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Circulation in Insect Wings

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Synopsis Insect wings are living, flexible structures composed of tubular veins and thin wing membrane. Wing veins can contain hemolymph (insect blood), tracheae, and nerves. Continuous flow of hemolymph within insect wings ensures that sensory hairs, structural elements such as resilin, and other living tissue within the wings remain functional. While it is well known that hemolymph circulates through insect wings, the extent of wing circulation (e.g., whether flow is present in every vein, and whether it is confined to the veins alone) is not well understood, especially for wings with complex wing venation. Over the last 100 years, scientists have developed experimental methods including microscopy, fluorescence, and thermography to observe flow in the wings. Recognizing and evaluating the importance of hemolymph movement in insect wings is critical in evaluating how the wings function both as flight appendages, as active sensors, and as thermoregulatory organs. In this review, we discuss the history of circulation in wings, past and present experimental techniques for measuring hemolymph, and broad implications for the field of hemodynamics in insect wings.

Introduction: What's a wing by any other name?

Wings are a key innovation in insects, enabling a myriad of ecologically important behaviors including predation, migration, and pollination (Dudley 2002). Although their composition of thin membranes and long tubular veins might superficially suggest otherwise, insect wings are dynamic, living structures that require a supply of vital substances. Veins not only provide mechanical integrity but also act as conduits, containing tracheal tubes for gas exchange, nerves that provide sensory information in flight, and perhaps most importantly, hemolymph that serves multiple functions. Hemolymph actively circulates through wing veins and is critical for maintaining sensory structures that populate the wing (Chintapalli and Hillyer 2016; Hillyer and Pass 2020). Movement of hemolymph, cells, and waste is also required for the proper development of the wing. At metamorphosis, movement of hemolymph in and out of the wing contributes to the expansion and unfolding of the wing (Pass et al. 2015). In flight, the arrangement of wing veins and flexures influences the mechanical integrity of the wing, and thereby aerodynamic performance (Wootton 1992; Combes and Daniel 2003a). Furthermore, without hemolymph circulation, wings desiccate and rapidly lose their flexibility (Dirks and Taylor 2012a). Despite its relevance to numerous physiological functions, the mechanics of hemolymph circulation in insect wings has been understudied, which motivates us to provide an overview of the field and point to current techniques for investigating flows in insect wings to encourage new studies of wing circulation.

It is well understood that the primary function of wings is to enable flight, and the structural properties of the wing help determine their aerodynamic performance. In flight, wings deform, bending, and twisting from aerodynamic and inertial forces as insects navigate complex environments (Wootton 1992; Combes and Daniel 2003b; Shyy et al. 2016). Both wing shape and composition influence flexibility, which can vary locally throughout the wing

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(Wootton 1981; Combes and Daniel 2003a; Vanella et al. 2009; Combes et al. 2010). An integrated approach to analyzing the functional mechanics of insect wings requires knowledge of (1) wing kinematics during wing deployment and flight, (2) detailed morphology of wings, (3) kinematics interpreted in aerodynamic terms, and (4) how morphology and kinematics vary across the phylogeny of insects (Wootton 1981). Whereas the kinematics of flapping flight and wing folding has received attention in the last few decades (Shyy et al. 2016; Bomphrey and Godoy-Diana 2018), the physiology of the actual structural supports, particularly the veins, is still largely a mystery. This lack of information is partially rooted in the difficulty of studying vein dynamics in living or recently sacrificed insects.

Recent reviews (Pass 2000; Pass et al. 2015; Pass 2018; Hillyer and Pass 2020) have repeatedly identified gaps in our knowledge of insect circulation, particularly in regard to how hemolymph moves into appendages including wings, legs, and antennae. However, misunderstanding about hemolymph circulation in insect wings persists. For instance, the presence of hemolymph in wings is sometimes ignored or is presumed to be absent or limited to certain veins (Dirks and Taylor 2012b). And, despite decades of work on the topic, we have found anecdotally that some biologists (and even entomologists) are unaware that flow occurs in the wings, part of our motivation for highlighting the role of wings as dynamic, living structures. This review focuses on circulation in insect wings, its history, and how hemolymph movement is coupled to an insect's multiple hearts, tracheal system, and gut system. Additionally, we identify challenges and open questions in wing hydraulics that are critical in shaping the future of how insect flight is measured, modeled, and engineered.

Evolutionary pressures on wing structure and sensory systems

Wing shape, venation pattern, and flight behavior are influenced by many selective pressures, including ecological niche, predator avoidance, and behavior, to name a few. Within the phylogeny of insects, examples of adaptive wing shapes and modified wing structures (e.g., elytra and halteres) abound. Butterflies in the genus *Morpho* exhibit different flight patterns, such as flapping and gliding, depending on habitat (forest understory versus canopy, respectively) (DeVries et al. 2010). Gliding male *Morpho* butterflies in the canopy exhibit longer forewings than those that inhabit the understory (DeVries et al. 2010). Rearing migratory insects in laboratory environments compared with natural, wild populations produce populations with wings that are "weaker, paler, and less elongated" (Davis et al. 2020). Luna moths and other Saturniid lepidopterans mitigate potential bat predator damage with "lures," which are elongated twisty tails that branch off the hind wing (Barber et al. 2015); the longer the hind wing, the more likely to escape a bat attack (Rubin et al. 2018). Bats and insects are engaged in an evolutionary predator–prey race, evolving structural and sensory strategies to effectively out-wit the other (Rubin et al. 2018). As modified hind wings, halteres are shaped as stalks topped with a knob-like end and a base (where stalk meets body) covered in an array of sensory structures. In more basal dipteran families such as in crane flies (Family: Tipulidae), halteres have much longer stalks and low asymmetry compared to more derived families (Yarger and Fox 2016). Haltere morphology changes with evolutionary derivation, generally increasing in asymmetry (Yarger and Fox 2016). Other selective pressures that might act on wing shape and function include auditory performance (related to signaling and sensing), sexual selection, chemosensory performance, visual signaling, and crypsis (Taylor and Krapp 2007). Sensory feedback is particularly critical to many of these functions, and sensory cells require hemolymph. These pressures form the basis of a major open question: how does hemolymph circulation evolve with wing structure and mechanosensory systems in the wing and modified wing structures?

