SYMPOSIUM

Handling and Use of Oxygen by Pancrustaceans: Conserved Patterns and the Evolution of Respiratory Structures

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Synopsis The handling and use of oxygen are central to physiological function of all pancrustaceans. Throughout the Pancrustacea, ventilation is controlled by a central oxygen-sensitive pattern generator. The ancestral condition was likely to achieve ventilation of the gills via leg-associated or mouth-associated muscles, but in insects and some air-breathing crustaceans, new muscles were recruited for this purpose, including intersegmental muscles likely used previously for posture and locomotion. Many aspects of the sensing of oxygen and the occurrence of responses to hypoxia (increased ventilation, depressed growth and metabolic rate, developmental changes that enhance the delivery of oxygen) appear common across most pancrustaceans, but there is tremendous variation across species. Some of this can be explained by habitat (e.g., ventilation of the internal medium occurs in terrestrial species and of the external medium in aquatic species; rearing under hypoxia induces tracheal proliferation in terrestrial insects and hemocyanin production in aquatic crustaceans); some plausibly by evolutionary origin of some responses to hypoxia within the Pancrustacea (the most basal arthropods may lack a ventilatory response to hypoxia); and some by the availability of environmental oxygen (animals adapted to survive hypoxia turn on the response to hypoxia at a lower PO_2). On average, crustaceans and insects have similar tolerances to prolonged anoxia, but species or life stages from habitats with a danger of being trapped in hypoxia can tolerate longer durations of anoxia. Lactate is the primary anaerobic end-product in crustaceans but some insects have evolved a more diverse array of anaerobic end-products, including ethanol, alanine, succinate, and acetate. Most clades of Pancrustacea are small and lack obvious respiratory structures. Gilled stem-pancrustaceans likely evolved in the Cambrian, and gills persist in large Ostracoda, Malacostraca, and Branchiopoda. Based on currently accepted phylogenies, invaginations of cuticle to form lungs or tracheae occurred independently multiple times across the Arthropoda and Pancrustacea in association with the evolution of terrestriality. However, the timing and number of such events in the evolution of tracheal systems remain controversial. Despite molecular phylogenies that place the origin of the hexapods before the appearance of land plants in the Ordovician, terrestrial fossils of Collembola, Archaeognatha, and Zygentoma in the Silurian and Devonian, and the lack of fossil evidence for older aquatic hexapods, suggest that the tracheated hexapods likely evolved from Remipedia-like ancestors on land.

Introduction

Insects and crustaceans represent critical, dominant animal groups (by biomass and species number) in terrestrial and aquatic systems, respectively (Price et al. 2011; Cisto 2013). Insects and crustaceans were historically grouped under separate taxonomic classes within the Phylum Arthropoda, and for many years it was thought that myriapods might be the closest relatives to insects, based on the morphology of their appendages and on their mutual terrestriality (Grimaldi and Engel 2005). New genetic data based on nuclear protein-coding genes (Regier et al. 2010), transcriptomics (von Reumont et al. 2012), and combinations of molecular and morphological data (Legg et al. 2013; Oakley et al. 2013) have made it clear that insects and other hexapods are nested within the crustaceans within the arthropod tree. While there remains debate on many aspects of the phylogeny, including which crustaceans are most closely related to insects, there is now a consensus that insects evolved from crustacean ancestors. Understanding the evolutionary relationships of the

Pancrustacea is catalyzing many new aspects of research both in insects and in crustaceans. One of the fundamental scientific questions raised by our new understanding of the phylogenetic relationships of the Pancrustacea is "how similar are their physiological systems?" Another type of question is "how did modern insect and crustacean morphology and physiology evolve from common crustacean ancestors?"

This article focuses on the handling and use of oxygen by the Pancrustacea, with a particular focus on responses to deficiencies in oxygen and on the evolution of structures used in the exchange of gases. One of the fundamental scientific questions raised by our new understanding of the phylogenetic relationships of the Pancrustacea is "how similar are their physiological systems?" Here I begin by identifying multiple, conserved characteristics related to responses to hypoxia that appear to be shared by most pancrustaceans. Second, I cover our current understanding of the evolution of gas-exchange structures within the Pancrustacea and encompassing groups (Mandibulata, Euarthropoda) in light of the most recent molecular and fossil data. Multiple prior reviews have covered aspects of the material covered here, although none has focused specifically on these aspects of the handling of oxygen by the Pancrustacea. The evolution of respiratory structures across animals recently was reviewed by Hsia et al. (2013); a review by Maina (2002) covers the evolution of respiratory structures across animals in the context of terrestrialization; and a review by Wirkner and Richter (2013) focuses on the respiratory and cardiovascular systems of crustaceans. McMahon and Wilkens have separately reviewed the evolution of cardiovascular systems in the Crustacea (Wilkens 1999; McMahon 2001a).

Many aspects of the sensing of oxygen and responses to a dearth of oxygen are conserved across the Pancrustacea

Oxygen-sensing pathways

Based on their occurrence in *Caenorhabditis elegans*, most aspects of oxygen-sensing are ancient pathways expected to be found in all animals, including the Pancrustacea. Hypoxia inducible factor (HIF) and prolyl hydroxylase response-elements occur in the worm *C. elegans*, the branchiopod *Daphnia*, and the insect *Drosophila* (Gorr et al. 2006). Direct evidence for close relationships of HIF and prolyl hydroxylase in the Pancrustacea comes from Piontkivska et al. (2011) who demonstrated that malacostracan and insect genes for these molecules group together. While the genes and basic response-elements may be common throughout animals, it is plausible that these hypoxia-response systems might vary in sequence, structure, and function among animals that differ in their likelihood and magnitude of exposure to hypoxia. Due to their diversity in life histories, size, and ecology, the Pancrustacea should be an excellent group for assessing how factors such as habitat, size, or temperature may affect the genetic and functional properties of these oxygen-sensing systems.

