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Blood Feeding Induces Hemocyte Proliferation and Activation in the African Malaria Mosquito, *Anopheles gambiae* Giles

Running Title: A blood meal activates mosquito immunity

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Summary

Malaria is a global public health problem especially in sub-Saharan Africa, where the mosquito *Anopheles gambiae* Giles serves as the major vector for the protozoan *Plasmodium falciparum* Welch. One determinant of malaria vector competence is the mosquito's immune system. Hemocytes are a critical component as they produce soluble immune factors that either support or prevent malaria parasite development. However, despite their importance in vector competence, understanding of their basic biology is just developing. Applying novel technologies to the study of mosquito hemocytes, we investigated the effect of blood meal on hemocyte population dynamics, DNA replication, and cell cycle progression. In contrast to prevailing published work, data presented here demonstrate that hemocytes in adult mosquitoes continue to undergo low basal levels of replication. In addition, blood ingestion caused significant changes in hemocytes within 24 h. Hemocytes displayed an increase in cell number, size, granularity, and Ras-MAPK signaling as well as altered cell surface moieties. As these changes are well-known markers of immune cell activation in mammals and *Drosophila melanogaster* Meig., we further investigated if a blood meal changes the expression of hemocyte-derived immune factors. Indeed, hemocytes 24 h post blood meal displayed higher levels of critical components of the complement and melanization immune reactions in mosquitoes. Taken together, this study demonstrates that the normal physiological process of a blood meal activates the innate immune response in mosquitoes. This process is likely in part regulated by Ras-MAPK signaling, highlighting a novel mechanistic link between blood feeding and immunity.

Key words: *Anopheles gambiae*, mosquito, hemocytes, immunity, blood meal

Introduction

The female *Anopheles gambiae* mosquito is the major vector of malaria in sub-Saharan Arica. One of the most important determinants of vector competence is the mosquito's humoral immune system (Mitri and Vernick, 2012), which includes the production of reactive oxygen species, melanin synthesis, and complement activation. The cellular arm of the mosquito immune system is represented by hemocytes, which are the insect's equivalent to the myeloblast lineage of blood cells. These cells perform phagocytosis and encapsulation, produce free radicals of nitrogen and oxygen, and express the majority of molecules in the melanization and complement-like pathways including prophenoloxidase (PPO) and the thioester-containing protein (TEP)-1, respectively (Hillyer, 2010). In addition, studies suggest hemocytes are recruited to the midgut and dorsal vessel during *Plasmodium sp.* infection (Volz et al., 2005; Rodrigues et al., 2010; King and Hillyer, 2012), and may mediate innate "immune memory" (Rodrigues et al., 2010). However, despite the multi-facetted contribution of mosquito hemocytes to immunity against malaria parasites, little is known about their basic biology.

Some mosquito hemocytes circulate freely in the hemocoel (Hall, 1983), while others are attached to a variety of tissues including midgut, trachea, muscles, dorsal vessel, Malpighian tubules, cephalic limbs, and maxillary palps (Barillas-Mury et al., 1999; Danielli et al., 2000; King and Hillyer, 2012, 2013). While true cell lineages have yet to be established, mosquito hemocytes are classified based on morphological and functional characteristics. Granulocytes represent 90% of the adult hemocyte population and are classified based on their granular appearance and their exclusive ability to spread on glass surfaces and phagocytize particles (Castillo et al., 2006). The remaining 10% are split equally into oenocytoids, which express factors required for melanization, and prohemocytes, which are believed to represent progenitor cells (Castillo et al., 2006). While generally it has been accepted that only 500-1000 hemocytes reside in an adult mosquito (reviewed in Hillyer, 2010), a recent study with improved *in-vivo* imaging techniques showed this number to be ~5,000 cells per mosquito two days posteclosion (King and Hillyer, 2013). Mosquitoes kept on sugar water exhibited a gradual decrease in hemocyte numbers (Hillyer et al., 2005).