Insect sensory systems are highly adaptable with respect to their functioning within the wing's mechanical systems (Taylor and Krapp 2007). Hemolymph circulation must also adapt accordingly, or features such as wings would lose sensory function. Wings are instrumented with an array of mechanosensors (Dickerson et al. 2014), some of which include stretch receptors, scolopidia (sound receiving), and campaniform sensilla (Taylor and Krapp 2007). Haltere bases (where the stalk connects to the body) are covered in campaniform sensilla, and these sensory structures are situated where they sense maximal strain during haltere oscillation (Yarger and Fox 2016). In some species, such as blow flies, 400 campaniform sensilla cover each haltere, making up the majority of said sensilla on the body (Yarger and Fox 2016). In both flight and non-flight wing movements (e.g., folding/unfolding, courtship displays, righting-maneuvers), wing mechanosensilla bend, providing active mechanosensation during wing movement (Pass 2018). Gustatory sensilla provide chemosensory information

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(Valmalette et al. 2015). Tympanal organs on the wing make and receive sound (Miller 1970; Sun et al. 2018). Scent patches produce mate-attracting pheromones. Sensory feedback is also tied to wing function (Taylor and Krapp 2007). For example, removing function of the campaniform sensilla on the forewing eliminates regulation of forewing twisting in *Schistocerca* locusts (Taylor and Krapp 2007). Throughout the body, the proper functioning of sensors requires a hemolymph supply. Thus all wing sensors and organs, epidermal cells, and branches of tracheae within the wing require hydration with hemolymph (Chintapalli and Hillyer 2016).

Moving hemolymph into the wings

Hemolymph is pumped throughout an insect's body and appendages by wave-like contractions of the dorsal vessel, assisted by additional "hearts" or accessory pulsatile organs (APOs) (Pass et al. 2015; Hillyer and Pass 2020). Flow within the head, thorax, and abdomen is open (unconstrained to vessels) and pulsatile. In these regions, hemolymph is moved in bulk by pumping of the tube-like dorsal heart (Lee and Socha 2009; Pass et al. 2015). APOs aid in circulating hemolymph, not only in wings, but also within appendages such as the antennae, legs, and ovipositors (Hustert 1999; Boppana and Hillyer 2014; Hustert et al. 2014). Antennal APOs, typically paired and of which there are several types, can function to pulse hemolymph into the antennae (ampulla-dilator type) (Boppana and Hillyer 2014). In contrast, insects can have one or more thoracic APOs that aspirate hemolymph from the wings, pulling it back into the body (from the posterior veins) several times faster than it enters the wing (Chintapalli and Hillyer 2016; Salcedo 2019). Unlike in the hemocoel, once it enters the wings, hemolymph is mostly constrained to a vessel network. Additionally, hemolymph flow may be affected by compression and reinflation of parts of the tracheal system, and possibly displacement of the gut system (Pendar et al. 2019). Assuming that the relatively stiff cuticle of the wing means that it maintains a fixed volume, the changes in air volume in tracheae within the wing may be coupled to the hemolymph volume within the wing veins. Therefore, open questions exist whether expansion and contraction of tracheal tubes may drive or be driven by changes in hemolymph volume, governed by fluid–structure interactions. Recent work suggests that a concerted effort of the thoracic APOs, the gut system, and the tracheal network is also necessary for overall circulation (Wasserthal 1982; Wasserthal 1996; Harrison et al. 2013; Pendar et al. 2019), which would represent a new, integrative view of insect circulation. The coordination of these parts, via both active and passive elements (pumps and veins, respectively), is necessary for maintaining a continuous circulatory flow throughout the body, and proper coordination appears to depend upon maintaining appropriate fluid pressure fields and patterned, synchronous pumping.

Winged insects tend to have thoracic APOs, also termed "wing hearts," with the exception of Thysanoptera (thrips) and Aphidina (aphids), whose lack may be due to their small size (Krenn and Pass 1994). Wing hearts can also be lacking in apterous insects and nymphs where wings are still developing. As accessory pumps that serve the wings, these APOs are located in the thorax and exist unpaired or in pairs surrounding or attached to the dorsal vessel. Wing hearts exhibit three forms: dorsal vessel modifications, attached pulsatile diaphragms, and unattached pulsatile diaphragms (Krenn and Pass 1994; Pass et al. 2015). Each pump utilizes a hemolymph space (subscutellar hemocoel), formed from a raised portion of exoskeleton, which acts as a pump casing to pull hemolymph from posterior wing veins and back into the body (Pass et al. 2015). Some debate revolves around whether pumping elements exist in the wing itself, and whether there is actively controlled flow in the wing. So-called "pumping elements" have been observed in the wing, but it is possible that passive wing tissues move in response to pumping of the thoracic wing heart (Arnold 1964; Tsai et al. 2020). To investigate active control of hemolymph flows in the wing, targeted imaging of these wing surfaces and measurement of pumping frequencies of the dorsal vessel, thoracic wing hearts, and hemolymph flow speeds are needed.