The response to hypoxia

Animals exposed to hypoxia usually demonstrate a set of physiological and behavioral responses sometimes termed collectively "the hypoxic response" (Guillemin and Krasnow 1997; Poellinger and Johnson 2004). Well-demonstrated elements of the hypoxic response in mammals include:

- Physiological responses that acutely increase the capacity to deliver oxygen to the tissues, e.g., increasing ventilation.
- Physiological and/or behavioral responses that reduce the need for oxygen, e.g., suppression of protein synthesis, reduced locomotion, or lowering of body temperature.
- With longer-term exposures, stimulation of developmental changes that enhance the delivery of oxygen.

Most of the elements of the hypoxia response have been demonstrated in some pancrustaceans, suggesting widespread conservation of its basic elements; however, there are intriguing data that within the Pancrustacea, it may be possible to identify key steps in the evolution of the hypoxic response, as well as important variation in this response among habitats.

Across the Pancrustacea, there is some evidence that animals regulate the delivery and use of oxygen in ways that maintain relatively low levels of PO_2 (1–3 kPa) in the tissues, perhaps reflective of conditions during the origin of animals and conditions that allow adequate oxygen for mitochondria without excessive reactive production of oxygen species (Massabuau 2001, 2003; Williams et al. 2011). In support of this concept, water-breathing crustaceans regulate arterial PO_2 at a low level relative to normoxic water (2–6 kPa) and maintain these levels across different temperatures and during exposure to moderate hypoxia, suggesting that these animals actively regulate a low level of oxygen in the blood and tissues, thereby minimizing the production of reactive oxygen species (Massabuau 2003).

Ostracods migrate through sediments to select moderately hypoxic conditions (Corbari et al. 2004), and both excessive and insufficient oxygen have caused major extinctions and expansions of crustacean lineages (Williams et al. 2011). Insects' levels of PO_2 in the trachea and blood are reported to be higher and more variable (Krogh 1913; Krolikowski and Harrison 1996; Matthews et al. 2012), but plausibly levels in the tissues may be lower, and more stable and crustacean-like, as suggested for birds and mammals (Massabuau 2001; Hetz and Bradley 2005).

Ventilatory response to hypoxia

Both in insects and crustaceans, ventilation is driven by oxygen-sensitive central-pattern oscillators located in thoracic ganglia; these can extend into abdominal ganglia in insects and to the subesophageal ganglia in crustaceans (Miller 1981; Taylor 1982; Kinnamon et al. 1984; Burrows 1996). However, the muscles and structures used for ventilation vary dramatically across the Pancrustacea.

Crustaceans ventilate their gills or lungs in different ways depending, on the size and taxon of the animal (Wirkner and Richter 2013). Ventilation can occur by waving of gill-epipodites in association with filter feeding, by waving of the thoracopods that bear the epipodites, and by the beating of other appendages that move water past the respiratory surface (Corbari et al. 2005; Wirkner and Richter 2013). In air-breathing crustaceans, ventilation usually is accomplished by scaphognathites moving air (McMahon and Wilkens 1983; Morris et al. 1988). Thus, the general pattern for crustaceans is for ventilation to be driven by muscles associated with modified legs or mouthparts. However, the amphibious crab *Holthuisana transversa* tidally ventilates its branchial chamber by lateral oscillations of the chamber walls (Greenaway et al. 1983), possibly accomplished by similar intersegmental muscles as those that drive most ventilation in insects.

In insects, the most common form of ventilation is abdominal pumping, which is driven by contractions of intersegmental muscles (Miller 1981); in general these muscles likely are used primarily for posture or locomotion in crustaceans. However, some insects ventilate their tracheal systems by the pulsing of mouthparts (Lehmann and Heymann 2005). In aquatic insects, ventilation can occur by waving of the gills, using muscles associated with the legs, as in crustaceans, or, in rectal breathers, by pulsations of the rectal muscles (Mill 1974; Resh et al. 2008; Chapman et al. 2013). Thus, in addition to evolving new respiratory structures (trachea), insects (and perhaps air-breathing crustaceans) likely repurposed muscles used in other capacities for a new function (ventilation) relative to their gilled ancestors, thereby, providing an interesting problem in the evolution of neuronal patterns of wiring.

Possibly, hypoxic stimulation of ventilation evolved within the Arthropoda. In the arthropods that have retained the most primitive traits, including the horseshoe crab *Limulus polyphemus* (Mangum and Ricci 1989) and the myodocopid ostracod *Cylindroleberis mariae* (Corbari et al. 2005), hypoxia does not stimulate ventilation. In contrast, moderate hypoxia stimulates both the frequency and force of ventilation by scaphognathites in most Malacostraca (Taylor 1982; McMahon 2001b). Similarly, hypoxia stimulates abdominal pumping in terrestrial insects (reviewed by Miller [1966] and Schmitz and Harrison [2004]), and ventilation of the gills in aquatic insects (Mill 1974; Kinnamon et al. 1984). Both for crustaceans and insects, it has been suggested that the primary location of the hypoxic ventilatory response is central rather than peripheral (Kinnamon et al. 1984).

