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The osmorespiratory compromise in marine flatfish: differential effects of temperature, salinity, and hypoxia on diffusive water flux and oxygen consumption of English sole (*Parophrys vetulus*) and Pacific sanddab (*Citharichthys sordidus*)

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Abstract

There have been few studies in seawater fish of the osmorespiratory compromise, the trade-off between the demands of high permeability of the gills for respiration but low permeability for osmoregulation. Flatfish are of particular interest because of their high tolerance to hypoxia, low metabolic rates, and partial reliance on the skin for O₂ uptake (MO₂). We studied the differential effects of acute challenges in temperature (12 °C to 7 °C, 12 °C to 17 °C), salinity (32 to16), and O₂ (> 80% to ~ 10% air saturation) on diffusive water flux and MO₂ of adult English sole (*Parophrys vetulus*) and Pacific sanddab (*Citharichthys sordidus*). Under acclimation conditions (normoxia, 12 °C, 32), the two species exhibited almost identical diffusive water flux rates (~0.2 ml/g/h) and MO₂ (~ 2 µmol/g/h). These values did not depend on the presence or absence of sand in which to bury. Under acute hypoxia (~10% saturation), both species exhibited substantial reductions in MO₂ (by 86–89%) and diffusive water flux (by 32–48%), similar to the osmorespiratory compromise responses of hypoxia-tolerant freshwater fishes. However, the species differed in their sensitivity to acute temperature and salinity challenges. Q_{10} values for diffusive water flux rates (2.99 overall) were higher than those for MO₂ (2.06) in the sole, whereas the opposite was true in the sanddab (2.31 versus 3.07). Both species increased diffusive water flux rate (by 26–34%) at salinity 16, but MO₂ remained unchanged in the sanddab and increased by 19% in the sole, suggesting uncoupling of the two processes.

Keywords Marine flatfish \cdot Oxygen consumption rate \cdot Diffusive water flux \cdot Osmorespiratory compromise \cdot Temperature \cdot Salinity and hypoxia

Introduction

The traditional osmorespiratory compromise has been described as a trade-off between conditions that favour O_2 uptake from the external environment (e.g. low water-toblood diffusion distance, high surface area, high effective permeability of the gill membranes) and those that minimize

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unfavourable ion and water fluxes (e.g. thick epithelia, small surface area, low effective permeability of the gill membranes) (Steen and Krusse 1964; Randall et al. 1967, 1972; Sundin and Nilsson 1997; Gonzalez 2011; Gilmour and Perry 2018). The osmorespiratory compromise has been well studied, mostly in freshwater fish, in the context of MO₂ versus ion loss (e.g. Randall et al. 1972; Wood and Randall 1973a, b; Nilsson 1986; Gonzalez and McDonald 1992; Gonzalez and McDonald 1994; Wood et al. 2009; Iftikar et al. 2010; Robertson et al. 2015; Giacomin et al. 2019; Giacomin et al. 2020). Somewhat less information is available with respect to MO₂ versus diffusive water flux rate, but it is in general accord with the ion flux data (Wood et al. 2009, 2019; Onukwufor and Wood 2018, 2020a, b; Giacomin et al. 2020). The emerging picture, summarized by Wood and Eom (2021), is more complex than originally believed. At the start of oxygen stress (e.g. exercise

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or hypoxia), highly aerobic fish such as rainbow trout and zebrafish exhibit the traditional osmorespiratory compromise with increased net ion losses and water fluxes—i.e. they sacrifice osmoregulatory homeostasis so as to improve MO₂. However, as the oxygen stress continues, they are able to bring these unfavourable fluxes back under control while maintaining MO₂. In contrast, less aerobic, hypoxia-tolerant fish such as Amazonian oscar and common killifish are able to selectively reduce ion and water fluxes almost as soon as hypoxia starts, and maintain this lowered permeability indefinitely, while still attempting to improve MO₂, as shown by continuing hyperventilation.

Diffusive water fluxes, measurable by tritiated water $({}^{3}\text{H}_{2}\text{O})$ efflux represent unidirectional water flux rates and are many-fold greater than net osmotic water flux rates, but are related to them as explained in greater detail by Wood et al. (2019). In general, earlier measurements of diffusive water flux rates in marine fish established that they are lower than in comparable freshwater fish; this may reflect an adaptation (lower water permeability) to deal with the higher osmotic gradients in seawater (Evans 1969; Motais et al. 1969; Potts and Fleming 1970; Motais and Isaia 1972; Isaia 1984). Nevertheless, this is surprising inasmuch as the mechanism of Na⁺ and Cl⁻ excretion in marine fish requires a much higher junctional permeability at the borders of branchial ionocytes than in freshwater fish, so as to facilitate the paracellular efflux of Na⁺ in equimolar amounts to the active transcellular efflux of Cl⁻, as originally proposed by Silva et al (1977) in a model that has withstood the test of time (Wood and Marshall 1994; Evans et al. 2005; Edwards and Marshall 2012). Furthermore, the ionocytes rapidly contract upon transfer to dilute salinities (reviewed by Marshall 2012). This apparent paradox could suggest that the paracellular pathways at the borders of ionocytes are unimportant in diffusive water permeability in seawater fish. There have been only a few studies on the osmorespiratory compromise with respect to diffusive water flux in marine fish, and patterns remain unclear. For example, in the hypoxia-tolerant common killifish, which is fully euryhaline, diffusive water flux rate was lower in seawater as expected, but did not decrease during acute hypoxia, in contrast to the marked reduction during hypoxia in freshwater. This difference occurred despite almost identical MO₂ responses in the two media (Wood et al. 2019; Giacomin et al. 2020). In the hypoxia-tolerant tidepool sculpin in seawater, diffusive water flux rate did decrease during acute hypoxia, but the response disappeared as hypoxia continued and was prevented by higher temperature (Somo et al. 2020).