Numerous studies in multiple insects clearly demonstrate the strong correlation between hemocyte numbers and immunity. *D. melanogaster* larvae parasitized by the parasitic wasp *Leptopilina boulardi* (Barbotin, Carton & Kelner-Pillault) show an increase in total number of hemocytes (Russo et al., 2001). Further, distinct genetic lines of *D. melanogaster* resistant to parasitoid wasps have almost twice the number of hemocytes as susceptible lines (Kraaijeveld et al., 2001). In tsetse flies, removal of their microbiota affects vector competence by decreasing hemocyte numbers, ultimately decreasing expression of important immune factors (Weiss et al., 2011; Weiss et al., 2012). Additionally, inoculation with *Dirofilaria immitis* (Leidy) microfilariae and *Escherichia coli* (Migula) results in hemocyte proliferation in *Aedes aegypti* L. and *An. gambiae*, respectively (Christensen et al., 1989; King and Hillyer, 2013). In contrast, infection with the rodent malaria parasite, *Plasmodium berghei* Fain, does not affect overall hemocyte number (Baton et al., 2009), but induces a number of changes. These include a relative increase in granulocytes compared to oenocytoids and prohemocytes (Baton et al., 2009; Rodrigues et al., 2010), TEP1 secretion (Frolet et al., 2006), changes in lectin binding (Rodrigues et al., 2010), changes in expression patterns (Pinto et al., 2009), and hemocyte aggregation near the ostia of the dorsal vessel of the mosquito (King and Hillyer, 2012). In the absence of infection, some studies show a blood meal induces an increase in circulating hemocyte numbers in *An. gambiae* and *Ae. aegypti* (Castillo et al., 2006; Baton et al., 2009). However, the consequences of a blood meal on different aspects of hemocyte biology have not been addressed.

The purpose of the present study was to determine whether the physiological event of a blood meal causes critical changes in *An. gambiae* hemocytes. Several highly conserved immune cell activation markers are detectable in hemocytes isolated from female mosquitoes 24 h after blood feeding. In addition, we found up-regulation of immune factors critical for the complement-like pathway and the melanization response demonstrating that a blood meal activates the immune response in mosquitoes.

Results

Blood Meal Induces Hemocyte Proliferation

To analyze hemocyte proliferation in *An. gambiae* we initially determined total hemocyte numbers using an established perfusion protocol (Castillo et al., 2006; Rodrigues et al., 2010). Hemocytes were collected from female mosquitoes that were either maintained on sugar solution or that had received a blood meal. Similar to the majority of previous reports in different mosquito species (Christensen et al., 1989; Hillyer et al., 2005; Castillo et al., 2006; Baton et al., 2009; Hillyer, 2010), we consistently observed hemocyte numbers in the range of ~500-2,500 cells per mosquito. In agreement with published data (Castillo et al., 2006; Baton et al., 2009), hemocytes numbers increased consistently and significantly by 2.1 fold 24 h post blood meal (pbm) (U test, P=0.0058, Fig. 1A).

To determine whether the increase in hemocyte numbers is due to cell division or remobilization of sessile hemocytes, we assayed DNA replication in hemocytes by monitoring EdU incorporation into DNA. Sugar fed and 20 h pbm mosquitoes were injected with EdU, and their hemocytes were harvested 4 h post injection. This experimental setup allowed us to label hemocytes for 4 h at the time of proposed hemocyte cell division for the blood fed group and determine basal levels of DNA replication in the sugar fed group. The fluorescence signal due to EdU incorporation was striking and easily detected in perfused hemocytes by fluorescence microscopy (Fig. 1C). The percentage of EdU-positive hemocytes obtained from sugar fed mosquitoes was low with a median of 1.45% (Fig. 1B). This number increased to 26.2% in hemocytes obtained 24 h pbm, resulting in an 18-fold increase in EdU-positive cells (U-Test P<0.0001, Fig. 1B). These data suggest that the increase of hemocytes after blood meal (Fig. 1A) is due to cell division rather than remobilization of sessile hemocytes.

To determine if EdU incorporation in hemocytes is a result of mitotic activity and not due to increasing ploidy levels due to endoreplication, we employed cell cycle analysis by flow cytometry. Propidium iodide (PI) staining was used to analyze DNA content of hemocyte populations from either sugar fed or blood fed mosquitoes. PI staining was displayed as fluorescence signal in area versus width (Fig. 1D). Cell aggregates were excluded by gating (Givan, 2001), and histograms of distinct cell populations were obtained (Fig. 1D). Experiments were performed in triplicate, and for each treatment group and sample, the fixed number of

20,000 events was counted. Thus the data present the relative number of cells and not absolute values. Both treatment groups contained distinct hemocyte populations with varying DNA content ranging from 2C to 16C. Three markers (M1-M3) were placed to delineate these populations based on their ploidy levels, and to determine the percentage of cells within these populations. M1 contained euploid cells within different phases of the canonical cell cycle, defined by the characteristic G0/G1 (2C) and G2 (4C) peaks, with S phase in between. The other two markers highlight distinct populations of aneuploid cells with peaks at 8C (M2) or 16C (M3). In sugar fed and blood fed mosquitoes, euploid cells represented the dominant hemocyte population (Figs. 1D, 2A). In three experimental replicates, an average of 37.8% of hemocytes in sugar fed mosquitoes were aneuploid, having either an 8C or 16C DNA content. However, in blood fed mosquitoes on average only 11.3% of hemocytes were aneuploid, marking a 28% relative increase in euploid cells in blood fed mosquitoes (Fig. 2A, Fisher's Exact Test, $P<0.0001$). Therefore, increase in EdU incorporation in hemocytes from blood fed mosquitoes was not due to endoreplication and increase in aneuploid cells, but rather a result of higher mitotic activity in the euploid cell population.

