

## RESEARCH ARTICLE

# Cuticular gas exchange by Antarctic sea spiders

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## ABSTRACT

Many marine organisms and life stages lack specialized respiratory structures, like gills, and rely instead on cutaneous respiration, which they facilitate by having thin integuments. This respiratory mode may limit body size, especially if the integument also functions in support or locomotion. Pycnogonids, or sea spiders, are marine arthropods that lack gills and rely on cutaneous respiration but still grow to large sizes. Their cuticle contains pores, which may play a role in gas exchange. Here, we examined alternative paths of gas exchange in sea spiders: (1) oxygen diffuses across pores in the cuticle, a common mechanism in terrestrial eggshells, (2) oxygen diffuses directly across the cuticle, a common mechanism in small aquatic insects, or (3) oxygen diffuses across both pores and cuticle. We examined these possibilities by modeling diffusive oxygen fluxes across all pores in the body of sea spiders and asking whether those fluxes differed from measured metabolic rates. We estimated fluxes across pores using Fick's law parameterized with measurements of pore morphology and oxygen gradients. Modeled oxygen fluxes through pores closely matched oxygen consumption across a range of body sizes, which means the pores facilitate oxygen diffusion. Furthermore, pore volume scaled hypermetrically with body size, which helps larger species facilitate greater diffusive oxygen fluxes across their cuticle. This likely presents a functional trade-off between gas exchange and structural support, in which the cuticle must be thick enough to prevent buckling due to external forces but porous enough to allow sufficient gas exchange.

**KEY WORDS:** Arthropod, Cuticle, Metabolism, Oxygen, Polar gigantism, Pycnogonids

## INTRODUCTION

Many animals exchange some or all of their respiratory gases across their outer integument (i.e. skin or cuticle), a process known as cutaneous respiration (Graham, 1988; Feder and Burggren, 1985). The proportion of total gas exchanged across the outer integument varies across animals, especially among those that live in marine habitats (Feder and Burggren, 1985). Small animals, with relatively thin integuments and relatively low metabolic rates (e.g. flatworms and most marine larvae), rely on diffusion alone (Graham, 1988). By contrast, many larger marine organisms (e.g. many adult vertebrates and arthropods) have relatively thick integuments (skin or cuticle), such that oxygen diffusion alone cannot supply their higher metabolic rates, which are distributed throughout larger

volumes. Instead, these groups often have specialized and highly ramified structures (e.g. gills), where most gas exchange occurs. Nevertheless, cutaneous respiration plays a role in all of these groups (Graham, 1988). For example, even in many adult fish, ~30% of oxygen uptake occurs across the integument (Weibel et al., 1998). Therefore, understanding the mechanisms of cutaneous respiration is important to understanding the physiology of marine animals.

We studied cutaneous respiration in sea spiders (phylum Arthropoda, class Chelicerata). Sea spiders are a basal group in the Arthropoda (Arango and Wheeler, 2007) that lack specialized respiratory structures such as gills, but nevertheless can grow to substantial sizes (Moran and Woods, 2012; Child, 1995; Arnaud and Bamber, 1987; Dell, 1972). Sea spiders have reduced thoraces and abdomens and many of their metabolically active tissues, like guts and gonads, are distributed into their legs (Davenport et al., 1987), which reduces the distance oxygen must travel through the body by diffusion. Although sea spiders have been studied for over 150 years (see reviews by Arnaud and Bamber, 1987; King, 1973), we still lack an understanding of the functional morphology of the cuticle as it relates to gas exchange. An obvious possibility is that oxygen diffuses via the pores that appear to cross the cuticles of most species (Fahrenbach, 1994; Davenport et al., 1987; King, 1973), but this idea has never been tested. Here, we tested the role of cuticular pores in oxygen diffusion across the cuticle of sea spiders using a combination of empirical measurements and mathematical modeling.

There are several different avenues by which oxygen may cross the cuticle. First, as hypothesized by Davenport et al. (1987), oxygen may diffuse in through pores that cross the cuticle. In insects, the diffusion coefficient of oxygen through chitin is ~4% that of the diffusion coefficient through water (Krogh, 1919), which is too low for adequate diffusive supply of oxygen unless the cuticle is extremely thin (e.g. ~0.2–1.2  $\mu\text{m}$  in the order Plecoptera; Resh et al., 2008). If the diffusion coefficient of oxygen through sea spider cuticle is similar to that of insect cuticle, then pores may permit much higher oxygen fluxes by reducing the amount of solid cuticle through which oxygen must diffuse. Pores in other cutaneous-respiring organisms appear to support almost all of the flux, especially when the integument is also used for support. For example, the pores in sea spiders are morphologically similar to those described from vertebrate and invertebrate eggshells (Rokitka and Rahn, 1987; Kern and Ferguson, 1997; Woods et al., 2005). These pores have been studied relatively extensively, and they do support almost all of the oxygen flux to the embryo (Wangenstein et al., 1971; Paganelli, 1980; Hinton, 1981; Tøien et al., 1988; Rahn and Paganelli, 1990).

Alternatively, cuticular pores may not facilitate oxygen flux; they may be incidental or have some other function (e.g. secretion). Cuticular pores have long been considered to have a secretory function (Fahrenbach, 1994), and it is possible they serve a dual function (secretion and gas exchange). Sea spiders are chelicerates, and the structure of chelicerate cuticle is, in general,

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**List of symbols and abbreviations**

