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ORIGINAL PAPER

The effect of salinity and calcium on diffusive water flux, oxygen consumption, and nitrogenous waste excretion rates in Pacific sanddab (*Citharichthys sordidus*) and Rock sole (*Lepidopsetta bilineata*)

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Abstract
Marine flatfishes have a low metabolic rate and routinely encounter large fluctuations in salinity, and are therefore of interest in the study of diffusive water flux (a proxy for transcellular water permeability), oxygen consumption ($\dot{M}O_2$), ammonia excretion and urea-N excretion as a function of salinity and seawater [Ca^{2+}]. These parameters were measured in two coastal marine flatfishes, Pacific sanddab and Rock sole acclimated to 31 ppt and exposed acutely (for up to 3 h), to environmentally relevant salinities of 45, 15.5, or 7.5 ppt. In both species, diffusive water flux and ammonia excretion rates increased as salinity decreased. $\dot{M}O_2$ and urea-N excretion rates remained relatively unchanged. Nitrogen quotient analysis indicated increased oxidation of protein at lower salinity. A second experimental series was performed on Rock sole to separate the effects of salinity from those of ambient [Ca^{2+}]. In direct contrast to the significant increase seen at 7.5 ppt, reducing salinity from 31 ppt to 7.5 ppt while maintaining [Ca^{2+}] at 10 mM or increasing it to 20 mM resulted in no change in diffusive water flux rate, demonstrating that reduced [Ca^{2+}], rather than reduced salinity itself, is the primary cause for the increases in diffusive water flux. However, ammonia excretion rate increased when salinity was decreased and [Ca^{2+}] was increased compared to 31 ppt with added [Ca^{2+}]. Our results demonstrate that both diffusive water flux and ammonia excretion rates are a function of salinity, that neither are coupled to $\dot{M}O_2$, and that ambient [Ca^{2+}] also plays a role in these rates.

Keywords Marine flatfish · Osmorespiratory compromise · Tritiated water · Ammonia excretion · Urea-N excretion

Introduction

As the world's climate gets warmer, ocean salinities are changing. Recent analyses show that in the past 50 years, the global water cycle has amplified by 2 ~ 4% per degree Celsius since 1960, resulting in greater salinity extremes, with the largest effects in surface waters (Stagl et al. 2014; Cheng et al. 2020). Coastal waters of the north-west Pacific are becoming less saline, and greater reductions will likely occur in estuarine areas as winter and spring runoff events become more extreme (Rogers et al. 1984; Cao et al. 2020). On the other hand, sea level rise drives the formations of coastal lagoons, which can quickly develop hypersalinity due to evaporation (Kjerfve 1994). Estuaries are prime habitats for many inshore fish species such as flatfishes (Order Pleuronectiformes), which in general have considerable capacity for euryhalinity (reviewed by Schreiber 2001; Ruiz-Jarabo et al. 2015). Both of these syntheses emphasize that there has been considerable research on salinity effects in flatfish early life stages, but very little on adults. Nevertheless, it is clear that salinity fluctuations exert considerable allostatic costs throughout the life cycle (Ruiz-Jarabo et al. 2015), and acute salinity challenges may elicit increases in oxygen consumption rates (e.g. Herrera et al. 2012; Onukwufor and Wood 2022). In the present study, we address the effects of acute decreases and increases in salinity on adults of two Pacific flatfish species that are known to move in and out of estuaries, thereby routinely encountering salinity challenges. Our experiments focused on water exchange, ammonia and

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urea-N excretion (indicative of protein metabolism), and aerobic metabolic rate as reflected in oxygen consumption ($\dot{M}O_2$) We also performed experiments to separate the effect of environmental calcium concentration, which usually, but not always, co-varies with salinity (Connors and Kester 1974), from those of salinity itself.

Water exchange in teleost fish is a complex and as yet relatively poorly understood process. As reviewed by Wood et al. (2019), it is presently believed that water moves across the gills through two distinct pathways, paracellularly through tight junctions where it is thought to move by bulk flow, and by diffusion across the entire gill surface, the latter largely by the transcellular pathway through aquaporins. Aquaporins are channels in cell membranes that greatly facilitate the diffusion of water. They were first identified by Preston et al. (1992), and several different types are now known to be abundant in fish gills (Cerdà and Finn 2010; Tingaud-Sequeira et al. 2010; Madsen et al. 2015). Diffusive water flux is measured by the unidirectional movement of tritiated water (^3H_2O) and is at least two orders of magnitude greater than net flux rate through the paracellular pathway, which can be measured only by indirect means. Therefore, measurement of diffusive water flux rate with ^3H_2O is considered a proxy for transcellular water permeability. Diffusive water flux rate is lower in seawater fishes compared to freshwater fishes which may represent an adaptation to combat the higher osmotic gradient between the external seawater environment and internal plasma (Evans 1969; Motais et al. 1969; Potts and Fleming 1970; Isaia 1984; Wood et al. 2019). This may also be due to the differences in specific ion concentrations such as Ca^{2+} (Isaia and Masoni 1976; Isaia 1984; Hunn 1985) which are higher in seawater than in freshwater. In both the Pacific sanddab and the English sole, a decrease in salinity resulted in an increase in diffusive water flux rate (Onukwufor and Wood 2022). In accord with this general pattern, many studies have reported lower expression of aquaporins in the gills in seawater fishes compared to freshwater fishes (e.g. Lignot et al. 2002; Cutler et al. 2007; Tipsmark et al. 2010; Madsen et al. 2015; Breves et al. 2016). However, some exceptions occur. For example, in the euryhaline killifish, Jung et al. (2012) and Ruhr et al. (2020) demonstrated that protein abundance and mRNA expression of aquaporin AQP3 respond in a complex fashion to differences in acclimation salinity, in the presence and absence of hypoxia.

It is well-established that water calcium concentration affects several aspects of gill permeability. For example, elevated environmental [Ca^{2+}] reduces the diffusive water permeability of the gills in both fish (Oduleye 1975; Isaia and Masoni 1976) and crustaceans (Rasmussen and Bjerregaard 1995). In mammals, both intracellular and extracellular [Ca^{2+}] regulate aquaporin function (Németh-Cahalan and Hall 2000; Valenti et al. 2005), but it is not known whether the [Ca^{2+}] in the external medium produces similar effects on branchial aquaporins in fish, thereby influencing the diffusive water flux rate. While we are aware of no evidence for the presence of aquaporins in tight junctions, it is also well established that elevated environmental [Ca^{2+}] helps to maintain the integrity of paracellular tight junctions (McWilliams and Potts 1978; McWilliams 1982). Both these effects may contribute to reductions in permeability to water when osmotic gradients are present (McWilliams and Potts 1978; Wendelaar Bonga and Van der Meij 1981; McWilliams 1982; McDonald 1983; Hunn 1985; McDonald and Rogano 1986). However, an additional complication is that paracellular permeability of the branchial tight junctions appears to increase at higher salinity (Marshall 2012), despite the higher water [Ca^{2+}] and lower water permeability in seawater fish.

In freshwater fish, ammonia excretion occurs predominantly by a transcellular route through Rh proteins, with contributions from a basolateral Na^+/NH_4^+-ATPase and an apical Na^+/H^+ antiporter coupled with NH_3 diffusion (Nawata et al. 2007; Wright and Wood 2009, 2012; Wood et al. 2014). In seawater fish, the Rh proteins and Na^+/H^+ antiporter are present (Wood and Nawata 2011), but the leakier paracellular tight junctions may also play an important role (Wilkie 1997, 2002; Wright and Wood 2009; Weihrauch et al. 2009). When three euryhaline species (rainbow trout–Wood and Nawata 2011; guppies–Zimmer et al. 2012; killifish–Giacomin et al. 2020), were acclimated to seawater, ammonia excretion rates were higher than in freshwater.

Urea produced by uricolysis and/or arginolysis circulates in adult ammoniotelic fish and is excreted continuously (McDonald et al. 2000; Walsh et al. 2001; McDonald and Wood 2003). A large proportion of these fish have urea transporter (UT) expression in their gills (Walsh et al. 2001; reviewed by McDonald et al. 2006) and while this may not always translate into functional gill UT, there is clear evidence that many ammoniotelic fish do have UTs that effectively excrete urea through the gills (reviewed by McDonald et al. 2006, 2012). Some freshwater and seawater teleosts may also have the ability to transport ammonia and/or urea through aquaporin subtypes found in the gill (Cutler et al. 2007; Tingaud-Sequeira et al. 2010; Tipsmark et al. 2010; Chen et al. 2010; Kolarevic et al. 2012; Ip et al. 2013). The extent of transport through these transcellular pathways in fish remains uncertain. An additional complication is that ammonia and urea-N excretion rates may also change in response to changes in the rate at which protein is being burned in oxidative metabolism. However, this can be detected by measuring the nitrogen quotient (NQ), the ratio of nitrogen excretion to $\dot{M}O_2$ (Van den Thillart and Kesbeke 1978; Van Waarde 1983; Lauff and Wood 1996).