Blood Meal Causes an Increase in Hemocyte Size and Granularity

The flow cytometry analyses indicated that cell aggregation was also more prominent in hemocytes isolated from blood fed mosquitoes. In the dot plots shown in Fig. 1D, hemocytes from sugar fed mosquitoes fell within the gate, with few cell aggregates outside the gate. In contrast, hemocytes form blood fed mosquitoes formed large cell aggregates as indicated by the blue dots outside the gate. To investigate these and other potential phenotypic changes in hemocytes after blood meal, size and granularity properties were assessed. For this analysis, we focused solely on the gated euploid cell population, as they represented the major cell type based on DNA content (Figs. 1D and 2A).

Backgating analysis revealed the effects a blood meal on both size and granularity of hemocytes. In density dot plots, nearly 75% of all circulating hemocytes from sugar fed mosquitoes were found in the lower left quadrant (small size, low granularity) compared to only 18.5% of hemocytes from blood fed mosquitoes (Fig. 2B, 2C). Nearly 60% of hemocytes from blood fed mosquitoes were found in the upper right quadrant, indicating larger cell size and increased granularity. This was confirmed by overlaying histograms of either FSC or SSC (Fig.

2D). Hemocytes from blood fed mosquitoes show a significantly increased average and range of size and granularity compared to sugar fed mosquitoes (Fig. 2D). The combined flow cytometry data clearly demonstrate that a blood meal causes significant changes in morphology of the euploid population of hemocytes.

Blood Meal Activates Hemocytes

The increase in cell number, aggregation, DNA replication, cell size and granularity of hemocytes after a blood meal are highly reminiscent of classical blood cell activation markers used in early immunological studies of mammalian leukocytes (Oppenheim and Rosenstreich, 1976). Based on these findings, we assessed additional potential molecular changes that have been linked to blood cell activation in insects.

Infection of *D. melanogaster* larvae by parasitic wasps, an established invertebrate model for blood cell activation, is characterized by increased cell aggregation, WGA lectin binding, and Ras-MAPK signaling. We asked whether blood meal induces similar changes in *An. gambiae.* Hemocytes from sugar fed mosquitoes exhibited low WGA binding, while cells from blood fed individuals exhibited a much stronger binding signal (Fig. 3A), demonstrating blood meal-induced changes in surface carbohydrate moieties.

Phosphorylated ERK, a commonly used and highly conserved marker for Ras-MAPK signaling (pAgERK, AGAP009207), was readily detected in hemocytes isolated from adult female *An. gambiae*. IFAs showed punctate staining around the perimeter of nuclei as well as diffuse signals within nuclei in hemocytes isolated from sugar fed mosquitoes (Fig. 3B). Stronger fluorescent signals of pERK were consistently observed in hemocytes isolated 24 h pbm (Fig. 3B), clearly illustrating elevated Ras-MAPK signaling in hemocytes after blood meal.

In *An. gambiae*, hemocytes are the only source of several immune factors critical for complement and melanization, including TEP1 and PPO. To determine if expression of these immune factors was altered by a blood meal, IFAs were performed using anti-TEP1 and anti-PPO6-specific antibodies. TEP1 signals were low or undetectable in hemocytes from sugar fed females, and significantly increased after a blood meal, resulting in punctate/granular staining (Fig. 3C). PPO6 antibody yielded low punctate staining in hemocytes from sugar fed females, which increased after blood meal (Fig. 3D). At the same time, blood meal did not significantly

alter global protein production, as measured by incorporation and detection of a methionine analog (Fig. S1).

The increase of WGA binding, pERK signal, as well as TEP1 and PPO6 expression 24 h after blood meal was highly reproducible (Fig. S2) and statistically significant, as compared to control treatments (U-test, $P<0.0001$, Fig. 3A-D).