Cardiac responses to hypoxia and anoxia

A logical hypothesis is that pancrustaceans that use hemolymph-born pigments for the transport of oxygen will show hypoxic stimulation of heart function while tracheated species will not. Many Malacostraca decrease, rather than increase, heart rate in response to hypoxia; however, cardiac output is increased due to increases in stroke volume (McMahon 2001b). Unfortunately, in insects, we currently have little data on cardiac output, and none on the effects of hypoxia on cardiac output, so the hypothesis cannot be tested at the present. Hypoxia has been reported to stimulate the heart rate of larval Trichoptera and the lepidopteran *Acentropus* (Wigglesworth 1972), both of which lack hemocyanin in the hemolymph (T. Burmester, this volume), suggesting that the transport of blood-borne oxygen in insects might be more important than usually thought. Interestingly, in *Daphnia* (Paul et al. 1998), hearts continue to beat during anoxia, suggesting that the heart has a strong capacity for anaerobic metabolism, and that functions of the circulatory system other than oxygen transport (e.g., waste removal, transport of hormones) may be triggered under hypoxia/anoxia.

Suppression of ATP demand by effects on growth and behaviors under mild hypoxia

The pancrustaceans examined to date exhibit suppression of growth and size when subjected to mild hypoxia, usually at PO_2 values above those that limit

metabolic rate. In the crustaceans, this has been well-demonstrated for *Daphnia* species (Weider and Lambert 1985; Hanazato and Dodson 1995). In insects, this has been shown for *Drosophila melanogaster* (Peck and Maddrell 2005), *Manduca sexta* (Harrison et al. 2013), *Tenebrio molitor* (Loudon 1988), *Cotinus* spp. (Harrison et al. 2009), and *Schistocerca americana* (Harrison et al. 2006). The fact that suppression of growth occurs without short-term reduction in metabolic rate suggests that the growth may be mediated by activation of oxygen-signaling systems such as HIF, acting either directly on protein synthesis and the cell cycle or via modulation of growth-affecting hormones (Gorr et al. 2006). Presumably, this reduction in growth reduces long-term demand for oxygen; this may also be a mechanism that allows the animal to more quickly reach maturity under possibly declining environmental conditions. The brineshrimp *Artemia franciscana* lengthened more quickly in 10 relative to 21 kPa oxygen, well above its critical PO_2 for metabolism (4 kPa PO_2), while shifting reproductive output from live offspring to cysts, demonstrating a different way that hypoxia can shift development (Spicer 1999). Some species that are more tolerant of anoxia can suppress locomotory behavior (rather than using more escape locomotion for escape), thereby reducing metabolic rate and extending survival. As another mechanism that reduces the metabolic demand for oxygen during hypoxia, the crayfish, *Procambarus simulans* (Dupré and Wood 1988), and the shore crab, *Carcinus maenas* (De Wachter et al. 1997), shift to lower temperatures when exposed to hypoxia.

Stimulation of developmental changes that enhance the delivery of oxygen during long-term hypoxia

The specific developmental responses to hypoxia seem to differ between air-breathing and water-breathing pancrustaceans. In insects there is strong evidence for compensatory morphological changes of the tracheal system in response to being reared under chronic hypoxia. Development under hypoxic conditions causes the tracheal trunks of insects to enlarge (Locke 1958; Loudon 1989; Henry and Harrison 2004; VandenBrooks et al. 2012), and increases tracheolar branching (Wigglesworth 1983; Jarecki et al. 1999). In insects, HIF signaling is responsible for hypoxic regulation of tracheal growth, reminiscent of hypoxic stimulation of capillary growth in vertebrates (Centanin et al. 2010). There is also some evidence that rearing under hypoxic conditions causes an increase in the conductance of the cuticles of insect eggs (Zrubek and Woods 2006).

In contrast, for gilled pancrustaceans there is strong evidence that hypoxia during development causes increased production of oxygen-transporting pigments in a variety of Malacostraca and Branchiopoda, and sometimes there are changes in the type and function of the particular pigments produced (McMahon 2001b; Zeis et al. 2003). However, there is little evidence for compensatory growth of the gills in pancrustaceans in response to hypoxia, partly due to lack of experiments that separate plastic and evolved responses. Crustaceans' gills areas vary with habitat and with the metabolic capacity of the species (Gray 1957), but apparently plastic responses of gills undergoing development under hypoxic conditions are unstudied. Mayflies from streams with lower oxygen content have larger gills, which could either be due to plastic or evolutionary effects (Dodds and Hisaw 1924). Similarly, populations of an amphipod inhabiting hypoxic sewage tanks have larger gills and greater tolerance to hypoxia, but it is not clear whether this is due to an evolutionary change or to plasticity (Roast and Jones 2003). It would be particularly interesting to study the effects of hypoxia on the development of gills or tracheal structures or on hemocyanin content over single and multiple generations of some of the hexapod clades that both utilize tracheae and contain hemocyanin.

Capacities for coping with hypoxia and anoxia

Although mobile animals, including many pancrustaceans, attempt to escape zones of severely declining oxygen (Phil et al. 1991; Wu 2002), it may be a relatively common occurrence for animals, especially small ones, to become trapped in conditions that lead to depletion of oxygen. For terrestrial, freshwater, or tidal species, becoming trapped under water or soil or in burrows is probably a relatively common occurrence. Miller et al. (2002) tested nine estuarine crustacean species for exposure to hypoxia over 1–4 days and found LC_{50} values (duration of exposure resulting in the death of 50% of the individuals) ranging from 7 to 42 kPa PO_2, with larvae significantly more sensitive than juveniles. Stickle et al. (1989) reported that 50% of individuals in five decapod species died within 1 day of exposure to anoxia at 30°C. Many meiobenthic copepods survive several days of anoxia, although a month of anoxia did eliminate most species (Grego et al. 2013).

