Determination of mosquito bloodmeal pH *in situ* by ion-selective microelectrode measurement: implications for the regulation of malarial gametogenesis

O. BILLKER[1]†, A. J. MILLER[2] *and* R. E. SINDEN[1]*

[1] *Department of Biology, Imperial College of Science, Technology and Medicine, London SW7 2AZ, UK*
[2] *Institute of Arable Crops Research, Rothamsted, Harpenden, Herts AL5 2JQ, UK*

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SUMMARY

Malarial gametocytes circulate in the peripheral blood of the vertebrate host as developmentally arrested intra-erythrocytic cells, which only resume development into gametes when ingested into the bloodmeal of the female mosquito vector. The ensuing development encompasses sexual reproduction and mediates parasite transmission to the insect. *In vitro* the induction of gametogenesis requires a drop in temperature and either a pH increase from physiological blood pH (*ca* pH 7·4) to about pH 8·0, or the presence of a gametocyte-activating factor recently identified as xanthurenic acid (XA). However, it is unclear whether either the pH increase or XA act as natural triggers in the mosquito bloodmeal. We here use pH-sensitive microelectrodes to determine bloodmeal pH in intact mosquitoes. Measurements taken in the first 30 min after ingestion, when malarial gametogenesis is induced *in vivo*, revealed small pH increases from 7·40 (mouse blood) to 7·52 in *Aedes aegypti* and to 7·58 in *Anophěles stephensi*. However, bloodmeal pH was clearly suboptimal if compared to values required to induce gametogenesis *in vitro*. Xanthurenic acid is shown to extend the pH-range of exflagellation *in vitro* in a dose-dependent manner to values that we have observed in the bloodmeal, suggesting that *in vivo* malarial gametogenesis could be further regulated by both these factors.

Key words: *Anopheles stephensi*, *Aedes aegypti*, midgut, pH, *Plasmodium berghei*, gametogenesis, xanthurenic acid.

INTRODUCTION

A temperature drop to at least 5 °C below that of the body temperature of the vertebrate host has been identified as an obligatory inducer of malarial gametogenesis *in vitro* and *in vivo* (reviewed by Sinden *et al*. 1996). However *in vitro* studies suggest the temperature stimulus alone is not sufficient, and either a pH increase from physiological blood pH (*ca* pH 7·4) to about pH 8·0, or the presence of a gametocyte-activating factor (GAF) present in mosquito extract are additionally required (Billker *et al*. 1997). GAF has recently been identified as xanthurenic acid (XA), a tryptophan metabolite of mosquitoes and vertebrates (Billker *et al*. 1998; Garcia *et al*. 1998). However, it is unclear whether either the pH increase or XA act as natural triggers in the mosquito bloodmeal. XA, although a prominent mosquito metabolite (Li & Li, 1997), is also present in mammalian plasma (Truscott & Elderfield, 1995). It remains to be shown therefore whether the gametocytes are induced by changes in XA levels that might occur in the bloodmeal.

* Corresponding author: Department of Biology, Imperial College of Science, Technology and Medicine, Sir Alexander Fleming Building, Imperial College Road, London SW7 2AZ, UK. Tel: 0171 594 5425. Fax: 0171 594 5424. E-mail: r.sinden@ic.ac.uk
† Present address: Max-Planck-Institut für Infektionsbiologie, Monbijoustraße 2, D-10117 Berlin, Germany.

It is well established that the loss of CO_2 from blood equilibrating with air leads to a pH increase that is sufficient to induce gametogenesis *in vitro* (Chorine, 1933; Carter & Nijhout, 1977; Nijhout & Carter, 1978). It is currently unclear what changes occur in bloodmeal pH *in vivo* in the mosquito. Using ion-selective microelectrodes (ISMs), commonly used for intracellular pH-measurements, we investigated the potential of changes in bloodmeal pH as a second (co-) inducer of gametogenesis *in vivo*.

MATERIALS AND METHODS

Mosquitoes

The refm strain of *Aedes aegypti* was obtained from Dr R. Lawrence, London School of Hygiene and Tropical Medicine. The SDA500 strain of *Anopheles stephensi* was maintained at Imperial College. Mosquitoes were reared as previously described (Sinden, 1996). Adults were maintained on autoclaved fructose/PABA (fructose 80 g/l; *p*-aminobenzoic acid 0·5 g/l in distilled water) at 24 °C, 70–80 % relative humidity and with a 12 h light/12 h dark cycle.