Discussion

The innate immune response of the female mosquito is a major obstacle faced by malaria parasites while traveling through and developing in their obligate vector. Within the first 18-48 h after arrival within the mosquito, ookinetes, the motile zygote life stage of the parasite, encounter epithelial immune responses, characterized by the production of reactive oxygen and nitrogen species (Luckhart et al., 1998; Han et al., 2000; Oliveira Gde et al., 2012). At the same time, the mosquito's complement-like system, characterized by TEP1 and leucine-rich repeat proteins binds to the ookinete surface and ultimately kills and lyses the parasite (Blandin et al., 2004; Fraiture et al., 2009; Povelones et al., 2009). In certain genetic backgrounds, melanization, the production and deposition of eumelanin on the surface of the parasite further decreases the ookinete population within the first 48 h of infection. In contrast, cellular encapsulation of malaria parasites, a classical anti-parasitic cellular immune response in insects does not occur. A second cellular immune response, phagocytosis of malaria parasites has been observed after 10 days post infection, but does not reduce significantly the parasite population (Hillyer et al., 2007). However, hemocytes contribute significantly to anti-malarial immunity (Ramirez et al., 2013), and they are the only source of many of the critical anti-parasitic humoral immune factors, including phenoloxidase (Muller et al., 1999) and TEP1 (Frolet et al., 2006).

Malaria parasite infection induces a number of significant molecular changes in hemocytes (Baton et al., 2009; Pinto et al., 2009; Rodrigues et al., 2010), which limit parasite development (Pinto et al., 2009; Ramirez et al., 2013). However, all studies to date have evaluated hemocyte responses to infection as compared to a noninfectious blood meal. Given that blood meal alone increases hemocyte numbers circulating in the hemolymph of *An. gambiae* (Castillo et al., 2006; Baton et al., 2009; Castillo et al., 2011), focusing on changes induced by infection alone may thus underestimate the contribution of hemocytes to anti-parasitic immunity. *An. gambiae* females are anautogenous, and must take a blood meal to produce eggs to complete its life cycle. Simultaneously, mosquitoes can encounter many distinct blood-borne pathogens, whose infection they have to overcome for at least the next two to three days in order to lay their eggs. Therefore, survival of the 48 h to 72h after blood ingestion is critical to the fitness of the

248 species. We thus set out to evaluate the putative role of a blood meal on hemocyte stimulation
249 and thus immune system activation.
250

251 Previous studies indicate that hemocytes in circulation increase after blood meal without
252 determining if they were dividing or if sessile hemocytes were detaching (Castillo et al., 2006;
253 Baton et al., 2009; Castillo et al., 2011). The base analog incorporation and flow cytometry data
254 obtained in this study illustrate clearly that the increase in hemocytes after blood meal was due to
255 mitosis instead of detachment of sessile hemocyte populations. Binucleated hemocytes
256 undergoing mitosis in adult mosquitoes has previously only be demonstrated after bacterial
257 infection (King and Hyllier, 2013). The EdU incorporation assay, which was established in this
258 current study and can be used as a proxy for hemocyte proliferation, proved not only more
259 convenient but also 10-fold more sensitive with a significantly wider dynamic range compared to
260 established cell counting methodologies (Castillo et al., 2006). Surprisingly, flow cytometry
261 analyses also revealed that up to 30% of hemocytes in naïve mosquitoes were aneuploid, with
262 DNA content at or above 8C. Aneuploid blood cells are well known in mammals, e. g.
263 megkaryocytes undergo endoreplication upon activation in mice, rats and humans (reviewed in
264 Lee et al., 2009). Aneuploid hemocytes in insects have been reported in *Manduca sexta* (L.)
265 (Nardi et al., 2003), however to our knowledge, this is the first description of their existence in
266 dipterans. Future studies will evaluate how DNA content complements existing morphological
267 classifications and how these criteria can be combined to discriminate hemocyte sub-populations.
268

269 As hypothesized, blood meal proved to induce significant changes in *An. gambiae*
270 hemocytes. The ratio of euploid to aneuploid cells increased significantly, suggesting that cell
271 division of the euploid cell population was stimulated rather than endoreplication. Similarly,
272 changes in the relative abundance of different hemocyte populations have been reported after
273 bacterial and parasite infection in different mosquito species (Christensen et al., 1989; Rodrigues
274 et al., 2010; King and Hillyer, 2013). The only other vector species for which changes in
275 hemocyte populations due to a blood meal have been observed is the kissing bug *Rhodnius*
276 *prolixus* Stål, an important vector of Chagas disease (Jones, 1967).
277

In *D. melanogaster*, parasitoid wasp infection induces a number of significant changes in larval hemocytes that are required for an encapsulation response. Hemocytes differentiate and increase in numbers in response to the presence of parasitoid wasp eggs, ultimately resulting in hemocyte activation (Russo et al., 2001). Consequently, hemocytes aggregate around the wasp egg to form a tight capsule around the parasite (Nappi and Streams, 1969). This cell adhesion is mediated by significant molecular changes on the hemocyte's cell surface, which is also demonstrated by increased WGA lectin binding (Nappi and Silvers, 1984; Mortimer et al., 2012). Our study shows that blood meal ingestion causes strikingly similar changes in mosquito hemocytes. Not only did the number of *An. gambiae* hemocytes double, but WGA binding and the propensity of hemocytes to form aggregates increased significantly. These data strongly suggest that a blood meal indeed induces hemocyte activation in mosquitoes and thus elicits an immune response. This is further supported by our observation that TEP1 and PPO6 protein abundance increased significantly in hemocytes isolated from blood fed mosquitoes. Their immune-fluorescence analyses revealed punctuate staining patterns, at least partially explaining the more granular appearance of hemocytes isolated from blood fed mosquitoes as compared to sugar fed controls.