Temperature is clearly an important factor in the osmorespiratory compromise. By raising metabolic rate, acute increases in temperature elevate MO₂, while acute decreases in temperature depress MO₂. Multiple investigations have demonstrated that the associated Q_{10} values for MO₂ are generally ≥ 2.0 in both freshwater and seawater fish, but there are only a few studies on the Q_{10} values for diffusive water flux, and even fewer where diffusive water flux rate and MO₂ were measured in the same animals (reviewed by Onukwufor and Wood 2020a). In general, Q_{10} values for diffusive water flux were also ≥ 2.0 , indicative of biological mediation rather than simple physicochemical processes. In freshwater rainbow trout (Onukwufor and Wood 2020a), marine tidepool sculpins (Somo et al. 2020), and marine dogfish sharks (Giacomin et al. 2017), Q_{10} values for diffusive water flux were greater than those for MO₂, whereas the freshwater zebrafish appeared to be an exception, with opposite Q_{10} differences (Onukwufor and Wood 2020b).

In the present study, we examined two marine flatfish species, the English sole (Parophrys vetulus) and the Pacific sanddab (Citharichthys sordidus) that reside in inshore coastal environments of the North-West Pacific (Boehlert and Mundy 1987; Rackowski and Pikitch 1989; Rooper et al. 2003, 2006). The two exhibit some subtle ecological and morphological differences, but share a benthic lifestyle, and co-occur in the same locations. Changes in temperature, dissolved oxygen, and salinity are frequent occurrences in coastal waters, especially in estuaries, which these species are known to enter. In general, flatfish have low rates of MO₂, are quite hypoxia-tolerant, and spend long periods buried in sand, all adaptations to their benthic, often hypoxic environment (Watters and Smith 1973; Duthie 1982; Wood et al. 1979; Kerstens et al. 1979; Steffensen et al. 1982; Maxime et al. 2000). Their flattened morphology provides a high cutaneous surface area that appears to be used as a supplementary respiratory epithelium, especially during hypoxia (Steffensen et al. 1981).

With this background in mind, we tested the effects of (i) the presence or absence of sand into which to bury; (ii) acute increases and decreases in temperature; (iii) acute decreases in salinity (50% seawater); and (iv) acute hypoxia (~10% air saturation) on MO₂ and diffusive water flux rates in the two species. Our overall hypotheses were that (i) sand would calm the fish resulting in lower diffusive water flux and MO₂ values; (ii) Q_{10} values ≥ 2.0 would be seen for both parameters, but with greater Q_{10} values for diffusive water flux than for MO₂; (iii) acute decreases in salinity would elevate both diffusive water flux rates and MO₂; (iv) acute hypoxia would decrease diffusive water flux rates as well as MO₂, in these hypoxia-tolerant fishes, and (v) responses patterns would be very similar in the two species, reflecting their similar niches, morphology, and environments.

Materials and methods

Animals

English sole (*Parophrys vetulus*) weighing 257.4 ± 10.8 g (N=40) and Pacific sanddab (*Citharichthys sordidus*) weighing 178.2 ± 1.0 g (N = 40) were caught by angling in Barkley Sound near the Bamfield Marine Sciences Center (BMSC), Bamfield, BC, Canada, under Department of Fisheries and Oceans Canada collection permit XR-204.18. Flatfish were transported to the holding facilities at BMSC where they were allowed to settle for at least 7 days before commencement of experiments. Seawater (12 °C, 32) was continuously pumped from the sea to the BMSC circulation system, and then to the holding and experimental tanks on a flow-through basis. Holding tanks were continuously aerated and enriched with sea sand into which the flatfish routinely buried during the day. Flatfish were fed to satiation every second day with small dead herring. On the day of loading with ³H₂O (see "Animals" section), experimental animals were first removed from the holding tank before the remaining fish were fed. Therefore, considering the subsequent overnight ³H₂O loading period, the flatfish had been fasted for about 60-72 h prior to experimental measurements. The BMSC Animal Care Committee (AUP RS-18-20) and the University of British Columbia Animal Care Committee (AUP 14-0251) approved all the procedures used in these investigations, in accordance with the Canadian Council on Animal Care guidelines.

General protocols

All protocols used in these investigations were based on the procedures outlined in Onukwufor and Wood (2020a), with minor modifications. In brief, to minimize disturbance, darkened, covered containers fitted with aeration and sampling ports were used in all experiments. To simulate the natural environment, sea sand was provided as a burrowing medium in most of the tests, and in the ${}^{3}\text{H}_{2}\text{O}$ loading protocol. All experimental tanks were placed into a water bath connected to a recirculation flow system that was used to maintain the desired temperature. For measurements at lower temperature (7 °C), ice was added to the outside water bath to cool it to the desired temperature. For measurements at higher temperature, a heater was employed.

