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RESEARCH ARTICLE

Do squid breathe through their skin?

Matthew A. Birk*, Agnieszka K. Dymowska and Brad A. Seibel

ABSTRACT

Squid are thought to obtain a large portion of their oxygen via simple diffusion across the skin in addition to uptake at the gills. Although this hypothesis has support from indirect evidence and is widely accepted, no empirical examinations have been conducted to assess the validity of this hypothesis. In this study, we examined cutaneous respiration in two squid species, *Doryteuthis pealeii* and *Lolliguncula brevis*, using a divided chamber to physically separate the mantle cavity and gills from the outer mantle surface. We measured oxygen consumption and ammonia excretion rates in the two compartments and found that, at rest, squid only obtain enough oxygen cutaneously to meet the demand of the skin tissue locally (12% of total) and excrete little ammonia across the skin. The majority of oxygen is obtained via the traditional branchial pathway. In light of these findings, we re-examine and discuss the indirect evidence that has supported the cutaneous respiration hypothesis.

KEY WORDS: Cutaneous respiration, Cephalopod, Metabolism, Oxygen, Ammonia, Gas exchange

INTRODUCTION

Cutaneous oxygen uptake, the acquisition of O_2 molecules from the environment into the skin, is likely to occur to some extent in nearly all animals by simple diffusion owing to an oxygen gradient from the environment into the skin tissue (Krogh, 1941). However, the acquisition of oxygen not just into the skin, but across the skin and into the underlying blood or tissues is less universal but still contributes notably to oxygen supply in a diversity of animals including most amphibians, several fishes (especially air-breathing fishes), copepods, many small terrestrial arthropods, echinoderms, pycnogonids, bryozoans, cnidarians, ctenophores, a variety of worms, and a number of embryonic and larval stages before respiratory structures have developed (Krogh, 1941; Feder and Burggren, 1985; Lane et al., 2018).

In addition to oxygen uptake, metabolically produced ammonia may also be excreted cutaneously. This has been demonstrated in several seawater and freshwater fishes including Pacific hagfish (*Eptatretus stoutii*; Clifford et al., 2016, 2017), mangrove killifish (*Kryptolebias marmoratus*; Frick and Wright, 2002; Cooper et al., 2013) and sea lamprey (*Petromyzon marinus*; Blair et al., 2017), as well as the fully aquatic African clawed frog (*Xenopus laevis*; Cruz et al., 2013) and freshwater leech (*Nephelopsis obscura*; Quijada-Rodriguez et al., 2015). However, the role of skin in the removal of nitrogenous waste has not been investigated in cephalopods to date.

College of Marine Science, University of South Florida, Saint Petersburg, FL 33701, USA.

*Author for correspondence (matthewabirk@gmail.com)

M.A.B., 0000-0003-0407-4077; B.A.S., 0000-0002-5391-0492

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Wells and Wells (1983) were the first to demonstrate cutaneous oxygen uptake in cephalopods. They found that in addition to branchial oxygen uptake (across the gills), *Octopus vulgaris* acquire roughly 13% of their resting O_2 requirement cutaneously (across the skin) on the web, arms and suckers. Soon thereafter, Wells et al. (1988) proposed that because squid have a higher surface area-to-volume ratio than octopuses, cutaneous O_2 uptake is likely to occur to a greater extent in squid. They estimated, in the complete absence of evidence, that it may contribute to 20% of oxygen acquisition.

In the 30 years since, the idea that squid obtain a notable portion of their O_2 cutaneously rather than branchially has pervaded the literature, with proposed contributions of the skin ranging from 20 to 73% of total O_2 uptake (Wells et al., 1988; Pörtner, 1994). The cutaneous respiration hypothesis has been incorporated into calculations of branchial oxygen extraction efficiency (Trübenbach et al., 2013a), used to explain calculations of surprisingly high cardiac output (O'Dor et al., 1990; Shadwick et al., 1990; Wells, 1992) and blood hemocyanin properties (Pörtner, 1990, 1994, 2002), and has been incorporated into discussion of skin morphology (Madan and Wells, 1997), muscle ultrastructure (Pörtner, 2002; Seibel, 2016), growth rates (O'Dor and Hoar, 2000; Moltschaniwskyj, 2004; Moltschaniwskyj and Carter, 2010), metabolic scaling (Seibel, 2007; Rosa et al., 2009; Trübenbach et al., 2013b) and hypoxia tolerance (Seibel, 2013, 2016).

This hypothesis was based on several lines of indirect evidence in addition to the direct measurements made in octopus. When examining the O_2-binding properties of the squid respiratory protein, hemocyanin, Pörtner (1990) observed that the Bohr coefficient (a metric of pH sensitivity) was less than –1, meaning that deoxygenated hemocyanin in tissue capillaries is capable of removing more CO_2 from solution than is produced during metabolism from the O_2 delivered. This suggests that venous blood should be less acidic than arterial blood, which would increase hemocyanin–O_2 affinity, thus inhibiting the delivery of oxygen to the tissues. In fact, such a venous alkalosis is not observed in squid (Redfield and Goodkind, 1929). Instead, they exhibit the typical venous acidosis, which supports O_2 delivery to the tissues. To explain this paradox, Pörtner (1990) supposed that if cutaneous oxygen acquisition were substantial, the CO_2 produced from this cutaneously derived oxygen could enter the blood and provide sufficient CO_2 into the blood to support a venous acidosis.