Throughout the body and appendages, hemolymph circulates to move nutrients, waste, and cells (Chapman 2012). Hemolymph contains plasma and hemocytes, and across a diverse range of insects differentiated hemocytes are typically termed as granulocytes (adhesive cells that attack foreign substances), plasmatocytes (strongly adhesive to pathogens, dead cells, and more), spherule cells (sources of cuticular compounds), and oenocytoids (which produce enzymes) (Strand 2008). Cells populations can be divided further into circulating and sessile hemocytes, the latter which are found attached to tissues (Hillyer and Strand 2014). Number of hemocytes and whether or not they circulate can depend on development stage and potential stress events (e.g., wounding, infection) (Strand 2008). For example, in adult mosquitoes, there may be 500–4000 circulating

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hemocytes, while in other dipteran, lepidopteran, and orthopteran insects, cell densities range from 500 to 900 hemocytes per microliter (Hillyer and Strand 2014). Hemocytes can range in size depending on species and if the cell is "active." The mosquito hosts three types of hemocytes: granulocytes (9 μm diameter, but can spread to 35 μm when attached to foreign surfaces), oenocytoids (9 μm diameter), and prohemocytes (4–6 μm diameter) (Hillyer and Strand 2014). In smaller insects, some appendages, such as wings, are too small to allow for cells to enter the wings, but hemolymph continues to circulate. For example, in mosquitoes, flowable space within the wing circuit is only 1 μm in diameter, whereas circulating hemocytes are typically 8 μm or larger in Diptera (Boppana and Hillyer 2014; Hillyer and Strand 2014; Chintapalli and Hillyer 2016). Smaller organisms such as *Plasmodium sporozoites*, bacteria, and other viruses have been found in mosquito wings, suggesting that active circulation is important for dissemination of immune factors (Akaki and Dvorak 2005; Hillyer et al. 2007; Moreira et al. 2009).

In insects with vein diameters large enough to accommodate cells, hemocytes can be visualized and used to identify flow direction (Arnold 1964). In fact, entomologists first recognized hemolymph in insects by tracking visible circulating hemocytes within larger insects (Arnold 1964). Recently observed in Nymphalid butterflies, hemocytes clearly circulate in veins and through major organs such as scent patches (Tsai et al. 2020). It is not known how hemolymph velocities and hemocyte circulation scale within insect wings of increasing size, nor how hemolymph movement changes with development and age. Flow metrics such as velocity, acceleration, and heart pumping frequency have been quantified for adult mosquitoes (*Anopheles gambiae*) and North American grasshoppers (*Schistocerca americana*) (Hillyer and Strand 2014; Chintapalli and Hillyer 2016; Salcedo 2019). Circulation in insects differs between species (tidal versus circuitous circulation, discussed below), but our knowledge of hemolymph fluid dynamics and wing morphology remains limited.

A brief history of wing hemodynamics

Circulation in insects has long been a subject of debate. Insect physiologists in the mid-1700s to the early 1900s disputed the presence of hemolymph not only in the wing, but also in the insect itself. In 1831, Carus first postulated a circulatory route in insect elytra and wings (an idea that would be known as the "Carus Rule"), whereby blood flows from the body to the anterior veins and then back toward the body in the posterior veins (Fig. 1A and C) (Yeager and Hendrickson 1933; Arnold 1964). As acceptance of hemolymph began to emerge, a debate sparked in 1841 as to the presence or absence of a circulatory system. In 1841, Léon Dufour, a scientist and naturalist, published the "History of Metamorphosis and the Pretended Circulation of Insects" (Timbs 1843). He garnered support of well-known physiologists (such as Italian biologist and physician Marcello Malpighi), who attested that with no obvious network of blood vessels, there was a clear "testimony against a circulatory system" (Timbs 1843). Despite a vigorous rebuttal by Verloren (1847), who documented 90 species of insects where circulation was observed by 17 authors, it took decades to reach consensus (Arnold 1964). In the 1850s, scientists explored the possibility that the tracheal system may transport hemolymph as a network of blood vessels. Interest in hemolymph circulation seemed to lapse in the latter 19th century and picked up with works by biologist Moseley (1871), the first to study wing circulation in cockroaches, and entomologist Brocher (1929), who studied the wings of lady beetles (Family: Coccinellidae) and also the legs, antennae, body, dorsal vessel, and pulsatile organs. Brocher became a key investigator of circulation in insects and their appendages, and in 1916 he complained that many naturalists denied that circulation existed in insects (Yeager and Hendrickson 1933). To clear up further confusion as to whether circulation existed in the elytron of an insect, Yeager and Hendrickson (1933) proposed a standardized method to study the circulatory route in cockroaches focusing on the elytra, wings, and wing pad of the American cockroach, *Periplaneta americana* (Yeager and Hendrickson 1933).

In response to an increase in circulatory studies, John Arnold gave a rigorous account of circulation in insect wings, its history, and particularly highlighted circulatory observations between 1900 and 1964 (Fig. 1) (Arnold 1964). His summaries and observations became a basis for much of what we know about insect wing circulation. Using an alternative method than that proposed by Yeager and Hendrickson, Arnold simply used a microscope and strong light source and ascertained hemolymph flow and directionality by visualizing the movement of hemocytes in 14 orders and 100 species. With new descriptions of flow patterns in wings, he challenged and confirmed nearly 200 years of publications on circulation in insects. His 72 drawings of flow patterns in insect wings highlighted a circuitous flow