The blood meal-induced hemocyte activation data presented here also draw strong parallels to mammalian blood cell activation. Upon activation, mammalian blood cells undergo mitotic replication (Oppenheim and Rosenstreich, 1976), increase in size and granularity (Cohn and Benson, 1965; Cook et al., 2004), and up-regulate vital immune factors (Bellingan, 1999; Wynn et al., 2013). The molecular mechanisms underlying blood cell activation are complex and include a number of highly conserved signaling pathways. For example, activation of macrophages can be stimulated by the granulocyte-macrophage colony stimulating factor, which binds to a receptor tyrosine kinase and signals through the Ras-mitogen activated protein kinase (MAPK) pathway (Cook et al., 2004). The same pathway also plays a critical role in lymphocyte activation (Downward et al., 1990; Cantrell, 2003). In *D. melanogaster*, Ras- MAPK signaling is activated in hemocytes after parasitic wasp infection (Sinenko et al., 2012) and plays a vital role in hemocyte homeostasis (Zettervall et al., 2004). Thus it was not surprising that we observed a consistent and statistically significant increase in Ras-MAPK signaling in mosquito hemocytes after a blood meal. Ras-MAPK signaling is likely not the only pathway required for hemocyte

proliferation and differentiation. Other likely candidates are the JAK/STAT, Jun kinase, and Toll pathways that control these processes in *D. melanogaster* (Zettervall et al., 2004). In addition, insulin signaling has recently been implicated in hemocyte proliferation after blood meal in the yellow fever mosquito, *Ae. aegypti* (Castillo et al., 2011). Another candidate is 20-hydroxyecdysone (20-E), which is circulating at increased levels in the mosquito's hemolymph after a blood meal (Clements, 2000; Bai et al., 2010). Expression of *PPO6* is increased by elevated levels of 20-E *in vitro* (Muller et al., 1999), suggesting that ecdysone signaling at least partially regulates blood meal-stimulated immune factor expression. Studies are on the way to identify the role of these signaling pathways in hemocyte proliferation and activation.

Taken together, this study identifies a blood meal as a significant immune system activator in *An. gambiae.* The factors that are upregulated upon blood ingestion have broad anti-pathogenic activity. TEP-1 and PPO can limit parasite and bacterial infections (Levashina et al., 2001; Blandin et al., 2004; Volz et al., 2006; Schnitger et al., 2007; Fraiture et al., 2009; Povelones et al., 2009), and PPO further aides in the defense against fungi (Yassine et al., 2012), and filarial worms (Guo et al., 1995). This suggests that the mosquito's immune system is primed to act against a broad range of putative pathogens which may be encountered in a blood meal. Furthermore, we provide a new mechanistic link between blood meal and immunity, which enables future molecular studies on trade-offs between mosquito immunity and fecundity.

Materials and Methods

Mosquito rearing and maintenance

The *An. gambiae* G3 strain was reared according to our standard protocol (An et al., 2011). Mosquitoes were starved for ~6-12 h before blood feeding. Heparinized horse blood (Plasvacc, Templeton, CA, USA) was provided through an artificial membrane feeding system.

Hemocyte collection and counts

Hemocytes were collected by perfusion using a modified protocol (Castillo et al., 2006; Rodrigues et al., 2010). Mosquitoes were injected with ~276 nl Anticoagulant buffer (60% of Schneider medium, 10% FBS, 30% citrate buffer [98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA, 41 mM citrate], 1µM DAPI) using a Nanoject II system (Drummond Scientific Company, Broomall, PA, USA) and incubated on ice for 10-15 minutes. Using forceps, a small tear was made into the penultimate abdominal segment and ~6-10 µl of anticoagulant buffer was perfused through the hemocoel at a rate of 1 µl/dispension using a Hamilton syringe system (see Fig. S3). The perfused hemolymph was loaded immediately into disposable hemocytometers (Incyto, Chungnam-do, Korea) to determine hemocyte numbers following manufacturer's instructions.

