RESEARCH ARTICLE

Hemolymph circulation in insect flight appendages: physiology of the wing heart and circulatory flow in the wings of the mosquito *Anopheles gambiae*

Ravi Theja V. Chintapalli and Julián F. Hillyer*

ABSTRACT

The wings of insects are composed of membranes supported by interconnected veins. Within these veins are epithelial cells, nerves and tracheae, and their maintenance requires the flow of hemolymph. For this purpose, insects employ accessory pulsatile organs (auxiliary hearts) that circulate hemolymph throughout the wings. Here, we used correlative approaches to determine the functional mechanics of hemolymph circulation in the wings of the malaria mosquito *Anopheles gambiae*. Examination of sectioned tissues and intravital videos showed that the wing heart is located underneath the scutellum and is separate from the dorsal vessel. It is composed of a single pulsatile diaphragm (indicating that it is unpaired) that contracts at 3 Hz and circulates hemolymph throughout both wings. The wing heart contracts significantly faster than the dorsal vessel, and there is no correlation between the contractions of these two pulsatile organs. The wing heart functions by aspirating hemolymph out of the posterior wing veins, which forces hemolymph into the wings via anterior veins. By tracking the movement of fluorescent microspheres, we show that the flow diameter of the wing circulatory circuit is less than 1 µm, and we present a spatial map detailing the flow of hemolymph across all the wing veins, including the costa, sub-costa, ambient costa, radius, media, cubitus anterior, anal vein and crossveins. We also quantified the movement of hemolymph within the radius and within the ambient costa, and show that hemolymph velocity and maximum acceleration are higher when hemolymph is exiting the wing.

INTRODUCTION

Many insects rely on their wings as a primary source of locomotion (Wootton, 1992; Chapman and Taylor, 2013). The mimicry conferred by the color patterns of some wings also provides protection against predators, and both wing interference patterns and the harmonics of wing beats can play roles in mate selection (Stevens, 2005; Cator et al., 2009; Gibson et al., 2010; Katayama et al., 2014). Wings are composed of thin membranes that are supported by a system of interconnected veins (Comstock and Needham, 1898; Chapman and Taylor, 2013; Pass et al., 2015). Veins are lined by a layer of epidermal cells, and traversing many veins are a trachea and a nerve, with the latter eventually connecting to sensory cells. The proper function of cells within the wings requires that nutrients, waste and signaling molecules be efficiently transported into and out of these appendages. This transport, along with processes that are required for wing inflation and maturation following eclosion, is dependent on the insect circulatory system (Arnold, 1964; Pass et al., 2015).

The circulatory system of insects is composed of a fluid known as hemolymph, an open body cavity known as the hemocoel, and a series of muscular pumps (Pass et al., 2006; Chapman et al., 2013; Wirkner et al., 2013; Hillyer, 2015). The primary pump is the dorsal vessel, which is a muscular tube that extends the length of the insect and is divided into a heart in the abdomen and an aorta in the thorax and head. While effective in disseminating molecules and immune cells to most parts of the body, the propulsion created by the dorsal vessel is insufficient to deliver hemolymph to some regions of the body that are either narrow or distant from the dorsal vessel. Thus, insects have evolved accessory pulsatile organs (APOs, or auxiliary hearts) that drive hemolymph into areas such as the antennae, the wings and the ventral abdomen (Pass, 2000; Pass et al., 2006; Togel et al., 2008; Andereck et al., 2010; Boppana and Hillyer, 2014; Hustert et al., 2014).

In most insects, wing APOs are present in a location of the thorax that is medial to the wings and immediately ventral of the scutellar cuticle (Arnold, 1964; Pass et al., 2015). In general, these circulatory organs function by periodically increasing the volume of a sinus located between the scutellar cuticle and a pulsatile diaphragm, with each expansion aspirating hemolymph out of the wings via posterior wing veins. This, in turn, draws hemolymph into the wings via veins in the anterior of the appendage. Although this mode of action is fairly conserved across taxa, the kinetics of contractions are largely unknown and the structure of these organs varies significantly (Krenn and Pass, 1994a,b; Pass et al., 2015). Some wing circulatory organs are extensions of the dorsal vessel. Others are structurally independent of the dorsal vessel and are called wing hearts. When independent from the dorsal vessel, wing hearts can be paired organs, in which case each wing has its own pulsatile diaphragm, or an unpaired organ, in which case a single pulsatile diaphragm circulates hemolymph in both wings. Within the order Diptera, which encompasses the flies, wing circulatory organs are structurally independent of the dorsal vessel, and can be either paired, as in *Drosophila* sp. and *Ceratitis capitata*, or unpaired, as in *Tipula* sp. and *Haematopoda pluvialis* (Krenn and Pass, 1994a). The functional mechanics of the wing heart of mosquitoes remains unknown. In the present study, we scrutinized the circulatory physiology of the wing of the malaria mosquito *Anopheles gambiae*. We describe an unpaired wing heart, demonstrate that it contracts independently from the dorsal vessel, and uncover the trajectory and velocity of hemolymph within the wing space.

