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SYMPOSIUM

Chelicerates and the Conquest of Land: A View of Arachnid Origins Through an Evo-Devo Spyglass

Prashant P. Sharma[1]

Department of Zoology, University of Wisconsin-Madison, 352 Birge Hall, 430 Lincoln Drive, Madison, WI 53706, USA

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[1]E-mail: prashant.sharma@wisc.edu

Synopsis The internal phylogeny of Chelicerata and the attendant evolutionary scenario of arachnid terrestrialization have a long and contentious history. Previous studies of developmental gene expression data have suggested that respiratory systems of spiders, crustaceans, and insects are all serially homologous structures derived from the epipods (outer appendage rami) of the arthropod ancestor, corresponding to an ancestral gill. A separate body of evidence has suggested that the respiratory systems of arachnids are modified, inverted telopods (inner rami, or legs). Here I review these dissonant homology statements and compare the developmental genetic basis for respiratory system development in insects and arachnids. I show that the respiratory primordia of arachnids are not positionally homologous to those of insects. I further demonstrate that candidate genes critical to tracheal fate specification in *Drosophila melanogaster* are expressed very differently in arachnid exemplars. Taken together, these data suggest that mechanisms of respiratory system development are not derived from homologous structures or mechanisms in insects and arachnids, and that different terrestrial arthropod lineages have solved the challenge of aerial respiration using different developmental mechanisms.

The history of arthropod terrestrialization

Terrestrialization is one of the most iconic and evocative phenomena in evolutionary biology for scientists and laypersons alike. The conquest of land and its concomitant expansion of ecological niche space are associated with a suite of requisite adaptations for such biological processes as locomotion, reproduction, and aerial respiration (Little 1990; Shear and Kukalová-Peck 1990; Vermeij and Dudley 2000; Shubin et al. 2004; Ward et al. 2006; Clack 2012). While partial or completely terrestrial life histories have evolved several times in Metazoa (e.g., vertebrates: Little 1990; Shubin et al. 2004; Ward et al. 2006; arthropods: Shear and Kukalová-Peck 1990; Ward et al. 2006; Dunlop et al. 2013; annelids: Struck et al. 2011; mollusks: Kocot et al. 2011; Smith et al. 2011), the phylum Arthropoda is exceptional in this regard, with at least seven major terrestrialization events occurring at various time scales and phylogenetic depths since the Paleozoic (Dunlop and Webster 1999; Dunlop 2010; von Reumont et al. 2012; Dunlop et al. 2013; Rota-Stabelli et al. 2013) (Fig. 1). The impact of terrestrialization on arthropod diversification is considerable, with terrestrial species outnumbering aquatic and marine counterparts by a factor of nearly 17 to 1 (Dunlop et al. 2013).

The diversity of terrestrial arthropod respiratory systems is remarkable. Insect gas exchange occurs via a branching tracheal tubule system, with segmentally repeated pairs of spiracles opening on the abdomen, and sometimes the thorax (Kennedy 1922; Hilken 1998; Ghabrial et al. 2003). A similar gas exchange system is observed in myriapods (e.g., centipedes and millipedes; Lewis 1981; Hopkin and Read 1992) and apulmonate arachnids (e.g., harvestmen, pseudoscorpions; Shultz 2007; Shultz and Pinto-da-Rocha 2007), although the number and placement of spiracles varies from one lineage to

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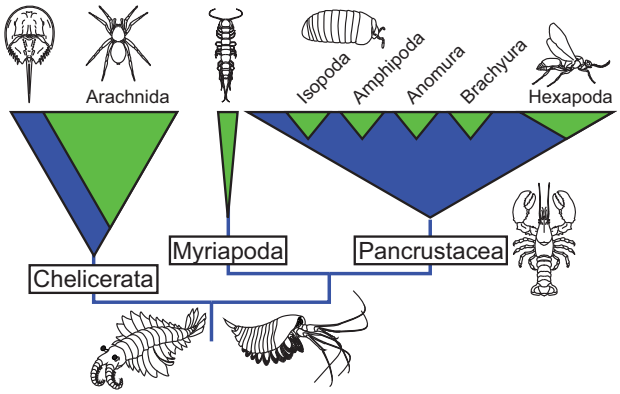

Fig. 1 Phylogenetic relationships of Arthropoda indicating seven major terrestrialization events (green). A marine ancestral condition (blue) is unambiguous.

the next (Shultz 2007). Pulmonate arachnids (e.g., scorpions, spiders) respire via book lungs, which are internalized gill-like organs with stacks of hemolymph-filled lamellae that facilitate gas exchange (Dunlop 1998; Scholtz and Kamenz 2006; Shultz 2007). As with insect spiracles, pairs of book lungs are segmentally iterated in the opisthosoma (the abdomen-like posterior tagma) of pulmonate arachnids. While basally branching spiders (i.e., the grade composed of Mesothelae, Mygalomorphae, and Hypochilidae) have two pairs of book lungs, a subset of derived spiders (i.e., most Araneomorphae; exceptions include Hypochilidae and Caponiidae) retain the anterior book lung pair only, and secondarily derived tracheal tubules in place of the posterior book lung pair (reviewed by Foelix 2010). Other strictly apulmonate arachnid orders (e.g., pseudoscorpions, harvestmen) possess only tracheal tubules, which are also segmentally iterated in the opisthosoma as spiracles (openings of the tracheae; Shultz 2007; Shultz and Pinto-da-Rocha 2007). A subset of arachnids also bears prosomal tracheae (e.g., the mite suborder Prostigmata; Solifugae), and some long-legged lineages also bear spiracles in the podomeres of the walking legs (e.g., phalangioid harvestmen) (Shultz 2007; Shultz and Pinto-da-Rocha 2007).

