

**Respiratory Responses to Hypoxia in Winter Flounder (*Pseudopleuronectes americanus*),
and Behavioral Correlation with Hemoglobin-Oxygen Affinity**

by

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Abstract

Climate change lower dissolved oxygen (hypoxic) by increasing the temperature; acidify the oceans by increasing dissolved carbon dioxide. Winter flounder (*Pseudopleuronectes americanus*) are native bottom-dwelling fish in the Long Island Sound (LIS). Parts of LIS are affected by sluggish circulation, municipal wastes, anthropogenic inputs; LIS may become hypoxic during hot summer months. Hemoglobin (Hb) is a protein found in the red blood cell, and responsible for oxygen transport from gills to tissue. In fish, oxygen is taken up into red blood cells at the gills and released to tissues due to the decreased pH and increased temperature. Little is known about the respiratory and behavioral responses of adult winter flounder to hypoxic water, nor the consequence or distribution of winter flounder. Winter flounder was exposed to increasing hypoxia from 8 mg O₂/L (150 torr pO₂) (normoxia) to 1 mg O₂/L. The goal of this study was to correlate the respiratory and behavioral responses of winter flounder to their hemoglobin oxygen-equilibrium curve (OEC).

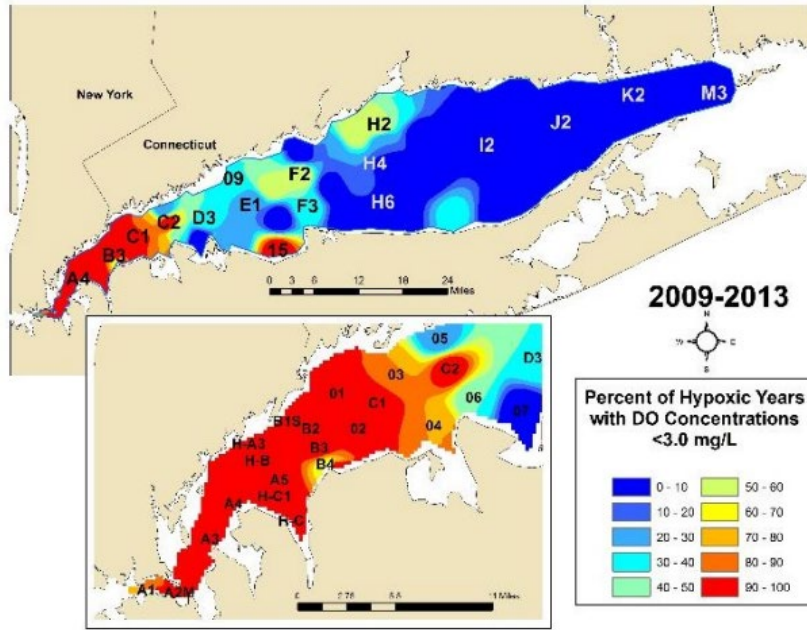
Introduction

Long Island Sound (LIS)

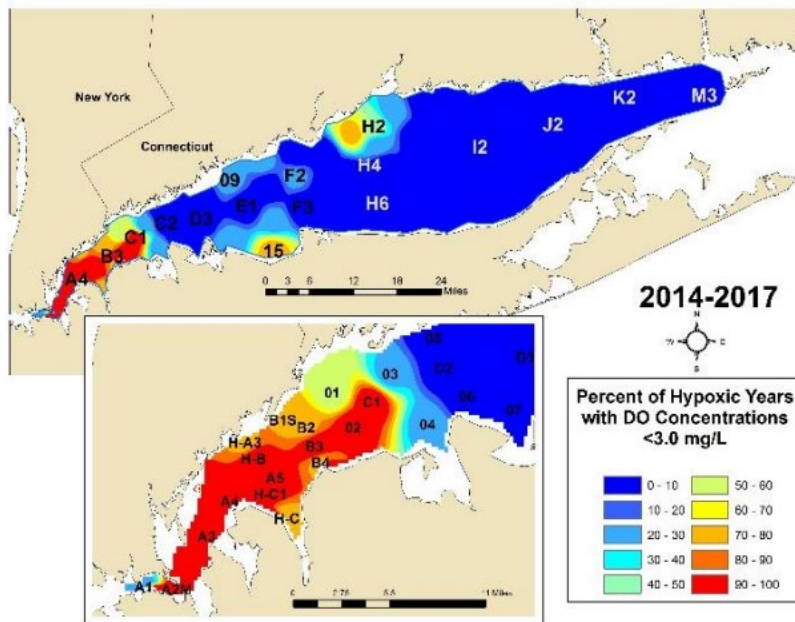
Long Island Sound (LIS) is an estuary that is bordered by the eastern shores of Bronx County, New York City, southern Westchester County, Connecticut and the North Shore of Long Island. Its watershed area extends to Massachusetts, Vermont, New Hampshire, Maine and Quebec. LIS is home to a diverse group of flora and fauna. With urbanization, organisms in LIS faces environmental stresses such as eutrophication, hypoxia, ocean acidification and climate change (Latimer *et al.*, 2014).

Hypoxia is when the concentration of dissolved oxygen (DO) is less than 2 to 3 milligrams of oxygen per liter of bottom water (Latimer *et al.*, 2014). Many fish species display physiological stress below 2 - 3 mg O₂/ L (Ritter & Montagna, 1999). Seasonal hypoxia during summer in the bottom waters is common in LIS. It usually starts in the western narrows of Long Island Sound (WLIS) near Throgs Neck Bridge and spreads eastward until the beginning of Fall (Latimer *et al.*, 2014; Parker and O'Reilly, 1991).

Seasonal weather patterns, especially from June to September, worsen the eutrophication and increase the impact of seasonal hypoxia. This effect is particularly prominent in WLIS (Latimer *et al.*, 2014). With climate change, increasing water temperatures may exacerbate hypoxic condition in LIS. In Western Long Island Sound (WLIS), locations with DO above 3 mg/ L had more diversity and quantity of fish; there were decreased abundance of Bluefish, Butterfish and Winter Flounder at location with DO less than 3 mg/ L (hypoxic).