Microelectrode construction and calibration

Double-barrelled solid pH-selective membranes were solvent-cast into the tips of glass micropipets as

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described previously (Miller & Smith, 1992; Miller, 1994). The proton-sensitive barrel was back-filled with 120 mM NaCl containing 10 mM KH_2PO_4, 20 mM MOPS, pH 6·0. One hundred mM NaCl with 300 U/ml heparin, pH 7·5, was used to back-fill the reference barrel. Immediately before use the back-filled, submicron-tipped electrodes were deliberately broken back slightly, taking care not to displace the ion-sensitive membrane, to give resistances of 6–12 GΩ. Such 'blunt' tips were sufficiently strong to penetrate the insect's cuticle without further electrode breakage. The reference barrel was connected to earth and recordings were made from the proton-sensitive barrel relative to ground with an FD 223 high-input differential electrometer (World Precision Instruments, USA). Voltage recordings were digitized at a sampling frequency of 10 Hz by an analogue/digital converter (Labmaster DMA/PGH; Scientific Solutions, USA) and processed on a personal computer running the VISER software developed by I. R. Jennings (Biology Department, University of York, UK). Electrical potential readings from the proton-selective barrel were recorded in mV and, after re-calibration of the electrode, automatically converted into pH values using a calibration curve that was obtained from the combined calibration points recorded before and after each impalement by a 3rd order polynomial curve fit performed by the VISER software.

ISMs were calibrated at 5 points between pH 6·50 and 8·50 using 120 mM NaCl with 10 mM KH_2PO_4, buffered by 20 mM of either MES (pH 6·50), MOPS (pH 7·00/7·50) or TAPS (pH 8·00/8·50). The pH of calibration solutions was adjusted to $\pm 0{\cdot}01$ pH units using a conventional bench-top pH meter. From the average reproducibility achieved for the calibration curves before and after impalement individual pH measurements were calculated to have an accuracy $\pm 0{\cdot}014$ pH units. Microelectrode measurements were rejected when calibration curves before and after use differed by more than 3 mV (*ca* 0·07 pH units). In preliminary experiments, mouse blood lysate was found not to interfere with electrode performance (data not shown).

pH measurements

Female mosquitoes (4–15 days old) were starved overnight and allowed to feed for 10 min on uninfected Theiler's Original (TO) mice (Harlan, UK) anaesthetized by intramuscular injection of 50 μl of a mixture of 1 vol. Rompun™ (Bayer, Germany), 2 vols Vetalar™ (Pharmacia & Upjohn, Luxembourg) and 3 vols sterile phosphate-buffered saline. Fed mosquitoes were maintained at 21 °C, 80–100 % relative humidity. Between 10 min and 48 h after the beginning of feeding, mosquitoes were individually collected and placed with their backs on adhesive tape. Their posterior midguts were immediately impaled with a pre-calibrated ISM through the lateral intersegmental cuticular membrane between the 3rd and 4th abdominal segment. For repeated recordings from different regions of the posterior midgut each mosquito was impaled between abdominal segments 1/2, 3/4 and 4/5 in random order.

To determine mouse blood pH small quantities of blood were collected from the tail of anaesthetized mice directly into *ca* 0·05 ml of mineral oil (Sigma, UK) to exclude any contact with air. ISMs inserted into the droplet gave stable pH readings which seemed unaffected by coagulation of the blood. Preliminary experiments using drops of calibration solution showed that mineral oil did not itself affect the pH measurements.

Statistical analysis

All calculations and statistical analyses by Student's *t*-test were performed with pH values rather than hydrogen ion concentrations since only the former were normally distributed at all time-points.

Parasites and in vitro *exflagellation assay*

Plasmodium berghei ANKA clone 2.34 was maintained by cyclic passage in TO mice and *An. stephensi* and used between the 1st and 4th infected blood passage after mosquito transmission. To produce test solutions for the *in vitro* exflagellation assay foetal bovine serum supplemented with additional HEPES (to 40 mM) and containing 0, 2·5 or 10 μM xanthurenic acid (Sigma, UK) were adjusted to the desired pH (pH 7·00–8·30). Then 5 μl of blood containing mature gametocytes were taken from the tail of an anaesthetized mouse and immediately mixed with 195 μl of the test solutions. After exactly 15 min at 20 °C, when the maximum number of microgametocytes were in the process of releasing gametes, 8 μl of the mixture were placed in a modified Neubauer haemacytometer. Cells were allowed to settle for 1 min, after which exflagellation centres were counted by phase-contrast microscopy and expressed as a percentage of exflagellation induced by a reference solution at pH 8·0. The pH measured in the cell suspensions after the experiments, confirmed that under the conditions used the desired pH was stable over the time of the experiment. Each test solution was tested in triplicate.