For each experimental run, 5–7 flatfish were placed simultaneously in a 10-L seawater volume (12 °C, 32, $PO_2 > 80\%$ air saturation) that contained 100 µCi of ³H₂O (PerkinElmer, Woodbridge, ON,). Preliminary

experiments determined that a 12-h loading period was more than sufficient to obtain full equilibration of the external ³H₂O radioactivity with the body water pool of the fish. At the end of the 12-h loading period, flatfish were removed, quickly rinsed with clean seawater, then transferred to individual chambers. The chambers were oblong plastic basin slightly larger than the length and breadth of the fish. Each chamber was served with continuous aeration and contained a 2-L seawater volume, sufficient to cover the fish by several cm, and was initially devoid of ${}^{3}\text{H}_{2}\text{O}$. The measurement of ${}^{3}\text{H}_{2}\text{O}$ efflux started immediately. The conditions in the chamber were the experimental conditions (e.g. sand or no sand, 7, 12, or 17 °C, salinity 32 or 16). PO₂ was kept at > 80% air saturation (normoxia) in all trials except for the hypoxia treatment (10% air saturation). After the initial sand versus no sand test, sand was present during all subsequent experiments. Seawater samples (1 ml) were drawn at time 0 and thereafter every 5 min for 60 min, with a final sample taken at 12 h when washout was complete—i.e. the ³H₂O radioactivity pool had equilibrated with the chamber water. The 12-h period for the final water sampling was based on our initial trials which showed that beyond this time, the water radioactivity was constant. The 0-60 min samples were used to compute the diffusive water flux rates while the 12-h sample was used to determine the original total amount of radioactivity in the fish (see "Effects of acute salinity change on diffusive water flux and oxygen consumption rates" section).

Oxygen consumption rates (MO_2) were measured either simultaneously with the diffusive water flux measurements, or shortly afterwards. Specifically, in the tests of the effects of the presence of sand, variations in temperature and differences in salinity, MO₂ measurements were completed 2 h after diffusive water flux measurements on the same fish. In the hypoxia tests, MO_2 measurement were carried out simultaneously with the diffusive water flux measurements on the same fish. In all but the hypoxia tests, the initial PO₂ was > 80% air saturation when aeration was stopped and the chamber was sealed with a layer of floating bubble-wrap. The subsequent decline in PO₂ was tracked until it reached 50% saturation, a value that is well above the critical PO_2 where MO_2 becomes dependent on PO₂ in these species (O.E. Johannsson and C.M. Wood, unpublished results). This period typically varied from 10 to 40 min in the various treatments. Preliminary tests demonstrated that the ventilation of the fish was adequate to ensure mixing, that the bubble-wrap seal prevented entry of O_2 from the air, and that the microbial blank was undetectable. The latter was ensured by baking the sand overnight at 70 °C immediately prior to its use in each test.

Effects of the presence of sand on diffusive water flux and oxygen consumption rates

Because these experiments were performed during the daytime when flatfish routinely bury in sand, we tested whether the presence or absence of a 2-cm layer of sand in the basins affected the measured rates. Both species were first loaded with ${}^{3}\text{H}_{2}\text{O}$ for 12 h under standard conditions (normoxia, salinity 32, 12 °C, sand present), then acutely transferred to chambers containing sand (N=8) or lacking sand (N=8), both under standard conditions (normoxia, 32, 12 °C).

Effects of acute temperature change on diffusive water flux and oxygen consumption rates

Both species were first loaded with ${}^{3}H_{2}O$ for 12 h under standard conditions (normoxia, salinity 32, 12 °C), then acutely transferred to 12 °C (control, N=7), or acutely decreased temperature (7 °C, N=7), or acutely increased temperature (17 °C, N=7), all under normoxia at 32, in the presence of sand.

Effects of acute salinity change on diffusive water flux and oxygen consumption rates

Here, after the two species were first loaded with ${}^{3}\text{H}_{2}\text{O}$ for 12 h under the standard conditions (normoxia, 32, 12 °C), flatfish were then transferred to the control salinity (32, N=6), or reduced salinity (16, N=6), both under normoxia at 12 °C, in the presence of sand. Salinity was adjusted by diluting the salinity 32 seawater with distilled water and was checked with a WTW Portable Conductivity Meter (Pro-fiLine Cond3310; Xylem Analytics, Welheim, Germany).

Effects of acute hypoxia on diffusive water flux and oxygen consumption rates

For this experiment, following loading with ${}^{3}\text{H}_{2}\text{O}$ for 12 h under the standard conditions (normoxia, salinity 32, 12 °C), the two species were then transferred to normoxic water that was already set at > 80% air saturation (N=6), or hypoxic water that was already set at 15% air saturation (N=6), both at 32, 12 °C. In the normoxia trial, MO₂ was measured in the standard fashion. In the hypoxia trial, the bubble wrap seal was left in place throughout 60-min, and therefore, the time-averaged PO₂ was approximately 10% air saturation. None of the fish died during or subsequent to these tests.

