FUNCTIONAL ASPECTS OF THE OSMORESPIRATORY COMPROMISE IN FISHES

by

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Abstract

The fish gill is a multipurpose organ that plays a central role in gas exchange, ion regulation, acid-base balance and nitrogenous waste excretion. Effective gas transfer requires a large surface area and thin water-to-blood diffusion distance, but such structures also promote diffusive ion and water movements between blood and water that challenge the maintenance of hydromineral balance. Therefore, a functional conflict exists between gas exchange and ionic and osmotic regulation at the gill.

The overarching goal of my thesis was to examine the trade-offs associated with the optimization of these different functions (i.e. the osmorespiratory compromise) in species with diverse osmoregulatory strategies, when exposed to environmental stressors such as hypoxia, changes in temperature and salinity. To address this I have used three species of fish that are phylogenetically, ecologically and physiologically diverse, the Atlantic killifish (teleost), the Pacific hagfish (myxine) and the Pacific spiny dogfish (elasmobranch).

My results show that salinity influences the capacity to regulate oxygen consumption at low oxygen and hypoxia tolerance in the killifish. Acclimation to fresh water resulted in a lowering of the lamellar respiratory surface area and a higher percentage of the gill lamellae covered by an interlamellar cell mass. These responses could be adaptations to aid survival in hypo-osmotic waters as freshwater-acclimated fish showed a greater ability to downregulate transcellular gill permeability to both ions and water when exposed to hypoxia in comparison to their seawater-acclimated counterparts. However, at salinities ranging from fresh water to 100% sea water, plasma ion concentration and osmolality were unaffected by hypoxia. I also found that there is a strong interaction between gill permeability to gases and to ions and water in hagfish, an osmoconforming marine species in which the osmorespiratory compromise had never been investigated. An increase in gill permeability to urea, ammonia, and water was also seen in the dogfish exposed to elevated temperature, indicating a disruption in the nitrogen conservation mechanisms at the gill.

In summary, this thesis has expanded the range of species in which the osmorespiratory compromise has been investigated, and has provided new insights into the mechanisms involved.
Lay Summary

The fish gill is an organ that plays a fundamental role in gas exchange and ion regulation. My thesis investigated how three different fish species deal with the trade-offs associated with optimizing these different functions when exposed to variation in environmental stressors such as salinity, oxygen content and temperature. My results show that there is a strong interaction between permeability of the gill to oxygen and to ions and water in the three species investigated. Adjusting to different salinities differentially influenced how fish handle the lack of oxygen in the water. Having to increase oxygen extraction led to different changes in gill permeability, which were salinity dependent. Understanding how environmental constraints affect fish physiology is of crucial importance as the world’s aquatic environments become increasingly more variable due to human impacts and climate change.
Preface

Chapter 1 is a general introduction providing relevant background information to the research chapters 2 - 6. Chapters 2 – 6 have been written as individual manuscripts. Chapter 7 provides a general discussion, summarizing the major findings of the 5 research chapters.

Chapter 2 has been written as a manuscript that is ready for submission as Giacomin, M., Bryant, H., Schulte, P.M. and Wood, C.M. The osmorespiratory compromise: physiological responses and tolerance to hypoxia exposure are affected by salinity acclimation in the euryhaline killifish (*Fundulus heteroclitus*). H. Bryant analyzed the histological images. I performed all experiments and analyzed all the remaining data. I wrote the manuscript under the supervision of Drs. P.M. Schulte and C.M. Wood.

Chapter 3 has been written as a manuscript that is intended for submission as Giacomin, M., Onukwufor, J., Schulte, P.M. and Wood, C.M. The osmorespiratory compromise: the ionoregulatory responses to hypoxia in the euryhaline killifish (*Fundulus heteroclitus*) acclimated to different salinities. J. Onukwufor performed the diffusive water flux experiments. I performed all remaining experiments and analyzed all the remaining data. I wrote the manuscript under the supervision of Drs. P.M. Schulte and C.M. Wood.

A version of Chapter 4 has been published as Giacomin, M., Eom, J., Schulte, P.M. and Wood, C.M. (2018). Acute temperature effects on metabolic rate, ventilation, diffusive water exchange, osmoregulation, and acid-base status in the Pacific hagfish (*Eptatretus stoutii*). Journal of Comparative Physiology (B). J. Eom collected and analyzed all the ventilatory and heart rate data. I collected and analyzed all the remaining data, with help from C.M. Wood. I wrote the manuscript under the supervision of Drs. P.M. Schulte and C.M. Wood. All co-authors provided editorial feedback to the manuscript.

A version of Chapter 5 has been submitted for publication as Giacomin, M., Dal Pont, G., Eom, J., Schulte, P.M. and Wood, C.M. The effects of salinity and hypoxia exposure on oxygen
consumption, ventilation, diffusive water exchange and ionoregulation in the Pacific hagfish (*Eptatretus stoutii*). Eom collected and analyzed all the ventilatory data. I collected and analyzed all the remaining data with the assistance of G. Dal Pont and C.M Wood. I wrote the manuscript under the supervision of Drs. P.M. Schulte and C.M. Wood. All co-authors provided editorial feedback to the manuscript.

A version of Chapter 6 has been published as Giacomini, M., Schulte, P.M. and Wood, C.M. (2017). Differential effects of temperature on oxygen consumption and branchial fluxes of urea, ammonia, and water in the dogfish shark (*Squalus acanthias suckleyi*). *Physiological and Biochemical Zoology* 90(6): 627 – 637. C.M. Wood and I designed the experiments. I collected and analyzed all the data, and wrote the manuscript under the supervision of Drs. P.M. Schulte and C.M. Wood. All co-authors provided editorial feedback to the manuscript.

All experiments performed for this thesis followed the guidelines of the Canada Council for Animal Care, under joint approval of the animal care committees at the University of British Columbia and Bamfield Marine Science Centre (AUP #: A14-0251 and RS–17-20, respectively).
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Figure 2.4 (A) Ventilatory index (cm H$_2$O/min), (B) ventilation frequency (breaths/min) and (C) ventilation pressure amplitude (cm H$_2$O/breath) in *Fundulus heteroclitus* acclimated to 0 (white circles), 11 (grey triangles) and 35 (black squares) ppt exposed to progressively decreasing water $PO_2$. Data have been plotted as averages every 5 Torr. Data are means ± 1 SEM (n = 11 - 12). .............................. 64

Figure 2.5 Representative oxygen equilibrium curves (OEC) for whole blood of *Fundulus heteroclitus* acclimated to (A,B) 0, (C,D) 11 and (E,F) 35 ppt exposed to (A,C,E) normoxia or (B,D,F) hypoxia (15 Torr for 3 h), assayed at a PCO$_2$ of 1.9 Torr (solid line) and at a PCO$_2$ of 7.6 Torr (dashed line)................................................................. 66
Figure 2.6 Hb-oxygen affinity (P50, Torr) in whole blood of Fundulus heteroclitus acclimated to 0, 11 and 35 ppt exposed to normoxia (white bars) or to hypoxia (15 Torr for 3 h) (black bars), assayed at (A) PCO$_2$ of 1.9 Torr and (B) PCO$_2$ of 7.6 Torr. Bars sharing the same lower case letters are not statistically different at the same oxygen level. [Two-way ANOVA p-values: (PCO$_2$ = 1.9 Torr: p$_{interaction}$ = .4772, p$_{oxygen}$ = .0046, p$_{salinity}$ = .2153; PCO$_2$ = 7.6 Torr: p$_{interaction}$ = .2447, p$_{oxygen}$ < .0001, p$_{salinity}$ = .0005]. Asterisks indicate significant differences between normoxia and hypoxia at the same salinity (Bonferroni post-hoc test). Data are means ± 1 SEM (n = 6 - 8).

Figure 2.7 (A) Blood hematocrit (HCT; % RBC), (B) hemoglobin concentration ([Hb]; mmol/L) and (C) mean cell hemoglobin concentration (MCHC; mmol/L) in Fundulus heteroclitus acclimated to 0, 11 and 35 ppt in normoxia (white bars) and exposed to hypoxia (15 Torr for 3 h) (black bars). Bars sharing the same lower case letters are not statistically different at the same oxygen level. [Two-way ANOVA p-values (HCT: p$_{interaction}$ = .9774, p$_{oxygen}$ = .0004, p$_{salinity}$ = .6009; [Hb]: p$_{interaction}$ = .5733, p$_{oxygen}$ = .1500, p$_{salinity}$ = .0069; MCHC: p$_{interaction}$ = .5865, p$_{oxygen}$ = .0001, p$_{salinity}$ = .08610)]. Asterisks indicate significant differences between normoxia and hypoxia at the same salinity (Bonferroni post-hoc test). Data are means ± 1 SEM (n = 6 - 8).

Figure 2.8 Lamellar surface area (x10$^7$ µm$^2$/g fish) of the second gill arch in Fundulus heteroclitus acclimated to 0, 11 and 35 ppt in normoxia. Bars sharing the same lower case letters are not statistically different (one-way ANOVA). Data are means ± 1 SEM (n = 5 - 6).

Figure 2.9 Representative histological sections of Fundulus heteroclitus gill filaments acclimated to (A) 0, (B) 11 and (C) 35 ppt in normoxia. (D) Illustration of gill filament detailing how morphological analysis were performed. Black arrows on micrographs indicate ionocytes. In 0 ppt acclimated fish, the epithelial cell layer covering the lamella is bulging and thicker than in 11 and 35 ppt. The epithelial cell coverage was measured as outlined in yellow on illustration D. The interlamellar cell mass (ILCM) is visible in all three salinities and highlighted in blue in illustration D. Scale bars are 10 µm in size.
**Figure 2.10** (A) Interlamellar cell mass area (µm²), (B) ionocyte density (#cells/µm²) and (C) epithelial cell coverage of the lamellae (µm²/µm) in *Fundulus heteroclitus* acclimated to 0, 11 and 35 ppt in normoxia. Bars sharing the same lower case letters are not statistically different (one-way ANOVA). Data are means ± 1 SEM (n = 6). ................................................................. 76

**Figure 2.11** The effect of water PO₂ on the relative extraction efficiency (ṀO₂/ventilatory index; µmol O₂/g/cm H₂O) of *Fundulus heteroclitus* acclimated to 0 (circles), 11 (triangles) and 35 (squares) ppt. Data were binned as 5 Torr averages. Data have been calculated from the mean values in Fig. 2.2A,B,C and 2.4A .......................................................... 78

**Figure 2.12** Representative framework for the analysis of the ṀO₂ versus PO₂ relationships as proposed by Alexander and McMahon (2004) and Mueller and Seymour (2011), and recommended by Wood (2018). The ṀO₂ data lie between two hypothetical lines of perfect regulation and conformation. The hatched area represents the area under the curve occupied by the relationship (regulation index, RI) relative to the total area between the two lines. A hyperbolic Michaelis-Menten relationship has been fitted to the hypothetical data set, yielding a Km of 12 Torr and a Routine O₂max of 8 µmol/g/h. The PO₂ at which the vertical distance is greatest between the data curve and the line of conformity yields the Pcrit = 32 Torr. ............. 80

**Figure 3.1** (A) Plasma sodium (mmol/L), (B) plasma chloride (mmol/L) and (C) plasma osmolality (mOsm/kg) in *Fundulus heteroclitus* acclimated to 0, 11 and 35 ppt in normoxia (white bars) and exposed to hypoxia (black bars). Bars sharing the same lower case letters are not statistically different at the same oxygen level. [Two-way ANOVA p-values (plasma Na⁺: p_{interaction} = .1221, p_{oxygen} = .5051, p_{salinity} < .0001; plasma Cl⁻: p_{interaction} = .8137, p_{oxygen} = .0909, p_{salinity} < .0001; plasma osmolality: p_{interaction} = .8878, p_{oxygen} = .0217, p_{salinity} < .0001)]. Data are means ± 1 SEM (n = 6 - 8). ........................................................................................................ 117

**Figure 3.2** (A) Na⁺/K⁺-ATPase and (B) H⁺-ATPase activity (µmol ADP/mg protein/h) in *Fundulus heteroclitus* acclimated to 0, 11 and 35 ppt in normoxia (white bars) and exposed to hypoxia (black bars). Bars sharing the same lower case letters are not statistically different at the
same oxygen level. [Two-way ANOVA p-values (\( \text{Na}^+/\text{K}^+\text{-ATPase}: p_{\text{interaction}} = .1356, p_{\text{oxygen}} = .0037, p_{\text{salinity}} = .2233; \text{H}^+\text{-ATPase}: p_{\text{interaction}} = .0608, p_{\text{oxygen}} = .0011, p_{\text{salinity}} = .2622)]). Asterisks indicate significant differences between normoxia and hypoxia at the same salinity (Bonferroni post-hoc test). Data are means ± 1 SEM (n = 6 - 8).

Figure 3.3 (A) Hematocrit (% RBC) and (B) plasma lactate (mmol/L) in Fundulus heteroclitus acclimated to 0, 11 and 35 ppt in normoxia (white bars) and exposed to hypoxia (black bars). Bars sharing the same lower case letters are not statistically different at the same oxygen level. [Two-way ANOVA p-values (\text{Hematocrit}: p_{\text{interaction}} = .1845, p_{\text{oxygen}} < .0001, p_{\text{salinity}} = .1239; \text{lactate}: p_{\text{interaction}} = .0051, p_{\text{oxygen}} < .0001, p_{\text{salinity}} = .0006)]. Asterisks indicate significant differences between normoxia and hypoxia at the same salinity (Bonferroni post-hoc test). Data are means ± 1 SEM (n = 6 - 8).

Figure 3.4 Water flux rate (% body water/h) in Fundulus heteroclitus acclimated to 0, 11 and 35 ppt in normoxia (white bars) and exposed to hypoxia (black bars). Bars sharing the same lower case letters are not statistically different at the same oxygen level. [Two-way ANOVA p-values (\text{water flux rate}: p_{\text{interaction}} < .0001, p_{\text{oxygen}} = .3608, p_{\text{salinity}} < .0001)]. Asterisks indicate significant differences between normoxia and hypoxia at the same salinity (Bonferroni post-hoc test). Data are means ± 1 SEM (n = 12).

Figure 3.5 (A) PEG-4000 clearance rate (\( \mu \text{L/g/h} \)) and (B) drinking rate (\( \mu \text{L/g/h} \)) in Fundulus heteroclitus acclimated to 0, 11 and 35 ppt in normoxia (white bars) and exposed to hypoxia (black bars). Bars sharing the same lower case letters are not statistically different at the same oxygen level. [Two-way ANOVA p-values (\text{clearance rate}: p_{\text{interaction}} = .0008, p_{\text{oxygen}} = .7808, p_{\text{salinity}} = .5193; \text{drinking rate}: p_{\text{interaction}} < .0001, p_{\text{oxygen}} < .0001, p_{\text{salinity}} < .0001)]. Asterisks indicate significant differences between normoxia and hypoxia at the same salinity (Bonferroni post-hoc test). Data are means ± 1 SEM (n = 5 - 8).

Figure 3.6 (A) Unidirectional sodium influx rate (\( J_{\text{Na}^+\text{influx}}; \text{nmol/g/h} \)), (B) unidirectional sodium efflux rate (\( J_{\text{Na}^+\text{efflux}}; \text{nmol/g/h} \)) and (C) sodium net flux (\( J_{\text{Na}^+\text{net flux}}; \text{nmol/g/h} \)) rate in Fundulus
*Fundulus heteroclitus* acclimated to 0 ppt. Fish were subjected to 3 h each of normoxia, hypoxia, normoxia. Asterisks indicate bars which are significantly different from the averaged starting normoxia period (Student’s t-test). Data are means ± 1 SEM (n = 15 - 17).

**Figure 3.7** The effect of hypoxia exposure on the (A) chloride net flux rate ($J^{\text{Cl}}_{\text{net flux}}$; nmol/g/h), (B) potassium net flux rate ($J^{\text{K}}_{\text{net flux}}$; nmol/g/h) and (C) ammonia net flux rate ($J^{\text{ammon}}_{\text{net flux}}$; nmol/g/h) in *Fundulus heteroclitus* acclimated to 0 ppt. Fish were subjected to 3 h each of normoxia, hypoxia, normoxia. Asterisks indicate bars which are significantly different from the averaged starting normoxia period (Student’s t-test). Data are means ± 1 SEM (n = 15 - 17).

**Figure 3.8** (A) unidirectional sodium influx rate ($J^{\text{Na}}_{\text{influx}}$; nmol/g/h) and (B) unidirectional sodium efflux rate ($J^{\text{Na}}_{\text{efflux}}$; nmol/g/h) in *Fundulus heteroclitus* acclimated to 0 ppt, 11 ppt and 35 ppt in normoxia (grey bars) and exposed to hypoxia (black bars). 0 ppt data are averaged normoxia and hypoxia data obtained in Fig. 3.6. [Two-way ANOVA p-values ($J^{\text{Na}}_{\text{influx}}$: $p_{\text{interaction}} < .0001$, $p_{\text{oxygen}} = .0004$, $p_{\text{salinity}} < .0001$; $J^{\text{Na}}_{\text{efflux}}$: $p_{\text{interaction}} = .1059$, $p_{\text{oxygen}} = .6178$, $p_{\text{salinity}} < .0001$)]. Asterisks indicate significant differences between normoxia and hypoxia at the same salinity (Bonferroni post-hoc test). Data are means ± 1 SEM (n = 15 - 17).

**Figure 3.9** Ammonia net flux rate ($J^{\text{ammon}}$; nmol/g/h) in *Fundulus heteroclitus* acclimated to (A) 0 ppt (B) 11ppt and (C) 35 ppt. Asterisks indicate significant differences between normoxia and hypoxia at the same salinity (Student’s t-test). Data are means ± 1 SEM (n = 15 - 17).

**Figure 4.1** (a) Oxygen consumption rate ($\dot{\text{MO}}_2$; µmol O$_2$/kg/h), (b) ammonia net flux rate ($J^{\text{ammon}}$; µmol/kg/h), (c) calculated ammonia quotient (AQ) and (d) diffusive water flux rate ($J^{\text{H}_{2}\text{O}}$; L/kg/h) in *Eptatretus stoutii* acutely exposed to 7, 12 (control) and 17 °C. The Q$_{10}$ values over the entire range (7 – 17°C) are displayed on the upper left corner. The lower range (7 – 12°C) Q$_{10}$ are shown above the 12°C bar and the higher range (12 – 17°C) Q$_{10}$ are shown above the 17°C bar. Means sharing the same lower case letters are not statistically different (one-way ANOVA; Tukey’s post hoc test). Data are shown as means ± 1 SEM (n = 8 - 16).
Figure 4.2 (a) Ventilation rate (frequency: breaths/min), (b) ventilation pressure amplitude (cmH₂O/breath), (c) calculated ventilatory index (cmH₂O/min) and (d) heart rate (beats/min) in *Eptatretus stoutii* acutely exposed (30 min) to 7, 12 (control) and 17 °C. The Q₁₀ values over the entire range (7 – 17°C) are displayed on the upper left corner. The lower range (7 – 12°C) Q₁₀ are shown above the 12°C bar and the higher range (12 – 17°C) Q₁₀ are shown above the 17°C bar. Means sharing the same lower case letters are not statistically different (repeated measures one-way ANOVA; Tukey’s post hoc test). Data are shown as means ± 1 SEM (n = 6 - 8).

Figure 4.3 Plasma ion concentration (a) Na⁺, (b) Cl⁻, (c) Mg²⁺, (d) Ca²⁺ (all in mmol/L) and (e) osmolality (mOsm/kg) in *Eptatretus stoutii* acutely exposed to 7, 12 (control) and 17°C. Means sharing the same lower case letters are not statistically different (one-way ANOVA; Tukey’s post hoc test). Data are shown as means ± 1 SEM (n = 7 - 8).

Figure 4.4 (a) Total plasma total ammonia (µmol/L), (b) and partial pressure of NH₃ (µTorr) in *Eptatretus stoutii* acutely exposed to 7, 12 (control) and 17°C. Means sharing the same lower case letters are not statistically different (one-way ANOVA; Tukey’s post hoc test). Data are shown as means ± 1 SEM (n = 6 - 8).

Figure 4.5 (a) Blood pH, (b) plasma PCO₂ (Torr) and (c) plasma HCO₃⁻ (mmol/L) in *Eptatretus stoutii* acutely exposed to 7, 12 (control) and 17°C. Means sharing the same lower case letters are not statistically different (one-way ANOVA; Tukey’s post hoc test). Data are shown as means ± 1 SEM (n = 6 - 8).

Figure 4.6 (a) Ammonia net flux rate (Jₐₚₙ: µmol/kg/h), (b) diffusive water flux rate (Jₕ₂ₒ : L/kg/h) in *Eptatretus stoutii* acutely exposed to hyperoxia (black bars) or normoxia (white bars) at 12 and 17°C. The normoxia Q₁₀ are shown in the 17°C normoxia bar while the hyperoxia Q₁₀ are shown in the 17°C bar. Normoxia and hyperoxia bars sharing the same lower case letters are not statistically different at the same temperature [Two-way ANOVA p-values (Jₐₚₙ: pinteraction= 0.601, p_oxygen=.443, p_temperature=.039; Jₕ₂ₒ : pinteraction=.014, p_oxygen=.001, p_temperature<.001)].
Asterisks indicate significant differences between 12 and 17°C at the same oxygen level (Bonferroni post hoc test). Data are shown as means ± 1 SEM (n = 6 - 8). ......................................................... 170

Figure 4.7 (a) Ventilation rate (frequency: breaths/min), (b) ventilation pressure amplitude (cmH₂O/breath), and (c) calculated ventilatory index (cmH₂O/min) in *Eptatretus stoutii* exposed to experimental conditions for 6 h. Normoxia and hyperoxia bars sharing the same lower case letters are not statistically different at the same temperature [Two-way ANOVA p-values (rate: \( p_{\text{interaction}} = .3597, p_{\text{oxygen}} < .0001, p_{\text{temperature}} = .0045; \) pressure: \( p_{\text{interaction}} = .3994, p_{\text{oxygen}} = .1707, p_{\text{temperature}} = .2259; \) index: \( p_{\text{interaction}} = .6268, p_{\text{oxygen}} = .0421, p_{\text{temperature}} = .3045 \)]. Data are shown as means ± 1 SEM (n = 6 - 8). ........................................................................................................ 172

Figure 4.8 Plasma ion concentrations (a) Na\(^+\), (b) Cl\(^-\), (c) Mg\(^{2+}\), (d) Ca\(^{2+}\) (mmol/L) and (e) osmolality (mOsm/kg) in *Eptatretus stoutii* acutely exposed to hyperoxia (black bars) or normoxia (white bars) at 12 and 17°C. Normoxia and hyperoxia bars sharing the same lower case letters are not statistically different at the same temperature [Two-way ANOVA p-values (Na\(^+\): \( p_{\text{interaction}} = .479, p_{\text{oxygen}} = .595, p_{\text{temperature}} = .762; \) Cl\(^-\): \( p_{\text{interaction}} = .037, p_{\text{oxygen}} = .626, p_{\text{temperature}} = .559; \) Mg\(^{2+}\): \( p_{\text{interaction}} = .012, p_{\text{oxygen}} = .001, p_{\text{temperature}} = .184; \) Ca\(^{2+}\): \( p_{\text{interaction}} = .067, p_{\text{oxygen}} = 0.001, p_{\text{temperature}} = .125; \) osmolality: \( p_{\text{interaction}} = .387, p_{\text{oxygen}} = .906, p_{\text{temperature}} = .948 \)]. Asterisks indicate significant differences between 12 and 17°C at the same oxygen level (Bonferroni post hoc test). Data are shown as means ± 1 SEM (n = 6 - 8). ........................................................................................................ 174

Figure 4.9 (a) Total plasma total ammonia (µmol/L), (b) and partial pressure of NH₃ (PNH₃: µTorr) in *Eptatretus stoutii* acutely exposed to hyperoxia (black bars) or normoxia (white bars) at 12 and 17°C. Normoxia and hyperoxia bars sharing the same lower case letters are not statistically different at the same temperature [Two-way ANOVA p-values (Total ammonia: \( p_{\text{interaction}} = .256, p_{\text{oxygen}} = .040, p_{\text{temperature}} = .340; \) PNH₃: \( p_{\text{interaction}} = .165, p_{\text{oxygen}} = .303, p_{\text{temperature}} = .466 \)]. Asterisks indicate significant differences between 12 and 17°C at the same oxygen level (Bonferroni post hoc test). Data are shown as means ± 1 SEM (n = 6 - 8). ........................................................................................................ 176
Figure 4.10 (a) Blood pH, (b) plasma PCO$_2$ (Torr) and (c) plasma HCO$_3^-$ (mmol/L) in *Eptatretus stoutii* acutely exposed to hyperoxia (black bars) or normoxia (white bars) at 12 and 17ºC. Normoxia and hyperoxia bars sharing the same lower case letters are not statistically different at the same temperature [Two-way ANOVA p-values (pH: $p_{\text{interaction}} = .403$, $p_{\text{oxygen}} = .005$, $p_{\text{temperature}} = .937$; PCO$_2$: $p_{\text{interaction}} = .011$, $p_{\text{oxygen}} = .025$, $p_{\text{temperature}} = .128$; HCO$_3^-$: $p_{\text{interaction}} = .010$, $p_{\text{oxygen}} = .548$, $p_{\text{temperature}} = .090$)]. Asterisks indicate significant differences between 12 and 17ºC at the same oxygen level (Bonferroni post hoc test). Data are shown as means ± 1 SEM (n = 7 – 8).

Figure 5.1 (A) Oxygen consumption rate (Ṁ$_{\text{O}_2}$: μmol O$_2$/kg/h), (B) ammonia net flux rate (J$_{\text{amm}}$: μmol/kg/h) and (C) Ammonia quotient in *Eptatretus stoutii* acclimated to 30 ppt or exposed to 25 and 35 ppt for 48h. Different lower case letters represent statistically different means detected by one-way ANOVA and a Tukey’s post hoc test. Data are shown as means ± 1 SEM (n = 7 - 8).

Figure 5.2 (A) Water flux rate (mL/kg/h) and (B) exchangeable water pool (% body weight) in *Eptatretus stoutii* acclimated to 30 ppt or exposed to 25 and 35 ppt for 48h. Different lower case letters represent statistically different means detected by one-way ANOVA and a Tukey’s post hoc test. Data are shown as means ± 1 SEM (n = 7 - 8).

Figure 5.3 Plasma ion concentration in *Eptatretus stoutii* acclimated to 30 ppt or exposed to 25 and 35 ppt for 48h. (A) Sodium: Na$^+$; (B) Chloride: Cl$^-$; (C) Magnesium: Mg$^{2+}$; (D) Calcium: Ca$^{2+}$ (mmol/L) and (E) Osmolality (mOsm/kg). Different lower case letters represent statistically different means detected by one-way ANOVA and a Tukey’s post hoc test. Data are shown as means ± 1 SEM (n = 7 – 8).

Figure 5.4 Plasma ion to seawater concentration differential in *Eptatretus stoutii* acclimated to 30 ppt or exposed to 25 and 35 ppt for 48h. (A) Sodium: Na$^+$; (B) Chloride: Cl$^-$; (C) Magnesium: Mg$^{2+}$; (D) Calcium: Ca$^{2+}$ (mmol/L) and (E) Osmolality (mOsm/kg). Data are shown as means ± 1 SEM (n = 7 – 8).
Figure 5.5 (A) Blood pH, (B) plasma PCO₂ (Torr) and (C) plasma HCO₃⁻ (mmol/L), (D) plasma total ammonia (µmol/L) and (E) partial pressure of ammonia (PNH₃ - µTorr) in *Eptatretus stoutii* acclimated to 30 ppt or exposed to 25 and 35 ppt for 48h. Different lower case letters represent statistically different means detected by one-way ANOVA and a Tukey’s post hoc test. Data are shown as means ± 1 SEM (n = 7 – 8).

Figure 5.6 (A) Oxygen consumption rate (ṀO₂: µmol O₂/kg/h), (B) ammonia net flux rate (J_{amm}: µmol/kg/h), (C) diffusive water flux rate (J_{H₂O}: mL/kg/h) and (D) Ammonia quotient in *Eptatretus stoutii* exposed to normoxia (white bars: 150 Torr for 6h; 30 ppt at 12ºC) and hypoxia (black bars: 30 Torr for 6h; 30 ppt at 12ºC). Different lower case letters represent statistically different means detected by Student’s t-test. Data are shown as means ± 1 SEM (n = 7 - 8).

Figure 5.7 (A) Ventilation rate (breaths/min), (B) ventilation pressure amplitude (cmH₂O/breath) and (C) ventilatory index (cmH₂O/min) in *Eptatretus stoutii* exposed to hypoxia (30 Torr for 6h; 30 ppt at 12ºC). Different lower case letters represent statistically different means detected by a paired Student’s t-test. Data are shown as means ± 1 SEM (n = 7 - 8).

Figure 5.8 (A) Blood pH, (B) plasma PCO₂ (Torr) and (C) plasma HCO₃⁻ (mmol/L) in *Eptatretus stoutii* exposed to hypoxia (30 Torr for 6h; 30 ppt at 12ºC). Different lower case letters represent statistically different means detected by Student’s t-test. Data are shown as means ± 1 SEM (n = 7 - 8).

Figure 6.1 (A) Oxygen consumption rates (ṀO₂: µmol O₂/kg/h); (B) ammonia excretion rates (J_{Amn}: µmol N/kg/h); (C) urea-N excretion rates (J_{Urea-N}: µmol N/kg/h); and (D) tritiated water turnover rates (% body water/h) in dogfish sharks (*Squalus acanthias suckleyi*) at 5 different temperatures. Numbers between data points are the Q₁₀ values for each increment in temperature, whereas the Q₁₀ value over the entire range from 7ºC to 22ºC is tabulated in the lower right of each panel. The same 8 animals were tested at 12ºC, 15ºC, 18ºC, and 22ºC, so these data were analyzed by a repeated-measures one-way ANOVA followed by a Tukey’s post hoc test. A different set of 6 fish was tested at 7ºC, so an unpaired Student’s two-tailed t-test was used to test...
the significance of differences between the 7ºC and 12ºC treatments. Means sharing the same upper case letters indicate the absence of statistical significance (p<0.05) through an unpaired Student’s t-test for 7.5 and 12ºC data points. Means sharing same lower case letters indicate the absence of statistical significance by a repeated-measures one-way ANOVA for 12 to 22ºC. Data are shown as means ± 1 SEM (n = 6 - 8).

**Figure 6.2** Nitrogen quotient in dogfish (*Squalus acanthias suckleyi*) at different temperatures (ºC). The same 8 animals were tested at 12ºC, 15ºC, 18ºC, and 22ºC, so these data were analyzed by a repeated-measures one-way ANOVA followed by a Tukey’s post hoc test. A different set of 6 fish was tested at 7ºC, so an unpaired Student’s two-tailed t-test was used to test the significance of differences between the 7ºC and 12ºC treatments. Means sharing the same upper case letters indicate the absence of statistical significance (p<0.05) through an unpaired Student’s t-test for 7.5 and 12ºC data points. Means sharing same lower case letters indicate the absence of statistical significance by a repeated-measures one-way ANOVA for 12 to 22ºC. Data are shown as means ± 1 SEM (n = 6 - 8).

**Figure 6.3** (A) Ammonia excretion rates (J\textsubscript{Amm}; µmol N/kg/h); (B) urea-N excretion rates (J\textsubscript{Urea-N}; µmol N/kg/h); and (C) tritiated water turnover rates (% body water/h) in dogfish sharks (*Squalus acanthias suckleyi*) at 12 (grey bars) and 22ºC (black bars) measured under normoxia and hyperoxia. Different lower case letters indicate statistical significance between 12 and 22ºC tested at the same oxygen tension, as determined by a paired Student’s two-tailed t-test (p < 0.05). Normoxia and hyperoxia data at a given temperature were compared through an unpaired Student’s two-tailed t-test (p < 0.05), and significance is indicated through an asterisk. Data are shown as means ± 1 SEM (n = 6 - 8).

**Figure 7.1** Conceptual diagram displaying effective gill permeability to O\textsubscript{2} (top line) versus the two very different accompanying changes (two lower lines) in effective gill permeability to ions and water seen in two species with very different strategies for the osmore Respiratory compromise. The rainbow trout exhibits increased gill permeability to ions and water, while the Amazonian oscar shows a regulated reduction in gill permeability. Contributions of my thesis include the
Pacific hagfish and the Atlantic killifish showing responses similar to the oscar, while the
responses of the spiny dogfish to increased temperature are similar to the rainbow trout model.

Figure 7.2 Comparative water to gas ratio (WGR) in the rainbow trout (*Oncorhynchus mykiss*),
Amazonian oscar (*Astronotus ocellatus*), Atlantic killifish (*Fundulus heteroclitus*), Pacific spiny
dogfish (*Squalus acanthias suckleyi*) and Pacific hagfish (*Eptatretus stoutii*). All bars are control
normoxic values in resting animals. *F. heteroclitus* have been acclimated to three salinities,
which are shown beside the bars. Data shown for the trout have been extracted from the literature
and added to the figure for comparison [\(\text{\(\dot{M}\)}_{\text{O}_2}\): Marvin and Heath (1968) and \(J_{\text{H}_2}\): Onukwufor
and Wood (2018)]. Data shown for the oscar have been extracted from the literature and added to
the figure for comparison [\(\text{\(\dot{M}\)}_{\text{O}_2}\): Sloman et al. (2012) and \(J_{\text{H}_2}\): Wood et al. (2009)]. The water
flux rate results for the killifish (Chapter 3) and dogfish (Chapter 6) were expressed as % of body
water/h and have been converted to mL/kg/h assuming that the fish’s body is made up of 80%
water. Then, using the molecular weight of water (MW 18.01), the diffusive water flux data
(mL/kg/h) for all five species was converted to \(\mu\text{mol H}_2\text{O/g/h}\). Therefore, the division between
the diffusive water flux (\(\mu\text{mol H}_2\text{O/g/h}\)) by the \(\dot{M}_2\) (\(\mu\text{mol O}_2/g/h\)) has no units.

Figure 7.3 Comparative water to gas ratio (WGR) in the rainbow trout (*Oncorhynchus mykiss*),
Amazonian oscar (*Astronotus ocellatus*), Atlantic killifish (*Fundulus heteroclitus*), and Pacific
hagfish (*Eptatretus stoutii*). Normoxia bars are shown in gray, while hypoxia exposed are shown
in black. *F. heteroclitus* have been acclimated to three salinities, which are shown beside the
bars. Data shown for the trout have been extracted from the literature and added to the figure for
comparison [\(\text{\(\dot{M}\)}_{\text{O}_2}\): Marvin and Heath (1968) and \(J_{\text{H}_2}\): Onukwufor and Wood (2018)]. Data
shown for the oscar have been extracted from the literature and added to the figure for
comparison [\(\text{\(\dot{M}\)}_{\text{O}_2}\): Sloman et al. (2006) and \(J_{\text{H}_2}\): Wood et al. (2009)]. The water flux rate results
for the killifish (Chapter 3) were expressed as % of body water/h and have been converted to
mL/kg/h assuming that the fish’s body is made up of 80% water. Then, using the molecular
weight of water (MW 18.01), the diffusive water flux data (mL/kg/h) for all four species was
converted to $\mu$mol H$_2$O/g/h. Therefore, the division between the diffusive water flux ($\mu$mol H$_2$O/g/h) by the $\dot{M}O_2$ ($\mu$mol O$_2$/g/h) has no units. 

Figure 7.4 Comparative water to gas ratio (WGR) in the rainbow trout (*Oncorhynchus mykiss*), Pacific spiny dogfish (*Squalus acanthias suckleyi*) and Pacific hagfish (*Eptatretus stoutii*). Control temperature bars are shown in gray, while acute warming temperature exposures are shown in black. Data shown for the trout have been provided by John Onukwufor and Chris Wood (personal communication). The water flux rate results for the dogfish (Chapter 6) were expressed as % of body water/h and have been converted to mL/kg/h assuming that the fish’s body is made up of 80% water. Then, using the molecular weight of water (MW 18.01), the diffusive water flux data (mL/kg/h) for all three species was converted to $\mu$mol H$_2$O/g/h. Therefore, the division between the diffusive water flux ($\mu$mol H$_2$O/g/h) by the $\dot{M}O_2$ ($\mu$mol O$_2$/g/h) has no units.
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Chapter 1: Introduction

1.1 Overview

The fish gill is a multipurpose organ that plays a central role in gas exchange, ion regulation, acid-base balance and nitrogenous waste excretion. The goal of my thesis was to examine the trade-offs associated with the optimization of these different functions in species with diverse osmoregulatory strategies, when exposed to environmental stressors such as hypoxia, temperature and salinity. To that end, I used three species of fish that differ greatly in both phylogeny and physiology, the Atlantic killifish (teleost), the Pacific hagfish (myxine) and the Pacific spiny dogfish (elasmobranch). In order to examine tradeoffs in gill function, I have performed experiments where I manipulated environmental variables, such as salinity in Chapters 2, 3 and 5, oxygen partial pressure in Chapters 2, 3, 4, 5, and 6, and temperature in Chapters 4 and 6. A goal common to all chapters was to evaluate how fish physiologically responded to those changes, in the context of the osmorespiratory compromise. To provide a conceptual background and framework for these data chapters, this Introduction provides an overview of the structure and function of the fish gill, as well as the trade-offs that occur when there is a need for optimization of certain functions. Additionally, it presents a short literature review on the development of the concept underlying the osmorespiratory compromise, while pointing out the many remaining knowledge gaps, as well as recent findings that have challenged our traditional understanding of this phenomenon. Finally, I present a brief review of the biology of the three species chosen as models, as well as their relationships with the diverse environments they inhabit. This introductory chapter ends with a brief summary of the goals, hypotheses and major findings of each data chapter.

1.2 The fish gill

The adult fish gill is the largest exchange surface of the fish’s body due to its thin, well vascularized epithelium. In most fishes, each of the branchial arches is subdivided into
multiple filaments, which further branch into lamellae, plate-like structures that are the site of the majority of gas exchange (Evans et al., 1999). Each arch supports two rows of filaments. Both filaments and lamellae are highly vascularized and they are perfused with the entire cardiac output before the blood enters the systemic circulation. Muscular pillar cells contain columns of collagen that hold open the thin lamellae, which can be adjusted to control and change blood flow patterns through the lamellae (Olson, 2002).

Fishes have evolved a suite of structural features that maximize the capacity for oxygen and carbon dioxide diffusion across the gill epithelium, such as a high surface area and a thin water-to-blood diffusion distance. Ventilation moves water through the interlamellar channels of the gill counter-current to the blood flow perfusing the lamellae, which maximizes oxygen uptake from the water into the blood, sustaining the gradients for gas exchange. The rate of gas diffusion is directly related to the partial pressure gradient between water and blood, and inversely proportional to the thickness of the barrier.

The thin gill epithelium is composed of distinct cell types including pavement cells (over 90% of the cell population), ionocytes (less than 10%, also known as mitochondrion-rich cells), mucous cells, neuroepithelial cells (chemosensory cells), and even stem cells (Hwang and Lin, 2014). Pavement cells are found throughout the entire branchial epithelium, and are the dominant cell type on the surface of the lamellae. Since they are more numerous, the majority of gas-exchange occurs through them, while ionocytes are responsible for the majority of the ion transport across the gills (Wilson and Laurent, 2002). Since transepithelial ion transport involves energy-intensive processes, all ionocytes are enriched with mitochondria to produce ATP to support the high energy demand of ion transport. Ionocytes possess specific transporters and channels on both the apical and basolateral membranes that allow for the directional movement of Na$^+$, Cl$^-$, K$^+$, Ca$^{2+}$, and other electrolytes (Dymowska et al., 2012).

1.3 The role of the gills in regulating osmotic and ionic balance

The maintenance of homeostasis of the body fluids is a substantial challenge for aquatic organisms, because they can face great fluctuations in the ionic composition and
osmolality of the surrounding environment. In most fish species, the gills, along with the kidney, are the major site of osmotic and ionic regulation (Evans, 2005).

Fishes are classified according to the strategy employed in order to maintain hydromineral balance when coping with environmental variation. Osmoconformers allow the osmolality of their internal fluids to follow that of the external environment, while osmoregulators maintain a relatively constant internal fluid osmolality that is largely independently of the external environment. The same is largely true for the ionic composition of their internal fluids. All teleost fishes are iono- and osmo-regulators, at least over the salinity ranges that they normally inhabit, while marine elasmobranchs are osmoconformers but ionoregulators, adjusting their plasma osmotic pressure through the use of organic osmolytes (nitrogenous compounds). Myxines are osmoconformers that are only found in marine environments, and while they regulate the concentration of divalent ions such as Ca$^{2+}$ and Mg$^{2+}$, they are generally believed not to regulate the major cation (Na$^+$) and anion (Cl$^-$) in their plasma when salinity changes (Smith, 1932).

Teleost fishes that inhabit fresh water are hypertonic to their environment, keeping their blood osmolality at approximately 250 – 300 mOsm, while the surrounding environment usually ranges from 0.5 to 15 mOsm. Thus, they face the continual gain of water by osmosis and loss of ions by diffusion (Edwards and Marshall, 2013). In order to maintain hydromineral balance, freshwater teleosts actively take up ions through the branchial epithelium while producing large quantities of dilute urine (reviewed by Hwang et al. 2011). Therefore, all freshwater teleosts are osmoregulators.

Most marine teleosts maintain the osmolality of their internal fluids at around 300 – 350 mOsm (Edwards and Marshall, 2013); therefore they are hypotonic to the surrounding marine environment, which has an osmolality of around 1000 mOsm. Thus, these fishes face the challenge of continual osmotic water loss, and diffusive ion gain. To counteract these challenges, seawater teleosts drink sea water and absorb water across their gut. Passive water absorption follows active Na$^+$ and Cl$^-$ uptake across the intestinal epithelium, while divalent ions (Ca$^{2+}$, Mg$^{2+}$, and SO$_4^{2-}$) are largely excluded. HCO$_3^-$ is secreted into the intestine and forms complexes with imbibed divalent cations, precipitating these as carbonate salts and providing another mechanism for water reabsorption at the gut (Wilson et al., 2002). Excess
ions are actively excreted against steep concentration gradients at the gills, as well as by the kidney (Smith, 1930; Evans, 2008).

Marine elasmobranchs are hypoionic osmoconformers. The gills play a primary role in ion regulation, while the kidney and the rectal gland have a minor contribution. While the osmotic pressure of their internal fluids is almost isosmotic to the environment, the concentration of ions reaches only about 300 mOsm, not dissimilar to that of teleosts. In order to maintain internal osmolality slightly greater than that of the environment, elasmobranchs accumulate organic osmolytes, mostly derived nitrogenous compounds such as urea and trimethylamine N-oxide (TMAO). The elasmobranch gill has a low permeability to urea, therefore playing an important role in the conservation of nitrogenous compounds (Fines et al., 2001). As internal osmolality is higher than the environment, a slow osmotic flux of water inwards is promoted, thereby eliminating the need to drink water (Smith, 1931; Smith, 1936), and avoiding excess accompanying salt load.

Myxines are marine osmoconformers, and to date, little is known about the mechanisms used by these fishes to maintain hydromineral balance. Internal osmolality is known to be symmetrically linked to water osmolality (Bellamy and Chester Jones, 1961; Sardella et al., 2009). Notably, divalent cations are approximately half that of seawater, while the concentration of major ions closely resembling that of sea water (Smith, 1932; Robertson, 1976). The somewhat permeable skin plays an important role in acid-base regulation (Clifford et al., 2014), nitrogenous waste regulation (Clifford et al., 2017) and nutrient acquisition (Glover et al., 2016).

1.4 The role of the gills in regulating oxygen uptake when demand is increased by increases in temperature

In order to maintain whole-body homeostasis, it is vital that a balance is reached between the available oxygen supply and the organism’s metabolic demand for oxygen. The gill has the capacity to modify oxygen uptake in order to match changes in oxygen demand (i.e. during exercise or in response to increased temperature), or oxygen supply (i.e. during hypoxia – see below).
Environmental variation in abiotic factors imposes constraints on organismal performance and determines distribution ranges based on the physiological ability to deal with, and thrive under such pressures. One of the key factors that can influence the ability of an organism to maintain this balance is temperature.

Temperature has profound effects on all levels of biological organization and can greatly affect organismal physiology (Hochachka and Somero, 2002). The influence of temperature on organisms is particularly important for aquatic ectotherms, in which internal body temperatures match those of the environment. As temperature rises, metabolic rate increases as a consequence of thermodynamic effects on biochemical reactions, resulting in an associated increase in oxygen demand (Schulte, 2015), and thus a need to increase oxygen supply to the body. The quantification of the thermal effects on biological rates is often done using the $Q_{10}$, which quantifies the fold change of a reaction rate over a 10ºC change in environmental temperature, where a high number indicates a high temperature sensitivity. $Q_{10}$ values close to 1.0 apply to simple physical processes such as diffusion, whereas most biochemical reactions and physiological processes have $Q_{10}$ values between 2.0 and 3.0. For example, metabolic rate increases with temperature in teleost fishes with a $Q_{10}$ of approximately 2.0 or lower (Clarke and Johnston, 1999).

Fish can increase oxygen supply by increasing uptake at the gills, boosting transport through cardiovascular modifications, or increasing extraction efficiency at the tissues (Farrell, 2009). Despite great variability among different species, fishes in general prioritize increases in ventilatory stroke volume rather than in ventilation frequency (Heath, 1973). At the gills, fishes can increase the vascular space within the lamellae which, coupled with erythrocyte swelling, leads to a shorter diffusion distance and has the potential to increase oxygen uptake (Soivio and Tuurala, 1981). It is well established that resting cardiac output changes in concert with temperature until the fish reaches its highest tolerable temperature (Farrell, 2009; Gamperl, 2011). When acutely exposed to higher temperatures, fishes increase cardiac output mainly through changes in heart rate rather than stroke volume (Sandblom and Axelsson, 2007), but the rationale underlying this response is still unknown (Gamperl, 2011).
Additionally, despite the fact that the gills adjust morphologically or physiologically in response to increases in temperature, no studies have examined the osmoresporatory compromise in the context of elevated temperature. One of the goals of this thesis is to address that gap.

1.5 The role of the gills in regulating oxygen uptake when environmental supply is reduced by environmental hypoxia

Environmental hypoxia can be simply defined as a decrease in the partial pressure of oxygen in the water below levels in the atmosphere at sea levels. In contrast to increases in temperature, hypoxia limits the availability of oxygen, making it challenging for the organism to meet the metabolic demand for oxygen. Hypoxia can occur across diverse aquatic environments such as shallow ponds, rivers, ice covered lakes, tidal pools, estuaries and salt marshes. The partial pressure of oxygen (PO$_2$) can decrease, especially at night, when oxygen consumption by vegetation and other aquatic organisms surpasses the available oxygen supply (Diaz and Breitburg, 2009). Hypoxia can also occur when the bottom layer of water is prevented from mixing with the upper highly saturated layer of water (stratification), due to differences in density or temperature. Even though hypoxia occurs naturally, anthropogenic activities have greatly increased the prevalence of hypoxia in many aquatic systems, especially in estuarine coastal areas, due to increases in nutrient loading (sewage and agricultural runoff) which leads to eutrophication (Diaz and Breitburg, 2009). The excess nutrient enrichment, especially when combined with physical conditions that limit water circulation, can cause hypoxic zones. The fundamental problem related to hypoxia exposure is the maintenance of the metabolic energy balance. A decrease in blood oxygen (O$_2$) content directly impacts mitochondrial ATP production given that without oxygen, the H$^+$ gradient at the mitochondria is dissipated, impairing the oxidative phosphorylation. Most animals possess mechanisms that enhance O$_2$ uptake and subsequent delivery to tissues as environmental O$_2$ is reduced (i.e., in response to hypoxia). In order to maintain aerobic metabolism, oxygen needs to be extracted and transported effectively from the water, across
the gills, and through the circulation until it reaches the tissues and ultimately the mitochondria.

Fish facing hypoxia usually increase the volume of water flowing across the gills per unit of time, also known as the hypoxic hyperventilatory response (Hughes and Saunders, 1970; Perry et al., 2009a). In most fishes, the majority of increases in ventilation volume come from increases in stroke volume (amplitude of the breath) rather than increases in ventilation frequency, which is thought to be less energetically costly (Perry, 2011). Hyperventilation increases the mean blood-to-water $PO_2$ gradient, elevating the rate at which oxygen diffuses to the blood, and minimizing the reduction in arterial $PO_2$ which is the inevitable consequence of hypoxia.

In addition, to improve the oxygen transfer from the water to the blood, fish can alter their functional respiratory surface area (Nilsson, 2007). The term functional surface area is used to describe the gill area that is actually being used for gas exchange in the living animal. Pillar cells link the two epithelial sheets that make up the outer surfaces of the lamellae, forming vascular channels in which erythrocytes are dispersed and slowed during their lamellar passage, promoting gas exchange through the counter-current respiratory mechanism (Nilsson et al., 1995). Increased blood pressure during hypoxia opens up more of the lamellar vasculature, resulting in higher lamellar recruitment (Booth, 1978; Farrell et al., 1980). Channels around the edge of the lamellae lack pillar cells and represent a circulatory shunt that allows for a rapid bypass for the erythrocytes (Pärt et al., 1984; Sundin and Nilsson, 1998). During periods of low oxygen demand (i.e., when fish are at rest), more blood will pass through the marginal channels and less between the pillar cells (Farrell et al., 1980), reducing the functional surface area of the gill. It has long been suggested that only 55–60% of the lamellae are used for gas exchange in resting fish. However, when treated with epinephrine or exposed to hypoxic water, rainbow trout increased the lamellar perfusion from basal levels by up to 80% (Booth, 1979). Hypoxia has been found to increase the lamellar vascular space (Soivio and Tuurala, 1981), causing the blood flow to shift from the marginal channels to the whole vascularized lamellar sheet. Sundin and Nilsson (1998) showed that endothelium-derived vasoactive peptide, endothelin-1, is able to constrict the vascular sheet within the lamellae, possibly through modulating the contraction of the pillar cells. While the
gill can increase functional surface area in order to maximize gas transfer, this may paradoxically lead to disruptions in the ionic and osmoregulatory functions of the gill, a physiological trade-off known as the osmorespiratory compromise.

1.6 The osmorespiratory compromise

A functional conflict exists between gas exchange and ionic and osmotic regulation in the gills of fishes, where effective gas transfer requires a large surface area, highly permeable gill epithelium and a thin water-to-blood diffusion distance. However, such structures will also promote diffusive ion and water movements between blood and water that challenge the maintenance of hydromineral balance. By increasing gill surface area, either functionally or morphologically, to promote O$_2$ transfer, undesirable ion and water exchange will also increase. Conversely, reducing surface area to minimize passive ion and water fluxes may impair the ability of the gill to exchange O$_2$ and carbon dioxide (CO$_2$). This functional tradeoff between high permeability to respiratory gases and low permeability to ions and water, or vice versa, has been called the osmorespiratory compromise. This phenomenon was first described by Randall et al. (1972), and later characterized by Wood and Randall (1973a; 1973b; 1973c) in a series of papers that looked at sodium and water balance in response to exercise in the model teleost rainbow trout (Oncorhynchus mykiss).

In salmonids, exercise is associated with a decrease in the vascular resistance of the gills, aiding increased blood flow (Stevens and Randall, 1967). Catecholamines released into the bloodstream dilate the branchial circulation and increase blood flow through the gills in order to increase the rate of gas transfer between blood and water. These vascular changes result in an increase in the functional surface area of the gills, enhancing the area available for gas exchange (Booth, 1979). Randall et al. (1972) tested the hypothesis that this enhanced gas transfer would also increase ion diffusion, by monitoring Na$^+$ fluxes in the freshwater rainbow trout after exercise. In accordance with their hypothesis, exercise increased the rate of Na$^+$ loss, and while catecholamine stimulation also increased Na$^+$ efflux, it was only about half of the efflux observed during exercise. Rainbow trout that were actively chased showed an increase in both ventilation and perfusion of the gills, and an impressive 70% increase in
Na\(^+\) loss, resulting in a negative Na\(^+\) balance (Wood and Randall, 1973c). These early findings from the rainbow trout have provided evidence for the typical nature of the “classic” osmorespiratory compromise.

In a later study, Gonzalez and McDonald (1992) simultaneously measured oxygen consumption rate (\(\dot{M}O_2\)) and efflux of Na\(^+\) in the model teleost rainbow trout, and were able to estimate the amount of ions lost to the water per unit of oxygen consumed. This proxy, the ion-gas ratio (IGR), was used as a practical measure of the effects of the osmorespiratory compromise. In fact, when rainbow trout were forced to swim, the IGR ratio increased acutely, suggesting a greater loss of ions relative to the amount of oxygen that was acquired, but returned to approximately routine levels with continued exercise (Gonzalez and McDonald, 1992). The IGR was also altered when animals were injected intraperitonially with epinephrine. Since the factors that increase functional surface area should also affect ion diffusion, the acute increase in IGR was attributed to an increase in the gill permeability to ions. External Na\(^+\) concentration did not affect ion loss, indicating that carrier mediated exchange diffusion and leaky pumps were not involved in the increased gill permeability (Gonzalez and McDonald, 1992). These workers concluded that most of the diffusive Na\(^+\) loss occurs via the paracellular pathway at the branchial surface, and could be exacerbated with increased blood pressure, which could disrupt the tight junctions, thereby resulting in an increase in ion loss (McDonald et al., 1989; Gonzalez and McDonald, 1992; Gonzalez and McDonald, 1994). More recently, using the paracellular marker polyethylene glycol (MW 4000), Robertson and Wood (2014) have confirmed the earlier findings of Gonzalez and McDonald (1992), demonstrating that paracellular permeability increased in parallel with \(\dot{M}O_2\) in trout subjected to sustained exercise. In contrast to all the studies looking at the effects of the osmorespiratory compromise on ion exchange, evidence for effects on water exchange are far less extensive. However, in three studies on freshwater rainbow trout, water fluxes were positively correlated with increases in oxygen consumption when fish were exercised (Wood and Randall, 1973b; Hofmann and Butler, 1979; Onukwufor and Wood, 2018).

Though the osmorespiratory compromise has been well studied in response to exercise, surprisingly few studies have investigated this trade-off in the face of hypoxia.
Rainbow trout exposed to hypoxia showed marked increases in gill permeability to ions, where \( \text{Na}^+ \) and \( \text{K}^+ \) losses to the water nearly doubled (Iftikar et al., 2010) and diffusive water fluxes increased markedly (Onukwufo and Wood, 2018). Additionally, using scanning electron microscopy, Iftikar et al. (2010) and Matey et al. (2011) identified in hypoxia-exposed trout a retraction of the PVCs which largely cover the ionocytes, thereby increasing the ionocyte area that is exposed to the water. This appears to provide a morphological explanation for the increased ionic losses seen in hypoxia-challenged salmonids (Iftikar et al., 2010; Matey et al., 2011; Robertson et al., 2015a). Increased ion and water fluxes at a time of increase oxygen permeability of the gills represents the “classic” osmorespiratory compromise (Fig. 1.1).

