OXYGEN UPTAKE AND DELIVERY IN COLD TEMPERATE MARINE TELEOSTS

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BY

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ABSTRACT

Aspects of respiratory and circulatory physiology were investigated in the winter flounder (*Pseudopleuronectes americanus*) and the sea raven (*Hemitripterus americanus*). The physical and physiological factors affecting oxygen uptake and delivery were the main foci of the research. Hemoglobin function, blood flow to the tissues, and the operation of cardiac muscle during different seasons were investigated. The influence of temperature on the acclimatization process was investigated.

A significant difference in oxygen uptake at the gills ($\dot{V}_{O_2}$) was found in the seasonally acclimatized flounder (Q_{10} = 2.4; winter temperature was 0.5°C, and summer temperature was 9.5°C). Comparable changes were observed in the cardiac output ($\dot{V}_b$) and ventilation volume ($\dot{V}_w$) of the winter flounder. The gills of winter fish were more resistant to oxygen uptake, yet the oxygen tension of arterial blood (P_{aO_2}) in winter fish did not differ from that of summer fish.

A number of findings point to or suggest the causes of changes in oxygen resistance of the gills during seasonal acclimatization. *In vitro* blood viscosity of the winter flounder, especially at temperatures below 10°C, shows a great sensitivity to changes in temperature. Viscosity measurements *in situ* indicate that at lower temperatures blood tends to flow through larger, less resistant blood vessels, so that the surface area for oxygen transfer to the blood is limited in winter fish gills. However, a slower blood flow rate during the

winter, and seasonal changes in blood components, assist in maintaining arterial oxygen tension. As indicated by studies on the sea raven heart *in situ*, winter acclimatized fish have diminished ability to produce a cardiac output. During the winter there is a significant increase in concentration of total hemoglobin and a significant shift toward higher hemoglobin-oxygen affinity.

Intraerythrocytic components such as H^+, Cl^-, and nucleotide triphosphates (NTP) influence hemoglobin-oxygen affinity in the winter flounder. Temperature influences the concentration of intraerythrocytic modifiers, and seasonal changes tend to support shifts in oxygen affinity.

It is concluded that temperature plays a significant role in effecting physiological changes during acclimatization to the seasons. The physical effects of temperature upon blood flow and oxygen uptake at the gills are opposed by alterations in blood components meeting metabolic demands while maintaining oxygen tension.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

ATP	adenosine triphosphate
β	oxygen solubility
C_{aO_2}	arterial oxygen content
C_{vO_2}	venous oxygen content
cpm_i	initial radioactive counts per minute
cpm_f	final radioactive counts per minute
cps	centipoise
f_H	heart frequency
f_R	ventilation frequency
Hb	hemoglobin
Ht	hematocrit
MHC	mean hemoglobin content of corpuscles
$\dot{M}_{O_2}$	molecular oxygen usage rate
NTP	nucleotide triphosphate
pH_e	extracellular pH
pH_i	intracellular pH
P_{50}	half saturation of Hb with oxygen
P_{aO_2}	arterial oxygen partial pressure
P_{vO_2}	venous oxygen partial pressure
P_{iO_2}	incurrent ventilatory water oxygen partial pressure
P_{eO_2}	effluent ventilatory water oxygen partial pressure
rbc	red blood cell
S_{aO_2}	arterial blood-oxygen saturation
S_{vO_2}	venous blood-oxygen saturation
SV_H	stroke volume of the heart
TO_2	oxygen transfer factor
U_{wO_2}	utilization of oxygen from ventilatory water
U_{bO_2}	utilization of oxygen from blood
$\dot{V}_b$	blood volume output rate of the heart (cardiac output)
$\dot{V}_w$	water volume flow rate at the gills
$\dot{V}_{O_2}$	oxygen volume usage rate

CHAPTER 1

INTRODUCTION

Poikilothermic vertebrates such as fish are affected internally by their external environment. During seasonal changes in the external environment the organism can maintain internal physiological variables at different levels than would be predicted from the conditions of the environment. This sort of physiological adjustment has been refered to as dynamic equilibrium by Prosser (1964). Dynamic equilibrium is necessary for the survival of organisms in widely changing environments, because the range of internal variables such as pH or P_{O_2} for survival are smaller than those that may be dictated by the external environment (Prosser, 1964). Fish have adapted many physiological mechanisms that allow them to maintain an internal equilibrium. Of particular interest are those fish which live in temperate regions that undergo broad temperature changes with changing seasons. The research of this thesis investigates some of the mechanisms employed by fish in achieving oxygen uptake and delivery during different seasons.

Temperature is recognized as an important influence on the physical environment, as well as on the physiological performance of animals (e.g. Hoar, 1983; Prosser, 1973). A number of physical challenges confront fish as they attempt to meet metabolic demands. They must be able to move water past the gills in order to breathe. The viscosity of water increases as it cools, thus ventilation becomes more difficult for the fish. Although the oxygen content of water is

higher at cooler temperatures, the absolute amount of oxygen present in water is only 3 per cent of the amount of oxygen found in air. The difficulty faced by water breathers during ventilation is more evident when the convection requirements for water breathers and air breathers are compared (Dejours, 1981). The convection requirement is the volume of ventilatory medium required per unit of oxygen uptake. The values are usually very high for water breathers.

Temperature also effects a number of changes in physiological processes in fish (e.g. Houston, 1973; Fry, 1971). The results of changes in fish physiology have been viewed in two contexts. When the seasons change, a change in temperature takes place in conjunction with other changes in the physical environment, and the alterations which take place as part of the physiological response of the animal are called conditions of acclimatization (Fry, 1958). In the laboratory it is possible to effect physiological alterations in response to changes in temperature alone; these create conditions of acclimation (Fry, 1958). Many of the early studies on the effects of temperature on fish used oxygen consumption as the main physiological indicator of acclimation or acclimatization (e.g. Scholander, Flagg, Walters, and Irving, 1953; and Fox, 1936). Measurements of oxygen consumption are useful in demonstrating the operation of cardiovascular and ventilatory systems. Knowledge of the oxygen consumption of a species can be used to predict the capability of the animal to tolerate differing environmental conditions. Precht (1958) found various levels of compensation for changing temperatures in some species of poikilotherms. Cold water fishes are particularly well known for their ability to compensate for temperature.

Polar aquatic poikilotherms, under the influence of year-round temperatures below 5°C, are thought to be adapted to cold, making comparisons with tropical aquatic animals difficult. Scholander et al. (1953) compared rates of oxygen consumption in arctic and tropical poikilotherms as a base for a theory of cold adaptation: that cold water species are able to maintain a higher rate of oxygen consumption at low temperatures than warm water fish at those temperatures.

Wohlschlag (1960; 1962a,b; 1964) concluded that nototheniid species exhibited cold adaptation, indicated by data on oxygen consumption. However, he found no cold adaptation in an Antarctic zoarcid (Wohlschlag, 1963). Oxygen consumption studies of a number of Antarctic fish species, including hemoglobinless types, by Hemmingsen and Douglas (1970), Grigg (1967), and Ralph and Everson (1968) reported rates comparable to those found by Wohlschlag (1964). In 1970 Holeton showed that oxygen consumption in nototheniids was much lower than that recorded by Wohlschlag in 1960 and 1964. In two later studies on Arctic species, Holeton challenged the validity of the cold adaptation theory (1973, 1974). Criticism of the cold adaptation studies has focused primarily on experimental procedures used by researchers, and their interpretations of the data. Cold adaptation still remains to be soundly demonstrated under laboratory conditions (Clarke, 1984). However, a recent study by Tetens, Wells, and DeVries (1984) supports the theory.

Studies of oxygen consumption are useful in illustrating the ability of certain species to adapt to environmental conditions. However, the functions of the several components of the oxygen delivery

system have yet to be elucidated. The effects of temperature on some aspects of circulatory and ventilatory physiology of fish have been studied, and results pertinent to the present research are reviewed below. The most striking examples of physiological alterations in response to temperature come from studies on Antarctic fish. Those fish endure extreme cold for their entire life. There are fewer reports on the effects of exposure to similar cold conditions on a seasonal basis.

Blood is the medium by which oxygen travels from the gills to the tissues in fish. Blood contains hemoglobin, an oxygen-binding protein basic to the oxygen delivery system. Exceptions of this general rule in fish species are found in the Antarctic, where many species of fish have no hemoglobin in their blood; this is thought to be due to the persistence of extremely cold temperatures. The cold water is rich in dissolved oxygen, and therefore it is thought that the presence of hemoglobin is not so essential. Due to the absence of hemoglobin, blood viscosity is decreased (Hemmingsen and Douglas, 1972). Some polar fishes which do have hemoglobin may still have blood of low viscosity (Graham, Fletcher, and Haedrich, 1985). Generally, it is recognized that total hemoglobin concentration ([Hb]) and total number of red blood cells (rbc's) of Arctic and Antarctic fish species are lower than [Hb] and rbc's in temperate and tropical fish (Wells, Ashby, Duncan, and MacDonald, 1980; Putnam and Freel, 1978; Everson and Ralph, 1968; Kooyman, 1963; Tyler, 1960; Scholander and Van Dam, 1957; Ruud, 1954). Similar trends to lowered hemoglobin and rbc's were observed in the pinfish (*Lagodon rhomboides*) and in the striped mullet (*Mugil*

cephalus) during acclimatization from 25°C to 7°C (Cameron, 1970), and in acclimation to low temperature in rainbow trout (Salmo gairdneri) from 21°C to 3°C (Houston and Cyr, 1974; DeWilde and Houston, 1967) and in carp (Cyprinus carpio) from 18°C to 2°C (Houston and Smeda, 1979).

The reduction in the capacity to store oxygen in the blood in conditions of low temperature seems reasonable because of the expected reduction in oxygen needed by the tissues under such conditions. However, in some species the reverse situation is seen. After acclimation to low temperature in three species, [Hb] was shown to have increased in rainbow trout from 18°C to 2°C, studied by Nikinmaa, Soivio, and Railo (1981) and by Nikinmaa, Tuurala, and Soivio (1980); in blackfish (Gadopsis marmoratus) from 20°C to 10°C, studied by Dobson and Baldwin (1982a); and in winter flounder from 15°C to 5°C, studied by Cech, Bridges, Rowell, and Balzar in 1976.

Denton and Yousef (1975) suggest that seasonal hematological changes in the rainbow trout can be due to factors others than temperature, such as diet and activity.

Responses of various blood components to temperature would be better understood if the physiology of cardiovascular and ventilatory systems of each species were fully known. Blood components meet the demands of tissues for oxygen, depending upon the operation of the cardiovascular system and the ventilatory system. Antarctic fish lacking hemoglobin, due to response to extremely low temperatures, exhibit conspicuous differences in the operation of the cardiovascular system from fish with hemoglobin containing rbc's. These Antarctic

hemoglobinless fish have larger blood volumes (Twelves, 1972; Holeton, 1970; Hemmingsen and Douglas, 1970) than 'red-blooded' fish (that is, fish with hemoglobin in their blood). They are therefore able to produce larger cardiac output with low peripheral resistance (Hemmingsen and Douglas, 1972 and 1977; Hemmingsen, Douglas, Johansen, and Millard, 1972; Holeton, 1970 and 1972). Reduced resistance was attributed to increase in size and/or number of capillaries carrying the blood (Hemmingsen and Douglas, 1972; Holeton, 1970) and decreased blood viscosity (Hemmingsen and Douglas, 1972; Twelves, 1972).

Cech et al. (1976) found that at high temperaures, there was a decrease in hemoglobin in the winter flounder, and oxygen demands were satisfied by a greatly increased cardiac output. Nikinmaa et al. (1981) found that temperature had no influence on the blood volume of rainbow trout; however, Hb levels changed at higher temperatures as a result of red blood cells moving into the tissues (tissue rbc shunt). Twelves (1972) found blood volumes of 'red-blooded' Antarctic fish species to be greater than those of other teleosts not exposed to conditions of constant cold.

The ventilation volume ($\dot{V}_w$) of the hemoglobinless Antarctic fish was very high, and gill tissues were more resistant to oxygen transfer (TO_2) than in other fish (Holeton, 1970). The TO_2 results in this extreme case are interesting. Seasonally cycling fish that receive exposure to extremely low temperatures would be useful models in understanding why fish in low temperature conditions have lower TO_2 values. An ideal model would be a fish which also undergoes seasonal hematological alterations, such as the winter flounder. The lower

TO_2 values in the hemoglobinless Antarctic fish may be due to diminished capacity of the blood to bind with oxygen. The winter flounder is an ideal species because the fish undergoes an increase in [Hb] during acclimatization to winter temperatures. That increase in [Hb] may act to counter increases in gill oxygen resistance. Diminished TO_2 may also be due to the physical effects of temperature on blood flow. Blood at lower temperatures is more viscous, and this may reduce the gill perfusion area in fish. In fish with red blood cells, more rbc's are likely to be in the secondary lamellae during conditions of high temperature (Nikinmaa et al., 1981 and 1980). This would provide a greater potential for Hb oxygenation during times of increased oxygen demand (Tuurala, Part, Nikinmaa and Soivio, 1984).

Research has been done on the effects of seasonal and temperature changes on hemoglobin function. If temperature is the only functional modifier, then Hb-oxygen affinity increases as the temperature decreases (Wells and Jokumsen, 1982). But the effects of temperature on whole blood can produce a number of variations of Hb-oxygen affinity, depending upon the species. For example, during exposure to high temperatures the Hb-oxygen affinity in winter flounder decreases (Hayden, Cech, and Bridges, 1975); values for Hb-oxygen affinity increase in the brown bullhead (Ictalurus nebulosus) (Grigg, 1969); while no detectable change in Hb-oxygen affinity were found in rainbow trout (Weber, Wood, and Lomholt, 1976).

Findings on the Hb-oxygen affinity of whole blood are more variable because [Hb] function is also influenced by intracellular components. Seasonal levels of H^+, Cl^-, Mg^{++}, and Ca^{++}, as well as

nucleotide triphosphates (NTP), which have all been shown to influence Hb function, have been observed in fish by Dobson and Baldwin (1982a), Powers (1980), Houston and Smeda (1979), and Houston (1973).

It is apparent that research has demonstrated the importance of temperature on parts of the oxygen delivery system in various fish species. It is clear that persistent, extreme cold can have radical affects on the oxygen delivery system in fish. However, fish exposed to the changing temperatures of the seasonal cycle exhibit more varied, but less extreme, changes in physiology during acclimatization to low temperatures. Even though the physiological changes of temperate and seasonal fish are less extreme than in some of the Antarctic fishes, some species such as the winter flounder are exposed to sub-zero temperatures for several months a year. The adaptation of the species to such a wide range of conditions in the physical environment must mean adjustment in many parts of the circulatory and respiratory systems of the fish.

In the present study, the investigation into the parameters mentioned in this discussion had three purposes:

1) to describe the function of various parts of the oxygen delivery system in the winter flounder;

2) to describe physiological changes which take place in the cardiovascular and respiratory systems during the process of acclimatization;

3) to test the hypothesis that temperature plays a major role in the process of acclimatization.

The specific tests are outlined in detail in Chapter 2. Each area

studied-seasonal changes in hematology and hemoglobin function, blood viscosity, cardiac performance, and respiratory performance - occupies a separate chapter, giving the results of the data collected, and discussing those results. Briefly, changes upon the physiology of the winter flounder, as a result of temperature change over the seasonal cycle, showed that the process of acclimatization affects all aspects of physiology. Chapter 6 attempts to integrate the data and make some conclusions that will assist in the prediction of seasonal physiological performance of fish under certain conditions.

The fish used in this study are distributed in the oceans of the north temperate latitudes, and normally encounter water temperatures up to 15°C during the summer, and winter temperatures as low as -1.0°C. The wide range of temperatures to which these species have had to adapt made possible the investigation of many aspects of their physiology. The changes of physiological functions during summer acclimatization were also investigated. Detailed accounts of the species studied can be found in Bigelow and Schroeder (1953), and Leim and Scott (1966).

The main species studied in the present research was the winter flounder, because of the accumulation of biological and physiological information available on this species (e.g. Fletcher, 1977 and 1975). The sea raven (Hemitripterus americanus) was used as part of the experiment to study the effects of seasonal and acute temperature changes on heart function. The sea raven had been thoroughly researched by Farrell, Macleod and Driedzic in 1982, and by Saunders and Sutterlin in 1971, therefore the investigative procedures used were straightforward. Consideration was given to using the winter flounder

for these studies of heart function, but due to the awkward location of that organ in the winter flounder it was felt that investigative procedures would be hampered by the technical difficulties. The sea raven experiences seasonal temperatures similar to those of the winter flounder, and is also a bottom-dwelling fish, so some general conclusions are possible.

The main objective of the present research was to provide experiments that would allow discussion about a thesis. The thesis which was investigated was that temperature has an important influence on many physiological functions which account for oxygen uptake and delivery in cold temperate marine teleosts.

CHAPTER 2

MATERIALS AND METHODS

2.1 Seasonal changes in hematology and hemoglobin function

2.1.1 Experimental animals

Winter flounder (*Pseudopleuronectes americanus*) were kept in flow-through seawater aquaria for at least one week prior to experimentation (Fletcher, 1975). Ambient seawater conditions were maintained at all times of the year: temperature range -0.5 to 15°C and salinity range 28-32 ‰ (Steele, 1974). During the months of April to October flounder feed actively, and at other periods the gut contents are diminished (Fletcher and King, 1978). During the feeding period fish were fed chopped capelin (*Mallotus villosus*) *ad libitum* 5 days per week.

2.1.2 Sample collection

All blood samples were taken by caudal puncture of unanesthetized fish. Blood samples were taken using 3 mL plastic syringes equipped with 21 gauge needles, and the samples placed into heparinized glass containers (dry heparin: Vacutainer). All samples were refrigerated during experiments and assay preparations. Summer blood samples were taken during July and August and winter samples were taken from

February to April for whole blood studies. Winter blood samples for preparation of hemoglobin solutions were taken during December.

2.1.3 Whole blood P_{50} protocol and hematological techniques

A number of blood aliquots were made from each animal sampled. One aliquot was used immediately to determine oxygen dissociation characteristics (P_{50}). Another was immediately used for measuring hematocrit (Ht), hemoglobin concentration ([Hb]), nucleotide triphosphate concentration ([NTP]), and pH. The remainder of the sample was used for rbc counts and sizing, and plasma and cell electrolyte concentrations.

The P_{50} determinations were made using the mixing technique (Torrence and Lenfant, 1969; Edwards and Martin, 1966). A blood sample (0.5-1.0 mL) was placed into a rotating glass container receiving nitrogen or air. The blood equilibrated within 30 minutes and readings were initiated 45-60 minutes after the start of gassing. Each sample was drawn into a 0.5 mL glass, gas-tight syringe (Hamilton) before being analysed for total content (C_{O_2}), and partial pressure (P_{O_2}) of oxygen. The needle deadspace was filled with mercury, which also acted as a device for mixing the blood sample. The C_{O_2} was determined using the LEX-- O_2 - CON_{TL} (Lexington Instruments, MA.). P_{O_2} measurements were taken with a Clark type electrode (Radiometer E5047, Copenhagen), and appropriately amplified (Radiometer PHM71, Copenhagen), before being recorded (Hewlett Packard 17501A). At least five nitrogenated-aerated mixtures from each individual sample were made in

determining P_{50}. The oxygen electrode was calibrated before each experiment, and the calibration was checked throughout the duration of experimentation. A zero P_{O_2} reading was set using a solution of 0.01 M sodium borate saturated with sodium sulfite. The high portion of the P_{O_2} range was calibrated with air saturated water as described in Hitchman (1983).

All of the percent saturation values pertain to hemoglobin only, therefore all oxygen carried in physical solution has been subtracted as follows:

$$C_{O_2,Hb} = C_{O_2,blood} - \frac{(C_{O_2,plasma\ max} \cdot P_{O_2,blood\ sample})}{P_{O_2,plasma\ max}}$$

Oxygen content values are in units of millilitres of O_2 per 100 ml of solution. Plasma max refers to the maximum partial pressure or content of oxygen in the plasma under the experimental conditions. The P_{50} value was determined using the Hill equation (Riggs, 1970); the P_{O_2}, and percent saturation values for the 25-75% oxygen saturated samples.

Hemoglobin concentration was assessed by means of the cyanmethemoglobin method, employing Sigma standards. Methemoglobin levels were measured using the methods outlined in Henry (1964). The NTP concentrations were measured using the methods outlined in Sigma bulletin #366 - UV. Winter acclimatized fish (February to April) and winter fish acclimatized to summer temperatures were sampled for blood NTP levels. The same tests were done on summer acclimatized fish (July and August) and summer fish acclimatized to winter temperatures. Fish

exposed to non-seasonal temperatures (0°C during the summer and 10°C during the winter) were acclimated for 30 days. Hematocrits were done in duplicate on a microhematocrit centrifuge (International Instruments model MB). A glass microelectrode (Radiometer E5021, Copenhagen) connected to a PHM71 acid-base analyser (Radiometer, Copenhagen) was used to measure pH.

Intraerythrocyte levels of Na^+, K^+, Mg^{++}, and Ca^+ were determined using flame atomic absorption (Varian-Tectron, model AA5 and Varian A-25 recorder) (Fletcher and King, 1978). Red blood cells were separated from plasma by centrifugation, and the plasma was pipetted off. Red cells were then digested in concentrated nitric acid. Analyses were done on clear digests. Plasma values for the four ions were also determined using flame atomic absorption. Chloride concentrations were measured from plasma and rbc digests by the Radiometer CMT-10 chloride titrator. Water content of rbc's was calculated after drying cell samples at 60°C until no further change in mass was noticed.

The pH of rbc's was measured on hemolysates (Steen and Turitzen, 1968). One mL blood samples were centrifuged for 2 minutes at 5000 X G, then immediately submersed in a mixture of ethanol and dry ice. The part of the centrifuge tube containing plasma and white cells was cut away, and the part of the tube containing rbc's was resealed before being thawed. Two subsequent freezing and thawing events ensured that cells had been disrupted. Measurements of pH on lysed rbc's were taken using a glass microelectrode.

Red blood cell sizes and numbers were obtained using a Coulter Channelyzer and Counter (Coulter Electronics, Ontario). Blood samples

were diluted to 50,000 times the original concentration in pH adjusted saline before counting and sizing with a 100 μm aperture. The following comprised the diluting saline: NaCl = 150 mM (summer), 175 mM (winter), KCL = 2.7 mM, $MgCl_2 \cdot 6\ H_2O$ = 1.0 mM, $CaCl_2 \cdot 2\ H_2O$ = 2.7 mM, D - Glucose = 2.2 mM, and TES buffer (N-tris[hydroxymethyl] methyl-2-aminoethane sulfonic acid) = 3.0 mM. Duplicate counts were done on 0.5 mL samples (20,000-50,000 cells per counting). Coincidence corrections were made where needed (Anonymous, 1970). The Channelyzer organizes a size scan of each sample into 100 channels, each representing an average volume. The average channel volume was calibrated with paper mulberry pollen (diameter = 13 μm). Mean corpuscular volume (MCV) was determined by dividing the total volume of a size scan by its number of cells. The total volume was determined as the sum of the products of the total number of cells per channel (or range of channels) and the average volume of the cells characteristic of those channels:

$$MCV = \frac{\sum_{1}^{100} \#\ \text{cells} * \text{average volume of channel(s)}}{\text{total}\ \#\ \text{of cells}}$$

All mean cell volumes were in units of cubic microns (μm^3).

2.1.4 Hemoglobin solution preparation

It was important to produce a hemoglobin solution that was free of substances that would influence its functions, such as nucleotide

triphosphates, Cl^-, Ca^{++}, Mg^{++}, and H^+. When this preparation is completed, known amounts of specific agents can be added and evaluated on their influence over hemoglobin function.

Blood from several fish was pooled together and used in preparing the hemoglobin solutions. Blood was obtained from the fish by the method described in 2.1.2. After the plasma was removed, blood cells were washed three times with ice cold saline (see 2.1.3). The blood cells received a final wash with 50 mM Trizma base buffer (Tris[hydroxymethyl]aminomethane at a pH of 7.95). The cell-buffer solution was then sonicated for 2 minutes at 70% of full scale power (Biosonik III, Bronwill Scientific, NY.) while on ice. The hemolysate was centrifuged (5000 rpm) twice for 20-30 min at 4°C to remove any cell debris. The resulting hemolysate (approximately 20 ml) was passed through a Sephadex G25 column twice before being concentrated. Sephadex G-25 is recommended for desalting purposes (Anonymous, 1984) and for the removal of nucleotides such as ATP and GTP (Jelkmann and Bauer, 1976). The column was 40 cm long with a 1.5 cm diameter. The elution volume was approximately 30 mL. The flow rate was 80 mL/hour. A 10 mM NaCl solution adjusted to a pH of 7.95 was used to elute the Hb solution from the column. Levels of NTP in the second elution of the Hb solution were undetectable. The final Hb solution was diluted with 5-8 times its volume using 50 mM Trizma base buffer (pH = 7.95). A high pressure filtering device (PM10 filter, 10,000 D cutoff; Amicon Corp., MA.) concentrated the hemolysate under an air pressure of 207 kPa (30 psi) (Jelkmann and Bauer, 1976). The column and concentration procedures were carried out at 4°C.

The P_{50} determinations on Hb solutions were done as described for whole blood. Concentrations of Cl^-, Ca^{++}, Mg^{++}, H^+, and ATP were added to the appropriate hemolysates in order to observe their effects on hemoglobin function (NaCl, $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$ and HCl were used). Stock solutions of the ions and ATP were made and Hb:ion ratios were based on an assumed Hb molecular weight of 68,000 grams/mole (Hoar, 1983).

2.1.5 Data analysis

Unpaired t-tests with $p < 0.05$ were used to compare mean values for summer and winter fish. Analysis of covariance was used to test for significant differences in the slopes of lines in figure 11 (Snedecor and Cochrane, 1967).

2.2 *In vitro* and *in situ* viscosity

2.2.1 Winter flounder *in vitro* study

Winter flounder (*Pseudopleuronectes americanus*) were regularly collected from Conception Bay, Newfoundland, by divers using SCUBA. Fish were kept at the laboratory in a 40,000 litre aquarium under seasonally ambient conditions of photoperiod, temperature and salinity (Fletcher, 1977). Measurements were carried out from October to May (temperature range -1 to 10°C).

Blood samples (5-15 mL) were taken from unanesthetized fish by

caudal puncture, immediately heparinized (100 units Na^+ heparin/ml blood), and then stored on ice.

Red cells or plasma were removed after centrifugation (4000 rpm) as Ht's were adjusted to desired levels ranging from 0% (plasma) to 80%. White blood cells constituted less than 3% of the rbc numbers, therefore no effort was made to remove them from the blood (Dintenfass, 1971).

Various concentrations of plasma protein were attained by dilution of normal plasma with flounder saline solution (as above), or by concentration in an AMICON ultrafiltration device (Lexington Inst. Inc., Missouri) with a UM2 filter under 207 kPa (30 psi) nitrogen pressure.

Hemoglobin and Ht values were determined as described above. Total plasma proteins were measured using the biuret reaction (Henry, 1964), with 0.1 ml of plasma.

The freezing point depression of the plasma was measured using an Advanced osmometer (Model 3D, Advanced Inst. Inc., MA.). This was done to ensure that only "winter type" fish were used (i.e. freezing point depression = approximately 1.12°C; see Fletcher, 1977). However, it was later discovered that fish which had atypical winter values (i.e. freezing point depression smaller than 1.12°C), did not have noticeably different blood plasma viscosities.

Viscosity was determined on 1 ml blood or plasma samples using a cone plate viscometer (LVT model 0.8° cone angle, Brookfield Ltd., Mississauga, Ontario). The temperature of the sample cup was regulated (±0.1°C) using a recirculating cooling unit (Neslab Inst. Inc., Portsmouth, N.H.). Calibration of the viscometer was checked using Brookfield standards; the gap between the cone and plate was adjusted

immediately prior to each test. The procedures outlined by Rand, Lacombe, Hunt, and Austin (1964) were used to determine viscosity. At least four sets of readings were made for each sample and the values obtained at each shear rate were averaged. In all instances, the measurements were made within 24 hours of blood collection.

2.2.2 The role of flounder plasma in *in vitro* viscosity: flounder-sculpin blood mixtures, and plasma and serum experiments

Flounder blood is much more viscous than sculpin blood, because of the plasma viscosity and the ability of the plasma to aggregate red blood cells (Graham and Fletcher, 1985). Blood from the shorthorn sculpin (*Myoxocephalus scorpius*) was used to evaluate the role of flounder plasma in red blood cell aggregation.

In the experiment, blood was taken from winter flounder and shorthorn sculpins, then each was divided into cells and plasma by centrifugation. The rbc's were washed three times with saline (recipe as above). The following mixtures of rbc's and plasma were prepared: (i) flounder cells in flounder plasma; (ii) flounder cells in sculpin plasma; (iii) sculpin cells in sculpin plasma; and (iv) sculpin cells in flounder plasma. The sculpin cells in the flounder plasma exhibited profuse clumping and could not be used for viscosity determinations.

The role of fibrinogen in the viscosity of flounder whole blood was assessed by the following experiment. Flounder red cells were suspended (Ht = 16%) in plasma and autologous serum (plasma and serum were pooled together from a group of seven fish). The viscosities of the plasma, serum, rbc's in plasma, and rbc's in serum were compared at 0°C.

2.2.3 *In situ* viscosity study

The *in vitro* viscosity investigations provide data for very specific conditions of flow: at one shear rate in a uniform, noncompliant vessel. The following set of experiments utilized the living vasculature of the winter flounder as a viscometer. The experimental parameters were temperature and flow rate; both influence blood viscosity. By comparing blood and saline flow through the vasculature it is possible to determine the average blood viscosity as it passes through the preparation. The object of the experiments was to elucidate the importance of temperature and flow rate to *in situ* viscosity.

Experiments were carried out during the winter (February to April). Anesthetized (0.76 mM Ethyl-M-Aminobenzoate, MS222, Sigma) winter flounder (200-600 g) were sectioned to remove the anterior body. The remaining trunk (50-75% of the body mass) had no kidney or peritoneal organs. The dorsal aorta and caudal vein were implanted with snugly fitting steel catheters (inside diameter = 1 mm). A tight ligature of umbilical tape was applied about the hemal arch of the vertebrae to secure the catheters in place. Approximately 25-50 mls of heparinized saline (10 iu/ml) flowed into the dorsal aorta from a 40 cm head and removed the blood from the trunk vasculature.