Madan and Wells (1996a) measured respiration rates of dissected skins from a variety of cephalopods including two squid species, *Lolliguncula brevis* and *Sepioteuthis lessoniana*, and found that they were comparable to those measures from the dissected skin of octopuses. In a separate experiment, they also found that intact *Octopus vulgaris* skin from the dorsal mantle could support up to 82% of O_2 demand. These findings are suggestive that squid skin may be able to support comparable rates, but *in vivo* measurements have still only been conducted in *Octopus* to date (Wells and Wells, 1983).

In addition to the evidence described above, an assortment of circumstantial evidence also supports the cutaneous respiration hypothesis in squid. The general body plan of a squid is a hollow

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tube, with inner and outer mantle surfaces exposed to seawater. The resultant high surface area-to-volume ratio could support a high contribution of cutaneous respiration. Unlike the body plans of most animals, the surface area-to-volume ratio of loliginid squid has been reported to scale isometrically over several orders of magnitude in size owing to allometric lengthening of the mantle and fins, such that large squid have just as much cutaneous area, relative to respiring mass, as small squid (O'Dor and Hoar, 2000).

Additionally, the composition of circular muscle in the mantle is arranged in a 'sandwich' pattern, where the outer layers of muscle tissue (both on the outside of the animal and the inner mantle cavity) are mitochondria-rich aerobic fibers and have high capillary density, and the central layer contains mitochondria-poor anaerobic fibers with lower capillary density (Bone et al., 1981; Mommsen et al., 1981). This arrangement may be conducive for cutaneous oxygen acquisition and the delivery of cutaneously derived CO_2 to the blood as proposed by Pörtner (1990). The active, water-column-dwelling lifestyle of squid and their jet propulsion biomechanics also provide ample ventilation across the skin, minimizing large boundary layers, which may limit cutaneous respiration in octopuses (Madan and Wells, 1996a). Finally, squid skin is quite thin among cephalopods: it is merely 300 µm in *Loligo vulgaris* and 150 µm in *Illex illecebrosus* compared with the ≈1300 µm skin of *O. vulgaris* (Madan and Wells, 1997). All else being the same, according to Fick's law (Fick, 1855; see Discussion), shorter diffusion distances allow a larger flow of respiratory gases. Moreover, octopus epidermal cells secrete a thin cuticle over the whole body while squid lack these secretions (Packard, 1988).

Despite the indirect and circumstantial evidence compiled above, to date, there have been no direct empirical measurements quantifying the magnitude of cutaneous oxygen uptake in squid or determining whether they 'breathe' through their skin. In this study, we provide the first measurements of *in vivo* cutaneous oxygen consumption in two squid species, *Doryteuthis pealeii* and *Lolliguncula brevis*. In addition to oxygen exchange, we also examine the potential role of squid skin in metabolic ammonia excretion for the first time.

MATERIALS AND METHODS

Animal capture and maintenance

Doryteuthis pealeii (Lesueur, 1821; n=17) were caught in Vineyard Sound, MA, USA, by benthic otter trawl by the R/V Gemma in October 2017 and held in a large aerated tank at the Marine Biological Laboratory (Woods Hole, MA, USA) at 19°C until experiments were performed. Trials were conducted within 24 h of capture for all but two animals that were fasted for up to 3 days. Only animals with skin in excellent condition were chosen for experiments. One individual was jigged aboard the R/V Gemma to determine whether capture by the trawl net had any effect on the results even in the absence of visible skin damage. *Lolliguncula brevis* (Blainville, 1823; n=4) were similarly caught by otter trawl in Tampa Bay, Florida, USA, from April through June 2017 by the Florida Fish and Wildlife Conservation Commission (FWCC) Fish and Wildlife Research Institute (FWRI) Fisheries Independent Monitoring program and tested within 24 h of capture. Mass and dorsal mantle length of both species are shown in Table 1. All *L. brevis* experiments were run at 20°C, whereas *D. pealeii* experiments were conducted at 11 and 19°C. Salinity was 30–34 in all trials.

Table 1. Characteristics of squid used in divided chamber experiments

Species	n	Mass (g)	Dorsal mantle length (cm)	Gender
Doryteuthis pealeii	17	94±24	15±2	F:3, M:14
Lolliguncula brevis	4	31±5	8±1	F:2, M:2

Values are means±s.d.