RESULTS

The pH of tail blood from different mice ranged from 7·32 to 7·44 (average pH 7·40, $n = 11$) when contact with air was excluded. In contrast, when a microelectrode was inserted into 2–3 μl drops of tail blood exposed to air, the pH reached values of *ca* 8·0 within 10 min (data not shown).

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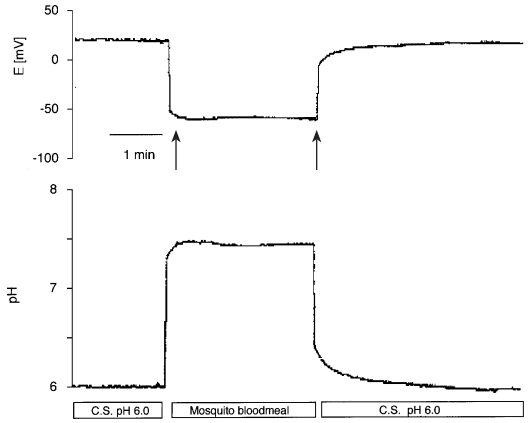

Fig. 1. A typical recording from a bloodmeal of *Anopheles stephensi* made 30 min after feeding on an anaesthetized mouse. The upper trace shows the output of the proton-sensitive barrel of the ISM, the lower trace the pH calculated by the software after re-calibration. Impalement of the gut and withdrawal of the electrode are indicated by arrows.

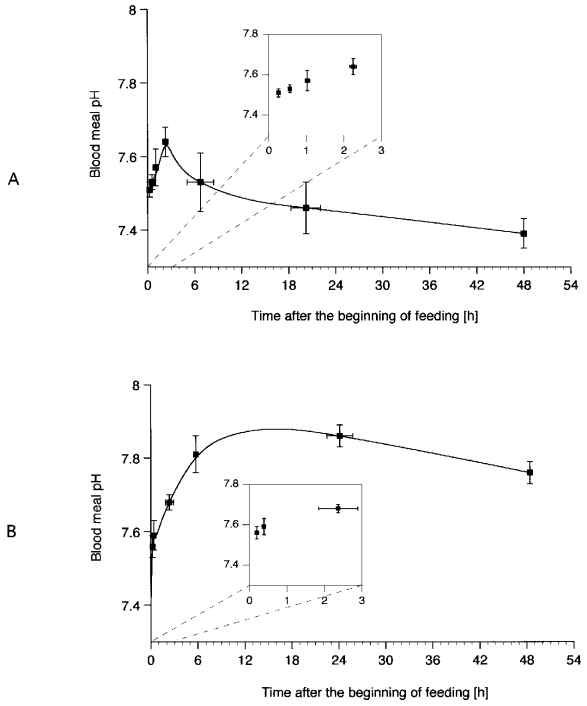

Fig. 2. Bloodmeal pH over time in (A) *Aedes aegypti* and (B) *Anopheles stephensi* fed on uninfected, anaesthetized mice. Insets show the detailed pH changes occurring in the first 3 h. Vertical error bars represent standard errors of 5–11 measurements from different mosquitoes except for the time point at 48 h at which only 3 and 4 independent measurements were taken in (A) and (B) respectively. The intricate techniques involved in the use of ISMs made measurements at exactly predetermined time-points impractical. Measurements were instead taken whenever a functional ISM and a fed mosquito were available. pH readings taken at similar time-points after feeding were pooled and horizontal error bars indicate the standard error along the time axis.

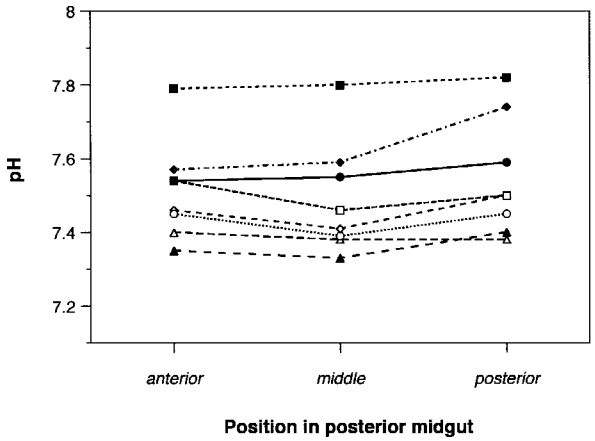

Fig. 3. pH in different regions of individual bloodmeals in *Aedes aegypti* fed on mice. Lines connect measurements from different regions of the same bloodmeal. Different individuals were measured between 15 min and 10 h after feeding.