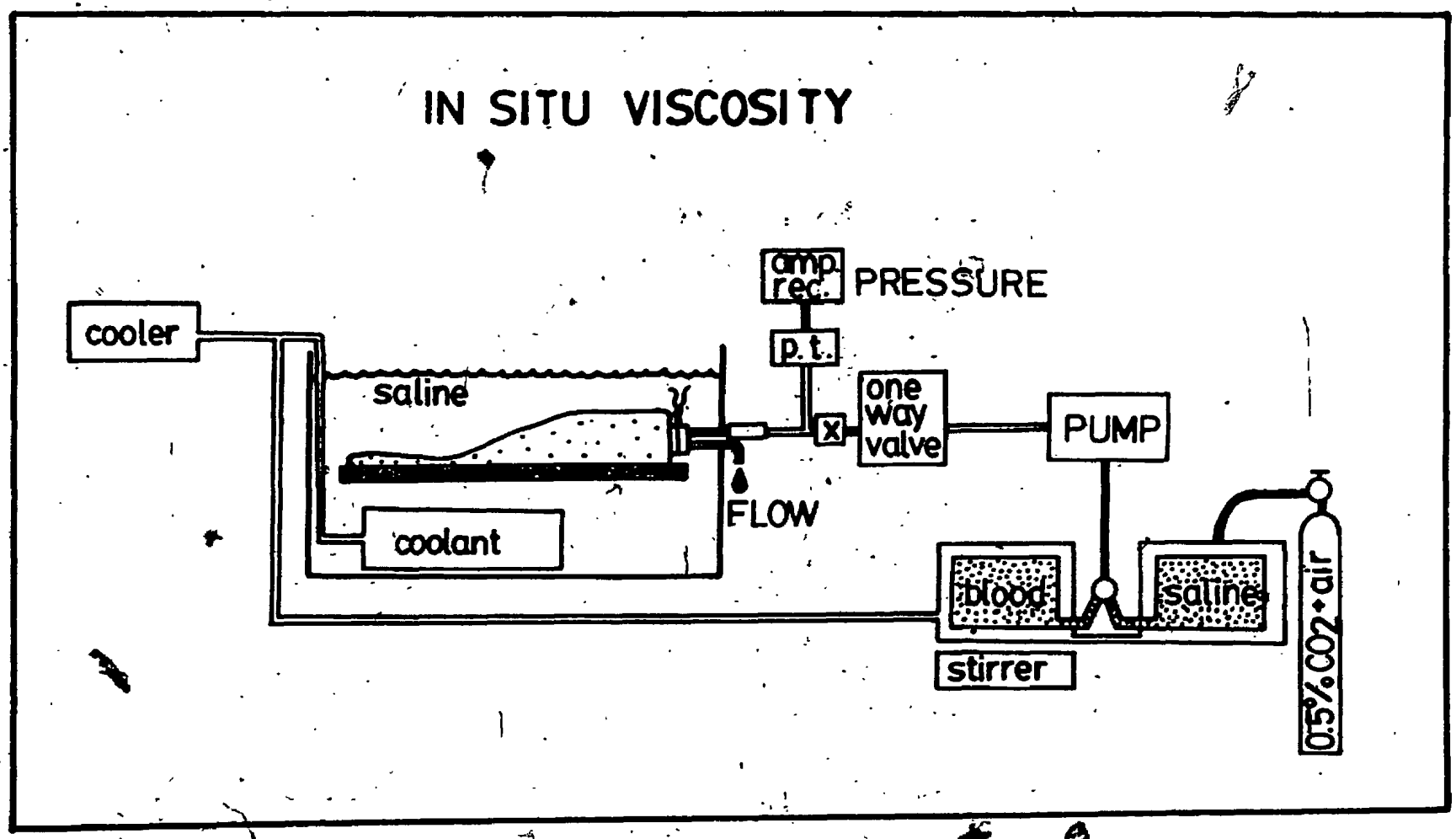
The sectioning, catheterization and rinsing procedure took no longer than 10 minutes. The fish was then submerged in a temperature controlled saline bath (saline same as perfusate minus the PVP; see below). A peristaltic pump delivered saline or blood from thermostated containers to the dorsal aorta. Pressure was measured with pressure

transducers (Microswitch, 126PC or National Semiconductor, LX1601 DF), and signals were amplified and recorded before fluid entered the trunk (Hewlett Packard preamplifier, 17402A and recorder 7402A). In addition to the conventional equipment listed, a custom made preamplifier was used with the Microswitch 126PC transducer, and a custom made interface was used with the National Semiconductor LX1601 DF transducer. Flow was altered by changing the pump rate. Flow was measured by collecting the volume of effluent leaving the caudal vein. There was no backflow of perfusate after it passed through a check valve (Figure 1).

The efficiency of volume recovery from the trunk was assessed after each flow change. Usually the outflow volume from the caudal vein catheter, as a percentage of the inflow (efficiency of volume recovery), declined with increased flow rates. Leaks at low flow pressures were from open tissues in the gut and the segmental vessels along the long section of the trunk. At elevated flows and pressures (e.g. 60-80 cm H_2O), the catheter insert area showed signs of leakage. Experiments were terminated at that point or flow was decreased to allow for perfusate switch (i.e. saline to blood). The preparation gave consistent flow-pressure relationships for up to 2.5 hours of saline perfusion.

Experiments were either conducted under high (blood = 10.12 $\pm$ 0.15°C, saline = 10.13 $\pm$ 0.15°C) or low temperature conditions (blood = 0.59 $\pm$ 0.15°C, saline = 0.78 $\pm$ 0.45°C). An initial perfusion with the following saline solution lasted 30 minutes for each preparation: 10^{-5} M yohimbine - HCl (competitive alpha adrenergic blocker, Sigma); 10^{-5} M papaverine - HCl (nonspecific smooth muscle

Figure 1. Diagramatic representation of the experimental setup for measuring blood viscosity. amp = amplifier, rec = recorder, p.t. = pressure transducer, x = position of flow meter and Windkessel.


IN SITU VISCOSITY
cooler
saline
coolant
FLOW
amp. rec.
PRESSURE
p.t.
x
one way valve
PUMP
blood
saline
stirrer
0.5% CO2 + air

relaxant, Sigma); 10.71 g/l NaCl, 0.41 g/l $CaCl_2 \cdot 2H_2O$, 0.22 g/l KCl, 0.13 g/l $MgSO_4$, 0.38 g/l Na_2HPO_4, 0.04 g/l NaH_2PO_4, 40 g/l polyvinylpyrrolidone (PVP, MW = 40,000; Sigma); 1.0 g/l glucose, and 2.0 g/l $NaHCO_3$. The perfusate was equilibrated with 0.5% CO_2 and air. Blood from 9-24 fish was pooled together, then pH and Ht adjusted at each experimental temperature (cold experiment: pH = 7.848 ± 0.025, Ht = 19.31 ± 0.61; warm experiments: pH = 7.936 ± 0.020, Ht = 19.24 ± 0.50). Saline pH values were: cold = 7.890 ± 0.028; and warm = 7.926 ± 0.012.

After the initial 30 minutes perfusion with saline, a stable pressure flow trace was evident and any leakage was detected. The initial perfusion was at 30-40 cmH_2O pressure, then flow was lowered or raised enough to change pressure by about 10 cmH_2O per shift. Once a stable trace was established, the flow was altered again, within the range of ~20 to 80 cmH_2O.

Blood was pumped through the same trunk as the saline. The blood was mixed continuously with a magnetic stirrer, and was fully oxygenated. Papaverine and yohimbine were added to the blood at 10^{-5} M concentration. Pressure-flow relationships were developed after caudal vein effluent Ht was equivalent to that in the thermostated reservoir.

Flow alterations due to catheter resistance were taken into account during all measurements.

2.3 Seasonal cardiac performance

2.3.1 Experimental animals and water conditions

Sea raven (Hemitripterus americanus) were caught in Passamaquoddy Bay, and kept in ambient surface seawater at the Huntsman Marine Laboratory, St. Andrews, New Brunswick. The fish were then transported to Mount Allison University, and held in seawater (30 $^{o}/oo$) until experimentation. No food was administered while the fish were in captivity.

Water temperature in the area of capture was 2-3°C during the winter (January, 1984), and 12-14°C during the summer (September, 1983) (Fletcher, Kao, and Haya, 1984). During the winter experiments, fish were held in the laboratory at 5°C for at least four weeks, and in the summer at 10°C for at least one week. The summer fish experiments were initiated at a temperature of 13.3°C, with $\dot{V}_b$ at approximately 15 $ml/kg*min^{-1}$. The preload (input) and output pressures of the summer fish were 0.95 $\pm$ 0.17 cmH_2O, and 39.90 $\pm$ 0.57 cmH_2O respectively. Winter fish experiments were initiated at a temperature of 3.4°C with $\dot{V}_b$ at approximately 9 $ml/kg*min^{-1}$. The initial $\dot{V}_b$ for winter fish was selected based on observations of cardiac output from summer fish after acute temperature decrease. The preload and output pressures for winter fish were 1.23 $\pm$ 0.21 cmH_2O, and 41.65 $\pm$ 0.71 cmH_2O respectively.

2.3.2 Experimental protocol

Anesthetized fish (MS222, Sigma) were weighed before surgery. Fish wet mass was used as a reference for setting $\dot{V}_b$. Surgery was conducted as described in Farrell et al. (1982), the procedure taking

10-20 minutes. The fish were placed ventral side up on an operating table, and the gills were irrigated with seawater. Stainless-steel cannulae were implanted and secured in the ventral aorta and the hepatic vein. The ducts of Cuvier, anterior jugular veins, and abdominal veins were ligated, so the only fluid entering the venous sinus was through the hepatic vein. The nerves going to the heart were destroyed.

The nerves, jugular veins, ducts of Cuvier and some of the abdominal veins were accessible through an incision in the opercular cavity. The ducts of Cuvier were left untied until the last moment so that the heart was supplied with blood during the procedure. After the ventral aorta cannula was secured a perfusion with heparinized saline was delivered through the hepatic vein cannula from a temporary reservoir (1 IU/ml; Na^+ heparin). The ducts of Cuvier were then tied closed, and the nerves were destroyed. After surgery, each animal was totally transected immediately caudal to the pectoral fins, then placed in a temperature controlled bath of Cortland saline. All abdominal viscera were removed. The perfusion saline composition was 150 mM NaCl, 2 mM $MgSO_4 \cdot 7H_2O$, 5 mM KCL, 2.3 mM $CaCl_2$, 2.3 mM Na_2HPO_4, 0.2 mM NaH_2PO_4, 3 g/l dextrose, and 40 g/l polyvinylpyrrolidone (MW = 40,000).

An initial perfusion of at least 15 minutes was allowed for tissue flushing and heart rate stabilization. Recordings were then made on a beat by beat basis of $\dot{V}_b$, f_H, and output pressure. The preload pressure was elevated slowly (<5 mm at each adjustment) until maximum $\dot{V}_b$ was attained. When maximum $\dot{V}_b$ stabilized (approximately 30 s),

another series of recordings were taken before restoring $\dot{V}_b$ and preload pressure to original levels. When original $\dot{V}_b$ had stabilized, an acute temperature change of about 10°C was made as follows: in winter fish, the perfusate and water jacket were warmed from 3.4 ± 0.2°C to 13.6 ± 0.3°C over 10.2 ± 0.8 minutes; in summer fish, the perfusate and water jacket were cooled from 13.3 ± 0.3°C to 4.1 ± 0.2°C over 19.9 ± 3.6 minutes. The temperature of the saline in the bath holding the preparation changed much more slowly than that of the perfusate. Therefore, heart tissue temperature was altered primarily by the perfusate passage. The temperature of the myocardium was not measured. It was assumed that the heart tissue temperature was stable at or near the perfusate temperature, because even though the lagging saline bath solution temperature was still changing, f_H was stable. Heart rate stability at the altered perfusate was required before the experiments proceeded. After the perfusate temperature change was completed, and heart rate was stable, the cardiovascular variables were recorded again. Preload was again elevated to achieve maximum $\dot{V}_b$ at the new temperature. The experiments were terminated shortly after the recordings at this stage. Ventricles were excised immediately, and after emptying contents and drying with tissue paper, wet mass was measured.

2.3.3 Experimental measurements and calculations

The preload and output pressures were measured with pressure transducers (Biotronex Laboratory, Kensington, Maryland). The volume

ejected with each heart beat (SV_H) was measured with an electromagnetic flow probe (Biotronex Laboratory, BL-5020). The flow probe was calibrated with known volumes of physiological saline at the experimental temperatures. Pressure and flow signals were amplified (Biotronex Laboratory, BL-630) and displayed on a chart recorder (Biotronex Laboratory, BL-882). Perfusate temperature was measured with a mercury thermometer immediately prior to entering the heart.

Heart rate was determined from pressure traces, and $\dot{V}_b$ was calculated as:

$$\dot{V}_b = SV_H * f_H.$$

Cadiac output values are in units of millilitres of blood per minute per kilogram wet mass of the fish ($ml/min \cdot kg^{-1}$). The power output of the ventricle was calculated as follows:

$$\text{power} = (\text{output pressure} - \text{preload pressure}) * \dot{V}_b,$$

and expressed as mW/kg ventricle wet mass. The resistance of inflow and outflow catheters of the heart were considered in all pressure measurements. All values presented are means $\pm$ standard error (SE), except where otherwise stated. Mean values were evaluated for significant differences using Student's t-test with $p < 0.05$ (unpaired tests were used for winter fish vs. summer fish comparisons; paired tests were used to compare temperature treatments within each seasonal group)

2.4 Blood volume, red blood cell distribution, and respiration studies

2.4.1 Experimental animals

Winter flounder (*Pseudopleuronectes americanus*) were captured by SCUBA divers from Conception Bay, Newfoundland, and kept in 40,000 liter seawater tanks for at least one week prior to experimentation (Fletcher, 1975). Respiration experiments were carried out from 30 September to 6 November (water temperature = 9 to 10°C), and 18 January to 28 March (water temperature = -0.5 to 1.6°C). The animals used for respiration experiments at 9-10°C cannot be refered to strictly as summer fish because at that time of the year day length is somewhat shortened. However, the temperatures which existed at that time were typical of those found in July and early August. For accuracy, the results from this group of experiments will be refered to as the high temperature values, except in Table 4, 6, and 7, and Figure 2 where the term summer is used for convenience. If a season must be refered to summer/fall would be appropriate. Animals were fed liberal amounts of chopped capelin daily during periods of high temperatures. Experimental fish were denied food at least 24 hours before surgery. After surgery, fish were allowed 1-3 days recovery before experiments commenced.

2.4.2 Animal preparations

All surgical manipulations were done on fish anesthetized with

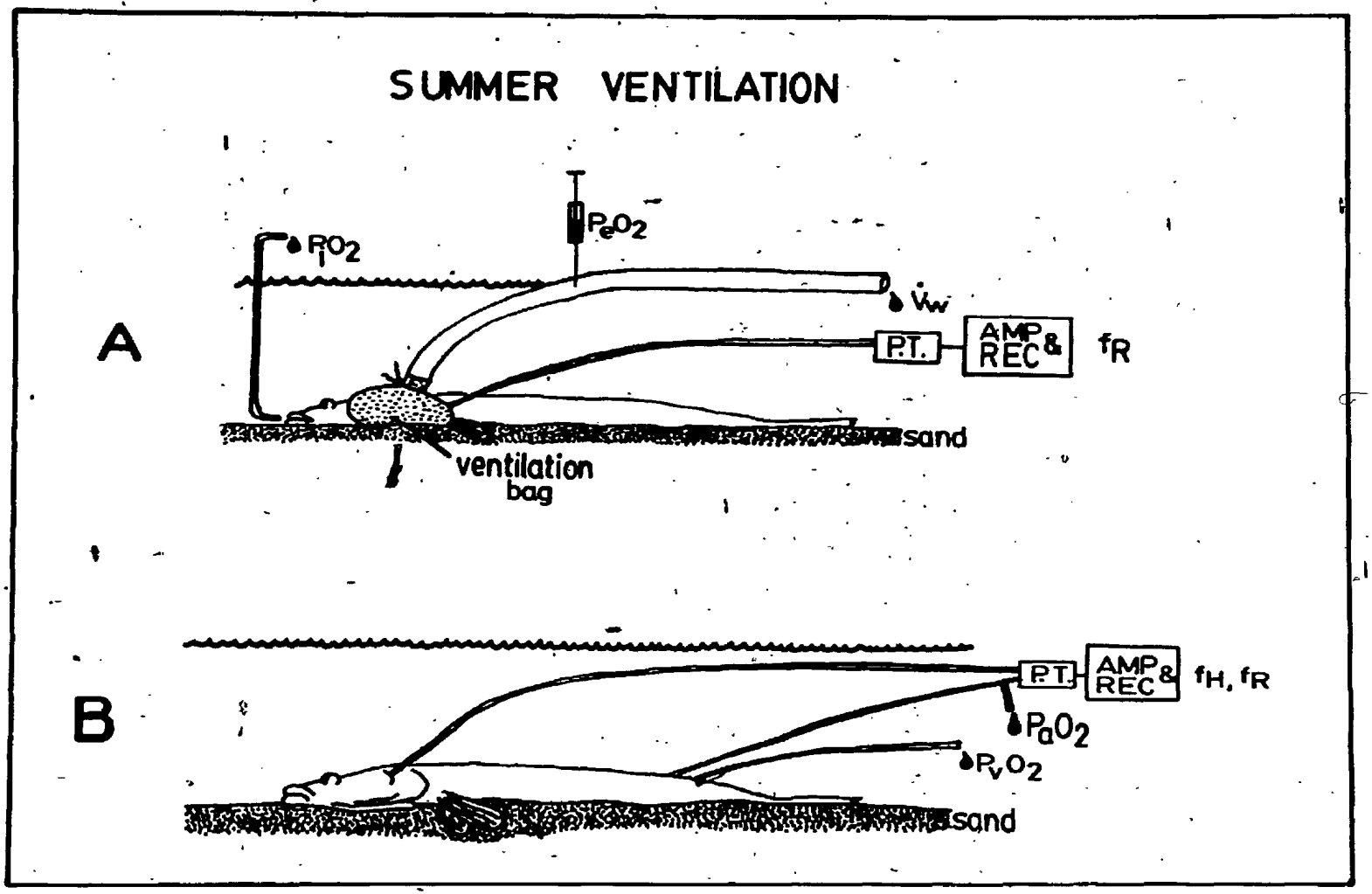
MS222. The fish at high temperatures were prepared in a different manner from the cold water fish. That precaution was essential, because during high temperatures the winter flounder does not tolerate excessive handling. The surgery at this time had to be as limited as possible, so two experimental groups were used. The preparations involved for each group are described below. All measurements were taken from fish resting on sandy bottomed tanks.

One of the groups used in high temperature experiments contained eight fish and was prepared for measurements of respiratory parameters. A water tight respiratory bag was stitched over the opercular apparatus of the fish (Figure 2). The bag collected all the water ventilating the gills ($\dot{V}_w$), but was applied loosely enough to minimize the restriction on breathing motions. The bags were made of pliable, strong dental-dam (Hygenic Corp., Ohio).

A small plastic tube attached the bag to a 6 mm wide vessel (inside diameter) leading to an overflow container. No significant backflow pressure was anticipated because of the low flow rate through the tube. The opening for the tube collecting $\dot{V}_w$ water was at the level of the water in the fish holding chamber. The partial pressure of oxygen for inflowing water to the gills (P_{iO_2}) was measured on samples taken directly beside the mouth (Radiometer microelectrode E5047 and amplifier, PHM71, Copenhagen). The oxygen tension from expiratory water (P_{eO_2}) was sampled from the collection tube.

A polyethylene cannula (PE90) was inserted 0.5 cm inside the opercular cavity at the junction of the opercular and preopercular bone on the dorsal side of the animal. The cannula was filled with seawater

Figure 2. Preparation for measuring the ventilation volume of the winter flounder during the summer. A. fish with ventilation bag attached. B. blood measurements. amp = amplifier, rec = recorder, p.t. = pressure transducer, all other symbols are described in the symbol legend (p. xi).


SUMMER VENTILATION
A
P_IO_2
P_eO_2
$\dot{V}_W$
P.T.
AMP & REC
f_R
sand
ventilation bag
B
P.T.
AMP & REC
f_H, f_R
P_aO_2
P_vO_2
sand

and attached to a pressure transducer (National Semi-conductor, LX1601 DF or Micro Switch, 126PC: see 2.2.3). Oscillation in opercular pressure was used to record the frequency of respiration (f_R). The amplified signals from the pressure transducer were displayed on a chart recorder (Hewlett Packard recorder, 7402A and preamplifier, 17402A).

The ventilation stroke volume (SV_R) was calculated as follows:

$$SV_R = \dot{V}_w/f_R.$$

Ventilation stroke volume values were in units of millilitres of water per breath per kilogram wet mass (ml/breath · kg^{-1}). The oxygen uptake at the gill ($\dot{V}_{O_2}$) was calculated as follows:

$$\dot{V}_{O_2} = (P_{iO_2} - P_{eO_2}) * \beta_w * \dot{V}_w,$$

where β_w was the solubility of oxygen in seawater at experimental temperature and salinity. The oxygen uptake values were in units of millilitres of O_2 per minute per kilogram wet mass of the fish (ml/min · kg^{-1}).

In a second group of fish at high temperatures (n = 9, Figure 2b) the caudal vein and artery were cannulated with polyethylene tubes (PE50). All fish were fitted with an arterial and a venous cannula. The arterial cannula was inserted into a lateral incision in the tail then fed anterior to the level of the dorsal aorta. The venous cannula was inserted from the incision anterior to the position of the kidney.

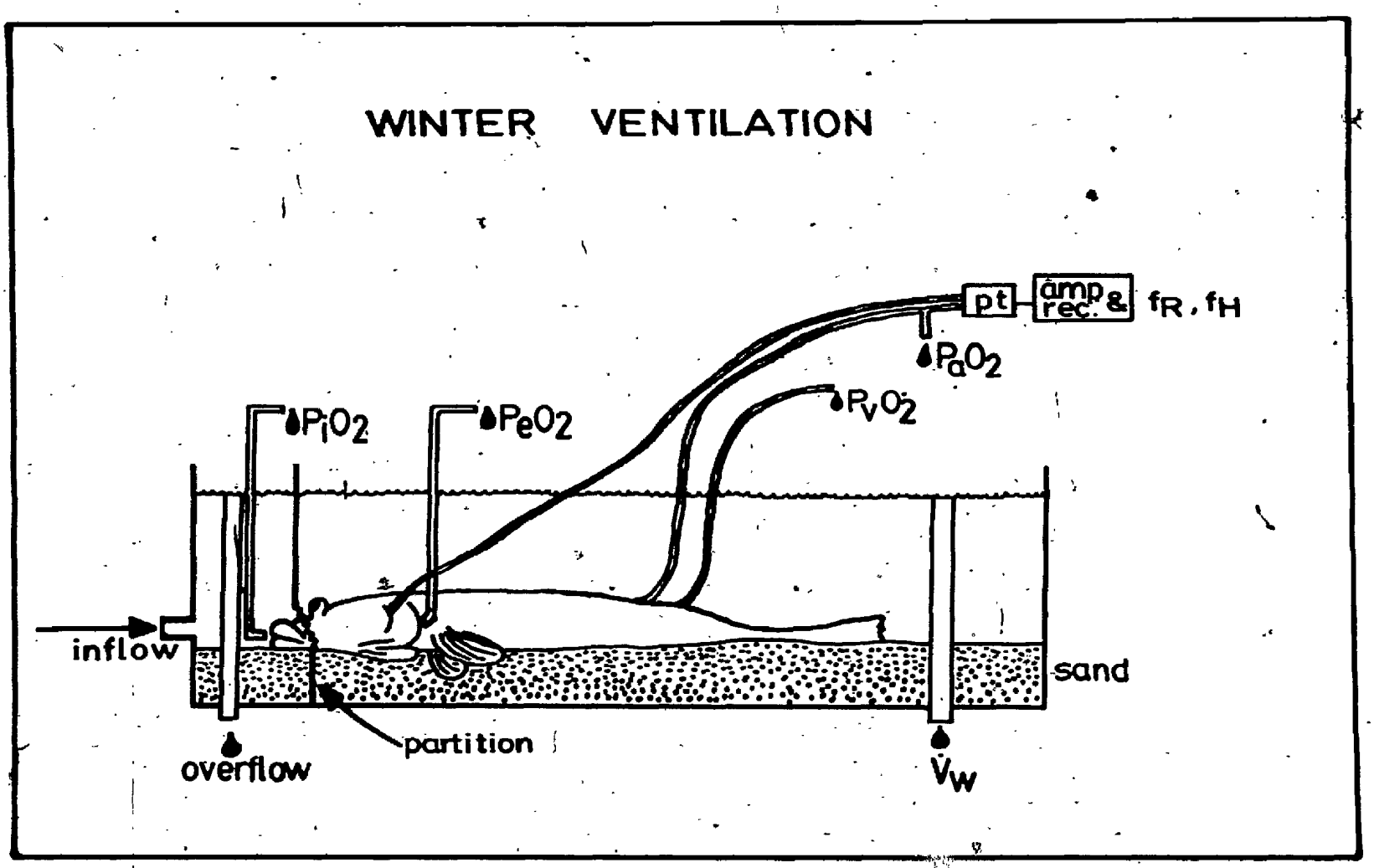
The cannulae were secured with silk sutures and filled with heparinized saline (100 units/mL, Sigma) (saline as above in section 2.1.2). Arterial and venous oxygen tension (P_{aO_2}, P_{vO_2}), and content (C_{aO_2}, C_{vO_2}) were measured. Heart rate (f_H) was monitored from pressure fluctuations after attaching the arterial catheter to a pressure transducer, and f_R was monitored as above. The fish in the two warm water groups had the following wet mass: 493 ± 61 g vs. 591 ± 44 g, $p < 0.05$. Cardiac output was estimated as:

$$\dot{V}_b = \frac{\dot{V}_{O_2}}{C_{aO_2} - C_{vO_2}}.$$

The units for $\dot{V}_b$ values are millilitres of blood per minute per kilogram wet mass of the fish (ml/min · kg^{-1}).

In winter fish (407.3 ± 36 g, n = 11) all of the above measurements were made on individual animals (Figure 3). The mouth and gill openings were isolated into separate water chambers (e.g. Heath, 1972). A thin membrane (dental-dam) was stitched about the fish's mouth and fitted into a partitioning box. Water level in the pre- and post-opercular chambers was equalised with the use of a manometry system. The $\dot{V}_W$ value was a measured volume of overflow water from the opercular compartment of the box. As with the warm water experiments, at least two measurements of the ventilation volume were made for each animal. The remainder of surgical and measurement procedures were the same as for the high temperature fish. The remaining caudal portion of the fish (approximately 75% of the body surface area), was isolated in

Figure 3. Preparation for measuring the ventilation volume of the winter flounder during the winter. All measurements for blood and water made on same fish. amp = amplifier, rec = recorder, p.t. = pressure transducer, all other symbols are described fully in the symbol legend (p. xi).


WINTER VENTILATION
pt
amp & rec.
f_R, f_H
P_aO_2
P_vO_2
P_iO_2
P_eO_2
inflow
sand
partition
overflow
$\dot{V}_W$

a separate compartment. When the ventilation measurements were finished the oxygen uptake by the skin was estimated over a 1-8 hour period. The surface area of each fish was estimated from tracings of the outline of the fish made on calibrated graph paper.

The two ventilation methods could only be compared on the winter acclimated animals. Three winter fish with ventilatory bags were used in the comparison.

2.4.3 Blood volume and red blood cell distribution

Winter (February-March) and summer (August-September) acclimatized fish were used to determine blood volume and the instantaneous distribution of red blood cells, following the methods outlined by Albert (1971). The blood volume of winter fish was examined at the seasonal temperature (0°C, 335.9 ± 61.4 g, n = 8), and after 8-15 days at 8.5°C (367.8 ± 45.9 g, n = 8). The blood volume of summer fish was examined at the seasonal temperature (10°C, 302.3 ± 68.1 g, n = 6), and after 20 days at 1°C (296.7 ± 49.8 g, n = 5).

Radioactive chromium (^{51}Cr) was used to tag rbc's which were later to be injected into the circulatory system. A concentration of 100 μCi ^{51}Cr/mL of heparinized blood was mixed in the presence of 3% CO_2/ balance air for 1.5- 2 hours. The cells were then washed three times with flounder saline (see section 2.1.3) before a 0.3 mL infusion (cells were reconstituted to approximately the same Ht as original blood sample with saline). Tagged rbc's were introduced into the caudal vein or artery in unanesthetized flounders. Any leftover cells

were used to determine predilution activity (cpm_i). A time period of 2.5-4 hours was allowed for cells to distribute throughout the circulatory system. Previous tests determined that 2 hours was sufficient to allow complete mixing for blood volume determinations at 1°C (G.L. Fletcher, personal communication).

After the equilibration period, a caudal puncture was used to draw blood from unanesthetized fish. The flounder was then totally immersed in liquid nitrogen which effectively stops all circulation rapidly. Liquid nitrogen was held in a heavily insulated container that restricted movement by the fish. The time of blood sampling to the time of total tissue freezing was never longer than 30 seconds. The blood sample was placed in a heparinized container for counting and hematocrit determinations. Hematocrit was determined as above. The activity of a 0.1 mL sample of the final blood sample was counted (cpm_f), then used in the following volume calculation:

$$\text{blood volume} = \frac{cpm_i}{cpm_f}.$$

The volume was then standardized to a percentage of the total wet mass of the fish.

Frozen tissues were sectioned from the flounder: total liver, spleen, kidney, intestine, gill filaments, and portions of the white muscle from the central part of the dark surface of the animal. Samples of dermis and epidermis were taken from the same region as the white muscle. Preweighed tissues were analysed for gamma emissions over a 20 minute period. The values presented are expressed as the

percent of the total tissue mass that was rbc's.

The mean values for high and low temperature experiments were compared using the unpaired Student's t-test with $p < 0.05$.

CHAPTER 3

SEASONAL CHANGES IN HEMATOLOGY AND HEMOGLOBIN FUNCTION

3.1 Introduction

The manner in which oxygen is delivered to the tissues in vertebrates has been described as a cascade, where oxygen flows along pressure gradients from regions of high oxygen values (arterial blood) to the working units of the cells (mitochondria) (Wood and Lenfant, 1979). At a given oxygen tension in the circulation (P_{O_2}), the percent of circulating hemoglobin that is bound to oxygen can be predicted. Such predictions are made from the results of *in vitro* tonometry, using the P_{50} (half saturation of hemoglobin with oxygen) as a common reference.

