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SHORT COMMUNICATION

It's all in the gills: evaluation of O_2 uptake in Pacific hagfish refutes a major respiratory role for the skin

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ABSTRACT

Hagfish skin has been reported as an important site for ammonia excretion and as the major site of systemic oxygen acquisition. However, whether cutaneous O_2 uptake is the dominant route of uptake remains under debate; all evidence supporting this hypothesis has been derived using indirect measurements. Here, we used partitioned chambers and direct measurements of oxygen consumption and ammonia excretion to quantify cutaneous and branchial exchanges in Pacific hagfish (*Eptatretus stoutii*) at rest and following exhaustive exercise. Hagfish primarily relied on the gills for both O_2 uptake (81.0%) and ammonia excretion (70.7%). Following exercise, both O_2 uptake and ammonia excretion increased, but only across the gill; cutaneous exchange was not increased. When branchial O_2 availability was reduced by exposure to anteriorly localized hypoxia (~4.6 kPa O_2), cutaneous O_2 consumption was only slightly elevated on an absolute basis. These results refute a major role for cutaneous O_2 acquisition in the Pacific hagfish.

KEY WORDS: Exercise, Metabolic rate, EPOC, Respiration, Agnathan, Ammonia

INTRODUCTION

Recent studies have highlighted the importance of cutaneous transport in Pacific hagfish (*Eptatretus stoutii*) for nutrient acquisition (Glover et al., 2011; Schultz et al., 2014), ammonia and base excretion (Clifford et al., 2014), and trace metal uptake (Glover et al., 2015); however, the role of hagfish skin in O_2 uptake ($\dot{M}_{O_2}$) is contentious. Cutaneous respiration has been demonstrated in the phylogenetically related lamprey (*Geotria australis*) in larval form (Potter et al., 1996) and the European eel (*Anguilla anguilla*) (Nonnotte and Kirsch, 1978), both of which share similar body plans to hagfish, as well as in several species of fish such as the inanga (*Galaxias maculatus*) (Urbina et al., 2012, 2014), flounder (*Platichthys flesus*) and sole (*Solea solea*) (Nonnotte and Kirsch, 1978). Steffensen et al. (1984), working with Atlantic hagfish (*Myxine glutinosa*), proposed that branchial ventilation can theoretically satisfy only ~20% of whole-animal $\dot{M}_{O_2}$ and that the skin is well suited for cutaneous respiration because of prominent dermal capillary networks. Moreover, when gill apertures were sutured shut, Atlantic hagfish retained 89% of $\dot{M}_{O_2}$, supporting this hypothesis (Lesser et al., 1996). Contrarily, Malte and Lomholt (1998) argued against the capacity for cutaneous O_2 uptake in hagfishes, citing the impracticality for O_2 exchange across the 70–100 µm epidermal layer, perfusion of capillaries with arterial blood of high P_{O_2} and the impact of skin boundary layers on diffusion.

Here, we used custom-designed respirometry chambers to isolate anterior (branchial+cutaneous) and posterior (cutaneous) regions of Pacific hagfish [*Eptatretus stoutii* (Lockington 1878)] to partition whole-animal $\dot{M}_{O_2}$ and ammonia excretion (J_{Amm}). Exercise typically leads to increases in $\dot{M}_{O_2}$ and J_{Amm} during post-exercise recovery; therefore, we employed exhaustive exercise to determine the relative contribution of the gills and skin to elevations in metabolic demand. Given that skin is proposed as the primary site for $\dot{M}_{O_2}$ in Atlantic hagfish (Steffensen et al., 1984), while J_{Amm} has been demonstrated to be gill dominant in Pacific hagfish (Clifford et al., 2014), we hypothesized that exercise would yield increases primarily in cutaneous $\dot{M}_{O_2}$ and branchial J_{Amm} during recovery.