Department of Biological Sciences, Vanderbilt University, Nashville, TN 37205, USA.

*Author for correspondence (julian.hillyer@vanderbilt.edu)

J.F.H., 0000-0002-3178-0201

MATERIALS AND METHODS
Mosquito rearing and maintenance
Anopheles gambiae Giles *sensu stricto* (G3 strain) were reared as described previously (Glenn et al., 2010). Eggs were hatched in distilled water, and larvae were fed yeast and koi food. Pupae were transferred to plastic containers with a tulle ceiling, and upon eclosion the adults fed on a 10% sucrose solution *ad libitum*. Mosquito rearing and maintenance was performed at 27°C, 75% relative humidity and a 12 h:12 h light:dark cycle. All experiments were conducted on adult, female mosquitoes at 5 days post-eclosion.

Histology of the wing APO
Thoraces were isolated using a razor blade to make two cuts. The first severed the head at the anterior-most region of the thorax and the second severed the abdomen immediately posterior of the thoraco-abdominal junction (thereby leaving the scutellum intact). The thoraces were fixed for 2 h by immersion in 4% formaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in 0.1 mol l^{-1} phosphate buffer (pH 7.0), dehydrated using a graded ethanol series (70%, 90%, 100%), infiltrated overnight in JB4-Plus resin (Electron Microscopy Sciences) and embedded in JB4-Plus resin using molding trays and block holders anaerobically sealed with wax. Sections of 2.5 μm thickness were cut using a glass knife on a JB-4 microtome (Sorvall, Newtown, CT, USA), and placed on glass slides. Sections were then stained with hematoxylin and eosin, dried and mounted under coverslips using Poly-Mount (Polysciences Inc., Warrington, PA, USA). Slides were imaged using a Nikon 90i compound microscope connected to a Nikon DS-Fi1 color CCD camera and Nikon Advanced Research NIS-Elements software (Nikon Corp., Tokyo, Japan).

Scanning electron microscopy
Whole mosquitoes were placed in ethanol and dehydrated using a graded ethanol series (70%, 90%, 100%). Mosquitoes were then dried by the critical point procedure using an E3100 drier (Quorum Technologies, East Sussex, UK), mounted on stubs in a dorsal-side-up position, sputter coated with gold using a Sputter Coater 108 (Cressington, Watford, UK) and viewed using a Tescan Vega-II scanning electron microscope (Brno, Czech Republic).

Intravital imaging of mosquito wing APOs and the dorsal vessel
Mosquitoes were anesthetized for less than a minute by placing them in a Petri dish resting on ice. Immediately prior to use, a mosquito was injected at the thoracic anepisternal cleft with 0.1 μl of 0.1% solids, 0.5 μm diameter, neutral-density fluorescent microspheres (Invitrogen, Carlsbad, CA, USA) in PBS, and was then restrained in a dorsal-side-up position on a Sylgard 184 silicone elastomer (Dow Corning, Midland, MI, USA) plate via a non-invasive pinning strategy previously described and pictured (Andereck et al., 2010; Glenn et al., 2010). This restraint prevented mosquito movement whilst preserving a natural body position during imaging. The mosquito wing APO was then video-recorded for 65 s through the scutellum (20 frames s^{-1}), under low-level fluorescence illumination, using a Nikon SMZ1500 stereomicroscope equipped with a Hamamatsu ORCA-Flash 2.8 digital CMOS camera (Hamamatsu Photonics, Hamamatsu, Japan) and Nikon Advanced Research NIS-Elements software. Then, another 65 s video of the same mosquito was taken, but this time imaging the heart portion of the dorsal vessel through the abdominal tergum. Following video acquisition, each wing heart contraction was manually counted by visualizing the spatial shift of microspheres that aggregated at the periphery of the pulsatile diaphragm. Similarly, each heart contraction was manually counted by visualizing the spatial shift of microspheres that aggregated on the surface of the heart, a process that occurs because of the phagocytic activity of periostial hemocytes (King and Hillyer, 2012). A total of 39 mosquitoes was assayed, with paired data obtained for the contractions of both the wing heart and the dorsal vessel.