In general, flatfishes have a low metabolic rate, a high tolerance for hypoxia and are able to withstand large

fluctuations in salinity (Wood et al. 1979; Steffensen et al. 1982; Dalla Via et al. 1997; Maxime et al. 2000; Schreiber 2001; Ruiz-Jarabo et al. 2015). Salinity influences flatfish distribution, with many species migrating to the lower salinity waters of estuaries for feeding, and during larval development and metamorphosis (Evans 1984; Burke et al. 1991, 1995; Minami and Tanaka 1992; Gibson 1994). Many also thrive in hypersaline lagoons (e.g. McNeil et al. 2013; Cruz et al. 2018); indeed the greenback flounder (*Rhombosolea tapirine*) has been caught in salinities above 81 ppt (McNeil et al. 2013). In the current study we investigated two northwest Pacific flatfishes that reside in benthic coastal environments. Both the Pacific sanddab (Thornburgh 1980; Durkin et al. 1981; Bottom et al. 1984; Armor and Herrgesell 1985; Rackowski and Pikitch 1989; Rooper et al. 2006; Sobocinski et al. 2018) and the Rock sole (Abookire et al. 2000; Essington et al. 2013; Beaudreau et al. 2022) are well documented to move in and out of estuaries.

Diffusive water flux, $\dot{M}O_2$, ammonia excretion and urea-N excretion rates were measured as a function of acute changes in water salinity and calcium concentration. With the preceding background in mind, we hypothesized that; i) diffusive water flux rates would increase in low salinity and decrease in high salinity; ii) oxygen consumption rates would become elevated during both of these challenges; iii) ammonia excretion would decrease at low salinity and increase in high salinity while urea excretion would remain stable throughout the experimental treatments; iv) changes in water [Ca^{2+}] would play an important role in these responses to salinity challenges; and (v) overall response patterns would be similar in these two benthic species.

In the course of answering these hypotheses, we report several novel findings. Notably, ammonia excretion increases as salinity decreases, similar to the pattern of diffusive water flux, and both are uncoupled from changes in $\dot{M}O_2$. Reliance on protein as an oxidative fuel increases as salinity decreases. Furthermore, reduced [Ca^{2+}], rather than reduced salinity itself, is the primary cause for the increases in diffusive water flux rates seen at low salinity. These make important contributions to our understanding of the ecophysiology of adult flatfishes when coping with fluctuations in salinity.

Methods

Animals

Flatfishes were caught by angling off Brady's beach in August and September near the Bamfield Marine Sciences Center (BMSC), Bamfield, BC, Canada, under Fisheries and Oceans Canada collection permit XR 136 2021. Pacific sanddab (*Citharichthys sordidus*) ($N = 20$, 0.185 ± 0.006 kg) and Rock sole (*Lepidopsetta bilineata*) ($N = 45$, 0.358 ± 0.018 kg) were transported to BMSC where they were held in aerated tanks supplied with flowing seawater (31 ppt, 12 ºC). Fishes were fed with small chunks of salmon every second day and were fasted for at least 72 h before experiments commenced. Experimental fishes were transferred to individual aerated vessels supplied with seawater (31 ppt) that rested in a water bath to maintain a control temperature of 12 °C. The night before the experiment, fishes were allowed to settle in the experimental vessels which were slightly longer and wider than each individual fish (35 cm X 25 cm). Seawater was pumped from the nearby ocean to both BMSC holding tanks and the experimental set-up. Sand was present in the holding tanks for flatfishes to bury in during the day but was absent in the experimental vessels, as a previous study showed no significant differences in diffusive water flux or oxygen consumption rates in the presence and absence of sand (Onukwufor and Wood 2022). All procedures used in this investigation were in accordance with the Canadian Council on Animal Care guidelines and were approved by the University of British Columbia Animal Care Committee (AUP A18-0271) and the BMSC Animal Care Committee (AUP RS-21(19)-01).

Experimental series

Two experimental series were performed, a salinity series and a salinity-Ca^{2+} series. In both series, diffusive water flux rates, oxygen consumption rates, and ammonia-N and urea-N excretion rates were measured. The salinity series tested the effect of 45 ppt, 31 ppt, 15.5 ppt, and 7.5 ppt in both Rock sole and Pacific sanddab. The salinity-Ca^{2+} series tested the effect of 31 ppt at 10 mM Ca^{2+}, 31 ppt at 20 mM Ca^{2+}, 7.5 ppt at 10 mM Ca^{2+}, and 7.5 ppt at 20 mM Ca^{2+} in Rock sole only.

Experimental solutions

All experimental solutions were maintained at 12 °C. Solutions of 45 ppt were made by adding an appropriate amount of Instant Ocean® Sea Salt (Blacksburg,VA, USA) to natural seawater (salinity 31). Solutions of 15.5 ppt and 7.5 ppt were made by diluting seawater with BMSC freshwater (Na^+ 0.3, Cl^-, 0.2, K^+ 0.005, Ca^{2+} 0.1, Mg^{2+} 0.05 mM) until the desired salinity was reached. Salinity was measured using a WTW Portable Conductivity Meter (ProfiLine Cond3310; Xylem Analytics, Welheim, Germany). At control salinity of 31 ppt, the background Ca^{2+} concentration was 10 mM Ca^{2+}, and at 7.5 ppt, the background Ca^{2+} concentration was 2.5 mM. Analytical grade $CaCl_2$ was added to the appropriate test salinity solution (i.e. 31 ppt or 7.5 ppt) to create four test solutions: 31 ppt at 10 mM Ca^{2+} (no $CaCl_2$ addition, so the same composition as in 31 ppt in the salinity series), 31 ppt at 20 mM Ca^{2+}, 7.5 ppt at 10 mM Ca^{2+}, and 7.5 ppt at

20 mM Ca^{2+}. The pH range among the various experimental solutions was less than 1.0 pH unit (Table S1).

Diffusive water flux

The experimental protocol used for measuring diffusive water flux rates follows the procedure outlined by Onukwufor and Wood (2020a). Briefly, the loading period began 12 h before the experiment started whereby 6–8 fish per experiment were loaded with ^3H_2O by exposure to 30 µCi of ^3H_2O (PerkinElmer, Woodbridge, ON, Canada) per litre of seawater (31 ppt) in individual aerated vessels, maintained at 12 °C. After the loading period, each individual fish and vessel was quickly rinsed with clean seawater. The fish was immediately placed back into the aerated, temperature-controlled vessel containing 2–4 L (exact volume recorded) of the experimental water without ^3H_2O. These were 7.5 ppt, 15 ppt, 31 ppt, or 45 ppt in the salinity series, and 7.5 ppt at 10 mM Ca^{2+}, 7.5 ppt at 20 mM Ca^{2+}, 31 ppt at 10 mM Ca^{2+}or 31 ppt at 20 mM Ca^{2+} in the salinity-Ca^{2+} series. Each fish was provided with enough experimental water such that they were covered to a depth of several cm. A 4 ml aliquot of the experimental solution was taken at time 0, marking the start of the washout period, and every 5 min thereafter for 60 min. A final sample was taken at 12 h when the external and internal ^3H_2O pools were once again in equilibrium. The 12 h sample was used to calculate the original amount of radioactivity in the fish and the 0–60 min samples were used to determine the diffusive water flux rates.

Oxygen consumption

Oxygen consumption rates ($\dot{M}O_2$) were measured as per Onukwufor and Wood (2022), directly following the end of the 60 min diffusive water flux experiment on the same fish. At the start of the measurements, PO_2 was > 80%. Aeration was removed, and the vessels were sealed with Styrofoam covers cut to the exact size of the chambers with a hole only big enough to fit the oxygen probe (DO 6 + galvanic oxygen electrode and meter, Oakton Instruments, Vernon Hills, IL, USA). The decrease in PO_2 was measured every 5 min until it reached 50% saturation, a PO_2 that is well above the critical PO_2 where $\dot{M}O_2$ becomes dependent on PO_2 in these species (O.E. Johannsson and C.M. Wood, unpubl data, Figure S1). This period ranged from 10–65 min within the various treatments. Preliminary experiments demonstrated that the Styrofoam covers adequately blocked the entry of O_2.

Ammonia and urea-N excretion

Water samples (5 ml) were taken from each vessel directly following the end of the $\dot{M}O_2$ measurements once aeration was re-established, representing time 0, and samples were taken every 30 min for 2 h thereafter. Preliminary experiments demonstrated that this time interval was adequate. Concentrations of total ammonia (salicylate hypochlorite assay, Verdouw et al. 1978) and total urea-N (diacetyl monoxime assay, Rahmatullah and Boyde 1980) in water samples were measured colorimetrically, with standards made up in the appropriate salinities.

Analytical procedures and calculations

As described by Onukwufor and Wood (2018), the concentration of ^3H_2O was determined by adding 8 ml of Optiphase 3 fluor (Perkin-Elmer, Wellesley, MA, USA) to the 4 ml water sample, shaking vigorously and loading it into a scintillation counter (LS6500, Beckman Coulter, Fullerton, CA, USA). Preliminary tests showed that quenching and chemiluminescence were negligible. The rate constant of ^3H_2O efflux (k) was calculated using the protocol outlined by Onukufor and Wood (2020a). Briefly, using the final 12 h wash-out sample, the initial amount of ^3H_2O in the fish was determined. It was then possible to calculate the amount of ^3H_2O left in the fish at each sampling time from 0–60 min based on the measured amounts that appeared in the external solution at each time. Next, the natural logarithm of the total ^3H_2O in the fish at each sample time was regressed against time in minutes on a linear scale. The fractional rate constant k for water turnover was the slope of this line. Multiplying the rate constant k by 60 yielded the fraction of the body water pool turned over per hour. Generally, the total exchangeable water pool of a fish is 800 ml/kg (Holmes and Donaldson 1969; Isaia 1984; Olson 1992). To calculate the diffusive water flux rate in ml/fish/h, the weight of the fish in kg was multiplied by 800 ml/kg and then by the fraction of the body water pool turned over per hour.