The Amazonian oscar (Astronotus ocellatus) is an extremely hypoxia-tolerant fish that can tolerate complete anoxia for up to 6 h and severe hypoxia for 1–2 days (Almeida-Val and Hochachka, 1995). The oscar shows a very different osmorespiratory compromise response than the highly athletic, hypoxia-intolerant rainbow trout, by decreasing branchial ion and water fluxes during hypoxia (Wood et al., 2007a; Wood et al., 2009; De Boeck et al., 2013; Robertson et al., 2015a). Notably, there is a decrease in ion and water permeability, which occurs even though there is an increase in ventilation and effective oxygen permeability at the gills, as measured by the oxygen transfer factor, an index of gill oxygen diffusion capacity (Scott et al., 2008a). This decrease in ion and water permeability is associated with a reduction in the exposed ionocyte surface area through pavement cell expansion and migration (Wood et al., 2009; Matey et al., 2011), markedly reducing ionocyte surface area and preventing ion loss without compromising oxygen uptake, a response opposite to the one seen for the hypoxia-intolerant, rainbow trout. Interestingly, again in contrast to the trout, the oscar exhibited a very low and unchanged gill paracellular permeability during exercise (Robertson et al., 2015b). The tambaqui (Colossoma macropomum) is another hypoxia-tolerant Amazonian species that has also been shown to decrease gill ion permeability when exposed to hypoxia (Robertson et al., 2015a). In summary, the oscar and tambaqui appear to show a “novel” osmorespiratory compromise
response, at least during hypoxia, where ion and water permeability can actually decrease while oxygen permeability increases (Fig. 1.1).

Rapid alterations of gill morphology can also be achieved through the reversible growth of interlamellar cell masses (ILCM) that partially or completely fill up interlamellar channels (Nilsson, 2007; Gilmour and Perry, 2018). Increases in the rate of apoptosis followed by a decrease in mitotic rate were proposed as the mechanisms responsible for the regression of the ICLM in Carassius carassius within a few hours of exposure to hypoxia (Sollid et al., 2003). Recently, Perry et al. (2012) has shown that the ILCM can retract after as little as 30 min of hypoxia exposure. Reversible gill remodeling has now been shown in more than 10 species (Gilmour and Perry, 2018) and it seems to be triggered by a variety of environmental factors such as hypoxia, temperature (Mitrovic and Perry, 2009; Barnes et al., 2014; McBryan et al., 2016), salinity (Blair et al., 2016; Gibbons et al., 2018), water contaminants (Blewett et al., 2017) and air exposure (Ong et al., 2007; LeBlanc et al., 2010).

The presence of an ILCM and the reduction in the functional surface area of the gill is predicted to lower the energetic cost of maintaining salt and water balance, by reducing passive ion and water fluxes at times of low O2 demand (Gilmour and Perry, 2018). Additionally, acclimation to soft water and to ion-poor waters promotes the proliferation of ionocytes and also, the redistribution of this cell type from the base of the lamellae to the actual lamellar bodies themselves (Perry, 1998). Since the rate at which gases diffuse across an epithelium is inversely related to the thickness of the water-to-blood diffusion barrier, ionocyte proliferation that causes thickening of the lamellar epithelium (Bindon et al., 1994a; Bindon et al., 1994b; Greco et al., 1996) could potentially impair gas transfer at the gills (Perry, 1998). Thomas et al. (1988) showed that trout acclimated to ion-poor waters dramatically increased ionocyte coverage of the lamellae, a strategy thought to improve whole-body ionic balance. In regular normoxic conditions, these trout exhibited a significant reduction of arterial PO2, and in the face of a hypoxia exposure, the oxygen extraction from the environment was impaired leading to a lower hypoxia tolerance in comparison with fish acclimated to regular ionic strength waters (Thomas et al. 1988). Similarly, thicker gills and lower respiratory surface area have been shown to impair hypoxia tolerance in a sculpin (Cottus asper), albeit conferring higher tolerance to freshwater exposure (Henriksson et al.,
2008). Thus not only can effective oxygen permeability affect osmoregulation, ionoregulatory needs can affect oxygen permeability, which represents the other side of the osmorespiratory compromise.

To date, the osmorepiratory compromise has been relatively well characterized in freshwater conditions but fewer studies have examined this phenomenon in sea water, likely due to the technical difficulties of measuring ion fluxes in saline waters. Because teleost fishes are hypo-osmotic to sea water, marine fishes would face osmotic water loss and diffusive ion gain across the gills during hypoxia or exercise. Stevens (1972) demonstrated that tilapia (Tilapia mossambica), acclimated to fresh water gained body weight after exercise, while those acclimated to sea water lost body water after exercise, likely due to increased osmotic movement of water. In salmonids, exercise in sea water has been linked to a disturbance in plasma Na\(^+\) and muscle water content (Randall and Brauner, 1991; Brauner et al., 1992; Nendick et al., 2011).

Recently, Zimmer and Wood (2014) showed that the osmorespiratory compromise also occurs in an elasmobranch (an osmoconformer that is not substantially hypo-osmotic to sea water), where urea loss rates increased in hypoxia-exposed dogfish (Squalus acanthias suckleyi). There have been many studies that have investigated the cost of osmoregulation through comparisons between resting and exercised fish at multiple salinities including their isosmotic point. For example, Febry and Lutz (1987) found that maintenance \(\text{MO}_2\) was highest in seawater-acclimated hybrid tilapia (Oreochromis mossambicus hornorum) in comparison to freshwater animals, and that the lowest \(\text{MO}_2\) (maintenance and swimming costs) were exhibited by fish acclimated to the isosmotic salinity. This lends support for the idea that the cost of ion/osmoregulation is lowest at the isosmotic salinity (Rao, 1968).

However, I am not aware of any studies that have directly quantified ion fluxes and/or water movements in seawater species in isosmotic conditions. Theoretically, no movement of ions and consequently water should occur at isosmotic salinities, therefore, fish should not experience an osmorespiratory compromise. Additionally, in hypoxia where oxygen acquisition and ATP production are challenging, isosmotic conditions would offer a metabolic advantage since less energy would be allocated to ion and osmoregulation.
However, no empirical studies have shown that and one of the goals of this thesis is to fill in this gap.

1.7 Study organisms and their environment

As one of the main objectives of the present thesis was to explore the gaps in our understanding of the osmorespiratory compromise, I have used three very different species as my experimental models: the euryhaline teleost *Fundulus heteroclitus* (“Atlantic killifish”), the largely stenohaline myxine *Eptatretus stoutii* (“Pacific hagfish”), and the largely stenohaline marine elasmobranch *Squalus acanthias suckleyi* (“Pacific spiny dogfish”).

The osmorespiratory compromise is as much of a constraint to marine fish as it is to freshwater fish, but as reviewed above, most of the information on the osmorespiratory compromise has been generated using freshwater teleost fish species, while very little has been done in seawater fishes, and virtually nothing in marine elasmobranchs or myxines. Additionally, to date, there have been no direct investigations of this phenomenon under isosmotic conditions. Details of the iono and osmoregulatory strategies in these different environments will be presented below on the model species chosen for this study.

1.7.1 The Atlantic killifish (*Fundulus heteroclitus*)

The Atlantic killifish, *Fundulus heteroclitus*, is a teleost that inhabits brackish water estuaries and salt marshes from northeastern Florida to the Gulf of St. Lawrence region, occasionally entering freshwater streams and rivers (Halpin, 2000; McMahon et al., 2005; Haas et al., 2009). Due to the usually shallow depths, salinity in estuaries varies daily and seasonally due to tidal regimes as well as due to varying river discharge, precipitation and evaporation rates. These environments can quickly become hypoxic due to stratification of the water column, and to high respiration rates of algae, macrophytes, and benthic microorganisms which deplete the oxygen stores produced by photosynthesis. *F. heteroclitus* is well known for its outstanding capacity for euryhalinity, and as such, has been used as a model organism (Burnett et al., 2007) for understanding a variety of iono- and osmo-
regulatory mechanisms in teleost fish, such as gill ionocyte structure and function (Wood and Marshall, 1994), acid-base regulation (Patrick et al., 1997; Patrick and Wood, 1999) and the role of the gut in osmoregulation (Marshall and Grosell, 2006; Scott et al., 2008b; Wood et al., 2010).

During acclimation to sea water, killifish rapidly increase active NaCl excretion at the gills (Marshall et al., 1999). Influx and efflux rates of both Na\(^+\) and Cl\(^-\) ions increased 5-6 fold when animals were transferred from 10% to 100% sea water, achieving stability after approximately 30 min and remaining stable for 7+ days (Wood and Laurent, 2003; Wood, 2011). Na\(^+\)/K\(^+\)-ATPase \(\alpha_{1a}\) gene expression increased 2-3 fold in killifish after 24 hours of transfer to sea water, and that result correlated with the increase in the enzyme activity in the same time frame (Scott and Schulte, 2005). *Fundulus heteroclitus* when acclimated to sea water are similar to most marine fishes in having a gill diffusive permeability which is cation-selective, i.e. higher permeability to cations than anions (Wood and Grosell, 2008). This characteristic supports the well-accepted model proposed by Silva et al. (1977) where the secondary active Cl\(^-\) excretion pathway energizes the passive efflux of Na\(^+\) via the paracellular pathway (intercellular space between an MRC and an accessory cell in gills of seawater-adapted teleosts). It has been shown that under seawater conditions, the gill and skin lining the opercular epithelium have similar transport characteristics (Wood and Laurent, 2003; Scott et al., 2008b), even though they differ in function in freshwater (Wood and Marshall, 1994; Patrick et al., 1997).

Within a few hours of transfer from brackish water (10% SW) to fresh water, influx and efflux rates of Na\(^+\) decreased almost 10-fold, Cl\(^-\) efflux rate was similarly decreased, while Cl\(^-\) influx was essentially abolished (Wood and Laurent, 2003; Wood, 2011). Over the same time frame, there was an apparent disappearance of the apical crypts of the ionocytes, suggesting that there is a coordinated expansion of PVCs in order to cover pit openings, and change the gill epithelium morphology from that typical of SW-acclimation to that typical of freshwater acclimation (Laurent et al., 2006). A similar mechanism has been described to explain the difference in tolerance to freshwater exposures between two different populations of *F. heteroclitus* (Scott et al., 2004a). It has also been proposed that killifish acclimated to fresh water possess a different type of ionocyte, which was termed “cuboidal” cell given its
cubical, or wedge-shape morphology (Laurent et al., 2006). Other examples of changes in gill morphology following transfer to fresh water include the deepening of tight junctions and disappearance of accessory cells (Marshall et al., 1999; Katoh et al., 2001; Katoh and Kaneko, 2003; Laurent et al., 2006) and increases in apoptosis and mitotic activity in the gill epithelium (Laurent et al., 2006). Gene and protein expression data (Scott, 2005; Edwards et al., 2005), together with pharmacological data (Brix et al., 2018) suggest that the uptake of Na\(^{+}\) from the environment occurs via an apical Na\(^{+}\)/H\(^{+}\) exchanger (NHE-2) in freshwater-adapted killifish. Cl\(^{-}\) uptake appears to occur exclusively from the diet in freshwater killifish (Wood et al., 2010; Bucking et al., 2013).

Killifish are also exceptionally tolerant to low environmental oxygen (Cochran and Burnett, 1996; Richards et al., 2008; McBryan et al., 2016), and therefore serve as a model species for hypoxia studies (Burnett et al., 2007). When exposed to chronic hypoxia, *F. heteroclitus* improves O\(_2\) uptake from the environment by increasing the O\(_2\) carrying capacity of the blood, due to increases in hematocrit and haemoglobin O\(_2\) affinity (Greaney and Powers, 1978; Wells, 2009), which is allosterically regulated by a decrease in red blood cell ATP and/or GTP content (Greaney and Powers, 1978). One of the other mechanisms involved in survival during acute severe hypoxia is a transient activation of substrate-level phosphorylation in muscle (Richards et al., 2008), as shown by increased lactate concentrations. Killifish are able to increase hypoxia tolerance as a result of acclimation to both constant and intermittent hypoxia, as evidenced by the suppression of resting metabolic rate and Pcrit, even though the mechanisms underlying such adaptations appears to differ between constant and intermittent hypoxia (Borowiec et al., 2015). In addition, acclimation to warmer temperatures increased hypoxia tolerance in *F. heteroclitus* likely to due a remodelling of the gill ILCM, which elevated the gill oxygen transfer factor (McBryan et al., 2016).

In summary, due to its exceptional euryhalinity and hypoxia tolerance, Atlantic killifish is an excellent model in which to study the physiological responses to hypoxia and salinity, and the responses to the physiological trade-offs posed by gill structure and function.
1.7.2 The Pacific hagfish (*Eptatretus stoutii*)

Hagfishes (family: Myxinidae) are strictly marine, jawless fishes that are extant representatives of the most basal craniates (Kardong, 2012), which are thought to share a common ancestry with all other fishes (Janvier, 1999; Wright, 2007). Pacific hagfish (*Eptatretus stoutii*) are commonly caught at depths of ~200 m, and while precise geographic distribution data are still lacking, hagfish have been caught off the Pacific coast of Canada and down as far as northern Mexico (Barss, 1993), often associated with soft muddy substrates (McInerney and Evans, 1970). Hagfishes are scavengers that feed on decaying carrion and often burrow into the coelomic cavities of the rotting carcasses (Axelsson et al., 1990; Forster et al., 1992; Clifford et al., 2015), where they can be exposed through periods of time to severe hypoxia or even anoxia. At present, Pacific hagfish populations are under pressure from increased commercial fishing for live food markets and leather-making in Asia (Barss, 1993; Grant, 2006).

Hagfishes exhibit very low oxygen consumption rates. For example, Pacific hagfish acclimated to 10°C showed an $\dot{M}O_2$ of about 350 μmol/kg/h (Munz and Morris, 1965), which is only about 10-20% of that in a comparably sized teleost fish at similar temperatures (Clarke and Johnston, 1999). Since they possess a high surface area to volume ratio and an extensive dermal capillary network, it was formerly thought that a substantial portion of the oxygen uptake of the animal occurred cutaneously (Steffensen et al., 1984; Lesser et al., 1997). However, Clifford et al. (2016) using a divided respirometric chamber, have shown that in both resting and post-exercise conditions, the gills contribute the vast majority of the animals’ $\dot{M}O_2$. Pacific hagfish have an incredible capacity to tolerate hypercarbia, mostly by modulating their plasma HCO$_3^-$ concentration through the exchange of Cl$^-$ ions (Baker et al., 2015), with evidence that the skin might play an important role in acid-base regulation (Clifford et al., 2014). Additionally, these animals possess an extreme tolerance to high external and internal ammonia (Clifford et al., 2015), anoxia (Cox et al., 2010) and hypercarbia (Baker et al., 2015) which again, might be related to their life style and feeding behaviours.
In the wild, hagfish can be routinely exposed to hypoxia, either due to low oxygen concentration of deep ocean waters, anoxic sediments, or due to their feeding behaviour (as mentioned above). Pacific hagfish are fairly tolerant to hypoxia, exhibiting periods of spontaneous apnea under normoxia (Eom and Wood, 2018) and are even able to sustain cardiac performance up to 70% of normoxic values during complete anoxia (Cox et al., 2010). Further adaptations to hypoxia include the ability to maintain oxygen uptake until very low PO$_2$ (Drazen et al., 2011) and rapid suppression of MO$_2$ (Cox et al., 2011). When exposed to hypoxia, hagfish are reported to increase ventilation volume by increasing only breathing frequency (Perry et al., 2009b), contrary to the characteristic increase in ventilation amplitude (i.e. ventilatory stroke volume) seen in most teleost fishes.

For a long time, hagfishes have been considered the only extant craniates that are both osmo- and ionoconformers, with their plasma [Na$^+$] and [Cl$^-$] concentrations closely resembling that of sea water (Smith, 1932; Robertson, 1976). While plasma osmolality has been shown to be symmetrically linked to water osmolality in many studies (e.g. Bellamy and Chester Jones, 1961; Sardella et al., 2009), several recent investigations have suggested that there may be a small offset between plasma and seawater levels of Na$^+$ and Cl$^-$, while divalent cations, such as plasma Ca$^{2+}$ and Mg$^{2+}$, are kept well below seawater concentrations (Sardella et al., 2009; Clifford et al., 2015). However, it is not yet known whether this is an actively regulated process. Evans (1984) has suggested that Atlantic hagfish (Myxine glutinosa) possesses both Na$^+$/H$^+$ and Cl$^-$/HCO$_3^-$ exchangers on the gill epithelium, functioning primarily for acid-base regulation. One of the many advantages of being an osmoconformer is the elimination of the need for drinking. Recently, using radioisotopic techniques, Glover et al. (2017) have confirmed that Pacific hagfish do not drink seawater in order to maintain hydromineral balance. They possess one of the highest water exchange rates ever reported for any fish (Rudy and Wagner, 1970; Glover et al., 2017), which is a trait likely linked to the primitive characteristics of these fishes, and a reflection of their osmoconforming strategy. The contribution of the skin to the high water permeability of Pacific hagfish remains to be elucidated.
In summary, as Pacific hagfish occupy an important research niche that has received little attention, and possess unique osmoregulatory strategies, they have been chosen as one of the model organisms for this study.

1.7.3 The Pacific dogfish (*Squalus acanthias suckleyi*)

The Pacific spiny dogfish (*Squalus acanthias suckleyi*) is a demersal shark found on the continental shelf, from the intertidal zone to the shelf slope, ranging from the Bering Sea to Baja California. Waters off British Columbia comprise a large portion of the core range of spiny dogfish in the northeast Pacific with high population density in the Strait of Georgia and the west coast of Vancouver Island. *S. acanthias* was chosen as our elasmobranch experimental model due to the large body of available information on the basic physiology of this species. The Pacific spiny dogfish is a migratory, long-lived, very important demersal resource that is under great threat due to overfishing and global climate change (Taylor and Gallucci, 2009).

The Pacific spiny dogfish, like most seawater elasmobranchs is an osmoconformer that has an internal osmolality equal to or greater than that of the surrounding seawater, thereby promoting the osmotic influx of water and eliminating the need for drinking (Smith, 1931; Smith, 1936). Water permeability at the elasmobranch gill is relatively high, given the usually low osmotic gradient from seawater to plasma (10 – 70 mOsm/L) and the need to take up water to form urine and rectal gland secretions (Wright and Wood, 2016). High internal osmolality is achieved through the synthesis and retention of high levels of organic osmolytes. The major organic osmolyte retained in tissues and plasma is urea (300 – 400 mmol/L), supplemented by lower concentrations of counteracting methylamines such as trimethylamine N-oxide (TMAO) which stabilize protein function (see Yancey, 1916). This osmoregulatory strategy, like ionoregulation in hyporegulating seawater teleosts, is energetically expensive. Urea is synthesized from ammonia through the ornithine-urea cycle (OUC), at the expense of 5 moles of ATP per mole of urea produced (Ballantyne, 1997). Therefore, in order to retain urea in the body fluids, urea permeability at the gills is kept low (Fines et al., 2001). The apical cell membrane surface area is very low relative to the
basolateral cell membrane surface area, and apical membrane permeability to urea is even lower than basolateral membrane permeability (Pärt et al., 1998; Hill et al., 2004). Although no urea transporting protein has ever been isolated in the elasmobranch gill (Wright and Wood, 2016), there is physiological evidence for an active urea back-transporter located basolaterally (Fines et al., 2001) and/or apically (Wood et al., 2013).

Hypoxia tolerance in elasmobranchs appears to be linked to the capacity for oxygen uptake and transport at low oxygen concentrations (Speers-Roesch et al., 2012). Hypoxia-exposed spiny dogfish showed marked increases in ventilation amplitude and frequency, while arterial partial pressure of O\textsubscript{2} decreased (Perry and Gilmour, 1996). Zimmer and Wood (2014) found that when spiny dogfish were exposed to hypoxia, urea-N loss rates at the gills increased substantially, likely due to increases in functional surface area and perhaps an inhibition of the urea retention mechanism (Fines et al., 2001). Using an artificially perfused whole animal preparation, that included suspending the fish’s head in air while the body remained submerged, Boylan (1967) exposed dogfish gills to water ranging from 2 to 20\degree\text{C}. He found that branchial urea-N excretion sharply increased when the water temperature reached 15 \degree\text{C}, suggesting the breakdown of some urea-N conservation mechanism. Despite the atypical methods used in that study, Boylan (1967) was the first to report a disruption of N-osmolyte retention in an elasmobranch species in relation to temperature.

In summary, as Pacific spiny dogfish are osmoconformers and ionoregulators, they make for great experimental models to study the osmorespiratory compromise in a species that differ in osmoregulatory strategy from that of teleost. Additionally, the stenohalinity characteristics of this species provides a contrasting feature to my teleost model.

1.8 Thesis objectives and chapter summaries

From the above background, it is clear that the osmorespiratory compromise has been well characterized in the face of exercise in freshwater teleost fish species, but much less is known about the osmorespiratory compromise during hypoxia, or at other salinities, or in other types of fish with different iono-/osmo-regulatory strategies. Therefore, the ultimate goal of the present thesis was to expand the range of species in which this phenomenon is
studied, while providing a mechanistic understanding of the osmorespiratory compromise under different environmental stressors in three very different groups of fishes with differing osmoregulatory strategies.

1.8.1 Summary of Chapter 2 – The respiratory responses to salinity in *Fundulus heteroclitus*

The goal of Chapter 2 was to determine if salinity acclimation affects whole-animal respiratory capacity and if that translates into differences in hypoxia tolerance in a euryhaline teleost. The overall hypothesis was that if acclimation to an isosmotic salinity confers a metabolic advantage through lower osmoregulatory costs, then hypoxia tolerance would be the greatest at this salinity. I also hypothesized that fish acclimated to both fresh- and seawater would have a lower permeability of the gills to ions in order to minimize diffusive ion gain/loss and improve active ion transport at the gill, and that this would result in a reduction in hypoxia tolerance. My first hypothesis was partially supported: there was a large increase in hypoxia tolerance between 0 and 11 ppt (isosmotic) acclimated fish, but no changes occurred between 11 and 35 ppt. My data also showed that acclimation to the isosmotic salinity (11 ppt) conferred the greatest capacity to regulate $\dot{M}O_2$ under progressive hypoxia, as indicated by three respiratory indices, followed by acclimation to 35 ppt. Furthermore, 0 ppt-acclimated fish, which showed the lowest tolerance to hypoxia, possessed the lowest lamellar surface area and highest interlamellar cell mass, thereby reducing the effective surface area for gas exchange. Additionally, they exhibited the highest ventilatory effort in order to maintain adequate oxygen uptake at the gills in comparison to other salinities. Small differences in blood $O_2$ transport characteristics were seen between salinities, particularly during hypoxia, and may also have contributed to the differences in hypoxia tolerance observed between salinities. In conclusion, many differences in the $O_2$ transport cascade were found between killifish acclimated to hypo-, iso- and hyper-osmotic salinities, in apparent association with the osmorespiratory compromise, and that of the three acclimation salinities tested, 11 ppt was the most advantageous, and 0 ppt was the most challenging for respiratory gas exchange.
1.8.2 Summary of Chapter 3 – The ionoregulatory responses to hypoxia in *Fundulus heteroclitus*

The goal of Chapter 3 was to examine the ionoregulatory aspects of the osmoregulatory compromise during hypoxia in killifish acclimated to fresh water, sea water, and to the isosmotic salinity. The hypothesis was that as a hypoxia-tolerant species, killifish would reduce gill permeability to ions and water in both fresh water and sea water, similar to the hypoxia-tolerant Amazonian oscar, but different from the hypoxia-intolerant rainbow trout (Fig. 1.1). I also hypothesized that since acclimation to the isosmotic salinity would essentially eliminate ionic and osmotic gradients between the fish and the environment, the effects of the osmoregulatory compromise would be minimized, or non-existent at 11 ppt. My first hypothesis was supported, but was dependent on salinity acclimation. Freshwater-acclimated fish decreased the passive efflux rates of ions (Na\(^+\), K\(^+\) and Cl\(^-\)) as well as ammonia. But perhaps more interestingly, active Na\(^+\) influx as well as the activities of key ionoregulatory enzymes were also reduced. Diffusive water flux rate also decreased during hypoxia in freshwater-acclimated killifish, suggesting that transcellular permeability was reduced during hypoxia as part of a regulated process. In summary, in fresh water, killifish did not exhibit the classic osmoregulatory compromise in hypoxia, but instead reduced gill permeability, as seen in other hypoxia-tolerant species. In full strength sea water, both Na\(^+\) influx and efflux declined, while a non-significant decrease in paracellular permeability was also seen, in accord with our hypothesis, though other indicators did not change. I also found that in killifish acclimated to the isosmotic salinity, both Na\(^+\) influx and efflux rates increased, as well as paracellular permeability, supporting our hypothesis. Common to all salinities, plasma ion concentrations were unaffected by hypoxia exposure, indicating that despite different strategies, killifish acclimated to hypo-, hyper-, or iso-osmotic salinities are able to maintain osmoregulatory homeostasis during severe hypoxia.
1.8.3 Summary of Chapter 4 – The respiratory and osmoregulatory responses to temperature in *Eptatretus stoutii*

The goal of Chapter 4 was to determine if temperature-induced increases in whole-animal oxygen demand would disrupt ionic and osmotic homeostasis in this osmoconforming myxinid. I hypothesized that if there was a permeability trade off at the gills following increases in metabolic demand, increased fluxes of osmolytes and water would be seen. Oxygen consumption increased linearly with temperature, while ventilation and heart rate were also elevated, although with different patterns. Increases in ammonia efflux and diffusive water flux rates were also observed, lending support to our hypothesis, and showing that an osmoconformer is also prone to the osmorespiratory compromise. Temperature also caused disturbances in the plasma concentrations of divalent cations, which are known to be actively regulated, while little change was seen in acid-base status. Some, but not all of the changes seen at elevated temperature were alleviated by experimentally increasing oxygen supply via water hyperoxia. These data clearly demonstrate that even an animal considered to be an osmoconformer shows interactions between osmoregulation and respiration.

1.8.4 Summary of Chapter 5 – The respiratory and osmoregulatory responses to salinity and hypoxia in *Eptatretus stoutii*

The goal of Chapter 5 was to examine the potential osmorespiratory compromise upon exposure to hypoxia and altered salinity in the hagfish. I hypothesized that in an osmoconformer such as the hagfish, exposure to salinities higher or lower than that of the sea water to which the animals were acclimated would elicit a reduction in metabolism, so that gill permeability could be reduced and osmo/ionoregulatory status maintained. I also hypothesized that as hypoxia-tolerant animals, hagfish would decrease gill permeability when exposed to hypoxia, thus preventing the unfavourable exacerbation of fluxes that arise due to the osmorespiratory compromise. My first hypothesis was partially supported as we observed a reduction in oxygen consumption that was accompanied by decreases in ammonia efflux and diffusive water flux rates when salinity was experimentally reduced. However,
when salinity was experimentally increased, oxygen consumption and ammonia efflux were not altered, though diffusive water flux rate was again reduced. I also observed that the concentration differences between plasma and external water were directly modified with increasing salinity for Ca$^{2+}$ and Mg$^{2+}$, two cations known to be actively regulated at levels well below those of sea water. Of particular note, I also identified a similar phenomenon for Cl$^-$. Upon exposure of hagfish to hypoxia, oxygen consumption was reduced by half, and despite an increase in ventilation, diffusive water flux rates were reduced. These results indicate that despite altering conditions to enhance oxygen flux at the gills, permeability to water was selectively reduced. This is a pattern that differs from the traditional model where an increase in gill permeability to respiratory gas exchange is associated with an increased permeability to ions, water and nitrogenous compounds.

1.8.5 Summary of Chapter 6 – The respiratory and osmoregulatory responses to temperature in *Squalus acanthias suckleyi*

The goal of Chapter 6 was to determine if temperature-induced changes in whole-animal oxygen demand would disrupt gill permeability to ammonia, urea and water in this osmoconforming marine elasmobranch. I hypothesized that, as metabolic demand for oxygen increased with temperature, the fluxes of ammonia, and urea, and diffusive water exchange at the gills would increase in parallel with that of oxygen. My hypothesis was only partially supported. The fluxes of ammonia, urea and water all increased with temperature, but only the changes in ammonia fluxes displayed a step-wise increasing pattern with temperature, while urea-N fluxes were maintained virtually constant until 15ºC. Beyond this temperature, a sharp increase in urea-N loss was observed, indicating that elevated temperature could have been directly disrupting specific N-conservation mechanisms at the gills. The effect of temperature on the diffusive water fluxes was similar to that seen for $\dot{MO}_2$. In contrast to the responses observed in hagfish, experimental hyperoxia did not prevent the effects of high temperature and indeed exacerbated the effluxes of ammonia and urea-N. Overall, oxygen consumption exhibited the lowest $Q_{10}$ while ammonia and urea efflux displayed the highest, indicating a greater temperature susceptibility for loss of nitrogenous compounds in this
species, where nitrogen is used as a valuable resource not just for growth, but also as an osmolyte for osmoregulation.
Oxygen

Ions and water

Effective gill permeability

Normoxia

Hypoxia

Normoxia/Recovery

Rainbow trout

Amazonian oscar
Figure 1.1 Conceptual diagram displaying effective gill permeability to O$_2$ (top line) versus the two very different accompanying changes (two lower lines) in effective gill permeability to ions and water seen in two species with very different strategies for the osmo respiratory compromise. The rainbow trout exhibits increased gill permeability to ions and water, while the Amazonian oscar shows a regulated reduction in gill permeability.
Chapter 2: The osmorespiratory compromise: physiological responses and tolerance to hypoxia exposure are affected by salinity acclimation in the euryhaline killifish (*Fundulus heteroclitus*)

2.1 Summary

The natural habitat of *F. heteroclitus* comprises estuaries and salt marshes where salinity and oxygen levels may vary greatly. The characteristics of the fish gill that maximize gas exchange are the same as those that promote diffusion of ions and water to and from the environment, therefore, physiological trade-offs are likely to occur. Here we used an integrative approach to investigate how salinity acclimation affects whole animal respiratory gas-exchange, particularly during hypoxia. Killifish were acclimated to 0 ppt (fresh water), 11 ppt (the isosmotic salinity), and 35 ppt (full strength sea water). Salinity acclimation had marked effects on hypoxia tolerance (measured as time to loss of equilibrium), where fish acclimated to 11 ppt and 35 ppt showed much greater ability to withstand hypoxia than 0 ppt fish. Killifish acclimated to 11 ppt exhibited the greatest capacity to regulate $\dot{M}O_2$ under progressive hypoxia, as measured through Michaelis-Menten analysis to yield oxygen affinity ($K_m$), critical oxygen tension ($P_{crit}$) and Regulation Index (RI). Killifish acclimated to 35 ppt exhibited a higher routine metabolic rate but a lower capacity to regulate $O_2$ uptake under hypoxia than the 11 ppt fish, but no substantial differences in gill morphology, ventilation, or blood $O_2$ transport. In contrast, the 0 ppt acclimated fish had the highest ventilation and lowest oxygen extraction efficiency in normoxia and hypoxia, pointing to a higher ventilatory workload in order to maintain similar levels of $\dot{M}O_2$. These differences were related to the alterations in gill morphology, where 0 ppt acclimated fish had the lowest lamellar surface area with greatest epithelial cell coverage (i.e. thicker lamellae, longer diffusion distance) and a larger interlamellae cell mass (ILCM), contrasting to 11 ppt acclimated fish that showed the highest lamellar surface area and thinnest lamellae. Salinity acclimation also affected whole blood $O_2$ transport properties, as measured by the $O_2$ affinity ($P_{50}$) and hematological parameters. Our data provides evidence for a compromise between salinity acclimation and hypoxia tolerance in killifish acclimated to fresh water. The
alteration of an array of physiological parameters indicates acclimation to fresh water leads to an impaired capacity to deal with hypoxia. Of the three acclimation salinities tested, 11 ppt was the most advantageous, and 0 ppt was the most challenging for respiratory gas exchange in this species.

2.2 Introduction

Diel cycles of aquatic respiration and photosynthesis are commonly associated with intermittent hypoxia in estuarine and coastal areas (Diaz and Breitburg, 2009), and in recent years, the occurrence and severity of aquatic hypoxia have greatly increased due to a variety of anthropogenic causes, including pollution, eutrophication and overall global climate change (Ficke et al., 2007; Diaz and Breitburg, 2011). The major threat posed by hypoxia is the potential mismatch between oxygen demand and supply within the organism. Fish facing hypoxia will often employ mechanisms to either increase O$_2$ uptake from the water, or to reduce utilization by depression of metabolic demand (Hochachka et al., 1996). Branchial oxygen uptake can be improved by increasing gill ventilation and perfusion (Perry et al., 2009a). Additionally, fish can also enhance gill oxygen transfer through increases in the functional respiratory surface area of the gill by redirecting blood flow from marginal circulatory shunts to central channels within the lamellae (Nilsson et al., 1995; Sundin and Nilsson, 1998). The blood O$_2$ carrying capacity can also be modified through increases in hematocrit and hemoglobin content, and hemoglobin oxygen affinity can be modified through changes in the concentration of allosteric modifiers (Val, 2000). A more recently discussed strategy is the rapid reversible alteration of gill morphology (Nilsson, 2007), where fish can quickly remove the interlamellar cell mass (ILCM) that in some species fills the channels between the lamellae, thereby decreasing the mean distance for diffusion between blood and water.

Since the gills are the primary sites for both ion and respiratory gas exchange, trade-offs between the optimal structures for these physiological processes occur, which has been called the osmorespiratory compromise (Randall et al., 1972; Gonzalez and McDonald, 1992), where the conflict between the need for high gill permeability for respiratory gas
exchange and the need for low gill permeability to limit ion diffusion has the potential for physiological impairment. Movement between salinities that range from freshwater to seawater poses a significant osmoregulatory challenge for euryhaline fishes, one which may have respiratory consequences. This challenge involves the transformation of the gill epithelium from a salt-absorbing surface in fresh water to a salt-secreting surface in seawater. This transformation is characterized by remarkable changes in ion transporter density (Katoh and Kaneko, 2003) and the morphology of the ion-transporting cells (ionocytes) in addition to their distribution (Laurent et al., 2006). This modulation of gill morphology may result in increased barrier thickness in order to facilitate favourable active ion transport and limit unfavourable passive ion diffusion. However, at the same time it is thought to be detrimental to whole animal respiratory capacity, because the blood-to-water diffusion distance for $O_2$ is also increased. This has been shown in several studies with freshwater fishes (Thomas et al., 1988; Greco et al., 1996; Perry, 1998; Sollid, 2003; Henriksson et al., 2008). For example, an investigation comparing two species of sculpin showed that the species with the higher freshwater tolerance had a greater diffusion distance and lower surface area, resulting in a compromised tolerance to hypoxia (Henriksson et al., 2008).

The overall goal of the present study was to use an integrative approach to investigate how salinity acclimation affects whole animal respiratory gas-exchange at multiple levels; in a parallel study we have investigated how acclimation to comparable conditions affects ionoregulatory performance (Giacomin et al., Chapter 3). To this end, we chose the Atlantic killifish *Fundulus heteroclitus* as our model. *F. heteroclitus* are native to estuaries and salt marshes from northeastern Florida to the Gulf of St. Lawrence, Canada, occasionally entering freshwater streams and rivers (Taylor et al., 1979). This species is well known for its remarkable euryhalinity, being able to adjust its physiology within hours of transfer from environments ranging from fresh water to much greater than full strength sea water (Griffith, 1974). As such, it has become a model for understanding ionoregulatory changes associated with salinity challenges (Wood and Marshall, 1994; Wood and Laurent, 2003; Marshall and Grosell, 2006; Marshall, 2013). Killifish are also exceptionally tolerant of hypoxia, for which they also serve as a model (Cochran and Burnett, 1996; Richards et al., 2008; McBryan et al., 2016). Despite their well-accepted status as model organisms in both fields (Burnett et al.,
2007), there are surprisingly few studies that have examined the interactive effects of respiratory gas exchange and ionoregulation under conditions of hypoxia and salinity variation (Blewett et al., 2013; Wood and Grosell, 2015). Therefore, an additional objective was to fill in this gap.

The salinity at which the internal osmotic pressure of the fish is the same as that of the water is known as the isosmotic point, where no net movement of ions and water from the plasma to the environment and vice versa should occur. Theory predicts that the energetic cost of osmoregulation should be the lowest in an isosmotic environment (Boeuf and Payan, 2001), since the ionic gradients between blood and water would be minimal. Thus the energy allocated for iono- and osmo-regulation should be negligible, allowing for more energy availability during low oxygen exposure. Additionally, as the need for active iono- and osmo-regulation should be minimized, the performance of the respiratory system for O\textsubscript{2} uptake should be maximized. Furthermore, due to the effects of the osmoresporatory compromise stated above, acclimation to other salinities should elicit changes in gill morphology with the potential to impair whole animal respiratory capacity and as a consequence, the ability to deal with hypoxia.

Thus, our first hypothesis was that acclimation to the isosmotic salinity (approximately 11 ppt for \textit{F. heteroclitus}) would result in the greatest hypoxia tolerance, with lower tolerances in fresh water and full strength sea water, in addition to the greatest capacity to regulate oxygen consumption under progressive hypoxia. Furthermore, we hypothesized that if fish acclimated to 0 ppt (fresh water) or 35 ppt (sea water) have a lower permeability of the respiratory surface in order to favor ionic homeostasis and improve active ion transport at the gill, then their hypoxia tolerance would be compromised. Additionally, we hypothesized that such differences would be reflected in their respiratory and ventilatory responses to hypoxia, in their blood O\textsubscript{2} transport characteristics, and in their gill morphology. Our approach involved evaluating hypoxia tolerance through loss of equilibrium (LOE) tests, assessing respiratory capacity through whole-animal respirometry, recording ventilatory dynamics, and determining whole blood O\textsubscript{2} dissociation curves in both normoxia and hypoxia, all as a function of acclimation salinity (0, 11, or 35 ppt). Several indices of gill
morphology (total surface area, interlamellar cell mass, and epithelial cell coverage of the lamellae as a proxy for diffusion distance) were also measured.

2.3 Material and Methods

2.3.1 Fish acclimation

*Fundulus heteroclitus macrolepidotus* (northern subspecies) were collected by Aquatic Research Organisms (Inc.) near Hampton, NH, USA (42°54′46″N) and shipped overnight to the University of British Columbia where they were held in groups of ~25 fish per 120-L tank at 18°C, under a photoperiod of 12-h light and 12-h dark. Salinity was achieved by mixing Instant Ocean Aquarium Salt (Instant Ocean, Spectrum Brands, Blacksburg, VA, USA) with Vancouver dechlorinated tap water, and was monitored with a conductivity meter (Cond 3310, WTW, Xylem Analytics, Weilheim, Germany). Fish were fed daily to satiation with commercial fish flakes (Nutrafin Max Tropical Flakes, Mansfield, MA, USA). All fish were acclimated for a minimum of 4 weeks at each salinity, and fasted for 24 h prior to all experimentation. Experiments were performed at the acclimation salinity, at 18 - 20°C. All experiments were conducted following the guidelines of the Canada Council for Animal Care, under approval of the animal care committee at the University of British Columbia (AUP A14-0251).

2.3.2 Time to loss of equilibrium in hypoxia

Separate groups of fish (n = 8 – 10 per salinity; mean body mass = 3.80 ± 0.22 g) were used for this experimental series, each acclimated to a different salinity (0, 3, 11, 15 and 35 ppt). Time to loss of equilibrium (LOE) in hypoxia was determined simultaneously for the five different salinities. Fish were placed in individual plastic chambers, where two of the sides had been replaced with a fine plastic mesh allowing the water to flow freely inside. The chambers were weighted down, so that the whole chamber was submerged, preventing killifish from performing aquatic surface respiration. A stick was fitted on the lid of each
chamber, allowing chamber manipulation without disturbing the fish inside. Chambers were placed in 30-L Plexiglas tanks, one for each salinity. Each 30-L tank contained a small submersible recirculating water pump and an air-stone. Fish were placed in the set-up overnight, and prior to the beginning of the trial, 50% of the water was replaced by gentle syphoning. Oxygen was reduced from normoxia (155 Torr) (note 1 Torr = 0.13332 kPa) to severe hypoxia (3.5 Torr) over approximately 1h, and maintained at this level by bubbling the tanks with either compressed N₂ or air. Water PO₂ was monitored throughout the whole duration of the trials using the same handheld oxygen meter (Accumet AP84A, Fisher Scientific, Toronto, ON, Canada), calibrated identically and checked repeatedly. Time to LOE was defined as the time (in hours) after the water reached 3.5 Torr until the fish lost equilibrium. LOE was determined as the point when a fish had settled at the bottom of the tank, on its side or upright, and was no longer responding to a gentle movement of the chamber. After removal from the chambers, fish were weighed, and recovered in a separate tank.

2.3.3 Oxygen consumption rate (ṀO₂)

Separate groups of fish (n = 8 per salinity; mean body mass = 3.83 ± 0.12 g) were used for this experimental series. Oxygen consumption rates (ṀO₂, µmol O₂/g/h) under routine conditions were determined using closed-system respirometry, at 18°C, with water at the salinity to which the fish had been acclimated (0, 11 and 35 ppt). Each respirometer consisted of a rectangular 220-mL glass jar containing a small magnetic stir bar, physically separated from the fish by a piece of mesh, plus two ports, each one closed with a rubber stopper. The chambers were placed inside a water bath to maintain a constant temperature of 18°C, over a stir-plate to ensure adequate mixing inside of the chambers. Fish were placed in the respirometers overnight under flow-through conditions. At the beginning of the trial, a probe (FOXY-R, Ocean Optics Ltd, Largo, FL, USA) was inserted in each chamber, water flow was stopped, the chamber was sealed, and water PO₂ was monitored over time (sampling rate every 20s), until PO₂ reached ~ 0 Torr. The chambers were rinsed daily with ethanol to eliminate any bacterial build-up. A blank (respirometer with no fish) trial was
performed for every 4 fish, and oxygen consumption was negligible. On average, trials lasted 175 ± 13 min. The average fish weight to respirometer volume ratio was 0.015 ± 0.0005 g/mL. The slope of decreasing water PO\textsubscript{2} versus time was computed every 5 min, and MO\textsubscript{2} (µmol/g/h) was calculated by:

\[
\text{MO}_2 = (\Delta \text{PO}_2 \times \alpha \text{O}_2 \times V) / W \quad \text{(Eq. 1)}
\]

where \(\Delta \text{PO}_2\) is the slope of PO\textsubscript{2} over time (h), \(\alpha \text{O}_2\) is the solubility constant (µmol O\textsubscript{2}/Torr/L) in water at 18°C and at the appropriate salinity (Boutilier et al., 1984), W is the body mass (g), and V is the respirometer volume (L). Data were binned at 5-Torr intervals.

The regulation index (RI) as described by Mueller and Seymour (2011), and originally conceived by Alexander and McMahon (2004), was calculated for the three salinities individually on an individual fish basis, using procedures recommended by Wood (2018). Briefly, the \(\dot{\text{MO}}_2\) vs. PO\textsubscript{2} plots were fitted with a Michaelis-Menten (M-M) curve:

\[
\dot{\text{MO}}_2 = \frac{\text{Routine O}_2\text{max} \times \text{PO}_2}{(\text{Km} + \text{PO}_2)} \quad \text{(Eq. 2)}
\]

where by analogy to enzyme kinetics, MO\textsubscript{2} is the rate, PO\textsubscript{2} is the substrate concentration, and the equation allows us to obtain Routine O\textsubscript{2max} and the affinity constant (Km) of the organism for oxygen. The M-M curve yielded the highest \(r^2\) when compared with other curve fits in Graph Pad Prism (v.5; Graph Pad Software, San Diego, CA), including a sigmoidal modification based on the Hill equation as another possibility suggested by Wood (2018).

This M-M curve was then compared with a linear regression constructed using the MO\textsubscript{2} value at the start of the curve (PO\textsubscript{2} of air saturation) and at the end of the curve (the PO\textsubscript{2} where \(\dot{\text{MO}}_2\) became 0). Both the M-M curve and the straight line were integrated and the area under the curve between both lines was calculated. Additionally, a straight horizontal line was plotted starting at the MO\textsubscript{2} at the highest PO\textsubscript{2}, representing a hypothetical situation where the fish regulates MO\textsubscript{2} at all PO\textsubscript{2} levels (Fig. 2.12). The area under the M-M curve was expressed as a proportion of the 100% regulation area, so a RI value of 1 would indicate
perfect regulation, while a value of 0 would indicate a perfect conformation. Therefore, the RI is used as an indication of the regulatory ability of the fish (Fig. 2.12).

The critical oxygen tension (Pcrit) is another indicator of the regulatory ability of the fish. The Pcrit is defined as the transition PO2 where MO2 is no longer independent of environmental PO2 and becomes dependent. In other words, the Pcrit is the inflexion point where a fish is no longer an oxyregulator and becomes an oxyconformer (Pörtner and Grieshaber, 1993). Pcrit was determined using the greatest difference method (Mueller and Seymour, 2011), where the PO2 at which the vertical distance between the M-M curve and the straight line describing 100% conformity was the greatest (Fig. 2.12). Routine metabolic rate (RMR) under non-limiting conditions was calculated as the average MO2 at the start of the trial, where PO2 was highest (> 90 Torr).

2.3.4 Ventilation during progressive hypoxia

Separate groups of fish (n = 8 – 10 per salinity; mean body mass = 5.58 ± 0.22 g) were used for this experimental series. Killifish were anaesthetized with MS-222 (Syndel Laboratories, Nanaimo, B.C., Canada) buffered with HCO3− and fitted with a buccal catheter for the recording of ventilatory pressure and amplitude. A short length of plastic tubing (~2 cm, PE 160 BD, Intramedic, Franklin Lakes, NJ, USA), flared on the buccal side, was inserted snugly into a hole made in the rostrum using a 19-gauge hypodermic needle. A slightly longer piece of PE50 tubing, also flared, was threaded through the PE160 sleeve, and both pieces were glued together with a drop of cyanoacrylate glue, and then secured in place with a silk suture on the outer side of the hole. Fish were allowed to recover from the procedure overnight, in the same individual plastic chambers as used in the LOE experiments (Section 2.3.2). The chambers were placed in a darkened bath filled with water of the salinity to which the fish had been acclimated.

For the measurements of ventilation, the water-filled internal PE50 catheter was bridged to a longer (50 cm) piece of tubing using the shaft of a 22-gauge blunted needle, and then connected to a pressure transducer (DPT-100, Utah Medical Products, Midvale, UT, USA) so that the ventilatory pressure amplitude (cm H2O) and frequency (breaths/min) could
be recorded. The pressure transducer was calibrated daily to a 4-cm water column. Water PO$_2$ was gradually lowered at a rate of ~1.15 Torr/min using compressed N$_2$ gas, bubbled into a reservoir from which water flowed to the water bath, so as to avoid any external source of stimuli and to decrease stress. PO$_2$ was lowered from ~150 Torr to ~2 Torr over a period of about 130 min, a rate similar to the one in the MO$_2$ series (Section 2.3.3), and ventilation was constantly recorded using a PowerLab Data Integrity system (ADInstrumets, Colorado Springs, CO, USA), connected to an amplifier (LCA-RTC, Transducer Techniques, Temecula, CA, USA), and visualized and analyzed using LabChart v. 7.0 (ADInstruments). Recordings of ventilation frequency (breaths/min) and pressure amplitude (cmH$_2$O/breath) were averaged every 5-min, and plotted against the average PO$_2$ at each time interval. The ventilatory index (cm H$_2$O/min) was calculated as follows:

\[
\text{Ventilatory index} = \text{frequency} \times \text{pressure amplitude} \quad \text{(Eq. 3)}
\]

where frequency is breaths/minute and amplitude is in cm H$_2$O. Data were binned at 5-Torr intervals. Water PO$_2$ was monitored throughout the whole duration of the trials using a handheld oxygen meter (Accumet AP84A, Fisher Scientific). All progressive hypoxia exposures were performed at the salinity of acclimation.

### 2.3.5 Hypoxia exposure for blood and tissue sampling

Separate groups of fish (n = 8 – 10 per salinity; mean body mass = 4.05 ± 0.18 g) were used for this experimental series. The same experimental chambers and apparatus described in Section 2.3.2 were used for the two experiments in this series. Fish were placed in the chambers and allowed to settle overnight. 50% of the water was replaced prior to the start of the trial. PO$_2$ was reduced from normoxia (155 Torr) to hypoxia (15 Torr) over 1 h and maintained at this level with compressed N$_2$ and air. Killifish were kept in hypoxia for 3 h, and then immediately anesthetized in 0.5 g/L buffered MS-222, and blood samples were quickly drawn by caudal puncture using a modified gas-tight Hamilton (100 µL) syringe (Reno, NV, USA). Normoxic fish underwent the same protocol, but PO$_2$ was kept in
normoxia. All fish used for the analysis of blood parameters and gill morphometrics underwent this regime. After blood sampling, the anaesthetized fish were euthanized.

2.3.5.1 Whole blood oxygen affinity, hematocrit, and hemoglobin concentration

Once withdrawn, blood samples were kept on ice until the analysis, and then transferred to a micro-tonometer at the experimental temperature, fashioned after the one described in Wood et al. (2010). Blood pH (~ 40 to 50 µL) was measured twice, after two different equilibration steps, where samples were equilibrated for 30 min to physiological PCO$_2$ (1.9 Torr and PO$_2$ = 155 Torr) and to a high PCO$_2$ (7.6 Torr and PO$_2$ = 155 Torr). Gas mixtures were obtained using Wösthoff DIGAMIX gas mixing pumps (H. Wösthoff Messtechnik, Bochum, Germany), and blood pH was measured using an esophageal micro pH electrode (MI-508; Microelectrodes Inc., Bedford, NH, USA).

Oxygen equilibrium curves (OEC) were generated using the spectrophotometric technique described by Lilly et al. (2013), adapted for a 96-well microplate spectrophotometer (SpectraMax 190; Molecular Devices, Sunnyvale, CA, USA). Fresh whole blood (1.8 µL) was sandwiched between two thin layers of gas-permeable low-density polyethylene, secured on a small aluminum ring by two plastic O-rings and placed in a gas-tight tonometry cell modified to fit into the microplate reader. Hb–O$_2$ saturation was determined spectrophotometrically at nine different PO$_2$ values obtained by mixing compressed O$_2$ and N$_2$ in various ratios using the Wösthoff pumps), starting from 0 Torr (fully deoxygenated), working towards 155 Torr (fully oxygenated). All spectrophotometric analyses were done at 18°C and at two separate PCO$_2$ levels (1.9 and 7.6 Torr). A logistic model designed to fit blood samples with multiple hemoglobin isoforms, was fitted through the % blood–O$_2$ saturation versus PO$_2$ data for each fish in order to determine blood P$_{50}$ in R (v. 3.1.2; Public domain):

$$y = \frac{d}{1 + \exp[\log_{10}(x / e)]} \quad \text{(Eq. 4)}$$
where y is the fractional O\textsubscript{2} saturation, x is the PO\textsubscript{2} at each equilibration step, and d, b, and e are the equation parameters: d is the upper asymptote of the model, b is the Hill’s slope and e is the inflexion point. After the determination of P\textsubscript{50}, Eq. 4 was derived and the Hill coefficient at P\textsubscript{50} (n\textsubscript{50}) was obtained:

\[
n50 = -b \left( \exp\left[ b \left( \log_{10} P_{50} \right) \right] \right) / \left[ -1 - e^{\left( b / \ln(10) \right)} \right] + \exp \left[ b \left( \log_{10} P_{50} \right) \right] \left( \ln(10) \right) \tag{Eq. 5}\]

The Bohr Coefficient (Φ) was calculated as follows:

\[
Φ = \left[ \log_{10} P_{50} (1.9 \text{ Torr}) - \log_{10} P_{50} (7.6 \text{ Torr}) \right] / \left[ \text{blood pH} (1.9 \text{ Torr}) - \text{blood pH} (7.6 \text{ Torr}) \right] \tag{Eq. 6}
\]

where \(\log_{10} P_{50} (1.9 \text{ Torr})\) is the logarithm of whole-blood P\textsubscript{50} assayed at PCO\textsubscript{2} of 1.9 Torr, \(\log_{10} P_{50} (7.6 \text{ Torr})\) is the logarithm of whole-blood P\textsubscript{50} assayed at PCO\textsubscript{2} of 7.6 Torr; blood pH \((1.9 \text{ Torr})\) is the blood pH after equilibration at 1.9 Torr PCO\textsubscript{2} and blood pH \((7.6 \text{ Torr})\) is the blood pH after equilibration at 7.6 Torr PCO\textsubscript{2}.

Hematocrit (HCT; %RBC) was measured as the percentage of packed RBCs within the total blood volume after centrifugation in micro-hematocrit tubes (2 x 5 µL) for 10 min at 10000 x g. Whole blood hemoglobin concentration ([Hb]: mmol/L blood) was measured spectrophotometrically at 540 nm using Drabkin’s reagent (Sigma-Aldrich, St. Louis, MO, USA), with a spectrophotometer (Shimadzu UV-160, Kyoto, Japan) and calculated with a millimolar extinction coefficient of 11 (Völkel and Berenbrink, 2000). Mean cell hemoglobin concentration (MCHC: mmol Hb/L RBC) was calculated as follows:

\[
MCHC = [\text{Hb}] / (\text{HCT/100}) \tag{Eq. 7}
\]

\textbf{2.3.5.2 Gill morphometrics and morphology}

Fish used for this experimental series underwent the same protocol as described in Section 2.3.5.1 but were kept in normoxia throughout the whole duration of the experiment. Once fish had lost equilibrium in anesthetic, fish were weighed, the spinal cord severed, and
the entire gill basket was carefully excised. The gills were fixed in 2 mL Karnovsky’s fixative (25% glutaraldehyde, 16% formaldehyde, 0.16 mol/L sodium phosphate buffer, pH 7.2) for 24 h at 4°C. Samples were then transferred to 0.1 M sodium cacodylate buffer (pH 7.2) and stored at 4°C until use. The second gill arch was isolated from the gill basket and its anterior hemibranch imaged using a light microscope (Olympus Stereomicroscope SZX10; Tokyo Japan) at 4 - 6.3x magnification, and images were captured using a cellSens Software (v.1.12; Olympus, Tokyo Japan), digitized and measured using ImageJ v.150i (Public domain). Care was taken to ensure that all images were taken at a consistent orientation that provided unbiased measurements. Total gill surface area was estimated according to Wegner (2011). After the total number of filaments had been counted on the intact hemibranch, the length of the gill arch was divided in five, and further measurements were made on the isolated central filament of each one of the five sections. Each filament was imaged from two different orientations (top and side), allowing clear views of its own length, additionally to the height (distance from base to the distal edge of the lamellae), length and width of the lamellae. More specifically, these were measured on three lamellae per filament (one located at the base, one from the middle and one from the tip), as well as the width of the water channels located in between two lamellae. Each filament’s lamellar frequency (number lamellae/µm) was estimated as:

\[
\text{Lamellar frequency} = \frac{F_{\text{length}}}{L_{\text{width}} + C_{\text{width}}} \quad \text{(Eq. 8)}
\]

where \(F_{\text{length}}\) is the total filament length (µm), \(L_{\text{width}}\) is the averaged lamellar width (µm) and \(C_{\text{width}}\) is the averaged channel width (µm). Individual lamellar surface area for each of the three lamellae measured was estimated as the product of lamellar height and lamellar basal length. The filament total lamellar surface (\(FL_{SA}: \mu m^2\)) area was obtained by multiplying the mean individual lamellar surface area by the total number of lamellae per filament by 4 as to account for the two faces of each lamella and the lamellae present on both sides of the filament. Finally, the mass-specific 2nd gill arch lamellar surface area (\(T_{SA}: \mu m^2/g\)) for both sides of the gill basket for each fish was calculated as:
\[ T_{SA} = \frac{(F_{LSA} \times F_{total} \times 4)}{W} \quad \text{(Eq. 9)} \]

where \(F_{LSA}\) is the mean lamellar surface area of the five analyzed filaments, \(F_{total}\) is the number of filaments per gill arch and 4 is to account for the 2 sides of the hemibranch multiplied by 2 sides of the gill basket, and \(W\) is the body mass (g).