Book lungs are unique to five extant arachnid orders (Scorpiones, Araneae, Amblypygi, Uropygi, and Schizomida) and the extinct orders Trigonotarbida, Haptopoda, and Uraraneida (Dunlop 1998; Dunlop 2010). These respiratory structures are considered by some workers to be the equivalent of internalized book gills of a horseshoe crab-like ancestor, and a waypoint toward the evolution of tubular tracheae in smaller arachnids (Dunlop 1998; Scholtz and Kamenz 2006; Waddington et al. 2015), an inference partly supported by comparative analyses of horseshoe crab and scorpion development (Farley 2011, 2012). While this transformational series is commonly favored in the literature, persistent conflicts in the phylogeny of Chelicerata obscure a clear reconstruction of the respiratory organ condition in the arachnid ancestor. In analyses of morphological data, scorpions are typically recovered near the base of the arachnid tree of life, in part due to their superficial similarities to the extinct marine order Eurypterida. By contrast, phylogenomic studies increasingly support a clade formed by arachnid orders that bear book lungs (Arachnopulmonata, *sensu* Sharma et al. 2014a), a topology now supported as well by evidence of (a) a shared (partial or whole) genome duplication in the common ancestor of Arachnopulmonata, (b) shared expression patterns of duplicated Hox paralogs, in comparison to single-copy Hox orthologs in apulmonate arachnids like mites and harvestmen, and (c) patterns of synteny in the genomes of a spider and a scorpion (Telford and Thomas 1998; Sharma et al. 2012a, 2014b; Schwager et al. 2007, 2017). On such a tree topology, an equal weights parsimony reconstruction of ancestral states invariably recovers the origin of the book lung as a derived state occurring on the branch subtending Arachnopulmonata. However, based on the morphological and embryological correspondences of book gills and book lungs, an alternative reconstruction that places the gain of the book lung at the base of Arachnida with concomitant losses in the branches subtending apulmonate arachnids (as originally favored by functional morphologists; Scholtz and Kamenz 2006; Shultz 2007) remains a strongly defensible hypothesis.

Support for the book lung as part of the ancestral arachnid groundplan is partly provided by the observation that a basal grade of spiders (Mygalomorphae and Mesothelae) bear two pairs of book lungs (i.e., the tetrapulmonate condition), whereas derived spiders retain only the anterior pair; the posterior pair is transformed into tubular tracheae in most Araneomorphae. Schizomida, another member of Tetrapulmonata and the sister group to Thelyphonida (vinegaroons), also retains only a single pair of book lungs; the posterior pair has been lost entirely in this lineage (Shultz 2007; Foelix 2010). Separately, paleontological data support an ancient origin of the book lung, with clear evidence of this organ in such Devonian arachnids as the fossil order Trigonotarbida (Dunlop 2010).

However, the ancient origin of the book lung and its morphological correspondence to the horseshoe crab book gill cannot be directly interpreted as evidence of a single terrestrialization event in the arachnid common ancestor. As I have previously discussed, even in phylogenies where Arachnida is recovered as monophyletic, the incidence of aquatic

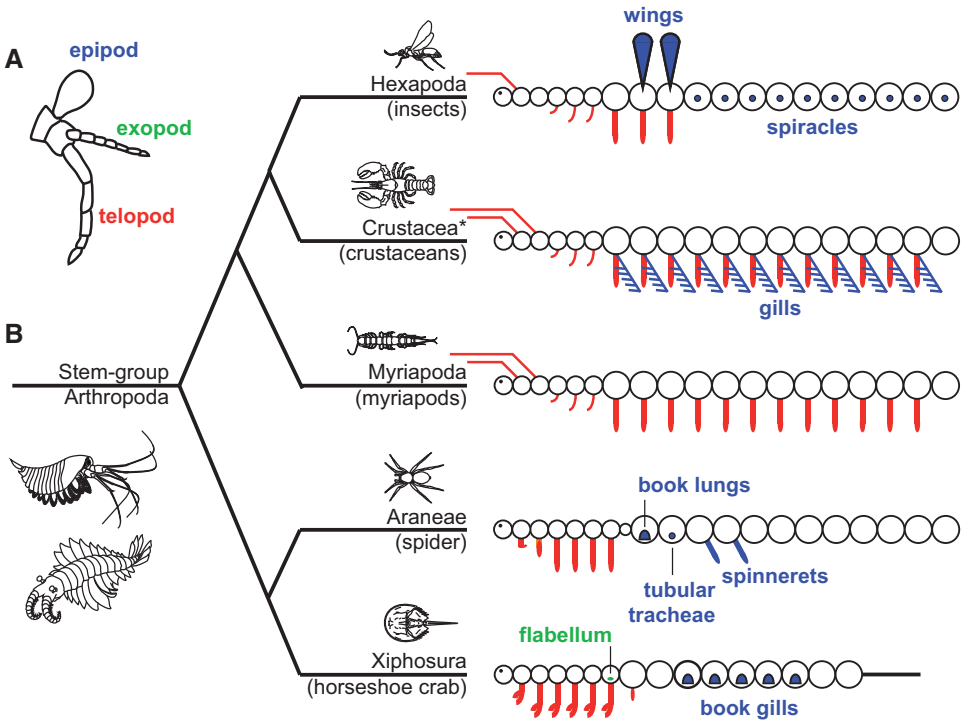

Fig. 2 Summary of the ancestral gill hypothesis *sensu* Damen et al. (2002). The homology of crustacean epipods and insect spiracles was separately postulated by Franch-Marro et al. (2006). Myriapods have not been included in the ancestral gill hypothesis and therefore no homology schema is shown for this group (tubular tracheae in Myriapoda are variable in number and placement; typically, segmentally iterated pairs of spiracles open dorsally to the walking legs).

scorpion fossils, together with the derived placement of scorpions in modern arachnid phylogenies, suggests that multiple terrestrialization events have transpired in the evolutionary history of Arachnida (Sharma et al. 2014a). In addition, the evolutionary history of such lineages as Onychophora and Hexapoda demonstrate that a pulmonate transitional form is not required for water-to-land transitions in various panarthropod groups. For these reasons, an alternative scenario of multiple terrestrialization events within Arachnida cannot be readily dismissed, regardless of phylogenetic tree topology.

An epipodal origin of respiratory organs in terrestrial arthropods?