For comparing tolerance to hypoxia/anoxia across pancrustaceans, the best data are for the duration of anoxia that is tolerated (Malacostraca: 16 species in four orders, Branchiopoda: two species in one order,

Copepoda: four species in one order; Insecta: 14 species in six orders; Supplementary Table S1). I excluded data for diapausing forms, and only included data for one *Cicindela* species, as data for six species were broadly similar (Brust and Hoback 2009). For crustaceans, data usually were presented as the number of hours required to kill 100% of the animals (LT_{100}). For insects, most data were given as durations that killed 50% of individuals (LT_{50}). To test the effects of taxon, habitat, and mass, missing LT_{100} values were calculated by multiplying the LT_{50} value by 2.9 (the average ratio of LT_{100}/LT_{50} for the eight species for which both values were available). LT_{100} values were then all corrected to 20°C, assuming a Q_{10} of 2, as found by Hoback et al. (1998). Habitat was classified as generally normoxic or escapable hypoxia (1) versus having a danger of inescapable hypoxia/anoxia (2). For example, the habitat of benthic copepods can be hypoxic, but these animals routinely migrate away from conditions of hypoxia, so they were placed in category "1" (Tinson and Laybourn-Parry 1985). If masses were not provided, I found data for that species in the literature. If only dry weights were presented, crustacean data were converted to wet weights on a class-specific basis using data from Ricciardi and Bourget (1998), except for copepods whose dry/wet weight ratio was estimated as 0.15 (van der Meeren et al. 2008). Wet weights were calculated from reported dry weights of beetles by multiplying by 0.5 (Costanzo et al. 1997). The duration of anoxia that can be survived by pancrustaceans (LT_{100}) was tremendously variable (Supplementary Table S1), ranging from 0.5 to 464 h.

Overall, crustaceans and insects did not differ in LT_{100}, but habitat had a significant effect on the duration of anoxia that could be tolerated (Fig. 1). Data did not meet assumptions of parametric tests. Crustaceans and insects did not differ significantly in their median LT_{100} (35 h, Mann–Whitney $U = 97$, $P = 0.07$). Pooling all data, there was a weak but significant negative relationship between body mass and LT_{100} (Spearman $r = 0.38$, $P = 0.025$, $n = 34$). Species from habitats with a danger of being trapped in hypoxia/anoxia could survive anoxia for a significantly longer time (median durations 80 versus 9 h, Mann–Whitney $U = 24$, $P < 0.0001$). The finding that the danger of being trapped in hypoxic conditions is associated with greater tolerance of anoxia is supported by the 5-fold to 10-fold greater period of anoxia that can be survived by larval relative to adult *Cicindela* tiger beetles, as these larvae live in occasionally
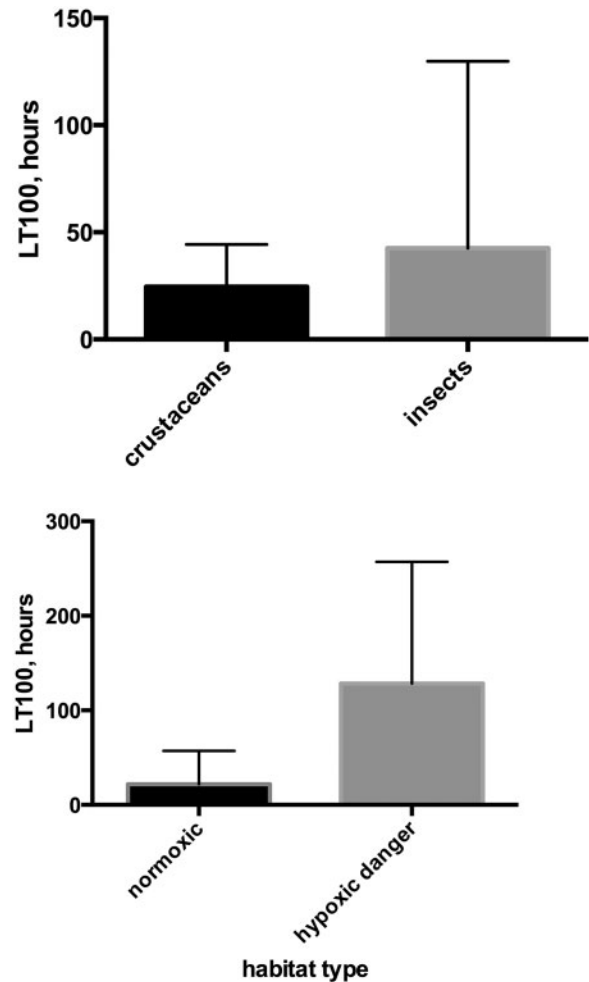

Fig. 1 Top: Duration of anoxia causing 100% mortality (LT_{100}) in crustaceans and insects. Bottom: LT_{100} in Pancrustaceans from normoxic habitats (terrestrial, marine, freshwater) versus those from habitats with danger of being trapped in hypoxia/anoxia (sediments, marshlands, stores of grain). Graphs show median and quartiles.

flooded soils while adults are terrestrial (Brust and Hoback 2009).

