The circulatory system of *Penaeus vannamei* Boone, 1931—Lacunar function and a reconsideration of the “open vs. closed system” debate

Torben Göpel | Christian S. Wirkner

Abstract
The morphology of hemolymph circulatory systems has been studied in many arthropod groups over the past decades. In most cases, however, the focus of these studies has been the vascular system, while its counterpart, the lacunar system, has often been neglected. To further understanding of the interrelationships between these two complementary subsystems, we investigated both, the hemolymph vascular system and the hemolymph lacunar system, of the decapod *Penaeus vannamei* using 3D-imaging techniques (micro-computed tomography and confocal laser scanning microscopy) in combination with 3D reconstruction. Major parts of the vascular and lacunar system are described. Our insights into their morphology are used to derive functional conclusions for a model illustrating the interrelationships between the two subsystems. The morphology of and the functional interaction between the vascular and lacunar systems are discussed in the context of the debate on “open vs. closed circulatory systems.”

KEYWORDS
Arthropoda, circulatory system, Decapoda, function, morphology

1 | INTRODUCTION
The morphology of the hemolymph circulatory systems of arthropods, especially of decapods, has been studied in great detail in recent years (e.g., Keller, Richter, & Wirkner, 2013, 2015; McGaw & Reiber, 2002; McGaw & Stillman, 2010). However, what has actually been studied in detail is the hemolymph vascular system (i.e., the heart and the arteries), only one of the two constituent subsystems of the hemolymph circulatory system (Wirkner et al., 2017; Wirkner, Tögel, & Pass, 2013). The hemolymph lacunar system (i.e., lacunae and sinuses) has mostly been neglected in studies on arthropod circulatory morphology.

In arthropods, the heart pumps hemolymph through arteries which emanate from it. These arteries have open endings (Dumont, Anderson, & Chomyn, 1965) through which the hemolymph leaves the hemolymph vascular system (which is therefore termed an open vascular system; Wirkner et al., 2013) and enters the hemolymph lacunar system. Through the lacunae and sinuses, the hemolymph is ultimately channeled back to the pericardial sinus which surrounds the heart. The hemolymph can then reenter the heart through ostia, that is, slit-like valves in the heart. There are no veins, that is, no afferent vessels, and the lacunar system therefore takes over venous function (Wirkner et al., 2013). The hemolymph within the vascular and lacunar system is constantly moved by systolic pumping and diastolic suction (Wirkner et al., 2013). The ostia and the arterial valves at the base of all arteries that leave the heart (Göpel & Wirkner, 2018) prevent backflow and thus ensure that the flow of hemolymph is unidirectional. The morphology of the lacunae and sinuses has mainly been
studied in arthropods lacking an elaborate vascular system, for example, branchiopods (Pirow, Wollinger, & Paul, 1999; Vehstedt, 1940) and insects (Jones, 1977; Snodgrass, 1935). Hemolymph flow in these taxa has also been observed and reconstructed (Jones, 1977; Vehstedt, 1940; Wigglesworth, 1974). Accounts of lacunar morphology and hemolymph flow in Malacostraca have been published for Anaspidacea (Siewing, 1959) and Isopoda (Silén, 1954), among a few other groups. In many cases, the reason for the neglect of the lacunae and sinuses might have been methodological. In dissections, lacunae are barely recognizable as distinct entities (Mayrat, McMahon, & Tanaka, 2006) and in histological sections, lacunae appear as “free space” between the organs and tissues, or are erroneously interpreted as a consequence of shrinkage artifacts. For this reason, lacunae and sinuses in arthropods have sometimes been considered ill-defined spaces or residual recesses rather than distinct morphological entities. The three-dimensional approach offered by modern morphological methods, however, allows the lacunar system to be documented properly and its functional importance to be highlighted.

Decapods are known to show the highest degree of complexity in vascular systems within Mandibulata (Keiler et al., 2013, 2015; McGaw & Reiber, 2002; Wirkner et al., 2013), which implies that hemolymph flow has to be accurately and precisely maintained in the various body regions. As the vascular system constitutes only half of the circulatory system, it can be assumed that a specialized and defined lacunar system is equally important for maintaining this precision of hemolymph flow. We investigated the hemolymph circulatory system of the decapod Penaeus vannamei Boone, 1931, using micro-computed tomography (μCT) and confocal laser scanning microscopy (cLSM) in order to describe the vascular and the lacunar systems in detail. We integrate the main vascular and lacunar constituents of the cephalothorax into a functional model of hemolymph flow derived from our three-dimensional morphological findings.

2 | MATERIALS AND METHODS

2.1 | Specimen preparation

Subadult specimens of *P. vannamei* Boone, 1931, with a total body length of 8–14 cm were obtained from a local aquaculture facility (Garnelenfarm Grevesmühlen GmbH, Grevesmühlen, Germany) and sacrificed using some drops of ethyl acetate. In total, 41 specimens have been studied. Specimens used for μCT were either fixed directly in 2–3% formaldehyde at a salinity of 15–20 PSU (n=5) or were injected with the casting resin Mercox II (Ladd Reserch, Williston, Vermont) into the heart prior to fixation (n=32). Additionally, specimens for μCT imaging were critical-point dried (Leica EM CPD300, Leica Microsystems, Wetzlar, Germany), while dissected parts of specimens were chemically dried using HMDS (Nation, 1983). Three specimens used for cLSM were fixed in 4% paraformaldehyde in 1x phosphate-buffered saline (PBS) for 12 to 48 hours. These specimens were dissected to obtain tissue samples for cLSM imaging. Samples were stained with 1:600 dilution of CellMask™ Green (ThermoFisher Scientific, Waltham, Massachusetts) in 1× PBS or a 1:200 dilution of Alexa Fluor 546 conjugated Phalloidin (Thermo Fisher Scientific) in 1× PBS for 2 hours. One specimen for stereomicroscopy was perfused with 20 ml of 3% formaldehyde solution with a salinity of 15 PSU dyed with congo red before fixation with 2% formaldehyde in 1× PBS.