For the histological analyses, the entire third gill arch was embedded in paraffin wax and serologically sectioned at 5-\(\mu\)m thickness, mounted and stained with Hematoxylin and Eosin at Wax-it Histology Services Inc. (UBC). Eighteen sections per fish were divided in 4 slides containing 4 - 5 sections each and imaged using an inverted microscope (Motic AE31; Hong Kong, China) at 400x magnification. Interlamellar cell mass area (ILCM: \(\mu\)m\(^2\)), epithelial cell layer area (\(\mu\)m\(^2\)) and ionocyte density at the filament (\#cells/\(\mu\)m\(^2\)) at randomly selected sections were measured using ImageJ v.1.52a while the observer was blind to treatment groups. For each fish, 40-60 ILCM measurements (15 per slide), 30-32 epithelial cell layer area measurements (8-17 per slide), and 8-10 ionocyte density measurements (2-5 per slide) were taken. Fig. 2.9D shows a diagram detailing the measurements. ILCMs, lamellae, and gill areas were randomly selected within each image for the respective measurements. Epithelial cell layer coverage was expressed per unit height of the lamellae (\(\mu\)m\(^2\)/\(\mu\)m).

### 2.3.6 Statistical analyses

All data are shown as means ± 1 SEM (n = number of animals). Detailed results of the statistical tests and post-hoc analyses are shown in specific Figure captions. ANOVA assumptions (data normality and homogeneity of variances) were checked, and if not achieved, data were transformed using a log transformation. For the one-way ANOVA, mean values were considered significantly different when \(p < 0.05\). For the two-way ANOVA post hoc tests, the Bonferroni correction for multiple comparisons was applied.
2.4 Results

2.4.1 Time to loss of equilibrium in hypoxia

Salinity acclimation had a significant effect on the time to LOE in hypoxia in killifish (p < 0.001; Fig 2.1). Fish acclimated to 0 ppt maintained their normal dorso-ventral orientation for 2.2 h and then showed a loss of equilibrium, while fish acclimated to 11 ppt (isosmotic point) maintained equilibrium for the longest time (~18 h; Fig. 2.1). Fish acclimated to 3 and 15 ppt exhibited intermediate tolerance. Time to LOE did not differ between 11 and 35 ppt acclimated fish.

2.4.2 Oxygen consumption rate (ṀO₂)

Fig. 2.2 shows the mean ṢO₂ versus PO₂ relationships of killifish acclimated to 0, 11 and 35 ppt. While fish at 0 and 11 ppt exhibited fairly similar patterns of ṢO₂ response with decreasing PO₂, the pattern seen for 35 ppt acclimated fish clearly differed. More detailed analyses of the relationships in individual fish revealed additional subtle differences; key aspects are summarized in Fig. 2.3.

Fish acclimated to 35 ppt had the highest RMR in normoxia (120 – 90 Torr), approximately 9 µmol O₂/kg/h, which was about 30% greater than for fish in the other two acclimation groups (Fig. 2.3A). The overall RI of the 35 ppt group was about 0.47 RI, substantially lower than the values of 0.70 and 0.81 (0 and 11 ppt respectively) in the other two treatments, indicating a lower degree of oxyregulation at the highest salinity (Fig. 2.3B). This conclusion was re-inforced by the Michaelis-Menten analysis, which revealed the highest Km (i.e. lowest O₂ affinity) in this group (~16 Torr). Notably, when acclimated to isosmotic salinity (11 ppt), killifish showed the lowest Km (~ 3 Torr), denoting a much higher affinity of the whole organism for O₂ (Fig. 2.3C). The Km was intermediate (~9 Torr) at 0 ppt. The Routine O₂max as derived from the M-M analysis was highest in fish acclimated to 35 ppt (9.7 µmol O₂/kg/h; Table 2.1), in accord with the RMR data. There were no striking differences between the Routine O₂max values in fish acclimated to 0 and 11 ppt (Table 2.1).
The Pcrit (as calculated by the greatest difference method) exhibited a somewhat different pattern than the Km. While the value was again lowest in 11 ppt (~21 Torr), it was highest (~41 Torr) at 0 ppt, and intermediate (~30 Torr) at 35 ppt (Fig. 2.3D).

2.4.3 Ventilation during progressive hypoxia

Fig. 2.4 shows the effect of water PO$_2$ on the ventilation dynamics (2.4A: ventilatory index; 2.4B: ventilation frequency; 2.4C: ventilatory pressure amplitude) of killifish acclimated to 0, 11 and 35 ppt. Table 2.2 summarizes specific comparisons of the ventilatory responses of fish acclimated under normoxia (> 90 Torr) and severe hypoxia (8 – 0 Torr). Clearly ventilation increased during hypoxia in all three groups and then declined at very low ambient PO$_2$. The onset for increases in ventilation upon PO$_2$ reduction was higher in fish acclimated to 0 ppt (approximately 56 Torr) and much lower for fish acclimated to 11 and 35 ppt (approximately 22 Torr) (Fig. 2.4A). The PO$_2$ at which fish reached maximum ventilation was also higher in 0 ppt acclimated fish (22 Torr) versus 15 and 16 Torr in fish acclimated to 11 and 35 ppt respectively (Fig. 2.4A). This pattern reflected increases and then decreases in both breathing frequency and ventilatory pressure amplitude in each treatment, although the absolute contribution of changes in amplitude was greater than that of changes in frequency to the alterations in ventilatory index.

Overall ventilation, as captured by the ventilatory index (Fig. 2.4A), was significantly greater in the 0 ppt group under normoxia, and this difference from the other two treatments increased progressively under hypoxia (Fig. 2.4A; Table 2.2). This effect was almost entirely due to a much greater pressure amplitude in the 0 ppt fish under both normoxia and hypoxia (Fig. 2.4C; Table 2.2); there were no significant differences in ventilation frequency among the treatment groups (Fig. 2.4B; Table 2.2). Furthermore, all ventilatory parameters were the same under both normoxia and hypoxia between the 11 and 35 ppt treatments.
2.4.4 Whole blood oxygen affinity, hematocrit, and hemoglobin concentration

Figure 2.5 shows representative oxygen equilibrium curves (OEC) of *Fundulus heteroclitus* acclimated to 0, 11 and 35 ppt in normoxia (Fig. 2.5A,C,E) and after exposure to hypoxia (Fig. 2.5B,D,F), assayed at two different PCO$_2$ tensions. When whole blood was assayed at a PCO$_2$ of 7.6 Torr, the OECs shifted to the right (Bohr effect) relative to the curves determined at PCO$_2$ = 1.9 Torr. The quantification of this effect is summarized in Table 2.5 and will be described below. Another interesting finding is that the OECs (independent of salinity) were slightly shifted to the right in animals that had been exposed to hypoxia (Fig. 2.5B,D,F) in comparison to fish in normoxia (Fig. 2.5A,C,E).

The cooperativity coefficients (Hill number: $n_{50}$) for all experimental treatments are summarized in Table 2.3. At both PCO$_2$s tested, two-way ANOVA revealed a strong significant overall effect of oxygen, while no significant effect of salinity was detected. At PCO$_2$ = 1.9 Torr there was a significant interaction, while at PCO$_2$ = 7.6 there was not. For all salinities, the Hill number was higher in fish exposed to hypoxia, regardless of PCO$_2$ (Table 2.3). Fish acclimated to all three salinities under normoxia showed a Hill number lower than or close to 1 (Table 2.3), indicating negligible cooperativity, which is evidenced by the hyperbolic shape seen in the OECs (Fig. 2.5A,C,E). In turn, the highest Hill numbers were seen in fish acclimated to 0 and 11 ppt, after exposure to hypoxia, when assayed at 7.6 Torr (Fig. 2.5B,D; Table 3), which were reflected visually in more sigmoidally shaped curves. At a PCO$_2$ of 1.9 Torr, there was a significant difference between the Hill number of fish in normoxia and the values of fish exposed to hypoxia at both 0 and 35 ppt (Table 2.3). At a PCO$_2$ of 7.6 Torr, the same was observed for fish acclimated to 0 ppt (Table 2.3).

When blood P$_{50}$ was determined at PCO$_2$ = 1.9 Torr (Fig. 2.6A), there was an overall significant effect of the oxygen regime, but not of salinity, and no significant interaction by two-way ANOVA. In normoxia, the blood-O$_2$ affinity (P$_{50}$) did not change with acclimation to different salinities (Fig. 2.6A). The P$_{50}$ was significantly higher (i.e. O$_2$ affinity was lower) in fish exposed to hypoxia at 35 ppt compared to those exposed to normoxia (Fig. 2.6A), while there was no difference at the other salinities. At PCO$_2$ = 7.6 Torr, both salinity and O$_2$ had significant effects, and as expected, P$_{50}$ was overall higher both in normoxia- and in
hypoxia-exposed fish compared to $P_{50}$ at $PCO_2 = 1.9$ Torr (Fig. 2.6B). There was no significant interaction effect. Interestingly when blood from 35 ppt acclimated fish was assayed at 7.6 Torr, there was no significant difference between normoxia and hypoxia.

Table 2.4 shows the effect of equilibration to a physiological (1.9 Torr) and to a higher (7.6 Torr) $PCO_2$ on the whole blood pH of killifish acclimated to different salinities in normoxia and exposed to hypoxia. At $PCO_2$ of 1.9 Torr, oxygen had a significant overall effect, while salinity did not (Table 2.4). For the $PCO_2$ of 7.6 Torr, there was a significant interaction between the parameters, while the separate effects of oxygen and salinity were not significant. Blood pH was consistently lower in fish exposed to hypoxia at all salinities (Table 2.4), and as expected, it was lower when blood was equilibrated to a higher $PCO_2$ (7.6 Torr). At 7.6 Torr, blood from fish acclimated to 35 ppt and exposed to hypoxia had a significantly lower pH than blood from fish in normoxia (Table 2.4).

There was a significant overall effect of oxygen, but no significant interaction between oxygen and salinity on the Bohr coefficient ($\Phi$; Table 2.5). Fish acclimated to 0 ppt in normoxia appear to have the smallest $\Phi$ (Fig. 2.5A; Table 2.5) and clearly a large but very variable significant increase occurred when fish were exposed to hypoxia (Fig. 2.5B; Table 2.5). Fish acclimated to 11 ppt exhibited a greater Bohr effect under normoxia when compared to 0 ppt, but the increase after exposure to hypoxia was highly variable and not significant (Fig. 2.5C). Fish acclimated to 35 ppt also did not show a significant difference between the OECs of fish in normoxia and exposed to hypoxia (Table 2.5). At all salinity acclimations, the $\Phi$ was higher in fish exposed to hypoxia (Table 2.5).

There was a significant overall effect of oxygen on blood hematocrit. Under normoxia, blood hematocrit (%RBC, Fig. 2.7A) was around 27-30% and did not differ among acclimation groups. Hematocrit was significantly elevated (approximately 1.3-fold) at all salinities when fish were exposed to hypoxia. However, there was no overall effect of salinity in either normoxia-, or hypoxia-exposed fish (Fig. 2.7A), in addition to no significant interaction. There was a significant overall effect of salinity on blood hemoglobin concentration ([Hb], Fig. 2.7B). In contrast to hematocrit, [Hb] (Fig. 2.7B) under normoxia was significantly greater in the 11 ppt group (about 1 mmol/L) than in the other two treatments (about 0.75 mmol/L). [Hb] increased slightly after hypoxia exposure at all
salinities, but there was no significant overall effect of oxygen was detected. Under normoxia, mean cell hemoglobin concentration (MCHC; Fig. 2.7C) did not vary significantly across salinities, but decreased in fish exposed to hypoxia, independent of the acclimation salinity (Fig. 2.7C), reflecting the elevated hematocrit and virtually unchanged [Hb]. There was an overall effect of oxygen, and at 11 and 35 ppt acclimation, MCHC was significantly lower in hypoxia than in normoxia.

### 2.4.5 Gill morphometrics and morphology

Fig. 2.8 shows the total lamellar surface area ($T_{SA}$) of the second gill arch in killifish acclimated to 0, 11 and 35 ppt in normoxia. $T_{SA}$ was the lowest in fish acclimated to 0 ppt (about $6.3 \times 10^7 \mu m^2$), and significantly different from $T_{SA}$ at the other two salinities. $T_{SA}$ did not change significantly between 11 and 35 ppt where SA was 12-19% greater (Fig. 2.8). Table 2.6 summarizes the effects of salinity acclimation on the quantitative metrics of gill morphology. Killifish acclimated to 11 ppt had longer filaments, higher number of lamellae per filament, higher number of filaments on the 2nd gill arch and a higher $FL_{SA}$ (Table 2.6), although not statistically significant.

Figure 2.9 shows three representative histological sections of killifish gills stained with Hematoxylin-Eosin. In killifish acclimated to 0 ppt (Fig. 2.9A), the epithelium covering the lamellae was protuberant and thicker than in fish acclimated to 11 and 35 ppt, which showed thinner, more compact lamellae (Fig. 2.9B,C). Additionally, ionocytes (mitochondria rich cells) are stained in light blue and can be seen scattered throughout the length of the filament, but not on the lamellae.

No ionocytes were observed located on the lamellae themselves at any of the three salinities. The interlamellar cell mass area (ILCM; Fig. 2.10A) was the smallest in fish acclimated to 35 ppt (about 167 $\mu m^2$), similar at 11 ppt, and significantly higher by about 22% in fish acclimated to 0 ppt. There was no significant effect of salinity acclimation on the ionocyte density (Fig. 2.10B). The ratio between the epithelial cell layer area and the lamellae height was measured as a proxy for the distance for gas diffusion at the lamellae (Fig. 2.10C). A higher ratio indicates thicker coverage of the exposed respiratory area. The ratio
was highest in fish acclimated to 0 ppt, and significantly different from fish acclimated to 11 ppt (Fig. 2.10C). Fish acclimated to 35 ppt had diffusion distance intermediate between that of 0 and 11 ppt acclimated fish (Fig. 2.10C).

2.5 Discussion

2.5.1 Overview

Our ultimate goal was to determine the relationship between salinity acclimation and hypoxia tolerance in the euryhaline killifish *Fundulus heteroclitus* and to elucidate the mechanisms by which salinity acclimation may influence hypoxia tolerance. Our first hypothesis, based on energetic considerations, was that *F. heteroclitus* acclimated to the isosmotic salinity (~ 11 ppt) would exhibit the greatest hypoxia tolerance, decreasing towards fresh and full strength sea water. Our data only partially supported this hypothesis, as we saw a large increase in hypoxia tolerance between 0 and 11 ppt acclimated fish, but no changes between 11 and 35 ppt. Our data also showed that 11 ppt acclimated fish exhibited the greatest capacity to regulate $\dot{M}O_2$ under progressive hypoxia, as indicated by three indices, the $RI$, the $K_m$, and the $P_{crit}$. We also hypothesized that fish acclimated to the ends of the spectrum (0 ppt or 35 ppt) would have respiratory surfaces of lower effective permeability than fish acclimated at 11 ppt, in order to favor ionic homeostasis, potentially at the expense of respiratory gas transfer (i.e. the osmorespiratory compromise). This was confirmed morphometrically inasmuch as the 11 ppt fish exhibited the highest lamellar surface area and lowest epithelial thickness (i.e. shortest diffusion distance). Furthermore, the 0 ppt acclimated fish, which had the lowest hypoxia tolerance, possessed the lowest lamellar surface area and highest ILCM, even relative to values in 35 ppt acclimated fish, thereby reducing the effective surface area for gas exchange. We also hypothesized that physiological adjustments would have to be made in light of this osmorespiratory compromise, and this was strongly supported, at least with respect to the fish acclimated to 0 ppt. Our data show that fish acclimated to 0 ppt had the highest ventilatory index in both normoxia and hypoxia, and initiated ventilatory increases at a higher $PO_2$ threshold, indicating a higher ventilatory
work in order to maintain satisfactory oxygen uptake at the gills. This was largely due to greater ventilatory pressure amplitudes (i.e. greater ventilatory stroke volumes) at 0 ppt. Subtle differences in blood O\textsubscript{2} transport characteristics, particularly during hypoxia, may also have been associated with the osmorespiratory compromise in these fish. Surprisingly, although the 35 ppt fish exhibited a lower capacity to regulate O\textsubscript{2} uptake under hypoxia than the 11 ppt fish, no substantial differences in gill morphology, ventilation, or blood O\textsubscript{2} transport were seen. We conclude that many differences in the O\textsubscript{2} transport cascade are associated with the osmorespiratory compromise in the killifish, and that of the three acclimation salinities tested, 11 ppt is the most advantageous, and 0 ppt is the most challenging for respiratory gas exchange.

2.5.2 Effects of salinity on time to loss of equilibrium (LOE)

The data presented here demonstrate that hypoxia tolerance (as measured as time to LOE) in *F. heteroclitus* is altered by salinity acclimation over a wide range of salinities (0, 3, 11, 15 and 35 ppt) all within the normal distribution of these animals in their natural habitats, which are estuaries and salt marshes (Griffith, 1974). We initially hypothesized that acclimation to the salinity that corresponds to the isosmotic point (~ 11 ppt) would yield the maximum hypoxia tolerance, since energy usage in iono- and osmo-regulation would be minimized. Our follow-up prediction was that hypoxia tolerance would decrease at the two salinity extremes, fresh water and full strength sea water. Fish acclimated to 11 ppt showed the highest time to LOE in hypoxia, in agreement with our initial hypothesis, but there was no significant difference between time to LOE between fish acclimated at 11 and 35 ppt (Fig. 2.1), contrary to our prediction based on the energetic costs of iono- and osmoregulation in full SW. Loss of the ability to maintain position in the water column and unresponsiveness to stimuli threatens survival in the wild, and therefore the time required to reach such endpoint is an important ecological indicator of tolerance to hypoxia (Mandic et al., 2012). However, the mechanisms underlying such loss of function are still poorly understood, although the ability to supply ATP to the brain may underlie patterns of LOE in fishes (Mandic et al., 2012). Times to LOE reported here are well above others found in the literature for *F.*
heteroclitus (Borowiec et al., 2015; McBryan et al., 2016). For example, killifish acclimated to 20 ppt and 15°C had a time to LOE of 75 min at 3 Torr (McBryan et al., 2016), versus 11 h reported here for fish at 15 ppt and 3.5 Torr (18°C), highlighting that small differences in methodology could result in large differences in time to LOE, and therefore difficulties in comparisons between different studies.

To our knowledge, this is the first study to look at time to LOE in killifish acclimated to fresh water and we found an 85% reduction in comparison to the 11 ppt acclimation. In some preliminary experiments, we evaluated time to LOE at a common assay salinity of 11 ppt in fish that had been acclimated to 0, 11 and 35 ppt (data not shown) in order to evaluate whether it was the exposure salinity, or the acclimation salinity that was of greater influence. No differences in time to LOE were observed between fish that were tested at their respective acclimation salinity and fish tested at 11 ppt, reinforcing the idea that it is the acclimation salinity, and not just exposure to hypoxia at the isosmotic salinity that alters hypoxia tolerance.

2.5.3 Effects of salinity on respiratory responses to hypoxia

It has been hypothesized that metabolic rates of fishes should reflect changes associated with osmoregulatory costs (Zadunaisky, 1984). A number of studies have attempted to quantify the amount of energy used towards ionoregulation, but different methodologies have yielded widely varying results (Kirschner 1995, Potts, 1954; Boeuf and Payan, 2001). Some theoretical calculations have placed the cost of ion transport at only 0.5 - 1% of resting metabolic rate (McCormick et al., 1989); however, direct measurement of these costs have yielded values as high as 27% (Rao, 1968). In F. heteroclitus, Kidder et al. (2006a) estimated the cost as 6 - 10% in sea water, and somewhat lower in fresh water, based on the higher ionic and osmotic gradients from water to blood in sea water. Measurements of the metabolic rates in isolated gill arch preparations in FW and SW acclimated cutthroat trout (Oncorhynchus clarki clarki) suggests that NaCl transport across the gills represents less than 4% of the whole animal’s energy budget in both salinities (Morgan and Iwama, 1999). Therefore, despite the uncertainties in the empirical and theoretical data, it is generally
agreed that osmoregulatory costs may occupy an important share of the animal’s energy budget. Potentially, this could be reflected in metabolic rate measurements. Our data agree in part with this idea. Our initial hypothesis was that the isosmotic salinity (11 ppt) would yield the lowest osmoregulatory costs and therefore the lowest RMR. Instead, we saw no differences in routine metabolic rate (RMR) in fishes acclimated to 0 and 11 ppt, and the greatest RMR in fishes acclimated to 35 ppt (Fig. 2.3A). This higher RMR at 35 ppt agrees qualitatively with the theoretical prediction of Kidder et al. (2006a). However, in contrast to the present data and to their own prediction based on theory, in a separate study, Kidder et al. (2006b) saw no differences in the RMR of *F. heteroclitus* acclimated to either fresh, sea water and water at 10 ppt. These authors suggested that differential ionoregulatory costs were hidden by adjustment of other expenditures. In two additional species of euryhaline fishes, *Galaxias maculatus* (inanga: Urbina and Glover, 2015) and *Sciaenops ocellatus* (Ern and Esbaugh, 2018), again no differences in RMR were observed among fish acclimated to a wide range of salinities.

Any environmental factor that has the potential to influence oxygen demand, such as temperature and salinity, will likely have implications for whole animal oxygen uptake and consequently, hypoxia tolerance. We had predicted that as the cost of osmoregulation would be minimum at the isosmotic salinity, fish would exhibit the highest ability to perform in hypoxia. A common predictor of hypoxia tolerance is the critical oxygen level (Pcrit). Pcrit is the oxygen level below which the animal can no longer sustain a constant rate of oxygen uptake (oxyregulation), and uptake becomes a function of environmental oxygen availability (oxyconformation). Pcrit has been widely used as an indication of the degree of hypoxia tolerance exhibited by a species (e.g. Mandic et al., 2009; Rogers et al., 2016). However, recent studies have pointed to the impracticality of translating Pcrit values into the real world, and have challenged its actual significance as a hypoxia tolerance trait (Mueller and Seymour, 2011; Wood 2018). Therefore, in our study we approached the MO₂ versus PO₂ relationship using the framework proposed by Alexander and McMahon (2004) and Mueller and Seymour (2011), where a nonlinear relationship is used to describe the PO₂ x MO₂ plots (Marshall et al., 2013). The analysis of the ability to regulate MO₂ over a range of PO₂s
(regulation index: RI) reveals the degree of a fish’s ability to maintain \( \dot{M}O_2 \) independently of environmental \( PO_2 \) (Fig. 2.12).

Our data show that fish acclimated to 35 ppt have the lowest RI, suggesting a lower ability to regulate \( \dot{M}O_2 \) in the face of declining \( PO_2 \), with a pattern closer to oxyconformation than at 11 ppt or 0 ppt (Fig. 2.3B). Blewett et al. (2013) identified the response pattern of \textit{F. heteroclitus} to declining \( PO_2 \) as almost entirely oxyconformation, contrasting with the results of several other studies including the present one, though the summary of Wood (2018) indicates that there exists a great diversity of patterns reported within this single species (see below). What was more remarkable in our study was the greater ability of 11 ppt acclimated fish to regulate \( \dot{M}O_2 \) down to very low \( PO_2 \)s as reflected in the higher RI, lower Pcrit, and lower Km values (Fig. 2.3), the latter representing the affinity constant derived from the Michaelis-Menten nonlinear function used (Marshall et al., 2013).

Our data clearly show that there is an influence of salinity on the \( \dot{M}O_2 \) responses to declining \( PO_2 \). We have calculated the Pcrit using the greatest difference method (Mueller and Seymour, 2011), and to our knowledge, this is the first report of either a Pcrit, or the description of the relationship between \( \dot{M}O_2 \) and \( PO_2 \), for the killifish when acclimated to fresh water (Fig. 2.3D). The Pcrit values reported here lie generally within the broad range of values reported for \textit{F. heteroclitus} at other salinities, although our value for 11 ppt acclimated killifish (20 Torr) is the lowest ever reported. The early study of Cochran and Burnett (1996), where a Pcrit of 35 Torr was found, was performed at 30\(^\circ\)C in “sea water”, though no precise value of salinity was reported. Richards et al. (2008) using fish acclimated to 10 ppt measured a Pcrit of 63 Torr, while both McBryan et al. (2016) and Borowiec et al. (2015) measured a Pcrit of 39 Torr, with fish acclimated to 20 and 4 ppt, respectively. We observed that the onset of significant increases in ventilation occurred at a \( PO_2 \) about 15 Torr higher than the measured Pcrit in fish acclimated to 0 ppt, while in fish at 11 and 35 ppt, Pcrit coincided with the start of ventilatory increase. Additionally, we noted that the onset of decline in ventilation in hypoxia occurred at \( PO_2 \)s below the measured Pcrit for all 3 salinities. This indicates that even at \( PO_2 \)s below what is often considered the onset of start of anaerobic metabolism, fish elect to maintain ventilation, an energetically expensive activity.
An important factor to consider when measuring $P_{crit}$ is the rate of $PO_2$ decline (Regan and Richards, 2017). In our study, we used fish of similar size across salinities as a way of standardizing the rate of $PO_2$ decline across treatments. The accumulation of $CO_2$ and associated decrease in water pH is another concern when using closed-system respirometry, though some have argued that this is most representative of the situation in nature, where water PCO$_2$ usually increases as $PO_2$ declines. Many, but not all studies have reported no effects of PCO$_2$ on $P_{crit}$ (reviewed by Rogers et al., 2016), and indeed Cochran and Burnett (1996) also reported a lack of effect of PCO$_2$ in $F. heteroclitus$. However, the latter investigation was performed in “sea water”, and it is important to consider that at 0 ppt the buffering capacity of the water would be lower and therefore any effects of $CO_2$ on water pH would be magnified.

2.5.4 Effects of salinity on ventilatory responses to hypoxia

Arguably, the single most important physiological response to hypoxia in water-breathing fishes is hyperventilation (Perry et al., 2009a). When faced with hypoxia, the majority of species increase ventilation volume, mainly through increases in stroke volume (amplitude of the breath) rather than through increases in ventilation frequency, as this strategy is known to be more energetically favourable (Perry, 2011). In normoxic conditions, fish acclimated to 0 ppt exhibited a higher absolute ventilatory index, driven by the ventilation amplitude, since there were no differences in ventilation frequency. Additionally, we saw no differences in both components of the ventilatory index (frequency and amplitude) between fish acclimated to 11 and 35 ppt (Fig. 2.4A). At a $PO_2$ of about 56 Torr, 0 ppt acclimated fish started to deviate from those at the other salinities, and markedly increased ventilation, through increases in ventilation amplitude, in qualitative accord with the pattern seen for several other species of fish (Perry et al., 2009a). It is clear from our ventilation data that fish acclimated to 0 ppt must input higher energetic work into ventilation, in order to achieve comparable levels of $\dot{M}O_2$.

In Fig. 2.11, we have estimated the relative oxygen extraction efficiency by dividing $\dot{M}O_2$ by the ventilatory index. In comparison to fish acclimated to 11 and 35 ppt, 0 ppt fish
showed a much lower extraction efficiency throughout the entire PO\textsubscript{2} range. Although the low extraction efficiency may help to explain the high Km and Pcrit values seen in 0 ppt acclimated fish, the lack of differences between 11 and 35 ppt does not fully explain the higher regulation capacity in seen in fish acclimated to 11 ppt.

Since ventilation is thought to be energetically costly (Perry et al., 2009a), at about 5 to 15\% of RMR (Cameron and Cech, 1970) we speculate that the lower hypoxia tolerance observed in fish acclimated to 0 ppt could be a reflection of the higher energy expenditure for ventilation alone. In fact, we saw no differences in the ventilatory output between fish acclimated to 11 and 35 ppt. On a recent examination of the effects of salinity acclimation (0 – 60 ppt) on ventilation in the euryhaline red drum (\textit{Sciaenops ocellatus}), Ern and Esbaugh (2018) saw no influence of salinity on gill ventilation, contrary to our data.

\subsection*{2.5.5 Effects of salinity on gill morpometers and morphology}

We observed no differences between the ionocyte density in fish acclimated to the three salinities (Fig. 2.10B), in contrast with previous reports for other species. Katoh and Kaneko (2003) observed a significant decrease in ionocyte density 3 days after transfer from sea water to freshwater, but after fish were kept at those salinities, these differences disappeared. It has been proposed that killifish acclimated to FW possesses a different type of ionocyte, which Laurent et al. (2006) termed the “cuboidal” cell given its cubical, or wedge-shape morphology. No morphological differences in cell shapes were identified between fresh and sea water acclimated fish (Fig. 2.9) through our histological techniques.

The disparity in hypoxia tolerance indices reported for \textit{F. heterooclitus} could be not only a function of the influence of salinity on metabolic costs of osmoregulation, but also on the morphological adjustments that happens at the gills when fish are acclimated to different salinities. We observed that killifish that had been acclimated to 0 ppt had a smaller lamellar surface area in relation to 11 and 35 ppt counterparts. Furthermore, fish acclimated to 0 ppt had thicker lamellae in addition to a thicker epithelial cell layer and the presence of an interlamellar cell mass (ILMC) that was greater than that observed in 11 and 35 ppt fish (Fig. 2.10A). Since the rate of gas diffusion across the lamellae is inversely related to the thickness
of the water-to-blood diffusion barrier (Evans, 2005), any morphological alterations that increase this distance have the potential to impair whole animal gas transfer (reviewed by Perry, 1998). Rainbow trout (*Oncorhynchus mykiss*) acclimated to ion-poor waters dramatically increased ionocyte proliferation and coverage of the lamellae (Greco et al., 1996), which led to a reduction in the tolerance to hypoxia (Thomas et al., 1988). In turn, freshwater adaptation in prickly sculpin (*Cottus asper*) was associated with thicker gills and a decrease in overall respiratory surface area, resulting in a higher Pcrit value (Henriksson et al., 2008). We had hypothesized that the same might be happening to the freshwater acclimated killifish. However, our morphological data do not support this hypothesis, as we did not observe the presence of ionocytes on the lamellae at any salinity (Fig. 2.9). In turn, what we observed was an increase in the thickness and size of the epithelial cell layer covering the lamellae, indicative of hypertrophy and/or hyperplasia of the epithelial lining (Mallatt, 1985). Additionally, the interlamellar cell mass (ILCM) filling the interlamellar channels (Fig. 2.9) was larger in fish acclimated to 0 ppt than fish in SW, which could have contributed to the reduction in respiratory surface area.

Since the ILCM was first reported (Sollid et al., 2003), many studies have looked at how different environmental factors can alter the ILCM in a variety of fish species (Nilsson, 2007; Mitrovic and Perry, 2009; LeBlanc et al., 2010; Nilsson et al., 2012; Blair et al., 2016). It is known that fish can quickly increase their respiratory surface area in circumstances of high oxygen demand (i.e. increased temperature) via the retraction or sloughing of the ILCM, and this has been documented for *F. heteroclitus* (Barnes et al., 2014; McBryan et al., 2016). To date, fewer studies have looked at the direct influence of salinity acclimation on variations of the ILCM (LeBlanc et al., 2010; Blair et al., 2016; Gibbons, 2017). In the mangrove killifish (*Kryptolebias marmoratus*), the ILCM decreased in animals acclimated to hypersaline conditions in comparison with freshwater acclimated fish (LeBlanc et al., 2010). A similar response was observed for Arctic grayling (*Thymallus arcticus*) a freshwater salmonid, which experienced a dramatic growth in ILCM, when transferred to salt water, nearly eliminating the interlamellar region for water flow and reducing respiratory surface area of the gills (Blair et al., 2016). Similar to our results, Gibbons (2018) observed an increase in ILCM area in response to a decrease in salinity, likely associated with freshwater
adaptation, in threespine stickleback (*Gasterosteus aculeatus*). However, in contrast to the present investigation, none of these earlier studies examined the possible correlation between ILCM and respiratory capacity. The morphological evidence presented here suggests that the decrease in total respiratory surface area and the presence of a greater ILCM contribute towards the elevated ventilatory work together with a marked reduction in hypoxia tolerance when killifish are acclimated to fresh water.

### 2.5.6 Blood oxygen transport characteristics as a function of salinity

We had hypothesized that changes in the respiratory surface area due to salinity acclimation could be reflected in functional changes in the blood O$_2$ transport characteristics. The OEC of the whole blood *F. heteroclitus* under normoxic conditions show characteristics of OECs from fish that are adapted to environmental hypoxia. Those include a relatively high O$_2$ affinity (low P$_{50}$) and a low cooperativity (Hill coefficient close to 1) (Wells, 2009). These characteristics are thought to improve O$_2$ uptake at the gills, and the hyperbolic shape of the OEC reflects the broad range where O$_2$ can be loaded or unloaded from the hemoglobin (Wells, 2009). Conversely, a high Bohr coefficient is thought to improve O$_2$ unloading at tissues. The Bohr coefficients reported here were calculated at a narrow range, since pH changes between low and high PCO$_2$ are fairly small, particularly in hypoxia, therefore, must be interpreted with caution. The pH values measured in our study fall within the predicted range for variations in pH with temperature, and are comparable with data from DiMichele and Powers (1982).

Upon exposure to hypoxia, we observed a rightward shift in the OECs, which resulted in an increase in whole blood P$_{50}$, regardless of salinity acclimation and PCO$_2$ equilibration (Fig. 2.5). Additionally, the Hill coefficient also increased for all salinities (at both PCO$_2$s) in fish that had been exposed to hypoxia, indicating an increase in cooperativity of the hemoglobin and a more sigmoidally shaped OEC, where the reduced O$_2$ affinity (i.e. higher P$_{50}$) favours O$_2$ unloading at the tissues (Wells, 2009). In fish acclimated to 35 ppt, a significant increase in P$_{50}$ was observed after exposure to hypoxia, accompanied by a significant decrease in blood pH. In hypoxia, where the O$_2$ supply for aerobic energy
production becomes limited, fish can resort to anaerobic pathways to obtain energy, often leading to the accumulation of lactic acid, a response common to several fish species, including *F. heteroclitus* (Cochran and Burnett, 1996; Kraemer and Schulte, 2004). The dissociation of protons from lactate, as well as protons released from ATP breakdown (Hochachka and Mommsen, 1983), could cause a generalized acidosis, enhancing the Bohr effect in order to favor O₂ unloading from the hemoglobin to the tissues, resulting in the higher P₅₀ seen in fish exposed to hypoxia. Blood pH decreased in all treatments in whole blood equilibrated with a PCO₂ of 7.6 Torr, relative to the lower PCO₂ (Table 2.4). In fish acclimated to 0 and 11 ppt (at a PCO₂ of 7.6 Torr), blood P₅₀ was substantially higher in fish that had been exposed to hypoxia, while no changes in blood pH were seen. The main erythrocyte organic phosphate compounds found in fish are adenosine and guanosine triphosphates (ATP, GTP), which decrease the Hb-affinity for oxygen (Val, 2000). *F. heteroclitus* is highly susceptible to organic phosphate allosteric modifications on Hb-O₂ affinity (Greaney and Powers, 1978). Since no significant change in blood pH was observed for fish acclimated to 0 and 11 ppt, we speculate that allosteric regulation from RBC organic phosphates could also be playing a role in modifying blood O₂ affinity (Fig. 2.6).

Whole blood P₅₀ values reported here (12.6 to 17.5 Torr) under normoxia are higher than previously reported values for *F. heteroclitus* O₂ binding affinity (Fig. 2.6). Chung et al. (2017) measured a P₅₀ of 3.7 Torr while working on RBCs that had been isolated and re-suspended in HCO₃⁻-free, HEPES buffer at 15 ºC, pH 7.8. Noteworthy, whole blood carries different elements that have the potential to alter O₂ binding affinity, such as various ions, most importantly HCO₃⁻, potential allosteric modifiers, and buffers (plasma proteins), and likely metabolic end products such as protons and lactate. This lack of a plasma environment, as well as the known effects of HEPES in interfering with HCO₃⁻ transport (Hanrahan and Tabcharani, 1990), may alter the blood O₂ binding characteristics, in comparison to measurements performed under normal-buffered conditions. Despite these considerations, whole-blood measurements provide a more realistic view of physiological responses to hypoxia in *F. heteroclitus*. DiMichele and Powers (1982) working with *F. heteroclitus* whole blood identified a P₅₀ of around 5 Torr (at 25°C and pH 7.4), though PCO₂ of the blood was...
apparently 0 Torr in their tests. Is not clear whether the values reported are higher exclusively due to low pH, or if other factors might be playing a role.

Upon exposure to hypoxia, we observed a significant increase in hematocrit independent of salinity acclimation. The increases in mean cell hemoglobin concentration accompanied by no change in [Hb] in fish acclimated to 11 and 35 ppt could indicate a swelling of the RBCs (Fig. 2.7). This could be beneficial for O$_2$ uptake at the gills, since a higher surface area of the RBC per unit of Hb would mean higher contact area of the RBC at the lamellae, and a higher diffusion rate of ambient O$_2$ into the RBC, although the compensatory adjustments of the cardiovascular system would have to be considered. Additionally, β-adrenergic stimulation of RBC swelling leading to increases in Hb-O$_2$ affinity is a known mechanism in teleost RBC (Nikinmaa and Huestis, 1984; Nikinmaa et al., 1984; Nikinmaa 2011). β-adrenergic stimulation of RBC membrane transporters is also present in *F. heteroclitus* (Dalessio et al., 1991). The same response was not observed in fish acclimated to 0 ppt. The lack of RBC swelling in 0 ppt acclimated killifish could be considered another negative aspect of freshwater acclimation (Fig. 2.7C). Taken altogether, our data suggest that the mechanisms for increasing overall O$_2$ carrying capacity of the blood differ between salinity acclimations.

### 2.5.7 Concluding remarks and future directions

We have shown that salinity acclimation had marked effects on the response to hypoxia in the euryhaline *Fundulus heteroclitus*. In a recent comprehensive review, Rogers et al. (2016) identified salinity as one of the parameters strongly correlated with respiratory responses in water-breathing fishes. In nature, stressors will often happen in combination.

In *F. heteroclitus*, acclimation to 11 ppt salinity showed the biggest advantages in relation to respiratory gas exchange, whereas acclimation to 0 ppt was clearly the most challenging. Killifish acclimated to 35 ppt exhibited a lower capacity to regulate O$_2$ uptake under hypoxia than the 11 ppt fish, but most other parameters did not differ substantially. We saw large differences in hypoxia tolerance, where 0 ppt acclimated fish had the lowest time to LOE relative to the 11 ppt fish. These differences were related to the higher capacity to
regulate MO$_2$ under progressive hypoxia at 11 ppt, as evidenced by the RI, the Km, and Perit. Additionally, 11 ppt acclimated fish exhibited the highest lamellar surface area and lowest epithelial coverage of the lamellae. Furthermore, the 0 ppt acclimated fish which had the lowest hypoxia tolerance, possessed the lowest lamellar surface area and highest ILCM, even relative to values in 35 ppt acclimated fish, thereby reducing the effective surface area for gas exchange. Fish acclimated to 0 ppt had the highest ventilatory work amongst the three salinities tested, probably necessitated by the lower respiratory surface area at the gills. Acclimation to different salinities has also led to some differences in blood O$_2$ transport characteristics, affecting O$_2$ affinity (P$_{50}$) and hematological parameters, especially upon exposure to hypoxia.

In nature, *F. heteroclitus* inhabits salt marshes which are highly dynamic environments, where they occupy deeper, more temperature stable areas in the winter, and move to shallower, lower salinity areas in the summer (Taylor et al., 1979). It has been experimentally shown that *F. heteroclitus* actively seeks different salinities based on their physiological and ecological needs (Bucking et al., 2012; Marshall et al., 2016). It could be predicted that in hypoxia, killifish would seek isosmotic salinities in order to alleviate the energetic needs of osmoregulation. Although, in normoxic conditions, killifish prefers salinities at around 20 ppt (Fritz and Garside, 1974; Bucking et al., 2012). It could be hypothesized that killifish avoid 0 ppt water due to the compromise between osmoregulation and respiratory capacity, evidenced by the present study.

Given the strong interactions seen between salinity acclimation and respiratory responses to hypoxia in the present study, there is clearly a need to investigate the other side of the osmorespiratory compromise. In the following chapter (Giacomin et al. Chapter 3), we explore how hypoxia exposure impacts iono-and osmoregulatory performance of *F. heteroclitus* at different salinities.
Time to LOE at 3.5 Torr (h)

- 0 ppt: a
- 3 ppt: ab
- 11 ppt: c
- 15 ppt: bc
- 35 ppt: c
Figure 2.1 Time (h) to loss of equilibrium (LOE) in hypoxia (3.5 Torr) in *Fundulus heteroclitus* acclimated to 0, 3, 11, 15 and 35 ppt salinity. Bars sharing the same lower case letters are not statistically different (one-way ANOVA). Data are means ± 1 SEM (n = 8).
Figure 2.2 The effect of water PO\textsubscript{2} on the oxygen consumption rate (\(\dot{\text{MO}}_2\): \(\mu\text{mol O}_2/\text{g/h}\)) of *Fundulus heteroclitus* acclimated to 0 (circles), 11 (triangles) and 35 (squares) ppt. Data are plotted as \(\dot{\text{MO}}_2\) averages every 5 Torr. Data are means ± 1 SEM (n = 7 - 8).
Figure 2.3 (A) Routine metabolic rate (RMR; μmol O$_2$/g/h), (B) Affinity constant (Km; Torr) (C) Regulation index and (D) Perit (Torr) in *Fundulus heteroclitus* acclimated to 0, 11 and 35 ppt, exposed to progressively decreasing water PO$_2$. Data have been calculated on an individual fish basis. Data are means ± 1 SEM (n = 7 - 8).
Figure 2.4 (A) Ventilatory index (cm H$_2$O/min), (B) ventilation frequency (breaths/min) and (C) ventilation pressure amplitude (cm H$_2$O/breath) in *Fundulus heteroclitus* acclimated to 0 (white circles), 11 (grey triangles) and 35 (black squares) ppt exposed to progressively decreasing water PO$_2$. Data have been plotted as averages every 5 Torr. Data are means ± 1 SEM (n = 11 - 12).
Figure 2.5 Representative oxygen equilibrium curves (OEC) for whole blood of *Fundulus heteroclitus* acclimated to (A,B) 0, (C,D) 11 and (E,F) 35 ppt exposed to (A,C,E) normoxia or (B,D,F) hypoxia (15 Torr for 3 h), assayed at a PCO$_2$ of 1.9 Torr (solid line) and at a PCO$_2$ of 7.6 Torr (dashed line).
**Figure 2.6** Hb-oxygen affinity ($P_{50}$, Torr) in whole blood of *Fundulus heteroclitus* acclimated to 0, 11 and 35 ppt exposed to normoxia (white bars) or to hypoxia (15 Torr for 3 h) (black bars), assayed at (A) PCO$_2$ of 1.9 Torr and (B) PCO$_2$ of 7.6 Torr. Bars sharing the same lower case letters are not statistically different at the same oxygen level. [Two-way ANOVA p-values: (PCO$_2$ = 1.9 Torr: $p_{\text{interaction}} = .4772$, $p_{\text{oxygen}} = .0046$, $p_{\text{salinity}} = .2153$; PCO$_2$ = 7.6 Torr: $p_{\text{interaction}} = .2447$, $p_{\text{oxygen}} < .0001$, $p_{\text{salinity}} = .0005$). Asterisks indicate significant differences between normoxia and hypoxia at the same salinity (Bonferroni post-hoc test). Data are means ± 1 SEM (n = 6 - 8).
Hematocrit (% RBC)

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[Hb] (mmol/L)

---

MCHC (mmol/L)
Figure 2.7 (A) Blood hematocrit (HCT; % RBC), (B) hemoglobin concentration ([Hb]; mmol/L) and (C) mean cell hemoglobin concentration (MCHC; mmol/L) in Fundulus heteroclitus acclimated to 0, 11 and 35 ppt in normoxia (white bars) and exposed to hypoxia (15 Torr for 3 h) (black bars). Bars sharing the same lower case letters are not statistically different at the same oxygen level. [Two-way ANOVA p-values (HCT: p_{interaction} = .9774, p_{oxygen} = .0004, p_{salinity} = .6009; [Hb]: p_{interaction} = .5733, p_{oxygen} = .1500, p_{salinity} = .0069; MCHC: p_{interaction} = .5865, p_{oxygen} = .0001, p_{salinity} = .08610)]. Asterisks indicate significant differences between normoxia and hypoxia at the same salinity (Bonferroni post-hoc test). Data are means ± 1 SEM (n = 6 - 8).
Lamellar surface area (x10^7 µm^2/g fish)

- 0 ppt
- 11 ppt (b)
- 35 ppt (b)

The values are compared across different salinity levels.
Figure 2.8 Lamellar surface area (x10^7 µm^2/g fish) of the second gill arch in *Fundulus heteroclitus* acclimated to 0, 11 and 35 ppt in normoxia. Bars sharing the same lower case letters are not statistically different (one-way ANOVA). Data are means ± 1 SEM (n = 5 - 6).
Figure 2.9 Representative histological sections of *Fundulus heteroclitus* gill filaments acclimated to (A) 0, (B) 11 and (C) 35 ppt in normoxia. (D) Illustration of gill filament detailing how morphological analysis were performed. Black arrows on micrographs indicate ionocytes. In 0 ppt acclimated fish, the epithelial cell layer covering the lamella is bulging and thicker than in 11 and 35 ppt. The epithelial cell coverage was measured as outlined in yellow on illustration D. The interlamellar cell mass (ILCM) is visible in all three salinities and highlighted in blue in illustration D. Scale bars are 10 µm in size.
**A**

ILCM area ($\mu m^2$)

0 ppt 11 ppt 35 ppt

**B**

Ionocyte density (# cells/$\mu m^2$)

0 ppt 11 ppt 35 ppt

**C**

Epithelial cell coverage ($\mu m^2/\mu m$)

0 ppt 11 ppt 35 ppt
Figure 2.10 (A) Interlamellar cell mass area ($\mu$m$^2$), (B) ionocyte density (#cells/$\mu$m$^2$) and (C) epithelial cell coverage of the lamellae ($\mu$m$^2$/µm) in *Fundulus heteroclitus* acclimated to 0, 11 and 35 ppt in normoxia. Bars sharing the same lower case letters are not statistically different (one-way ANOVA). Data are means ± 1 SEM (n = 6).
Relative $O_2$ extraction efficiency ($\mu$mol $O_2$/g/cm $H_2O$) vs $PO_2$ (Torr) for different salinity levels (11 ppt, 35 ppt, 0 ppt).

- 11 ppt
- 35 ppt
- 0 ppt
Figure 2.11 The effect of water PO$_2$ on the relative extraction efficiency ($\dot{\text{MO}}_2$/ventilatory index; $\mu$mol O$_2$/g/cm H$_2$O) of *Fundulus heteroclitus* acclimated to 0 (circles), 11 (triangles) and 35 (squares) ppt. Data were binned as 5 Torr averages. Data have been calculated from the mean values in Fig. 2.2A,B,C and 2.4A
Routine $O_{2}^{\text{max}} = 8 \mu\text{mol/g/h}$

$K_{m} = 12 \text{Torr}$

$P_{c}^{\text{crit}} = 32 \text{Torr}$

$R_{I} = 0.7$

Perfect regulation

Perfect conformity
**Figure 2.12** Representative framework for the analysis of the \( \dot{M}O_2 \) versus \( PO_2 \) relationships as proposed by Alexander and McMahon (2004) and Mueller and Seymour (2011), and recommended by Wood (2018). The \( \dot{M}O_2 \) data lie between two hypothetical lines of perfect regulation and conformation. The hatched area represents the area under the curve occupied by the relationship (regulation index, RI) relative to the total area between the two lines. A hyperbolic Michaelis-Menten relationship has been fitted to the hypothetical data set, yielding a \( K_m \) of 12 Torr and a Routine \( O_2 \text{max} \) of 8 \( \mu \text{mol/g/h} \). The \( PO_2 \) at which the vertical distance is greatest between the data curve and the line of conformity yields the \( P_{\text{crit}} = 32 \) Torr.
Table 2.1 Mean Michaelis-Menten equation parameters as calculated from the \( \text{MO}_2 \) versus \( \text{PO}_2 \) relationships obtained in single individuals in *Fundulus heteroclitus* acclimated to 0, 11 and 35 ppt. Data are means ± 1 SEM (N = 7 -8).

<table>
<thead>
<tr>
<th></th>
<th>Michaelis-Menten parameters</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Routine ( \text{O}_2^{\text{max}} ) (µmol ( \text{O}_2 )/kg/h)</td>
<td>Km (Torr)</td>
</tr>
<tr>
<td>0 ppt</td>
<td>7.72 ± 0.49</td>
<td>8.8 ± 3.1</td>
</tr>
<tr>
<td>11 ppt</td>
<td>7.20 ± 0.32</td>
<td>3.1 ± 1.4</td>
</tr>
<tr>
<td>35 ppt</td>
<td>9.69 ± 0.62</td>
<td>16.1 ± 4.3</td>
</tr>
</tbody>
</table>
Table 2.2 Ventilatory frequency, ventilatory pressure amplitude and ventilation index in *Fundulus heteroclitus* acclimated to 0, 11 and 35 ppt in normoxia (> 90 Torr), and in severe hypoxia (8 - 0 Torr). Ventilation data sharing the same lower case letters are not statistically different among the salinities (one-way ANOVA) within each oxygen level. Different ventilation parameters were not compared. Data are means ± 1 SEM (n = 11 - 12).

<table>
<thead>
<tr>
<th></th>
<th>Normoxia (&gt; 90 Torr)</th>
<th>Hypoxia (8 - 0 Torr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 ppt</td>
<td>11 ppt</td>
</tr>
<tr>
<td><strong>Frequency</strong></td>
<td>78.73 ± 3.24(^a)</td>
<td>76.35 ± 3.55(^a)</td>
</tr>
<tr>
<td>(breaths/min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Amplitude</strong></td>
<td>1.33 ± 0.13(^a)</td>
<td>0.82 ± 0.13(^b)</td>
</tr>
<tr>
<td>(cmH(_2)O/breath)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Index</strong></td>
<td>109.28 ± 11.49(^a)</td>
<td>66.27 ± 11.41(^b)</td>
</tr>
<tr>
<td>(cmH(_2)O/min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Frequency</strong></td>
<td>83.03 ± 4.29(^x)</td>
<td>85.52 ± 3.78(^x)</td>
</tr>
<tr>
<td>(breaths/min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Amplitude</strong></td>
<td>1.59 ± 0.07(^x)</td>
<td>1.03 ± 0.08(^y)</td>
</tr>
<tr>
<td>(cmH(_2)O/breath)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Index</strong></td>
<td>146.29 ± 9.63(^x)</td>
<td>90.74 ± 14.29(^y)</td>
</tr>
<tr>
<td>(cmH(_2)O/min)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.3 Hill cooperativity coefficients ($n_{50}$) in the whole blood of *Fundulus heteroclitus* acclimated to 0, 11 and 35 ppt, in normoxia or exposed to hypoxia and assayed at a PCO$_2$ of 1.9 Torr or a PCO$_2$ of 7.6 Torr. Means sharing the same lower case letters are not statistically different at the same oxygen level. [Two-way ANOVA p-values: (PCO$_2$ = 1.9 Torr: p$_{interaction}$=.0041, p$_{oxygen}$<.0001, p$_{salinity}$=.0241); (PCO$_2$ = 7.6 Torr: p$_{interaction}$=.1329, p$_{oxygen}$=.0003, p$_{salinity}$=.0264)]. Asterisks indicate significant differences between normoxia and hypoxia at the same salinity (Bonferroni post-hoc test). Normoxia and hypoxia data were only compared within a single PCO$_2$. Data are means ± 1 SEM (n = 6 - 8).

<table>
<thead>
<tr>
<th></th>
<th>PCO$_2$ = 1.9 Torr</th>
<th>PCO$_2$ = 7.6 Torr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normoxia</td>
<td>Hypoxia</td>
</tr>
<tr>
<td>0 ppt</td>
<td>0.91 ± 0.03$^a$</td>
<td>1.21 ± 0.08$^x*$</td>
</tr>
<tr>
<td>11 ppt</td>
<td>1.02 ± 0.07$^{ab}$</td>
<td>1.03 ± 0.03$^y$</td>
</tr>
<tr>
<td>35 ppt</td>
<td>0.78 ± 0.03$^b$</td>
<td>1.04 ± 0.05$^{xy}$</td>
</tr>
</tbody>
</table>
Table 2.4 Effect of PCO$_2$ on the whole blood pH of *Fundulus heteroclitus* acclimated to 0, 11 and 35 ppt, in normoxia or exposed to hypoxia. Means sharing the same lower case letters are not statistically different at the same oxygen level. [Two-way ANOVA p-values: (PCO$_2$ = 1.9 Torr: $p_{interaction}$=.1812, $p_{oxygen}$=.0064, $p_{salinity}$=.1438); (PCO$_2$ = 7.6 Torr: $p_{interaction}$=.0457, $p_{oxygen}$=.0984, $p_{salinity}$=.1785)]. Asterisks indicate significant differences between normoxia and hypoxia at the same salinity (Bonferroni post-hoc test). Normoxia and hypoxia data were only compared within a single PCO$_2$. Data are means ± 1 SEM (n = 6 - 8).