Anaerobic end-products

Invertebrates such as molluscs and a variety of worms are known to use a much wider variety of anaerobic end-products than vertebrates, including succinate, acetate, and propionate (Müller et al. 2012). These accumulate during exposure to severe hypoxia or anoxia, and during burst activity. In contrast to this broad invertebrate pattern, evidence to date suggests that crustaceans, like vertebrates, seem to rely more narrowly on conversion of glycogen or other stored carbohydrate to lactate as the primary anaerobic end-product. This evolutionary pattern has been suggested to be due to the relatively high mobility

of crustaceans in comparison to molluscs and annelids, and therefore the greater capacity to behaviorally escape hypoxia/anoxia (Hervant et al. 1996). Lactate is the primary end-product of anaerobic metabolism in decapod crustaceans, and it is virtually the only major anaerobic end-product during exercise in these species (McDonald et al. 1979; Wood and Randall 1981; Hill et al. 1991). In two species of decapod crabs exposed to 12–24 h of anoxia, glycogen was the only significant fuel, and lactate was the primary end-product, with alanine accounting for 4–14% of the accumulation of anaerobic end-products; there were no significant changes in succinate (Zebe 1982). Similar results, with lactate being the primary aerobic end-product with some minor accumulation of alanine, have been found for the crayfish *Orconectes limosus* during exercise or anoxia (Gade 1984), the stone crab, *Menippe mercenaria* exposed to severe hypoxia (Albert and Ellington 1985), and the green crab *C. maenas* exposed to anoxia (Hill et al. 1991). Within a different group of the Malacostraca, the conversion of glycogen to lactate, alanine, and succinate accounted for 82%, 13%, and 4% (respectively) of the anaerobic production of ATP in the marine isopod, *Cirolana borealis*, exposed to 18 h of anoxia (De Zwaan and Skjoldal 1979). Similarly, in four species of amphipods, lactate accounted for the majority of anaerobic production of ATP, with alanine accounting for an additional 8–12% and succinate for 0–4% (Hervant et al. 1995, 1996). Lactate production during anoxia has also been shown in the branchiopod *Daphnia* (Paul et al. 1998), branchiopod *Artemia* (cysts) (Ewing and Clegg 1969), marine copepods (Thuesen et al. 1998), and barnacles (López et al. 2003).

Primary reliance on lactate as an anaerobic end-product observed in crustaceans is consistent with observations in chelicerates. Lactate is the primary anaerobic end-product in spiders (Prestwich 1983) and the chelicerate horseshoe crab *Limulus* (Gäde 1983). Thus, the stem arthropod lineage may have been primarily dependent on lactate as an aerobic end-product. The dependence of crustaceans on lactate production may be explained by their lack of mitochondrial rhodoquinone, which is essential for mitochondrial production of succinate during anaerobic conditions (Holman and Hand 2009).

Although relatively few insects have been reported to exhibit anaerobic metabolism during activity, in all cases, lactate is reported as the major end-product. Lactate accumulates in the extensor tibia muscle of grasshoppers during jumping (Harrison et al. 1991). The intersegmental muscles of many coleopteran and lepidopteran larvae have high levels of lactate dehydrogenase, suggesting that these insects

may utilize anaerobic lactate production for the generation of ATP during burst activity (Gäde 1985).

However, during anoxia, hexapods, especially dipteran insects, appear to have evolved more diverse anaerobic end-products than have crustaceans. Lactate has been reported to be the primary anaerobic end-product of the Collembola during drowning (Zinkler and Russbeck 1986). However, two species of larval tiger beetles accumulated near-equal amounts of lactate and alanine during anoxia, and together these accounted for anaerobic heat production (Hoback et al. 2000); a similar pattern was observed for the larval mosquito, *Culex pipiens* (Redecker and Zebe 1988). Near-anoxic adult *D. melanogaster* accumulated twice as much alanine as lactate, as well as considerable acetate (Feala et al. 2007). The larval midge *Chaoborus* accumulates more succinate and alanine than lactate during anoxia (Englisch et al. 1982), and larval chironomid larvae produce mostly ethanol (excreted to the ambient water), and also significant quantities of lactate, alanine, and succinate (Wilps and Zebe 1976; Redecker and Zebe 1988). The genetic and biochemical mechanisms responsible for evolution of these anaerobic metabolic patterns of insects remain unknown.

Evolution of respiratory structures in the Arthropoda

Here I focus on questions related to the evolution of respiratory structures in the Pancrustacea, and also consider the Arthropoda, partly because the relationships of clades within these groups remain controversial (Wägele and Kück 2014), and also because hexapods, myriapod, and chelicerates are arthropods that evolved from aquatic ancestors.