$A_c$	cross-sectional area of cuticle segment at top of pore ( $\text{cm}^2$ )
$A_n$	cross-sectional area of segment within pore ( $\text{cm}^2$ )
$d$	diameter of pore segment within model (cm)
$d_c$	diameter of top of pore
$d_{20}$	diameter of bottom of pore
$D$	diffusion coefficient
$D_c$	diffusion coefficient of oxygen in cuticle ( $\text{cm}^2 \text{s}^{-1}$ )
$D_t$	diffusion coefficient of oxygen in tissue ( $\text{cm}^2 \text{s}^{-1}$ )
$D_w$	diffusion coefficient of oxygen in seawater ( $\text{cm}^2 \text{s}^{-1}$ )
$G_{\text{pore}}$	conductance through single pore ( $\mu\text{mol s}^{-1} \text{kPa}^{-1}$ )
$J_a$	oxygen flux through pores across whole body ( $\mu\text{mol s}^{-1}$ )
$J_{\text{pore}}$	oxygen flux through a single pore ( $\mu\text{mol s}^{-1}$ )
$k$	constant, $RT/D$
$k_c$	$k$ for diffusion coefficient of oxygen in cuticle, $RT/D_c$ ( $\text{cm kPa s } \mu\text{mol}^{-1}$ )
$k_t$	$k$ for diffusion coefficient of oxygen in muscle tissue, $RT/D_t$ ( $\text{cm kPa s } \mu\text{mol}^{-1}$ )
$k_w$	$k$ for diffusion coefficient of oxygen in seawater, $RT/D_w$ ( $\text{cm kPa s } \mu\text{mol}^{-1}$ )
$l_c$	thickness of segment of cuticle above pore (cm)
$l_p$	thickness of segment within pore (cm)
$M$	body mass
OLS	ordinary least squares regression
PD	pore density (pores $\text{cm}^{-2}$ )
PGLS	phylogenetic least squares regression
$R$	gas constant ( $\text{cm}^3 \text{kPa } \mu\text{mol}^{-1} \text{K}^{-1}$ )
$R_{\text{bottom}}$	boundary layer resistance at bottom of pore ( $\text{kPa s } \mu\text{mol}^{-1}$ )
$R_{\text{cuticle}}$	resistance of thin piece of cuticle at top of pore ( $\text{kPa s } \mu\text{mol}^{-1}$ )
$R_{\text{seg}}$	resistance of each segment within pore ( $\text{kPa s } \mu\text{mol}^{-1}$ )
$R_{\text{top}}$	boundary layer resistance at top of pore ( $\text{kPa s } \mu\text{mol}^{-1}$ )
$R_{\text{tot}}$	total summed resistance ( $\text{kPa s } \mu\text{mol}^{-1}$ )
$S_3$	surface area of whole animal based on 3D shape ( $\text{cm}^2$ )
$T$	temperature (K)
$V_{\text{pore}}$	pore volume across whole animal ( $\text{cm}^3$ )
$x$	cuticle thickness or pore length (cm)
$\Delta P_{\text{O}_2}$	oxygen gradient (kPa)

morphologically and chemically similar to the cuticles of insects and other arthropods (Davenport et al., 1987; Nentwig, 1987). The thickness of sea spider cuticle ranges from 20 to 150  $\mu\text{m}$ , depending on body size (Lane et al., 2017), which is similar to observed thicknesses of insect cuticle (1 to more than 200  $\mu\text{m}$ ) (Vincent and Wegst, 2004) and slightly thicker than *Limulus* gill cuticle (3–10  $\mu\text{m}$ ) (Henry et al., 1996). Sea spiders, however, have unsclerotized and non-calcified cuticle and a microstructure that resembles the thin cuticle of the gills of *Limulus* and Malacostraca (Fahrenbach, 1994). These observations suggest that even non-porous sea spider cuticle may support high rates of oxygen diffusion. Therefore, sea spider cuticle may support relatively high diffusion coefficients of oxygen or, at least in small sea spider species, it may be thin enough to permit adequate direct diffusion of oxygen even through chitin, as has been observed in many aquatic insects (Weis-Fogh, 1964; Eriksen, 1986; Kehl and Dettner, 2009; Seymour and Matthews, 2013).

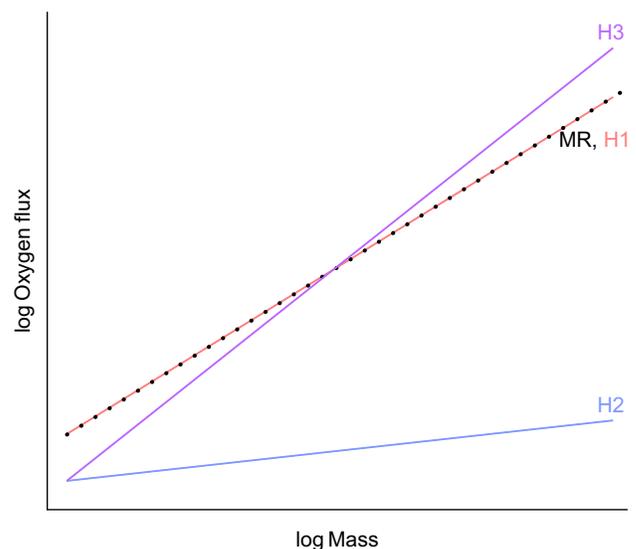
Finally, there is an intermediate scenario: each of the two pathways, pores and cuticle, supports some flux. If so, then a third hypothesis is that their relative importance changes in a size-dependent way, where smaller species with relatively thin cuticle take up oxygen directly across the cuticle and through pores while larger species take up oxygen only through pores.

To determine whether pores permit most or all of the inward flux across a range of taxa and body sizes, we measured the density and size of pores and estimated the diffusive fluxes of oxygen across sea

spider cuticle. Diffusive fluxes were estimated using a mathematical model based on Fick's law (Fick, 1855; Toïen et al., 1988) that incorporated the shape of the pores and the oxygen gradient between the external and internal environments. This model allowed us to quantitatively estimate the rates of flux across individual pores, which, along with estimates of pore density, we scaled up to whole-animal fluxes of oxygen. We then compared the scaling of oxygen flux to the scaling of metabolic rate, to test the following three hypotheses (Fig. 1). (1) If pores facilitate most of the oxygen movement, then the total flux attributable to pores should closely match metabolic demand across a range of taxa and body sizes. (2) If the pores do not facilitate oxygen diffusion, then the total flux through pores should be lower than that of metabolic demand across a range of taxa and body sizes. (3) If the relative importance of pores varies in a size-dependent way, then small individuals with thin cuticles may obtain sufficient oxygen directly across the cuticle, but large individuals with relatively thick cuticles may obtain larger fractions through their pores.

**MATERIALS AND METHODS**

We collected sea spiders while SCUBA diving at sites in McMurdo Sound, Antarctica, in 2015 and 2016. Animals were brought to Cray Lab, McMurdo Station, and kept in seawater tables at  $-1$  to  $0^\circ\text{C}$  (ambient seawater temperature  $\sim -1.8^\circ\text{C}$ ). Individuals were used within 2 weeks of collection. We collected data from 10 different species of sea spiders in five different families [Ammonotheidae: *Ammonothea glacialis* (Hodgson 1907), *Ammonothea longispina* Gordon 1932, *Ammonothea* sp.; Colossendeidae: *Colossendeis hoeki* Gordon 1944, *Colossendeis megalonyx* Hoek 1881, *Colossendeis scotti* Calman 1915; Nymphonidae: *Nymphon australe* Hodgson 1902, *Pentanyphon antarcticum* Hodgson 1904; Pallenopsidae:



**Fig. 1. Hypothesized scaling of measured metabolic rate and modeled rates of oxygen flux across pores.** Metabolic rate (MR; dotted line) was taken from Lane et al. (2017). Hypothesis 1 (H1; red line): if pores facilitate most of the oxygen movement, then the total flux attributable to pores should closely match metabolic demand across a range of taxa and body sizes. Hypothesis 2 (H2; blue line): if the pores do not facilitate oxygen diffusion, then the total flux through pores should be lower than metabolic demand across a range of taxa and body sizes. Hypothesis 3 (H3; purple line): if the relative importance of pores varies in a size-dependent way, then small individuals with thin cuticles may obtain sufficient oxygen directly across the cuticle, but large individuals with relatively thick cuticles may obtain larger fractions through their pores.