Assessing hemolymph flow in the wing
Mosquitoes were anesthetized, injected with microspheres and restrained as detailed above, except that only one wing was held in the extended position while the other wing was allowed to remain resting on the mosquito's dorsum. A 60 s video of the resting wing was recorded using the same method used to image the wing heart, and the movement of microspheres as they traveled across the wing was measured and used as a proxy for the flow of hemolymph in a manner similar to what we have previously done for the antennae, the dorsal vessel and the hemocoel (Andereck et al., 2010; Glenn et al., 2010; Boppana and Hillyer, 2014; League et al., 2015). Initially, videos were used to qualitatively determine the directional flow of hemolymph across each wing vein, and later, they were used to quantitatively monitor the trajectory, distance, velocity and maximum acceleration of microspheres as they traveled in the wing space (Fig. S1). Quantitative measurements were performed using the Object Tracker module of NIS-Elements, with hemolymph entry into the wing being measured within the radius and hemolymph exit out of the wing being measured within the ambient costa. For quantitative analyses, 20 mosquitoes were imaged, and for each mosquito, five microspheres were tracked while they flowed into the wing and five microspheres were tracked while they flowed out of the wing (100 microspheres in each vein). Microspheres were tracked for an average distance of 227±46 μm (mean±s.d.) and 276±56 μm as they flowed into and out of the wing, respectively.

Statistical analyses
Data comparing the contraction rates of the wing heart and the dorsal vessel were tested for normality and analyzed using a paired, two-tailed *t*-test. Correlation analyses were performed by plotting the contraction rate of the wing heart of a mosquito against the contraction rate of the dorsal vessel of the same mosquito, and then calculating the correlation coefficient (R) as well as the Pearson correlation *P*-value of the entire dataset. Data on hemolymph velocity and maximum acceleration were tested for normality. Then, data on velocity were compared using an unpaired, two-tailed *t*-test, and data on maximum acceleration were compared using the Mann–Whitney test.

RESULTS
The wing heart of mosquitoes is unpaired and located in the scutellum
To identify the presence and location of the wing heart of *A. gambiae*, we utilized an approach similar to the one we previously used to identify the location of the antennal hearts of this mosquito (Boppana and Hillyer, 2014). Injection of fluorescent microspheres into the hemocoel resulted in their dissemination throughout the body, and their aggregation in specific sites. One of the locations where the microspheres aggregated was within the scutellum, and fluorescence imaging of this area revealed a pulsatile structure and the vigorous movement of hemolymph (Movie 1, Fig. 1A). Then, examination of sectioned thoraces by light
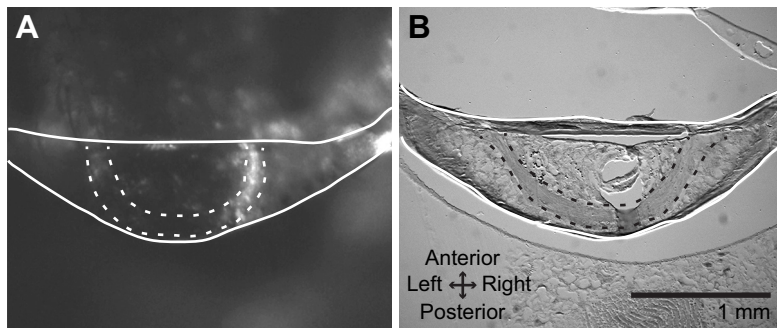
Fig. 1. The pulsatile diaphragm of the unpaired wing heart of mosquitoes. (A) Fluorescence microscopy image through the thorax of a mosquito showing the outline of the scutellum (solid lines) and the outline of the single pulsatile diaphragm of the wing heart (dashed lines). The unpaired pulsatile diaphragm can be observed because of the aggregation of fluorescent microspheres around its periphery. (B) Bright-field microscopy image of a sectioned scutellum (solid lines) showing the pulsatile diaphragm of the wing heart (dashed lines).

microscopy uncovered an unpaired wing heart whose diaphragm forms a posterior-facing semicircle that anchors in two points of the anterior of the scutellar hemocoel (Fig. 1B). Further examinations of intravital video recordings, together with inferences from light and scanning electron microscopy, showed that when the diaphragm contracts it assumes a slightly more flattened form. This expands the scutellar sinus and aspirates hemolymph out of both wings (Figs 1, 2, Movies 1, 2). When the pulsatile diaphragm relaxes, the volume of the scutellar sinus is reduced, which results in the expulsion of hemolymph into the thoracic hemocoel.

Hemolymph being aspirated by the wing heart exits the wings via veins located at the posterior of each appendage. This hemolymph moves from the wing to the wing heart via channels called axillary cords that connect to scutellar arms (diagrammed in a general sense in Pass et al., 2015), with the latter extending medially from each wing and being visually manifested in whole-mount preparations as protrusions from the thoracic cuticle (Fig. 2). Visualization of hemolymph flow within the wings and scutellum revealed that, unlike what is seen in the heart of adult mosquitoes (Andereck et al., 2010; Glenn et al., 2010; League et al., 2015), hemolymph flow in the wings and scutellum does not reverse direction, and no backflow was observed.

