Immune challenge induces DNA synthesis and nuclear fragmentation in Aedes aegypti fat body cells

Bernardo Pérez-Zamorano, Verónica Valverde-Garduño

Abstract
Some biological processes require a rapid increase in the production of specific proteins. A temporal or permanent increase in the number of copies of the relevant gene contributes to achieving high levels of protein product in short periods of time. Polyploid cells may arise as a result of injury, stress, developmental and environmental signals. In insects, the innate immune response is a demanding process that requires the rapid synthesis of antimicrobial effectors. Here we investigated the potential involvement of DNA synthesis in Aedes aegypti response to immune challenge. An efficient way to selectively analyze genomic regions undergoing active nucleotide incorporation is to label DNA in vivo while it is being synthesized. First, a reliable, practical technique to achieve in vivo BrdU-labelling of culicid DNA was developed. This BrdU feeding method allows in vivo incorporation that was detected by Southern blot and immunofluorescence. Both detection techniques confirmed BrdU incorporation into DNA from specific mosquito tissue. Labelling tools described in this work provide an integrative approach for the in vivo study of DNA replication and repair related phenomena in the culicid. Here we show that Aedes aegypti adult female fat body cells undergo de novo DNA synthesis upon injury and that immune challenge triggers DNA synthesis and subsequent formation of a pattern consistent with multiple nuclei in these cells. This multinuclear pattern suggest fat body cells may undergo endomitosis.

Keywords: Aedes aegypti, innate immunity, fat body, DNA replication, immune challenge, in vivo, DNA labelling

1. Introduction
Increasing cell’s DNA content in the absence of mitosis is involved in some key physiological and developmental processes. In some cases only specific genetic loci are replicated (amplification) [1], in others the whole genome undergoes various rounds of replication in the absence of mitosis. Three mechanisms involving active DNA replication have been described for developmentally programmed polyploidy: acytokinetic mitosis, endomitosis and endoreplication. These mechanisms provide multiple gene copies available for a rapid increase in transcription and translation for specific protein products [1-5]. As a consequence, high protein concentrations are achieved for highly demanding biological processes. Endoreplication, where a cell undergoes multiple rounds of S phase without entering mitosis, and gene amplification are well documented in Drosophila melanogaster [6-8].

Mosquitoes are vectors of a variety of infectious diseases; Aedes aegypti in particular, is competent for the transmission of viruses including Dengue virus, chikungunya virus, West Nile virus and Yellow Fever virus. This species is able to transmit these viruses owing to its blood feeding habits. Blood feeding is necessary for egg development and the fat body produces massive amounts of vitellogenin for this process. It has been shown that in Ae. aegypti adult female mosquitoes, the fat body undergoes at least two cycles of DNA synthesis (with subsequent polyploidization) [9]. The first cycle of postemergence DNA synthesis occurs up to 72 hrs after emergence. Another cycle of de novo DNA synthesis in the adult fat body occurs after a blood meal and is dependent upon juvenile hormone levels [10,11].

In mosquitoes the fat body is also the main innate immune responsive tissue. It contributes to mounting a strong response against invading microorganisms. This response is characterized by induction and release of effector proteins, including antimicrobial peptides, into the hemolymph [12,13]. An efficient mechanism to achieve high concentration of proteins is the generation of a large quantities of the corresponding mRNAs [14-17]. One possible way to
generate these mRNAs is to increase the number of copies for each relevant gene through de novo DNA synthesis. Therefore we set out to investigate the possible involvement of DNA synthesis in Ae. aegypti adult fat body response to immune challenge.

To follow active synthesis of DNA, radioactively labelled nucleotides have been used before in Ae. Aegypti [9]. More recently the use of 5- bromo -2'- deoxyuridine (BrdU), a synthetic nucleoside analog of thymidine (Fig. 1), has substituted radiactively labelled nucleotides in a diversity of biological systems. It has been particularly useful for in vivo studies, extensively used in biology and medicine as a diagnostic tool [18,20]. Because of its chemical structure, BrdU is incorporated easily into DNA, substituting for thymidine during DNA replication or repair. Therefore BrdU can be incorporated into newly synthesized DNA in actively replicating cells. It is commonly used for detection of proliferating cells in living tissues [21]. BrdU-specific antibodies are then used to detect the incorporated chemical, revealing cells that are actively replicating their DNA. Binding of anti-BrdU antibodies to newly synthetized DNA, containing BrdU, requires DNA denaturation in order to expose nitrogenated bases, including BrdU. In Drosophila BrdU-based experimental techniques for labelling of newly synthesized DNA have been used to analyze endoreplication and gene amplification.