(a)



(b)

Figure 1. Percent of hypoxic years when dissolved oxygen concentrations is lower than 3.0 mg per liter in Long Island Sound bottom water. (a) From 2009 to 2013. (b) From 2014 to 2017.

Images from (CT DEEP and IEC. 2017).

Hypoxia in fish

Winter flounder (*Pseudopleuronectes americanus*) is a demersal flatfish native along the Atlantic coast from Labrador, Canada to Georgia, United States. They are most abundant from Nova Scotia to New Jersey (Fairchild et al., 2008); their habitats in Long Island Sound may become hypoxic during hot summer days with sluggish circulation, anthropogenic phosphorus and nitrogen wastes and fertilizer runoffs.

Many coastal benthic species evolved to survive mild hypoxia for 1 to 2 weeks. Fishes have the lowest tolerance to hypoxia, followed by crustaceans, polychaetes and bivalves (Diaz and Rosenberg, 1995; Gray *et al.*, 2002; McEnroe, 1991). Fish usually escape from hypoxic water. However, it is common for many coastal benthic fish, such as Winter flounder, to experience periodic hypoxic conditions, especially in intertidal zones (Lopez *et al.*, 2014).

Over 95% of oxygen consumption by fish is for oxidative phosphorylation of ATP in mitochondria. To maintain homeostasis and metabolic functions, fish develop various behavioral, physiological, biochemical and genetic responses to cope with hypoxia (Richards, 2009). Biochemical responses to hypoxia include increasing hemoglobin (Hb) concentration, concentrations of some Hb components and decreasing intraerythrocytic nucleoside triphosphate (NTP) concentration to increase Hb affinity for oxygen (Brix *et al.*, 2004; De Souza and Bonilla-Rodriguez, 2007). Behavioral responses to hypoxia include escaping hypoxic area, modification of activity level, air-breathing and aquatic surface respiration (ASR). The two most common behavioral responses of hypoxia in osteichthyes are air-breathing and ASR. These two behaviors are not observed in Chondrichthyes or Agnathans (Chapman and McKenzie, 2009). Some Teleostei also modify the operculum ventilation patterns under hypoxia (Johnsson *et al.*, 2001. McEnroe and Krozlowitz, 1996).

Aquatic surface respiration (ASR) refers to the behavior when fish swim to the surface and ventilate the top water layer, where the DO concentration is higher. Some fish can hold air bubbles during ASR to increase oxygen level in the buccal and opercular cavities, as well as buoyancy (Gee and Gee, 1991; McEnroe and Krozlowitz, 1996). ASR is positively correlated to higher temperature in some species, and the triggering threshold of ASR varies with season (Love and Rees, 2002).

Decreased oxygen concentration can lower net metabolic rates. During hypoxia, some species decrease metabolic rate and employ anaerobic pathways (Burnett, 1997); some species do not feed when the metabolic rate is low. Hypoxia is associated with decreased growth rate, physical fitness, reproduction, endocrine system, ability to withstand disease and environmental stress. Thus, hypoxia increases the mortality rate (Eby *et al.*, 2005; Howell and Simpson, 1994; Lopez *et al.*, 2014; Pihl *et al.*, 1991; Roussel, 2007; Tyler & Targett, 2007; McEnroe, 1992; Wu, 2002).

Hemoglobin (Hb)

540 million years ago, multicellular Metazoa began to get bigger during the early Cambrian. Oxygen supply by simple diffusion is insufficient to meet the metabolic needs of large and active animals. Virtually all multicellular animals evolved respiratory proteins to enhance oxygen uptake and transport. Three types of respiratory proteins have been discovered: hemerythrin, hemocyanin (Hc) and hemoglobin (Hb). Hemerythrins are found in a few invertebrate phyla; copper-containing hemocyanins (Hc) are blue respiratory proteins found in the hemolymph of many arthropods and mollusks; hemoglobins (Hb) are the most common respiratory proteins (Burmester and Hankeln, 2007; Markl, 2013).

Many invertebrates, such as annelids, mollusks, echinoderms and flatworms use either Hc or Hb as an oxygen carrier. In vertebrates, Hb are produced in either the hematopoietic part of the kidney in fishes or in bone marrow of terrestrial animals by erythrocytes (also known as the red blood cell or RBC). Each red blood cell contains around 280 million Hb, which transports oxygen from the lungs or gills to body tissues, where oxygen is released for aerobic respiration (Hardison, 1999). Hb is an ancient protein. All extant vertebrates, except the Antarctic icefishes (Family Channichthyidae), have Hb inside red blood cells (Sidell and O'Brien, 2006). Since the close relatives of the icefish all produce hemoglobin, it was secondarily lost in this group which can survive without Hb because of their low oxygen demand at their supercooled state.

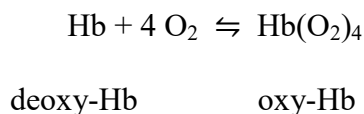
The facilitation of oxygen transport by Hb represents an important breakthrough in physiological evolution, because it vastly increases the amount of O₂ transported per unit of fluid. Each gram of Hb can transport 1.34 ml of oxygen, while 100 ml of plasma only carries 0.3 ml oxygen (De Villota *et al.*, 1981; Pittman, 2011). Hemoglobin evolved more than once in vertebrates. Hoffmann *et al.* (2010) suggested that Hb evolved separately from distinct lineages of ancient protein in gnathostomes (jawed vertebrates) and cyclostomes (jawless fish, such as hagfishes and lampreys). Hemoglobins in both cyclostomes and gnathostomes are multimeric. Such quaternary structures lay the foundation for oxygen binding cooperativity and allosteric regulation. In living cyclostomes, the oxygen binding cooperativity of Hb comes from the polymerization and dissociation of subunits. When oxygenated, Hb multimers dissociate into monomers; when deoxygenated, monomers polymerize into dimer or tetramer (Fago *et al.*, 2001).