To understand the oxygen delivery system, one must begin with a clear description of the hematological parameters of the organism. For example, the amount of hemoglobin in the blood will directly affect the amount of circulating oxygen. Some hematological features have been found to change with the alterations in demand for oxygen during strenuous exercise (Wood, Turner, and Graham, 1983; Yamamoto, Itazawa, and Kobayashi, 1980), hypoxia (Wood and Johansen, 1973), and temperature change (Houston and Cyr, 1974; Cameron, 1970; DeWilde and Houston, 1967). The changes in hematological variables such as Ht, rbc count or corpuscular hemoglobin content (MHC) are relatively slow compared with the speed at which alterations in cardiovasuclar, ventilatory and

hemoglobin function occur (Weber, 1982).

This section investigates some possible functional modifiers of hemoglobin, and seasonal changes in hematological variables in the winter flounder. The affinity of hemoglobin to bind with oxygen can be affected by a number of factors found within the red blood cells; some organic, others inorganic. Intraerythrocytic concentrations of Mg^{++}, Ca^{++}, H^{+}, Cl^{-}, and nucleotide triphosphates (NTP) were measured in the rbc's of winter acclimatized (0°C) and summer acclimatized (10°C) fish. Inorganic ions and adenosine triphosphate (ATP) were then added to prepared hemoglobin solutions from which concentrations of other modifying agents had been removed; the temperature of the hemoglobin solutions was at an intermediate 5°C. The levels of hemoglobin modifiers added covered the seasonal range of values observed *in vivo*. This experiment enabled a comparison to be made of the relative importance of each modifier as it affected oxygen affinity. The direct effects of temperature on hemoglobin within rbc's and in prepared solutions were also observed. Red blood cells from winter acclimatized, and summer acclimatized fish were subjected to tonometry at 0° and 10°C.

3.2 Results

3.2.1 Seasonal hematology

Table 1 is a summary of the rbc data on the winter flounder. There is significant difference between summer fish and winter fish

Table 1: Hematological data for the winter flounder. [Hb] = hemoglobin concentration, Ht = hematocrit, MHC = mean hemoglobin content per cell (nanograms), total ions = sum in mM/l cells for Na^+, Cl^-, K^+, Ca^{++}, Mg^{++} in the red blood cells. Values are means ± SE. () = n.

	[Hb] (g/100 ml)	% met Hb	Ht (%)	cell #'s (ml^{-1})	MHC (ng/cell)	cell volume (μm^3)	% water	total ions (mM/l cells)
Winter	7.58 ±.48 (10)	13.52 ±0.59 (15)	25.00 ±1.28 (10)	2.62×10^9 ±.10 $\times 10^9$ (9)	31.17 ±1.08 (9)	101.4 ±3.09 (9)	65.9 ±0.3 (16)	199.4 8.35 (10)
Summer	5.65 ±.32 (10)	13.08 ±0.96 (21)	20.93 ±1.33 (10)	2.18×10^9 ±.15 $\times 10^9$ (10)	28.19 ±0.66 (10)	90.30 ±3.03 (10)	69.6 ±0.7 (10)	199.3 ±3.47 (10)
	$p < 0.05$	N.S.	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	N.S.

Note: N.S. is the abbreviation for not significant. Mean values for winter and summer were compared by using the unpaired Student's t-test. Significance was at the 95% confidence level.

with respect to rbc size and number, total [Hb], MHC, and Ht. The summer fish have fewer, smaller rbc's with less hemoglobin in each cell, resulting in a decrease in [Hb] and Ht for the group. A large percentage of the total [Hb] (13%) is in the form of methemoglobin during the winter and summer sampling times.

3.2.2 Seasonal red blood cell ion and NTP levels

Table 2 summarizes the intraerythrocytic ion levels found in the winter flounder. Chloride values are significantly lower in winter fish than in summer fish, but Mg^{++} and Ca^{++} levels are higher in winter fish. The Ca^{++}/Hb molar ratios increase significantly in winter fish. The lowest ion values are those of Ca^{++}, followed by Mg^{++} and Cl^{-}. The extracellular (pH_e) and intracellular (pH_i) pH values are inversely related to temperature (Figure 4). Observed pH_e changes resemble those of water (pH_{H2O}), while pH_i is relatively stable throughout the year.

The NTP/Hb molar ratios are significantly higher in summer fish rbc's than in those of winter fish (Table 2). Temperature is responsible for the total seasonal shift in the ratios (Figure 5). During exposure of the summer fish group to cold water, the NTP/Hb levels actually fall below those for winter acclimatized fish.

3.2.3 Water content of red blood cells

The water content of summer rbc's is significantly greater than

Table 2: Seasonal difference in red blood cell contents for the winter flounder. Mean values are averages ± SE. () = number of animals used. N.S. = not significant.

	Summer (μmoles/mL cells)	Winter (μmoles/mL cells)	Significance	Summer (X/HB)	Winter (X/HB)	Significance
K^+	97.6 ±6.06 (16)	94.3 ±8.52 (10)	NS	22.04 ±0.82 (10)	20.24 ±0.55 (10)	$p < 0.05$
Cl^-	69.2 ±1.8 (16)	59.5 ±4.4 (10)	$p < 0.05$	18.44 ±1.1 (10)	12.9 ±0.8 (10)	$p < 0.05$
Na^+	29.3 ±6.13 (16)	41.7 ±10.2 (10)	NS	7.50 ±0.68 (10)	8.96 ±0.74 (10)	$p < 0.05$
Ca^{++}	0.29 ±0.02 (16)	0.55 ±0.06 (10)	$p < 0.05$	0.09 ±0.01 (10)	0.13 ±0.01 (10)	$p < 0.05$
Mg^{++}	4.88 ±0.13 (16)	5.65 ±0.20 (10)	$p < 0.05$	1.22 ±0.09 (10)	1.30 ±0.07 (10)	NS
NTP	6.25 ±0.51 (10)	6.01 ±0.25 (10)	NS	1.58 ±0.09 (10)	1.36 ±0.03 (10)	$p < 0.05$

Note: N.S. is the abbreviation for not significant. Mean values for winter and summer were compared using the unpaired Student's t-test. Significance was at the 95% confidence level.

Figure 4. Plasma and red blood cell pH (pH_e and pH_i) for the winter flounder at different times of the year. Symbols are means ± SE. The buffer line shows the pH-temperature dependence of a phosphate buffer system (Radiometer, Copenhagen, S1500). The pH temperature dependence of H_2O is also given.

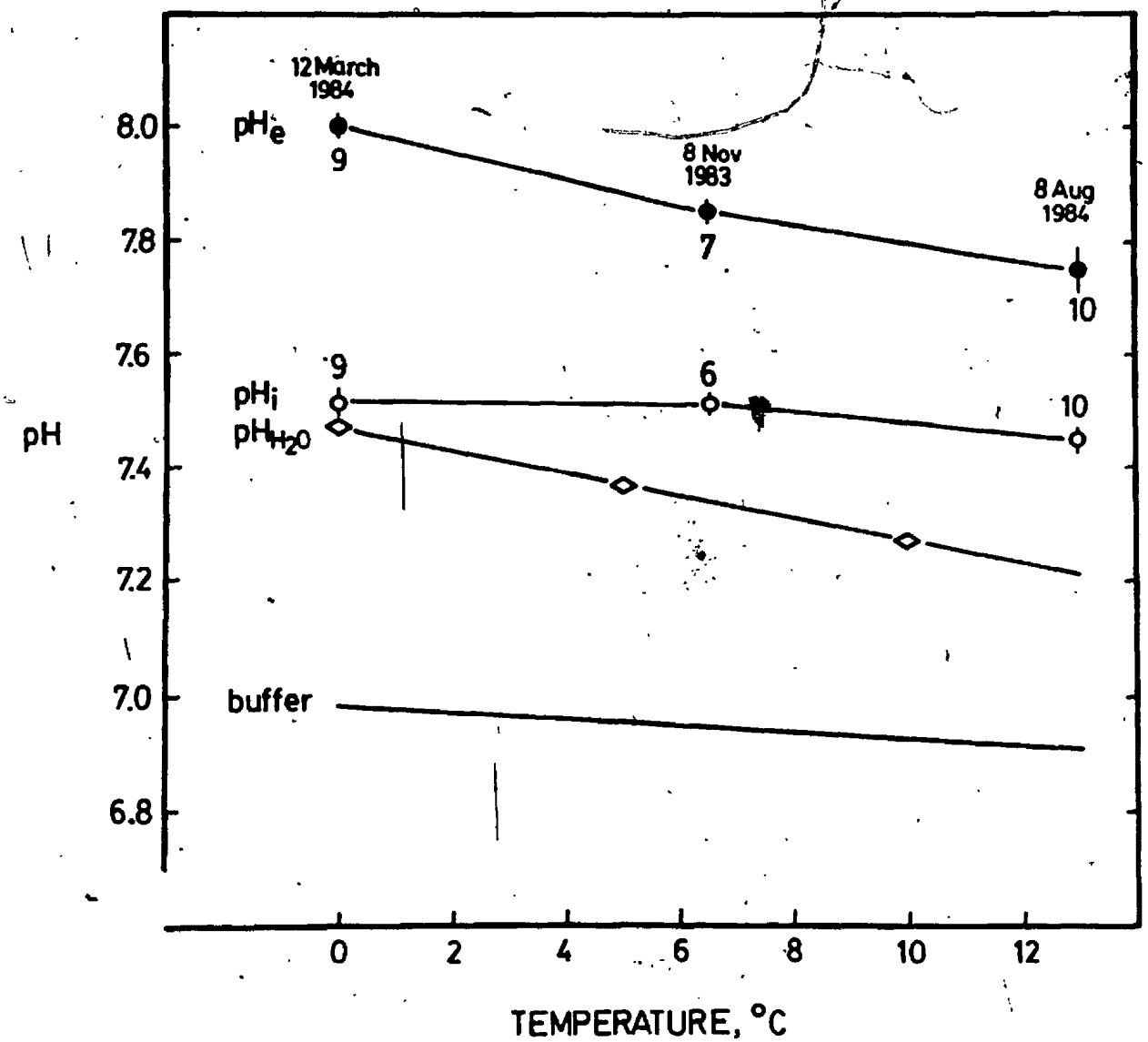
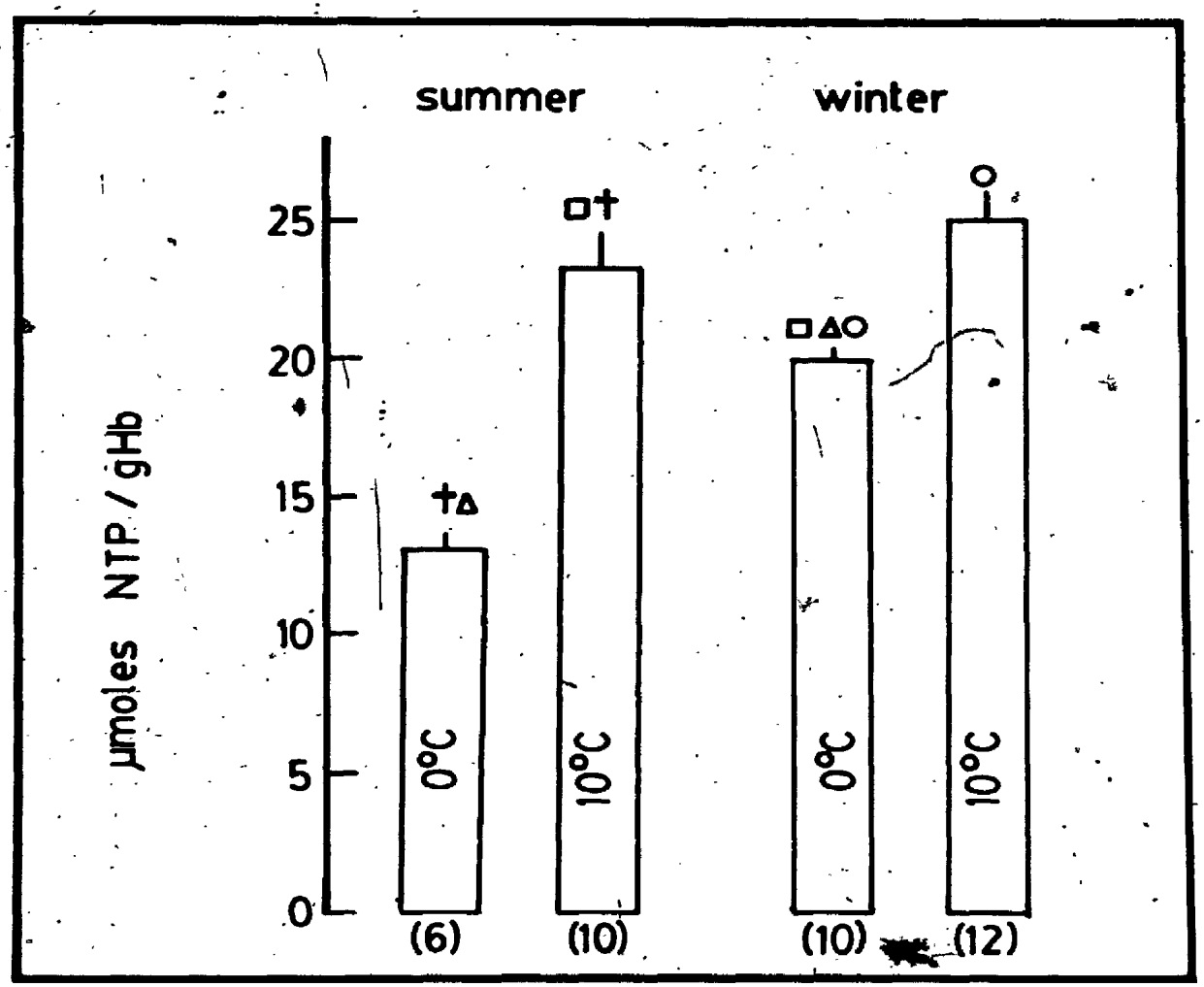

12 March 1984
8 Nov 1983
8 Aug 1984
pHe
pHi
pH$_{H_2O}$
buffer
9
7
10
9
6
10
pH
8.0
7.8
7.6
7.4
7.2
7.0
6.8
0
2
4
6
8
10
12
TEMPERATURE, °C

Figure 5. Summer and winter red blood cell nucleotide triphosphate concentrations for the winter flounder. Height of the bars equals the mean value and the vertical lines are SEM. ( ) = n. Symbols above bars match experimental groups that were significantly different ($p < 0.05$). Significance testing was done by using the unpaired Student's t-test.


summer
winter
µmoles NTP / gHb
25
20
15
10
5
0
0°C
10°C
0°C
10°C
(6)
(10)
(10)
(12)

of winter rbc's (Table 1). There is no difference between the ion concentrations of summer fish and winter fish rbc's (Table 1).

3.2.4 The effect of temperature on whole blood oxygen affinity

Figure 6 shows the effects of temperature on the Hb-oxygen dissociation of whole blood. The P_{50} values from low temperature experiments are always lower than values from high temperature tests. The average seasonal change in the P_{50} of whole blood is 5.7 torr (Figure 6). The full seasonal P_{50} shift is demonstrated during an acute temperature change to winter and summer fish blood. Summer fish blood brought to winter temperatures has P_{50} values that are not significantly different from the values of P_{50} in blood from winter acclimatized fish. Winter fish blood brought to summer temperatures has P_{50} values that are not significantly different from the P_{50} values in blood from summer acclimatized fish.

3.2.5 The effect of temperature on the oxygen affinity of hemoglobin solutions

The influence of temperature alone accounted for 65 per cent of the total seasonal P_{50} shift for whole blood (Table 3, Figure 7). Temperature is approximately four times more influential than ATP, the next most important functional modifier. Hemoglobin from summer acclimatized (July-August) fish and winter acclimatized (December) fish show the same P_{50} values when tested at 5°C (Figure 7).

Figure 6. The effect of temperature upon hemoglobin dissociation characteristics for the whole blood of the winter flounder during winter and summer periods. Open symbols were for 10°C tests (n = 3 for winter and n = 3 for summer tests). Closed symbols were tests at 0°C (n = 3 for winter and 4 for summer tests). Hemoglobin concentrations were: summer (0°C) = 5.21 ± 0.32 g/100 ml (mean ± SE); summer (10°C) = 5.65 ± 0.96 g/100 ml; winter (0°C) = 4.82 ± 0.42 g/100 ml; and winter (10°C) = 5.06 ± 0.27 g/100 ml. Lines were fit to points by eye.

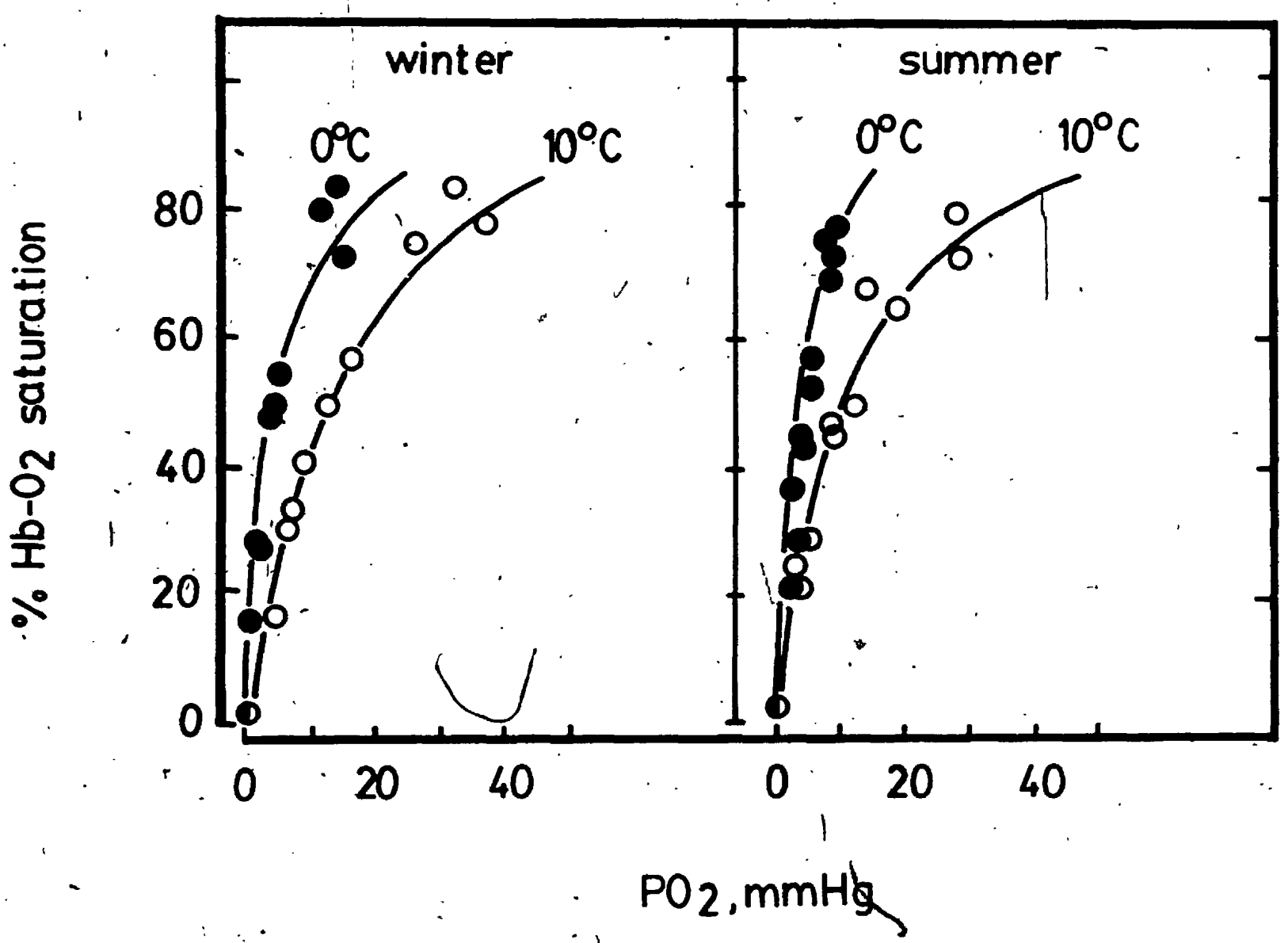
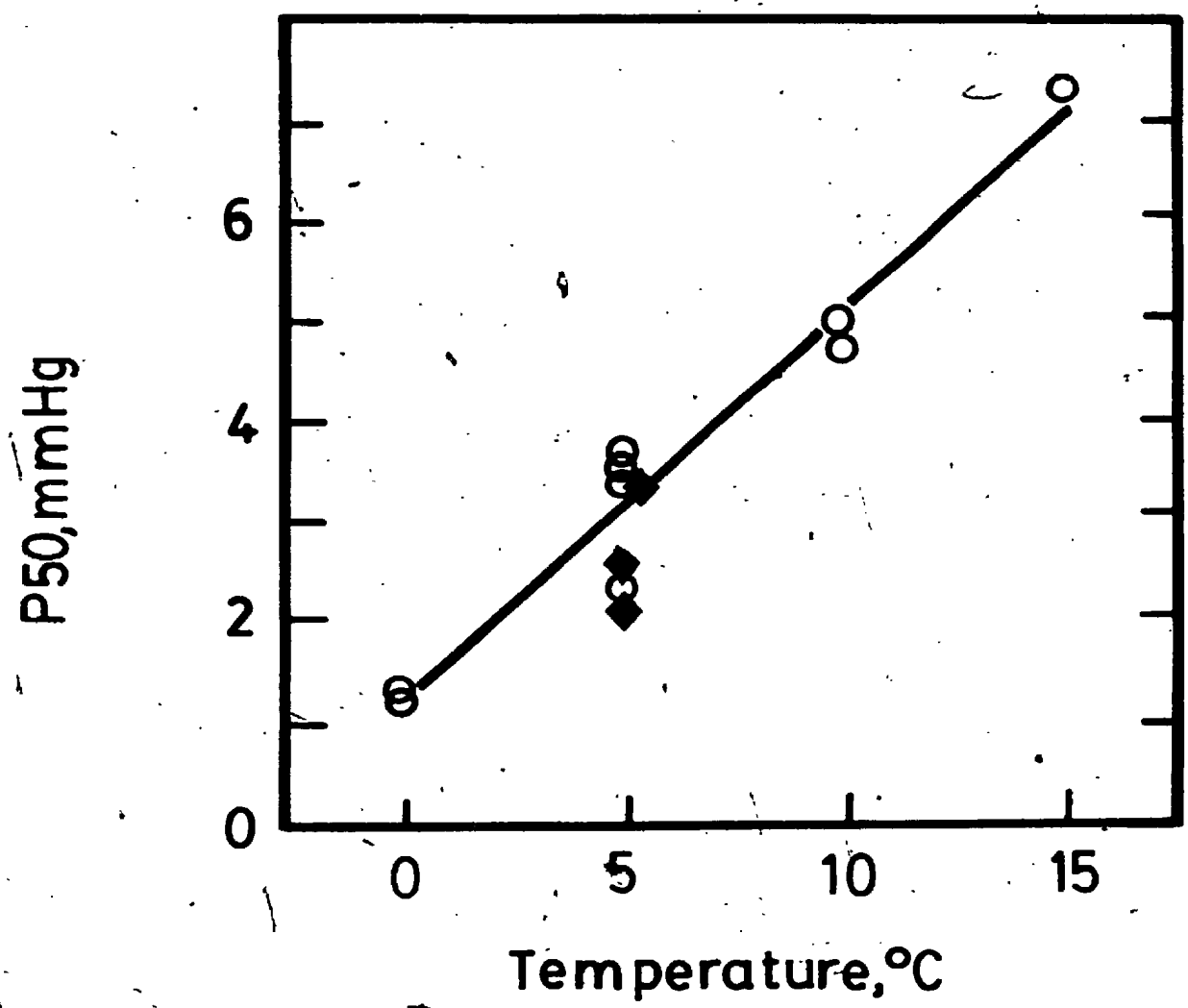

winter
summer
0°C
10°C
0°C
10°C
% Hb-O2 saturation
0
20
40
60
80
0
20
40
0
20
40
PO2, mmHg

Figure 7. The effect of temperature upon oxy-hemoglobin dissociation characteristics for hemoglobin solutions of the winter flounder. Symbols are individual values from pooled blood samples (n = 5 to 10 fish). Open circle values were from summer acclimatized fish (July-August) and closed symbols were from winter acclimatized fish (December). No ATP was present. Regression equation was $P_{50} = 0.37$ (temperature) + 1.33 (r = 0.99). Hemoglobin concentration = 7.14 $\pm$ 2.28 g/100 ml.


P50,mmHg
0
2
4
6
0
5
10
15
Temperature,°C

3.2.6 Intracellular modulators of hemoglobin function

The most important intracellular modifier of these tests is ATP (Table 3). The effect of ATP on Hb-oxygen affinity is given in Figure 8. The effect of ATP is the same at both high and low experimental temperatures. The seasonal range of [NTP] (shaded region in Figure 8) causes the P_{50} to change by 1.0 mmHg (approximately 17.5 per cent of the total seasonal P_{50} shift for whole blood; Table 3).

Chloride ions are the next most influential intracellular Hb functional modifier. The seasonal change in Cl^- concentration causes a P_{50} shift of 0.6 mmHg, or 10.5 per cent of the total seasonal value for whole blood (Figure 9, Table 3). Chloride ions act in the same manner as ATP, so P_{50} values increase as Cl^-/Hb values increase. An increase in Cl^- concentration in the presence of ATP decreases the effectiveness of the nucleotide in altering Hb function (Figure 9). The Cl^-/Hb interaction suggests a competition for Hb binding sites.

Calcium and magnesium have no direct effect upon hemoglobin function in the absence of all other functional modifiers (Figure 10). Both influence the effectiveness of ATP in altering Hb function. Calcium is effective at a much higher concentration than is found *in vivo*. The effect of Mg^{++} on ATP occurred within the seasonal range of Mg^{++} values in the rbc's.

Hydrogen ions have a small effect upon the Hb function of the winter flounder. The Bohr and Root effects are small, which corresponds to the small change in pH_i values between winter and summer

Table 3: Intracellular modulators of hemoglobin function. Total seasonal P_{50} shift was 5.7 torr (see text section 3.24 and figure 6): % of total seasonal P_{50} shift values were calculated from this. Direct influence on Hb-oxygen affinity (P_{50}) = results obtained in the absence of all other Hb functional modifiers (eg. NTP and ions). The total direct influence was taken as the total P_{50} shift occurring over the observed seasonal concentration of ions and NTP (shaded bars on figures 8-11; at 5°C) or over the temperature range 0-10°C (figure 7).

Modulator	Direct influence on oxygen affinity (torr)	% of total
Temperature	3.7	64.9
ATP	1.0	17.5
Cl^-	0.6	10.5
H^+	0.3	5.3
Ca^{2+} & Mg^{2+}	0.0	0.0
Total	5.6	98.2

Figure 8. The effect of ATP on the P_{50} of winter flounder hemoglobin solutions at 0°C (●) and 10°C (○). Hemoglobin concentration was 4.18 g/100 ml. Shaded bar = seasonal range of NTP/Hb values. The left margin of the bar represents an average winter (w) ratio, and the right margin represents an average summer (s) value. For specific winter and summer ratios see Table 2. All values are from a single pooled sample (n = 10).

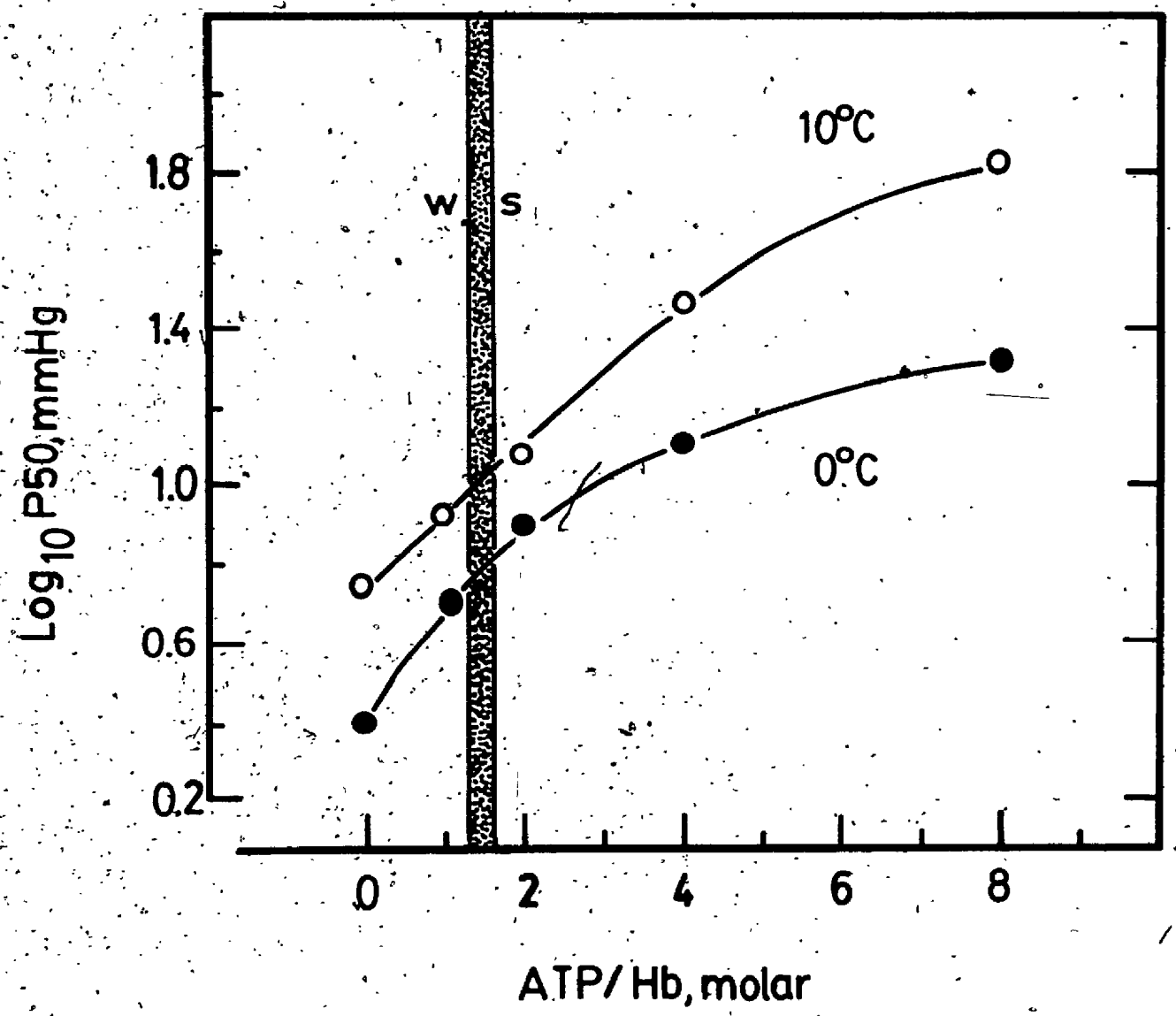

10°C
0°C
w
s
1.8
1.4
1.0
0.6
0.2
0
2
4
6
8
Log10 P50, mmHg
ATP/ Hb, molar

Figure 9. Cl^-/Hb molar ratio and the effect on the oxy-hemoglobin dissociation values of winter flounder hemoglobin solutions with ATP (ATP/Hb = 1.5:1) (●) and without (○). Shaded bar and letters as in Figure 8. n = 5 to 10 fish. Hemoglobin concentration was 5.50 g/100 ml.