Analytical procedures and calculations

A scintillation counter (LS6500, Beckman Coulter, Fullerton, CA, USA) was used to determine the concentration of ${}^{3}\text{H}_{2}\text{O}$ in the water as described (Onukwufor and Wood

2018). In brief, 2 ml of Optiphase 3 fluor (Perkin-Elmer, Wellesley, MA, USA) was added to the 1-ml water sample and vortexed before loading into the scintillation counter. Our internal standardization tests showed that quenching was constant, thereby removing the need for quench correction. To calculate the rate constant (k) of the ${}^{3}\text{H}_{2}\text{O}$ efflux, we adopted the method used by Onukwufor and Wood (2020a). Briefly, we used the last water sample at the end of 12 h of the washout period (i.e., when the fish water pool was at equilibrium with the external water pool) to back-calculate the initial amount of ${}^{3}\text{H}_{2}\text{O}$ in the fish. Thus, it was feasible to calculate the remaining amount of ${}^{3}\text{H}_{2}\text{O}$ in the fish at every sample time from 0 to 60 min in the flux period. The natural logarithm (ln) of the total ${}^{3}\text{H}_{2}\text{O}$ in the fish at each time was then regressed against time on a linear scale. The slope of this line yielded the fractional rate constant k for water turnover. By multiplying rate constant $k \times 100$, the percent body turnover per hour was obtained. The total exchangeable water pool of fish is typically 0.8 ml/g (Holmes and Donaldson 1969; Isaia 1984; Olson 1992), and this value was multiplied by the percent body turnover per hour to get the diffusive water flux rate in ml/h. To account for the differences in body mass, the logarithm of diffusive water flux rate was regressed against the logarithm of fish weight (Onukwufor and Wood 2020a). The resultant allometric mass scaling coefficient obtained was then used to adjust the diffusive water flux rate of each individual fish to that of a standard 100-g of flatfish and expressed as ml/g/h.

PO₂ measurements were performed using a YSI Model 55 m and dissolved O₂ probe (Yellow Spring, Ohio, USA). MO₂ calculations followed the procedure of Onukwufor and Wood (2020a). In brief, PO₂ values were first converted to O₂ concentrations (in µmol/L) using salinity- and temperature-dependent solubility coefficients (Boutilier et al. 1984). Then the change in O₂ concentration was multiplied by respirometer volume (2 L) and divided by time (in h) to yield MO₂ in µmol O₂/h. By regressing the logarithm of MO₂ against the logarithm of fish weight, we were able to obtain the allometric mass scaling coefficient. We then used this coefficient to adjust each individual fish MO₂ to that of a 100-g flatfish, with the final rate expressed as µmol O₂/g/h. The scaling coefficients are reported in the Results.

Temperature coefficients (Q_{10} values) for diffusive water flux and MO₂ over the ranges 7–12 °C, 12–17 °C and 7–17 °C were calculated as described by Onukwufor and Wood (2020a):

$$Q_{10} = (R_2/R_1)^{[10/(T2-T1)]}$$

where R_2 and R_1 are the rates at the higher temperature (*T*2) and lower temperature (*T*1), respectively. Since different fish were used in each trial, we employed mean values of diffusive water flux rates and MO₂ at each temperature for

these calculations; error estimates and statistical comparisons were not possible.

Statistical analyses

All the data passed normality and homogeneity of variance tests. Data were analyzed using unpaired Student's twotailed *t* test (single comparisons), or One-Way ANOVA plus Tukey's post hoc test (multiple comparisons), with temperature, salinity or hypoxia as independent variables. Significance was accepted at p < 0.05. Statistical analyses, linear regressions, and curve fitting were done using SigmaPlotTM11 (Systat Software, San Jose, CA, USA) and GraphPadTM Prisms v7 (GraphPad Software, San Diego, CA, USA).

Results

Scaling coefficients of diffusive water flux rates and MO₂

When the logarithms of diffusive water flux rates for English sole (*Parophrys vetulus*) were plotted against the logarithms of fish weights, the relationship was significant (p=0.0092) with R^2 =0.65 and a high allometric mass scaling coefficient of b=0.84 (Fig. 1A). Similar to the diffusive water flux rates, when the logarithms of MO₂ were plotted against the logarithms of fish weight, the relationship was again significant (p=0.0017) with R^2 =0.83 and a somewhat lower allometric mass scaling coefficient of b=0.66 (Fig. 1B). For the Pacific sanddab (*Citharichthys sordidus*), similar plots for diffusive water flux rates were significant (p=0.0003) with



Fig. 1 English sole allometric mass scaling coefficients for diffusive water flux rates and MO_2 in **A**, **B** the English sole (*Parophrys vetulus*) and **C**, **D** the Pacific sanddab (*Citharichthys sordidus*) Measurements were all done at the acclimation temperature of 12 °C, salinity 32, under normoxic conditions (> 80% air saturation). **A** log diffusive water flux rate (ml/h) vs log fish weight (g) in *P. vetulus* (*N*=9), **B** log MO_2 (µmol O_2 /h) vs log fish weight (g) in *P. vetulus* (*N*=8), **C**

log diffusive water flux rate (ml/h) vs log fish weight (g) in *Citharichthys sordidus* (N=12), and **D** log MO₂ (µmol O₂/h) vs log fish weight (g) in *Citharichthys sordidus* (N=8). Note that these 12 °C, salinity 32 measurement values were used to derive the coefficients for all experiments for the two species separately. The b values are the slopes of the regression lines. See "Materials and methods" for details

 $R^2 = 0.75$ and a high coefficient value of b = 0.95 (Fig. 1C), while for MO₂, the relationship was not statistically significant (p = 0.056) with a moderate $R^2 = 0.48$ and a lower coefficient value of b = 0.50 (Fig. 1D). In all cases, the 12 °C rates were used to obtain the coefficients as this temperature was the acclimation temperature. For both species, their respective b coefficients for diffusive water flux and MO₂ were used to adjust all experimental rates of individual fish to those for a 100-g flatfish. These rates were then divided by 100 g, yielding a rate in ml/g/h for diffusive water flux or µmol O₂/g/h for MO₂. All data reported in the subsequent Results are these mass-corrected values.