Physiological importance of Hemoglobin

Hemoglobin-Oxygen binding is reversible and dependent on the partial pressure of CO₂, pH, and temperature. Hemoglobin has higher O₂ affinity at lower blood temperature, lower P_{CO2}, higher pH and vice versa. This reversible binding facilitates O₂ unloading in acidic tissue capillaries; promotes O₂ loading at the cooler respiratory surfaces.

In most living gnathostomes, the oxygen binding cooperativity of Hb comes from the changes in quaternary structure. Most Hb in gnathostomes is a tetramer composed of four polypeptide chains: two α chains and two β chains. The four subunits are positioned roughly at the corners of a tetrahedron, forming a cavity at the center of the molecule. Each polypeptide chain is associated with a heme prosthetic group. Vertebrate hemoglobin is found in erythrocytes, and in most fish, erythrocytes are oval and nucleated. Although some fish have asymmetric hemoglobin with at least three different globin chains in a single hemoglobin, most fish have symmetrical hemoglobin with two pairs of identical globin chains (Dafré and Reischl, 1997).

Each heme group has a central iron atom, which can bind to an oxygen molecule. Hence, each Hb molecule can bind to a maximum of four oxygen. The chemical interaction between oxygen and the iron in the heme group gives Hb and blood its red color. Based on the partial pressure of O₂, Hb picks up O₂ from the respiratory surfaces and releases it in tissue capillaries, and this reaction can be represented as follows (Perutz, 1978):



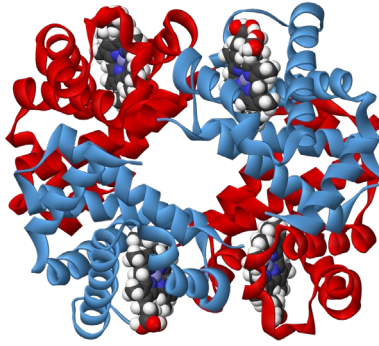


Figure 2. Structure of a hemoglobin. α subunits are in red and β subunits are in blue. The iron containing heme groups are in black. (Haemoglobin-3D-ribbons by Benjah-bmm27. CC-BY 2.0.)

Cooperativity

Due to the positive cooperativity of the four O_2 binding sites in hemoglobin, the oxygen equilibrium curve (OEC) is sigmoid shape. When one or two oxygen is bound to Hb, conformational changes will occur, and the oxygen affinity of the remaining sites will increase. A common way to compare O_2 affinity is P_{50} , which is the oxygen partial pressure (pO_2) at which 50% of Hb is saturated. P_{50} and oxygen affinity are inversely related. As oxygen affinity increases, P_{50} decreases (Hill *et al.*, 2012).

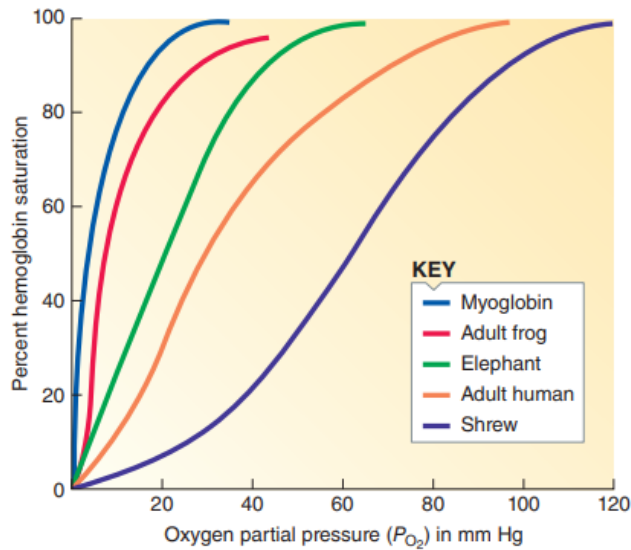


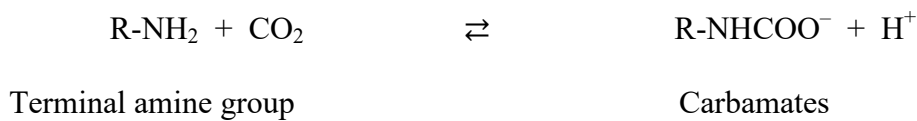
Figure 3. Oxygen–hemoglobin dissociation (saturation) curve. The curve of myoglobin is hyperbolic. With multiple subunits, the curve of hemoglobin is sigmoidal. The percentage of hemoglobin saturation on the ordinate is depends on the partial pressure of oxygen on the abscissa (Sherwood *et al.*, 2012).

Carbon Dioxide Transport and chloride shift

There are three ways of carbon dioxide transport in blood: dissolved in the plasma, transported as bicarbonate, or bound to Hb as carbaminohemoglobin. Water and carbon dioxide diffuse freely into red blood cell (RBC), where carbonic anhydrase (CA) catalyzes the reaction of water and carbon dioxide to form carbonic acid. Carbonic acid (H₂CO₃) further dissociates into bicarbonate ions (HCO₃⁻) and hydrogen ion (H⁺).



Carbon dioxide acts as an acid, as it reacts to form protons, and bicarbonate ions are the major form of carbon dioxide in blood. As bicarbonate ions in RBC increase and move across the RBC membrane to plasma, charge balance is maintained by anion exchange. Band 3 anion transport protein exchanges bicarbonate for chloride ions. This is also known as the chloride shift. Other than forming bicarbonate, CO₂ can bind to N-terminal amino groups on hemoglobin forming carbamino compound.



However, the N-terminals of the alpha-chain of teleosts' hemoglobins are acetylated, blocking carbamates formation (Randall, 2011). While organophosphates bind to the N-terminals of the beta-chain of teleosts' hemoglobin, competing for carbamates formation. Teleosts are less likely to transport carbon dioxide in form of carbamates (Randall, 2011).

Haldane effect and Bohr Effect

The Haldane effect describes how the carbon dioxide equilibrium and the affinity of hemoglobin for hydrogen ions depend on Hb-O₂ saturation. High Hb-O₂ saturation facilitates CO₂ release, while low Hb-O₂ saturation facilitates CO₂ uptake by Hb (Brauner and Rummer, 2011). It can be expressed as follows:

$$\text{Haldane coefficient} = \Delta \text{H}^+$$

where ΔH^+ is the moles of hydrogen ions released per mole of oxygen bound to hemoglobin.