2.2 | Micro-computed tomography

Specimens for μCT imaging were selected after evaluation of injection quality under a stereomicroscope. Eleven injected and three non-injected specimens (as well as three dissected gills) were used for μCT imaging. Specimens were mounted onto a specimen holder. X-ray imaging was performed on a ZEISS Xradia Versa 410 X-ray microscope (ZEISS; Oberkochen, Germany) using the software Scout and Scan v.11. Acquisition properties were 30 or 40 kV, 150–200 μA, 1.601–3,201 projections, 3–10 s acquisition time per single transmission image.

2.3 | Confocal laser scanning microscopy

Stained samples were mounted in RapiClear 1.47 (SunJin Lab Co., Taiwan) in chambers made of two coverslips and iSpacers (SunJin Lab Co., Taiwan) which were sealed with clear nail polish. Imaging was performed using a Leica DMI6000 CFS microscope equipped with a Leica TCS SP5 II confocal laser scanning unit (Leica Microsystems, Wetzlar, Germany). Stained samples were imaged by excitation with the appropriate wavelength for the dye, and autofluorescence was recorded additionally at multiple wavelengths.

2.4 | Stereomicroscopy

The specimen dyed with congo red was analyzed using a ZEISS Discovery.V12 (ZEISS Microscopy, Jena, Germany).

2.5 | 3D-reconstruction and 3D-data processing

Image stacks from μCT and cLSM were analyzed and reconstructed using the software packages Amira 6.4 (Thermo Fisher Scientific) and Imaris 7.0 (Bitplane AG, Zurich, Switzerland). Desired parts of the volume were segmented and masked, 3D-mesh surface objects were exported. 3D-meshes were further processed using MeshLab (www.meshlab.net; last accessed: March 3, 2020) and Adobe 3D-Reviewer (Adobe Systems Inc., San José, California).

2.6 | Image processing

All figures were arranged and labeled using the software package CorelDraw Graphics Suite X3 (Corel Corp., Ottawa, Canada).
2.7 | Terminology

All terms used to refer to morphemes of the hemolymph circulatory system in *P. vannamei* are based on terms and classes in the Ontology of Arthropod Circulatory Systems (OArCS, accessible via http://oarcs.speciesfilegroup.org, last accessed March 3, 2020; Wirkner et al., 2017).

3 | RESULTS

3.1 | Hemolymph vascular system

The hemolymph vascular system of *P. vannamei* consists of a globular heart and six emanating artery systems: the paired anterior lateral artery systems, the paired hepatic artery systems, the unpaired ventral vessel system, and the unpaired posterior aorta system (Figures 1 and 2a). Our visualization and description of the vascular system focuses on larger branches, very fine vessels are not described and may not be visualized by the methods used.

3.1.1 | Heart

The globular heart is located dorsally in the posterior cephalothorax (Figures 1 and 2a) and appears roughly pentagonal in dorsal view and roughly triangular in sagittal section (Figure 3a, b). It is made up of a rather thick, spongious outer epicard and an inner myocard (Figure 3a). As distinctive for globular hearts, the myocard is a thick, with a peripheral layer of cardiomyocytes which run in various directions, and bundles of cardiomyocytes traversing the heart lumen (Figure 3a, d). The heart is equipped with five pairs of ostia (Figure 3b, c). The first pair (counted from anterior to posterior) is situated anterolaterally, the second and third pairs are situated dorsally, the fourth is situated ventrolaterally, and the fifth posterolaterally. The fifth pair of ostia is distinctly larger in diameter than the other four pairs. In some specimens, one of the four dorsal ostia is rudimentary. Which of the four, however, appears to be subject to intraspecific variation (Figure 3b, d).

3.1.2 | Anterior lateral artery systems

The paired anterior lateral arteries originate from the anterior apex of the heart, close to each other (Figure 2a, b; ala), yet clearly distinguishable from each other, as indicated by separate arterial valves. The two arteries diverge at an angle of approximately 30 degrees anterolaterally and then continue to run anteriorly along the dorsolateral margin of the cephalothorax into the head region (Figures 1 and 2). Along their course, the anterior lateral arteries give rise to a number of arteries, of which only the larger ones will be described in more