Dispute persists in the literature regarding the evolutionary origins of arthropod respiratory systems. An early gene/protein expression survey of the transcription factors *nubbin/pdm* and *apterous* (*ap*) has been interpreted to suggest that (a) insect wings, (b) crustacean gills, (c) spider book lungs and tracheal tubules, (d) spider spinnerets (the silk spinning appendage-like organs), and (e) horseshoe crab book gills are all homologous structures derived from the epipod (outermost appendage ramus; Fig. 2A) of the ancestral arthropod appendage, inferred to represent the ancestral gill (Averof and Cohen 1997; Damen et al. 2002). This interpretation, summarized in Fig. 2B, was first based on strong expression of cross-reactive antibodies against Nubbin and Apterous, two wing markers, in the putative epipodal derivatives of insects and crustaceans (wings and gills), relative to weak rings of expression in the walking legs (telopods, or inner appendage ramus) of those lineages (Averof and Cohen 1997). A logical bridge was subsequently applied to the opisthosomal (abdominal) organs of two chelicerates. Strong expression of Nubbin was reported in the book gill primordia of horseshoe crabs, as well as the book lung, tracheal tubule, and spinneret primordia of spiders. Comparable gene expression patterns were reported for two *apterous* orthologs of the spider *Cupiennius salei*. Thus, these too were taken to be derivatives of the epipodal ramus (Damen et al. 2002). No developmental data exist for myriapods in this regard.

For the more closely related insects and crustaceans, the interpretation of homology in wings and gills, respectively, is partly supported by similar dynamics of transcription factors that initiate tubulogenesis in *Drosophila melanogaster*. The epipodal gills of crustaceans, the tracheal placodes of insects, and the legs (telopods, or major ramus) of both lineages all arise from a common pool of primordial cells (Franch-Marro et al. 2006). However, whereas pancrustacean telopods are

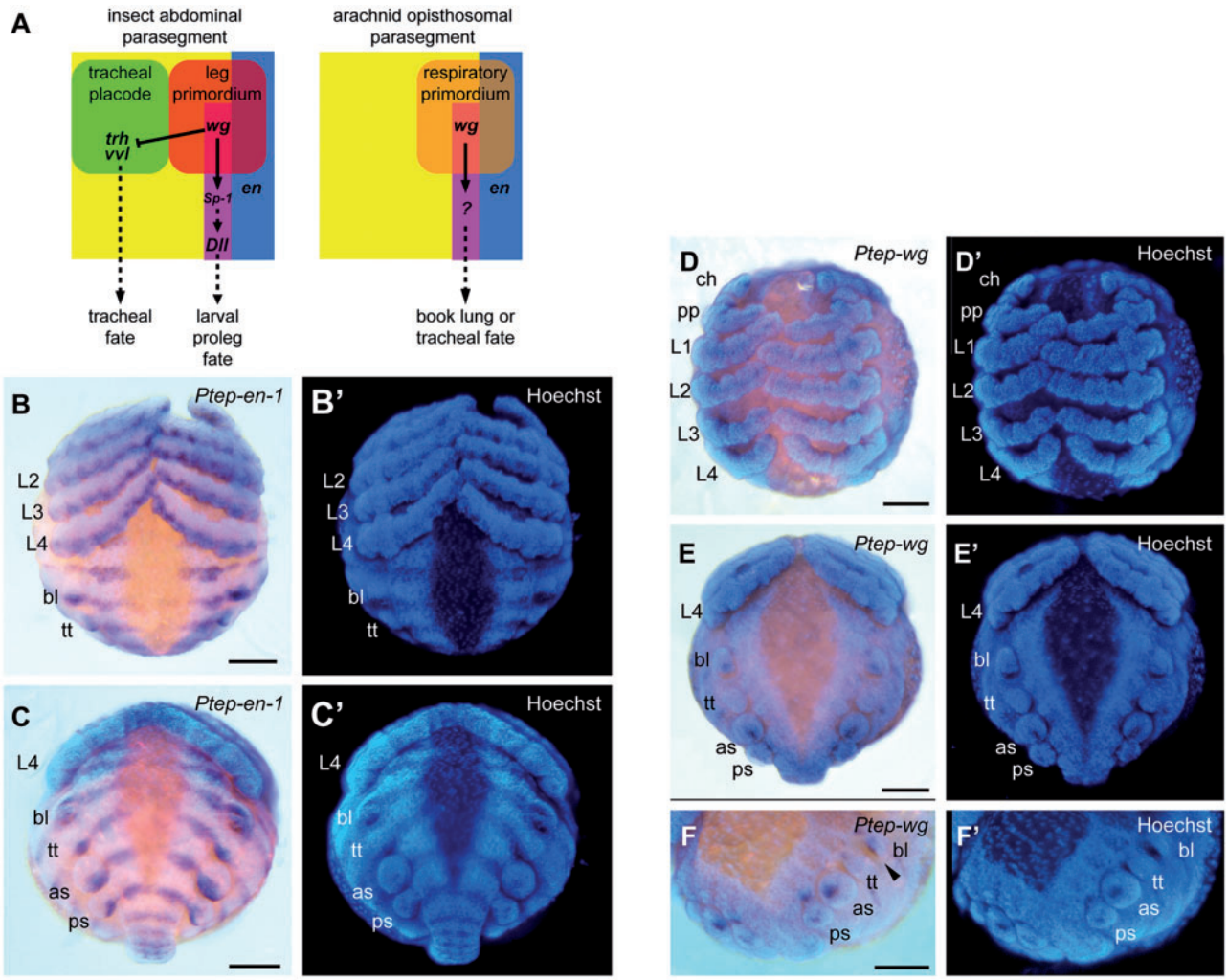

Fig. 3 (**A**) Positional relationships of respiratory organ placodes and walking leg primordia in the abdominal segment of an holometabolous insect larva insect (left) and a spider (right). Anterior is to the left, dorsal is up. *wg* (purple) and *en* (blue) demarcate the posterior compartment of the segment. (**B**) Expression of *Ptep-en-1* in the prosoma of a stage 10 spider embryo. (**C**) Expression of *Ptep-en-1* in the opisthosoma of a stage 10 spider embryo. Note the ventrally positioned limb bud-like anlage of the book lungs, tubular tracheae, and spinnerets all express *Ptep-en-1* at their posterior margin, comparably to prosomal appendages. (**D**) Expression of *Ptep-wg* in the prosoma of a stage 10 spider embryo. (**E**) Expression of *Ptep-wg* in the opisthosoma of a stage 10 spider embryo. At this stage, *Ptep-wg* is expressed in the book lung and spinneret primordia. (**F**) Expression of *Ptep-wg* in the opisthosoma of a stage 13 embryo. In the book lung, *Ptep-wg* is expressed in invaginating cells that form the presumptive book lung lamellae (arrowhead). (B′–F′) Counterstaining of (B–F) with Hoechst 33342. as, anterior spinneret; bl, book lung; ch, chelicera; L1, first walking leg; pp, pedipalp; ps, posterior spinneret; tt, tracheal tubule. All scale bars 100 μm.