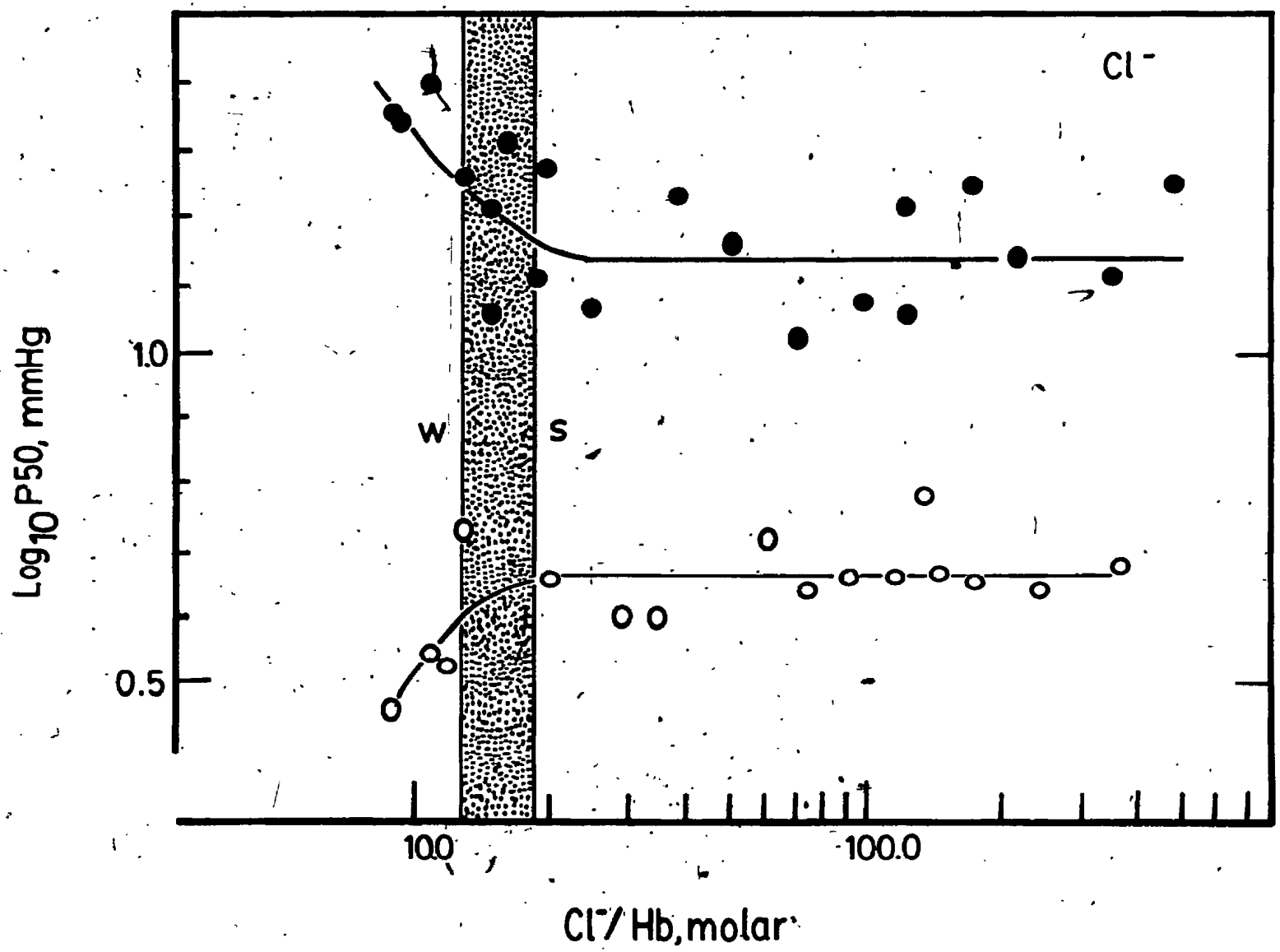
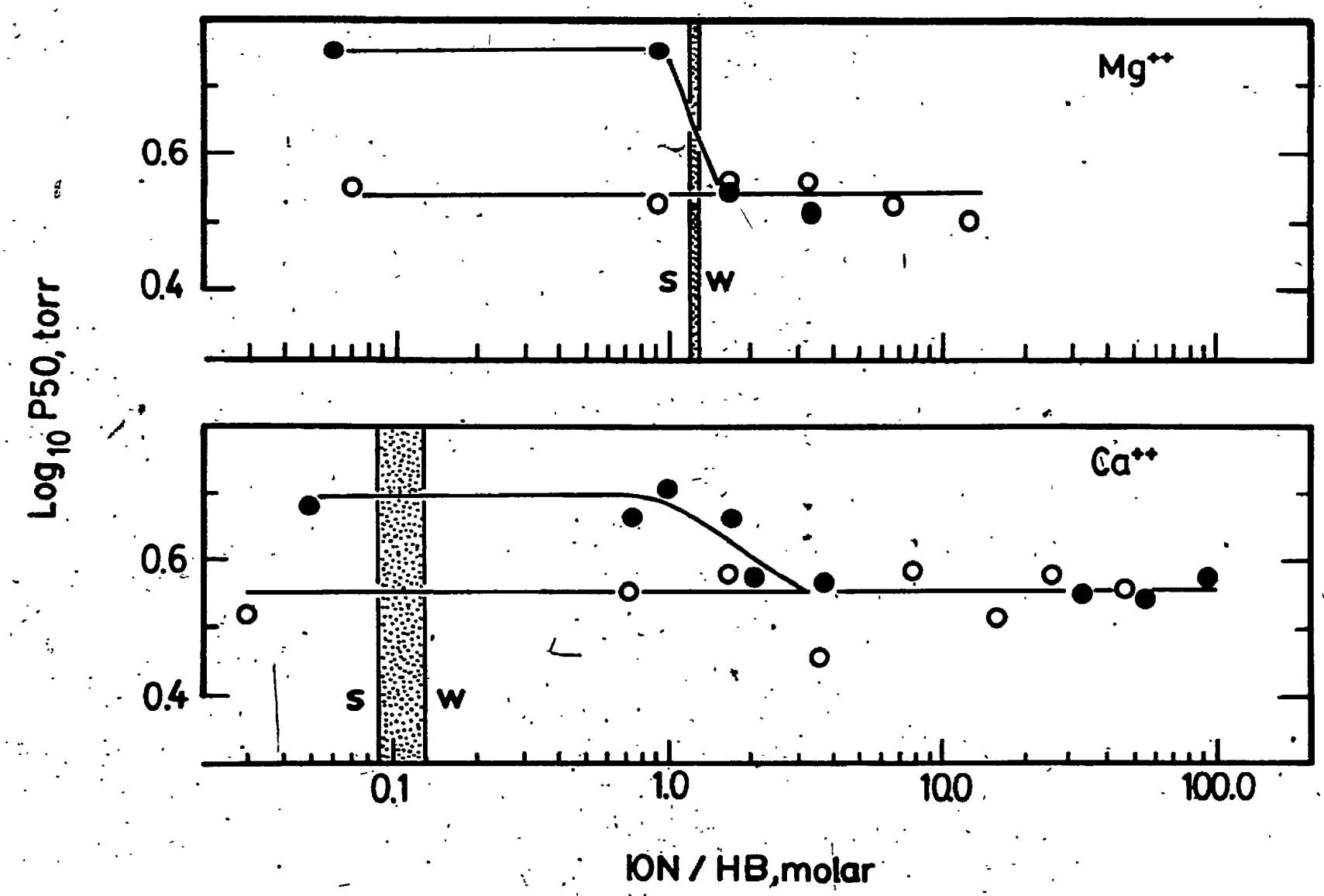

Cl⁻
W
S
1.0
0.5
Log10 P50, mmHg
10.0
100.0
Cl⁻/Hb, molar

Figure 10. Mg^{++} and Ca^{++}/Hb molar ratios and the effects on oxy-hemoglobin dissociation values of winter flounder hemoglobin solutions, with and without ATP. The right margin of the shaded bar represents an average winter (w) ratio, and the right margin represents an average summer (s) value. For specific winter and summer ratios see Table 2. Hemoglobin concentration was 5.15 g/100 ml.


Mg++
Ca++
0.6
0.4
0.6
0.4
s
w
s
w
Log10 P50, torr
0.1
1.0
10.0
100.0
ION / HB, molar

acclimatized fish (Figure 11 and 12). There is no significant difference between the Bohr shift ($\Delta \log_{10} P_{50}/\Delta pH$) at 0°C (-0.66) and 10°C (-0.43) ($F = 4.63$, d.f. = 1, 11, $p < 0.05$). The Bohr shift is responsible for 5.3 per cent of the total seasonal P_{50} change for whole blood (Table 3).

The influence of pH on oxy-hemoglobin saturation is the same at 0° and 10°C (Figure 12). The seasonal change in pH_i for winter flounder rbc's alters oxy-hemoglobin saturation by 4.5 per cent.

3.3 Discussion

3.3.1 General

During seasonal change there are a number of physical alterations in the environment of winter flounder. One important change is temperature. Temperature change is able to alter significantly the metabolic processes of poikilotherms and thus the demand for oxygen by the living tissues. Hemoglobin is recognized as being an important oxygen carrier. The following discussion describes the effect of season and temperature upon the quantitative and functional aspects of hemoglobin.

3.3.2 Seasonal hematology of the winter flounder

Summer acclimatized winter flounder have reduced capacity to carry oxygen in the blood. The findings in this study agree with the Hb

Figure 11. The Bohr effect of flounder hemoglobin at 0°C (●) and 10°C (○). No ATP was present during tests. The shaded area = seasonal range of rbc pH_i where w = winter mean value and s = summer mean value. Symbols are individual readings from pooled samples (n = 5 to 10 fish). Regressions were: 10°C, $\log_{10} P_{50} = -0.43\ (pH_i) + 4.19$ ($r = -0.97$); and 0°C, $\log_{10} P_{50} = -0.66\ (pH_i) + 5.49$ ($r = -0.92$). Hemoglobin concentrations were: 10°C = 4.75 ± 0.11 g/100 ml; and 0°C = 4.56 ± 0.10 g/100 ml.

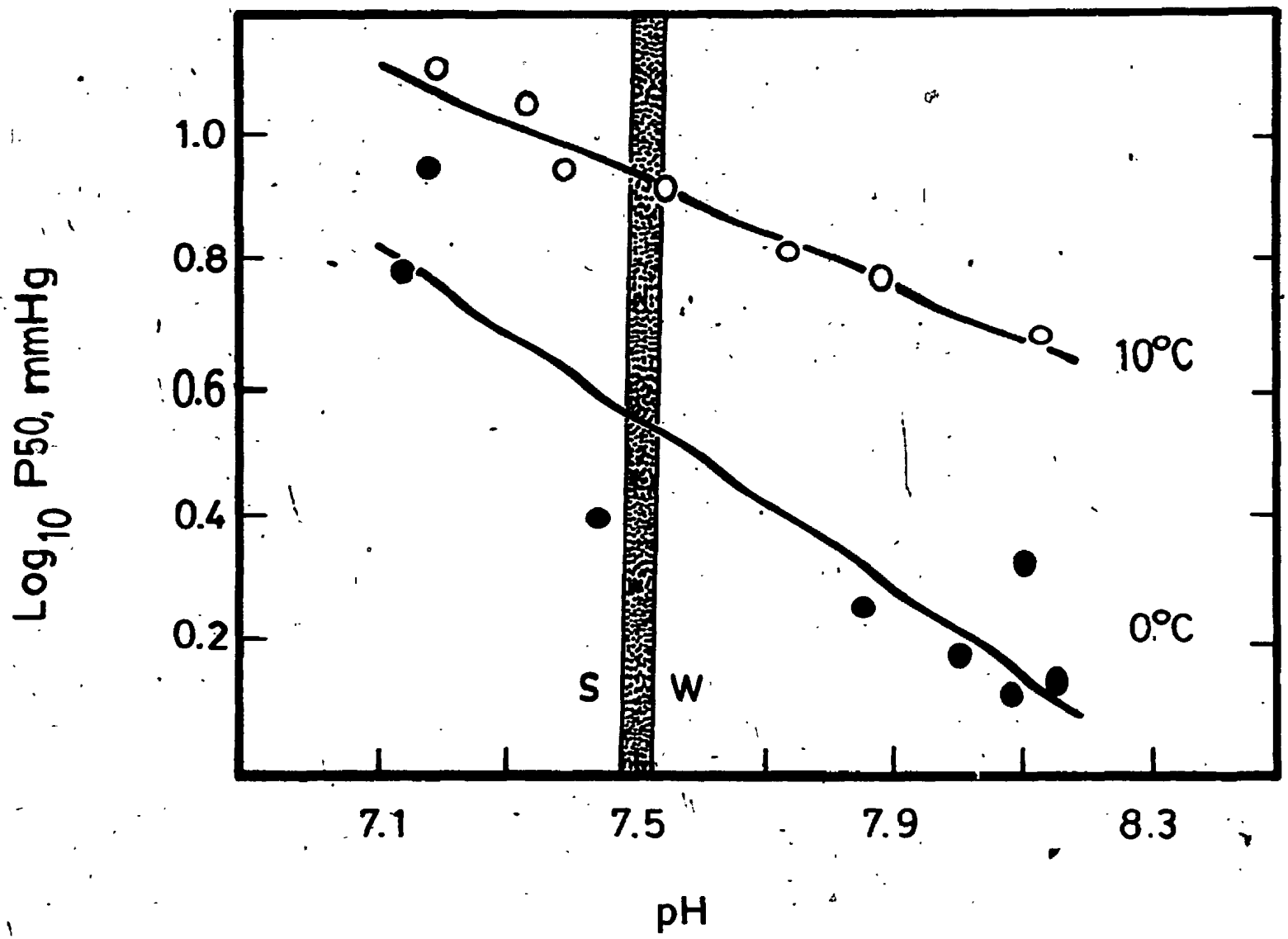
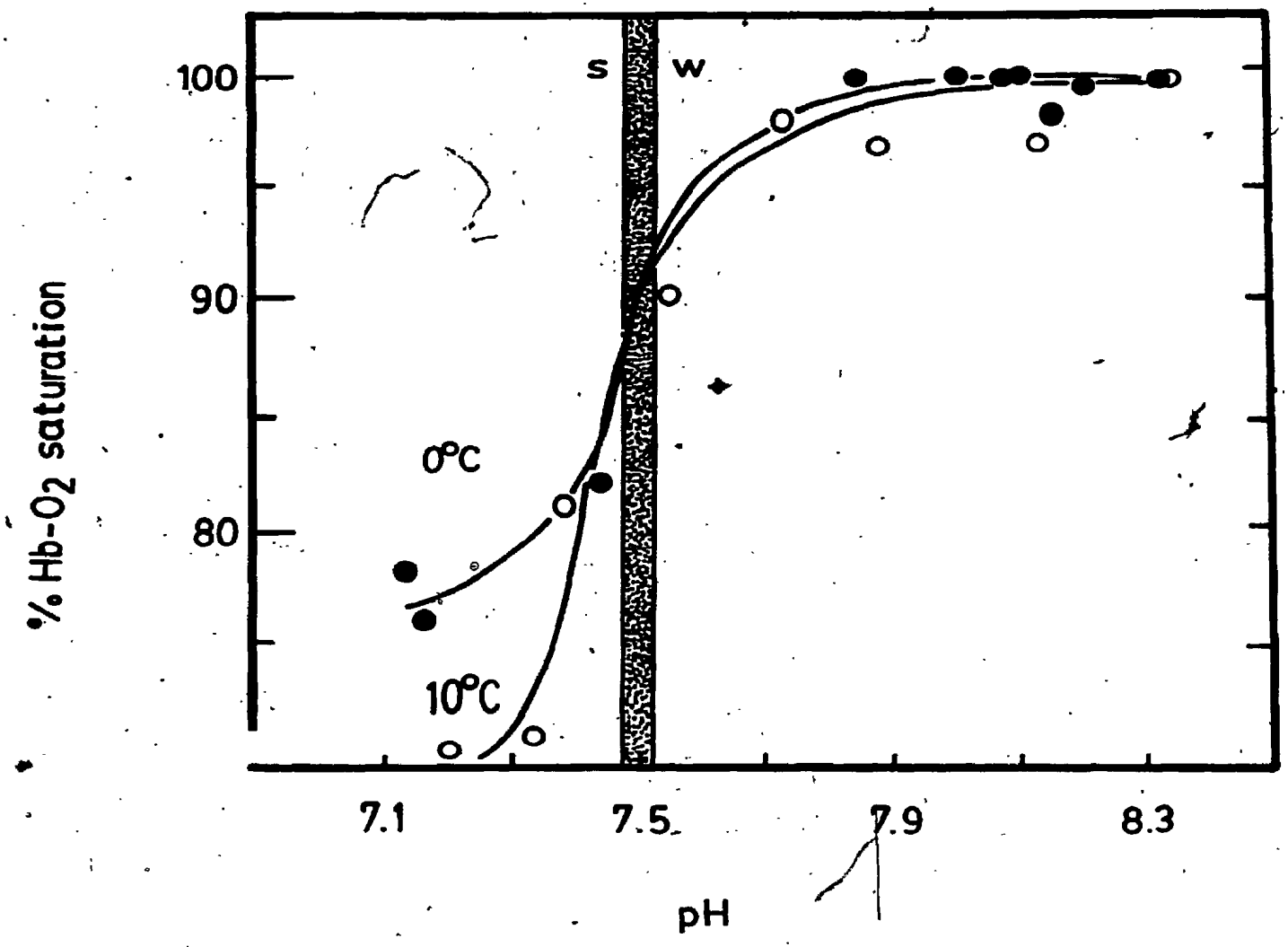

Log10 P50, mmHg
1.0
0.8
0.6
0.4
0.2
S
W
10°C
0°C
7.1
7.5
7.9
8.3
pH

Figure 12. Root effect of flounder hemoglobin at 0°C (●) and 10°C (○). Symbols are individual readings from pooled samples (n = 5 to 10 fish). The shaded area = seasonal range of rbc pH_i, where w = winter mean value and s = summer mean value. Hemoglobin concentrations were: 10°C = 4.75 ± 0.11 g/100 ml, and 0°C = 4.56 ± 0.10 g/100 ml.


% Hb-O2 saturation
100
90
80
S
W
0°C
10°C
7.1
7.5
7.9
8.3
pH

values reported by Cech et al. (1976) for the winter flounder. The hematocrit of the brown bullhead (Ictalurus nebulosus) was found by Grigg (1969) to decrease during high temperature acclimation. However, the pinfish (Lagodon rhomboides) and the striped mullet (Mugil caphalus) demonstrated [Hb] increases during exposure to high temperature (Cameron, 1970). In studies by Nikinmaa et al. (1981 and 1980), the Ht and [Hb] of rainbow trout were shown to decrease during high temperature acclimation; but in other studies by Houston and Cyr, in 1974, and by DeWilde and Houston, in 1967, the rainbow trout demonstrated [Hb] increases during exposure to high temperature.

There are two obvious patterns in [Hb] adjustment to temperature change. At this point, it is difficult to determine why such patterns develop. The differences are species specific, with the exception of the rainbow trout. In the case of the trout, Nikinmaa et al.'s fish were a Scandinavia variety and Houston and coworkers fish were from hatcheries in Ontario, Canada. Perhaps there are real physiological differences between these strains of trout. Further research into the circulatory and respiratory physiology of the winter flounder (Chapter 4, 5 and 6) will assist in understanding the seasonal hematology of the flounder at least.

The red blood cell volume of summer acclimatized winter flounders was significantly smaller than that of winter acclimatized fish. Holland (1970) determined that smaller rbc's become oxygenated more readily. Therefore, the summer acclimatized flounder have less hemoglobin contained within rbc's that may become oxygenated more readily.

An interesting feature of the hematology of winter flounder is the

large amount of methemoglobin (Table 1, Appendix 1). Methemoglobin does not assist in the transport of oxygen, and in humans methemoglobin levels are usually less than 1 per cent of the total hemoglobin (Dijkhuizen, Buursma, Fongers, Gerding, Oesburg, and Zijlstra, 1977; Henry, 1964). The presence of high concentrations of methemoglobin in the winter flounder would reduce the capacity of the blood to carry oxygen. High values (2 per cent to 28 per cent) of methemoglobin have been found in several other species of fish (Dobson and Baldwin, 1982a; Heuy and Beitinger, 1982; Cameron, 1971, Appendix 1).

The presence of methemoglobin tends to make the shape of oxyhemoglobin dissociation curves more hyperbolic instead of the sigmoidal shape expected when hemoglobin has not been displaced by large amount of methemoglobin (Darling and Roughton, 1942). Thus, the ability of heme groups to cooperate in oxygen binding is less apparent in the presence of such high levels of methemoglobin.

3.3.3 The seasonal variability of the intraerythrocytic environment and the effects on hemoglobin function

The oxygen affinity of ~~Hb in winter~~ flounder whole blood was greater in winter fish than in summer fish (Figure 6). There are numerous studies on the blood oxygen affinity of fish and the quantitative results vary. However, Hb-oxygen affinity results can be grouped according to the qualitative response to temperature change. When temperature is decreased, this study found an increased Hb-oxygen affinity in the winter flounder, agreeing with the results of the

study by Hayden et al. (1975). Other species showing the same Hb-oxygen affinity increase during temperature decrease were several Antarctic species (Tetens et al., 1984; Qvist et al., 1977; Grigg, 1967), the carp (Cyprinus carpio) studied by Albers et al. (1983), and several Amazon species (Powers, Martin, Garlick, Fyhn, and Fyhn, 1979; Powers, Fyhn, Fyhn, Martin, Garlick and Wood, 1979). When temperature is increased, an increase in Hb-oxygen affinity occurred in the brown bullhead (Grigg, 1969). Several catostomid species (Powers, 1974), and the killifish Fundulus heteroclitus (Powers, 1980) do not demonstrate Hb-oxygen affinity changes after temperature alterations. Again, the findings on rainbow trout by Nikinmaa et al. (1980) showing an increase in Hb-oxygen affinity when temperatures are decreased, do not agree with the findings by Weber et al. (1976) that rainbow trout do not demonstrate Hb-oxygen affinity changes after temperature alterations.

The direct effect of temperature on hemoglobin in the absence of other functional modifiers is to increase oxygen affinity as temperature decreases (Wells and Jokumsen, 1982; Weber et al., 1976; Weber and DeWilde, 1975). The predictability of the temperature influence on Hb solutions suggests that the variability between species whole blood oxygen affinity during seasonal aclimatization may be due to intra-erythrocytic modifiers.

All of the changes in the seasonal concentration of intracellular components from flounder rbc's assisted in creating a greater Hb-oxygen affinity in the winter-acclimatized fish. Increased Hb-oxygen affinity during the winter would assist oxygen uptake at the gills. The decreased Hb-oxygen affinity during the summer would facilitate oxygen

unloading to the tissues.

The most influential fntracellular modifier is ATP. It is assumed that in the winter flounder, ATP would be more influential than GTP, as was found for the European flatfish (Pleuronectes platessa) in a study by Wood, Johansen, and Weber (1975). The NTP/Hb changes were similar to those seen after seasonal acclimatization and can be demonstrated after temperature acclimation. While temperature is important because of its direct effects on hemoglobin function (see 3.2.5), it is also important for its indirect effects in influencing NTP levels.

It could be argued that NTP/Hb varied becasue of changes in oxygen related to temperature. However, Greaney and Powers (1977) found that in killifish, ATP/Hb values were independent of the oxygen content of the water. An intersting feature of killifish NTP/Hb results is that the values decresase when temperatures increase, an opposite trend from the present study. The NTP/Hb results of the present study had a similar response to temperature as those for Pagothenia borchgrevinki (Tetens et al., 1984), and the blackfish (Dobson and Baldwin, 1982a).

The influence of temperature to Ca^{++}, Mg^{++}, and Cl^- levels in the winter flounder was not inyestigated, although there are significant seasonal changes in the amounts of ions present in the blood. Houston and Smeda (1979), and Koss and Houston (1981) documented the change in rbc ions during temperature acclimation in the rainbow trout, carp, and goldfish (Carrasius auratus). Data for ion levels of rbc's in goldfish acclimated to 10° and 30°C (Koss and Houston, 1981) are qualitatively similar to those found in this study. The rainbow trout and carp showed the same trends in rbc Cl^-/Hb ratios

(Houston and Smeda, 1979) as the winter flounder. The carp showed no changes in Ca^{++}/Hb in response to temperature change. Grigg (1967) also found no temperature effects on rbc Ca^{++} levels for brown bullheads acclimated to 9° and 24°C. The Mg^{++}/Hb ratios for rainbow trout studied at 2° and 18°C, and carp at 16° and 30°C were highest during exposure to low temperature (Houston and Smeda, 1979); those findings therefore differed from those in the present study.

Lykkeboe, Johansen, and Maloiy (1976) investigated the direct effects of Cl^- upon the performance of hemoglobin in Tilapia grahami. The action of Cl^- in that species is similar to that which has been found in the present research; as the Cl^-/Hb ratio increases, the oxygen affinity of Hb decreases. Magnesium has been shown to interfere with the effect of ATP on Hb, effectively increasing the Hb-oxygen affinity (Weber and Lykkeboe, 1978; Weber, 1978; Lykkeboe et al., 1976). In the present study Mg^{++} was also found to interact with ATP in altering Hb function. However, the seasonal variation in rbc Mg^{++} is so small in the flounder that there is no significant alteration in Hb-oxygen affinity. During handling, winter flounder become stressed (Fletcher, 1975), and have been known to produce greatly elevated plasma Mg^{++} levels (M. King and G. Fletcher, unpublished data). If this increase is translated intracellularly, then when fish are stressed, Mg^{++} would tend to increase Hb-oxygen affinity to a greater degree. Presumably this would be true at any time of the year, and the increase in Hb-oxygen affinity would be dependent upon the level of Mg^{++} increase and the mode of translation of Mg^{++} intracellularly. In the present study Ca^{++} was

found to influence Hb-oxygen affinity at levels far from the *in vivo* Ca^{++} concentrations. Calcium has no significant role in the control of seasonal Hb-oxygen affinity in the winter flounder.

Hydrogen ions can influence Hb function via the Bohr or Root shifts. Hydrogen ions tend to stabilize the T-state hemoglobin (tense or deoxy-Hb), thus decreasing Hb-oxygen affinity (Perutz, 1970a and b). The pH_i changes for winter flounder rbc's are small (0.003 units/°C) compared to those for blackfish, where pH_i parallels changes in pH_e (0.016 units/°C, Dobson and Baldwin, 1982b). The rbc pH is known to be regulated by cell membrane exchangers sensitive to β-adrenergic mediation (Nikinmaa, 1983; Nikinmaa and Huestis, 1984). Adrenaline administration in high doses increases the pH of fish red blood cells, therefore increasing Hb-oxygen affinity (Nikinmaa, 1983). The pH_i of summer fish was expected to decrease more than it did. However, there may have been greater β-adrenergic activity at the rbc membrane during the summer. Peyraud-Waitzenegger, Barthelemy, and Peyraud (1980) showed that β-agonists are more effective on summer acclimatized eels (*Anguilla anguilla*).

The values of the Bohr effect found in the present study were in the range of values found in other studies on the winter flounder (Hayden *et al.*, 1975) and in other flatfish species (Weber and DeWilde, 1975). The Bohr effect on winter flounder Hb was the same at 0° and 10°C, so is not temperature dependent. That is consistent with Bohr effect studies done on Antartic fish Hb over a 5°C range (Tetens *et al.*, 1984).

The Root effect of H^+ on Hb is commonly associated with the

regulation of buoyancy in fish. The winter flounder, however, has no swim bladder, but does have a choroid rete (Wittenberg and Haedrich, 1974). The Root effect may therefore be important to the physiology of the winter flounder eye. Such an assumption has been made previously for fish without swim bladders by Ingermann and Tewilliger (1982).

The ion and NTP levels in winter flounder can account for approximately 35 per cent of the total seasonal change in whole blood oxygen affinity. Temperature has a direct effect on hemoglobin and therefore influences the remainder of the change in seasonal oxygen affinity. Temperature may therefore be considered to be the most influential Hb functional modifier. Temperature is also instrumental in controlling [NTP], the most important intracellular modifier.

3.4 Summary of seasonal hematology findings

It has been shown that the seasonal change from winter to summer brings about a decrease in the capacity of the blood to store oxygen and a decrease in Hb-oxygen affinity in the winter flounder. These physiological responses to seasonal change are apparently due to the physical changes in the environment. Temperature was found to be responsible for much of the change in seasonal oxygen affinity in the winter flounder. The change in Hb-oxygen affinity in the summer results in improved delivery of oxygen to the tissues at a time when aerobic demands are highest. Even though seawater P_{O_2} is lower during the summer, it is still greatly elevated above limiting values which might cause oxygen affinity to increase. The reason for a decrease in

[Hb] during the summer is not apparent at this point.

The results of this study on seasonal changes in hematology and hemoglobin function, in conjunction with the results of the studies on blood viscosity and cardiac performance (Chapter 4 and 5), will provide a greater knowledge of the physical and physiological factors which influence oxygen uptake and delivery in the winter flounder.

Many of the results in this chapter appear in published form. Figures 13, 14, 15, and 17 are reproduced from a paper by M.S. Graham and G.L. Fletcher (1983) entitled, " Blood and plasma viscosity of winter flounder: influence of temperature, red blood cell concentration, and shear rate ". That paper is in the Canadian Journal of Zoology (61(10):2344-2350). Figures 16 and 18 are reproduced from the paper by M.S Graham and G.L. Fletcher (1985), and a complete reference is given on page 151.

CHAPTER 4

BLOOD VISCOSITY

4.1 Introduction

The resistance of a fluid to flow, or its viscosity, is inversely related to temperature. The viscosity of a given fluid remains uniform at any flow for most fluids as long as temperature remains stable. These fluids are said to be Newtonian in this respect (Burton, 1972). Blood is a complex tissue made up of many components, and as such does not behave as a Newtonian fluid. It is known that as the flow rate of blood increases, the viscosity decreases (Chien, 1975; Burton, 1972; Dintenfass, 1971). Viscosity also changes as changes take place within the blood components (amount of rbc's and type of plasma proteins) according to studies by Chien (1975) and Guyton and Richardson (1961).

