Evolutionary Origins of the Blood Vascular System in Metazoans – A Microbial Perspective

François Papale[1] and Louis-Patrick Haraoui[2,3,4*]

[1] Department of Philosophy, McGill University, Montréal, Québec, Canada.

[2] Department of Microbiology and Infectious Diseases, Université de Sherbrooke, Sherbrooke, Québec, Canada.

[3] Centre de recherche Charles-Le Moyne, CISSS Montérégie-Centre, Greenfield Park, Québec, Canada.

[4] Humans & the Microbiome Program, Canadian Institute for Advanced Research, Toronto, Ontario, Canada.

[*] Correspondence: louis.patrick.haraoui@usherbrooke.ca

Abstract

This opinion piece posits two intertwined major evolutionary transformations – the advent of the circulatory system in animals and the subsequent emergence of sterile[a] organismal compartments it enabled – as promoted by microbial communities. These transformations should be considered significant in that they shaped the ability of downstream multicellular organisms to diverge and attain new levels of specialization. We rely on evolutionary, metabolic, developmental and biophysical considerations to argue for the essential roles played by microbial communities in the emergence of vascular systems in animals. We also briefly allude to similar phenomena occurring independently in plants[2,3], suggesting a form of parallel evolution. In developing our arguments, we highlight issues with the uses of the sterile vs. non-sterile dichotomy in scientific disciplines focusing on animal-microbial interactions, demonstrate how adopting this distinction has promoted inaccurate frameworks about how to conceptualize these interactions, and offer important correctives to better understand the role of microbes in shaping animal evolution and development.

[a] We use the term "sterile" to refer to tissues, organs or compartments whose cells and fluids have no direct physical contact with free-living microbes [1]. Endosymbiotic bacteria are not free-living and can therefore be found in "sterile" sites.

Introduction

In an article published shortly after his passing, John T. Bonner explored what he termed "the evolution of evolution"[4]. To achieve his objective, that is, to compare the evolutionary trajectories of micro- and macro-organisms from the beginnings of life to today, Bonner opens by charting the increasing volumes of living organisms through time, correlating this trend with rising atmospheric oxygen levels[5,6]. From there, his analysis highlights "four essential transformations in the evolution of life: the emergence of the eukaryotic cell, meiosis, multicellularity, and the nervous system"[7] (p. 307). In an accompanying commentary, Scott F. Gilbert adapts Bonner's perspective through the lens of holobiont evo-devo[7]. In doing so, Gilbert aptly re-integrates the intertwined life cycles of micro- and macro-organisms which were dichotomized in Bonner's piece. Indeed, macrobes[8] are part of interdependent metaorganisms[9], also called holobionts, resulting from mutual scaffolding and co-construction[10]. It is therefore futile to attempt to understand evolutionary transformations without considering the influence of microbial communities in the process of holobiont co-construction and in shaping these transformations. This is true even if, under the sustained well informed skepticism of many researchers[11,12], we leave the status of holobionts as units of selection open to debate, as we do in this paper. Whether they are units of selection or not, multispecies assemblages are functional systems that shape biological phenomena[13], including natural selection[14–17].

Using contemporary observations as a guide[18], we aim to answer the following question: given the growing appreciation that animals are dependent on microbial communities for

a variety of developmental and physiological processes, is there evidence that microbes were key factors in shaping the evolutionary emergence of the blood vascular system?

We argue that the origin of the circulatory system should be considered a major evolutionary transformation, yet not for the roles we usually perceive it to have played. Indeed, the common view presents the circulatory system as enabling an increase in body size based on its ability to feed oxygen and nutrients to distant animal cells and tissues more efficiently and across greater distances than the process of diffusion. While we partially agree with this common view, we believe it is incomplete as it obscures the ubiquitous microbial influence on multicellular organisms.

We claim that the advent of the circulatory system primarily played another role, enabling the physical expansion of interspecies interactions that compose metaorganisms by providing conduits to circulate microbial products across scales that would be impossible through diffusion alone. This development promoted the emergence of cells, tissues, organs and compartments with no direct physical contact with microbes – traditionally categorized as sterile body sites – while maintaining the necessary multi-kingdom metabolite cross-talk[19] between microbiota and distant animal cells.

In short, the reasoning put forward in this paper goes as follows:

- Diploblastic animals, whose tissues arise from one of only two germ cell layers (the ectoderm and the endoderm), lack circulatory systems and rely on the process of diffusion to exchange nutrients and gases[4,20]. Reliance on diffusion and the need to maintain metabolic interactions between cells of diploblasts and microbial communities, we posit, imposes constraints on anatomic configurations.

- The emergence of triploblastic animals via the development of a third germ cell layer (the mesoderm) is a precursor to the appearance of numerous iterations of circulatory systems (derived from mesodermal cells) as well as to the diversification of body plans and the expansion of animal size[4,21]. According to the received view, these developments occurred thanks to the advantages conferred by circulatory systems in overcoming the limitations imposed by diffusion on animal cells' needs for gas and nutrient exchanges. Absent from this perspective, we argue, is a critical novelty: the capacity of blood vascular systems to maintain metabolic cross-talk between microbial communities and distant, physically non-contiguous tissues and organs (so-called "sterile" body sites).
- Not only do circulatory systems allow microbial communities to influence cells, tissues and organs with which they lack direct contact, but compelling evidence also suggests that microbes play a central role in the development of circulatory systems, e.g. in angiogenesis. This developmental role suggests that, evolutionarily speaking, the development of the circulatory system might also have been dependent on the benefits it provides to microbes.