The Bohr effect describes how the oxygen affinity of hemoglobin depends on the partial pressure of carbon dioxide (P_{CO2}) and hydrogen ions concentration (pH) in blood. When pH

decreases or P_{CO_2} increases, Hb-O₂ affinity also decreases and the O₂-Hb dissociation curve (oxygen equilibrium curve, OEC) shifts to the right. There are two types of Bohr effects. The first is a fixed-acid Bohr effect, where proton concentration directly affects Hb. The second is a CO₂ Bohr effect, where increased in CO₂ partial pressure partly lowers the pH, and CO₂ also acts as an allosteric inhibitor of hemoglobin (Hill *et al.*, 2012). The Bohr Effect can be expressed as follows:

$$\text{Bohr coefficient } (\Phi) = - \Delta \log P_{50} / \Delta \text{pH}$$

where P_{50} is the partial pressure of oxygen (P_{O_2}) when 50% of the Hb are oxygenated.

The Haldane effect and the Bohr effect are like the opposite processes of each other. The Bohr effect describes how Hb-O₂ affinity is affected by pH (H^+); the Haldane effect describes how Hb- H^+ affinity is affected by Hb-O₂ saturation and P_{O_2} (Brauner and Rummer, 2011). Oxygenation at the gills promotes carbon dioxide removal by the Haldane effect; oxygenation of hemoglobin promotes hydrogen ion release from Hb. Hydrogen ion binds to bicarbonate to form carbon dioxide, which will be released into the environment. At the same time, oxygen uptake at the gills is promoted by the Bohr effect. This is because carbon dioxide removal and the corresponding decrease in H^+ lead to an increase in oxygen affinity of hemoglobin. At active tissues, the Bohr effect promotes oxygen delivery due to high P_{CO_2} and H^+ ; the Haldane effect promotes carbon dioxide removal from the tissues as low Hb-O₂ saturation promotes CO₂ uptake by Hb (Brauner and Rummer, 2011).

Root Effect

Many teleost fish also display Root effect, where increased in CO₂ partial pressure or proton concentration leads to decreased Hb affinity for O₂. Root effect is commonly observed in the swim bladder and the eyes (Hill *et al.*, 2012). Bohr effect shows the horizontal shift of OEC

around P_{50} due to changes in pH and Hb- O_2 affinity. Root effect shows the decrease in O_2 affinity of hemoglobin under low pH (Waser, 2011). Even at high O_2 concentration, Root effect prevents a full O_2 saturation of hemoglobin. In LIS, with seasonal hypoxia, temperature fluctuation in shallow intertidal zone, and climate changes, both Bohr Effect and Root Effect are expected. However, data are limited on these studies.

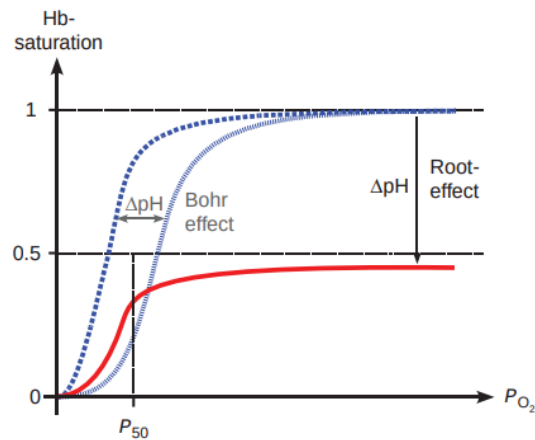


Figure 4. Oxygen binding curves with the Bohr effect and Root effect (Waser, 2011).

Increased temperature and lower Hb- O_2 affinity

During hot summer days with low water flows, the water temperature can increase significantly in intertidal zone at LIS. Like many ectotherms, fish are influenced by the temperature of the surrounding. As temperature increases, solubility of gases, such as oxygen, decreases in water. At the same time, as temperature rise, fish's aerobic metabolism typically increases two to three-fold for every $10^{\circ}C$ (Jensen *et al.*, 1993; Kaufman *et al.*, 2007). In order to maintain the O_2 transport at a higher water temperature, heart rate and cardiac output increases in most ectothermic fish. Interaction of O_2 and Hb is also exothermic. Thus, Hb- O_2 affinity decreases as water temperature rises and fish take up less oxygen from warm water (Jensen *et al.*, 1993).

Both increased temperature and acidity lower Hb-O₂ affinity and compromise oxygen transport, limiting aerobic metabolism. McEnroe and Krozlowitz (1996) found that juvenile winter flounder, when exposed to hypoxia at summertime temperatures, employed a sequence of behaviors, including heads-up, ASR and air-gulping.

Materials and Methods

A. Fish Collection and Care

Adult winter flounder (364 g) was purchased from Marine Biological Laboratory (MBL) in Woods Hole, Massachusetts, and transported to the laboratory at Purchase College in a cooler. The Winter flounder was held in a 20-gallon aquarium in the Animal Care facility for two days at salinity of 35 parts per thousand (ppt) and 10°C. Then, the fish was acclimated to 15°C by increasing 1°C per day with a chiller (Arctica Model DBI-050-D, JBJ Aquariums), and was allowed to acclimate for several days before experiments were taken place. The aquarium was fitted with charcoal Tetra Whisper Power Filters and aerated. Instant Ocean was used to make up artificial sea water of the same salinity and the water was renewed regularly.