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**FIGURE 1** *Penaeus vannamei*, schematic representation of the main parts of the hemolymph vascular system (red) and hemolymph lacunar system (blue). Right thoracopodal arteries and right posterior lateral arteries are indicated by dashed lines. Only three gills and their respective branchio-pericardial sinuses are shown in order to prevent the drawing from being cluttered. ag1l: first-order afferent gill lacuna; ala: anterior lateral artery; ana: antennal artery; aua: antennular artery; bps: branchio-pericardial sinus; bstga: branchiostegal artery; da: descending artery; eg1l: first-order efferent gill lacuna; h: heart; ha: hepatic artery; mp3: third maxillipedal artery; oa: optic artery; ost: ostium; p3: third pereiopodal artery; pa: posterior aorta; pcs: pericardial sinus; pla3: third posterior lateral artery; pnl: perineural lacuna; rar: rostral arch; tll: trough-like lacuna; upa: uropodal artery; vll: ventrolateral lacuna; vv: ventral vessel [Color figure can be viewed at wileyonlinelibrary.com]
Shortly after originating from the heart, each of the anterior lateral artery gives rise to an artery extending in a ventromedian direction. One of the two contralateral arteries is larger and more ramified and continues anteroventrally to supply the stomach from its posterior side, and the midgut (Figure 2b). The other median branch, however, is finer and runs ventrally to supply the midgut and the anterior portion of the midgut gland. Which anterior lateral artery gives rise to the larger artery supplying the stomach, however, is subject to intraspecific variability. A little more distally, each anterior lateral artery gives rise to the branchiostegal artery (Figures 1 and 2b; bstga) which first runs ventrolaterally and then bends posteriorly to run into the branchiostegite. The branchiostegal artery gives rise to several arteries along its course before actually running posteriorly in the direction of the branchiostegite. Before it bends in a posterior direction, one branchiostegal artery gives rise to an artery which runs ventromedially and supplies the foregut (Figure 2b; left bstga). More anteriorly, the antennal artery branches off from the anterior lateral artery in an anteroventral direction (Figure 2b; ana). The proximal part of the antennal artery gives rise to several smaller arteries which supply the extrinsic musculature of the second antenna. A little more distally, the antennal artery bifurcates into the two arteries which run into the endopodite and the scaphocerite, respectively. Anterior to the junction of the antennal artery, the anterior lateral arteries bend slightly anteromedially giving rise to the rostral arch (Figure 2b; rar), an anastomosis between the two contralateral anterior lateral arteries which runs dorsally to the cerebral region, and at its crest gives rise to the rostral artery. After this junction, the anterior lateral arteries curve anteroventrally around the brain and give rise to numerous
fine brain arteries. Ventrolaterally, the antennular artery (Figure 2b; aua) emanates from each anterior lateral artery and soon bends in a directly anterior direction. The last major branch to emanate from each anterior lateral artery is the optic artery (Figure 2b; oa), which runs into the respective eyestalk. Anteriormost, the final tributaries of the anterior lateral arteries anastomose in the anterior cerebral region.

3.1.3 | Hepatic artery systems

The paired hepatic arteries originate from the anteroventral portion of the heart extending anteroventrally at a 45° angle to the longitudinal axis (Figure 2a). The hepatic arteries are found between the diverticles of the midgut gland and soon bifurcate into an anterior and a posterior branch each. These branches give rise to numerous smaller arteries which continue between the diverticles of the midgut gland to supply every region of this organ. Anteriorly, some smaller branches of the hepatic artery system come close to branches of the anterior lateral artery systems, though anastomoses between the two artery systems do not occur.

3.1.4 | Ventral vessel system

The descending artery emanates at the posteroventral apex (Figure 2a; da) of the heart right beside the posterior aorta (see below). The descending artery runs ventrally and curves around the gut. Whether the descending artery passes by the gut on the right or the left side is subject to intraspecific variability. The descending artery merges with the longitudinal ventral vessel (Figure 2a, d; vv) at the point where the latter gives rise to the third pereiopodal arteries (Figure 1). The ventral vessel follows the ventral longitudinal line of the cephalothorax right underneath the ventral nerve cord. Posterior to the junction with the descending artery, the ventral vessel gives rise to the fourth pereiopodal arteries and ultimately bifurcates into the fifth pereiopodal arteries. Anterior to the junction with the descending artery, the ventral vessel gives rise to the second and first pereiopodal arteries, as well as the third and second maxillipedal arteries, which share a common stem (Figure 2d; mp3, mp2). Anterior to the second maxillipedal arteries, the ventral vessel bifurcates into branches from which the first maxillipedal arteries and the arteries running into the two maxillae emanate (Figure 2d; mp1, mx2, mx1). Close to the bifurcation of the ventral vessel, from one of the two branches, an unpaired artery originates which runs anteriorly and bifurcates into the two mandibular arteries (Figure 2d; md). In the two specimens in which this unpaired branch could be seen best, it originated from the right branch, though close to the bifurcation. Furthermore, the ventral vessel gives rise to several fine arteries dorsally which supply the ventral nerve cord.

3.1.5 | Posterior aorta system

The posterior aorta originates from the posteroventral apex of the heart and runs longitudinally through the dorsal portion of the pleon (Figures 1 and 2a; pa). In the posterior part of the first to fifth pleonal segment, a pair of posterior lateral arteries emanates (Figure 2a, c;
pla1–5) which follow the lateral margin of the pleon to run ventrally and supply the pleonal musculature via small branches along their course, and ultimately to supply the pleopods. In the anterior part of the sixth pleonal segment, the posterior aorta bifurcates (Figure 1 and 2c). While one of the resulting branches is rather small and with its ramifications supplies only the muscles within the segment itself, the other branch is substantial and at the posteriormost part of the segment bifurcates into the left and right uropodal arteries (Figure 2c; upa).