Respiratory structures in the Pancrustacea

A chitinous cuticle covers arthropods. In small or thin species with thin exoskeletons, transport of gas can occur across the general exoskeleton and no respiratory organs are present, although often there are regions of thin cuticle specialized for exchange of gas. For example, the small branchiopod *Daphnia*, which does not have an obvious gill, uses a thin-walled epithelium under the carapace as the primary site of gas exchange, with this surface ventilated by the feeding legs (Pirow et al. 1999). Examination of the Pancrustacea and Euarthropoda (Fig. 2) shows that 7 of 19 of the clades of these taxa lack an obvious respiratory structure. This is because all species in these clades are small (under a few millimeter in length) and can obtain adequate oxygen without the elaborations of surface area associated with formation of a respiratory organ. This is plausibly the
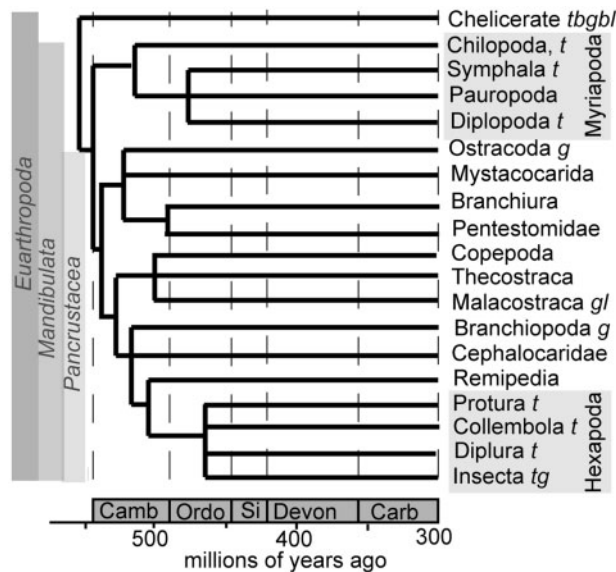
Fig. 2 Phylogeny of arthropods, focused on the Mandibulata and Pancrustacea, based on (Edgecombe and Legg 2014). "g" indicates that members of the clade exhibit gills, "bg" indicates the occurrence of book gills, "bl" the occurrence of book lungs, "l" the occurrence of lungs, and "t" the occurrence of a tracheal respiratory system. If there is no letter, most members of the clade lack respiratory organs, though they may have regions of thin cuticle specialized for gas exchange.

ancestral condition, although miniaturization and loss of respiratory structures have likely also occurred in many taxa.

Today, most large/thick arthropod species have a specialized respiratory structure, with a thin cuticle and a large surface area for gaseous exchange. It is unclear to what extent this is driven by the decrease in surface-area:volume ratio with larger size versus the fact that the exoskeleton may become too thick to allow adequate exchange of gas in larger species. In aquatic species, the exoskeleton is calcified and sclerotized; in insects, the exoskeleton is sclerotized and covered with lipids that reduce loss of water. The most common respiratory structures in Arthropoda are gills (cuticular evaginations of legs or abdominal appendages), book gills or lungs (lamellar cuticular evaginations), lungs (invaginated cuticular pouches), and tracheae (tubular air-filled invaginations of the cuticle). Book gills and book lungs are confined to the Chelicerata; gills occur in the Ostracoda, Malacostraca, Branchiopoda, and Insecta; lungs in the Malacostraca; and tracheae in the Chelicerata, Myriapoda, Hexapoda, and Malacostraca (Fig. 2).

Within the gilled, non-hexapod pancrustaceans (Ostracoda, Malacostraca, Branchiopoda), gills are evaginated, thin, cuticle-covered epithelia, usually located on legs (epipodites) or on the inner side of the lateral portions of the carapace (often called branchiostegites) (Wirkner and Richter 2013). In the interior, a thin layer of hemolymph flows by the epithelium. Gills likely evolved as elaborations from areas of thin cuticle specialized for gas exchange in small crustaceans.

Large, modern, air-breathing crustaceans have modified gas exchangers usually called lungs; most commonly these are invaginated pouches under the carapace (Wirkner and Richter 2013). Terrestrial isopods have lungs that are thin-walled, invaginated, air-filled sacs, often with one or more circular entrances (spiracles) (Hornung 2011; Edney 2012). Some terrestrial isopods, including the Porcellionidae and the Armadillidiidae, have hollow, tuft-like invaginations of the pleopods called pseudotracheae, somewhat similar to insect tracheae that function in gas exchange (Edney 2012). Whether gas is exchanged by diffusion or convection, the invaginated cavity will tend to lower the ratio of water loss to oxygen uptake because the gradient in water loss from the cavity will be relatively constant (with air being nearly saturated with water within the invaginated cavity), whereas the gradient in oxygen transport can be increased by reducing the conductance of the system. Thus, animals with lungs or tracheae can close spiracles or reduce ventilation, thereby reducing respiratory loss of water when the need for oxygen is low.

Tracheal respiratory systems of hexapods have ramifying, air-filled cuticle-lined tubes, mostly with gated spiracles (Chapman et al. 2013). Tracheal systems combine the advantages of an in-folded cavity (lung) with the further benefits of air-based transport of gases between the tissues and atmosphere, thereby elevating maximal rates of metabolism. Tracheal systems are thought to have evolved multiple times within the Ecdysozoa, with independent evolution from aquatic ancestors in arthropods, onychophorans, and tardigrades (Bradley et al. 2009). Within the Arthropoda, tracheae occur in all the major clades of Hexapoda, in three of the major clades of Myriapoda, within the Malacostraca (if the pseudotracheae of terrestrial isopods are considered tracheae), and, outside the Mandibulata, within many of the clades of Chelicerata (Fig. 2).

Evolution of air-breathing in Pancrustacea

Data from molecular clocks suggest that gilled, marine arthropods and pancrustaceans evolved in the Ediacaran or Cambrian (Lin et al. 2006; Legg et al. 2013; Oakley et al. 2013; Rota-Stabelli et al. 2013; Edgecombe and Legg 2014). Fossils of gilled

arthropods are known from the Cambrian. *Marrella* (up to 2 cm long) is one of the most abundant fossils in the Cambrian Burgess Shale; it is considered an arthropod with gills (Whittington 1971). Trilobites were common, now extinct, gilled marine arthropods (Whittington 1997). Fossils from the Malacostraca appeared at this time (and in the Devonian); some were larger than a centimeter (Collette and Hagadorn 2010) and likely required gills. Within the chelicerates, gilled aquatic forms (Xiphosura, Eurypterida, Scorpiones) are known from the Cambrian (Xiphosura), Ordovician (Eurypterida), and Silurian (Scorpiones) (Seldan and Jeram 1989).