Hagfish often partially burrow into decaying carcasses (Hardisty, 1979), where conditions are hypoxic (Clifford et al., 2015b). We further hypothesized that under such conditions, cutaneous O_2 exchange would become more important. We exposed Pacific hagfish to anteriorly localized hypoxia to examine whether hagfish can compensate for reduced branchial O_2 availability by elevating cutaneous O_2 uptake. This represents the first assessment of cutaneous contributions to O_2 uptake in Pacific hagfish under resting and metabolically challenging conditions.

MATERIALS AND METHODS

Experimental animals

Pacific hagfish (N=32, 86.53±4.13 g) were captured near Bamfield, BC, Canada, and held at Bamfield Marine Sciences Centre (BMSC) as previously described (Clifford et al., 2014) under licences from Department of Fisheries and Oceans Canada (permit no. XR-310 2015) and animal care protocols from BMSC (RS-15-31) and University of Alberta (AUP00001126).

Apparatus details and insertion of hagfish into partitioned chambers

Specially constructed hagfish compartmentalizing flux chambers (58×6.3×6.1 cm L×W×H; schematic diagram available in Fig. S1 and photos available in Clifford et al., 2014) were used to partition anterior and posterior contributions to $\dot{M}_{O_2}$ and J_{Amm}. Anterior (20 cm) and posterior (32 cm) chambers were separated by a collar assembly made from two square Plexiglas plates with 2.5 cm diameter central holes sandwiching a latex sheath (finger from non-powdered latex glove; 7 cm long×2 cm unstretched diameter) and bolted together.

Hagfish were anaesthetized (MS-222; 0.5 g l^{-1} neutralized with NaOH; 0.15 g l^{-1}), and then blotted dry 2–4 cm posterior to the most posterior branchiopore and inserted into the sandwiched sheath. This anaesthetic dose lightly sedated hagfish long enough for them to be fitted into partitioned chambers. Cyanoacrylate glue

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was applied around the circumference of the dried area and the latex was glued to the skin to form a watertight seal. The area of skin covered by the sheath (~10% of the animal length) was removed in data corrections. Hagfish were immersed in fresh seawater to clear anaesthetic (resumption of movement within ~2 min), and then placed into the chamber by fitting the collar assembly into slots. Silly Putty (Crayola LLC, Easton, PA, USA) lined the perimeter of the Plexiglas plates to ensure a watertight seal between chambers. Seawater was added to the anterior chamber (~650 ml) and a check for leaks was conducted. Only those preparations that demonstrated no leakage against the head pressure of the anterior region were used. Following the leak test, seawater was added to the posterior chamber (~950 ml) and stir-bars were added to both chambers.

Experimental series

Series 1: post-exercise metabolism in hagfish

Hagfish were transferred from holding tanks to 200 l wet-tables receiving flowing seawater. Hagfish were either subjected to 25 min of exercise induced by chasing and tail-pinching or left undisturbed for the same duration (control group).

Following exercise/control treatments, hagfish were immediately transferred to individual 1.5 l respirometers containing magnetic stir-bars and filled with normoxic seawater. Acclimation to respirometers was not technically possible for all experiments as we were interested in examining the immediate recovery from exercise; therefore, animals from the two treatments were handled identically. An initial 10 ml sample of water was immediately drawn for analysis of oxygen tension (P_{O_2}) via a Clarke electrode ($P_{O_2,initial}$: ~20.8 kPa) and the remainder was stored at −20°C for later determination of total ammonia (T_{Amm}) concentration using the colorimetric salicylate-hypochlorite method (Verdouw et al., 1978); the remainder was stored at −20°C. Immediately following sampling, respirometers were sealed. After 2 h, each respirometer was stirred, a final water sample was drawn and analysed as above ($P_{O_2,final}$: control ~15.5 kPa; exercise ~8.4 kPa), the remainder of which was stored at −20°C for later [T_{Amm}] determination, and respirometer volume and hagfish mass were recorded.

