Mosquito hemocytes preferentially aggregate and phagocytose pathogens in the periostial regions of the heart that experience the most hemolymph flow

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Abstract

When a mosquito acquires an infection in the hemocoel, dedicated immune cells called hemocytes aggregate around the valves of the heart. These sessile hemocytes are called periostial hemocytes. In the present study we scrutinized the immune response mounted by the periostial hemocytes of the malaria mosquito, *Anopheles gambiae*, against bacterial pathogens, and tested the relationship between periostial hemocyte aggregation, immune activity, and hemolymph flow. Initially, we quantified the process of periostial hemocyte aggregation and found that hemocytes migrate to the periostial regions in response to infection with *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Micrococcus luteus* (all infections tested). Then, we investigated whether the periostial hemocytes are evenly distributed along the six periostial regions of the heart, and found that they preferentially aggregate in the periostial regions of the mid-abdominal segments (4, 5 and 6). This distribution perfectly correlates with the spatial distribution of phagocytic activity along the surface of the heart, and to a lesser extent, with the distribution of melanin deposits. Finally, experiments measuring circulatory physiology found that the majority of hemolymph enters the heart through the ostia located in the periostial regions of abdominal segments 4, 5, and 6. These data show that periostial hemocytes aggregate on the surface of the heart in response to diverse foreign stimuli, and that both hemocytes and immune activity preferentially occur in the regions that experience the swiftest hemolymph flow. Thus, these data show that two major organ systems – the immune and circulatory systems – interact to control infections.

Keywords: Immunity; Phagocytosis; Melanization; Bacteria; *Anopheles gambiae*; Insect.

1. Introduction

Mosquitoes have evolved robust innate immune responses that destroy pathogens (Bartholomay, 2014; Blair and Olson, 2014; Hillyer, 2010; Severo and Levashina, 2014). These immune responses act via phagocytic, lytic, and melanization pathways, and are driven by hemocytes, the fat body, and other tissues. Hemocytes are immune cells that are present in the hemocoel, and can be found both in circulation with the hemolymph as well as attached to tissues (Hillyer and Strand, 2014). Humoral factors that are secreted by hemocytes also circulate in the hemolymph, as well as pathogens that have penetrated the cuticle or have crossed the gut epithelium (Hillyer et al., 2007; Paulson and Grimstad, 1989; Povelones et al., 2011). While many pathogens succumb to immune responses, others escape or survive these assaults, as is the case of malaria parasites in susceptible mosquitoes (Mitri and Vernick, 2012). A further understanding of the dynamics of the immune processes that confer the resistance of mosquitoes to infection may lead to the development of new strategies to control vector-borne disease (Dong et al., 2011; Franz et al., 2006).

Because hemocytes, humoral immune factors and pathogens are present in the hemocoel, their biology is impacted by the circulation of hemolymph. Mosquitoes have an open circulatory system that propels hemolymph via the contractile action of a muscular tube called the dorsal vessel (Glenn et al., 2010; League et al., 2015). This dorsal vessel extends the length of the body and is comprised of two contiguous sections: the aorta in the thorax and the heart in the abdomen. Wave-like contractions of the heart propel the hemolymph, and these contractions periodically reverse the direction in which they propagate. When the contractions propagate toward the head (anterograde), hemolymph enters the dorsal vessel through 6 pairs of ostia (valves) that are sequentially located in the anterior portion of abdominal segments 2 through 7, and exits the vessel in the head. When the contractions propagate toward the posterior of the abdomen (retrograde), which occurs a minority of the time, hemolymph enters

the dorsal vessel through a single pair of ostia located at the thoraco-abdominal junction and exits the vessel in the 8th abdominal segment (Glenn et al., 2010; League et al., 2015).

Pathogens in the hemocoel, such as malaria parasites and arboviruses, circulate with the hemolymph as they attempt to complete an obligate migration across the mosquito hemocoel (Hillyer et al., 2007; Paulson and Grimstad, 1989). Recently, a novel, anti-malarial and antibacterial cellular immune response that occurs on the surface of the heart was described (King and Hillyer, 2012). Specifically, in the absence of infection, mosquitoes possess sessile hemocytes that are attached to the heart and heart-associated tissues at the location of the ostia. Because of their location at the periostial regions, these sessile hemocytes were named periostial hemocytes. Periostial hemocytes rapidly phagocytose *Escherichia coli* and *Plasmodium berghei* that are circulating with the hemolymph, and the periostial regions are the only locations where sessile hemocytes increase in number in response to infection (King and Hillyer, 2012, 2013). This increase in cell number is at least partly due to the directed migration of hemocytes to the periostial regions, which is advantageous as it places immune cells in the areas of highest hemolymph flow, thus increasing the probability that they will encounter circulating pathogens.

In this study we scrutinized the immune response mounted by the periostial hemocytes of the malaria mosquito, *Anopheles gambiae*, against multiple bacterial pathogens. We found that hemocytes aggregate at the periostial regions of the heart in response to all infections tested, and that periostial hemocytes preferentially aggregate in the mid-abdominal segments. This asymmetric distribution of hemocytes correlates with spatial differences in immune activity on the surface of the heart and with spatial differences in the rate of hemolymph flow through the ostia. Thus, these data show the correlative activities of the mosquito circulatory and immune systems, and support the hypothesis that periostial hemocyte aggregation is a basal immune response employed by mosquitoes against foreign invaders.