<table>
<thead>
<tr>
<th></th>
<th>PCO$_2$ = 1.9 Torr</th>
<th></th>
<th>PCO$_2$ = 7.6 Torr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normoxia</td>
<td>Hypoxia</td>
<td>Normoxia</td>
</tr>
<tr>
<td>0 ppt</td>
<td>7.53 ± 0.08$^a$</td>
<td>7.35 ± 0.05$^xy$</td>
<td>7.18 ± 0.07$^a$</td>
</tr>
<tr>
<td>11 ppt</td>
<td>7.48 ± 0.07$^a$</td>
<td>7.45 ± 0.04$^x$</td>
<td>7.28 ± 0.05$^a$</td>
</tr>
<tr>
<td>35 ppt</td>
<td>7.48 ± 0.06$^a$</td>
<td>7.16 ± 0.07$^x$</td>
<td>7.26 ± 0.08$^a$</td>
</tr>
</tbody>
</table>
**Table 2.5** Bohr coefficient ($\Phi$) in *Fundulus heteroclitus* acclimated to 0, 11 and 35 ppt in normoxia and exposed to hypoxia. Means sharing the same lower case letters are not statistically different at the same oxygen level. [Two-way ANOVA p-values: (Bohr: $p_{interaction} = .0300$, $p_{oxygen} = .0004$, $p_{salinity} = .1265$)]. Asterisks indicate significant differences between normoxia and hypoxia at the same salinity (Bonferroni post-hoc test). Data are means ± 1 SEM (n = 6 - 8).

<table>
<thead>
<tr>
<th></th>
<th>Bohr coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normoxia</td>
</tr>
<tr>
<td>0 ppt</td>
<td>-0.75 ± 0.09$^a$</td>
</tr>
<tr>
<td>11 ppt</td>
<td>-1.08 ± 0.39$^a$</td>
</tr>
<tr>
<td>35 ppt</td>
<td>-1.14 ± 0.19$^a$</td>
</tr>
</tbody>
</table>
Table 2.6 Gill morphometrics in *Fundulus heteroclitus* acclimated to 0, 11 and 35 ppt in normoxia. Morphometrics data sharing the same lower case letters are not statistically different among the salinities (one-way ANOVA). Different morphometrics parameters were not compared. Data are means ± 1 SEM (n = 5 - 6).

<table>
<thead>
<tr>
<th>Acclimation salinity</th>
<th>0 ppt</th>
<th>11 ppt</th>
<th>35 ppt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamellar height</td>
<td>43.89 ± 0.67&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44.32 ± 2.76&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.08 ± 3.33&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lamellar width</td>
<td>17.68 ± 0.69&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.73 ± 0.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.42 ± 0.47&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Filament length</td>
<td>1.56 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.60 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.51 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lamellar frequency</td>
<td>25.37 ± 1.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.33 ± 0.83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.24 ± 0.75&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(lamellae/mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Filamental lamellar surface area</td>
<td>1.38 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.58 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.40 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(FL&lt;sub&gt;SA&lt;/sub&gt;; x10&lt;sup&gt;6&lt;/sup&gt; µm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number filaments 2&lt;sup&gt;nd&lt;/sup&gt; gill arch</td>
<td>47.66 ± 0.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50.66 ± 0.93&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47.16 ± 1.51&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Chapter 3: The osmorespiratory compromise: the ionoregulatory responses to hypoxia in the euryhaline killifish (*Fundulus heteroclitus*) acclimated to different salinities

3.1 Summary

The classic osmorespiratory compromise is a physiological trade-off between the characteristics of the gill that promote respiratory gas-exchange *versus* those that limit passive fluxes of ions and water. In hypoxia, changes in gill blood flow and functional surface area that increase gas transfer can exacerbate unfavourable ion and water fluxes. We have characterized the ionoregulatory responses of *Fundulus heteroclitus* to acute hypoxia (15 Torr = 2 kPa), under acclimation to fresh water (FW, 0 ppt), isosmotic salinity (11 ppt), and sea water (SW, 35 ppt). Plasma ion concentrations increased only slightly with salinity acclimation, and exposure to hypoxia had minimal effects, indicating maintenance of osmoregulatory homeostasis. Branchial Na$^+$/K$^+$ ATPase and vH$^+$-ATPase activity were reduced by hypoxia in FW, but not at 11 or 35 ppt. Similarly, diffusive water flux rates, measured with $^3$H$_2$O, were reduced in hypoxia only in FW fish. Gill paracellular permeability, measured as [$^3$H]PEG-4000 clearance, was reduced in 0 and 35 ppt acclimated fish, but increased in 11 ppt acclimated fish. Unidirectional Na$^+$ influx and Na$^+$ efflux were both decreased during hypoxia in FW, so that net flux did not change. The net fluxes of Cl$^-$, K$^+$ and ammonia were also attenuated. These reductions appeared to be regulated phenomena of both transcellular and paracellular origin, and fluxes were restored as soon as normoxia was reinstated. In SW (35 ppt), there were small reductions in unidirectional Na$^+$ fluxes, suggesting a similar response to hypoxia as in FW. In summary, the FW and SW killifish do not exhibit the classic osmorespiratory compromise during hypoxia, but rather a reduction in gill permeability, as seen in other hypoxia-tolerant species. However, fish acclimated to the isosmotic salinity increased Na$^+$ influx and efflux rates, as well as [$^3$H]PEG-4000 clearance rates in hypoxia, responses in accord with the concept of the classic osmorespiratory compromise. The salinity-dependent pattern of permeability change is in accord with our
earlier study showing that 0 ppt is the most challenging for respiratory gas exchange during hypoxia, and 11 ppt the most advantageous in this species.

3.2 Introduction

The teleost fish gill is a multifunctional organ that, in addition to playing a central role in acid-base balance and nitrogenous waste excretion, is the primary site for ion and respiratory gas exchange (Evans, 2005). The structural features that enable optimal performance for respiratory gas exchange, such as large surface area and thin diffusion distance, are the very same characteristics that can lead to osmoregulatory impairment. Branchial oxygen exchange can be improved by increasing water flow across the gills (ventilation) and changing the rate and pattern of blood flow through the gills (perfusion). Increased perfusion, often accompanied by a reduced heart rate (bradycardia) and increased cardiac stroke volume, will lead to a recruitment of otherwise unperfused lamellae (Booth, 1978), increasing the functional surface area for diffusion and reducing the blood-to-water diffusion distance, which will tend to increase ion fluxes. The functional trade-off that exists in gill optimal design is termed the osmorespiratory compromise (Randall et al., 1972; Gonzalez and McDonald, 1992).

To date, the osmorespiratory compromise has been relatively well characterized in response to exercise where the metabolic demand for oxygen is increased. Most studies have used highly aerobic species such as the freshwater rainbow trout (*Oncorhynchus mykiss*), where increased swimming activity has led to increased ion and water fluxes (Wood and Randall, 1973a; Wood and Randall, 1973b; Wood and Randall, 1973c; Hofmann and Butler, 1979; Gonzalez and McDonald, 1992; Gonzalez and McDonald, 1994; Postlethwaite and McDonald, 1995; Onukwufo and Wood, 2018) and gill paracellular permeability has been shown to increase in proportion to oxygen uptake (Robertson and Wood, 2014). Surprisingly few studies have investigated this trade-off in the face of hypoxia where oxygen supply is depleted. Rainbow trout, a known hypoxia-intolerant fish, increased gill permeability to ions and increased exposure of ionocytes due to pavement cell retraction when subjected to low oxygen levels, thereby exacerbating ionic losses (Iftikar et al., 2010; Matey et al., 2011;
Robertson et al., 2015b). Increased ion losses during acute hypoxia were also seen in a number of other freshwater species (Robertson et al., 2015a). In marked contrast, the Amazonian oscar (*Astronotus ocellatus*), which is an extremely hypoxia-tolerant fish, exhibits a very different response, in which branchial ion and water fluxes are reduced during hypoxia exposure (Wood et al., 2009; De Boeck et al., 2013; Robertson et al., 2015a). Notably, there is a decrease in ion and water permeability, which occurs even though there is an increase in ventilation and effective oxygen permeability at the gills, as measured by the oxygen transfer factor (Scott et al., 2008a). This decrease in ion and water permeability is associated with a reduction in the exposed surface area of branchial ionocytes through pavement cell modifications (Wood et al., 2009; Matey et al., 2011; De Boeck et al., 2013), thus preventing ion loss without compromising oxygen uptake. The tambaqui (*Colossoma macropomum*) is another hypoxia-tolerant Amazonian species that has been shown to decrease gill ion permeability when exposed to hypoxia (Robertson et al., 2015a).

In sea water, the basis of the osmorespiratory compromise is essentially the same as in fresh water, except that the concentration differences for major ions and osmolality are reversed (internally lower than in the environment). It is thought that fish would face the opposite (net ion loading and water loss) during hypoxia and exercise, given the reversed nature of the ionic gradient between fish and environment (Gonzalez, 2011). To date, very few studies have evaluated this phenomenon in sea water, mostly due to methodological challenges and constraints in measuring ionic fluxes, that require the use of radiotracer techniques which are more complicated and expensive in sea water. Stevens (1972) showed that SW-acclimated *Tilapia mossambica* lost body water after exercise, likely due to increased osmotic movement of water given that the effective exchange area of the gills would have increased. Acute hypoxia exposure reduces the transepithelial potential (TEP) in *Fundulus heteroclitus* acclimated to both 0 and 35 ppt, independently of salinity (Wood and Grosell, 2015). Additionally, hypoxia prevents the rise in TEP that happens upon transfer from fresh water to sea water, a trait that has been suggested to be of adaptive significance (Wood and Grosell, 2009). Recently, it has been shown that the osmorespiratory compromise also occurs in elasmobranchs, where an increased loss of urea (a valuable osmolyte) was seen
when dogfish (*Squalus acanthias suckleyi*) were exposed to either acute hypoxia (Zimmer and Wood, 2014) or acute temperature increases (Giacomin et al., 2017; Chapter 6).

Therefore, the overall goal of this study is to increase our mechanistic understanding of the ionoregulatory aspects of the osmorespiratory compromise during hypoxia in both fresh water and sea water. We chose the Atlantic killifish *Fundulus heteroclitus* (northern subspecies) as our model system, primarily due to its outstanding euryhalinity (Marshall, 2013), in addition to its exceptional tolerance of hypoxia (Cochran and Burnett, 1996; Richards et al., 2008). Killifish are native to estuaries and salt marshes along the east coast of North America from northern Florida to the Gulf of St. Lawrence, Canada, where they often enter freshwater rivers and creeks (Fish and Wildlife Service, 1985). In these environments, these fish can be subjected to frequent, sometimes daily, variations in environmental conditions, including changes in salinity and dissolved oxygen. Therefore, this species has become a model for studies understanding ionoregulatory changes associated with rapid salinity challenges (Wood and Laurent, 2003; Wood, 2011), as well as adaptation to freshwater environments (Scott et al., 2004a). In a previous parallel study (Giacomin et al., Chapter 2) we found that acclimation to fresh water resulted in a marked reduction in hypoxia tolerance, likely due to a decrease in respiratory surface area, increased thickness of the lamellar epithelium, and the presence of a greater interlamellar cell mass in the gills. Acclimation to 0 ppt resulted in a compromise of whole-animal respiratory capacity. Additionally, killifish acclimated to 35 ppt exhibited a higher routine metabolic rate and a lower capacity to regulate O$_2$ uptake under hypoxia than the 11 ppt fish. Of the salinities tested in that study, 11 ppt (essentially isosmotic) proved to be the most advantageous for respiratory gas exchange while 0 ppt was the most challenging.

Our first hypothesis was that killifish, a euryhaline hypoxia-tolerant species, would decrease gill ion permeability in acute hypoxia, reducing the fluxes of ions and other substances in both fresh and sea-water, as seen in the other two hypoxia-tolerant species that have been studied (oscar and tambaqui, discussed earlier), both of which are stenohaline freshwater fish. An additional goal was to evaluate the osmorespiratory compromise at the isosmotic salinity (11 ppt), where theoretically, no net movement of ions and water from the plasma to the environment, and vice versa, should occur. Therefore, we hypothesized that in
killifish acclimated to 11 ppt the negative effects of the osmorespiratory compromise would be minimal, allowing the animal to more fully expand its gills for respiratory gas exchange during hypoxia.

Since the nature of the osmorespiratory compromise may be different in fresh and seawater, we employed a broad range of techniques looking at both transcellular and paracellular gill permeability responses as a function of salinity. We examined plasma ion composition, the activity of key osmoregulatory enzymes, diffusive water flux rates, and an index of paracellular gill permeability (PEG-4000 clearance; Robertson and Wood, 2014). Additionally, we used $^{22}$Na to evaluate the unidirectional Na$^+$ flux rates in fish acclimated to 0, 11, and 35 ppt. These are easier to measure in fresh water (requiring less radiotracer and allowing longer periods of flux measurement; Wood and Laurent, 2003; Wood, 2011). Furthermore, our previous study (Chapter 2) indicated that the impacts of the osmorespiratory compromise are most intense in freshwater-acclimated killifish. Therefore, a more detailed investigation of the unidirectional Na$^+$ flux responses to hypoxia has been carried out in freshwater conditions, for theoretical, technical, and practical reasons.

### 3.3 Material and Methods

#### 3.3.1 Experimental animals

All procedures were approved by the University of British Columbia animal care committee (AUP A14-0251) and complied with the guidelines of the Canada Council for Animal Care. *Fundulus heteroclitus macrolepidotus* (northern subspecies) were collected by beach seine from a brackish estuary near Hampton, NH, USA by Aquatic Research Organisms (Inc.). They were shipped overnight to the University of British Columbia where they were held for at least 6 weeks prior to experimentation, in 120-L glass tanks, containing recirculating, charcoal-filtered dechlorinated Vancouver tap water (0 ppt; [Na$^+$] = 0.09, [Cl$^-$] = 0.10, [Ca$^{2+}$] = 0.13, [Mg$^{2+}$] = 0.01 mmol/L) or fresh water mixed with Instant Ocean Aquarium Salt (Instant Ocean, Spectrum Brands, Blacksburg, VA, USA) up to 11 ppt ([Na$^+$] = 146, [Cl$^-$] = 196, [Ca$^{2+}$] = 4.3, [Mg$^{2+}$] = 21.0 mmol/L) or 35 ppt ([Na$^+$] = 459, [Cl$^-$] = 663,
[Ca^{2+}] = 12.4, [Mg^{2+}] = 53.6 mmol/L). Fish were fed every day with commercial flakes (Nutrafin Max Tropical Flakes) and fasted for at least 24 h prior to experiments. Salinity in the tanks and in the water prepared for experiments was monitored with a conductivity meter (Cond 3310, WTW, Xylem Analytics, Weilhem, Germany). The photoperiod was 12-h light and 12-h dark, and the temperature during both acclimation and experimentation was 18 - 20ºC. Water PO$_2$ was monitored throughout all experiments using a handheld oxygen meter (Accumet AP84, Fisher Scientific, Toronto, ON, CA).

### 3.3.2 The influence of hypoxia and salinity acclimation on blood ion composition and gill ionoregulatory enzymes

Fish (n = 8 at each salinity and O$_2$ level) were held in individual plastic containers modified so that the sides were replaced with a plastic mesh for free water flow. These were placed in 30-L darkened Plexiglas tanks overnight, one for each acclimation salinity (0, 11, and 35 ppt). The water in each 30-L tank was aerated and continuously recirculated by a small submersible water pump. Water in the tanks was replaced prior to the start of the trial. PO$_2$ was either maintained at normoxia (approximately 155 Torr, = 20.7 kPa) or reduced from normoxia to hypoxia (15 ± 2 Torr, = 2 kPa) quickly and maintained at a constant level with bubbling of N$_2$ or air. Fish were kept in hypoxia for 3 h, and at the end of the experiment were immediately euthanized with an overdose of neutralized MS-222 (Syndel Laboratories, Nanaimo, B.C., Canada). Normoxic fish underwent the same protocol, but PO$_2$ was kept at normoxia. Blood samples (~100 µL) were quickly drawn using a modified gastight Hamilton 100 µL-syringe (Reno, NV, USA) by caudal puncture. Two 5-µL microhematocrit tubes were filled with blood and centrifuged at 10000 g for 10 min. The remainder of the blood was also centrifuged (5 min, 10000 g), plasma was separated and then flash-frozen in liquid N$_2$. Additionally, the entire gill basket was excised and also flash-frozen in liquid N$_2$. Plasma and tissue samples were kept at -80ºC until analysis of plasma ion concentrations and osmolality, plasma lactate concentration, and gill Na$^+$/K$^+$ and v-type H$^+$-ATPase activities (described below).
3.3.3 The influence of hypoxia and salinity acclimation on diffusive water flux rates

Separate groups of fish (n = 12 at each salinity and O2 level) were used for this series. Fish were loaded to equilibrium with tritiated water in groups of 6 in 1 L of water at the acclimation salinity, labeled with 20 μCi/L of $^3$H$_2$O (Amersham Pharmacia Biotech, Little Chalfont, UK) overnight. The loading chamber was aerated and shielded from light. Preliminary trials demonstrated that equilibration was in fact complete within 6 h. The fish were quickly rinsed in non-radioactive water, then transferred to individual darkened 250-mL containers, each filled with water at the appropriate salinity. These served as the experimental chambers. Water PO$_2$ had been pre-equilibrated to normoxia (155 Torr) or hypoxia (15 ± 2 Torr) and was maintained at these levels for the following 60 min. Water samples (5 mL) were taken every 5 min for the initial 0.5h, and then every 10 min until the end of the experimental period of 1h. Normoxia was restored and a final water sample was taken at 24 h, a period when the radioactivity in the fish had completely equilibrated with the external water. Scintillation fluor (Optiphase, Perkin Elmer, Waltham, MA, USA) was added to the water samples in a 2:1 (fluor:water) ratio, and samples were stored in the dark for a minimum of 12 h to eliminate chemiluminescence prior to scintillation counting for beta emissions (Beckman Coulter, LS6500, Wellesley, MA, USA). Tests showed that quench was constant.

The diffusive water flux rates were calculated following methods described in Giacomin et al. (2017; Chapter 6) and Onukwufor and Wood (2018). The rate of disappearance of $^3$H$_2$O from the fish was calculated from the $^3$H$_2$O washout from the animal as measured by its appearance in the water. The rate constant of $^3$H$_2$O turnover (k: % body water/h) was calculated as:

$$k = (\ln R_1 - \ln R_2) / (t_1 - t_2) \quad \text{(Eq. 1)}$$

where $R_1$ and $R_2$ are total $^3$H$_2$O radioactivities (cpm) in the fish (see below) at times $t_1$ and $t_2$ (h). Data were only used over the range where efflux was linear (30 - 40 min of the start of experiment). The total volume of the system was estimated as the measured volume of
external water (experimental container) plus the volume of the fish (assuming 1 g = 1 mL). The water sample that was taken at 24 h (after complete washout and equilibration had occurred) plus all radioactivity removed by sampling during the experiment were used to calculate the total amount of $^3$H$_2$O radioactivity that had been loaded into the fish ($R_{\text{total}}$). Therefore, the radioactivity remaining in the fish at each time interval ($R_1, R_2, R_3$) were then calculated by subtracting the $^3$H$_2$O measured in the water from $R_{\text{total}}$ at each time interval. Based on the estimates of Holmes and Donaldson (1969), Isaia (1984) and Olson (1992) where water comprises about 80% of body composition, the percent of body water turned over per hour, was calculated as the product of k multiplied by 80%.

3.3.4 The influence of hypoxia and salinity on gill paracellular permeability and drinking rate

Separate groups of fish (n = 8 at each salinity and O$_2$ level) were used for this series. Gill paracellular permeability was measured as the clearance of radiolabeled polyethylene glycol ([$^3$H]PEG-4000) from the external water, following methods developed by Robertson and Wood (2014). Appearance of radioactivity in the carcass was used as a measurement of gill paracellular permeability, while appearance of radioactivity in the gut was used to measure drinking rate. In preliminary experiments, we found that the minimum period over which reliable measurements could be made was 3 h. To minimize radioisotope usage, experiments were performed on fish in groups of 8, one for each O$_2$ and salinity treatment. The groups were exposed for 3 h in 1 L of water at the acclimation salinity labeled with 25 µCi/L of [$^3$H]PEG-4000 (Amersham). Fish were allowed to settle in the aerated light-shielded experimental container for 2 h in normoxia prior to the addition of radioactivity. After that, PO$_2$ was either maintained at 155 Torr by air bubbling, or quickly reduced to 15 ± 2 Torr by N$_2$ bubbling, and when PO$_2$ reached hypoxic levels, radioisotope was added. Water samples (5 mL) were taken at 0 h, 1.5 h, and 3 h for scintillation counting. At the end of the 3 h, all fish were euthanized by an overdose of neutralized MS-222, rinsed in radioisotope-free water, blotted dry and kept on ice until dissection. The gastrointestinal tract was tied at both ends (to avoid content loss), and excised. The gut and remaining carcass were weighed.
separately, and digested in sealed vials with 2 N HNO\textsubscript{3} for 48 h at 60ºC, with periodic vortexing. The digests were then centrifuged (5000 g for 5 min) and the clear supernatant was aliquoted for scintillation counting. Water and carcass digest samples (2 mL), and gut digest samples (0.2 mL) were dosed with 10 mL of scintillation cocktail (Ultima Gold AB; Perkin Elmer) and read on a scintillation counter (LS6500, Beckman Coulter).

Radioactivities of the tissue digests were quench-corrected to the same counting efficiency as water samples based on a quench curve constructed previously with different amounts of tissue digest.

\[^{\text{3}}\text{H}\text{PEG-4000 gill clearance rate (CR, } \mu\text{L/g/h) as a proxy for branchial paracellular permeability was calculated as follows:}]

\[^{\text{3}}\text{H}\text{PEG-4000 CR} = \frac{\text{cpm in carcass}}{\text{mean water cpm/} \mu\text{L x W x T}} \]  \quad (Eq. 2)

where cpm in carcass is the total amount of radioactivity in the carcass, W is the fish body mass (g) and T is time (h).

\[^{\text{3}}\text{H}\text{PEG-4000 drinking rate (DR, } \mu\text{L/g/h) was calculated in a similar manner as follows:}]

\[^{\text{3}}\text{H}\text{PEG-4000 DR} = \frac{\text{cpm in gut}}{\text{mean water cpm/} \mu\text{L x W x T}} \]  \quad (Eq. 3)

where cpm in gut is the total amount of radioactivity in the gut.

3.3.5 Unidirectional and net sodium flux rates in \textit{Fundulus heteroclitus} acclimated to 0, 11 and 35 ppt

Separate groups of fish (n = 15 – 17 at each salinity and O\textsubscript{2} level) were used for this series. For all experimental flux measurements, fish were held in individual 250-mL plastic containers, with a hole drilled at the 250-mL mark to facilitate water changes. Each container was served with aeration tubing, darkened with black plastic and partially submerged in a wet table to ensure temperature control. The surface of the water was covered in plastic so to
avoid oxygen diffusion from the air, and to prevent the fish from performing aquatic surface respiration. All approaches described below were employed to meet the criteria outlined in Wood (2011) where the specific activity (SA: ratio of radioisotopic Na to “cold” Na) in the compartment where radioactivity appearance was being monitored, either water or fish, remained less than 10% of the SA in the compartment to which radioactivity was added. The difficulty of meeting this criterion when measuring $^{22}$Na fluxes in sea water have constrained our abilities to replicate in sea water, all the experiments performed in fresh water. Therefore, our investigation of the unidirectional flux responses to hypoxia is more detailed in fresh water than in sea water. All $^{22}$Na measurements (gamma counting) were made with a gamma counter (1470 Wallac-Wizard, Perkin Elmer).

3.3.5.1 Unidirectional, net Na$^+$ fluxes and net Cl$, K^+$ and ammonia fluxes in freshwater-acclimated killifish

In fresh water, Na$^+$ influx ($J_{\text{Na}^+\text{influx}}$) was measured by monitoring the disappearance of radioactivity from the water. To that end, fish were transferred to the containers described above and allowed to acclimate overnight under flow-through conditions. Prior to the start of the experiment, flow-through ceased, water level was set at 250 mL, and 5 µCi $^{22}$Na was added to the water. Water samples (5 mL) were collected hourly for 3 h and stored for measurements of radioactivity, total Na$^+$, Cl$, K^+$ and ammonia concentrations.

At 3 h, the water was replaced with water that had been pre-equilibrated with N$_2$ to 15 ± 2 Torr (hypoxia) and spiked with the same amount of $^{22}$Na. Water samples (5 mL) were collected again hourly for another 3 h. At the end of the hypoxia exposure time, the containers were quickly bubbled with air and normoxia was restored within 5 min. Recovery trials lasted for another 3 h. A control group was also performed, where all the experimental procedures were conducted similarly, with the exception that the animals were maintained in normoxia throughout the whole 9-h duration of the trial. Fish were weighed at the end of the experiment. At each 1-h period, the unidirectional Na$^+$ influx rate (nmol/g/h) was calculated as:
\[ J^{Na^+}_{\text{influx}} = [(cpm_i - cpm_f) \times V] / (\text{mean } SA_{\text{ext}} \times T \times W) \]  
(Eq. 4)

where, cpm\(_i\) and cpm\(_f\) are the \(^{22}\text{Na}\) counts per mL in the water at the start and end of each 1 h period respectively, \(V\) is the volume in each period (L), mean \(SA_{\text{ext}}\) is the mean external specific activity of \(Na^+\) (cpm/nmol), \(T\) is time (h) and \(W\) is the body mass (g).

\(Na^+\) net flux rates (nmol/g/h) were determined from the concentrations of total “cold” \(Na^+\) in the water (nmol/L) and calculated using the following equation:

\[ J^{Na^+}_{\text{net flux}} = [(\left[Na^+\right]_i - \left[Na^+\right]_f) \times V] / (T \times W) \]  
(Eq. 5)

where \(\left[Na^+\right]_i\) and \(\left[Na^+\right]_f\) are the \(Na^+\) concentration the water (nmol/L) at the start and end of each 1 h period respectively, \(V\) is the volume in each period (L), \(T\) is time (h) and \(W\) is the body mass (g).

Unidirectional \(Na^+\) efflux rates (nmol/g/h) were calculated as follow:

\[ J^{Na^+}_{\text{efflux}} = J^{Na^+}_{\text{net flux}} - J^{Na^+}_{\text{influx}} \]  
(Eq. 6)

where \(J^{Na^+}_{\text{net flux}}\) was calculated by equation 5 and \(J^{Na^+}_{\text{influx}}\) by equation 4.

\(Cl^-\), \(K^+\), and total ammonia (\(NH_3 + NH_4^+\)) net flux rates (nmol/g/h) were determined from the concentrations in the water (nmol/L) and calculated using an equation analogous to equation 5.

3.3.5.2 Unidirectional \(Na^+\) influx in seawater-acclimated (11 and 35 ppt) killifish

Separate groups of fish (\(n = 15 - 17\) at each \(O_2\) and salinity level) were used. In killifish acclimated to 35 ppt or 11 ppt, Na influx (\(J^{Na^+}_{\text{influx}}\)) was determined by monitoring the appearance of radioactivity in the fish. In order to avoid radiotracer cycling and maintain the 10% SA criterion (see above), a flux period of 0.5 h was employed (Wood and Laurent, 2003; Wood, 2011). Fish were allowed to settle in the containers described above overnight under flow-through conditions, and water volume was set to 250 mL prior to start of the
experiment with normoxic (155 Torr) or hypoxic (15 ± 2 Torr) water. Fish were exposed to these conditions for 2.5 h in order to mimic the duration of the hypoxia exposure employed in fish acclimated to 0 ppt. At 2.5 h, 10 μCi $^{22}$Na was added to the water. Water samples were taken and at the start and at the end of the 0.5 h flux period and stored for measurements of radioactivity and total Na$^+$ concentration. The fish were then rinsed in non-radioactive sea water of the appropriate salinity for 1 min, euthanized in neutralized MS-222 (0.5 g/L), blotted dry, weighed and processed for measurements of whole body radioactivity ($^{22}$Na). Unidirectional influx rates (nmol/g/h) were calculated as follows:

$$J_{Na^+ in flux} = \frac{\Sigma_{(cpm \text{ in fish})}}{\text{(mean } SA_{ext} \times T \times W)} \quad (\text{Eq. 7})$$

where $\Sigma_{(cpm \text{ in fish})}$ is the total $^{22}$Na radioactivity in the fish (cpm), mean $SA_{ext}$ is the mean external specific activity of Na$^+$ (cpm/nmol), T is time (h) and W is the body mass (g).

### 3.3.5.3 Unidirectional Na$^+$ efflux in seawater-acclimated (11 and 35 ppt) killifish

Separate groups of fish ($n = 15 - 17$ at each O$_2$ and salinity level) were used. Similar procedures were carried out in fish acclimated to 11 and 35 ppt. Na$^+$ efflux ($J_{Na^+ efflux}$) was determined by loading the fish with $^{22}$Na, and then monitoring the appearance of radioactivity in the water. Fish were incubated overnight in batches of 8 in 80 μCi $^{22}$Na/L at the salinity of acclimation, in a 1.5-L Erlenmeyer glass container, in order to load radioactivity into the fish. The loading chamber was aerated and shielded from light. Previous tests show that a minimum of 10 h was needed for complete equilibration between the fish and the external media. After incubation, fish were quickly rinsed in non-radioactive water, then transferred to the same experimental chambers as described above, each containing 250 mL of sea water at the appropriate salinity. Water samples were taken every 0.5 h for 1.5 h, and then either normoxic (155 Torr) or hypoxic (15 ± 2 Torr) water was used to replace the water in the chambers. The volume was again adjusted to 250 mL, and additional water samples were taken every 0.5 h for a further 3 h. This was done as to provide the fish with the same time frame of hypoxia exposure as described for fish.
acclimated to 0 ppt. At the end of the experiment, fish were rinsed in non-radioactive water for 1 min, euthanized in neutralized MS-222 (0.5 g/L), blotted dry, weighed and processed for measurements of whole body radioactivity (\(^{22}\)Na). Unidirectional efflux rates were calculated as follows (Wood and Laurent, 2003):

\[
J_{Na^{+}\text{efflux}} = \left[\frac{k \times \Sigma Z \times F}{W}\right]
\]  
(Eq. 8)

where \(k\) is the rate constant of radioactivity lost (% exchangeable whole body Na\(^{+}\) pool/h) by the fish per unit time, \(\Sigma Z\) is the total internal Na\(^{+}\) pool (nmol/g), \(F\) is the fractional labeling of that pool by the radioisotope and \(W\) is the body mass (g). \(\Sigma Z\) and \(F\) were obtained from Wood and Laurent (2003). The total radioactivity appearance in the water, including that removed in water sampling, when added to the radioactivity measured in the fish carcass, yielded the total radioactivity in the fish at the start of the experiment. The slope of the natural logarithm of the loss of this radioactivity versus time provides the rate constant (\(k\)) of radioactivity lost, and it was calculated using an equation similar to Eq. 1. Only data obtained after the water replacement were used.

Ammonia net flux rate (nmol/g/h) was determined from the measured concentrations of ammonia (NH\(_3\) and NH\(_4^{+}\)) in the water and calculated as described for freshwater trials above. Net flux rates of Na\(^{+}\), K\(^{+}\), and Cl\(^{-}\) could not be measured in seawater-acclimated fish due to the background concentrations being too high to reliably detect changes caused by the fish.

### 3.3.6 Analytical techniques

Water Na\(^{+}\) and K\(^{+}\) concentrations (nmol/L), and plasma Na\(^{+}\) concentrations (mmol/L) were determined by atomic absorption (1275 Atomic Absorption Spectrophotometer, Varian, Mulgrave, Victoria, Australia) using certified commercial standards (Fluka Analytical, Sigma-Aldrich, St. Louis, MO, USA). Plasma osmolality (mOsm/kg) was measured using a Wescor vapor pressure osmometer and standards (Wescor 5100C, Logan, UT, USA). Water (\(\mu\)mol/L) and plasma (mmol/L) Cl\(^{-}\) were determined colorimetrically using the mercury-
thiocyanate method described by Zall et al. (1956). Ammonia concentration (µmol/L) in the water was measured using the colorimetric method of Verdouw et al. (1978).

Plasma lactate concentration (mmol/L) was determined as described by Bergmeyer (1983). Briefly, plasma samples were deproteinized with 8% perchloric acid, centrifuged briefly to precipitate proteins, and assayed in triplicate on a microplate spectrophotometer (SpectraMAX Plus; Molecular Devices, Menlo Park, CA, USA) with a reaction buffer solution (0.18 M glycine, 0.15 M hydrazine, 0.015 βNAD+, 15 U/mL lactate dehydrogenase, pH 9.4). The reaction mixture was incubated for 60 minutes at 37ºC, and lactate concentration was determined against a standard curve.

Na⁺/K⁺-ATPase and v-type H⁺-ATPase activities (µmol ADP/mg protein/h) were measured in gill tissues according to protocols described in McCormick (1993) and Lin and Randall (1993) respectively. Tissues were homogenized (1:10 w/v) in ice-cold buffer solution [50 mM imidazole, 125 mM sucrose and 5 mM EGTA (pH 7.3)], then centrifuged (5000 g, 3 min, 4 ºC), and supernatants separated and kept on ice throughout the analysis. All samples were assayed within 2 h of thawing and homogenization. Ouabain and N-ethylmaleimide (NEM) were used as inhibitors of Na⁺/K⁺-ATPase and H⁺-ATPase, respectively, with sodium azide (NaN₃) as inhibitor of ATP synthase. Enzyme activity was calculated by the difference between the amount of adenosine diphosphate (ADP) produced in control and inhibited reactions. Absorbance was monitored at 10 s intervals over 5 min (25ºC) on the same microplate spectrophotometer. Protein concentration on homogenates was determined using Bradford Reagent (Sigma Aldrich).

For the measurement of hematocrit, micro-hematocrit tubes (2 x 5 µL) were centrifuged (10000 g, 10 min,) and hematocrit (HCT) was measured as the percentage of packed RBCs (%RBC) within the total blood volume.

3.3.7 Statistical analyses

All data are shown as means ± 1 SEM (n = number of animals). Assumptions of parametric statistics (data normality and homogeneity of variances) were checked, and if not achieved, data were transformed using a log transformation. Detailed results of the statistical
tests and post-hoc analysis are shown in specific figure captions. One-way ANOVA was followed by a Tukey post hoc test. For the one-way ANOVA and t-test, mean values were considered significantly different when p < 0.05, while for the Bonferroni post-hoc tests following two-way ANOVA, the Bonferroni correction for multiple comparisons was applied.

3.4 Results

3.4.1 The influence of hypoxia and salinity on plasma ions and enzyme activity

Salinity acclimation had small but significant overall effects on plasma ions and osmolality (Fig. 3.1A,B,C). Under normoxia, plasma [Na\(^+\)] was significantly higher (~150 mmol/L) in fish acclimated to 35 ppt, than in fish acclimated to 0 ppt (~135 mmol/L) (Fig. 3.1A). Plasma [Na\(^+\)] was not significantly different between 11 and 35 ppt acclimated fish. Plasma [Na\(^+\)] was not affected by 3 h of hypoxia exposure at any of the acclimation salinities (Fig. 3.1A). There was no significant effect of hypoxia on plasma [Cl\(^-\)] at all salinities (Fig. 3.1B). In normoxia, plasma Cl\(^-\) followed a similar trend as plasma [Na\(^+\)], where 11 ppt acclimated fish showed a intermediate value between 0 and 35 ppt acclimated fish (~110 and ~140 mmol/L respectively), which were statistically different from each other (Fig. 3.1B).

There was no significant interaction between salinity and oxygen for both plasma ion concentrations (Fig. 3.1A,B). Plasma osmolality was similar in fish acclimated to 11 and 35 ppt (~ 375 mOsm/kg) and both were significantly higher than fish at 0 ppt (~ 340 mOsm/kg; Fig. 3.1C). There was no significant effect of hypoxia and no significant interaction between oxygen and salinity on plasma osmolality (Fig. 3.1C).

Hypoxia exposure had a significant overall effect on both gill Na\(^+\)/K\(^+\)- and H\(^+\)-ATPase activities (Fig. 3.2A,B). Although fish acclimated to 0 ppt showed the highest Na\(^+\)K\(^-\)-ATPase activity (~1.9 µmol ADP/mg protein/h) in normoxia, there was no overall significant effect of salinity (Fig. 3.2A). Na\(^+\)K\(^-\)-ATPase activity was significantly inhibited by 3 h of hypoxia exposure in fish acclimated to 0 ppt; decreases at the other two salinities were not significant (Fig. 3.2A). There was no overall significant effect of salinity on H\(^+\)-
3.4.2 The influence of hypoxia and salinity on blood hematocrit and plasma lactate

Hypoxia exposure had a significant overall effect on blood HCT, while salinity acclimation did not (Fig. 3.3A). In normoxia, fish acclimated to 11 ppt had the lowest HCT levels, and there was a small significant difference in hematocrit levels between fish acclimated to 11 and 35 ppt. At all salinities, hypoxia exposure caused an increase in blood HCT, which was significantly different than normoxia at 11 and 35 ppt (Fig. 3.3A). At 11 ppt, fish exposed to hypoxia increased blood HCT by about 1.3 fold. At 0 ppt acclimation, the non-significant increase in HCT caused by hypoxia was the smallest (1.1-fold) in comparison to other salinities (Fig. 3.3A).

There were significant overall effects of both salinity and hypoxia on plasma lactate concentration (Fig. 3.3B). Plasma lactate increased after hypoxia exposure in fish acclimated to all three salinities, although these differences were significant only at 0 and 11 ppt (Fig. 3.3B). Fish acclimated to 11 ppt showed the largest elevation in plasma lactate between normoxia and hypoxia exposure (60%), followed by fish acclimated to 0 ppt (46%) and 35 ppt (31%; Fig. 3.3B).

3.4.3 The influence of hypoxia and salinity acclimation on the diffusive water flux rates

Salinity acclimation had a significant overall effect on the diffusive water flux rate (Fig. 3.4). Water flux rate was the highest (~75% of the body water pool/h) in fish acclimated to 0 ppt, and significantly different from fish acclimated to 11 and 35 ppt, which had similar rates (~46 - 38%/h; Fig. 3.4). There was a significant decrease (25%) with hypoxia exposure
in the water flux rate of fish acclimated to 0 ppt, but no effect of hypoxia in fish acclimated to 11 and 35 ppt (Fig. 3.4). While there was no significant overall effect of oxygen on the diffusive water flux rates, there was a strong significant interaction between oxygen and salinity (Fig. 3.4).

3.4.4 The influence of hypoxia and salinity on gill paracellular permeability and drinking rate

There was no overall effect of salinity on gill clearance rates of PEG-4000, an index of gill paracellular permeability, and under normoxia, fish acclimated to 0 and 35 ppt exhibited very similar rates (~ 2.5 µL/g/h), while 11 ppt acclimated fish had a significantly lower rate than the other two salinities (~ 1.0 µL/g/h) (Fig. 3.5A). Hypoxia exposure did not have an overall effect on the PEG-4000 gill clearance rate (Fig. 3.5A). In 0 and 35 ppt acclimated fish, hypoxia exposure slightly reduced gill clearance rate while at 11 ppt, hypoxia exposure significantly elevated gill clearance rate (Fig. 3.5A).

Both salinity acclimation and hypoxia exposure had significant overall effects on drinking rate (Fig. 3.5B). Under normoxic conditions, drinking rate was the lowest at fish acclimated to 0 ppt (~ 0.15 µL/g/h), and approximately 40-fold higher (~ 6.1 µL/g/h) in fish acclimated to 35ppt (Fig. 3.5B). At both 11 and 35 ppt, drinking rate was decreased with hypoxia exposure (by 76% at 11 ppt), but this was only statistically significant at 35 ppt (by 88%) (Fig. 3.5B).

3.4.5 The influence of hypoxia and salinity on unidirectional flux rates of Na$^+$, and net flux rates of Cl$^-$, K$^+$, and ammonia

In fresh water, there were no significant changes in unidirectional flux rates of Na$^+$ or the net flux rates of Cl$^-$, K$^+$, and ammonia in the fish subjected to the normoxic control sham procedures (data not shown). Killifish acclimated to 0 ppt exhibited an average unidirectional Na$^+$ influx rate of 310 nmol/g/h in normoxia (Fig. 3.6A). In the first hour after exposure to hypoxia, Na$^+$ influx rate did not change, but it decreased significantly by about 55% for the
remainder of the hypoxia exposure (Fig. 3.6A). \( \text{Na}^+ \) influx rates returned to pre-exposure levels as soon as fish were returned to normoxic \( \text{PO}_2 \) (Fig. 3.6A). The unidirectional \( \text{Na}^+ \) efflux rate showed a similar response pattern as seen for the influx, with an overall reduction in the second and third hours of exposure to hypoxia, with recovery back close to control levels upon re-instatement of normoxia (Fig. 3.6B). In normoxia, the average efflux rate of \( \text{Na}^+ \) was about -300 nmol/g/h, and during exposure to hypoxia, it decreased significantly to about -150 nmol/g/h (50% reduction) at the second and to about 190 nmol/g/h (36% reduction) at third hour (Fig. 3.6B). The resulting net flux rate of fish in normoxia was a loss of ~20 nmol/g/h while in the second and third hours of hypoxia exposure it was a loss of ~25 nmol/g/h – i.e. essentially unchanged from the normoxic control rate (Fig. 3.6C). There were no significant differences among the net flux rates in hypoxia, normoxia, and recovery (Fig. 3.6C).

In normoxia, the average net \( \text{Cl}^- \) flux rate was -39 nmol/g/h, while in the second and third hour of exposure to hypoxia, this rate was significantly reversed to about +100 nmol/g/h (Fig. 3.7A). In the first hour of exposure to hypoxia, the \( \text{Cl}^- \) flux rate did not change. \( \text{Cl}^- \) net flux rates returned to pre-exposure levels once normoxic \( \text{PO}_2s \) were restored (6 – 9h; Fig. 3.7A).

In normoxia, the net \( \text{K}^+ \) flux was about -20 nmol/g/h and during hypoxia exposure, it followed the same pattern as seen for unidirectional rates of \( \text{Na}^+ \) flux, and net \( \text{Cl}^- \) flux rate, where the rates were unchanged in the first hour, but decreased substantially (by about 70%) in the second and third hours (Fig. 3.7B). In recovery, the \( \text{K}^+ \) flux rate was restored to levels similar to the ones in normoxia (Fig. 3.7B).

The ammonia excretion rate was about -350 nmol/g/h in fish in normoxia, and it significantly decreased by about 60% in hypoxia-exposed fish, immediately after transfer to hypoxia (Fig. 3.7C). During the 3 h of hypoxia exposure, ammonia excretion rates did not change, and increased immediately back to control levels after normoxia was restored (Fig. 3.7C).

In sea water, due to the technical constraints mentioned earlier, only single values were obtained for unidirectional flux rates of \( \text{Na}^+ \) during normoxia and hypoxia, and net flux rates of \( \text{Na}^+ \), \( \text{K}^+ \), and \( \text{Cl}^- \) could not be measured. Therefore for reference purposes, in Fig. 3.8,
we have shown the overall time-averaged unidirectional Na\(^+\) flux rates for normoxia and hypoxia of killifish acclimated to 0 ppt (from Fig. 3.6A,B) in order to make comparisons with fish acclimated to 11 and 35 ppt (Fig. 3.8A,B). Note that the scale is broken to accommodate the wide range of values.

On average, 0 ppt acclimated fish had a unidirectional Na\(^+\) influx rate of +310 nmol/g/h while in hypoxia this rate decreased to +210 nmol/g/h (Fig. 3.8A). The average Na\(^+\) efflux rate was -300 nmol/g/h while in hypoxia it decreased to -250 nmol/g/h (Fig. 3.8B). Fish acclimated to 11 ppt in normoxia showed a many-fold higher unidirectional Na\(^+\) influx rate of +6400 nmol/g/h while in hypoxia this rate increased to +16000 nmol/g/h (150% increase) (Fig. 3.8A). There was also a 92% increase in the unidirectional Na\(^+\) efflux rate in 11 ppt fish exposed to hypoxia (-5400 nmol/g/h) in comparison to normoxic fish (-2800 nmol/g/h; Fig. 3.8B). Fish acclimated to 35 ppt had even higher unidirectional Na\(^+\) flux rates. The Na\(^+\) influx rate in normoxia was about +22000 nmol/g/h, which decreased significantly by 13% when fish were exposed to hypoxia (Fig. 3.8A). In normoxia, the unidirectional efflux rate of Na\(^+\) was approximately -12000 nmol/g/h, and when exposed to hypoxia, it decreased by 16% to about -10000 nmol/g/h, although this change was not significant (Fig. 3.8B). Note that although the absolute unidirectional Na\(^+\) flux rates were more than an order of magnitude higher in fish acclimated to 35 ppt, the overall patterns of response to hypoxia were similar to those in fish acclimated to fresh water (Fig. 3.8). However, they were different from those in fish acclimated to the isosmotic salinity (11 ppt), where fluxes increased rather than decreased during hypoxia.

Figure 3.9 shows the averaged ammonia excretion rates in normoxia versus hypoxia of killifish acclimated to 0 ppt (Fig. 3.9A) in order to facilitate comparison with fish acclimated to 11 ppt (Fig. 3.9B) and 35 ppt (Fig. 3.9C). Rates did not differ significantly among the three salinities, and similar overall reductions were seen during hypoxia (54% at 0 ppt, 33% at 11 ppt, and 45% at 35 ppt.)
3.5 Discussion

3.5.1 Overview

Our initial hypothesis was that the killifish, a euryhaline and hypoxia-tolerant species, would decrease gill permeability upon exposure to hypoxia, reducing the fluxes of ions and water in both fresh water and full strength (35 ppt) sea water. We assessed gill permeability through measurements of the fluxes of ions (Na\(^+\), Cl\(^-\), K\(^+\), ammonia, \(^{3}\text{H}_2\text{O}\)), and a paracellular permeability marker (\([^{3}\text{H}]\text{PEG-4000}\)). Our hypothesis was confirmed for fish acclimated to 0 and 35 ppt. Na\(^+\) efflux rate decreased rather than increased in hypoxia in the freshwater-acclimated killifish. Additionally, active Na\(^+\) influx rate, gill Na\(^+\)/K\(^+\)-ATPase and \(\nu\)-type H\(^+\)-ATPase activities, ammonia efflux rate, net K\(^+\) and Cl\(^-\) loss rates, and diffusive water flux rate were all significantly reduced in hypoxia while gill \([^{3}\text{H}]\text{PEG-4000}\) clearance rate exhibited a non-significant decline. As discussed subsequently, the responses of this suite of markers suggests that both transcellular and paracellular permeability were reduced during hypoxia as part of a regulated process in freshwater animals. Earlier, we have shown that fresh water is the most challenging of the three salinities for O\(_2\) uptake during hypoxia in *F. heteroclitus* (Chapter 2), so it is perhaps not surprising that this coincides with the greatest modifications of gill iono- and osmoregulatory functions during hypoxia. At 35 ppt, the Na\(^+\) gradients are reversed, and Na\(^+\) fluxes are more complicated due to the presence of a large exchange diffusion component, but again both Na\(^+\) influx and efflux declined, and there was a non-significant decrease in \([^{3}\text{H}]\text{PEG-4000}\) permeability in accord with our hypothesis, though other indicators did not change. Our second hypothesis was that killifish acclimated to the isosmotic salinity (11 ppt) would be able to more fully expand their gills for respiratory gas exchange during hypoxia and would show minimal effects of the osmoregulatory compromise. Unidirectional Na\(^+\) influx and efflux rates increased in accord with our hypothesis, as well as \([^{3}\text{H}]\text{PEG-4000}\) clearance rate. Diffusive water flux rate did not change with hypoxia exposure. Furthermore, plasma lactate accumulation was greatest at this salinity, contrary to expectation. Interestingly, at all three salinities, plasma ions and osmolality were largely unaffected by hypoxia exposure, indicating that despite the various
disturbances seen in the ion and water fluxes, *F. heteroclitus* are capable of maintaining osmoregulatory homeostasis under hypoxic challenge in diverse environments.

### 3.5.2 The effects of salinity acclimation and hypoxia exposure on plasma ion homeostasis

Salinity acclimation had only a small overall effect on the plasma concentrations of Na\(^+\) and Cl\(^-\) and on plasma osmolality (Fig. 3.1). In their natural habitat, *F. heteroclitus* can encounter transient fluctuations in salinity as well as seasonal variations, therefore the ability to maintain ionoregulatory homeostasis is likely an adaptation for this unpredictable environment. Plasma [Na\(^+\)] and [Cl\(^-\)] values reported here are in accordance with previous reports of killifish acclimated to 10 ppt and then transferred to fresh or full strength sea water, where after 30 days of transfer, differences in plasma ions were minimal (Scott et al., 2004a; Scott et al., 2004b).

Plasma ion concentration and osmolality were not significantly affected by hypoxia exposure in killifish at the three salinities tested (Fig. 3.1), indicating a remarkable homeostatic regulation of these parameters, despite diverse changes in associated parameters such as flux rates and transport enzyme activities. In previous studies, an increase in plasma [Na\(^+\)] with hypoxia was observed for two contrasting species, the Amazonian oscar (*Astronotus ocellatus*), a hypoxia-tolerant species (Wood et al., 2007a), and the rainbow trout (*Oncorhynchus mykiss*), a hypoxia-intolerant species (Thomas et al., 1986). In both studies, the slight increase in plasma [Na\(^+\)] was attributed to a fluid shift towards the intracellular compartment as well as a movement of Na\(^+\) in order to balance the increased lactate in the plasma (Thomas et al., 1986). In our study, we observed an increase in plasma lactate concentration in response to hypoxia in all three salinities (Fig. 3.3B). Although blood acid-base status was not measured in the present study, Giacomin et al. (Chapter 2) reported that comparable hypoxia exposure caused a reduction in blood pH. Therefore it is likely that killifish are able to deal with a hypoxia-induced acidosis by employing mechanisms that are independent of the modulation of plasma ions. Blood hematocrit increased with exposure to hypoxia at all three salinities, consistent with findings of our previous study where it was
shown to be almost entirely due to RBC swelling rather than recruitment of stored RBCs. (Giacomin et al., Chapter 2). This serves to improve the intracellular conditions for Hb-O$_2$ binding, and is typical of hypoxia-exposed fish (Wells, 2009).

3.5.3 The effects of salinity acclimation and hypoxia exposure on branchial Na$^+/K^+$- and v-type H$^+$-ATPase activities

The fundamental problem imposed by hypoxia exposure is the maintenance of metabolic energy balance. A decrease in blood O$_2$ content directly impacts mitochondrial energy production (in the form of ATP) from oxidative phosphorylation (Boutilier and St-Pierre, 2000). Hochachka et al. (1996) suggested that maintenance of electrical gradients through Na$^+/K^+$-ATPase is amongst the most energetically costly processes performed by a cell. Therefore, the marked reduction in both Na$^+/K^+$- and v-type H$^+$-ATPase upon hypoxia exposure (Fig. 3.2A,B) would have clear adaptive value in terms of energy conservation. Notably killifish at 11 and 35 ppt did not show this response (Fig. 3.2A,B), suggesting another fundamental difference between fish acclimated to fresh and sea water. Once more we see similarities between the freshwater-acclimated killifish and the Amazonian oscar (A. ocellatus), where Na$^+/K^+$-ATPase activity was similarly downregulated after 3 h of exposure to hypoxia (Richards et al., 2007; Wood et al., 2007a). In our previous study (Chapter 2), we have shown that 0 ppt acclimated fish have a reduced respiratory capacity in hypoxia relative to their seawater-acclimated counterparts, therefore it is expected that a different strategy is employed in dealing with the osmorespiratory compromise. We suggest that the downregulation in enzyme activities shown in fish acclimated to 0 ppt could be a reflection of the compromised respiratory ability in these fishes (Chapter 2), a strategy to conserve energy, possibly in concert with a simultaneous downregulation of gill permeability, so that plasma ion concentrations were maintained and whole-animal ion balance remained unaffected. The absence of this phenomenon in killifish acclimated to 11 ppt and 35 ppt (Fig. 3.2) is in accord with the lesser respiratory difficulties that these animals face during hypoxia (Giacomin et al., Chapter 2).
3.5.4 The effects of salinity acclimation and hypoxia exposure on diffusive water flux rates, paracellular permeability, and drinking rate

Our starting hypothesis was that *F. heteroclitus*, a euryhaline and hypoxia-tolerant species, would decrease gill ion and water permeability when exposed to hypoxia. Indeed, in confirmation, we have seen a reduction in the flux of tritiated water across the gills, which was salinity-dependent, occurring only in fresh water (Fig. 3.4). Tritiated water fluxes represent unidirectional diffusive water fluxes, and are approximately 100-fold larger than osmotic water fluxes, which are net water fluxes usually estimated from body weight changes and urine flow (Stevens, 1972; Isaia, 1984). Diffusive water fluxes are thought to mainly occur mainly by the transcellular route, through cell membranes (Evans, 1969; Isaia, 1984), very likely through aquaporins (Evans et al. 2005), whereas osmotic water fluxes may occur by both routes. In general, marine teleosts have lower gill permeability to water than fresh water teleosts (Evans, 1969), and the water permeability of euryhaline fishes decreases with transfer to sea water (Potts and Fleming, 1970). This appears to correlate with lower expression of levels of aquaporins at higher salinities (reviewed by Cutler et al., 2007; Madsen et al., 2015; Breves et al., 2016). Our data are in agreement with this trend, where in normoxia, fish that were acclimated to 0 ppt showed higher water permeability than their seawater-acclimated counterparts; surprisingly, there was no difference between fish acclimated to the isosmotic salinity (11 ppt) and 35 ppt (Fig. 3.4). The decrease in diffusive water flux at 0 ppt during hypoxia but not at 11 ppt and 35 ppt may suggest the involvement of aquaporins in this response.

Gill permeability and consequently diffusive water flux rates increased markedly in both goldfish (*Carassius auratus*) and the rainbow trout (*Oncorhynchus mykiss*) during exposure to moderate hypoxia - the classic osmorespiratory compromise response (Loretz, 1979; Onukwufor and Wood, 2018). Interestingly, when freshwater rainbow trout were exposed to continuing severe hypoxia, water flux rates returned to control levels, indicating some regulatory ability, perhaps involving aquaporins (Onukwufor and Wood, 2018). Our data for the freshwater-acclimated killifish are comparable to observations on the Amazonian oscar, which was able to reduce both diffusive and osmotic water fluxes during hypoxia.
(Wood et al., 2009), while maintaining effective branchial oxygen permeability high (Scott et al., 2008a). The explanation offered by the authors was that there was an adaptive reduction in transcellular permeability, due to the observed paving over of the ionocytes by the pavement cells (PVCs), in addition to channel (e.g. aquaporin) closure (Wood et al., 2009; Matey et al., 2011). Thus, we speculate that this ability to similarly downregulate gill water permeability in the freshwater-acclimated killifish could be an adaptation to frequent variations in O$_2$ in the freshwater environments inhabited by these fishes (Layman et al., 2000; Smith and Able, 2003).