*Pallenopsis patagonica* (Hoek 1881); Pycnogonidae: *Pycnogonum gaini* Bouvier 1910]. Because we do not know the age of the animals or time since last molt, the animals used in this study were chosen without bias to cuticle condition to try and minimize any variation in cuticle condition based on age or time since last molt.

### Morphological measurements

We weighed and photographed each individual. Each individual was blotted dry, to remove excess water, and then weighed on a microbalance ( $\pm 0.001$  g). Photographs were taken of the dorsal side, with the animal placed flat with its legs fully extended, using a Nikon D7100 digital camera (Nikon Inc., Melville, NY, USA) attached to a tripod. Surface area was estimated in ImageJ (v1.49, <http://imagej.nih.gov/ij/>) by tracing around the individual and multiplying by two. To account for the three-dimensional shape of the animal, we treated the body of a sea spider as an open cylinder with a calculated surface area of  $S_3 = 2\pi rL$ , where  $r$  and  $L$  are the radius and length of a leg segment, versus the surface area of a two-dimensional rectangle ( $S_2$ ) of the same diameter and length ( $S_2 = 2 \times 2rL$ ). These two estimates of surface area are related by the expression  $S_3/S_2 = \pi/2$ , and so  $S_3 = 1.57 \times S_2$ , where  $S_2$  was the surface area found using ImageJ.

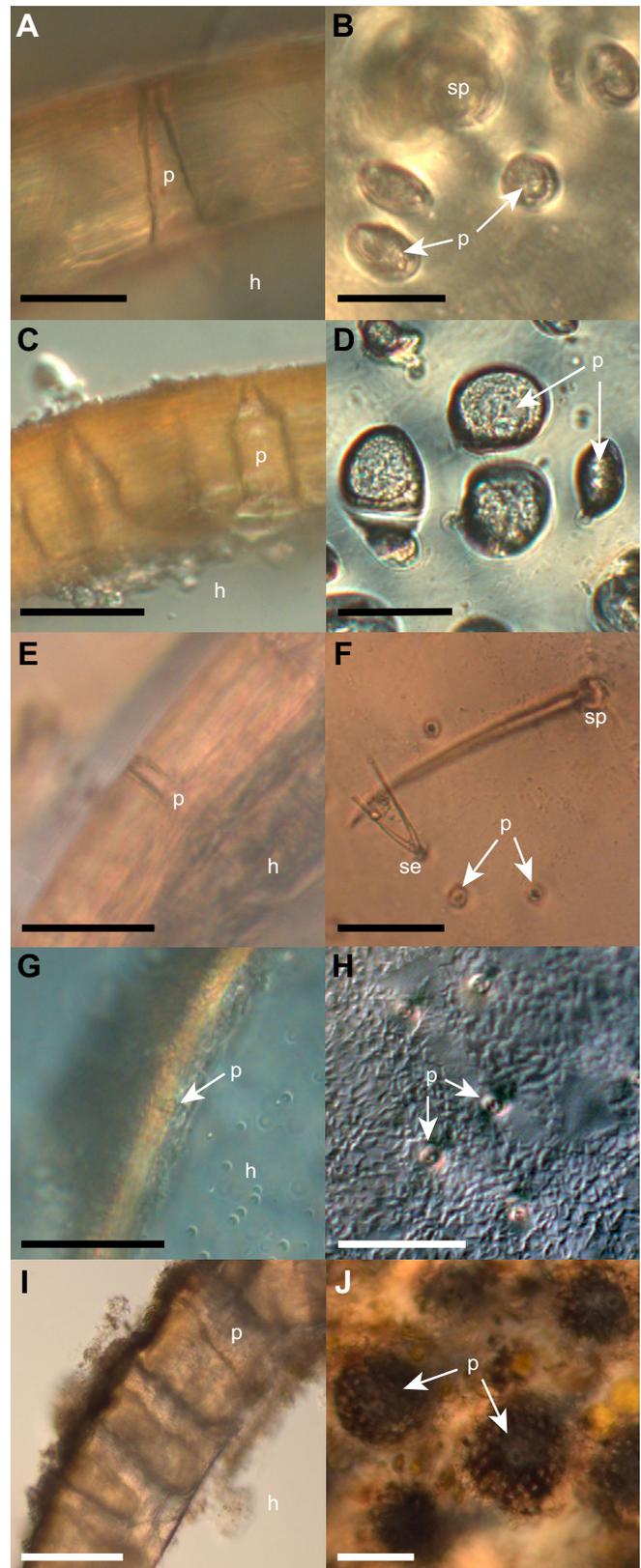
To measure cuticle thickness, or pore length ( $x$ , cm), we prepared multiple thin sections ( $< 1$  mm each) of a single femur per individual using a razor blade. The thin sections were then mounted under a compound microscope and imaged (Zeiss AxioScope, Zeiss International, Oberkochen, Germany). From those images, we extracted cuticle thickness, which closely approximates pore length. Values were averaged per individual and then per species. Images of each cross-section (e.g. Fig. 2A,C,E,G,I) were taken using a camera mounted on a compound microscope and then analyzed in ImageJ.

We estimated pore density by taking multiple longitudinal sections of a single femur from each animal using a razor blade. Longitudinal images (e.g. Fig. 2B,D,F,H,J) were taken using a camera mounted on a compound microscope (Zeiss AxioScope). In ImageJ, pore density (PD, pores  $\text{cm}^{-2}$ ) was calculated as the total number of pores in a measured area, which were counted and averaged per individual and then per species. To calculate pore volume ( $V_{\text{pore}}$ ,  $\text{cm}^3$ ), the total volume of the cuticle that is porous, we calculated the average volume of a single pore and then multiplied it by the total number of pores for each animal. The total number of pores was estimated by taking pore density (PD) and multiplying it by the surface area ( $S_3$ ). As with cuticle thickness above, these values were averaged per species.

The above traits were measured in the femur of 10 different species. To determine whether cuticle and pore structure varied by leg segment, however, we also used the same methods to measure these traits on the 1st and 2nd tibias of three of those species: *Ammothea glacialis* ( $N=8$ ), *Colossendeis megalonyx* ( $N=8$ ) and *Nymphon australe* ( $N=8$ ).

### Oxygen gradient

Internal oxygen levels were measured within a single femur from each individual sea spider ( $n=46$ , 2–8 of each species) using a 100  $\mu\text{m}$  tip Clark-style oxygen electrode (Unisense, Aarhus, Denmark) positioned using a micromanipulator. The leg was removed underwater at the proximal end of the third coxa, and the electrode tip was immediately advanced into the center of the femur. Each measurement took place within a water-jacketed glass platform. Seawater temperature was maintained at  $-1^\circ\text{C}$  using a recirculating water bath. We also measured external oxygen levels 1 cm away from the leg segment. The electrode was connected to a



**Fig. 2.** Pore structure for one representative of each sea spider family in the study. Images on left side are from cross-sections and those on the right side are from longitudinal sections. (A,B) Ammotheidae, (C,D) Colossendeidae, (E,F) Nymphonidae, (G,H) Pallenopsidae, (I,J) Pycnogonidae. p, pore; h, hemocoel; sp, cuticular spines; se, cuticular setae. Scale bar: 50  $\mu\text{m}$  in A–F, H and J; 25  $\mu\text{m}$  in G; 100  $\mu\text{m}$  in I.