Blood viscosity is only a matter of concern under pathophysiological conditions for humans. However, research has been done on mammals that must endure cold temperatures regularly. In hibernators such as the ground squirrel (Maclean, 1981), and in the extremities of polar animals such as seals (Guard and Murrish, 1975) and reindeer (Halikas, 1971), blood is subjected to extreme cold. Although adaptations have been made in all of the above-mentioned animals to minimize the effects of cold on the blood, the viscosity of the blood in these animals does increase greatly. For non-hibernators, most of the blood volume is kept near the core of the animal, so is at normal temperature. The

hibernators tolerate the refrigeration of the entire circulatory system. The blood components of ground squirrels have adapted so as not to be as temperature sensitive as those of non-hibernating rodents (Maclean, 1981).

Fish are poikilotherms that are able to live through significant seasonal and acute temperature changes. In polar and cold temperate fishes, extreme cold predictably dictates highly viscous blood, and reduced cardiac output (or blood flow rate) at the same time. It is obvious that fish like the winter flounder survive these highly viscous conditions in their blood year after year. The manner of that survival is not apparent. Perhaps they are like the mammalian hibernators and possess blood constituents that have adapted to the physical environment, having blood viscosity which show only minor temperature dependence. It is also possible that there are very great changes in blood viscosity related to temperature which will in turn demand compensatory changes in the respiratory and cardiovascular systems, in order to facilitate oxygen uptake and delivery in the animal. Only after the basic rheological properties of the blood are described, and compared with those of other animals also faced with physical challenges, can these possibilities be discussed in full, and some general conclusions made.

Some work has already been done on the viscosity of fish blood. Hemmingsen and Douglas (1972), using a glass tube viscometer, found the viscosity of blood of hemoglobinless Antarctic fish to be very temperature dependent between 0° and 10°C. Cameron and Davis (1970) investigated the relative viscosity of blood from rainbow trout at

20°C. Milligan and Wood (1982) observed the relative blood viscosity of rainbow trout following exposure to acidified water at 14°C. Wood (1974) studied blood pressure-flow in the trout branchial system, and measured the relative viscosity of blood and plasma at 5°C. Blaxter, Wardle and Roberts (1971) measured relative viscosity of plasma from deep sea fishes at 0°, 10°, and 20°C, and found that temperature had considerable influence. Blaxter et al. used a method known as the descending sphere technique, whereby the time taken for a hard spherical object to sink through a viscous fluid is recorded.

None of the preceding studies provide information about the influence of shear rate on blood viscosity. The object of this study is to quantify some of the basic rheological properties of blood of winter flounder, including the effects of shear rate. Where possible these results will be compared with findings from other studies. Information on temperature influence on flow resistance of blood will be mentioned again in a later chapter when the results of data on oxygen dynamics between water and blood at the gills of seasonally acclimatized flounders are discussed. If temperature is found to influence blood viscosity to a large degree, the routing of blood through the vasculature of the gills may differ between winter fish and summer fish. That difference would be apparent in respiratory measurements at the gills.

Finally, experiments were done to observe the effects of temperature on blood viscosity in living tissues.

A short discussion on the operation of the cone-plate viscometer is warranted. This is a precision instrument that can be used on small

blood samples. The cone is attached to a spring, and both are turned by a variable-speed motor. The cone is brought into contact with the fluid which has been placed in a metal cup. The fluid resists the movement of the cone. However, once the speed is set, the motor provides a constant movement of the cone. The viscosity of the fluid, or the resistance to the rotation of the cone, is measured by the torque on the spring. The speed of rotation of the cone is directly proportional to the shear rate through the sample.

4.2 Results

4.2.1 The effect of temperature and shear rate on viscosity

The blood and plasma of the winter flounder are greatly affected by temperature (Figure 13) and shear rate (Figure 14). Most of the effect of temperature is demonstrated in the -1° to 10°C range. Viscosity values of blood and plasma measured at 10°C show an increase of about four-fold over a temperature decrease to -1°C. Blood and plasma are shear rate dependent at all temperatures (Figure 14). The lowest viscosity values occur at high shear rates. Viscosity changes at shear rates greater than 23 s^{-1} are very small for flounder blood and plasma. For flounder blood at normal-Ht (22%), the viscosity change per unit shear rate becomes larger as the temperature decreases.

Figure 13. The effects of temperature on blood (Ht = 22.26 ± 0.20%), and plasma (total protein = 4.32 ± 0.12 g/100 ml) viscosity of the winter flounder. n = 4 to 15 fish. Values are means ± SE.

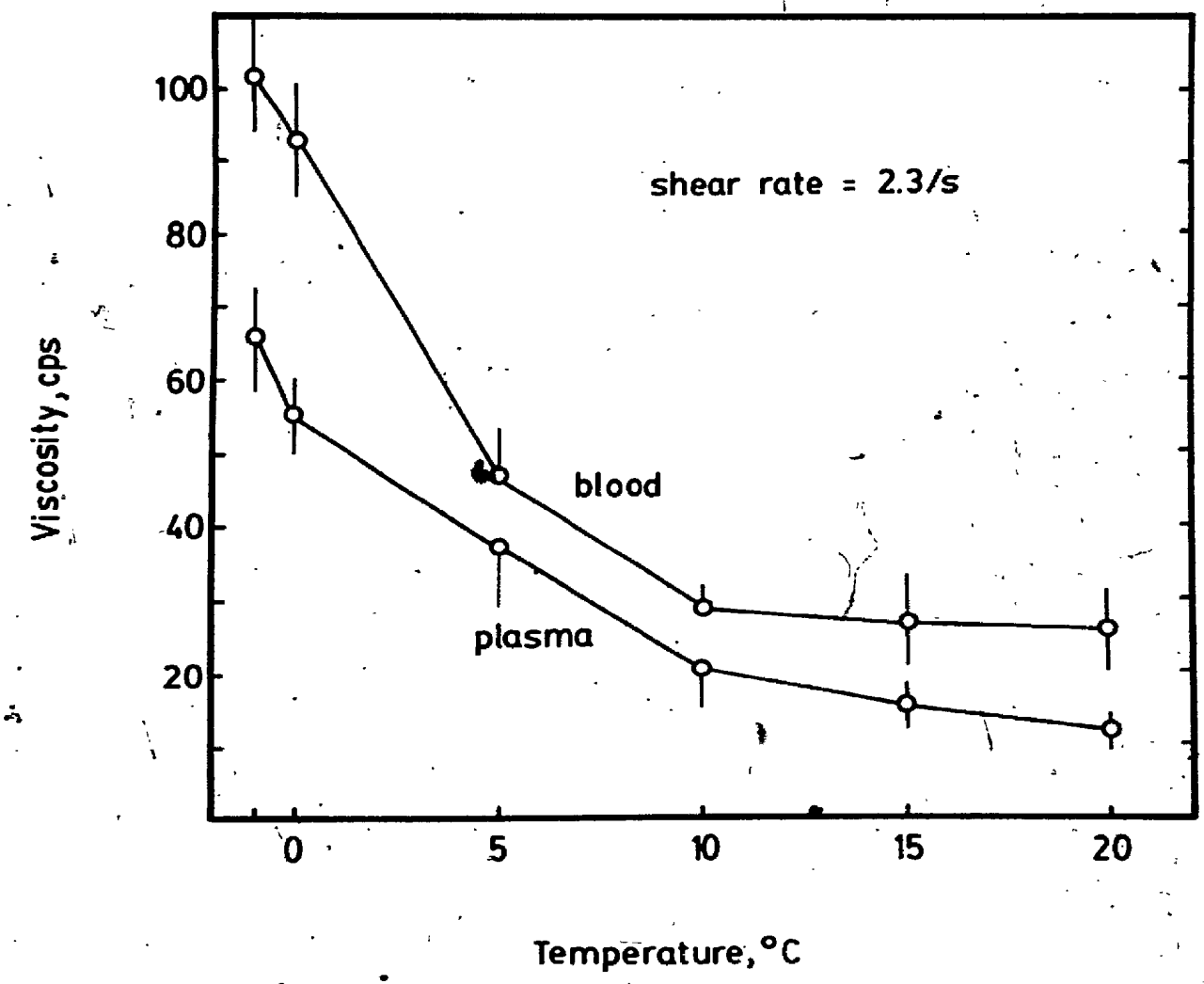
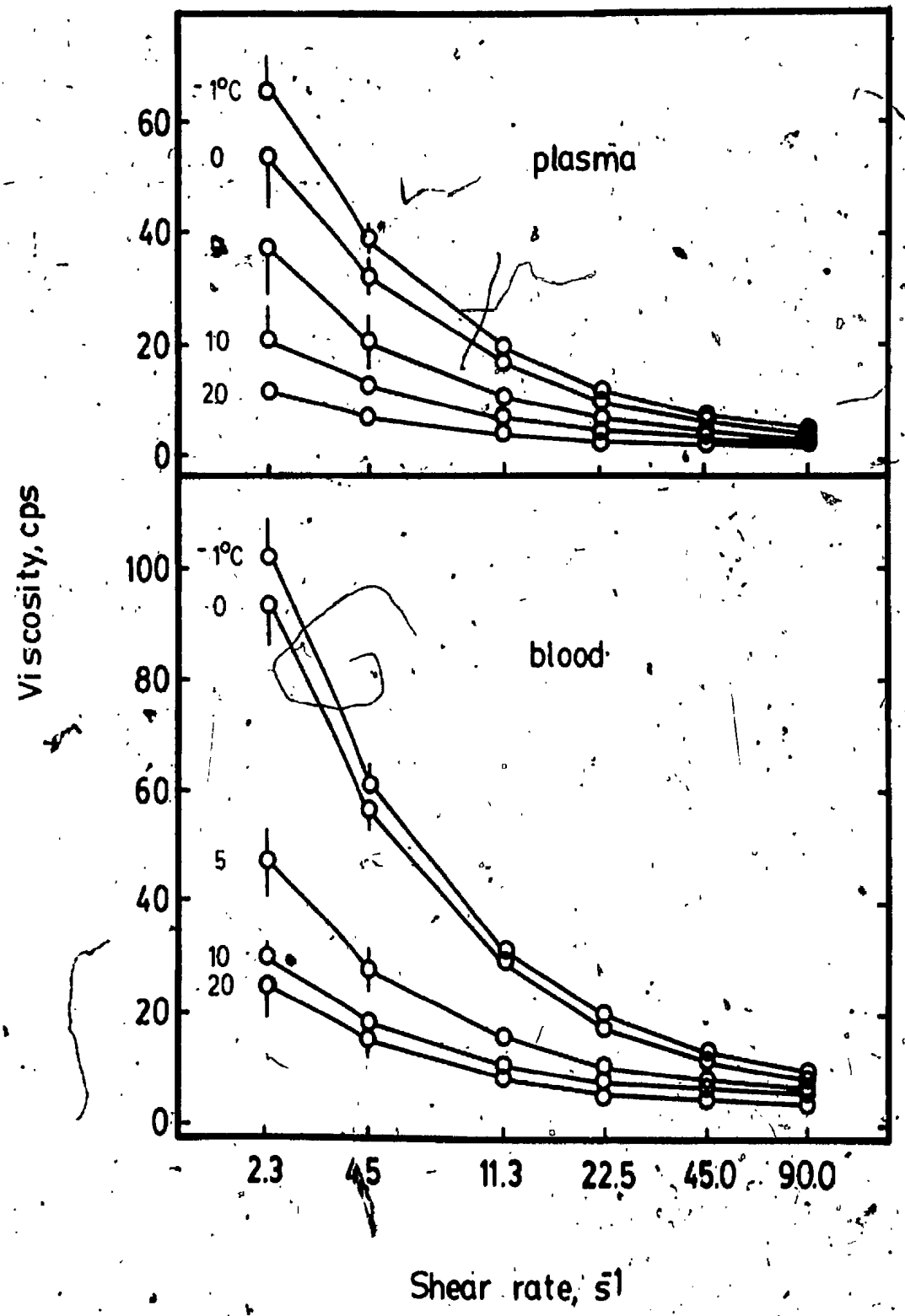

shear rate = 2.3/s
blood
plasma
Viscosity, cps
100
80
60
40
20
0
5
10
15
20
Temperature, °C

Figure 14. The effect of temperature and shear rate on blood and plasma viscosity of the winter flounder. Total protein concentration = 4.32 ± 0.12 g/100 ml; average [Hb] = 4.93 ± 0.18 g/100 ml; average Ht = 22.26 ± 0.20 %. Values are means ± SE. n = 4 to 15 fish.


plasma
blood
-1°C
0
5
10
20
Viscosity, cps
Shear rate, s⁻¹
2.3
4.5
11.3
22.5
45.0
90.0
0
20
40
60
80
100

4.2.2 Viscosity of blood and plasma after altering plasma contents

The following experiments were undertaken in order to illustrate the importance of components in winter flounder plasma to the viscosity of the blood and plasma. Plasma protein concentration alters the viscosity of the plasma over a wide range of shear rates (Figure 15). However, only small changes in viscosity occur over the normal range of plasma protein concentrations (3-6 g/100 ml; Fletcher, 1975).

The blood serum is less viscous than the autologous plasma (Figure 16). The viscosity of plasma and serum is shear rate dependent. The viscosity of a rbc-serum mixture is less than that of a rbc-plasma mixture (Figure 16). The difference in viscosity seen between the plasma and serum rbc mixtures was greater than the difference in viscosity between plasma and serum.

4.2.3 The effect of hematocrit on blood viscosity

The effect of different concentrations of red blood cells on blood viscosity were tested at various temperatures. The results at each temperature are qualitatively similar. Data for 0° and 15°C have been selected to illustrate the relationships between temperature, shear rate, and Ht (Figure 17). The viscosity of whole blood at 11 per cent Ht is greater than plasma viscosity at all temperatures. At higher temperatures (>10°C), increases in Ht (11 to 75%) resulted in exponential increases in viscosity. At lower temperatures (<5°C), and shear rates (<4.5 s^{-1}), viscosity did not increase significantly over the

Figure 15. The relationship between plasma viscosity and total protein concentration for the winter flounder. A pooled plasma sample was analysed at 5°C (n_o = 6).

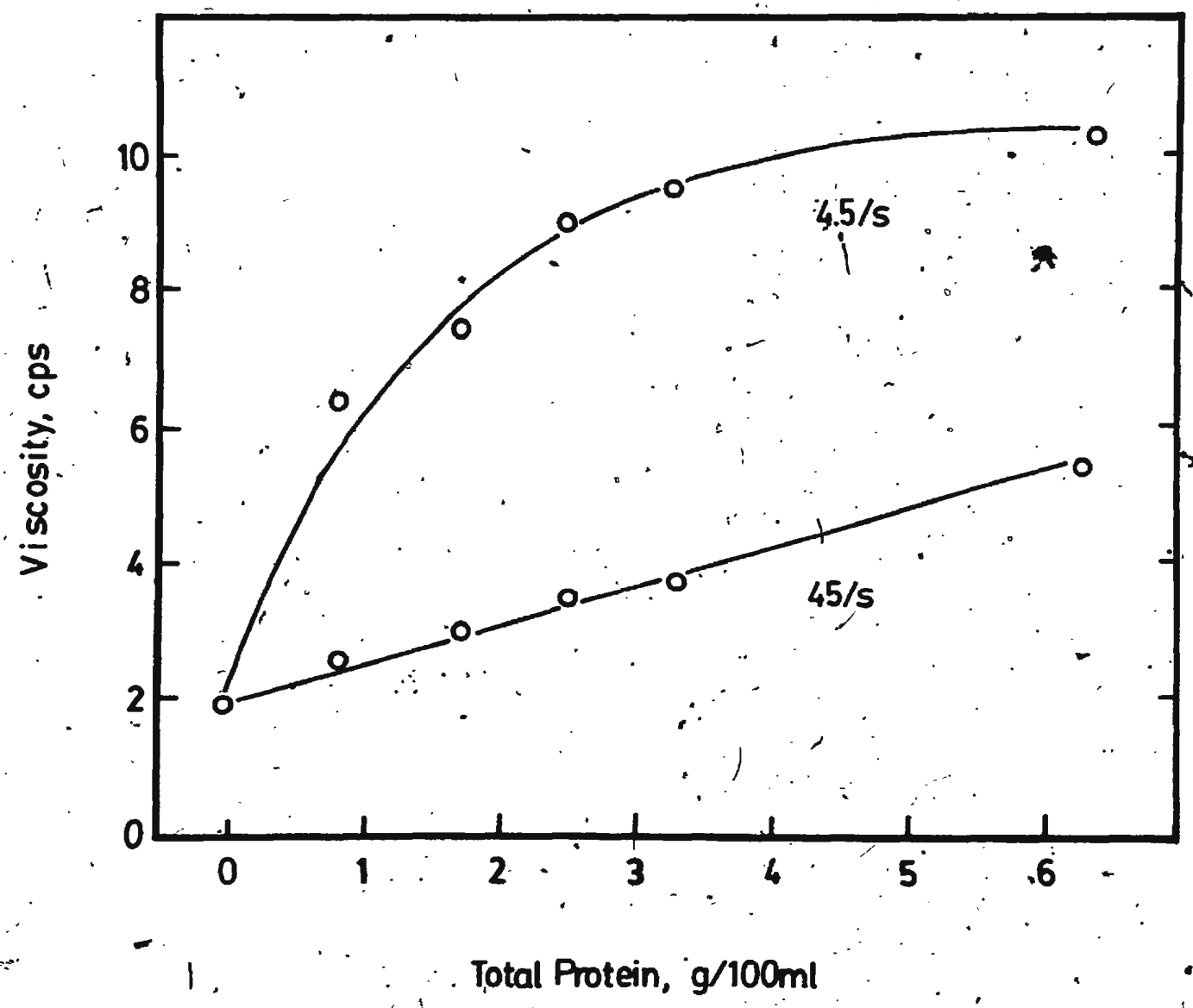

Viscosity, cps
0
2
4
6
8
10
4.5/s
45/s
0
1
2
3
4
5
6
Total Protein, g/100ml

Figure 16. Data from a pooled flounder blood sample (n = 7) showing whole blood (▲), cells and serum (△), plasma (●), and serum (○) viscosity at 0°C and a range of shear rates.

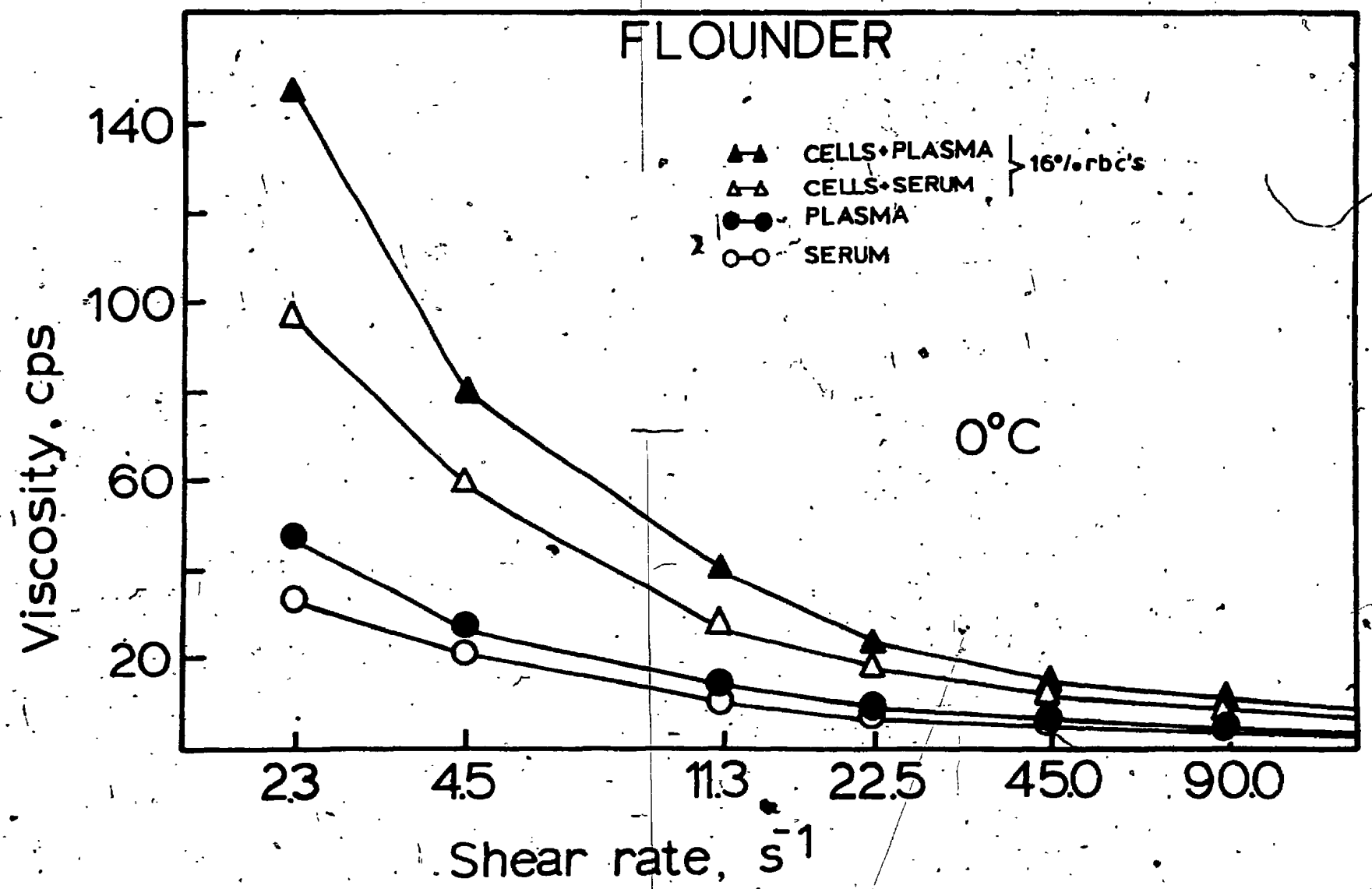
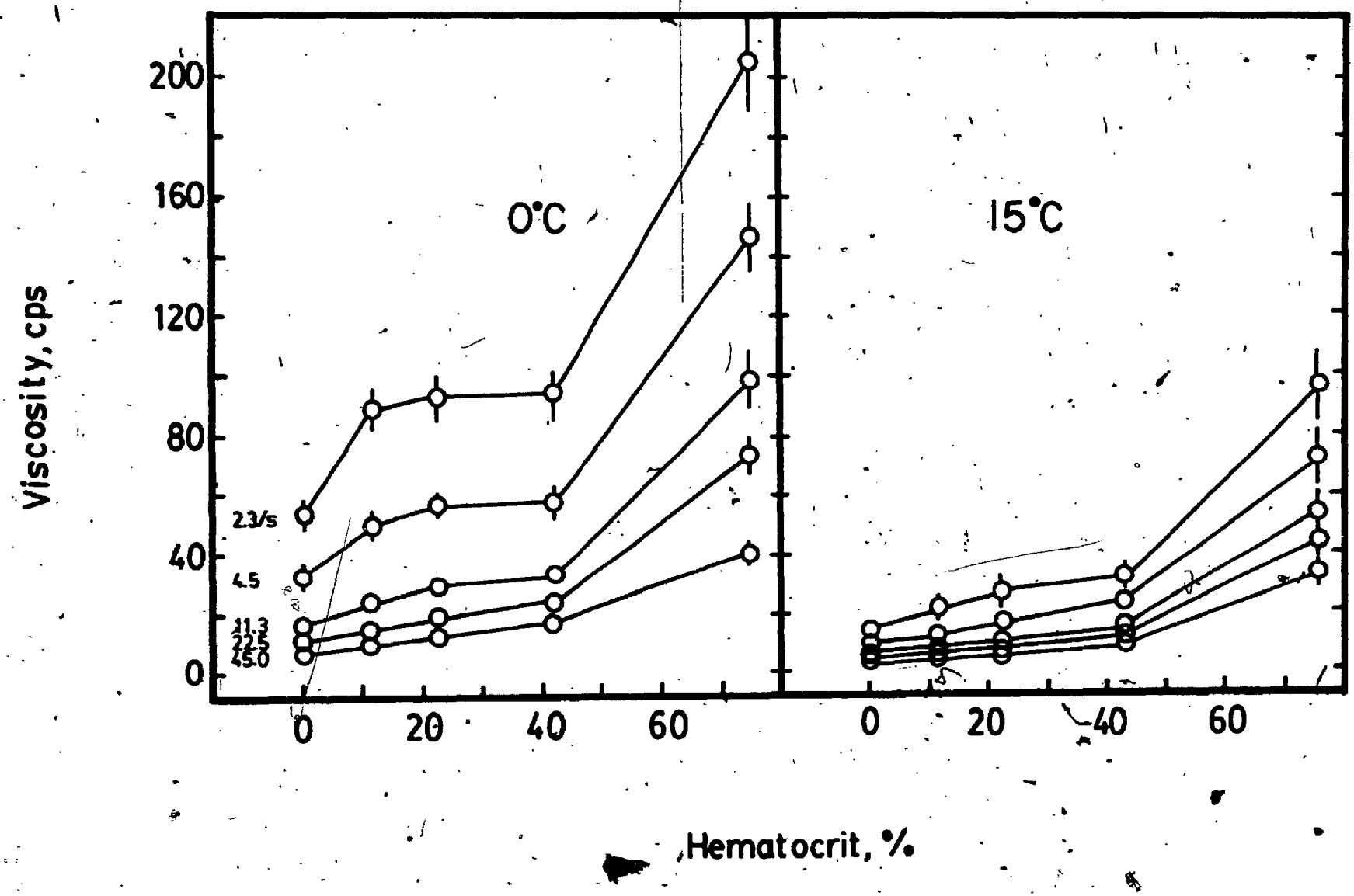

FLOUNDER
CELLS+PLASMA
CELLS+SERUM
16% rbc's
PLASMA
SERUM
0°C
Viscosity, cps
140
100
60
20
2.3
4.5
11.3
22.5
45.0
90.0
Shear rate, s⁻¹

Figure 17. The effect of hematocrit on winter flounder blood viscosity at 0 and 15°C. Average Ht and MCHC are as follows: 0% (plasma), 11.5 and 24.8; 22.3 and 20.6; 43.1 and 26.3; 75.0 and 25.7. Each line represents a different shear rate as indicated to the left. Values are means ± SE. n = 4 to 15 fish.


Viscosity, cps
200
160
120
80
40
0
0°C
15°C
2.3/s
4.5
11.3
22.5
45.0
0
20
40
60
0
20
40
60
Hematocrit, %

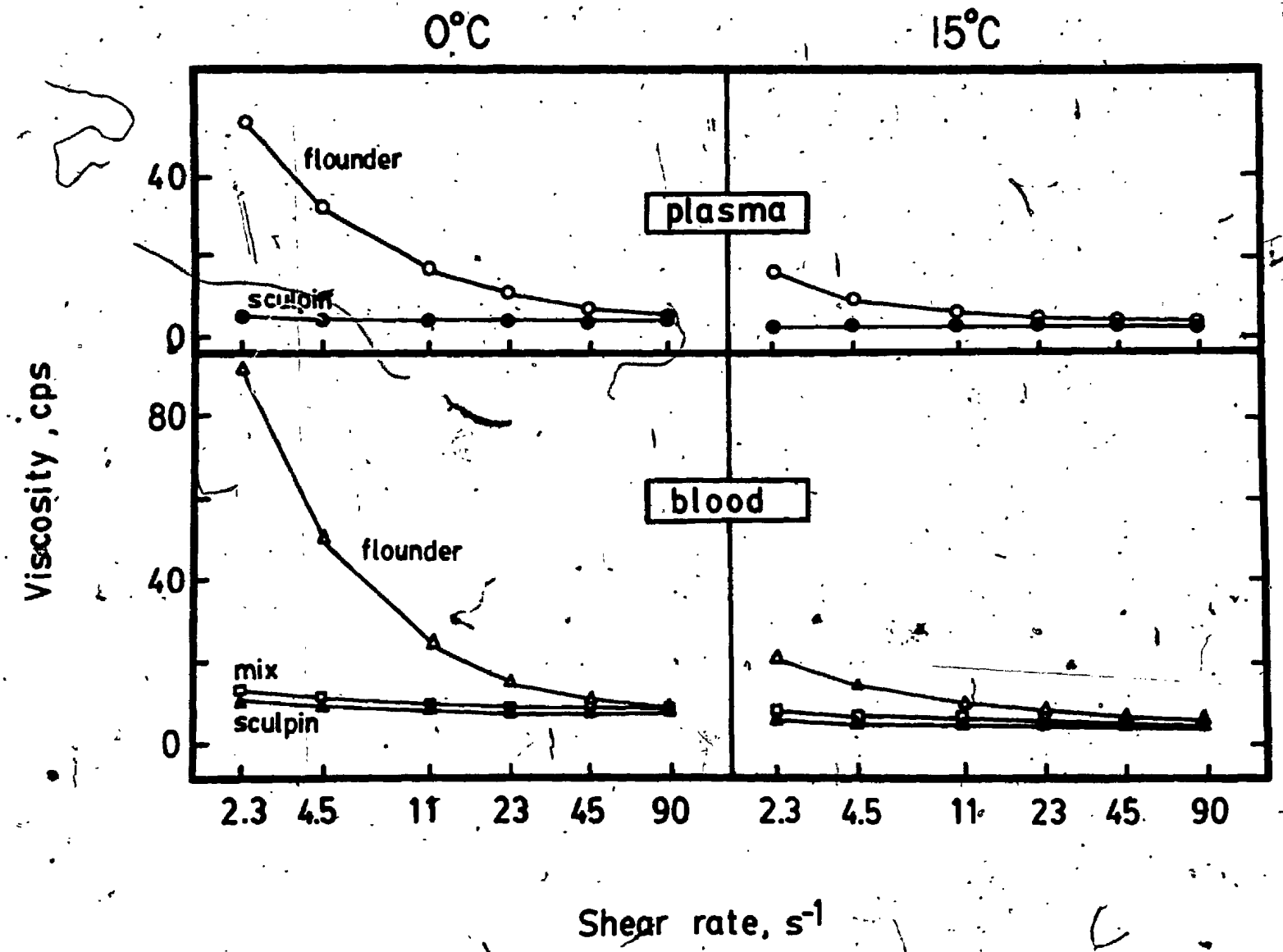
11 to 40 per cent Ht range. At higher shear rate and temperatures the viscosity values increased steadily over the 11-40 per cent Ht range.