PO_2 was measured using the oxygen probe described above (Oxygen consumption). $\dot{M}O_2$ calculations followed the procedure of Onukwufor and Wood (2020a). Briefly, using salinity and temperature-dependent solubility coefficients (Boutilier et al. 1984), PO_2 values were converted to O_2 concentrations (µmol/L). The rate of decline of O_2 concentration was then multiplied by the known volume of the experimental vessel, and divided by time (h), to give $\dot{M}O_2$ in µmol/fish/h.

Ammonia-N and urea-N excretion rates (note: 1N per ammonia molecule, 2N per urea molecule) were calculated as per Wood (1992) whereby the change in concentration in the water (µmol-N/L) was divided by the time of the flux period (h), multiplied by the volume of the experimental container (L) to give the excretion rate as µmol-N /fish/h. The four 30 min rates were averaged to yield a single value for each fish.

To account for the differences in body mass, the logarithms of each experimental measurement (diffusive water flux, $\dot{M}O_2$, ammonia-N and urea-N excretion rates (expressed as units/fish/h) were regressed against the logarithm of fish weight (Table 1). For each statistically significant result (Table 1), an allometric mass scaling exponent was obtained by using the power equation $Y = aM^b$ where Y is the rate of interest (units/fish/h), M is the body weight (kg) and b is the scaling exponent. When the power equation is transformed into a linear function, a, representing the proportionality constant, is the y-intercept and b, representing the scaling exponent is the slope (Huang and Riviere 2014).The allometric mass scaling exponents were used to adjust the diffusive water flux rates, $\dot{M}O_2$, ammonia or urea excretion rates of each individual fish to that of a standard 0.25 kg of flatfish, and then divided by 0.25 kg so as to yield rates expressed per kg per h. In cases where no scaling was applied (see Results), each individual rate expressed per fish per h was divided by weight in kg, to yield rates expressed per kg per h.

The nitrogen quotient (NQ) is the ratio of the total measured nitrogenous waste excretion ($\dot{M}_N$), the sum of the ammonia-N excretion and the urea-N excretion, to the $\dot{M}O_2$. Therefore, the NQ was calculated as $NQ = (\dot{M}_N/\dot{M}O_2)$ for each individual fish at the control salinity and in the experimental solutions. The unscaled rates were used for each measurement in the calculations.

Statistical analyses

Data have been expressed as means ± standard error (SEM; n) where n represents the number of animals sampled. All data passed normality tests. Data that also passed the homogeneity of variance tests were analysed using one-way analysis of variance (ANOVA) with Tukey's post hoc test (multiple comparisons). If data did not pass the Brown-Forsythe test for homogeneity of variances, data were analysed using Brown-Forsythe and Welch ANOVA test with Dunnett's T3 multiple comparison test. The effects of water salinity on each measurement (diffusive water flux, $\dot{M}O_2$, ammonia and urea-N excretion) were analysed by linear regression of the appropriate measurement versus the salinity of the water, and the significance of the regressions were assessed. Differences in mean control rates at salinity 31 between species, and also within the Rock sole in the salinity series versus the salinity-Ca^{2+} series were assessed by unpaired Student's t-tests.

All statistical analyses and data plots were done using GraphPad™ Prism 7 (GraphPad Software, San Diego, CA, USA). A significance level of $p < 0.05$ (i.e. two-tailed) was used throughout, except in the case of allometric regressions where $p < 0.10$ (i.e. one-tailed) was used, because for these, we predicted a priori that positive relationships should occur for all parameters.

Results

Allometric scaling exponents

To calculate each of the scaling exponents for diffusive water flux rates, $\dot{M}O_2$, ammonia excretion and urea-N excretion rates, the control data at salinity 31 (i.e. the acclimation salinity) were used. The logarithms of individual diffusive water flux rates, $\dot{M}O_2$, ammonia-N excretion or urea-N excretion rates (expressed as units per fish per h) for each species, were plotted against the logarithms of individual fish weights. The relationships were significant at $p < 0.10$ for all parameters in both species, except for $\dot{M}O_2$ in Rock sole, and urea-N excretion rates in both species, as indicated

Table 1 Allometric mass scaling for diffusive water flux, oxygen consumption, ammonia and urea-N excretion rates for Rock sole (*Lepidopsetta bilineata*) and Pacific sanddab (*Citharichthys sordidus*)

Measurement	Species	Regression equation	R2	*P* values	Proportionality constant	Scaling coefficient
Diffusive water flux rate*	Rock sole	$Y = 0.673 \times X - 0.06229$	0.3380	0.0182	− 0.06229	0.673
Diffusive water flux rate*	Pacific sanddab	$Y = 1.069 \times X - 0.9391$	0.9786	0.0002	− 0.9391	1.069
Oxygen consumption rate	Rock sole	$Y = 0.441 \times X + 1.500$	0.08543	0.2720	+ 1.500	0.441
Oxygen consumption rate*	Pacific sanddab	$Y = 0.862 \times X + 0.6207$	0.7064	0.00361	+ 0.6207	0.862
Ammonia excretion rate*	Rock sole	$Y = 0.747 \times X + 0.8316$	0.4446	0.0906	+ 0.8316	0.747
Ammonia excretion rate*	Pacific sanddab	$Y = 1.401 \times X - 1.485$	0.6121	0.066	− 1.485	1.401
Urea-N excretion rate	Rock sole	$Y = 0.718 \times X - 0.9624$	0.112	0.4631	− 0.9624	0.718
Urea-N excretion rate	Pacific sanddab	$Y = 0.470 \times X - 0.3002$	0.0404	0.7457	− 0.3002	0.470

Y = log rate and X = log body mass

$p < 0.10$ (i.e. one-tailed) was used for statistical significance

*indicates parameters for which allometric scaling was applied

in Table 1. Therefore, allometric scaling, based on the power equation $Y = aM^b$ (see Analytical Procedures and Calculations), was applied in subsequent analyses for all species-specific parameters except the three non-significant ones. The scaling exponents (Table 1) were used to adjust the individual experimental rates to that of a 0.25 kg flatfish. Only parameters that yielded significant relationships in the regressions were scaled, and scaling exponents were in general agreement with those previously published across species and salinities (Discussion, Scaling exponents of diffusive water flux, $\dot{M}O2$ and ammonia excretion rate). The appropriately scaled or unscaled rates were finally expressed as ml/kg/h for diffusive water flux rates, µmol O_2/kg/h for $\dot{M}O_2$, µmol ammonia-N /kg/h for ammonia excretion rates and µmol urea-N/kg/h for urea excretion rates.

The scaling exponents were consistently lower for Rock sole than for Pacific sanddab and were highest for ammonia excretion rate (0.747, 1.401), intermediate for diffusive water flux rate (0.673, 1.069) and lowest for $\dot{M}O_2$, (0.441, 0.862) (Table 1).

The effect of salinity on diffusive water flux rates

At control salinity, 31 ppt, the mean diffusive water flux rates in Pacific sanddab were 168 ml/kg/h, significantly higher than in Rock sole which were 136 ml/kg/h ($p = 0.0248$) (Table S2). In Rock sole, the acute transfer from control salinity, 31 ppt, to 7.5 ppt, resulted in a significant 37% increase in diffusive water flux rates within the first hour (Fig. 1A). In Pacific sanddab, rates were likewise significantly higher by 36% at 7.5 ppt when compared to the control salinity (Fig. 1B). When diffusive water flux rates were regressed against salinity, there were significant negative relationships for both Rock sole ($p = 0.0100$) and Pacific sanddab ($p = 0.0005$) (Fig. 1C, D).

The effect of salinity on $\dot{M}O_2$

The $\dot{M}O_2$ values were not significantly different between species at 31 ppt (Table S2). Mean $\dot{M}O_2$ values in Rock sole were 1549 µmol O_2/kg/h and 1437 µmol O_2/kg/h in Pacific sanddab. There were no significant differences in $\dot{M}O_2$ values among salinity treatments in either species (Fig. 2A, B). When $\dot{M}O_2$ was plotted against salinity, there was a significant positive relationship for Rock sole ($p = 0.0108$) but not in Pacific sanddab (Fig. 2C, D).

The effect of salinity on ammonia and urea-N excretion rates

The average ammonia excretion rates for both Rock sole and Pacific sanddab were about 305 µmol ammonia /kg/h and were not significantly different between species (Table S2). The acute transfer of Rock sole from 31 to 45 ppt resulted in a significant 47% decrease in ammonia excretion rates, and no significant changes were observed compared to control salinity in response to transfers to 15.5 ppt or 7.5 ppt (Fig. 3A). In Pacific sanddab, the acute transfer from 31 ppt to 7.5 ppt resulted in a significant increase of 92% in ammonia excretion rates, but there were no significant changes in response to transfers to other salinities (Fig. 3B). When the ammonia excretion rates were regressed against salinity there were significant negative relationships in both Rock sole ($p = < 0.0001$) and Pacific sanddab ($p = 0.0014$) (Fig. 3C, D).

At 31 ppt, mean urea-N excretion rates were not significantly different between Rock sole (44.9 urea-N /kg/h) and Pacific sanddab (69.1 urea-N /kg/h) (Table S2). In Rock sole, there were no significant differences in urea-N excretion rates in response to any of the salinity treatments tested (Fig. 4A). In Pacific sanddab, the acute transfer from 31 ppt to 15.5 ppt yielded a significant increase of 115%, but there was no significant effect of transfer to 7.5 ppt or 45 ppt (Fig. 4B). There were no significant relationships between urea-N excretion rates and salinity in either species when urea-N excretion rates were regressed against salinity (Fig. 4C, D).