formed at the expression boundary of the segmental polarity gene *wingless* (*wg*), the respiratory primordia of *D. melanogaster* lie anterior to the expression domain of *wg*. *wg* demarcates the posterior parasegment boundary and specifies leg (telopod) fate through *Distal-less* (*Dll*) and D-*Sp1* (orthologous to *Sp6-9* of other arthropods; Estella et al. 2003; Franch-Marro et al. 2006; Estella and Mann 2010). It has been shown that *wg* represses expression of *ventral veins lacking* (*vvl*) and *trachealess* (*trh*), two inducer genes required for tracheal tree formation in *D. melanogaster* (de Celis et al. 1995; Isaac and Andrew 1996; Wilk et al. 1996; Sánchez-Higueras et al. 2014) (Fig. 3A, left diagram). Franch-Marro et al. (2006) showed that orthologs of *vvl* and *trh* are comparably expressed in epipodal gills of two crustaceans and in the tracheal placodes of *D. melanogaster*, and considered these data to be consistent with epipodal origins of respiratory structures in both crustaceans and insects (Franch-Marro et al. 2006). Thus, a broad swath of posterior arthropod appendages has now been homologized with the epipods of the arthropod ancestor. By transitive reasoning, the internalized respiratory structures of arachnids and insects are putatively homologous as epipodal derivatives as well. This hypothesis (Fig. 2B) is referenced henceforth as the "ancestral gill hypothesis".

In spite of the attractiveness of this deep homology paradigm, there are multiple reasons to doubt the putative homology of all of these structures (insect wings, crustacean gills, horseshoe crab book gills, and spider book lungs, tracheae, and spinnerets). A growing body of morphological and developmental evidence has supported an alternative hypothesis of wing origin, favoring the interpretation of the wing as a paranotal extension incorporating elements of the upper pleuron (lateral body wall), the tergum (dorsal body wall), and modules of appendage-patterning genes (Jockusch and Nagy 1997; Niwa et al. 2010; Engel et al. 2013). By comparison to the insect wing, an epipodal origin of chelicerate opisthosomal organs has received much less scrutiny. Here, I describe inconsistencies between the ancestral gill hypothesis and other data classes, with respect to the posterior organs of Chelicerata. I also provide new gene expression data to test the homology of arachnid and insect respiratory organs implied by the ancestral gill hypothesis (Damen et al. 2002; Franch-Marro et al. 2006).

Functional morphology and expression of leg gap genes suggest that spider spinnerets are structurally homologous to telopods

Whereas some of the developmental genetic literature has treated horseshoe crab book gills and spider spinnerets as epipodal derivatives, functional morphologists have held that these structures constitute (at least in part) walking legs. While the ancestral arthropod appendage was almost certainly polyramous, a *bona fide* epipod (defined as an unsegmented outer appendage ramus attached to the proximal-most segment, the coxa) is uncommon in the evolutionary history of Arthropoda, likely having evolved independently in derived crustacean lineages (Boxshall 2004). Specifically, no true epipod (or any other outer ramus, other than the exopod) is observed in any Paleozoic marine arthropod, including in extraordinarily preserved specimens from the Burgess Shale, Chengjiang and Orsten faunas (Boxshall 2004; Boxshall and Jaume 2009). Modern inferences of pancrustacean phylogeny based on hundreds of loci additionally suggest that epipods are an unlikely element of the ancestral arthropod appendage (von Reumont et al. 2012; Oakley et al. 2012). By contrast, the exopod ("middle" appendage ramus) has been shown to be phylogenetically widespread in arthropods (Boxshall 2004), and its developmental origins are better understood. It has been shown that exopods and telopods results from the subdivision of the main appendage axis distally of the protopodite (Wolff and Scholtz 2008), and both exopods and telopods of various lineages bear true segments and internal muscle attachment sites (Boxshall and Jaume 2009). Intriguingly, a similar distal subdivision of an initially uniramous spinneret primordium appears to give rise to the median (accessory) spinnerets in *C. salei* (Pechmann et al. 2010). By contrast, epipods represent an entirely different axis during crustacean development, and lack PD segmentation and musculature. Accordingly, epipods have traditionally been considered as secondary branches by various authors (e.g., Thiele 1905; Borradaile 1926; Snodgrass 1958; Boxshall 2004; Wolff and Scholtz 2008).

In Chelicerata, while the discovery of key fossils has corroborated the biramous nature of the ancestral appendage of Chelicerata, the additional external rami found in synziphosurines (stem group horseshoe crabs) are also definitively exopods, not epipods (Sutton et al. 2002; Briggs et al. 2012). Within extant chelicerates, the postembryonic book gills of modern Xiphosura are considered to retain elements of a telopod, as evidenced by segmentation of the inner ramus along the proximo-distal (PD) axis. Much of the gill surface itself is interpreted to be of exopodal, but not epipodal origin (Boxshall 2004; Legg 2014; but see Suzuki and Bergström 2008 for a different interpretation).

Another dissonance exists in the arachnological literature between functional morphologists who hold that spider spinnerets represent derived walking legs (Shultz 1987), and developmental geneticists who favor epipodal origins of spinnerets (Damen et al. 2002, reviewed by Pechmann et al. 2010). In embryonic stages of spiders, the spinnerets occur as two pairs of uniramous appendages on the fourth (O4) and fifth (O5) opisthosomal segments. In basally branching spider lineages (Mesothelae and Mygalomorphae), the spinnerets are segmented to various degrees, and clearly bear internal musculature throughout the spinneret PD axis (Shultz 1987). More recently, it has been shown that the spinneret primordia express a cascade of appendage-patterning genes (*Dll*, *dachshund*, *extradenticle*, and *homothorax*) during development comparably to the prosomal appendages (i.e., true telopods; Prpic et al. 2003; Pechmann and Prpic 2009; Pechmann et al. 2010). These aspects of spider spinnerets suggest that they are structurally homologous to telopods, not epipods.