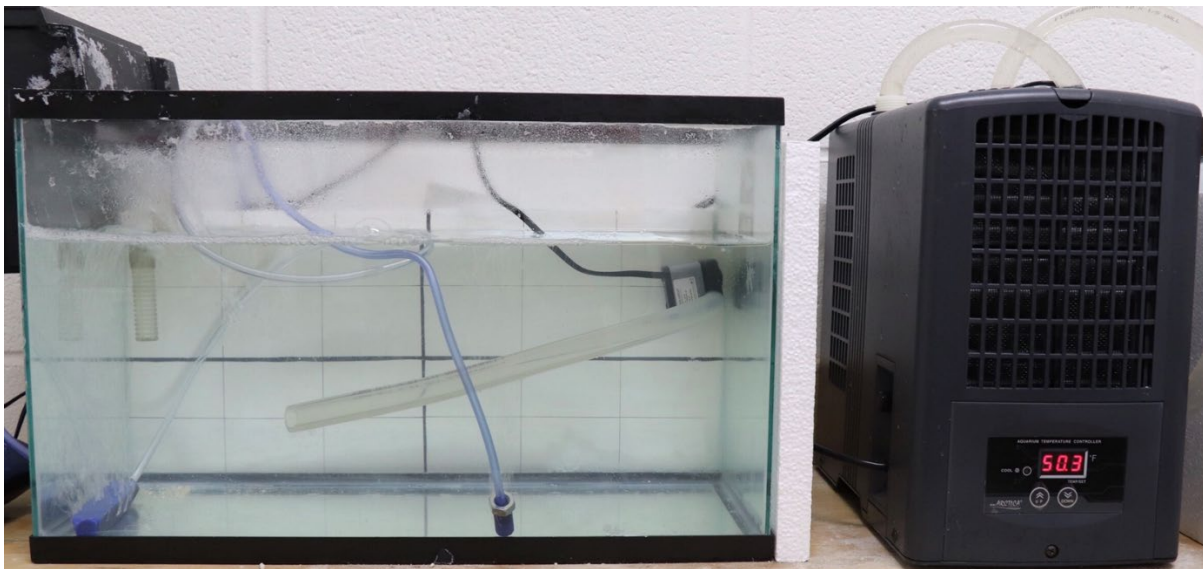


Figure 5. The experimental setup for winter flounder was prepared with Mini-Arctica Titanium Chiller, charcoal filter and air stone. Instant Ocean was used to prepare artificial sea water of 35 ppt salinity.

B. Respiratory Behaviors Experiment

Dissolved oxygen (DO) level were continuously monitored by FireStingO₂ fiberoptic oxygen meter with robust probe (OXROB10) and temperature probe. Data was logged using the FireSting software. The FireStingO₂ fiberoptic oxygen meter was calibrated with distilled water bubbled with air for 20 minutes.

Desired DO level in the tank was controlled by bubbling with N₂ for hypoxia, or air for normoxia. To decrease the DO, the water filter and air pumped were turned off; airstone connecting to a nitrogen gas tank was added; N₂ was bubbled into to the aquarium to displace O₂.

Respiratory responses to hypoxia were documented when dissolved oxygen concentration (DO) were decreased from normoxia (about 8 mg O₂/L) to 1 mg/L. Experiments were conducted at 15°C and 20°C, 35 ppt salinity, and water depth of about 25 cm. The top of the aquarium was covered with a polystyrene foam to minimize gas diffusion from air current.

Behavioral measurements were made at nomoxia, 5, 4, 3, 2 and 1 mg O₂/L. Nitrogen flow was stopped when the desired DO level was reached, and the fish was allowed to acclimate for 30 minutes before observations were videotaped by a SONY Handycam video camera. The types of behavior, and the DO level at which the behavior first occurred were monitored:

- a) Opercular ventilation rate was measured by averaging the number of opercular movement over several one-minute intervals.
- b) The types of ventilation (rapid shallow breath versus slow deep breath) and the DO level at which the behavior first occurred.
- c) Heads-up behavior.

- d) Horizontal movements and the DO level at which the behavior first occurred. A grid was placed behind the tank, dividing the aquarium into six vertical and four horizontal sections. Movements were measured by counting the frequency the fish crossed the vertical lines.
- e) Vertical movements, such as ASR, air-gulping, and jumping out of water, were measured using the grid. The DO level, at which the behavior first occurred, was also monitored.

C. Calculation

1. Calculation of water percent saturation with oxygen and partial pressure (PO₂) in torr.

Dissolved oxygen (DO) concentration for O₂ saturated water at different salinity DO_(salt) in milligrams per liter (mg/L) was calculated using:

$$DO_{(salt)} = DO - (k \times S)$$

where DO is the dissolved oxygen concentration for O₂-saturated distilled water, S is the salinity value in ppt, k is the salinity correction constant. k is equal to 0.05602 at 15°C; 0.04964 at 20°C

Tank oxygen was monitored as dissolved oxygen (DO) in milligrams oxygen per liter (mg/L). For comparison to P_{O₂} and Hemoglobin P₅₀, the mg/L was converted to millimeter of mercury (mmHg) or Torr. At the beginning of the experiment, when the oxygen probe was calibrated with O₂-saturated distilled water, the initial oxygen was recorded in both mg/L and torr. At 35 ppt, O₂-saturated water, the DO level was 8.20 mg/L and 157 torr at 15°C; 8.17 mg/L and 157.7 torr at 20°C. Therefore, the 50% saturation was $\frac{8.2}{2} = 4.1$ mg/L or $\frac{157}{2} = 78.5$ torr at 15°C and $\frac{157.7}{2} = 78.75$ torr at 20°C.

2. Calculations on Blood-oxygen Affinity

To compare the temperature effects on Hb-O₂ affinity, the apparent heats of oxygenation (ΔH) in kilocalorie per mole (kcal/mol) were calculated from the Van't Hoff Equation (Hayden *et al.*, 1975; Kaufman *et al.*, 2007; Mislan *et al.*, 2016):

$$\Delta H = 4.578 \times 10^{-3} \left(\frac{\Delta \log P50}{\Delta 1/T} \right)$$

where temperature (T) is in Kelvin.

To compare the effects of pH and CO₂ on Hb-O₂ affinity, the Bohr coefficients (Φ) were calculated using:

$$\Phi = \frac{\Delta \log P50}{\Delta pH}$$

Results

Escape responses were observed when the dissolved oxygen concentration (DO) was below normoxia. A series of respiratory and behavioral responses were observed: below 90 torr pO_2 , the opercular ventilation became rapid and shallow (Table 1). At 60 torr, the opercular ventilation became slow and deep. At 28 torr, the fish began to lift its head up. At 16 torr, the flounder swam to the surface, gulped air and jumped out of the aquarium.