In a second protocol, hagfish were exercised or left undisturbed as described above. However, they were then fitted into partitioned chambers (see above). Following insertion, normoxic seawater ($P_{O_2,initial}$: ~20.7 kPa) was added to both anterior and posterior chambers and initial water samples were drawn before sealing as above. After 2 h, chambers were stirred and final water samples taken and analysed for P_{O_2} (anterior and posterior $P_{O_2,final}$: control ~5.5 and 20.5 kPa; exercise ~3.0 and 20.6 kPa, respectively); the remainder was stored at −20°C for later [T_{Amm}] determination. Anterior and posterior chamber volumes, hagfish mass and relative (anterior and posterior) lengths were then recorded.

Series 2: effects of hypoxia on whole-animal and partitioned metabolic rate

O_2 uptake was characterized during whole-animal and anteriorly localized hypoxia. Hagfish were transferred into 1.5 l respirometers containing hypoxic seawater (P_{O_2} ~3.9 kPa; bubbled with 100% N_2 gas for 20 min) and experiments were performed as above. In a second protocol, hagfish were fitted into partitioned chambers as above. Hypoxic seawater (P_{O_2} ~2.7 kPa) was added to the front compartment while the rear compartment contained normoxic seawater. The minor differences in $P_{O_2,initial}$ between trials should have negligible effect on $\dot{M}_{O_2}$ (Drazen et al., 2011). Sample collection and respirometry trials were conducted as above.

Calculations and statistical analysis

$\dot{M}_{O_2}$ (μmol kg^{-1} h^{-1}) was calculated as previously described (Guffey and Goss, 2014) based on measured P_{O_2} values using the following equation:

$$\dot{M}_{O_2} = ((P_{O_2,initial} - P_{O_2,final}) \cdot \alpha_{O_2} \cdot V) \cdot \frac{1}{m} \cdot \frac{1}{\Delta t}, \quad (1)$$

where P_{O_2} is the initial or final O_2 content in the water (Torr, where 1 Torr≈133 Pa), α_{O_2} is the solubility constant for O_2 (μmol O_2 l^{-1} Torr^{-1}) derived by Boutilier et al. (1984), V is the volume of water (l), m is the animal mass (g) and Δt is the duration of the flux period.

J_{Amm} (μmol kg^{-1} h^{-1}) was calculated as previously described (Clifford et al., 2015a) based on T_{Amm} accumulation in the water using the following equation:

$$J_{Amm} = (([T_{Amm}]_{initial} - [T_{Amm}]_{final}) \cdot V) \cdot \frac{1}{m} \cdot \frac{1}{\Delta t}, \quad (2)$$

where [T_{Amm}] is the initial or final concentration of ammonia in the water (μmol l^{-1}).

Anterior and posterior $\dot{M}_{O_2}$ rates ($\dot{M}_{O_2,ant}$ and $\dot{M}_{O_2,post}$) were converted to branchial and cutaneous rates ($\dot{M}_{O_2,branc}$ and $\dot{M}_{O_2,cutan}$) as follows:

$$\dot{M}_{O_2,cutan} = \dot{M}_{O_2,post} + \left(\left(\frac{\dot{M}_{O_2,post}}{L_{post}}\right) \cdot L_{ant}\right), \quad (3)$$

$$\dot{M}_{O_2,branc} = \dot{M}_{O_2,ant} - \left(\left(\frac{\dot{M}_{O_2,post}}{L_{post}}\right) \cdot L_{ant}\right), \quad (4)$$

where L_{ant} and L_{post} are the measured body lengths (cm) in the anterior and posterior compartments. Eqns 3 and 4 were used similarly to calculate branchial and cutaneous J_{Amm} ($J_{Amm,branc}$ and $J_{Amm,cutan}$, respectively). Our calculations assume that anterior skin behaves similarly to posterior skin, based on scanning electron microscopy with vascular corrosion casts (Lametschwandtner et al., 1989) and light microscopy (Potter et al., 1995) studies demonstrating that dermal capillaries are similarly structured and uniform in anterior, median and posterior sections in three species of hagfish (*M. glutinosa*, *Paramyxine atami* and *E. stoutii*; Welsch and Potter, 1998). All data are expressed as the mean±1 s.e.m. Whole-animal and combined branchial and cutaneous rate comparisons were made using unpaired *t*-tests, while comparisons between anterior and posterior rates within the same group were made using paired *t*-tests. The fiducial limit of significance was $P<0.05$.