EdU Incorporation Assay

EdU assays were performed with the Click-iT® EdU Alexa Fluor® 488 Imaging kit (Invitrogen, Grand Island, NY, USA) following manufacturer's instructions. Mosquitoes were injected with 138 nl of 20mM EdU in PBS and allowed to recover under normal rearing conditions. Four hours after injection, hemocytes were collected by perfusion (see above) onto PTFE printed glass slides (Electron Microscopy Sciences, Hatfield, PA, USA). After 1 h incubation at 4ºC, perfusion buffer was replaced with fixative (4% formaldehyde in PBS), and incubated for 15 min at room temperature (RT). Cells were washed twice with 3% BSA in PBS, and permeabilized in 0.5% Triton X-100 in PBS for 20 min. Subsequently, cells were washed twice with 3% BSA in PBS, incubated in the dark with the Click-iT® Reaction Cocktail for 30 min at RT, and washed again with 3% BSA in PBS. Cells were mounted in VectaShield medium (Vector Laboratories Inc., Burlingame, CA, USA), slides were sealed with nail polish, and stored at 4° C. EdU

incorporation was determined by fluorescence microscopy using an Axioplan2 fluorescent light microscope (Zeiss, Jena, Germany), and expressed as fraction of positive cells in a pool of at least 300-400 hemocytes.

Cell Cycle Analysis by Flow Cytometry

Hemocytes were collected into anticoagulant buffer without DAPI by perfusion as described above. Hemocytes were pooled from 60-75 mosquitoes. Each pool was collected on ice within 1 h and immediately centrifuged at 2350 x g at 4ºC. Cells were resuspended in 200 µl PBS with 0.1% FBS. 700 µl of 70% ethanol was added drop wise to the cells and incubated for 1 h at RT. Cells were centrifuged again at 2350 x g at 4ºC and resuspended in 200 µl of PI solution (50 µg/ml Propidium Iodide, 100 µg/ml RNAseA, 0.1% Triton X-100, 0.1 mM EDTA in PBS). Cells were incubated for at least 1 h at 4ºC, and pushed through a 40 µm nylon filter (Becton Dickinson Falcon, San Jose, CA, USA) to remove large cell aggregates. Cells were analyzed with a FACSCalibur cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), obtaining 20,000 events per sample at a rate not exceeding 150 events/s. Data were analyzed using WinList software (Verity Software House, Topsham, ME, USA). Events were gated and their corresponding histograms were obtained. Additionally, cell populations with differences in DNA content were backgated to determine size (FSC-H) and granularity (SSC-H). To backgate cells, first a dot plot based on PI (DNA) signal was drawn up for both sugar- and blood-fed mosquitoes. Gated populations of interest were analyzed for size (FSC-H) and granularity (SSC-H) in density dot plots and overlaying histograms.

Immunofluorescence analysis (IFA)

Hemocytes from two mosquitoes were collected and pooled onto PTFE-printed glass slides (Electron Microscopy Sciences, Hatfield, PA, USA) as described above. Cells were incubated at 4ºC for 1 h. Cells were fixed with 4% formaldehyde for 15 min at room temperature. Cells were incubated with blocking buffer (5% BSA, 0.3% Triton X-100 with PBS as diluent) for 1 h at RT. Cells were exposed to primary antibodies in antibody dilution buffer (1% BSA, 0.3% Triton X-100 in PBS) overnight at 4ºC. The following primary antibodies were used at the indicated dilutions: rabbit anti-TEP-1, 1:350 (Povelones et al., 2009); rat anti-PPO6, 1:1000 (Muller et al., 1999); rabbit anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), 1:30 (cat#4370, Cell

Signaling, Boston, MA, USA). Cells were washed in PBS three times, and incubated with secondary antibody in antibody dilution buffer in the dark for 1-2 h at RT. The following secondary antibodies were used at the indicated dilutions: IgG (H + L) Alexa Fluor® 594 (Invitrogen, Grand Island, NY, USA) goat anti-rabbit at 1:500 (TEP1) or 1:100 (pERK); IgG (H + L) Alexa Fluor® 594 (Invitrogen, Grand Island, NY, USA) goat anti-rat at 1:1000 (PPO6). Cells were rinsed in PBS three times and mounted in Vectashield (Vector labs, Burlingame, CA). Slides were sealed using nail polish and stored at 4° C until further analysis.

Lectin staining

To determine lectin staining of hemocytes, we followed protocols developed by (Rizki and Rizki, 1983). Hemocytes from two mosquitoes were collected by perfusion and pooled onto PTFE printed glass slides (Electron Microscopy Sciences, Hatfield, PA, USA) as described above. Cells were incubated at 4ºC for 1 h, and washed with PBS. Cells were fixed with 4% formaldehyde in PBS for 15 min at room temperature. Fixative was removed and cells were washed twice with PBS. Cells were stained with 10 µg/ml of Wheat Germ Agglutinin (WGA, Vector Labs, Burlingame, CA) in PBS for 10 min at room temperature in the dark. Cells were subsequently washed twice with PBS containing 0.3% Triton X-100 to remove unbound lectin, and mounted in Vectashield (Vector labs, Burlingame, CA). Slides were sealed with nail polish and stored at 4° C until further analysis.