The effect of salinity on the nitrogen quotient

The nitrogen quotient, the ratio between total nitrogenous waste excretion rate and $\dot{M}O_2$, was about 0.23 at control salinity, 31 ppt, and not significantly different between Rock sole and Pacific sanddab (Table S2). In Rock sole, there were no significant differences compared to the control salinity in response to exposure to the three other test solutions (Fig. 5A). However, compared to 45 ppt, NQ was significantly greater by 128% in 15.5 ppt (Fig. 5A). In Pacific sanddab, there was a significant 124% increase in NQ in 7.5 ppt compared to the control ratio (Fig. 5B). When the NQ values were regressed against salinity there were significant negative relationships in both Rock sole ($p = 0.0004$) and Pacific sanddab ($p = 0.0007$) (Fig. 5C, D).

The effect of Ca^{2+} during a salinity challenge in Rock sole

In Rock sole, there were no significant differences (Table S3) between the salinity series control rates presented above (diffusive water flux, $\dot{M}O_2$, ammonia excretion and urea-N excretion) and the salinity-Ca^{2+} series control rates presented below. Note that in both, the seawater composition was identical (31 ppt at 10 mM Ca^{2+}).

The goal of this series was to separate the effects of salinity from those of external [Ca^{2+}]. Doubling [Ca^{2+}] from 10 to 20 mM at control salinity, 31 ppt, appeared to decrease

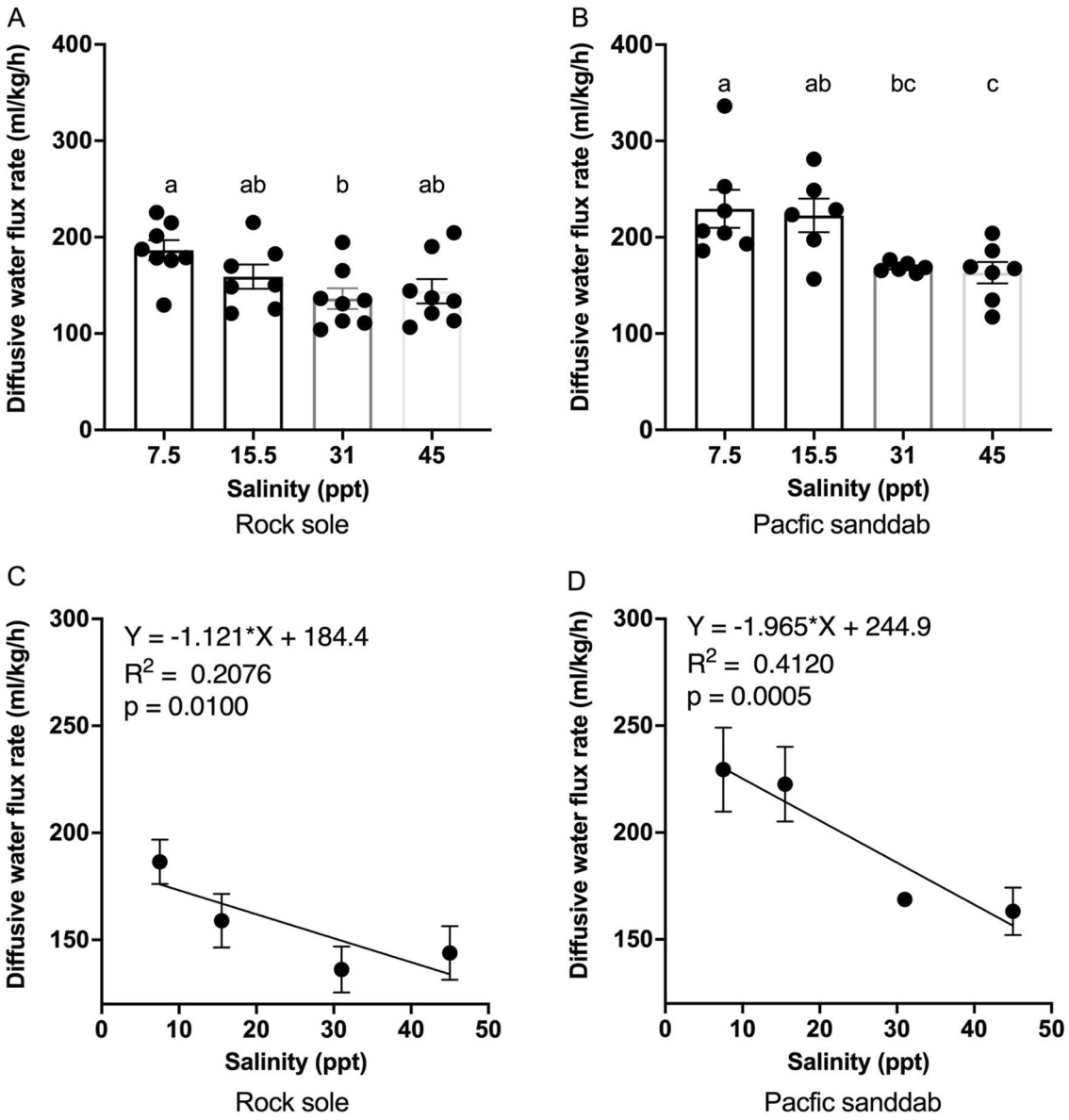

Fig. 1 The effect of salinity (7.5 ppt, 15.5 ppt, 31 ppt, 45 ppt) on diffusive water flux rate (ml/kg/h) in **A** Rock sole (*Lepidopsetta bilineata*), $n = 7–8$ and **B** Pacific sanddab (*Citharichthys sordidus*), $n = 6–7$. Data (means ± S.E.M) not sharing the same letters within a species are significantly different ($p < 0.05$) (analysis of variance with Tukey's test). The relationship between salinity (7.5 ppt, 15.5 ppt, 31 ppt, 45 ppt) and diffusive water flux rate (ml/kg/h) in **C** Rock sole (*Lepidopsetta bilineata*), $n = 7–8$ and **D** Pacific sanddab (*Citharichthys sordidus*), $n = 6–7$

the mean diffusive water flux rate by 38%, though this difference was not significant ($p = 0.1213$) (Fig. 6A). Reducing salinity from 31 ppt to 7.5 ppt while maintaining [Ca^{2+}] at 10 mM ($p = 0.5980$) or increasing it to 20 mM ($p = 0.1060$) resulted in no change in diffusive water flux (Fig. 6A), in direct contrast to the significant increase ($p = 0.0205$) in diffusive water flux rate seen earlier at 7.5 ppt when [Ca^{2+}] was not adjusted (Fig. 1A).

There were no significant differences in $\dot{M}O_2$ in response to any of the experimental solutions tested (31 ppt at 10 mM Ca^{2+}, 31 ppt at 20 mM Ca^{2+}, 7.5 ppt at 10 mM Ca^{2+} or 7.5 ppt at 20 mM Ca^{2+}) (Fig. 6B). This was similar to the constancy of $\dot{M}O_2$ observed earlier when this same salinity challenge was performed without adjustments of [Ca^{2+}] (Fig. 2A).

Doubling [Ca^{2+}] from 10 to 20 mM at control salinity 31 ppt appeared to decrease the mean ammonia excretion rate by 28%, though this difference was not significant ($p = 0.0967$). There were also no significant differences from control rates at 31 ppt with 10 mM Ca^{2+} in response to any