Effects of sand enrichment on diffusive water flux rates and MO₂

Diffusive water flux rates in both English sole and Pacific sanddab were around 0.2 ml/g/h at 12 °C and 32 and did not differ (p > 0.05) with and without sand (Fig. 2A). Similarly, the MO₂ values were about 2 µmol/g/h and did not differ (p > 0.05) with and without sand (Fig. 2B). Although our results showed no effects, we noted that both species seemed to be calmer when sand was present, so we decided to use sea sand as a substrate for burrowing in all the subsequent experiments.

Effects of acute temperature challenge on diffusive water flux and MO_2

Both species exhibited marked responses to acute temperature change (p < 0.0001) overall for both diffusive water flux and MO₂. For MO₂, the greatest relative changes were in the lower temperature challenges.

In English sole, an acute decrease in temperature from 12 to 7 °C resulted in a significant 43% decrease in diffusive water flux rate, whereas an acute temperature rise from 12 to 17 °C resulted in a significant 41% increase (Fig. 3A). The overall Q_{10} for diffusive water flux rate over 7–17 °C was 2.99, with a slightly higher Q_{10} (3.08) at the lower range of 7–12 °C, and a slightly lower Q_{10} (2.91) at the higher range of 12–17 °C (Table 1). An acute decrease in temperature from 12 to 7 °C was accompanied by a significant 40% decrease in MO₂, whereas a rise from 12 to 17 °C was accompanied by a more modest but still significant increase of 19% (Fig. 3B). Therefore, the overall Q_{10} value for MO₂ in English sole was 2.06 (7–17 °C), with a higher value (2.75) in the lower temperature range of 7–12 °C, and a lower value (1.54) at the higher temperature range of 12–17 °C (Table 1).

In Pacific sanddab, an acute drop in temperature from 12 to 7 °C resulted in a significant 35% decrease in diffusive water flux rate, whereas an acute temperature rise from 12 to 17 °C caused a significant increase of 36% (Fig. 3C). The associated Q_{10} values were fairly stable—2.36, 2.27



Fig. 2 Effects of sea sand enrichment on English sole (*Parophrys vetulus*) and Pacific sanddab (*Citharichthys sordidus*). A diffusive water flux rates (ml/g/h) (N=8) and **B** MO₂ (µmol O₂/g/h) (N=8). "+" indicates the presence of sand, "-" indicates the absence of sand. Measurements were all done at the acclimation temperature of 12 °C, salinity 32, using separate fish for each trial. Data are means ± SEM. There were no significant differences (ns, p > 0.05)

and 2.31 for temperature ranges of 7–12 °C, 12–17 °C and 7–17 °C respectively (Table 1). Pacific sanddab responded to an acute reduction in temperature from 12 to 7 °C, with a significant 54% decrease in MO₂ and to an acute elevation in temperature from 12 to 17 °C with a significant 29% increase in MO₂ (Fig. 3D). Therefore, MO₂ was highly sensitive to acute temperature changes in this species with an overall Q_{10} value of 3.07 for temperature range of 7–17 °C. At the lower temperature range of 7–12 °C, the Q_{10} was particularly high (4.81), while at the higher temperature range of 12–17 °C, it was considerably lower (1.96) (Table 1).

Effects of acute salinity challenge on diffusive water flux and MO₂

When English sole were acutely transferred from salinity 32 to salinity16 their diffusive water flux rates increased



Fig.3 Effects of acute temperature changes on English sole (*Parophrys vetulus*) and Pacific sanddab (*Citharichthys sordidus*) **A** English sole diffusive water flux rate (ml/g/h) (N=7), **B** English sole MO₂ (µmol O₂/g/h) (N=7), **C** Pacific sanddab diffusive water flux rate (ml/g/h) (N=7) and **D** Pacific sanddab MO₂ (µmol O₂/g/h)

(*N*=7). Measurements were done at the acclimation temperature of 12 °C, salinity 32, and after acute downward transfer from 12 to 7 °C and acute upward transfer from 12 to 17 °C using separate fish for each trial. Data are means \pm SEM. Means with asterisk (*) within a species are significantly different (*p* < 0.05)

Table 1 The mean Q_{10} values of English sole (*Parophrys vetulus*) and Pacific sanddab (*Citharichthys sordidus*) for diffusive water flux rates and MO₂ during expose to acute temperature challenges from the acclimation temperature of 12 °C to 7 °C and from 12 to 17 °C

	Treatment	Q ₁₀ 7–12	Q ₁₀ 12–17	Q ₁₀ 7–17
English sole	Diffusive water flux rate (ml/g/h)	3.08	2.91	2.99
	$MO_2 \ (\mu mol \ O_2/g/h)$	2.75	1.54	2.06
Pacific sanddab	Diffusive water flux rate (ml/g/h)	2.36	2.27	2.31
	$MO_2 \ (\mu mol \ O_2/g/h)$	4.81	1.96	3.07

Values have been calculated from the group mean rates for N=6-7 fish at each temperature. As different fish were tested at each temperature, it was not possible to calculate error estimates or make statistical comparisons

significantly by 34% (p = 0.0096) (Fig. 4A), whereas MO₂ also increased significantly by 19% (p = 0.0014) (Fig. 4B). When Pacific sanddab were subjected to the same acute salinity challenge from 32 to 16, their diffusive water flux rates increased significantly by 26% (p = 0.0233) (Fig. 4C) but their MO₂ did not change (p = 0.812) (Fig. 4D).