![Fig 1](image1.png)

**Fig 1:** (A) Thymidine and (B) 5-bromo-2’-deoxyuridine (BrdU)

In order to analyse possible involvement of de novo DNA synthesis in Ae. aegypti fat body immune response, we devised a method for in vivo DNA labelling. To illustrate the potential utility of this method for other mosquito species, it was also tested on Anopheles albimanus. Therefore, for this species, only general BrdU incorporation on genomic DNA was demonstrated by southern blot. This labelling method is based on an ad libitum diet from a BrdU containing gel and is described in detail in the methods section (see below). Here, using such protocol, we show that injury, concomitant with injection of a sterile foreign substance induces de novo DNA synthesis in adult female Ae. aegypti fat body cells. Further, we show that immune challenge stimulation leads not only to DNA synthesis, but also induces fat body cells nuclear fragmentation. DNA synthesis reported here is independent from the postemergence cycle previously reported, since we used 5 day old female mosquitoes [9]. It is also independent from the blood feeding induced DNA synthesis cycle, since female mosquitoes were not blood fed in our experiments. These results suggest that Ae. aegypti fat body may undergo two distinct types of polyploidization mechanisms in response to injury and immune challenge. We propose that these mechanisms may correspond to endoreplication and endomitosis, respectively.

### 2. Materials and Methods

#### 2.1 Mosquitoes

Mosquitoes were obtained from already established insectary colonies. Male and female Ae. aegypti and An. albimanus mosquitoes were obtained from the insectary at the Instituto Nacional de Salud Pública, Cuernavaca, México. Five day-old mosquitoes were used in our assays. They were reared under a photoperiod cycle of 12 h light: 12 h dark, at 28°C and 70–80% relative humidity. They were fed ad libitum with 5% sucrose-soaked pad cottons.

#### 2.2 Cell lines and reagents

Yeast and reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. Cultures of Aedes albopictus derived C636 cell line were used as positive controls for active DNA replication.

#### 2.3 In Vivo Bromodeoxyuridine (BrdU) Incorporation

Five day old Ae. aegypti and An. Albimanus mosquitoes were fed for 48 hours with 5-bromo -2'- deoxyuridine (Boehringer Mannheim GmbH, Germany). This was performed using 15ml, 0.5% agarose gels in 60 x 15 mm disposable petri dishes (Corning). Mosquitoes were allowed to feed from these gels (Fig. 2). Gels contained 8% sucrose, 100 µg/ml BrdU, 200µ/ml penicillin, 200 µg/ml streptomycin sulphate and 0.5 µg/ml amphotericin B. Antibiotic and anti-fungal cocktail was added in order to avoid microorganism growth which could affect the DNA replication signal. Feeding was performed in the same conditions aforementioned in section 2.1. In addition, containers holding mosquitoes and BrdU gels were placed inside wet chambers, in order to avoid gel desiccation. Efficient wet chambers were made by placing containers on trays bearing 1 inch of water and setting everything up inside plastic bags. Feeding gels were replaced every 24 hours with freshly made ones. The employed BrdU concentration (100 micrograms/ml) was chosen because it produced the best detection signal vs survival rate ratio. BrdU toxicity became an issue at higher concentrations. BrdU incorporation mechanism can cause mutations, and its use is therefore a potential health hazard [22].

![Fig 2](image2.png)

**Fig 2.** Feeding mosquitoes with BrdU containing gel

Mosquitoes were fed for 48 hours with 5-bromo-2'-deoxyuridine (BrdU), using 0.5% agarose gels in 60 x 15 mm disposable petri dishes. Mosquitoes were allowed to feed ad linitum. Gels' contents: 8% sucrose, 100 µg/ml BrdU, 200µ/ml penicillin, 200 µg/ml streptomycin sulphate and 0.5 µg/ml amphotericin B. The containers holding the mosquitoes and
the BrdU gels were placed inside wet chambers in order to avoid gel desiccation.