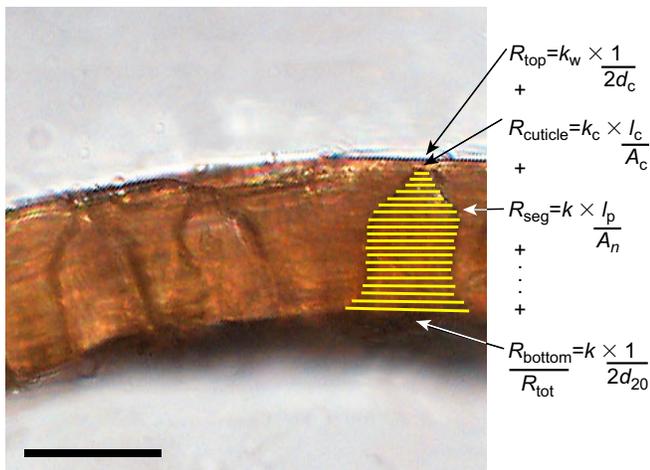
picoammeter (PA2000, Unisense), and data were recorded onto a computer running Expedata (v1.8.4, Sable Systems, North Las Vegas, NV, USA). Prior to each measurement, the electrode was calibrated in  $N_2$  and air-saturated seawater at the measurement temperature ( $\sim -1^\circ C$ ). Internal  $P_{O_2}$  was then subtracted from external  $P_{O_2}$  to estimate the transcuticular oxygen gradient for each animal.

### Oxygen consumption

We used data on oxygen consumption from Lane et al. (2017), which was obtained using closed-system respirometry. See Lane et al. (2017) for methods and data values.

### Model of oxygen flux through pores

We estimated resistance to oxygen across the cuticular pores following the approach of Tøien et al. (1988), who analyzed diffusive resistance of bird eggshells using Fick's first law (Fick, 1855). We made 20 cross-sectional measurements across a single pore from each individual (Fig. 3). These measurements were made in at least three pores from each individual and then the estimated resistances were averaged. We used ImageJ to perform a segment analysis of pore morphology. Pore shape can vary substantially within an individual (e.g. Fig. 2C,I). For this analysis, however, we measured only pores that had the most common pore shape (shape



**Fig. 3. Oxygen flux through cuticular pores.** Diagram of pore divided into 20 equally separated segments (yellow lines). Resistance was calculated as the sum of the resistances for all 20 segments and the layer of cuticle immediately above the pore (Tøien et al., 1988).  $R_{top}$  ( $kPa\ s\ \mu mol^{-1}$ ), which represents boundary layer resistance at the top of the pore, is inversely proportional to two times the segment diameter immediately below the layer of cuticle ( $d_c$ , cm). Constant  $k_w$  ( $cm\ kPa\ s\ \mu mol^{-1}$ ) was calculated based on the oxygen diffusion coefficient through seawater.  $R_{bottom}$ , conversely, represents the boundary layer resistance at the bottom of the pore and is inversely proportional to two times the segment diameter at the bottom of the pore ( $d_{20}$ ) multiplied by  $k$ , which was calculated based on the diffusion coefficient of oxygen through seawater or muscle tissue.  $R_{cuticle}$  represents the resistance through the portion of cuticle above the pore.  $l_c$  (cm) is the thickness of the cuticular cap above the pore and was divided by the cross-sectional area ( $A_c$ ,  $cm^2$ ) of the first segment, assuming the segment is circular. The following middle segment resistances ( $R_{seg}$ ) were calculated using the thickness of each segment through the pore ( $l_p$ ), which was the same for each segment, divided by the cross-sectional area ( $A_n$ ).  $A_n$  was calculated by measuring the segmental pore diameter ( $d_n$ ) and assuming each segment is circular. The constant  $k$  was calculated as  $k=RT/D$  ( $cm\ kPa\ s\ \mu mol^{-1}$ ), where  $R$  is the gas constant,  $T$  is temperature and  $D$  is the diffusion coefficient of the material (water, cuticle or tissue). See Materials and methods for more details. Scale bar: 50  $\mu m$ .

shared by at least 65% of total pores per species) for each given species (e.g. identified with 'p' in Fig. 2A,C,E,G,I). We calculated resistance for a given cross-sectional segment using Fick's law:

$$R_{seg} = k \times \frac{l_p}{A_n}, \quad (1)$$

where  $R_{seg}$  is the resistance of each segment within the pore in  $kPa\ s\ \mu mol^{-1}$  (Fig. 3),  $l_p$  is the thickness of each cross-sectional segment in cm and  $A_n$  is the cross-sectional area of each segment in  $cm^2$ .  $k$  is a constant equal to  $R \times T/D$  in  $cm\ kPa\ s\ \mu mol^{-1}$ , where  $R$  is the gas constant in  $cm^3\ kPa\ \mu mol^{-1}\ K^{-1}$ ,  $T$  is temperature in K, and  $D$  is the diffusion coefficient of oxygen in the body fluid within the pores in  $cm^2\ s^{-1}$ . Because we did not know the diffusion coefficient of oxygen in the fluid within the pores, we bracketed our results by running two separate models with different estimates of the oxygen diffusion coefficient. One model was run by calculating  $k$  using the diffusion coefficient of oxygen in seawater ( $9.86 \times 10^{-6}\ cm^2\ s^{-1}$ ) at the experimental temperature ( $\sim 0^\circ C$ ). This model used  $k_w$  in place of  $k$  to calculate resistance of each cross-sectional segment ( $R_{seg}$ , Eqn 1). The second model assumed that the pores are filled with muscle tissue. Although pores likely do not contain muscle, the diffusion coefficients of oxygen in muscle are relatively well known and provide a lower limit for possible diffusion coefficients in our system. For this model, we set  $k$  as  $k_t$  for calculating resistance at each cross-sectional segment ( $R_{seg}$ , Eqn 1).  $k_t$  was calculated using the diffusion coefficient of oxygen in muscle tissue ( $3.95 \times 10^{-6}\ cm^2\ s^{-1}$ ). We estimated the diffusion coefficient of oxygen through muscle tissue based on Krogh (1919) and Davenport et al. (1987), who estimated that the diffusion coefficient of oxygen in muscle tissue is approximately 40% of that in seawater.