The wing heart contracts at 3 Hz and is independent of the dorsal vessel

Analysis of the spatial shift of the pulsatile diaphragm of the wing heart by intravital video imaging showed that it contracts at an average rate of 3.01±0.68 Hz (mean±s.d.; Fig. 3A). Analysis of the dorsal vessel of the same mosquitoes showed that this organ contracts at an average rate of 2.28±0.21 Hz, and thus, the rate of contraction of the dorsal vessel is 24% slower than that of the wing heart (paired, two-tailed t-test, $P<0.0001$). A Pearson correlation analysis did not yield a significant correlation between the contraction rates of the wing heart and the dorsal vessel ($R=0.160$, $P=0.329$), which together with visual analysis of the data illustrates that these two circulatory organs do not

contract in synchrony (Fig. 3B). Finally, for each video, the contraction of the wing heart was measured at both the left and the right regions of the pulsating diaphragm. When these two contraction rates were compared, they displayed a linear relationship and a near-perfect correlation ($R=0.999$, Pearson correlation $P<0.0001$; Fig. S2), further confirming that the wing heart is unpaired (a single pulsatile diaphragm).

Hemolymph enters the wing via anterior veins and exits via posterior veins

To map hemolymph flow within the wing, we intrathoracically injected fluorescent microspheres and tracked their movement across the network of wing veins. For the purpose of this exercise, we used the wing vein nomenclature described by Knight and Laffoon (1970). However, we refer to the previously unnamed vein that travels along the distal and posterior periphery of the wing as the ambient costa, based on what for other insects has been described as the 'ambient extension of the costa' (Arnold, 1964). Light and scanning electron microscopy images of a wing of *A. gambiae*, as well as a diagram detailing the wing vein nomenclature used in this study, are presented in Fig. 4A–C.

Initial experiments aimed to determine the flow diameter of the circulatory circuit of the wing. For this, we intrathoracically injected microspheres of different sizes, and found that 2 μm diameter microspheres did not circulate in the wing whereas 1 μm diameter microspheres entered the wing but only flowed in the costa, sub-costa, radius, radius 1 and ambient costa. When 0.5 μm diameter microspheres were injected, these particles flowed smoothly and unimpeded throughout the wing veins, indicating that the flow diameter of the wing circulatory circuit is between 1 and 0.5 μm.

Having established 0.5 μm diameter microspheres as the proper tool for mapping hemolymph flow in the wing, we injected these microspheres and visualized their directional flow (Fig. 4D, Movie 2). Hemolymph enters each wing and proceeds distally (afferent flow) via veins that emanate from the thorax at a location
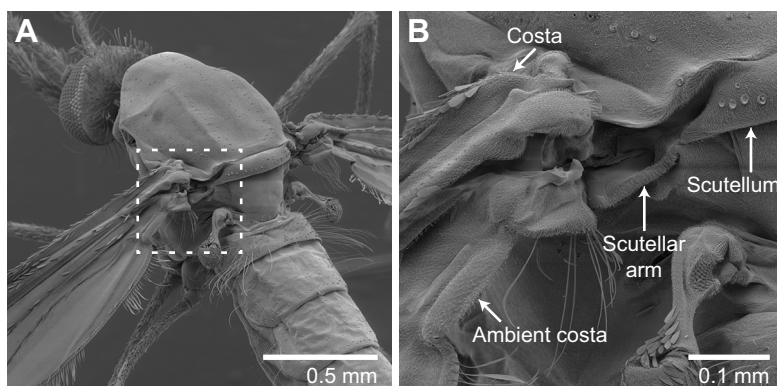

Fig. 2. Relationship between the wing heart, the scutellar arms and the wings of mosquitoes. (A,B) Scanning electron microscopy images showing a scutellar arm extending from the scutellum (and hence, the wing heart) to the posterior margin of the left wing. Also noted are the location of the costa (an anterior wing vein with afferent flow; inflow) and the ambient costa (a posterior wing vein with efferent flow; outflow). The area in the square in A is magnified in B.
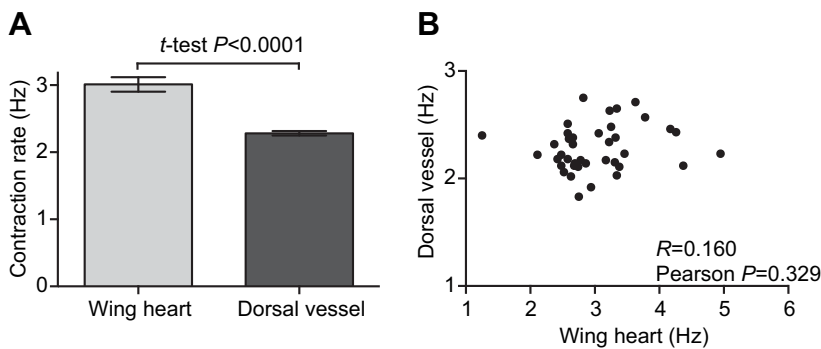
Fig. 3. Contraction rates of the wing heart and dorsal vessel of mosquitoes. (A) Average contraction rates of the wing heart and the dorsal vessel (n=39). Error bars represent ± s.e.m. (B) Correlation between the contraction rates of the wing heart and the dorsal vessel, where each point represents the values obtained for an individual mosquito (n=39). For the dorsal vessel, rates were determined by visualizing the abdominal portion of the vessel, also known as the heart.