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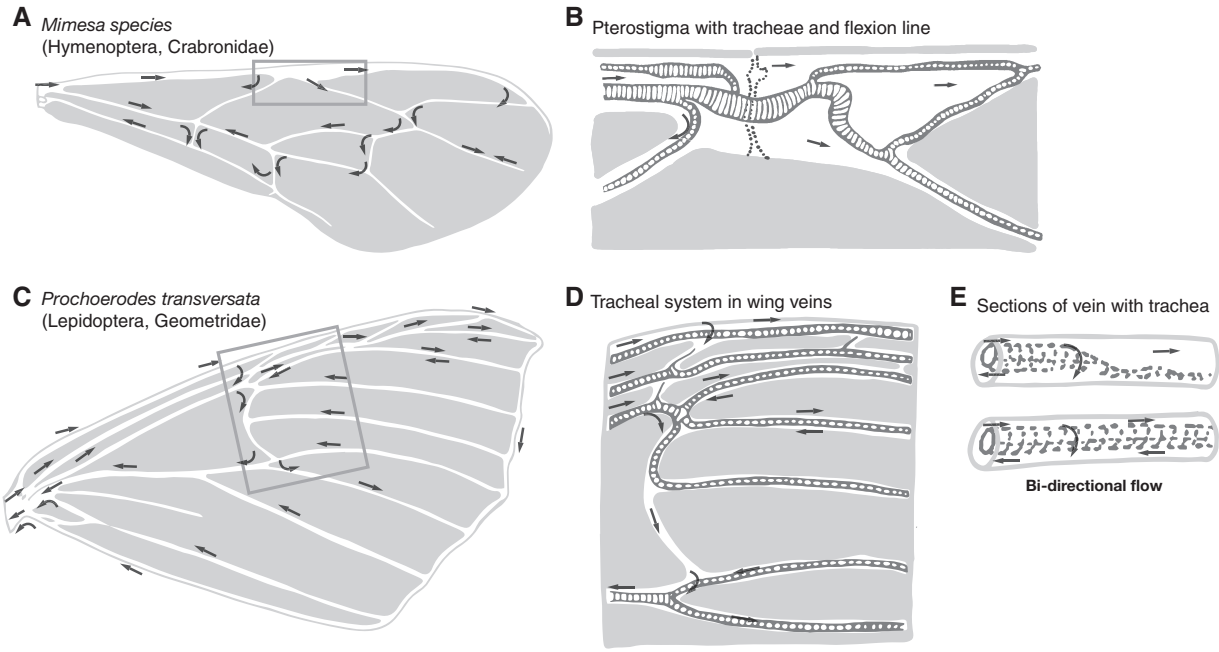

Fig. 1 Circulation patterns and examples of tracheae in insect wings (adapted from Arnold 1964). Arnold observed hemolymph and hemocyte movement in 100 insect species across 14 insect orders. Veins can contain tracheal tubes, and hemolymph can move across and around such tracheae. Direction of hemolymph flow is indicated by arrows, which are placed in the orientation and location as Arnold drew them. (**A**) Circulation in the forewing of *Mimesa* species (Hymenoptera) shows a circuitous pattern. (**B**) In the expanded box from A showing a costal break, tracheae (ribbed tubes) extend into the wing veins. Costal breaks, which can occur within the pterostigma (a sinus), are specialized joints for bending found in some hymenopteran species (Mountcastle and Combes 2014). (**C**) Another pattern of circuitous hemolymph movement is in the forewing of *Prochoerodes transversata* (Lepidoptera). (**D**) Due to partitioning flow by tracheae, hemolymph moves in two directions within the same vein. This pattern is not observed in all Lepidoptera. (**E**) An example of how tracheal branches partition flow within sections of wing vein. Hemolymph here moves bidirectionally and also across the tracheal branch. Wings are not shown to scale.

pattern, in which hemolymph circulates into the costal and subcostal veins (which are located at the leading edge of the wing), moves toward the wing tip, and eventually returns, flowing back toward the wing base (Fig. 1A and C). In many insects (with the exception of some Lepidoptera), flow moves out to the wing tip through anterior veins near the leading edge, and back toward the body through more posterior veins near the trailing edge. Shorter, smaller-diameter cross-veins shunt flow across the wing in the chordwise direction (specifically from the leading to the trailing edge), ultimately forming a circuitous pattern of fluid flow (Arnold 1964). Arnold identified flow directions with clear afferent (to wing tip) and efferent (to wing base) directions (Fig. 1). He also noted structural features that serve to redirect hemolymph, such as tracheal branches, crossveins, and species-specific venation features (e.g., the arculus, a basal crossvein in dragonflies) (Fig. 1B, D, and E). Detailed drawings of wing bases described some flows that do not enter the wing, but circulate around the wing hinge, flowing slightly into the wing and immediately back into the body. This observation has also been seen in *S. americana* (Salcedo 2019). In some beetles, hemolymph in and around the wing hinge can be used to hydraulically open the wing (*Dorcus titanus platymelus*) (Sun et al. 2014).

Arnold also first identified regions of "leaky" flow, where hemolymph moved from wing vein into membrane (Arnold 1963). This type of flow was common not only in thickened wings like elytra (in beetles and true bugs) and tegmen (in grasshoppers and crickets), but also within what appeared to be "normal" thin wing membrane. It is unknown how leakiness occurs; Arnold did not speculate, but one possibility is that pores exist in veins to allow for hemolymph to move between vein tubules to membrane. How hemolymph might be directed into the membrane, and what functions it serves, are also open questions. However, a year before his major treatise on circulation, Arnold (1963) described the leakiness of the pterostigma, a thickened portion of the wing typically seen as a melanated rectangle near the wing tip of dragonflies. The pterostigma plays a functional role in gliding flight by reducing flutter and acting as an inertial regulator of pitch angle (Norberg 1972). In some insects, the pterostigma is a discrete sinus where hemolymph is contained in a

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semi-rectangular box. In others, however, the region is less well-defined and termed a "pseudostigma," where leakiness occurs between the two major leading edge veins (costa and subcosta). Arnold described pterostigmas and pseudostigmas in Odonata, Neuroptera, Pscoptera, Hemiptera, and Hymenoptera (Arnold 1963).