All studied species had a small layer of cuticle separating the top of the pore from the external environment. We estimated the resistance of this thin piece of cuticle as:

$$R_{cuticle} = k_c \times \frac{l_c}{A_c}, \quad (2)$$

where  $k_c$  is the constant  $k$  described above calculated using the diffusion coefficient of oxygen in chitin. This diffusion coefficient was also estimated based on Krogh (1919), where he calculated that the diffusion coefficient of oxygen through chitin is approximately 4% that of seawater ( $3.95 \times 10^{-7}\ cm^2\ s^{-1}$ ).  $l_c$  is the thickness of the cuticle layer (measured in ImageJ) and  $A_c$  is the cross-sectional area of the cuticle directly above the pore.

Like Tøien et al. (1988), we also included boundary layer resistance at the top and bottom of the pore ( $R_{top}$  and  $R_{bottom}$ , respectively; Fig. 2) using Stefan's law (Meidner and Mansfield, 1968), as shown for  $R_{top}$ :

$$R_{top} = k_w \times \frac{1}{2 \times d_c}, \quad (3)$$

where  $d_c$  is the diameter of the top of the pore (top cross-sectional measurement; Fig. 3).  $R_{bottom}$  was calculated similarly, except it used the constant  $k_w$  or  $k_t$  (depending on the model), and  $d_{20}$  in place of  $d_c$ , which was the diameter of the bottom of the pore. We estimated total resistance ( $R_{tot}$ ) by summing the resistances for all the middle segments ( $R_{cuticle}$  and  $R_{seg}$ ) and the boundary layer resistances ( $R_{top}$  and  $R_{bottom}$ ) (Fig. 3). From  $R_{tot}$ , we calculated

conductance through a single pore ( $G_{\text{pore}}$ ,  $\mu\text{mol s}^{-1} \text{kPa}^{-1}$ ):

$$G_{\text{pore}} = \frac{1}{R_{\text{tot}}}, \quad (4)$$

where conductance is a measure of how fast oxygen moves across a material given a difference in partial pressure (Dejours, 1981).

The diffusive oxygen flux through a single pore ( $J_{\text{pore}}$ ,  $\mu\text{mol s}^{-1}$ ) was then calculated using Fick's first law (Dejours, 1981):

$$J_{\text{pore}} = G_{\text{pore}} \times \Delta P_{\text{O}_2}, \quad (5)$$

where  $\Delta P_{\text{O}_2}$  is the oxygen gradient in kPa, and  $J_{\text{pore}}$  is the number of moles of oxygen moving across a single pore per unit time. We then scaled this up to the whole body ( $J_{\text{a}}$ ) by multiplying the diffusive oxygen flux across a single pore by the density of pores (PD, pores  $\text{cm}^{-2}$ ) and then the total calculated surface area of the animal ( $S_3$ ,  $\text{cm}^2$ ):

$$J_{\text{a}} = J_{\text{pore}} \times (\text{PD} \times S_3). \quad (6)$$

We then compared whole-body oxygen flux for a given species with the species' oxygen consumption (Lane et al., 2017) to determine how well the total diffusive oxygen flux through the pores matched whole-animal oxygen uptake.

Because most oxygen uptake occurs across the sea spider's legs (Davenport et al., 1987), we focused our analyses on the pore morphology and physiology of the major leg segments. To determine whether there was any variation among segments within individuals and species, we estimated oxygen conductance through an average pore on the femur, first tibia and second tibia of three species [*Ammothea glacialis* ( $n=8$ ), *Colossendeis megalonyx* ( $n=8$ ) and *Nymphon austral* ( $n=3-4$ )] following the methods described above. Using a linear mixed effects model, with individual as a random factor, we did not find any differences between leg segments for any of the three species for any of the measured variables (Tables S1 and S3). We therefore used pore characteristics from just the femur of each species, which were extrapolated to the whole animal using Eqn 6.

### Statistical analyses

For all scaling analyses, we measured body mass, pore diameter, pore length, pore density, pore volume, oxygen flux and oxygen consumption in 10 different species (from five different families) of sea spiders (Tables S4 and S5). To compare how those traits changed with body size, we  $\log_{10}$ -transformed the data and fitted ordinary least squares regressions (OLS). For each trait, we took the average measurements from 2–8 individuals, which varied depending on the trait. However, for several species (*Ammothea* sp., *Colossendeis hoeki*, *Pallenopsis patagonica*, *Pentanyphon antarcticum* and *Pycnogonum gaini*), we collected data on pore density from only one individual.

To account for potential phylogenetic effects on traits, we also conducted phylogenetic least squares regressions (PGLS) on the data following the procedures of Lane et al. (2017). We constructed trees using mitochondrial cytochrome *c* oxidase (CO1) sequences collected from our samples and then conducted PGLS analyses using: (1) an unconstrained phylogeny built from our CO1 data (PGLS-mtCO1) and (2) a constrained phylogeny following the tree topology of Arango and Wheeler (2007) in which branch lengths were free to vary based on our CO1 data (PGLS-var. brlens). The tree from Arango and Wheeler (2007) was constructed using three nuclear and three mitochondrial genes from 63 different species, representing all 9 sea spider families. We also created a third tree

using the constrained topology above but with equal branch lengths (PGLS-equal brlens). As in the OLS models described above, we used species averages and  $\log_{10}$ -transformed the data prior to running each PGLS model.

We then used Pagel's lambda (Pagel, 1999) to test for phylogenetic signal. Only two variables showed a significant phylogenetic signal (pore diameter and pore density; Table S2). In the two cases where we detected a phylogenetic signal, the PGLS model results are reported in the text and figures (only PGLS-equal brlens are reported, for brevity); otherwise, the OLS results were reported (see Table 1 and Table S2 for full results).

All statistical analyses were conducted in R (v3.3.0, <http://www.R-project.org/>) and the PGLS models were conducted using the R package 'ape' (v3.5) (Pagel, 1992). Data are reported as means $\pm$ s.e.m.

## RESULTS

### Interspecific variation in pore morphology

A phylogenetic signal was detected for pore diameter ( $d$ ,  $\mu\text{m}$ ), pore density (PD, pores  $\text{cm}^{-2}$ ) and pore volume ( $V_{\text{pore}}$ ,  $\text{cm}^3$ ) with the PGLS-equal brlens model (Table S2), so the PGLS results are presented here, while no signal was detected for pore length ( $x$ ,  $\mu\text{m}$ ) (Table S2), so the OLS results are presented here (see Table 1 for regression summary of all models). Pore length and pore diameter both increased with body size (Fig. 4A,B, respectively, Table 1). The relationship between pore length ( $x$ ,  $\mu\text{m}$ ) and body mass ( $M$ , g) is  $x = -2.30 \times M^{0.37}$ . For pore diameter ( $d$ ,  $\mu\text{m}$ ) the relationship with body size is  $d = 1.17 \times M^{0.30}$ . Pore density decreased slightly with increasing body size as  $\text{PD} = 4.32 \times M^{-0.23}$  (Fig. 4C). *Pycnogonum gaini*, which had substantially fewer pores per square centimeter than expected for its body size (over one order of magnitude lower), was treated as an outlier and was not included in the pore density analysis. Total pore volume of the entire cuticle ( $V_{\text{pore}}$ ,  $\text{cm}^3$ ) increased with increasing body size as  $V_{\text{pore}} = -2.56 \times M^{1.35}$  (Fig. 4D).