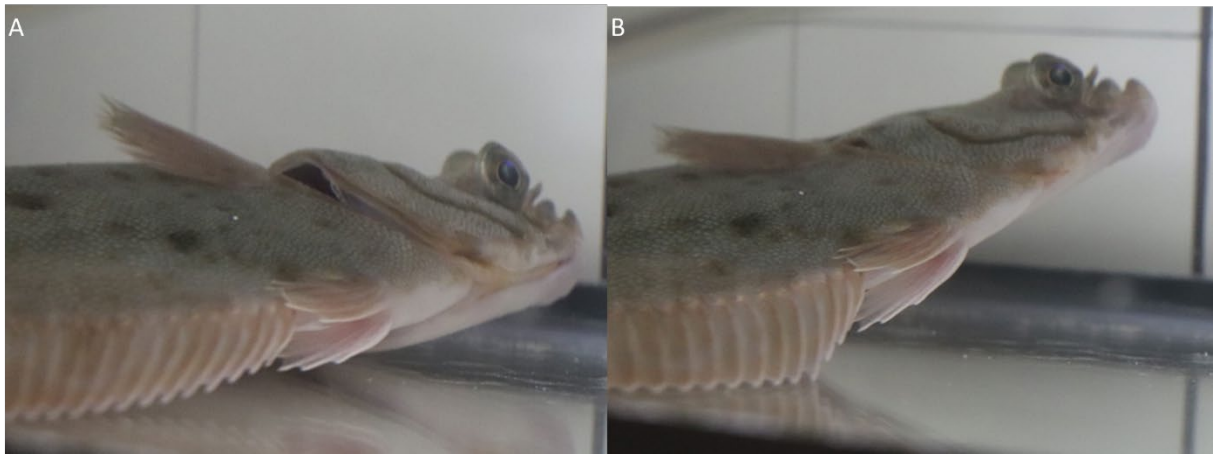


Figure 6. A) Normal body position of winter flounder at normoxic environment. B) Heads-up behavior of winter flounder in hypoxic environment. This allow better ventilation of the gills on the ventral side of the body.

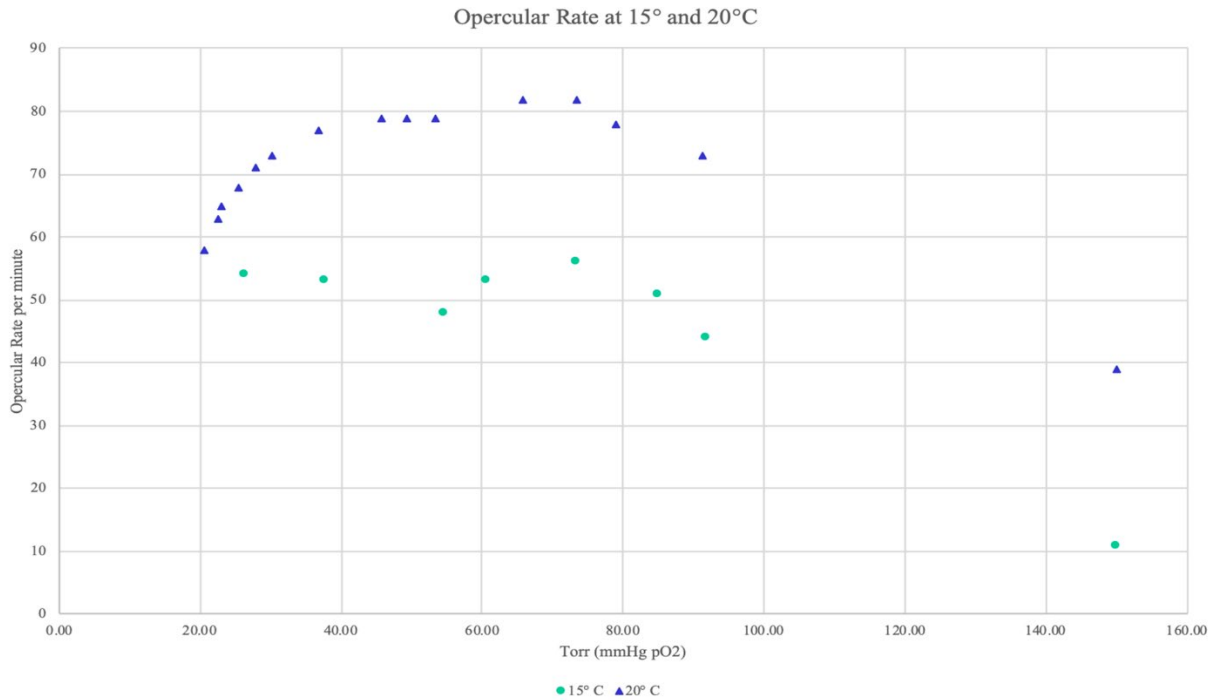


Figure 7. At 15°C, the opercular rate increased as oxygen concentration decreased from normoxia and leveled off below 74 torr PO₂. At 20°C, with decreasing DO from normoxia, the opercular rate first increased, leveled off at 74 torr PO₂, and declined below 40 torr PO₂.

Table 1: Oxygen levels at which respiratory behaviors were observed.

| | At 15°C | At 20°C |
|------------------------------|-----------------------------|-----------------------------|
| Lateral movement | 150 (normoxia) - 60.62 torr | 150 (normoxia) - 65.81 torr |
| Rapid shallow breath | 91.86 – 60.62 torr | 91.37 – 53.39 torr |
| Slow deep breath | < 60.62 torr | < 53.39 torr |
| Heads up begin at | 27.94 torr | 45.71 torr |
| ASR and air-gulping begin at | 16.17 torr | 27.71 torr |
| Jumping out of water | < 16.17 torr | < 27.71 torr |