Interestingly, 11 ppt acclimated fish in normoxic conditions had a significantly lower gill paracellular permeability in comparison to their 0 and 35 ppt acclimated counterparts. At the isosmotic salinity, it is predicted that the need for active Na$^+$ excretion would be minimized as gradients are lower. Therefore, maintenance of a pathway of high paracellular permeability for active Na$^+$ efflux (Wood and Marshall, 1994) would not be necessary. Upon hypoxia exposure, in fish acclimated to 0 and 35 ppt there appeared to be a reduction in paracellular permeability, while fish acclimated to the isosmotic medium (11 ppt) significantly elevated paracellular permeability (Fig. 3.5A). Polyethylene glycol MW-4000 ([³H]PEG-4000) is a classic gill paracellular marker (Wood and Part, 1997) and recently, its use in whole animal experiments in the influx direction has been validated (Robertson and Wood, 2014). Our measured branchial PEG-4000 clearance rate for the freshwater-acclimated killifish is slightly higher but comparable to that obtained by Scott et al. (2004a) using different methodology. In hypoxia, no change in PEG-4000 clearance rate was seen for the Amazonian oscar, and all reductions in gill permeability during hypoxia appeared to be transcellular in nature (Wood et al., 2009), contrary to our data (Fig. 3.5A). Noteworthy, the absolute branchial clearance rate in the oscar was less than 50% of that of the killifish in 0 and 35 ppt. This suggests that the oscar may maintain a tighter regulation of overall gill permeability, while the freshwater killifish with normally higher paracellular permeability may employ reductions in both paracellular and transcellular permeability during hypoxia (discussed below).

As expected, drinking rates in seawater were much higher than in freshwater-acclimated killifish (Fig. 3.5B), in accord with several previous studies on F. heteroclitus
(Potts and Evans, 1967; Scott et al., 2006), reflecting the fact that marine teleosts must ingest water to compensate for net osmotic losses across the gills (Takei and Balment, 2009). Seawater-acclimated fish clearly reduced drinking when exposed to hypoxia, which indicates that in a situation of probable energy shortage, drinking must be curtailed as it is an energetically costly process. The stimulation of drinking in sea water and its cessation in fresh water are both thought to be mediated by the renin-angiotensin system (Malvin et al., 1980). Possibly the reduction of drinking in hypoxia is also mediated by the same pathway; further studies on this present a fruitful area for future investigations.

3.5.5 The effects of salinity acclimation and hypoxia exposure on ammonia excretion

Salinity acclimation had no effect on ammonia net flux rates in *F. heteroclitus* in accordance with previous observations by Blanchard and Grosell (2006) and ammonia net flux rates were inhibited by hypoxia at all three salinities (Fig. 3.9). In freshwater, the pattern of inhibition was slightly different than the one observed for Na⁺, Cl⁻ and K⁺, given that ammonia net fluxes decreased immediately after hypoxia exposure (Fig. 3.7C). It is widely accepted now that in freshwater fishes the majority of ammonia excretion occurs transcellularly, most likely via Rh proteins (Wright and Wood, 2009; Weihrauch et al., 2009). Therefore, the reduction in net ammonia flux is likely another indication of a reduction in transcellular permeability in response to hypoxia exposure in freshwater-acclimated killifish. In addition to transcellular ammonia transport, paracellular ammonia flux is also probably an important pathway in seawater fishes, given the leakier nature of junctions in SW fish gills (Wilkie, 1997; Wilkie, 2002). In turn, Giacomin et al. (Chapter 2) have shown that at PO₂s around 15 Torr (the O₂ level used in the hypoxia exposures here), *F. heteroclitus* would exhibit a reduced MO₂ at all salinities, and therefore reduction in aerobic metabolism could be an additional cause of decreased ammonia excretion.
3.5.6 The effects of hypoxia and salinity on unidirectional and net ionic flux rates

The discrepancies in absolute values between unidirectional Na\(^{+}\) influx and efflux rates in 11 ppt and 35 ppt fish (Fig. 3.8) were likely of technical origin, reflecting the different measurement techniques for influx and efflux, but the relative differences between normoxia and hypoxia remain comparable as they were determined by the same techniques within a salinity.

In freshwater acclimated *F. heteroclitus*, the normoxic Na\(^{+}\) influx rate was ~300 nmol/kg/h, which are comparable to the measurements of Scott et al. (2004a) and Wood (2011), but slightly lower than those of Wood and Laurent (2003). Fish that were acclimated to sea water (11 or 35 ppt) had more than one order of magnitude higher fluxes than in freshwater-acclimated fish (Fig. 3.8A), in agreement with previous studies (Motais et al., 1966; Potts and Evans, 1967; Maetz et al., 1967; Wood and Laurent, 2003; Wood, 2011). Fluxes at 11 ppt acclimation were 20-fold higher than FW, but 3.5-fold lower than full strength SW (Fig. 3.8A). This is explained by the fact, that in contrast to FW-acclimated killifish, at higher salinities, a large percentage of the Na\(^{+}\) fluxes is due to an exchange diffusion component (Wood and Marshall, 1994), which is proportional to external [Na\(^{+}\)] (Wood and Marshall, 1994), and not present in FW-acclimated animals (Patrick et al., 1997).

Based on the earlier investigations on the Amazonian oscar (Wood et al., 2007a; Wood et al., 2009), we had hypothesized that killifish, a euryhaline hypoxia-tolerant species, would reduce gill permeability upon exposure to hypoxia (i.e. opposite to the classic osmorespiratory compromise), decreasing the fluxes of ions in both fresh and sea water. Indeed, the freshwater-acclimated *F. heteroclitus* reduced both J\(^{\text{Na}^{+}}\) influx and J\(^{\text{Na}^{+}}\) efflux during hypoxia, so that no resulting changes were seen in net fluxes (Fig. 3.6). In light of the absence of an exchange diffusion component in the gills of the FW-acclimated killifish (Patrick et al., 1997), the very similar reductions of J\(^{\text{Na}^{+}}\) influx and J\(^{\text{Na}^{+}}\) efflux during hypoxia (Fig. 3.6) were probably independent of each other. The reduction in both unidirectional fluxes appear to be regulated phenomena, since they were only significantly inhibited at the second hour of exposure, remaining depressed until normoxia was restored (Fig. 3.6A). Interestingly, net K\(^{-}\) flux was reduced with an identical time-dependent profile (Fig. 3.7B).
As K\(^+\) concentrations are much higher inside cells than in blood plasma, K\(^+\) flux is often considered a marker of transcellular permeability (Laurén and McDonald, 1985; Wood et al., 2007a; Wood et al., 2009; Robertson et al., 2015a), so a role for the transcellular pathway in Na\(^+\) flux reduction (Fig. 3.6) is implicated, as in the oscar (Wood et al., 2009). This would also be in accord with the transcellular origin of the reduction in diffusive water permeability (Fig. 3.4) as well as the decrease in ammonia excretion (Fig. 3.7C), both discussed earlier. Freshwater killifish do not take up Cl\(^-\) at the gills (Patrick et al., 1997; Patrick and Wood, 1999; Wood, 2011), only at the opercular epithelium (Wood and Laurent, 2003), so the apparent reversal of net Cl\(^-\) loss during hypoxia (Fig. 3.7A) suggests that that the diffusive loss pathway at the gills for Cl\(^-\) was also down-regulated. Finally, similar to the oscar (Wood et al., 2007a; Richards et al., 2007), branchial Na\(^+\)/K\(^+\)- and H\(^+\)-ATPase activities were also significantly reduced during hypoxia (Fig. 3.2), pointing to an overall strategy of depression of transcellular active transport processes, likely aimed at conserving energy.

In our previous work (Chapter 2), we concluded that in comparison with other salinities, freshwater-acclimated fish showed the biggest compromise in respiratory capacity. Therefore, it was expected that the largest compensatory responses in order to maintain ionoregulatory balance would be seen in freshwater fish. A similar response pattern was observed for the Amazonian oscar, where a simultaneous inhibition of active Na\(^+\) uptake and passive ion losses was speculated to be an adaptive response (Wood et al., 2009), since these fish are frequently exposed to hypoxia in their natural habitat (Val and Almeida-Val, 1995). Vancouver city dechlorinated tap water (0 ppt used in this study) is undoubtedly a very dilute media. It is possible that the responses seen here are a reflection of the low ion concentration of this water that this fish may never experience in their natural enviroments. Altogether, our results for the killifish point to response different from the classic osmorespiratory compromise seen in salmonids, where ion and water fluxes are exacerbated when gas transfer needs to be upregulated (Wood and Randall, 1973a; Wood and Randall, 1973b; Iftikar et al., 2010).

Increases in blood perfusion patterns of the gills are a classical response to hypoxia in teleost fishes (Farrell et al., 1980; Pärt et al., 1984; Sundin, 1995; Nilsson, 2007), and killifish seem to be able to maintain \(O_2\) extraction efficiency until \(PO_2\)s lower than the ones
used here (Giacomin et al., Chapter 2). Therefore, we propose that a mechanism similar to that described for the Amazonian oscar (Wood et al., 2009), whereby about 80% of the the ionocyte surface area was reduced due to covering by pavement cells (Wood et al., 2009), occurs in killifish exposed to hypoxia. This simultaneous increase in effective gill surface area (due to increased perfusion) and decrease in ionocyte surface area, is believed to maintain O₂ transfer in hypoxia (Scott et al., 2008a) while concomitantly reducing gill permeability to ions.

We (Giacomin et al., Chapter 2) showed that acclimation to 35 ppt also placed constraints on respiratory performance during hypoxia, though not as severe as those at 0 ppt. At 35 ppt, the concentration gradients of major ions are reversed (fish are hypoosmotic), so the classical osmorespiratory compromise would predict increased Na⁺ loading. This did not occur - both Na⁺ influx and efflux rates were reduced by about 15%, though only the change in the former was significant (Fig. 3.8A,B), and there was a non-significant reduction in the PEG-4000 clearance rate (Fig. 3.5). While the Na⁺ efflux component contains the active pathway through leaky paracellular junctions (Evans, 2005; Evans, 2008), it must be remembered that the major components of both unidirectional Na⁺ fluxes are exchange diffusion (Motais et al., 1966; Potts and Evans, 1967; Maetz et al., 1967; Wood and Marshall, 1994). Overall, these observations suggest that as in freshwater, there was a decrease in branchial permeability during hypoxia. Wood and Grosell (2015) have reported that acute hypoxia exposure reduced branchial transepithelial potential (TEP) in F. heteroclitus acclimated to both 0 and 35 ppt, providing additional evidence of decreased ion fluxes at both salinities.

Killifish acclimated to the isosmotic salinity (11 ppt) had the greatest respiratory capacity during hypoxia (Giacomin et al., Chapter 2), and this is in accord with the idea that the osmorespiratory compromise at the isosmotic salinity is of minimal concern. The observed increases in both Na⁺ influx and efflux rates coupled with the increase in the [³H]PEG-4000 clearance rate likely reflected an increase in functional surface area of the gills for respiratory gas exchange, thereby providing support for this hypothesis. On the other hand, the unchanged diffusive water flux rate (Fig. 3.4), and the greatest lactate accumulation (Fig. 3.3B) at this salinity all argue against our hypothesis. Unfortunately, no data are
available for TEP in 11 ppt-acclimated fish exposed to hypoxia, which might clarify the
responses seen. Perhaps there are other constraints on gill function at the isosmotic salinity of
which we are currently unaware.

3.5.7 Conclusions and future directions

As the prevalence and severity of hypoxia in the world’s aquatic environments are
increasing, a comprehensive understanding of the physiological responses to hypoxia has
become extremely important. Collectively, the present data elucidate the nature of the
osmorespiratory compromise accompanying acute hypoxia exposure in *Fundulus heteroclitus*
at different salinities. As a euryhaline estuarine teleost, killfish were able to acclimate to
fresh water (0 ppt), full strength sea water (35 ppt), and isosmotic salinity (11 ppt). However,
salinity acclimation had a strong effect on how killfish responded to hypoxia as measured by
a wide range of parameters. Overall our results show that the FW-acclimated killfish is
similar to the Amazonian oscar model of response to the osmorespiratory compromise when
acclimated to 0 and 35 ppt, where a regulated reduction of active Na\(^+\) uptake and
downregulation of Na\(^+\) efflux result in no change in the net Na\(^+\) balance. Additionally,
important ion transport enzyme activities were markedly reduced indicating a cost saving
strategy in hypoxia. Together with a reduction of ammonia excretion, K\(^+\) efflux and diffusive
water exchange, our results point to an overall reduction in gill transcellular permeability.
Paracellular permeability also tended to decrease at 0 and 35 ppt. Plasma ion concentrations
were unaffected by hypoxia exposure at the three salinities, indicating that despite different
strategies, fish acclimated to all salinities were able to maintain osmoregulatory balance. At
35 ppt, there were small reductions in unidirectional Na\(^+\) fluxes, a response that resembles
that of freshwater fish. In contrast, fish acclimated to 11 ppt increased both Na\(^+\) influx and
efflux rates in hypoxia, as well as paracellular permeability, responses that follow the
predictions for the classical osmorespiratory compromise. Since hypothetically, ionic
gradients are minimal at the isosmotic point, this increase in gill permeability should not be
of great physiological concern. Future studies should examine at cellular and molecular
levels how these adjustments in gill permeability are achieved.
Figure 3.1 (A) Plasma sodium (mmol/L), (B) plasma chloride (mmol/L) and (C) plasma osmolality (mOsm/kg) in *Fundulus heteroclitus* acclimated to 0, 11 and 35 ppt in normoxia (white bars) and exposed to hypoxia (black bars). Bars sharing the same lower case letters are not statistically different at the same oxygen level. [Two-way ANOVA p-values (plasma Na\(^+\): p\(_{interaction}\) = .1221, p\(_{oxygen}\) = .5051, p\(_{salinity}\) < .0001; plasma Cl\(^-\): p\(_{interaction}\) = .8137, p\(_{oxygen}\) = .0909, p\(_{salinity}\) < .0001; plasma osmolality: p\(_{interaction}\) = .8878, p\(_{oxygen}\) = .0217, p\(_{salinity}\) < .0001)]. Data are means ± 1 SEM (n = 6 - 8).
**Na⁺/K⁺-ATPase activity** (µmol ADP/mg protein/h)

- A
- 0 ppt
- 11 ppt
- 35 ppt

**H⁺-ATPase activity** (µmol ADP/mg protein/h)

- B
- 0 ppt
- 11 ppt
- 35 ppt
Figure 3.2 (A) Na\textsuperscript{+}/K\textsuperscript{+}-ATPase and (B) H\textsuperscript{+}-ATPase activity (\(\mu\)mol ADP/mg protein/h) in Fundulus heteroclitus acclimated to 0, 11 and 35 ppt in normoxia (white bars) and exposed to hypoxia (black bars). Bars sharing the same lower case letters are not statistically different at the same oxygen level. [Two-way ANOVA p-values (Na\textsuperscript{+}/K\textsuperscript{+}-ATPase: \(p_{\text{interaction}} = .1356\), \(p_{\text{oxygen}} = .0037\), \(p_{\text{salinity}} = .2233\); H\textsuperscript{+}-ATPase: \(p_{\text{interaction}} = .0608\), \(p_{\text{oxygen}} = .0011\), \(p_{\text{salinity}} = .2622\)]. Asterisks indicate significant differences between normoxia and hypoxia at the same salinity (Bonferroni post-hoc test). Data are means ± 1 SEM (n = 6 - 8).
**Figure 3.3** (A) Hematocrit (% RBC) and (B) plasma lactate (mmol/L) in *Fundulus heteroclitus* acclimated to 0, 11 and 35 ppt in normoxia (white bars) and exposed to hypoxia (black bars). Bars sharing the same lower case letters are not statistically different at the same oxygen level. [Two-way ANOVA p-values (Hematocrit: p_{interaction} = .1845, p_{oxygen} < .0001, p_{salinity} = .1239; lactate: p_{interaction} = .0051, p_{oxygen} < .0001, p_{salinity} = .0006)]. Asterisks indicate significant differences between normoxia and hypoxia at the same salinity (Bonferroni post-hoc test). Data are means ± 1 SEM (n = 6 - 8).
Figure 3.4 Water flux rate (% body water/h) in *Fundulus heteroclitus* acclimated to 0, 11 and 35 ppt in normoxia (white bars) and exposed to hypoxia (black bars). Bars sharing the same lower case letters are not statistically different at the same oxygen level. [Two-way ANOVA p-values (water flux rate: $p_{\text{interaction}} < .0001$, $p_{\text{oxygen}} = .3608$, $p_{\text{salinity}} < .0001$)]. Asterisks indicate significant differences between normoxia and hypoxia at the same salinity (Bonferroni post-hoc test). Data are means ± 1 SEM (n = 12).
PEG-4000 clearance rate (µL/g/h)

Drinking rate (µL/g/h)

A

B

124
Figure 3.5 (A) PEG-4000 clearance rate (µL/g/h) and (B) drinking rate (µL/g/h) in *Fundulus heteroclitus* acclimated to 0, 11 and 35 ppt in normoxia (white bars) and exposed to hypoxia (black bars). Bars sharing the same lower case letters are not statistically different at the same oxygen level. [Two-way ANOVA p-values (clearance rate: p_{interaction} = .0008, p_{oxygen} = .7808, p_{salinity} = .5193; drinking rate: p_{interaction} < .0001, p_{oxygen} < .0001, p_{salinity} < .0001)]. Asterisks indicate significant differences between normoxia and hypoxia at the same salinity (Bonferroni post-hoc test). Data are means ± 1 SEM (n = 5 - 8).
A

\[ J_{Na^{+}_{\text{influx}}} \text{ (nmol/g/h)} \]

B

\[ J_{Na^{+}_{\text{efflux}}} \text{ (nmol/g/h)} \]

C

\[ J_{Na^{+}_{\text{net flux}}} \text{ (nmol/g/h)} \]

Normoxia | Hypoxia | Normoxia
Figure 3.6  (A) Unidirectional sodium influx rate ($J_{Na^{+}\text{influx}}$; nmol/g/h), (B) unidirectional sodium efflux rate ($J_{Na^{+}\text{efflux}}$; nmol/g/h) and (C) sodium net flux ($J_{Na^{+}\text{net flux}}$; nmol/g/h) rate in *Fundulus heteroclitus* acclimated to 0 ppt. Fish were subjected to 3 h each of normoxia, hypoxia, normoxia. Asterisks indicate bars which are significantly different from the averaged starting normoxia period (Student’s t-test). Data are means ± 1 SEM (n = 15 - 17).
Figure 3.7 The effect of hypoxia exposure on the (A) chloride net flux rate ($J_{\text{Cl}^- \text{net flux}}$; nmol/g/h), (B) potassium net flux rate ($J_{\text{K}^+ \text{net flux}}$; nmol/g/h) and (C) ammonia net flux rate ($J_{\text{ammonia net flux}}$; nmol/g/h) in *Fundulus heteroclitus* acclimated to 0 ppt. Fish were subjected to 3 h each of normoxia, hypoxia, normoxia. Asterisks indicate bars which are significantly different from the averaged starting normoxia period (Student’s t-test). Data are means ± 1 SEM (n = 15 - 17).
Figure 3.8 (A) unidirectional sodium influx rate ($J_{Na^{+}}^{influx}$; nmol/g/h) and (B) unidirectional sodium efflux rate ($J_{Na^{+}}^{efflux}$; nmol/g/h) in *Fundulus heteroclitus* acclimated to 0 ppt, 11 ppt and 35 ppt in normoxia (grey bars) and exposed to hypoxia (black bars). 0 ppt data are averaged normoxia and hypoxia data obtained in Fig. 3.6. [Two-way ANOVA p-values ($J_{Na^{+}}^{influx}$: $p_{interaction} < .0001$, $p_{oxygen} = .0004$, $p_{salinity} < .0001$; $J_{Na^{+}}^{efflux}$: $p_{interaction} = .1059$, $p_{oxygen} = .6178$, $p_{salinity} < .0001$)]. Asterisks indicate significant differences between normoxia and hypoxia at the same salinity (Bonferroni post-hoc test). Data are means ± 1 SEM (n = 15 - 17).
Figure 3.9 Ammonia net flux rate ($J_{\text{am}}$; nmol/g/h) in *Fundulus heteroclitus* acclimated to (A) 0 ppt (B) 11ppt and (C) 35 ppt. Asterisks indicate significant differences between normoxia and hypoxia at the same salinity (Student’s t-test). Data are means ± 1 SEM (n = 15 - 17).
Chapter 4: Acute temperature effects on metabolic rate, ventilation, diffusive water exchange, osmoregulation, and acid-base status in the Pacific hagfish (*Eptatretus stoutii*)

4.1 Summary

The Pacific hagfish (*Eptatretus stoutii*) is a representative of the most basal extant craniates, and is a marine osmoconformer with an extremely low metabolic rate (\(\dot{M}O_2 = 475\ \mu\text{mol O}_2/\text{kg h at 12°C}\)). We investigated potential physiological trade-offs associated with compensatory changes in gill ventilation and perfusion when 12°C-acclimated hagfish were acutely exposed to 7 °C or 17°C, as reflected in diffusive unidirectional water flux (\(J_{H_2O}\), measured with tritiated water: \(3H_2O\)), net ammonia flux (\(J_{amn}\)), and plasma ion and acid-base status. \(J_{H_2O}\) was high (~1.4 L/kg/h at 12°C) in comparison to marine teleosts and elasmobranchs. \(\dot{M}O_2\) increased linearly with temperature (\(R^2 = 0.991\)), and was more sensitive (\(Q_{10} = 3.22\)) in the 12 - 7°C range than either \(J_{amn}\) (1.86) or \(J_{H_2O}\) (1.35), but the pattern reversed from 12 - 17°C (\(Q_{10}\): \(\dot{M}O_2 = 2.77, J_{amn} = 2.88, J_{H_2O} = 4.01\)). Heart rate, ventilatory index (a proxy for total ventilation), and coughing frequency also increased but with different patterns. At 17°C, plasma [Ca\(^{2+}\)] and [Mg\(^{2+}\)] decreased, although osmolality increased, associated with elevations in plasma [Na\(^+\)] and [Cl\(^-\)]. Blood pH and PCO\(_2\) were unaffected by acute temperature changes while [HCO\(_3^-\)] increased. Hyperoxia (PO\(_2 > 300\) Torr) attenuated the increase in \(J_{H_2O}\) at 17°C, did not affect \(J_{amn}\), and had diverse effects on plasma ion and acid-base status. Our results suggest a clear osmorepiratory compromise occurring for the diffusive water fluxes as a result of acute temperature changes in this osmoconformer.

4.2 Introduction

Hagfish are marine jawless vertebrates that are considered to be osmoconformers, maintaining plasma [Na\(^+\)] and [Cl\(^-\)] at concentrations that closely resemble those of sea water (Robertson, 1976), although they have the ability to regulate plasma [Ca\(^{2+}\)] and [Mg\(^{2+}\)] when
acclimated to salinities above and below that of normal sea water (Sardella et al., 2009). They are also known to be excellent regulators of acid-base status (Clifford et al., 2014; Baker et al., 2015). In teleost fishes, which are osmoregulators, a phenomenon termed the osmorespiratory compromise occurs (Randall et al., 1972; Nilsson, 1986; Gonzalez, 2011). The osmorespiratory compromise represents a tradeoff between osmoregulation and oxygen uptake. Under conditions when energy demand increases, physiological mechanisms such as increased gill ventilation, perfusion, and effective gas permeability must be activated to increase oxygen uptake, but these changes may cause undesirable passive fluxes of ions, osmolytes and water. Although the osmorespiratory compromise is well documented in teleost fishes, the extent to which this phenomenon would be expected to occur in osmoconformers remains unclear. There is some evidence for an osmorespiratory compromise in Chondrichthyan fishes, which are osmoconformers but ionoregulators (Wright and Wood, 2016). For example, fluxes of ammonia, urea, and water (diffusive water flux) in the Pacific spiny dogfish shark (*Squalus acanthias suckleyi*) all increase to a greater extent than does \( \dot{M}O_2 \) in response to acute increases in temperature (Giacomin et al., 2017).

However, the presence or extent of an osmorespiratory compromise has not previously been examined in species, such as hagfishes, whose plasma \([Na^+]\) and \([Cl^-]\) are similar to those in seawater. In addition, hagfishes exhibit very low oxygen consumption rates (\( \dot{M}O_2 \): Munz and Morris, 1965; Clifford et al., 2016), often less than 20% of those in comparably sized jawed fishes (Clarke and Johnston, 1999), again opening the possibility of a minimal osmorespiratory compromise in these animals.

Here, we investigate the osmorespiratory compromise in Pacific hagfish *Eptatretus stoutii*, through increasing their metabolic rate by exposing them to acute temperature increases in the laboratory. Very little is known about the physiological responses of hagfishes to changes in temperature, although when acclimated to 10°C, Pacific hagfish linearly increase \( \dot{M}O_2 \) when exposed acutely from 5°C to 15°C with a \( Q_{10} \) of 2.4 (Munz and Morris, 1965), which is relatively high compared to that of most teleost fishes (Wood, 2001; Lea et al., 2016). In nature, hagfish are thought to be found in thermally stable environments. For example, Pacific hagfish have been captured at depths where temperature varied only between 10 and 11°C, and thus their tolerance to temperature fluctuations is thought to be
low (McInerney and Evans, 1970). However, *Eptatretus stoutii* tolerated short exposures to 29°C when maintained at 22°C for a few days (Worthington, 1905). Hagfish also may swim near the surface (Adam and Strahan, 1963) where they could potentially be exposed to sharp changes in temperature, as well as hyperoxic conditions during phytoplankton blooms. Recently a novel species (*Eptatretus strickrotti*) has been found near hydrothermal vents, raising the question of whether hagfishes are confined to low temperature environments (Moller and Jones, 2007). In addition, the increased commercial demand for hagfish and the current use of fishing practices that result in hagfish being exposed to large acute changes in temperature (as well as in salinity and oxygen concentration; Olla et al., 1998) necessitate an understanding of how hagfish react to such challenging conditions.

Our initial goal was to evaluate the effects of acute temperature changes on the rates of oxygen consumption, ammonia excretion, diffusive water flux (measured with tritiated water), and on ventilation and heart rate in the Pacific hagfish *Eptatretus stoutii*, an animal which is known to be an ionoconformer for major ions (Na\(^+\) and Cl\(^-\)) but an excellent regulator of plasma [Ca\(^{2+}\)], [Mg\(^{2+}\)], and acid-base status. We hypothesized that if there was a permeability trade off at the gills accompanying increases in ventilation or heart rate as \(\dot{M}O_2\) increased with temperature, the fluxes of ammonia and water would also increase. We also hypothesized that unfavorable fluxes of ions and water would be reflected in perturbed plasma homeostasis. Additionally, we investigated the possible role of environmental hyperoxia (PO\(_2 > 300\) Torr) in attenuating the effects of exposure to high temperature. Our hypothesis here was that by increasing oxygen supply at the gills (through hyperoxia), the hypothesized trade-offs for ion regulation at high temperature would be minimized.

### 4.3 Material and Methods

#### 4.3.1 Animal housing and acclimation

Pacific hagfish (*Eptatretus stoutii*; average mass = 46.5 ± 0.8 g) were caught using bottom-dwelling traps (rotting hake *Merluccius productus* was used as bait), from Trevor Channel, near Bamfield Marine Sciences Centre (BMSC), Bamfield, British Columbia,
Canada, under Fisheries and Oceans Canada collecting permit XR1942017. The traps consisted of 22-L plastic buckets, fitted with a modified lid and containing a conical entrance, which allowed the hagfish to enter the bucket but prevented exit. The traps were placed at approximately 80 m depth, and recovered after 8 h. At BMSC hagfish were held in 200-L darkened tanks, served with flow-through fully aerated sea water (12 – 13°C, 30 ppt). During acclimation, food was offered in the form of hake strips, but the fish did not feed. Therefore, fish were fasted for at least one week, and for a maximum of three weeks prior to experimentation. All experiments were performed following the guidelines of the Canada Council for Animal Care, under joint approval of the animal care committees at the University of British Columbia and BMSC (AUPs A14-0251 and RS–17-20, respectively). After completion of experiments, animals were euthanized by an overdose of anesthetic (MS-222, Syndel Labs., Parksville, BC, Canada; 5 g/L neutralized to pH 7.8 with 5 M NaOH), followed by evisceration to ensure death.

4.3.2 Effects of acute temperature changes

4.3.2.1 Oxygen consumption, ammonia excretion, and blood parameters

All experiments were carried out using darkened, 1-L glass jars topped with a fine plastic mesh, fitted with fine tubing providing gentle aeration and served with flow-through sea water at 50 mL/min. The effect of acute temperature changes on hagfish O₂ consumption rates (ṀO₂) and ammonia flux rates (Jₐmm), as well as blood acid-base and ion status, were assessed at 12°C (control = acclimation temperature), and after acute transfer to 7°C and 17°C, 5°C lower and higher than the control temperature respectively. Fish were first allowed to settle in the experimental chambers for a minimum of 6 h before the start of the experiment. For the 12°C experiments, the water flow to the jar was stopped and the volume of water was set to exactly 1 L. For the acute 7°C or 17°C exposure, the jars were gently tipped so that almost all the control water was drained, and new water at the prepared temperature was poured inside until 1 L of water at the exact temperature was reached. This procedure was chosen as it avoided touching the hagfish and prevented the formation of slime.
Temperatures of 7, 12 and 17°C were maintained by partially submerging the jars in a thermostatted water bath.

Experiments started 0.5 h after an adjustment period to the new temperature. Water samples (5 mL) were taken at 0, 3, and 6 h (for the 7 and 12°C experiments), and at 0, 2, 4 and 6 h (for the 17°C experiments), and immediately frozen at -20°C for later analyses of ammonia concentration. During the water sampling intervals, the jars were sealed with gas-impermeable dental dam, right after an initial PO$_2$ value was taken using an Accumet AP84 handheld O$_2$ meter (Fisher Scientific, Toronto, ON, Canada). The detection limit of the oxygen probe was ~0.5 Torr. Simultaneous with water sampling at the end of an interval, a final PO$_2$ was measured, and then the jars were lightly bubbled with air for re-oxygenation, the volume was replaced, and the procedure was repeated until the end of the experiment. During the periods where the chamber was sealed, the rate of PO$_2$ decrease varied from 0.15 to 0.45 Torr/min, and the final value never fell below 75 Torr. The differences between the initial and final PO$_2$ or total ammonia values in each interval were used to calculate $\dot{M}$O$_2$ and $J_{amn}$ (see below), which are reported as an average of the multiple measurements (2 or 3 depending on temperature). $\dot{M}$O$_2$ was not measured in the hyperoxia series as we did not have an O$_2$ probe that measured PO$_2$ accurately at that high background. At the end of an experimental day, all jars were rinsed with 70% ethanol in order to prevent any bacterial build-up. A blank trial (respirometer with no fish in it) was run in the same fashion as the real experiments, every time a respirometry trial was run. No significant changes in PO$_2$ or ammonia were observed throughout the duration of the blank trials.

At the end of the experiment, fish were quickly transferred to MS-222 (0.6 g/L neutralized with 5 M NaOH) solution, and once fish were anesthetized, a blood sample (~500 µL) was drawn from the posterior sinus using a heparinized gas-tight syringe (Hamilton, Reno, NV, USA). Fresh blood was immediately transferred to a temperature-controlled water bath, and blood pH was measured using an MI-4156 Micro-Combination pH probe (Microelectrodes Inc., Bedford, NH, USA) and Accumet pH meter (Fisher Scientific, Toronto, ON, Canada). After that, plasma was separated by centrifugation (12,000 g for 3 min), aliquoted, flash frozen in liquid N$_2$ and stored at -80°C for later analyses. Fish were then immediately weighed and euthanized as described above (section 2.1). Sea water
samples were also taken in all trials and similarly frozen for comparison to plasma ionic composition and osmolality.

4.3.2.2 Ventilation and heart rate

A separate group of fish was used for this experimental series. The surgical procedures used here are the same as those outlined in detail in Eom and Wood (2018). Briefly, hagfish were anesthetized in neutralized MS-222 (0.6 g/L sea water) for <5 min and transferred to an operating table. A small piece of flexible plastic tubing was inserted snugly into the duct of the single nostril and stitched to the skin (26 mm 1/2C taper, Perma-Hand Silk, Ethicon, Somerville, NJ, USA). A ~2 cm PE160 tubing (BD, Intramedic, Franklin Lakes, NJ, USA) was secured inside the plastic tubing and this was then bridged to a connecting PE 160 catheter using a blunt #18-gauge needle when necessary for recording. The water-filled PE 160 tubing was connected to a pressure transducer (DPT-100, Utah Medical Products, Midvale, UT, USA) so that pressure amplitude (cm H$_2$O) and frequency (breaths/min) could be recorded at the nostril duct. The pressure transducer was calibrated against a 4-cm water column. Two laminated copper wires (AWG #32, Belden, Chicago, IL, USA) were stripped of their insulation for about 1 cm at the recording end, and placed slightly under the skin (as “fish hook” electrodes), in the abdomen, posterior to the 12$^{th}$ gill pouch so as to monitor the branchial heart rate. The wires were stitched to the skin as described above. Heart beat frequency was collected by connecting the wires to an impedance converter (Model 2991, UFI, Morro Bay, CA, USA). Both analog signals were amplified by an amplifier (LCA-RTC, Transducer Techniques, Temecula, CA, USA), and digitalized in a PowerLab Data Integrity system (ADInstruments, Colorado Springs, CO, USA), and visualized and analyzed using LabChart v. 7.0 (ADInstruments).

Following surgery, fish were allowed to recover overnight in darkened plastic containers supplied with flow-through control seawater. Two different series of experiments were conducted. First, ventilation and heart rate were recorded for 5 min at 12$^\circ$C, then the water was gently replaced with flowing water at either 7 or 17$^\circ$C, until the total volume (1 L) of the containers had reached a stable temperature. Animals were allowed to adjust to the
new temperature for 30 min, and ventilation and heart rate were recorded for additional 5 min.

In the second experimental series, ventilation alone was recorded only at 12°C and 17°C, in normoxic and hyperoxic conditions, and a longer term (6 h) exposure was employed to match the time of blood sampling. Similar protocols as stated above were used to change the water and adjust temperature. For the hyperoxia experiments, water that had been bubbled with pure O₂ to a PO₂ > 300 Torr was introduced at the proper temperature. Animals were allowed to adjust to the new conditions for 6 h, then ventilation was recorded for 5 min. Note that in this series, heart rate could not be recorded as the animals invariably displaced the impedance leads over this longer (6-h) period.

The ventilation frequency (breaths/min) reported here represents the frequency of the beat of the velum, and the pressure amplitude (cm H₂O) represents the difference between average maximum and average minimum pressure in the ventilatory cycle. Eom and Wood (2018) have shown that there is a direct correlation between pressure amplitude and ventilatory stroke volume, and that ventilatory index (cm H₂O/min), calculated as the product of frequency and pressure amplitude, is directly correlated with the rate of total ventilatory flow. The software measured frequency and pressure amplitude in the short periods in between coughs, and the frequency of coughing (coughs/min) was tabulated separately.

4.3.2.3  **Diffusive water exchange**

A separate group of fish was used for the assessment of diffusive water exchange. Water exchange rates were measured using radiolabelled tritiated water (³H₂O). Preliminary experiments demonstrated that water exchange rates were high, such that complete equilibration of ³H₂O between the internal compartment of the hagfish and the external sea water occurred within 6 h. In each experimental treatment, hagfish (N = 8) were incubated as a group in 20 μCi/L of ³H₂O (Amersham Pharmacia Biotech, Little Chalfont, UK) in control sea water at 12 °C under normoxia for 6 h in a darkened 4-L glass container placed in a water bath for temperature control. At the end of the loading period, duplicate 5-mL water samples were taken to assess water specific activity at equilibrium. The fish were removed from the
incubation container, quickly placed in an intermediate basin containing sea water free of radioactivity for a cold displacement rinse, and then transferred to individual experimental chambers (darkened 1-L glass jars identical to those described above). The jars contained normoxic sea water at the desired water temperature (7, 12 or 17 ºC) according to the trial being performed. Temperature control was achieved as described above. Water samples (5 mL) were taken at 5-min intervals from 0 to 30 min, and 10-min intervals thereafter from 30 to 70 min. Final samples were taken at the next day at 9-14 h to assess specific activity of the system (hagfish plus external water) at equilibrium after complete \(^3\)H\(_2\)O washout. Scintillation fluor (Optiphase, PerkinElmer, Waltham, MA) was immediately mixed to the water samples in a 2:1 ratio (fluor:water), and samples were stored in the dark for a minimum of 12 h to eliminate chemiluminescence prior to counting for beta emissions on a Triathler portable counter (Hidex, Helsinki, Finland). Tests showed that quench was constant.

4.3.3 Interactive effects of temperature changes and hyperoxia

A separate group of hagfish were used for these tests. In the first set of experiments, the effects of acute hyperoxia exposure (PO\(_2\) > 300 Torr) on J\(_{\text{amm}}\) and blood parameters were assessed at 12ºC and after acute exposure to 17ºC. In a second set, the effects of the same treatments on diffusive water exchange rates were determined. The experimental apparatus and procedures used to reach and maintain temperature were the same as those described earlier, the only difference being that the sea water was bubbled with pure O\(_2\) until it reached a PO\(_2\) > 300 Torr in the hyperoxia tests. PO\(_2\) was monitored throughout the experiments using the same handheld O\(_2\) meter and the container was reoxygenated as necessary.

In the first set of experiments, procedures were identical to those described earlier, though it proved to be impractical to measure O\(_2\) consumption under hyperoxia. In the second set, procedures were again similar. Note that the fish were loaded with \(^3\)H\(_2\)O under normoxic conditions at 12ºC, and then acutely transferred to the experimental treatments (12 or 17ºC, with or without hyperoxia).
4.3.4 Analytical techniques and calculations

For all calculations involving volume (V), the weight of the fish was subtracted from the 1-L respirometer volume, assuming 1 g = 1 mL.

Ammonia concentration in the water was measured colorimetrically according to Verdouw et al. (1978). Ammonia (J_{amm}, µmol/kg/h) flux rate was calculated using the following equation:

\[
J_{amm} = \frac{[(Amm(f) - Amm(i)) \times V]}{(W \times t)} \quad \text{(Eq. 1)}
\]

where Amm(f) and Amm(i) are the final and initial water ammonia (µmol/L) concentrations respectively; V is the volume of water used (L); W is the body mass of the fish (kg); t is the duration of the flux period (h).

Oxygen consumption rates (\(\dot{M}O_2\): µmol O₂/kg/h) were calculated with the following equation:

\[
\dot{M}O_2 = \frac{[((PO_2(i) - PO_2(f)) \times \alpha_{O_2}) \times V]}{(W \times t)} \quad \text{(Eq. 2)}
\]

where PO_2(i) and PO_2(f) are the initial and final water PO₂ (Torr) respectively; \(\alpha_{O_2}\) (µmol/Torr/L) is the oxygen solubility constant from Boutilier et al. (1984) at the appropriate temperature and salinity; W is the body mass of the fish (kg); t is the duration of the measurement period (h).

Since ammonia excretion rate (J_{amm}) and oxygen consumption (\(\dot{M}O_2\)) were measured concomitantly, the ammonia quotient (AQ) was calculated:

\[
AQ = \frac{J_{amm}}{\dot{M}O_2} \quad \text{(Eq. 3)}
\]

where J_{amm} was calculated using Eq. 1 and \(\dot{M}O_2\) calculated using Eq. 2.

The temperature coefficient (Q_{10}) was calculated using the average values for the physiological rate of interest at each experimental increment as:
\[ Q_{10} = \left( \frac{R_2}{R_1} \right)^{\frac{10}{(T_2 - T_1)}} \]  \hspace{1cm} (Eq. 4)

where \( R_1 \) and \( R_2 \) are the rates measured at temperature 1 (\( T_1, ^\circ C \)) and temperature 2 (\( T_2, ^\circ C \)).

The ventilatory index (cm H\(_2\)O/min) was calculated using the following equation:

\[ \text{ventilatory index} = \text{frequency} \times \text{pressure amplitude} \] \hspace{1cm} (Eq. 5)

where frequency represents velar breaths/minute and amplitude is in cm H\(_2\)O.

Plasma total ammonia (\( T_{\text{amm}}, \mu\text{mol/L} \)) was determined using a commercial kit (Raichem, Cliniqa, San Marcos, CA, USA). Due to background interference by hagfish plasma, a standard curve was created using hagfish plasma as a matrix spiked with increasing concentrations of NH\(_4\)Cl, in order to compensate for the matrix effect. The concentration of ammonia in the plasma was calculated by the standard addition method. Plasma TCO\(_2\) content was measured using a total CO\(_2\) analyzer (Corning 965 CO\(_2\) analyzer, Ciba Corning Diagnostic, Halstead, Essex, UK). Plasma ion concentrations (mmol/L) were determined by a chloridometer (Radiometer CMT10, Copenhagen, Denmark) for [Cl\(^-\)], or by atomic absorption (1275 Atomic Absorption Spectrophotometer, Varian, Mulgrave, Victoria, Australia) for [Na\(^+\)], [Ca\(^{2+}\)], and [Mg\(^{2+}\)] using certified commercial solutions as standards (Fluka Analytical, Sigma-Aldrich, St. Louis, MO, USA). Plasma osmolality was measured using a Wescor vapor pressure osmometer and standards (Wescor 5100C, Logan, UT, USA).

The solubility coefficient of carbon dioxide in hagfish (\( \alpha_{CO_2}, \text{mmol/L/Torr} \)) plasma was calculated for the three different temperatures using the following equation from Heisler (1984):

\[ \alpha_{CO_2} = 0.1008 - 9.8 \times 10^{-3} (M) + (1.218 \times 10^{-3} (M) - 3.639 \times 10^{-3}) \times T - (19.57 \times 10^{-6} (M) - 69.59 \times 10^{-6}) \times T^2 + (71.71 \times 10^{-9} (M) - 559.6 \times 10^{-9}) \times T^3 \] \hspace{1cm} (Eq. 6)

where \( M \) is the measured osmolality (mOsm/kg) and \( T \) is temperature (ºC).
The apparent pK of CO$_2$ ($pK_{app}$) in hagfish plasma was calculated using the following equation from Heisler (1984):

\[
pK_{app} = 6.583 - 13.41 \times 10^{-3}(T) + 228.2 \times 10^{-6}(T^2) - 1.516 \times 10^{-6}(T^3) - 0.341M^{0.323} - \\
\log\{1 + 0.00039[Pr] + 10^{pH - 10.64 + 0.011T} + (0.737M^{0.323}) x (1 + 10^{1.92} - 0.01T - (0.737M^{0.323}) + \log[Na^+] + (-0.494M + 0.651)(1 + 0.0065[Pr]))\} \quad (Eq. 7)
\]

where M is the measured osmolality (mOsm/kg), T is temperature (°C), [Na$^+$] is the measured sodium concentration (mol/L) and [Pr] is a constant amino acid concentration of 1 g/L (Cholette and Gagnon, 1973; Currie and Edwards, 2010). With those two calculated parameters, plasma PCO$_2$ (Torr) was calculated from measured TCO$_2$ and pH values using a modified Henderson-Hasselbalch equation:

\[
PCO_2 = TCO_2 / [\alpha_{CO_2} x (1 + \text{antilog} (pH - pK_{app}))] \quad (Eq. 8)
\]

where $\alpha_{CO_2}$ (mmol/L/Torr) was calculated using Eq. 6, and $pK_{app}$ was calculated using Eq. 7.

Plasma [HCO$_3^-$] (mmol/L) was calculated as:

\[
[HCO_3^-] = TCO_2 - (\alpha_{CO_2} \times PCO_2) \quad (Eq. 9)
\]

where PCO$_2$ was calculated using Eq. 8.

Plasma [NH$_4^+$] was calculated using a modified Henderson-Hasselbalch equation and $pK'$ values obtained from Cameron and Heisler (1983) adjusted to hagfish plasma NaCl concentration:

\[
[NH_4^+] = T_{amm} / [1 + \text{antilog} (pH - pK')]} \quad (Eq. 10)
\]

where $T_{amm}$ is the measured total plasma ammonia (µmol/L). The partial pressure of NH$_3$ (PNH$_3$; µTorr) was calculated using the following equation:
$PNH_3 = \frac{(T_{amm} - [NH_4^+])}{\alpha NH_3}$  \hspace{1cm} (Eq. 11)

where $\alpha NH_3$ is the solubility coefficient of NH$_3$ in hagfish plasma (µmol/L/Torr) plasma, was obtained from Cameron and Heisler (1983) for the three temperatures tested in the present study.

The rate constant of $^3$H$_2$O turnover ($k$) was calculated from the exponential rate of decline in total $^3$H$_2$O radioactivity in the fish (Evans, 1967):

$$k = \frac{(\ln R_1 - \ln R_2)}{(t_1 - t_2)}$$  \hspace{1cm} (Eq. 12)

where $k$ is the rate constant of the efflux (in h$^{-1}$), and $R_1$ and $R_2$ are total $^3$H$_2$O radioactivity (in cpm) in the fish at times $t_1$ and $t_2$ (in h). The product of $k \times 100$ yields the percent of body water turned over per hour. In practice, the rate constant $k$ for $^3$H$_2$O efflux was calculated by regression of the natural logarithm of R against time over the range of linearity (generally 5 to 30 min after transfer to the experimental chambers). By measuring the radioactivity in the water after 9-14 h, when complete $^3$H$_2$O washout and equilibration between the fish and the water had occurred, multiplying by the known volume of the system (fish plus water), and then adding radioactivity removed in sampling, it was possible to accurately calculate the total amount of $^3$H$_2$O radioactivity ($R_{total}$, cpm) that the fish had originally taken up during the loading period. Therefore, from $R_{total}$ and from measurements of $^3$H$_2$O radioactivity appearance in the water at each time interval, it was possible to back-calculate $R$ in the fish at each time during the experiment for use in the regression of $\ln R$ against time in equation 12. It was also possible to calculate the exchangeable internal water volume of each fish ($V_{H2O}$, mL/kg) as:

$$V_{H2O} = \frac{R_{total}}{SA_{H2O} \times W}$$  \hspace{1cm} (Eq. 13)
where $SA_{H2O}$ is the specific activity (cpm/mL) of the external water at the end of the original $^3$H$_2$O loading period, and $W$ is body mass in kg. The unidirectional diffusive flux rate of water ($J_{H2O}$, mL/kg/h) could then be calculated as:

$$J_{H2O} = k \times V_{H2O}$$  \hspace{1cm} (Eq. 14)

### 4.3.5 Statistical analyses

All data are shown as means ± 1 SEM (N = number of animals). Requirements for using parametric statistics (normality and homogeneity of variances) were tested, and in case of failure, data were transformed either using a square root or log transformation. Data from Series 2.2 were compared using one-way analysis of variance (ANOVA). Data from Series 2.3, where the effects of temperature and hyperoxia were tested in combination, were evaluated using two-way ANOVA. Detailed results of the statistical tests and post-hoc analysis are shown in specific Figure captions. For the one-way ANOVA, mean values were considered significantly different when $p < 0.05$, while for the two-way ANOVA, the Bonferroni correction for multiple comparisons was applied to define the alpha values.

### 4.4 Results

#### 4.4.1 Acute temperature exposures

$\dot{M}O_2$ at the control temperature of 12°C was 475 µmol O$_2$/kg/h and increased by about 65% after an acute warming to 17°C, and decreased by about 48% after an acute cooling to 7°C (Fig. 4.1a). The $Q_{10}$s for both 5°C changes were high, being greater from 7 to 12°C ($Q_{10} = 3.75$), and slightly lower at 12 to 17°C ($Q_{10} = 2.77$), while the overall 10°C $Q_{10}$ was 3.22. The $J_{amm}$ at the control temperature was only about 15 µmol/kg/h. $J_{amm}$ showed a similar pattern of temperature response as $\dot{M}O_2$, but the $Q_{10}$s were, on average, lower (Fig. 4.1b). Differently from $\dot{M}O_2$, the $J_{amm}$ Q$_{10}$ from 7 to 12°C (1.86) was lower than the Q$_{10}$ from 12 to 17°C (2.88). The overall 10°C Q$_{10}$ for $J_{amm}$ was 2.31. We were not able to measure the
urea-N excretion rate, and previous studies have shown that in Pacific hagfish it is anywhere between 6 – 60% of the ammonia excretion (Walsh et al., 2001; Braun and Perry, 2010; Wilkie et al., 2017). Therefore, we calculated the ammonia quotient (AQ) using only ammonia excretion rate as a proxy for total nitrogen (N₂) excretion. The AQ (0.03 at control 12°C) was very low and did not vary over the temperature range tested (Fig. 4.1c). Over the temperature range from 7 to 12°C, J_{H2O} was the most conserved of the three rates measured, (Q_{10} = 1.35); this increase was not significant (Fig. 4.1d). However, the Q_{10} from 12 to 17°C was the highest of all (Q_{10} = 4.01). At the acclimation temperature, diffusive water flux rate was about 1.4 L/kg of water per hour, and when hagfish were exposed to 17°C, the diffusive water flux rate increased to almost 3 L/kg per hour. The full range Q_{10} (7 to 17°C) was 2.36. The mean exchangeable internal water pool (V_{H2O}; Eq. 13) of all the fish used in this study was 621.5 ± 14.3 mL/kg (n = 55), obtained from all fish loaded at 12°C. Table 4.1 shows the rate constants of $^3$H₂O turnover (as calculated in Eq. 12).

The ventilation frequency at the control temperature was ~ 25 breaths/min and it did not differ at the three temperatures tested (Fig. 4.2a). This was reflected in very low Q_{10}s, where the full range Q_{10} was only 1.09 (Fig. 4.2a). Pressure amplitude showed a different response pattern with temperature (Fig. 4.2b), where there was a steady increase with temperature, however due to high variability, there was no statistical significance detected. Since this measurement is not a rate, Q_{10}s were not calculated (Fig. 4.2b). The ventilatory index, a proxy for total ventilatory flow (Eom and Wood, 2018), is the product of ventilation rate and pressure amplitude (Fig. 4.2c). Over 7 to 12°C, the ventilatory index was very temperature sensitive, showing a Q_{10} of 6.53. There was no significant change in the ventilatory index between 12 and 17°C (Q_{10} = 1.29), but the ventilatory indices at both those temperatures were significantly higher than at 7°C. The Q_{10} over the full range was 2.90. The coughing frequency (i.e. the transient reversal of ventilatory flow; data not shown) seemed to be the most temperature sensitive metric with an overall Q_{10} of 49.5 (7-17 °C). It decreased by 92% from 12 to 7°C, and increased by 75% from 12 to 17°C. The heart rate (~ 15 beats/min at 12 °C; Fig. 4.2d) showed a very different pattern from the various ventilatory measurements. It did not change significantly from 7 to 12°C (Q_{10} = 1.38), but it was significantly elevated (about 1.6-fold) at 17°C, with a Q_{10} of 2.58 (Fig. 4.2d).
Plasma [Na\(^+\)] tended to rise but did not change significantly with temperature (Fig. 4.3a), while plasma [Cl\(^-\)] was significantly elevated at 17°C (Fig. 4.3b). While mean plasma [Na\(^+\)] values (551 - 600 mmol/L) at all three temperatures were significantly above the mean concentration measured in SW (465 ± 4 mmol /L), all plasma [Cl\(^-\)] values (452 - 486 mmol/L) were significantly below the mean SW concentration (515 ± 7 mmol/L). In contrast to [Na\(^+\)] and [Cl\(^-\)], plasma [Mg\(^{2+}\)] (Fig. 4.3c) and [Ca\(^{2+}\)] (Fig. 4.3d) decreased at 17°C. Both plasma [Mg\(^{2+}\)] (8 -10 mmol/L) and [Ca\(^{2+}\)] (3 - 4 mmol/L) showed values well below those of seawater ([Mg\(^{2+}\)] = 51.2 ± 0.2 and [Ca\(^{2+}\)] = 8.8 ± 0.1 mmol/L), highly significant differences. Plasma osmolality decreased by about 50 mOsm/kg with an acute exposure to 7°C, but did not change significantly with a 5°C increase in temperature (Fig. 4.3e). With the exception of the 7°C treatment, mean plasma osmolarities (890 - 948 mmol/L) in all treatments (Figs. 4.3e and 4.8e) were not significantly different from the seawater osmolality (958 ± 4 mOsm/kg).

Plasma total ammonia concentration decreased by about 60% from 12 to 7°C but it was not altered at 17°C (Fig. 4.4a) despite the 1.7-fold increase in J\(_{\text{amm}}\) at this temperature (Fig. 4.1b). However the calculated partial pressure of NH\(_3\) (PNH\(_3\)) increased with temperature, and was significantly different between 7 and 17°C (Fig. 4.4b). Plasma [NH\(_4^+\)] did not statistically change at the three temperatures tested (data not shown), however the data pattern resembled that of total plasma ammonia. Blood pH and plasma PCO\(_2\) were not altered at the three temperatures tested (Fig. 4.5a,b). However, there was a significant elevation in plasma HCO\(_3^-\) between 7 and 17°C (Fig. 4.5c).

### 4.4.2 Interactive effects of temperature changes and hyperoxia

Animals exposed to hyperoxia at 12°C showed no significant change in J\(_{\text{amm}}\) relative to normoxia at the control temperature (Fig. 4.6a). At 17°C, J\(_{\text{amm}}\) in normoxia was also not different than in hyperoxia. However the increase in J\(_{\text{amm}}\) in hyperoxia (12-17°C Q\(_{10}\) = 4.94) was greater than the one seen in normoxia (Q\(_{10}\) = 2.14). Although temperature had a significant overall effect on J\(_{\text{amm}}\) (p = 0.03), oxygen did not (p = 0.44), and temperature and oxygen did not have a significant interaction (p = 0.60) (Fig. 4.6a).
However, in contrast to the pattern in $J_{\text{amm}}$, hyperoxia had no effect on $J_{\text{H2O}}$ at 12°C but greatly attenuated the large increase in $J_{\text{H2O}}$ which occurred in normoxia at 17°C (Fig. 4.6b). As a result, the 12-17°C $Q_{10}$ in hyperoxia was also much smaller than the one in normoxia (1.86 and 4.00, respectively). There was a significant overall effect of oxygen ($p = 0.01$) and temperature ($p < 0.001$), as well as significant interaction between temperature and oxygen ($p = 0.01$).

There were significant main effects of both oxygen ($p < 0.0001$) and temperature ($p = 0.004$) on the ventilation rate (velar frequency) of longer term (6-h) exposed hagfish (Fig. 4.7a), and there was no significant interaction ($p = 0.35$). Longer term exposure to 17°C tended to increase ventilation rate (Fig. 4.7a), although this difference was not statistically significant in the post hoc test. At 12°C, hyperoxia significantly increased ventilation rate by more than 2-fold, and similarly, at 17°C, exposure to hyperoxia significantly increased velar rate (Fig. 4.7a). There was no significant overall effect of either oxygen ($p = 0.17$) or temperature ($p = 0.22$) on ventilation pressure amplitude (Fig. 4.7b). Exposure to hyperoxia slightly decreased ventilation amplitude at both 12 and 17°C; however, these changes were not statistically significant. Oxygen had a significant overall effect ($p = 0.04$) increasing the calculated ventilatory index (Fig. 4.7c), while the overall stimulatory effect of temperature was not significant ($p = 0.30$). In normoxia, longer term ventilatory index did not increase with temperature (Fig. 4.7c), similarly to short term measurements (Fig. 4.2c). Longer term measurements in hyperoxia suggest that while the pattern of ventilatory increases is reversed in comparison to short term acute measurements, its absolute rates did not change.