Impaling the centre of the bloodmeal bolus through the abdomen of fed mosquitoes produced stable pH readings after an equilibration period of 1–3 min (Fig. 1). Bloodmeal pH measurements taken in the first 30 min after ingestion revealed small pH increases from 7·40 (mouse blood value) to 7·52 (range 7·46–7·58; $n = 7$) in *Ae. aegypti* (Fig. 2A) and to 7·58 (range 7·48–7·73; $n = 11$) in *An. stephensi* (Fig. 2B). At time-points between 10 and 30 min bloodmeal pH in both mosquito species was significantly different from the initial mouse blood pH (t_{Ae} 10–30 min/blood = 5·79, 16 D.F., $P < 0{\cdot}001$; t_{An} 10–30 min/blood = 6·70, 20 D.F., $P < 0{\cdot}001$) but the mosquito species did not differ significantly from each other ($t_{Ae\ 10\text{–}30\ \mathrm{min}/An\ 10\text{–}30\ \mathrm{min}} = 1.87$, 16 D.F., $P > 0{\cdot}05$). Over the next 2 h average bloodmeal pH further increased in both mosquito species. In *An. stephensi* this slow increase continued for 24 h and bloodmeal pH remained significantly elevated at all times. In marked contrast, in *Ae. aegypti*, the initial increase continued for only about 3 h thereafter the bloodmeal pH decreased again to *ca* 7·4. A between-species comparison of measurements taken later than 10 h after ingestion demonstrates this difference: the average pH was 7·82 (range 7·68–8·03; $n = 11$) in *An. stephensi* and 7·43 (range 7·26–7·60; $n = 9$) in *Ae. aegypti*. This species difference was highly significant ($t_{Ae\ 10\mathrm{h}\text{–}48\mathrm{h}/An\ 10\mathrm{h}\text{–}48\mathrm{h}} = 8{\cdot}41$, 18 D.F., $P \ll 0{\cdot}001$).

Multiple readings taken in the anterior, central and posterior regions of the bloodmeal of *Ae. aegypti* found no significant pH gradients along the anterior–posterior axis of the bloodmeal between 15 min and 10 h after feeding (Fig. 3).

In an infected bloodmeal gametogenesis is induced within the first 30 min of ingestion (Billker *et al.* manuscript in preparation). The frequency distribution of bloodmeal pH values we observed in *An. stephensi* during this period is shown in Fig. 4A. To determine whether these observed pH values might

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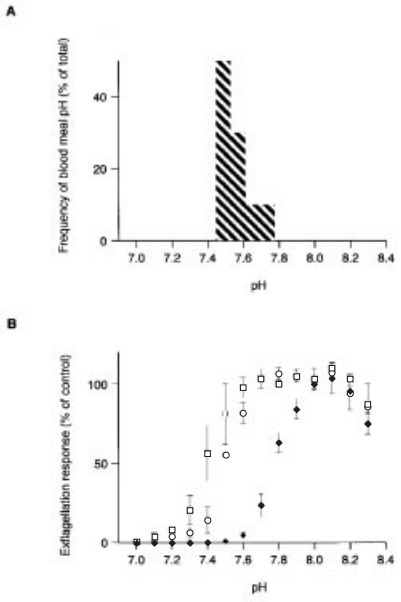

Fig. 4. The frequency distribution of midgut pH in *Anopheles stephensi* is compared to the pH response of exflagellation in *Plasmodium berghei in vitro*. (A) The shaded area shows the frequency distribution of pH among 10 bloodmeals of *An. stephensi* measured between 10 and 30 min after the beginning of feeding. (B) Exflagellation response in foetal bovine serum in the absence of additional xanthurenic acid (◆) or in the presence of 2·5 μM xanthurenic acid (○) or 10 μM xanathurenic acid (□) expressed as a percentage of the pH 8·0 condition. Error bars represent standard errors of 3 experiments.

induce gametogenesis in a malaria parasite transmitted by this mosquito species we measured the pH-response curve of *P. berghei* exflagellation *in vitro* in the absence and presence of exogenous xanthurenic acid. There was hardly any overlap between observed bloodmeal pH in *An. stephensi* and the *in vitro* pH response of the microgametocytes of *P. berghei* in the absence of added xanthurenic acid (Fig. 4B). This situation changed dramatically when the pH response of exflagellation was determined in the presence of synthetic xanthurenic acid (Fig. 4B), which extended the lower pH range of exflagellation *in vitro* in a dose-dependent manner to values that we have observed in the bloodmeal.