Evolution toward a terrestrial habitat has occurred in several subgroups of the Pancrustacea. Some crustacean species take air bubbles into their branchial chambers when their water is hypoxic, a possible first step toward the behavioral utilization of air (McMahon and Wilkes 1983). Among decapods, some brachyuran crabs exhibit varying degrees of terrestriality, with hermit crabs being particularly terrestrial. While terrestriality has occurred relatively often in Malacostraca, even in the most terrestrial species, the integument is relatively permeable to water, and these species generally are restricted to habitats with good access to high humidity or to free water (Edney 2012). Evolution to this level of terrestriality can occur relatively rapidly; Jamaican land crabs have evolved from fully aquatic ancestors in 4 million years (Shubart et al. 1998). Completely terrestrial crustaceans, capable of living and reproducing entirely on land, are within the Oniscidia (Malacostraca: Isopoda: Oniscidia) and the Taltridae (Malacostraca: Amphipoda: Taltridae); however, with a few exceptions, most of these species are still limited to moist environments (Friend and Richardson 1986; Hornung 2011). In both cases, terrestrialization included changes in respiratory structures that likely decreased rates of water loss (Spicer et al. 1987; Hornung 2011). Terrestriality in the oniscideans is estimated to have originated in the Carboniferous (Hornung 2011; Broly et al. 2013). In contrast to the relatively weak capacity of modern crustaceans, terrestrialization within the Chelicerata occurred in the Devonian and Carboniferous for multiple lineages, and many of these are highly resistant to desiccation (Seldan and Jeram 1989).

In contrast to the other Mandibulata, Hexapoda are primarily terrestrial and Myriapoda totally terrestrial; most species within these groups are tracheated. Molecular phylogenies suggest independent and very early evolution of terrestriality and tracheation in these two groups (Fig. 2) (Rota-Stabelli et al. 2013). Myriapods appear to have arisen from a stem group related to modern chelicerates that also gave rise to the pancrustaceans in the Cambrian (Fig. 2). Hexapods descend from an aquatic group that also gave rise to the Remipedia, with this divergence occurring in the Ordovician (Fig. 2). If these dates are correct, both groups arose and terrestrialization occurred nearly coincidently with the first land plants (Rota-Stabelli et al. 2013) and before the evolution of vascular plants (Silurian to Devonian) (Wellman 2014).

The evidence currently available suggests that hexapods and myriapods did not evolve in oceans. Based on phylogeography, Shelly and Golavatch (2011) postulated that the earliest diplopodans arose in the Cambrian, utilizing the land even before the evolution of terrestrial plants. There have been reports of marine hexapods (Haas et al. 2003), but that conclusion was rejected upon later examination (Kühl and Rust 2009). There are Cambrian fossils that have been interpreted as marine myriapods, but this remains controversial (Edgecombe 2004). The earliest known myriapod fossils (diplopods) are from Silurian water-lain deposits, but it is not definitive that these were aquatic animals (Almond 1985). A recent synthetic analysis suggests, based on the lack of marine fossils and on morphological evidence, that the evolution of marine Myriapoda in the Cambrian is improbable (Wägele and Kück 2014). The earliest known accepted hexapod and insect fossils are from the Devonian and are terrestrial (Grimaldi and Engel 2005; Grimaldi 2010; Garrouste et al. 2012). The lack of fossilized marine hexapods and myriapods, as well as the fact that most members of these groups are terrestrial, suggests that both of these taxa evolved on land.

Supporting the fossil evidence for a terrestrial origin of the hexapods and myriapods, several other lines of evidence suggest that tracheal systems must have evolved in air. Most modern, aquatic, gill-breathing insects are secondary derivations from terrestrial forms in holometabolous insects (Wootton 1988; Pritchard et al. 1993; Grimaldi 2010). Possession of an air-filled tracheal system causes insects to be so buoyant that they have limited capacities to utilize deep waters, possibly explaining why they are virtually excluded from off-shore marine waters, and further reducing the likelihood of their having evolving trachea before they became terrestrial (Maddrell 1998). Invagination of an air-filled cuticle is developmentally easy to imagine as happening in air, but perhaps challenging to conceive of it as having occurred under water (Pritchard et al. 1993). Together, these arguments suggest that tracheal systems may have evolved

as early members of the Hexapoda and Myriapoda adapted to terrestriality.

