteins regulated upon blood ingestion

Comparison of protein patterns from sugar-fed and blood-fed female *An. gambiae* revealed a limited number of differences. Five of the 23 female-specific proteins (Figure 2) were produced in the midguts of both sugar-fed and blood-fed females; these proteins were classified as constitutive proteins. Eight proteins were present only in sugar-fed females (SDP). The ten remaining proteins were present in the midguts of females only after a blood meal (BDP). In addition to differences in the presence/absence of protein spots, we also observed reproducible differences in spot intensity for another small set of proteins. The amount of these proteins either decreased (spots A & B panel A) or increased (spots C-F, panel B) upon blood-feeding.

Proteins specifically produced in blood-fed mosquitoes and proteins overproduced upon blood-feeding are likely to be involved in blood digestion or in synthesis of the

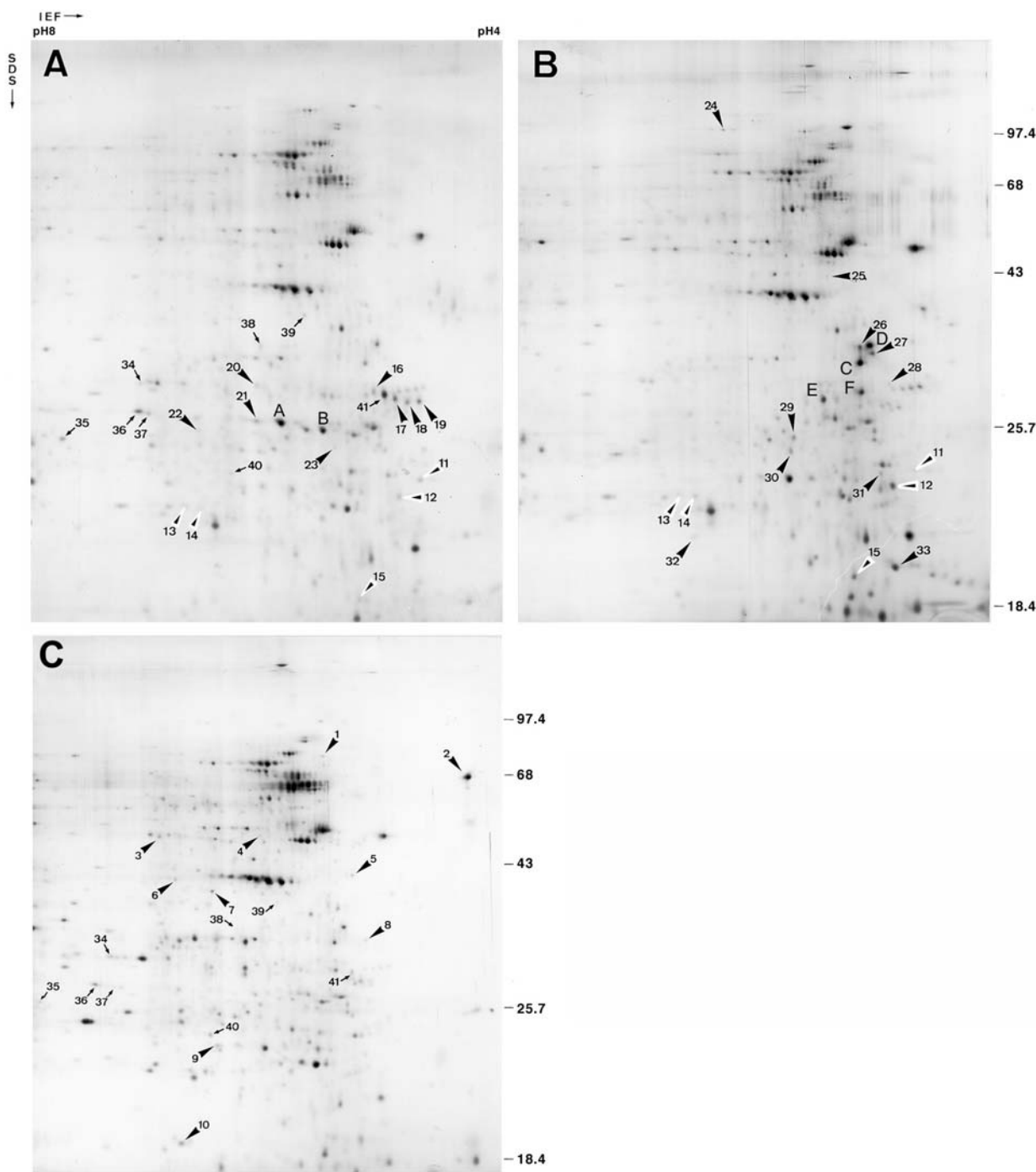


Figure 1

Silver-stained 2-D gels of *Anopheles gambiae* midgut proteins: **(A)** Sugar-fed females. **(B)** Blood-fed females. **(C)** Males. Black arrowheads: sugar-meal-dependent proteins (panel A), blood-meal-dependent proteins (panel B), male-specific proteins (panel C); white and black arrowheads: constitutive female-specific proteins (panels A and B); thin arrows: proteins detected in both sugar-fed females and males (panels A and C). Numbers refer to proteins described in Figure 2; lettered spots are discussed in the main text. The molecular weights of standards (kDa) are indicated on the right-hand side of the gels.

Male	Sugar-fed Female	Blood-fed Female	Number of spots	Effect of the meal	Spot Number
-	+	+	5	Constitutive	11 - 15
-	+	-	8	SDP	16-23
-	-	+	10	BDP	24-33
+	-	-	10	-	1-10
+	+	-	8	SDP	34-41
+	+	+	358	None	not numbered

Figure 2

Midgut protein differences between male and female *Anopheles gambiae* mosquitoes. Spot number refers to the labelling used in Figure 1. SDP denotes a protein detected in sugar-fed mosquitoes that was absent from blood-fed mosquitoes; BDP: absent from sugar-fed mosquitoes and present in blood-fed mosquitoes; constitutive: present in both blood-fed and sugar-fed mosquitoes [Female-specific proteins: dark shaded fields; Male-specific proteins: light shaded fields].