4.2.4 The importance of red blood cell aggregates to flounder blood viscosity

Flounder red blood cells were mixed with plasma from the shorthorn sculpin. The viscosity of the mixture was compared with results from readings on flounder and sculpin whole blood (Figure 18). Shorthorn sculpins have low blood viscosity compared to flounder (4.3.5).

Substitution of flounder plasma with sculpin plasma in a mixture with flounder rbc's gives viscosity values in the range of sculpin whole blood levels. At 0°C and a shear rate of 2.3 s^{-1}, flounder whole blood viscosity is 91 cps, while flounder plasma is 53 cps. The difference (91-53 = 38 cps) is due to the blood cells and blood cell aggregates. In the same experimental conditions, the viscosity of the flounder rbc's-sculpin plasma mixture is 13 cps, and sculpin plasma is measured at 5 cps. The difference (13-5 = 8 cps) is due to the viscosity of the flounder rbc's and any aggregates. If the assumption is made that under these experimental conditions flounder rbc's do not aggregate, then 8 cps (from the preceding calculation) must be due to the presence of individual founder rbc's. Therefore, the contribution of rbc aggregates to the total blood viscosity of the flounder is 38-8 = 30 cps, or 33 per cent (30/91 = 0.33).

Figure 18. The results of viscosity experiments using shorthorn sculpin and winter flounder blood mixtures. Results were obtained at 0° and 15°C, and at various shear rates. Flounder (○) and shorthorn sculpin (●) plasma viscosity are in upper panels. Flounder (△) and shorthorn sculpin (▲) autologous whole blood viscosity are in lower diagrams. Mix = flounder cells and shorthorn sculpin plasma. Ht's were: flounder = 19.0%; sculpin = 16.8%; and mix = 18.8%.


0°C
15°C
plasma
blood
flounder
sculpin
mix
Viscosity, cps
40
0
80
2.3
4.5
11
23
45
90
Shear rate, s-1

4.2.5 Aspects of *in situ* blood viscosity

The *in situ* viscosity experiments employed living tissue as a flow through viscometer. The results can be used to estimate flow resistance and viscosity within the body vasculature.

The flow of saline through vasculature conditioned to 10°C has the same pressure-flow relationships as saline through vasculature conditioned to 0.6°C (Figures 19 and 20). However, at any given pressure, the flow rate of blood through vasculature conditioned to low temperature is greater than the flow rate through vasculature conditioned to high temperature. These trends of the saline and blood are further supported by calculations of resistance (Figure 21).

From *in situ* estimates of viscosity, average shear rates can be interpolated from findings on *in vitro* viscosity (using Figure 14). At 10°C and at 40 cmH_2O flow pressure (ΔP), the average vascular shear rate is 10 s^{-1}. At 0°C, but otherwise similar conditions, the shear rate is >90 s^{-1}. These shear rates would produce an average blood viscosity of about 10 cps at 0° and 10°C.

4.3 Discussion

4.3.1 General

Temperature affects the viscosity of all fluids. Blood is a complex fluid which has non-Newtonian flow characteristics. Because temperature can also influence heart performance, it can bring about

Figure 19. *In situ* pressure-flow results for blood (●) and saline (o) perfusions at 0.6°C in the winter flounder. Symbols are the individual results from 11 separate perfusions. Regression lines were: blood inflow pressure = 46.25 (Fs) + 13.91 (r = 0.87); and saline inflow pressure = 28.79 (Fs) + 10.59 (r = 0.94).

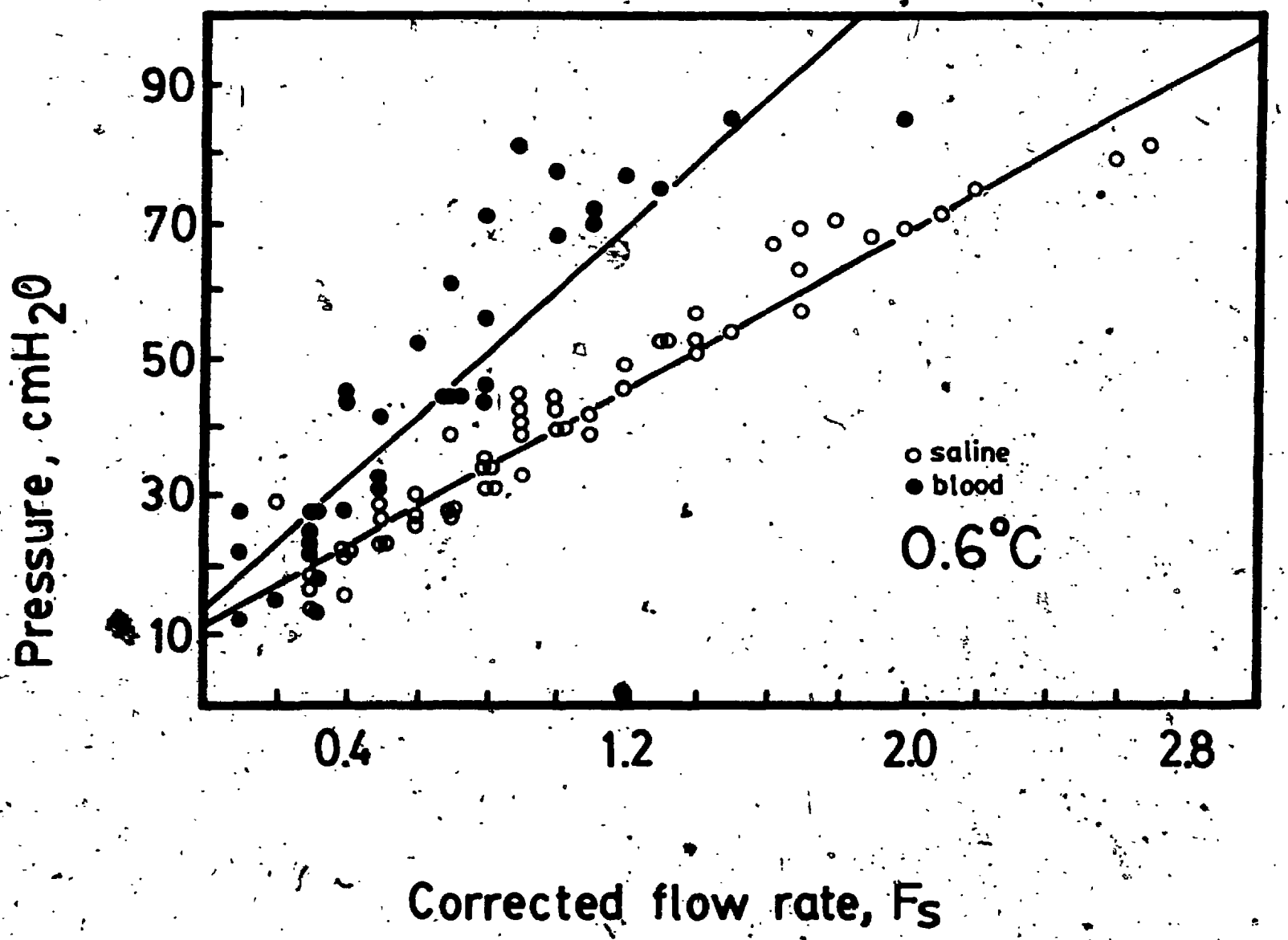

Pressure, cmH2O
90
70
50
30
10
0.4
1.2
2.0
2.8
Corrected flow rate, Fs
o saline
● blood
0.6°C

Figure 20. *In situ* pressure-flow results for blood (●) and saline (o) perfusions at 10°C in the winter flounder. Symbols are the individual results of 13 separate perfusions. Regression lines were: blood inflow pressure = 43.27 (Fs) + 22.74 (r = 0.81); and saline inflow pressure = 25.70 (Fs) + 13.26 (r = 0.86).

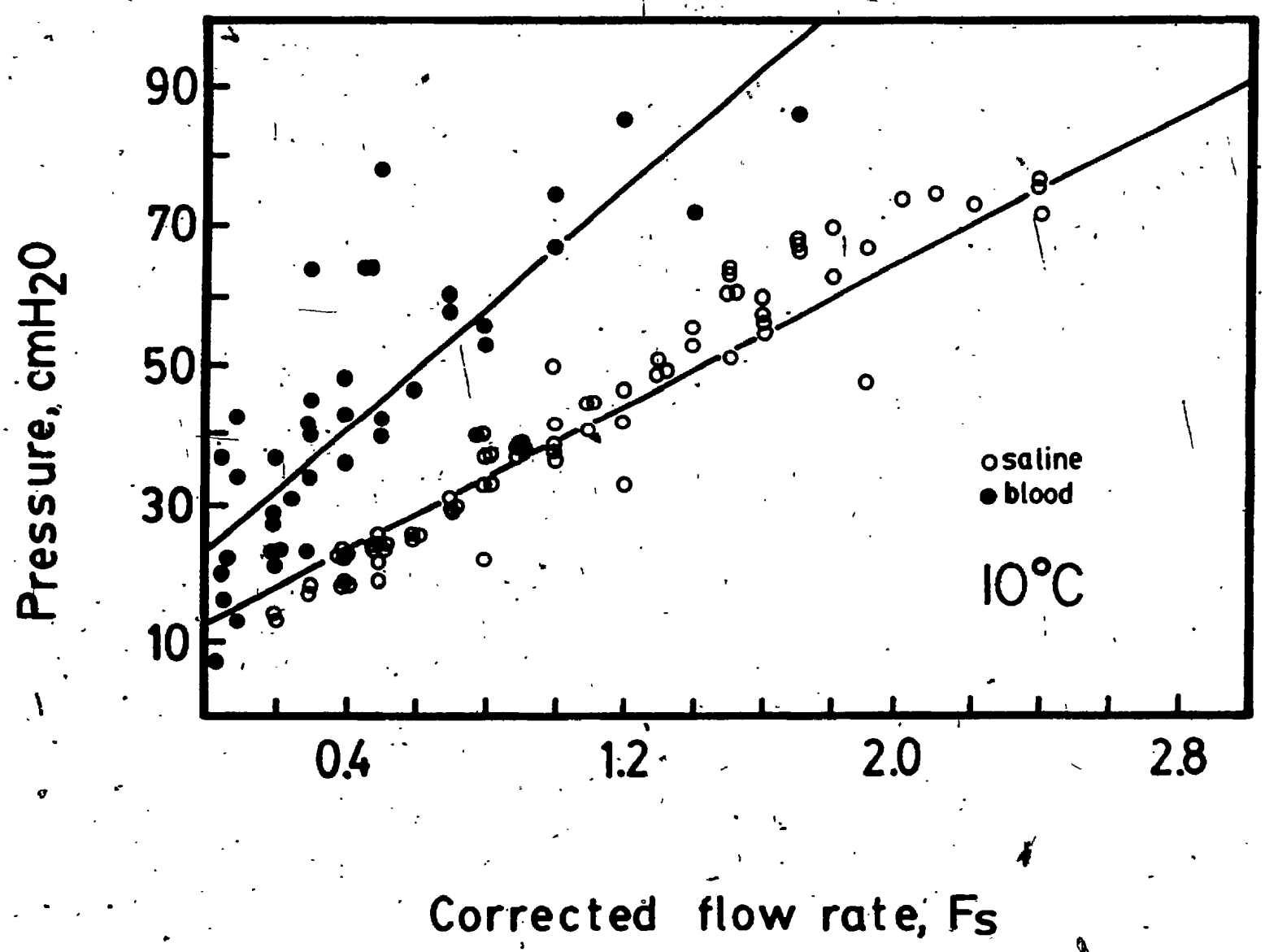
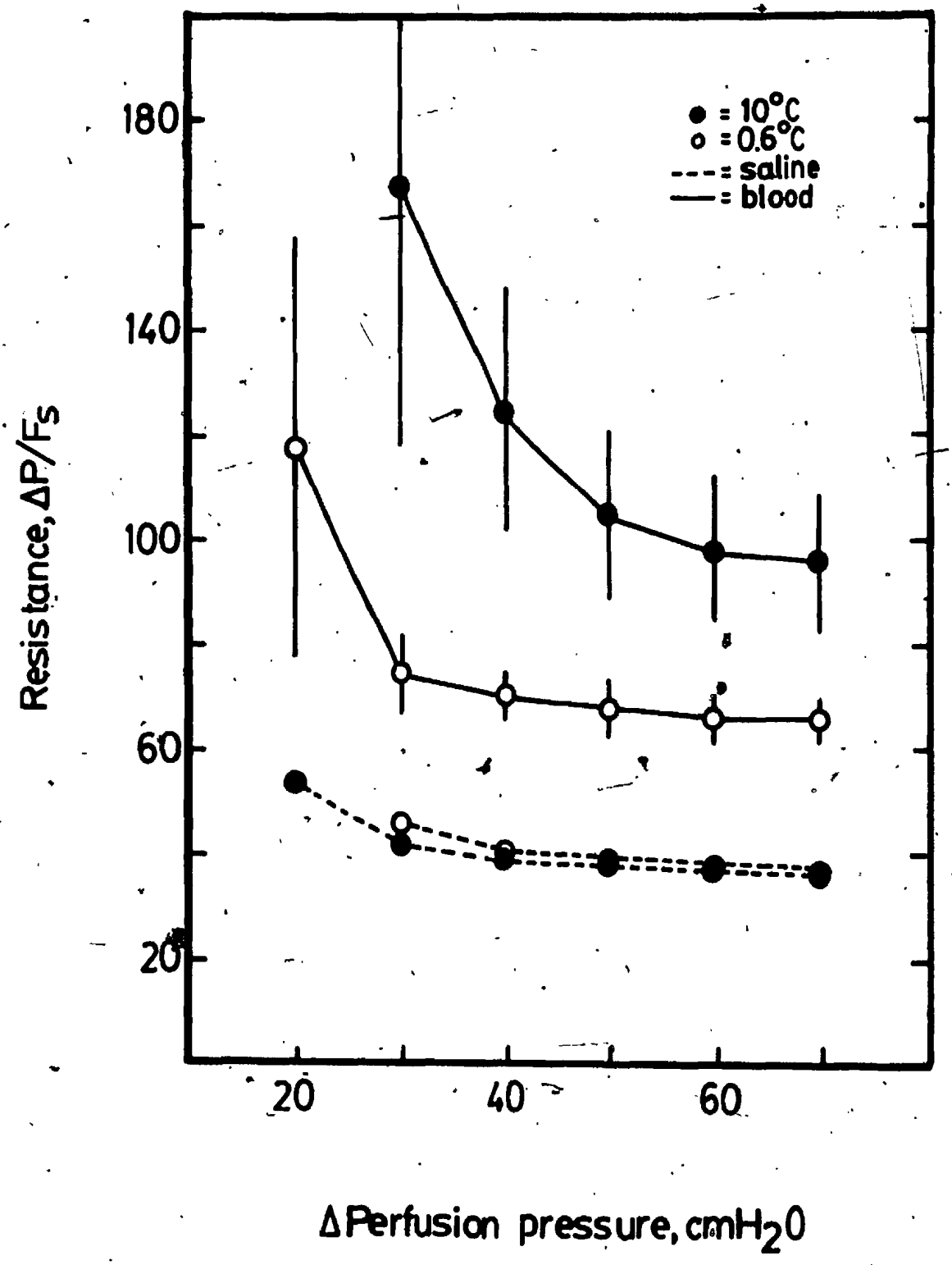

Pressure, cmH2O
90
70
50
30
10
0.4
1.2
2.0
2.8
Corrected flow rate, Fs
o saline
● blood
10°C

Figure 21. *In situ* flow resistance for blood (—) and saline (----) at 10°C (●) and 0.6°C (○). Values are calculated averages ± SE. n = 13 for 10°C experiments and 11 for 0.6°C experiments.


● = 10°C
o = 0.6°C
---- = saline
—— = blood
180
140
100
60
20
Resistance, ΔP/Fs
20
40
60
ΔPerfusion pressure, cmH2O

changes in viscosity by altering the rate of blood flow. Blood flow rate is directly proportional to shear rate (Chien *et al.*, 1971; Wells and Merrill, 1961). This section discusses the characteristics of temperature influence on the *in vitro* viscostiy of flounder blood, and goes on to investigate the importance of various blood components to the viscosity of whole blood. Finally, the implications of these findings are discussed in relation to their effects on the physiology of the seasonally acclimatized winter flounder.

4.3.2 The effects of shear rate on blood viscosity

In general, low flow velocities result in reduced cell deformation, increased cell aggregation, and increased viscosity. As flow increases, cell aggregation is reduced, while cell deformation increases, resulting in reduced viscosity. The aggregation of rbc's occurring under low flow conditions is due to the bridging effect of large plasma proteins adsorbed to the rbc surfaces (Chien, 1975).

Winter flounder plasma, in the absence of rbc's, also has viscosity values which are shear rate dependent, especially at low temperatures. Plasma shear rate dependency has been observed under hypothermic conditions in the leopard seal (Guard and Murrish, 1975), and the reindeer (Halikas, 1971). The ratio of plasma viscosity to whole blood viscosity in the winter flounder is the same over a broad range of temperatures and shear rates. These ratios indicate that plasma accounts for up to 50 per cent of the viscosity of whole blood within the normal Ht range.

4.3.3 The importance of plasma components to blood and plasma viscosity

Plasma viscosity in humans is primarily a function of protein concentration, particularly the albumins and globulins (Lawrence, 1950). The lipids and cholesterols were found to be less important (Larcan, Streiff, Peters, and Genetet, 1965). When the total concentration of plasma protein is altered in samples from the winter flounder, only small alterations in viscosity are apparent (see Figure 15). Small changes in plasma viscosity are also seen when fibrinogen is selectively removed (see Figure 16). Fibrinogen also is a factor in the aggregate formation, therefore fibrinogen makes a contribution to viscosity. Red blood cell aggregates are responsible for up to 33 per cent of the total blood viscosity in the winter flounder.

4.3.4 The effect of red blood cell concentration on viscosity

Studies on mammalian blood indicate that the increases in viscosity which accompany increases in Ht, up to a normal value (about 45 per cent), are due to the interaction between rbc's and plasma proteins, and the fluid properties of the rbc's (Chien, 1975; Dintenfass, 1971). The lack of an increase in viscosity values for flounder at low temperature and shear rates over the 11-40 per cent Ht range was not apparent in other species (Chien, Usami, Daellenback, and Bryant, 1971; Halikas, 1971). By increasing the rbc concentration in a sample, the volume of plasma decreases. The plasma accounts for 50

per cent of the blood viscosity on its own, and then contributes further by its ability to aggregate cells. The addition of cells (increased Ht) would tend to increase viscosity while the displacement of plasma and the plasmas ability to create aggregates would tend to decrease viscosity. In the Ht range of 11-40%, and under low temperature and flow conditions, these two tendencies seem to balance until Ht goes above 40%. At Ht's above 40%, the rbc's become concentrated enough for frequent interaction with each other (crowding), causing high viscosity values. Viscosity increases sharply upon further addition of rbc's above a Ht of 40%.

Another possibility may explain the flounder viscosity results with changing Ht. At approximately 10 per cent Ht, near maximum cell aggregation may occur. This means that all aggregating plasma proteins are in use. Further addition of rbc's causes no further increases in viscosity until the crowding of rbc's at very high Ht values.

Red blood cells are normally highly deformable, a characteristic which is essential to circulation through the capillaries (Schmid-Schonbein, 1976; Prothero and Burton, 1962). The cell membranes and the viscosity of the cytoplasm contribute to rbc deformability. Winter acclimatized fish, used in these studies on viscosity, tend to have fatty acids in the cell membranes that are more unsaturated (Hochachka and Somero, 1971; Kuiper, Livne, and Meyerstein, 1971). A larger amount of unsaturated fatty acids would allow a greater degree of cell membrane flexibility. The rbc membranes of winter acclimatized flounder may be more flexible than those of summer fish.

4.3.5 Physiological implications of viscosity to seasonally acclimatized fish

A number of poikilotherms demonstrate seasonal physiological alterations that would help to offset thermally induced changes in viscosity. The iguanid lizard has Ht-dependent blood viscosity, and undergoes decreasing Ht with acclimation to lower temperatures (Maclean, Lee, and Withers, 1975; Snyder, 1971). Some hibernating rodents have low viscosity blood due to more flexible rbc membranes, and plasma characteristics that minimize aggregation at low flow rates (Maclean, 1981).

The blood viscosity of two sculpin species (*Myoxocephalus scorpius* and *Myoxocephalus octodecemspinosus*), and the Arctic char (*Salvelinus alpinus*) is much less than that of winter flounder (Graham and Fletcher, 1985; Graham, Fletcher, and Haedrich, 1985). The blood viscosity of the Arctic char was not even temperature dependent (Graham, Fletcher, and Haedrich, 1985). The main difference between sculpin and flounder blood viscosity is the role of plasma, and its interaction with rbc's (Graham and Fletcher, 1985). Since blood viscosity is a major determinant of blood flow resistance, low blood viscosity should confer a circulatory advantage in cold water species. *Myoxocephalus* spp. are believed to have their origin in the Arctic (Cowan, 1972), therefore circulatory adaptations to low temperatures are expected. In contrast, the winter flounder is more temperate in its distribution, with Newfoundland approaching the northern limit of its range (Leim and Scott, 1966). Most populations of winter flounder

therefore inhabit waters warmer than those inhabited by sculpins (Van Guelphin and Davis, 1979; McCracken, 1963).

It is apparent from *in vitro* results that the winter flounder is faced with greatly increased blood viscosity during exposure to low temperatures. Such high values may also be the case *in vivo*, because reduced rates of blood flow during the winter (e.g. Cech *et al.*, 1976) would decrease shear rate, thus promoting higher viscosity. The apparent stasis in blood viscosity over the 10-40 per cent Ht range may be functionally significant to the winter flounder. Unlike many other fish species, the Ht of winter founder increases during the winter. The stasis in blood viscosity allows for an increased capacity of the blood to carry oxygen, without concomitant changes in rheology.

4.3.6 Aspects of *in situ* blood viscosity

Results from *in vitro* tests suggest large differences in blood viscosity values due to temperature influences. However, the results of blood flow resistance *in situ* suggest that the average viscosity of blood in the winter flounder vascular bed is approximately equal at all temperatures. That indicates the possibility of homeoviscous control through alterations in the flow path of blood. A review of some pertinent information will be useful before the flow alterations are described. Needleman and Johnson (1980) found papaverine to be a powerful smooth muscle dilator, so all of the vessels in each *in situ* winter flounder preparation is expected to be fully perfused. The pressure flow relationships for saline are the same in high and low

temperature preparations. Therefore, during perfusion with saline, a Newtonian fluid (5.06 cps at 0°C, and 3.74 cps at 10°C), the *in situ* vasculature is functionally the same at either experimental temperature. Finally, it is known that at any shear rate, the viscosity of blood at a low temperature is always greater than that of warmer blood.

Since the vasculature in the high and low temperature preparations is functionally the same, the pressure-flow difference during blood perfusion must have been due to differences in blood viscosity. The increased viscosity of low temperature blood would create a greater flow through larger, low resistance vessels. The result is a greater net flow rate from the cold preparations at any given perfusion pressure. This effect of temperature on blood flow may have important physiological implications. Viscosity may limit the perfusion rate of the highly resistant vessels of the secondary lamellae of the gills during low temperatures, hindering oxygen transfer. This limitation may be accompanied by circulatory features that act to help maintain oxygen uptake from the water. The possible reasons for the uncommon hematological findings for the winter flounder pointed out in Chapter 3 may become clear when the trends in seasonal oxygen uptake at the gill are viewed in light of the present viscosity findings.

The results of this chapter will appear in published form in the Journal of Experimental Biology, in a paper by M.S. Graham and A.P. Farrell entitled, "The seasonal intrinsic cardiac performance of a marine teleost ". (in press Spring, 1985)

CHAPTER 5

SEASONAL INTRINSIC CARDIAC PERFORMANCE

5.1 Introduction

The experiments described in this section were performed on the sea raven due to the technical difficulties of reaching the winter flounder heart *in vivo*. Cardiac preparation of the sea raven has been done before (Farrell, 1984; Farrell *et al.*, 1982).

In this chapter, the function of the sea raven heart during seasonal change will be described. The sea raven (*Hemitripterus americanus*) is a bottom-dwelling fish that lives in seasonal conditions similar to those for the winter flounder. There are also indications that the sea raven has similar feeding habits to the winter flounder. As described in 5.3.1, the sea raven feeds poorly, if at all, during the winter. Winter flounder also do not feed during the winter (see 2.1.1). These considerations lead to the assumption that the standard oxygen demand for the flounder and the sea raven will be similar. The biological and environmental similarities of the sea raven to the winter flounder permit some general conclusions on heart performance in the sea raven being applied to the winter flounder. A better understanding of heart performance will contribute, in turn, to an understanding of seasonal oxygen uptake and delivery in the flounder.

The previous two chapters deal with seasonal aspects of circulatory physiology in the winter flounder. The hematology, Hb function,

and resistance to blood flow were altered significantly by temperature. This chapter investigates the seasonal contractile ability of the heart muscle.

Extrinsic factors, such as neural and humoral substances, have been implicated in inotropic and chronotropic control of cardiac function. Cholinergic input is of prime importance in control of f_H (Laurent, Holmgren, and Nilsson, 1983; Gannon and Burnstock, 1969); while adrenergic factors alter the inotropic state of the heart, and can exert an excitatory influence on f_H (Holmgren, 1977; Randall, 1970; Gannon and Burnstock, 1969). The relative importance of adrenergic and cholinergic cardiac control is also affected by environmental temperatures (Seibert, 1979; Wood, Pieprzak, and Trott, 1979; Priede, 1974).

Aside from the extrinsic control factors, influences dependent upon intracellular characteristics (i.e. intrinsic) are evident. For example, SV_H can undergo marked alteration with small changes in inflow pressure to the heart (the Starling response) (Stuart, Hedtke, and Weber, 1983; Farrell, MacLeod, Driedzic, and Wood, 1983; Farrell et al., 1982; Randall, 1970; Johansen, 1962). Also, the heart can generate greater pressures when outflow resistance increases while maintaining $\dot{V}_b$ (homeometric regulation; Farrell, 1984).

The chronotropic effect of temperature is easily recognized in intact fish. However, the precise impact on the heart is complicated by extrinsic factors. As animals acclimate to different temperatures, a number of cell membrane and intracellular adjustments take place (Hazel and Prosser, 1974; Prosser, 1958). It is likely that cellular

alterations occurring in cardiac tissue during acclimation will affect muscle contractility. The changes, if present, may provide different contractile responses than those seen during acute temperature alterations.

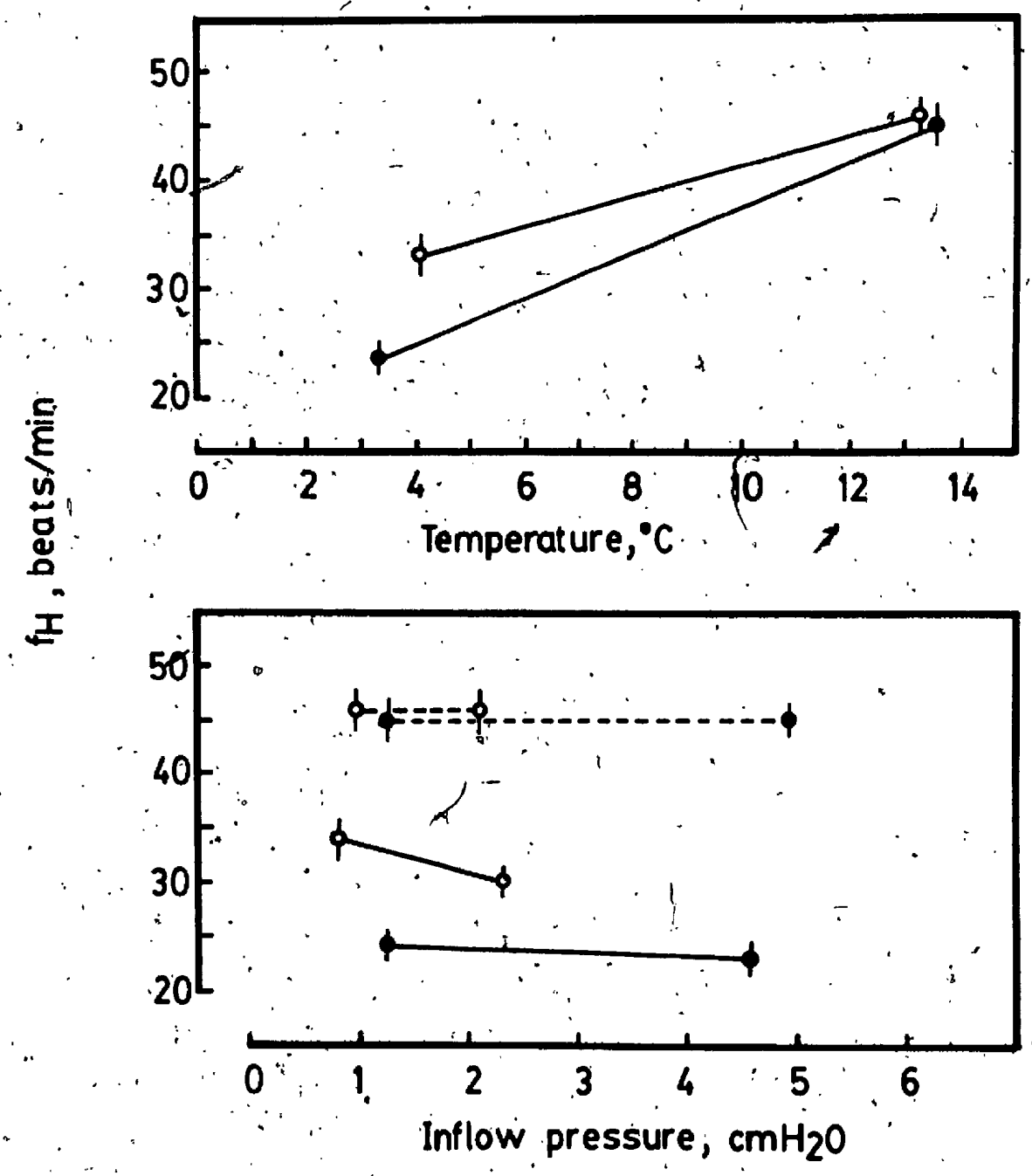
Inflow pressure to the heart and temperature were the two experimental parameters used in the present study to investigate the intrinsic state of the fish heart. The investigation quantifies the direct effects of acute temperature changes on *in situ* heart preparations from winter and summer fish. Fish taken during the winter and during the summer were used to assess the maximum SV_H influenced by inflow pressure as an index of muscle contractility.