Kukalova-Peck (1983) argued for a freshwater "amphibious" route for insect evolution, with insects first evolving with freshwater juvenile stages and a terrestrial adult stage, as occurs in modern Odonata and Ephemeroptera. With this scenario, one can hypothesize that invaginations that became tracheae and spiracles first developed in the terrestrial adult forms, with embryonic and aquatic juveniles having tracheal systems without spiracles as in most modern insect embryos and some aquatic species. Juvenile Odonata and Ephemeroptera utilize "tracheal gills" to obtain oxygen; these are evaginations of the body wall or within the rectum that are well supplied with tracheae (Wigglesworth 1972). Some dragonfly nymphs have spiracles, but water does not pass into the air-filled tracheal system. Trachea form and become air-filled in the embryos of many modern insects without direct access of the tracheae to air due to capillary and cavitation forces (Woods et al. 2009), and trachea become air-filled underwater in Odonata after each molt of juveniles (Wigglesworth 1953), so this scenario is developmentally and physiologically possible. Thus, the physiological evidence does not refute the Kukalova-Peck hypothesis. However, while Ephemeroptera and Protodonata have an extensive fossil record in the Carboniferous, the oldest hexapod fossils (from the Silurian and Devonian) are collembolans, with the oldest insect fossils likely being from the wingless insect groups Archaeognatha, Zygentoma, and possibly Paleodictyoptera, strongly suggesting that hexapods and insects evolved on land without an amphibious stage (Grimaldi and Engel 2005; Garrouste et al. 2012). If Silurian or Devonian fossil Odonata and Ephemeroptera are found to have had aquatic larvae, this could possibly rejuvenate Kukalova-Peck's creative hypothesis.

Open questions regarding the evolution of tracheal systems

Key open questions remain as to how many times tracheal systems evolved in the Mandibulata, and when. Plausibly, the first terrestrial Myriapod and Hexapod stem-groups might have had gills or been small skin-breathers and been restricted to moist environments like most modern terrestrial crustaceans. Tracheae might have evolved independently within the four hexapod clades and the three tracheated myriapods clades; alternatively, tracheal systems may have evolved at the origin of the Myriapoda, and also at the origin of the Hexapoda. According

to the first scenario, the myriapod stem group diverged into Chilopoda, Symphyla, Pauropoda, and Diplopoda, with the evolution of tracheae (and possibly terrestrialization) occurring independently in each line. Similarly, the hexapod stem group would have diverged into Protura, Collembola, Diplura, and Insecta, with later evolution of terrestriality and tracheation, independently in each group. According to the second scenario, the first myriapod and hexapod would have become terrestrial, and evolved a tracheal system, followed by later diversification. The most definitive evidence supporting independent evolution of tracheal systems in the various lineages (e.g., Collembola, Insecta, Diplopoda, and Chilopoda) would be to find ancestral fossils of these clades with gills. At the moment, the lack of such evidence provides some support for the parsimonious conclusion that tracheal systems evolved just once within the Pancrustacea, i.e., at the origin of the Hexapoda.

In another evolutionary scenario, previously suggested, e.g., Snodgrass (1938) and recently reproposed, tracheal systems evolved only once in the group termed the Tracheata, a subgroup of Mandibulata and including myriapods and hexapods (Wägele and Kück 2014). Wägele and Kück acknowledged that current molecular trees (e.g., Fig. 2) do not find the Myriapoda and Hexapoda to be sister groups, but they argued that systematic errors plague current molecular phylogenies, and that both fossil and morphological data strongly support the alternative hypothesis that Myriapoda and Hexapoda are both descended from a Remipedia-like ancestor that conquered land. These authors base their conclusion on the fact that Myriapoda and Hexapoda share 19 derived characters (many not occurring in crustaceans), including ectodermally-derived Malpighian tubules, a tracheal system with paired, segmental, pleural spiracles, maxillae with two terminal, frontally directed endites, long protopod, and uniramous palps, maxillae two basally fused to form a labrum, a cephalic endoskeleton with anterior tentorial arms, and pleural sclerites forming ringed subcoxa surrounding the coxal insertion.

Clearly, many fundamental questions remain concerning the evolution of the Pancrustacea that will need to be addressed by further molecular and paleontological research. However, further exploration of the physiology of the various clades of Hexapoda and Myriapoda may also help resolve these questions. Like the current Remipedia that likely descended from ancestors of Hexapoda, most of the basal clades of Hexapoda and Insecta (Collembola, Archaeognatha, Zygentoma), as well as some Myriapoda, have blood-borne hemocyanin in addition to tracheal

systems (T. Burmester, this volume). As yet, we understand little about how hemocyanin is used in insects, although in an aquatic, juvenile Plecoptera, the oxygen-binding affinity is appropriate for oxygen transport (Hagner-Holler et al. 2004). As in modern crustaceans, it is reasonable to imagine that the ancestor of terrestrial hexapods relied on gills or on skin perfused with hemocyanin-containing blood for oxygen exchange. During the early evolution of the tracheal system, as today in some insects and spiders, it is likely that not all tissues received a tracheal supply, and these tissues may have been reliant on hemocyanin-born oxygen. Study of oxygen transport in the basal clades of Hexapoda that utilize mixed tracheal and hemocyanin-based transport of oxygen may be particularly helpful in understanding which scenarios might be plausible for the evolution of terrestriality. In addition, it would be very useful to have more information on the structure of the tracheal systems of Mandibulata; plausibly, structural features of the tracheal system could be found that could help determine whether tracheal systems are homologous or convergent in Myriapoda and Hexapoda, as well as within Insecta, Protura, Collembola, and Diplura.

Study of the developmental genes and processes associated with the production of respiratory structures could also be very enlightening. In arachnids, several developmental genes commonly are expressed in book lungs and tracheae, suggesting common developmental processes (Damen et al. 2002). There is great variation in the location of spiracles and other structures of the tracheal system, with variation within clades being similar to that across clades, even across the Ecdysozoa (Klass and Kristensen 2001). The developmental biology of respiratory structures is an almost unexplored, important area of comparative biology that may provide answers to questions about evolutionary history and how species adapt to different environments.

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Supplementary data

Supplementary Data available at *ICB* online.

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