that is anterior of the scutellum. Specifically, hemolymph enters each wing via the costa, sub-costa and radius, and travels toward the distal end of the appendage. Partial flow of hemolymph from the sub-costa to the costa occurs via a humeral crossvein that is located near the proximal end of the appendage, and the sub-costa joins the costa at a location that is approximately halfway into the anterior margin of the appendage. Hemolymph entering each wing via the radius also proceeds distally until this vein divides into the radius 1 and the radial sector. The radius 1 is a longitudinal extension of the radius, and hemolymph flows in an afferent direction until it joins the costa at the distal margin of the appendage. The junction of the costa and the radius 1 marks the beginning of the ambient costa, where hemolymph flows in a posterior and then proximal direction (efferent flow) along the periphery of the appendage.

The other vein that bifurcates from the radius, the radial sector, briefly extends toward the posterior before proceeding distally. The radial sector ends when it divides into the radiomedial crossvein, which extends toward the posterior, and the radius 2+3, which is a longitudinal extension of the radial sector and proceeds distally. The radius 2+3 then divides into two veins, the radius 2 and the radius 3, and both of these connect to the ambient costa. Hemolymph within the radial sector flows distally until it reaches the radiomedial crossvein, at which point the hemolymph flows toward the posterior via this crossvein. The radius 2 and the radius 3 receive hemolymph from the ambient costa, and this hemolymph moves proximally, converges into the radius 2+3 and then moves toward the posterior via the radiomedial crossvein. Thus, the anterior end of the radiomedial crossvein represents a point of directional conversion, where hemolymph flowing distally (afferent) via the radial sector meets hemolymph flowing proximally (efferent) via the radius 2+3, and the converged hemolymph proceeds toward the posterior.

The radiomedial crossvein extends from the junction of the radial sector and the radius 2+3 to the junction of the media and the media 1+2. Also in this region is the radius 4+5, which extends from the ambient costa to the midline of the radiomedial crossvein. Hemolymph from the ambient costa enters the radius 4+5 and flows proximally until it reaches the radiomedial crossvein, at which point it proceeds toward the posterior and enters the media. Similarly, the media 1 and media 2 accept hemolymph from the ambient costa, and this efferent flow converges into the media 1+2, and then proceeds into the media. Hemolymph in the media flows proximally until it merges with the cubitus anterior at a location that is near the proximal end of the appendage, and exits the wing.

Also extending from the media to the ambient costa is the media 3+4. At the proximal and anterior end of the media 3+4, some hemolymph from the media proceeds toward the posterior and then flows proximally via the mediocubital crossvein. At the posterior and distal end of the media 3+4, hemolymph from the ambient costa flows proximally and enters the mediocubital crossvein (on a few occasions,

hemolymph in the distal section of the media 3+4 was observed to flow in the opposite direction). The mediocubital crossvein deposits hemolymph into the cubitus anterior, and this hemolymph flows to the proximal end of the wing and exits the appendage. In addition to receiving hemolymph from the mediocubital crossvein, the cubitus anterior also receives hemolymph from the ambient costa, and this hemolymph flows in the proximal direction until it exits the wing. Although Knight and Laffoon (1970) mention the presence of a cubitus posterior, flow was not observed at that location.

Also intersecting with the ambient costa, at a location that is at the posterior of the wing and proximal to the junction between the ambient costa and the cubitus anterior, is the anal vein. The anal vein accepts hemolymph from the ambient costa and delivers it to the cubitus anterior at a location that is near the proximal end of the appendage. Hemolymph in the ambient costa that does not enter the anal vein continues its efferent flow along the posterior margin of the wing until it exits the appendage. Hemolymph exiting each wing enters a scutellar arm (Fig. 2) and deposits hemolymph into the sinus of the wing heart.