5.2 Results

5.2.1 The effect of temperature on heart rate

During surgical preparation, nerves supplying the heart of the sea raven were severed, thus f_H became dependent upon the sino-atrial pacemaker (Farrell *et al.*, 1982). Since f_H was stable under control conditions, any change in response to alterations in temperature was interpreted as a variation in the intrinsic f_H rate. The effects of acute temperature change on f_H are shown in Figure 22. After rapid cooling to 4.1°C, the heart tissues of the summer fish had an average f_H of 34 min^{-1}. The average winter value was 23 min^{-1} (at 3.4°C). Rapid warming of the heart tissues of winter fish to 13.6°C created f_H values that were not significantly different from the

Figure 22. The effect of temperature and inflow pressure on heart rate (f_H) for winter (●) and summer (○) acclimatized sea ravens. In lower diagram (—) was low temperature experiments and (----) was high temperature experiments. Values are means ± SE.


50
40
30
20
0
2
4
6
8
10
12
14
Temperature, °C
f_H, beats/min
50
40
30
20
0
1
2
3
4
5
6
Inflow pressure, cmH_2O

average f_H values of fish acclimatized to summer conditions.

5.2.2 The effect of temperature on aspects of seasonal cardiac output

Under conditions of low inflow pressure, the cardiac output of summer fish is always greater than that of winter fish (Figure 23). The alterations in $\dot{V}_b$ during acute temperature change are parallel in the summer fish and winter fish. When summer and winter fish $\dot{V}_b$ values are compared at similar low inflow pressures and temperatures, no significant difference is apparent (Figure 23).

While f_H is directly related to temperature (Figure 22), SV_H is inversely related (Figure 24). The seasonal winter fish therefore have the greatest SV_H values, while the seasonal summer fish have the lowest. A slight increase occurs in the average SV_H during acute cooling of summer fish. Summer fish at low temperature have significantly lower SV_H values than those for seasonal winter fish. When winter fish are exposed to warm water, a large decrease occurs in SV_H. Winter fish acutely exposed to high temperatures show SV_H values that are not significantly different from average SV_H values for seasonal summer fish.

5.2.3 The effect of inflow pressure on seasonal cardiac performance

Alterations in inflow pressure to the heart changes SV_H values. They increase with increases in inflow pressure under all seasonal and

Figure 23. The effects of temperature and inflow pressure on cardiac output ($\dot{V}_b$) for winter (●) and summer (○) acclimatized sea ravens. In lower diagram (—) was low temperature experiments and (---) was high temperature experiments. Values are means ± SE.

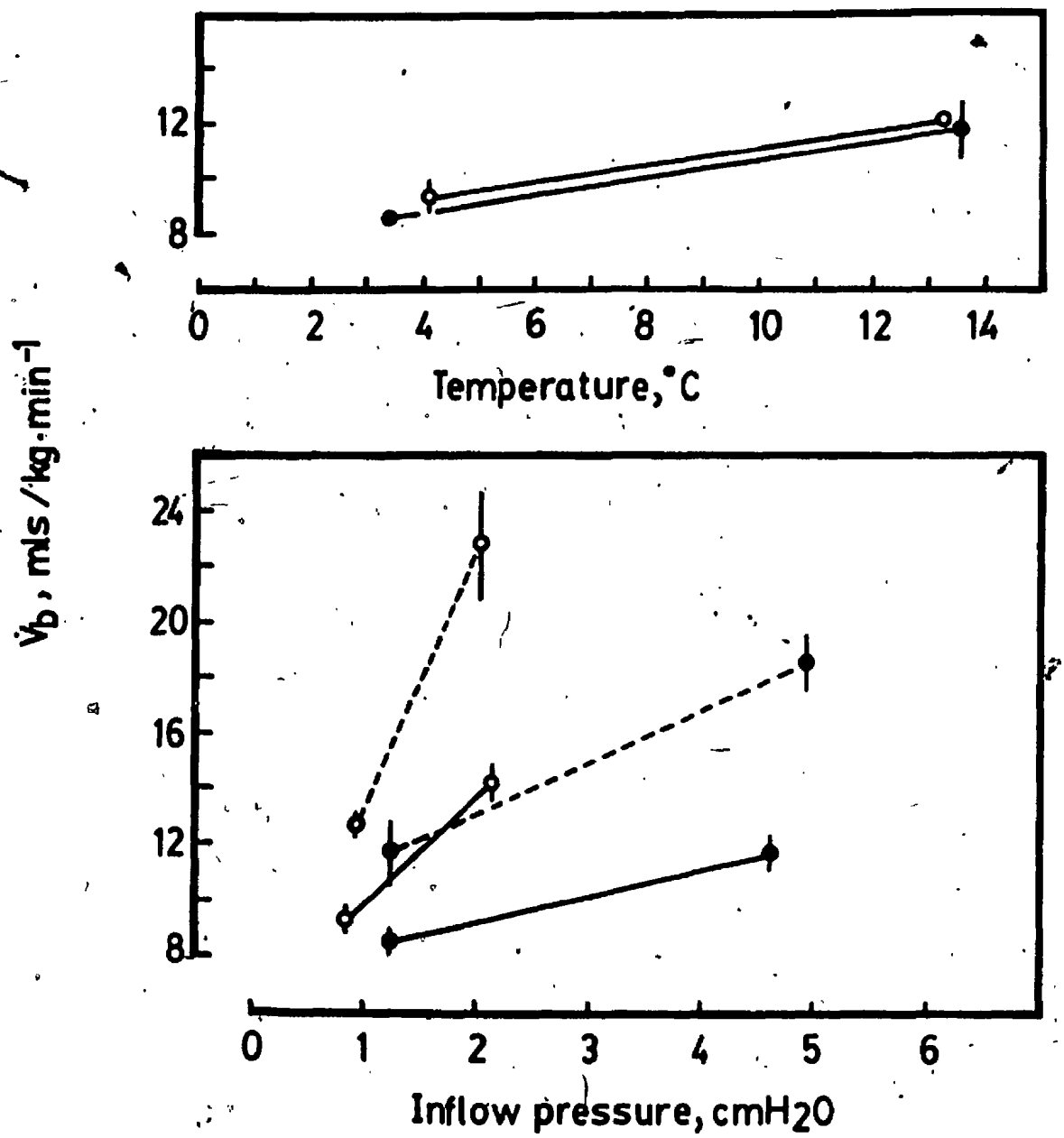
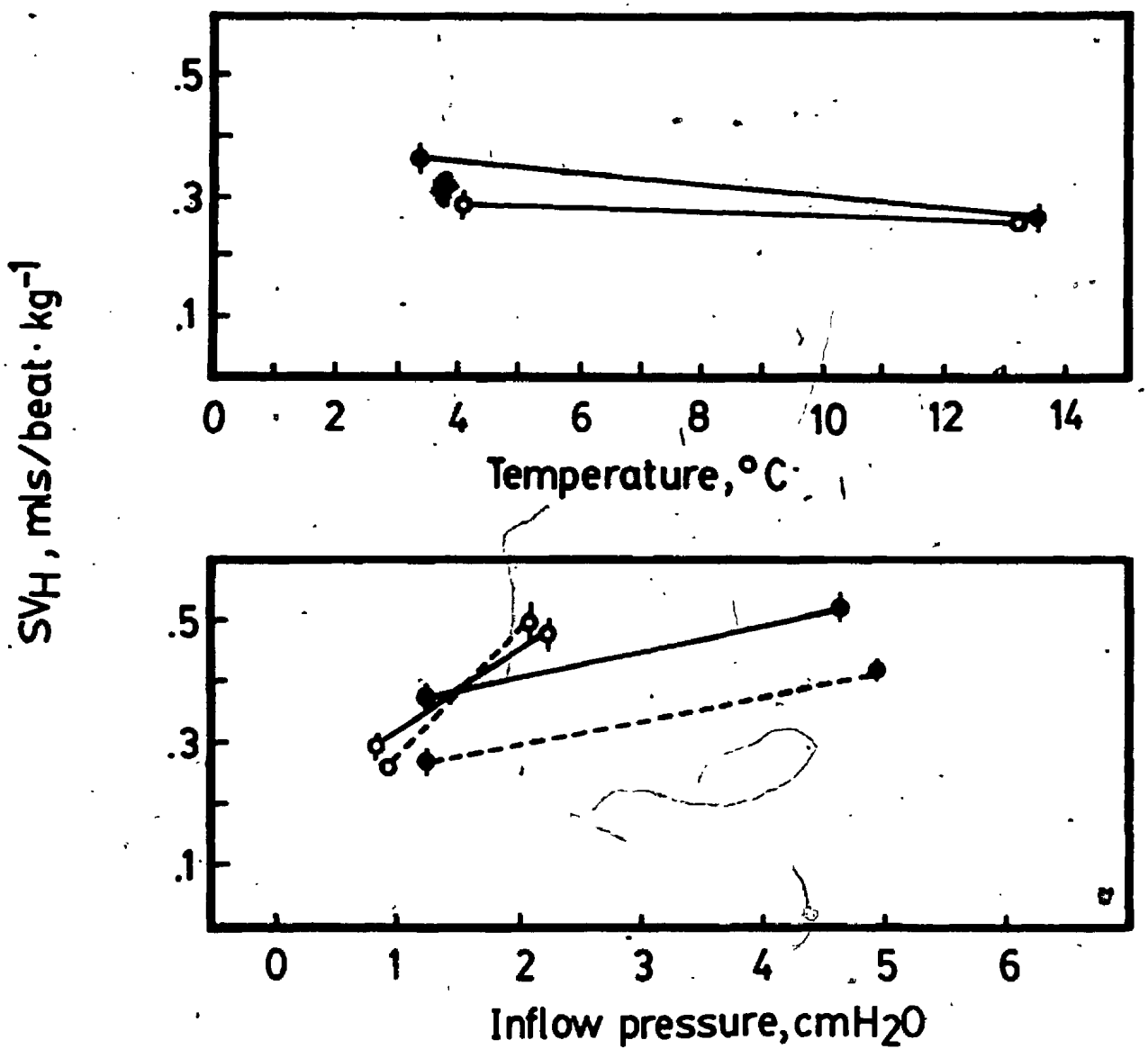

12
8
0
2
4
6
8
10
12
14
Temperature, °C
$\dot{V}_D$, mls/kg·min^{-1}
24
20
16
12
8
0
1
2
3
4
5
6
Inflow pressure, cmH2O

Figure 24. The effects of temperature and inflow pressure on the stroke volume of the heart (SV_H) for winter (●) and summer (○) acclimatized sea ravens. In lower diagram (—) was low temperature experiments and (---) was high temperature experiments. Values are means ± SE.


SV_H, ml/beat · kg^-1
.5
.3
.1
0
2
4
6
8
10
12
14
Temperature, °C
.5
.3
.1
0
1
2
3
4
5
6
Inflow pressure, cmH2O

experimental temperature conditions (Figure 24). The magnitude of SV_H change is greater in summer fish than in winter fish at high (13.3°C) and low (4.1°C) experimental temperatures. The total SV_H change requires a much smaller alteration in inflow pressure in summer fish.

Power (Figure 25) and $\dot{V}_b$ data at higher inflow pressure reflect the higher SV_H values of seasonally acclimatized fish. The $\dot{V}_b$ values for winter fish were significantly lower during conditions of high inflow pressure. The $\dot{V}_b$ changes due to the effects of inflow pressure are a function of SV_H, since f_H is not affected (Figure 22).

5.2.4 Aspects of contractility in seasonally acclimatized fish

In this study a Starling response (measure of contractility) is regarded as an increase in SV_H per unit inflow pressure, per unit ventricular mass (Figure 26). The Starling response of summer fish was considerably greater than that of winter fish at both experimental temperatures. The maximum contractility of summer fish heart muscle was about five times greater than that of winter fish.

5.3 Discussion

5.3.1 Seasonal changes in cardiac muscle performance

Summer and winter fish have the ability to increase $\dot{V}_b$ by

Figure 25. The effects of temperature and inflow pressure on power output of the heart for winter (●) and summer (○) acclimatized sea ravens. In lower diagram (—) was low temperature experiments and (---) was high temperature experiments. Values are means ± SE.

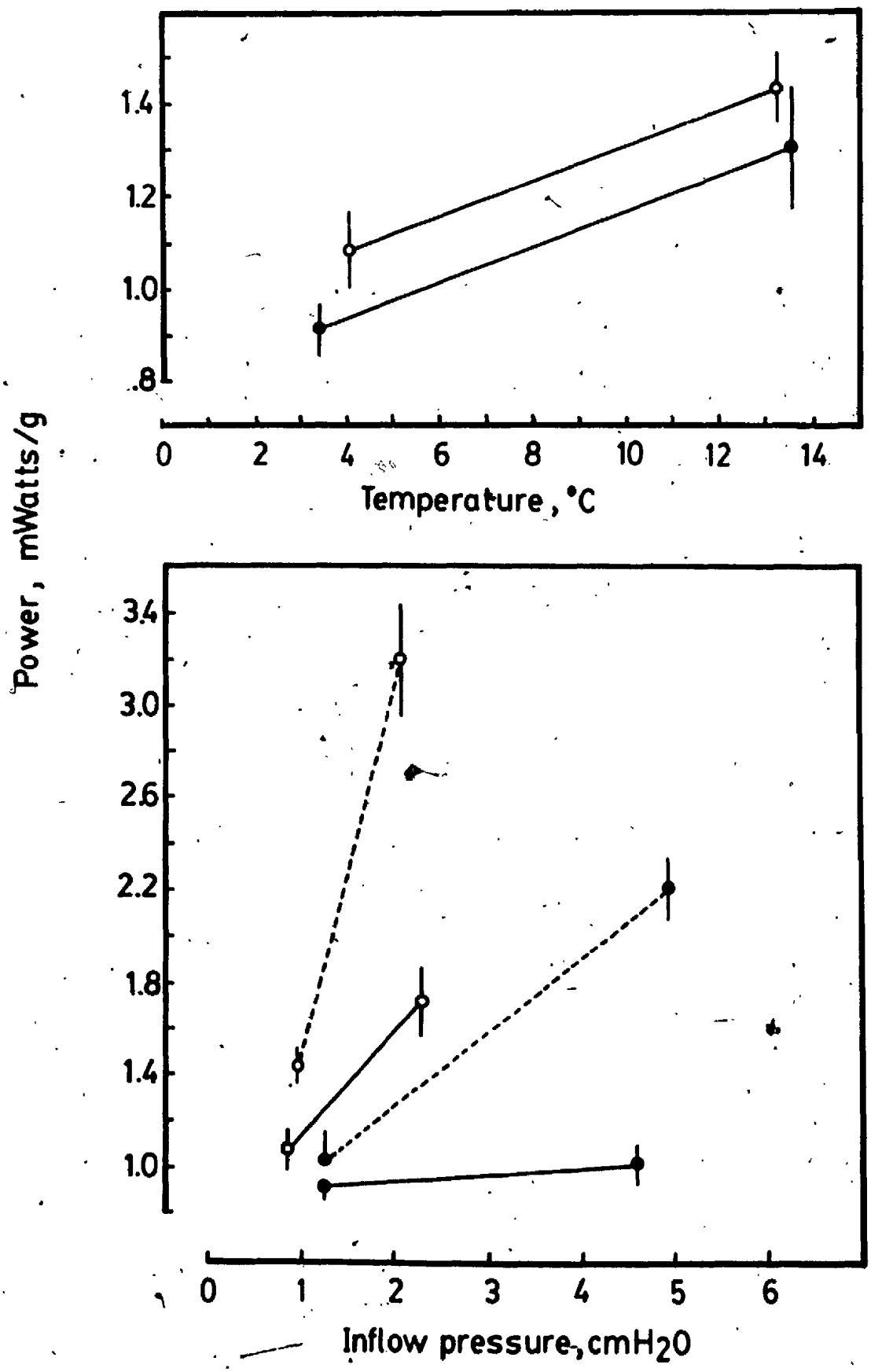
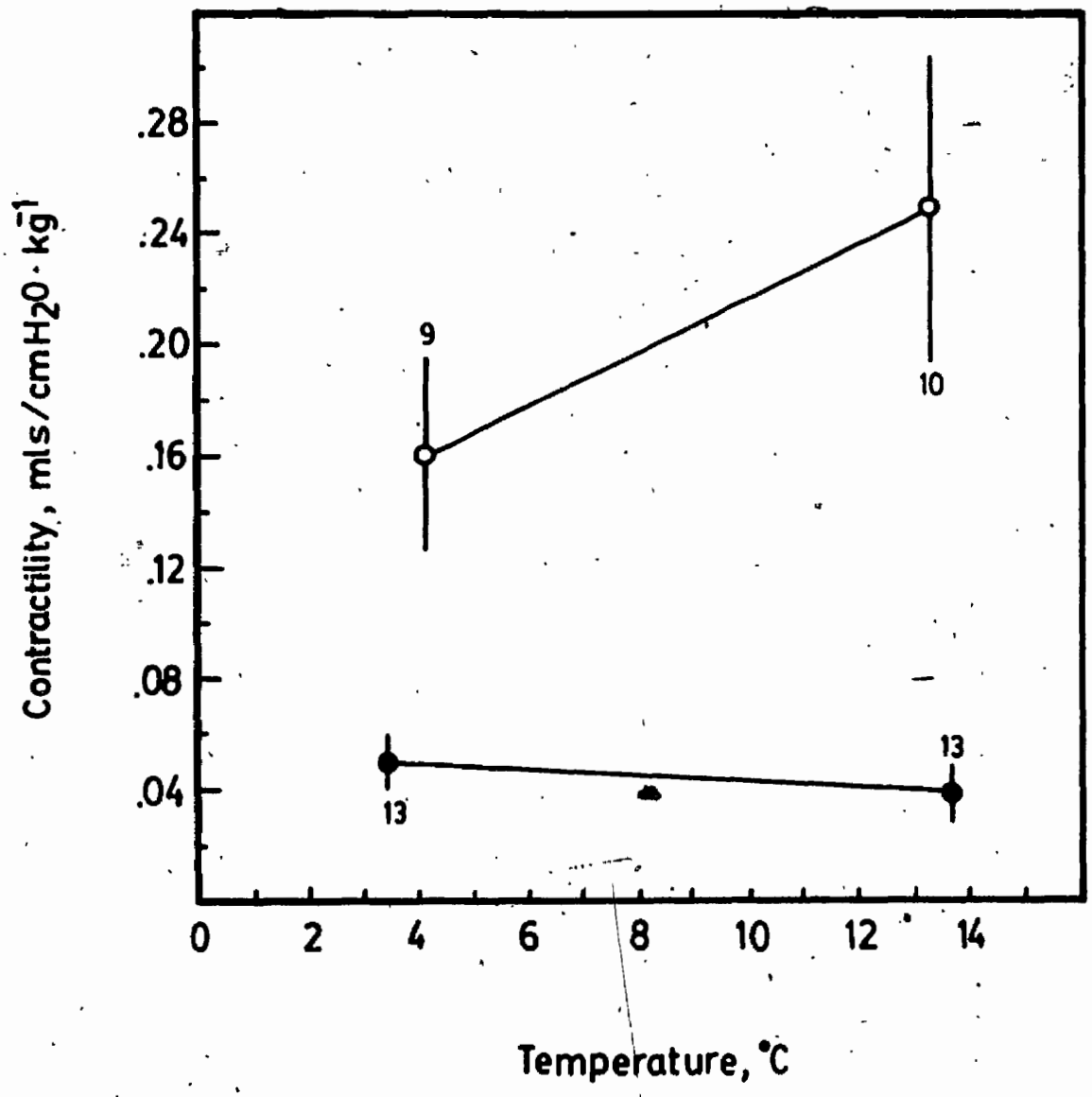

1.4
1.2
1.0
.8
0
2
4
6
8
10
12
14
Temperature, °C
Power, mWatts/g
3.4
3.0
2.6
2.2
1.8
1.4
1.0
0
1
2
3
4
5
6
Inflow pressure, cmH2O

Figure 26. The Starling response of the heart (contractility) for winter and summer acclimatized sea ravens. Symbols are means ± SE, and n is placed near each symbol. (○) = summer acclimatized fish and (●) = winter fish.


Contractility, mls/cmH2O · kg-1
.28
.24
.20
.16
.12
.08
.04
9
10
13
13
0
2
4
6
8
10
12
14
Temperature, °C

chronotropic and inotropic means. The seasonal differences in the Starling response indicate that the inotropy of the heart varies. The inotropic state of the summer fish heart shows a broad range in adjustment of contractility in response to temperature change. Summer fish heart muscle is also more sensitive to changes in inflow pressure. It therefore appears that summer fish are more capable of meeting blood flow requirements during conditions of changing demands for oxygen.

Observations on intrinsic f_H point to different temperature sensitivity during different seasons; summer fish show less sensitivity. This may have adaptive significance, as, for example, a summer fish being able to maintain a high f_H when moving into colder water.

When inshore water cools, often to near-freezing temperatures, the sea raven may move into deeper water (as deep as 192 m; Bigelow and Schroeder, 1953), although there is no conclusive evidence. The sea raven has been known, however, to overwinter in the near-freezing shallows around the Magdalen islands in the Gulf of St. Lawrence.

Winter temperatures depress metabolic activity. From casual observations of winter fish, greatly reduced liver size was apparent, indicating the likelihood of limited feeding at this time of year. The nutritive status of the winter fish can only be speculative without more biological data. If feeding is limited during the winter, production of high-energy compounds necessary to fuel contraction of the heart muscle may be affected. This may limit any circulatory compensations made for seasonal temperature changes. However, if feeding and swimming are diminished in winter, then seasonal compensation in circulatory performance would not be necessary. Conversely, in summer,

the heart of the summer fish undergoes positive inotropic and chronotropic alterations, effectively producing elevated $\dot{V}_b$ even when confronted by intermittent cold temperatures.

Despite the fact that the sea raven heart exhibits little compensation for seasonal temperature change, it appears that intrinsic heart performance is suited for the oxygen demands of the fish.

A definitive comparison between heart contractility in the sea raven and the winter flounder is not possible until more information is known. However, some comparisons can be made between the species. The winter flounder, also a bottom dweller, does not feed during the winter months. Swimming activity is also reduced during the cold months. The similarities in temperatures to which the two species are exposed, and the feeding and swimming habits of the two species in cold temperatures, would tend to lead to similar shifts in seasonal oxygen demand. Since heart performance generally can be related to overall demand for oxygen, it is conceivable that winter flounder also undergo similar changes in cardiac contractility related to season.

5.3.2 General remarks on heart performance

It is known from previous studies on intact winter flounder that cardiac output in the resting fish is greatly reduced in cold temperatures (5°C) (Cech *et al.*, 1976). The present research on the sea raven also suggests that the intrinsic potential to increase blood flow is much reduced in winter acclimatized fish. A combination of decreased flow capacity and increased blood viscosity in winter acclimatized fish

would tend to inhibit oxygen transfer at the gills. It will be interesting to consider the oxygen uptake at the gills of the winter flounder ($\dot{V}_{O_2}$) in view of the hematological, respiratory, and cardiovascular information on seasonally acclimatized fish.

CHAPTER 6

SEASONAL RESPIRATORY PERFORMANCE

6.1 Introduction

The previous chapters describe the effects of temperature and seasonal changes on portions of the respiratory and circulatory systems of the winter flounder and sea raven. In this chapter, using findings discussed in the previous chapters, an attempt is made to explain the seasonal physiology of the winter flounder. This discussion is therefore meant to be both an interpretation and a summary of this research on this species. The flounder is emphasized because most of the research was done on that species. Indeed, all of the work would have been done with the winter flounder, if technical difficulties were not present. However, the sea raven was used to investigate the effects of season and temperature upon heart performance (see Chapter 5). A number of biological considerations were made in comparing the flounder and the sea raven (5.3.1). It was concluded on that preliminary basis that sea raven cardiac performance would likely be similar to that of the winter flounder. On these grounds some of the findings for the sea raven will be used in the interpretation of seasonal oxygen uptake and delivery in the winter flounder.

In this section the oxygen dynamics between the water and the blood are investigated. Measurements of oxygen uptake are useful, and reflect the aerobic metabolic needs of the fish. Oxygen uptake at the

gills is dependent on the convective properties of the water and blood, the amounts of oxygen present in the fluids, the distance that oxygen from the water travels in diffusing to the blood (diffusion distance), the surface area of the exchange site, and the ability of the blood to bind and store oxygen. Seasonal changes in blood physiology (including the function and quantity of the blood components, and the flow characteristics of the blood) contribute toward seasonal differences in the oxygen uptake at the gills. The contributions of the individual components have been discussed in previous sections. In the previous chapters it was shown that temperature was responsible for much of the seasonal change in blood physiology. In this chapter, many components of the ventilatory and circulatory system are considered together. In observing the characteristics of oxygen uptake at the gills during different seasons, along with some of the circulatory and ventilatory parameters during those periods, a better understanding of the interaction of physiological components is gained.

The literature on hematological changes due to seasonal changes points to differences even within fish species. In most cases, rises in temperature result in increases in [Hb] (Houston and Smeda, 1979; Cameron, 1970), and [NTP] of the red blood cells (Tetens et al., 1984; Dobson and Baldwin, 1982a; Greaney and Powers, 1977). It is thought that these changes assist in improving oxygen supply to the tissues by increasing the capacity of the blood to store oxygen, and increasing the potential of Hb to release oxygen. The changes in [Hb] and [NTP] occur more gradually in response to temperature change (acclimation), compared to the immediate response of the cardiovascular system (Weber,

1982; Cech et al., 1976; Heath and Hughes, 1973).

Species demonstrating decreases in [Hb] during acclimation to high temperature include the winter flounder (Cech et al., 1976), a Scandinavian variety of rainbow trout (Nikinmaa et al., 1981 and 1980); and the brown bullhead (Grigg, 1969). A decrease in [Hb] under high temperature conditions seems inappropriate, since demand for oxygen is greater. Similarly, an increase in blood oxygen storage capacity during the winter seems inappropriate since metabolic oxygen demand is decreased at that time. Oxygen uptake in the resting winter flounder was studied to clarify further the function of seasonal changes in the hematology of the species. Measurements were made of seasonal changes in red blood cell distribution, and seasonal changes in the respiratory and cardiovascular systems. Results of data analysis point to the importance of the maintenance of a balance between hemodynamics and blood composition during the seasonal changes in oxygen uptake.

6.2 Results

The avergae rate of oxygen uptake at the gills of the winter flounder is considerably lower in winter fish (Q_{10} = 2.4), as can be seen in Table 4. There are proportional changes in $\dot{V}_w$ and $\dot{V}_b$ (Q_{10} = 2.2 and 2.5, respectively). The changes in $\dot{V}_w$ and $\dot{V}_b$ are the result of large alterations in frequency and modest shifts in SV_H.

Even though some blood loss in the winter flounder occurred during surgical manipulation, the seasonal trends in Ht and [Hb] of

Note for Table 4

The average values for winter and summer were compared using the unpaired Student's t-test. Significance was decided at the 95% confidence level.

The P_{iO_2} values given in the table exceed saturation values. This is a common occurrence at the Marine Sciences Research Laboratory, Logy Bay, Newfoundland. The supersaturation with oxygen occurs at all times of the year, and is related to the extensive mixing during the pumping procedure from the ocean to the laboratory. These conditions are not deleterious to the fish unless temperature conditions change rapidly. For example, water saturated at low temperatures and then rapidly heated produces gas bubbles which are lethal to the fish. The temperature conditions during the time of winter and summer experiments were very stable. The P_{iO_2} conditions of inflowing water during these tests does not alter the interpretation of the results and calculations.

Table 4: Respiratory and circulatory *in vivo* data from the winter flounder. Values are means ±SE. () = number of animals used. NS = not significant.

	Summer	Winter	Significance
Water temperature, T_{H_2O} °C (°C)	9.51 + 0.19 (17)	0.44 + 0.21 (11)	$p < 0.005$
Total wet mass, grams	493 + 61 (9)	407 + 36 (11)	N.S.
Hematocrit, Ht % red blood cells	8.0 + 1.1 (9)	11.2 + 0.8 (11)	$p < 0.025$
Hemoglobin concentration, [Hb] g/100 mLs blood	2.25 + 0.24 (6)	3.10 + 0.30 (11)	$p < 0.05$
Arterial oxygen tension, P_{aO_2} mm Hg	82.3 + 7.8 (9)	100.3 + 7.6 (11)	N.S.
Venous oxygen tension, P_{vO_2} mm Hg	30.2 + 3.7 (8)	33.6 + 5.9 (10)	N.S.
Arterial oxygen content, C_{aO_2} mLs O_2/100 mLs blood	2.33 + 0.19 (9)	3.90 + 0.35 (11)	$p < 0.005$
Venous oxygen content, C_{vO_2} mLs O_2/100 mLs blood	1.14 + 0.20 (8)	1.80 + 0.26 (10)	N.S.
Arterial Hb-O_2 saturation, S_{aO_2} %	69.32 + 6.77 (6)	79.71 + 5.05 (11)	N.S.
Venous Hb O_2 saturation, S_{vO_2} %	38.10 + 2.27 (6)	39.82 + 6.01 (10)	N.S.
Oxygen tension, inflow water, P_{iO_2} mm Hg	159.5 + 1.9 (8)	169.6 + 3.0 (11)	$p < 0.01$
Oxygen tension, expired water, P_{eO_2} mm Hg	49.1 + 6.8 (8)	99.6 + 6.4 (11)	$p < 0.005$
Oxygen consumption across gill, $\dot{V}_{O_2}$ mLs O_2/kg . min^{-1}	0.33 + 0.021 (8)	0.16 + 0.02 (10)	$p < 0.005$
Volume water flow at respiration, $\dot{V}_w$ mLs H_2O/kg . min^{-1}	73.4 + 9.8 (8)	46.7 + 10.3 (10)	$p < 0.02$
Frequency of respiration, f_R breaths/min	46.2 + 3.5 (9)	22.78 + 7.75 (11)	$p < 0.005$
Water volume per respiration, SV_R mLs H_2O/kg . $breath^{-1}$	1.9 + 0.15 (6)	2.05 + 0.45 (10)	N.S.
Volume blood flow, $\dot{V}_b$ mLs blood/kg . min^{-1}	26.74 + 3.54 (7)	17.7 + 2.0 (8)	$p < 0.005$
Frequency of heart beat, f_H beats/min	26.8 + 1.8 (6)	18.1 + 1.3 (11)	$p < 0.005$
Stroke volume of the heart, SV_H mLs blood/kg . $beat^{-1}$	1.05 + 0.21 (4)	0.67 + .12 (8)	N.S.

experimental fish are consistent with results from uncannulated fish when comparing data in Tables 1 and 4. In experimental fish, a change from high temperatures to winter results in significantly higher Ht and [Hb], and C_{aO_2} in winter fish (Table 4). Other variables in venous and arterial oxygen are not significantly different when winter and high temperature values are compared.