RESULTS AND DISCUSSION

Routine $\dot{M}_{O_2}$ in the current study (583.4±162.3 μmol kg^{-1} h^{-1}; Fig. 1A) was consistent with other reported values for *E. stoutii* (Cox et al., 2011; Munz and Morris, 1965). To our knowledge, this study is the first to describe $\dot{M}_{O_2}$ and J_{Amm} in Pacific hagfish following exercise. Excess post-exercise O_2 consumption (EPOC; Wood, 1991), whereby $\dot{M}_{O_2}$ nearly doubled (1085.0±148.0 μmol kg^{-1} h^{-1}) compared with routine rates (P=0.03; Fig. 1A), probably served to replenish phosphagen stores and oxidize lactate (Wood, 1991). Increased $\dot{M}_{O_2}$ in post-exercise hagfish was accompanied by a ~4-fold increase in J_{Amm} (126.1±17.4 versus 33.2±6.4 μmol kg^{-1} h^{-1} under resting conditions, $P<0.01$; Fig. 1B), a response associated with post-exercise recovery (Wood, 1991, 2001) as a result of adenylate deamination (Dobson and Hochachka, 1987).

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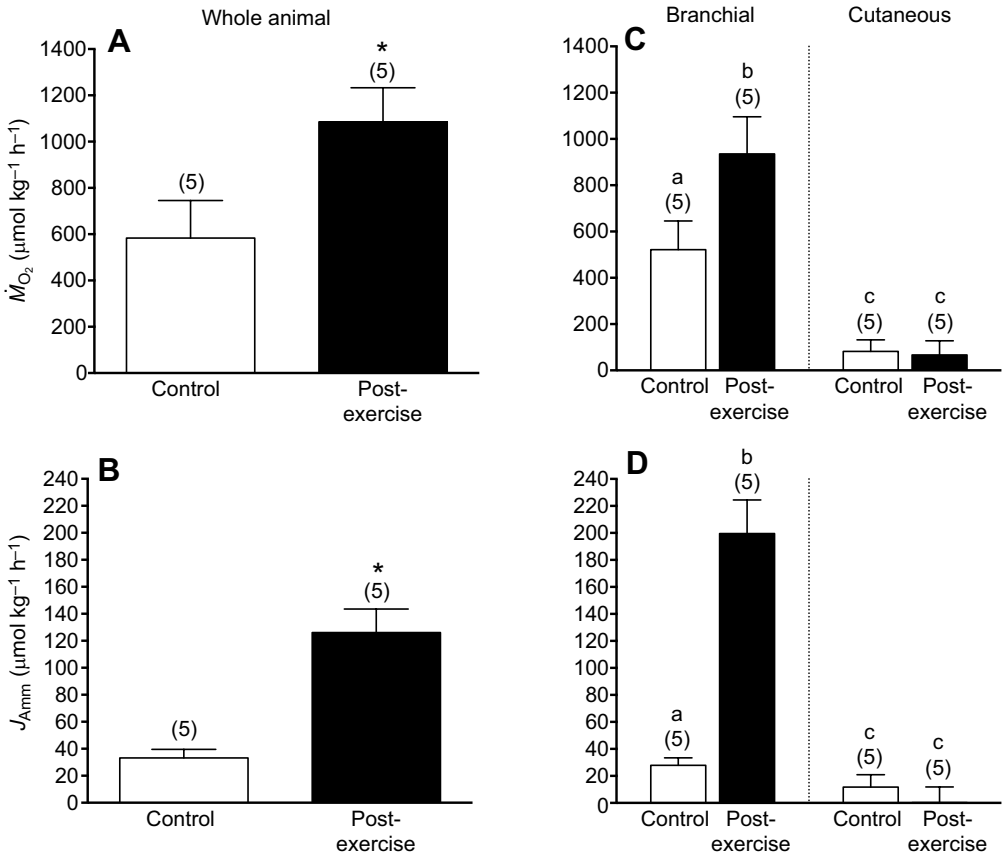