Other observations from Arnold (1963) include tracheation of these sinuses (Fig. 1 B) and other structural features in the wing (e.g., the dragonfly arculus), indicating live tissue or areas of dense sensory demand (Arnold 1963). Throughout the wing, tracheal branches, besides delivering oxygen, serve to re-route flow and act as septa, diverting flow in antagonistic directions within the same vein (Fig. 1B, D, and E), a common feature across taxa (Arnold 1964). In some species of large grasshoppers, compression and re-inflation of thoracic air sacs at the wing hinges can influence flow into the wing (Salcedo 2019). Tracheation within the wing membrane occurs, but has been described as "unusual", as it is found in somewhat unpredictable locations within and across species (Guillermo-Ferreira et al. 2017). Arnold also tracked hemocyte movement within modified wings such as elytra and halteres (Arnold 1964). In both structures, hemolymph flow moves in an apparent circuit. Currently, it is difficult to measure live tissues continuously throughout a wing, especially in larger wings with dense venation. Harkening back to Arnold's history lesson, perhaps it should not be unusual to find tracheae within the membrane. Multiple authors have called for focused research attention on this topic (Pass 2018; Hillyer and Pass 2020), reflecting on the fact that there is still not widespread recognition of the insect wing as living tissue.

Tidal versus circuitous: relationship between circulation and respiration

Insect wings exhibit two main hemolymph flow patterns: tidal (also known as "oscillatory") and circuitous (as defined above) (Fig. 2). In the 1980s, Lutz Wasserthal challenged the historical assumption that all flows were circuitous and discovered that lepidopterans employ tidal flow, and that tracheal expansion and compression influences wing hemodynamics (Wasserthal 1982). Generally, a tidal pattern involves hemolymph moving into all wing veins, and then reversing direction, coordinated by wing hearts, the dorsal vessel, thoracic air sacs, and wing tracheae (Fig. 2B). Quantitative measurements in Lepidoptera, Diptera, and Coleoptera suggested that tidal flows of hemolymph are possible with vigorous pumping of the wing hearts (pulling hemolymph from the wing), dorsal vessel reversals, coupling of wing tracheal tubes (expansion and compression), and inflation of thoracic air sacs. Large air sacs expand and compress between the hind wing and forewing hinges. In addition, visible through microscopy, sections of tracheal branches near the wing tip expand and contract, also affecting local flows (Salcedo 2019).

Wasserthal studied the extremely large Atlas moth (*Attacus atlas*, wing span, 30 cm), showing a four-part bulk movement of hemolymph in which hemolymph is pulled in and out of the wing due to coordinated pumping from thoracic APOs and tracheal distension. Thoracic APOs pump vigorously preceding air intake, creating a pressure difference that pulls hemolymph out of the wing as tracheal volume increases. Thus, as the pressure differential reverses, hemolymph moves from wing to wing with each breath (Wasserthal 1996), demonstrating that in the Atlas moth the circulatory and respiratory fluid systems are coupled.

Not unexpected, the actions of both systems serve to increase the overall rate of hemolymph circulation. It has been observed in the abdomen of grasshoppers (*S. americana*) that the dorsal vessel and compressions of the tracheal system operate at slightly different pumping frequencies (Lee and Socha 2009). In large rhinoceros beetles, hemolymph moves with each flow reversal of the dorsal heart, where anterograde (posterior to anterior) flow is correlated with hemolymph movement into the wing, and retrograde (anterior to posterior) correlated with outflow (Wasserthal 1998). However, in mosquitoes, which employ a circuitous flow pattern in the wing, reversals within the dorsal heart do not change the wing's circulation direction nor affect flow velocities (Fig. 2A) (Chintapalli and Hillyer 2016). Although some studies have examined qualitative patterns of hemolymph flow in the dorsal heart, wing hearts, and wings of particular species, few studies have quantified the overall hemodynamics and net circulatory performance arising from the combination of wing morphology, venation patterning, and fluid dynamics, or have examined variation of wing hemodynamics across the insect phylogeny.

Experimental systems to study circulation in insect wings

Exploring and quantifying complex fluid movement in insect wings requires an understanding both of the venation pattern and how/where hemolymph is flowing. To study multiple fluid systems in parallel,

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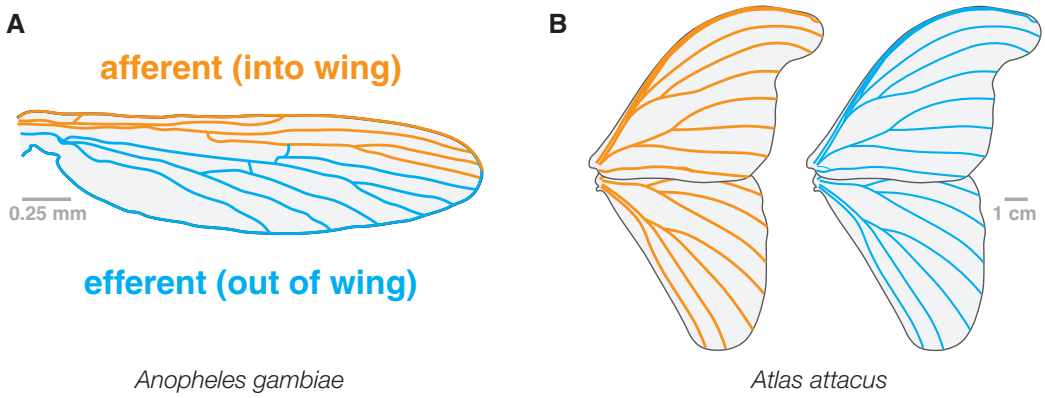

Fig. 2 Circuitous versus tidal flow patterns in insect wings. Main currents of hemolymph flow are indicated as afferent hemolymph movement, which moves into the wing, and efferent movement, which returns back into the insect body. (**A**) Circuitous flow, measured in *A. gambiae* (Chintapalli and Hillyer 2016), moves into the leading edge veins of a wing (costa, subcosta) and reverses in the wing tip, returning to the body through trailing edge veins (cubitus, annals, etc.). (**B**) Tidal flow, known to occur in *Atlas attacus* (Wasserthal 1982), moves into all veins and moves out of all veins.