There was no significant main effect ($p = 0.59$) of oxygen or interaction ($p = 0.47$) between temperature and oxygen on plasma $[\text{Na}^+]$ (Fig. 4.8a). At 12°C there was a small, but significant increase in plasma $[\text{Cl}^-]$ with hyperoxia, but this same increase was not seen at 17°C (Fig. 4.8b). Hyperoxia did not alter plasma $[\text{Mg}^{2+}]$ at 12°C, but significantly increased it at 17°C (Fig. 4.8c). Overall, there was no main effect of temperature ($p = 0.18$), but the overall effect of oxygen ($p < 0.001$) and the interaction term ($p = 0.01$) were both significant. At both temperatures tested, plasma $[\text{Ca}^{2+}]$ was significantly higher in hyperoxia than in normoxia (Fig. 4.8c). In normoxia only there was a significant decrease in plasma $[\text{Ca}^{2+}]$ at 17°C in comparison to 12°C. Overall, there was a significant main effect of oxygen ($p <
0.001) on plasma [Ca\(^{2+}\)], but no significant main effect of temperature (p = 0.12) or interaction term (p = 0.07). Plasma osmolality was not significantly affected by either temperature or oxygen, and there was no significant interaction (Fig. 4.8e).

At 12°C, plasma total ammonia concentration was not different between animals in normoxia, or acutely exposed to hyperoxia (Fig. 4.9a). However, at 17°C, there was a significant decrease in plasma ammonia between normoxia and hyperoxia. There was a significant main effect of oxygen (p = 0.04) but there was no significant overall effect of temperature (p = 0.34), and the interaction term (p = 0.26) was also not significant (Fig. 4.9a). Plasma [NH\(_4^+\)] (data not shown) exhibited the same trends as plasma total ammonia concentration. There was no significant effect of either of the main factors, temperature or oxygen, on the partial pressure of ammonia, and the interaction term was also not significant (Fig. 4.9b). PNH\(_3\) seemed to be reduced with exposure to hyperoxia at 17°C, however the effect was not significant.

Blood pH increased with exposure to hyperoxia at 12°C (Fig. 4.10a), while at 17°C, although it was elevated, it was not significantly different from the normoxic value. The overall effect of oxygen (p = 0.005) was significant, while those of temperature (p = 0.94) and the interaction term (p = 0.40) were not. Plasma PCO\(_2\) decreased markedly with exposure to hyperoxia at 12°C, but it was not altered by hyperoxia at 17°C (Fig. 4.10b). However, there was a significant increase in PCO\(_2\) at 17°C in hyperoxia only (Fig. 4.10b). There was a significant overall effect of oxygen (p = 0.03) but not temperature (p = 0.13), and the interaction term was significant (p = 0.01). Like PCO\(_2\), plasma [HCO\(_3^-\)] also tended to fall with hyperoxia at 12°C, and it also tended to rise with hyperoxia at 17°C but neither difference was significant. However, plasma [HCO\(_3^-\)] was significantly higher in animals exposed to hyperoxia at 17°C in comparison to hyperoxic hagfish at 12°C. (Fig. 4.10c). The overall effects of oxygen (p = 0.55) and temperature (p = 0.09) were not significant, but there was a significant interaction (p = 0.01) between the two factors.
4.5 Discussion

4.5.1 Overview

Inasmuch as both ammonia and diffusive water flux rates increased as $\dot{M}O_2$ and ventilation increased with temperature, and the water flux response was attenuated by hyperoxia, there was clear evidence of an osmorespiratory compromise in the Pacific hagfish. The differing $Q_{10}$s observed for $\dot{M}O_2$, $J_{amm}$ and $J_{H2O}$ indicate that these are independently regulated, and this is likely because they move through different pathways at the gills and skin. Plasma ions and osmolality were also affected, whereas there was little change in plasma acid-base status. These data clearly demonstrate that even an animal commonly considered as an osmoconformer shows interactions between osmoregulation and respiration.

4.5.2 Temperature effects on the rates of oxygen consumption, ventilation, ammonia and water fluxes

The osmorespiratory compromise is a phenomenon that can occur at any gas exchange surface, and while we usually think of the gills, it could also happen at the skin. The debate on the major site of oxygen uptake in hagfish spans almost 30 years, and recently it has been shown in $E. stoutii$, that the bulk of the animal’s oxygen uptake occurs through their gills (Clifford et al., 2016a). Our results confirm previous reports (Munz and Morris, 1965; Steffensen et al., 1984; Perry et al., 2009b) that hagfish $\dot{M}O_2$ values are amongst the lowest reported for vertebrates. As metabolic demand for oxygen increases with temperature, oxygen uptake from the environment must be elevated and/or increased anaerobic metabolism must occur. In our study, $\dot{M}O_2$ increased linearly with temperature ($R^2 = 0.991$; data not shown), with an overall $Q_{10}$ of 3.22 (Fig. 4.1a), which is greater than in marine elasmobranchs (Butler and Taylor, 1975; Giacomin et al., 2017) and teleosts where the $Q_{10}$s are usually around 2.0 or lower (Clarke and Johnston, 1999; Clark et al., 2011; Lea et al., 2016). Munz and Morris (1965) reported $Q_{10}$s of 2.4 – 2.8 for $\dot{M}O_2$ in $E. stoutii$, similar to the results shown here, further confirming the temperature sensitivity of this species. In nature,
Hagfish generally inhabit the ocean floors, known to be nutrient-poor but very stable environments (Martini, 1998). Similarly, E. stoutii is found in deep areas where temperature varies only between 10 and 11ºC (McInerney and Evans, 1970). Importantly, the high temperature sensitivity $\dot{M}O_2$ of this species ($Q_{10}^{7-12\degree C} = 3.75$) at the low range tested (close to temperature range of their natural habitat) suggests that the impacts of temperature challenges on these fishes (as occurs during commercial fishing) may be of special concern.

Hagfish extract $O_2$ from the water through a counter-current mechanism, resulting in high diffusive conductance of the gill (Malte and Lomholt, 1998). There are very few reported measurements of hagfish ventilatory dynamics, mostly due to the technical difficulties involved in measuring ventilation on these complex animals (Steffensen et al., 1984; Perry et al., 2009b). Eom and Wood (2018) have recently developed methods for measuring ventilation and heart rate in hagfish, which were employed here, and have shown that increases in both ventilatory pressure amplitude (reflecting velar stroke volume) and ventilatory frequency (velar rate) lead to increases in ventilatory water flow. In our study, ventilation frequency did not vary with temperature when the challenges were done acutely (Fig. 4.2a). In contrast, ventilation pressure amplitude increased linearly, resulting in a ventilatory index (a proxy for total ventilatory water flow, Eom and Wood, 2018) that decreased substantially between 12 and 7ºC, but exhibited only a slight increase between 12 and 17ºC (Fig. 4.2c). The greatly increased coughing frequency at 17ºC suggests pathological impacts on ventilation at 17ºC; at high flows, perhaps more particles are encountered, resulting in more coughing. The function of the coughs in the ventilatory dynamics has yet to be elucidated and the possible contributions of these flow reversals to $\dot{M}O_2$ are unknown. The constancy of ventilatory frequency suggests that immediate temperature-induced changes in ventilatory water flow are achieved mainly by changes in the stroke volume of the velar apparatus. This contrasts with the effects of hypoxia and hypercapnia, where most of the reported changes resulted from alterations in respiratory frequency in this same species (Perry et al., 2009b). However, our results differ greatly from the report of Coxon and Davison (2011) on the congeneric New Zealand hagfish (Eptatretus cirrhatus) where velar frequency increased with a $Q_{10}$ of 3.2 during exposure to temperatures between 7 and 19ºC.
Notably, the temperature protocols were very different in the two studies, and Coxon and Davison (2011) did not measure ventilation, but rather velar frequency by electromyography.

Measured heart rate values in control conditions (~15 beats/min) were somewhat higher than those reported by Cox et al. (2010) (~10 beats/min). In contrast to the ventilatory index, heart rate did not change between 12 and 7°C but was elevated at 17°C (Fig. 4.2e), suggesting that at the lower temperature range, rather than solely relying on changes in heart rate, modulation of cardiac stroke volume could be responsible for maintaining the ČO₂ at the gills. This response was observed when *E. stoutii* was exposed to anoxia, as cardiac stroke volume increased to compensate for the bradycardia that resulted from anoxia (Cox et al., 2010).

Ammonia transporting proteins Rhbg and Rhcg have been identified in both the gills and the skin of the Atlantic hagfish *Myxine glutinosa* (Edwards et al., 2015) and the Pacific hagfish *E. stoutii* (Clifford et al., 2017). They were likely an important route of ammonia excretion in the present study. In *E. stoutii*, 30% of the animal’s resting J_{amn} can occur through the skin (Clifford et al., 2016a), therefore, rates of J_{amn} reported here should be interpreted as a summation of branchial and cutaneous contributions. The present control rates of J_{amn} (~15 μmol/kg/h; Fig. 4.1b) were generally lower than those previously reported in the literature (30 - 50 μmol/kg/h) (Walsh et al., 2001; Braun and Perry, 2010; Clifford et al., 2015; Wilkie et al., 2017). Overall, J_{amn} seemed to be less sensitive to temperature than ČO₂, but this was only apparent in the 12 to 7°C range where the J_{amn} Q₁₀ = 1.86 was much lower than the ČO₂ Q₁₀ = 3.75 (Fig. 4.1a). In ammoniotelic teleosts, J_{amn} is more sensitive to temperature than ČO₂ (Wood, 2001), and the same was seen for the ureotelic spiny dogfish (Giacomin et al., 2017). The elevation in plasma ammonia at 12 ºC and 17ºC in comparison to 7ºC (Fig. 4.4a) was possibly a result of the general increase in metabolic rate (reflected in ČO₂) and the accompanying breakdown of amino acids. This would lead to an elevation in the gradient for ammonia diffusion (PNH₃; Fig. 4.4b). Since ČO₂ and J_{amn} were measured simultaneously in the same fish, we have calculated the ammonia quotient (AQ) in order to estimate the metabolic fuel used in aerobic metabolism (Lauff and Wood, 1996a). AQ values ranged from 0.03 to 0.04, which are very low numbers, indicating that these fasting hagfish were utilizing only 13% protein to fuel aerobic metabolism (Fig. 4.1c). This may be an
important adaptation for protein conservation in an animal that lives in a nutrient-poor environment where feeding opportunities are scarce. The AQ did not vary over the range of temperatures tested, similar to results seen with NQ in the ureotelic spiny dogfish shark (Giacomin et al., 2017), but in that animal, aerobic metabolism was fueled by approximately 100% somatic protein.

Pacific hagfish have very high permeability to water, with a rate constant \( k \) of 2.28 \( h^{-1} \), exchanging about 1.4 L water/kg/h at 12°C, and that nearly doubled after a 5°C increase in temperature \( (Q_{10} = 4.01) \) (Fig. 4.1d). The high diffusive water flux rates measured here are in close proximity to values measured in a recent study by Glover et al. (2017) on E. stoutii and to those of Rudy and Wagner (1970) on the same species (previously known as Poliostrema stouti). Both were performed at the same control temperature (12°C). Diffusive water flux rates are significantly lower in seawater teleosts compared to freshwater teleosts (Motais et al., 1969). Results from the current study indicate that hagfish possesses water flux rates that are higher than even those in freshwater fishes (e.g. Evans, 1969; Onukwufor and Wood, 2018) and substantially greater than those in marine teleosts (Motais et al., 1969) and elasmobranchs (Carrier and Evans, 1972; Wright and Wood, 2016; Giacomin et al., 2017). It is thought that this high water permeability is a trait linked to the primitive characteristics of these fishes, and likely a reflection of their osmoconforming strategy, where maintenance of the isosmotic balance is a key requirement (Rudy and Wagner, 1970). To our knowledge, this is the first study that looked at the effects of temperature on the diffusive water flux rates in a species of hagfish. When fish were exposed to a 5°C decrease in temperature, \( J_{H2O} \) was mildly affected \( (Q_{10} of 1.35) \), differing from \( \dot{M}O_2 \) and \( J_{amm} \), which were greatly affected. However, a 5°C increase in temperature led to a significant increase in \( J_{H2O} \), a much more marked response than that seen with \( \dot{M}O_2 \) \( (J_{H2O} Q_{10} of 4.01 \text{ versus } \dot{M}O_2 Q_{10} of 2.77) \). A comparable response was also seen in the spiny dogfish (Giacomin et al., 2017). In hagfish gills, the expression of at least one aquaporin (AQP4) has been identified, and localized in the basolateral membrane of pavement cells (Nishimoto et al., 2007). Two aquaporin homologs (AQP3 and AQP4) have also been identified at the slime glands throughout the skin (Herr et al., 2014). Although the functional role of aquaporins in the
hagfish is yet to be characterized (Nishimoto et al., 2007; Madsen et al., 2015), it is very likely that they played a role in the changes in water permeability shown in the current study.

4.5.3 Temperature effects on ionoregulation and acid-base status

Hagfishes are unusual among aquatic vertebrates exhibiting a unique strategy of both iono and osmoconformation (Wright, 2007). More recently, two different studies have shown that they actually maintain plasma [Na\(^+\)] slightly higher and plasma [Cl\(^-\)] slightly lower than sea water (Sardella et al., 2009; Clifford et al., 2018). The results presented here confirm that finding (Fig. 4.3a,b). Despite the significant evolutionary importance of hagfishes, little is known about the mechanisms that are involved in maintaining hydro-mineral balance in these animals (Currie and Edwards, 2010). Interestingly, early findings by Bellamy and Chester Jones (1961), more recently confirmed by Sardella et al. (2009) and by the present study, show that Pacific hagfish have the capacity to regulate divalent ions ([Ca\(^{2+}\)] and [Mg\(^{2+}\)]) in plasma well below environmental levels. In our study, while plasma [Na\(^+\)] did not statistically vary within the three temperatures tested (Fig. 4.3a), there was a tendency to rise with temperature, and there was a significant elevation in plasma [Cl\(^-\)] at 17ºC, bringing the level closer to that of sea water (Fig. 4.3b). The absolute increases in mean plasma [Na\(^+\)] and [Cl\(^-\)] were sufficient to account for the absolute increase in plasma osmolality, since Na\(^+\) and Cl\(^-\) are the major ions constituting the blood plasma. We are aware of only a single study (unpublished) that investigated the plasma ion status of E. stoutii after acute exposure to different temperatures (Hastey, 2011). After 6 h of exposure to 6 and 12ºC increases in temperature, plasma Na\(^+\) did not change, while plasma Cl\(^-\) seemed to be reduced, a result opposite to the ones reported here. However, Hastey (2011) reported that plasma osmolality did tend to increase at high temperatures, similarly to what we found (Fig. 4.3e). It is somewhat curious that both cations (Mg\(^{2+}\) and Ca\(^{2+}\)) that hagfish regulate at concentrations far lower than in sea water were maintained further away from the seawater levels as temperature and MO\(_2\) increased (Fig. 4.3c,d), contrary to the results seen by Hastey (2011), where plasma [Mg\(^{2+}\)] slightly increased with temperature. Our results support the previous data showing regulation of divalent cations, but the explanation for a higher excretion rate
and/or lower entry rate for these ions at higher temperature remains unknown and is an interesting avenue for future research.

Baker et al. (2015) demonstrated the remarkable ability of hagfish in correcting blood pH disturbances, through an unprecedented capacity to accumulate HCO$_3^-$ in the blood plasma, likely due to exchanges with Cl$^-$ at the gills (Evans, 1984). The skin may also play a role in acid-base exchange (Clifford et al., 2014). Intriguingly, in our study, at 17ºC we observed an elevation in both plasma HCO$_3^-$ and plasma Cl$^-$, while plasma pH was not altered (Fig. 4.5a,b,c). According to the relative alkalinity (Rahn and Baumgardner, 1972) or alphastat (Reeves, 1977) hypotheses, blood pH falls with increases in temperature, while in our study, pH remained unchanged through the range of temperatures tested. At present, it is unclear whether the present data represent an alternative strategy to maintain blood pH constant, independent of temperature, or whether it is because the time frame of the temperature challenge (6 h) was too short to see the pattern typical of most fishes. In future, longer-term exposures to altered temperatures will be informative.

4.5.4 Does hyperoxia alleviate the effects of temperature on Pacific hagfish?

We had hypothesized that by increasing the oxygen supply at the gills through environmental hyperoxia, the disturbances caused by elevated temperature in the fluxes of other moieties with the surrounding environment would be minimized. Our predictions were only partially supported. Certainly, at 17ºC, exposure to hyperoxia significantly reduced the diffusive flux of water ($J_{H2O}$) to almost control normoxic levels (Fig. 4.6a), whereas at 12ºC (control), hyperoxia did not have any effect on $J_{H2O}$ or $J_{amm}$ (Fig. 4.6a,b). Surprisingly, contrary to our predictions, hyperoxia did not depress ventilation (Fig. 4.7). In fact, at both 12ºC and 17ºC, hyperoxia caused an increase in ventilation frequency (Fig. 4.7b), but since pressure amplitude was reduced (Fig. 4.7b), total ventilation did not change (Fig. 4.7c). While the two-way ANOVA showed no significant differences on the ventilatory index between normoxia and hyperoxia at 12ºC, a pairwise comparison demonstrated that hyperoxia significantly increase ventilatory index. Hyperoxia also did not alleviate the increase in $J_{amm}$ at 17ºC, despite a decrease in PNH$_3$ and a significant decrease in total plasma.
ammonia (Fig. 4.9a,b). This could indicate that hyperoxia is playing a role in decreasing the concentration gradient driving ammonia diffusion at the gills, but not affecting the metabolic production rate of ammonia. Our results differ from those of Giacomin et al. (2017) in the spiny dogfish, where the fluxes of ammonia and urea-N were actually exacerbated by hyperoxia at high temperature, whereas the diffusive water flux ($J_{H2O}$) was not affected. In the present study, plasma [$Na^+$], [$Cl^-$] and osmolality were largely unaffected by hyperoxia at the two temperatures tested (Fig. 4.8a,b). However, hyperoxia increased plasma [$Mg^{2+}$] and [$Ca^{2+}$] at the two temperatures tested (Fig. 4.8c,d). As these ions are normally regulated substantially below concentrations in sea water, then the increases caused by hyperoxia could be detrimental rather than beneficial.

In most fishes, hyperoxia usually depresses ventilation, so the increased CO$_2$ retention in the blood causes a respiratory acidosis and consequently, decrease blood pH (Heisler, 1984; Heisler et al., 1988; Wood, 1991). This hyperoxia-induced hypercarbia usually elicits slower compensatory responses than environmental hypercapnia (Heisler, 1993). In our study, hyperoxia tended to increase rather than decrease ventilation, so the accompanying decrease in plasma PCO$_2$ and elevation in pH at 12ºC (Fig. 4.10a,b) could be a reflection of this effect. Additionally, there was no change in plasma Cl$^-$ during hyperoxia at either 12 or 17ºC (Fig. 4.8b), which could indicate that the HCO$_3^-/Cl^-$ exchanger mechanisms had not been activated yet. Work by Perry et al. (2009) has shown that hagfish gills likely possess both internal and external O$_2$ chemoreceptors involved in the control of breathing. A more detailed study of ventilatory control, oxygen transfer capabilities, and the influence of hyperoxia on blood O$_2$ and CO$_2$ in hagfish is required.

### 4.5.5 Future directions and perspectives

Hagfish populations all over the world have been commercially exploited for about 30 years now. More recently, the international demand for hagfish skin for the production of leather, as well as a growing market for live hagfish in Korea, has added pressure for the harvest of these animals in both Pacific and Atlantic coasts of North America (Barss, 1993; Grant, 2006). Additionally, given their demersal habitat, hagfish are common by-catch items
resulting from bottom-trawls that target other species. Current targeted fishing practices involve pulling baited traps full of hagfish rapidly to the ocean surface, where they can be exposed to abrupt changes in pressure, salinity and temperature (Olla et al., 1998). Hagfish that do not match size requirements are discarded. Very little is known about the general ecology and physiology of hagfish, knowledge which is usually a basic requirement for the establishment of a sustainable commercial fishery. Our results indicate that hagfish are quite susceptible to acute changes in ambient temperature. The higher temperatures characteristic of surface waters and “on-deck” conditions will lead to elevations in the diffusive permeability of the gills to water, as well as marked increases in metabolic rate that likely result in increases in the rate of production of nitrogenous waste. Additionally, plasma ions and osmolality are disturbed at high temperature. We were able to show that hyperoxia can have some beneficial effects counteracting the temperature effects (reduced diffusive water fluxes), but for the majority of parameters analyzed here, hyperoxia led to adverse effects, such as a disruption of plasma ion and acid-base balance. Future studies should specifically focus on the mechanisms employed to regulate divalent cations and blood acid-base status in hagfishes facing temperature challenges. Also, since reactive oxygen species (ROS) can be generated both by temperature and hyperoxia, it is of great importance to characterize the responses of these fishes to ROS, as well as the potential for oxidative damage, and their antioxidant defense mechanisms.

The present results are similar to those reported by Giacomin et al. (2017) in the dogfish shark, another marine osmoconformer, where \( \dot{M}O_2 \) and the fluxes of ammonia, urea-N and water were differentially affected by temperature. These response patterns were likely due to the different transport pathways of these moieties. In summary, a complex osmorespiratory interaction occurs as a result of acute temperature changes, even in this extant craniate which is commonly believed to be an osmoconformer.
Figure 4.1 (a) Oxygen consumption rate ($\dot{\text{MO}_2}$: $\mu$mol O$_2$/kg/h), (b) ammonia net flux rate ($J_{\text{amm}}$: $\mu$mol/kg/h), (c) calculated ammonia quotient (AQ) and (d) diffusive water flux rate ($J_{\text{H}_2\text{O}}$: L/kg/h) in Eptatretus stoutii acutely exposed to 7, 12 (control) and 17 ºC. The $Q_{10}$ values over the entire range (7 – 17ºC) are displayed on the upper left corner. The lower range (7 – 12ºC) $Q_{10}$ are shown above the 12ºC bar and the higher range (12 – 17ºC) $Q_{10}$ are shown above the 17ºC bar. Means sharing the same lower case letters are not statistically different (one-way ANOVA; Tukey’s post hoc test). Data are shown as means ± 1 SEM (n = 8 - 16).
\( Q_{10} (7\text{ - }17^\circ C) = 1.09 \)

- Ventilation rate (breaths/min)
  - Temperature (°C): 7, 12, 17
  - Values: 20, 20, 20
  - Differences:
    - 7 vs 12: a
    - 12 vs 17: a

\( Q_{10} (7\text{ - }17^\circ C) = 2.90 \)

- Ventilatory index (cm H\(_2\)O/min)
  - Temperature (°C): 7, 12, 17
  - Values: 6.53, 4.0, 3.0
  - Differences:
    - 7 vs 12: a
    - 12 vs 17: b

\( Q_{10} (7\text{ - }17^\circ C) = 1.89 \)

- Pressure amplitude (cm H\(_2\)O/breath)
  - Temperature (°C): 7, 12, 17
  - Values: 0.1, 0.2, 0.3
  - Differences:
    - 7 vs 12: a
    - 12 vs 17: a

\( Q_{10} (7\text{ - }17^\circ C) = 1.38 \)

- Heart rate (beats/min)
  - Temperature (°C): 7, 12, 17
  - Values: 10, 10, 10
  - Differences:
    - 7 vs 12: a
    - 12 vs 17: a
Figure 4.2 (a) Ventilation rate (frequency: breaths/min), (b) ventilation pressure amplitude (cmH\textsubscript{2}O/breath), (c) calculated ventilatory index (cmH\textsubscript{2}O/min) and (d) heart rate (beats/min) in *Eptatretus stoutii* acutely exposed (30 min) to 7, 12 (control) and 17 °C. The Q\textsubscript{10} values over the entire range (7 – 17°C) are displayed on the upper left corner. The lower range (7 – 12°C) Q\textsubscript{10} are shown above the 12°C bar and the higher range (12 – 17°C) Q\textsubscript{10} are shown above the 17°C bar. Means sharing the same lower case letters are not statistically different (repeated measures one-way ANOVA; Tukey’s post hoc test). Data are shown as means ± 1 SEM (n = 6 - 8).
**Figure 4.3** Plasma ion concentration (a) Na\(^+\), (b) Cl\(^-\), (c) Mg\(^{2+}\), (d) Ca\(^{2+}\) (all in mmol/L) and (e) osmolality (mOsm/kg) in *Eptatretus stoutii* acutely exposed to 7, 12 (control) and 17°C. Means sharing the same lower case letters are not statistically different (one-way ANOVA; Tukey’s post hoc test). Data are shown as means ± 1 SEM (n = 7 - 8).
**Figure 4.4** (a) Total plasma total ammonia (µmol/L), (b) and partial pressure of NH$_3$ (µTorr) in *Eptatretus stoutii* acutely exposed to 7, 12 (control) and 17ºC. Means sharing the same lower case letters are not statistically different (one-way ANOVA; Tukey’s post hoc test). Data are shown as means ± 1 SEM (n = 6 - 8).
Blood pH

Plasma PCO₂ (Torr)

Plasma HCO₃ (mmol/L)

Temperature (°C)
Figure 4.5 (a) Blood pH, (b) plasma PCO$_2$ (Torr) and (c) plasma HCO$_3^-$ (mmol/L) in *Eptatretus stoutii* acutely exposed to 7, 12 (control) and 17ºC. Means sharing the same lower case letters are not statistically different (one-way ANOVA; Tukey’s post hoc test). Data are shown as means ± 1 SEM (n = 6 - 8).
Ammonia net flux (µmol/kg/h)

Temperature (ºC)

Normoxia

Hyperoxia

Water flux rate (L/kg/h)

Temperature (ºC)
Figure 4.6 (a) Ammonia net flux rate ($J_{\text{amm}}$: µmol/kg/h), (b) diffusive water flux rate ($J_{\text{H2O}}$: L/kg/h) in *Eptatretus stoutii* acutely exposed to hyperoxia (black bars) or normoxia (white bars) at 12 and 17°C. The normoxia Q_{10} are shown in the 17°C normoxia bar while the hyperoxia Q_{10} are shown in the 17°C bar. Normoxia and hyperoxia bars sharing the same lower case letters are not statistically different at the same temperature [Two-way ANOVA p-values ($J_{\text{amm}}$: $p_{\text{interaction}} = 0.601$, $p_{\text{oxygen}} = .443$, $p_{\text{temperature}} = .039$; $J_{\text{H2O}}$: $p_{\text{interaction}} = .014$, $p_{\text{oxygen}} = .001$, $p_{\text{temperature}} < .001$)]. Asterisks indicate significant differences between 12 and 17°C at the same oxygen level (Bonferroni post hoc test). Data are shown as means ± 1 SEM (n = 6 - 8).
**Graph A:**
- y-axis: Ventilation rate (breaths/min)
- x-axis: Temperature (°C)
- Two groups: Normoxia (white bars) and Hyperoxia (black bars)
- Bars marked with 'a', 'b', 'x', 'y' indicate significant differences among groups.

**Graph B:**
- y-axis: Pressure amplitude (cm H$_2$O/breath)
- x-axis: Temperature (°C)
- Bars marked with 'a', 'b', 'x', 'y' indicate significant differences among groups.

**Graph C:**
- y-axis: Ventilatory index (cm H$_2$O/min)
- x-axis: Temperature (°C)
- Bars marked with 'a', 'b', 'x', 'y' indicate significant differences among groups.
Figure 4.7 (a) Ventilation rate (frequency: breaths/min), (b) ventilation pressure amplitude (cmH₂O/breath), and (c) calculated ventilatory index (cmH₂O/min) in *Eptatretus stoutii* exposed to experimental conditions for 6 h. Normoxia and hyperoxia bars sharing the same lower case letters are not statistically different at the same temperature [Two-way ANOVA p-values (rate: $p_{\text{interaction}} = .3597$, $p_{\text{oxygen}} < .0001$, $p_{\text{temperature}} = .0045$; pressure: $p_{\text{interaction}} = .3994$, $p_{\text{oxygen}} = .1707$, $p_{\text{temperature}} = .2259$; index: $p_{\text{interaction}} = .6268$, $p_{\text{oxygen}} = .0421$, $p_{\text{temperature}} = .3045$)]. Data are shown as means ± 1 SEM (n = 6 - 8).
Plasma ion concentration (mmol/L)

- **Panel A**: Sodium ($\text{Na}^+$)
- **Panel B**: Chloride ($\text{Cl}^-$)
- **Panel C**: Magnesium ($\text{Mg}^{2+}$)
- **Panel D**: Calcium ($\text{Ca}^{2+}$)

Plasma osmolality (mOsm/kg)

- **Panel E**: Osmolality

Comparison groups:
- a
- b
- x
- y

Notes:
- Temperature (°C) values: 12, 17
- Plasma ion concentration and osmolality are shown with error bars indicating standard deviation.
Figure 4.8 Plasma ion concentrations (a) Na$^+$, (b) Cl$^-$, (c) Mg$^{2+}$, (d) Ca$^{2+}$ (mmol/L) and (e) osmolality (mOsm/kg) in *Eptatretus stoutii* acutely exposed to hyperoxia (black bars) or normoxia (white bars) at 12 and 17ºC. Normoxia and hyperoxia bars sharing the same lower case letters are not statistically different at the same temperature [Two-way ANOVA p-values (Na$^+$: $p_{interaction}=.479$, $p_{oxygen}=.595$, $p_{temperature}=.762$; Cl$^-$: $p_{interaction}=.037$, $p_{oxygen}=.626$, $p_{temperature}=.559$; Mg$^{2+}$: $p_{interaction}=.012$, $p_{oxygen}=.001$, $p_{temperature}=.184$; Ca$^{2+}$: $p_{interaction}=.067$, $p_{oxygen}=0.001$, $p_{temperature}=.125$; osmolality: $p_{interaction}=.387$, $p_{oxygen}=.906$, $p_{temperature}=.948$)]. Asterisks indicate significant differences between 12 and 17ºC at the same oxygen level (Bonferroni post hoc test). Data are shown as means ± 1 SEM (n = 6 - 8).
Plasma ammonia (µmol/L)

Temperature (°C)

P NH₃ (µTorr)

Temperature (°C)
Figure 4.9 (a) Total plasma total ammonia (µmol/L), (b) and partial pressure of NH$_3$ (PNH$_3$: µTorr) in *Eptatretus stoutii* acutely exposed to hyperoxia (black bars) or normoxia (white bars) at 12 and 17°C. Normoxia and hyperoxia bars sharing the same lower case letters are not statistically different at the same temperature [Two-way ANOVA p-values (Total ammonia: p$_{interaction}$ = .256, p$_{oxygen}$ = .040, p$_{temperature}$ = .340; PNH$_3$: p$_{interaction}$ = .165, p$_{oxygen}$ = .303, p$_{temperature}$ = .466)]. Asterisks indicate significant differences between 12 and 17°C at the same oxygen level (Bonferroni post hoc test). Data are shown as means ± 1 SEM (n = 6 - 8).
Figure 4.10 (a) Blood pH, (b) plasma PCO$_2$ (Torr) and (c) plasma HCO$_3^-$ (mmol/L) in *Eptatretus stoutii* acutely exposed to hyperoxia (black bars) or normoxia (white bars) at 12 and 17ºC. Normoxia and hyperoxia bars sharing the same lower case letters are not statistically different at the same temperature [Two-way ANOVA p-values (pH: $p_{\text{interaction}} = .403$, $p_{\text{oxygen}} = .005$, $p_{\text{temperature}} = .937$; PCO$_2$: $p_{\text{interaction}} = .011$, $p_{\text{oxygen}} = .025$, $p_{\text{temperature}} = .128$; HCO$_3^-$: $p_{\text{interaction}} = .010$, $p_{\text{oxygen}} = .548$, $p_{\text{temperature}} = .090$)]. Asterisks indicate significant differences between 12 and 17ºC at the same oxygen level (Bonferroni post hoc test). Data are shown as means ± 1 SEM (n = 7 - 8).
Table 4.1 Rate constant of $^3$H$_2$O turnover (k) in Pacific hagfish exposed to 7, 12 and 17ºC in normoxia (series 1), and to 12 and 17ºC in both normoxia and hyperoxia (series 2). Series 1 data were compared through a one-way ANOVA. Different lower case letters indicate statistical significance (Tukey’s post hoc test). Series 2 data were compared through a two-way ANOVA. Similar upper case letters indicate lack of significant differences between normoxia and hyperoxia at the same temperature. [Two-way ANOVA p-values (k: $p_{interaction} = .058$, $p_{oxygen} = .002$, $p_{temperature} = .194$)]. Asterisks indicate significant differences between 12 and 17ºC at the same oxygen level (Bonferroni post hoc test) (Series 2 only). Data are means ± 1 SEM (n = 7).

<table>
<thead>
<tr>
<th>Temperature (ºC)</th>
<th>Normoxia</th>
<th>Normoxia</th>
<th>Hyperoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>$1.92 \pm 0.17^a$</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td>12</td>
<td>$2.28 \pm 0.15^a$</td>
<td>$2.04 \pm 0.21^A$</td>
<td>$2.20 \pm 0.22^A$</td>
</tr>
<tr>
<td>17</td>
<td>$3.38 \pm 0.28^b$</td>
<td>$3.38 \pm 0.28^{*x}$</td>
<td>$2.55 \pm 0.19^x$</td>
</tr>
</tbody>
</table>
Chapter 5: The effects of salinity and hypoxia exposure on oxygen consumption, ventilation, diffusive water exchange and ionoregulation in the Pacific hagfish (Eptatretus stoutii)

5.1 Summary

Hagfishes (Class: Myxini), are marine jawless craniate fishes that are widely considered to be osmoconformers whose plasma [Na$^+$], [Cl$^-$], and osmolality closely resemble that of sea water, although they have the ability to regulate plasma [Ca$^{2+}$] and [Mg$^{2+}$] below seawater levels. We investigated the responses of Pacific hagfish to changes in respiratory and ionoregulatory demands imposed by a 48-h exposure to altered salinity (25 ppt, 30 ppt (control) and 35 ppt) and by an acute hypoxia exposure (30 Torr; 4 kPa). When hagfish were exposed to 25 ppt, oxygen consumption rate ($\dot{M}O_2$), ammonia excretion rate ($J_{\text{am}}$) and unidirectional diffusive water flux rate ($J_{\text{H}_2\text{O}}$, measured with $^3\text{H}_2\text{O}$), were all reduced, pointing to an interaction between ionoregulation and gas exchange. At 35 ppt, $J_{\text{H}_2\text{O}}$ was reduced, though $\dot{M}O_2$ and $J_{\text{am}}$ did not change. As salinity increased, so did the difference between plasma and external water [Ca$^{2+}$] and [Mg$^{2+}$]. Notably, the same pattern was seen for plasma Cl$^-$, which was kept below seawater [Cl$^-$] at all salinities, while plasma [Na$^+$] was regulated well above seawater [Na$^+$], but plasma osmolality matched seawater values. $\dot{M}O_2$ was reduced by 49% and $J_{\text{H}_2\text{O}}$ by 36% during hypoxia, although overall ventilation was slightly elevated. Thus despite an attempt to increase O$_2$ permeability, permeability to water was selectively reduced. Our results depart from the “classical” osmorespiratory compromise but are in accord with responses in other hypoxia-tolerant fish; instead of an exacerbation of gill fluxes when gas transfer is upregulated, the opposite occurs.

5.2 Introduction

Gas exchange across the gill can be modulated under conditions that require increased O$_2$ uptake and CO$_2$ excretion, such as exercise, or environmental hypoxia. The osmorespiratory compromise is the trade-off between the need for high gill permeability to
promote respiratory gas exchange, and low gill permeability to limit ion and water diffusion (Randall et al., 1972; Nilsson, 1986). The majority of the studies on the osmorespiratory compromise thus far have focused on how freshwater teleosts (e.g. the rainbow trout) deal with the permeability trade-off at the gills following increases in metabolic demand caused by exercise (Randall et al., 1972; Wood and Randall, 1973a; Gonzalez and McDonald, 1992), or a reduction in environmental oxygen supply, i.e. hypoxia (Wood et al., 2009; Iftikar et al., 2010; Robertson et al., 2015b). The classic response is an increase in the fluxes of ions and osmolytes as a consequence of the increases in functional branchial surface area, ventilation and perfusion rates, needed to elicit oxygen consumption increases (Gonzalez and McDonald, 1992; Gonzalez, 2011). There are, however, a few examples, exclusively in hypoxia-tolerant fish, where hypoxia challenges cause the opposite response, a decrease in the fluxes of ions and water across the gills, despite increases in effective $O_2$ permeability (Robertson et al., 2015b; Scott et al., 2008; Wood et al., 2009; Chapters 2 and 3). To date, investigations on the osmorespiratory compromise have been focused largely on freshwater species, mostly for technical and practical reasons, even though seawater fishes are also susceptible to such phenomena (e.g. Stevens, 1972; Chapters 2 and 3).

More recently, it has been shown that the osmorespiratory compromise also occurs in two non-teleost marine species. These include sharks (Class Chondrichthyes), where a failure of the urea retention mechanism occurs, evidenced by exacerbated urea efflux rates when dogfish ($Squalus acanthias suckleyi$) are exposed to either hypoxia (Zimmer and Wood, 2014) or increased temperature (Giacomin et al., 2017; Chapter 6). Hagfishes (Class: Myxini), which are widely accepted as osmoconformers, are benthic scavengers that feed on decaying carrion and often burrow themselves into the coelomic cavities of their prey (Martini, 1998) where they can be exposed for long periods of time to severe hypoxia or even anoxia. Pacific hagfish ($Eptatretus stoutii$) inherently possess extremely low oxygen consumption rates (Munz and Morris, 1965; Giacomin et al. 2018; Chapter 4). When exposed to higher temperatures (as a modulator of oxygen demand), these animals show a high sensitivity of $\dot{M}O_2$ ($Q_{10} = 3.22$), accompanied by marked increases in the diffusive fluxes of ammonia and water (Giacomin et al. 2018; Chapter 4). Therefore these animals also appear to suffer from an osmorespiratory interaction. The present study aims to follow up on the
osmorespiratory compromise responses of Pacific hagfish using two experimental treatments: short term salinity alteration and an acute hypoxia exposure, in order to elicit changes in gill respiratory and ionoregulatory demands.

One may question whether an ionoregulatory challenge is relevant for a hagfish, since for a long time these animals have been considered the only extant craniates that are both osmo- and ionoconformers, with plasma [Na$^+$], [Cl$^-$], and osmolality closely resembling those of sea water (Smith, 1932; Robertson, 1976). However, it is now known that divalent cations, such as [Ca$^{2+}$] and [Mg$^{2+}$] are kept well below seawater levels (Sardella et al. 2009; Giacomin et al. 2018; Chapter 4). Very recently, it has been shown that there is a small offset between plasma and seawater levels of Na$^+$ and Cl$^-$, where the former is regulated slightly above sea water, while the latter is regulated slightly below sea water (Clifford et al., 2015; Giacomin et al. 2018; Chapter 4). A second goal of the present study was to delve deeper into this phenomenon of apparent ionoregulation, using this same two challenges in order to elicit plasma ion concentration changes.

Our first hypothesis was that as salinity is altered away from the control (seawater) condition, $\dot{M}O_2$ would be depressed so that permeability could be reduced to preserve osmo/ionoregulatory status. Secondly, if plasma ions really are actively regulated to maintain constant internal concentrations, we predicted that exposure to different external ion concentrations would elicit compensatory changes, that is, the plasma-to-water gradients would become smaller with low salinity and greater with high salinity. Thirdly, we hypothesized that as hypoxia-tolerant animals, hagfish would decrease gill permeability and ventilation when exposed to hypoxia, thus preventing the unfavourable exacerbation in the fluxes that arise due to the osmorespiratory compromise. Our approach involved measurements of the rates of oxygen uptake ($\dot{M}O_2$), ammonia excretion ($J_{\text{amm}}$), and diffusive water flux, as well as plasma ions, ammonia, acid-base status, and osmolality. Ventilatory rates were also recorded during the hypoxia challenge.
5.3 Material and Methods

5.3.1 Animal collection and housing

Pacific hagfish (*Eptatretus stoutii*) were caught in two batches from Trevor Channel, near Bamfield Marine Sciences Centre (BMSC), Bamfield, British Columbia, Canada, under Fisheries and Oceans Canada collecting permit XR1942017, using bottom-dwelling traps baited with rotting hake (*Merluccius productus*). Fish were immediately transferred to BMSC, where they held in 200-L darkened tanks, served with flow-through fully aerated sea water (12 – 13°C, 30 ppt) for a period of 1-4 weeks. Two collections were performed, and the second were held for a lesser period (2-3 weeks) than the first (4-5 weeks). For both, fish were fasted for at least one week prior to experimentation. At the end of all experimentation and sampling, fish were euthanized by an overdose of anesthetic (MS-222, Syndel Labs, Parksville, BC, Canada; 5 g/L neutralized to pH 7.8 with 5 M NaOH), followed by evisceration to ensure death. All experiments were performed following the guidelines of the Canada Council for Animal Care, under joint approval of the animal care committees at the University of British Columbia and BMSC (AUPs A14-0251 and RS–17-20) respectively.

5.3.2 The effects of salinity on rates of oxygen consumption, ammonia excretion, and diffusive water exchange

Hagfish from the first collected batch were transferred in groups of n = 16 from the main holding tanks to 80-L darkened plastic containers where they were exposed for 48 h to 25 ppt (obtained by diluting seawater with dechlorinated BMSC tap water), 30 ppt (plain filtered seawater), or 35 ppt (filtered seawater where salinity was raised by the addition of Instant Ocean artificial salts; Spectrum Brands, Blacksburg, VA, USA), all under normoxia. Salinity was monitored using a portable conductivity meter (Cond 3110, WTW, Weilheim, Germany). Water in the acclimation tanks was renewed every 24 h.

All flux experiments were carried out using 1-L glass jars as the experimental containers, covered with dark plastic, topped with a fine mesh, and fitted with fine tubing for
aeration. At 42 h of salinity exposure, fish were transferred to the flux containers filled with water at the appropriate salinity and were allowed to settle in for a minimum of 6 h before the start of the experiment. Water temperature was maintained by placing the jars in a water bath kept at 12°C and at the target salinity. For measurements of ammonia concentration in the water, samples (5 mL) were taken at 0, 3, and 6 h and immediately frozen at -20°C for later analyses. During the water sampling intervals (0, 3 and 6 h), the jars were sealed with gas-impermeable dental dam, immediately after an initial PO2 value was taken using an Accumet AP84 handheld O2 meter (Fisher Scientific, Toronto, ON, Canada). A final PO2 measurement was obtain simultaneous with water sampling at the end of each 3-h interval, and then the jars were lightly bubbled with air for re-oxygenation, and the procedure was repeated once until the end of the experimental period. Oxygen consumption (MO2) and ammonia excretion (Jamm) rates did not vary between the two periods, so averages of the two measurements have been reported. Chamber sterilization and blank measurements were performed as described by Giacomini et al. (2018, Chapter 4).

At the end of the experiment, fish were quickly transferred to a neutralized MS-222 (0.6 g/L) solution, prepared at the appropriate salinity, and a blood sample (~ 500 µL) was drawn from the posterior sinus using a heparinized gas-tight syringe (Hamilton, Reno, NV, USA). The sample was immediately transferred to a tube placed in a temperature-controlled water bath, and pH was measured using an MI-4156 Micro-Combination pH probe (Microelectrodes Inc., Bedford, NW, USA) and Accumet pH meter (Fisher Scientific, Toronto, ON, Canada). After that, plasma was separated by centrifugation (12,000 g for 3 min) aliquoted, flash frozen in liquid N2 and stored at -80°C for later analyses. Fish were then immediately weighed and euthanized as described above (Section 5.3.1).

Diffusive water exchange rates were measured using radiolabelled tritiated water (3H2O) in different groups of fish. After 42 h of exposure to each salinity, hagfish (N = 8) were incubated as a group in 20 µCi/L of 3H2O (Amersham Pharmacia Biotech, Little Chalfont, UK) in sea water at the target salinity under normoxia for 6 h in a shielded glass container placed in a water bath for temperature control. At the end of the incubation period, duplicate water samples (2 x 5 mL) were taken to measure the water specific activity. Preliminary experiments showed that complete equilibration of 3H2O between the internal compartment
of the hagfish and the external sea water occurred within 6 h (Giacomin et al, 2018; Chapter 4).

After incubation, the fish were rinsed with radioactivity free water, and then placed in individual experimental chambers (darkened 1-L glass jars identical to those described above) with water at the appropriate salinity. Water samples (5 mL) were taken every 5 min from 0 - 30 min, and at 10 min interval thereafter from 30-70 min. Final samples were taken at approximately 12 h to assess specific activity of the system (hagfish plus external water) once equilibrium had been reached. Ten mL of scintillation fluor (Optiphase, PerkinElmer, Waltham, MA) was added to the samples, and they were stored in the dark for a minimum of 12 h prior to counting for beta emissions on a Triathler portable counter (Hidex, Helsinki, Finland). Tests showed that quench was constant. After the final water sample was taken, fish were immediately weighed and euthanized as described above (Section 5.3.1)

5.3.3 The effect of hypoxia on rates of oxygen consumption, ammonia excretion, diffusive water exchange and ventilation

Hagfish from the second collected batch, acclimated to normoxia, 30 ppt and 12°C, were used to examine the effects of hypoxia on \( \dot{M} O_2 \), \( J_{amn} \) and diffusive water flux rates. All flux experiments were carried out using the experimental chambers described above. Fish were transferred to and allowed to settle in the chambers for 6 h. After that, measurements were taken under normoxia, or alternately, water was gently siphoned and replaced with hypoxic sea water at 35 Torr (4.7 kPa). Water samples (5 mL) were taken at 0, 3, and 6 h and frozen at -20°C for later analyses of ammonia concentration. Similarly to the procedures described above, the jars were sealed with gas-impermeable dental dam, immediately after a \( PO_2 \) value had been measured. At the end of the 3-h flux period, the jars were opened, a final \( PO_2 \) value and a water sample were collected, the \( PO_2 \) was reset to 35 Torr through light \( N_2 \) bubbling, and the procedure was repeated once more until 6 h. The average \( PO_2 \) during hypoxia was about 30 Torr (4 kPa), and during normoxia about 130 Torr (17.3 kPa).

For the diffusive water flux measurements, fish were loaded for 6 h with \( ^3 H_2O \) under normoxic conditions at 12°C and duplicate water samples (2 x 5 mL) were taken to determine
specific activity (cpm/mL) of the external water at the end of the loading period. The fish were rinsed, and then acutely transferred to the experimental chambers containing either normoxic water (120 – 150 Torr) or hypoxic water at 25-35 Torr. PO₂ was monitored throughout the duration of the experiment, and if necessary, water was bubbled with N₂ to reduce PO₂ or with plain air to increase PO₂. After 70 min, full aeration was restored and final samples were taken at approximately 12 h to assess specific activity of the system at equilibrium.

In order to measure ventilation, fish were anesthetized, fitted with a device in the single nostril for measurements of ventilatory pressure and frequency, and allowed to recover overnight with flow-through control sea water before experiments were performed. The procedures used here were the same as those employed by Giacomin et al. (2018, Chapter 4) and described in detail by Eom and Wood (2018). Briefly, a flexible plastic tube was inserted snugly into the nostril, and stitched to the skin (Ethicon, Somerville, NJ, USA). PE160 tubing (BD, Intramedic, Franklin Lakes, NJ, USA) was inserted through the tubing and then bridged using a blunt needle to an extra piece of PE 160 tubing (both water-filled) when recordings were performed. Frequency (breaths/min) and pressure amplitude (cm H₂O) were recorded at the nostril duct using a pressure transducer (DPT-100, Utah Medical Products, Midvale, UT, USA), which had been previously calibrated against a 2-cm water column. Signals were amplified (LCA-RTC, Transducer Techniques, Temecula, CA, USA) and digitized using a PowerLab Data Integrity system (ADInstruments, Colorado Springs, CO, USA), visualized and analyzed using LabChart v. 7.0 (ADInstruments).

Fish were then transferred to the same experimental containers described above, and allowed to settle for several hours. Ventilation was recorded for 5 min, and then the water was replaced with water that had been pre-equilibrated with N₂, until the total volume (1 L) reached a stable PO₂ of 30 Torr (4 kPa). Animals were kept in hypoxia for 6 h, and then ventilation was recorded for 5 min. During the experiment, water PO₂ was monitored, and if needed, adjusted by lightly bubbling with either N₂ or pure air.
5.3.4 Analytical techniques and calculations

For all calculations involving volume (V), the weight of the fish was subtracted from the 1-L respirometer volume, assuming 1 g = 1 mL. Since closed-system respirometry was used, oxygen consumption rates (\(\dot{\text{MO}}_2\): \(\mu\text{mol O}_2/\text{kg/h}\)) were calculated with the following equation:

\[
\dot{\text{MO}}_2 = \left[\left(\text{PO}_2^{(i)} - \text{PO}_2^{(f)}\right) \times \alpha_{\text{O}_2}\right] \times V / \left(\text{W} \times t\right) \tag{Eq. 1}
\]

where \(\text{PO}_2^{(i)}\) and \(\text{PO}_2^{(f)}\) are the initial and final water \(\text{PO}_2\) (Torr) respectively; \(\alpha_{\text{O}_2}\) is the oxygen solubility coefficient (Boutilier et al., 1984) at 12°C at the appropriate salinity; \(V\) is the water volume (L); \(W\) is the body mass of the fish (kg); \(t\) is the duration of the measurement period (h).

Ammonia concentrations in sea water were measured spectrophotometrically following the method described in Verdouw et al. (1978), using \(\text{NH}_4\text{Cl}\) standards made up at the appropriate salinity. Ammonia flux rate \((J_{\text{amm}}, \mu\text{mol/kg/h})\) was calculated using the following equation:

\[
J_{\text{amm}} = \left[\left(\text{Amm}^{(i)} - \text{Amm}^{(f)}\right) \times V\right] / \left(\text{W} \times t\right) \tag{Eq. 2}
\]

where \(\text{Amm}^{(f)}\) and \(\text{Amm}^{(i)}\) are the final and initial water ammonia (\(\mu\text{mol/L}\)) concentrations respectively, and the other parameters are the same as in Equation 1. The ammonia quotient was calculated from the simultaneous measurements of \(\dot{\text{MO}}_2\) and \(J_{\text{amm}}\) using the following equation:

\[
\text{AQ} = J_{\text{amm}} / \dot{\text{MO}}_2 \tag{Eq. 3}
\]

For the diffusive water flux measurements, the total amount of \(^3\text{H}_2\text{O}\) radioactivity \((R_{\text{total}}, \text{cpm})\) that the fish had originally taken up during the loading period (i.e. the total present at the start of the washout period) was calculated by the addition of all radioactivity.
removed during sampling to the total radioactivity in the system (water + fish) after complete equilibration between fish and water had occurred at 12 h.

Using $R_{\text{total}}$ and values of $^3$H$_2$O radioactivity appearance in the water at each time interval, radioactivity left in the fish ($R_{\text{time}}$) was back-calculated for each time during the experiment and used in a regression of natural log of $R_{\text{time}}$ against time. Therefore, the rate constant of $^3$H$_2$O efflux ($k$) was calculated from the exponential rate of decline in total $^3$H$_2$O radioactivity in the fish (Evans, 1967):

$$k = \frac{(\ln R_{\text{time1}} - \ln R_{\text{time2}})}{(t_1 - t_2)}$$  \hspace{1cm} (Eq. 4)

where $k$ is the rate constant of the efflux (in h$^{-1}$), and $R_{\text{time1}}$ and $R_{\text{time2}}$ are total $^3$H$_2$O radioactivity (in cpm) in the fish at times $t_1$ and $t_2$ (h). The product of $k$ x 100 yields the percent of the total body water pool turned over per hour. The rate constant ($k$) for $^3$H$_2$O efflux was only calculated over the range where efflux was linear (usually up to 30 min after the start of the experiments).

It was also possible to calculate the exchangeable internal water volume of each fish ($V_{\text{H2O}}$; ml/kg) as:

$$V_{\text{H2O}} = \frac{R_{\text{total}}}{SA_{\text{H2O}} \times W}$$  \hspace{1cm} (Eq. 5)

where $SA_{\text{H2O}}$ is the specific activity (cpm/ml) of the external water at the end of the original $^3$H$_2$O loading period, and $W$ is body mass in kg. The unidirectional diffusive water flux rate ($J_{\text{H2O}}$; mL/kg/h) could then be calculated as:

$$J_{\text{H2O}} = k \times V_{\text{H2O}}$$  \hspace{1cm} (Eq. 6)

where $k$ was calculated using Equation 4 and $V_{\text{H2O}}$ was calculated using Equation 5.

The ventilatory index (cm H$_2$O/min) was calculated using the following equation:

$$\text{Ventilatory Index} = \text{frequency} \times \text{pressure amplitude}$$  \hspace{1cm} (Eq. 7)
where frequency represents velar breaths/min and amplitude is in cm H₂O.

Plasma total ammonia ($T_{\text{amm}}; \mu\text{mol/L}$) was determined using a commercial kit (Raichem, Cliniqa, San Marcos, CA, USA), and background correction was done as described in Giacomini et al. (2018; Chapter 4). Plasma chloride concentration (mmol/L) was determined using a chloridometer (Radiometer CMT10, Copenhagen, Denmark), and plasma cations (sodium, calcium and magnesium) by atomic absorption spectroscopy (Varian, Mulgrave, Victoria, Australia) using certified commercial solutions as standards (Fluka Analytical, Sigma-Aldrich, St. Louis, MO, USA). Plasma osmolality was determined using a Wescor vapor pressure osmometer and standards (Wescor 5100C, Logan, UT, USA).

Plasma TCO₂ content was measured using a total CO₂ analyzer (Corning 965 CO₂ analyzer, Ciba Corning Diagnostic, Halstead, Essex, UK). The solubility coefficient of carbon dioxide ($\alpha_{\text{CO}_2}: \text{mmol/L/Torr}$) in hagfish plasma was calculated using an equation from Heisler (1984), where measured values for plasma osmolality (mOsm/kg) were used. The apparent pK of CO₂ ($pK_{\text{app}}$) in hagfish plasma was calculated using a second equation from Heisler (1984). Both equations are described in Giacomini et al. (2018, Chapter 4).

With those two calculated parameters ($\alpha_{\text{CO}_2}$ and $pK_{\text{app}}$), plasma PCO₂ (Torr) was calculated from measured TCO₂ and pH values using a modified Henderson-Hasselbalch equation:

$$PCO_2 = \frac{TCO_2}{[\alpha_{\text{CO}_2} \times (1 + \text{antilog (pH} - pK_{\text{app}}))] \quad \text{(Eq. 8)}$$

Plasma $[\text{HCO}_3^-]$ (mmol/L) was calculated as:

$$[\text{HCO}_3^-] = TCO_2 - (\alpha_{\text{CO}_2} \times PCO_2) \quad \text{(Eq. 9)}$$

where PCO₂ was calculated using Eq. 8.