Fig. 1. Routine and post-exercise metabolism in Pacific hagfish. Whole-animal (A,B) and partitioned branchial and cutaneous (C,D) oxygen consumption ($\dot{M}_{O_2}$; A,C) and ammonia excretion (J_{Amm}; B,D) of hagfish under routine conditions (control) and following exhaustive exercise. Means+s.e.m. (*N* values are given in parentheses). Significant differences are noted by asterisks in A and B and by different letters in C and D (P<0.05).

Whole-animal metabolic metrics did not significantly differ from combined partitioned rates for either control ($\dot{M}_{O_2}$: 583.3±162.3 versus 604.0±107.26 µmol kg^{-1} h^{-1}, P=0.92; J_{Amm}: 33.2±6.4 versus 39.6±7.8 µmol kg^{-1} h^{-1}, P=0.54; whole-animal versus combined, respectively) or post-exercise ($\dot{M}_{O_2}$=1085.0±148.0 versus 1001.6±139.8 µmol kg^{-1} h^{-1}, P=0.69; whole-animal versus combined, respectively) hagfish with the exception of J_{Amm} in post-exercise animals, which was slightly elevated in partitioned experiments (199.8±19.7 versus 126.1±17.4 µmol kg^{-1} h^{-1} in whole-animal experiments, P=0.03). Thus, we concluded that partitioned chambers did not interfere with normal metabolism.

In control animals, branchial $\dot{M}_{O_2}$ averaged 521.7±123.8 µmol kg^{-1} h^{-1}, while cutaneous rates were much lower (82.3±22.2 µmol kg^{-1} h^{-1}, P=0.04; Fig. 1C). Following exercise, the increase in O_2 uptake was solely a function of branchial exchange, which nearly doubled to 935.1±160.8 µmol kg^{-1} h^{-1} (P=0.04) comprising 92±3.1% (Table 1) of total $\dot{M}_{O_2}$, further confirming the dominant role of gills over skin in O_2 uptake by Pacific hagfish. Cutaneous $\dot{M}_{O_2}$ remained unchanged following exercise at 66.5±27.5 µmol kg^{-1} h^{-1} (P=0.33). Interestingly, while ~30% of total resting J_{Amm} in control hagfish was cutaneous, the 6-fold increase in post-exercise animals (P<0.01) was due solely to an increase in branchial J_{Amm}, comprising 99.3±0.7% of total ammonia excretion (Fig. 1D, Table 1). Pacific hagfish did not demonstrate a dominant role for cutaneous O_2 uptake in either control (19±7.3%) or post-exercise (8.0±3.1%) animals in contrast to previous reports with Atlantic hagfish, where cutaneous O_2

Table 1. Calculation of skin-specific rates and corrected distribution of $\dot{M}_{O_2}$ and J_{Amm} in branchial and cutaneous components

	Uncorrected rate (µmol kg^{-1} h^{-1})		Skin length in chamber (cm or %)			Corrected rate (%)		
Treatment	Anterior	Posterior	Anterior	Posterior	Skin length-specific rate (µmol kg^{-1} h^{-1} cm^{-1})	Branchial	Cutaneous	s.e.m.
$\dot{M}_{O_2}$								
Control	560.0±116.1	44.0±11.5	18.74±7.05	22.14±15.07	2.0±0.59	81.0	19.0	±7.3
Post-exercise	966.3±150.7	35.3±14.7	17.96±10.59	20.24±12.52	1.66±0.70	92.0	8.0	±3.1
J_{Amm}								
Control	33.3±6.4	6.3±2.2	41.7±1.1	49.0±1.4	0.30±0.12	70.7	29.3	±6.9
Post-exercise	199.7±23.3	0.04±2.8	42.1±0.7	47.4±1.0	−0.01±0.14	99.3	0.7	±2.4