### 3.2 | Hemolymph lacunar system

#### 3.2.1 | Sinuses

**Pericardial sinus**

The pericardial sinus (Figures 1 and 4a; pcs) is situated in the posterior dorsal region of the cephalothorax and encloses the heart. It is bordered ventrally by the pericardial septum (Figure 4a; pcsp) and dorsally by the integument. Medially, the pericardial septum rests on the midgut gland. Laterally it bends in a ventral direction, so that that the pericardial sinus has approximately a crescent-shape in cross-section. The pericardial septum is attached to the integument to form a closed space (except for the inlets of the branchio-pericardial sinuses), and it is therefore penetrated by the arteries emanating from the heart. Ventrolaterally, the branchio-pericardial sinuses merge into the pericardial sinus (Figure 4a; bps). Posteriorly on each side, the branchioostegal–pericardial lacunae open into the pericardial sinus (Figure 4a; bpl).

**Branchio-pericardial sinuses**

The branchio-pericardial sinuses (Figure 4a; bps) connect the efferent gill lacunae with the pericardial sinus. However, there are not 18 inlets into the pericardial sinus on each side, rather the sinuses merge before opening into the pericardial sinus. The branchio-pericardial sinuses are located laterally and enclosed by a sinus septum and the lateral integument. The sinuses are elliptical in horizontal section. On each side, three major systems of branchio-pericardial sinuses open into the pericardial sinus, with the second efferent to the largest group of gills (9 of 18 gills; three proximal sinuses efferent to 3 gills each). The anteriormost of the three major sinus systems extends posterodorsally, while the posteriormost extends anterodorsally (Figure 4a). The transition from the branchio-pericardial sinuses to the pericardial sinus is smooth as the sinus septa merge with the pericardial septum.

#### 3.2.2 | Major lacunae of the cephalothorax

**Trough-like lacuna**

The trough-like lacuna (Figures 1 and 4b; tll) is located in the posterior cephalothorax directly underneath the midgut gland. Ventrally, it is bordered by the extensive ventral musculature of the cephalothorax. In cross section, this lacuna resembles a compressed “u.” It runs along the longitudinal axis, that is, it has a trough-like shape in 3D view. It is connected dorsally to the numerous small lacunae between the diverticula of the midgut gland. Ventrally, the trough-like lacuna connects to the smaller lacunae which interlace the ventral thoracic musculature. The trough-like lacuna is connected ventrally to the perineural lacuna (Figure 4b; pnl) along the median line via straight, ventrally oriented lacunae which occur predominantly in its anterior portion.

**Perineural lacuna and ventrolateral lacunae**

The perineural lacuna (Figures 1 and 4b; pnl) is located in the ventral longitudinal line and encloses the ventral nerve cord dorsally and laterally (at some points also ventrally). Dorsally, the perineural lacuna is connected to the numerous small lacunae which interlace the ventral thoracic musculature and to the ventrally oriented lacunae arising from the trough-like lacuna. At the point where the ventral nerve cord diverges anteriorly to curve around the pharynx, the perineural lacuna resolves into the complex head lacuna (which, however, could not be reconstructed satisfactorily). Laterally, the perineural lacuna gives rise

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**FIGURE 4** *Penaeus vannamei*, hemolymph lacunar system of the cephalothorax. (a) Lateral view of the pericardial sinus and respective inlets; (b) lateral view of major lacunae of the cephalothorax. bpl: branchiostegal-pericardial lacuna; bps: branchio-pericardial sinus; pcs: pericardial sinus; pcsp: pericardial septum; pnl: perineural lacuna; tll: trough-like lacuna; vll: ventrolateral lacuna. The PDF version contains interactive 3D content. Open in Adobe Reader and click on the figure to activate [Color figure can be viewed at wileyonlinelibrary.com]
to the ventrolateral lacunae (Figures 1 and 4b; vll). The ventrolateral lacunae are superficially segmental in their arrangement but cannot actually be assigned to a specific segment as they are so difficult to differentiate. Six “clusters” of afferent gill lacunae which emanate from the ventrolateral lacunae could be distinguished (more or less).

### 3.2.3 | Gills

There are 18 dendrobranchiate (sensu Calman, 1909) gills on each side. Each gill is made up of a main stem pointing dorsally (Figure 5). The main stem gives rise to second-order branches on each side which, except for the ones at the dorsal tip of the gill, have the shape of stacked horseshoes (Figure 5c,d). From the outer side of the second-order branches, the terminal branches of the gill emanate. All terminal branches except the most distal bifurcate one last time. The terminal branches of the gill point dorsally. Each part of the gill contains one afferent and one efferent gill lacuna (Figure 5c–e); the afferent gill lacunae are located more internally and the efferent gill lacunae more externally. In some gills, the first-order efferent gill lacuna curves around one side (or both) of the proximal part of the first-order afferent gill lacuna. Within each terminal branch of the gill, the third-order afferent gill lacuna runs along the dorsal margin into the distal tip and then transitions into the third-order efferent gill lacuna which runs along the ventral margin of the terminal gill branch (Figure 5e). The third-order efferent gill lacuna merges into the second-order efferent gill lacuna, which in turn merges into the first-order efferent gill lacuna. The first-order efferent gill lacuna runs through the stalk of the gill into the cephalothorax, where it opens into the branchio-pericardial sinus. In the terminal branches of the gill, hemolymph flows not only through the loop formed by the third-order afferent and efferent gill lacunae but also through a fine network of lacunae which connects them (Figure 5e).