2.4 Microbial inoculations
Inoculation needles were fabricated from glass capillaries; with an 1.1-1.2 mm internal diameter; by heating them on an open flame in the middle and pulling by the ends to obtain two needles from each capillary. The injection process was performed with the aid of a Drummont Captral III Microdispenser and a stereoscopic microscope. Mosquitoes were anesthetized by putting them at 4 °C for 10 minutes and keeping them on ice during the inoculation process. Each mosquito was injected with 0.25 microlitres of a heat-inactivated Saccharomyces cerevisiae suspension, prepared in sterile RPMI 1640 culture medium (Gibco BRL, Grand Island, NY). The suspension had an approximate concentration of 4 x 10⁹ cells/ml. Control mosquitoes were inoculated with 0.25 ml of sterile RPMI medium. Inoculations were made through the pleural membrane, between the fourth and fifth abdominal segments [23]. Mosquitoes were allowed to recuperate for 24 hours, period during which they were still fed BrdU.

2.5 DNA extraction and quantification
DNA was extracted from whole mosquitoes with a Zymo Research Tissue & Insect DNA MiniPrep™ kit (Zymo Research, California, USA), and quantified at 230/260 nm in a nanodrop spectrophotometer (NanoDrop 2000, Thermo Scientific).

2.6 Basal BrdU incorporation detection by Southern Blotting
500 ng of each extracted DNA sample from BrdU-fed and non-inoculated mosquitoes (An. albimanus and Ae. aegypti) were electrophoresed in a 1% agarose gel and transferred to a nitrocellulose membrane. The membrane was incubated at room temperature for 1.5 h with a 1:1,000 dilution of anti-BrdU horseradish peroxidase conjugated antibody (Roche Mannheim GmbH, Germany) in PBS, followed by chemiluminescent detection.

2.7 Mosquito dissections and immunofluorescence analysis
Inoculated and non-inoculated Ae. aegypti mosquitoes were dissected, their viscera removed (intestines, ovaries, Malpighian tubules) and their abdomens collected. Each abdomen was split in half to expose each pleural membrane and abdominal fat body. Samples were then subjected to the following immunofluorescence protocol: samples were left overnight in a 3.65% paraformaldehyde solution. The paraformaldehyde was removed afterwards and the abdomens were placed in methanol for 10 minutes; this was followed by 6 washes in PBS Tween 1%. Samples were hydrolyzed in 2N HCl, 1 h at 37 °C, and then neutralized by three changes of Hank’s solution (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 1.3 mM CaCl₂, 1.0 mM MgSO₄, 4.2 mM NaHCO₃), each wash lasting 10 minutes. Specimens were subsequently washed three times with PBS- 1% Tween and blocked 1 h at 37 °C with 2% bovine serum albumin in PBS. They were then incubated at 4 °C overnight with a FITC-labeled-mouse anti-BrdU monoclonal antibody, 1:100 in PBS - Albumin (Roche, Indianapolis, IN). After removing the antibody solution samples were washed 4 times in PBS- 1% Tween and a 0.025 mg/ml propidium iodide solution was added and left at room temperature for 20 minutes. After rinsing 4 times in PBS- 1% Tween, samples were mounted on slides using 80% glycerol. Fluorescence was recorded using a photomicroscope (Nikon E-600, Tokyo, Japan).