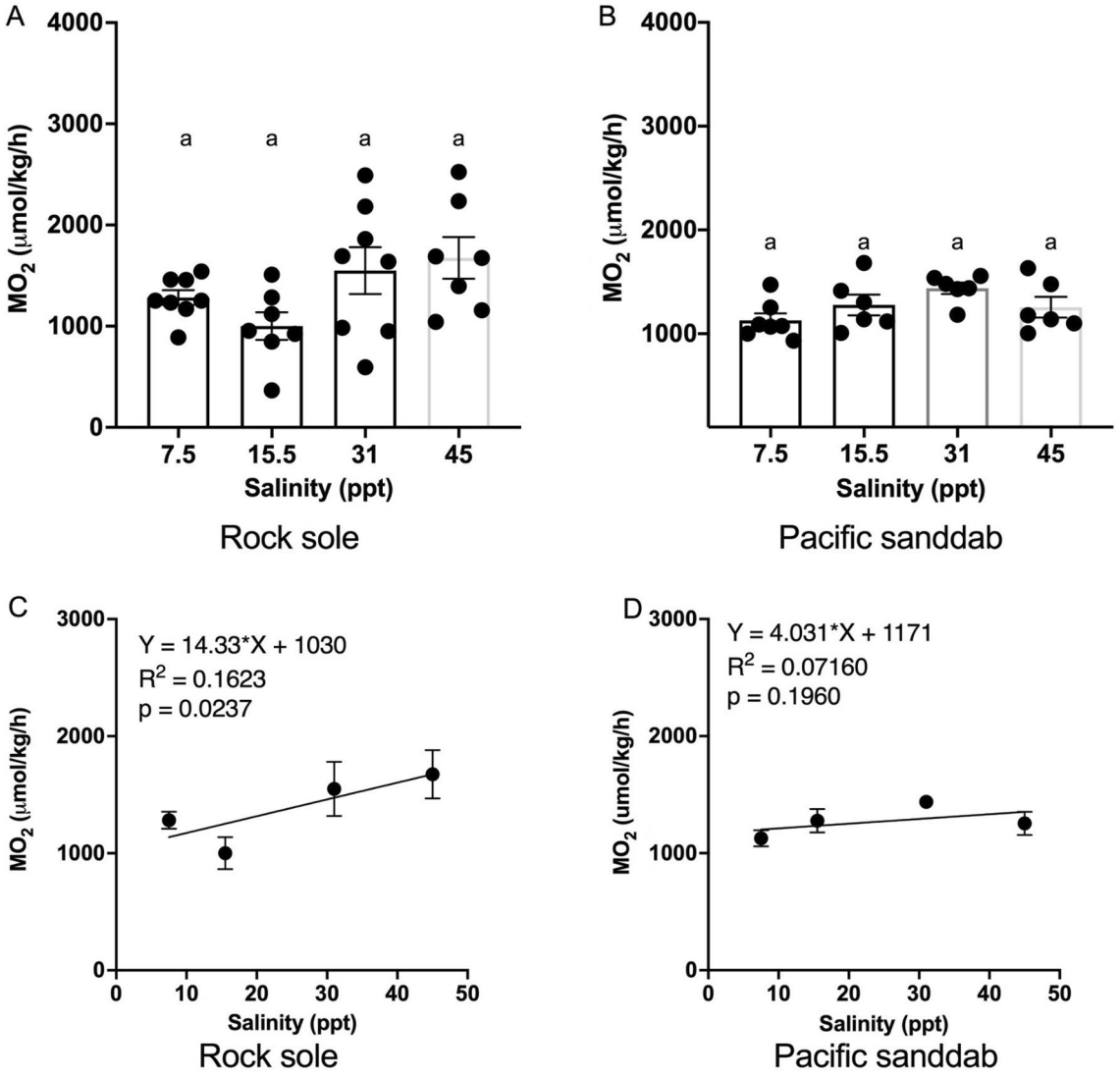

Fig. 2 The effect of salinity (7.5 ppt, 15.5 ppt, 31 ppt, 45 ppt) on oxygen consumption rate (MO_2) (µmol/kg/h) in **A** Rock sole (*Lepidopsetta bilineata*), $n = 7–8$ and **B** Pacific sanddab (*Citharichthys sordidus*), $n = 6–7$. Data (means ± S.E.M), are not significantly different ($p < 0.05$) (analysis of variance with Tukey's test). The relationship between salinity (7.5 ppt, 15.5 ppt, 31 ppt, 45 ppt) and oxygen consumption rate (MO_2) (µmol/kg/h) in **C** Rock sole (*Lepidopsetta bilineata*), $n = 7–8$ and **D** Pacific sanddab (*Citharichthys sordidus*), $n = 6–7$

of the other test solutions (Fig. 6C). Ammonia excretion rates increased significantly by 70% in response to 7.5 ppt at 10 mM Ca^{2+} compared to 31 ppt at 20 mM Ca^{2+}. Again, in comparison to 31 ppt at 20 mM Ca^{2+}, ammonia excretion rates increased by 67% in response to 7.5 ppt at 20 mM Ca^{2+} (Fig. 6C). Earlier, we observed no significant change in ammonia excretion rate when this same salinity challenge was performed without adjustments of [Ca^{2+}] (Fig. 3A).

There were no significant differences in urea-N excretion rates in response to any of the solutions tested (Fig. 6D), similar to the pattern observed earlier when this same salinity challenge was performed without adjustments of [Ca^{2+}] (Fig. 4A).

There were no significant differences in NQ at control 31 ppt between Rock sole in the salinity series and Rock sole in the salinity-Ca^{2+} series (Table S3). There were also no significant changes to NQ in the salinity-Ca^{2+} series (Fig. 6E), similar to the result when the same salinity challenge was performed without adjustments of [Ca^{2+}] (Fig. 5A).

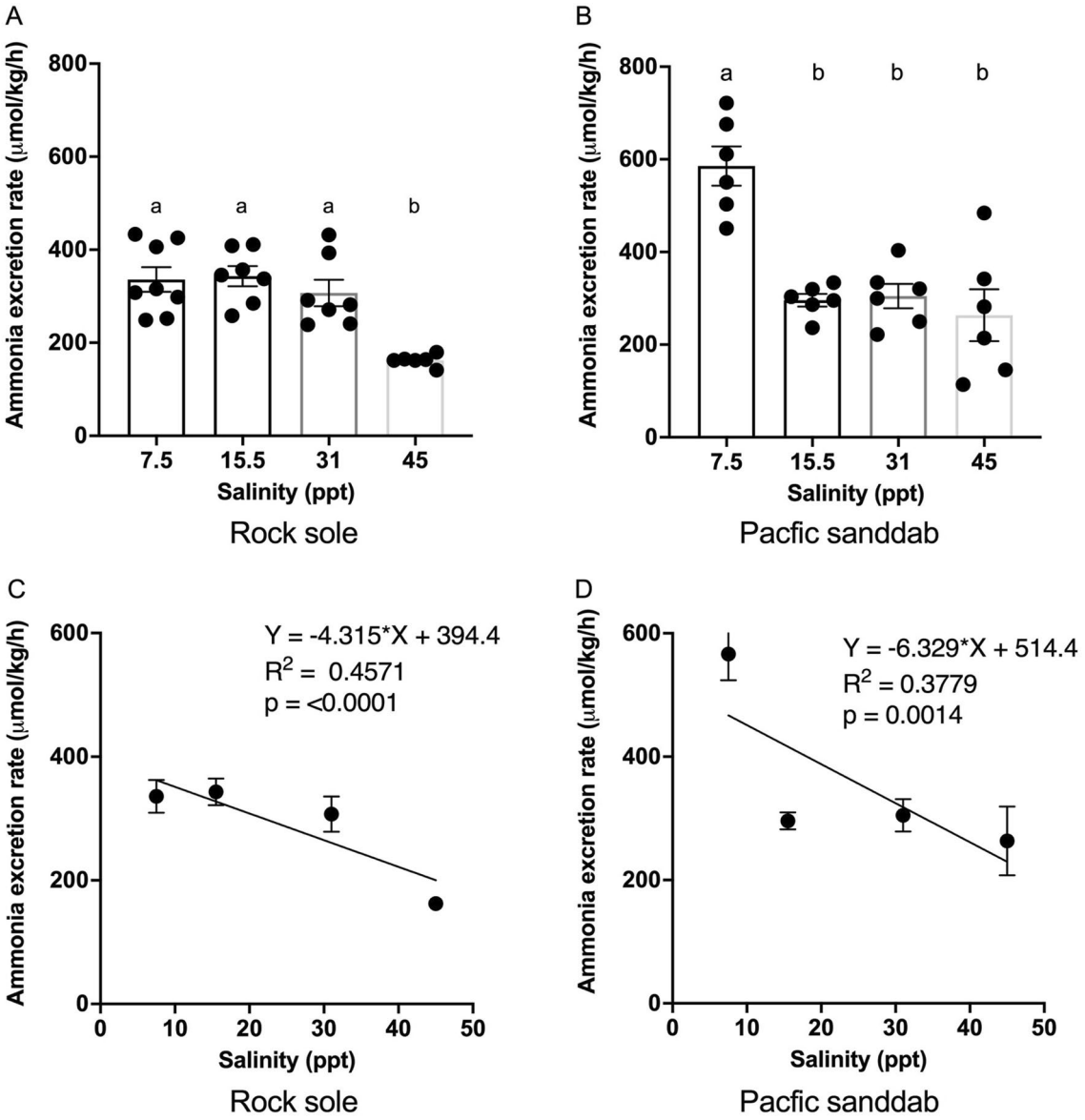

Fig. 3 The effect of salinity (7.5 ppt, 15.5 ppt, 31 ppt, 45 ppt) on ammonia-N excretion rate (µmol/kg/h) in **A** Rock sole (*Lepidopsetta bilineata*), $n = 6$–8 and **B** Pacific sanddab (*Citharichthys sordidus*), $n = 6$. Data (means ± S.E.M), not sharing the same letters within a species are significantly different ($p < 0.05$) (analysis of variance with Tukey's test **A** or Brown-Forsythe and Welch analysis of variance with Dunnett's T test **B**). The relationship between salinity (7.5 ppt, 15.5 ppt, 31 ppt, 45 ppt) and ammonia-N excretion rate (µmol/kg/h) in **C** Rock sole (*Lepidopsetta bilineata*), $n = 6$–8 and **D** Pacific sanddab (*Citharichthys sordidus*), $n = 6$.