### Oxygen flux and oxygen consumption

We did not observe significant differences when diffusive oxygen flux was estimated using an assumed diffusion coefficient for water or tissue (Table 1). Therefore, for brevity, only the tissue model is described here.

Diffusive oxygen flux across the cuticular pores and oxygen consumption both increased with body size (Fig. 5, Table 1). The relationship between oxygen flux ( $J_{\text{a}}$ ,  $\mu\text{mol s}^{-1}$ ), using the tissue model, and body size is  $J_{\text{a}} = -4.13 \times M^{1.01}$ . The relationship between oxygen consumption (OC,  $\mu\text{mol s}^{-1}$ ) and body size is  $\text{OC} = -3.96 \times M^{0.80}$ . Furthermore, the regression coefficient for slope and intercept did not vary between oxygen flux and oxygen consumption based on overlapping confidence intervals (Table 1).

## DISCUSSION

Although we cannot formally disprove hypothesis 2, that gas exchange occurs across solid cuticle, our data support hypothesis 1, that sea spiders take up oxygen primarily via cuticular pores. Broadly speaking, there was a close match between the scaling of known rates of oxygen uptake (based on our measurements of metabolic rate; Lane et al., 2017) and the scaling of calculated rates of oxygen flux via pores. Those fluxes match closely over two orders of magnitude in body size. Nevertheless, our data also suggest some size dependence of the relative contribution of pore-based fluxes (H3, Fig. 1). In large-bodied species, estimated pore-based fluxes were higher than measured metabolic rates, whereas in

Table 1. Summary of OLS and PGLS regression analyses

Model	<i>N</i>	<i>a</i>	95% CI	<i>P</i>	<i>b</i>	95% CI	<i>P</i>	<i>R</i> <sup>2</sup>
<i>x</i> vs <i>M</i>								
OLS	10	-2.30	-2.53, -2.06	<0.001	0.37	0.00, 0.74	0.049	0.33
PGLS-mtCO1	10	-2.44	-2.93, -1.94	<0.001	0.18	-0.19, 0.56	0.286	
PGLS-var. brlens	10	-2.35	-2.74, -1.96	<0.001	0.20	-0.20, 0.59	0.281	
PGLS-equal brlens	10	-2.28	-2.74, -1.82	<0.001	0.23	-0.15, 0.60	0.196	
<i>d</i> vs <i>M</i>								
OLS	10	1.14	0.93, 1.35	<0.001	0.42	0.08, 0.76	0.020	0.45
PGLS-mtCO1	10	1.06	0.63, 1.49	<0.001	0.29	-0.03, 0.61	0.069	
PGLS-var. brlens	10	1.12	0.80, 1.44	<0.001	0.30	-0.02, 0.63	0.064	
PGLS-equal brlens	10	1.17	0.82, 1.52	<0.001	0.30	0.02, 0.58	0.038	
PD vs <i>M</i>								
OLS	9	4.33	4.25, 4.41	<0.001	-0.12	-0.23, 0.00	0.053	0.35
PGLS-mtCO1	9	4.31	4.14, 4.48	<0.001	-0.23	-0.35, -0.11	0.003	
PGLS-var. brlens	9	4.31	4.18, 4.44	<0.001	-0.23	-0.35, -0.11	0.003	
PGLS-equal brlens	9	4.32	4.17, 4.48	<0.001	-0.23	-0.35, -0.11	0.003	
<i>V</i> <sub>pore</sub> vs <i>M</i>								
OLS	10	-2.51	-3.03, -1.99	<0.001	1.79	0.97, 2.61	0.001	0.73
PGLS-mtCO1	10	-2.89	-4.04, -1.75	<0.001	1.25	0.40, 2.11	0.010	
PGLS-var. brlens	10	-2.72	-3.60, -1.84	<0.001	1.28	0.39, 2.17	0.010	
PGLS-equal brlens	10	-2.56	-3.54, -1.57	<0.001	1.35	0.55, 2.14	0.005	
<i>J</i> <sub>a</sub> vs <i>M</i> <sup>*</sup>								
OLS	10	-3.84	-4.09, -3.60	<0.001	0.99	0.60, 1.37	<0.001	0.79
PGLS-mtCO1	10	-3.80	-4.38, -3.22	<0.001	1.04	0.60, 1.48	<0.001	
PGLS-var. brlens	10	-3.86	-4.31, -3.41	<0.001	1.04	0.59, 1.48	<0.001	
PGLS-equal brlens	10	-3.89	-4.46, -3.32	<0.001	1.03	0.56, 1.49	<0.001	
<i>J</i> <sub>a</sub> vs <i>M</i> <sup>†</sup>								
OLS	10	-3.94	-4.17, -3.70	<0.001	1.01	0.64, 1.39	<0.001	0.81
PGLS-mtCO1	10	-3.91	-4.48, -3.34	<0.001	1.01	0.59, 1.44	<0.001	
PGLS-var. brlens	10	-3.96	-4.40, -3.53	<0.001	1.01	0.57, 1.44	<0.001	
PGLS-equal brlens	10	-3.99	-4.55, -3.43	<0.001	1.00	0.55, 1.45	0.001	
OC vs <i>M</i>								
OLS	10	-3.96	-4.15, -3.77	<0.001	0.80	0.52, 1.08	<0.001	0.83
PGLS-mtCO1	10	-4.03	-4.47, 3.59	<0.001	0.77	0.46, 1.07	<0.001	
PGLS-var. brlens	10	-4.01	-4.34, -3.69	<0.001	0.77	0.46, 1.07	<0.001	
PGLS-equal brlens	10	-3.99	-4.39, -3.60	<0.001	0.75	0.47, 1.04	<0.001	

*N*, number of species used in each analysis. Regression coefficients: intercept (*a*) and scaling exponent (*b*).

*x*, pore length (cm); *M*, body mass (g); *d*, pore diameter (cm); PD, pore density (pores cm<sup>-2</sup>); *V*<sub>pore</sub>, pore volume (cm<sup>3</sup>); *J*<sub>a</sub>, whole-animal flux (μmol s<sup>-1</sup>); OC, oxygen consumption (μmol s<sup>-1</sup>); OLS, ordinary least squares regressions; PGLS, phylogenetic least squares regressions. PGLS models are as follows: mtCO1, tree built with unconstrained topology; var. brlens, tree with variable branch lengths; equal brlens, same tree topology but with all branch lengths set to 1. Individual oxygen consumption rates were taken from Lane et al. (2017).