2. Materials and Methods

2.1 Mosquito rearing and maintenance

Anopheles gambiae Giles sensu stricto (G3 strain) were reared and maintained at 27°C and 75% relative humidity under a 12 h: 12 h light/dark photoperiod, and adults were fed a 10% sucrose solution as previously described (Estévez-Lao et al., 2013). All experiments were carried out using female mosquitoes at 5 or 6 days post-eclosion.

2.2 Mosquito injections and bacterial infections

For injections, mosquitoes were anesthetized on ice, a finely pulled glass needle was inserted through the thoracic anepisternal cleft, and 0.15-0.20 µl of a solution was injected into the hemocoel. For bacterial infections, tetracycline resistant/GFP-expressing *Escherichia coli* (DH5 alpha), *Micrococcus luteus*, *Staphylococcus aureus* (RN6390), and *Staphylococcus epidermidis* (HIP 04645) were grown and injected into mosquitoes as previously described (Coggins et al., 2012). Across experimental trials, infection doses per mosquito averaged 54,720 for *E. coli*, 45,733 for *S. epidermidis*, and 67,789 for *S. aureus*. Because *M. luteus* does not form individual colonies under our growing conditions, the exact dose per mosquito is unknown. However, all *M. luteus* infections were performed with cultures at OD₆₀₀ = 5.0. Control mosquitoes were injected with sterile LB broth (injured) or were left untreated (naïve).

2.3 Hemocyte labeling and mosquito dissections

Periostial hemocytes were stained and observed as previously described (King and Hillyer, 2012). Briefly, mosquitoes were anesthetized and injected 75 mM Vybrant CM-Dil Cell-Labeling Solution (Invitrogen, Carlsbad, CA, USA) and 0.75 mM Hoechst 33342 (Invitrogen) in PBS. Live mosquitoes were incubated at 27°C for 20 min, which allows the dye to incorporate into the hemocytes. Mosquitoes were anesthetized again, injected 16% formaldehyde, and the dorsal abdomens were placed in PBS containing 0.1% Tween-20 before being bisected along

the coronal plane. The dorsal abdomens, which contain the heart and the periostial hemocytes, were then either mounted between a microscope slide and a coverslip using Aqua Poly/Mount (Polysciences; Warrington, PA, USA) or further processed to stain muscle tissue. For the staining of muscle (including the heart), dorsal abdomens were incubated in 4% formaldehyde for 5 min, suspended in 0.6 μ M phalloidin-Alexa Fluor 488 (Invitrogen) in PBS for 10 hours at 4°C, washed twice in PBS, and mounted on microscope slides.

2.4 *Bright field and fluorescence microscopy: acquisition of still images*

A Nikon 90i compound microscope (Nikon Corp., Tokyo, Japan) equipped with a Nikon Intensilight C-HGFI fluorescence illumination unit and a Nikon DS-Qi1Mc CCD camera was used for the acquisition of still images. Samples were visualized under bright field and/or epi-fluorescence illumination, and Z-stacked images were acquired using a linear encoded Z-motor and Nikon Advanced Research NIS-Elements software. For the rendering of two-dimensional images from three-dimensional Z-stacks, all images in a Z-stack were combined to form a single focused image using the Extended Depth of Focus tool of NIS-Elements.

2.5 *Counting of periostial hemocytes*

Immediately after a dorsal abdomen was mounted on a microscope slide, it was visualized at 200-400X magnification and the periostial hemocytes present in the second through seventh abdominal segments were counted. A cell was considered a periostial hemocyte if it resided in a region immediately adjacent to an ostium or was aggregated with hemocytes that were associated with an ostium. Furthermore, for a cell to be considered a periostial hemocyte it had to be 9-18 μ m in diameter, and it had to be labeled with both CM-Dil and Hoechst 33342. For each treatment group (naïve, injured, and infected), a minimum of 20 mosquitoes were analyzed across 11 independent trials.

2.6 Quantification of phagocytosis and melanization of live *E. coli*

At 4, 9 and 24 h post-treatment, live mosquitoes were anesthetized and the dorsal abdomens were dissected and mounted on microscope slides. Fluorescence and brightfield Z-stacked images were acquired sequentially at 40x magnification, using identical microscope and camera settings for all samples.

Phagocytosis of *E. coli*-GFP was quantified by measuring fluorescence pixel intensity. Briefly, extended depth of focus images from naïve, injured and infected mosquitoes were used to determine the threshold of background fluorescence, and pixels with values below the threshold were assigned a value of zero as they represent noise, whereas pixels above the threshold were assigned values based on their brightness. Then, for each abdomen, the 6 periostial regions of the heart (one each in abdominal segments 2-7) were individually delineated by boxes of standardized size using the regions of interest (ROI) tool in NIS-Elements software, and the sum pixel intensity of GFP fluorescence in each ROI was measured. To normalize the phagocytosis readings, the average value for each periostial region in injured mosquitoes was subtracted from the corresponding value in *E. coli* infected mosquitoes. This final value represents the level of phagocytosis of *E. coli* by periostial hemocytes.