### 3.3 | Branchiostegites

The branchiostegites are traversed by a mesh of both arteries and lacunae (Figure 6a). The branchiostegal artery, a part of the anterior lateral artery system, enters the branchiostegite approximately halfway along it and ramifies multiple times (Figure 6a–c). The ramifications of the branchiostegal artery supply most of the branchiostegite via a large number of small terminal arteries in the distal portion close to its margin. Another smaller artery enters the branchiostegite from

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**FIGURE 5** *Penaeus vannamei*, gill morphology. (a) Anterior view of a single gill; red-cyan anaglyph, use glasses; (b) posterior view of a single gill; red-cyan anaglyph, use glasses; (c) virtual section of a single gill showing first-order afferent (blue) and efferent (red) gill lacunae; (d) shadow projection of second-order gill branches; red-cyan anaglyph, use glasses; (e) schematic representation of a terminal gill filament and the oxygenation of hemolymph; blue: afferent gill lacunae and deoxygenated hemolymph; red: efferent gill lacunae and oxygenated hemolymph; arrows indicate hemolymph flow. egl1: first-order efferent gill lacuna; agl1: first-order afferent gill lacuna; egl2: second-order efferent gill lacuna; agl2: second-order afferent gill lacuna; egl3: third-order efferent gill lacuna; agl3: third-order afferent gill lacuna. The PDF version contains interactive 3D content. Open in Adobe Reader and click on figure (e) to activate [Color figure can be viewed at wileyonlinelibrary.com]
its posteriormost connection to the cephalothorax (Figure 6a; asterisk). This artery runs along the posterior edge of the branchiostegite and gives rise to smaller branches which mostly run into the central region of the branchiostegite.

The branchiostegal lacunae connect to the lacunar system of the cephalothorax as follows: the major branchiostegal lacuna (Figure 6a; mbl) enters the branchiostegite near the branchiostegal artery at the notch around halfway along the branchiostegite. The branchiostegal–pericardial lacuna (Figures 4a and 6a; bpl) runs along the edge of the branchiostegite and into the posterior region of the pericardial sinus. These two larger lacunae are connected by a network of highly ramified smaller tributary lacunae. The small lacunae diverge fan-like from the major branchiostegal lacuna to then merge again and join the branchiostegal–pericardial lacuna. Congo red staining revealed concentric loops (Figure 6a; arrowhead) in the branchiostegite, crossing the path of arteries and lacunae. Confocal microscopy, however, showed that these loops are not hemolymph channels but presumably cuticular crests of some kind (Figure 6d, e).

4 | DISCUSSION

4.1 | Morphology of the vascular system

In general, the hemolymph vascular system of *P. vannamei* resembles those described for other decapod crustaceans (Brody & Perkins, 1930; Keiler et al., 2013; McGaw & Stillman, 2010; Scholz, Richter, & Wirkner, 2018). The single difference in the vasculature is the absence of an anterior aorta. While other decapod groups such as anomurans (Keiler et al., 2013), crayfish (Scholz et al., 2018), and caridean shrimps (Pillai, 1965), and actually most arthropods in general (Göpel & Wirkner, 2018; Wirkner et al., 2013), exhibit an anterior aorta, *P. vannamei* is lacking such an artery. Young (1959) described a fine unpaired anterior artery for *Penaeus setiferus* which he concluded might be a vestige of the anterior aorta (an idea taken up and generalized for Dendrobranchiata by McLaughlin, 1980), but Mayrat (1958) explicitly noted the lack of an anterior aorta for *Peneus kerathurus*. It is unambiguous in *P. vannamei* that the only arteries emanating from the anterior apex of the heart are the anterior lateral arteries. There is, however, a proximal side branch of one anterior lateral artery which runs dorsomedially and gives rise to a fine longitudinal artery which in turn follows the course one would expect from a rudimentary anterior aorta. Because of its relative vicinity to the heart, this artery might be confused with an anterior aorta. The anterior lateral arteries take on the functions of the anterior aorta and supply the eyes and the brain, for example, via a remarkably complex set of ramifications (Figure 2a, b). As in other decapods (Keiler et al., 2015), the hepatic arteries and their branches only supply the midgut gland, so although they exhibit complex ramifications, they are restricted to a rather narrow region. The descending artery shows some intraspecific variability in whether it passes the gut on the right side or the left, a phenomenon that has also been observed in marbled crayfish (Scholz et al., 2018; Vogt, Wirkner, & Richter, 2009), among other species. In general, the

FIGURE 6  *Penaeus vannamei*, branchiostegite. (a) Photograph of left branchiostegite injected with congo red; asterisk indicates artery entering the branchiostegite posteriorly; arrowhead indicates concentric loop (see [d,e] for detail); (b) confocal laser scanning microscopy (cLSM) image of arteries (orange) and lacunae; CellMask™ staining; (c) oblique view of an artery (green) embedded in the branchiostegal tissue and thus crossing the lacunae; detail of (b), solid line indicates perspective; dashed ellipse indicates truncated artery; (d, e) cLSM images of concentric loops (autofluorescence at multiple wavelengths) depicting those loops most likely not part of the circulatory system. bpl: branchiostegal-pericardial lacuna; bstga: branchiostegal artery; mbl: major branchiostegal lacuna [Color figure can be viewed at wileyonlinelibrary.com]
morphology of the vascular system of *P. vannamei* bears a strong resemblance to that of *P. kerathurus* (Mayrat, 1958), including features such as the rostral arch (Figure 2b, rar).