3. Results and Discussion
3.1 Non-stimulated mosquitoes incorporate BrdU into their DNA
Southern blot assays in Fig. 3 show that 100 micrograms/ml was the optimal BrdU concentration in order to obtain the best in vivo basal DNA labelling (no immune challenge). Both male and female mosquitoes of the Ae. aegypti (Fig. 3A) and An. albimanus (Fig. 3B) species incorporated BrdU in their DNA. This concentration produces a clean and reproducible signal in both male and female mosquitoes, without any saturation that could interfere with further analyses. As it can be appreciated in the images (Fig. 3), female mosquitoes show a distinct higher chemiluminescent signal, implying that there is a higher BrdU incorporation in their DNA. This comes as no surprise since BrdU could be incorporated in the ovaries. It is known that in some strains of Ae. aegypti, the autogeny phenomenon can occur, which is the ability to start the first gonadotropic cycle without ingesting blood [20]. Also, BrdU incorporation into, both, female and male DNA in the absence of any stimuli could be a consequence of genome maintenance processes such as DNA repair mechanisms. Our in vivo labelling protocol shows improvement over other published techniques. First, we found that feeding time with BrdU for proper labelling could be reduced to two days. The BrdU signal proved to be clear and reproducible after this time. In contrast, previously reported methods required feeding on BrdU for four days, hence reducing the time left to carry out further in vivo experimental treatments [24]. Also, the previously reported BrdU concentration of 1mg/ml for DNA labeling [24] created some difficulties. In our assays this concentration produced more than 80% mortality rate in BrdU fed, non-stimulated mosquitoes. In contrast, here, a concentration of 100 micrograms per millilitre of BrdU was used, sharply increasing mosquitoes survival. Therefore, the protocol developed in this work is efficient in the use of the main reagent, reduces time required for effective labelling, increases mosquito survival and is reproducible.
Ae. aegypti genomic DNA, 1: control; 2: + control; 3: Male (M) 0 mg/ml BrdU; 4: Female (F) 0 mg/ml BrdU; 5: M 0.001 mg/ml BrdU; 6: F 0.001 mg/ml BrdU; 7: M 0.01 mg/ml BrdU; 8: F 0.01 mg/ml BrdU; 9: M 0.1 mg/ml BrdU; 10: F 0.1 mg/ml BrdU; 11: M 0.2 mg/ml BrdU; 12: F 0.2 mg/ml BrdU; 13: M 0.5 mg/ml BrdU; 14: F 0.5 mg/ml BrdU. (B) An. albimanus genomic DNA, 1: + control; 2: - control; 3: Male (M) 0 mg/ml BrdU; 4: Female (F) 0 mg/ml BrdU; 5: M 0.001 mg/ml BrdU; 6: F 0.001 mg/ml BrdU; 7: M 0.01 mg/ml BrdU; 8: F 0.01 mg/ml BrdU; 9: M 0.1 mg/ml BrdU; 10: F 0.1 mg/ml BrdU; 11: M 0.2 mg/ml BrdU; 12: F 0.2 mg/ml BrdU.

3.2 Injury and immune challenge induce BrdU incorporation into fat body cells DNA

Our results show that Ae. aegypti mosquitoes inoculated with sterile RPMI incorporate BrdU in the nuclei of their fat body cells (Fig. 4 A). Staining fat body tissue DNA with propidium iodide showed that incorporated BrdU co-localizes with DNA in these cells. This activity could be induced by trauma (injection wound) or by the introduction of a foreign substance (RPMI). Incorporation of BrdU in fat body cells from BrdU fed, non stimulated mosquitoes (not injected) was not observed.

4. Conclusion

The evident in vivo incorporation of the nucleotide analog BrdU into Ae. aegypti fat body cells DNA, upon immune challenge, indicate that these cells enter active DNA synthesis. This shows that methods developed in this work allow for an efficient in vivo labelling of DNA molecules under active de novo synthesis. We anticipate that this set of tools would be useful for further studies on DNA replication and repair-related phenomena in vivo in culicides. The BrdU intake protocol described here represents a non-invasive, low-toxicity process, that improves mosquito survival with efficient DNA labelling. It also reduces potential physiological stress that could interfere with further experimental stimuli. Two distinct patterns of newly synthesised DNA were found in mosquito fat body cells. The first pattern, induced by injury and injection of sterile RPMI into the hemocele, consisted of fat body cells with a single nucleus, where de novo DNA synthesis was evident by the presence of incorporated BrdU. The second pattern, induced specifically by immune challenge, consisted of the presence of multiple newly synthesized DNA containing bodies where BrdU was incorporated. These two patterns of newly synthesised DNA, discovered here in fat body cells, may correspond to at least two distinct polyploidization mechanisms. We suggest the first pattern may arise by endoreplication, and the second, triggered by immune challenge, may arise by endomitosis. Alternatively the first pattern may represent an intermediate phase of the endomitosis process, arising from a less stressful challenge. Results presented here warrant further studies to dilucidate molecular mechanisms involved in DNA synthesis triggered by immune challenge in Ae. aegypti abdominal fat body cells.

5. Acknowledgements

Funding was provided by CONACYT, México, grant 62389 and grant 84012.

6. References