capturing both spatial and temporal resolution of fast-moving flows requires high speed videography combined with microscopy, technologies that are not often available together. Also, complex internal wing morphology is typically captured with histological sections or using scanning electron microscopy (SEM) to observe discrete slices of vein morphology across a wing (Song et al. 2020). Most descriptions of whole-venation patterns have largely been qualitative, focusing on 2D information from wings that have been scanned or hand-drawn (Hoffmann et al. 2018; Salcedo et al. 2019). These descriptions assume that wing veins are cylindrical, which is often contradicted by measurements from SEM, which have revealed variations in cross-sectional shape from circular to kidney-shaped (Appel et al. 2015). This complex system thus requires a multifaceted approach. In this section, we discuss how methods to observe and measure wing circulation have advanced over recent decades. Four experimental methods (detailed in Fig. 3) in particular have contributed the most: direct observation with microscopy (Fig. 3A), fluorescent staining (Fig. 3B), contact thermography (Fig. 3C), and fluorescent particle tracking with high-speed microscopy (Fig. 3D). We also highlight other experimental techniques for observing wing geometries and potential solutions to examining wing structures continuously.

As discussed previously, Arnold observed circuitous flow in wings across the insect phylogeny (Fig. 1). In the 1960s, he used a light source and microscope to observe hemocyte movement, choosing insects with mostly translucent wings (due to low pigmentation) (Arnold 1964). He laid the wings flat and pinned them with glass to observe cell movement in a 2D venation pattern. Smaller insects were left unrestrained and a glass coverslip was used to spread the wings, with a drop of water and detergent sandwiched between wings and glass (Fig. 3A). Larger insects were restrained with elastic material. In smaller insects, wing vein diameter is a limiting factor and can prevent hemocyte entry into the wing. Because hemocyte movement is most easily seen in larger insects (wider veins), he focused on larger species and identified patterns of the flow direction by eye. Although this pioneering work was largely qualitative and relied on 2D data, it laid the groundwork for future quantitative studies of flow.

In contrast to direct visual observation, fluorescent staining can be used to quantify flow movement, independent of hemocyte tracking. Wasserthal extended and developed this method, using diluted fluorescent stains (specifically pyrrolidino-methyltetracycline [TC]) and ultra-violet illumination to identify tidal flow in pierid butterflies (Wasserthal 1983). After stain injection in the abdomen, the stain begins to circulate in the body, eventually being pulled through the wing by aspirating thoracic APOs. Butterflies were then mounted with wings pinned between glass slides and photographed under UV (scales on wings were gently removed due to slight fluorescence of the scales and wing themselves) (Fig. 3B). The staining first appeared in the wing veins within the first 5–10 min of application. Wasserthal took photographs at relatively rapid intervals (1–15 min) in the first hour, and then at broader intervals (1–6 h) each day afterward until the end of their lives. The fluorescent stain accumulates and dilutes over time (likely as a function of heart rate, thoracic APOs, and general circulation), but the stain front showed a clear step-wise advancement from wing base to tip, showing an oscillatory

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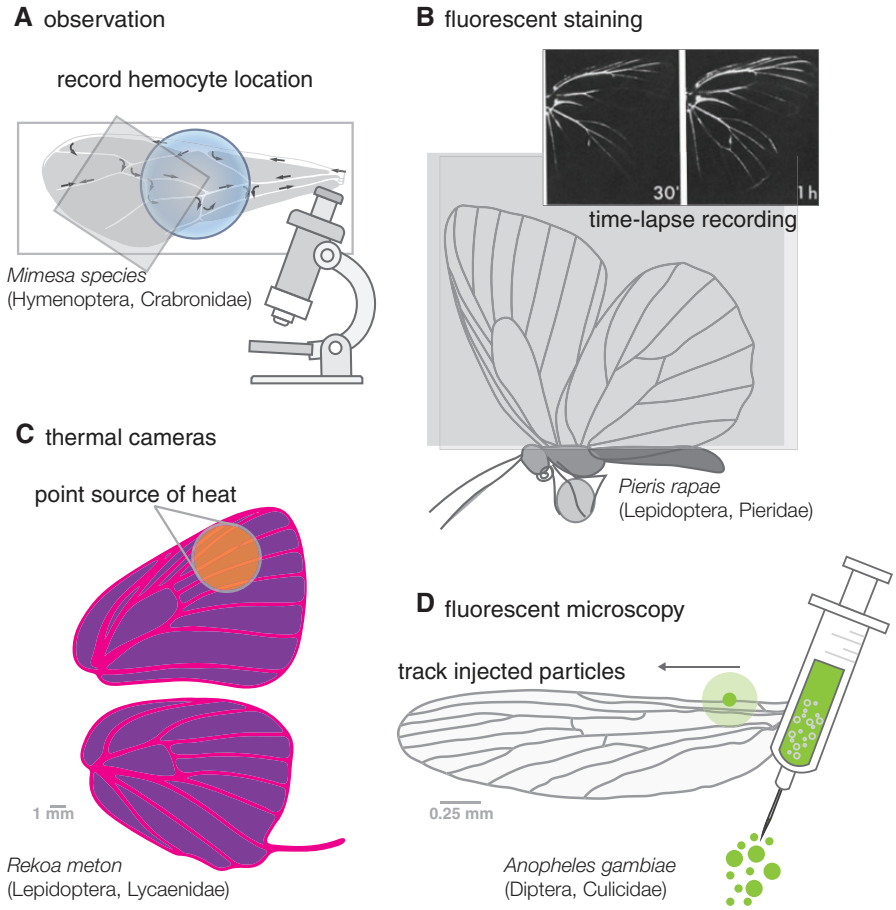