Divided chamber setup

A divided chamber was developed to physically isolate cutaneous and branchial oxygen consumption in squid. This setup was conceptually similar to that used by Wells and Wells (1983) to measure cutaneous respiration in *Octopus*. Each squid was gently patted dry around the anterior edge of the mantle and a custom-sized rubber collar was attached to the anterior margin of the mantle with cyanoacrylate glue. The entire procedure typically lasted less than 1 min. Collars were constructed with 0.4 mm thick, 70 duro nitrile (West American Rubber Company LLC, Orange, CA, USA). The collars were truncated cones arranged so that they widened posteriorly and were attached to one end of a transparent acrylic tube (9×17 cm; 1 liter). The other end of the acrylic tube was covered with a flexible rubber oxygen-impermeable membrane so that ventilatory inspirations were not impeded by pressure inside the tube. With the collar attached, animals were observed to ventilate at a rate similar to that of unrestrained animals (Birk et al., 2018). This tube, the 'mantle respirometer', contained water in contact with the exterior mantle and fins (Fig. 1). For *D. pealeii* and *L. brevis*, 35±3% and 45±6% of the skin was contained inside the 'mantle' respirometer, respectively.

The mantle respirometer and squid were then placed into a larger respirometer (37 liters), the 'gill respirometer', which contained water in contact with the gills, mantle cavity, head, arms and tentacles. Magnetic stir beads were placed inside both respirometers to ensure uniform mixing (Fig. 1). In one of the trials, food coloring was added to the mantle respirometer water to demonstrate that there was no water exchange between the mantle and gill respirometers. The dissolved oxygen partial pressure (P_{O_2}) was measured with oxygen-sensitive spots adhered to the inside of both respirometers [PreSens Fibox 3 (Regensburg, Germany) and Loligo Systems Witrox O_2 meters (Viborg, Denmark)]. Oxygen meters for both respirometers were calibrated simultaneously with the same

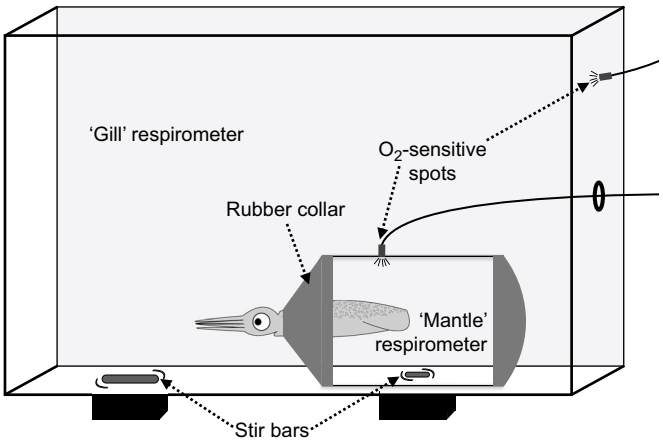

Fig. 1. Divided chamber setup for cutaneous respirometry experiments. Squid were adhered to a rubber collar and placed inside a small cylindrical 'mantle' respirometer to isolate cutaneous from branchial gas exchange. The 'mantle' respirometer measured gas exchange across the outer mantle and fins whereas the 'gill' respirometer measured exchange across the gills, mantle cavity, head, and appendages. Both chambers were mixed with magnetic stir bars. The partial pressure of oxygen (P_{O_2}) was measured with oxygen-sensitive spots via fiber optic cables to oxygen meters.

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Table 2. Gas exchange rates and skin arrangements of squid in divided chamber experiments

	Doryteuthis pealeii (11°C)	*Doryteuthis pealeii* (19°C)	*Lolliguncula brevis* (20°C)
Total cutaneous surface area (cm^2)	435±77	416±82	113±13
Cutaneous $\dot{M}_{O_2}$ (μmol O_2 cm^{-2} h^{-1})	0.23±0.04	0.32±0.09	0.43±0.2
Branchial $\dot{M}_{O_2}$ (μmol O_2 h^{-1})	1053±347	1135±455	343±83
Mass-specific $\dot{M}_{O_2}$ (μmol O_2 g^{-1} h^{-1})	11.33±2.41	14.28±4.09	12.74±2.59
$\dot{M}_{O_2}$ from cutaneous uptake (%)	9±1	11±4	12±4
Cutaneous $\dot{M}_{NH_3}$ (μmol NH_3 cm^{-2} h^{-1})	12.5±4.52	11.07	87.36, 27.09
Branchial and renal $\dot{M}_{NH_3}$ (μmol NH_3 h^{-1})	69±20	50	38, 20
$\dot{M}_{NH_3}$ from cutaneous uptake (%)	7±2	7	23, 14

Measurements were made for the outer mantle and fins separately from the gills, mantle cavity, head and appendages. All reported values are corrected to account for the skin exposure in both chambers. Values are means±s.d. $\dot{M}_{O_2}$, oxygen consumption rate; $\dot{M}_{NH_3}$, NH_3 excretion rate.

samples of air-saturated seawater and concentrated $NaSO_3$ solution (P_{O_2}=0) before beginning experiments for both species.