Effects of acute hypoxia on diffusive water flux rates and MO_{2}

English sole responded to transfer from normoxia $(PO_2 > 80\% \text{ air saturation})$ to acute hypoxia $(PO_2 = ~10\% \text{ air saturation})$ with a significant reduction (p < 0.0001) in



Fig. 4 Effects of acute salinity change on English sole (*Parophrys vetulus*) and Pacific sanddab (*Citharichthys sordidus*). **A** English sole diffusive water flux rate (ml/g/h) (N=6), **B** English sole MO₂ (µmol O₂/g/h) (N=6), **C** Pacific sanddab diffusive water flux rate (ml/g/h) (N=6) and **D** Pacific sanddab MO₂ (µmol O₂/g/h) (N=6). Measure-

ments were done at the acclimation salinity of 32, 12 °C, and after acute transfer to salinity 16 using separate fish for each trial (see "Materials and methods"). Data are means \pm SEM. Means with asterisk (*) within a species are significantly different (p < 0.05)

diffusive water flux rate of 32% (Fig. 5A). MO₂ was reduced (p = 0.0017) more dramatically by 89% (Fig. 5B). Pacific sanddab significantly (p < 0.0001) reduced their diffusive water flux rates by 48% during acute hypoxia (Fig. 5C). MO₂ in the sanddab followed a similar pattern as in the sole with a huge reduction (p = 0.0006) of 86% following transfer from > 80 to ~ 10% air saturation (Fig. 5D).

Discussion

Overview

Overall, only some of our hypotheses were confirmed. Surprisingly, despite our perception that both species were calmer in the presence of sand, into which they would bury with their mouths exposed, there were no differences in either MO_2 or diffusive water flux rates under control conditions, in contrast to our first prediction. Nevertheless, since the experiments were carried out during the day when flatfish were mostly buried under the sand in their holding tank, we incorporated sand into all of the subsequent studies, in case it helped them to deal with the more stressful challenges (low salinity, altered temperature, hypoxia). In partial support of our second hypothesis, Q_{10} values in English sole were ≥ 2.0 (except for MO₂ between 12 and 17 °C) and were greater (actually closer to 3.0) for diffusive water flux rate than for MO_2 (Table 1), in agreement with studies on several other species (see "Introduction"). However, for Pacific sanddab, Q_{10} values for diffusive water flux rate were lower, though still > 2.0 and generally below those for MO_2 , whereas Q_{10} values for MO_2 were very high in the 12 to 7 °C range (Table 1). Our third hypothesis that an acute decrease in salinity would elevate both diffusive water flux rates and MO₂ in both species was also partially supported. This proved to be true in English sole, whereas in Pacific sanddab diffusive water flux rate increased but MO_2 did not change upon 50% reduction in salinity (Fig. 4), suggesting that the two processes can be uncoupled. Our fourth hypothesis was confirmed for both species, wherein diffusive water flux rates and MO₂ were both reduced during acute hypoxia stress, though the latter to a much greater



Fig. 5 Effects of transfer from normoxia (PO₂>80% air saturation) to acute hypoxia (PO₂=10% air saturation) on English sole (*Parophrys vetulus*) and Pacific sanddab (*Citharichthys sordidus*). **A** English sole diffusive water flux rate (ml/g/h) (N=6), **B** English sole MO₂ (µmol O₂/g/h) (N=6), **C** Pacific sanddab diffusive water flux rate (ml/g/h)

extent (Fig. 5). This result is similar to those for tidepool sculpins in seawater (Somo et al. 2020) but different from those for trout (Onukwufor and Wood 2018) and zebrafish (Onukwufor and Wood 2020b) in freshwater where diffusive water flux went up, as well as for the killifish in seawater (Wood et al. 2019; Giacomin et al. 2020), where diffusive water flux did not change during acute hypoxia. Our final hypothesis that response patterns would be very similar in the two species was confirmed overall, and can be evaluated by reference to Fig. 6. Despite the differences in Q_{10} values noted above, there were strong positive correlations between diffusive water flux rates and MO₂ in both species during the temperature challenge (Fig. 6A) and hypoxia experiments (Fig. 6C). However, this was less clear during the low salinity challenge trials, where the correlations were much weaker in both species, and indeed not significant in the Pacific sanddab (Fig. 6B). As discussed in "Salinity challenge elicits differential responses of diffusive water flux and MO₂" section, different mechanisms are likely at play during salinity challenge.

(N=6) and **D** Pacific sanddab MO₂ (µmol O₂/g/h) (N=6). Measurements were all done at the acclimation temperature of 12 °C, salinity 32 using separate fish for each trial. Data are means ± SEM. Means with asterisk (*) within a species are significantly different (p < 0.05)

Scaling coefficients of diffusive water flux rates and \mbox{MO}_2

The scaling coefficients for diffusive water flux rates (0.84-0.95) were higher than those for MO₂ (0.50-0.66) in both species. Our scaling coefficients for MO₂ are on the low end of previously reported values for other flatfish species (0.63-0.85) (Duthie 1982; Fonds et al. 1992), which are typical for teleost fish in general (Clarke and Johnston 1999). However, similarly high coefficients for diffusive water flux (0. 81–1.05) have been reported in two other flatfish (sole and European flounder; Evans 1969), stickleback (Evans 1969), and tilapia (Potts et al. 1967) in seawater, as well as in zebrafish (Onukwufor and Wood 2020b), rainbow trout (Onukwufor and Wood 2020a), tilapia (Potts et al. 1967), brown trout and yellow eel (Evans 1969) in freshwater, suggesting that this trait maybe shared across multiple species and salinity. The consistently higher scaling coefficients suggest that as body mass changes, different principles govern diffusive water flux relative to those determining MO_2 ,