Discussion

Overview

Our results demonstrate that both diffusive water flux and ammonia excretion rates are a function of salinity in Rock sole and Pacific sanddab and that in Rock sole, $[Ca^{2+}]$ also plays a role in these rates. With regard to our original hypotheses (see Introduction), we predicted that diffusive water flux rates would increase in low salinity and decrease in high salinity which was only partially confirmed. Diffusive water flux rates did increase in response to low salinity, but did not decrease in response to high salinity (Fig. 1A, B). Nevertheless, the overall relationships between diffusive water flux and salinity were significant (Fig. 1C, D). In contrast to our prediction, $\dot{M}O_2$ was not significantly altered by acute increases or decreases in salinity (Fig. 2). However, as postulated, urea-N excretion rates also remained relatively unchanged during the salinity challenges (Fig. 4). Our hypothesis that ammonia excretion rates would decrease in low salinity and increase in high salinity was falsified (Fig. 3). The

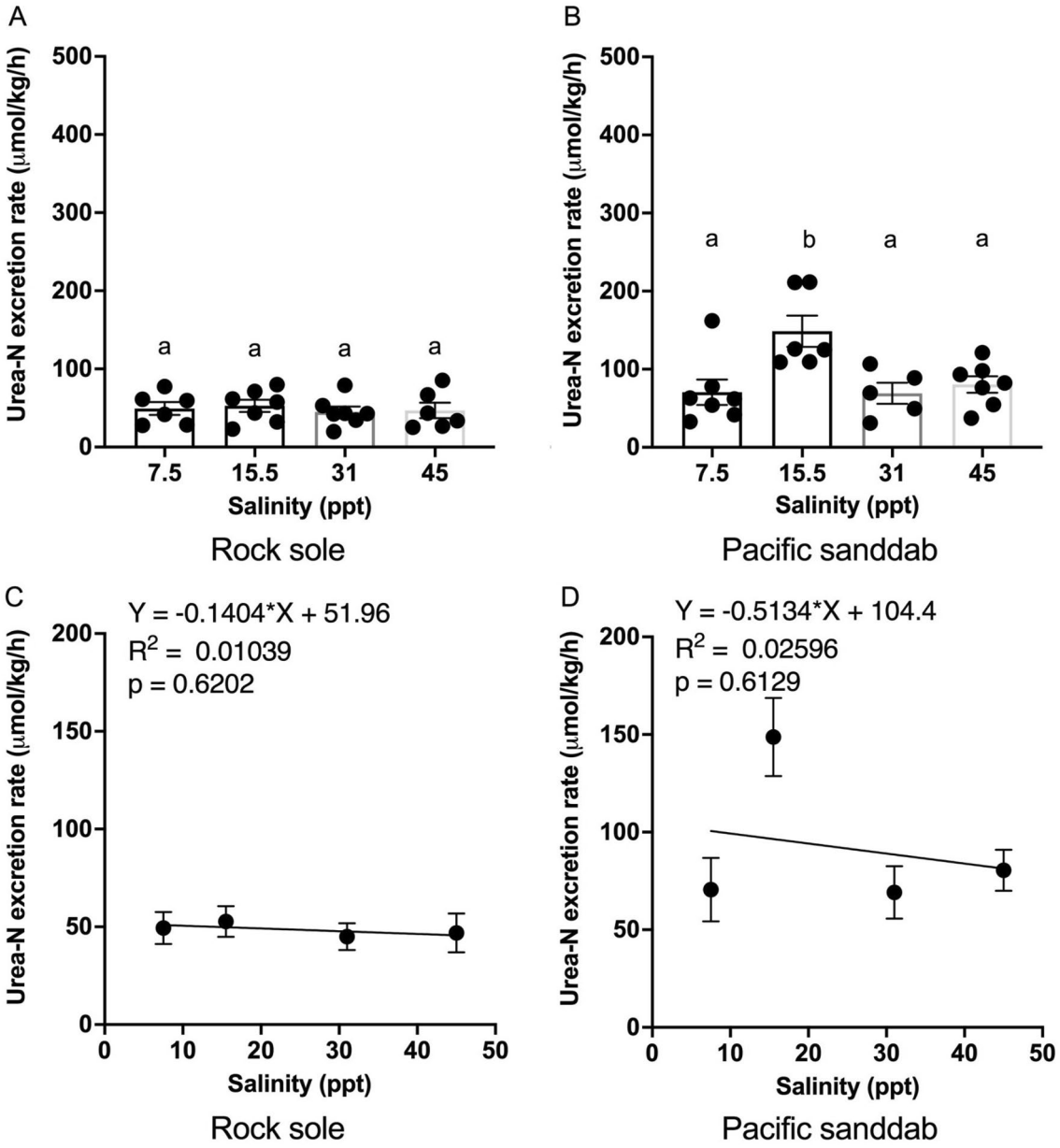

Fig. 4 The effect of salinity (7.5 ppt, 15.5 ppt, 31 ppt, 45 ppt) on urea-N excretion rate (μmol/kg/h) in **A** Rock sole (*Lepidopsetta bilineata*), $n = 6–7$ and **B** Pacific sanddab (*Citharichthys sordidus*), $n = 5–7$. Data (means ± S.E.M), not sharing the same letters within a species are significantly different ($p < 0.05$) (analysis of variance with Tukey's test). The relationship between salinity (7.5 ppt, 15.5 ppt, 31 ppt, 45 ppt) and urea-N excretion rate (μmol/kg/h) in **C** Rock sole (*Lepidopsetta bilineata*), $n = 6–7$ and **D** Pacific sanddab (*Citharichthys sordidus*), $n = 5–7$

opposite occurred, and the NQ analysis (Fig. 5) suggested a greater contribution of protein oxidation to constant $\dot{M}O_2$ as salinity decreased. Our prediction that changes in water $[Ca^{2+}]$ would play an important role in the responses to a salinity challenge in Rock sole was confirmed (Fig. 6). Thus, maintaining or elevating water $[Ca^{2+}]$ prevented the increase in diffusive water flux associated with low salinity exposure. However, effects of water $[Ca^{2+}]$ versus salinity on ammonia excretion were less clear. Our final hypothesis that overall response patterns would be similar in these two benthic species was confirmed.

Scaling exponents of diffusive water flux, $\dot{M}O_2$ and ammonia excretion rate

The scaling exponents were lower for Rock sole than for Pacific sanddab for diffusive water flux, $\dot{M}O_2$ and ammonia excretion rate, though the reason for these consistent

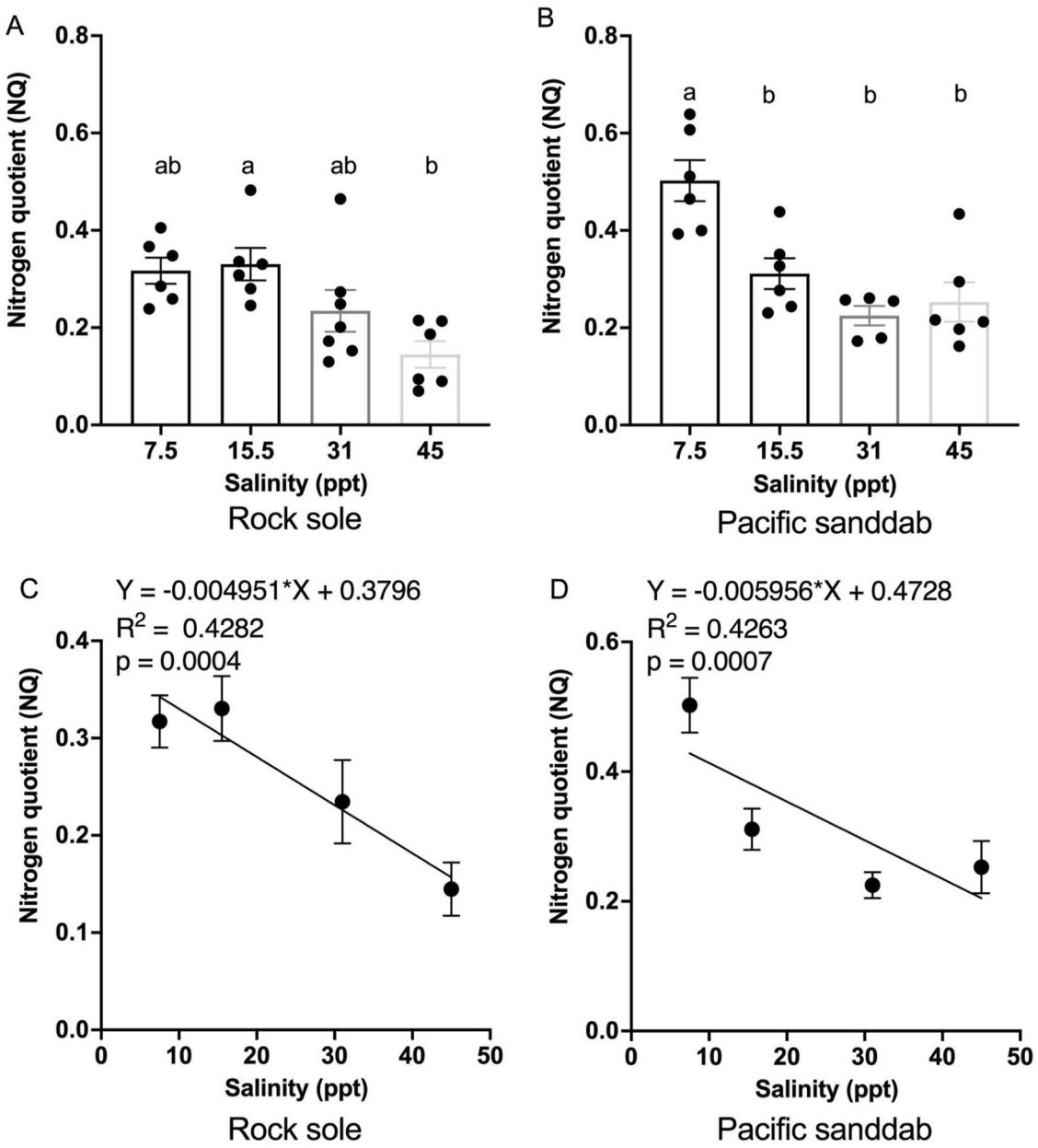

Fig. 5 The effect of salinity (7.5 ppt, 15.5 ppt, 31 ppt, 45 ppt) on nitrogen quotient ($NQ = \dot{M}_N / \dot{M}O_2$) on **A** Rock sole (*Lepidopsetta bilineata*) $n = 6–7$, **B** Pacific sanddab (*Citharichthys sordidus*), $n = 5–6$. Data (means ± S.E.M), not sharing the same letters within a species are significantly different ($p < 0.05$) (analysis of variance with Tukey's test). The relationship between salinity (7.5 ppt, 15.5 ppt, 31 ppt, 45 ppt) and nitrogen quotient **C** Rock sole (*Lepidopsetta bilineata*), $n = 6–7$ and **D** Pacific sanddab (*Citharichthys sordidus*), $n = 5–7$