The preceding arguments regarding the development and evolution of circulatory systems lead us to challenge the received view of sterility and the existence of an aposymbiotic stage in some metazoan life cycles[22]. The distinction established between sterile and non-sterile organs and compartments is helpful in certain disciplines such as medicine and clinical microbiology. However, its adoption across scientific domains, notably in developmental biology and holobiont/symbiosis research, has generated

unwarranted differentiations, including the reification of the idea that placental mammals, among other animals, have an aposymbiotic phase at the start of their life cycles. Regarding these ideas, our argument goes as follows:

- The sterile/non-sterile dichotomy obscures the fact that microbial communities act on and influence both physically contiguous and non-contiguous organs and tissues, including "sterile" ones (e.g., the brain), through microbially-derived molecules (metabolites) circulating in blood vascular systems[19]. These metabolic interactions have been observed during embryonic development in placental mammals (metabolites from the maternal gut microbiota crossing the placenta to mice embryo *in utero*[23–25]) and oviparous organisms (metabolites from the external microbiome present on the surface of fertilized eggs passing through the chorion in zebrafish embryos[26]).
- These observations[23–26] therefore challenge currently held views that these animals transition from an aposymbiotic phase in their life cycle (during embryonic development) to a post-embryonic symbiotic phase. There is no aposymbiotic phase as these metabolic interactions extend throughout all developmental stages, from embryonic development until death. The emphasis on sterility as physical absence of microbes obscures this fact.

In the following sections, we will detail the evidence and rationale that sustains this alternative view of the evolutionary origins and roles of the circulatory systems in enabling microbial influence across larger and more complex bodies. In later sections, we explore the consequences of adopting this reconfigured perspective, namely the

117 unraveling of the medical concept of "sterility"[27] and the challenge this entails regarding
118 the existence of aposymbiotic phases in metazoan life cycles.
119
120 **Emergence of the mesoderm – transition from diploblasty to triploblasty**
121 Organogenesis in animals – with the exception of sponges (phylum Porifera) – derives
122 from germ cell layer differentiation taking place very early in embryological development.
123 Animals other than sponges can be divided into a) diploblasts, whose tissues are derived
124 from two primary germ cell layers, the ectoderm and the endoderm, and b) triploblasts,
125 who produce a third layer, the mesoderm. From an evolutionary perspective, triploblasts
126 arose after diploblasts, and most of them harbor circulatory systems[21,28].
127 The emergence of the mesoderm was a transformative event, driving multiple key
128 developments[21,29–31]:
129 1. The formation of entirely **new mesoderm-derived structures**, such as the
130 circulatory and musculoskeletal systems.
131 2. The **expansion of endoderm- and ectoderm-derived organs absent from**
132 **diploblasts**, including the liver, pancreas, brain, and spinal cord.
133 3. The **modification and specialization of preexisting endodermal and ectodermal**
134 **tissues** (e.g., gut; nervous system), which had evolved in diploblasts before the
135 advent of the mesoderm.
136 In this article, we focus on the development of circulatory systems from the mesoderm
137 germ layer, a major evolutionary transition which increased anatomical complexity and
138 led to greater functional specialization, thereby setting the stage for the diversity and

increased sizes observed in triploblastic organisms. We argue that microbial communities were crucial actors driving the emergence and evolution of circulatory systems, and remain to this day essential to their optimal development.

Defining the circulatory system

We use the term circulatory system to refer to an internal network of structures that distributes extracellular fluids produced by an animal[21]. Similar structures, termed transporting tissues, are found among the vast majority of land plant species[3].

Most vertebrate animals possess three internal circulatory systems whose cellular components are derived from the mesoderm[21,32]: a blood vascular system, in which fluid is transported under the impulse of cellular contraction "(myoepithelial cells) and/or muscular pumps"[18] (p. 50); the lymphatic circulatory system, a unidirectional conduit of liquid composed of extravasated arterial fluid which the lymphatics return to the blood vascular system; and a coelomic circulatory system, which lacks a pump and relies on cilia on the surface of mesoderm-derived mesothelial cells to circulate fluids. Examples of coelomic cavities in humans include the pleural, pericardial and peritoneal spaces. In addition to these three mesoderm-derived circulatory systems, vertebrates also have a cerebrospinal fluid (CSF) circulatory system, whose germ cell layer origins include both ectodermal and mesodermal cells.

This article will focus primarily on the blood vascular system among placental mammals, and specifically on the role of microbial communities in enabling its evolution and development. We believe similar arguments regarding the role of microbes in the

161 developmental origins of circulatory systems can be extended to other triploblastic
162 animals with a blood vascular system, as well as to land plants with transporting tissues.
163
164 **Origins of the blood vascular system**
165 The emergence of the blood vascular system can be traced phylogenetically and
166 embryologically following the development of the mesoderm germ cell layer, and
167 therefore of triploblasty. Anatomically, we observe important variations and changes in
168 the development and configuration of circulatory systems among triploblasts[21]: present
169 vs absent (the latter in acoelomates and pseudocoelomates); open vs closed circulation;
170 propulsive organ vs contractile vessels; number of heart chambers, ranging from 1 to 4.
171 This diversity can be tracked phylogenetically as follows.
172 Invertebrate triploblastic animals such as acoelomates (flatworms) lack both a coelom
173 and a blood vascular system; their mesoderm-derived cells form a meshwork called a
174 parenchyma. Other invertebrate triploblastic animals, such as nematodes, are
175 categorized as pseudocoelomates as their meshwork of mesoderm cells contain fluid-
176 filled clefts. The development of the blood vascular system is believed to have initially
177 arisen in invertebrate triploblastic animals such as annelids and mollusks. Among
178 vertebrate animals (the most recently evolved group of metazoans), the blood vascular
179 system undergoes further iterations in addition to being coupled with a lymphatic system
180 and a CSF circulatory system, which are absent among invertebrates.