Book lungs, tubular tracheae, and spinnerets are positionally homologous to walking legs in the embryonic segment

Key to the specification of leg and respiratory organ fates in insects is the activity of *wg*, which is required for leg patterning in the posterior compartment of

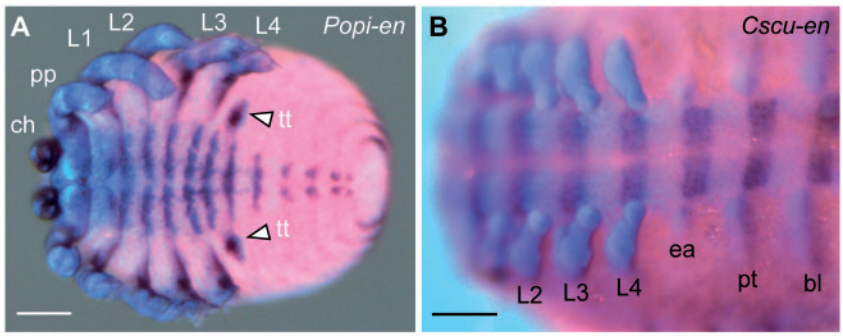

Fig. 4 (**A**) Expression of *Popi-en* in a harvestman stage 18 embryo, with legs dissected away for clarity. (**B**) Expression of *Cscu-en* in the scorpion. ea, embryonic O2 appendage; pt, pectine. Other abbreviations as in Fig. 3. All scale bars 100 μm.

the segment, and inversely, represses tracheal fate in this territory. Adjacent to *wg* is the expression domain of another segment polarity gene, *engrailed* (*en*), which demarcates the posterior boundary of the postembryonic segment. In insects, the tracheal placode is therefore localized in the region of the segment where neither *en* nor *wg* are expressed, i.e., in the anterior compartment (Fig. 3A).

While functional data for *en* and *wg* orthologs are lacking for Chelicerata, the expression patterns of these genes are strongly conserved across arthropods, with reference to postembryonic segment boundaries, as demarcated by stripes of *en* and *wg* expression in all surveyed arthropod embryos (Damen 2002). As with insects, the prosomal appendages of spiders also express *en* and *wg* throughout the posterior and ventral parts of each walking leg, respectively, suggesting a conservation of function across arthropods (Damen 2002).

Contrary to the position of insect tracheal placodes, however, the respiratory organ primordia (both book lungs and tubular tracheae) of the spider *C. salei* are positioned within the expression domain of *wg* and *en-1*, like typical telopods (Damen 2002). Both the anterior and posterior spinneret pairs share this condition as well. In older stages of spiders, *en-1* is expressed in discrete stripes within the book lungs, corresponding to the developing lamellae (Pechmann et al. 2010). This observation is striking, given the functional role of the *wg* ortholog in suppressing tracheal placode fate in *D. melanogaster*.

To test whether spiders are atypical in this regard, I compared expression data for *en* and/or *wg* orthologs of the theridiid spider *Parasteatoda tepidariorum* (a derived spider with a pair of book lungs on the O2 segment and a pair of tubular tracheae opening as spiracles on the O3 segment) with the harvestman *Phalangium opilio* (an apulmonate arachnid with a single pair of spiracles on the O2 segment) and the scorpion *Centruroides sculpturatus* (four pairs of book lungs on segments O4–O7). Methods for generating and imaging gene expression data followed previously published works (Damen 2002; Sharma et al. 2012a, 2012b, 2014b). Primer sequences are available upon request, or have been published previously.

As shown in Fig. 3B–F, expression data for *Ptep-en-1* and *Ptep-wg* are conserved with respect to *C. salei*. In addition, the single pair of tubular tracheae in the harvestman (Fig. 4A), and the O2 embryonic appendage, pectines, and book lungs of the scorpion (Fig. 4B) are also positioned within the posterior compartment of the segment that is marked by *en* expression, not the anterior region where insect spiracles would open (Fig. 3A, right diagram). These data refute the homology of respiratory organs of spiders and insects, and favor instead the positional homology of arachnid opisthosomal organs and telopods. In the specific case of the leg-like spinnerets, the position of the spinneret primordia within the segment further support for a telopodal origin of this organ.

Chelicerate orthologs of tracheal inducer genes are not dispositive of epipodal origin

One intriguing element of the ancestral gill hypothesis is the putative deep homology of mandibulate and arachnid respiratory organs (gills, tracheal tubules, book gills, and book lungs) as epipodal derivatives (Damen et al. 2002; Franch-Marro et al. 2006). In the case of the three major terrestrial arthropod groups (arachnids, myriapods, and hexapods), tracheal tubules have evolved repeated as bilaterally symmetrical, segmentally iterated organs (with secondary fusion in scutigeromorph centipedes; and secondary losses in miniaturized lineages of various groups; Hilken 1998). The similarity of the tracheal tubule network of myriapods and hexapods was once considered evidence of their sister group relationship (together with the uniramous

condition of their appendages; the reduced tritocerebral intercalary segment; the gnathobasic mandible; and the hexapodous condition of some myriapod postembryonic stages). Apropos, the clade Myriapoda + Hexapoda was termed Tracheata *sensu* Pocock 1893 (alternatively, Atelocerata *sensu* Heymons 1901; or Uniramia *sensu* Weygoldt 1986).

Given the discrepant position of the spiracles of the insect *D. melanogaster* and the respiratory organ primordia of arachnids (with respect to the expression of *wg* and *en*), I surveyed expression of homologs of the tracheal inducer genes *trh* and/or *vvl* in four other species, in order to test whether the co-expression of these genes previously observed in crustaceans and insects is conserved beyond Pancrustacea. Both of these inducers are repressed by *wg* in *D. melanogaster* and thus restricted to the anterior of the abdominal segments, but comparative data for these genes are limited outside of *D. melanogaster* and two crustacean species (Franch-Marro et al. 2006). Notably, the internal logic of this homology statement is identical to that of the Nubbin and *apterous* expression surveys of Damen et al. (2002), in that comparable expression is taken to mean conservation of function and/or homologous origin. However, functional data for *trh* and *vvl* do not exist in crustaceans and this premise remains to be tested.