Because melanization results in dark deposits, this immune process was quantified by measuring the area of pixels with intensities below a threshold. This was done using the brightfield images in the same Z-stacks used to quantify phagocytosis. Briefly, by visualizing preparations from naïve, injured and infected mosquitoes, a threshold of pixel intensity was determined to distinguish melanized areas (below the threshold) from non-melanized areas (above the threshold). Subsequently, pixels with intensities that were below the threshold were assigned a value of 1 and pixels with intensities that were above the threshold were assigned a value of 0. The total area of melanization was then measured in each ROI. To normalize the melanization readings, the average value for each periostial region in injured mosquitoes was

subtracted from the corresponding value in *E. coli* infected mosquitoes. This final value represents the level of melanized *E. coli* at each periostial region. For the phagocytosis and melanization experiments, each treatment group contained a minimum of 24 mosquitoes that were assayed across 3 independent trials.

2.7 Quantification of phagocytosis of dead *E. coli*-pHrodo and *S. aureus*-pHrodo

E. coli or *S. aureus* bacterial bioparticles conjugated to pHrodo-Red (pHrodo; Invitrogen) were reconstituted in PBS at 1 mg/ml and injected into mosquitoes. Mosquitoes were returned to 27°C, and at 4, 9 and 24 h post-treatment, the dorsal abdomens were dissected, incubated in 4% paraformaldehyde for 5 min, washed in PBS for 5 min, and mounted on microscope slides. Images were captured and processed using the sum pixel intensity protocol described above for the measurement of phagocytosis. Injured and naïve mosquitoes served as controls. For each treatment group, a minimum of 37 mosquitoes were analyzed across 4 independent trials.

2.8 Intravital imaging by fluorescence microscopy

Hemocyte aggregation, phagocytosis, and hemolymph flow were visualized through the dorsal abdominal cuticle of live mosquitoes. For intravital observations of hemocytes and the process of phagocytosis, mosquitoes were injected pHrodo-conjugated bacteria to induce phagocytosis. Then, mosquitoes were restrained on microscope slides using a non-invasive method previously used to measure circulatory physiology (Boppana and Hillyer, 2014), and time-lapse series were acquired on the Nikon 90i compound microscope.

For intravital video imaging of hemolymph flow, naïve mosquitoes and mosquitoes that had been infected with *E. coli* for 24 h were restrained on Sylgard 182 silicone elastomer plates using a different non-invasive method also previously used to measure circulatory physiology (Andereck et al., 2010; Glenn et al., 2010). Mosquitoes were then injected 0.001% solids 2 µm diameter red fluorescent microspheres in PBS, and starting at 10 sec post-injection, a 70 sec

real-time video (8-9 frames s⁻¹) of the entire length of the abdomen was acquired under epifluorescence illumination on a Nikon SMZ1500 stereomicroscope connected to a CoolSNAP HQ² digital camera (Photometrics, Tucson, AZ). The usage of each ostial pair was quantified by manually counting the number of microspheres entering the lumen of the heart at each abdominal segment (League et al., 2015). For each mosquito, a minimum of 54 microspheres were counted as they entered the heart, and 30 and 25 mosquitoes that were distributed across 3 independent trials were analyzed for the naïve and infected groups, respectively.

2.9 Statistical analysis

Data were tested for normality using the Kolmogorov-Smirnov test and nonparametric data were transformed (natural log or square root) before using parametric tests. ANOVA followed by Tukey's post-hoc test, or repeated measures ANOVA (RM of segments) followed by Sidak's post-hoc test, were used to compare data from experiments with one variable, and RM two-way ANOVA (RM of segments) followed by Sidak's post-hoc test was used to compare data from experiments with two variables. Statistical analyses were performed using Prism 6 Software (GraphPad, La Jolla, CA, USA), and groups were deemed significantly different at $P < 0.05$.

3. Results

3.1 Periostial hemocytes aggregate in response to different bacterial infections

Following infection with *E. coli*, periostial hemocytes aggregate at the periostial regions of the heart in abdominal segments 2-7, where they phagocytose and kill pathogens (Figure 1A-D). To further characterize the range of foreign stimuli that induce the aggregation of hemocytes at the periostial regions, we counted the number of hemocytes on the surface of the heart in naïve mosquitoes, injured mosquitoes, and mosquitoes that had been infected with *Escherichia coli*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Micrococcus luteus* for 24 h. Periostial hemocyte aggregation was induced by all infections tested, but not by injury (Figures

1E-J, 2). More specifically, the average number of periostial hemocytes in naïve and injured mosquitoes was 63 and 79, respectively, and periostial hemocyte numbers increased by 2.3-fold, 1.8-fold, 1.7-fold, and 1.5-fold following *E. coli*, *S. epidermidis*, *S. aureus*, and *M. luteus* infection, respectively. Thus, periostial hemocyte aggregation is induced by diverse foreign stimuli.