Fig. 3 Methods to measure hemolymph movement in insect wings. Tracking hemolymph movement in insect wings typically involves microscopy, fluorescence, or thermography. Four methods stand out as foundations for observing circulation and wing morphology. (**A**) Well-lit microscopy: By applying water between glass slides, direct observation can be made through gently restrained insects with clear or semi-clear wings. Arnold established this method, tracking hemocytes in 100 species of insects across 14 insect orders (Arnold 1964). (**B**) Fluorescent staining and UV fluorescence show the presence of hemolymph in the wings. Hemolymph carries the stain into the wings, which accumulates and dilutes over time, showing bulk movement (Wasserthal 1983). (**C**) Thermography: using thermal cameras and selective heating (e.g., using a laser or contact thermistors), bulk hemolymph movement can be visualized under changing environmental conditions (Wasserthal 1980; Schmitz and Wasserthal 1993; Tsai et al. 2020). (**D**) Fluorescent microscopy: injecting fluorescent particles in the abdomen or thorax allows for visualization of flow in the wings and throughout the body. Particles are pumped into the wings via APOs and can be tracked (Hillyer and Strand 2014; Chintapalli and Hillyer 2016; Salcedo 2019). Wings in (A) and (B) are not shown to scale.

pattern of hemolymph movement in the wing. These studies were the first to report tidal flow in wings and a unidirectional flow mechanism quantitatively. To test flows of hemolymph into membrane and study potential mechanisms of water loss through the wings, staining was conducted at varying humidity. In some butterfly species at high humidity, fluorescence seeped into the wing membrane driven by diffusion and evaporation, suggesting one-way flow into the wings. This research was the first to describe bulk movement of hemolymph, an important precursor to understanding instantaneous flow in the wing.

Thermal techniques have been used to observe sensory properties and hemolymph movement. Wings, especially those with dark patches, are highly effective radiation-absorbing surfaces. Thermoreceptors along wing veins sense heat, and under intense temperatures or selective heating (such as a laser beam or thermistor), insects will close their wings (basking as a protective posture) or leave for a cooler location (Wasserthal 1975; Schmitz and Wasserthal 1993; Tsai et al. 2020). With the advancement of low-cost infrared cameras, thermal imaging has become a reliable way to record temperature fluctuations corresponding to hemolymph movement (Fig. 3C). Such techniques are relatively non-invasive because the insect can move semi-freely, and also provide instantaneous measurements of how insects thermoregulate. Because most

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insects have thoracic APOs and other hemolymph sinuses that lie just below the cuticle, applied thermistors can be used to both heat and record pulsations of hemolymph flow (Wasserthal 1980). Such contact thermography with surface probes can be used to quantify directionality of hemolymph and bulk flow rate, and indirectly indicate behavioral responses (such as basking).

With advances in high-speed microscopy and fluorescent techniques, hemolymph movement can now be visualized instantaneously throughout the wing and body. Recent work in mosquitoes with injected fluorescent particles measured velocities and accelerations of hemolymph flow in antennae and antennal APOs (Boppana and Hillyer 2014) and described circulation in wings, and how thoracic APOs and dorsal vessel pumping frequencies vary (Chintapalli and Hillyer 2016). By injecting fluorescent particles within the abdomen or thorax, hemolymph is seeded with particles that can be tracked (as seen in Fig. 3D) throughout the insect using a fluorescent microscope, but especially in appendages such as wings and antennae (Boppana and Hillyer 2014; Chintapalli and Hillyer 2016). Particle sizes are critical: for the mosquito *A. gambiae*, particles with diameters of 2 μm did not flow through the veins; those with diameters of 1.0 μm did flow, but only in certain veins; and in contrast, those with diameters of 0.5 μm flowed freely (Boppana and Hillyer 2014). The particle tracking showed that flow leaves mosquito wings four times faster than how it enters, and an unpaired (single wing heart) thoracic APO beats at frequencies independent of those of the dorsal vessel (Chintapalli and Hillyer 2016). This technique is a powerful method to observe flow across an insect wing and its pumping organs. It can be used more broadly to examine flow in insects across the phylogeny, and has been applied successfully to the North American grasshopper, *S. americana* (Salcedo 2019). However, with particle sizes of a similar order to the diameter of the vein, this technique only provides information on bulk flow patterns. For finer details of flow within a vein, micro-particle image velocimetry (PIV) (Wereley and Meinhart 2010) is needed; studies such as these are currently being planned in our lab.

Combined, each of these techniques (observation, fluorescent staining, thermography, fluorescent particles) has informed the other and led to the ability to observe venation pattern as a whole, not just in separate pieces. There is still a need to understand these systems from a three-dimensional perspective. One option is to use synchrotron x-ray imaging, which enables visualization of internal structures with resolutions on the scale of 1 μm (Socha et al. 2007; Socha and De Carlo 2008). Perhaps the most striking demonstration of the power of this technique is the three-dimensional visualization of muscles, tracheae, and tendon recorded in the thorax during tethered flight of flies (Walker et al. 2014). Similarly, complex systems such as the pumping of the dorsal vessel and compressions of the tracheal system in the abdomen of the grasshopper *S. americana* can be tracked using injected microbubbles (Lee and Socha 2009), but thus far, only two-dimensional imaging has been successful. If combined with the previously mentioned techniques (such as adding fluorescent particles), live microtomographic imaging may be able to reveal not only the fluidic dynamics of multiple flows (hemolymph and air), but also active and passive morphology (muscles and cuticle). Although access to a synchrotron facility in the United States requires proposal acceptance, the cost to a research team in travel, lodging, and supplies can be relatively low when compared to a microscopy set-up in a lab. Synchrotron facilities can also be found in other locations throughout the world (https://lightsources.org).