Expression data of arachnid orthologs of *trh* and *vvl* demonstrate different expression domains when compared with insect orthologs. With respect to *trh*, expression of the harvestman single copy *Popi-trh* ortholog is restricted to the prosomal appendages; no expression was observed in the opisthosoma during embryogenesis, and specifically not in the developing spiracles (Fig. 5A, B). A similar expression pattern of the *trh* ortholog is observed in the scorpion (Fig. 5D). Two copies of *trh* occur in spiders. The more strongly expressed *trh* homolog (*Ptep-trh-1*) is expressed as rings in the prosomal appendages in late state embryos, and no expression is observed in the respiratory organ primordia (Fig. 5C). The expression pattern of the *Ptep-trh-1* paralog is thus comparable to the expression observed in the harvestman. The second spider paralog of *trh* (*Ptep-trh-2*) is weakly expressed as rings in the prosomal appendages, and was also not detected in the opisthosoma (data not shown). These data suggest that a role for *trh* in patterning respiratory structures may not be conserved across Arthropoda.

With respect to *vvl* orthologs, in the scorpion, *Cscu-vvl* is segmentally expressed throughout the body as strong expression domains in all appendage types, as well as in the central nervous system; expression is not limited to the respiratory organ primordia (Fig. 5E). As with *trh*, two *vvl* paralogs occur in the spider. *Ptep-vvl-1* is expressed in the developing brain and in the neurectoderm (Fig. 5F, G). In later stages, *Ptep-vvl-1* is additionally expressed in both book lung and tracheal tubule primordia (Fig. 5H). By contrast, *Ptep-vvl-2* is expressed in the labrum (not shown), as rings in the prosomal appendages (Fig. 5I, J), and in all opisthosomal organ types (book lungs, tubular tracheae, and spinnerets; Fig. 5K).

Taken together, these expression surveys suggest that the patterning of tracheal fate may not be genetically homologous between insects and arachnids, given the presence of *wg* in the tracheal and book lung primordia of arachnids, as well as the absence of *trh* in arachnid respiratory primordia. The expression pattern of *vvl* homologs is not dispositive of the ancestral gill hypothesis homology statement, as *vvl* is expressed in respiratory primordia, but also coexpressed with *wg* in several different tissues during arachnid embryogenesis (compare with Fig. 3A, left diagram). Functional data are required as a next step toward understanding the evolution of *trh* and *vvl* expression domains beyond *D. melanogaster* tracheal placodes.

The *D. melanogaster* model may be generalizable to other insects, however. In the abdomen of the hemimetabolous insect *Oncopeltus fasciatus*, *Ofas-trh* is expressed comparably to *D. melanogaster*, around the spiracles at the anterior end of each abdominal segment (Fig. 5L). *Ofas-trh* is not expressed in the posterior of the segment, which corresponds to the expression domain of *wg* (Fig. 5 of Angelini and Kaufman 2005). While the regulatory interaction of *trh* and *wg* is not known in *O. fasciatus*, it is intriguing that *Ofas-en* and *Ofas-wg* RNAi result in segmentation defects in the abdomen and enlarged spiracles (compare Figs. 4A, B, F, and 6B of Angelini and Kaufman 2005). The *wg* ortholog of the beetle *Tribolium castaneum* is also absent from the spiracle (Fig. 1C of Ober and Jockusch 2006), and RNAi against *Tcas-wg* results in enlarged or fused spiracles (Fig. 2D of Ober and Jockusch 2006). These data suggest that inhibition of *trh* by *wg* may be a conserved element of this gene regulatory network as well, at least within a subset of winged insects. A future test of this hypothesis should emphasize the effect of *wg* RNAi on the size of abdominal *trh* expression domains in embryonic stages prior to the formation of spiracles, in both *O. fasciatus* and *T. castaneum*.

Are arachnid respiratory organs serially homologous to walking legs?

In the myriapods and crustaceans, respiratory organs (tracheae and gills) occur dorsally with respect to the