The heart of *P. vannamei* is equipped with five pairs of ostia, which are identical in position to the five pairs in *P. kerathurus* (Mayrat, 1958). Five pairs of ostia have also been found in the caridid shrimp *Cardina laevis* (Pillai, 1965) and thus might well represent the plesiomorphic condition within Decapoda. Young (1959) described only three pairs of ostia for *P. setiferus*, as have been found in other decapods (e.g., Keiler et al., 2015; Scholz et al., 2018). Although a difference of this nature between two species of dendrobanchiate shrimp is possible, it is also imaginable that the anterior and the ventrolateral pairs of ostia (pairs one and four in *P. vannamei*) might have been overlooked in *P. setiferus*. These two pairs of ostia are rather small and are located in regions of the heart that are difficult to examine by dissection. Another distinctive feature of the heart in *P. vannamei* is the intraspecific variability of the dorsal ostia. In most specimens, one of the four dorsally situated ostia (Pairs 2 and 3) is only rudimentarily developed, sometimes to the extent that it is not recognizable as an ostium from the outside at all. Which of the four this applies to appears to follow no obvious rule. However, because in the µCT volume the missing ostium can be identified as vestigial and is thus not lacking entirely (Figure 3b, d), it is possible that some unspecified and yet undescribed developmental processes might be the reason for the reduction of one of the four dorsal ostia.

### 4.2 | Branchiostegite

The branchiostegites of *P. vannamei* are amply supplied with hemolymph, mirroring the situation in *P. setiferus* as described by Young (1959). The carapaces of various malacostracans are ascribed osmoregulatory (Cieluch, Charmantier, Grousset, Charmantier-Daures, & Anger, 2005; Lignot, Charmantier-Daures, & Charmantier, 1999) and respiratory functions and are also supplied with hemolymph through an elaborate system of lacunae (Farrelly & Greenaway, 1993, 2005; Wirkner & Richter, 2013). While respiratory regions of the carapace in Lophogastrida, Mysida, and Brachyura are supplied via afferent lacunae (Gruner, 1993), the branchiostegites of *P. vannamei* are supplied simultaneously via afferent lacunae and afferent arteries. The afferent arteries are the branchiostegal artery, which enters the branchiostegite approximately halfway along its length, and a smaller artery which enters the branchiostegite at its posterior margin (Figure 6a). Both arteries ramify, with their side branches ultimately ending openly to let hemolymph into the lacunae. Separate afferent lacunar supply to the branchiostegite is provided by the major branchiostegal lacuna, which enters the branchiostegite close to the branchiostegal artery and then ramifies radially into an elaborate system of small lacunae. Closer to the margins of the branchiostegite, the lacunae merge and then join the branchiostegal-pericardial lacuna, the only efferent lacuna of the branchiostegite, which channels hemolymph into the pericardial sinus. In his description of hemolymph supply in *P. setiferus*, Young (1959) termed all the hemolymph channels "vessels," without distinguishing between vascular and lacunar elements. Nevertheless, our results clearly show that both afferent vessels and afferent lacunae are present in *P. vannamei*.

Young (1959) distinguished two types of channels in the branchiostegite: the radially ramifying channels and a set of channels running parallel to the margin of the branchiostegite (figure 77 in Young, 1959). However, as described above, the only lacuna running parallel to the margin of the branchiostegite is the branchiostegal–pericardial lacuna. What Young (1959) might have mistaken for hemolymph channels are the concentric loops described above (Figure 6a, arrowhead) which cLSM image data clearly show not to be any kind of hemolymph channel (Figure 6d, e).

### 4.3 | A functional model of hemolymph flow in the cephalothorax

As described above, the hemolymph lacunar system exhibits a definite morphology and major lacunae of the cephalothorax can be identified. Siîén (1954) described well-defined lacunae and sinuses in Oniscoidea and derived a model of the main hemolymph currents in the posterior thorax. However, the internal organization of oniscoid isopods, especially with regard to lacunae and sinuses, apparently differs substantially from that of *P. vannamei* (Gruner, 1993). Thus, implications for lacunar hemolymph flow are expected to differ as well.

In bringing together our findings on the morphology of the vascular and lacunar systems in *P. vannamei*, we hope to achieve a better functional understanding of the relationships between these two subsystems of the circulatory system. As a first step, morphology is used to derive function (Bock & von Wahlert, 1965). In the following, we seek to integrate the vascular system and the major lacunae and sinuses of the cephalothorax into a functional model of hemolymph flow in the (posterior) cephalothorax (Figure 7) and to highlight the importance of lacunar structures in terms of oxygen and nutrient supply.