Trials lasted 2 and 11 h for *D. pealeii* and *L. brevis*, respectively. P_{O_2} fell at similar rates in both respirometers (owing to volume differences) from air saturation to 15 kPa on average across the trials and never lower than 8 kPa. These levels are well above the hypoxic threshold for aerobic metabolism in this species (Birk et al., 2018). Oxygen consumption rates ($\dot{M}_{O_2}$) in both respirometers were calculated with the R package 'respirometry' (https://cran.r-project.org/web/packages/respirometry/index.html). Only $\dot{M}_{O_2}$ values derived from P_{O_2}–time relationships with an R^2>0.8 were used. Background oxygen consumption rates were ≤1% of animal rates in both respirometers and were ignored. Statistical comparisons of cutaneous O_2 uptake as a percentage of total uptake were arcsine square-root transformed.

Calculation of cutaneous oxygen uptake

Cutaneous surface area was measured for each squid by photographing: the flattened mantle after the viscera and gladius were removed, the fins, the head, the arms and the tentacles. Surface area was quantified from the images via ImageJ (Abràmoff et al., 2004). Total measured cutaneous oxygen uptake was then calculated according to Eqn 1:

$$\text{Measured cutaneous } \dot{M}_{O_2} = \frac{SA_{total}}{SA_{MR}} \times \dot{M}_{O_2,MR}, \quad (1)$$

where SA_{total} is total surface area, SA_{MR} is the surface area inside the mantle respirometer and $\dot{M}_{O_2,MR}$ is the oxygen uptake of the mantle respirometer. The amount of cutaneous oxygen consumption expected for exclusively localized use within the skin tissue was then calculated according to Eqn 2 by multiplying the total surface area, skin thickness (T_{skin}) of 300 μm (Madan and Wells, 1997), a tissue density (D_{tissue}) of 1.05 g cm^{-3} (Packard, 1972) and the skin's mass-specific metabolic rate ($\dot{M}_{O_2,mass}$):

$$\text{Local cutaneous } \dot{M}_{O_2} = SA_{total} \times T_{skin} \times D_{tissue} \times \dot{M}_{O_2,mass}. \quad (2)$$

To date, the $\dot{M}_{O_2,mass}$ of *in vivo* skin tissue has not been measured in a cephalopod. Therefore, two metrics were considered. Firstly, the $\dot{M}_{O_2,mass}$ of the skin tissue was considered to be equivalent to the average $\dot{M}_{O_2,mass}$ of the whole animal as determined by the combined oxygen consumption in both the mantle and gill respirometers. Secondly, the $\dot{M}_{O_2,mass}$ of the skin was calculated from *in vitro* measurements of *L. brevis* and *Sepioteuthis lessoniana* skin by Madan and Wells (1996a; mean=8.45 μmol O_2 g^{-1} h^{-1} given 300 μm thick skin). The measured and local cutaneous rates were then compared using a paired *t*-test.

The total $\dot{M}_{O_2}$ was the sum of measured $\dot{M}_{O_2}$ in both the mantle and gill respirometers. The percentage of total $\dot{M}_{O_2}$ from cutaneous uptake was calculated from the measured cutaneous $\dot{M}_{O_2}$ (Eqn 1) as a proportion of total $\dot{M}_{O_2}$, and accounted for cutaneous uptake from both respirometers (i.e. inner and outer mantle, fins, head and appendages). The remaining percentage of total $\dot{M}_{O_2}$ was considered branchial uptake.

Ammonia excretion rates

To quantify ammonia excretion rates, water samples were collected from both respirometers at the end of the experiments and stored at −80°C until processed. The concentration of excreted ammonia was measured using a phenol method adapted from Ivančič and Degobbis (1984). Briefly, water samples (2 ml) were treated with phenol, nitroprusside, alkaline citrate and dichloro-iso-cyanuric acid, and incubated for 12 h in the dark. All reagents were purchased from Sigma-Aldrich (St Louis, MO, USA). Blue-colored indophenol was formed in the presence of ammonia in the sample. Ammonia concentration in duplicate water samples was measured with a spectrophotometer (Shimadzu UV-1700, Kyoto, Japan) by observing absorbance at 635 nm, and the values were averaged. Ammonia excretion measurements were conducted for six and one *D. pealeii* at 11 and 19°C, respectively, and two *L. brevis*.

RESULTS

Oxygen consumption

The proportion of whole-animal oxygen consumption rate ($\dot{M}_{O_2}$) that was acquired from cutaneous uptake ranged from 5 to 19% (Table 2, Fig. 2). The cutaneous uptake of the one jigged specimen

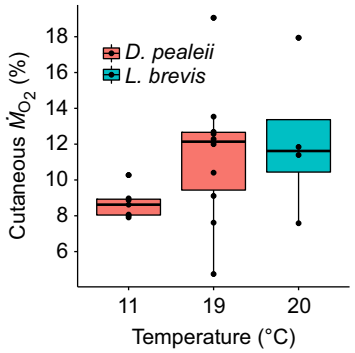

Fig. 2. Contributions of cutaneous O_2 uptake ($\dot{M}_{O_2}$) to whole-animal $\dot{M}_{O_2}$ in two species of squid, *Doryteuthis pealeii* (11°C, *n*=7; 19°C, *n*=10) and *Lolliguncula brevis* (*n*=4). Values were calculated from the measured cutaneous $\dot{M}_{O_2}$ (Eqn 1) as a proportion of total $\dot{M}_{O_2}$, and accounted for cutaneous uptake from both respirometers (i.e. inner and outer mantle, fins, head and appendages). The contribution of cutaneous uptake was indistinguishable between temperatures (d.f.=15, *P*=0.107) and species (d.f.=19, *P*=0.333).