3.2 *Periostial hemocytes preferentially aggregate in the periostial regions of abdominal segments 4, 5 and 6*

Although hemocytes aggregate in the periostial regions of all abdominal segments, initial observations suggested that the mid-abdominal segments contain the largest aggregations of periostial hemocytes (Figure 1D). We investigated this further by counting the number of periostial hemocytes in abdominal segments 2-7 of naïve mosquitoes, injured mosquitoes, and mosquitoes that had been infected with *E. coli*, *S. epidermidis*, *S. aureus*, and *M. luteus* for 24 h. In response to all infections, hemocyte aggregation was favored in the periostial regions of abdominal segments 4, 5 and 6 (Figure 3). The distribution of hemocytes formed a bell-shaped pattern that peaked in abdominal segments 4 or 5, progressively decreased in the adjacent abdominal segments, and bottomed in abdominal segments 2 and 7 (on average, segment 5 contained 2.7 times the number of periostial hemocytes than segment 2). Furthermore, the same pattern was observed in naïve and injured mosquitoes, indicating that infection induces a change in the number of periostial hemocytes but does not change their relative distribution (Figure 3). Across all treatments, in 84% of mosquitoes the largest periostial hemocyte aggregates were present in abdominal segments 4, 5, or 6, and these 3 abdominal segments contained 64% of the periostial hemocytes. Within an abdominal segment, periostial hemocytes occasionally aggregated unevenly among the ostial pair (Supplementary File 1: Figure S1). In summary, periostial hemocytes are preferentially distributed along the mid-abdominal segments.

3.3 *Phagocytosis by periostial hemocytes is highest in abdominal segments 4, 5 and 6*

Because most periostial hemocytes aggregated in the mid-abdominal segments, we hypothesized that immune activity on the surface of the heart follows a similar pattern. To spatially quantify periostial hemocyte immune activity, we measured the phagocytosis of GFP-expressing *E. coli* at 4, 9 and 24 h following infection. Similar to the distribution of periostial hemocytes, at all timepoints tested the phagocytosis of *E. coli* along the surface of the heart preferentially occurred in abdominal segments 4, 5 and 6 (Figures 4, 5A), with the highest level of phagocytosis occurring in abdominal segment 5. In 76% of mosquitoes the highest level of phagocytosis by periostial hemocytes occurred in abdominal segment 4, 5 or 6, and these mid-abdominal segments contained 66% of the phagocytic activity that occurs on the surface of the heart.

To rule out the possibility that bacteria co-aggregate with hemocytes but are not phagocytosed, we quantified phagocytosis using *E. coli* and *S. aureus* bacterial bioparticles that were conjugated to pHrodo, a pH sensitive dye that only fluoresces when inside the acidic confines of a phagolysosome. Similar to the distribution of periostial hemocytes and the phagocytosis of live *E. coli*-GFP, phagocytosis of *E. coli*-pHrodo and *S. aureus*-pHrodo along the surface of the heart was highest in abdominal segments 4, 5 and 6 (Figures 4, 5B-C). This spatial pattern of phagocytosis was similar at 4, 9, and 24 h post-injection (no interaction between time and segment), and the highest level of phagocytosis occurred in abdominal segment 5 (on average, segment 5 contained 4 times and 2.8 times the phagocytic activity of segment 2 following *E. coli*-pHrodo and *S. aureus*-pHrodo injection, respectively). In 79% of mosquitoes, the highest level of phagocytosis occurred in abdominal segment 4, 5 or 6, and these abdominal segments contained 64% of the phagocytic activity that occurs on the surface of the heart.

More detailed analysis of the kinetics of the phagocytosis response at the periostial regions revealed the rapid nature and effectiveness of this immune process. *E. coli*-GFP and

pHrodo bioparticles were detected in all abdominal segments and at all timepoints tested. In the experiments using live *E. coli*-GFP, the highest signal of phagocytosis was observed 4 h after infection, and this signal progressively decreased by 9 and 24 h after infection, indicating that the bacteria were being killed. However, pHrodo signal inside of the peristial hemocytes remained stable or increased over time, remaining visible at 5 days post-challenge (Supplementary File 2: Figure S2), indicating that unlike the bacteria that express GFP, this fluorescent dye is not degraded by the peristial hemocytes. Taken altogether, these data show that the regions of the heart with the greatest numbers of peristial hemocytes (Figure 3) are also the regions of the heart with the highest capacities for phagocytic activity (Figures 4, 5).

3.4 *Peristial hemocyte preference for the mid-abdominal segments is observed immediately following infection*

To visualize the phagocytic activity of peristial hemocytes in real-time, we performed fluorescence-based intravital video imaging through the dorsal cuticle of intact mosquitoes immediately following injection of *E. coli*-pHrodo. Phagocytosis was observed at all of the peristial regions within seconds of injection. By 1 min post-injection, the mid-abdominal segments had measurable quantities of phagocytosis, and the signal increased by 4 min post-injection (Figure 6, Supplementary File 3: Movie S1). This continued over time as more pHrodo was internalized by hemocytes and more hemocytes aggregated along the heart. Video analyses also revealed that hemocyte aggregation is dynamic; during the course of an infection hemocytes migrate into and out of the peristial regions. That is, peristial hemocytes (individually and as aggregates) can leave the peristial regions and reenter circulation, and circulating hemocytes can leave circulation and actively aggregate along the surface of the heart (Supplementary File 4: Movie S2, Supplementary File 5: Movie S3). Circulating hemocytes can also attach to non-peristial tissues (e.g., the cuticle and the trachea), detach from these tissues, re-enter circulation, and then attach to the peristial regions (Supplementary

File 5: Movie S3). This movement of hemocytes appears to be either by active migration or adhesive capture (and not passive transport), because, for example, whereas hemolymph moves at approximately 6-8 mm/sec (Estévez-Lao et al., 2013; Glenn et al., 2010; League et al., 2015), it can take upwards of 5 min for hemocytes to complete the directed movement from a trachea to the periostial regions (Supplementary File 5: Movie S3). Together with earlier quantitative measurements of hemocyte migration to the periostial regions (King and Hillyer, 2012), these observations show the dynamic nature of the hemocyte population, and that despite the continual transitions from a sessile to a circulating state (and vice versa), the periostial regions retain a distinct pattern of hemocyte distribution along the surface of the heart.