Plasma $[\text{NH}_4^+]$ was calculated using a modified Henderson-Hasselbalch equation and $pK^+$ values obtained from Cameron and Heisler (1983) adjusted to hagfish plasma NaCl concentration:
\[ [\text{NH}_4^+] = \frac{T_{\text{amm}}}{1 + \text{antilog} (\text{pH} - \text{pK}')} \] (Eq. 10)

where \( T_{\text{amm}} \) is the measured total plasma ammonia (\( \mu \text{mol/L} \)). The partial pressure of \( \text{NH}_3 \) (\( \text{PNH}_3; \mu\text{Torr} \)) was calculated using the following equation:

\[ \text{PNH}_3 = \frac{(T_{\text{amm}} - [\text{NH}_4^+])}{\alpha_{\text{NH}_3}} \] (Eq. 11)

where \( \alpha_{\text{NH}_3} \), the solubility coefficient of \( \text{NH}_3 \) in hagfish plasma (\( \mu \text{mol/L}/\mu\text{Torr} \)) was obtained from Cameron and Heisler (1983), and \( \text{NH}_4^+ \) was calculated in Eq. 10.

### 5.3.5 Statistical analyses

All data were tested for normality and homogeneity of variances, and in case of failure, were transformed either using a log transformation, or a square root transformation. Data from Section 5.3.2 (effect of salinity) were compared using one-way analysis of variance (ANOVA), followed by a Tukey’s post-hoc test, while data from Section 5.3.3 (effect of hypoxia), were evaluated using Student’s two-tailed t-test, paired or unpaired as appropriate (details are shown in figure captions). Mean values were considered significantly different when \( p < 0.05 \). All data are shown as means ± 1 SEM (\( n = \) number of animals).

### 5.4 Results

#### 5.4.1 Responses to altered salinity

There was an overall increase in \( \text{MO}_2 \) with increasing salinity exposure (Fig. 5.1A). While \( \text{MO}_2 \) of hagfish exposed to 25 ppt was 16% lower than at the control salinity (581 \( \mu \text{mol O}_2/\text{kg/h} \)), \( \text{MO}_2 \) did not vary significantly between 30 and 35 ppt salinity (Fig. 5.1A). \( J_{\text{amm}} \) was highest at the control salinity (31 \( \mu \text{mol/kg/h} \)), and decreased significantly by 64% in fish exposed to 25 ppt (Fig. 5.1B). Similar to \( \text{MO}_2 \), there was no significant
differences between $J_{\text{amn}}$ at 30 and 35 ppt (Fig. 5.1B). The ammonia quotient (AQ) was ~0.04 in control fish at 30 ppt, and decreased significantly by 44% in fish acclimated to 25 ppt (Fig. 5.1C). AQ did not differ significantly between 30 and 35 ppt exposed fish (Fig. 5.1C). Diffusive water flux rate ($J_{\text{H2O}}$) was 1580 mL/kg/h at the control salinity and significantly decreased in fish exposed to both to 25 and 35 ppt (Fig. 5.2A). As expected, the exchangeable water pool, which was 62% of body weight under control conditions, increased by 11% at 25 ppt, and decreased by 11% at 35 ppt. Although the pool at 25 ppt was significantly different than that at 35 ppt, both values were not significantly different than the control (Fig. 5.2B).

The measured seawater ion concentrations at each salinity are summarized in supplementary Table 5.1. Plasma $[\text{Na}^+]$ and $[\text{Cl}]$ were 552 mmol/L and 463 mmol/L, respectively, under control conditions, and varied greatly with salinity, being highest at 35 ppt (Fig. 5.3A,B). Plasma $[\text{Na}^+]$ was significantly higher than the seawater $[\text{Na}^+]$ values at all three salinities (Fig. 5.4A), while plasma $[\text{Cl}]$ was significantly lower than seawater $[\text{Cl}]$ values at all salinities (Fig. 5.4B). Interestingly, for $[\text{Cl}]$, the concentration differential between plasma and water increased as salinity increased (Fig. 5.4B). This pattern was not seen for $[\text{Na}^+]$, but for both ions, the differentials were greatest (+148 mmol/L for $[\text{Na}^+]$, -73 mmol/L for $[\text{Cl}]$) though opposite in sign, at 35ppt. While plasma $[\text{Mg}^{2+}]$ was unaffected by salinity acclimation (Fig. 5.3C), the plasma-to-water concentration differential for $[\text{Mg}^{2+}]$ increased as salinity increased, reaching -42 mmol/L at 35 ppt (Fig. 5.4C). Plasma $[\text{Ca}^{2+}]$ was significantly reduced at 25 ppt, and did not change between 30 and 35 ppt (Fig. 5.3D). Similar to $[\text{Mg}^{2+}]$, the plasma to seawater difference for $[\text{Ca}^{2+}]$ increased as salinity increased, reaching -5.4 mmol/L at 35 ppt (Fig. 5.4D). Plasma osmolality increased in proportion to salinity (Fig. 5.3E), with values that were not significantly different from seawater osmolalities, as seen by the plasma-to-water concentration difference ranging from -6 to +10 mOsm/kg (Fig. 5.4E).

Under control conditions, plasma pH was 7.77 and no changes were seen in fish exposed to higher or lower salinity (Fig. 5.5A). Plasma $\text{PCO}_2$ was 4.35 Torr at 30 ppt, while plasma $\text{HCO}_3^-$ was 11.3 mmol/L (Fig. 5.5B,C). Overall, the measured blood acid-base parameters were mostly unaffected by salinity, with the exception of plasma $[\text{HCO}_3^-]$, which
significantly decreased at 35 ppt in comparison to 25 ppt (Fig. 5.5C). The total plasma ammonia concentration (Fig. 5.5D) and \([\text{NH}_4^+]\) (data not shown), as well as plasma \(P_{\text{NH}_3}\) (Fig. 5.5E), all tended to increase with salinity, but none of the differences were significant.

### 5.4.2 Responses to hypoxia exposure

\(\mathcal{M}_\text{O}_2\) under control conditions in this series of experiments was 80% higher relative to the first batch of fish used in experiments from 5.4.1 (Fig. 5.6A versus Fig. 5.1A), though there were no significant differences in \(J_{\text{amn}}\) (Fig. 5.6B versus Fig. 5.1B) or \(J_{\text{H}_2\text{O}}\) (Fig. 5.6C versus Fig. 5.2A).

Exposure to hypoxia for 6 h resulted in a 49% reduction in \(\mathcal{M}_\text{O}_2\) from the control level of 872 \(\mu\text{mol/kg/h}\) (Fig. 5.6A). However, there was no change in \(J_{\text{amn}}\) from the control rate (31 \(\mu\text{mol/kg/h}\)) under hypoxia (Fig. 5.6B). In normoxia, the ammonia quotient (AQ) was 0.04 and despite an increase with exposure to hypoxia, it was not significantly different (Fig. 5.6D). Diffusive water flux rate (1338 mL/kg/h) under normoxia was significantly reduced by 36% with hypoxia exposure (Fig. 5.6C).

Ventilation rate was ~11 breaths/min under normoxia, and increased 4.8 fold when hagfish were exposed to hypoxia (Fig. 5.7A), while ventilation pressure amplitude, which was 0.14 cm\(\text{H}_2\text{O}/\text{breath}\) under normoxia decreased by 75% (Fig. 5.7B). Thus, the ventilatory index, which in normoxia was 1.56 cm\(\text{H}_2\text{O}/\text{min}\), increased in hypoxia by 24%, a small but significant increase on a paired basis (Fig. 5.7C).

Plasma pH did not change with hypoxia exposure (Figure 5.8A), while plasma PCO\(_2\) and HCO\(_3^-\) both decreased significantly by about 57 and 60% respectively (Figure 5.8B,C). Plasma ion concentrations ([Na\(^+\)], [Cl\(^-\)], [Mg\(^{2+}\)] and [Ca\(^{2+}\)]), and osmolality were largely unaffected by hypoxia exposure, with no significant differences between normoxic control and hypoxia exposed fish (Table 5.2). Additionally, despite being reduced, total plasma ammonia, plasma [NH\(_4^+\)] (data not shown), and the partial pressure of ammonia (\(P_{\text{NH}_3}\)) were highly variable, and did not statistically change with exposure to hypoxia (Table 5.3).
5.5 Discussion

5.5.1 Overview

Our ultimate goal was to examine how Pacific hagfish respond to changes in respiratory and ionoregulatory demands, elicited by a relatively short (48h) exposure to altered salinity, and by acute hypoxia challenge. We predicted that as salinity departed from the control condition, as a natural adaptive response, hagfish would depress aerobic metabolism, and consequently reduce gill permeability. The data were partly consistent with our prediction, as hagfish kept for 48h at a lower salinity (25 ppt) exhibited a reduction in $\text{MO}_2$, $J_{\text{amm}}$ and $J_{\text{H}_2\text{O}}$. Also, when exposed for 48 h to elevated salinity (35 ppt), $J_{\text{H}_2\text{O}}$ was again reduced, though $\text{MO}_2$ and $J_{\text{amm}}$ did not change. Also, we hypothesized that as salinity was changed, hagfish would exhibit compensatory adjustments of plasma ions. We observed that the concentration difference between plasma and external water was directly modified with increasing salinity for the divalent cations ($[\text{Ca}^{2+}]$ and $[\text{Mg}^{2+}]$) previously known to be actively regulated at levels well below those of sea water, and we identified a similar phenomenon for $[\text{Cl}^-]$. The salinity–dependent pattern was less clear for plasma $[\text{Na}^+]$, but nevertheless, at all salinities, this cation was regulated at a level well above those in the external sea water. Finally, when exposed to hypoxia, hagfish decreased $\text{MO}_2$, even though overall ventilatory water flow, as represented by the ventilatory index, was slightly elevated. The observed reductions in $J_{\text{H}_2\text{O}}$ and $J_{\text{amm}}$ at this time indicate that, despite the fact that hagfish were attempting to increase $\text{O}_2$ uptake, they were selectively reducing gill permeability. This points to a strategy of dealing with the osmorespiratory compromise that differs from the traditional model of freshwater teleosts where gill permeability to respiratory gas exchange increases, and consequently gill permeability also increases to ions, water and nitrogenous compounds.
5.5.2 Effects of exposure to altered salinity on $\dot{M}O_2$ and gill permeability

We had predicted that altered salinity exposure would elicit a metabolic depression, thereby avoiding associated compensatory costs, since nutrients are scarce giving the environment these fish inhabit. At the lower salinity (25 ppt), $\dot{M}O_2$ was indeed moderately depressed (by 16%) while ammonia excretion exhibited a more pronounced (64%) decrease (Fig. 5.1). This further downregulation of $J_{\text{amm}}$ independent of the reduction in $\dot{M}O_2$, with no change in plasma ammonia concentrations (Fig. 5.5D, E), could suggest a selective decrease in gill permeability for ammonia, while the system remained in balance. However, we cannot identify if this reduction occurred mostly at the gills or the skin. Using immunohistochemistry, Braun and Perry (2010) demonstrated that Pacific hagfish express ammonia transporting proteins (Rhbg and Rhcg1) at the gills. More recently, Clifford et al. (2017) have shown that the skin of hagfish expresses Rhcg proteins, providing a mechanism by which ammonia can be excreted across the thick epidermal layer. Moreover, when exposed to high environmental ammonia, these fish are able to differentially regulate the ammonia permeability of the gills and the skin, revealing a wide-scope of ammonia handling strategies (Clifford et al., 2017). The ammonia quotient (Fig. 5.1C) is a proxy for the nitrogen quotient, which is an index of the use of protein/amino acids as an aerobic fuel (Lauff and Wood, 1996b). In control hagfish, this index indicates that only ~16% of aerobic metabolism is fueled by proteins/amino acids, a conclusion consistent with previous findings that hagfish mostly utilize carbohydrates (Hansen and Sidell, 1983; Sidell et al., 1984). At 25 ppt, the AQ was significantly reduced, indicating that during this metabolic depression, protein oxidation was further decreased from the already low numbers.

Recently, using radioisotopic techniques, Glover et al. (2017) confirmed that Pacific hagfish do not drink sea water in order to maintain hydromineral balance, yet surprisingly, they possess one of the highest water exchange rates ever reported for any fish (Rudy and Wagner, 1970; Glover et al., 2017). This was confirmed by the present study (Fig. 5.1B) and earlier studies on the same species (Giacomin, et al. 2018; Chapter 4). This high water permeability is probably a reflection of their osmoconforming strategy. Although the contribution of the skin to the high water permeability remains to be elucidated, the presence
of two aquaporin homologs (AQP3 and AQP4) has been identified throughout the skin and on the slime glands of Pacific hagfish (Herr et al., 2014). AQP4 was also identified at the gills of another Pacific hagfish (*Eptatretus burgeri*), possibly located at the basolateral membrane of pavement cells (Nishimoto et al., 2007). Several earlier studies where environmental salinity has been manipulated have pointed to the fact that hagfish in general are better able to cope with a transfer to a hypotonic medium rather than to a hypertonic one, as evidenced by weight changes, tissue water content and blood pressure (Foster and Forster, 2007; Mcfarland and Munz, 1958; 1965; Toop and Evans, 1993). However, no investigation so far has examined aquaporin expression and distribution in relation to environmental salinity changes.

Diffusive water fluxes (measured as $^{3}$H$_2$O fluxes) are unidirectional and are known to be about 100-fold higher than osmotic water fluxes which are net fluxes. The latter are usually measured indirectly through body weight changes, drinking rate and urine flow. It is argued that such measurements underestimate osmotic water fluxes (Isaia, 1984). When hagfish are exposed to low salinity, the decreased external osmotic pressure would drive the osmotic influx of water, and this likely led to the observed elevation in the internal exchangeable water pool (Fig. 5.2B) in the present study. Conversely, exposure to higher salinity would create an outwardly directed gradient for osmotic water flux, explaining the lower internal exchangeable water pool seen at 35 ppt (Fig. 5.2B). At both lower and higher salinity, we observed a decreased diffusive water flux rate (Fig. 5.2A) despite opposing changes in the size of the exchangeable water pool (Fig. 5.2B). At the lower salinity, it is possible that this decrease is a result of lowered MO$_2$, which would be in accord with our previous study (Giacomin et al. 2018; Chapter 4), where high temperature and hyperoxia treatments indicated parallel changes in O$_2$, ammonia, and water permeability, reflective of an osmorespiratory compromise. Additionally, an independent downregulation of water permeability may have occurred, through possible post-translational modifications affecting membrane aquaporins. Certainly, a downregulation of aquaporin function may be a more likely explanation for the decrease in diffusive water flux at 35 ppt (Fig.5.2A), where MO$_2$ (Fig. 5.1A) and $J_{\text{amn}}$ (Fig. 5.1B) remained unchanged.
5.5.3 Effects of exposure to altered salinity on plasma ion concentrations

Preceding studies on the Pacific hagfish have suggested that these fish are stenohaline, seeming to lack the capacity to regulate either plasma [Na\(^+\)] or [Cl\(^-\)] upon acute salinity changes (Mcfarland and Munz, 1965; Sardella et al., 2009). However, several previous investigations have indicated that plasma [Na\(^+\)] is kept slightly below the levels in external sea water while plasma [Cl\(^-\)] is maintained slightly above the concentrations in sea water (Mcfarland and Munz, 1965; Sardella et al., 2009; Clifford et al., 2018; Giacomin et al., 2018; Chapter 4). Furthermore, when exposed to 80% SW, hagfish were able to elevate plasma [Na\(^+\)] to seawater levels after 2 days (Mcfarland and Munz, 1965). There is clear evidence that divalent cations (Mg\(^{2+}\) and Ca\(^{2+}\)) are maintained at levels much lower than in the sea water, even when hagfish are acclimated and/or exposed to salinities above and below that of normal sea water (Sardella et al., 2009; Hastey, 2011). The present results (Figs. 5.3 and 5.4) confirm all these previous observations, and additionally reveal that the differences between plasma and water for Cl\(^-\), Mg\(^{2+}\), and Ca\(^{2+}\) (and possibly Na\(^+\)) all increase as salinity increases (Fig. 5.4). These results indicate that not only do hagfish possess some ability to regulate all four ions, but also that this regulation becomes greater as environmental salinity increases. Giacomin et al. (2018; Chapter 4) have shown that increases in environmental temperature induced an increase in both plasma [Na\(^+\)] and [Cl\(^-\)], while plasma [Ca\(^{2+}\)] and [Mg\(^{2+}\)] significantly decreased. Thus there was increased internal regulation of [Na\(^+\)], [Ca\(^{2+}\)] and [Mg\(^{2+}\)] but decreased regulation of [Cl\(^-\)] at high temperature. The presence of ionocytes on both the gills and skin of hagfish is well established (Mallatt et al., 1987; Choe et al., 1999; Tresguerres et al., 2006; Herr et al., 2014); future studies should address their potential roles in the ionoregulatory phenomena identified here, and the adaptive significance of this regulation.

We also observed a decrease in plasma [HCO\(_3^-\)] in fish exposed to 35 ppt for 48h (Fig. 5.5C), though there was no change in plasma pH (Fig. 5.5A) because of the accompanying non-significant fall in PCO\(_2\) (Fig. 5.5B). In marine fishes, HCO\(_3^-\) is secreted into the intestine of fishes and forms complexes with imbibed Ca\(^{2+}\) and Mg\(^{2+}\), precipitating these salts and providing protection from potential divalent cation toxicity, and this process is
elevated at higher salinities (for review: Wilson et al., 2002). Sardella et al. (2009) suggested that this mechanism is also present in hagfishes, though there appears to be no direct evidence, and the lack of drinking (Glover et al., 2017) in these animals would seem to make this process unnecessary.

5.5.4 Effects of hypoxia exposure on \( \dot{\text{M}}O_2 \), ventilation, gill permeability and plasma homeostasis

\( \dot{\text{M}}O_2 \) decreased by 49% in hagfish during hypoxia exposure (Fig. 5.6A), similar to previous observations in both the Pacific hagfish (Clifford et al., 2016a) and the New Zealand hagfish (Forster, 1990). The pattern of ventilation changed to higher frequency (Fig. 5.7A) and lower pressure amplitude (Fig. 5.7B, i.e. lower stroke volume, Eom and Wood, 2018), and total ventilatory water flow, as indicated by the ventilatory index (Fig. 5.7C), was moderately elevated in hypoxia. Thus, contrary to part of our initial hypothesis, the animals did not reduce ventilation, but rather attempted to increase \( O_2 \) uptake under hypoxia by increasing ventilation. It appears that hagfish utilize a strategy to increase breathing under low \( O_2 \) supply that differs from that of most teleosts, where ventilation amplitude is elevated and frequency is decreased, an adjustment that is thought to conserve energy (Perry, 2011).

Our results are in agreement with Perry et al. (2009) where using a similar \( P_{\text{O}_2} \) level (20 Torr vs. 30 Torr in the present study) for hypoxia, hagfish markedly increased ventilation frequency. However, in their study, \( \dot{\text{M}}O_2 \) was not altered by hypoxia exposure, whereas in our study, we saw a significant reduction in \( \dot{\text{M}}O_2 \) (Fig. 5.6A). As Perry et al. (2009b) mentioned, it is possible that the metabolic cost of hyperventilation in hagfish, where flow is generated through the velum pump, is lower than in species where ventilation is powered by both buccal and opercular pumps. Hagfishes often encounter hypoxia in the wild, either by inhabiting oxygen-depleted environments, such as anoxic sediments, or through their feeding style of burrowing their heads into the coelomic cavities of decaying carrion (Axelsson et al., 1990; Forster et al., 1992; Clifford et al., 2015). Likely due to their life history, hagfishes are extremely tolerant to \( O_2 \) starvation, exhibiting periods of spontaneous apnea under normoxia.
(Eom and Wood, 2018) and being able to maintain up to 70% of normoxic cardiac performance even during complete anoxia (Cox et al., 2010).

We had also hypothesized that hagfish, when exposed to hypoxia, would reduce gill permeability, differing from the prediction (increased permeability) of the “classic” osmorespiratory compromise (see Section 5.2). Our hypothesis was confirmed inasmuch as $J_{\text{H2O}}$ was again reduced (Fig. 5.6C), as it had been during the salinity challenges, and there were no disturbances in plasma ions or osmolality (Table 5.1). Recent studies have identified several teleost species that also exhibit responses during hypoxia that differ from those of the “classic” osmorespiratory compromise. Robertson et al. (2015), investigating 12 species of temperate and tropical teleosts, found that only 5 exhibited the typical predicted osmorespiratory compromise response of increased fluxes when exposed to hypoxia. Wood et al. (2007a; 2009) exposed the hypoxia-tolerant Amazonian oscar (*Astronotus ocellatus*) to hypoxia, and observed a downregulation of both transcellular and paracellular permeability of the gills to ions and ammonia, suggesting that this species attempts to maintain $O_2$ uptake at the gills without compromising ionoregulatory balance. Moreover, the oscar hyperventilated during hypoxia (Scott et al., 2008a; Wood et al., 2009). More recently, Giacomin et al. (Chapters 2 and 3) have identified traits similar to those of the Amazonian oscar in another hypoxia-tolerant teleost, the euryhaline killifish (*Fundulus heteroclitus*). In both fresh water and sea water, the killifish reduced gill permeability to ions and water, and maintained ionoregulatory homeostasis, while still attempting to increase $O_2$ uptake by hyperventilation during hypoxia. The present results indicate that the Pacific hagfish, a representative of the oldest extant craniates, can be added to the list of species that are capable of reducing gill permeability to ions and water during hypoxia, thereby avoiding the resulting effects of the osmorespiratory compromise. In future, it will be of interest to determine whether hagfish exhibit morphological responses of the branchial epithelium similar to those of the oscar (Wood et al., 2009; Matey et al., 2011; De Boeck et al., 2013) and killifish (Giacomin et al., Chapter 3) under hypoxia.
5.5.5 Conclusions and future directions

We have provided new evidence supporting the idea that hagfish possess some ability to regulate plasma $[\text{Na}^+]$ and $[\text{Cl}^-]$, in addition to their previously known ability to regulate $[\text{Ca}^{2+}]$ and $[\text{Mg}^{2+}]$, when exposed to different salinities, although the mechanisms involved are still unknown. Future studies should focus on the role of already identified ion transporting proteins at the gills and skin in this ionoregulation, as well as its adaptive significance. We have also shown that exposure to altered salinity changes the metabolic demand of hagfish, with accompanying effects on the rates of ammonia excretion and diffusive water flux. Benthic habitats are often hypoxic, and hagfishes are additionally exposed to hypoxia in the wild due to their feeding style, where they burrow their heads into putrefying carcasses. Therefore, we evaluated the responses to acute hypoxia exposure within the context of the osmorespiratory compromise and found that despite an elevation of ventilation, permeability to water and ammonia excretion were reduced. Altogether, these results point to a strategy to deal with the osmorespiratory compromise that is typical of hypoxia-tolerant species. Since the commercial exploitation of hagfish has increased drastically in recent years, understanding its basic physiology and general ecology are crucial for the development of sustainable fisheries practices.
Oxygen consumption (µmol O₂/kg/h)

Ammonia net flux (µmol/kg/h)

Ammonia quotient

A

B

C

25 ppt 30 ppt 35 ppt

25 ppt 30 ppt 35 ppt

25 ppt 30 ppt 35 ppt
Figure 5.1 (A) Oxygen consumption rate ($$\dot{M}O_2$$: µmol O$_2$/kg/h), (B) ammonia net flux rate ($$J_{amm}$$: µmol/kg/h) and (C) Ammonia quotient in *Eptatretus stoutii* acclimated to 30 ppt or exposed to 25 and 35 ppt for 48h. Different lower case letters represent statistically different means detected by one-way ANOVA and a Tukey’s post hoc test. Data are shown as means ± 1 SEM (n = 7 - 8).
Water flux rate (mL/kg/h)

Exchangeable water pool (% body weight)
Figure 5.2 (A) Water flux rate (mL/kg/h) and (B) exchangeable water pool (% body weight) in *Eptatretus stoutii* acclimated to 30 ppt or exposed to 25 and 35 ppt for 48h. Different lower case letters represent statistically different means detected by one-way ANOVA and a Tukey’s post hoc test. Data are shown as means ± 1 SEM (n = 7 - 8).
Plasma ion concentration (mmol/L)

A. Na⁺
B. Cl⁻
C. Mg²⁺
D. Ca²⁺
E. Osmolality

Osmolality (mOsm/kg)

25 ppt 30 ppt 35 ppt

Statistical significance indicated by different letters.
Figure 5.3 Plasma ion concentration in *Eptatretus stoutii* acclimated to 30 ppt or exposed to 25 and 35 ppt for 48h. (A) Sodium: Na$^+$; (B) Chloride: Cl$^-$; (C) Magnesium: Mg$^{2+}$; (D) Calcium: Ca$^{2+}$ (mmol/L) and (E) Osmolality (mOsm/kg). Different lower case letters represent statistically different means detected by one-way ANOVA and a Tukey’s post hoc test. Data are shown as means ± 1 SEM (n = 7 – 8).
**Figure 5.4** Plasma ion to seawater concentration differential in *Eptatretus stoutii* acclimated to 30 ppt or exposed to 25 and 35 ppt for 48h. (A) Sodium: Na\(^+\); (B) Chloride: Cl\(^-\); (C) Magnesium: Mg\(^{2+}\); (D) Calcium: Ca\(^{2+}\) (mmol/L) and (E) Osmolality (mOsm/kg). Data are shown as means ± 1 SEM (n = 7 – 8).
Figure 5.5 (A) Blood pH, (B) plasma PCO$_2$ (Torr) and (C) plasma HCO$_3^-$ (mmol/L), (D) plasma total ammonia (µmol/L) and (E) partial pressure of ammonia (PNH$_3$ - µTorr) in *Eptatretus stoutii* acclimated to 30 ppt or exposed to 25 and 35 ppt for 48h. Different lower case letters represent statistically different means detected by one-way ANOVA and a Tukey’s post hoc test. Data are shown as means ± 1 SEM (n = 7 – 8).
Figure A: Oxygen consumption (µmol O₂/kg/h) in Normoxia and Hypoxia conditions.

Figure B: Ammonia net flux (µmol/kg/h) in Normoxia and Hypoxia conditions.

Figure C: Water flux rate (mL/kg/h) in Normoxia and Hypoxia conditions.

Figure D: Ammonia quotient in Normoxia and Hypoxia conditions.
Figure 5.6 (A) Oxygen consumption rate (ṀO₂: µmol O₂/kg/h), (B) ammonia net flux rate (Jamm: µmol/kg/h), (C) diffusive water flux rate (JH₂O: mL/kg/h) and (D) Ammonia quotient in Eptatretus stoutii exposed to normoxia (white bars: 150 Torr for 6h; 30 ppt at 12°C) and hypoxia (black bars: 30 Torr for 6h; 30 ppt at 12°C). Different lower case letters represent statistically different means detected by Student’s t-test. Data are shown as means ± 1 SEM (n = 7 - 8).
Figure 5.7 (A) Ventilation rate (breaths/min), (B) ventilation pressure amplitude (cmH₂O/breath) and (C) ventilatory index (cmH₂O/min) in *Eptatretus stoutii* exposed to hypoxia (30 Torr for 6h; 30 ppt at 12°C). Different lower case letters represent statistically different means detected by a paired Student’s t-test. Data are shown as means ± 1 SEM (n = 7 - 8).
Figure 5.8 (A) Blood pH, (B) plasma PCO$_2$ (Torr) and (C) plasma HCO$_3^-$ (mmol/L) in *Eptatretus stoutii* exposed to hypoxia (30 Torr for 6h; 30 ppt at 12ºC). Different lower case letters represent statistically different means detected by Student’s t-test. Data are shown as means ± 1 SEM (n = 7 - 8).
Table 5.1 Measured ion concentrations in sea water at 25, 30 and 35 ppt prepared as described in section 5.3.2 of Methods (Sodium: Na\(^+\); Chloride: Cl\(-\); Magnesium: Mg\(^{2+}\); Calcium: Ca\(^{2+}\) (mmol/L) and Osmolality (mOsm/kg)).

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<th>Ion concentration (mmol/L)</th>
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<td>Na(^+)</td>
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<tr>
<td>Cl(-)</td>
<td>393 ± 9</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>40.6 ± 0.1</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>7.0 ± 0.1</td>
</tr>
<tr>
<td>Osmolality (mOsm/kg)</td>
<td>778 ± 7</td>
</tr>
</tbody>
</table>
**Table 5.2** Ion concentration in blood plasma of *Eptatretus stoutii* exposed to hypoxia (30 Torr for 6 h; 30 ppt at 12°C) [Sodium: Na⁺; Chloride: Cl⁻; Magnesium: Mg²⁺; Calcium: Ca²⁺ (mmol/L) and Osmolality (mOsm/kg)]. No statistical differences were found between animals in normoxia versus hypoxia exposure. Data are shown as means ± 1 SEM (n = 7 - 8).

<table>
<thead>
<tr>
<th></th>
<th>Plasma ion concentration (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normoxia</td>
</tr>
<tr>
<td>Na⁺</td>
<td>555 ± 11</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>465 ± 2</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>10.6 ± 0.3</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td>Osmolality (mOsm/kg)</td>
<td>964 ± 4</td>
</tr>
</tbody>
</table>
Table 5.3 Plasma total ammonia (µmol/L) and partial pressure of ammonia (PNH₃: µTorr) of *Eptatretus stoutii* exposed to hypoxia (30 Torr for 6 h; 30 ppt at 12°C). No statistical differences were found between animals in normoxia versus hypoxia exposure. Data are shown as means ± 1 SEM (n = 7 - 8).

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia (µmol/L)</td>
<td>323 ± 68</td>
<td>159 ± 61</td>
</tr>
<tr>
<td>PNH₃ (µTorr)</td>
<td>56 ± 11</td>
<td>31 ± 14</td>
</tr>
</tbody>
</table>
Chapter 6: Differential effects of temperature on oxygen consumption and branchial fluxes of urea, ammonia, and water in the dogfish shark (*Squalus acanthias suckleyi*).

6.1 Summary

Environmental temperature can greatly influence the homeostasis of ectotherms through its effects on biochemical reactions and whole animal physiology. Elasmobranchs tend to be N-limited and are osmoconformers, retaining ammonia and urea-N at the gills, and using the latter as a key osmolyte to maintain high blood osmolality. However, the effects of temperature on these key processes remain largely unknown. We evaluated the effects of acute exposure to different temperatures (7, 12, 15, 18, 22ºC) on oxygen consumption, ammonia, urea-N and diffusive water fluxes at the gills of *Squalus acanthias suckleyi*. We hypothesized that as metabolic demand for oxygen increased with temperature, the fluxes of ammonia, urea-N and ^3^H_2^O at the gills would increase in parallel with those of oxygen. Oxygen consumption rate (overall; Q_{10} = 1.76 from 7.5 to 22ºC) and water fluxes (overall Q_{10} = 1.96) responded to increases in temperature in a similar, almost linear manner. Ammonia-N efflux rates varied the most, increasing almost 15-fold from 7.5 to 22ºC (Q_{10} = 5.15). Urea-N efflux was tightly conserved over the 7.5 – 15ºC range (Q_{10} ~ 1.0), but increased greatly at higher temperatures, yielding an overall Q_{10} = 1.45. These differences likely reflect differences in the transport pathways for the four moieties. They also suggest the failure of urea-N and ammonia-N conserving mechanisms at the gill above 15ºC. Hyperoxia did not alleviate the effects of high temperature. Indeed, urea-N and ammonia-N effluxes were dramatically increased when animals were exposed to high temperatures in the presence of hyperoxia, suggesting that high partial pressure of oxygen may have caused oxidative damage to gill epithelial membranes.
6.2 Introduction

Environmental temperature has profound effects on multiple levels of biological organization, and can greatly affect organismal homeostasis (Hochachka and Somero, 2002). In a classic study on the lesser-spotted dogfish (Scyliorhinus canicula), Butler and Taylor (1975) showed that oxygen uptake (ṀO₂), cardiac output and heart rate all increased with water temperature, with Q₁₀'s between 2.1 and 2.5 (Butler and Taylor, 1975). More recent studies on metabolic rate in other elasmobranchs have confirmed that they exhibit a temperature sensitivity that is typical of ectotherms, with Q₁₀’s usually ranging from 2 to 3 (e.g. Miklos et al., 2003; Di Santo and Bennett, 2011), although Di Santo (2016) recently reported much higher values (5 to 11.5) in a species of skate. However, few studies have focused on the effects of temperature on the excretion of nitrogenous-waste products. Nitrogenous waste handling is particularly important in this group, because of their ureotelism and their unique urea-based osmoregulatory strategy, which requires some retention of waste-N. In contrast, this topic has been well studied in ammoniotelic teleost fish where Q₁₀’s for metabolic rate are somewhat lower (usually below 2.0), and there is general agreement that nitrogen-waste excretion is much more sensitive than ṀO₂ to increases in temperature (i.e. higher Q₁₀’s, for review see Wood, 2000). For example in three model teleosts (goldfish - Maetz, 1972; trout - Kieffer et al., 1998; tilapia - Alsop et al., 1999), Q₁₀’s for ammonia excretion were well above the values for ṀO₂. The one elasmobranch study of which we are aware is that of Boylan (1967) on the spiny dogfish (Squalus acanthias). Using an unusual artificially ventilated whole animal preparation, Boylan reported a surprising conclusion that has never been confirmed: branchial urea-N excretion was independent of temperature up to 15 ºC, and then increased sharply at higher temperatures, suggesting the breakdown of some urea-N conservation mechanism.

Urea-N conservation is important in seawater elasmobranchs because they are osmoconformers and utilize organic osmolytes in order to maintain internal osmolality slightly greater than that of their environment, thereby promoting the osmotic influx of water and eliminating the need for drinking seawater (Smith, 1931; Smith, 1936). The major organic osmolyte retained is urea, supplemented by much lower concentrations of
counteracting methylamines such as trimethylamine N-oxide (TMAO) which stabilize protein function (see Yancey, 1916). Urea is synthesized from ammonia through the ornithine-urea cycle (OUC), at the expense of 5 moles of ATP per mole of urea (2 moles of urea-N) produced (for review see Ballantyne, 1997), accounting for approximately 10% of the overall MO2 of elasmobranchs (Kirschner, 1970). This urea retaining strategy results in a massive urea gradient across the gills (300- 400 mmol L\(^{-1}\) inside versus 0 mmol L\(^{-1}\) outside).

The mechanism of urea-N retention at the gills against this enormous gradient remains controversial. Dogfish (Squalus acanthias) gill basolateral membranes possess unusually high cholesterol-to-phospholipid and phosphatidylcholine-to-phosphatidylethanolamine ratios that are thought to contribute to low urea permeability (Fines et al., 2001). The apical cell membrane surface area is very low relative to the basolateral cell membrane surface area, and apical membrane permeability to urea is even lower than basolateral membrane permeability (Pärt et al., 1998; Hill et al., 2004). A “back-transport” mechanism appears to trap urea which leaks through the gills (Wood et al., 1995; Pärt et al., 1998). Although no urea transporting protein has ever been isolated in the elasmobranch gill (Wright and Wood, 2016), there is physiological evidence for an active urea back-transporter located basolaterally (Fines et al., 2001) and/or apically (Wood et al., 2013).

The elasmobranch gill is also even less impermeable to ammonia than it is to urea, keeping ammonia losses at just a few percent of urea-N losses (Wood et al., 1995; Wood et al., 2007b). Recently, Nawata et al. (2015) and Wood and Giacomin (2016) showed that nitrogen-limited Squalus acanthias are capable of actively scavenging ammonia-N from the water by a branchial transporter exhibiting saturation kinetics, resulting in increased synthesis of urea. The mechanism of ammonia uptake is unknown, but appears to involve Rh proteins.

Another important aspect of the elasmobranch osmoregulatory strategy is the branchial transport of water. Water permeability at the elasmobranch gill has to be kept relatively high, given the usually low osmotic gradient from seawater to plasma (10 – 70 mOsmol L\(^{-1}\)) and the need to take up water to form urine and rectal gland secretions (Wright and Wood, 2016). The few available measurements of diffusive water turnover rates (with
\( ^3 \text{H}_2 \text{O} \) (Payan and Maetz, 1971; Payan et al., 1973; Haywood, 1975) indicate branchial permeabilities about 5-fold greater than in marine teleosts.

Against this background, the goal of this study was to evaluate the effects of acute exposure to different temperatures on \( \text{MO}_2 \), on the excretion of nitrogen-wastes (ammonia and urea-N), and on diffusive water transport at the gills of the Pacific spiny dogfish (\textit{Squalus acanthias suckleyi}). \textit{S. acanthias} was chosen as our experimental model due to the extensive body of literature on the physiology of this species, which is a migratory, long-lived, very important demersal resource that is under threat due to overfishing and global climate change (Taylor and Gallucci, 2009), like many other marine elasmobranchs (Dulvy et al., 2014). The present investigation was part of a larger ongoing study on the osmorespiratory compromise and gill function in this species (Zimmer and Wood, 2014; Nawata et al., 2015; Wood and Giacomin, 2016). The osmorespiratory compromise is the functional trade-off between respiratory gas exchange \textit{versus} osmolyte and water exchange that has been well-documented in freshwater teleosts (Randall et al., 1972; Nilsson, 1986), but never examined in marine elasmobranchs. The osmorespiratory compromise predicts that permeability changes at the gills that favour increased \( \text{O}_2 \) consumption may cause unfavourable passive fluxes of osmolytes and water. A particular focus was to replicate the unusual results obtained by Boylan (1967), while using a less invasive experimental technique. Ammonia, urea-N and tritiated water (\( ^3 \text{H}_2 \text{O} \)) fluxes were assessed following exposure to 7.5, 12 (acclimation temperature, used as control), 15, 18 and 22ºC. Based on fishery data and capture records, this temperature range is environmentally relevant for this species which occurs between extremes of 4ºC and 24ºC in the wild (Bangley and Rulifson, 2014; Sagarese et al., 2014). Our general hypothesis was that as metabolic demand for oxygen increased with temperature, the fluxes of ammonia, urea-N, and \( ^3 \text{H}_2 \text{O} \) at the gill would increase in parallel with that of oxygen. We performed follow-up experiments where the animals were exposed to control (12ºC) and high (22ºC) temperature in the presence of hyperoxia. We hypothesized that increasing oxygen availability would minimize or prevent the associated effects of rising temperature on gill permeability.
6.3 Material and Methods

6.3.1 Animal housing and acclimation

Spiny dogfish (*Squalus acanthias suckleyi*) were caught by angling near Bamfield Marine Sciences Centre (BMSC), Bamfield, British Columbia, Canada (48.8355 °N 125.1355 °W) under Fisheries and Oceans Canada collecting permit XR2392015. At BMSC, they were held for approximately 20 days prior to experimentation in a large (150,000 L) indoor concrete tank. The tank was served with flowing sea water (12 – 13°C, 30 ppt salinity) pumped directly from the nearby ocean, and dissolved O$_2$ was kept at > 80 % saturation. Dim lighting was maintained in the room for approximately 10 h a day. During this period, fish were fed twice a week with a 3% ration of commercially purchased frozen hake (*Merluccius productus*). Feeding was stopped at least 1 week before experimentation began. All animal experimentation was carried out under the guidelines of the Canada Council for Animal Care under approval of animal care committees at BMSC and the University of British Columbia (joint AUP A14-0251). In total, 20 adult male dogfish weighing $1.62 \pm 0.05$ kg were used in this study. Animals were not fed during the experimentation period (maximum of two weeks).

6.3.2 Experimental set up

Animals were transferred from the main holding tank to individual 40-L wooden boxes, coated with polyurethane, which have been previously used for studies with dogfish (Wood et al., 1995; Zimmer and Wood, 2014). Each box was served with perimeter aeration and fed with flow-through seawater. All animals were acclimated to the boxes for at least 12 h before experiments were conducted. In all experiments, O$_2$ and temperature levels were monitored using a YSI 55 handheld oxygen meter fitted with a temperature probe (YSI Incorporated, Yellow Springs, OH, USA) which was referenced to a precision thermometer, serial 210620, traceable to NIST standards (H-B Instrument, Trappe, PA, USA).
6.3.3 Experimental series

6.3.3.1 Series 1: acute temperature exposure

The effect of acute temperature changes on dogfish $O_2$ consumption rates, ammonia and urea-N fluxes were assessed using temperatures of 7.5, 12 (control = acclimation temperature), 15, 18 and 22°C. The same dogfish ($n = 8$) were tested at 12, 15, 18 and 22°C on different days, with a minimum 24-h interval back at 12 °C between each experiment; the order of temperature testing was sequential rather than random in these animals. A different set of animals ($n = 6$) was tested at 7.5°C.

For flux measurements, the water flow to the box was stopped, and the volume of water was set to 35 L by the removal of a rubber stopper located near the bottom of the box. The control temperature of 12°C was maintained by placing the wooden box in a water bath served with flow-through ambient sea water at 12°C. The 7.5°C temperature was achieved by the addition of several 250-mL, -80°C-frozen seawater blocks, which were mixed into the water in the box. After the target temperature of 7.5°C was reached, ice chips were added to the bath surrounding the box, in order to maintain the 7.5°C inside without altering or diluting the box volume. The increased temperature treatments of 15, 18 and 22°C were performed by gradually mixing heated seawater with the water in the box, until the desired temperature was reached, and thereafter the experimental temperature was maintained using immersed aquarium heaters. Water mixing inside the boxes was ensured by constant, well-spread perimeter aeration. The temperature in each box was monitored every 0.25 h, and never differed from the target temperature by more than ± 0.25 ºC. The desired temperatures (7.5, 15, 18 and 22°C) were always achieved within 0.5 h, and all animals were left to adjust to the new temperature for an additional 0.5 h prior to the start of the experiment. Water samples (5 mL) were taken at 0 h, 2 h, 4 h, and 6 h, and then frozen at -20°C for later analyses of ammonia and urea concentrations. During the final 2 h of the experiment, shortly after the 4-h water sample, air flow inside the box was stopped, and an initial $PO_2$ value was taken using the YSI 55 handheld $O_2$ meter. After that, the box was sealed with a floating lid to prevent $O_2$ diffusion from the air into the water and after 0.25-0.5 h, depending on the
temperature, a final PO$_2$ value was measured. O$_2$ saturation never fell below 120 Torr. The difference between the initial and final PO$_2$ values was used to calculate the O$_2$ consumption rate. This procedure was repeated once after a 1-hour re-aeration period. At the end of the 6-h experiment, a final 5-mL water sample was taken, ending the experimental period. After that, the seawater flow-through and aeration were re-established to the box, and the temperature was gradually brought back to control levels.

The effect of acute temperature changes on tritiated water ($^3$H$_2$O) turnover rates in dogfish was measured in separate experiments, using some of the same animals from the preceding trials ($n = 6$). Animals used for this series remained in the experimental chambers for no longer than 2 weeks. The same experimental protocol as described above for changing and maintaining the temperature was used. Pilot experiments demonstrated that stable $^3$H$_2$O efflux rates could be measured from about 0.5 to 1.5-2.5 h after injection, depending on temperature, after which recycling of the radioisotope became a problem, because external specific activity exceeded 10% of internal specific activity (Kirschner, 1970).

Once the fish had settled at the experimental temperature for 0.25 h, they were injected intraperitoneally with 20 µCi of $^3$H$_2$O (Perkin-Elmer Wellesley, MA, U.S.A.) diluted in 10 mL of isotonic NaCl (500 mmol/L). For the injection, the fish were not anaesthetized and spent less than 1 min out of water; this protocol was adopted to avoid a long post-anaesthesia recovery time which would have compromised the measurement window for tritiated water efflux. The fish was allowed to settle for 0.25 h before an initial 5-mL water sample was taken, marking the start of the experiment. Water samples were taken at 0.25-h intervals for 2.5 h, with a final sample at 4-6 h, after which seawater flow-through was re-established to the box, bringing the temperature gradually back to control levels. The final sample was taken to ascertain the exact dose of $^3$H$_2$O which had been administered to each fish, because by this time the radio-isotope had completely equilibrated between the fish and the water (see Section 6.3.4). The experiment was repeated at different temperatures on subsequent days after at least 24-h recovery back at 12 ºC. Water samples were immediately dosed with scintillation fluor (Optiphase, PerkinElmer, Waltham, MA) in a 2:1 ratio (fluor:water). Samples rested for 12 h in the dark to eliminate chemiluminescence before
being counted for beta-emissions (Tri-Carb 2900TR Liquid Scintillation Analyzer; PerkinElmer, Waltham, MA). Tests showed that quench was constant.

6.3.3.2 Series 2: hyperoxia experiments

The effects of hyperoxia (PO₂ > 310 Torr) on the responses of ammonia and urea-N fluxes, as well as on ³H₂O turnover rates, to acute temperature changes were assessed. A new set of fish (n = 6) was tested at 12°C (control) and 22°C under hyperoxia. The protocol used to reach and maintain the targeted temperature was similar to that of Series 1. Once the fish had settled in experimental temperature for approximately 30 min, aeration was stopped and pure oxygen gas (O₂) was bubbled in the box until the PO₂ reached > 310 Torr (hyperoxia). The PO₂ and temperature were checked every 0.25 h with a YSI 55 handheld meter. For ammonia and urea-N flux measurements, the fish were allowed to settle at the experimental temperature for 0.5 h, and an initial 5-mL water sample was then taken to start the experiment (0 h), with additional samples at 2 h and 4 h. Water samples were frozen at -20°C for later analyses of ammonia and urea concentrations. After 4 h, normoxic seawater flow-through was re-established, and fish were allowed to recover for at least 24 h. Data from these fish were compared with data from normoxic fish of Series 1, measured over the same 4-h period. The same two sets of fish (normoxia and hyperoxia exposed) were used subsequently for the ³H₂O turnover measurements at the two temperatures, using a protocol identical to that outlined in Section 6.3.3.1.

6.3.4 Analytical procedures and calculations

Ammonia-N (Jₐmm; µmol N/kg/h) and urea-N (Jₜₚurea-N; µmol N/kg/h) flux rates were calculated using the following equations:

\[ Jₐmm = \frac{[(\text{Amm-f} - \text{Amm-i}) \times V]}{(W \times T)} \]  
\[ \text{(Eq. 1)} \]

and
\[ J_{\text{urea-N}} = \{[(\text{Urea-f} - \text{Urea-i}) \times V] / (W \times T)\} \times 2 \quad \text{(Eq. 2)} \]

where Amm-f and Urea-f are the final water ammonia (\(\mu\text{mol/L}\)) and urea (\(\mu\text{mol urea/L}\)) concentrations; Amm-i and Urea-i are the initial water ammonia (\(\mu\text{mol/L}\)) and urea (\(\mu\text{mol urea/L}\)) concentrations; V is the volume of water during the experiment (L); W is the weight of the animal (kg); T is the duration of the flux period (h). Ammonia and urea concentrations in the water were measured colorimetrically according to Verdouw et al. (1978) and Rahmatullah and Boyde (1980) respectively. The total nitrogen excretion rate (\(J_{N\text{-total}}; \mu\text{mol N/kg/h}\)) was then calculated as:

\[ J_{N\text{-total}} = J_{\text{Amm}} + J_{\text{Urea-N}} \quad \text{(Eq. 3)} \]

Note that the molar urea excretion rate is multiplied by 2 in Eq. 2 so the two nitrogen atoms of urea are taken into account, therefore \(J_{N\text{-total}}\) is expressed in units of N.

The rate constant of \(^3\text{H}_2\text{O}\) turnover was calculated from the rate of decline in total \(^3\text{H}_2\text{O}\) radioactivity in the fish, which was approximately exponential with time (Evans, 1967):

\[ k = (\ln \text{CPM}_1 - \ln \text{CPM}_2) / (T_1 - T_2) \quad \text{(Eq. 4)} \]

where k is the rate constant of the efflux (in \(\text{h}^{-1}\)), \(\text{CPM}_1 = \text{total } ^3\text{H}_2\text{O radioactivity (in cpm) in the fish at time } T_1\) (in h), and \(\text{CPM}_2 = \text{total } ^3\text{H}_2\text{O radioactivity (in cpm) in the fish at time } T_2\) (in h). The product of \(k \times 100\%\) yields the percent of body water turned over per hour.

In practice, \(^3\text{H}_2\text{O}\) efflux rates were calculated by regressing the natural logarithm of CPM measurements against time over the range of linearity (generally 0.5 h to 1.5-2.5 h after injection), depending on temperature, to yield the slope k. By measuring the \(^3\text{H}_2\text{O}\) radioactivity in the water after 4-6 h, when complete equilibration between the fish and the water had occurred, it was possible to calculate accurately the total amount of radioactivity (\(\text{CPM}_{\text{total}}\)) in the system. The volume of the system was taken as the measured volume of
external water plus the volume of the fish. Therefore, from \( CPM_{\text{total}} \) and from measurements of \(^3\text{H}_2\text{O} \) radioactivity appearance in the water at each time interval, it was possible to back-calculate the CPM in the fish at each time during the experiment.

\( O_2 \) concentrations in the water were obtained by converting the partial pressure \( (PO_2) \) values using solubility constants from Boutilier et al. (1984). Oxygen consumption rates \( (\dot{MO}_2: \mu\text{mol O}_2/\text{kg/h}) \) were calculated using the following equation:

\[
\dot{MO}_2 = \frac{[(O_2-\text{i} - O_2-\text{f}) \times V]}{(W \times T)} \quad \text{(Eq. 5)}
\]

where \( O_2-\text{i} \) and \( O_2-\text{f} \) are oxygen concentrations in the water \( (\mu\text{mol/L}) \) at the start and at the end of the experiment and the other symbols are as defined above.

The temperature coefficient \( (Q_{10}) \) for every 10 °C increment in temperature was calculated using the mean values for each physiological parameter at each experimental increment as:

\[
Q_{10} = \left( \frac{R_2}{R_1} \right)^{10/(T_2 - T_1)} \quad \text{(Eq. 6)}
\]

where \( R_1 \) and \( R_2 \) are the rates of interest measured at temperature 1 \( (T_1, ^\circ\text{C}) \) and temperature 2 \( (T_2, ^\circ\text{C}) \).

The relative protein use as a fuel for aerobic metabolism was calculated according to Lauff and Wood (1996). Firstly, the nitrogen quotient \( (NQ) \) was calculated as:

\[
NQ = \frac{J_{N-\text{total}}}{\dot{MO}_2} \quad \text{(Eq. 7)}
\]

The percentage of metabolism fueled by the oxidation of protein was then determined by:

\[
\% \text{ Protein} = \frac{NQ}{0.27} \quad \text{(Eq. 8)}
\]

because 0.27 represents the maximum theoretical value when protein is the only fuel being metabolized (Van Den Thillart and Kesbeke, 1978).
6.3.5 Statistical analyses

All data are represented as means ± 1 SEM. Normality and homoscedasticity were tested through Shapiro’s and Bartlett’s tests respectively, prior to all parametric statistical analyses. All data reported here passed the two criteria for using parametric tests. Data from experiments where measurements were made on the same animals under different experimental treatments were analyzed by either a repeated-measures one-way ANOVA followed by a Tukey’s post hoc test (for multiple comparisons) or by a paired Student’s two-tailed t-test (for binary comparisons). Means originating from independent experiments were compared through a one-way ANOVA followed by a Tukey’s post hoc test (for multiple comparisons) or an unpaired Student’s two-tailed t-test (for binary comparisons). Where data were used in two comparisons, the Bonferroni correction was applied. Significance was accepted at the level of 0.05. The specific statistical analyses employed for each data set are detailed in the Figure Legends.

6.4 Results

Oxygen consumption rate (Ṁ$\text{O}_2$) at the acclimation temperature of 12ºC was approximately 2140 £mol O$_2$/kg/h, and varied with acute changes in temperature in a close to linear fashion (Fig. 6.1A). Ṁ$\text{O}_2$ fell significantly at 7.5ºC and increased significantly at 18ºC and 22ºC. The Q$_{10}$ values for each temperature increment were low (1.36-1.64), whereas the Q$_{10}$ for the decrease from 12ºC to 7.5ºC was somewhat higher (2.59). The overall Q$_{10}$ (7.5-22ºC) was 1.76.

Ammonia excretion rates (J$_\text{Amm}$; Fig. 6.1B) exhibited a different pattern from Ṁ$\text{O}_2$. At both 12ºC and 15ºC, J$_\text{Amm}$ was approximately 10 µmol N/kg/h, thereafter increasing to a 3-fold higher value at 22ºC. However J$_\text{Amm}$ dropped by about 70% when temperature was acutely decreased to 7.5ºC. In accord with this pattern, Q$_{10}$ values were very high between 12ºC and 7.5ºC (19.33), as well as between 15ºC and 18ºC (9.36), and 18ºC and 22ºC (3.19). The overall Q$_{10}$ (7.5-22ºC) was 5.15.
Urea-N excretion rate ($J_{\text{Urea-N}}$) at 12°C was approximately 430 µmol N/kg/h (Fig. 6.1C) – i.e. 43-fold greater than $J_{\text{Amm}}$. In contrast to $J_{\text{Amm}}$, $J_{\text{Urea-N}}$ was tightly conserved over the range from 7.5°C to 15°C (i.e. $Q_{10}$ values close to 1.0) but thereafter increased significantly at 18°C ($Q_{10} = 4.22$) with a further small increase at 22°C ($Q_{10} = 1.32$). The overall $Q_{10}$ (7.5-22°C) was 1.45, the lowest for the four flux parameters which were monitored.

At the acclimation temperature of 12°C, dogfish exchanged approximately 60% of their body water per hour (Fig. 6.1D) based on tritiated water turnover rate, which would translate to about 450 ml/kg/h, assuming a body water content of 750 mL/kg. An acute decrease in temperature to 7.5°C resulted in only a small, non-significant reduction in water exchange rate ($Q_{10} = 1.60$), whereas acute elevations in temperature caused moderate, approximately linear increases ($Q_{10}$ values = 1.90 – 2.52) over the range from 12°C to 22°C. Only at the latter temperature was the water exchange rate significantly different from the value at 12°C. The overall $Q_{10}$ (7.5-22°C) was 1.96.

The simultaneous measurements of $\dot{\text{M}}O_{2}$, $J_{\text{Amm}}$, and $J_{\text{Urea-N}}$ allowed calculation of the Nitrogen Quotient (NQ) which provides information on aerobic fuel usage (see Methods). NQ values were not significantly different at any of the experimental temperatures, ranging from 0.20 to 0.33 (Fig. 6.2). By standard metabolic theory, an aerobic metabolism based entirely (i.e. 100%) on protein oxidation would yield an NQ of 0.27 (see Material and Methods) and none of the experimental means were significantly different from this reference value. Therefore, at all temperatures, aerobic metabolism in these fasting dogfish was based largely on the oxidation of somatic protein.