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(8%) fell within the range of those collected by trawl, suggesting that trawl damage was not a factor. The contribution of cutaneous uptake was indistinguishable between species (d.f.=19, P=0.333) or temperature treatments (d.f.=15, P=0.107). *Doryteuthis pealeii* ranged in size from 51 to 150 g and 10.8 to 18.1 cm dorsal mantle length (Table 1). Across this size spectrum, the squid's surface area-to-volume ratio decreased with increasing size according to $15.6\times\text{mass}^{-0.28}$ (d.f.=15, P=0.004), and with it, the contribution of cutaneous $\dot{M}_{O_2}$ to total $\dot{M}_{O_2}$ (d.f.=15, P=0.002).

To determine whether the oxygen taken up by the skin was in excess of that used locally by the skin, the measured cutaneous $\dot{M}_{O_2}$ was compared with the expected cutaneous $\dot{M}_{O_2}$ from local (the skin tissue only) oxygen demand, which, in turn, was derived from cutaneous surface area and $\dot{M}_{O_2,mass}$. Total cutaneous surface area was 424±78 cm^2 and 113±13 cm^2 for *D. pealeii* and *L. brevis*, respectively (Fig. 3A). The $\dot{M}_{O_2,mass}$ of *D. pealeii* was 11.3±2.4 µmol O_2 g^{-1} h^{-1} at 11°C and increased to 14.3±4.1 µmol O_2 g^{-1} h^{-1} at 19°C (Fig. 3B). *Lolliguncula brevis* $\dot{M}_{O_2,mass}$ at 20°C was 12.7±2.6 µmol O_2 g^{-1} h^{-1}, which was similar to that of *D. pealeii* at 19°C (d.f.=12, P=0.502; Fig. 3B).

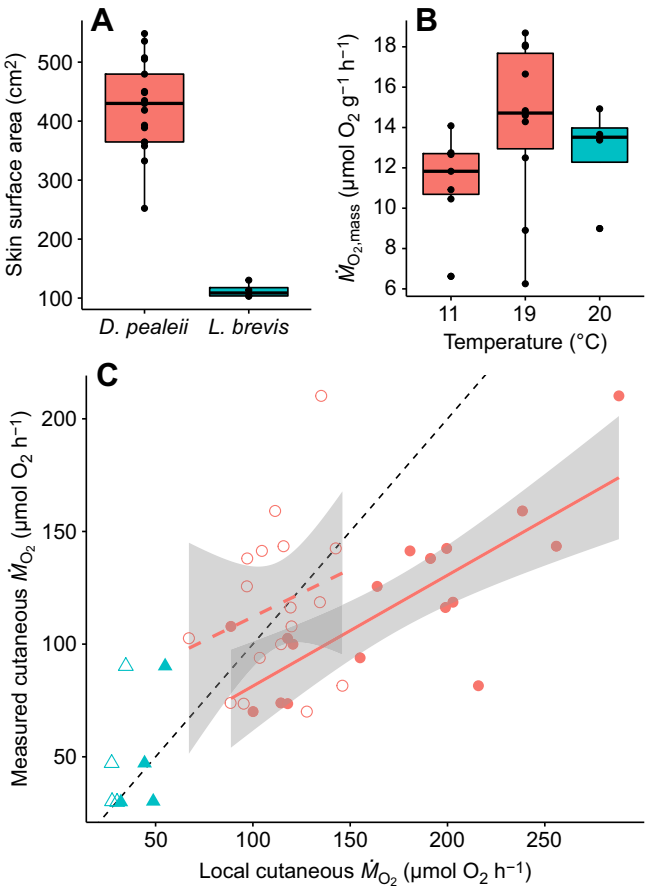

Fig. 3. Comparison of measured and local cutaneous oxygen uptake. (A) Total cutaneous surface area. (B) Mass-specific metabolic rate ($\dot{M}_{O_2,mass}$) from combined oxygen consumption in both the mantle and gill respirometers. (C) Measured versus local cutaneous $\dot{M}_{O_2}$ in squid. Red circles represent *Doryteuthis pealeii* (n=17) and blue triangles represent *Lolliguncula brevis* (n=4). Filled shapes are data from the present study (B), while outlined shapes are data from Madan and Wells (1996a; 8.45 µmol O_2 g^{-1} h^{-1}). Gray dashed line is the unity line. Red lines and shaded bands are the regressions and 95% confidence bands for *D. pealeii*. Inter-individual variation is primarily due to body size and measurement temperature.