3.5 *Infection induces the deposition of melanin at the periostial regions*

Another immune process that we observed to be associated with the periostial regions was melanization. Melanization is a humoral immune response that is driven by a tightly regulated phenoloxidase cascade, and the enzymes involved in this pathway are produced by hemocytes (Christensen et al., 2005; Hillyer, 2010). To determine if the deposition of melanin on the surface of the heart is influenced by the distribution of periostial hemocytes, we quantified the relative amount of melanin associated with the heart at 4, 9 and 24 h following infection with *E. coli*-GFP (Figure 7A-B). At 4 and 9 h after infection, there was a trend for melanin to be primarily present in abdominal segment 4. By 24 h after infection, the amount of melanin increased significantly, which was accompanied by a change in the relative distribution of melanin along the periostial regions (interaction between time and segment). This interaction was manifested by a dramatic increase in melanin deposition within the periostial regions of abdominal segments 5, 6, and 7. Moreover, between 9 and 24 h after infection the levels of melanin increased by at least 16-fold in all abdominal segments except for segment 4, where melanin only increased by 3-fold.

While phagocytosis by periostial hemocytes commenced immediately following infection, melanin deposition on the heart took significantly longer to occur, indicating that differences exist in the spatial and temporal dynamics of these two immune responses. Furthermore, although dark pigments were often observed within the pericardial cells, these pigments were clearly different from melanin deposits as they were lighter in color and did not differ between naïve, injured and infected mosquitoes (Figure 7C). In summary, infection induces the deposition of melanin in the periostial regions. The kinetics of melanization is slower than the kinetics of phagocytosis, and the spatial distribution of melanization, while favoring the mid-abdominal segments, is variable.

3.6 *The distribution of periostial hemocytes correlates with the patterns of hemolymph entry into the heart*

Hemolymph flows through the periostial regions immediately prior to entering the heart (Glenn et al., 2010; King and Hillyer, 2012; League et al., 2015). To determine whether the pattern of hemolymph entry into the heart correlates with the distribution of periostial hemocytes, neutral-density fluorescent microspheres were injected into the hemocoel of naïve and *E. coli* infected mosquitoes, and their movement was used to measure the flow of hemolymph (Andereck et al., 2010; Boppana and Hillyer, 2014; Glenn et al., 2010; League et al., 2015). Particle tracking revealed that the rate of hemolymph entry into the heart through each ostial pair is different between abdominal segments (Figure 8). Specifically, in both naïve and infected mosquitoes a larger percentage of hemolymph entered the heart through the ostia located in abdominal segments 4, 5 and 6. Although the trend in both experiments was similar, the asymmetric usage of the ostia was less pronounced in infected mosquitoes, suggesting that the large mass of periostial hemocytes in the mid-abdominal segments may slightly occlude the ostia, thus redirecting some of the hemolymph to the anteriormost and posteriormost abdominal segments. Regardless, these experiments show that the patterns of hemolymph entry into the

heart correlate with the spatial distribution of hemocytes (and immune processes) on the surface of the heart.

4. Discussion

Upon infection, circulating hemocytes migrate to the regions surrounding the heart's ostia, and these are the only locations where sessile hemocytes increase in number as an infection progresses (King and Hillyer, 2012, 2013). The periostial regions of the heart comprise a very small portion of the entire abdomen, yet hemocytes in these locations sequester greater than 40% of the bacteria present on the abdominal wall (King and Hillyer, 2012). Thus, the specific aggregation of hemocytes in the periostial regions, together with the heightened immune activity present within these locations, suggests that deploying immune cells to fight an infection in regions of high hemolymph flow is advantageous for the survival of the insect. In the present study we expand on these findings and show that (1) periostial hemocyte aggregation is a basal immune response that is activated by diverse stimuli; (2) periostial hemocyte aggregation is dynamic and occurs in a consistent, asymmetric pattern on the surface of the heart; and (3) the spatial pattern of periostial hemocyte aggregation correlates with spatial patterns of immune activity and hemolymph flow (Figure 9).

Prior to the initiation of the present project it was known that periostial hemocyte aggregation is induced in mosquitoes by infection with *E. coli* and *Plasmodium* parasites (King and Hillyer, 2012, 2013), and subsequent structural analyses observed the presence of hemocytes at the periostial regions of the heart (Leódido et al., 2013). Other papers that describe the immune response of mosquitoes also contain images that show the accumulation of pathogens and melanin at locations now referred to as the periostial regions (Hernández-Martínez et al., 2013; Hillyer et al., 2007; Michel et al., 2005; Schnitger et al., 2007; Yassine et al., 2014; Yassine et al., 2012). Additionally, hemocytes have been observed on or in the heart of *Drosophila melanogaster*, *Baculum extradentatum* and *Galleria mellonella* (da Silva et al.,