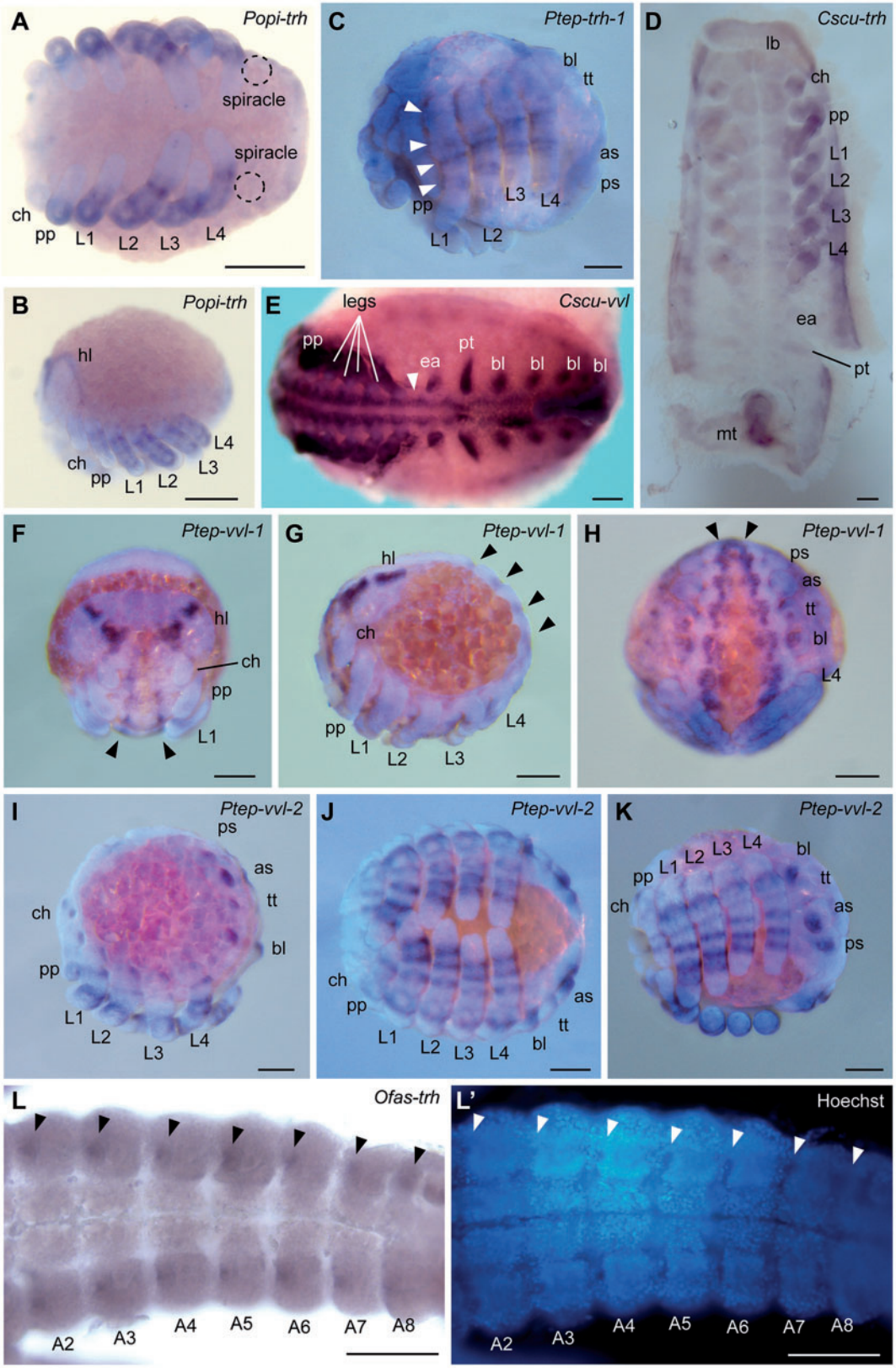

Fig. 5 (**A**) Expression domains of *Popi-trh* occur in the prosomal appendages of stage 12 embryos as heterogeneous rings proximal of the metatarsus. No expression of *Popi-trh* is observed in the invaginating cells that form the spiracles of the tracheal tubules. (**B**) *Popi-trh* is also expressed in the anterior margin of the head lobes. (**C**) Expression of *Ptep-trh-1* in a stage 13 embryo as heterogeneous rings in the prosomal appendages. No expression is observed in the book lungs or tracheal primordia. (**D**) Expression of *Cscu-trh* in the scorpion is detected in all prosomal appendages; weak expression is detected in the pectinal limb buds. The expression in the

telopod. In these lineages, the ontological relationship between the respiratory organs and the telopod is therefore unambiguous; we can reject the serial homology of dorsal respiratory structures and walking legs, because both elements coexist simultaneously in the same segment and are metamerically repeated. It is only in insects and arachnids that respiratory organs occur on a posterior tagma lacking true telopods, thereby obscuring the ontological relationship of tracheal tubules and walking legs. However, in several insect models, serial homology has been well explored using Hox gene misexpression studies. As examples, RNAi the against posterior Hox genes *Ultrabithorax* (*Ubx*) and *abdominal-A* (*abdA*) in *O. fasciatus* results in derepression of appendages on abdominal segments (Angelini and Kaufman 2005). Crucially, the spiracles are not affected by the homeotic transformation and retain their position in every segment, dorsal to the derepressed leg (Figs. 4 and 5 of Angelini and Kaufman 2005). This experiment has *de facto* replicated the myriapod or crustacean condition, wherein the serial homology of tracheal primordia and leg primordia is falsified by the induction of their coexistence in each segment. Furthermore, in *D. melanogaster*, recent work has demonstrated the serial homology of tracheal placodes, and two endocrine organs, the corpora allata (located in the maxillary segment) and the prothoracic glands (located in the labial segment; Sánchez-Higueras et al. 2014). These data are all internally consistent with one another in that tubular tracheae are shown not to be homologous to walking legs.

Only one comparable experiment has been conducted to date in an arachnid. Remarkably, RNAi against *Antennapedia* (*Antp*) in the spider *P. tepidariorum* results in an ectopic leg on the first opisthosomal (pedicel) segment, which has no legs or respiratory organs in wild type spiders (Khadjeh et al. 2012). RNAi against both *Antp* and *Ubx* additionally results in a transformation of the O2 book lung primordia to a small outgrowth that expresses *Dll* and prosomal Hox genes, indicating homeotic transformation to anterior identity. These results were taken to mean that the book lung of spiders is serially homologous to walking legs, and *prima facie* stand in contradiction to the interpretation of book lungs as epipodal derivatives under the ancestral gill hypothesis.

While consistent with the positional homology of book lungs and walking legs (see above), this datum must nevertheless be interpreted with caution, for two reasons. First, as previously mentioned, the book gill of horseshoe crabs (the putative homolog and ancestral condition of the book lung) is putatively composed of two rami (a medial telopod and a large outer exopod; Boxshall 2004; but see Suzuki and Bergström 2008). Both rami of the book gill express *Dll* during the embryogenesis of *Limulus polyphemus*, as does the flabellum, the putative exopod of the L4s segment (Fig. 3B, G of Mittmann and Scholtz 2003). Therefore, while the homeotically transformed O2 appendage in the spider Hox knockdown experiment could represent a complete walking leg, this is not something that can be assessed using *Dll* and prosomal Hox gene expression alone.