Circulatory systems and the rise of sterile body sites

Central to the perspective we present in this article is the idea that circulatory systems enable microbial communities to maintain metabolic interactions with all tissues and cells as macrobial organisms expanded in size. These co-metabolic interactions persist notwithstanding i) the absence of physical contact between the tissues and cells of macrobes with microbes and ii) the distance separating microbes and macrobial cells extending beyond the reach of diffusion.

In disciplines such as medicine and clinical microbiology, body sites devoid of physical contact with microbes are referred to as "sterile". The body sites classified as sterile include some that were not sterile in diploblasts and early triploblasts (e.g., the nervous system) as well as organs and tissues emerging in later triploblasts (e.g., liver, pancreas).

For example, *Hydra* are diploblastic organisms, members of the Cnidaria phylum, the sister group of Bilateria (triploblasts)[28]. They are commonly used as model organisms to study various developmental and physiological processes, including interactions with symbiotic microbial communities[33,34]. Anatomically, *Hydra* develop into hollow tubes composed of two epithelial cell layers (ectoderm and endoderm) separated by an acellular matrix called the mesoglea. Microbes are in contact along the surface of both cell layers, including parts of the nervous system, and circulate within the mesoglea[28,34]. Evidence from *Hydra* (as well as other diploblasts and early triploblasts) demonstrate that prior to the emergence of the mesoderm-derived blood vascular system, there were no "sterile" body sites.

While the absence of sterile body sites also characterizes early triploblasts, later ones developed tissues and organs where cells do lack any direct contact with microorganisms. We believe that the sterile/non-sterile dichotomy should nonetheless be challenged and reconsidered. Indeed, this dichotomy suggests that sterile body sites are devoid of microbial presence, which is misleading. Using a wide range of evidence from research in the development and maintenance of "sterile" body sites in placental mammals, we argue, in the next section, that microbes make their presence felt in these structures, albeit from afar. Describing them as sterile therefore misrepresents the frequency and intensity of cross-kingdom interactions.

Placental mammals

We focus our argument on placental mammals due to the breadth of studies investigating the role of these metazoans' resident microbiota in angiogenesis throughout their life cycles (in embryological and post-natal development[23–25,35–38]) as well as the association of these microbial communities with various disease states[39–43]. The unique role of the maternal and fetal blood vascular systems in mediating transgenerational exchanges during pregnancy also makes placental mammals telling case studies[37,44–47]. Indeed, they differ from other animals in their reliance on interconnected blood vascular systems throughout gestation, serving as an ideal group to explore the role of microbes in shaping the circulatory system from fetal to post-natal periods.

In what follows, we refer to three key developmental features of placental mammals as well as physiological functions[b] associated with their blood vascular system for which there is evidence of significant contribution by microbes: i) placental angiogenesis; ii) the development of the intestinal vasculature; iii) the permeability and integrity of blood brain interfaces (the blood-brain and blood-CSF barriers).

Placental angiogenesis

Among animals, placental mammals carry their progeny to relatively late stages of development. During gestation, the placenta, a fetal organ, enables exchange of gases and nutrients between the mother and fetus, including the transfer of metabolites originating from the maternal microbiome. Compelling evidence suggests these gut microbial metabolites from the mother influence fetal development as well as offspring phenotype (risks of being obese and developing metabolic syndrome)[24]. More recently, data has emerged supporting the role of the maternal gut microbiome in placental development[37]. Specifically, this influence is most pronounced on placental vascularization. Both pregnant germ-free mice and pregnant mice whose gut microbiome was depleted with antibiotics were found to carry placentae of lower weights and volumes as compared to two other groups of mice, i.e. conventionally colonized pregnant mice, and germ-free mice colonized with the microbiota of conventionally colonized mice. Alterations in placentae leading to reduced weights and volumes were primarily observed

[b] We use the term "function" throughout this paper to refer to the causal role some entities (e.g., organs) may play in a system. Except if stated otherwise, we do not imply that the traits have been selected for this function throughout their evolutionary history (even if that might be the case).