2012; Ghosh et al., 2015; Horn et al., 2014; Pereira et al., 2015), and the perioistal accumulation of pathogens and melanin has been detected in both dipteran and lepidopteran insects (Akbar et al., 2011; Bao et al., 2011; Chambers et al., 2012; Cuttall et al., 2008; Elrod-Erickson et al., 2000; Kocks et al., 2005; Pereira et al., 2015; Shiratsuchi et al., 2012; Stone et al., 2012). Analogous interactions between immune and circulatory processes also occur in crustaceans, which like insects are members of the phylum Arthropoda (Hillyer, 2015). Thus, our finding that perioistal hemocyte aggregation is induced by all the immune insults tested so far, together with the observation that strong immune responses occur at the perioistal regions of a diverse group of insects, suggests that perioistal hemocyte aggregation is a primary and basal immune response in this arthropod group.

Perioistal hemocytes are not evenly distributed amongst the perioistal regions; regardless of the infections status, we observed that the distribution of perioistal hemocytes forms a bell-shaped curve that peaks in abdominal segment 4 or 5 and bottoms in segments 2 and 7. Furthermore, our quantitative analyses show that the strength of the phagocytosis response mimics the spatial distribution of hemocytes, and images from earlier qualitative studies show a pattern of pathogen aggregation along the surface of the mosquito heart that is similar to the one we observed here (Hillyer et al., 2007; King and Hillyer, 2012). Finally, qualitative analyses of images published in papers examining immune responses in *D. melanogaster* suggest that the spatial distribution of hemocytes along the heart of this insect is also asymmetric, but in this case it is the anteriormost abdominal segments that contain both the highest number of immune cells (Ghosh et al., 2015; Horn et al., 2014) and the largest aggregations of pathogens (Akbar et al., 2011; Cuttall et al., 2008; Elrod-Erickson et al., 2000; Shiratsuchi et al., 2012). Thus, although perioistal immune responses appear to occur in both of these dipterans, the activities are spatially dissimilar, which could be due to differences in the overall anatomy and circulatory physiology between these two insect families. For example, mosquitoes have an elongated and slender abdomen, whereas the abdomen of fruit flies is

more compact. Additionally, the number of ostia are different between mosquitoes and fruit flies, and relative to mosquitoes, fruit flies contain an exaggerated conical heart chamber in the anteriormost portion of the abdomen (Glenn et al., 2010; League et al., 2015; Wasserthal, 2007).

While infection-induced periostial hemocyte aggregation and phagocytosis were consistent and rapid responses, melanin deposition at the periostial regions was more variable and took longer to develop. Phenoloxidase-based melanization is an extracellular, enzymatic process used by insects to kill a wide variety of pathogens (Christensen et al., 2005; Nappi and Christensen, 2005). Mosquito hemocytes called oenocytoids produce and secrete the enzymes that drive the melanization cascade (Castillo et al., 2006; Hillyer and Christensen, 2002; Hillyer and Christensen, 2005; Hillyer et al., 2003a), and melanized bacteria are often phagocytosed by hemocytes called granulocytes (Hillyer et al., 2003a; Hillyer et al., 2003b, 2004). Thus, the differences in the kinetics and spatial organization of melanization versus phagocytosis at the periostial regions could be because of the humoral nature of the melanization response. Specifically, we hypothesize that pathogens are first melanized in non-periostial regions, are then swept by the hemolymph, and are finally phagocytosed by periostial hemocytes. Alternately, the differences observed could be due to the lower resolution of our melanization assay, as this response was weak in comparison to phagocytosis. The difference in the strength of these two pathways was not unexpected, as relative to melanization, phagocytosis is the primary immune pathway employed by mosquitoes to kill *E. coli* (Hillyer et al., 2003a; Hillyer et al., 2003b, 2004). Regardless, images published in the only other study that, to our knowledge, has shown melanin deposits along the length of the mosquito abdomen qualitatively display a pattern of distribution that is similar to the one observed in our study (Michel et al., 2005). There was also a difference in the timing of the phagocytosis response, as *E. coli*-pHrodo became internalized at a faster rate than *S. aureus*-pHrodo. This difference, observed

in the periostial regions, loosely parallels the difference previously reported when examining the lateral abdomen of *A. gambiae* (Moita et al., 2005).

By aggregating around the ostia, periostial hemocytes have a higher probability of encountering pathogens than non-periostial hemocytes because of the higher flow of hemolymph at these locations, and because all hemolymph flows through these areas. The dorsal vessel, and more specifically the heart, is the primary organ responsible for the propulsion of hemolymph (Andereck et al., 2010; Glenn et al., 2010; League et al., 2015). In mosquitoes, the heart spends the majority of the time contracting in the anterograde direction, and extracardiac hemolymph enters this vessel through ostia located in abdominal segments 2-7. Given that there are multiple locations where hemolymph can enter the dorsal vessel, the bell-shaped spatial distribution of periostial hemocytes would be especially advantageous if it placed the majority of hemocytes at the ostia that experience the highest levels of hemolymph flow. Our experiments quantifying the entry of hemolymph through the different ostial pairs – the first such experiment performed in any insect – show that this is indeed the case; the majority of hemolymph enters the heart via the ostial pair located in abdominal segment 5, and the least amount of hemolymph enters via the ostial pairs in segments 2 and 7. This bell-shaped pattern occurs in both naïve and infected mosquitoes, although the inflection of the curve is slightly smaller in infected mosquitoes. This minor decrease in inflection could be due to the slight obstruction of hemolymph flow by the periostial hemocytes, but regardless of this, the spatial correlation of hemocyte aggregation, immune activity and hemolymph flow demonstrates the advantages of placing immune cells in the areas of highest hemolymph flow. Because the structure of the heart is conserved across mosquito genera (Glenn et al., 2010; League et al., 2015; Leódido et al., 2013; Martins et al., 2011), the mid-abdominal segments are likely areas of high cellular immune activity across Culicidae.