Fig. 6 The relationships between MO₂ (µmol O₂/g/h) and diffusive water flux rate (ml/g/h) for **A** temperature challenges (12 °C to 7 °C and 12 °C to 17 °C, N=7), **B** salinity challenges (32 to 16) N=6 and **C** hypoxia challenges (PO₂>80% to 10% air saturation) N=7, in English sole (*Parophrys vetulus*, green dots) and Pacific sanddab (*Citharichthys sordidus*, red dots). R^2 and significance levels are given for the regressions, with the following equations, **A** temperature (English sole: Y=0.1211 * X - 0.02722 and Pacific sanddab: Y=0.07405 * X+0.07673), **B** salinity (English sole: Y=0.1502 * X - 0.09697 and Pacific sanddab: Y=0.05030 * X+0.1066). In the above equations *Y* is diffusive water flux rate (ml/g/h) and *X* is MO₂ (µmol O₂/g/h). Values are means ± SEM

as discussed in "Acute temperature challenges alter gill membrane permeability for diffusive water flux and MO₂" section.

It should be noted that potential ${}^{3}\text{H}_{2}\text{O}$ recycling due to drinking was not a concern in this study. Reported drinking rates in flatfish (Carroll et al. 1994, 1995), including the English sole (Webb and Wood 2000), are relatively low, in the order of 0.001–0.003 ml/g/h relative to our recorded diffusive water flux rates of 0.1–0.3 ml/g/h. Furthermore, the specific activity of the water in the external bath was at most only a few percent of that in the fish.

Acute temperature challenges alter gill membrane permeability for diffusive water flux and MO₂

Like many other fish in both freshwater and seawater that increase gill permeability with increases in temperature (Evans 1969; Loretz 1979; Giacomin et al. 2017; Onukwufor and Wood 2018; Onukwufor and Wood 2020a; Onukwufor and Wood 2020b; Somo et al. 2020), English sole and Pacific sanddab increased both diffusive water flux rate and MO₂ at high temperature and reversed these trends at low temperature (Fig. 3). This suggests that a universal influence of temperature occurs across different fish species for these two parameters. The increase in effective gill permeability would help increase the rate of oxygen flux from the environment to the mitochondria to meet increased demand for ATP production at higher temperature (Onukwufor et al. 2015, 2016, 2017). This increase is mediated in part by the modulatory effects of temperature on the primary components of the osmorespiratory compromise such as water and blood flow distribution, water-to-blood diffusion distance and effective gill surface area (Randall et al. 1972; Nilsson 1986). However, the overall MO₂ will be determined by the entire transport pathway to the mitochondria, including circulation, oxygen loading and unloading by erythrocytes, and diffusion from capillaries to mitochondria. On the other hand, diffusive water flux rate may be determined only by events at the gill (and perhaps skin) surface, where it is believed that aquaporins play a major role (Tingaud-Sequeira et al. 2010; Cerda and Finn 2010; Madsen et al. 2015). This may explain not only the different allometric scaling coefficients (see "Scaling coefficients of diffusive water flux rates and MO_2 " section), but also different Q_{10} values for diffusive water flux versus MO₂. Notably, in both species, the Q_{10} values for MO₂ were much greater in the 12 °C to 7 °C range than in the 12 °C to 17 °C range (Table 1), a phenomenon that is interpreted as a general decline in metabolic performance once the optimal temperature is exceeded (Sinclair et al. 2016). This was not seen for diffusive water flux Q_{10} values (Table 1), again indicating the different mechanisms involved. There are similar reports of higher Q_{10} values for MO_2 at lower temperatures in a variety of fish species (e.g.

Roberts 1964; Walsh et al. 1983; Lemons and Crawshaw 1985).

In accord with several previous studies on diverse species [trout in freshwater (Onukwufor and Wood 2020a), dogfish sharks (Giacomin et al. 2017) and tidepool sculpins (Somo et al. 2020) in seawater], diffusive water flux rates in the English sole had a higher temperature sensitivity (greater Q_{10} values) than did MO₂ (Table 1, Fig. 3). This may reflect the fact that diffusive water flux rate is mainly a facilitated diffusion process mediated by aquaporins (Ruhr et al. 2020). On the other hand, the opposite pattern in Pacific sanddab $(MO_2 Q_{10})$ > diffusive water flux Q_{10}) was similar to that in the freshwater zebrafish (Onukwufor and Wood 2020b). Perhaps, aquaporins make a lesser contribution in sanddab and zebrafish. Worthy of note, is the fact that these two flatfish appear to inhabit similar ecological niches (Boehlert and Mundy 1987; Rackowski and Pikitch 1989; Rooper et al. 2003, 2006), but with different temperature sensitivities of the two processes. Nevertheless, in both species, there were strong positive correlations of MO2 and diffusive water flux associated with acute temperature challenges (Fig. 6A), in accord with both the general effects of temperature on biologically mediated processes (Hazel and Prosser 1974; Cossins and Prosser 1978), and at least partial coupling through the osmorespiratory compromise at the gills.