Metabolic protein labeling using Fluorescent noncanonical amino acid tagging (FUNCAT)

To quantify total protein production in hemocytes in sugar-fed and blood fed mosquitoes, we labelled proteins through incorporation of the amino acid analog, L-azidohomoalanine (AHA) *in vivo* and detected of the incorporated azide by click-it chemistry (Hinz et al., 2012). FUNCAT assays were performed with the Click-iT® AHA Alexa Fluor® 488 Protein Synthesis assay (Invitrogen, Grand Island, NY, USA) following manufacturer's instructions. Mosquitoes were injected with 138 nl of 2.5mM AHA in PBS and allowed to recover under normal rearing conditions. As expected, intensity of signal was strongly time dependent (Fig. S1). Virtually no AHA incorporation was detectable at 10 min after injection, while a strong signal was observed after four hours post injection. All subsequent assays were thus performed using a four hour labeling period. Hemocytes were collected, fixed and processed as described above for the EdU

assays and according to the manufacturer's protocol. Cells were mounted in VectaShield medium (Vector Laboratories Inc., Burlingame, CA, USA), slides were sealed with nail polish, and stored at 4° C until further analysis.

Quantification of Immunofluorescence, Lectin, and AHA Staining

To quantify pERK, TEP1, PPO6, WGA, and AHA signals of hemocytes from sugar fed and blood fed mosquitoes, TIFF images were obtained with an Axioplan2 fluorescent light microscope (Zeiss, Jena, Germany) equipped with a camera and processed using the imaging software Image J (http://rsb.info.nih.gov/ij/). All images were taken with identical magnification. In addition, optimal fluorescence intensities and exposure times were empirically determined for each marker and kept constant between the two treatment groups. TIFF files were imported into Image J, where circles were drawn around cells and raw intensity values were obtained. To determine background fluorescence, the same circle was used to measure raw intensity values of a blank space in the image, which was subtracted from foreground values. Between 69 to 180 cells were analyzed per biological replicate and treatment group. Experiments were performed in triplicate with three independent biological replicates (Fig. S1 and 2).

Confocal microscopy and image analysis

Representative images were obtained using a LSM700 Confocal Microscope (Zeiss, Jena, Germany) using identical laser and microscope settings between samples. Images were processed using ZEN 2010 software (Zeiss, Jena, Germany), and figures were prepared with Photoshop and Illustrator software (Adobe Systems, San Jose, CA, USA).

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Author Contributions

Conceived and designed experiments: WBB, KM. Performed experiments: WBB. Analyzed data: WBB, KM. Wrote paper: WBB, KM.

Competing Interests

The authors declare no competing interests.

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Figure Legends

Figure 1. Blood Feeding Induces Hemocyte Proliferation.

(**A**) Hemocyte numbers are increased significantly in blood fed (BF) as compared to sugar fed (SF) mosquitoes (Mann-Whitney U-Test). *n* = 11 for each group from two independent biological replicates, graphed as median with interquartile range. (**B**) EdU incorporation is increased significantly in hemocytes from BF females (Mann-Whitney U test). *n* = 14 for each group, graphed as median with interquartile range. For each data point, 300-400 cells were assessed from a hemocyte pool collected from two mosquitoes. Confocal images of representative EdU-positive hemocytes are shown in panel (**C**). Blue, DAPI; green, EdU; scale bar is 10μm. (**D**) Flow cytometry analysis of PI-stained hemocytes from sugar fed and blood fed mosquitoes. Dot plots of PI fluorescent signal area over width (expressed in arbitrary units) illustrate DNA content per cell. Gates were drawn to eliminate putative aggregates based on high signal width from further analysis. Histograms show measurements for PI staining and thus DNA content for the gated cells. Markers designate three hemocyte populations based on DNA content, M1 representing euploid, M2 and 3 representing aneuploid cells. Percentages of cells within the three markers are indicated above the brackets. The figure shows a representative result from three independent biological replicates.