Relative to naïve and injured mosquitoes, mosquitoes that have suffered an infection retain an elevated number of periostial hemocytes for the rest of their life (King and Hillyer,

2012). Whether the same hemocytes remain at the peristial regions throughout this time is not completely understood, but our data suggest that this is not the case. Intravital imaging showed that peristial hemocytes, and hemocytes in general, are dynamic; hemocytes attach and detach from the peristial regions, thus switching from a circulating to a sessile state and vice versa. Similar switching from sessile to circulating states has also been observed in *Drosophila* (Babcock et al., 2008; Makhijani et al., 2011; Márkus et al., 2009). However, a switch in spatial state may not be the only mechanism by which peristial hemocytes increase in number in response to infection. In *A. gambiae*, an infection induces not only an increase in peristial hemocytes, but also an increase in circulating hemocytes (Coggins et al., 2012; King and Hillyer, 2013). A hematopoietic gland in mosquitoes has yet to be discovered, but the hemocytes in adult-stage insects from both major subfamilies of mosquitoes undergo cell division, and this replication occurs at least in part while the cells are in circulation (Bryant and Michel, 2014; Castillo et al., 2011; King and Hillyer, 2013). In *D. melanogaster*, the hematopoietic lymph gland degenerates around the time of eclosion, but the hemocytes in adults continue to replicate while positioned along the surface of the heart (Ghosh et al., 2015). Perhaps a similar process occurs in mosquitoes.

5. Conclusions

Mosquito-borne pathogens such as malaria and arboviruses undergo an obligate intrahemocoelic migration from the midgut to the salivary glands prior to being transmitted. During the course of this migration these pathogens are subject to both immune responses and circulatory currents. The present study illustrates the strength and kinetics of an immune response that occurs on the surface of the heart of mosquitoes, and shows how two major organ systems – the immune and circulatory systems – interact to control infections.

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Figures Legends

Figure 1. Perioistial hemocytes aggregate on the surface of the mosquito heart. (A)

Fluorescence image of a perioistial region of an uninfected mosquito, showing the heart (outlined; muscle is stained green with phalloidin-Alexa Fluor 488), the location of the ostia (asterisks), the diamond shaped alary muscles (e.g., arrows), and the perioistial hemocytes (labeled red with CM-Dil). (B-C) Fluorescence (B) and bright field (C) images of a perioistial region of a mosquito that had been infected with *E. coli*-GFP (green) for 24 h, showing perioistial hemocytes (red) that had phagocytosed *E. coli*. (D) Fluorescence image of the dorsal abdomen of a mosquito that had been infected with *E. coli*-GFP (green) for 24 h, showing the perioistial regions of abdominal segments 2 through 7 (circled; the abdominal sutures are marked with vertical lines) and the phagocytic perioistial hemocytes (red). (E-J) Fluorescence images showing perioistial hemocytes (red) in a perioistial region of a naïve mosquito (E), an injured mosquito (F), and mosquitoes that had been infected with *E. coli* (G), *S. epidermidis* (H), *S. aureus* (I), and *M. luteus* (J) for 24 h. For all fluorescence images, Hoechst 33342 (blue) labeled the cell nuclei. Horizontally oriented dashed lines outline the heart (except for D, where the horizontal line marks the lumen of the heart), and asterisks denote the locations of the ostia. Bars: A-C and E-J, 100 µm; D, 200 µm. A, anterior; P, posterior; L, lateral.

Figure 2. Hemocytes aggregate in the perioistial regions following bacterial infection.

Graph shows the number of perioistial hemocytes in naïve mosquitoes, injured mosquitoes, and mosquitoes that had been infected with *E. coli*, *S. epidermidis*, *S. aureus*, and *M. luteus* for 24 h. Column heights mark the mean and whiskers denote the standard error of the mean. P-value results from ANOVA, and asterisks denote treatment groups that are significantly different from the reference group (R; Tukey's test).

Figure 3. Regardless of treatment, periostial hemocytes preferentially aggregate in abdominal segments 4, 5 and 6. Graphs show the number of periostial hemocytes in abdominal segments 2 through 7 of naïve mosquitoes (A), injured mosquitoes (B) and mosquitoes that had been infected with *E. coli* (C), *S. epidermidis* (D), *S. aureus* (E), and *M. luteus* (F) for 24 h. In all treatment groups, periostial hemocytes were preferentially present in abdominal segments 4, 5, and 6. Column heights mark the mean and whiskers denote the standard error of the mean. P-values result from repeated measures ANOVA, and asterisks denote treatment groups that are significantly different from the reference group (R; Sidak's test).