Salinity challenge elicits differential responses of diffusive water flux and MO₂

It is well established that diffusive water flux rates are generally lower in seawater fish than in freshwater fish, possibly an adaptation to the higher osmotic gradient in seawater (see "Introduction"). Several reports have also established that acute transfer of marine fish to lower salinity tends to increase both diffusive water flux rate (Motais et al. 1969; Evans 1969) and MO₂ (Barton and Barton 1987; Dalla Via et al. 1998; Herrera et al. 2012). The latter appears to be a short-term stress effect lasting several hours, as over the longer term (days) MO_2 returns to either pre-transfer or lower levels. However, to our knowledge, the present study is the first to investigate simultaneous changes in diffusive water flux rate and MO₂ during the acute phase of transfer to lower salinity. The results indicate that this treatment decouples diffusive water flux rate, which increased in both species, from MO₂, which did not change in Pacific sanddab (Fig. 4D), and increased to a lesser extent than MO_2 in English sole (Fig. 4B). The weaker, more shallow regression relationships (Fig. 6B) also illustrate this point. The species difference in the MO₂ response also indicates that the sanddab may be better able to cope with entry into lower salinities in the inshore environment.

Our findings reinforce the point made in the Introduction that the diffusive water flux increases upon acute exposure to reduced salinity appear to be de-coupled from changes in the paracellular permeability of the junctions bordering the ionocytes that would be decreasing at this time (Edwards and Marshall 2012; Marshall 2012). These results also indicate that the diffusive water flux increases are de-coupled from the trans-gill osmotic gradient which is sharply decreased at salinity 16 relative to salinity 32, and also de-coupled to some extent from changes in the respiratory pathway for O_2 uptake. The latter predominantly involves the secondary lamellae which generally have a sparse distribution of ionocytes (Evans et al. 2008). Other explanations remain possible, such as rapid changes in the apical exposure of gill ionocytes at lower salinity (Marshall and Nishioka 1980; Wood and Marshall 1994; Evans et al. 1999).

However, we suggest that future investigations should focus on two possible explanations. The first would be that the aquaporin pathway (largely transcellular) is quickly upregulated in response to decreased salinity, independent of any osmorespiratory compromise changes in the gills contributing to increased MO_2 as a result of stress. There is abundant evidence that branchial expression (at both mRNA and protein levels) of aquaporins increases upon transfer to dilute salinities (e.g. Lignot et al. 2002; Cutler et al. 2007; Tipsmark et al. 2010; Madsen et al. 2015; Breves et al. 2016). It is problematic whether aquaporin expression could change within the 1-h time course of our transfers, so a functional change in the channel proteins would be more likely. The second explanation could be related to this possibility-a virtually instantaneous increase in gill diffusive water permeability caused by the 50% reduction in environmental calcium concentration when salinity is reduced from 32 to 16. Calcium, and to a lesser extent magnesium, are known to have a potent, almost immediate effects in reducing many aspects of gill permeability, including the osmotic permeability to water (reviewed by Wendelaar Bonga and Van der Meij 1981; Hunn 1985), so parallel effects on diffusive water permeability are likely.

Acute hypoxia reduces both diffusive water flux and MO₂

Both species reduced diffusive water flux rates (Fig. 5A, C) and MO₂ (Fig. 5B, D) in response to acute hypoxia exposure, resulting in strong correlative relationships (Fig. 6C). This reduction in diffusive water flux is exactly opposite to the traditional osmorespiratory compromise response (see "Introduction") in which diffusive water flux rates and ion flux rates are elevated during acute hypoxia stress, as seen in goldfish (Loretz 1979), trout (Iftikar et al. 2010; Robertson et al. 2015; Onukwufor and Wood 2018), and zebrafish (Robertson et al. 2015; Onukwufor and Wood 2020b). However, it is similar to a growing number of observations on hypoxia-tolerant species such as Amazonian oscars and

tambaqui (Wood et al. 2009; De Boeck et al. 2013; Robertson et al. 2015) and killifish (Wood et al. 2019; Giacomin et al. 2020) in freshwater, where exactly the opposite occurs, as well as the tidepool sculpin in seawater (Somo et al. 2020). Many hypoxia-tolerant fish, including some flatfish (Maxime et al. 2000; Chu and Gale 2017; Tunnicliffe et al. 2020), have a great capacity for metabolic suppression during hypoxia (Richards 2009, 2011). Thus, an entirely different regulatory mechanism that reduces osmoregulatory costs at times of oxygen shortage, without impeding respiratory gas exchange (Wood and Eom 2021) may be part of this strategy, and would be clearly beneficial. To date, the mechanism has been worked out only in the Amazonian oscar, where it involves an extension of the gill pavement cells over the ionocytes, thereby reducing the functional exchange area for ion and water exchange, without compromising the conditions for respiratory gas exchange (Scott et al. 2008; Wood et al. 2009; Matey et al. 2011; De Boeck et al. 2013). Whether this same mechanism applies in these marine flatfish, and whether at least part of the adjustment occurs in the skin which may serve as an accessory gas exchange surface in flatfishes (Steffensen et al. 1981), are key topics for future investigation.

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Data availability Data are available upon request.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethics approval The BMSC Animal Care Committee (AUP RS-18-20) and the University of British Columbia Animal Care Committee (AUP 14-0251) approved all the procedures used in these investigations, in accordance with the Canadian Council on Animal Care guidelines.

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