242 in the placental labyrinth, a highly vascularized subregion of the placenta responsible for
243 maternal-fetal exchanges.
244 In other words, the placentae in pregnant germ-free and pregnant mice exposed to
245 antibiotics displayed significant decreases in feto-placental vasculature, implying a role
246 for maternal gut microbiome metabolites (including short-chain fatty acids, SCFAs) in
247 regulating placental angiogenesis[37]. This claim was further supported by experiments
248 demonstrating that supplementing SCFAs to pregnant mice exposed to antibiotics
249 prevented impairments in placental growth and microvasculature. There is therefore
250 evidence that from early gestation, microbes, and more specifically maternal gut
251 microbial metabolites acting transgenerationally on embyros, influence the development
252 of the blood vascular system in an organ at the interface of maternal and fetal exchanges.
253
254 *Intestinal angiogenesis*
255 Among all body sites associated with microbiota, the gut harbors the greatest abundance.
256 The gut lining is the primary site enabling gut microbial metabolites to enter the blood
257 vascular system and influence the development of organs. Unsurprisingly, the
258 gastrointestinal (GI) tract of more recently evolved animal species and its associated
259 circulatory system serve as an important interface enabling nutrient intake to reach
260 distant cells beyond the limits of diffusion.
261 Some of the earliest studies pointing to the role of microbiota in animal
262 development investigated intestinal angiogenesis. These studies demonstrated that
263 germ-free mice had arrested capillary network formation in gut microvasculature

compared to conventionally raised mice [36]. Moreover, small intestine angiogenesis could be rapidly induced by colonizing ex-germ-free mice with either the microbiota of conventionally raised mice or with *Bacteroides thetaiotaomicron*. Numerous subsequent studies[35,38] have demonstrated the role of the gut microbiome in influencing tissue development and their associated vasculature in and beyond the gut. This includes organs with no direct contact with microbes (liver, eye, placenta, central nervous system, etc.). Microbial influence via direct contact, seen in diploblasts and some triploblasts, is maintained in vascularized triploblasts through circulatory systems. This observation underscores the deep evolutionary relationship between microbiota, tissue development, and circulatory integration: microbes shape tissue formation by contributing to the development of circulatory systems which they use to maintain this influence.

Blood-brain interfaces

In this section, we discuss the emergence of blood-brain interfaces, essential developmental features of the nervous system of some triploblasts. These interfaces appear in the context of two intertwined evolutionary developments: i) the transformation from a mostly decentralized nervous net in diploblasts to numerous iterations of nervous systems in triploblasts, some with progressively more centralized structures (brain, spinal cord); ii) the development of a blood vascular system, complemented in vertebrates with a cerebrospinal fluid (CSF) circulatory system. Below,

285 we focus on the role played by microbiota in the development and maintenance of the
286 blood-brain barrier and the blood-CSF barrier.

287 These two blood-brain barriers are one of many ways in which the nervous system
288 of triploblasts became gradually shielded from direct physical contact with microbes,
289 while remaining accessible to microbial metabolites. Indeed, the development of a
290 "sterile" nervous system stands in contrast to what is observed in organisms lacking a
291 circulatory system, including diploblasts such as *Hydra*, and early triploblasts such as the
292 nematode *C. elegans*. In both these organisms, neuronal receptors known as pacemaker
293 cells physically interact with their microbiota, a feature which disappears in later
294 triploblasts such as mice and humans[48].

295 The influence of the gut microbiota on the development and function of the
296 central nervous system (CNS) in placental mammals has been the subject of extensive
297 study and reviews. Multiple gut-brain axis pathways have been documented, most
298 notably the vagus nerve and the blood vascular system. To transit from the gut to the CNS
299 using the blood vascular system, gut microbial metabolites need to cross distinct barriers.
300 The first two, the intestinal epithelial barrier and the gut-vascular barrier, limit the
301 passage of microbial metabolites between microbiota in the gut lumen and the blood
302 vascular system. Numerous studies have demonstrated the influence of gut microbiota in
303 altering the integrity of these first two barriers located along the gastrointestinal tract[49,50]
304 with ongoing research assessing the impacts of intestinal hyperpermeability (i.e., leaky
305 gut) on various health conditions.

Once in the blood vascular system, microbial metabolites (or other molecules induced by signaling cascades) can reach the CNS microvasculature (capillaries) where further barriers limit their diffusion to either one of two CNS structures acting as blood-brain interfaces: i) the blood-brain barrier (BBB), separating the capillaries from the brain; ii) the blood-CSF barrier, regulating the passage of substances between blood and CSF. The structures of the BBB and blood-CSF barriers play crucial roles in regulating the transfer of gut microbial metabolites (and other substances) to the CNS. Alterations in the permeability and integrity of these barriers have been shown to predispose to various CNS conditions, ranging from neurodevelopmental disorders to neurodegenerative diseases[51–53]. Specifically, increased permeability of these barriers allows harmful substances to reach the brain (or to reach it in higher amounts).

Blood-brain barrier

Gut microbiota and metabolites have been shown to alter BBB permeability and integrity[39,40]. In one study, the influence of gut microbiota on BBB permeability was shown in mice to begin during gestation (development of the BBB begins during the early intrauterine period), and to continue throughout life[23]. Specifically, the BBB of embryos of germ-free mice were found to display the unfavorable phenotype of increased permeability as compared to what was observed in embryos of mice reared in an environment with germs but free of known mice pathogens. Increased BBB permeability was also observed in germ-free adult mice compared to “pathogen-free” mice. Additionally, following either the colonization of adult germ-free mice with certain

bacterial strains that produce SCFAs or their supplementation with SCFAs via oral gavage, the impacts of increased BBB permeability were shown to revert to an equivalent state of decreased permeability as seen in pathogen-free adult mice. Once again, this means that important macrobial phenotypes are co-constructed by microbes and macrobes, the influence of the former being mediated by the vascular system. This generates a feedback loop: microbes contribute to the formation of the vascular system (see previous case studies), enabling them to influence further developments in macrobial tissues and organs.