The velocity of hemolymph increases as it exits the wing
Visual examination of flowing microspheres illustrated that a major afferent vein is the radius and a major efferent vein is the ambient costa. To determine whether hemolymph velocity changes within the wing space, we quantitatively measured the flow of 0.5 μm diameter microspheres within proximal regions of the radius and the ambient costa (Fig. 5A, Movie 2). Using microspheres as a proxy for hemolymph flow, we found that the velocity of hemolymph increases as it exits the wing. Specifically, hemolymph flows into the wing at an average velocity of 99 μm s^{-1} whereas it flows out of the wing at an average velocity of 458 μm s^{-1} (Fig. 5B; unpaired, two-tailed t-test, P<0.0001). Similarly, the maximum acceleration of hemolymph increases from 370 μm s^{-2} as it enters the wing to 5108 μm s^{-2} as it exits the wing (Fig. 5C; Mann–Whitney, P<0.0001). It is important to note that 0.5 μm diameter microspheres are likely to occasionally interact with the epithelium of the veins as they flow within the wings, and thus the flow of smaller components of the hemolymph may be even faster. Taken altogether, these data show that hemolymph flow throughout the wing is swift, and that the flow of hemolymph is faster and more forceful as it exits the wing.

DISCUSSION
The structure of the wing accessory pulsatile organ varies amongst taxa. It can be a modification of the dorsal vessel, as is seen in most hemimetabolous (Exopterygota) and some holometabolous (Endopterygota) insects, or it can be separate from the dorsal vessel, as is seen in Hemiptera and many holometabolous insects (Krenn and Pass, 1994a,b). Furthermore, the wing hearts that are separate from the dorsal vessel can either be paired, in which case
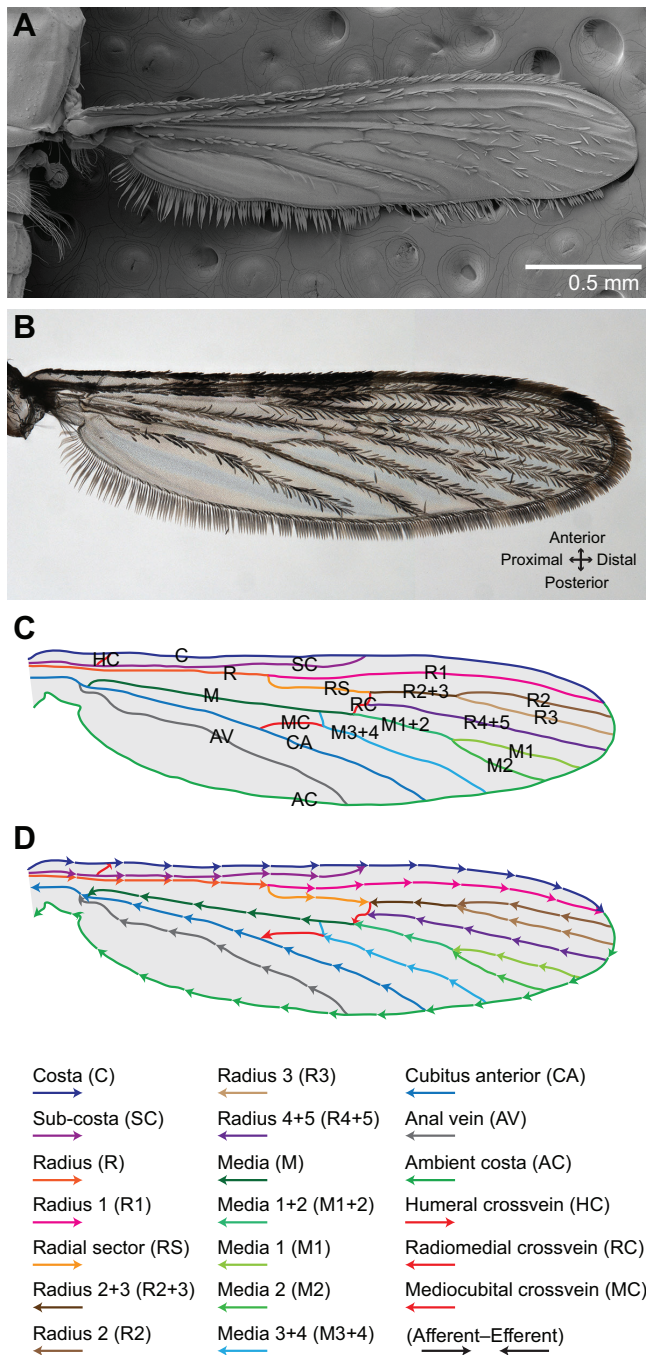
Fig. 4. Hemolymph flow across the wing. (A) Scanning electron microscopy image and (B) bright-field microscopy image of a mosquito wing. (C) Nomenclature of the veins of a mosquito wing. (D) Map of hemolymph flow across the veins of a mosquito wing. Acronyms are defined at the bottom of the figure, and the direction of the arrows indicates the direction of flow (afferent, toward the distal end of the wing; efferent, toward the thorax).