Measured anterior and posterior oxygen consumption ($\dot{M}_{O_2}$) and ammonia excretion (J_{Amm}) from series 1 experiments were converted to corrected branchial and cutaneous rates (Fig. 1). Rates were calculated assuming similar $\dot{M}_{O_2}$ and J_{Amm} per cm and based on individual animal skin lengths using Eqns 3 and 4. Average skin lengths are presented in cm (adjacent to $\dot{M}_{O_2}$), and were converted into % length (presented adjacent to J_{Amm}). N=5.

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uptake was proposed to account for 75–89% of total uptake (Lesser et al., 1996; Steffensen et al., 1984).

While feeding on decaying carrion, hagfish immerse their heads in the flesh (Hardisty, 1979), subjecting the gills to a localized environment that is hypoxic, hypercapnic and with a high [T_{Amm}] (Clifford et al., 2015b), while the tail remains outside. We hypothesized that significant cutaneous $\dot{M}_{O_2}$ might only occur when branchial $\dot{M}_{O_2}$ was impaired. Indeed, Lesser et al. (1996) found that whole-animal $\dot{M}_{O_2}$ was maintained when branchial pores of Atlantic hagfish were sutured shut, and therefore concluded that hagfish must recruit the skin to maintain $\dot{M}_{O_2}$ in the absence of a branchial contribution. Based on these observations, we had predicted that anteriorly localized hypoxia would have minimal effects on whole-animal $\dot{M}_{O_2}$. The asymmetric conditions in this experiment prevented branchial and cutaneous contribution corrections and data are thus presented as anterior and posterior $\dot{M}_{O_2}$.

We found that whole-animal $\dot{M}_{O_2}$ decreased by 90% in hagfish during non-localized, whole-animal hypoxia, as noted in previous reports (Forster, 1990). Contrary to our hypothesis, hagfish that had been anteriorly exposed to hypoxia also had a dramatically reduced $\dot{M}_{O_2}$, whereby summed anterior and posterior $\dot{M}_{O_2}$ during localized hypoxia was not significantly different from $\dot{M}_{O_2}$ during whole-animal hypoxic exposure (87.3±9. versus 54.8±61.4 µmol kg^{-1} h^{-1}, P=0.67, respectively; Fig. 2). Nevertheless, during anteriorly localized hypoxia, posterior $\dot{M}_{O_2}$ was significantly elevated (81.8±6.7 µmol kg^{-1} h^{-1}) compared with control posterior $\dot{M}_{O_2}$ rates (44.0±11.5 µmol kg^{-1} h^{-1}, P<0.01), thereby accounting for ∼94% of summed anterior and posterior $\dot{M}_{O_2}$ in animals exposed to anteriorly localized hypoxia. The increase in posterior $\dot{M}_{O_2}$ during anteriorly localized hypoxia probably occurred as a result of reduced arterial P_{O_2} augmenting the driving gradient for O_2 transport at the skin. Indeed, measurements in the anterior chamber during anteriorly localized hypoxia revealed increases in P_{O_2} from baseline hypoxia for some (3 of 6) experimental trials, suggesting loss of O_2 across the gills. Importantly, on an absolute basis, this posterior increase was small, so these data clearly demonstrate that even when branchial O_2 acquisition is greatly impaired and transcutaneous water–blood P_{O_2} gradients are likely to be optimal, the skin is not recruited to any great extent to maintain whole-animal $\dot{M}_{O_2}$ at control rates, contrary to observations made in the burrowing Atlantic hagfish (Lesser et al., 1996).