Hemolymph is ejected from the heart through the hepatic arteries, which ramify between the diverticles of the midgut gland. At this point, the hemolymph leaves the arteries of the hepatic artery systems and enters the lacunar system, flowing around the midgut and the diverticles of the midgut gland through a number of fine lacunae. Here, it is likely that nutrients are taken up into the hemolymph (while the midgut gland is supplied with oxygen) to be transported to the target organs. The hemolymph collects in the trough-like lacuna ventral to the midgut gland. From the trough-like lacuna, hemolymph is channeled to the perineural lacuna either via the direct dorsoventral connection between the two lacunae or via the small lacunae in the interstices of the bundles of the ventral musculature (where nutrients can also be given off to the muscles). The hemolymph in the perineural lacuna can now supply the ventral nerve cord directly with the nutrients taken up earlier, before entering the ventrolateral lacunae which emanate laterally from the perineural lacuna. From the ventrolateral lacunae, the hemolymph enters the afferent gill lacunae to be channeled to the terminal branches of the gill, where in small lacunae...
between the third-order afferent and efferent gill lacunae the gas exchange takes place. The oxygenated (and still nutrients carrying) hemolymph flows back into the cephalothorax through the efferent gill lacunae, which open into the branchio-pericardial sinuses. The branchio-pericardial sinuses bring the hemolymph back to the pericardial sinus, from where it can reenter the heart through the ostia. The oxygen and nutrients rich hemolymph is pumped out of the heart again and flows into the ventral vessel via the descending artery. Through the pereiopodal arteries, the hemolymph is pumped into the pereiopods to supply the legs with nutrients and oxygen, from where it returns through the lacunae in the legs back to the ventrolateral lacunae. The ventral vessel also gives rise to short arteries dorsally which open directly at the ventral nerve cord to supply it with oxygen before the hemolymph is channeled from the perineural lacuna into the ventrolateral lacunae from where it can flow through the gills and ultimately back to the pericardial sinus.

This theoretical model of hemolymph flow is intended to facilitate understanding of the functional relationships between sinuses and lacunae in terms of nutrient and oxygen supply to tissues and organs. However, it is self-evident that the processes described above all happen simultaneously and that nutrient-enriched hemolymph and oxygenated hemolymph thus cannot be separated as strictly as the model might imply when interpreted literally. What is also obvious, though, is the functional importance of the lacunar system. Ultimately, it is the lacunae which are responsible for oxygen and nutrient supply to the various organs. The ventral nerve cord, for example, is supplied by the hemolymph in the perineural lacuna, branchial gas exchange takes place in the third-order gill lacunae, and nutrient uptake happens in the small lacunae around the midgut and the diverticles of the midgut gland.

Accounts of lacunar morphology and hemolymph flow in malacostracans are often restricted to certain parts of the body (just as our model is restricted to the posterior cephalothorax). Klövekorn (1934), for instance, described hemolymph flow in the thoracopods of *Gammarus pulex* in detail, while Silén (1954) derived a model of hemolymph currents in the major thoracic lacunae of oniscoid isopods. Siewing (1959) schematized hemolymph flow from the body into the legs and back for the thorax and pleon of *Anaspides tasmaeae*. Each of these accounts of lacunar function constitutes a building block of which are more needed if we are to functionally understand the complex interrelationships between the hemolymph vascular system and the hemolymph lacunar system in crustaceans (or arthropods in general).

### 4.4 Not an open circulatory system?

The arthropod circulatory system, which is traditionally considered an "open circulatory system" (Gruner, 1993), has often been derided as a "primitive," poorly designed system. The point of reference for this implicit comparison is always the vertebrate (basically mammalian and avian) "closed circulatory system," whose highest expression of
complexity is held to be its separate systemic and pulmonary circuits (McMahon, 2012).

However, the definitions of “open” and “closed” in this context are not the same as in thermodynamics, for example, where closed systems can only exchange energy, not matter, with their environment (Khonsari & Amiri, 2012, p. 11). The definition of “closed” in terms of biological circulatory systems is that such systems are completely separated from the body cavity and bounded by an endothelial layer across which substance exchange takes place (Reiber & McGaw, 2009). Though vessels with an endothelial lining are indeed a peculiarity of vertebrates, the dense specialized arterial walls found in arthropods (e.g., Göpel & Wirkner, 2015; Lane, Harrison, & Bowerman, 1981), while not endothelial per se, are also assumed to similarly seal the arteries (Lane et al., 1981). On the other hand, even vertebrate circulatory systems are not completely “closed” as fenestrated and discontinuous endothelia in capillaries allow not only blood plasma and dissolved molecules but also (in the case of discontinuous endothelia) blood cells to pass the capillary wall (e.g., Noble, Johnson, Thomas, & Bass, 2013, pp. 126–127).