The proportion of oxygen derived cutaneously was unrelated to $\dot{M}_{O_2,mass}$ (P>0.05) at all temperatures for both species.

The measured cutaneous $\dot{M}_{O_2}$ was significantly less than that expected from local tissue demand when local demand was calculated from whole-animal $\dot{M}_{O_2,mass}$ from this study (d.f.=20, P<0.001; Fig. 3C, filled shapes). When assuming a local demand of 8.45 µmol O_2 g^{-1} h^{-1} from the *in vitro* measurements from Madan and Wells (1996a), the measured cutaneous $\dot{M}_{O_2}$ was indistinguishable from that expected from local demand (d.f.=20, P=0.346; Fig. 3C, outlined shapes).

The area-specific cutaneous $\dot{M}_{O_2}$ (µmol O_2 cm^{-2} h^{-1}) was similar between the two species at similar temperatures (d.f.=12, P=0.188) and increased with increasing temperature in *D. pealeii* (d.f.=15, P=0.015) with a Q_{10} of 1.6, which was similar to the Q_{10} for $\dot{M}_{O_2,mass}$ of 1.3. In fact, the area-specific cutaneous $\dot{M}_{O_2}$ was related to $\dot{M}_{O_2,mass}$ within both temperature treatments for *D. pealeii* (11°C: d.f.=5, P=0.003; 19°C: d.f.=8, P=0.025) and for all animals from both species (d.f.=19, P=0.019).

Ammonia excretion

Doryteuthis pealeii skin excreted 7.5±1.6% of the metabolically produced ammonia, with the remainder excreted via the gills and renal sac (Table 2). The atomic ratio of O_2 consumed to NH_3 excreted by the whole animal (O:N) varied widely, from 17 to 59 with a median of 27. The animal exhibiting the highest O:N was fasted for 3 days before the trial. The O:N ratio in the mantle respirometer was significantly higher than in the gill respirometer (d.f.=8, P=0.033; Fig. 4).

DISCUSSION

Contrary to popular conjecture, we found that the uptake of O_2 across the skin in squid is slow and likely consumed locally by the skin tissue rather than being incorporated into the blood for systemic utilization. The proportion of total O_2 uptake across the skin was less than any previous estimates (Wells et al., 1988; Pörtner, 1994). The measured cutaneous uptake was either indistinguishable from or, on average, 23% less than the estimated amount required by the skin tissue locally, depending on the method of predicting local tissue demand. The most realistic estimate is likely between these values given that *in vivo* skin tissue likely has higher demand (e.g. active chromatophore use) than excised tissue, but may be lower than the whole-animal average composed of oxygen-demanding brain,

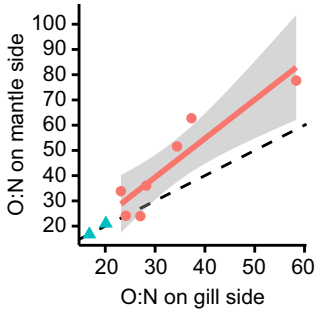

Fig. 4. Metabolic quotient of O_2 consumed and NH_3 excreted. The gill side of the respirometer was in contact with the gills, viscera and skin from the mantle cavity, head, arms and tentacles. The mantle side was in contact with the outer surface of the mantle and fins. Red circles represent *Doryteuthis pealeii* (n=7) and blue triangles represent *Lolliguncula brevis* (n=2). Gray dashed line is the unity line. Red line and shaded area are the regression and 95% confidence band for *D. pealeii*. The O:N ratio on the mantle side was significantly higher than on the gill side (d.f.=8, P=0.033).

heart, viscera and muscle tissue. Therefore, squid skin does not seem to be a net source of O_2 to the animal, but rather, if anything, a sink, at least partially dependent on blood-borne oxygen.

We also found that the O:N ratio was higher across the skin than the gills, suggesting that the capacity of ammonia excretion across the skin is lower than that across the gills. This is not surprising given the presence of ammonia transport proteins in the gills (Hu and Tseng, 2017). The gill side of the respirometer was also in contact with the renal sac in the mantle cavity, which is also involved in ammonia excretion (Boucher-Rodoni and Mangold, 1994), though to a lesser extent than the gills (Hu et al., 2017).

Madan and Wells (1996a) found that isolated skin from both *L. brevis* and *Sepioteuthis lessoniana* had an $\dot{M}_{O_2}$ of 0.12±0.04 µmol O_2 cm^{-2} h^{-1} at 20–24°C. These experiments, however, were in an unstirred chamber such that a hypoxic boundary layer was allowed to form. When performing similar experiments on *Octopus vulgaris* skin, they found that adding a magnetic stir bar more than doubled the *in vitro* rate of O_2 consumption. When the same factor is applied to the squid skin they examined, isolated squid skin should have an $\dot{M}_{O_2}$ of 0.26–0.27±0.09 µmol O_2 cm^{-2} h^{-1}. The *in vivo* results for *L. brevis* and *D. pealeii* at 19°C from the present study are indistinguishable from these values (P>0.05 for all species-wise comparisons), which suggests that the O_2 consumed in our experiments can be fully explained by localized consumption without any uptake by the blood or other body tissues.