Blood-CSF barrier

Although not as extensively studied as the BBB, the blood-CSF barrier has gained increasing attention in recent years, especially as it pertains to the impacts of its structural integrity on the pathogenesis and progression of neurodegenerative disorders such as Alzheimer's disease[53]. In a recent study, Xie *et al.* found an increase in blood-CSF barrier permeability (again, a detrimental phenotype) in young adult mice lacking a normal gut microbiota (germ-free and post antibiotic-treatment). This outcome was mostly compensated for by subsequent restoration of normal fecal microbiota or SCFA supplementation. The authors also found that in a mouse model of Alzheimer's disease with baseline disruptions in the blood-CSF barrier, SCFA supplementation improved various parameters involved with progression of disease resulting from improved integrity of both the BBB and blood-CSF barrier.

Taken together, these and other studies[23,49,51,52,55,56] demonstrate that the influence of gut microbiota on maintaining the integrity and permeability of blood-brain interfaces is: i) a dynamic process extending from embryological development (at least for the BBB) to late adult life, rather than one leading to a fixed phenotype; ii) affected by fluctuating inputs from microbial metabolites. Among these metabolites, SCFAs have been most studied, and their influence on blood-brain interfaces takes place either directly in the CNS, as demonstrated by their interaction with various receptors present on endothelial cells of the BBB (e.g., free fatty acid receptors)[57], or indirectly, through various pathways (immune, endocrine, vagal)[58]. With the exception of the vagus nerve, all of these pathways depend on the blood vascular system circulating (from the gut to the CNS) SCFAs or other molecules (e.g., hormones, interleukins) downstream of signaling cascades initiated by SCFAs[53,58].

Exit Aposymbiosis

In previous sections, we reviewed empirical evidence that supports an argument in favor of dispensing with the concept of sterility in research domains attentive to metabolic interactions between microbes and macrobes. As a result, we challenge the widespread adoption of the use of the concept of sterile body sites in these research domains as it creates an exaggerated distinction between tissues and cells in direct contact with microbes and those which are devoid of such physical contact.

A consequence of this exaggerated distinction has been for the field of holobiont research to consider the initiation of interaction between microbiota and certain animals

371 to begin at birth (e.g., in placental mammals)[22]. The fetus of placental mammals is
372 considered “sterile”, with newborns colonized with microbes at birth. Considering fetuses
373 as sterile has led some researchers to distinguish between aposymbiotic (embryonic) and
374 symbiotic (post-embryonic) phases in the life cycles of animals whose fetuses are not in
375 contact with symbionts (including endosymbionts)[22]. Placental mammals and many
376 oviparous animals are thus described as having an aposymbiotic phase.

377 One corollary of the claim that placental mammals have an aposymbiotic phase is
378 to overemphasize the importance of early exposures of newborns such as the birthing
379 process (vaginal vs Caesarean delivery) and food intake (maternal milk vs formula). In
380 contrast, and except in rare studies, there is a tendency to ignore the pivotal role that
381 microbial metabolites play during gestation and whose influence can have impacts well
382 beyond pregnancy[23–25,37]. This situation gains to be rectified.

383 Further research, however, will have to be carried out to generalize our approach
384 adequately beyond placental mammals. Indeed, among animals, placental mammals have
385 evolved such that they make the most extensive use of the blood vascular system as it is
386 the conduit through which fetal growth is supported. This contrasts with other common
387 modes of reproduction such as oviparity where a fertilized egg (supplied with abundant
388 yolk) has no ongoing connection with microbial metabolites from the maternal gut
389 (although interactions with external microbes have been observed). Notwithstanding
390 these differences, we believe similar analyses done on triploblastic animals using distinct
391 modes of reproduction (other forms of viviparity; oviparity; etc.[59]) will confirm the reach
392 of our claims and our argument to dispense with sterility and aposymbiosis. Indeed, some

393 have hypothesized that egg-associated microbiomes may be tied to developmental
394 functions, potentially serving as a source of metabolites traversing the shell and
395 influencing various features of embryological development[60]. A recent pre-print[26]
396 appears to lend credence to this claim, opening additional avenues to further question
397 and to reassess the concept of aposymbiosis.
398
399 **Assessing blood vascular systems as "an adaptation for" microbes**
400 The perspective we present offers a picture of the evolution of vascular systems that
401 differs drastically from the received view according to which the vascular system evolved
402 to sustain increases in body size as something that is beneficial to the macrobial
403 organisms increasing in size (if only because it makes new niches available to them).
404 Indeed, according to our approach, the vascular system can be conceived as the result of
405 microbial niche construction. Assuming that bigger macrobes have a higher carrying
406 capacity, increased body size can be conceived as the result of microbes constructing a
407 better (bigger) niche for themselves. Given the extensive evidence (presented throughout
408 this paper) that microbes are, still to this day, actively involved in the development of the
409 vascular system and, through it, that of other tissues and organs, it is likely that they were
410 involved in the positive feedback loops that would foster increases in macrobial body size.
411 Yet this works only if microbes can sustain their interactions with macrobial organs
412 necessary to the macrobes' functioning across space and beyond the reach of diffusion.
413 This is where the vascular system steps in, as a niche-constructed feature that facilitates
414 further niche construction by microbes. Hence, while the received view assumes that

increased body size favors the organism whose body size is increasing, our view complements it by positing microbes as beneficiaries of this process. Ultimately, the two explanations are compatible, in a multilevel selection rationale[61,62]: acquiring new niches through increased body size is undoubtedly beneficial to the macrobe, while any microbial phyla that can contribute to the proper functioning of bigger organisms will benefit from the increased room for proliferation.