Another problem with the terms “open” and “closed” is that they have been applied to the circulatory system as a whole (see McGaw, 2005 and literature therein), hence “open circulatory system” of arthropods. However, the circulatory system as a whole, consisting of the vascular system and the lacunar system, is closed to the same degree as the circulatory system of vertebrates. As pointed out previously (Wirkner et al., 2013), it is the vascular (sub)system (and the lacunar system respectively) of arthropods which should be considered open as the hemolymph never leaves the circulatory system; it just moves between the vascular subsystem and the lacunar subsystem. To cloud the matter even further, the vascular systems of decapod crustaceans have been assigned the exceptional status “incompletely closed” due to their complexity (McGaw, 2005; Reiber & McGaw, 2009). Although it recognizes the complexity of decapod vascular systems (horseshoe crabs and pulmonate arachnid taxa should be regarded as equally complex; Huckstorf, Kosok, Seyfarth, & Wirkner, 2013; Klußmann-Fricke, Pomrehn, & Wirkner, 2014; Göpel & Wirkner, 2015), the term “incompletely closed” dilutes the conceptual pair of “open” vs. “closed”. Apart from the arbitrariness of the decision regarding the degree of complexity (e.g., in terms of ramifications) above which an arthropod circulatory system can be called “incompletely closed,” the abstract situation in decapods is no different from that in other arthropod taxa: the hemolymph always leaves the arteries, no matter how much they ramify, and passes into the lacunar system and then ultimately back to the heart, that is, back to the vascular system again.

To uphold the conceptual pair of “open” vs. “closed” vascular systems, a new and ubiquitously applicable definition of these concepts is called for, a definition independent of the histological properties of the arterial lining, the fuzzy parameter of separation from the body cavity or the inevitably arbitrary degree of complexity. We suggest that a vascular system should be considered closed if fluid (including cells) can complete a full heart-back-to-heart circuit within the lumen of vessels (although a fraction of the fluid might leave the lumen, for example, through discontinuous capillaries). In open vascular systems, on the other hand, the fluid inevitably leaves the vascular lumen at some point and the vessels end (Dumont et al., 1965). This definition fits the perception of the mammalian vascular system as closed despite its discontinuous capillaries, as not all the blood leaves via the fenestrated capillaries, and most of it will leave and reenter the heart within the pulmonary and systemic circuit without leaving the vascular lumen. This conceptual definition also fits taxa other than arthropods and vertebrates. Some molluscs, for example, unlike arthropods, have arteries and veins, but in pulmonate gastropods, for example, hemolymph passes through a lacunar system between the arterial and venous systems. This would match the definition of an open vascular system, while the circulatory systems of cephalopods would match the definition of a closed vascular system (Bourne, Redmond, & Jorgensen, 1990).

It is worthwhile considering the complexity of the vascular systems found in some arthropod taxa in terms of functional aspects. The open vascular systems of arthropods have been derided as “primitive” and poorly designed in comparison to the elaborate closed vascular systems of vertebrates (Gruner, 1993; Pyle & Cronin, 1950). However, some arthropod taxa (e.g., Xiphosura, Decapoda) possess a vascular system that, despite being open, exhibits a level of complexity in terms of ramification that is comparable to that in smaller vertebrates. This is the main argument for Reiber and McGaw (2009) to consider the decapod vascular system “incompletely closed.” Apparently, the view persists that a closed vascular system, as found in vertebrates, represents the “ideal” or “best” condition which arthropods have unfortunately not been able to evolve. However, the functional importance of the vascular systems in arthropods is likely to differ from that in their vertebrate counterparts. The key functions of circulatory systems are, of course, to supply the body with nutrients and oxygen, remove waste material and carbon dioxide, provide immunocompetent cells, and distribute hormones (Wirkner et al., 2013). In arthropods, exchange between tissues and hemolymph mainly takes place in the lacunar system, which “bathes” the organs in hemolymph (the only prominent exception being the perineural vascular sheath in Xiphosura and apulmonate arachnid taxa; Göpel & Wirkner, 2015, Klußmann-Fricke & Wirkner, 2018). The overarching functional purpose of the vascular system is to circulate and replace hemolymph which has already interacted with organs and tissues. This is a different function than that fulfilled by the vascular system in vertebrates, and it is entirely possible that a closed vascular system in arthropods would not be the “desirable” condition at all. The vascular system provides various hemolymph inlets into the interstitial lacunae and permits constant hemolymph flow to all parts of the body. If the vascular system in P. vannamei were to consist only of a heart and one short anterior aorta (e.g., as in copepods), there would most likely be no significant hemolymph flow in the pleon or the appendages and homeostasis could not be upheld. Such simple vascular systems are thus only found in minute arthropods, independent of their phylogenetic position (Göpel & Wirkner, 2018; Wirkner et al., 2013). Larger arthropods have evolved a remarkable disparity of vascular systems, with those in horseshoe crabs, pulmonate arachnids, and decapod crustaceans—the
three taxa boasting the largest arthropod organisms—doubtlessly exhibiting the highest complexity (Göpel & Wirkner, 2015; Klüßmann-Fricke et al., 2014; McGaw & Reiber, 2015). This high degree of ramification is likely to ensure steady circulation even in remote body regions. Organs with high nutrient and oxygen consumption are equipped with extensive vasculature to permit faster exchange of hemolymph in the adjacent lacunae to keep nutrient and oxygen concentration as high as possible (Wilkens, 1999). Furthermore, many arthropod taxa have centralized respiratory organs such as gills or book lungs which demand an elaborate vascular system to evenly distribute oxygenated hemolymph (Wirkner et al., 2013 and literature therein).

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Data Availability Statement
The data that support the findings of this study are available from the corresponding author upon request.

ORCID
Torben Göpel https://orcid.org/0000-0002-6726-6319
Christian S. Wirkner https://orcid.org/0000-0001-9528-5106

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