DISCUSSION

Blood pH is regulated by the equilibrium between dissolved CO_2 and bicarbonate ions. The loss of CO_2 that occurs when infected blood is exposed to ambient conditions has long been known to result in a pH increase large enough to induce malarial gametogenesis *in vitro* (Chorine, 1933; Bishop & McConnachie, 1956; Nijhout & Carter, 1978). This conclusion was supported by the present study which described a shift from pH 7·4 to 8·0 in just 10 min. That a similar pH shift functions as a natural trigger in the mosquito midgut has, however, been doubted on the evidence that gut contents dissected from mosquitoes showed a 'suboptimal pH' (Micks, de Caires & Franco, 1948; Bishop & McConnachie, 1956; Chege & Beier, 1998). Despite the diligence of these workers, all the assays described potentially were compromised by the inclusion of other body components (gut wall and haemocoelomic fluid) in the samples measured. Use of precisely located ISMs overcomes such potential limitations. We show that, in contrast to the *in vitro* situation, blood ingested by a mosquito undergoes a small initial pH shift of only 0·1–0·2 pH units that is followed by a slow sustained increase over the ensuing 3 h. This suggests that the mosquito cuticle and tissues form a sufficiently tight barrier to reduce significantly the loss of CO_2 from the gut content.

Bishop *et al.* (1956) have shown that, within the limitations of their technique, bloodmeal pH in *Ae. aegypti* was not changed by *P. gallinaceum* infection. We have previously shown that the low asexual parasitaemias early in the infection (when *P. berghei* shows maximal infectivity) have no measurable impact on host blood pH (Butcher, Sinden & Billker, 1996). Thus for the purposes of this study, although pH measurements were obtained from uninfected bloodmeals we believe that the results adequately reflect what *infectious P. berghei* gametocytes experience as they pass from the blood stream into the mosquito gut. We note, however, that the acidosis associated with high asexual parasitaemias in *P. berghei*-infected mice reduces blood pH significantly, and would render conditions in the bloodmeal even less favourable for the induction of exflagellation than those recorded here (Butcher *et al.* 1996).

Exflagellation of *P. berghei* has a narrow pH optimum *in vitro* and requires at least pH 7·8 for half-maximal levels. This is very similar to results obtained with *P. gallinaceum* (Carter & Nijhout, 1977; Nijhout & Carter, 1978). ISM measurements show that bloodmeal pH in both mosquito species studied clearly remains below pH 7·8 during the 30 min period in which the induction of malarial gametogenesis is completed *in vivo* (Sinden *et al.* 1996). During this period only 1 out of 10 *An. stephensi* examined had reached a bloodmeal pH of 7·7. These observations confirm the notion that in the large majority of mosquitoes bloodmeal pH at the time of exflagellation is suboptimal for the induction of gametogenesis (when observed *in vitro*). The disparity between the pH optimum for exflagellation *in vitro* and the observed pH of the bloodmeal is intriguing, and suggests that gametocyte activating factor(s) such as XA (Billker *et al.* 1998) that can reduce the size of the pH rise required for the induction of gametogenesis may be important *in vivo*.

In *An. stephensi* the slow and steady pH increase until 24 h after feeding probably results from a slow loss of CO_2 via the haemolymph or the tracheoles of

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the midgut epithelium. Alternatively this pH change might be mediated by ATPase-mediated changes in ionic and water concentrations (MacVicker, Billingsley & Djamgoz, 1992). In *Ae. aegypti* a different, unknown mechanism of bloodmeal pH regulation is apparent 3–6 h after feeding leading to a decrease in bloodmeal pH. This decrease may be linked to bloodmeal concentration, which in *Anopheles* and *Aedes* is achieved by different mechanisms (Briegel & Rezzonico, 1985) or to the secretion of digestive components of bloodmeal. Twenty-four h after the bloodmeal is ingested the midgut of *Aedes* and *Anopheles* differ significantly. Two previously published factors which could be important to the survival of *Plasmodium* include differences in the composition of the peritrophic matrix (Billingsley & Rudin, 1992), and in the activities of midgut proteases (Rudin, Billingsley & Saladin, 1991). The present study indicates, that the midgut pH also differs significantly between these mosquito genera. Recognizing that it is at this time the ookinete matures, crosses the peritrophic matrix and invades the midgut epithelium, it would be interesting to determine whether these differences in pH are critical to the success and failure of ookinetes from avian and mammalian parasites to differentiate into oocysts in their respective vectors.

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