Our approach has one advantage for explaining evolutionary dynamics at work, however. Increased body size might only become selectable at the macrobial level once it opens a new niche, but reaching this threshold requires the gradual accumulation of slight increases in body size (which may or may not be beneficial). If these smaller increases fail to offer significant fitness advantages to their bearers, macrobe-based selection cannot explain how the threshold that opens new selective niches is reached. In contrast, gradual increases in size and carrying capacity confer direct advantages to the microbes that contribute to it (e.g., microbes that increase vascularization of placental tissues). It could explain how body size gradually increases over evolutionary time until important thresholds are met (opening of new macrobial niches). In other words, our view better corresponds to the gradualism that is inherent to the theory of evolution by natural selection than the received view regarding circulatory systems, while still being compatible with a multilevel selection perspective.

Conclusion and Outstanding Questions

From an evolutionary and physiological outlook, the dominant perspective has been to present the emergence of the blood vascular system from the point of view of the animal. That is, the process of diffusion imposes physical limitations on the distance that gases, nutrients and waste can spread, and therefore on the ability of animals to attain certain sizes and shapes[63,64]. An alternative to diffusion emerged in the form of the blood vascular system. In this paper, we presented the emergence of circulatory systems from the viewpoint of microbiota, arguing that adopting a microbial perspective provided a novel and essential theoretical shift to reassess the evolutionary and functional interplay between microbial communities and macrobes.

We conclude by pointing out how this reconceptualization of the circulatory system and the notion of sterility will foster novel approaches to think about the co-evolution of microbes and multicellular organisms. Specifically, increases in body size can now be approached in two distinct and complementary manners. First, it may have conferred fitness-related advantages to macrobes, allowing the exploration of new ecological niches. Second, increased body sized means larger niches for microbes that thrive in such environments (e.g., microbial taxa that are associated with human guts)[65]. It can therefore be conceived as the result of natural selection acting on both microbes and macrobes.

There are other key points that should be explored in future research stemming from this novel outlook:

- *Development of the immune system*: the emergence and expansion of "sterile" compartments required an adaptation on the part of organisms in responding to breaches of sterility. At the same time, blood vascular systems enabled the circulation of components of the immune system such as dedicated cells (T cells, B cells) and antibodies, the latter being transferred from mother to fetus in placental mammals. What are the roles of microbiota in the evolution of components of the immune system and how did circulatory systems shape such developments? Could the immune system, just like the vascular system, have evolved for the benefit of microbes?

- *Interconnected microbiomes*: our discussion essentially focused on how the circulatory systems maintained co-metabolism between microbiota and macrobes. Could circulatory systems also allow signaling not just between microbes and non-microbial cells, but also between microbiota at a distance from each other (e.g., gut and skin microbiota)? Would this be an instance of Networked Collective Microbiomes[15] within a network of niches found within a single macrobe?

- *Vascular Plants*: most land plants also contain transporting tissues, structures similar to the circulatory systems of animals[3,66]. To our knowledge, no similar attempt at adopting a microbial perspective on the emergence of transporting tissues in plants has been undertaken. What would such an analysis yield and could any parallels be drawn? Are these structures enabling increases in plant size while maintaining microbe/microbe co-metabolism, as is the case for animals? Can this be conceived as a case of convergent evolution?

References

1 Tsegay E, Hailesilassie A, Hailekiros H, Niguse S, Saravanan M, Abdulkader M. Bacterial Isolates and Drug Susceptibility Pattern of Sterile Body Fluids from Tertiary Hospital, Northern Ethiopia: A Four-Year Retrospective Study. *J Pathog* 2019; **2019**: 1–6.

2 Lu K-J, van 't Wout Hofland N, Mor E, *et al.* Evolution of vascular plants through redeployment of ancient developmental regulators. *Proc Natl Acad Sci* 2020; **117**: 733–40.

3 Woudenberg S, Renema J, Tomescu AMF, De Rybel B, Weijers D. Deep origin and gradual evolution of transporting tissues: Perspectives from across the land plants. *Plant Physiol* 2022; **190**: 85–99.

4 Bonner JT. The evolution of evolution. *J Exp Zoolog B Mol Dev Evol* 2019; **332**: 301–6.

5 Payne JL, Boyer AG, Brown JH, *et al.* Two-phase increase in the maximum size of life over 3.5 billion years reflects biological innovation and environmental opportunity. *Proc Natl Acad Sci* 2009; **106**: 24–7.

6 Payne JL, McClain CR, Boyer AG, *et al.* The evolutionary consequences of oxygenic photosynthesis: a body size perspective. *Photosynth Res* 2011; **107**: 37–57.

7 Gilbert SF. Evolutionary transitions revisited: Holobiont evo-devo. *J Exp Zoolog B Mol Dev Evol* 2019; **332**: 307–14.

8 O'Malley MA, Dupré J. Size doesn't matter: towards a more inclusive philosophy of biology. *Biol Philos* 2007; **22**: 155–91.

9 Bosch TCG, McFall-Ngai MJ. Metaorganisms as the new frontier. *Zoology* 2011; **114**: 185–90.

10 Chiu L, Gilbert SF. The Birth of the Holobiont: Multi-species Birthing Through Mutual Scaffolding and Niche Construction. *Biosemiotics* 2015; **8**: 191–210.