Fig. 5. Quantitative analysis of hemolymph flow in the radius and the ambient costa of the mosquito wing. (A) Image of a wing, where the rectangles indicate the locations where hemolymph flow was measured within the radius (for hemolymph entry into the wing; afferent flow) and the ambient costa (for hemolymph exit out of the wing; efferent flow). (B) Average velocity and (C) maximum acceleration of hemolymph entering and exiting the wing (n=100 microspheres for each direction). Error bars represent ±s.e.m.

there is one wing heart for each wing, or unpaired, in which case one wing heart aspirates hemolymph from both wings. A phylogenetic reconstruction of the evolution of the wing circulatory organ revealed that dorsal vessel modifications are the ancestral state, and that the evolution of paired and unpaired diaphragms occurred multiple times during the course of insect evolution (Pass et al., 2006, 2015). Furthermore, the secondary loss of wings, as has occurred in Siphonaptera, coincides with the loss of the scutellum and the scutellar arms, suppor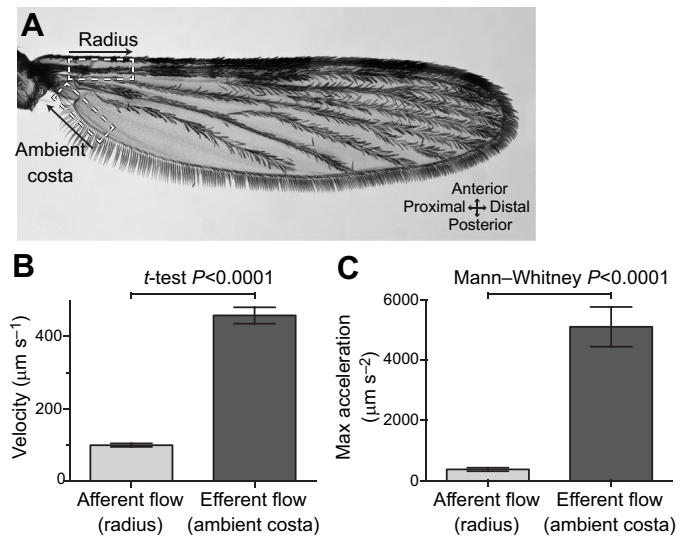ting the hypothesis that these cuticular structures evolved to house the machinery required for moving hemolymph across the wings (Krenn and Pass, 1994a,b). Dipteran insects – the flies – possess wing hearts that are separate from the dorsal vessel and are either paired or unpaired. The fruit fly, *Drosophila melanogaster* (Diptera: Brachycera), for example, has paired wing hearts located toward the lateral edges of the scutellum (Togel et al., 2008; Lehmacher et al., 2009). In the present study we show that mosquitoes (Diptera: Nematocera) have an unpaired wing heart that circulates hemolymph throughout both wings.

Hemolymph flow within the wings of insects occurs by one of two general routes: circulatory flow or oscillating flow. Circulatory flow is most common and involves the afferent flow of hemolymph via anterior wing veins and the efferent flow of hemolymph via the posterior wing veins (Moseley, 1871; Arnold, 1959, 1964). In this case, the hemolymph moves through the veins via a defined circulatory circuit. Oscillating flow occurs in some Lepidoptera, and involves the unidirectional flow of hemolymph into and out of the wing (Wasserthal, 1980, 1982, 1983). In this case, a switch in afferent to efferent flow, and vice versa, occurs simultaneously in all wing veins with every switch in the direction in which the dorsal vessel is contracting. In mosquitoes, hemolymph flow within the wings occurs via circulatory flow, and the direction and physical path of this flow is consistent across time. Given this consistency, and that the mosquito heart reverses contraction direction an average of 11 times per minute (Estevez-Lao et al., 2013; Hillyer et al., 2014; League et al., 2015), flow through the wings appears to be unaltered by the direction in which heart contractions propagate.

To our knowledge, prior to the present study, the contraction rate of the wing heart had only been measured in a scorpionfly [Order: Mecoptera; a sister group of Diptera (Misof et al., 2014)], where the paired wing hearts were found to not contract in synchrony but to contract at approximately the same rate: 1.3 Hz (Krenn and Pass, 1993). Our study found that the unpaired wing heart of mosquitoes contracts faster, at approximately 3 Hz, which is also 24% faster than the contraction rate of the dorsal vessel of mosquitoes. The difference in the contraction rate between the