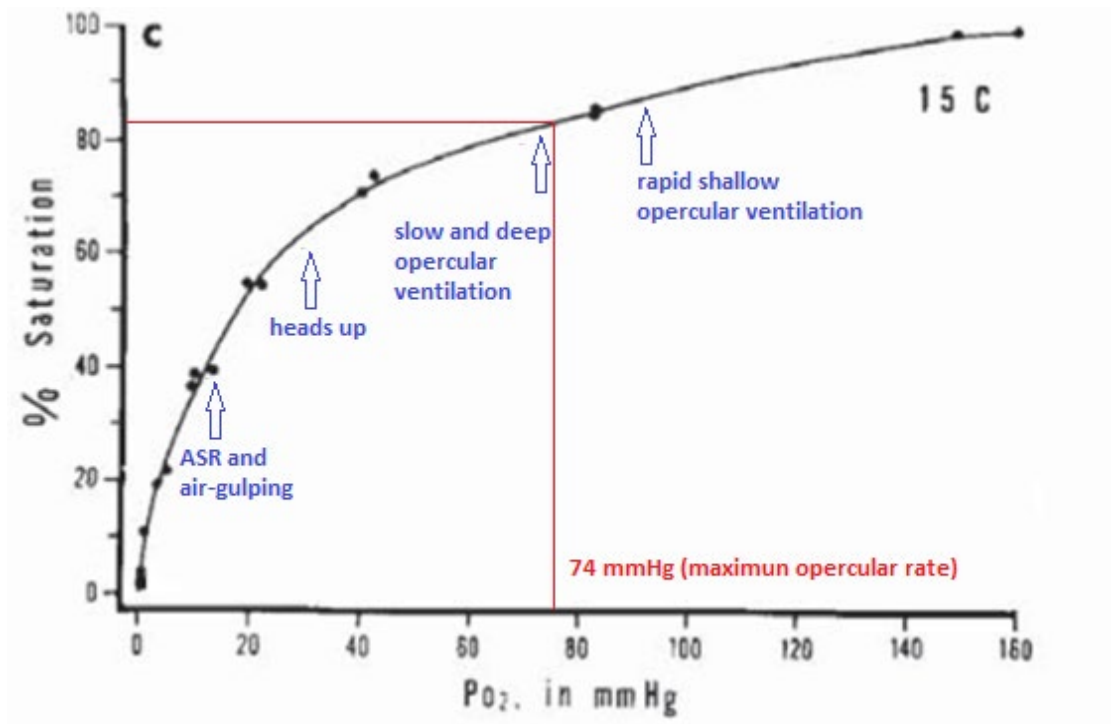


Figure 8. Blood-oxygen equilibrium curve (OEC) of Winter Flounder blood at 15°C, with a P₅₀ of 18 mmHg (torr) pO₂ at a pH of 8.0. Arrows indicating the oxygen concentration on OEC at which various respiratory behaviors of winter flounder were observed at 15°C. The hemoglobin OEC was adapted from Hayden et al. (1975).

Discussion

A. Behavioral responses and OEC correlation

The critical pO_2 (P_{crit}) can be defined as the DO level at which aerobic metabolic rate decreases due to limiting oxygen supply. P_{crit} can also be defined as the water pO_2 at which the animal transits from oxyregulation to oxyconformation. An oxyconformer is an animal whose oxygen consumption (metabolic rate) decreases with ambient pO_2 . An oxyregulator makes physiological adaptations, such as increasing heart rate and ventilation rate, to meet metabolic demand (McEnroe and Krozlowitz, 1996; Seibel 2011; Speers-Roesch *et al.*, 2012).

Opercular rate has a significant linear relationship with metabolic rate (Millidine *et al.*, 2008). Decline in opercular rate occurs at the P_{crit} for aerobic metabolism. The maximum opercular rate at both 15°C and 20°C was at about 74 torr. When comparing P_{crit} (assessed as the decline in opercular rate) to OEC of the blood of winter flounder (Figure 8), P_{crit} occurred at about 83% O₂-Hb saturation. This is the region on OEC where the curve begins to decline steeply, indicating O₂ will be released readily. Below 40 torr at 20°C, the opercular rate declined with decreasing pO_2 (Figure 7), suggesting the fish was limited by O₂. It is likely that the P_{crit} (83% Hb-O₂ saturation) happened at a DO slightly below or near the venous pO_2 , where decreased pO_2 stimulates the chemoreceptors in the gills for compensatory respiratory responses, such as maximizing the opercular ventilation rate. Observation of opercular rate may shed light on the Hb-O₂ affinity characteristic and potentially be an alternative indicator for O₂ affinity analysis. P_{crit} (for opercular rate) analysis may have a greater physiological significance than the traditional P_{50} comparison in OEC.

The order of behavioral responses to hypoxia indicated the preferred strategies (Figure 8). From normoxia to about 60 torr, the first behavioral change was lateral movements (escape

responses), followed by rapid shallow opercular ventilation. There were few lateral movements possibly due to the small size of the tank. Below 61 torr at 15°C and 53 torr at 20°C, slow and deep opercular ventilation began. As pO₂ continued to decrease, the flounder began to lift its head up and eventually swam to the surface to reach the more oxygenated surface water, gulped air, and even jumped out of the aquarium as a “last-effort” to survive extreme hypoxia.

In a species of flounder, speckled sanddab (*Citharichthys stigmaeus*) displayed altered posture and activity after visual exposure to a model predator (Andrade *et al.*, 2018). In our study, we noticed that as we entered the room, the winter flounder returned to normal posture from heads-up position. The presence of experimenters in the proximity may skew the results, delay and lower the frequency of “risk prone” behaviors. Future studies should minimize visual disturbance to the fish. Other factors, such as aquarium's background color, light intensity and turbidity, may also impact the perception of predator risk and thus, delay the hypoxic behaviors. Similarly, the mere presence of experimenters may elevate the opercular respiratory rate, and skew the Hb-O₂ correlation. Behavioral responses may also correlate the age or the length of the fish. Future experiments should increase the sample size and number of trials. The fish should also be studied in decreasing DO (from normoxia to hypoxia), as well as in increasing DO (from hypoxia to normoxia).

B. Comparison of Prior Studies

Hemoglobin-oxygen affinity

The O₂ affinity of Hb is often compared using P₅₀ value in lieu of OEC, where P₅₀ is the pO₂ at which Hb is 50% saturated with O₂. P₅₀ values of Winter flounder are summarized (Table 2) from data in Hayden *et al.* (1975).