The O:N ratios observed here were higher than is typical for squid (11–17), which have a well-known protein-rich diet (Ikeda, 2016). One animal was fasted for 3 days and had a notably higher O:N ratio than any other individual, suggesting a use of lipid reserves. This matches well the lipid utilization by *Sepia officinalis* after 3–5 days of starvation (Speers-Roesch et al., 2016).

Doryteuthis pealeii in this study exhibited metabolic rates that were on average roughly twice the basal rates (Birk et al., 2018), likely owing to handling stress (although *L. brevis* showed no such response relative to unrestrained *L. brevis*; Bartol et al., 2001). We found no relationship between the whole-animal metabolic rate (which varied three-fold between individuals and temperature treatments) and the proportion of oxygen acquired cutaneously. Any stress-induced rise in the measured cutaneous oxygen consumption would have overestimated the contribution of cutaneously derived oxygen to supplying oxygen demand when compared with *in vitro* skin tissue measurements from Madan and Wells (1996a; Fig. 3C, outlined shapes).

Limitations to cutaneous uptake

Diffusion distance is likely the most important factor limiting cutaneous respiration in squid. Squid skin is thinner than *Octopus* skin, but unlike octopods, it does not seem to be vascularized (Madan and Wells, 1997). Blood vessels are commonly found in the skin of cutaneously respiring animals (Krogh, 1941; Feder and Burggren, 1985). Thus, O_2 from seawater would have to diffuse across the entire squid skin thickness (150–300 µm) before reaching capillaries. Oxygen diffusion across animal tissue is slow, however: three times slower than in water and one million times slower than in air (Krogh, 1941). Oxygen transport may be limited by diffusion even on an intracellular scale, influencing mitochondrial placement within myocytes (Kinsey et al., 2007).

A simple calculation of diffusion capacity reveals the situation. In another loliginid squid, *Alloteuthis subulata*, total branchial surface area and diffusion distance for a 100 g individual are 1063 cm^2 and 6.2 µm, respectively (Eno, 1994), yielding a diffusion capacity of 519 µmol O_2 kPa^{-1} h^{-1} according to Eqn 3:

$$\text{Diffusion capacity} = K \times \frac{\text{surface area}}{\text{diffusion distance}}. \quad (3)$$

At 20°C, K is ≈3.03 µmol O_2 cm^{-2} µm kPa^{-1} h^{-1} (Krogh, 1919). This mass-specific branchial surface area is comparable to that of many marine fishes (Gray, 1954) and other cephalopods (Madan and Wells, 1996b). With a skin thickness of 300 µm (Madan and Wells, 1997), the *D. pealeii* in our study had a cutaneous diffusion capacity of only 4 µmol O_2 kPa^{-1} h^{-1}. As a useful comparison, the inter-capillary distance in aerobic circular muscle of the squid mantle is no more than 80 µm (Bone et al., 1981; Kier and Thompson, 2003). Thus, the furthest distance that oxygen must travel from capillaries to mitochondria is 40 µm, far less than the skin thickness.

The estimates of total cutaneous $\dot{M}_{O_2}$ in the present study were derived from cutaneous uptake across the outer mantle and fins and extrapolated to the mantle cavity, head and appendages. Cutaneous diffusion capacity is inversely proportional to the distance from the skin surface to the blood vessels or underlying tissue. Therefore, differences in skin thickness across the body could lead to differences in estimated cutaneous O_2 uptake. However, Madan and Wells (1996a) found no difference between *in vitro* cutaneous $\dot{M}_{O_2}$ on the web and the dorsal mantle of *Octopus*. In addition, Wells and Wells (1982) demonstrated through a series of manipulative experiments in *Octopus* that there is no measurable extraction of oxygen within the mantle cavity once water has passed the gills, suggesting that there is no cutaneous uptake across the inner mantle either. These findings suggest that variations in skin thickness that may exist across the body are unlikely to notably affect the estimates measured here.

Why is octopus skin vascularized while squid skin is not? One plausible explanation is that octopus skin requires vascularization because it is more metabolically active than squid skin. Octopus skin contains both muscular papillae that can alter the skin's three-dimensional texture (Allen et al., 2014) and one to two orders of magnitude more chromatophores than squid skin (Messenger, 2001). Such active muscular structures in the skin may require greater O_2 supply than can be supplied by diffusion from the skin surface.

Three species of loliginid squids have now been examined through a combination of *in vitro* (Madan and Wells, 1996a) and *in vivo* (present study) measurements, and all show similar area-specific cutaneous O_2 uptake rates. The ommastrephid squid *Illex illecebrosus* has thinner skin (150 µm) than most loliginids but it also lacks cutaneous vascularization (Madan and Wells, 1997) and thus likely also has a low cutaneous diffusion capacity. As part of this study, we conducted a short preliminary experiment on another ommastrephid, *Sthenoteuthis oualaniensis*. Its cutaneous respiration was 12.7% of total $\dot{M}_{O_2}$, similar to our results for loliginid squid. Although further examination is needed, it seems likely, given our current knowledge, that ommastrephid squid do not breathe through their skin either.