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wing heart and the dorsal vessel, together with the lack of correlation between the contraction rates of these two organs, strongly suggests that these circulatory pumps are not physically linked. This independence is reminiscent of the independence between the dorsal vessel and another type of accessory pulsatile organ: the antennal heart. In both mosquitoes and cockroaches, there is no correlation between the contraction rate of the antennal hearts and the dorsal vessel, but different from what we observed for the wing heart, the antennal hearts contract at a significantly slower rate than the dorsal vessel (Hertel et al., 1985; Boppana and Hillyer, 2014; Suggs et al., 2016). Furthermore, some cardioacceleratory peptides, such as CCAP, FMRFamides and proctolin, are known to modulate the contraction dynamics of both the dorsal vessel and the antennal hearts of insects (Cuthbert and Evans, 1989; Predel et al., 2004; Ejaz and Lange, 2008; Hertel et al., 2012; Estevez-Lao et al., 2013; Hillyer et al., 2014; Suggs et al., 2016). Whether these same factors also modulate the rhythmicity of the wing heart remains to be determined.

In the present study, we measured hemolymph velocity and maximum acceleration in the wing space. Specifically, hemolymph flow as it exits the wing via the ambient costa is 4.6 times faster than when it enters the wing via the radius. Similarly, the maximum acceleration of hemolymph is nearly 14 times greater when it exits the wing than when it enters it. For insects, data on hemolymph flow dynamics are sparse, but the data in the present study follow a comparable pattern to what we have observed for the antennal space of mosquitoes: velocity and maximum acceleration are highest near the contractile pump (Boppana and Hillyer, 2014; Suggs et al., 2016). In the case of the antennal heart, hemolymph velocity is fastest near the pump because the diameter of the antennal vessel that extends from the pump is narrower than the diameter of the antennal hemocoel into which it empties. By that logic, we hypothesize that the combined cross-sectional area of the afferent wing veins is larger than the combined cross-sectional area of the efferent wing veins. Even though the relative patterns of velocity and maximum acceleration are similar between the wing heart and the antennal hearts of mosquitoes, a significant difference between these two pulsatile organs pertains to the mechanics of flow: the wing heart aspirates hemolymph out of the appendages whereas the antennal hearts propel hemolymph into the appendages (Boppana and Hillyer, 2014; Suggs et al., 2016).

Our approach for determining the directional flow of hemolymph was to visualize the movement of fluorescent microspheres that were 0.5 µm in diameter. Almost 150 years ago, H. N. Mosely inferred the directional flow of hemolymph within the wings of cockroaches by observing the movement of corpuscles – bodies now known to be immune cells called hemocytes – across the veins (Moseley, 1871). Subsequently, researchers used this hemocyte-based method to describe the directional flow of hemolymph in the wings of insects from over 10 taxonomic orders (Yeager and Hendrickson, 1934; Arnold, 1959, 1964). One of these studies remarked that this methodology proved difficult for the study of circulatory physiology in the wings of Diptera because these flies contained few hemocytes in the wings (Arnold, 1964). This is in agreement with subsequent studies that fluorescently labeled hemocytes in different parts of the body. In fruit flies, for example, numerous hemocytes are present in the wings during eclosion, but by the time the wing cuticle layers have bonded, the hemocytes have largely left these appendages or become degraded (Kiger et al., 2007). Similarly, we reported that fewer than 30% of adult mosquitoes have hemocytes in their wings, and those that contain hemocytes have fewer than four hemocytes in these appendages (King and Hillyer, 2013). Given our finding that

the flow diameter of the wing circulatory circuit is less than 1 µm, and that circulating hemocytes in Diptera are usually 8 µm in diameter or larger (Meister and Lagueux, 2003; Ribeiro and Brehelin, 2006; Hillyer and Strand, 2014), it is not surprising that hemocytes are seldom observed in the wings of these small insects. In larger insects, such as many of those studied by Arnold (1964), gross examination of the wing venation suggests that the flow diameter of their wing circulatory circuit is significantly larger, and thus hemocytes can easily enter and exit these appendages. Because *Plasmodium* sporozoites, *Wolbachia* bacteria and viruses have been observed or detected in the wings of mosquitoes (Dobson et al., 1999; Akaki and Dvorak, 2005; Hillyer et al., 2007; Bolling et al., 2012), and because pathogens can undoubtedly populate the wings of other insects, we hypothesize that proper circulation of hemolymph within the wings is important for the dissemination of humoral immune factors that quell or limit infections, and that this process is especially important in small insects whose wing venation precludes the entry of hemocytes into the flight appendages.

Mosquitoes are of great epidemiological importance because they transmit deadly and debilitating diseases to humans and vertebrate animals (Becker et al., 2010). In the present study, we provide the first description of hemolymph circulation in the wings of mosquitoes. We show that this circulation is controlled by the aspirational force created by an unpaired wing heart located underneath the thoracic scutellum, and we physically map the systemic entry and exit of hemolymph into the wing space via a network of afferent and efferent wing veins.

Supplementary information
Supplementary information available online at
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