peritrophic matrix that surrounds the blood meal. Alternatively, some of these proteins may be involved in defence reactions directed against micro-organisms ingested along with the blood meal. We conducted our analysis 19 h after feeding. At this time, *Plasmodium* ookinetes interact with midgut cells if the mosquito fed on *Plasmodium* gametocyte-containing blood; some of the BDP may therefore be involved in host-parasite interactions as well. Indeed, in a well-adapted host-parasite system, such as that constituted by *P. falciparum* and *An. gambiae*, the parasite probably makes use of pre-existing host molecules to ensure its development at a lower cost to the host. If this is indeed the case, then some of the BDP identified here may be of relevance to *P. falciparum*/*An. gambiae* midgut interactions.

In previous studies of differences between *An. stephensi* strains refractory and susceptible to infection by *P. falciparum*, we also detected sugar-meal and blood-meal-de-

pendent proteins in female mosquitoes [26]. The main difference between the refractory and susceptible *An. stephensi* lines was the larger number of BDP in the susceptible line. This observation is consistent with the possible involvement of some midgut BDP in *Plasmodium*/*Anopheles* midgut interactions.

Conclusions

Overall, we found 16 SDP in the midguts of *An. gambiae* females: eight were female-specific and the other eight were present in both males and females. We also found ten BDP. All the BDP were female-specific, as males do not feed on blood. The characterisation of BDP by mass spectrometry of protein spots isolated from gels will be greatly facilitated by the recent completion of the whole genome sequence of *An. gambiae* [36]. This will undoubtedly provide additional information as to whether these proteins are involved in blood digestion or in the development of the malaria parasite. The characterisation of female-specif-

ic proteins may also be useful for the identification of blood meal-inducible, female-specific promoters, which could be used to drive the expression of genes encoding proteins impairing the development of *Plasmodium* parasites in transgenic mosquitoes [37].

Authors' contributions

GP carried out mosquito manipulation and dissection, protein preparation and gel analyses. C.L.-W. carried out 2-D gel electrophoresis. FR provided the scientific environment and CB conceived and supervised the experimental work and the writing of the manuscript.

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