11 Moran NA, Sloan DB. The Hologenome Concept: Helpful or Hollow? *PLoS Biol* 2015; **13**: e1002311.

12 van Vliet S, Doebeli M. The role of multilevel selection in host microbiome evolution. *Proc Natl Acad Sci* 2019; **116**: 20591–7.

13 Koskella B, Hall LJ, Metcalf CJE. The microbiome beyond the horizon of ecological and evolutionary theory. *Nat Ecol Evol* 2017; **1**: 1606–15.

14 Papale F, Doolittle WF. Towards a More General Theory of Evolution by Natural Selection: A Manifesto. *Philos Theory Pract Biol* 2024; **16**. DOI:10.3998/ptpbio.5563.

15 Haraoui L-P. Networked collective microbiomes and the rise of subcellular 'units of life'. *Trends Microbiol* 2022; **30**: 112–9.

16 Papale F, Not F, Bapteste É, Haraoui L. The evosystem: A centerpiece for evolutionary studies. *BioEssays* 2024; **46**: 2300169.

17 Lloyd, E.A. (2018) Holobionts as units of selection: holobionts as interactors, reproducers, and manifestors of adaptation. In Landscapes of collectivity in the life sciences. (Snait B. Gissis, Ehud Lamm and Ayelet Shavit.), pp. 351–368, MIT Press. .

18 Harrison SA, Rammu H, Liu F, Halpern A, Nunes Palmeira R, Lane N. Life as a Guide to Its Own Origins. *Annu Rev Ecol Evol Syst* 2023; **54**: 327–50.

19 Krautkramer KA, Fan J, Bäckhed F. Gut microbial metabolites as multi-kingdom intermediates. *Nat Rev Microbiol* 2021; **19**: 77–94.

20 Martín-Durán JM, Vellutini BC, editors. Old Questions and Young Approaches to Animal Evolution. Cham: Springer International Publishing, 2019 DOI:10.1007/978-3-030-18202-1.

21 Monahan-Earley R, Dvorak AM, Aird WC. Evolutionary origins of the blood vascular system and endothelium. *J Thromb Haemost* 2013; **11**: 46–66.

22 Bright M, Bulgheresi S. A complex journey: transmission of microbial symbionts. *Nat Rev Microbiol* 2010; **8**: 218–30.

23 Braniste V, Al-Asmakh M, Kowal C, *et al.* The gut microbiota influences blood-brain barrier permeability in mice. *Sci Transl Med* 2014; **6**. DOI:10.1126/scitranslmed.3009759.

24 Kimura I, Miyamoto J, Ohue-Kitano R, *et al.* Maternal gut microbiota in pregnancy influences offspring metabolic phenotype in mice. *Science* 2020; **367**: eaaw8429.

25 Vuong HE, Pronovost GN, Williams DW, *et al.* The maternal microbiome modulates fetal neurodevelopment in mice. *Nature* 2020; **586**: 281–6.

26 Green EM, Harishchandra A, Lickwar CR, Rawls JF, Di Giulio RT, Jayasundara N. The External Microbiome Communicates with the Developing Zebrafish (*Danio rerio*) Embryo Through the Chorion and Influences Developmental Trajectory. 2024; published online May 30. DOI:10.1101/2024.05.28.596134.

27 Stedman TL. Stedman's medical dictionary, 28th ed. Philadelphia: Lippincott Williams & Wilkins, 2006.

28 Klimovich AV, Bosch TCG. Rethinking the Role of the Nervous System: Lessons From the *Hydra* Holobiont. *BioEssays* 2018; **40**: 1800060.

29 Northcutt RG. Evolution of centralized nervous systems: Two schools of evolutionary thought. *Proc Natl Acad Sci* 2012; **109**: 10626–33.

30 Steinmetz PRH. A non-bilaterian perspective on the development and evolution of animal digestive systems. *Cell Tissue Res* 2019; **377**: 321–39.

31 Lammert E, Cleaver O, Melton D. Role of endothelial cells in early pancreas and liver development. *Mech Dev* 2003; **120**: 59–64.

32 Oliver G, Kipnis J, Randolph GJ, Harvey NL. The Lymphatic Vasculature in the 21st Century: Novel Functional Roles in Homeostasis and Disease. *Cell* 2020; **182**: 270–96.

33 Klimovich et Bosch - 2018 - Rethinking the Role of the Nervous System Lessons.pdf. .

34 Schröder K, Bosch TCG. The Origin of Mucosal Immunity: Lessons from the Holobiont *Hydra*. *mBio* 2016; **7**: e01184-16.

35 Khandagale A, Reinhardt C. Gut microbiota - architects of small intestinal capillaries. *Front Biosci Landmark Ed* 2018; **23**: 752–66.

36 Stappenbeck TS, Hooper LV, Gordon JI. Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. *Proc Natl Acad Sci* 2002; **99**: 15451–5.

37 Pronovost GN, Yu KB, Coley-O'Rourke EJL, *et al.* The maternal microbiome promotes placental development in mice. *Sci Adv* 2023; **9**: eadk1887.

38 Kiouptsi K, Pontarollo G, Reinhardt C. Gut Microbiota and the Microvasculature. *Cold Spring Harb Perspect Med* 2023; **13**: a041179.