Figure 4. Phagocytosis by periostial hemocytes is highest in the mid-abdominal segments. Fluorescence images of the dorsal portion of dissected abdomens, showing the phagocytosis of *E. coli*-GFP (left column), *E. coli*-pHrodo (middle column) and *S. aureus*-pHrodo (right column) at 4 h (top row), 9 h (middle row), and 24 h (bottom row) following treatment. Quantitative analyses of these and other images are presented in Figure 5. Regardless of treatment and time, the highest level of phagocytosis was detected in the periostial regions of the mid-abdominal segments. Bar, 500 μ m. A, anterior; P, posterior; L, lateral.

Figure 5. Phagocytosis by periostial hemocytes is highest in abdominal segments 4, 5, and 6. Graphs show quantitative analyses measuring the phagocytosis of *E. coli*-GFP (A), *E. coli*-pHrodo (B) and *S. aureus*-pHrodo (C) at 4 h, 9 h, and 24 h following treatment. Regardless of treatment and time, the highest levels of phagocytosis were detected in the periostial regions of abdominal segments 4, 5, and 6. Column heights mark the mean and whiskers denote the standard error of the mean. P-values result from repeated measures two-way ANOVA.

Figure 6. Phagocytosis by periostial hemocytes commences immediately following an immune challenge. Still images from intravital imaging experiment presented in Supplementary File 3: Movie S1. In the movie, phagocytosis of *E. coli*-pHrodo was visualized through the dorsal abdomen for 30 min, beginning immediately following the introduction of the foreign agent. Still images were extracted at 1, 4, 10, 20 and 30 min post-treatment, and show that phagocytic activity by periostial hemocytes begins immediately following treatment and preferentially takes place in the periostial regions of the mid-abdominal segments. Bar, 500 μ m. A, anterior; P, posterior; L, lateral.

Figure 7. Following *E. coli* infection, melanin accumulates in the periostial regions. (A) Bright field and fluorescence image overlay of the dorsal portion of an abdomen, showing *E. coli*-GFP (green) and melanin (black) on the heart at 24 h after infection. The periostial regions of abdominal segments 2 through 7 are circled. (B) Graph shows the quantitative analysis of melanin deposition in the periostial regions of abdominal segments 2 through 7 at 4, 9, and 24 h following infection with *E. coli*. The pattern of melanin deposition along the surface of the heart changed with time, and there was an interaction between time and segment, indicating that as time progressed there were changes in the spatial distribution of melanin deposits. Column heights mark the mean and whiskers denote the standard error of the mean. P-values result from repeated measures two-way ANOVA. (C) Bright field images of the dorsal portion of the fifth abdominal segment in naïve, injured and *E.coli* infected mosquitoes at 24 h post-treatment, showing natural pigments in uninfected mosquitoes and dark melanization deposits in an infected mosquito. Bars: A, 200 μ m; C, 50 μ m. A, anterior; P, posterior; L, lateral.

Figure 8. Hemolymph preferentially enters the heart through the ostia in abdominal segments 4, 5 and 6. Graphs show the percent of hemolymph entering the heart through the ostia in abdominal segments 2 through 7, as quantified by measuring the movement of

neutrally-buoyant, 2 μm diameter fluorescent microspheres. In naïve mosquitoes (A) and mosquitoes infected with *E. coli* for 24 h (B), the majority of hemolymph entered the heart through the ostia located in abdominal segments 4, 5 and 6. Column heights mark the mean and whiskers denote the standard error of the mean. P-values result from repeated measures ANOVA, and asterisks denote treatment groups that are significantly different from the reference group (R; Sidak's test).

Figure 9. Schematic of hemolymph flow and infection-induced periostial hemocyte aggregation in *Anopheles gambiae*. Hemocytes exist in two states: in circulation and in sessile form.

In naïve mosquitoes (top), some of the sessile hemocytes are aggregated around the ostia (valves) of the heart, and are called periostial hemocytes. Hemolymph flow is swift in the regions surrounding the ostia (the periostial regions), and the majority of hemolymph enters the heart through the ostia located in abdominal segments 4, 5, and 6. Upon infection (bottom), circulating hemocytes undergo mitosis and increase in number, and many hemocytes migrate to, and aggregate in, the periostial regions of the heart where they phagocytose pathogens (King and Hillyer, 2012, 2013). The periostial regions are the only location of the body where sessile hemocytes increase in number in response to infection (King and Hillyer, 2013). Infection does not significantly alter the proportion of hemolymph that flows through each periostial region, and periostial hemocytes – including their immune activity – preferentially aggregate around the ostia that experience the most hemolymph flow.

A. Naive

$P < 0.0001$

B. Injured

$P < 0.0001$

C. *E. coli*

$P = 0.0001$

D. *S. epidermidis*

$P = 0.0074$

E. *S. aureus*

$P < 0.0001$

F. *M. luteus*

$P < 0.0001$

Abdominal segment

A. *E. coli*-GFP **B. *E. coli*-pHrodo** **C. *S. aureus*-pHrodo**

E. coli-pHrodo

A. Bright field and fluorescence (green) at 24 h: *E. coli*-GFP

B. Quantification of melanization: *E. coli*-GFP

C. Bright field of abdominal segment 5 at 24 h:

A. Naive

$P < 0.0001$

B. *E. coli*

$P < 0.0001$

Abdominal segment

Infection-induced migration of hemocytes to the mosquito heart