\*Oxygen flux calculated using the diffusion coefficient of seawater (*D*<sub>w</sub>). †Oxygen flux calculated using the diffusion coefficient of muscle tissue (*D*<sub>t</sub>).

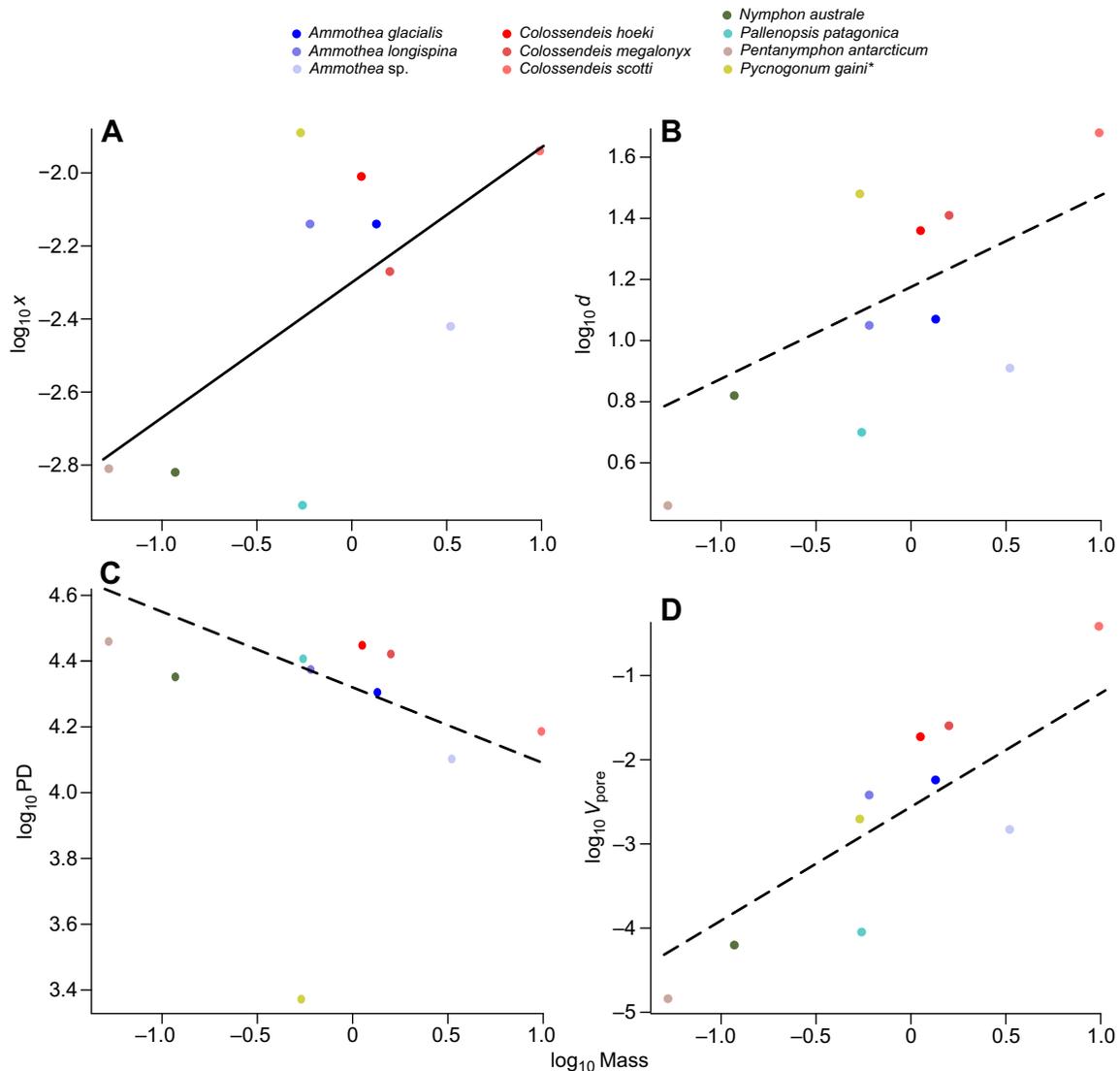
small-bodied species they were lower (Fig. 4). This pattern suggests that large-bodied species rely more exclusively on pore-based fluxes of oxygen. The broad confidence intervals in our data (Table 1) preclude distinguishing hypotheses 1 and 3 more quantitatively.

The size dependence of oxygen uptake via pores emerges from the scaling of pore morphology. Larger species have thicker cuticle (as described by pore length), which decreases flux by increasing the distance that oxygen must move. Cuticle thickness and pore diameter scaled approximately as expected for geometric similarity (*b*=0.37 and *b*=0.30, respectively). Pore density decreased slightly with increasing body size (*b*=-0.23). Pore volume, however, scaled with a larger coefficient (*b*=1.35) than expected for geometric similarity (*b*=1.00; Schmidt-Nielsen, 1984). This means that to offset the decrease in flux associated with thicker cuticle, larger species have wider pores, which results in greater total pore volume.

Rather than possess high pore density, one of the species in our study – *Pycnogonum gaini* – maintains adequate diffusive oxygen flux with relatively few but large pores. For its body size, *Pycnogonum gaini* (Fig. 2E) has substantially fewer pores per square area than expected, which would convey relatively low oxygen flux. Nevertheless, it meets its required diffusive fluxes of oxygen by also having much wider pores than expected for its body size.

The boundary layer resistance above and below the pores conferred little resistance to oxygen movement (Table S6). Therefore, flow conditions across the outer cuticle or within the hemolymph likely have little effect on total oxygen flux. Conversely, in most species, the cuticular cap conferred the greatest resistance to oxygen diffusion. For example, in the three *Colossendeis* species, resistance across the cuticular cap accounted for over 80% of the total resistance. The thickness of this cap can play a large role in restricting oxygen flux. Colonizing organisms, such as bryozoans, could increase the functional thickness of the cuticle and greatly decrease oxygen flux. We are currently testing the effects of different colonizing organisms on sea spider topology gas exchange.