39 Jie Z, Xia H, Zhong S-L, *et al.* The gut microbiome in atherosclerotic cardiovascular disease. *Nat Commun* 2017; **8**: 845.

40 Koeth RA, Wang Z, Levison BS, *et al.* Intestinal microbiota metabolism of l-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat Med* 2013; **19**: 576–85.

41 Robles-Vera I, Toral M, De La Visitación N, Aguilera-Sánchez N, Redondo JM, Duarte J. Protective Effects of Short-Chain Fatty Acids on Endothelial Dysfunction Induced by Angiotensin II. *Front Physiol* 2020; **11**: 277.

42 Sajib S, Zahra FT, Lionakis MS, German NA, Mikelis CM. Mechanisms of angiogenesis in microbe-regulated inflammatory and neoplastic conditions. *Angiogenesis* 2018; **21**: 1–14.

572 43 Wan X, Song M, Wang A, Zhao Y, Wei Z, Lu Y. Microbiome Crosstalk in
573 Immunotherapy and Antiangiogenesis Therapy. *Front Immunol* 2021; **12**: 747914.

574 44 Elliot MG, Crespi BJ. Placental invasiveness and brain–body allometry in eutherian
575 mammals. *J Evol Biol* 2008; **21**: 1763–78.

576 45 Rossant J, Cross JC. Placental development: Lessons from mouse mutants. *Nat Rev*
577 *Genet* 2001; **2**: 538–48.

578 46 Silva JF, Serakides R. Intrauterine trophoblast migration: A comparative view of
579 humans and rodents. *Cell Adhes Migr* 2016; **10**: 88–110.

580 47 Watson ED, Cross JC. Development of Structures and Transport Functions in the
581 Mouse Placenta. *Physiology* 2005; **20**: 180–93.

582 48 Klimovich A, Giacomello S, Björklund Å, *et al.* Prototypical pacemaker neurons
583 interact with the resident microbiota. *Proc Natl Acad Sci* 2020; **117**: 17854–63.

584 49 Brescia P, Rescigno M. The gut vascular barrier: a new player in the gut–liver–brain
585 axis. *Trends Mol Med* 2021; **27**: 844–55.

586 50 Barbara G, Barbaro MR, Fuschi D, *et al.* Inflammatory and Microbiota-Related
587 Regulation of the Intestinal Epithelial Barrier. *Front Nutr* 2021; **8**: 718356.

588 51 Aragón-González A, Shaw PJ, Ferraiuolo L. Blood–Brain Barrier Disruption and Its
589 Involvement in Neurodevelopmental and Neurodegenerative Disorders. *Int J Mol Sci*
590 2022; **23**: 15271.

591 52 Sweeney MD, Sagare AP, Zlokovic BV. Blood–brain barrier breakdown in Alzheimer
592 disease and other neurodegenerative disorders. *Nat Rev Neurol* 2018; **14**: 133–50.

593 53 Xie J, Bruggeman A, De Nolf C, *et al.* Gut microbiota regulates blood-cerebrospinal
594 fluid barrier function and Aβ pathology. *EMBO J* 2023; **42**: e111515.

595 54 Logsdon AF, Erickson MA, Rhea EM, Salameh TS, Banks WA. Gut reactions: How the
596 blood–brain barrier connects the microbiome and the brain. *Exp Biol Med* 2018; **243**:
597 159–65.

598 55 Parker A, Fonseca S, Carding SR. Gut microbes and metabolites as modulators of
599 blood-brain barrier integrity and brain health. *Gut Microbes* 2020; **11**: 135–57.

600 56 Knox EG, Lynch CMK, Lee YS, *et al.* The gut microbiota is important for the
601 maintenance of blood–cerebrospinal fluid barrier integrity. *Eur J Neurosci* 2023; **57**:
602 233–41.

57 Hoyles L, Snelling T, Umlai U-K, *et al.* Microbiome–host systems interactions: protective effects of propionate upon the blood–brain barrier. *Microbiome* 2018; **6**: 55.

58 Dalile B, Van Oudenhove L, Vervliet B, Verbeke K. The role of short-chain fatty acids in microbiota–gut–brain communication. *Nat Rev Gastroenterol Hepatol* 2019; **16**: 461–78.

59 Lodé T. Oviparity or viviparity? That is the question…. *Reprod Biol* 2012; **12**: 259–64.

60 Nyholm SV. In the beginning: egg–microbe interactions and consequences for animal hosts. *Philos Trans R Soc B Biol Sci* 2020; **375**: 20190593.

61 Damuth J, Heisler IL. Alternative formulations of multilevel selection. *Biol Philos* 1988; **3**: 407–30.

62 Okasha S. Multilevel Selection and the Major Transitions in Evolution. *Philos Sci* 2005; **72**: 1013–25.

63 Kempes CP, Koehl MAR, West GB. The Scales That Limit: The Physical Boundaries of Evolution. *Front Ecol Evol* 2019; **7**: 242.

64 West GB, Brown JH, Enquist BJ. A General Model for the Origin of Allometric Scaling Laws in Biology. *Science* 1997; **276**: 122–6.

65 Baquero F, Coque TM, Galán JC, Martinez JL. The Origin of Niches and Species in the Bacterial World. *Front Microbiol* 2021; **12**: 657986.

66 West GB, Brown JH, Enquist BJ. A general model for the structure and allometry of plant vascular systems. *Nature* 1999; **400**: 664–7.