differences is unknown. For diffusive water flux rate, the scaling exponent for Rock sole (0.673) was slightly lower than previously published values, however, high scaling exponents, similar to that of Pacific sanddab (1.069), have been reported for several other species across salinities (Potts et al. 1967; Evans 1969, 1984; Onukwufor and Wood 2020b). This suggests that this trait may be shared across multiple species and salinities. The scaling exponents for $\dot{M}O_2$ (0.441, 0.862) were in reasonable agreement with previously reported values for other flatfish species (0.50–0.85) (Duthie 1982; Fonds et al. 1992; Onukwufor and Wood 2022) which are generally typical for teleost fish (Clarke and Johnston 1999). The scaling exponents were highest for ammonia excretion rate. For Rock sole, the exponent (0.747), was within range of those previously reported for other teleost fish (0.724–0.99) including, pirarucu (Pelster et al. 2020), yellowtail kingfish (Moran and Wells 2007) and Atlantic cod larvae (Finn et al. 2002) whereas for Pacific

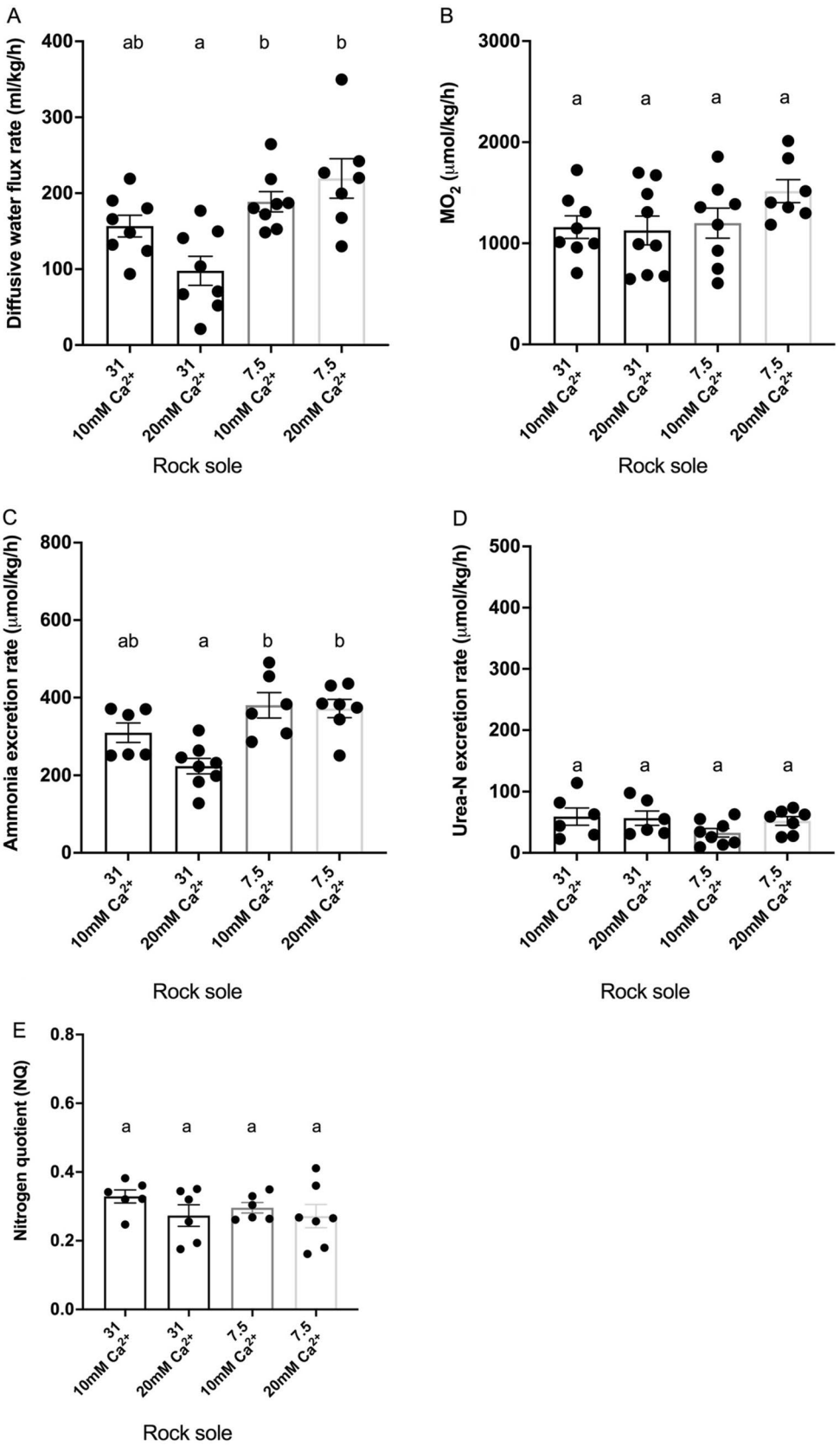

Fig. 6 The effect of $[Ca^{2+}]$ and salinity (31 ppt and 7.5 ppt) in the salinity-Ca^{2+} series on Rock sole (*Lepidopsetta bilineata*) on **A** Diffusive water flux rate, $n = 7–8$, **B** MO_2, $n = 7–9$, **C** ammonia excretion rate, $n = 6–8$, **D** urea-N excretion rate, $n = 6–8$, and **E** nitrogen quotient (NQ), $n = 6–8$. Data (means ± S.E.M), not sharing the same letters are significantly different ($p < 0.05$) (analysis of variance with Tukey's test)

sanddab (1.401), the exponent was higher. The high scaling exponents for ammonia excretion could indicate that with increasing body mass there are different principles that govern ammonia excretion relative to those determining diffusive water flux and $\dot{M}O_2$. Overall, the scaling exponents presented in this study are in general accord with those previously reported in the literature.

Diffusive water flux and oxygen consumption rates can be independently regulated in response to a salinity challenge

Several studies have demonstrated that acute exposure of seawater fish to low salinity increases diffusive water flux rate (Motais et al. 1969; Evans 1969; Onukwufor and Wood 2022) while other reports indicate that $\dot{M}O_2$ may also be elevated for several days after exposure to lower salinity before returning to original rates (Barton and Barton 1987; Dalla Via et al. 1998; Herrera et al. 2012). Recently, Onukwufor and Wood (2022) investigated the simultaneous changes in diffusive water flux rate and $\dot{M}O_2$ in two species of marine flatfish, Pacific sanddab and English sole. In agreement with our results in Pacific sanddab and Rock sole (Fig. 1A, B), they found that diffusive water flux rates increased in response to low salinity. The $\dot{M}O_2$ in Pacific sanddab did not change with altered salinity in both the current study (Fig. 2B) and Onukwufor and Wood (2022). We observed that $\dot{M}O_2$ was also unchanged in Rock sole (Fig. 2A) while in English sole $\dot{M}O_2$ was elevated at low salinity (Onukwufor and Wood 2022). The difference in $\dot{M}O_2$ response among the three species may indicate that some species (i.e. Pacific sanddab and Rock sole) are better able to cope with lower, inshore salinities, possibly due to life history traits (see Introduction). It should be noted that our study explored a larger range of salinities (45 ppt, 31 ppt, 15.5 ppt and 7.5 ppt) compared to Onukwufor and Wood (2022) (31 ppt and 16 ppt).

Therefore, acute changes in diffusive water flux rate can be uncoupled from acute changes in $\dot{M}O_2$. Our findings also suggest that diffusive water flux rate is uncoupled from the decreasing paracellular permeability in the tight junctions bordering ionocytes that occurs at low salinity (Marshall 2012), which may be the case for many euryhaline species including Pacific sanddab and Rock sole. The decrease in salinity from 31 ppt to 7.5 ppt reflects a large decrease or even slight reversal in the trans-gill osmotic gradient which also appears to be independent from the observed increase in diffusive water flux rate (Fig. 1A, B). Low salinity could rapidly elicit changes in exposure of gill ionocytes (Marshall and Nishioka 1980; Wood and Marshall 1994), possibly either through ionocyte proliferation or through changes in the morphological relationships between ionocytes and adjacent pavement cells (reviewed by Wood and Eom 2021). Lower expression of aquaporins in the gill has been correlated with lower diffusive water flux rate in seawater fish compared to freshwater fish and the protein abundance and mRNA expression of aquaporins increases upon transfer or acclimation to dilute waters (Lignot et al. 2002; Cutler et al. 2007; Tipsmark et al. 2010; Jung et al. 2012; Madsen et al. 2015; Breves et al. 2016; Ruhr et al. 2020). This upregulation of the aquaporin-mediated transcellular pathway may be independent of the osmorespiratory compromise associated with altered $\dot{M}O_2$ (Wood and Eom 2021). Our study investigated acute changes (within 1 h) in diffusive water flux rate so changes in the function of existing protein in the channels rather than changes in mRNA expression associated with new protein synthesis seems a more probable explanation. Nevertheless, Ruhr et al. (2020) reported that changes in AQP3 mRNA expression and cellular protein distribution were apparent within 3 h of an experimental treatment (acute hypoxia), so this topic should be investigated further in the future. Another possible explanation is the reduction in water $[Ca^{2+}]$ associated with a decrease in salinity, which is known to affect many aspects of gill permeability and will be discussed in [$[Ca^{2+}]$ plays a role in diffusive water flux and ammonia excretion rates in Rock sole].