Table 2. P₅₀ values of Winter flounder (*Pseudopleuronectes americanus*) whole blood in literature (Hayden *et al.*, 1975).

| Species | Temperature | pH | P ₅₀ (Torr) |
|--|-------------|------|------------------------|
| Winter flounder (<i>Pseudopleuronectes americanus</i>) | 10 °C | 8.31 | 7 |
| | 10 °C | 7.57 | 37 |

The P₅₀ at 10 °C for Windowpane flounder (33.4 Torr at pH 7.5) from Khan (2018) is close to the P₅₀ of Winter flounder (37 Torr at pH 7.57) (Table 2) from Hayden *et al* (1979). At 10 °C, the oxygen transport capacity of both Windowpane flounder and Winter flounder are similar and may explain the fact that they both occupy a similar ecological niche at the seafloor.

Temperature effects

Binding of O₂ to Hb is exothermic. In most fish, higher temperature will lower Hb-O₂ affinity, facilitating O₂ unloading in active tissues. The Hb-O₂ affinity in some tunas and sharks are independent of temperature, or even increase with higher temperature. These fishes do not unload O₂ at high temperature to minimize O₂ loss (Nikinmaa, 2011).

Using the equation listed in the Methods section, the apparent heats of oxygenation (ΔH) of two flounder species were re-calculated based on existing data (Table 3). A negative ΔH value means that energy is released when O₂ binds to Hb. A negative ΔH indicates Hb-O₂ binding is favorable (higher affinity) at low temperatures, and vice versa.

Table 3. Temperature effect and re-calculation of ΔH for winter flounder whole blood data in Hayden *et al.* (1975). A negative ΔH indicates a right shift in OEC.

| Species | Temperature 1 | Temperature 2 | P ₅₀ 1 (torr) | P ₅₀ 2 (torr) | ΔH (kcal/mol) |
|---|-----------------------|-----------------------|--------------------------|--------------------------|-----------------------|
| Winter flounder (<i>Pseudopleuronectes americanus</i>) | 5 °C (at pH 8.33) | 10 °C (at pH 8.31) | 5 | 7 | -10.54 |
| | 10 °C (at pH 8.31) | 15 °C (at pH 8.02) | 7 | 18 | -30.64 |

As temperature increases, Hb-O₂ affinity is expected to decrease (less Hb-O₂ exothermic binding), and ΔH is expected to be less negative (less exothermic) as in most fish. Surprisingly in Windowpane flounder, the data in Khan (2018) shows a positive ΔH from 10 to 13°C, signifying a left shift in OEC. When comparing the ΔH at 10-25°C (-8.067 kcal/mol) and 24-33°C (-1.242 kcal/mol), the ΔH becomes less negative as expected, suggesting a right shift in OEC, and the Hb-O₂ affinity decreases with increasing temperature. It also suggests a greater temperature sensitivity in 10-25°C than in 24-33°C range.

The blood of winter flounder showed a greater temperature sensitivity at 10-15°C ($\Delta H = -30.64$ kcal/mol) than at 5-10°C ($\Delta H = -10.54$ kcal/mol) (Table 3). However, Hb-O₂ affinity increases with increasing temperature, which is unusual in most fish. There were notably more exothermic Hb-O₂ binding at a higher temperature (10-15°C) than at a lower temperature (5-10°C).

Bohr Effect

Using the equations in the Methods section, Bohr coefficients (Φ) were re-calculated from data in Hayden *et al.* (1975).

Table 4. Calculation of Bohr Coefficient in Winter flounder whole blood from data in Hayden *et al.* (1975).

| Species | Temperature (°C) | pH1 | pH2 | P ₅₀₁ (Torr) | P ₅₀₂ (Torr) | Bohr Effect (Φ) |
|--|---------------------|------|------|----------------------------|----------------------------|--------------------|
| Winter flounder | 10 | 8.31 | 7.57 | 7 | 37 | -0.977 |
| <i>(Pseudopleuronectes americanus)</i> | 15 | 8.02 | 7.48 | 18 | 45 | -0.737 |

There is a strong Bohr effect in Winter flounder (Table 4). With a large Bohr coefficient, a small change in H⁺ (P_{CO2} and lactic acid increases) can hugely improve oxygen delivery in active tissues. Bohr effect in Winter flounder at 10 °C (Φ = -0.977) is greater than at 15 °C (Φ = -0.737) (Table 4). This suggests that Winter flounder may have greater activity and metabolic rate at lower temperature. Khan (2018) found the opposite in Windowpane flounder, where the Bohr effect is stronger at a higher temperature (13°C. Φ = -0.89) than at a lower temperature (10°C. Φ = -0.56).

Lactic acid or CO₂ buildup shifts OEC to the right. Bohr effect facilitates both O₂ and CO₂ transport. In acidic tissue capillaries, Hb-O₂ affinity decreases, and the deoxygenated blood takes up more HCO₃⁻ (due to increased H⁺ uptake by Hb, and shift in HCO₃⁻ equilibrium). Bohr effect evolved multiple times in vertebrates. In the last common ancestor of jawed vertebrates, the Bohr effect was small and resembled to those in extant sharks and lungfish; in tetrapods and the last common ancestor of Actinopterygii, the Bohr effect became greater and resembled to those in extant sturgeons and gars; in the last common ancestor of bowfin and Teleostei, the Bohr effect elevated even more. This evolutionary inflation in Bohr effect was accompanied by a weakening Hb buffering capacity. This allowed Teleostei to take further advantage of Bohr

effect. As the blood became more sensitive to pH changes, it evolved to have greater pH responses in Hb-O₂ affinity (Bohr effect) (Berenbrink *et al.*, 2005; Berenbrink, 2011).

Overall, this study suggested that as the average water temperature in LIS gradually increases, certain species may adapt better than others. Some species may migrate to areas with lower temperature and higher DO; if escape is impossible, a series of “last-effort” behavioral responses may be displayed to survive hypoxia and climate change.

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