A temperature of 22°C is likely close to the upper critical temperature ($CT_{\text{max}}$) of *Squalus acanthias suckleyi*, at least without prior acclimation, because most of the present animals lost orientation, turning laterally or completely over during this treatment. Note however that they have been caught in the wild at 24°C (Bangley and Rulifson, 2014). In order to test the hypothesis that gill permeability increases because dogfish become oxygen-limited at 22°C, sharks were exposed to this high temperature (22°C) in the presence of hyperoxia ($PO_{2} > 309$ Torr), and compared with fish maintained at the control temperature (12°C) but also exposed to hyperoxia. These results were also compared to data from the
parallel normoxic treatments (data from Section 6.3.3.1; Series 1), which were calculated over a 4-h period to allow direct comparison with data from Section 6.3.3.2 (Series 2). Surprisingly, exposure to hyperoxia significantly increased both $J_{\text{Amm}}$ (Fig. 6.3A) and $J_{\text{Urea-N}}$ (Fig. 6.3B) by more than 2-fold at 12°C, and to an even greater extent (by 2.7 to 4.2-fold) at 22°C. Most importantly, hyperoxia exacerbated rather than reduced the influence of high temperature on $J_{\text{Amm}}$ (Fig. 6.3A) and $J_{\text{Urea-N}}$ (Fig. 6.3B). In contrast, small increments (6-24%) in water exchange rates at both temperatures associated with the hyperoxic treatments were not significant (Fig. 6.3C). $Q_{10}$ values under hyperoxia over the range from 12°C to 22°C were 4.02 for $J_{\text{Amm}}$ (versus 3.82 under normoxia), 3.77 for $J_{\text{Urea-N}}$ (versus 1.88 under normoxia), and 2.04 for water exchange rate (versus 2.14 under normoxia).

6.5 Discussion

6.5.1 Overview

Our initial hypothesis was that as metabolic demand for $O_2$ increased with temperature, the fluxes of ammonia, urea-N and water at the gill would increase in parallel with those of $O_2$. We thought that this would happen through the osmorespiratory compromise due to the normal physiological adjustments, such as increases in gill ventilation, perfusion, and effective branchial surface area, that promote an enhancement of $O_2$ transfer, thereby supplying the increased demand. Overall, the hypothesis was only partially supported. The fluxes of all four moieties ($O_2$, ammonia, urea, and $^3H_2O$) increased with temperature, but only the changes in $^3H_2O$ exchange roughly paralleled those in $MO_2$. The relative changes in $J_{\text{Urea-N}}$, and in particular in $J_{\text{Amm}}$, were much larger overall than those in $MO_2$, and displayed step-wise patterns indicative of temperature effects on specific N-conservation mechanisms, as discussed subsequently. In this regard, our results confirmed the surprising findings of Boylan (1967) that branchial urea-N excretion is independent of temperature up to 15 °C, and then increases sharply at higher temperatures. Finally, our hyperoxia treatments provided no support for the hypothesis that increased $O_2$ availability would minimize or prevent the effects of rising temperature on gill permeability. Rather, the
results suggested that hyperoxia induced detrimental effects in the gills, thereby exacerbating the influence of temperature on gill permeability.

However, there are several important caveats in the interpretation of our data. The first is that animals were exposed to increased temperature for only 6.5 h; therefore, we cannot assess whether the animals were in long-term steady-state, or whether undershoot or overshoot phenomena may have occurred. Nevertheless, for urea, ammonia, and oxygen fluxes where 2-3 replicate measurements were made on each fish within this 6.5-h period, there was at least a short-term steady-state as rates were constant over time. Therefore while our data do not cast light on responses to global warming, they are very relevant to the daily life of this animal as it moves into warmer surface or inshore waters to feed, or migrates through the thermocline. The second caveat is that no internal concentrations were measured and therefore we do not know if the effects of temperature seen here were due to changes in internal concentrations, which would affect diffusion gradients. In addition, the different group of fish exposed to 7.5 ºC was not tested at 12 ºC, therefore we cannot rule out the possibly of greater variability in the responses over this range. Finally, time controls were not performed to check the potential effects of prolonged fasting and confinement. Nevertheless, we believe that such effects would be negligible because Wood et al. (2007b) found that the rates of oxygen consumption did not change in comparably fasted dogfish kept in the boxes for 5 days, while Kajimura et al. (2008) reported that ammonia-N and urea-N flux rates were stable in dogfish kept under the same fasted conditions for up to 56 days.

6.5.2 Temperature effects on O₂ consumption

In ectotherms, metabolic demand for O₂ increases with temperature, and then rapidly declines at higher temperatures (for review see: Schulte, 2015). In our study, \( \dot{M}O_2 \) increased with increases in temperature in an almost linear fashion, with \( Q_{10} \)’s ranging from 1.36 to 2.59, with an overall value of 1.76 (Fig. 6.1A). This temperature sensitivity lies at the lower end of the range reported in previous studies on both sharks and rays, where the temperature coefficient is usually around 2 -3 (Butler and Taylor, 1975; Hopkins and Cech, 1994; Miklos et al., 2003; Di Santo and Bennett, 2011b), or occasionally higher (Di Santo, 2016). Notably,
in the current study, the $Q_{10}$ was greatest (2.59) at the lower end of the temperature range (7.5°C-12 °C), which may be most representative of the normal annual range experienced by *Squalus acanthias suckleyi* in the north Pacific Ocean.

### 6.5.3 Temperature effects on urea-N excretion

One of our initial goals was to replicate the study done by Boylan (1967), where using an unusual ventilated whole animal technique in which the head was air-exposed, the author found that the urea-N excretion at the gills was independent of temperature from 1 to 15 °C, and then increased dramatically at temperatures higher than 15 °C. Boylan (1967) apparently studied only 3 animals at a wide range of temperatures, whereas we elected to study a larger number of dogfish (6-8) at only five fixed temperatures. Nevertheless, our results are very similar to those of Boylan (1967) and were obtained with a much less invasive and therefore less stressful experimental technique. $J_{\text{Urea-N}}$ did not vary from 7.5 to 15°C but increased greatly thereafter, reaching a 2-fold increase at 22 °C (Fig. 6.1C). This pattern was completely different from the linear increase in $\text{Ṁ}_2$ over the entire range (Fig. 6.1A). Even though urea-N production rates (via the OUC cycle in liver and muscle) could have been upregulated due to increases in aerobic metabolic rate, it seems very unlikely that upregulation of the OUC cycle alone could explain the increase in urea-N excretion, given that $J_{\text{Urea-N}}$ was unchanged up to 15 °C. Furthermore, in the study of Boylan (1967), it appears that only the temperature of the irrigated gills was manipulated, and not the temperature of the whole animal.

Urea is retained at the elasmobranch gill mainly by two important factors: (1) an unusual gill membrane composition (Fines et al., 2001), and (2) the presence of an activetransporter in the branchial epithelium (Wood et al., 1995; Pärt et al., 1998; Fines et al., 2001; Wood et al., 2013), the exact location of which is in debate (see 6.2; Introduction). Is it possible that high temperatures may have affected one, or, both of the factors cited above? Boylan (1967) hypothesized that the breakpoint of urea-N excretion could be interpreted as a phase change in the ultrastructure of the gill cell membranes, i.e., increases in temperature may have influenced the lipids in the membranes leading to an increase in membrane
fluidity. This is an interesting area for future investigation. Additionally, it is also possible that the activity of the mechanism responsible for re-capturing urea back to the plasma (Pärt et al., 1998; Fines et al., 2001) could have decreased drastically at temperatures higher than 15ºC.

6.5.4 Temperature effects on ammonia excretion

The gills of elasmobranchs are thought to be very impermeable to ammonia leakage relative to teleosts, thereby contributing to N-conservation (Wood et al., 1995; Wright and Wood, 2016). Nevertheless, amongst the variables tested, ammonia excretion rate seemed to be the most temperature sensitive with an overall Q₁₀ from 7.5 to 22 ºC equal to 5.15 (Fig. 6.1B). Different from the results obtained for urea-N, it seems that ammonia excretion exhibits two breakpoints. We observed an increase in ammonia excretion from 7 to 12 ºC, no change from 12 to 15 ºC, and then a marked increase from 15 to 22 ºC. These results are not in agreement with previous data on the lesser-spotted dogfish (Scyliorhinus stellaris) by Heisler (1978), where a 10 ºC increase in temperature was reported to cause no changes in ammonia excretion rates. It is important to note that even though we recorded a 15-fold increase in ammonia excretion rates over the whole temperature range, our absolute values ranged only from 2 to ~30 µmol N/kg/h. Heisler (1978) did not report any absolute values of ammonia excretion rates because they were thought to be negligible, so it is possible that his animals could have experienced similar large-fold changes in very small rates. Nawata et al. (2015) reported that Squalus acanthias are N-limited and, when presented with high concentrations of ammonia in the water, they have the ability to scavenge ammonia-N, convert it into urea, and elevate urea-N excretion rates. This uptake of ammonia exhibits classic Michaelis-Menten saturation kinetics resulting in a net retention of nitrogen (Wood and Giacomin, 2016). Along with other transformations at the gill, it is possible that at high temperatures the ammonia-conserving uptake mechanism could have been impaired, leading to the high temperature sensitivity seen. In contrast to the scarcity of data on the N-waste excretion sensitivity to temperature of elasmobranchs, this topic has been thoroughly explored in ammoniotelic teleosts where it is well-established that N-waste excretion is far
more sensitive than \( \dot{M}O_2 \) to increases in temperature. However, in this case, the explanation appears to be that as temperature increases, a greater percentage of aerobic metabolism is fueled by protein oxidation (Wood, 2001).

### 6.5.5 Temperature effects on the nitrogen quotient

We measured oxygen consumption rates, as well as urea-N and ammonia-N excretion rates in the same experimental animals, and therefore we were able to calculate the \( \text{N}/\dot{M}O_2 \) ratio, or the nitrogen quotient (NQ), which is an index of fuel utilization supplying aerobic metabolism (Lauff and Wood, 1996a). Despite a slight decrease from 7.5 to 12°C, the NQ did not vary significantly across all temperatures tested. It appears that these fasted dogfish are metabolizing almost entirely protein (amino acids) in aerobic respiration. This finding is in accord with one previous study on fasted members of the same species, in which measurements were made at only 12 ± 1°C (Wood et al., 2007b). Interestingly, in that study, the NQ tended to fall after feeding as \( \dot{M}O_2 \) increased and the OUC was activated, yet branchial urea-N excretion fell as part of the animal’s post-prandial N-retention strategy. We are aware of no other elasmobranch studies where the NQ has been measured, but in teleosts, NQ values tend to be much lower as fuels other than protein are burned during fasting (Lauff and Wood, 1996a; Wood, 2001).

The implications of temperature increases for energy budgets are of interest in an organism which supports aerobic respiration almost entirely by the oxidation of protein. Total N-efflux rates (i.e. urea-N + ammonia-N losses) approximately doubled between 12°C and 22°C (Fig. 6.1B,C). Based on measurements made during feeding and fasting in this species and the protein content of a typical meal of teleost fish (Kajimura et al., 2006; Kajimura et al., 2008), this would double the need for food consumption, or reduce the time that a single meal would last from about 5 to 2.5 days so as to maintain body mass. Alternately, in a fasting dogfish, it would double the rate at which muscle protein was broken down so as to support this elevated rate of N-loss while maintaining constant blood urea-N concentrations for osmoregulatory homeostasis.
6.5.6 Temperature effects on tritiated water fluxes

Using tritiated water (\(^3\text{H}_2\text{O}\)) we measured the diffusive water exchange rates at the gills, which are thought to be about 100-fold greater than the net osmotic flux of water, which depends on the low osmotic gradient from seawater to plasma (10 – 70 mOsmol L\(^{-1}\)) (Wright and Wood, 2016). At the control temperature, 12ºC, the fish exchanged approximately 60% of their body water per hour (Fig. 6.1D). To our knowledge, this is the first report of diffusive water exchange rates for *Squalus acanthias suckleyi*, and our results fall at the low end of the range of the very few available measurements for seawater elasmobranchs, where rates can vary from 81 to 157% body water h\(^{-1}\) in sharks (Payan and Maetz, 1971; Carrier and Evans, 1972; Haywood, 1975) and from 64 to 167% body water h\(^{-1}\) in rays (Payan and Maetz, 1971; Payan et al., 1973), values which are about 5-fold higher than those in marine teleosts (Wright and Wood, 2016). Water exchange rates increased with temperature in a linear fashion (Fig. 6.1D), similar to the response pattern seen for \(\dot{M}_\text{O}_2\) (Fig. 6.1A), with an overall Q\(_{10}\) (7.5 – 22ºC) of 1.96. \(^3\text{H}_2\text{O}\) is the only moiety for which the measured flux pattern fit clearly with our overall hypothesis based on the osmorespiratory compromise. This might suggest that the pathway of diffusive water flux across the gills is the same as that of diffusive O\(_2\) flux. The role, if any, played by aquaporins in facilitating the diffusion of water across the gills of elasmobranchs is not yet fully understood. Initial research on isolated gill cell membranes of *Squalus acanthias* ruled out the participation of aquaporins (Hill et al., 2004). However, more recently Cutler et al. (2012a) have detected the presence of aquaporin AQP4 mRNA in the gills of *Squalus acanthias*, and AQP4 protein expression was localized to branchial ionocytes (Cutler et al., 2012b). Furthermore, after acclimation to 120% SW, branchial mRNA expression of AQP4 declined, perhaps indicating an important role for aquaporins in salinity tolerance. The time frame of our acute temperature change experiments was short, and it is unclear whether aquaporin message and protein function could have been adjusted in that period.
6.5.7 The impact of hyperoxia on temperature effects

In our study, we hypothesized that by supplying excess amounts of oxygen in the water (hyperoxia), the animals would not need to upregulate gill ventilation, perfusion, and functional area to the same extent in order to obtain the same amount of O$_2$ or even more O$_2$, thereby minimizing increases in nitrogen and water fluxes. Hyperoxia is an important and relevant environmental circumstance to dogfish sharks, which are well known for making foraging and breeding incursions into estuaries and river mouths (Ulrich et al., 2007). Estuaries are very dynamic ecosystems, exhibiting large daily variations in water parameters such as salinity, temperature, and dissolved oxygen, the latter due to photosynthesis and respiration rates (Kennish, 1986).

Our data clearly do not support this hypothesis. Rather than attenuating N-waste fluxes, exposure to hyperoxia at 22 ºC led to a dramatic increase in ammonia (Fig. 6.3A) and urea-N fluxes (Fig. 6.3B) in comparison to animals that were exposed to high temperatures in normoxic conditions. To our knowledge, this is the first report of increased N-waste effluxes during exposure to hyperoxia in any elasmobranch species. Surprisingly enough, the same response was also seen at 12 ºC. The fluxes of $^3$H$_2$O were not significantly elevated by hyperoxia; nevertheless, the response to temperature was clearly not reduced (Fig. 6.3C).

Hyperoxia is known to cause an elevation of blood PCO$_2$, leading to a decrease in blood pH and consequently to a respiratory acidosis. This phenomenon can be rapidly compensated by retention of bicarbonate (HCO$_3^-$) in the blood. In the larger spotted dogfish (*Scyliorhinus stellaris*), Heisler et al. (1988) reported that acute hyperoxia exposure caused a decrease in gill ventilation, increased arterial blood PO$_2$ (PaO$_2$), and hypercapnia which was compensated by an increased net uptake of HCO$_3^-$ equivalent ions at the gill epithelia. Although an acid-base disturbance could have exacerbated the N-compound effluxes, there are not enough available data to support such inferences.

An alternative, and perhaps more likely, explanation for the increased urea-N and ammonia fluxes at the gills of animals exposed to hyperoxia is the production of reactive oxygen species (ROS) in the water and subsequent accumulation in tissues, causing oxidative damage to gill cell membranes, and leading to an elevated leakage of N-waste compounds.
The presence of ROS in the water has been shown to increase with natural diel increases in the O$_2$ concentration in the water (Johannsson et al., 2014). Johannsson et al. (2014) and Pelster et al. (2016) have reported that two different species of facultative air-breathing teleost fishes increased the frequency of air-breathing events when exposed to aquatic hyperoxia. These behavioural alterations are thought to be an adaptation to prevent ROS damage caused by increased ROS concentration in hyperoxic waters (Johannsson et al., 2014; Pelster et al., 2016). The oxidative stress response in teleost fish exposed to hyperoxia has been well reported, with studies showing downregulation of both enzymatic and non-enzymatic antioxidant defenses, accumulation of oxyradicals in tissues, and oxidative damage to lipids and proteins (Lygren et al., 2000; Lushchak and Bagnyukova, 2006; Johannsson et al., 2014). Hypoxaemia at high temperatures may also result in internal ROS production (Lushchak, 2011) which could be synergistic with ROS originating from exposure to hyperoxic water. In contrast to teleosts, studies investigating the hyperoxia-induced oxidative stress response in elasmobranch are still lacking. This is an important area for future investigation.

6.5.8 Implications

Based on the data in Fig. 6.1, above 15ºC, the large temperature-dependent increases in ammonia and urea-N excretion will pose the greatest physiological challenges for the organism, especially the latter because urea-N losses comprise such a large component of the N-budget of sharks. However it is the 12-15ºC range that is the most relevant for the day-to-day movements of *Squalus acanthias suckleyi* during the summer. The temperature at depth close to Bamfield Marine Sciences Centre (from which the acclimation water was pumped, and at which most of the dogfish were caught by angling) is about 12ºC, and 15ºC is very representative of normal sea-surface temperatures in the area (where some of the animals were caught by angling; personal observations). In light of this, we have compared our control data (12ºC) with data on animals that were exposed to 15ºC (Table 6.1). The physiological aspect that seems to be most sensitive to an increase of 3ºC in water temperature was the water exchange rate, increasing significantly by 32%; $\text{MO}_2$, $J_{\text{Urea-N}}$, and
$J_{\text{Amm}}$ were not significantly altered. Clearly, future studies on water balance in this species will be valuable, especially since fluctuations in temperature are often coupled with fluctuations in salinity in inshore regions due to the influence of river inflows. Overall, the current data are useful in characterizing the thermal sensitivity of branchial processes that may be involved in setting thermal constraints to whole organism.
Figure 6.1 (A) Oxygen consumption rates (\(\dot{M}O_2\): µmol O\(_2\)/kg/h); (B) ammonia excretion rates (\(J_{\text{Amm}}\): µmol N/kg/h); (C) urea-N excretion rates (\(J_{\text{Urea-N}}\): µmol N/kg/h); and (D) tritiated water turnover rates (% body water/h) in dogfish sharks (\(Squalus acanthias suckleyi\)) at 5 different temperatures. Numbers between data points are the Q\(_{10}\) values for each increment in temperature, whereas the Q\(_{10}\) value over the entire range from 7ºC to 22ºC is tabulated in the lower right of each panel. The same 8 animals were tested at 12ºC, 15ºC, 18ºC, and 22ºC, so these data were analyzed by a repeated-measures one-way ANOVA followed by a Tukey’s post hoc test. A different set of 6 fish was tested at 7ºC, so an unpaired Student’s two-tailed t-test was used to test the significance of differences between the 7ºC and 12ºC treatments. Means sharing the same upper case letters indicate the absence of statistical significance (p<0.05) through an unpaired Student’s t-test for 7.5 and 12ºC data points. Means sharing same lower case letters indicate the absence of statistical significance by a repeated-measures one-way ANOVA for 12 to 22ºC. Data are shown as means ± 1 SEM (n = 6 - 8).
Figure 6.2 Nitrogen quotient in dogfish (*Squalus acanthias suckleyi*) at different temperatures (°C). The same 8 animals were tested at 12°C, 15°C, 18°C, and 22°C, so these data were analyzed by a repeated-measures one-way ANOVA followed by a Tukey’s post hoc test. A different set of 6 fish was tested at 7°C, so an unpaired Student’s two-tailed t-test was used to test the significance of differences between the 7°C and 12°C treatments. Means sharing the same upper case letters indicate the absence of statistical significance (p<0.05) through an unpaired Student’s t-test for 7.5 and 12°C data points. Means sharing same lower case letters indicate the absence of statistical significance by a repeated-measures one-way ANOVA for 12 to 22°C. Data are shown as means ± 1 SEM (n = 6 - 8).
**A**

$J_{\text{Amm}}$ (µmol N/kg/h)

**B**

$J_{\text{Urea-N}}$ (µmol N/kg/h)

**C**

Water turnover rate (% body water/h)

- **Normoxia**
- **Hyperoxia**

Legend:
- **a**, **b**, **x**, **y**
- * indicates significant difference compared to Normoxia.
Figure 6.3 (A) Ammonia excretion rates ($J_{Amm}$: µmol N/kg/h); (B) urea-N excretion rates ($J_{Urea-N}$: µmol N/kg/h); and (C) tritiated water turnover rates (% body water/h) in dogfish sharks (*Squalus acanthias suckleyi*) at 12 (grey bars) and 22°C (black bars) measured under normoxia and hyperoxia. Different lower case letters indicate statistical significance between 12 and 22°C tested at the same oxygen tension, as determined by a paired Student’s two-tailed t-test ($p < 0.05$). Normoxia and hyperoxia data at a given temperature were compared through an unpaired Student’s two-tailed t-test ($p < 0.05$), and significance is indicated through an asterisk. Data are shown as means ± 1 SEM ($n = 6 - 8$).
Table 6.1 Ammonia efflux rate, urea-N efflux rate, oxygen consumption rate, water turnover rate and nitrogen quotient at 12 and 15°C. Asterisk indicate significant differences between 12 and 15°C obtained through a paired Student’s t-test (p < 0.05). Means ± 1 SEM (n = 6 - 8)

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<th>Temperature (°C)</th>
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<td></td>
<td>12</td>
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<tr>
<td>Ammonia Efflux (µmol N/kg/h)</td>
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<tr>
<td></td>
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<td>Oxygen Consumption (µmol O₂/kg/h)</td>
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<td>SEM</td>
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<td>Water Turnover Rate (% body water/h)</td>
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<tr>
<td>Nitrogen Quotient (NQ)</td>
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<td>SEM</td>
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Chapter 7: General discussion and conclusions

The overarching goal of my thesis was to expand our knowledge of the osmorespiratory compromise, by diverging from the current body of literature that has been dominated by work using freshwater fishes such as rainbow trout, and exploring this phenomenon in a range of species that exhibit diverse ionic-/osmo-regulatory strategies. To address this issue I have used three species of fish that are diverse phylogenetically and that occupy different environmental niches, the Atlantic killifish (teleost), the Pacific hagfish (myxine) and the Pacific spiny dogfish (elasmobranch). Because the natural environment to which these species are adapted differ greatly, it was not possible to perform experiments using a factorial design, where every species was exposed to every stressor. Therefore, these three species of fish were exposed to one or more of the following stressors: hypoxia, salinity and temperature, provided that the manipulation of the abiotic factor was within the range of tolerance for each species. Below, I highlight some of the key findings of the work presented in this thesis, and discuss some of the technical challenges associated with studying the osmorespiratory compromise in these diverse species.

7.1 Thesis highlights and main contributions

To date, the vast majority of our understanding of the osmorespiratory compromise came from studies with two very different species, the athletic rainbow trout (*Oncorhynchus mykiss*) and the highly hypoxia-tolerant Amazonian oscar (*Astronotus ocellatus*). When this phenomenon was first described using the rainbow trout, the picture that emerged was that when fish were exercised, a hormonally-modulated increase in functional surface area of the gills elevated O$_2$ uptake, but also led to an impairment of hydromineral balance (Randall et al., 1972; Wood and Randall, 1973a; 1973b; 1973c). When rainbow trout were exposed to hypoxia, a very similar increase in gill permeability to ions and water was observed (Iftikar et al., 2010; Onukwufor and Wood, 2018). In contrast to the trout, the Amazonian oscar decreased branchial ion and water fluxes during hypoxia (Wood et al., 2007a; Wood et al., 2009) while maintaining effective oxygen permeability at the gills (Scott et al., 2008a),
suggesting that the responses seen in trout may not be representative of all teleosts. In the Introduction, these two general patterns of response were plotted on a conceptual diagram of the changes in effective gill permeability to O₂ and also to ions and water (Fig. 1.1). My thesis adds to this diagram with responses by the three model species investigated here (Fig. 7.1). While the killifish and hagfish responses to hypoxia generally follow the model represented by the oscar, the dogfish responses to temperature follow the model represented by the trout.

7.1.1 The two faces of the osmorespiratory compromise

In Chapters 2 and 3 I used the euryhaline killifish to perform a thorough investigation of the osmorespiratory compromise, by approaching this phenomenon from two different angles. In Chapter 2 I investigated the respiratory responses to hypoxia and thus hypoxia tolerance, in fish acclimated to different salinities. In Chapter 3, I looked at the ionoregulatory responses of fish acclimated to the same salinities, during exposure to hypoxia.

From the results obtained in Chapter 2, it is clear that the freshwater-acclimated killifish exhibits the lowest ability to deal with hypoxia, likely due to a low capacity to regulate oxygen consumption at low PO₂s. In fresh water, fish also exhibit the lowest lamellar respiratory surface area, and the highest diffusion distance for O₂ at the gills. At all salinities, killifish show the presence of an ILCM, which is greater in freshwater fish. The higher percentage of the gill lamellae covered by an ILCM could be one of the adaptations to aid survival in hypo-osmotic waters, since the ILCM could act as a barrier preventing ionic loss and water gain with respect to the environment. In their recent review, Gilmour and Perry (2018) argued that the reversible remodelling of fish gills, i.e. the presence/absence of an ILCM, could be an alternative to the osmorespiratory compromise. More specifically, by keeping surface area low and diffusion distance high at rest, fish would maintain low gill permeability and avoid the metabolic cost of ion pumping and the regulation of water balance. It has been shown that when fish need to upregulate oxygen uptake at the gills, either due to increased demand or environmental hypoxia, they can “shed” the ILCM in
approximately 30 min (Perry et al., 2012). Therefore, the rapidly reversible ILCM could be a clever solution to deal with the permeability trade-off at the gills. However, it is not yet clear whether maintaining an ILCM has deleterious effects on the maintenance of hydromineral balance, acid-base regulation, and nitrogenous waste excretion (Gilmour and Perry, 2018).

In my thesis, I have also examined the ionoregulatory responses of killifish to hypoxia, aiming to determine which mechanisms are used to deal with this compromise. In Chapter 3, I discovered that regardless of the acclimation salinity, killifish are capable of maintaining plasma ion concentrations unchanged, but that the mechanisms employed to achieve this are different. More specifically, in freshwater-acclimated fish, a reduction in gill ion permeability was seen, so that both Na\(^+\) influx and efflux were reduced. This reduction appears to be part of a regulated response, inasmuch as it only happens after the first hour of hypoxia exposure, and normal flux rates are restored immediately after fish are brought back to normoxia. Permeability to water was also greatly reduced in freshwater-acclimated killifish, perhaps indicating a coordinated movement of gill ionocytes and neighbouring pavement cells, and their associated water and ion transporters. Additionally, ammonia efflux rate, which likely happens transcellularly through Rh proteins, was immediately depressed with exposure to hypoxia. Along with those two key indicators, K\(^+\) efflux, which has been used as a classical transcellular permeability marker, also decreased in hypoxia. Although paracellular permeability was mildly reduced in both fresh water and sea water (but interestingly increased at the isosmotic salinity), overall the results point to a strategy of reduction in gill transcellular permeability. Additionally, the activity of key iono- and osmo-regulatory enzymes was markedly reduced during hypoxia, pointing to a strategy that might be related to energy conservation during hypoxia, when oxygen acquisition and energy production are challenged. This result reinforces the findings of Chapter 2, where the freshwater killifish showed the lowest capacity to deal with hypoxia. Altogether, results from both Chapters 2 and 3 suggest that in order to acclimate to fresh water, killifish compromise whole-animal oxygen uptake capacity, which results in a poor ability to deal with hypoxia.
7.1.2 The osmorespiratory compromise in sea water

One of the major contributions of my thesis was the direct quantification of ion fluxes during hypoxia in a teleost fish in sea water, in both hypo and isosmotic conditions, in addition to the investigation of this phenomenon in two species of marine fish, the Pacific hagfish and Pacific spiny dogfish.

The measurements of Na\(^+\) uptake and Na\(^+\) loss in the killifish during normoxia and hypoxia were performed using the radiotracer \(^{22}\)Na. Due to the inherent differences in the ionic composition of fresh and sea water, different techniques were employed. In fresh water, the fact that the external [Na\(^+\)] is much lower than the internal [Na\(^+\)] of the fish, allows for the use of a method where radiosotope disappearance from the water is quantified. This method permits measurements over a number of successive periods of time. In contrast, in seawater, techniques based on the ones developed in Wood and Laurent (2003) and Wood (2011) were used, which are adapted for the high ionic concentration of sea water, with the goal of minimizing radiosotope usage as well as background noise, but these methods do not allow successive measurements. Therefore, in my thesis I have presented a more detailed examination of Na\(^+\) fluxes in fresh, than at both seawater concentrations in the teleost killifish.

In Chapter 2 I established that the isosmotic salinity (11 ppt) in comparison to fresh and full-strength seawater, was the one that conferred the greatest ability to regulate oxygen consumption in hypoxia, reflected in the highest RI, and consequently led to a higher hypoxia tolerance. Based on these results, in Chapter 3 I hypothesized that this greater respiratory capacity would minimize or eliminate the osmorespiratory compromise, since theoretically, at 11 ppt the concentration gradients between internal and external media should be minimal. In other words, since the gradients should not exist, killifish would be able to increase permeability to oxygen without deleterious ionoregulatory effects. My results only partially support this hypothesis. Unidirectional Na\(^+\) influx and efflux rates both increased, and paracellular permeability was elevated, while plasma ion concentration was unaltered, consistent with the hypothesis. However, in contrast to the hypothesis, diffusive water flux rate did not change. Classical studies have concluded that the isosmotic salinity confers a
great metabolic advantage, by preventing the fish from having to use anywhere from 2 to
30% of its energy budget in osmoregulation (Rao, 1968; McCormick et al., 1989; Boeuf and
Payan, 2001). Febry and Lutz (1987) found that the lowest resting MO2 was obtained at the
isosmotic salinity. From my results in Chapter 2, I was not able to replicate this finding.
Instead, I found no differences in resting MO2 between fish at 0 ppt and the isosmotic point.
My results point to different ways of regulating gill permeability in the isosmotic salinity
from the two other salinities tested (0 and 35 ppt). Unfortunately, there are no data available
for TEP in 11 ppt-acclimated fish exposed to hypoxia, which could help tease apart these
differences.

My results suggest that at 35 ppt, similarly to fish acclimated to fresh water, there is a
decrease in branchial permeability during hypoxia. Both unidirectional Na+ influx and active
Na+ efflux rates were reduced, while water permeability did not change. However, contrary
to freshwater killifish, in seawater fish the major components of both unidirectional Na+
transport are involved in exchange diffusion (Motais et al., 1966; Potts and Evans, 1967;
Maetz et al., 1967; Wood and Marshall, 1994).

Another major contribution of my thesis was the investigation of the osmorespiratory
compromise in a species of marine osmoconformer, the Pacific hagfish, which is actually a
strong regulator of plasma divalent cations (Sardella et al., 2009). Up to now, studies on the
osmorespiratory compromise had been restricted to iono- and osmo-regulators. Given the
hagfish’s osmoconformation strategy, the extent to which the osmorespiratory compromise
was expected to occur was still unclear. In order to get at this question, I exposed hagfish to
changes in water temperature (Chapter 4), salinity and oxygen concentration (Chapter 5).
Since hagfish are very hypoxia-tolerant, my predictions were that they would respond
similarly to other hypoxia-tolerant species studied, such as the Amazonian oscar (Wood et
al., 2007a; Wood et al., 2009; Robertson et al., 2015b), and the killifish (present thesis).
Overall, my data show that there is a strong interaction between gill permeability to gases
and to ions and water in hagfish.

In Chapter 4, when exposed to acute increases and decreases in temperature, hagfish
showed different Q10s for MO2, ammonia efflux and diffusive water flux rates, the latter
being the highest of the three, indicating not only that temperature elicits responses for
parameters that are differentially regulated, but also that water fluxes are the most
temperature sensitive parameter analyzed. The increased water flux at high temperature was
attenuated by experimental hyperoxia, again showing interactive effects of gas exchange and
water exchange. These results have implications for the hagfish commercial fishery. The
increasing demand for hagfish products has elevated the fisheries pressure on this species.
The current fishing practices involve pulling traps rapidly to the ocean surface, where they
are exposed to quick changes in pressure, salinity and temperature (Olla et al., 1998). Results
from Chapter 4 and 5 indicate that hagfish are very susceptible to acute changes in ambient
temperature and salinity, so that rapid exposure to surface waters can lead to increases in the
diffusive water permeability of the gill, as well as marked increases in MO₂ that can result in
elevations in the rate of production of nitrogenous waste (ammonia), and its plasma
accumulation. When hagfish were exposed to hypoxia, MO₂ was reduced by half, although
overall ventilation slightly increased. These results indicate that despite an attempt to
improve MO₂, the permeability of the respiratory epithelium to water was selectively
reduced. Additionally, ammonia efflux rates were also reduced. Altogether these results show
that hagfish depart from the “classical” osmorespiratory compromise exemplified by the
trout, but are in accord with responses in other hypoxia-tolerant fish (Fig. 6.1). Additionally,
results from experiments where temperature and external salinity were manipulated showed
that hagfish possess some capacity to regulate monovalent ions (Na⁺ and Cl⁻), as well as
divalent ions (Ca²⁺ and Mg²⁺) at concentrations that differ from those in the external sea
water. These results suggest that hagfish might not be true ionoconformers for Na⁺ and Cl⁻,
laying the groundwork for some interesting future research on the mechanisms involved.
Results from both Chapters 4 and 5 show a very minimal disturbance in the acid-base status,
after exposure to all three environmental stressors, confirming previous investigations
showing that these fishes possess great ability to regulate acid base status (Baker et al., 2015;
Clifford et al., 2018).

Zimmer and Wood (2014) found that when dogfish are exposed to hypoxia, they
display a response that is similar to the trout model (Fig. 6.1), with increased osmolyte fluxes
at the gill during hypoxia exposure. Since measuring the ion fluxes on a seawater species that
weighs on average 1.5 kg is essentially impossible due to the large amounts of radioisotope
needed, the quantification of the effects of the osmorespiratory compromise was done through the measurement of urea loss. In elasmobranchs, urea is used as a valuable osmolyte (see section 1.7.3), as it raises the osmolality of the body fluids (Yancey, 2016), and its loss would confer great energetic expense (Ballantyne, 1997). Since that work opened an avenue for researching the osmorespiratory compromise in sharks, I chose to investigate this phenomenon upon exposure to high temperature, when the metabolic demand for oxygen is increased. The Pacific spiny dogfish is a very important resource that is under great threat due to overfishing and global climate change (Taylor and Gallucci, 2009), in addition to its ecological characteristics of being long-lived, and very slow to mature to reproductive age (Dulvy et al., 2014). When dogfish were exposed to temperatures above 15ºC, urea fluxes increased sharply, indicating a disruption in the nitrogen conservation mechanisms at the gill membranes. Urea comprises a large component of the nitrogen budget of sharks, both for protein growth and for its osmoregulatory strategy. Interestingly, in contrast to hagfish exposed to high temperature, experimental hyperoxia was not protective but exacerbated gill permeability in sharks at high temperature, perhaps due to oxidative stress.

7.1.3 The water-to-gas ratio

As outlined in Chapter 1, Gonzalez and McDonald (1992) measured oxygen consumption rate (ṀO₂) and efflux of Na⁺ in the rainbow trout, and estimated the amount of ions lost to the water per unit of oxygen consumed. This practical measure of the effects of the osmorespiratory compromise was called the ion to gas ratio (IGR). Due to the practical and technical reasons outlined above, I was not able to perform measurements of ion fluxes in the hagfish and the dogfish. However, using tritiated water (³H₂O), I measured the diffusive water flux rates in the three model species investigated in this thesis, when exposed to hypoxia and/or increased temperature. The use of radiolabelled water (tritiated water) in order to measure diffusive water fluxes originated many years ago (Potts and Evans, 1967; Evans, 1969; Loretz, 1979), and the hypothesis that diffusive water fluxes at the gills occurred through the transcellular route (Isaia, 1984) was formulated years before the discovery of aquaporins. Aiming to replicate the concept developed by Gonzalez and
McDonald (1992) in order to compare multiple species and experimental treatments used in this thesis, I calculated the water to gas ratio (WGR) from measurements of $\dot{M}O_2$ and diffusive water flux rates. These values for species I investigated in this thesis were compared to values calculated for other species obtained from the literature. Figures 7.2, 7.3 and 7.4 show the comparative plots of the three species used in this thesis and the effects of salinity acclimation, hypoxia, and temperature exposure, as well as comparative data from the literature for the two model teleosts (rainbow trout and Amazonian oscar) discussed previously. Temperature exerts great effects on the metabolism of ectotherm animals, and its general effects on gill function and fish physiology have been reviewed in the Introduction (Chapter 1). Therefore, I opted to separate the results of the WGR into three different figures, one regarding effects of salinity only (Fig. 7.2), a second comparing the effects of hypoxia exposure (Fig. 7.3), and a third regarding the effects of temperature (Fig. 7.4).

When making comparisons among the killifish, hagfish and dogfish, which are diverse in terms of phylogeny, physiology and ecology, a few cautions should be noted. Sharks are long-lived, usually large animals, with low mass-specific $\dot{M}O_2$, while small, short-lived killifish have comparatively much higher mass-specific $\dot{M}O_2$s. The hagfish of intermediate size and indeterminate age arguably possesses one of the lowest mass-specific $\dot{M}O_2$s (Munz and Morris, 1965; Forster, 1990) yet highest water exchange rates (Rudy and Wagner, 1970; Glover et al., 2017; Chapters 4 and 5) of any vertebrate. Both $\dot{M}O_2$ (Clarke and Johnston, 1999) and diffusive water flux rate (Potts and Evans, 1967; Evans, 1969; Onukwufor and Wood, 2018) are known to scale allometrically with body mass within species, though the scaling coefficients may differ. Nevertheless, both processes are diffusion-mediated, so the ratio of the two rates should remain informative. Finally, hagfish are known to use the skin for several physiological functions that include ammonia excretion (Clifford et al., 2017; Wilkie et al., 2017), acid-base regulation (Clifford et al., 2014; Clifford et al., 2018) and to a small extent oxygen uptake (Clifford et al., 2016b). However, to my knowledge, the contribution of the skin to the high water permeability has never been investigated, so hagfish WGR values should be interpreted with the caveat of a possible involvement of the skin.
Comparisons of the WGR values among species and acclimation salinities, under control normoxic conditions reveal some interesting differences (Fig. 7.2). Firstly, in fresh water, the WGRs in the two “model” species (trout and oscar) with very different strategies of osmorespiratory compromise did not differ much under these control conditions – i.e. the resting WGR is not predictive of the response under hypoxia. Therefore, not surprisingly, the WGR of resting killifish in fresh water was also similar to the values in resting trout and oscar. Overall, marine teleosts are known to have lower gill permeability to water than freshwater teleosts (Evans, 1969), and when euryhaline fishes are transferred to sea water, water permeability is reduced (Potts and Fleming, 1970). Recent studies suggest that these differences relate to lower expression of aquaporins in marine teleosts (Cutler et al., 2007; Madsen et al., 2015; Breves et al., 2016). Therefore, not surprisingly, under control conditions, the diffusive water flux rate of killifish acclimated to 0 ppt was higher than those of fish acclimated to 11 and 35 ppt, and since \( \dot{MO}_2 \) was only slightly higher in seawater (at 35 ppt), the WGR, calculated from the data of Chapters 2 and 3, was higher in freshwater-acclimated killifish than in seawater-acclimated killifish (Fig. 7.2). Jung et al. (2012) have found that upon transfer from fresh to sea water, AQP3 mRNA levels in the gills of killifish decreases dramatically, but the total gill aquaporin protein abundance does not change significantly. These results could add to the explanation of the decrease in water permeability in seawater-acclimated killifish. In relation to marine teleosts, marine elasmobranchs have been reported to exhibit much higher water permeability (Payan and Maetz, 1971; Carrier and Evans, 1972; Haywood, 1975), and this was confirmed by my findings in Chapter 6. Thus the WGR in the dogfish under control, normoxic conditions at 35 ppt was higher than in the killifish at 35 ppt. The very high diffusive water flux rates of Pacific hagfish at 35 ppt, measured in Chapters 4 and 5, are in accordance with the recent work by Glover et al. (2017). When divided by the simultaneous measurements of very low \( \dot{MO}_2 \) of these animals, this resulted in an extremely high WGR in hagfish under control normoxic conditions, indeed the highest seen in any species under any salinity. As outlined in Chapters 4 and 5, it is hypothesized that the high water permeability is related to the osmoconforming strategy of hagfish.
As discussed previously, the concept of the “typical” response to the osmorespiratory compromise has been developed from studies with the rainbow trout (see Introduction). Similar to the ion-to-gas ratio (Gonzalez and McDonald, 1992), the WGR shows a marked increase in water permeability relative to O₂ consumed when trout are exposed to hypoxia (Fig. 7.3). Interestingly, the oscar which exhibits the opposite osmorespiratory compromise, does not show a decrease in the WGR during hypoxia, but rather a modest increase – i.e. water flux rate is not reduced to quite the same extent as Ō₂. Gonzalez and McDonald (1992) have attributed in increase in IGR to increases in intralamellar pressure, which was thought to occur due to increases in cardiac output. In turn, this was thought to disrupt the regulation of paracellular junctions in the gill epithelium. It is not clear whether this same idea applies to the WGR, since modern theory holds that water transport occurs mainly via the transcellular route, through aquaporins.

In Chapters 2 and 3, I have found similarities between the Amazonian oscar and the freshwater-acclimated killifish in their responses to hypoxia. Therefore, it was not expected that their WGR responses in hypoxia would differ. At 0 ppt, the lack of change in the WGR of killifish in hypoxia reflected equal decreases in water permeability and Ō₂, a slightly more effective response than in the oscar where the WGR increased moderately during hypoxia. Resting Ō₂ in the Amazonian oscar is relatively low (Sloman, 2006; Wood et al., 2009) when compared to the killifish (Borowiec et al., 2015; McBryan et al., 2016; Chapter 2). Therefore, having the ability to maintain WGR in hypoxia does not seem to be related with having a low resting Ō₂. At 11 ppt and 35 ppt, the WGRs of killifish increased during hypoxia exposure, because water permeability remained unchanged (Chapter 3) while Ō₂ fell (Chapter 2). Studies that investigate aquaporin expression and abundance in killifish gills upon hypoxia exposure are still lacking and would help to clarify some of the differences seen here. Despite the greater absolute magnitude of the hagfish WGR, the relative increases seen in hypoxia are comparable to the ones seen in both seawater-acclimated killifish, and the oscar. When exposed to hypoxia, hagfish decreases its Ō₂ to a greater extent than it decreases its water flux rate.

A common denominator between the oscar, killifish, and hagfish is the likelihood of hypoxia exposure in their natural environment, and accompanying this, their outstanding
capacity to withstand hypoxia. Recently, Mandic and Regan (2018) outlined that the severity and duration of the hypoxia exposure in the environment can shape a species’ response to hypoxia. Indeed, the study of Robertson et al. (2015a) showed that environmental conditions correlate more strongly than does phylogeny with the osmorespiratory compromise exhibited in several species of temperate and tropical teleost. In my thesis, although all species included in this comparison can frequently be exposed to hypoxia, some particularities exist. Based on the WGR it is not possible to establish a relationship between capacity to tolerate hypoxia, and that of decreasing water permeability during hypoxia.

Despite some individual differences, all three species shown in Figure 7.4 are considered temperate, or species that inhabit cold-water environments. Surprisingly, there is no change in WGR between 13 (control) and 18°C-exposed trout. This result reflects the fact that \( \dot{M}O_2 \) and water permeability both increase upon acute exposure to 18°C with similar \( Q_{10} \). In Chapter 6 I found that the increases in water permeability at the gills of the dogfish paralleled the increases in \( \dot{M}O_2 \), however, a slightly higher \( Q_{10} \) was seen for water fluxes, explaining the increases in WGR (Fig. 7.4). In hagfish, the temperature-induced increases in water flux rates resulted in a \( Q_{10} \) that was 30% higher than the \( Q_{10} \) for \( \dot{M}O_2 \), and thus an increase in the WGR. Dogfish and hagfish are known to inhabit environments that are thermally stable, while salmonids might be more frequently exposed to sharp changes in temperature, such as when they migrate through the thermocline to feed (Brett, 1971). The ability of the trout to control the WGR more effectively could be a reflection of the more variable environment they inhabit. In ectotherms, temperature-induced perturbations in membrane organization can pose a serious challenge to the ability to maintain physiological functions, as changes in membrane fluidity can affect the activity of membrane-embedded proteins and enzymes (Hazel, 1995). It is possible that temperature-induced changes in the WGR could be related to a disruption in cell membrane organization, that in turn would increase gill permeability for water and possibly, osmolytes such as ammonia and urea (Chapters 4 and 6).
7.2 Future directions

The findings of this thesis served to further advance the physiological understanding of the osmorespiratory compromise triggered by several environmental factors, in three distinct fish species that differ in phylogeny and physiological characteristics. However, as in all research, the results provided here have opened several new avenues for future research.

One of the overarching goals of this thesis was to improve our understanding of the osmorespiratory compromise in seawater conditions. Over time, one of the biggest constraints in measuring ion fluxes in seawater species has been the cost of radiolabelled materials (Wood and Laurent, 2003). I was able to overcome some of those constraints, by using a model species which is small in size, thereby drastically reducing the costs associated with using radioisotopes. But even though measuring ion fluxes in sea water was possible, the resolution of the results is not as fine as that of experiments conducted in fresh water. Therefore, there is a pressing need for the development of novel tools that can be used for the investigation of the osmorespiratory compromise in marine fish.

More specifically, methodological tools are needed that can distinguish with precision between transcellular versus paracellular routes of transport. In Chapter 6, I have shown that the fluxes of O$_2$, ammonia, urea and tritiated water all increased with temperature. This suggests that there may be also be different transcellular permeabilities to molecules with different transport proteins (e.g. Rh proteins, aquaporins and urea transporters). $[^3]$H]PEG-4000 has been used as a marker of paracellular transport for many years now (Robertson and Wood, 2014), however, the need for a broader range of molecules that will give us better insight into the transcellular transport route is increasing.

Another interesting area that lacks research is the regulation of tight junction permeability. For example, are changes in physical factors such as oxygen tension, internal blood pressure, and temperature more or less important than changes in hormonal signals in this respect? It has become clear that the degree of leakiness of the tight junctions can be modified according to osmoregulatory needs, such as exposure to hypersaline environments (Cozzi et al., 2015). Species-specific differences clearly exist at the gills, such as the modification of tight junctions, which reduce the extent to which an elevation in metabolic
rate could impair osmoregulation. The roles of prolactin and cortisol, hormones known to alter tight junction function and morphology (Chasiotis et al., 2010; Chasiotis et al., 2012) also remain relatively unknown in the context of the osmorespiratory compromise. Additionally, it may be an oversimplification to just talk about one kind of paracellular permeability, as there may be different permeabilities to molecules of different size and different charge due to the permselectivity of the tight junctions.

Additionally, in the future, a more extensive examination of the interactions between ionocytes and pavement cells in the gills of fish in normoxia and hypoxia, using detailed microscopy techniques such as scanning electron and transmission electron microscopy (Iftikar et al., 2010; Matey et al., 2011), could shed some light into the mechanisms of reduction in gill permeability to ions and water while maintaining permeability to O₂ high.

My thesis has shown that hagfish are quite susceptible to changes in temperature and hypoxia. Giving that the skin of hagfish is known to adopt several physiological roles, teasing apart the contributions of the skin and gills to the high water permeability displayed by these animals would be helpful in providing further insights into the osmorespiratory compromise in this species. In Chapters 4 and 5 I was able to show that, contrary to what was previously believed, hagfish do regulate plasma Na⁺ and Cl⁻ concentrations, at levels that differ from those in the surrounding water, even across sharp changes in temperature and salinity, while conforming total osmolality. The identification of the mechanisms involved in the regulation of plasma divalent cations (Mg²⁺ and Ca²⁺) is long overdue, and there is now a need to add to this by examining the mechanisms by which hagfish also regulate plasma Na⁺ and Cl⁻. With the results provided in my thesis, another fruitful avenue of research has opened up, in a species that has great evolutionary and physiological significance.

In Chapters 4 and 6 I hypothesized that by providing excess O₂ supply in the water, fish would not have to face the complex physiological and morphological alterations of the gills usually needed to increase O₂ uptake, and subsequently, would not display an osmorespiratory compromise. This hypothesis only held true for a decrease in water flux rates in the hagfish at high temperature. The dogfish in contrast, showed exacerbated osmolyte permeability when exposed to hyperoxia, especially at high temperature. One of the possible explanations for those results is that hyperoxia can cause oxidative stress (Lushchak
and Bagnyukova, 2006), which may damage lipid membranes, leading to increases in membrane fluidity and consequently a possible leak of osmolytes (Lushchak, 2011). Studies investigating the hyperoxia-induced oxidative stress response and damage in marine fishes are still lacking. In shallow waters (i.e. tide pools) daily variations in photosynthesis and respiration rates can cause water PO$_2$ to increase sharply (Mandic et al., 2009), exposing stranded fish to hyperoxia. In open waters, phytoplankton blooms can also be a source of increased O$_2$ in the water. Therefore this is an important research area waiting to be explored.

7.3 Concluding remarks

My thesis investigated how fish deal with the trade-offs associated with the optimization of gill respiratory and osmoregulatory functions. I have used three species that differ in physiological traits, phylogeny and ecology. To address the questions asked in the research chapters, I have exposed these fishes to variations in environmental parameters that are likely to be encountered in their natural environments, such as hypoxia, temperature and salinity. In the Atlantic killifish, I provided strong evidence that there is a compromise between respiratory capacity and ionoregulatory functions, where acclimation to fresh water resulted in impaired tolerance to hypoxia. My thesis has shown that elasmobranchs are susceptible to sharp changes in temperature, and urea loss can increase drastically, possibly due to a failure of an important urea retention mechanisms at the gills, and likely leading to an impairment of the whole animal nitrogen budget. Before the results obtained in my thesis, the osmorespiratory compromise had never been investigated in an osmo and ioneconforming species. I have shown that when exposed to both hypoxia and changes in temperature, Pacific hagfish shows disturbances in gill permeability to water and osmolytes, opening up a fruitful avenue for further research. In summary, the three species investigated in this thesis exhibit a different pattern of the osmorespirical compromise. In a scenario where human influences have the potential to change environmental variables, and increasing the likelihood of extreme events, understanding how environmental variables affect animal physiology is of extreme importance.
Effective gill permeability

Oxygen

Ions and water

Normoxia

Hypoxia/ Temperature

Normoxia/Recovery

Spiny dogfish

Rainbow trout

Amazonian oscar

Atlantic killifish

Pacific hagfish

Rainbow trout

Amazonian oscar

Spiny dogfish

Atlantic killifish

Pacific hagfish
Figure 7.1 Conceptual diagram displaying effective gill permeability to O$_2$ (top line) versus the two very different accompanying changes (two lower lines) in effective gill permeability to ions and water seen in two species with very different strategies for the osmorespiratory compromise. The rainbow trout exhibits increased gill permeability to ions and water, while the Amazonian oscar shows a regulated reduction in gill permeability. Contributions of my thesis include the Pacific hagfish and the Atlantic killifish showing responses similar to the oscar, while the responses of the spiny dogfish to increased temperature are similar to the rainbow trout model.
Figure 7.2 Comparative water to gas ratio (WGR) in the rainbow trout (*Oncorhynchus mykiss*), Amazonian oscar (*Astronotus ocellatus*), Atlantic killifish (*Fundulus heteroclitus*), Pacific spiny dogfish (*Squalus acanthias suckleyi*) and Pacific hagfish (*Eptatretus stoutii*). All bars are control normoxic values in resting animals. *F. heteroclitus* have been acclimated to three salinities, which are shown beside the bars. Data shown for the trout have been extracted from the literature and added to the figure for comparison [\( \text{\( \dot{M} \)} \text{O}_2 \): Marvin and Heath (1968) and \( J_{\text{H}_2\text{O}} \): Onukwufor and Wood (2018)]. Data shown for the oscar have been extracted from the literature and added to the figure for comparison [\( \text{\( \dot{M} \)} \text{O}_2 \): Sloman et al. (2012) and \( J_{\text{H}_2\text{O}} \): Wood et al. (2009)]. The water flux rate results for the killifish (Chapter 3) and dogfish (Chapter 6) were expressed as % of body water/h and have been converted to mL/kg/h assuming that the fish’s body is made up of 80% water. Then, using the molecular weight of water (MW 18.01), the diffusive water flux data (mL/kg/h) for all five species was converted to \( \mu \)mol \( \text{H}_2\text{O}/\text{g/h} \). Therefore, the division between the diffusive water flux (\( \mu \)mol \( \text{H}_2\text{O}/\text{g/h} \)) by the \( \text{\( \dot{M} \)} \text{O}_2 \) (\( \mu \)mol \( \text{O}_2/\text{g/h} \)) has no units.
**Figure 7.3** Comparative water to gas ratio (WGR) in the rainbow trout (*Oncorhynchus mykiss*), Amazonian oscar (*Astronotus ocellatus*), Atlantic killifish (*Fundulus heteroclitus*), and Pacific hagfish (*Eptatretus stoutii*). Normoxia bars are shown in gray, while hypoxia exposed are shown in black. *F. heteroclitus* have been acclimated to three salinities, which are shown beside the bars. Data shown for the trout have been extracted from the literature and added to the figure for comparison [\( \dot{M}O_2 \): Marvin and Heath (1968) and \( J_{H2O} \): Onukwufor and Wood (2018)]. Data shown for the oscar have been extracted from the literature and added to the figure for comparison [\( \dot{M}O_2 \): Sloman et al. (2006) and \( J_{H2O} \): Wood et al. (2009)]. The water flux rate results for the killifish (Chapter 3) were expressed as % of body water/h and have been converted to mL/kg/h assuming that the fish’s body is made up of 80% water. Then, using the molecular weight of water (MW 18.01), the diffusive water flux data (mL/kg/h) for all four species was converted to \( \mu \text{mol} \ H_2O/\text{g/h} \). Therefore, the division between the diffusive water flux (\( \mu \text{mol} \ H_2O/\text{g/h} \)) by the \( \dot{M}O_2 \) (\( \mu \text{mol} \ O_2/\text{g/h} \)) has no units.
Figure 7.4 Comparative water to gas ratio (WGR) in the rainbow trout (*Oncorhynchus mykiss*), Pacific spiny dogfish (*Squalus acanthias suckleyi*) and Pacific hagfish (*Eptatretus stoutii*). Control temperature bars are shown in gray, while acute warming temperature exposures are shown in black. Data shown for the trout have been provided by John Onukwufor and Chris Wood (personal communication). The water flux rate results for the dogfish (Chapter 6) were expressed as % of body water/h and have been converted to mL/kg/h assuming that the fish’s body is made up of 80% water. Then, using the molecular weight of water (MW 18.01), the diffusive water flux data (mL/kg/h) for all three species was converted to µmol H₂O/g/h. Therefore, the division between the diffusive water flux (µmol H₂O/g/h) by the MO₂ (µmol O₂/g/h) has no units.
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