The blood volumes of seasonally acclimatized winter fish and cold acclimated summer fish are significantly lower than the blood volume of fish under all warm water conditions (Figure 27).

The only significant difference in seasonal red blood cell distribution was found in samples of dermis and epidermis. They showed a higher rbc content in the summer sample (Table 5). Oxygen uptake of the skin is about 33 per cent of the total oxygen uptake in winter acclimatized fish (Table 6).

6.3 Discussion

6.3.1 Experimental procedures

As explained in 2.4.2, due to the fragility of fish at high temperatures, two different procedures were used to obtain data on changes in respiratory physiology. Measurements on winter fish fitted with ventilatory masks gave values within the range of those derived by using the isolated box technique. However, some apparent differences are noted. The change in P_{O_2} across the gills and f_R was greater, while $\dot{V}_W$ was lower for fish with ventilatory masks. These findings

Figure 27. The results of seasonal blood volume measurements for the winter flounder. Matched symbols indicate values that are significantly different ($p < 0.05$). S and W are mean values $\pm$ SE. ( ) = n. Significance testing was done by using the unpaired Student's t-test.

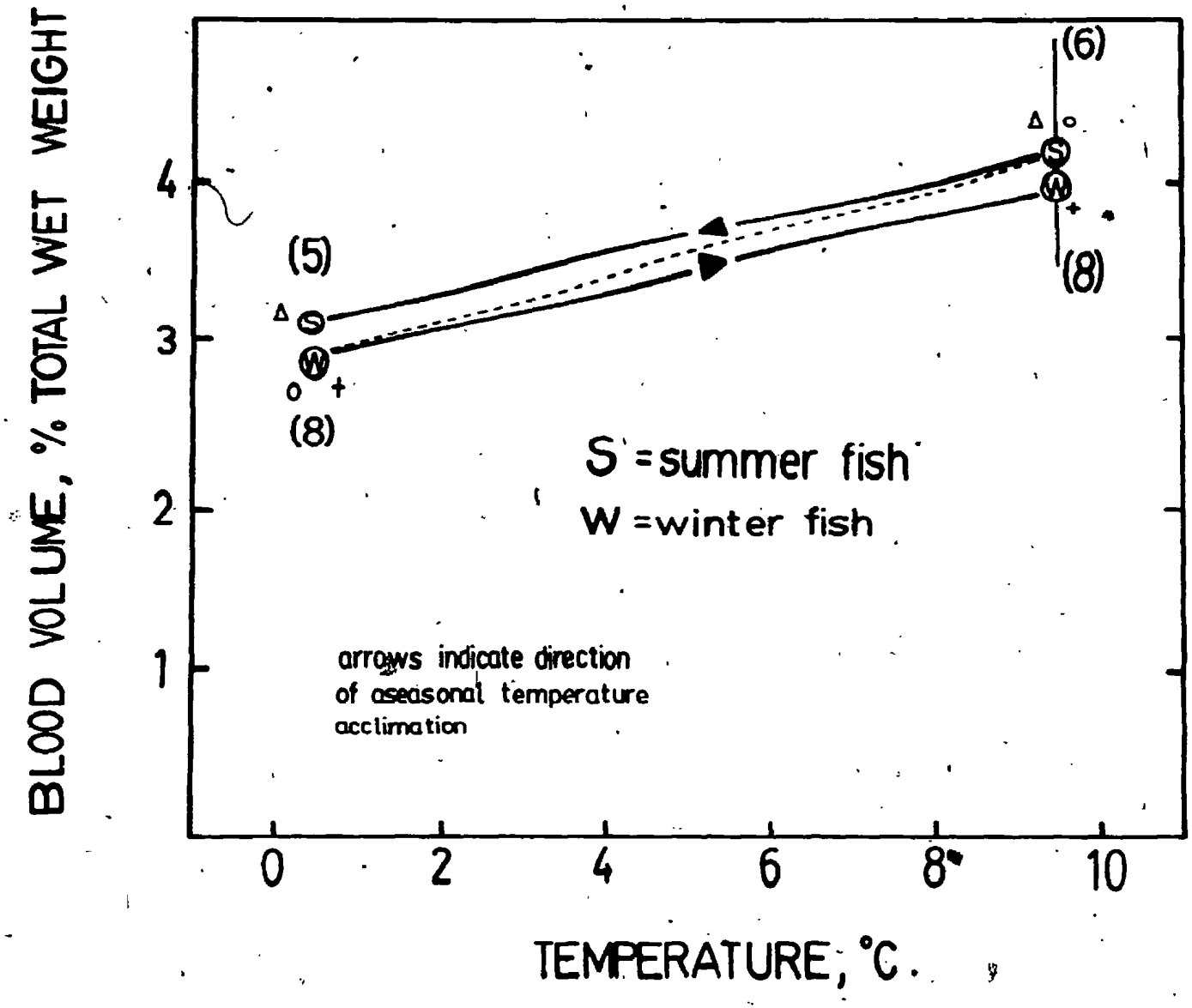

BLOOD VOLUME, % TOTAL WET WEIGHT
4
3
2
1
0
2
4
6
8
10
TEMPERATURE, °C.
(5)
(8)
(6)
(8)
S = summer fish
W = winter fish
arrows indicate direction
of aseasonal temperature
acclimation

Table 5: Red blood cell distribution in the winter flounder during various temperature exposures. Values are percent tissue mass as red blood cells (means ± SE). () = number of animals used. Condition = season/temperature, Fil = filament, and W. muscle = white muscle.

Condition	Spleen	Kidney	Liver	Intestine	Gill Fil.	W. Muscle	Dermis & Epidermis
Summer warm	3.579 1.029 (6)	0.801 .273 (6)	0.476 .127 (6)	0.034 .011 (6)	0.574 0.169 (6)	0.005 .002 (6)	0.017 .006 (6) + Δ
Summer cold	3.239 0.442 (5)	1.195 0.249 (5)	0.522 .089 (5)	0.050 .007 (5)	0.692 0.168 (5)	0.011 .005 (5)	.014 .006 (5)
Winter cold	4.261 .730 (8)	0.719 .280 (8)	0.348 .138 (8)	0.053 .016 (8)	0.670 0.344 (8)	0.007 0.002 (8)	.006 .003 (8) Δ
Winter warm	2.486 .660 (8)	0.822 .125 (8)	0.416 .069 (8)	0.034 .005 (8)	0.339 0.084 (8)	0.012 .005 (7)	.00[illegible] .00[illegible] (8) +

NOTE: + compares s/w vs. w/w
Δ compares s/w vs. w/c

All comparisons were for $p < 0.05$

Note: statistical comparisons were done by using the unpaired Student's t-test.

Table 6: Respiration data from flow through respirometry on unrestrained winter flounders (Kiceniuk, unpublished), and the present study for winter and summer animals. Values are means + SE. Kiceniuk's values are for whole animals (=total) and those for the present study are for gills, skin, and whole body. All values are in units of $mlO_2/kg \cdot min^{-1}$.

	Kiceniuk		Present Study					
	Summer	Winter	Summer	Winter				
	(total)	(total)	(gill)	(gill)	(skin)	(total)	$\frac{skin}{total} \times 100$	$\frac{nMole\ O_2}{cm^2}$
	0.28 + .03 (8)	0.20 + .01 (6)	0.33 + .04 (8)	0.15 + .02 (10)	0.07 + .02 (9)	0.22 + .03 (9)	33.2 + 7.03 (9)	2.42 + .67 (9)
°C	9.90 ± .03	1.20 ± .04	9.51 ± .19	0.44 ± .21	0.44 ± .21	0.44 ± .21	0.44 ± .21	0.44 ± .21

suggest a greater degree of restriction of ventilation using the mask technique, therefore Q_{10} for $\dot{V}_W$ should be considered conservative. Similar comments have been put forward by other researchers (Piiper and Schumann, 1967). Although the $\dot{V}_W$ for fish at high temperatures is significantly greater than that of winter fish under the present conditions, if it were possible to use the isolated box technique, $\dot{V}_W$ would likely be even higher for summer acclimatized fish.

Anemia in the experimental fish was due to arterial and venous cannulations during summer and winter experiments. Wood, McMahon, and McDonald (1979a) provide respiration data for the starry flounder (Platichthys stellatus) during various states of experimentally induced anemia. In the study by Wood et al. (1979a), the levels of anemia comparable to the values found in the present study brought about only a decrease in C_{vO_2}. That adjustment maintained the C_{aO_2}-C_{vO_2} difference at the same value for the anemic and the control group. A similar trend is expected for the winter flounder of the present study.

6.3.2 Cutaneous oxygen uptake of the winter flounder

The present $\dot{V}_{O_2}$ estimates for fish under restraint are similar to those for unrestrained winter flounder (Kiceniuk, unpublished data, Table 6). The Kiceniuk data were obtained by flow-through respirometry on winter flounder resting in a sandy-bottomed tank. The whole-body $\dot{V}_{O_2}$ for summer fish is equal to the oxygen uptake at the gills in the high temperature fish used in the present study. The addition of skin $\dot{V}_{O_2}$

would create a higher whole-body $\dot{V}_{O_2}$ in the fish at high temperatures. Whole body $\dot{V}_{O_2}$ values during low temperatures did not differ from those found by Kiceniuk (Table 6), even though in the present study, fish were restrained. This agreement in $\dot{V}_{O_2}$ values for fish at cold temperatures seems to suggest that winter acclimatized fish do not respond as readily to the stress of restraint as summer fish.

A high $\dot{V}_{O_2}$ value in the skin of winter fish (one-third of the total $\dot{V}_{O_2}$) is due, in part, to the low rate of oxygen taken up by the gills at that time. It is expected that $\dot{V}_{O_2}$ in the skin of summer fish would increase in absolute value because of increased vascularization (Table 5). High values of cutaneous $\dot{V}_{O_2}$ have been described in other fish at high temperatures (10°C) (Steffensen, Lomholt, and Johansen, 1981; Nonnotte and Kirsch, 1978).

At 10°C, circulation to the skin can affect cutaneous oxygen uptake by as much as 10 per cent (Steffensen and Lomholt, in press). If the increased rbc contents of the skin indicated by data in the present study caused a 10 per cent increase over winter values in skin $\dot{V}_{O_2}$, a cutaneous $\dot{V}_{O_2}$ of approximately 20 per cent would be expected for high temperature values. At 10°C, Steffensen _et al._ (1981) found skin $\dot{V}_{O_2}$ to be 27 per cent of the total value in _Pleuronectes platessa_, while Nonnotte and Kirsch (1978) found skin $\dot{V}_{O_2}$ to be 33 per cent of the total value in _Platichthys flesus_. Even though oxygen uptake across the skin is high, most of it is used by the skin tissues (Steffensen and Lomholt, in press; Kirsch and Nonnotte, 1977). The oxygen taken up directly from the water by the skin is expected to be higher during the summer due to a smaller diffusion distance (thinner epidermis, see

6.3.3) and greater vascularization. The oxygen uptake from the water by the skin suppliments the demand for oxygen by the cutaneous tissues.

It is not certain if the skin vasculature of the winter flounder has any respiratory significance for the rest of the body. It is apparent from Table 5 that the alteration in cutaneous circulation requires more than just temperature acclimation, and that the skin has relatively little vasculature when compared with other tissues (Table 5).

6.3.3 Seasonal oxygen dynamics at the gills: Interaction of blood components and hemorheology

Blood volumes of summer and winter fish under warm and cold conditions were measured. One of the important components of the oxygen delivery system is Hb. As mentioned in 3.3.3, temperature has a major influence on the function of Hb. In this section, the role of temperature in controlling Hb concentration will be briefly discussed.

Blood taken from the major caudal vessels of the winter flounder indicated a greater blood volume in all fish exposed to high water temperatures. Nikinmaa et al. (1981) explained that the blood volume of the rainbow trout does not change with temperature acclimation. He showed that the [Hb] of blood from the dorsal aorta decreased during high temperature acclimation (18°C) apparently due to shunting of red blood cells to the tissues. In the present study, it was found that blood volume did show a significant change in response to seasonal and temperature acclimation. However, seasonal differences in the amount

of rbc's in the tissues (a tissue rbc shunt) were not apparent in the winter flounder (Table 5), except in the dermis/epidermis samples. Determinations of blood volume were not affected by changes in circulation in the skin, thus, the increased blood volume, decreased [Hb], and rbc concentration of P. americanus in the high temperature fish is due to increased plasma volume.

It is clear that the oxygen demands of the winter flounder change from winter to summer season. The oxygen-carrying capacity of the blood also changes seasonally, presumably best to answer that demand. Summer fish have a decreased blood oxygen carrying capacity, indicated by the lower [Hb] and Ht values. This finding is somewhat anomolous since use of oxygen by the tissues would be greatest at that time. A closer examination of Table 4, and some calculations (Table 7) enable some observations to be made on oxygen dynamics at the gills of seasonally acclimatized winter flounder.

A number of calculations were made to arrive at an estimate of the characteristics of gill oxygen transfer under different seasonal conditions and temperatures. Decreased cardiac output in winter fish would increase the time necessary for blood-gas exchange at the tissues. Even though $\dot{V}_{O_2}$ is lower in winter fish, the slower blood flow through tissues is indicated in higher C_{aO_2}-C_{vO_2} differences. The high arterial-venous O_2 difference in winter fish is also indicated by the greater effectiveness of oxygen transfer from the blood to the tissues. The transfer factor (TO_2) is a measure of conductance, giving the rate of oxygen movement per unit P_{O_2} gradient across the gills (Dejours, 1981). The lower TO_2 of winter fish indicates a resistance to oxygen transfer. That idea is supported because of the greater

oxygen diffusion gradient across the gills in winter fish, and a signifciantly lower utilization of ventilatory water. The effectiveness of oxygen transfer from water to blood is also lower in winter fish. Finally, there is an increase of the average ventilation-perfusion ratio in winter fish.

Several of the calculations in Table 7 are presented without error estimates. That short-coming was due to the experiments on summer fish. Blood measurements were done on one group of fish at high temperatures, while the oxygen measures on water were done on another group (2.4.2). When the values from both experimental groups were needed in a calculation (e.g. ΔP_{O_2}, $\dot{V}_b/\dot{V}_w$, $\dot{V}_b/\dot{M}_{O_2}$, capacity-rate ratio, TO_2, and effectiveness of water O_2 transfer), average values from each group were used. Because no error is known for the high temperature values, no statistical testing between winter and high temperature values was done. The TO_2 values indicate that the gills of winter fish are more resistant to oxygen passage. However, there is no statistical evidence to support the large difference in the average TO_2 values. Similar results are indicated by the effectiveness of water O_2 transfer, capacity-rate ratio, and ΔP_{O_2} values. In the following arguments it is assumed that the increased gill resistance to O_2 does exist in winter fish. That assumption is based on the highly significant difference seen between winter and high temperature fish when the utilization of O_2 from ventilation water is compared. That consideration on its own indicates a very real difference in the gill resistance to oxygen transfer in seasonally acclimatized fish.

Seasonal differences in gill oxygen conductance may be due in part

Table 7: Oxygen transfer parameters for the winter flounder. Values are means + SE. () = number of animals used. N.S. = not significant.

	Summer	Winter	Significance
$C_{aO_2} - C_{vO_2}$ (mLs O_2/100 mLs blood)	1.18 0.19 (8)	1.98 0.33 (10)	$p < 0.05$
Mean P_{O_2} gradient across gill; ΔP_{O_2} $[(P_{iO_2} + P_{eO_2})/2] - [(P_{aO_2} + P_{vO_2})/2]$ (mm Hg)	48.1 - (-)	67.2 9.1 (10)	-
Oxygen consumption across gill, $\dot{V}_{O_2}$ (mLs O_2/kg . min^{-1})	0.33 0.04 (8)	0.16 0.02 (10)	$p < 0.05$
utilization of O_2 from water; U_{wO_2} $(P_{iO_2} - P_{eO_2})/P_{iO_2}$	0.69 0.04 (8)	0.41 0.03 (11)	$p < 0.005$
utilization of O_2 from blood, U_{bO_2} $(C_{aO_2} - C_{vO_2})/C_{aO_2}$	0.48 0.07 (8)	0.52 0.07 (10)	N.S.
Ventilation-perfusion ratio $\dot{V}_w/\dot{V}_b$	2.7 - (-)	4.4 1.2 (8)	-
Convection requirement, water $\dot{V}_w/\dot{M}_{O_2}$ (L, H_2O/mMoles O_2)	5.0 0.3 (8)	6.1 0.6 (10)	N.S.
Convection requirement, blood $\dot{V}_b/\dot{M}_{O_2}$ (L, Blood/mMoles O_2)	1.8 - (-)	1.7 0.3 (8)	-
Transfer factor; T_{O_2} $\dot{V}_{O_2}/\Delta P_{O_2}$ (mLs O_2/kg . min^{-1} . mm Hg^{-1})	0.007 - (-)	0.003 0.001 (9)	-
Capacity-rate ratio $(P_{aO_2} - P_{vO_2})/(P_{iO_2} - P_{eO_2})$	0.43 - (-)	1.05 0.13 (10)	-
Effectiveness of water O_2 transfer $(P_{iO_2} - P_{eO_2})/(P_{iO_2} - P_{vO_2})$	0.85 - (-)	0.52 0.05 (10)	-
Effectiveness of blood O_2 transfer $(C_{aO_2} - C_{vO_2})/(C_{maxO_2} - C_{vO_2})$	0.58 0.07 (5)	0.78 0.09 (7)	N.S.

Note: Where applicable, the mean values for winter and summer were compared using the unpaired Student's t-test. Significance was at the 95% confidence level.

to a combination of rbc shunting within the gill filaments, and a change in the rate of blood perfusion. Whole gill filaments were analysed in this study and no seasonal nor temperature influences on rbc content were discovered. However, Nikinmaa et al. (1980) found that rainbow trout have a greater number of rbc's in the secondary lamellae at 18°C than at 2°C. The perfusion rate through the filaments ($\dot{V}_b$) is also greater in fish at high temperatures (present study; and Cech et al., 1976), facilitating oxygen uptake at the gills (Daxboeck, Davie, Perry, and Randall, 1982). The variation in distribution of rbc's over the filaments depending upon season and temperature, along with the changes in rate of blood perfusion, would bring about seasonal differences in utilization of oxygen from the water (U_{wO_2}).

A simple explanation of the seasonal differences in gill oxygen resistance in the winter flounder would be the alteration in the diffusion distance across the gill epithelium. Other studies on the winter flounder have shown that the epithelium of the trunk is thicker in the winter (Burton and Fletcher, 1983). The thickening was found to be most noticeable in male flounders. However, both sexes demonstrated significant epithelial thinning during the summer. These seasonal changes can be correlated with the levels of 11-ketotestosterone in the blood (Campbell, Walsh, and Idler, 1976). Treatment with 11-ketotestosterone caused increased skin thickness in the sockeye salmon (Idler, Bitners, and Schmidt, 1961). Although no observations were made of gill tissue by Burton and Fletcher in their 1983 study, it is possible that thickening occurs in winter flounder gill tissue too. Wood, McMahon, and McDonald (1979b) have noted that TO_2 differences

between the flatfish Platichthys stellatus and the tench Tinca tinca may be due to thickness of the gill epidermis.

Another explanation of seasonal blood oxygenation involves many of the physiological changes which occur as a result of changes in temperature. The blood of winter fish has a greater oxygen-binding capacity (Tables 1 and 4), but, as mentioned in 4.2.3, this does not result in increased flow resistance. However, the effect of temperature and season on blood viscosity (see 4.3.5), and heart performance (see 5.3.1 and Table 4) is to decrease the rate of blood flow through the respiratory vasculature of winter-acclimatized fish. Increased blood viscosity in winter fish may limit the surface area for gas exchange at the gill. During the winter, viscous blood would flow through the larger, less resistant basal and marginal channels (Hughes and Morgan, 1973) to a greater extent. The findings on the effects of temperature on in situ blood flow resistance in the winter flounder support this suggestion (see 4.3.6). Such a flow pattern would expose the blood to a smaller surface area for oxygen uptake, and in the case of the basal channel, deep in epidermal tissue (Tuurala, Part, Nikinmaa and Soivio, 1984; Cooke, 1980; Smith and Johnson, 1977). Farrell, Sobin, Randall, and Crosby (1980) studied the variation of intralamellar blood flow under different perfusion conditions. They found that during conditions of low blood flow, blood was distributed nearer the base of the lamella. Blood distribution moved distally as flow increased through the lamella, and gas transfer at the gills could therefore be facilitated.

The lower U_{wO_2} and TO_2 of winter-acclimatized fish appears to

be due, therefore, to increased diffusion distance and/or the rheological performance of the blood. Even though the gills of winter fish showed a greater resistance to oxygen transfer, a number of aspects of the hematology assist in maintaining P_{aO_2}. Blood of winter fish is known to have a significantly higher affinity for oxygen, so oxygen uptake is enhanced at the gills. The increased storage capacity for oxygen in the blood of winter fish acts to increase the amount of arterial oxygen. An increased average ventilation-perfusion ratio in winter fish means that more water moves across the gill surface per unit blood flow. An increased P_{O_2} gradient across the gills of winter fish facilitates a greater diffusion of oxygen towards the blood. In the winter fish then, a number of factors could effect a greater potential for blood oxygenation. From the present study, these factors are: an elevated Hb-oxygen affinity, a greater blood oxygen storage capacity, a higher ventilation-perfusion ratio, and a greater P_{O_2} gradient across the gills. It therefore seems that the resistance to oxygen transfer in winter fish is overcome largely by seasonal changes in the blood components.

Seasonally-acclimatized winter flounder showed differences in oxygen demand. Seasonal differences in oxygen dynamics at the gills, the primary organ answering the demand of the fish for oxygen, were also noted. Temperature is believed to be responsible for the seasonal change in oxygen demand, and the alterations in the process of oxygen transfer to the blood. Even though oxygen conductance of the gills is clearly variable on a seasonal basis, the winter flounder is able to maintain P_{aO_2} levels at all times (see above discussion and 3.3). The

P_{aO_2} values of fish at high temperature may seem unnecessarily high for winter fish with significantly lower $\dot{V}_{O_2}$ levels. However, the maintenance of P_{aO_2} may be required by the winter fish during periods of stress. As indicated by the findings of Chapter 5, winter acclimatized fish such as the sea raven and the winter flounder appear to have a poor potential for increasing cardiac output. The inotropic and chronotropic ability of the heart to meet increased demands for oxygen may be very limited in winter acclimatized fish. The flounder may have adapted by having an oxygen reserve stored in circulating hemoglobin. The larger hemoglobin concentration during the winter has been shown to occur due to the influence of season (e.g. Table 1 and 4), and specifically the influence of temperature (Figure 27). The converse is true for summer acclimatized winter flounders. In those fish hemoglobin values are reduced, and cardiac output potentials are increased (Table 1 and 4, and Figure 23).

The Hb function of seasonally acclimatized fish also seems well adapted. The red blood cell components that influence Hb function, and the direct effects of temperature, produce Hb-oxygen affinity that favours delivery of oxygen to the tissues during the summer, and increased oxygen uptake at the gills during the winter. The trend in oxygen affinity change is then appropriate for the higher demand for oxygen during the summer, and the high resistance to oxygen passage at the gill during the winter. Oxygen release rate to the tissues is not as critical during the winter because demand is low, and the passage of blood is slower at that time (Table 4).

The circulatory physiology of the summer acclimatized fish aids

the blood in passing over a larger respiratory surface area, which is less resistant to oxygen uptake. While the capacity of summer fish blood to store oxygen is diminished, the blood is returned to the respiratory surface at a greater rate.

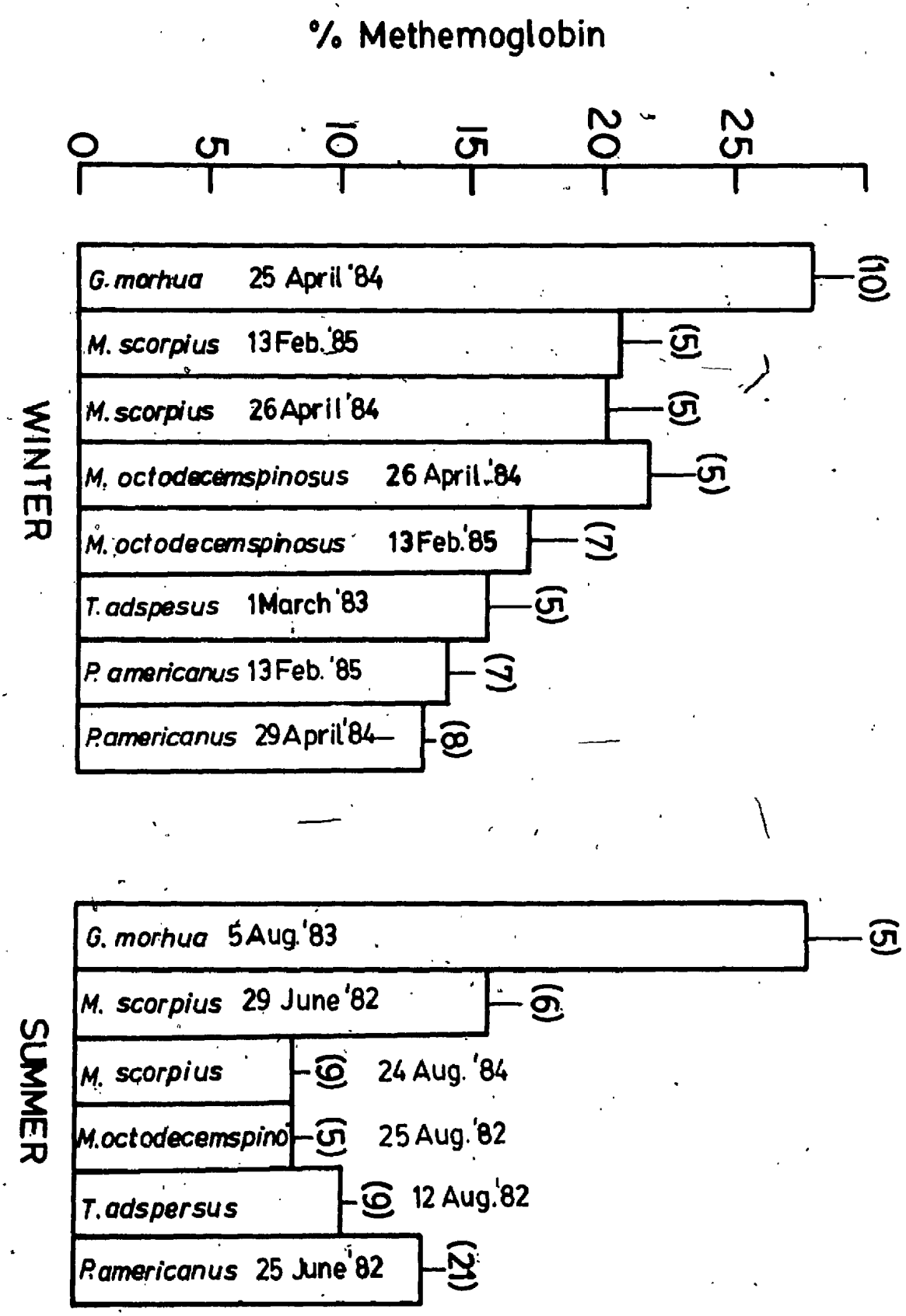
It is clear that fish located in temperate zones that encounter seasonal environmental changes, undergo marked physiological alterations. Many aspects of the respiratory and circulatory physiology are directly influenced by temperature. The results of the present study focus primarily on the changes which take place in the winter flounder. However, similarities with these results can be seen when compared with examples from other species. Initially, it should be recognized that oxygen uptake and delivery is dependent upon many aspects of an organisms physiology. The several aspects necessarily interact in providing oxygen for the living tissues, and some parts of the system are effected by temperature more than others. The fact that the system is built upon interacting parts allows for compensation if change occurs to any of those components. The most obvious examples of such component interactions come from the Antarctic. Some fish species in that region have no hemoglobin in their blood. The persistent extreme cold is thought to be responsible for that rather radical change to the circulatory system. The absence of hemoglobin, limits the oxygen storage capacity of the blood. However, the loss in oxygen capacity is somewhat compensated by changes in other components of the oxygen delivery system: larger heart, more blood capillaries, and larger blood volume. The oxygen storage capacity of blood from fish in temperate regions changes on a seasonal basis. Presumably the change in oxygen-capacity

is ultimately to satisfy the tissue oxygen demands. It is apparent though, that the physical environment has different effects on various species. The flounder, and other species mentioned in Chapter 1, show increases in hemoglobin when acclimatized to winter conditions. It is a change that would assist in maintaining P_{aO_2} during a time when blood convection through the gill, and oxygen passage into the blood are less effective. The alterations in blood components can be looked upon as compensation for the effects of the physical environment on other physiolgical features at that time. In those fish which show decreased [Hb] during low temperature conditioning, it is likely that the other components of the oxygen delivery system also respond in a different fashion than that of the flounder.

Although fish are poikilotherms, the notion of an equilibrium during seasonal changes in the physical environment is quite apparent, especially in the winter flounder. Even though there are marked seasonal changes in the cardiovascular and ventilatory system, the functions of those systems are able to stabilize during winter and summer acclimatization. As those physiological systems perform, the interaction of the blood components with water via the gills maintains homeostasis in blood oxygen levels.

Appendix 1

The percentage of the total [Hb] that was metHb for a number of marine teleosts. Date of sampling and species names are given. ( ) = number of animals sampled. Bars represent average values and lines are SEM.


% Methemoglobin
0
5
10
15
20
25
WINTER
G. morhua 25 April '84
(10)
M. scorpius 13 Feb. '85
(5)
M. scorpius 26 April '84
(5)
M. octodecemspinosus 26 April '84
(5)
M. octodecemspinosus 13 Feb. '85
(7)
T. adspesus 1 March '83
(5)
P. americanus 13 Feb. '85
(7)
P. americanus 29 April '84
(8)
SUMMER
G. morhua 5 Aug. '83
(5)
M. scorpius 29 June '82
(6)
M. scorpius
(9)
24 Aug. '84
M. octodecemspino
(5)
25 Aug. '82
T. adspersus
(9)
12 Aug. '82
P. americanus 25 June '82
(21)

Appendix 2

The units for the measurements done in the present study are commonly used in the literature, however some are not SI units. The following is a conversion table to allow for quick understanding of all values.

1 atmosphere	=	760 mmHg = 760 torr (torricelli)
1 cmH_2O	=	980.64 dyne/cm^2 = 0.10 KPa
1 pound/in^2	=	68947 dyne/cm^2 = 6.89 KPa
1 dyne/cm^2	=	1×10^{-4} KPa = 0.1 Pa (Pascal)
1 kPa	=	7.5 mmHg

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