The cutaneous diffusion capacity of deep-water squid also remains poorly studied. Morphological measurements of branchial diffusion capacity have been made in a number of taxa (Madan and Wells, 1996b) and generally seem sufficient to support their lower metabolic rates (Seibel, 2007). Additionally, the hypoxic waters that many deep-sea squid inhabit lowers the diffusion gradient from the

environment into the skin compared with aerated surface waters, making notable cutaneous uptake unlikely.

Where did previous studies go wrong?

If squid do not breathe through their skin, how are we to interpret previously reported findings that have been used to support this hypothesis? The first indirect evidence was Pörtner's (1990) paradox of blood pH rising at the tissue capillaries unless some additional source of CO_2 or other acid is provided independently of blood-delivered O_2. There are two theoretical issues with this prediction, however. Firstly, for the hypothesis to work, CO_2 produced in the tissue mitochondria would have to diffuse into the blood rather than diffuse out into seawater. Seawater P_{CO_2}, however, is ∼7× lower than that of blood (Redfield and Goodkind, 1929; Pörtner, 1990; Hu et al., 2014), which should result in a higher diffusion rate out into seawater than into the blood. In fact, it is common among skin-breathing animals for much larger quantities of CO_2 to be released than O_2 absorbed across the skin (Krogh, 1919; Feder and Burggren, 1985).

Secondly, Pörtner (1990)'s hypothesis that extra CO_2 from the skin enters the blood could only function for capillaries very near the skin. If CO_2 produced by the consumption of cutaneously sourced O_2 were required for hemocyanin to function efficiently, how would blood-borne oxygen be efficiently delivered across capillaries in internal organs, such as the brain, that are far removed from the skin? This leaves the very high Bohr coefficients found in many squid and octopuses unexplained.

Madan and Wells (1996a) found that *in vitro* squid skin has similar oxygen uptake rates as *in vitro* octopus skin. However, caution must be used before predicting *in vivo* estimates from their experiments. Blood convection is a highly important factor in facilitating cutaneous respiration (Feder and Burggren, 1985). Intact *Octopus* skin tissue had an oxygen uptake rate over 5× higher than dissected skin tissue (Madan and Wells, 1996a), likely because blood convection through the tissue capillaries in the intact animal could maintain a steep diffusion gradient in the skin tissue. Because squid lack cutaneous blood vessels, they lack this ability to draw oxygen deeper into the body. Also, as noted above, *Octopus* skin is likely more metabolically active than squid skin owing to the higher density of chromatophores (Messenger, 2001) and muscular papillae (Allen et al., 2014).

Although squid do indeed have a higher surface area-to-volume ratio than most animals (O'Dor and Hoar, 2000), we found that, at least for *D. pealeii* over the very limited size ranges examined here, the surface area-to-volume ratio declines with increasing size, scaling at a similar rate as expected for geometric solids (b=−0.33). Thus, the small contribution of cutaneous oxygen uptake found here should be even smaller in larger individuals.

Finally, even though squid mantle circular muscle is arranged such that O_2-dependent aerobic muscle fibers are on the exterior margins (Bone et al., 1981; Mommsen et al., 1981), these are ultimately not relevant to cutaneous respiration because the diffusion limitation is on a much smaller scale within the skin than across the mantle. When Mommsen et al. (1981) originally described the 'sandwich' pattern of muscle fibers in the mantle, they proposed an entirely biomechanical explanation for this arrangement. They proposed that when only a small portion of circular muscles are being utilized, as during breathing or routine swimming contractions (Bone et al., 1981), this arrangement allows the passive central mantle muscle to be pressurized as a hydrostatic working fluid.

This study demonstrates that, contrary to a commonly held but untested hypothesis, squid do not acquire large quantities of oxygen through their skin for systemic use. This finding has important implications for our understanding of branchial and cardiac performance in squid. The squid cardiovascular system is already believed to be delivering near-maximal quantities of oxygen (O'Dor et al., 1990; Shadwick et al., 1990; Wells, 1992). Based on this research, cutaneous oxygen uptake does not alleviate the oxygen delivery demand on the cardiovascular system. This makes the physiological adaptations of active squid to provide sufficient oxygen supply to meet their high oxygen demand (Seibel, 2007) all the more interesting.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.A.B.; Methodology: M.A.B., A.K.D.; Validation: M.A.B., A.K.D.; Formal analysis: M.A.B., A.K.D.; Investigation: M.A.B., A.K.D.; Resources: M.A.B., B.A.S.; Data curation: M.A.B.; Writing - original draft: M.A.B., A.K.D.; Writing - review & editing: M.A.B., A.K.D., B.A.S.; Visualization: M.A.B.; Supervision: B.A.S.; Project administration: M.A.B., B.A.S.; Funding acquisition: M.A.B., B.A.S.

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