Second, the development of the homeotically transformed O2 structure was followed until the first postembryonic stage of spiders, but key landmarks of spider distal leg identity (namely, tarsal claws) do not appear until the second postembryonic stage. In addition, while the O2 book lung primordium was externally everted, it was not assessed whether a wild type internal book lung was retained in addition to the protrusion. Recently, my colleague Zhiyong Di and I encountered a spontaneously occurring mutation in a Southeast Asian scorpion species with homeotically transformed genital opercula (O2 segment) and pectines (O3 segment), and a flattened "gill-like" protrusion of the book lungs (O4–O7 segments). Intriguingly, the book lung was retained and mostly wild type with respect to its internal position within the segment and the morphology of the lamellae; the protrusion was medially

Fig. 5 Continued

metasoma represents non-specific staining that was also observed in the sense probe negative control (not shown). (**E**) Expression of *Cscu-vvl* occurs in all prosomal and opisthosomal limb buds, as well as in the ventral ectoderm. (**F**) In stage 9 spider embryos, *Ptep-vvl-1* is expressed in the developing brain and in the ventral ectoderm (arrowheads). (**G**) Same embryo as in (F), showing neuroectodermal expression domains of all opisthosomal segments (arrowheads). (**H**) In stage 10 embryos, *Ptep-vvl-1* is expressed in a complex pattern in the neuroectoderm (arrowheads), as well as in the book lung and tracheal tubule primordia. (**I**) In stage 10 embryos, *Ptep-vvl-2* expressed as rings in all prosomal appendages and as discrete domains in all opisthosomal organ primordia. (**J**) In stage 13 embryos, *Ptep-vvl-2* retains the expression domains observed in (I), but expression in the tracheal limb buds is diminished. (**K**) Stage 13 embryo in ventrolateral view, showing expression of *Ptep-vvl-2* in the book lung and spinneret primordia, but not in the tracheal tubule limb bud. (**L**) Abdomen of a milkweed bud embryo at 75–80 h after egg-laying, showing expression of *Ofas-trh* around the spiracles (arrowheads) at the anterior end of each segment. (L′) Counterstaining of (L) with Hoechst 33342. A1, first abdominal segment; hl, head lobe. All other abbreviations as in Figs. 3 and 4. All scale bars 100 μm.

occurring and attached to the book lung, approximately reflecting the condition observed in horseshoe crabs and putatively aquatic Paleozoic scorpion fossils. By contrast, all of the pectinal axis of this mutant was transformed to leg identity, as evidenced by segmentation along the PD axis, distal tarsal claws, and telotarsal macrosetae on the ventral margin of the tarsal territory (Z. Di and P. P. Sharma, submitted for publication).

It is therefore imperative to analyze the homeotically transformed O2 appendages of spiders more closely to understand the ontological relationship of book lungs and walking legs. Specifically, *Antp* and *Ubx* double knockdown experiments in the spider should be followed until the second postembryonic stage to test for the presence of tarsal claws at the distal end of the O2 protrusion. If keeping these specimens alive through the first molt is not possible, then their embryos should be assessed for (a) the retention of an internal book lung in addition to the everted structure, using *en* or *wg* to mark the developing lamellae; (b) the expression of medial and/or distal telopod markers, such as *extradenticle-1* (medial ring domain in arachnid pedipalps and legs), *dachshund*, and *aristaless*; and/or (c) the presence of either segmentation markers (e.g., *Notch*, *Serrate*) or muscle attachment sites in the homeotically transformed appendage.

Due to the relative inefficiency of RNAi in *P. tepidariorum* during late development (e.g., Pechmann et al. 2011; Turetzek et al. 2015), as well as redundancy of appendage repression functions in multiple posterior Hox genes, triple knockdowns were shown to have no effect on tracheal tubules or spinnerets in that study (Khadjeh et al. 2012). Another explanatory variable for low penetrance is the incidence of two paralogs of every opisthosomal Hox gene in the common ancestor of spiders and scorpions, with demonstrable divergence of expression pattern in each paralog pair (Schwager et al. 2007, 2017; Sharma et al. 2014b, 2015), relative to the expression of the single-copy orthologs of non-Arachnopulmonata orders (e.g., mites and harvestmen; Telford and Thomas 1998; Sharma et al. 2012a; Barnett and Thomas 2013). Thus, the serial homology of tracheal tubules and spinnerets has yet to be tested from the perspective of functional genetics.

Summary

As recent phylogenomic works increasingly support the monophyly of Arachnopulmonata, the traditional interpretation of book lungs as an ancestral feature of the arachnid common ancestor has come into question. Developmental genetic data have the potential to be highly adjudicative of alternative evolutionary scenarios, but contradictory interpretations of book gill and book lung origins must first be addressed. Examining different definitions of homology as they pertain to the ancestral gill hypothesis, I note that spider spinnerets are structurally homologous to telopods, not epipods. I observe that arachnid respiratory organs are not positionally homologous to insect respiratory organs, as inferred using segment polarity gene expression as landmarks within segments. My survey of gene expression of arachnid orthologs of tracheal inducer genes suggests that *vvl* may be regulated in a different way by *wg* in comparison to insects, whereas *trh* may not be involved in patterning arachnid respiratory organs at all. Thus, there is presently no evidence for evolutionary conservation (i.e., genetic homology) of the gene regulatory network that specifies insect tracheal fate in arachnids. While Hox gene misexpression data addressing the opisthosoma are limited to one landmark study, the interpretation of homeotic book lung-to-leg transformation in Khadjeh et al. (2012), if taken at face value, refutes the homology of book lungs and epipods as well. There is presently little empirical evidence supporting the deep homology of various respiratory organs as epipodal derivatives.

Instead, an accruing body of evidence presently supports the interpretation that arachnid opisthosomal organs are (at least in part) derived from walking legs. This interpretation reconciles the homology concepts of disparate approaches, such as functional morphology (e.g., Shultz 1987) and developmental genetics (Pechmann et al. 2010; Khadjeh et al. 2012), and further underscores the unparalleled range of adaptations facilitated by the modularity of the arthropod leg. If validated, this interpretation would suggest that different terrestrial arthropod lineages have made landfall using different evolutionary solutions to the physiological challenge of aerial respiration. Future avenues of inquiry should therefore emphasize (a) the discovery of the gene regulatory networks underlying fate specification of book gills, book lungs, tracheal tubules, and spinnerets; (b) a comparison of the mechanisms whereby the book lungs of Arachnopulmonata and the gills of terrestrial crustaceans (e.g., isopods) are internalized; and (c) a comparison of the mechanisms whereby the tracheal tubules of apulmonate arachnids (e.g., harvestmen), derived spiders (e.g., the O3 tracheae of non-hypochilid araneomorphs), myriapods, and insects begin invagination and elongation during embryogenesis, toward understanding whether the

tracheal tubules of apulmonate arachnids can be homologized with the secondarily derived tracheae of spiders.

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Supplementary data

Supplementary data available at *ICB* online.

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