Ammonia excretion rate is a function of salinity

Our ammonia and urea excretion rate values at 31 ppt are in agreement with previously published values in English sole and related species (Walsh et al. 2001). In both Pacific sanddab and Rock sole, the general trends in ammonia excretion (Fig. 3C, D) were similar to those in diffusive water flux (Fig. 1C, D), with rates increasing as salinity decreased. These trends were opposite to the greater ammonia excretion rates seen in seawater-acclimated rainbow trout (Wood and Nawata 2011), guppies (Zimmer et al. 2012) and killifish (Giacomin et al. 2020) all of which are euryhaline species. This disagreement may reflect species-specific differences, but a fundamental difference is that in the present study, the animals were acutely exposed to the salinity challenges, whereas in the cited studies, they were long-term acclimated to freshwater and seawater. Bucking (2017) suggested that the greater ammonia excretion at higher salinities in the latter studies was a reflection of greater ammonia production by protein catabolism. In our study, this explanation appears to explain the opposite trend because the NQ, which is an indicator of reliance on amino acids to fuel oxidative metabolism, as discussed subsequently, increased significantly in both species as salinity decreased (Fig. 5), whereas $\dot{M}O_2$ did not change (Fig. 2). Additionally, the lower pH at lower salinities (Table S1) would favour ammonia excretion, but the lower ionic strength would also decrease both NH_3 solubility and the pK of the ammonia equilibrium reaction,

which would oppose ammonia excretion (Cameron and Heisler 1983; Ip et al. 2001; Bucking 2017).

As predicted, urea-N excretion rates generally did not change in response to acute exposure to altered salinities with the exception of an increase at 15.5 ppt in Pacific sanddab (Fig. 4). The reason for this is unknown. In general urea-N excretion rates tend to make a larger contribution to total N-excretion rates in seawater fish than in freshwater fish (Wood 1993), though many exceptions exist (Altinok and Grizzle 2004). Branchial UT expression also tends to be higher in seawater-acclimated fish (Mistry et al. 2001; Wood and Nawata 2011), but this would be unlikely to change in the short time frame of our exposures.

The nitrogen quotient (NQ), the ratio of the total measured nitrogenous waste excretion (i.e. the sum of the ammonia-N excretion and the urea-N excretion) to the $\dot{M}O_2$, is used to estimate metabolic fuel use. Historically it has been believed that protein was the dominant fuel source for fish (Driedzic and Hochachka 1978; van Waarde 1983; Jobling 1994) however, it is now generally accepted that the dominant fuel in most species is lipid (Brett and Zala 1975; Lauff and Wood 1996; Alsop and Wood 1997; Kieffer et al. 1998; Alsop et al. 1999), followed by carbohydrate (Lauff and Wood 1996). From the knowledge of the metabolism of fish protein, an NQ = 0.27 represents aerobic respiration being 100% fueled by protein (van den Thillart and Kesbeke 1978). In control salinity, 31 ppt, the NQ in Rock sole was 0.23 (Fig. 5A) and 0.22 in in Pacific sanddab (Fig. 6B) indicating that 83–86% of aerobic respiration is fueled by protein. Although we did not measure the respiratory quotient, this appears to be contrary to the notion that lipids are the primary source of fuel but to our knowledge these are the first presented NQ values for wild caught marine flatfish. In low salinity there was a significant increase in NQ in both species (Fig. 5A, B), which suggests a shift to even greater protein oxidation. Fat snook showed an increase in reliance on lipids over amino acids when acclimated to saltwater (Rocha et al. 2005, 2007), which fits with this scenario. Notably, at 7.5 ppt, the NQ became significantly greater than 0.27 (one sample t-test) in Pacific sanddab (Fig. 5B), which could indicate that protein metabolism had increased so much that non-steady state conditions prevailed.

$[Ca^{2+}]$ plays a role in diffusive water flux and ammonia excretion rates in Rock sole

This series was performed to separate the effects of salinity from those of water $[Ca^{2+}]$. There were marked differences in the trends of diffusive water flux and ammonia excretion rates between the salinity series and the salinity-Ca^{2+} series. Note that in contrast to these effects of water $[Ca^{2+}]$ on diffusive water flux and ammonia excretion, there were no confounding effects on $\dot{M}O_2$ or urea excretion. There was no change in diffusive water flux rate in response to a decrease in salinity when $[Ca^{2+}]$ was maintained at the concentration of control salinity (i.e. 31 ppt with 10 mM Ca^{2+}) or elevated to 20 mM (Fig. 6A). This demonstrates that reduced $[Ca^{2+}]$ is the primary cause for the increases in diffusive water flux rates seen at low salinity (Fig. 1). To our knowledge, this has never been shown before. The effects of water $[Ca^{2+}]$ versus salinity on ammonia excretion are less clear. In the salinity series there were no significant changes in ammonia excretion rate in Rock sole between 31 ppt and 7.5 ppt (Fig. 3A), however in the salinity-Ca^{2+} series when Ca^{2+} was adjusted there were significant increases between 31 ppt at 20 mM Ca^{2+} and 7.5 ppt at both 10 mM Ca^{2+} and 20 mM Ca^{2+} (Fig. 6C).

The modulation of aquaporins between low permeability and high permeability in mammals, specifically in kidney and brain tissue, is dependent on both external $[Ca^{2+}]$ and pH. The effects of $[Ca^{2+}]$ and pH are separable and arise from processes on opposite sides of the membrane (Németh-Cahalan and Hall 2000; Németh-Cahalan et al. 2004; Valenti et al. 2005). Low external $[Ca^{2+}]$ increases permeability of aquaporins, a process which is dependent on functioning calmodulin that binds to the aquaporin (Németh-Cahalan and Hall 2000; Németh-Cahalan et al. 2004). As the calmodulin binding site is on the cytoplasmic side of the membrane, it has been suggested that both Ca^{2+} and calmodulin may alter the interactions of water with the cytoplasmic vestibule (Németh-Cahalan et al. 2004). It is not yet clear if Ca^{2+} similarly regulates branchial aquaporin pathways in fish. Our results are in accord with previous studies that measured the effect of water $[Ca^{2+}]$ on diffusive water flux, where an increase in ambient $[Ca^{2+}]$ reduced the diffusive water permeability of the gills in fish (Odulye 1975; Isaia and Masoni 1976) and crustaceans (Rasmussen and Bjerregaard 1995), though in contrast to the present study, these reports did not separate the effects of water $[Ca^{2+}]$ from those of salinity. Some aquaporin subtypes found in the gill (e.g. AQP3) may have the ability to transport ammonia and/or urea (Cutler et al. 2007; Tingaud-Sequeira et al. 2010; Tipsmark et al. 2010; Chen et al. 2010; Kolarevic et al. 2012; Ip et al. 2013) and could likewise be affected by ambient calcium concentrations. Overall, the relationship between salinity and $[Ca^{2+}]$ on diffusive water flux and ammonia excretion rates and the role of aquaporins should be further investigated at the levels of transcription, protein function and distribution, and whole animal physiology.

Ecophysiological significance

As noted in the Introduction, these two flatfish species, as well as many others, exploit estuarine areas for feeding, reproduction, and nurseries, where they are routinely exposed to a range of reduced salinities near those tested

here. As the ocean of the north-west Pacific coast becomes more dilute and "atmospheric river" run-off events become more extreme, they will likely encounter even more extreme variations. Sea- level rise will also cause the formation of more lagoons, where flatfish can be temporarily or permanently trapped, and where hypersaline conditions can develop rapidly though evaporation. Such dilution and concentration events may also change the ratio of $[Ca^{2+}]$ to total salinity (Connors and Kester 1974). To our knowledge the salinities we evaluated (7.5–45 ppt) represent the largest range tested for these physiological endpoints in marine flatfishes, and our study is one of the very few to examine these effects in adult flatfishes. Understanding how these fish alter gill function and metabolic fuel use in response to acute, environmentally relevant, salinity challenges can inform the mechanisms used to thrive within their life cycle that requires them to enter waters of these salinities. It can also give an indication of their physiological ability to endure the extreme salinity changes that climate change will inevitably bring.

Key findings include the marked increases in diffusive water flux rates, ammonia excretion rates, and the reliance on protein as a metabolic fuel that occur with acute exposure to reduced salinities (and vice versa) and the fact that none of these are coupled to changes in $\dot{M}O_2$. Furthermore, reduced $[Ca^{2+}]$, rather than reduced salinity itself, is the primary cause for the increases in diffusive water flux at low salinity. Environmental $[Ca^{2+}]$ may also play a role in modulating ammonia excretion, a topic that deserves further study.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00227-023-04245-w.

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Authors contributions The project was jointly conceived by CM and CMW. CM did the experiments under the supervision of CMW. CM wrote the first draft of the paper, and CMW edited it.

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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.

Ethical approval Animals were collected under Fisheries and Oceans Canada collection permit XR 136 2021. The University of British Columbia Animal Care Committee (AUP A18-0271) and the Bamfield Marine Science Centre Animal Care Committee (AUP RS-21(19)-01)) approved all experimental procedures, in accordance with the Canadian Council on Animal Care guidelines.

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