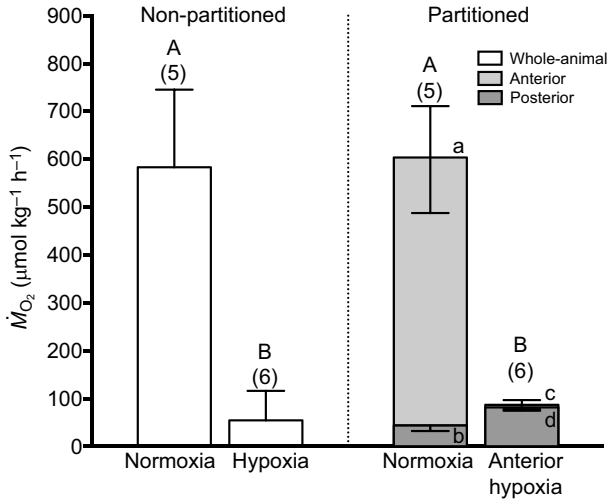

Fig. 2. Measured $\dot{M}_{O_2}$ in hagfish exposed to either normoxia or hypoxia. Hagfish were exposed to hypoxia in non-partitioned chambers (left) or normoxia and anteriorly localized hypoxia in partitioned chambers (right). Bars on the left for non-partitioned data and those on the right for combined anterior and posterior data are presented as means+s.e.m. (*N* values are given in parentheses); bars on the right for partitioned anterior and posterior data are given as means−s.e.m. Bars sharing common letters are not significantly different (P<0.05). Uppercase letters denote significant differences for whole-animal/combined anterior and posterior measurements; lowercase letters denote significant differences for partitioned rates.

The contrasting cutaneous O_2 exchange contributions presented here versus those reported previously (Steffensen et al., 1984; Lesser et al., 1996) may be explained by species-specific differences and/or differences in experimental design. Notably, there may be more extensive vascularization in burrowing hagfish species (Potter et al., 1995; Welsch and Potter, 1998). Moreover, Steffensen et al. (1984) concluded that over 80% of $\dot{M}_{O_2}$ occurred cutaneously in Atlantic hagfish based on measuring branchial ventilation rates and assuming that gill oxygen extraction could not account for the whole-animal $\dot{M}_{O_2}$ measured in their study. Malte and Lomholt (1998) have challenged these findings, claiming that Steffensen et al. (1984) probably underestimated gill oxygen extraction, leading to overestimates of cutaneous contributions. Lesser et al. (1996) similarly did not directly assess branchial and cutaneous $\dot{M}_{O_2}$ but rather estimated cutaneous $\dot{M}_{O_2}$ by measuring $\dot{M}_{O_2}$ in Atlantic hagfish whose branchial pores had been sutured shut. The methods used in the current study directly measured branchial and cutaneous $\dot{M}_{O_2}$ in Pacific hagfish. Indeed, partitioned chambers have been effectively utilized by our group (Clifford et al., 2014; Zimmer and Wood, 2015; Zimmer et al., 2014) and others (Urbina and Glover, 2015; Urbina et al., 2014, 2012) to tease apart the remarkably specialized roles of skin in physiological function in fish. Our study thus demonstrates that in Pacific hagfish, the skin does not contribute significantly to gas exchange. This is in contrast to studies conducted in the smaller, burrowing Atlantic hagfish, in which the epidermal capillary network (Potter et al., 1995) may support cutaneous exchange observed in previous work (Steffensen et al., 1984; Lesser et al., 1996). Perhaps the high tolerance to hypoxia/anoxia in Pacific hagfish (Cox et al., 2011) renders cutaneous respiration unnecessary in this species. Our findings highlight the need for comparative studies in different hagfish species examining the roles of the skin and the underlying morphological and physiological factors driving differences in cutaneous exchange.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
The study was conceived and conducted by A.M.C. and A.M.Z.; A.M.C. and A.M.Z. analysed the data; A.M.C. wrote the manuscript; all authors revised and approved the final manuscript.

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