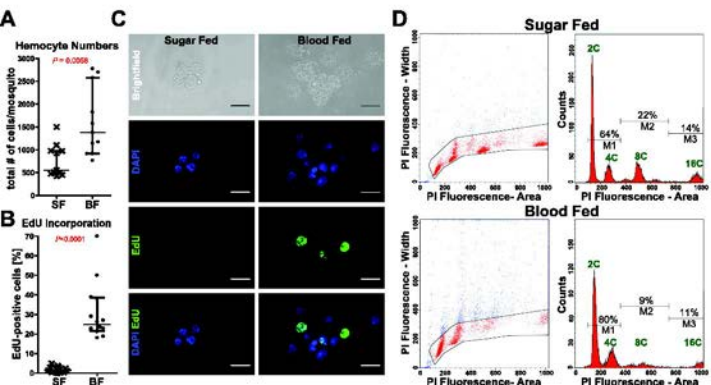
Figure 2. Blood Feeding Induces Hemocyte –Population and –Morphological Changes

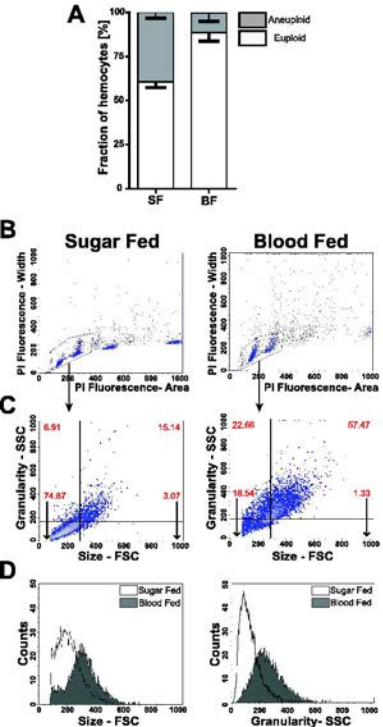
(**A**) The percentage of euploid (white) and aneuploid cells (gray) obtained from the flow cytometry analyses of PI-stained hemocytes are graphed as mean +/- s.e.m.. Euploid cells were gated (**B**) and analyzed for their size (FSC) and granularity (SSC). Axes are shown in arbitrary units. Density dot plots (**C**) illustrate size and granularity (expressed as arbitrary units) and their intensity on a blue to white color scale. (**D**) Overlaying histograms of sugar fed (open) and blood fed (filled) euploid hemocytes reveal an increase in average cell size and granularity after a blood meal. Data shown are representative of three independent experiments.

Figure 3. Blood Feeding Increases Expression of Several Cell Activation Markers

Hemocytes from sugar fed and blood fed females were analyzed for blood meal-induced activation markers. *n* = 91 for WGA (**A**), *n* = 126 for pERK (**B**), *n* = 103 for TEP-1 (**C**), *n* = 136

for PPO6 (**D**). Confocal maximum intensity projections are shown for all stains, blue, DAPI; green, WGA; red, pERK (B), TEP-1 (C), and PPO6 (D). Scale bar is 10μm. Quantification of activation markers was graphed as median with interquartile range. Blood feeding led to significant increase in fluorescence for all IFA analyses (Mann-Whitney U test, P <0.0001). Experiments were performed in triplicate with one representative experiment image and graph shown for each hemocyte activation marker. Results from all replicates are shown in Fig. S2.


A
Hemocyte Numbers
P = 0.0008
total # of cells/mosquito
3000
2500
2000
1500
1000
500
0
SF
BF
B
EdU Incorporation
P=0.0001
EdU-positive cells [%]
70
60
50
40
30
20
10
0
SF
BF
C
Sugar Fed
Blood Fed
Brightfield
DAPI
EdU
DAPI, EdU
D
Sugar Fed
PI Fluorescence - Width
PI Fluorescence- Area
Counts
2C
64%
M1
4C
22%
M2
8C
14%
M3
16C
Blood Fed
80%
M1
9%
M2
11%
M3


A
Aneuploid
Euploid
Fraction of hemocytes [%]
100
75
50
25
0
SF
BF
B
Sugar Fed
Blood Fed
PI Fluorescence - Width
PI Fluorescence- Area
C
Granularity - SSC
Size - FSC
6.91
15.14
74.87
3.07
22.66
57.47
18.54
1.33
D
Sugar Fed
Blood Fed
Counts
Size - FSC
Granularity- SSC

A
Brightfield
DAPI
WGA
DAPI
WGA
Sugar Fed
Blood Fed
arbitrary fluorescence (x10⁶)
SF
BF
B
Brightfield
DAPI
pERK
DAPI
pERK
Sugar Fed
Blood Fed
arbitrary fluorescence (x10⁶)
SF
BF
C
Brightfield
DAPI
TEP1
DAPI
TEP1
Sugar Fed
Blood Fed
arbitrary fluorescence (x10⁶)
SF
BF
D
Brightfield
DAPI
PPO6
DAPI
PPO6
Sugar Fed
Blood Fed
arbitrary fluorescence (x10⁶)
SF
BF