A look to the future: Wing material and flexibility

A wing's mechanical properties, particularly stiffness and toughness, depend highly on hydration status and how proteins interact with chitin (Vincent and Wegst 2004). Insect wings, with their broad surface area, are sensitive to evaporative water-loss. Although evaporation can be positive, aiding in the release of pheromones and other specific scents found on the surfaces of wings (Pass 2018), water loss can greatly influence wing flexibility, a largely unsolved problem. If a wing is removed from an insect, it desiccates and decreases in mass by 21% and increases in stiffness by 46% in just 24 h (Mengesha et al. 2010). In fact, upon wing removal, wings begin to immediately dessicate, and over 36 h, damping properties decrease (Norris et al. 2013). As an insect ages, wings become brittle and more likely to fracture. Once wings lose flexibility, flight performance declines, and individual survival is reduced (Arnold 1964; Combes et al. 2010). Wing flexibility plays a major role in aerodynamic force production and improves both inertial and aerodynamic power economy (Vanella et al. 2009; Mountcastle and Combes 2013; Hedrick et al. 2015; Reid et al. 2019). These effects strongly suggest that hydration is essential to maintaining the mechanical function

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of wings, as well as for maintaining local sensory structures (Dickerson et al. 2014; Pass 2018; Hillyer and Pass 2020). Wing hydration and mechanical performance, and thus behavior, are inextricably related.

Insect cuticle is a composite material, composed of crystalline chitin nanofibers embedded in a matrix of proteins, polyphenols, water (Chapman 2012). Wing veins are layered with resilin, an extremely resilient elastic protein that exhibits energy return as high as 96–97% (in dragonflies up to 99%) (Wainwright et al. 1982; King 2010). Found configured around most wing joints and occasionally the wing membrane, the presence of resilin allows for dynamic deformation in flapping flight by storing energy and resisting long-lasting damage (Donoughe et al. 2011; Mountcastle and Combes 2014; Appel et al. 2015). Acting as a fail-safe against permanent damage, resilin requires a water content of 50–60% in order to function at its highest resilience (Weis-Fogh 1960; Wainwright et al. 1982). Although resilin is highly hygroscopic and a type of hydrogel (Kovalev et al. 2018), the importance of hemolymph circulation to its performance is far less appreciated.

Wings can be foldable, and to reduce damage, insects often store neatly pleated membrane and veins under a modified, toughened forewing, the elytra. Resilin is often embedded in each of the major pleats of folded wings, which is well-recognized in dermapterans such as the earwig, where it enables wing expansion from their shortened elytra to 10 times their folded wing area (Haas et al. 2000a). Unfolding wings can depend on built-in (embedded) folding patterns that are often dependent on structural elastic proteins such as resilin (Haas et al. 2000b). To unfurl their wings, earwigs depend on bistability of a resilin-embedded wing, where the wing is most stable either closed or open (Faber et al. 2018). This viscoelastic behavior of resilin at the wing hinge in extending and retracting a wing is best described by a duality with fast (elastic) and slow (viscous) responses (Kovalev et al. 2018). In other beetles, unfolding wings are opened with a pulse of hemolymph into wing veins (Sun et al. 2014). A dynamic system, wing motions such as unfolding/folding, flapping, and gliding depend on structural integrity of a hydrated resilin system.

Conversely, it is possible that the wing motion during flight itself could influence how hemolymph is circulated in the wing. Few studies have examined how hemolymph mass affects overall wing mass and influences torsional characteristics of the wing (Hou et al. 2015; Song et al. 2020). Many models of insect wing aerodynamics fail to include not only wing flexibility, but also overlook the existence of fluid within the wing veins, modeling veins as hollow (air-filled) tubes (Hou et al. 2015; Song et al. 2020). Consider the mosquito, where pumping organs pulse at frequencies of >3 Hz and flapping frequencies can range between 500 and 700 Hz. Fluid could shift during flight, with high tracheal ventilation (contribution of thoracic or abdominal air sacs) and flapping frequencies. However, hemolymph circulation in the wing likely operates at low Reynolds numbers. Using a flow speed of 100 μm/s and diameter of 1 μm from *A. gambiae* (Hillyer and Strand 2014), and dynamic viscosity of 3 cP and density of 1 g/mL from hemolymph of *Manduca sexta* larvae (Kenny et al. 2018), the Reynolds number of flow in small insects can be estimated to be on the order of 0.003. It is unclear in theory how wingbeat patterns would change such viscous-dominated flows in the wing. Recently, a model of hemolymph hydraulics in flapping wings of Asian ladybeetles (*Harmonia axyridis*) suggested that presence of hemolymph acts to shift the center of mass along the wing veins, suppressing unfavorable wing flutter (Song et al. 2020). Further results indicate even stronger effects: removing the hemolymph to make a hollow hind wing reduced the wing inertia and rotational inertia by 64% relative to a fluid-filled hind wing (Song et al. 2020). This result suggests that hemolymph-filled wing veins may play a significant role in active flapping flight. While flow experiments measure an insect at rest, high-speed cameras and extensions of described methods could be used to determine how moving fluid mass affects the flexibility of the wing and its dynamics during flapping flight.

Lastly, all winged insects at metamorphosis vigorously pump hemolymph to hydraulically expand their wings (Salcedo 2019). A critical bottleneck in development, this expansion phase must be successful to produce viable adults. Successful wing expansion requires a concerted effort by the circulatory and tracheal systems, which are highly dependent upon each other during this period. When wings develop, tracheae branch into the imaginal disc, supplying oxygen to the forming wing tissue (Comstock 1918). Tracheae can be found within a wing vein, but during development, are not active predictors of where wing veins will form (Comstock 1918). The mechanics of insect wing expansion, and how an expanding wing undergoes rapid changes in stiffness, can be further informed by new understanding of flows in the wing during metamorphosis.

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Data availability

Data sharing not applicable – no new data generated.

Authors' contributions

M.K.S. conceived and wrote manuscript. J.J.S. contributed significant edits and conceptual development.

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