To calculate resistance at the top and bottom of each pore, we used Stefan's law. This is appropriate when the diffusing molecule enters or leaves the pore from a concentration gradient distributed hemispherically around the pore opening. This is possible only if the pore openings are spaced far enough apart – otherwise, the hemispherical concentration gradients interfere with one another, which has the effect of raising the total resistance (Brown and Escombe, 1900; Ting and Loomis, 1963). In general, Stefan's law is thought to hold if pore openings are spaced more than 10 diameters apart. In our study, pore openings on the external cuticle met this



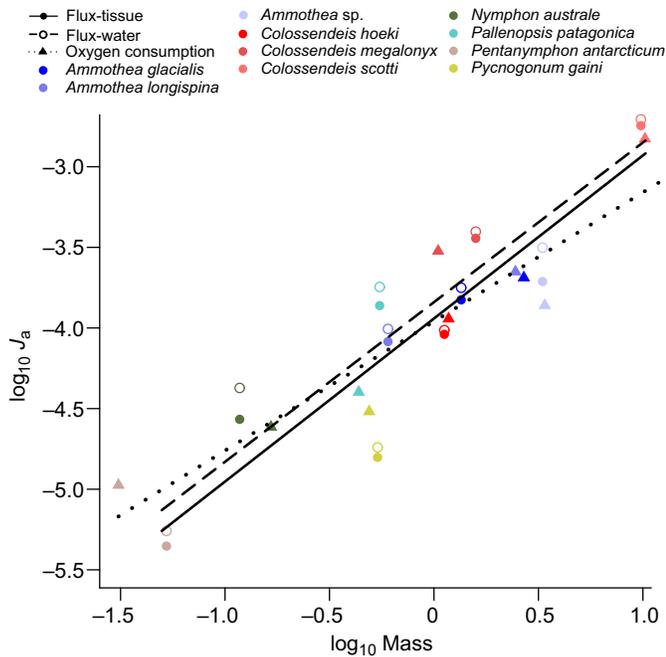
**Fig. 4. Regression lines for scaling relationships to body mass.** (A) Pore length ( $x$ , measured as cuticle thickness in  $\mu\text{m}$ ;  $n=10$ ), (B) pore diameter ( $d$ ,  $\mu\text{m}$ ;  $n=10$ ), (C) pore density (PD, pores  $\text{cm}^{-2}$ ;  $n=9$ ) and (D) pore volume across the entire cuticle ( $V_{\text{pore}}$ ,  $\text{cm}^3$ ;  $n=10$ ). Body mass was measured in g. In A, the line indicates the fit using ordinary least squares regression (OLS); in B–D, lines indicate the fit using phylogenetic least squares regressions (PGLS) with equal branch length. See Results for details. See Table 1 for regression coefficients. \**Pycnogonum gaini* appears to be an outlier and was not used in the pore density regression.

assumption (data not shown). Conversely, pore openings on the internal side of the cuticle generally were closer than 10 diameters from one another, thus violating the Stefan assumption. Theoretically, this raises the total resistance provided by the internal layers of hemolymph. We decided, however, to ignore this problem – both because the calculated internal resistances are of the order of 1% of the total resistance, and because agitation of the hemolymph by gut peristalsis (Woods et al., 2017) probably reduces this resistance anyway.

The presence of pores in the cuticle likely presents a functional trade-off between gas exchange and structural support: a greater total pore volume supports greater fluxes of oxygen but likely also weakens the cuticle. Alternatively, thicker cuticle provides greater strength, but it reduces the rate of diffusive oxygen flux by lengthening the pores. While many studies have discussed the relationship between shell thickness, shell material composition and structural support in eggshells (Ar et al., 1979; Board and Scott, 1980; Board, 1980), few have tested the relationship between

structural support and porosity. Tyler (1955) discussed whether the distribution of pores in avian egg shells,  $\sim 2$  pores per square millimeter, helps reduce the number of weak areas in an egg shell as areas of greater pore density would be weaker and act as sites for crack propagation. The relationship between structural support and pore shape has also been studied in vertebrate bones and engineered materials. In vertebrate bones, increasing porosity lowers fracture strength, the ability of a material to withstand breaking, because pores reduce the load-bearing area of the bone (Yeni et al., 1997). In metallic glass, an amorphous metal used in electronics and medical devices, and ceramic microsieves, material used in microfiltration, the diameter of the pore affects material strength; wider pores yield weaker material strength because the edge of the pore is the weakest point, so pores with larger diameters will be weaker than those with smaller diameters (Gao et al., 2016; Kuiper et al., 2002).

The structure of sea spider cuticle may therefore present an evolutionary compromise to minimize strength reduction while



**Fig. 5. Oxygen flux and consumption.** Regression lines comparing total diffusive oxygen flux ( $J_a$ ) through pores using the tissue-diffusion model, the water-diffusion model and oxygen consumption to body mass. Oxygen flux was measured in  $\mu\text{mol s}^{-1}$  and body mass in g. Regression lines were calculated based on OLS models.  $N=10$ . Oxygen consumption rates were taken from Lane et al. (2017). See Table 1 for regression coefficients.

maintaining sufficient oxygen flux, as we have hypothesized recently (Lane et al., 2017). In an absolute sense, large sea spiders have thicker cuticle, which provides greater strength but also long distances over which oxygen must diffuse. Large species, therefore, must have larger average diameter pores to offset the resistance to oxygen flux arising from these long distances, which may weaken the cuticle. The species of *Colossendeis* and *Ammothea* are the largest-bodied individuals in our analyses, and they have many conical pores, with the small aperture near the surface of the cuticle. This pore design may allow these species to grow to relatively large sizes by minimizing the structural weakness associated with pores, because conical pores concentrate chitinous material away from the central axis and therefore offer greater polar moments of area (Vogel, 2013) relative to the same pore areas distributed by cylinder pores. We are presently testing the structural integrity of sea spider cuticles with different pore shapes and densities.

In conclusion, sea spider cuticle is not solid but, rather, contains many pores (Fahrenbach, 1994; Davenport et al., 1987). The volume and density of pores both scale with body size to allow sufficient oxygen into the body to meet the sea spider's metabolic demands, especially for larger-bodied species. Future studies should examine sea spiders from different environments, such as those living in temperate or tropical locations or those living in the intertidal zone, to see whether pore structure changes with temperature or the likelihood of strong external forces. For instance, because higher temperatures stimulate metabolic rate more than they do rates of diffusive flux (Woods, 1999; Verberk et al., 2011), we predict that, for their small body sizes, tropical species will have higher conductance cuticles, which could be achieved by thinner or more porous cuticle. The trade-off discussed above may be more acute for warm-water species, as those living in the intertidal zone and other areas with higher current may be more

at risk from external forces (e.g. tide cycles, current surges) and may need proportionately thicker cuticle with fewer pores to prevent structural damage.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: S.J.L., A.L.M., B.W.T., H.A.W.; Methodology: S.J.L., A.L.M., H.A.W.; Software: S.J.L., H.A.W.; Validation: S.J.L., A.L.M., B.W.T., H.A.W.; Formal analysis: S.J.L., H.A.W.; Investigation: S.J.L., C.M.S., H.A.W.; Resources: S.J.L., A.L.M., C.M.S., B.W.T., H.A.W.; Writing - original draft: S.J.L., A.L.M., B.W.T., H.A.W.; Writing - review & editing: S.J.L., A.L.M., C.M.S., B.W.T., H.A.W.; Visualization: S.J.L., A.L.M.; Supervision: A.L.M., B.W.T., H.A.W.; Project administration: A.L.M., B.W.T., H.A.W.; Funding acquisition: A.L.M., B.W.T., H.A.W.

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#### Supplementary information

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